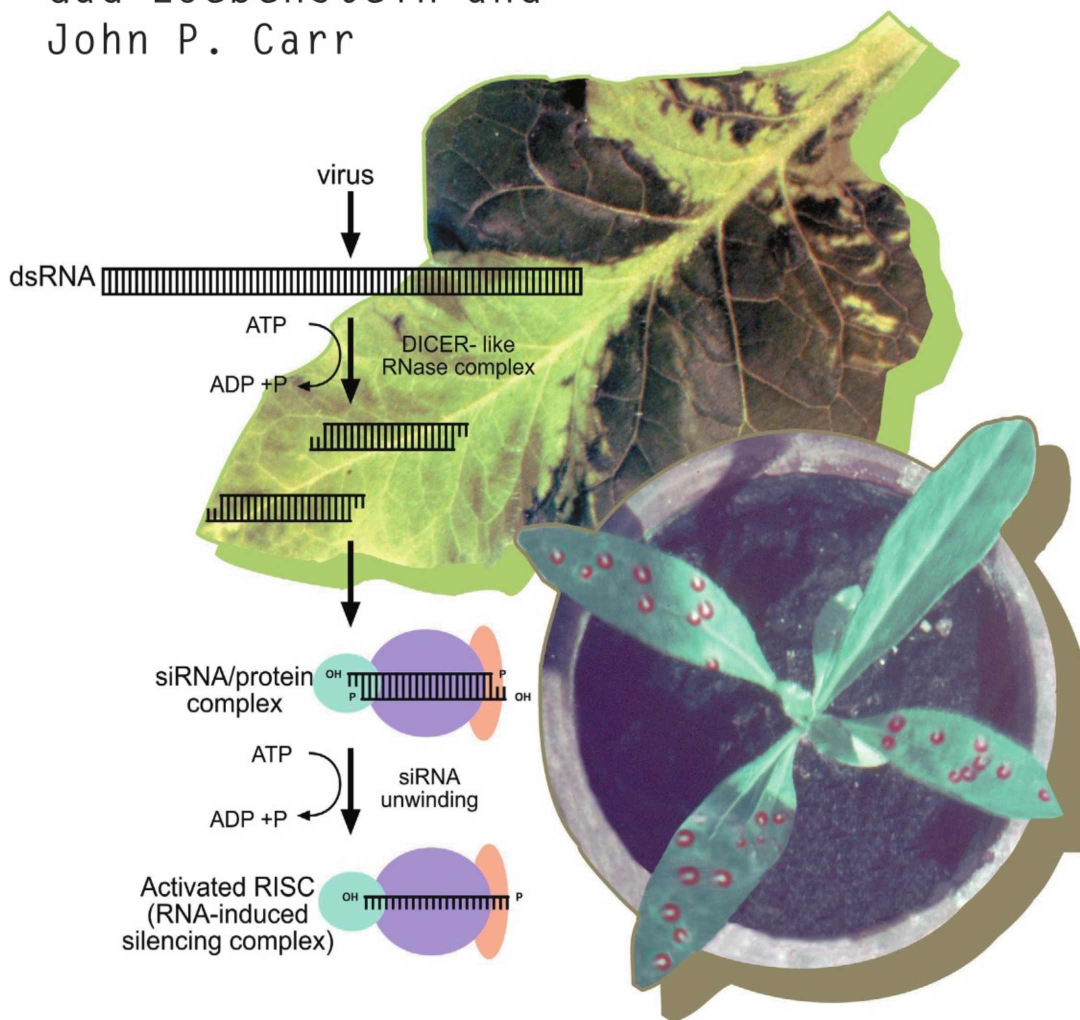
Natural Resistance Mechanisms of Plants to Viruses

Edited by
Gad Loebenstein and
John P. Carr



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NATURAL RESISTANCE MECHANISMS
OF PLANTS TO VIRUSES

Natural Resistance Mechanisms of Plants to Viruses

Edited by

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Preface

Over the course of evolution most plants have acquired the ability to defend themselves against most groups of pathogens, including the viruses. Many antiviral resistance phenomena have been known and studied for decades but, until recently, understanding of their underlying mechanisms has lagged behind. These phenomena include resistance to infection, resistance to virus translocation through the plant, recovery from infection and genetically defined resistance, together with the associated phenomena of the local lesion response, and induced, or acquired, resistance.

The identification and cloning of plant resistance genes, characterization of downstream signaling components, and especially the explosion of data regarding gene-silencing mechanisms, has led to rapid progress in the investigation of natural resistance phenomena. Meanwhile, in plant virology there has been remarkable progress in the arenas of replication, movement proteins and plasmodesmatal gating, and in the discovery of gene silencing suppressors. Therefore, it seemed timely and appropriate to link older but still important data on the well known, 'classical' resistance phenomena with the new information that has emerged during the last decade or so.

We hope that this book will inspire further research in this area, as resistance presents the most economical and environmentally sound approach to control plant virus diseases. Future technologies that emerge from this research might include an improved ability to introduce resistance genes into virus-susceptible, agronomically important cultivars, to improve current pathogen-derived resistance strategies using our new knowledge of small interfering and microRNAs, or to develop targeted chemical treatments. Most likely the technologies that emerge from this work will not be easily predictable from our present standpoint any more than the feasibility of pathogen-derived resistance, the workings of resistance genes, or the existence of small RNAs would have been foreseeable from the standpoint of a writer in say, 1980, when the junior editor began his PhD work.

This book is presented in two parts. The first discuses the more general findings concerning the various resistance mechanisms, which have been obtained for the most part using 'model' plant and virus systems. The second part of the book deals with resistance phenomena in a selection of crop plants. We constructed the book in this way because it is evident that many of the new principles discovered with model systems and presented in the

first part have not yet been applied to the 'real world' of crop protection. It is our hope that they soon will be.

The editors warmly thank all the contributing authors for their efforts in the timely preparation of their chapters with the most up-to-date information. We also want to thank D. Ronen for preparing the cover design and some of the photographic illustrations; and Springer (formerly Kluwer) for their help in preparing this book, with special thanks going to Ing. Zuzana Bernhart, Publishing Editor, for her consistent help.

Gad Loebenstein John P. Carr
April 2005

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Colorplates

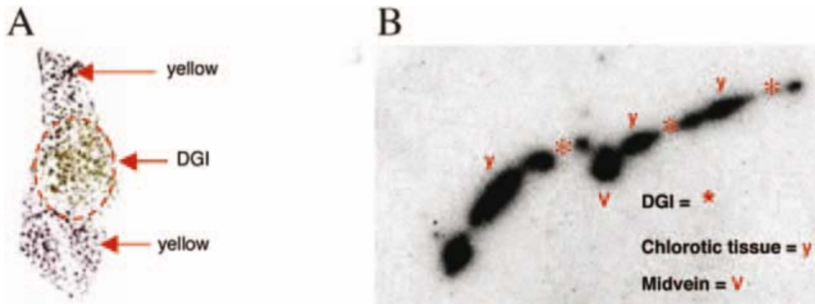

Fig. 3 Tissue prints of TamMV-infected le aves containing DGIs shows viral protein and RNA exclusively in yellow tissue. Both viral protein and RNA are present in the yellow, chlorotic tissue surrounding DGIs and are not detected or are present at low levels within DGI tissue. (A) Detection of TamMV CP in a *Cyphomandra betacea* (tamarillo) leaf with anti-PVA (TamMV) antibody results in a purple color whereas those regions not positive for the presence of CP remained green (chlorophyll). The single visible DGI is indicated with a dotted line, the yellow surrounding tissue was chlorotic in the sampled leaf and corresponds to the purple hue. (B) Detection of TamMV CP RNA in a section of TamMV-infected N. benthamiana leaf cut traverse to three DGIs and the mid-vein. The probe detects the TamMV CP RNA,which is part of the full-length TamMV RNA genome (black = positive for TaMV CP RNA). Figure from Moore (2002). (See also Chapter A9, p. 198)

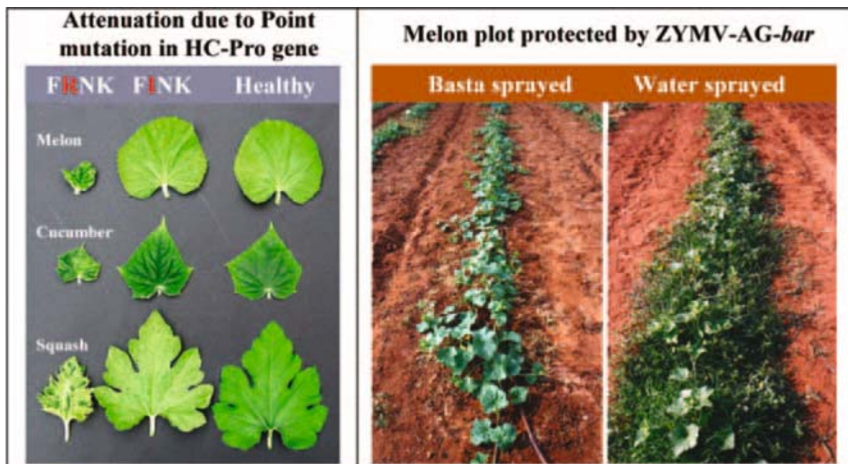

Fig. 2. ZYMV-AGII as an attenuated, aphid non-transmissible, potentially cross-protecting expression vector. A single mutation in the potyviral suppressor gene HC-pro from FRNK to FINK attenuates symptoms in various cucurbits (left). Functional expression of bar via AGII-Bar in cucurbits confers resistance to glufosinate ammonium herbicide (Basta®) (right). Melons were sprayed with 0.5% Basta 14 days after planting and photographed 5 days later. (See also Chapter A12, p. 278)

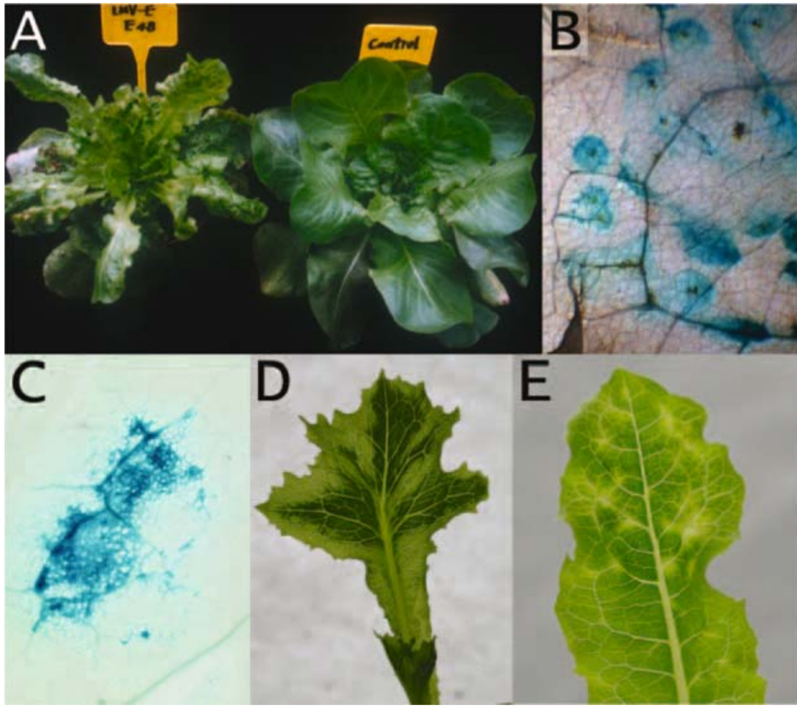

Fig. 1. Phenotypes of LMV infection in lettuce. A: typical mosaic symptoms induced by LMV-E in a susceptible butterhead lettuce (Trocadéro). **B**: necrotic local lesions limit the spread of a GUS-tagged LMV in rub-inoculated leaves of *Lactuca virosa* containing the *Mo4* gene. **C**: accumulation of a GUS-tagged LMV-Common (non-resistance-breaking) isolate in an inoculated leaf of a *mo1*^{2} crisphead variety (Vanguard 75). **D**: mosaic symptoms induced by LMV-Most (resistance-breaking) in a *mo1*^{2} crisphead lettuce (Vanguard 75). **E**: vein clearing and "star" symptoms induced by LMV-E (resistance-breaking) in a *mo1* crisphead breeding line. (See also Chapter B3, p. 387)

Fig. 1. A *Turnip mosaic virus* infected cabbage plant in the field showing severe necrotic symptoms. (See also Chapter B5, p. 416)

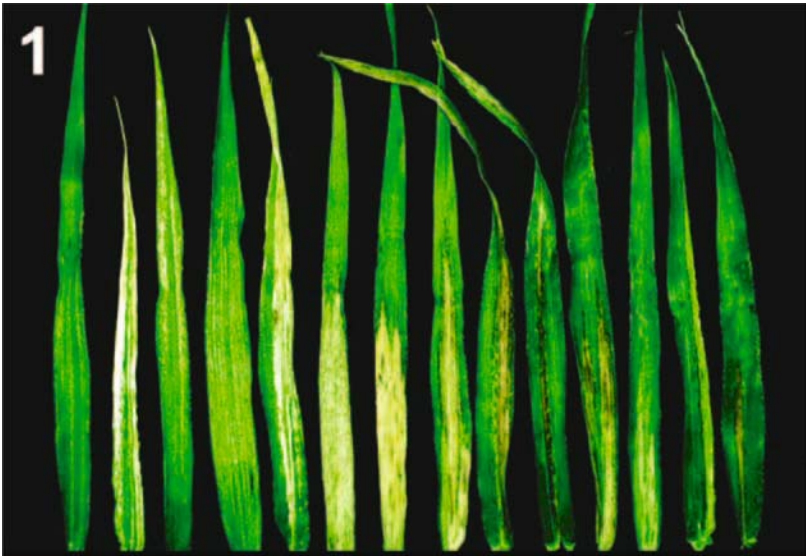

Fig. 1. Typical leaf symptoms induced by *Barley stripe mosaic virus* on 'Dickson' barley on leaves of plants in a field planted with infected seed. The symptoms range from mild mosaic to severe necrosis, often form a V-shaped pattern or chevron. (See also Chapter B8, p. 468)

Fig. 3. Barley yellow dwarf focus in a field near Purdue University photographed by Richard Lister in 1968. Note the yellowing plants and the circular ring of infection that probably resulted from movement of apterous aphids from plant to plant. (See also Chapter B8, p. 476)

Fig. 5. Symptoms induced by *Barley yellow mosaic virus* on barley in Japan. Yellow-green spots and short streaks are typically produced on leaves, and as yellowing increases, large yellow patches appear in affected fields. Courtesy B. Steffenson, University of Minnesota. (See also Chapter B8, p. 483)

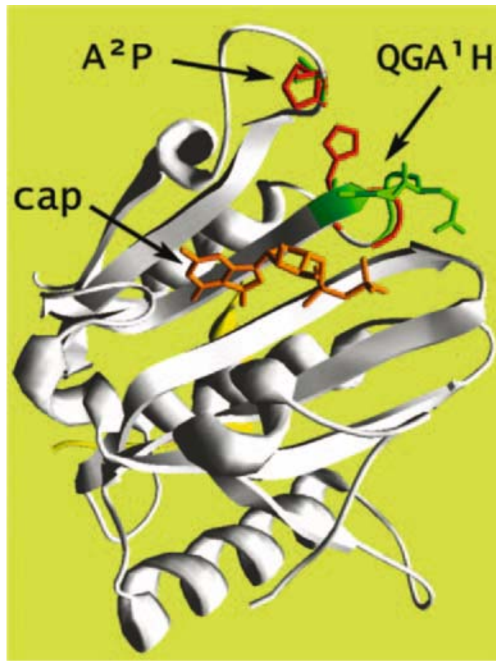

Fig. 2. 3D modelling of lettuce eIF4E. The structure is predicted based on that of its mouse homologue (see Nicaise et al. 2003). The predicted differences between the *mo1*^{1} ("QGA^{1}H") or *mo1*^{2} ("A^{2}P") forms and the susceptibility allele are shown in red versus green. The cap analogue ("cap") is shown bound in the cap-binding pocket. The yellow helix in the background is the portion of the eIF4G protein known to interact with eIF4E for translation initiation. (See also Chapter B3, p. 389)

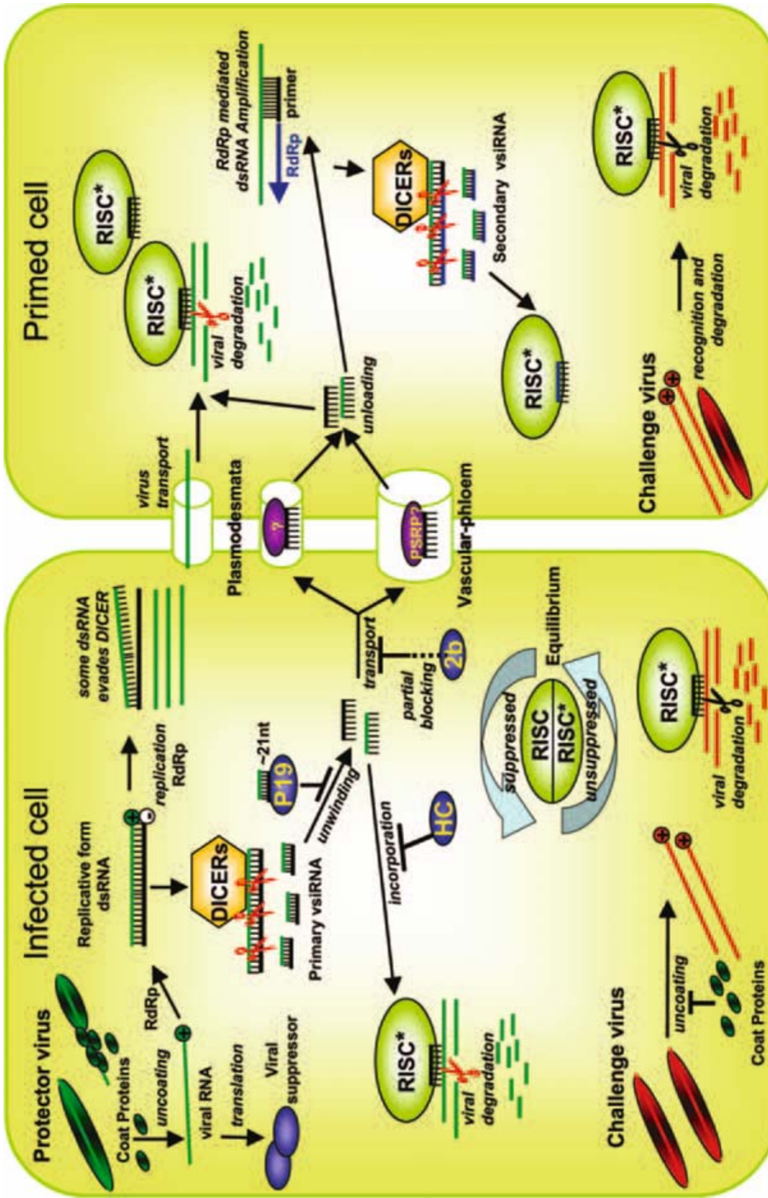
Infected cell
Protector virus
Coat Proteins
uncoating
viral RNA
RdRp
translation
Viral suppressor
Replicative form dsRNA
replication RdRp
some dsRNA evades DICER
DICERs
Primary vsiRNA
~21nt
P19
unwinding
incorporation
HC
partial blocking
transport
2b
RISC*
viral degradation
suppressed
RISC
RISC*
unsuppressed
Equilibrium
Challenge virus
uncoating
Coat Proteins
RISC*
viral degradation
Challenge virus penetrates cell containing protector virus
virus transport
Plasmodesmata
?
PSRP?
Vascular-phloem
Primed cell
RISC*
RISC*
viral degradation
unloading
RdRp mediated dsRNA Amplification
RdRp
primer
DICERs
Secondary vsiRNA
RISC*
Challenge virus
recognition and degradation
RISC*
viral degradation
Challenge virus penetrates primed cell and is degraded by activated RISC

Fig. 1. Model of RNA-mediated viral cross-protection in RNA viruses. Initially the cell is infected with the protector virus (green) (**left**). The protector virus uncoats and its coding sense strand is translated, producing, among other things, a suppressor of silencing. Viral RNA-dependent RNA polymerase (RdRp) makes replicative form dsRNA which is cleaved by Dicer RNAse III type enzymes into ~21 and ~25 class viral siRNAs (vsiRNAs). Some dsRNAs must evade Dicer cleavage for virus establishment to take place. vsiRNA is unwound by a helicase and its separate strands are incorporated into the RNA-Induced Silencing Complexes (RISC), to form "activated RISCs" (RISC*s). RISC*s can now recognize and cleave any complementary viral RNA, of either strand, in the infected cell. A race now takes place between active transport of viral RNA, and transport of vsiRNA through the plasmodesmata and the vascular phloem [with a putative Phloem Small RNA-binding Protein (PSRP)]. If a cell has been reached first by vsiRNA, (**right**) then this cell will have been "primed" for viral degradation through the direct activation of RISC. Upon entry of viral RNA a priming reaction is performed on any complementary viral template that enters, causing the production of secondary vsiRNAs. Secondary vsiRNAs are Dicer-cleaved amplification products of plant RdRps based on viral templates. This secondary amplification occurs in the infected cell also (not drawn). These primed cells could be a possible explanation for "green islands", mosaics and the recovery phenomenon. In the infected cell (**left**) the viral suppressors of different viruses (blue ovals) act at different stages; for example, *Tomato bushy stunt virus* P19 binds ~21nt dsRNA, thus blocking transport, CMV 2b blocks transport, and HC-Pro of potyviruses might interfere with the activation of RISC. Suppression is partial, not total, and attains a state of equilibrium (see text). On the entry of a challenge virus (red) into the infected cell, RISC that has been activated with the related sequence of the protector can cause its degradation. Additionally a CP-mediated protection mechanism may operate when challenge virus enters a virus-infected cell. On the entry of a challenge virus into a primed cell (right) immediate degradation by pre-activated RISC will occur. (See also Chapter A12, p. 267)

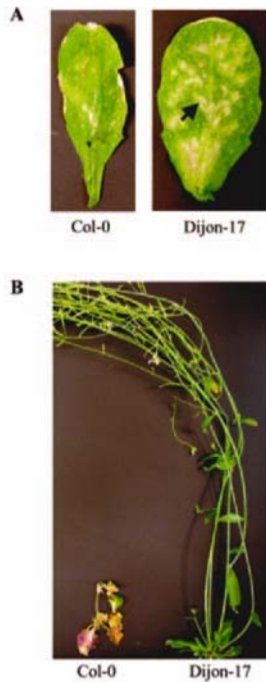

Figure 1. Morphological phenotypes of TCV-infected Col-0 and Dijon-17 plants. (**A**) TCV-inoculated leaves at four days post infection. The small specks seen on the inoculated leaves of Col-0 plants are damaged tissue or dried inoculation buffer. The TCV inoculated resistant plant, Dijon-17, show development of discrete lesion known as hypersensitve response (marked by an arrow). (**B**) The morphological phenotypes of the TCV-inoculated plants at three-weeks post infection. The susceptible plants show severe crinkling of their leaves, remain stunted and show dropping bolts. By comparison, the resistant plants develop normally. (See also Chapter A7, p. 149)

Chapter A1

Applied Aspects of Induced Resistance to Plant Virus Infection

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Introduction

Plant virus diseases occur worldwide in cultivated plant species as well as many native (weed) plants. A plant virus is dependent on host and vector for its "survival". The efficiency and extent of spread of infection within a plant are important factors for allowing the virus to be accessible to its vector(s), which in turn allows for dispersal of the virus to new plants and crops. If dispersal by a vector, such as an insect, is not part of the virus' infection cycle, it may adapt alternative, and seemingly clever, approaches. For example, *Tomato mosaic virus* is not dispersed in nature by a recognized vector but its ability to persist in the environment allows it to be transmitted from soil bound decaying plant tissues, and virus occurred in clouds and fog which may have served as a source of virus for transmission to spruce trees (Castello et al. 1995). Plant viruses appear to represent extreme ends of a life spectrum; they are genetically and structurally simple infectious agents but their obligatory and intimate relationship with a host plant is quite complex.

Plant viruses seem nearly impossible to control, instead, practical attempts are made to keep them in check, to reduce losses, basically to manage their existence within a crop. The availability of genetically resistant varieties is clearly the best approach for all cultivated crops; however, such varieties are often not available, and even when they are

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available, there is the possibility for the occurrence of other viruses or viral strains that are not affected by the resistance. Much effort has been devoted to development of management schemes that integrate with growers' cultivation practices, e.g., altering planting dates to avoid vector migrations, various mulches to deter vectors and use of trap crops. The basic premise behind these approaches is to deter or delay the introduction of virus into the crop thereby allowing the plant to mature to a stage of development that will essentially tolerate the infection. Virus infection of a more mature plant typically results in delayed movement of virus throughout the plant, reduced virus accumulation, reduced symptom severity and losses in yield.

A complicating factor for virus management schemes is the unpredictability of the timing and extent of the virus out-break. The complexity of most viral pathosystems requires that numerous factors align themselves for a serious out-break to occur. Susceptible hosts must be available during (to become infected) and between (to serve as sources of inoculum) crop seasons. The appropriate vectors must be available and able to feed on the virus infected source plants. The vector must be able to acquire the virus from that particular host plant and then deliver the virus into the crop of concern. Secondary spread within that crop may be equally complicated but with much greater uniformity among virus source plants.

Genetic and engineered forms of resistance to virus infection are the best options economically and environmentally for a grower; however, each has limitations in availability. Interestingly, most plant species appear to have evolved inducible defence mechanisms for protection against plant pathogens and herbivorous insects. These inducible forms of resistance are "turned on" upon attack and may be localized or systemic in their response (Kessman et al. 1994). Chester (1933) initially described the ability of plants to develop resistance in response to infection. An induced resistance response to virus infection was first described by Yarwood (1960). Ross (1961) showed that localized infection by *Tobacco mosaic virus* (TMV) resulted in resistance throughout the plant, referred to as 'systemic acquired resistance'. Since that initial report, much effort has focused on mechanisms associated with induced resistance to virus infection (Kessmann et al. 1994; Murphy et al. 1999; Pennazio and Roggero, 1998; Singh et al. 2004; van Loon et al. 1998). The application of this knowledge to reduce losses caused by virus disease, however, has been limited. While induced resistance has been demonstrated in response to simple wounding, one might expect induced protection against pathogen attack when plants are grown in natural conditions since they are under almost continual challenge from wound-inducing wind-blown soil particles and herbivorous insects. Induced resistance is, in some cases, thought to be a general response but perhaps

costs to plant fitness from a continually engaged induced resistance response may not benefit plant survival.

Bacterial resistance-inducing agents

The treatment of plants with biological agents has evolved from rather simple bacterial preparations that induced systemic resistance to combinations of bacterial strains mixed with carriers that result in induced systemic resistance and enhanced plant growth. Loebenstein and Lovrekovich (1966) injected heat-killed *Pseudomonas syringae* into intercellular spaces of Samsun NN plants to reduce the number of local lesions induced by infection with TMV. Mann (1969) showed that addition of *Bacillus uniflagellatus* to the soil of Xanthi tobacco plants prior to inoculation with TMV caused a reduction in the number of local lesions. Inoculation of cucumber leaves 1 and 2 with *Pseudomonas lachrymans* followed by challenge inoculation of leaf 3 with *Cucumber mosaic virus* (CMV) caused reduced lesion number on the CMV inoculated leaf and a delay in the appearance of systemic symptoms (Bergstrom et al. 1982).

Bacterial populations that colonize the plant rhizosphere sometimes exert beneficial effects on plant growth and are referred to as plant growth-promoting rhizobacteria (PGPR) (Kloepper et al. 1980). Similar bacterial preparations have been used in China and referred to as ‘yield-increasing bacteria’ (Chen et al. 1996). PGPR were shown to suppress soil-borne and foliar plant pathogens in different plant species (Alstrom, 1991; Broadbent et al. 1977; Kloepper, 1996; van Peer et al. 1991; Wei et al. 1996). Treatment of cucumber with selected PGPR strains reduced bacterial wilt disease and the feeding of spotted and striped cucumber beetles which serve as vectors for the bacterial pathogen (Zehnder et al. 1997; Zehnder et al. 2001).

The initial approaches to application of PGPR treatments to manage plant virus infection involved single bacterial strains used to coat seed or treat roots by a drench or root dip (Maurhofer et al. 1994; Raupach et al. 1996). Treatment of tobacco roots with *P. fluorescens* strain CHA0 resulted in a similar level of reduction in the number and size of *Tobacco necrosis virus* (TNV)-induced local lesions as with the well demonstrated approach of induction of resistance by TNV itself (Maurhofer et al. 1994). Similarly, treatment involving *P. aeruginosa* strain 7NSK reduced the size of lesions caused by TMV infection in tobacco plants (De Meyer et al. 1999). In a greenhouse study, CMV was mechanically inoculated onto the cotyledons of cucumber seedlings treated with either PGPR strain 89B-27 or 90-166 (Raupach et al. 1996). A delay in the development of CMV-induced symptoms of up to 7 days occurred in PGPR-treated plants relative to a non-treated control. In addition, significantly fewer symptomatic plants occurred among PGPR treatments compared with the non-treated control at 2 weeks after inoculation. CMV was not detected in non-inoculated leaves of those

plants not expressing symptoms. In that same study (Raupach et al. 1996), the area under the disease progress curve (AUDPC) was significantly less in PGPR-treated tomato plants inoculated with CMV relative to a non-treated control.

Zehnder et al. (2000) evaluated additional PGPR strains under greenhouse conditions for their ability to induce resistance against CMV in tomato with the intention of selecting treatments to be evaluated under field conditions. Four PGPR strains were selected, SE34, IN937a, IN937b and IN114, and used to treat tomato seedlings that were mechanically inoculated with CMV just prior to being transplanted to the field. These treatments resulted in significant reductions in percentage of plants infected, amount of CMV in young tissues (based on serological tests) and AUDPC values compared with non-treated, CMV inoculated controls. Furthermore, the PGPR-treated plants had significantly greater height and yield than the control. Using a similar approach whereby selected PGPR strains were first screened in the greenhouse and then plants were mechanically inoculated with CMV for the field trial, Jetiyanon and Kloepper (2003) showed that PGPR treatments of cucumber resulted in protection against CMV. These experiments illustrated that the protection observed for some PGPR treatments performed under the controlled environmental conditions of a greenhouse was maintained when plants were artificially challenged with CMV but grown using conventional practices in the field.

The use of PGPR to induce systemic resistance to virus infection evolved from greenhouse-based studies to field trials, all involving artificial application of the challenge virus. When the selected PGPR strains used by Zehnder et al. (2000) were tested in natural conditions where a severe CMV epidemic had essentially devastated a local tomato industry (Sikora et al. 1998), induced systemic resistance was not observed compared with non-treated controls (Murphy and Sikora, unpublished data). In an effort to progress further into commercial application of PGPR treatments, preparations of industrially formulated seed treatments consisting of selected PGPR strains were evaluated for induced systemic resistance against the whitefly vectored *Tomato mottle virus* (ToMoV) under high levels of disease pressure (Murphy et al. 2000). ToMoV disease severity and virus incidence were significantly reduced with corresponding increases in yield for one of the three trials. Protection against ToMoV was most pronounced up to 40 days after plants were transplanted to the field with little or no protection by 80 days. These data offered promise that commercially prepared biological control agents could induce resistance to high levels of ToMoV disease pressure with virus delivered by its natural vector.

While treatments involving single PGPR treatments illustrated the ability to induce systemic resistance to virus infection, other research indicated that mixtures of PGPR strains offered more consistent and broader spectrum

protection (Raupach and Kloepper, 1998). Combinations of PGPR strains offered protection in cucumber against CMV as well as plant growth promotion (Jetiyanan and Kloepper, 2003). The addition of chitosan to the two strains PGPR formulation resulted in dramatic plant growth promotion (Kloepper et al. 2004). This combination apparently offers a combined effect on plants involving induced systemic resistance with plant growth promotion. Tomato plants subjected to the PGPR plus chitosan treatments were significantly larger than non-treated plants of the same age, while being phenotypically and developmentally similar to plants 10 days older (Figure 1) (Murphy et al. 2003). The PGPR plus chitosan treated tomato plants responded to CMV infection in a similar manner to the older control plants with significantly greater plant height, fresh weight and flower and fruit numbers relative to non-treated control plants of the same age as plants subjected to PGPR treatments. CMV disease severity was significantly less for all PGPR treatments than the non-treated, same age control at 14 and 28 days post-inoculation (dpi) (Figure 2, left panel). When plants shown to be infected with CMV by ELISA were compared, all PGPR treatments had significantly lower disease severity ratings at 14 dpi with three of the PGPR treatments remaining significantly less at 28 dpi (Figure 2, right panel). Similar reductions in CMV accumulation in non-inoculated leaves and percent infection within a treatment were observed among PGPR treatments and the non-treated older control. These experiments involved inoculation of plants with CMV when the tomato plants in the non-treated control treatment (those that were the same age as the plants in the PGPR treatments) were at a fairly early stage of development (as seen in Figure 1), whereas the PGPR plus chitosan treated plants were dramatically larger. When the PGPR plus chitosan treated tomato plants were inoculated at an early developmental stage (e.g., similar to the size of the non-treated, same age control plants in Figure 1), protection against CMV infection was not observed. These findings suggest that the PGPR plus chitosan treatment resulted in a form of resistance similar to mature plant resistance rather than induced systemic resistance. If induced resistance is not a factor in the protection afforded plants treated with PGPR plus chitosan, the rapid and enhanced growth provide an important form of protection by shortening the time during which plants are young and highly vulnerable to infection and development of severe disease.

Fig. 1. Representative tomato plants for control (left), older control (center) and PGPR (right) treatments. The control (left) and PGPR-treated (right) plants are the same age, whereas the older control (center) is 10 days older. These photographs were taken five days prior to *Cucumber mosaic virus* inoculation. Reprinted with permission from Murphy, J. F., Reddy, M. S., Ryu, C. M., Kloepper, J. W., and Li, R., 2003, Rhizobacteria-mediated growth promotion of tomato leads to protection against *Cucumber mosaic virus*, *Phytopathology* 93:1301-1307.

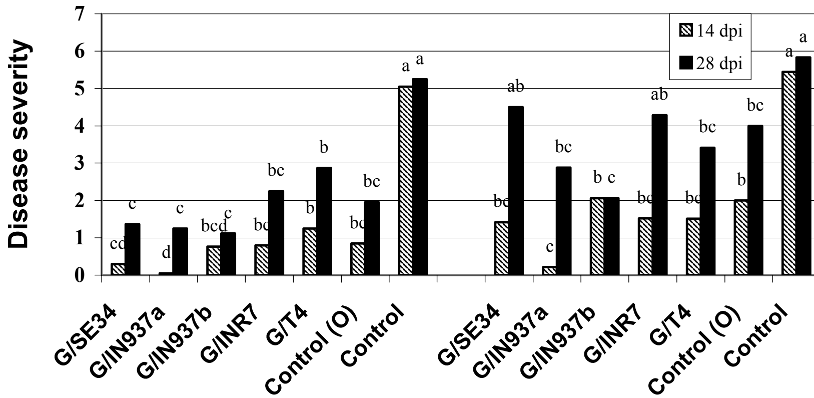

Fig. 2. Mean disease severity ratings at 14 and 28 dpi for all 40 plants in each treatment (left panel) or only for plants determined by ELISA to be infected with *Cucumber mosaic virus* (right panel). Disease severity was rated using the following scale: 0 = no symptoms, 2 = mild mosaic symptoms on leaves, 4 = severe mosaic symptoms on leaves, 6 = mosaic and deformation of leaves, 8 = severe mosaic and deformation of leaves, and 10 = severe mosaic and deformation of leaves with stunted growth. Treatments are listed along the x-axis consisted of PGPR treatments G (GB03, *Bacillus subtilis*), SE34 (*B. pumilus*), IN937a (*B. amyloliquefaciens*), IN937b (*B. subtilis*), INR7 (*B. pumilus*) and T4 (*B. pumilus*). Control treatments included plants that were the same age as plants in the biopreparation treatments (designated control) and plants that were 10 days older than those in the control and biopreparation treatments (designated control (O)). Statistical comparisons were made among treatments within each date of disease assessment. Different letters represent a significant difference of the means at *P*=0.05 according to Fisher's protected LSD test. Reprinted with permission from Murphy, J. F., Reddy, M. S., Ryu, C. M., Kloepper, J. W., and Li, R., 2003, Rhizobacteria-mediated growth promotion of tomato leads to protection against *Cucumber mosaic virus, Phytopathology* 93:1301-1307.

Chemical resistance-inducing agents

The induction of systemic acquired resistance (SAR) results from the systemic release of signal molecules that activate a broad-spectrum resistance. A search to identify the molecule(s) or chemical(s) involved in SAR offered the potential for a commercial product that would protect plants from multiple types of pathogens as well as herbivorous insects. The plant activator, CGA-245704 (benzo[1,2,3]thiadiazole-7-carbothioc acid S-methyl ester), was commercially produced and shown to induce SAR in plants. CGA-245704 was marketed under the trade name Bion™ in Europe and Actigard™ in the United States. Since treatment of plants with CGA-245704 results in induced resistance, which requires 2 to 4 days for activation, it was shown to be most effective when applied preventatively prior to transplant. It has since been shown to be effective at protecting plants from numerous different types of pathogens in distinctly different crops (Tally et al. 1999).

The application of Actigard to manage plant virus disease under natural conditions has been tested quite thoroughly against *Tomato spotted wilt virus* (TSWV) in flue-cured tobacco (Csinos et al. 2001; Pappu et al. 2000). Tobacco plants treated with Actigard had significantly less TSWV incidence (based on occurrence of symptomatic plants) than non-treated controls in one of four trials (Pappu et al. 2000). When using ELISA to identify TSWV-infected plants, the Actigard treatment significantly reduced TSWV incidence in two of four trials. Csinos et al. (2001) applied Actigard as four weekly sprays starting at four weeks post-transplant with no apparent reduction in TSWV incidence but also no phytotoxic effects on tobacco growth and development. When similar applications of Actigard were used to treat tobacco plants prior to transplant, significant phytotoxic affects resulted (Pappu et al. 2000). The application of Actigard at reduced amounts prior to transplant showed no phytotoxicity and significantly reduced TSWV incidence (Csinos et al. 2001). Thus, Actigard may significantly reduce incidence of TSWV in tobacco under natural conditions with application prior to transplant being most effective.

Momol et al. (2004) examined the integration of different plastic mulches with and without use of Actigard or an insecticide to reduce TSWV in tomato. While treatment of tomato plants with Actigard reduced TSWV incidence, the most effective approach was the combined treatment of UV-reflective plastic mulch, Actigard and insecticide.

Summary

The potential for broad-spectrum resistance resulting from both biological and chemical inducing agents holds promise for the practical use of induced resistance to manage diseases caused by different types of pathogens as well as herbivorous insects. Biological inducing agents that colonize plant roots may offer a more long-term protection since they may persist during the course of the plant's life; however, there may be factors that negatively affect the viability of biological agents reducing consistency in their effectiveness. This lack of consistency may be overcome through the use of multiple bacterial strains within a single treatment, which appears to be the direction taken for some commercial products. In contrast, chemical inducers may offer consistency but tend to lack persistence thereby requiring repeated applications. While the broad-spectrum nature of induced resistance should offer a practical advantage, the triggering of the induced state may need to be highly specific in order to avoid a continual state of resistance from elements of nature such as the physical effects of wind. A continual state of resistance may have cost trade-offs that negatively affect physiological and developmental processes of an otherwise healthy plant. Along similar lines, the rapid and enhanced growth of some combinations PGPR treatments may offer an opportunity to greatly shorten the window of time during which young plants are vulnerable to infection and development of severe disease. This enhanced growth of plants, however, may lead to an increased drain on soil fertility.

Induced resistance may not effectively serve as a sole means to manage plant virus disease, but rather, an important component in an integrated system. Most importantly, perhaps, is a need for more holistic approaches that integrate the best management practices for crop health, from soil to fruit and management of pathogens and pests.

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Chapter A2

Viral Determinants of Resistance Versus Susceptibility

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Introduction

The ability of a plant virus to systemically infect its hosts can be considered the consequence of a series of interactions between the viral genome and its gene products with the host. Once a virus enters the cell, it must be able to express each of its proteins, replicate its genome, move from one cell to another, and then gain access to the host vascular system to move out of the initially infected leaf to other parts of the plant. At each step of this process, there must be a basic level of compatibility between the virus and its host; otherwise, the infection process ends prematurely. Hull (2002) discusses how mismatches between host and virus at different points of the infection process can define the host range of a virus. For example, stringent requirements for cell-to-cell movement in the plant can provide one important barrier that limits the host range of a virus. A virus may be able to replicate in an individual cell, but its cell-to-cell movement protein may be non-functional in that host, thereby limiting its movement into adjacent cells. In this instance, there would be no visible response of the plant to the inoculation of the virus.

Plants also have the capacity to recognize and actively defend themselves from virus infections. This concept of plant defense was first developed by H. H. Flor in his 'gene-for-gene' model (Flor, 1971). He proposed that plant

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pathogens have a series of avirulence (*Avr*) genes whose protein products can be recognized by a surveillance system of plant resistance (*R*) gene products. Later studies showed that this recognition event sets in motion a plant defense cascade that limits the spread of the pathogen (Baker et al. 1997; Hammond-Kosack and Jones, 1996; Martin et al. 2003). Consequently, the viral proteins that are recognized by plant *R* gene products can be considered *Avr* gene products. The manifestation of the host resistance response may take several forms (Loebenstein, 1972). In some instances, viral *Avr* gene products may trigger a classic hypersensitive response (HR), in which case a necrotic lesion forms in the inoculated leaf. However, viral *Avr* genes can trigger other responses as well as the HR. For example, resistant plants may respond with chlorotic local lesions rather than necrotic lesions, and in some cases, there may be no visible response at all in the resistant plant. Furthermore, the recent cloning of recessive genes conferring resistance to potyviruses indicates that host resistance may also reflect the inability of that host protein to support a key step in the virus infection process. In this chapter, I discuss the development of the concept that virus gene products can act as Avr proteins and the contributions that viral *Avr* genes have made to our understanding of gene-for-gene resistance and plant defenses.

Early investigations into the identification of plant virus host range determinants

The year 1984 serves as a benchmark for plant pathologists in understanding how plants recognize and defend themselves from pathogen attack, as this was the year that Staskawicz and coworkers (1984) cloned and identified the first bacterial *Avr* gene. This bacterial gene, designated *AvrA*, was cloned from race 6 of *Pseudomonas syringae* pv. *glycinea*. Its discovery was remarkable for both technical and theoretical reasons. The paper was a remarkable advance in techniques, because the authors essentially gambled that they could identify an avirulence determinant by chopping up the genomic DNA of an avirulent race, transforming each DNA segment into a virulent race, and observing a conversion of a compatible interaction into an incompatible interaction. The gamble was a success, as one transformant out of 680 tested was shown to contain the *Avr* gene from Race 6. Perhaps more importantly were the theoretical implications, because the physical isolation of an *Avr* gene provided tangible proof of the gene-for-gene theory. Soon after this was reported, several other bacterial *Avr* genes were isolated and cloned from other bacterial pathogens. Today, the existence of *Avr* genes is a well-established tenet of plant pathology, and studies have shifted towards understanding the functions of bacterial *Avr* genes, including whether they may have a role in virulence.

What has not been as readily recognized are the studies that describe the identification of virus host range factors. Many of these studies were published before the advent of infectious clones of viruses, and in many instances before the nucleotide sequences were known. The first plant virus gene to be implicated as an *Avr* gene was the coat protein of *Tobacco mosaic virus* (TMV), a protein that triggers a hypersensitive response in tobacco plants that carry the *R* gene, N' (reviewed in Culver, 2002). These early studies were conducted in the late 50's and early 60's and, at the time, researchers were not interested in the gene-for-gene theory, but instead were using this system to characterize the genetic code.

A series of mutagenesis studies eventually showed that 18 amino acids, out of a total of 152 in the TMV coat protein sequence, could individually convert TMV from a virulent to an avirulent form (summarized in Funatsu and Fraenkel-Conrat, 1964). It could not be proven until later that specific mutations in the amino acid sequence of the coat protein were responsible for elicitation of the HR, because other TMV genes had not yet been sequenced and mutations in other viral genes might have been responsible for eliciting the HR. However when infectious clones of TMV became available, they were used to formally prove that the coat protein does indeed elicit the HR in tobacco plants that carry the *N'* gene (Knorr and Dawson, 1988; Saito et al. 1987). For example, Knorr and Dawson (1988) identified a point mutation within the coat protein coding sequence of an infectious TMV clone that was sufficient to trigger the HR in *N. sylvestris*. This change occurred at amino acid 148, converting it from serine to phenylalanine, and interestingly, it had also been noted previously by Funatsu and Fraenkel-Conrat (1964). One year later Culver and Dawson (1989) showed that mutations at coat protein amino acids 11, 20, 25 and 46 could also elicit the HR in *N. sylvestris*; each of these locations had been identified in the mutagenesis studies conducted in the early 60's.

What is intriguing about this host/virus interaction is that a wealth of information about this viral avirulence gene product had been developed in the early 60's. However, it took more than 20 years before researchers could state categorically that the TMV coat protein was an *Avr* gene product. Limitations in technology were largely responsible for this delay, as the development of an infectious TMV clone was essential for the identification of the coat protein as the avirulence determinant. The delay also might be partly attributed to conceptual limitations. Very few investigators appeared to consider this host/virus interaction in terms of the gene-for-gene theory, at least in the published record, until Knorr and Dawson's paper was published in 1988.

From a further review of the literature, it is clear that viral *Avr* genes were also characterized in the genomes of several multicomponent viruses in the early 70's and 80's (Table 1). Each of the viruses in Table 1 has a

divided genome, which meant that *Avr* genes could be identified by swapping RNA components between a strain that elicited a resistance response in a particular host and one that was able to systemically infect that host. For example, *Alfalfa mosaic virus* (AMV) strain 425 elicited pinpoint necrotic lesions in the bean variety "Berna" and failed to move systemically, whereas AMV strain YSMV induced a chlorotic symptom and was transported to the trifoliate leaves (Dingjan-Versteegh et al. 1972). An exchange of nucleoprotein components between the two strains showed that RNA 2 of strain 425 was responsible for eliciting defense responses in bean. The authors were not aware then that RNA 2 of AMV encoded a single protein involved in replication of the virus, as the nucleotide sequence of RNA 2 of AMV was not determined until 1983 (Cornelisson et al. 1983). However, the HR is a classic indicator of gene-for-gene resistance, and on the virus side of this interaction, the HR was induced by an RNA encoding a single AMV gene. Consequently, it would not be overly speculative to suggest now that the replicase protein component encoded by RNA 2 of AMV of strain 425 acts as an *Avr* gene product in bean, and that early studies on the multicomponent nature of AMV laid the foundation for this observation.

There are several other examples in Table 1 in which resistance could be described as an HR elicited by a single viral protein. In particular, *Cucumber mosaic virus* (CMV) was ideal for these types of RNA component-swapping experiments because of its simple genome structure and diversity of strains. Consequently both replicase proteins encoded by RNAs 1 and 2 were shown to act as Avr determinants, depending on the type of host tested (Edwards et al. 1983; Hanada and Tochihara, 1980; Lakshman et al. 1985). In other cases, the RNA component identified as the host range determinant encoded more than one virus protein. Consequently, Avr determinants were not localized to a single protein in *Raspberry ringspot virus* or *Cherry leafroll virus* (Harrison et al. 1974; Jones and Duncan, 1980), among others.

Although individual *Avr* genes can be identified in many of the viruses listed in Table 1, the gene-for-gene concept was not introduced in any of these studies. The very earliest studies were concerned with proving the multicomponent nature of that virus, and the local lesion response was a convenient marker to track infectivity. Later studies were concerned with mapping symptom determinants, and an inability to infect a host systemically was considered one type of symptom determinant. There is a subtle difference in designating a viral gene product as a symptom determinant rather than an *Avr* gene; consequently these studies generally were not included in early reviews that discuss the characterization of *Avr* genes (Keen and Staskawicz, 1988). However, they clearly laid the

TABLE 1: Identification of virus genetic components that act as host range determinants: studies conducted before the availability of infectious virus clones.

Virus (strain)	Host	RNA Component	Viral Proteins	HR?	Reference
AMV (425)[a]	bean	2	Replicase	Yes	Dingjan-Versteegh et al. 1972
CLRV (G)	*G. globosa*	1	Replicase & Protease	No	Jones & Duncan 1980
CCMV (T)	Cowpea	1	Replicase	No	Wyatt & Kuhn, 1980
CMV (Y)	Cowpea	2	Replicase	Yes	Hanada & Tochihara 1980
CMV (U & M)	Corn	2	Replicase	No	Rao & Francki 1983
CMV (LsS)	Pea, cowpea, & bean	2	Replicase	Yes No	Edwards et al. 1983
CMV (NL#1)	Pumpkin & Tobacco	2	Replicase	Yes No	Lakshman et al. 1985
RCNMV (H)	Cowpea	2	Movement	Yes	Osman et al. 1986
RCNMV	Sweet clover	1	Replicase & Coat Protein	Yes	Okuno et al. 1983
RpSRV (S & LG)	Raspberry & Bean	1	Replicase & Protease	No No	Harrison et al. 1974
TMV	*N. sylvestris*	N/A[b]	Coat Protein	Yes	Funatsu & Fraenkel-Conrat 1964
TRV (Z)	*P. hybrida*	1	Replicase & Movement	Yes	Ghabrial & Lister 1973
TSWV	Tomato	M	Movement & Coat Protein	Yes	Hoffmann et al 2001

[a]the virus strain that could not infect the host is listed in parenthesis. Viruses are: AMV-*Alfalfa mosaic virus*, CLRV-*Cherry leafroll virus*, CCMV-*Cowpea chlorotic mottle virus*, CMV-*Cucumber mosaic virus*, RCNMV-*Red clover necrotic mosaic virus*, RpSRV-*Raspberry ringspot virus*, TMV-*Tobacco mosaic virus*, TRV-*Tobacco rattle virus*, TSWV, *Tomato spotted wilt virus*.
[b]not applicable.

foundation for later studies that involved the exchange of genetic information between infectious virus clones.

Identification of plant virus avirulence genes in infectious virus clones

The nucleotide sequences of hundreds of plant viruses have been determined, and in many instances, an infectious clone has been developed for one or more members of a plant virus genus. With the development of infectious clones, gene-swapping experiments have been devised to identify which viral gene product triggers resistance and even to pinpoint the nucleotides within the gene that are responsible for triggering resistance. The first virus *Avr* gene identified in this manner was P6 of *Cauliflower mosaic virus* (CaMV), the type member of the caulimovirus group. The genome of CaMV consists of circular, double-stranded (ds) DNA approximately 8000 bp in length. It was the first plant virus genome to be completely sequenced (Franck et al. 1980; Gardner et al. 1981), and the first plant virus to be cloned in infectious form (Howell et al. 1980; Lebeurier et al. 1980). It is fairly simple to initiate infections in turnip leaves from cloned, viral DNA, as the full length viral DNA can be released from the plasmid vector through digestion with the appropriate restriction enzyme. This mixture is applied to a glass rod and then gently rubbed onto turnip leaves to introduce the viral DNA into the plant cell (Howell et al. 1980; Lebeurier et al. 1980). Once inside, the viral DNA is re-circularized by plant DNA ligases, and the infection ensues. To identify P6 as an Avr determinant, DNA segments delimited by common restriction enzyme sites were swapped between CaMV strains D4 and CM1841 to construct a series of reciprocal chimeric viruses. The inoculation of the chimeric viruses to solanaceous hosts revealed that P6 of CM1841 was responsible for eliciting the HR in *Datura stramonium* and a non-necrotic resistance response in *Nicotiana bigelovii* (Daubert et al. 1984; Schoelz et al. 1986). In contrast, chimeric viruses that contained P6 of D4 induced a systemic mosaic in both hosts.

As infectious clones were developed for other RNA and DNA virus groups, one priority was to determine which viral gene products triggers resistance. Plant viruses have very small genome sizes; most have the capacity to encode anywhere from three to ten proteins to fulfill the requirements for replication, gene expression, cell-to-cell movement and encapsidation. To date, every viral gene that has been shown to be an *Avr* gene has always had an essential role in the viral disease cycle. Table 2 lists some well-characterized viral *Avr* genes as well as the hosts that contain resistance genes. Although in many cases, the viral coat protein has been found to elicit the resistance response, it is generally accepted that virtually any viral gene product may trigger resistance in plants (Culver, 1997). This observation is underscored in Table 2, as viral proteins involved in genome expression, replication, movement and encapsidation have all been identified as *Avr* proteins.

Although gene-swapping experiments between infectious clones might be considered the primary method for the identification of viral *Avr* genes, other techniques have also proven valuable to identify viral *Avr* genes and to probe their function. For example, virulent and avirulent forms of the TMV coat protein gene have been expressed in transgenic plants to show that viral avirulence genes can elicit the HR independently of the viral genome (Culver and Dawson, 1991; Pfitzner and Pfitzner, 1992). In contrast, transgenic plants that expressed the virulent form of the TMV coat protein developed normally. The study by Culver and Dawson (1991) also highlighted that viral *Avr* genes could be characterized as "weak elicitors" and "strong elicitors". TMV variants that acted as weak elicitors evoked a slow developing HR in *N. sylvestris*, and transgenic plants that expressed this coat protein variant became stunted and chlorotic. TMV variants that acted as strong elicitors evoked a rapid HR in *N. sylvestris*; transgenic plants that expressed this coat protein variant developed large necrotic patches and eventually died. The authors suggested that TMV coat protein variants that acted as strong elicitors might have a stronger affinity for the product of the *N'* gene than the coat proteins that acted as weak elicitors.

Agroinfiltration provides a more rapid alternative to screen for *Avr* genes relative to the development of transgenic plants. In this technique, a putative *Avr* gene is placed under the control of a constitutive promoter and into the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*. Expression of this gene is achieved when *Agrobacterium* containing this Ti plasmid is treated with acetosyringone and 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 has been shown to elicit the HR upon agroinfiltration into tobacco varieties that contain the *N* gene (Abbink et al. 1998; Erickson et al. 1999), and the coat protein of *Potato virus X* (PVX) elicited the HR upon agroinfiltration into potato plants that contain the *Rx* resistance gene (Bendahmane et al. 1999).

Agroinfiltration has two advantages over the inoculation with viruses. First, it allows for the identification of an *Avr* gene when no resistance breaking strain of the virus is available. Second, it is possible to probe the structure of *Avr* genes in a manner that would not be possible in the context of the viral genome. For example, agroinfiltration was used to show that the helicase domain of the TMV 126/183 kDa replicase protein was responsible for eliciting the HR in N-gene tobacco (Abbink et al. 1998; Abbink et al. 2001; Erickson et al. 1999). This experiment could not have been done in the context of the viral genome, as the deletions would have destroyed its infectivity.

In addition, viral *Avr* genes may also be expressed from an unrelated virus vector. Scholthof and coworkers (1995) utilized a PVX vector to identify *Avr* genes in the genome of *Tomato bushy stunt virus* (TBSV). They found that p19 of TBSV elicits a systemic cell death symptom in *N. clevelandii* and the HR in *N. tabacum,* whereas the TBSV p22 protein elicits the HR in *N. edwardsonii*. They also could use this system to dissect the functions of Avr proteins from other functions associated with either p19 or p22. Mutations in the N-terminal portion of p19 abolished the elicitation of cell death in both *N. clevelandii* and *N. tabacum*, but the resistance pathway remained intact in *N. tabacum* against the mutant viruses (Chu et al. 2000). In contrast, mutations in p22 that abolished the HR in *N. edwardsonii* also influenced systemic movement, an indication that cell death was important for effective defense in this host/virus combination (Chu et al. 1999).

Structure/function studies of viral Avr gene products

Through the years, numerous *Avr* genes have been identified in viral, bacterial and fungal pathogens, and many excellent reviews have been written on the subject (Alfano and Collmer, 2004; Culver, 1997; Culver, 2002; Gabriel, 1999; Lauge and De Witt, 1998; Staskawicz et al. 2001; White et al. 2000). As *Avr* genes have been identified for each pathogen group, similar types of research questions have emerged. For example, what are the biochemical functions of *Avr* gene products? What roles do *Avr* gene products play in pathogenicity? And, how are *Avr* gene products recognized by the host?

It is instructive at this point to compare what has been learned about bacterial *Avr* genes in order to illustrate the contributions viral *Avr* genes have made to this area of research. Until recently, little was known about the functions of the bacterial *Avr* gene products, but several studies have now shown that many of them are types of bacterial 'effector' proteins injected into plant cells through a type III secretion system (Alfano and Collmer, 2004). By convention, bacterial *Avr* genes are recognized by host *R* genes, and this recognition event triggers an active defense response. For many years there was no evidence that they contributed anything of value to the bacterium, and the mystery was why bacteria would retain them as part of their genome (Gabriel, 1999). However there is now good evidence that in the absence of recognition, bacterial *Avr* gene products can function as virulence factors or "effectors" (Alfano and Collmer, 2004). Effectors promote disease through suppression of plant defenses or by contributing to growth of the bacteria in the host. The discovery of many effectors has been facilitated through the completion of genome sequences of bacteria such as *Ralstonia solanacearum* (Salanoubat et al. 2002), *Xanthomonas* species (Da

Silva et al., 2002), and *Pseudomonas syringae* (Fouts et al. 2002). Genome sequencing projects have also facilitated the discovery of the enzymatic activities of several effector proteins, as well as key elements of the type III secretion system. Bacterial effectors are thought to act in concert to cause disease, and perhaps not too surprisingly, they are also considered to have multiple functions (Alfano and Collmer, 2004).

In comparison, the characterization of viral *Avr* genes has had some built-in advantages. For example, the small genome sizes of plant viruses have made it relatively easier to identify functions for each of the genes in a viral genome, in addition to their role in avirulence. In many instances, we have moved beyond the understanding of a single function for viral proteins, as multiple functions have now been identified for many virus gene products. Furthermore, it is now well accepted that any viral protein can have a role as an *Avr* gene product. In the sections below, I discuss what is known about the functions of several well-characterized virus avirulence genes.

The coat protein of Tobacco mosaic virus

The TMV coat protein is recognized by the *N'* gene of *N. sylvestris* (Knorr and Dawson, 1988; Saito et al. 1987), and the *L* gene in pepper (Berzal-Herranz et al. 1995), as well as by as-yet uncharacterized *R* genes in other hosts such as eggplant (Dardick and Culver, 1997). Of all pathogen *Avr* gene products that have been identified, the coat protein of TMV has been the best characterized at the structural level. Not only has its three dimensional structure been solved (Bhyravbhatla et al. 1998; Bloomer et al. 1978; Namba et al. 1989), there is also extensive information on how it self aggregates to form virions (Butler, 1984; Butler, 1999), and how virions disassemble upon entry into the host (Wilson, 1984). In addition to its role in encapsidation, at least two other functions are associated with the TMV coat protein. It is necessary for systemic movement of the virus through the vascular system (reviewed in Derrick and Nelson, 1999; and Chapter A14) and it also plays an essential role in the phenomenon of cross protection by inhibiting the disassembly of the challenge virus (Sherwood and Fulton, 1982).

Through a detailed structural analysis, Culver and collaborators have characterized the molecular features of the coat protein that are recognized by the N' protein. It consists of a central hydrophobic core surrounded by polar and charged residues; amino acids on the right face of the coat protein molecule are thought to comprise the binding site for the N' protein (reviewed in Culver, 2002). The same basic structure is also recognized by *R* gene products in pepper and eggplant (Dardick et al. 1999). Surprisingly,

both virulent and avirulent forms of the viral coat protein contain the same sequence. In fact, this structure is highly conserved among tobamoviruses, and mutations that affect recognition by the N' protein also affect the ability of the virus to assemble into virions (Taraporewala and Culver, 1996; Taraporewala and Culver, 1997).

So, how do virulent TMV strains avoid recognition by the host? In this scenario, the coat protein subunits are aggregated in a manner such that the HR-determinant is not accessible to the product of the *N'* gene. Consequently, the amino acid mutations that converted a virulent TMV strain to an avirulent form were responsible for subtle changes in structure that exposed the recognition site. Neither the whole virion nor the coat protein monomer appear to be recognized by the N' protein (Culver et al. 1994; Toedt et al. 1999). Instead, recognition may reside in the quaternary structure, the formation of coat protein dimers, trimers and tetramers. Furthermore, small shifts in the ratios of these lower order aggregates can be the deciding factor between recognition and subsequent elicitation of the HR versus the evasion of host defenses and development of a systemic infection (Toedt et al. 1999).

The coat protein of TMV has also been useful for illustrating that pathogen *Avr* gene products have a significant role in causing disease, as the TMV coat protein has been implicated in the development of chlorotic and necrotic symptoms. It is not known exactly how the coat protein induces chlorosis, but mutations at specific amino acids in the coat protein can modulate the severity of this symptom. For example, a point mutation that altered amino acid 19 in the coat protein resulted in a TMV variant that induces bright yellow chlorosis (Banerjee et al. 1995). The evidence indicates that TMV may induce chlorosis via several mechanisms, but the common thread is that chloroplast functions are impaired. Several studies have shown that the TMV coat protein is imported into the chloroplast, where it becomes associated with the thylakoid membrane (Banerjee et al. 1995; Hodgson et al. 1989; Reinero and Beachy, 1989). Reinero and Beachy (1989) found that chloroplasts from plants infected with a severe strain of TMV contained significantly higher levels of coat protein than chloroplasts of plants infected with milder strains. Furthermore, the TMV coat protein inhibits electron transport in the photosystem II (PSII) complex (Hodgson et al. 1989). However, other TMV coat protein mutants induce chlorosis and cause significant degradation of chloroplasts, but do not accumulate in the chloroplast (Lindbeck et al. 1991; Lindbeck et al. 1992). These authors hypothesized that the TMV coat protein might interfere with the synthesis of nuclear encoded chloroplast proteins or their translocation into the chloroplast, thereby affecting chloroplast structure. In a third variation, Lehto and coworkers (2003) have suggested that the coat protein

of the flavum strain of TMV triggers the destruction of the PSII complex and a corresponding increase in reactive oxygen species. This mechanism was specific to the flavum strain, as other TMV strains did not have an affect on the PSII protein levels.

The TMV coat protein may induce a systemic cell death symptom through at least two mechanisms. The first mechanism might be considered a failure of containment after the induction of the HR. Culver and Dawson (1989) found that some mutations in the coat protein of TMV could alter its recognition by the product of the *N'* gene, converting an HR to a spreading, systemic cell death symptom. However, several other coat protein mutants have been described that induce necrosis, but the underlying mechanisms remain obscure. For example, some coat protein deletion mutants elicit local necrotic lesions in the absence of any known resistance gene (Dawson et al. 1988; Saito et al. 1989). In addition to deletions within the coat protein, some insertions of foreign sequences into the TMV coat protein may also unexpectedly elicit necrotic local lesions. TMV vectors that displayed epitopes of hepatitis and rabies viruses on the outer surface of the coat protein triggered necrotic local lesions in a tobacco variety that did not carry any known resistance to TMV (Bendahmane et al. 1999). It could be that alterations in TMV coat protein structure reveal some interaction with a previously unknown *R* gene that is common to many different plants. However, Bendahmane et al. (1999) suggest that these alterations in coat protein structure may be destabilizing cellular membranes, and this would trigger host cell death.

The P6 protein of Cauliflower mosaic virus

The P6 protein of CaMV is recognized by resistance genes in *D. stramonium, N. bigelovii, N. glutinosa*, *N. edwardsonii,* and *Arabidopsis thaliana* ecotype Tsu-0 (Agama et al. 2002; Daubert et al. 1984; Király et al. 1999; Schoelz et al. 1986; Wintermantel et al. 1993), albeit none of these *R* genes have been characterized genetically yet. Examinations of P6 of CaMV have contributed to the concept that viral *Avr* gene products have multiple roles in the viral infection cycle, and that viral *Avr* gene products can promote disease. The primary role of P6 is to modify the host translation machinery to facilitate the translation of the polycistronic CaMV 35S RNA, described as the translational transactivator (TAV) function (Bonneville et al. 1989; Fütterer and Hohn, 1991; Gowda et al. 1989; Scholthof et al. 1992). It is not known exactly how P6 facilitates translation of complex mRNAs, although recent evidence indicates that it interacts with ribosomes to allow reinitiation of translation of polycistronic messages (Bureau et al. 2004; Leh et al. 2000; Park et al. 2001). The discovery of the TAV function of P6 has

illustrated how a complex, polycistronic mRNA such as the CaMV 35S RNA could be translated after it is transcribed in plant cells. CaMV P6 has been shown to transactivate the expression of reporter genes in plant protoplasts derived from host and non-host plants (Bonneville et al. 1989; Gowda et al. 1989), in transgenic plants (Zijlstra and Hohn, 1992), in the yeast, *Saccharomyces cerevisiae* (Sha et al. 1995), and in *in vitro* systems (Ranu et al. 1996).

P6 also self aggregates to form the amorphous inclusion bodies seen in infected cells (Li and Leisner, 2002). These inclusion bodies may be considered pathogen organelles, as they are thought to serve as the sites for replication of the viral nucleic acid, as well as translation of the 35S RNA and assembly of the virions. P6 may contribute to long distance movement of the virus (Schoelz et al. 1991). Additionally, recent evidence indicates that it is essential for replication of the virus (Kobayashi and Hohn, 2003; Kobayashi and Hohn, 2004).

Considerable effort is now being directed towards teasing apart the various functions ascribed to P6. Given that one function of CaMV P6 is to regulate the translation of other genes, there had been a possibility that the avirulence phenotype associated with P6 of strains such as W260 might actually be attributed to altered expression of either a different CaMV gene or a host gene. However, the elicitation of the HR can be uncoupled from the TAV function of P6 (Palanichelvam and Schoelz, 2002), which indicates that the interaction of P6 with ribosomes might not contribute directly to pathogenesis or avirulence. Interestingly, the Avr function of P6 is very sensitive to any perturbations in amino acid sequence. A deletion of only 10 amino acids on the amino-terminus, or 39 amino acids on the carboxy-terminus, completely abolishes the avirulence function of P6, whereas TAV function was unaffected by these deletions (Palanichelvam and Schoelz, 2002). This indicates that maintenance of the three-dimensional structure of P6 must be critical for recognition by the host resistance gene product. It is not known if the self aggregation properties of P6 contribute to its function as an *Avr* gene product.

CaMV P6 also acts as a symptom determinant in several hosts. It has been shown to play a key role in the formation of chlorotic symptoms in turnips (Daubert et al. 1984; Stratford and Covey, 1989). This virulence function was first associated with P6 through gene-swapping experiments, but it was confirmed when P6 was transformed into several species of plants, and in most cases, they exhibited virus-like symptoms (Bálasz, 1990; Baughman et al. 1988; Goldberg et al. 1991; Schoelz et al. 1991; Takahashi et al. 1989). Transgenic *Arabidopsis* plants that express P6 have emerged as one host that can be used to investigate the development of chlorotic symptoms (Geri et al. 1999; Yu et al. 2003; Zijlstra and Hohn, 1992). Geri

and coworkers (1999) used differential display techniques to compare the changes in gene expression that occur in transgenic plants that express P6 to those that occur in virus infections. Although there were some differences in the expression patterns of the two types of plants, there were also many similarities. Geri et al. (1999) concluded that transgenic plants that express CaMV P6 could serve as a model system to investigate symptom development.

CaMV P6 is also responsible for triggering systemic cell death in *N. clevelandii*, as well as a non-necrotic resistance response in *N. glutinosa*, and the HR in *N. edwardsonii* (Király et al. 1999; Palanichelvam et al. 2000). Cole et al. (2001) hypothesized that the interaction between CaMV and *Nicotiana* species may represent a variation of a gene-for-gene system in which the HR is conditioned by the interaction of a single viral protein, CaMV P6, with two host gene products; one host gene product may trigger resistance, whereas the other may regulate the development of cell death. If the gene conditioning cell death is present and the resistance gene product is absent, as with *N. clevelandii*, then the outcome is systemic cell death. On the other hand, if both of these genes are present in the plant, then the outcome is an HR.

The coat protein of Turnip crinkle virus (TCV)

The coat protein of TCV is recognized by the *HRT* gene of *Arabidopsis thaliana* (Dempsey et al. 1997; Oh et al. 1995). The TCV-coat protein/*HRT* model system is valuable because it has provided new insights into how host *R* gene products may physically recognize the cognate *Avr* gene products. In the most straightforward model for gene-for-gene resistance, *Avr* and *R* gene products would be expected to physically interact, and this would set in motion the plant defense response (Flor, 1971). However, this physical interaction has only been demonstrated in a few cases: the *Pto* gene of tomato, which recognizes *AvrPto* or *AvrPtoB* in *Pseudomonas syringae* (Tang et al. 1996); the *Pita* gene in rice, which recognizes *AVR-Pita* in *Magnaporthe grisea* (Jia et al. 2000); and the *RPS2* gene in *Arabidopsis thaliana*, which recognizes *AvrRpt2* in *P. syringae* (Leister and Katagiri, 2000).

The apparent absence of a physical interaction between most *R* gene products with *Avr* gene products has led to the development of alternative models, such as the guard hypothesis (Dangl and Jones, 2001; van der Biezen and Jones, 1998). This model was originally formulated to explain the requirement of the tomato gene *Prf*, in addition to *Pto*, for the activation of host defenses against *P. syringae*. In this model, the bacterial effector *AvrPto* interacts with *Pto* to promote disease or perhaps to suppress basal

plant defenses. *Prf* has the capacity to recognize or stabilize this interaction, and consequently plays an essential role in the activation of plant defenses. *Prf* is thought to guard against the interaction of *Pto* and *AvrPto*.

Similar to the situation seen with *Pto, AvrPto*, and *Prf* a third *Arabidopsis* protein is required to activate resistance against TCV, in addition to the TCV coat protein and *HRT*. This protein, designated *TIP* (for TCV-interacting protein), is a member of a family of proteins that regulate the development of plant embryos and flowers (Ren et al. 2000). *TIP* is thought to be a plant transcription factor, and it is expressed in TCV-resistant and TCV-susceptible *Arabidopsis* ecotypes (Ren et al. 2000), so it is distinctly different from what would be considered a typical *R* gene. In contrast, *HRT* has been cloned and its protein sequence contains the nucleotide binding site and leucine-rich repeat motifs that are characteristic of many other *R* genes (Cooley et al. 2000). The TCV coat protein physically interacts with *TIP*, and mutations in the coat protein that disrupt this interaction also block the resistance response (Ren et al. 2000).

Furthermore, a recent study has shown that the TCV coat protein blocks the import of *TIP* into the nucleus (Ren et al. 2005). Consistent with the guard hypothesis, it has been hypothesized that *TIP* may have a role in the activation of basal defenses against TCV. The interaction of the TCV coat protein would prevent this activation of basal defenses by blocking the movement of *TIP* into the nucleus. *Arabidopsis* has evolved a counter-counter-defense mechanism in the form of the *HRT* protein, which has the capacity to recognize and guard against the formation of the TCV-CP/*TIP* complex; this recognition event sets in motion plant defenses mediated through a hypersensitive response (Ren et al. 2005).

The TCV coat protein also has recently been shown to suppress posttranscriptional gene silencing (PTGS) (Qu et al. 2002). Consequently, it was speculated that *TIP* might be a necessary component of the PTGS machinery, and that the TCV coat protein might interfere with this function (Choi et al. 2004). This hypothesis was attractive because it would confirm that *TIP* contributed to host basal defenses and would explain how the TCV coat protein is able to block these defenses. However, coat protein mutants that could not interact with *TIP* retained the ability to suppress RNA silencing (Choi et al. 2004). The authors concluded that the ability of the TCV coat protein to interact with *TIP* was unrelated to its role as a silencing suppressor. It does confirm, though, that plant virus proteins have multiple, and possibly independent, roles in promoting infections, eliciting symptoms, triggering plant defenses, and suppressing plant defenses.

The replicase protein of Tobacco mosaic virus

The helicase domain of the TMV 126/183-kDa replicase proteins is recognized by the *N* gene of *N. glutinosa*, and this interaction results in the development of a hypersensitive response (Abbink et al. 1998; Erickson et al. 1999; Holmes, 1938; Padgett and Beachy, 1993; Padgett et al. 1997). Two experimental approaches have been used to show that the helicase domain of the replicase is the Avr determinant. Padgett and coworkers (1997) exchanged domains between infectious clones of virulent and avirulent TMV isolates, whereas other groups expressed TMV genes individually by agroinfiltration to identify the helicase domain of the replicase as the HR determinant (Abbink et al. 1998; Erickson et al. 1999). Helicases participate in the replication of the viral nucleic acid by promoting the unwinding of duplex nucleic acids (Hull, 2002; Kadarei and Haenni, 1997). Since this process is energy dependent, requiring the hydrolysis of ATP, Erickson et al. (1999) investigated whether the ATPase activity of the helicase contributed in some way to its role as an Avr protein. They found that the two functions acted independently of each other; a helicase that lacked ATPase activity could still elicit the HR in an agroinfiltration assay.

The *N* gene was one of the first resistance genes to be cloned (Whitham et al. 1994). Since the corresponding *Avr* gene product has also been identified, this system has provided an opportunity to investigate how resistance proteins recognize Avr proteins. However, studies completed to date have failed to indicate how recognition of the helicase domain is mediated. Yeast two-hybrid screens have yielded several host proteins that interact with the helicase domain of TMV, but none of the proteins identified appear to have a role in recognition by the *N* gene product. For example, Abbink et al. (2002) identified two host proteins that each physically interacted with the TMV helicase domain; one belonged to a family of ATPases, whereas the other was a 33-kDa subunit of the oxygen-evolving complex of photosystem II. To investigate the role of these genes in TMV infections, they were silenced in *N. benthamiana* and in transgenic *N. benthamiana* that expressed the *N* gene. Silencing of either the ATPase or the photosystem II subunit had no effect on *N* gene function; these plants remained resistant to TMV. Interestingly, silencing of these proteins in susceptible *N. benthamiana* had an effect on viral titer. Silencing of the ATPase resulted in a modest decrease in the titer of TMV, whereas silencing of the photosystem II subunit resulted in a 10-fold increase in TMV titer (Abbink et al. 2002).

Bilgin et al. (2003) identified a third protein that physically interacts with the TMV helicase domain, the double-stranded RNA-dependent protein kinase inhibitor $P58^{IPK}$. Interestingly, silencing of $P58^{IPK}$ in *N. benthamiana*

resulted in the death of the plant upon infection by TMV or *Tobacco etch virus* (Bilgin et al. 2003). Similarly, plant virus infections killed *Arabidopsis* knockouts that lack $P58^{IPK}$. However, $P58^{IPK}$ was not involved in mediating virus resistance, as silencing of the $P58^{IPK}$ transcript had no effect on *N*-mediated resistance to TMV in transgenic *N. benthamiana* plants that express the *N* gene product. The authors also showed that loss of $P58^{IPK}$ had a negative effect on viral titer, and they concluded that it might function as a susceptibility factor.

A fourth protein that interacts with the TMV helicase domain has been identified through a mutant hunt in *Arabidopsis*. In contrast to the other three genes, this gene, designated *TOM1*, was isolated through a map based cloning strategy and subsequently shown to physically interact with the helicase domain (Yamanaka et al. 2000). Mutations in *TOM1* suppress the replication of TMV in single cells, but have no effect on the replication of other viruses, such as CMV, TCV, or *Turnip yellow mosaic virus*. The authors showed that the *TOM1* protein is associated with membranes and they proposed that it functions in the TMV replication complex by anchoring the complex to host membranes. The discovery of this particular role for a host protein is satisfying because it is well established that the TMV replicase complex could only function in association with host membranes (Heinlein et al. 1998). Homologs of *TOM1* can be found in tobacco, but there is no evidence at this time for an involvement of *TOM1* in the recognition of TMV by the *N* gene product.

As a group, the four proteins that interact with the TMV helicase domain are intriguing because they illustrate the complexity of interactions that can occur between host and virus. Each of these proteins has been shown to physically associate with the helicase domain of the TMV replicase proteins, and each has been shown to influence the titer of TMV. Yet none of them appear to participate in recognition events by the *N* product that result in the HR. If there is a bridge protein between *N* and the helicase domain, it remains to be found.

The 126/183-kDa replicase proteins of TMV have also been shown to influence symptoms and phloem dependent movement of the virus. The attenuated "masked" strain of TMV elicits very mild symptoms in tobacco (Holmes, 1934). Gene-swapping experiments conducted between the M strain and the more severe U1 strain showed that the mild symptoms and effects on phloem-dependent accumulation could be attributed to eight amino acids in the 126/183-kDa replicase proteins (Bao et al. 1996; Derrick et al. 1997; Shintaku et al. 1996). New studies now indicate that the 126-kDa protein is a suppressor of RNA silencing, and that suppression is correlated with severity of symptoms (Ding et al. 2004; Meshi et al. 2003). These studies further underscore that viral proteins that act as *Avr* gene

products have the capacity to suppress host defenses, as well as facilitate virus infections and contribute to symptom development.

Identification of divergent Avr genes by R gene homologs

As host *R* genes have been cloned and the cognate pathogen *Avr* genes identified, it has been possible to begin to examine the issue of specificity; in particular, how do *R* gene products of similar sequence and structure recognize different *Avr* gene products? The three examples below illustrate how closely related *R* genes can recognize *Avr* gene products that differ by only a few amino acids, as well as *Avr* gene products from widely divergent pathogen groups.

The potato *R* genes *Rx1* and *Rx2* both recognize the same domain of the PVX coat protein (Bendahmane et al. 1995; Querci et al. 1995) (Table 2). Two amino acids in the PVX coat protein, located at positions 121 and 127, are involved in breaking the resistance specified by *Rx1* and *Rx2*. Consequently, it was considered that *Rx1* and *Rx2* might have a high degree of similarity (Bendahmane et al. 2000; Querci et al. 1995). Although the *Rx1* and *Rx2* genes were considered to be homologs, they are not allelic, as *Rx1* is located on chromosome XII (Bendahmane et al. 1997), whereas *Rx2* is located on chromosome V (Ritter et al. 1991).

The *Rx1* gene was physically isolated before *Rx2*, through a map-based cloning strategy (Bendahmane et al. 1999). Based on the sequence of *Rx1*, a transient expression assay was then developed to identify and clone the *Rx2* gene. In this assay, *Rx* homologues were cloned into an *Agrobacterium* vector and infiltrated into transgenic tobacco that expressed the PVX coat protein. The *Rx* homologue that elicited the HR upon agroinfiltration into the transgenic tobacco was confirmed to be *Rx2* (Bendahmane et al. 2000). A sequence comparison between *Rx1* and *Rx2* revealed a close evolutionary relationship that the two *R* genes may have evolved through repeated sequence exchanges between these unlinked loci. Interestingly, amino acid 121 in the PVX coat protein also plays a critical role in recognition by an unspecified *R* gene in *Gomphrena globosa* (Goulden and Baulcombe, 1993). The implication is that this *R* gene may have homology to *Rx1* and *Rx2*.

Host *R* genes that have a high degree of homology may also recognize viral *Avr* genes that have similar functions but share very little or no sequence similarity. This can be seen in comparing the *Arabidopsis R* gene *HRT*, which recognizes the coat protein of TCV (Oh et al. 1995), to the *Arabidopsis R* gene *RCY1*, which recognizes the coat protein of CMV (Takahashi et al. 2001) (Table 2). *HRT* and *RCY1* are allelic and their predicted amino acid sequences differ by only 8.7% (Cooley et al. 2000; Takahashi et al. 2002), yet the coat proteins of TCV and CMV have no

similarity at the amino acid level. Takahashi et al (2002) suggests that *RCY1* and *HRT* may have evolved to recognize completely different sequences within viral coat proteins, or alternatively, specificity might reside in the three dimensional structure of the coat proteins. The *HRT* and *RCY1* genes are themselves part of a cluster of related *R* genes that include *RPP8*, a gene that conditions resistance to *Peronospora parasitica* (McDowell et al. 1998). The predicted amino acid sequence of *RCY1* differs by only 8.0% from that of *RPP8*, yet it would be very surprising if their respective *Avr* gene products had any similarity at the primary amino acid level.

Finally, host *R* genes that have similarities in sequence may recognize *Avr* genes from completely different pathogen groups. The tomato *R* gene *SW-5*, which conditions resistance to *Tomato spotted wilt virus* (TSWV), is considered a homolog of the tomato gene *Mi*, which conditions resistance to Root-knot nematode (Brommonschenkel et al. 2000; Milligan et al. 1998). The degree of similarity between *Mi* and *Sw-5* (30% identity in predicted amino acid sequence) is not nearly as close as the relationship between *HRT* and *RCY1*, but Brommonschenkel and coworkers (2000) speculate that the two *R* genes might share a common signal transduction pathway. The avirulence determinant of TSWV has been mapped to the M RNA segment (Table 1) (Hoffmann et al. 2001), which encodes a 34-kDa protein involved in cell-to-cell movement and a 127-kDa protein that is processed into two polypeptides that form the spikes on the surface of the virus particles (Hull, 2002; Moyer, 1999). In contrast, the avirulence determinant in the root-knot nematode has not yet been identified.

At present, only a handful of viral *Avr-R* gene combinations exist in which both components have been cloned and sequenced, largely because the identification of *R* genes has lagged behind the identification of viral *Avr* genes. However, as more plant genomes are sequenced and more tools are developed for their analysis, the pace of discovery of *R* genes will accelerate. Their discovery holds the promise for unlocking the mystery of *Avr-R* gene specificity.

The interaction of the Potyvirus genome-linked protein (VPg) with eukaryotic translation factors: a model for recessive resistance

Many of the dominant *R* genes function as part of a surveillance system in the host to recognize specific pathogen *Avr* genes, and this recognition event activates a large number of plant defense pathways, which collectively protect the plant from infection. However, some recessive *r* genes may function in a different manner; they may contain defects such that the host cellular machinery is unable to provide functions essential for virus

replication and spread. One example that illustrates this type of resistance can be found in the interaction of potyvirus VPg proteins with their recessive host resistance genes (Table 2). Several recessive potyvirus resistance genes have recently been cloned from a variety of hosts, including *pvr2*^{1} and *pvr2*^{2} in pepper, *mo1* in lettuce, and *sbm1* in pea (Gao et al. 2004, Nicaise et al. 2003; Ruffel et al. 2002). These genes condition resistance to *Potato virus Y* (PVY), *Lettuce mosaic virus* (LMV) and *Pea seedborne mosaic virus* (PSbMV), respectively. Each of these host *r* genes were revealed to be eIF4E, a protein component of a complex involved in translation initiation of mRNAs. Furthermore *Arabidopsis* mutants that could not support the replication of *Tobacco etch virus* (TEV), *Turnip mosaic virus* (TuMV) and LMV were shown to have defects in the mRNA cap-binding protein eIF(iso)4E, another protein that participates in translation initiation (Lellis et al. 2002; Duprat et al. 2002).

On the virus side of this interaction, mutations in the potyvirus VPg have been shown to be responsible for overcoming recessive *r* gene resistance from many different hosts (Table 2). The VPg protein has been considered to participate in translation of the viral genome and to also influence cell-to-cell and long distance movement of the virus (Revers et al. 1999). In contrast to studies involving dominant *R* genes, physical interactions between potyvirus VPg and host translation initiation factors have been readily detected. For example, the VPg of TuMV has been shown to bind to eIF4E and eIF(iso)4E of *A. thaliana* (Léonard et al. 2000; Léonard et al. 2004; Wittmann et al. 1997), and the VPg of TEV binds to eIF4E from tomato or tobacco (Schaad et al. 2000). Léonard and coworkers (2000) also showed that mutations in the TuMV VPg that disrupt the interaction with eIF(iso)4E abolish the infectivity of TuMV in *Brassica perviridis*. However, protein-protein interactions have not been observed in all cases, as an interaction has not been found between the VPg of PSbMV and pea eIF4E (Gao et al. 2004).

A direct protein-protein interaction between VPg and host translation factors could facilitate the understanding for how recessive resistance is mediated. One attractive feature of this host-virus system is that a large number of recessive potyvirus *r* genes have been characterized in a variety of hosts (Khetarpal et al. 1998), and indications are that many of these *r* genes will be components of the host translational apparatus. As more recessive anti-potyvirus *r* genes are cloned and their Avr counterpart identified, it should be possible to resolve the questions regarding how this class of Avr proteins is recognized.

Conclusions

Throughout this review I have sought to emphasize several themes that are common to the study of viral *Avr* gene products. Given the small genome size of viruses, it may not be surprising that virtually any type of viral protein may act in the capacity of an *Avr* gene product. A large number of viral proteins can be considered to function as *Avr* genes (Tables 1 and 2), and many more will likely be identified in the coming years. Viral *Avr* gene products have multiple roles in the infection cycle, both in facilitating virus infections and in eliciting disease symptoms. Recent studies have shown that some viral *Avr* gene products can also act as silencing suppressors, and there has been speculation that this suppressor function might be directly related to gene-for-gene-resistance. However in the one instance where this hypothesis has been investigated, the suppressor function of the TCV coat protein appears to be independent of its role in elicitation of the HR by HRT (Choi et al., 2004).

So, what does the future hold for the study of viral *Avr* genes? One challenge will be to gain a more thorough understanding of how viral *Avr* gene products are recognized by host resistance gene products. Clearly, the viral coat proteins have led the way, both in terms of the resolution of tertiary and quaternary structures, and in terms of the discovery of host proteins such as TIP, a host protein that bridges the interaction between the TCV coat protein and the HRT protein. Will other viral *Avr*/*R* gene combinations require a third interactor to facilitate recognition? Furthermore, plant viruses may be especially valuable for investigating the molecular basis for specificity, how host *R* genes are able to target a wide array of pathogens. With their small genome size, the *Avr* genes can be placed into a finite number of distinct categories: coat proteins, movement proteins, replicases, and genome expression proteins. For example, do diverse proteins within a category have features in common that allow them to be recognized by host *R* genes?

A second challenge will be to identify host gene products that directly interact with viral gene products for the development of symptoms, i.e. how they act as virulence factors. Many viral *Avr* gene products also influence the severity of systemic host symptoms, such as chlorosis or necrosis. In many instances, it will likely be found that within a viral protein, the symptom determinant is unrelated to its Avr determinant. However, it may be that some forms of systemic necrosis may actually represent a failure of gene-for-gene resistance. The identification and cloning of these *R* gene variants may yield a convergence of several themes that relate to how *R* genes recognize viral Avr gene products, how necrotic symptoms develop, and how plant defense signal transduction pathways are activated.

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Chapter A3

RNA Silencing: A Natural Resistance Mechanism in Plants

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Introduction

Over the last years RNA silencing in plants and its animal counterpart RNA interference (RNAi) have become intensively studied biological systems. While initially being discovered as a side effect of transgene expression in plants and a process by which transgenic virus resistance could be obtained, it has since been implicated in natural virus resistance and basic biological processes such as development, gene regulation and chromatin condensation. RNA silencing related mechanisms are not only limited to plants, but also play a role in a variety of eukaryotic organisms. Due to the biochemical dissection of components of the silencing pathway in several model organisms, such as *Arabidopsis thaliana*, *Caenorhabditis elegans* and *Drosophila melanogaster,* the general understanding of how RNA silencing works has greatly increased in recent years. The revelation of a striking level of conservation of the RNA silencing pathway between most eukaryotic organisms strengthens its importance. Nowadays, RNA silencing induced by double stranded RNA (dsRNA) molecules such as short hairpins, short interfering RNAs (siRNAs) and long dsRNAs has developed into a standard tool in gene function studies (gene knock-down). It is being applied in large automated genome screens, where a majority of genes of certain organisms (e.g. *C. elegans* and *Homo sapiens*) are knocked-down and analyzed using different assays depending on the research interests. In plants RNA silencing is used as a generally applicable antiviral strategy.

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In this chapter we will describe the RNA silencing process with emphasis on the functioning of the mechanisms and its role in natural virus infection in plants. In addition, applications of RNA silencing in plants and implications of RNA silencing for research in other organisms will be discussed.

The discovery of RNA silencing

The first recognized encounter with RNA silencing was when van der Krol, Napoli and their respective co-workers (Napoli 1990; van der Krol et al. 1990) reported their inability to over-express chalcone synthase (CHS) in transgenic petunia plants. In order to obtain an increase of flower pigmentation, petunia plants were transformed with the CHS gene using different constructs that should have led to over-expression. However, instead of observing an increase of flower pigmentation, the opposite effect was observed: some plants completely lacked pigmentation in the flowers and others showed patchy or reduced pigmentation. It was shown that even though an extra copy of the transgene was present, the CHS mRNA levels were strongly reduced in the white sectors. Since the transgene RNA was suppressing not only its own expression, but also the endogenous gene this observation was called 'co-suppression'.

Not much later, another encounter with RNA silencing was made in the field of virus resistance where the concept of pathogen-derived resistance (PDR) was being exploited to produce virus resistant plants. Using different viral systems, three reports demonstrated that in contrast to the original notion, the expression of viral proteins was not required for virus resistance, but untranslatable viral RNA sufficed (de Haan et al. 1992; Lindbo and Dougherty 1992; van der Vlugt et al. 1992). Since the virus resistance in the recovered plant parts correlated with reduction of transgene mRNA in the cytoplasm, Lindbo and co-workers (1993) proposed this phenomenon to be similar to co-suppression. The observation that a silenced GUS transgene could prevent virus accumulation of *Potato virus X* (PVX) carrying GUS sequences pointed toward an actual role of, what was then called post-transcriptional gene silencing (PTGS), as a sequence specific antiviral defense mechanism (English et al. 1996). Supporting evidence of the more general nature of this plant response to viral infection was provided by the finding that the recovered parts of virus infected plant would not only be resistant against the initially inoculated virus, but would also cross-protect the plant against other viruses carrying homologous sequences (Ratcliff et al. 1999). In addition, this work showed that viral RNA-mediated cross protection was caused by the same mechanism as transgene induced PTGS. These phenomena are now generally known as virus-induced gene silencing (VIGS). The identification of different *Arabidopsis* mutants exhibiting

impaired RNA silencing revealed more details about the mechanisms involved in this process (Elmayan et al. 1998; Dalmay et al. 2000). Certain mutants affected in the silencing pathway showed enhanced susceptibility to virus infection, confirming their involvement in antiviral activity (Dalmay et al. 2000; Mourrain et al. 2000). Over recent years, many components of the plant silencing pathways (Fig. 1) have now been uncovered and will be further discussed later in this chapter.

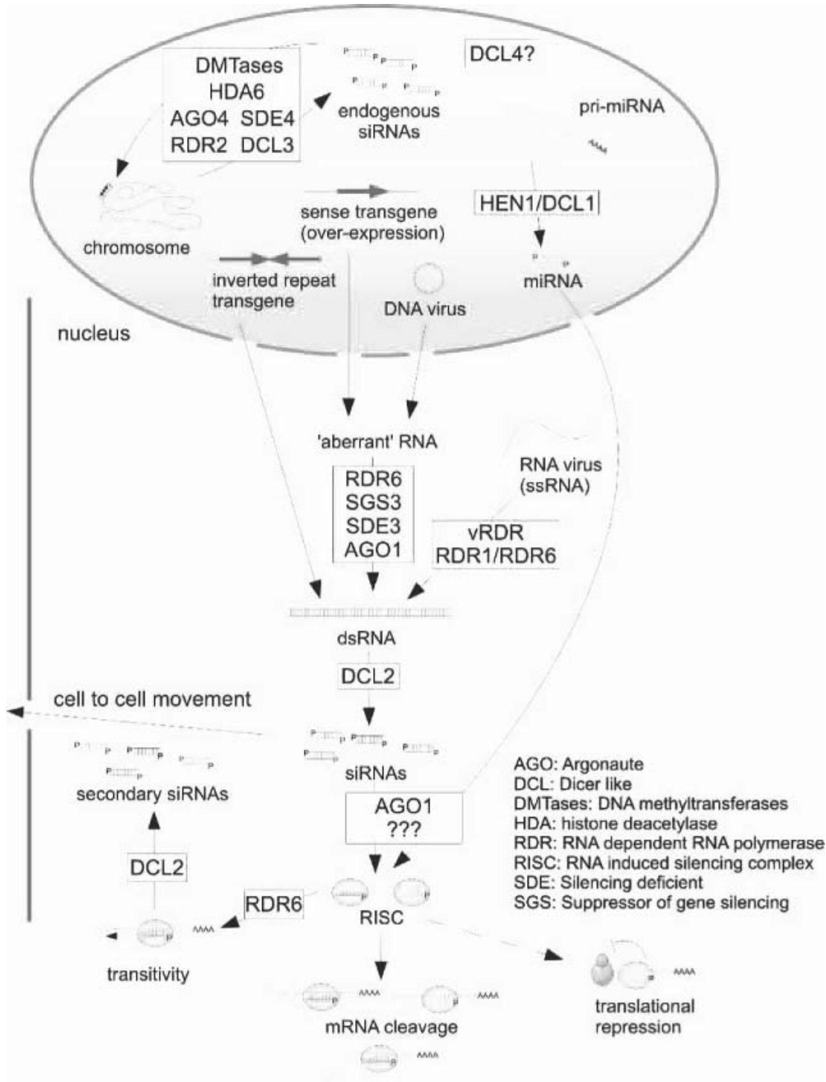

Fig. 1. A model of the RNA silencing pathways in plants. The squares indicate identified proteins or genes involved in the different silencing processes. ??? indicates the position of proteins associating with DICER such as R2D2 that have been identified in animals but not (yet) in plants.

Silencing of endogenous and viral genes has now become a commonly used method. Transgene constructs can be arranged as inverted repeats, producing double-stranded RNA (dsRNA), which efficiently trigger silencing of homologous genes (Smith et al. 2000). This can be used to obtain transgenic virus resistance or endogenous gene knock-down. For gene knock-down VIGS is often preferred to the production of transgenic plants, as this fast method can give a first indication on whether a gene knock-down produces the expected phenotype (reviewed in Lu et al. 2003).

To explain the extreme sequence specificity of the RNA silencing process, small RNA molecules had been envisaged in models throughout the second half of the nineties. However, it was not until 1999 that Hamilton and Baulcombe (1999) unequivocally proved that plants containing a silenced transgene indeed accumulated small (ds)RNA molecules whose sequence was identical to the transgene. They observed the same kind of approximately 25 bp sequence-specific small RNAs in PVX infected plants, suggesting a role of these molecules in a sequence specific antiviral defense mechanism. A further breakthrough pointing to the involvement of RNA silencing in antiviral defense was the discovery of virus specific RNA silencing suppressors (Anandalakshmi et al. 1998; Brigneti et al. 1998; Voinnet et al. 1999). This will be discussed in detail in subsequent paragraphs.

A next step to an increased general notion of RNA silencing was achieved in animal research. In *C. elegans* sense and anti-sense transcripts were already being used for quite some time to knock-down gene expression. However the real break-through came when Fire and co-workers (1998) discovered that injection of very low amounts of dsRNA into *C. elegans* could induce what they called RNAi. Like in plants, this method of RNA silencing was much more efficient than just using single-stranded sense or anti-sense RNA. Building blocks of the gene silencing pathway proved to have remarkable similarities in different organisms and hence suggest an ancient role of RNA silencing in development, gene regulation, pathogen resistance, and chromatin structure.

Mounting the plant antiviral defense

In plants, the control of virus replication is considered as one of the primary roles of RNA silencing. Although expressing viral transgene RNAs can precondition this response, the natural response is adaptive and requires recognition of ‘foreign’ molecules for initiation. This recognition is subsequently converted into ‘effector’, ‘memory’ and ‘warning’ signals to alert the systemic parts of the plant. DsRNA molecules have been shown to

be most potent initiators of RNA silencing (Smith et al. 2000). As most plant viruses are RNA viruses that replicate *via* double stranded replication intermediates, it is tempting to suggest that these molecules are a trigger for RNA silencing. This is, however, put too simply. Most, if not all plant RNA viruses may replicate via dsRNA. The chance that these RNAs appear as naked RNA in the cell is very small since replication complexes are protected by viral replication and/or capsid proteins. Viral replication often takes place inside specialized replication structures and dsRNA can immediately be unwound by viral and host RNA helicases (Ahlquist 2002). Though we do not dismiss the possibility of detection of these structures by RNA silencing, we think viral mRNAs, which might be recognized by the plant as being 'aberrant' (e.g. non-capped or non-polyadenylated mRNAs), are (also) an important target which can be converted into double stranded RNA by plant RNA-dependent RNA polymerases (RdRps). This would explain the generation of virus specific siRNAs in plants infected with geminiviruses (single-stranded DNA viruses) (Vanitharani et al. 2003).

The *Arabidopsis* genome encodes four Dicer-like enzymes that have the ability to process dsRNA into siRNA molecules (Schauer et al. 2002). In a normal virus infection, plants contain a significant amount of siRNAs originating from the virus (Hamilton and Baulcombe 1999). These siRNAs can subsequently be used in two ways: either they are unwound and one strand is incorporated into the RNA induced silencing complex (RISC) to target and degrade RNAs homologous to the siRNA, or a plant RdRp uses the siRNA as a primer on homologous mRNAs and synthesizes dsRNA that then is processed by Dicer into secondary siRNAs (a phenomenon called 'transitivity') (Vaistij et al. 2002). This latter step leads to the amplification of the intracellular silencing signal. In plants, RNA silencing generated secondary siRNAs can originate from 5' and 3' parts of the targeted site in the messenger, indicating that the transitivity is bidirectional. This is in contrast to *C. elegans* where secondary siRNAs only originate from the 5' side of the target mRNA in relation to the inducer molecule (Sijen et al. 2001). This may be related to the fact that both siRNA strands seem to be stable in plants (Hamilton and Baulcombe 1999), while in *C. elegans*, only the antisense strand is maintained. In mammals and insects, transitivity was reported not to be present. Indeed no endogenous RdRp, which would be required for this activity, has been identified (Schwarz et al. 2002). Next to the predominant 21 nt species of siRNAs observed in all eukaryotes, the plant silencing machinery has the unique ability to produce, a second size class of siRNAs, of around 24 nt (Hamilton et al. 2002). The longer class of siRNAs has been correlated with the long-distance spread of RNA silencing. This ability allows the viral siRNAs produced by the plant silencing

machinery to move to adjacent cells advancing the spread of the virus. RISC is thought to be pre-programmed with these siRNAs allowing an immediate recognition and elimination of incoming viruses. The shorter class of siRNAs is thought to operate in local RNA silencing (Hamilton et al. 2002). This size class has also been reported to be able to move from cell to cell, however, spreading no further than up to 15 cells (Himber et al. 2003).

Of great interest and confirming the biological role of RNA silencing in antiviral defense, was the discovery that nearly all plant viruses investigated so far encode RNA silencing suppressors. The interference of plant viruses with the RNA silencing machinery will be discussed in more detail in the next paragraph.

Suppressor proteins: Viral counter measures against RNA silencing

Even though an RNA-based sequence-specific defense against virus infection may be efficient, there are still many viruses that successfully infect plants. The discovery of viral RNA silencing suppressors gave a first hint on how viruses could counteract the plant defense. An indication that these counter measures were developed as an answer to RNA silencing is their great diversity. None of the RNA silencing suppressors discovered so far share any significant sequence homology with those from other viruses. In addition, the RNA silencing antagonists encoded by different plant viruses appear to suppress this virus defense pathway at different points.

It has long been known that certain proteins expressed by viruses played an important role in their virulence (Pruss et al. 1997). It was observed that co-infection of combinations of viruses could cause increased symptom severity compared to each of the viruses alone. These mixed infections indicated that at least one of the viruses possessed a character that could support the replication and spreading of the other virus. Potyviruses were reported early on to increase the virulence levels of another virus (see Chapter by Palukaitis and MacFarlane). The actual underlying mechanism started to become understood in studies of mixed infections of PVX with different potyviruses. Mutational analysis of the *Tobacco etch virus* (TEV) revealed that the helper component-proteinase (HC-Pro) was required for the synergistic activity of TEV (Shi et al. 1997). A first indication that HC-Pro could actually block a general plant antiviral pathway was found when transgenic plants constitutively expressing HC-Pro were produced. Heterologous viruses such as TMV and CMV showed enhanced accumulation and pathogenicity in these plants (Pruss et al. 1997). In the case of CMV, virulence could be linked to its 2b protein (Brigneti et al. 1998). These results were later confirmed by studies where the 2b gene of

different CMV subgroups were replaced (Shi et al. 2002). Indication that RNA silencing is indeed involved in virus resistance came with the reports that HC-Pro can enhance virulence of heterologous viruses by directly suppressing RNA silencing (Anandalakshmi et al. 1998; Brigneti et al. 1998; Kasschau and Carrington 2001).

Assays used to identify suppressors of RNA silencing

Following the discovery of HC-Pro as a suppressor of RNA silencing many other viruses were shown to express proteins capable of inhibiting this antiviral mechanism (Table 1). The establishment of relatively simple and reliable functional assays to detect suppressors of RNA silencing greatly accelerated their discovery.

Currently, one of the most commonly used methods for the identification of potential suppressors of RNA silencing is a transient assay using *Agrobacterium tumefaciens* (Voinnet et al. 2000; Llave et al. 2000; Johansen and Carrington 2001). In this assay, two *Agrobacterium* strains are used to deliver a reporter gene (often the gene encoding the green fluorescent protein, GFP) and a putative suppressor protein. The *Agrobacterium* culture mix is infiltrated into a *Nicotiana benthamiana* leaf and reporter gene expression is monitored. Typically, without a suppressor of RNA silencing, the reporter gene becomes silenced after three to five days. However, if an *Agrobacterium* strain carrying a strong suppressor of RNA silencing between the T-DNA borders is mixed with the ones carrying the reporter gene and co-infiltrated, the reporter gene expression will remain at its high level or even increase during the six days. Using different reporter constructs, such as genes arranged as inverted repeats, one has the possibility to assess at which step of RNA silencing the suppressor protein acts (Takeda et al. 2002).

Another method makes use of GFP or GUS (beta-glucoronidase) silenced transgenic plants. Plants expressing a reporter gene are systemically silenced by the infiltration of *Agrobacterium* expressing (a fragment of) the RNA of that reporter gene, or plants are genetically silenced (e.g. using inverted repeats). Subsequently, these plants are infected with different viruses or virus constructs and the reporter gene expression is monitored. Restoration of reporter gene expression indicates that the tested virus encodes a suppressor of RNA silencing. PVX encodes a suppressor of RNA silencing that cannot restore the reporter gene expression in this assay and makes it a good vector to test other viral genes for their silencing suppression capability (Brigneti et al. 1998). Additionally it has been

Table 1. Suppressors of RNA silencing of different plant viruses that have been identified so far. *Different results have been reported by different groups.

Genome	Genus	Virus	Suppressor protein	Suppressed RNA silencing mechanism	Reference
DNA	Begomovirus	ACMV	AC2	-	Voinnet, et al. 1999; Vanitharani, et al. 2004
			AC4	local	Vanitharani, et al. 2004
		TGMV	AL2	-	Wang, et al. 2003
		TYLCV-C	C2	local and systemic	Dong, et al. 2003; Van Wezel. et al. 2003
(+) RNA	Curtovirus	BCTV	L2	-	Wang, et al. 2003
	Carmovirus	TCV	CP (P38)	local	Thomas, et al. 2003; Qu, et al. 2003
	Closterovirus	BYV	p21	local	Reed, et al. 2003
		CTV	p20	local	Lu, et al. 2004
			p23	local and systemic	Lu, et al. 2004
			CP	systemic	Lu, et al. 2004
		BYSV	p22	local	Reed, et al. 2003
	Comovirus	CPMV	S coat protein	local	Voinnet, et al. 1999; Canizares; et al. 2004; Liu, et al. 2004
	Cucumoviruss	CMV	2b	local* and systemic	Brigneti, et al. 1998; Lucy et al. 2000
	Furovirus	PCV	P15	local and systemic	Dunoyer, et al. 2002
	Hordeivirus	BSMV	γb	-	Yelina, et al. 2002
	Polerovirus	BWYV	P0	local and not	Pfeffer, et al. 2002
	Potexvirus	PVX	P25	systemic	Voinnet, et al. 2000
	Potyvirus	PVY	HC-Pro	local and systemic*	Brigneti, et al. 1998; Anandalakshmi, et al. 1998
	Sobemovirus	RYMV	P1	-	Voinnet, et al. 1999
	Tobamovirus	TMV	126-kDa protein	-	Voinnet, et al. 1999 ;Ding, et al. 2004
		ToMV	130-kDa protein	local	Kubota, et al. 2003
	Tombusvirus	TBSV	P19	local and systemic (binds siRNAs)	Voinnet, et al. 1999; Lakatos, et al. 2004
			P19	local and systemic	Silhavy, et al. 2002
	Tymovirus	TYMV	p69	local	Chen, et al. 2004
(-) RNA	Tenuivirus	RHBV	NS3	local	Bucher, et al. 2003
	Tospovirus	TSWV	NS_S	local	Bucher. et al. 2003; Takeda, et al. 2002

observed that if PVX expresses a heterologous suppressor of RNA silencing it causes more severe symptoms compared to the empty vector (Pruss et al. 1997; Brigneti et al. 1998).

Finally, one can produce transgenic plants that constantly express a suppressor of RNA silencing. A significant drawback with this method is that (high) expression of suppressors of RNA silencing often leads to developmental defects in the plants (Anandalakshmi et al. 2000). Nevertheless, some successes have been reported (Kasschau et al. 2003; Chapman et al. 2004; Dunoyer et al. 2004).

RNA silencing suppressor proteins

Even though many viral suppressors of RNA silencing have been described so far (Table 1), extensive research was focused on a selection of these proteins.

HC-Pro of potyviruses

The first and best described suppressor of RNA silencing is the potyviral HC-Pro protein (Anandalakshmi et al. 1998). It was shown to suppress RNA silencing in experiments where plants, in which a reporter gene was silenced, were infected with PVX carrying HC-Pro. Upon systemic infection by this chimeric virus, reversal of the silenced state of the reporter gene was observed. Additionally, Anandalakshmi and co-workers (1998) showed that crossing a GUS silenced plant line and a HC-Pro expressing plant line could restore GUS expression. On the molecular level it was shown that HC-Pro prevented the degradation of the reporter gene mRNA (Anandalakshmi et al. 1998; Brigneti et al. 1998). Further analysis revealed that HC-Pro could prevent the degradation of the reporter mRNA into siRNAs (Hamilton et al. 2002). This means that HC-Pro could inhibit, for instance, an RNase III-like enzyme involved in the processing of dsRNA into the siRNAs or a component of the RNA silencing effector complex RISC. Interestingly, HC-Pro did not affect the silencing signal from moving through the plant, even though all siRNAs were eliminated (Mallory et al. 2001). However, HC-Pro was shown to efficiently prevent the plant from responding to the silencing signal in grafting experiments. It is noteworthy that Hamilton and co-workers (2002) reported that HC-Pro could interfere with the silencing signal. These conflicting observations could be a result of different assays

being used by the different groups (*Agrobacterium* infiltration versus grafting). Additionally, there are conflicting reports on whether or not HC-Pro affects the methylation of a silenced transgene locus in the plant genome (Llave et al. 2000; Mallory et al. 2001).

A first indication on how HC-Pro actually suppresses RNA silencing was shown by protein-protein interaction studies using the yeast two hybrid assay. Anandalakshmi and co-workers (2000) identified a calmodulin related protein rgs-CaM (regulator of gene silencing-calmodulin-like protein) that directly interacts with HC-Pro. In addition, its expression is up-regulated by the suppressor protein. It was found that rgs-CaM could act like an endogenous suppressor of RNA silencing. Transgenic plants over-expressing rgs-CaM showed phenotypic changes very similar to HCpro transgenic plants such as tumor-like structures at the stem-root junction. From that, it was concluded that HC-Pro suppresses silencing, at least in part, by stimulating the expression of rgs-CaM.

Recently, HC-Pro has been shown to influence microRNA (miRNA)-mediated gene regulation, explaining in part the developmental defects observed in transgenic plants (Mallory et al. 2002; Kasschau et al. 2003). This effect will be discussed further in a later section of the review.

A recent report on the structure of the HC-Pro protein confirmed earlier reports that it can form dimers (Plisson et al. 2003). Additionally the structure reveals three domains that correlate with three different functions of that protein. Interestingly, the domain involved in RNA-binding correlates with the domain required for silencing suppression (Kasschau and Carrington 2001).

Taken together, the data indicates that HC-Pro suppresses RNA silencing downstream of dsRNA and miRNA formation. However, it also acts upstream of the siRNA production and possibly interferes with the systemic silencing signal.

2b of cucumoviruses

While HC-Pro had a direct and strong effect on the maintenance of RNA silencing, *Cucumber mosaic virus* (CMV) 2b was shown to affect the RNA silencing pathway differently. 2b cannot suppress RNA silencing in tissues where RNA silencing is already established. However, it was shown to be able to prevent the initiation of RNA silencing in newly emerging tissue (Beclin et al. 1998; Brigneti et al. 1998). This suggested that 2b might be involved in inhibiting the systemic spreading of the silencing signal. Further analysis revealed that CMV 2b carries a monopartite nuclear localization

signal (NLS) that is required for the 2b silencing suppression activity (Lucy et al. 2000). This was very surprising, since at that time components of RNA silencing were thought to operate in the cytoplasm only. How 2b prevents the silencing signal from spreading throughout the plant remains to be investigated.

Guo and Ding (2002) showed that 2b interferes with the restoration of transgene methylation, giving a first hint on the function of 2b in the nucleus. It was also postulated that 2b was not able to prevent signal-independent RNA silencing initiation of transgene and virus silencing. Additional observations showed that CMV suppresses RNA silencing in mixed infection experiments on transgenic plants expressing dsRNA targeting PVY. The PVY derived dsRNA expressed in these plants renders them immune to PVY infection. However, when PVY was co-inoculated with CMV these plants showed a transient PVY accumulation (Mitter et al. 2003). Additionally, CMV caused a high increase of transgene mRNA levels by preventing its degradation into siRNAs. From these investigations it can be concluded that 2b inhibits the systemic propagation of a silencing signal which would be sent out from the initially infected loci to the rest of the plant and prevent further spreading of the virus.

Finally it is interesting to add that some experiments showed that 2b could also reduce the inhibitory effect of salicylic acid (SA) on virus accumulation (Ji and Ding 2001). Even though a recent finding reported that a SA inducible RdRp (RDRP1 in *Arabidopsis*) is involved in TMV resistance, this RdRp had no effect on CMV accumulation (Yu et al. 2003). This indicates that different silencing pathways may be involved in the antiviral defense depending on the infecting virus. Furthermore Yang et al. (2004) recently showed that the high susceptibility of *N. benthamiana* to viruses in general could at least in part be explained by the fact that its RDRP1 homologue is mutated.

P19 of tombusviruses

One of the most immediate suppressors of RNA silencing is P19 of the tombusviruses, such as *Cymbidium ringspot virus* (CymRSV). P19 was found to suppress RNA silencing by binding siRNAs in their double stranded form (Silhavy et al. 2002). P19 only very inefficiently binds single-stranded siRNAs, long dsRNAs, or blunted 21 nucleotide (nt) dsRNAs. However, a 2 nt overhang at the 3’ end is sufficient for P19 to bind 21 nt RNA duplexes (Silhavy et al. 2002). The step of the RNA silencing pathway upon which P19 has an effect on was indicated by biochemical experiments

performed in *Drosophila* cell extracts. It was found that P19 activity prevents siRNAs from incorporating into RNA silencing effectors such as RISC (Lakatos et al. 2004). Furthermore, specific binding of siRNAs by P19 efficiently blocks the development of systemic spreading of silencing. This substantiates the previously suggested involvement of siRNAs in the spreading of RNA silencing. Either P19 suppresses systemic silencing by binding the siRNAs, preventing them from moving through the plant, or it inhibits the activity of an siRNA-primed RdRp which is thought to be involved in the formation of the systemic signal (Voinnet 2001).

The elucidation of the crystal structure of P19 binding a 21 nt siRNA duplex finally provided information on the property of the physical interaction between P19 and siRNAs. The structure of P19 elegantly shows how dimers of this protein are capable of recognizing RNA duplexes with the length of 21 nt and overhanging 3' nucleotides that are typical for siRNAs (Vargason et al. 2003). The finding that P19 specifically binds siRNAs, the molecule conserved among all silencing-capable organisms, makes it a very potent tool to be used in all kinds of organisms. Indeed P19 has been reported to be active in insect (Lakatos et al. 2004) and mammalian cells (Dunoyer et al. 2004).

Like HC-Pro, P19 was shown to affect the processing and activity of miRNAs, a feature that will be discussed in a later section of this review.

RNA silencing suppressors of negative strand viruses

The first suppressors of RNA silencing of negative stranded RNA viruses to be found were NS_S of *Tomato spotted wilt virus* (TSWV) and NS3 of *Rice hoja blanca virus* (RHBV) (Takeda et al. 2002; Bucher et al. 2003). The accumulation of the NS_S protein had long since been shown to coincide with symptom severity of the virus (Kormelink et al. 1991), but it was not until 2002 that NS_S was proven to be an efficient suppressor of RNA silencing. For its identification the transient expression system using *Agrobacterium* and the viral infection GFP silenced plants were used. Although NS_S efficiently suppresses RNA silencing of sense transgenes by preventing the production of siRNAs it is not able to suppress RNA silencing mediated by dsRNA (Takeda et al. 2002). This indicates that NS_S acts upstream of the plant RdRp. While its activity appears to be similar to HC-Pro at the molecular level, only further analysis will reveal how NS_S exactly suppresses RNA silencing and whether it has any effect on systemic silencing or the miRNA pathway.

The NS3 of the distantly related *Tenuivirus* RHBV also efficiently suppresses RNA silencing but it is intriguing that even though it inhibits the mRNA degradation, it does not prevent the accumulation of siRNAs (Bucher et al. 2003). An interesting feature of negative stranded plant viruses is that insects do not only transmit them, but they also replicate in their insect vectors. This may suggest that NS_S and NS3 suppress RNA silencing in both hosts, possibly in a step of the pathway, which plants and insects have in common.

Consistent with the idea that silencing suppressors can function in both insect and plant cells, it has been shown for the insect-infecting positive-sense RNA virus *Flock house virus* (FHV) that it encodes a suppressor of RNA silencing that is active both in plants and in *Drosophila* cells (Li et al. 2002).

RNA silencing suppressors of DNA viruses

In addition to the silencing suppressors of RNA viruses described above, DNA viruses have also been shown to encode suppressors of RNA silencing. This is interesting considering the fact that these viruses replicate in the nucleus and their genomes consist of DNA. Hence geminivirus-derived dsRNA intermediates never occur during replication. It has, however, been reported that geminiviral mRNAs in the plant are targeted by RNA silencing in a plant RdRp (RDR6, previously named SGS2/SDE1) dependent manner (Muangsan et al. 2004). In GFP-silenced plants the bipartite geminivirus *African cassava mosaic virus* (ACMV) was shown to weakly suppress RNA silencing and AC2 was identified to be its suppressor of RNA silencing (Voinnet et al. 1999; Hamilton et al. 2002; Vanitharani et al. 2004). Further investigation revealed that AC4 of ACMV was a strong suppressor of RNA silencing (Vanitharani et al. 2004). However, for the *East African cassava mosaic Cameroon virus* (EACMCV) the unrelated AC2 encodes a strong suppressor of RNA silencing. Similar to the synergism observed for PVX and PVY, mixed infections of ACMV and EACMCV revealed enhanced virulence. AC2 and AC4 were shown to be involved in this synergism. AC4 of ACMV could enhance EACMCV DNA accumulation and reciprocally AC2 increased the accumulation of ACMV DNA (Vanitharani et al. 2004). Although RNA silencing was originally regarded as entirely cytoplasmic, there is evidence that elements of the mechanism also have effects in the nucleus (Fig. 1). The fact that AC2 requires a DNA-binding domain and an NLS for its activity as a suppressor of RNA silencing might fit this notion (Dong et al. 2003). Also the AL2 and L2 proteins of the bipartite *Tomato golden mosaic virus* and the monopartite *Beet curly top virus*, respectively, were reported to act as suppressors of RNA silencing (Wang et al. 2003).

The way these proteins exercise their function is unclear, although they have been shown to increase susceptibility to virus infection by inactivating the SNF1 and ADK kinases (Hao et al. 2003; Wang et al. 2003). Whether and how these inactivated endogenous proteins are involved in RNA silencing is not known.

Considering their range of activities and lack of sequence homology, it appears that RNA silencing suppressors of the geminiviruses evolved independently even within the genus. It remains to be discovered, whether this is a mere reflection of the renowned plasticity of geminivirus genomes, or an indication of a powerful selection pressure (even on DNA viruses) to be able to counteract RNA silencing

The role of RNA silencing in antiviral defense in animals

Evidence that RNA silencing plays a role in antiviral defense in insect cells came from experimental infections of FHV in insect cells (Li et al. 2002). Replication of the virus in *Drosophila* cells, similar to the situation observed in plants, leads to the production of siRNAs originating from the virus. This strongly indicates that RNA silencing in insect cells actively targets the virus. Furthermore it was found that FHV encoded a suppressor of RNA silencing (B2) which was not only functional in insects but also plants. Recent reports show that the NS1 protein encoded by *Influenza A virus* acts as a suppressor of RNA silencing in plants and insects (Bucher et al. 2004; Delgadillo et al. 2004; Li et al. 2004). It was also shown that NS1 efficiently binds siRNAs and that the dsRNA binding domain that is involved in the siRNA binding is required for the suppression of RNA silencing (Bucher et al. 2004). Further work is required to show that NS1 suppresses RNA silencing in mammalian cells and that indeed RNA silencing is an antiviral mechanism used to counter influenza.

Other functions of RNA silencing

As important as it is, the antiviral activity of RNA silencing is certainly is not its only function in plants. By using components of the RNA silencing machinery several other processes are supported. These processes play an important role in plants and perhaps even more so in other multicellular organisms. Among these processes are transposon silencing, transcriptional gene silencing due to sequence specific DNA methylation, chromatin

condensation and (developmental) gene regulation by miRNAs. Perhaps more so than the other RNA silencing functions, the latter process, one of the most recent sapling of the RNA silencing tree, has turned out to be of major consequence for molecular biology as it influences gene expression in an unforeseen way and scale.

Transcriptional gene silencing

One of the first indications that RNA is involved in transcriptional gene silencing (TGS) in the nucleus was done by Wassenegger and co-workers (1994). Upon viroid infection of plants transformed with T-DNAs containing viroid cDNA sequences, the latter became methylated, while other parts of the T-DNA insertion remained unaffected. They concluded from this that the replicating viroid RNA had lead to specific methylation of homologous sequences in the plant genome. This phenomenon was termed RNA dependent DNA methylation (RdDM). Expression of dsRNA of promoter sequences was shown to be a trigger for sequence-specific RdDM of these promoters and subsequent TGS (Mette et al. 2000). The fact that the promoter-derived dsRNA was processed to siRNAs suggests a role for the siRNAs in the sequence specific targeting of DNA methylation in the nucleus. Endogenous repeat-associated small RNAs possess the ability to trigger *de novo* methylation of cognate genomic DNA sequences and may thereby contribute to heterochromatin formation (Xie et al. 2004). Recently several components of the RdDM pathway have been identified. While the DNA methyltransferases (DMTase) DRM1 and DRM2 were reported to be involved in the *de novo* RNA-directed methylation, the DMTase MET1 and the putative histone deacetylase HDA6 maintain or enhance methylation. Recruitment of HDA6 then reinforces CG methylation and finally heterochromatin is formed at the specific targeted loci (reviewed in Matzke et al. 2004). Recent reports imply that AGO4 is also involved in long siRNA directed DNA methylation and its maintenance (Zilberman et al. 2003). DCL1, which is required for miRNA processing, was shown not to be required for TGS (Finnegan et al. 2003). The fact that siRNA induced TGS has also been found in human cell lines confirms the importance of RNA silencing in gene regulation through TGS (Morris et al. 2004).

Chromatin modeling

A second role of methylation of perhaps a greater magnitude than TGS was recently discovered in *Schizosaccharomyces pombe*, where RNA silencing was shown to play a role in chromatin structure, centromeric cohesion and cell division. Mutational analyses showed that RNA silencing compounds were required for the pericentromere organization in *S. pombe* (Volpe et al. 2003). Three genes that encode key enzymes of the RNA silencing machinery, Argonaute ($ago1^+$), Dicer ($dcr1^+$) and an RdRp ($rdp1^+$), were shown to be essential for this process. The RdRp is required for the production of the dsRNA from transcripts originating from the pericentromeric heterochromatin composed of complex repeats. These RNA duplexes are rapidly processed by Dicer and incorporated into what was termed the RNAi-induced transcriptional gene silencing (RITS) complex, a complex with high biochemical similarity to RISC (Verdel et al. 2004). Ago1 of *S. pombe* is a key component of these complexes and binds the siRNA. RITS activity is exerted in the dividing cell leading to the recruitment of the chromodomain protein Swi6, sequence specific methylation of centromeric regions and ultimately to chromosome condensation (Noma et al. 2004). Though discovered in yeast, these features seem to be conserved among all eukaryotes including vertebrates (reviewed by White and Allshire 2004 and Dawe 2003).

Transposon and endogenous repeat associated gene silencing

Like viruses, transposons represent a nucleic acid-based threat to plants. Movement of transposons to new insertion sites can cause major damage to the plant genome. To fight against transposons, plants have evolved a defense system based on RNA silencing. Indeed, it has been shown that plants produce the longer type of siRNAs derived from transposons (Hamilton et al. 2002; Llave et al. 2002; Xie et al. 2004). As discussed earlier these siRNAs can then lead to sequence specific RdDM and therefore transcriptional silencing of the transposons. Since transposon-derived siRNAs are present in plants, it must be concluded that transposon-derived dsRNA is being produced. Indeed, *Arabidopsis* mutant studies revealed the involvement of RDR2 and other RNA processing factors to be required for

transposon silencing (reviewed in Bender 2004). Similarly, a great body of work in *C. elegans* revealed that several factors involved in RNAi (mut-7 an RNaseD homolog, mut-14 an RNA helicase and mut-16) are required for transposon silencing (Sijen and Plasterk 2003).

Cloning and sequencing of endogenous naturally occurring siRNAs of *A. thaliana* showed that these originate not only from transposons or retroelements, but also from highly repeated ribosomal DNAs (rDNAs: 5S, 18S and 25S) (Llave et al. 2002; Xie et al. 2004). Quite a number of sequenced siRNAs were found to be homologous to expressed and predicted genes. For the majority of these small RNAs it still remains to be investigated whether they act as miRNAs, which will be discussed in the next paragraph, or whether they are implicated in other biological processes yet to be identified.

Development: miRNAs regulating timing and patterning

One of the recent major discoveries in developmental biology was the finding that many higher organisms produce endogenous small RNAs that are essential for the regulation of genes, of which many are involved in development. The most notable of these are the miRNAs. miRNAs are characterized by their phylogenetic conservation across species and their involvement in basic biological processes of development, such as cell death and patterning. Typically, miRNAs are encoded by the genome as more or less imperfect inverted repeats as part of (much) larger processed transcripts, which are actively transported to the cytoplasm (Meister and Tuschl 2004). Depending on the degree of homology to the target mRNAs in the cytoplasm, these miRNAs guide the RISC complex for the cleavage or inhibition of translation of mRNAs homologous to the miRNA. Most miRNAs in plants studied so far have a (near) perfect match with their target mRNA in the open reading frame leading to mRNA cleavage (Rhoades et al. 2002). Translational inhibition by miRNA binding but not cleavage was so far only observed in one case {APETALA2, (Aukerman and Sakai 2003)}, while this is the main mode of action for miRNAs in animals (Ambros 2004).

First hints on the involvement of miRNAs in development were observed in *C. elegans* mutant screens. Worms carrying mutations in the genes producing non-coding small temporal RNAs (stRNAs) *lin-4* and *let-7* (Lee et al. 1993; Reinhart et al. 2000) were found to modulate developmental timing. The miRNAs encoded by *lin-4* or *let-7* are incorporated into a miRNA-ribonucleoprotein complex (miRNP) and inhibit the translation of mRNAs containing partial complementarity with the miRNA in the 3' UTR.

By this mechanism miRNAs derived from the *lin-4* and *let-7* transcripts were shown to modulate the translation of their target genes *lin-14*, *lin-28* and *lin 41*, *hbl-1* respectively.

Many miRNAs have been cloned and sequenced in both plants and animals and a great number of genes have in the meantime been identified as being regulated by these miRNAs (an *Arabidopsis* small RNA database can be found at: http://cgrb.orst.edu/smallRNA/db/). Using computational methods, potential targets of these miRNAs were also indicated in plants (Rhoades et al. 2002). It was found that many predicted miRNA targets are transcription factors involved in development. One group of transcription factors recently found to be regulated by miRNAs in an AGO1 dependent manner are of the Class III HD-Zip gene family. This family directs the polarity establishment in leaves and vasculature (Kidner and Martienssen 2004; Juarez et al. 2004). Interestingly, these authors propose the miRNAs to be a mobile signal during the establishment of the polarity of developing leaves.

Finally it should be noted that miRNAs are not only involved in development since predicted miRNAs also target genes involved in abiotic stress (Sunkar and Zhu 2004). The involvement of miRNAs in so many different biological processes underlines its importance in biology (reviewed in Ambros 2004 and Baulcombe 2004).

Plant viral RNA silencing suppressors interfere with miRNA action

As mentioned earlier, the expression of viral suppressors of RNA silencing in transgenic plants was shown to lead to strong developmental defects (Kasschau et al. 2003; Chapman et al. 2004; Dunoyer et al. 2004). Further research revealed that these proteins interfere with the action of miRNAs on the regulation of genes involved in plant development. For instance transgenic plants stably expressing HC-Pro over-accumulate miRNAs and show developmental defects (Mallory et al. 2002; Kasschau et al. 2003). Not only does HC-Pro change the accumulation levels of miRNAs, it also prevents their activity. It has been shown that HC-Pro could prevent the miRNA- guided cleavage of certain mRNAs and therefore cause a higher accumulation of these mRNAs. It appears that HC-Pro might affect the activity and the turnover of the miRNAs by interfering with one of the factors involved in their biogenesis or their cellular localisation.

Also P19 has been shown to interfere with the production of active miRNAs. Since P19 is capable of binding siRNA duplexes it was suggested that P19 could also bind the miRNA/miRNA* duplexes (miRNA* being the partly anti-sense strand of the active miRNA), thereby preventing its incorporation into RISC (Dunoyer et al. 2004).

Whether the inhibition of miRNA function by RNA silencing suppressors, which leads to enhanced virulence, is a genuine role of these proteins in virus infection or a mere side effect of their inhibition of siRNA-mediated RNA silencing remains to be established.

The biochemistry of the RNA silencing machinery

Since the discovery of RNA silencing in animal model systems, the dissection of the RNA silencing machinery has caught up considerable speed. Though the RNA silencing mechanism in plants is the major focus of this chapter, knowledge on the RNA silencing machinery in plants also builds on information gathered from several animal model systems. Parts of the conserved RNA silencing machinery have been studied in many organisms ranging from plants to insects to mammals and back to protozoans. A comprehensive model encompassing the many-shared features is represented in Fig. 1.

The key action of RNA silencing involves a sequence-specific cytoplasmic degradation of RNA molecules. It can be induced in a variety of ways. For instance plant viral RNAs can be targeted after the transgenic expression of over-abundant or dsRNA. The key intermediary element in the RNA silencing pathway is dsRNA, which is recognised by a dsRNA-specific nuclease called Dicer, to yield small (21-23 nucleotides long) siRNAs. These siRNAs subsequently serve as guides for cleavage of homologous RNA molecules, mediated by RISC.

Dicer

In plants, several molecular processes can generate small RNAs. Naturally occurring small RNAs can be: (1) miRNAs involved in gene regulation; (2) endogenous siRNAs (also known as repeat associated siRNAs); (3) transposon-derived, and (4) virus-derived siRNAs. DsRNAs can also be produced artificially by the expression of constructs arranged as inverted repeats which will result in the production of siRNAs processed from long dsRNA precursors and destruction of mRNAs with a homologous sequence (Smith et al. 2000). All siRNAs are products of cleavage of dsRNA by members of an RNase III-like enzyme family, first discovered in

Drosophila (Bernstein et al. 2001) and termed Dicer in animals or Dicer-like (DCL) in plants. Dicers are multi-domain proteins that typically contain one or more dsRNA binding domain(s), a DExH RNA helicase, a PIWI/ARGONAUTE/ZWILLE (PAZ) domain and two neighbouring RNase III-like domains. It has been reported that human Dicer works as an intra-molecular dimer of its two RNase III domains (Zhang et al. 2004). The products of the endonucleic cleavage by Dicer enzymes are RNA duplexes that have 5' phosphates and 2 nt 3' overhangs, mostly around 21 nt in size. It is interesting to note that while many animals only encode a single Dicer, *Drosophila* encodes two (Lee et al. 2004), and *Arabidopsis* has evolved four Dicer homologues (DCL1, DCL2, DCL3 and DCL4) (Schauer et al. 2002). It would appear that the multiple roles Dicer plays in the different branches of the RNA silencing in animals are divided over the different homologues in plants.

In the case of *Arabidopsis*, the role of DCL4 is yet unknown, while DCL3, in concert with RDR2, plays a role in the production of endogenous siRNAs. As mentioned earlier, these endogenous siRNA are involved in the initiation or maintenance of a heterochromatic state (Matzke et al. 2004), DCL2 was found to be involved in the production of siRNAs derived from viruses (Xie et al. 2004). The fact that viral siRNA accumulation was not completely abolished in DCL2 mutant plants, but just delayed, suggests the existence of another redundant DCL enzyme. In addition to DCL2, the production of virus-derived siRNAs requires two RdRps (RDR1 and RDR6), depending on which kind of virus infects the plant (Muangsan et al. 2004; Xie et al. 2004). DCL1, together with other factors, such as HEN1 and HYL1 (a dsRNA binding protein), was shown to be responsible for the generation of miRNAs (Vazquez et al. 2004; Xie et al. 2004). The processing of the primary miRNA (pri-miRNA) to the miRNA duplex most probably occurs in the nucleus, but is also guided by DCL1. Interestingly, HEN1 is not only involved in miRNA biogenesis but also in transgene silencing and natural virus resistance as was shown by a CMV based sensitivity assay (Boutet et al. 2003).

Compared to plants, processing of miRNA precursors in animals is different. The pri-miRNAs, synthesised by the RNA polymerase II, are first processed by a nucleus-specific enzyme, *Drosha*, initially discovered in *Drosophila* (Filippov et al. 2000), into precursor miRNAs (pre-miRNAs) (Lee et al. 2003). These pre-miRNAs, imperfect hairpins of approximately 70 nt in length, are then exported to the cytoplasm and processed into miRNAs by the cytoplasmic Dicer.

RISC

Regardless of the way different Dicer enzymes produce siRNAs and miRNAs and their final destination, single strands of siRNA or miRNA duplexes are incorporated into RISC, the effector of RNA silencing. RISC provides the different (catalytic) functions such as mRNA cleavage and translational inhibition. RISC is a multi-protein complex of which several components have been identified. Small RNA molecules provide sequence-specificity to RISC. Like these small RNAs, ARGONAUTE (AGO) proteins have been found to be part of RISC in all organisms studied and are essential for its mRNA slicing activity. The term "Argonaute" refers to the squid-like appearance of the leaves of *Arabidopsis* mutants lacking AGO1 gene function (Bohmert et al. 1998). To date, 10 members of the Argonaute family have been identified in plants. Two of them, AGO1 and AGO4, have been studied extensively. AGO1 mutant plants have been found to develop distinctive developmental defects. miRNAs accumulate normally in these plants, but their target mRNAs are no longer cleaved. Interestingly, the expression of AGO1 itself is regulated by a miRNA (miR168) indicating that the AGO1 protein regulates its own expression in a negative feedback loop (Vaucheret et al. 2004). AGO4 has a role in the production of the 'long' siRNAs of 24 bp. While it is not known yet whether AGO4 mutants are affected in systemic RNA silencing, it was reported that AGO4 is involved in long siRNA mediated chromatin modification (histone methylation and non-CpG DNA methylation) (Zilberman et al. 2003).

In *Drosophila,* AGO2 is part of RISC and essential for siRNA-directed RNA silencing. AGO2 is not required for the miRNA biogenesis, but a role for AGO1 was indicated (Okamura et al. 2004). R2D2, a Dicer-2 associated protein, was shown to play an important role in binding and strand discrimination of siRNAs and miRNAs for incorporation of the proper RNA strands into RISC (Liu et al. 2003). Though, R2D2 is not involved in the endonucleic cleavage of dsRNA to siRNAs, it stabilizes the association of Dicer-2 to the siRNA.

Generally, it can be concluded that most if not all AGO proteins are involved in different parts of the RNA silencing and possibly define the mode of action of the RISC in which they are incorporated (Baulcombe 2004).

Concluding remarks

Taking into account all the information discussed in this chapter it is possible to conclude that RNA silencing has evolved as an efficient, general

way of counteracting the deleterious influence of foreign nucleic acids. However, it is very interesting that RNA silencing is not only involved in this defensive process, but also in very basic biological processes such as gene regulation and development. That is why this research has reached great momentum. Certainly, more surprising discoveries will be revealed.

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Chapter A4

Recognition and Signal Transduction Associated with *R* Gene-mediated Resistance

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1.1 Introduction

Plants are constantly challenged by a wide array of pathogens, including viruses. For any specific plant species most viruses cannot surmount basal defenses that include physical barriers like a waxy layer covering the plant and post-transcriptional gene silencing (PTGS). However, in those instances when a virus is able to infect a plant, host survival relies on quick recognition of the invading virus and rapid signaling of a defense response. One form of resistance termed gene-for-gene type of resistance relies on the interaction of a plant *R* gene and a pathogen-encoded avirulence (*Avr*) gene. If a plant has a specific R protein that can recognize a pathogen Avr product, the plant will mount a defense response and thwart an infection. Therefore, plant R proteins have a dual role. Not only must they recognize a pathogen directly or indirectly, they must also initiate signaling that leads to a defense response. One of the earliest defense responses is the hypersensitive response (HR), a type of programmed cell death (PCD) that occurs at the pathogen's infection site. HR is correlated with the signaling of an *R*-gene-mediated disease resistance response and containment of the pathogen at the infection site (For details, see Chapter A5). Following HR, a systemic acquired resistance (SAR) response results in an enhanced resistance to further infection by a variety of pathogens. In this chapter we will discuss the major

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advances in understanding how R proteins recognize different viruses and the intricacies of the defense-signaling network that leads to HR and SAR.

1.2 R Genes and Recognition

Viral resistance genes

The disease resistance field has advanced quickly with the advent of cloned *R* genes. The tobacco *N* gene that confers resistance to *Tobacco mosaic virus* (TMV) was the first antiviral *R* gene cloned (Whitham et al. 1994). The cloning of *N* was a major breakthrough because it was one of the first R protein containing domains with a nucleotide binding site (NBS) and leucine-rich repeats (LRR). The NBS-LRR class represents the vast majority of *R* genes that confer resistance to viruses and other pathogens (Martin et al. 2003). The *N* gene belongs to a subclass of NBS-LRR genes that contain a Toll interleukin-1 receptor domain at the N-terminus (TIR-NBS-LRR). The only other cloned antiviral *R* gene that belongs to this subclass is the potato *Y-1*, which has 57% identity to the *N* gene (Vidal et al. 2002). Although the *Y-1* locus is known to confer resistance to *Potato virus Y* (PVY), it is yet unclear if this confers complete resistance.

The remaining cloned antiviral *R* genes belong to the NBS-LRR class, but contain a coiled-coiled domain at their N-terminus (CC-NBS-LRR). Potato *Rx1* and *Rx2* are two unlinked genes but they are functionally identical and confer extreme resistance to *Potato virus X (*PVX) without inducing HR (Bendahmane et al. 1999; Bendahmane et al. 2000). Tomato *Sw-5* confers resistance to *Tomato spotted wilt virus* (TSWV) and belongs to a seven-member gene family (Brommonschenkel et al. 2000). However, the pathogens recognized by the other family members are currently unknown. *Tm-2* and *Tm-2*^{2} provide resistance to *Tomato mosaic virus* (ToMV) (Lanfermeijer et al. 2003). Interestingly, the *Tm-2* allele of *Tm-2*^{2} is easily overcome by resistant strains of ToMV even though there are only 38 amino acid differences between the two alleles (Lanfermeijer et al. 2003).

The two antiviral *R* genes cloned from *Arabidopsis* include *HRT* from the Dijon-17 ecotype and *RCY1* from the C24 ecotype. *HRT* and *RCY1* confer resistance to *Turnip crinkle virus* (TCV) (Cooley et al. 2000) and the yellow strain of *Cucumber mosaic virus* (CMV-Y) (Takahashi et al. 2002) respectively. Interestingly, *HRT* and *RCY1* are allelic to *RPP8* from the Landsberg errecta ecotype that confers resistance to the fungus *Peronospora parasitica* (McDowell et al. 1998; Cooley et al. 2000). *RCY1* is 92.1% and 91.3% homologous to *HRT* and *RPP8* respectively (Takahashi et al. 2002). This is the first example of three alleles of the same gene conferring resistance to three different pathogens. This suggests that there must be

conserved mechanisms for both recognition and signaling by NBS-LRR genes.

The range of pathogens recognized by *RPP8/HRT/RCY1* family of *R* genes suggests plants may have evolved allelic series that can recognize, and therefore, confer resistance to a vast array of pathogens (Cooley et al. 2000). Alternatively, *R* genes may have diversified by duplication followed by a divergence of recognition. For example, *Gpa2* in potato is adjacent to *Rx1* but confers resistance to a nematode (van der Vossen et al. 2000).

Viral products recognized by R proteins

The diversity of recognition and the Tobamoviruses

R proteins recognize different types of virally encoded proteins. Tobamoviruses like TMV and ToMV encode three proteins: replicases, a movement protein (MP), and a coat protein (CP). *R* genes have evolved to recognize all three of these viral proteins. Additionally, R proteins appear to recognize viral proteins via protein-protein interactions rather than by detecting their function.

The N protein recognizes the helicase domain of the TMV replicases. *N* confers resistance to all tobamoviruses examined except the Ob strain of TMV (Tobias et al. 1982). Analysis of the Ob strain showed that amino acid changes in the helicase domain of the replicase proteins allowed it to surmount *N*-mediated resistance (Padgett and Beachy, 1993; Padgett et al. 1997). Expression of the helicase domain in plants containing the *N* gene induces resistance response proving that the helicase domain alone is necessary and sufficient to elicit an *N*-mediated response (Erickson et al. 1999). Furthermore, the N protein might recognize TMV helicase via protein-protein interactions because ATPase activity of the helicase domain is not required for recognition (Erickson et al. 1999).

The allelic genes *Tm-2* and *Tm-2*^{2} recognize the MP of ToMV (Meshi et al. 1989; Weber et al. 1993). Resistance breaking strains contain mutations in the variable C-terminus of the MP that is dispensable for ToMV movement or replication (Gafny et al. 1992). This suggests that Tm-2 alleles do not recognize the function of the MP, but rather, the recognition probably occurs via protein-protein interactions.

The tobacco *N'* gene has not been cloned, but extensive studies have shown that it recognizes the TMV CP (Saito et al. 1989). Domain swaps between the TMV-L strain that induces HR and the TMV-OM strain that does not induce HR has shown that the CP of TMV-L strain is necessary to elicit a response in the presence of N'. Deletion analysis shows that the entire CP except 13 amino acids at the C-terminus are needed for recognition by N'

(Saito et al. 1989). It appears that proper formation of the tertiary structure of assembled coat proteins is necessary for N' to recognize TMV (Toedt et al. 1999).

Recognition of coat proteins

The CP is most common viral product recognized by cloned R proteins. For example, antiviral *R* genes such as *Rx1, Rx2, N', HRT* and *RCY1* all recognize CP.

Mutations in the CP of resistance breaking strains of PVX were responsible for their evasion of *Rx1*-mediated recognition. The protein, and not the RNA of CP, is recognized by Rx1 because sequence differences that did not change amino acids were unnecessary for PVX resistance breaking strains to evade Rx1 detection (Kohm et al. 1993). To determine if the CP alone is sufficient to elicit resistance by Rx1, recombinant TMV expressing the PVX CP was expressed in protoplasts containing *Rx1*. TMV normally can infect and replicate in protoplasts containing *Rx1*. However, TMV expressing the PVX CP could not replicate in protoplasts containing *Rx1* (Bendahmane et al. 1995). Therefore the PVX CP is sufficient to elicit an *Rx1*-mediated resistance response to PVX.

The *R* genes *HRT* and *RCY1* recognize the CP of TCV and CMV respectively (Zhao et al. 2000; Takahashi et al. 2001). The *HRT* and *RCY1* genes are highly homologous, but the CPs they recognize contain no sequence homology. Domain swaps between CMV-Y that induces an *RCY1*-mediated defense and CMV-B2 that cannot be detected by RCY1, suggest that the CP is necessary for recognition (Takahashi et al. 2001). However, it remains unknown if the CP alone is sufficient for recognition by *RCY1*, or if other CMV proteins are required.

Recognition of proteases

The *Ry* gene has not been cloned, but it confers a durable, extreme resistance to PVY. The nuclear inclusion a protease (NIaPro) from PVY can elicit *Ry*-mediated resistance response. Although an intact protease site is necessary for NIaPro to elicit a defense response, the protease activity of NIaPro is not sufficient for the elicitation of *Ry*-mediated resistance (Mestre et al. 2003). A mutant with close to wild-type protease activity is unable to elicit a defense response, indicating that NIaPro is either recognized via a protein-protein interaction or has another protease activity that cleaves a host protein.

1.3 The mode of recognition

The guard hypothesis

How do R proteins recognize specific Avr products from viruses? Direct interaction between an elicitor from the virus and a corresponding antiviral R protein has not been shown. However, in a few cases direct interactions between R proteins and Avr ligands has been demonstrated for interactions between plants and non-viral pathogens (Scofield et al. 1996; Tang et al. 1996; Jia et al. 2000; Deslandes et al. 2003). Since most cloned R proteins fail to interact directly with cognate Avr proteins, alternative hypotheses have been proposed. The most popular model is the "guard hypothesis" (Dangl and Jones, 2001). The guard hypothesis states that R proteins act as "guards" that monitor key host cellular factors called "guardees" that are modified by a pathogen's Avr product. R proteins recognize the pathogen by perceiving a change in the status of the cellular factor. The guard hypothesis may explain some non-viral host-pathogen interactions. For example, in the interactions between avirulent strains of *Pseudomonas syringae* and *Arabidopsis thaliana*, two different *R* gene products guard the cellular protein RIN4. Rpm1 recognizes hyperphosphorylation of RIN4 caused by AvrRpm1 or AvrB and then induces a resistance response (Mackey et al. 2002). Another bacterial R protein, RPS2, recognizes the rapid degradation of RIN4 (Axtell and Staskawicz, 2003; Mackey et al. 2003). Likewise, RPS5 recognizes the proteolytic cleavage of PBS1 kinase by the bacterial protein, AvrPphB (Shao et al. 2003).

There is currently one virus-plant interaction that supports the guard hypothesis as the model for viral recognition. A yeast two-hybrid screen conducted with the TCV CP yielded the TCV-interacting protein (TIP) from *Arabidopsis* (Ren et al. 2000). The interaction between TIP and TCV-CP is necessary for *HRT*-mediated resistance and therefore, HRT may be guarding the host protein TIP. However, it has not been directly shown that HRT is guarding TIP. An alternative hypothesis is HRT is guarding a protein that binds to TIP or is regulated by TIP. TIP belongs to the NAC family and has the ability to induce transcription in yeast. TIP-regulated transcription of genes may change when TCV CP binds TIP, and HRT might indirectly recognize that change in transcription.

R protein-containing complexes

The guard hypothesis suggests that the host-pathogen interaction is more likely an interaction between the Avr protein and a host recognition complex. This complex must be able to recognize the pathogen and signal a defense response. Complex levels and activation of signaling must be tightly regulated and the recognition complex must be poised to perceive and respond to pathogens. To understand the function of protein complexes during disease resistance, we must determine the components of the complex, how the complex forms, and how the complex is activated to signal defense.

The Hsp90-Sgt1-Rar1 complex

Recently, a few proteins that may belong to R protein-containing signaling complexes have been discovered. The 90 kDa heat shock protein (Hsp90) was the first protein discovered to interact directly with an antiviral R protein. The LRR domain of N directly interacts with Hsp90 in a yeast two-hybrid assay (Liu et al. 2004b). In addition, Hsp90 coimmunoprecipitates with the full length N protein *in vivo* (Liu et al. 2004b). Two independent research groups have shown that silencing of *Hsp90* compromises *N*-mediated resistance to TMV (Lu et al. 2003; Liu et al. 2004b). *Hsp90* has also been shown to be necessary for the function of the *R* genes *Rpm1*, *RPS2*, and *Pto* that confer resistance to different strains *Ps. syringe* and *Rx1*, which confers resistance to PVX (Hubert et al. 2003; Lu et al. 2003; Takahashi et al. 2003).

The exact role of *Hsp90* during disease resistance is unknown. Hsp90 is a highly conserved eukaryotic ATP-dependent chaperone that facilitates protein folding and activation of proteins (Picard, 2002). Therefore, it is possible that Hsp90 has a non-specific role during protein folding of R proteins and other components involved in *R* gene-mediated resistance (Shirasu and Schulze-Lefert, 2003). The Rx1 protein levels decrease when *Hsp90* is silenced, and therefore Hsp90 may control the stability of Rx1 (Lu et al. 2003). However, two pieces of evidence suggest that Hsp90 plays a more direct role during disease resistance. Rare Hsp90 mutants in *Arabidopsis* do not have severe morphological phenotypes, but do have an attenuated *Rpm1*-mediated resistance response (Hubert et al. 2003). Additionally, Hsp90 directly interacts with the resistance protein N and other components of disease resistance signaling.

Hsp90 interacts with two defense signaling components, Rar1 and SGT1 (Takahashi et al. 2003; Liu et al. 2004b). *Rar1* was originally identified as a gene required by multiple *Mla* resistance genes in barley (Shirasu et al. 1999). Silencing of *Rar1* in *NN* plants compromises *N*-mediated resistance to TMV (Liu et al. 2002a). SGT1 interacts directly with RAR1 and Hsp90 and

silencing of *SGT1* compromises *Rx1*-mediated resistance to PVX (Peart et al. 2002b) and *N*-mediated resistance to TMV (Liu et al. 2002b; Peart et al. 2002b). SGT1 also plays a second role downstream of recognition during defensive signal transduction (see above). It is tempting to speculate that Hsp90 is a chaperone that regulates the folding and formation of an R protein-containing complex, and recruits SGT1 and Rar1 as co-chaperones. Thus, a possible role for the Hsp90-SGT1-Rar1 complex might be to finely adjust the abundance or activation status of R protein-containing complexes (Hubert et al. 2003).

Intramolecular interactions of R proteins

Changes in the recognition complex may be initiated by changes in intramolecular interactions of R protein domains. There is convincing evidence that such interactions occur in R proteins that belong to the CC-NBS-LRR subclass. The CC-NBS domain and the LRR domain of Rx1 physically interact with each other (Moffett et al. 2002). This interaction is disrupted only in the presence of an *Rx1*-eliciting PVX CP but not in the presence of a non-eliciting PVX CP. This suggests that recognition may occur by the viral elicitor by directly or indirectly disrupting the intramolecular domain interactions of R proteins. However, a more likely model places the disruption of intramolecular interactions directly downstream of recognition. The abrogation of domain interactions may alter the components of the R protein complex by exposing or activating signaling domains, such as the CC or NBS, which then recruit signaling components to the complex. Alternatively, a signaling component that constitutively belongs to the complex may be activated and released to initiate signaling pathways.

Signal transduction

After a virus is recognized, the function of an R protein complex must switch from recognition to signal transduction. Intramolecular interactions, activation of the NBS domain, and changes in signaling components that may associate with the CC or TIR domain and LRR domain have all been implicated during early signaling. However, their precise roles during the initiation of signal transduction remain elusive. On the contrary, researchers have made major advances in identifying crucial small signaling molecules, defense-related signaling pathways, and shared signaling components.

Early signaling in the hypersensitive response

Reactive Oxygen Intermediates

The HR is a type of localized cell death that occurs at the infection site of viruses and is correlated with, but not always required for, the restriction of viruses (For a detailed discussion, see Chapter A5). HR is dependent on the production of reactive oxygen intermediates (ROIs), mainly in the form of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\cdot}OH$) (Grant and Loake, 2000). The formation of ROIs upon pathogen infection has been identified during infection with bacteria, fungi, and viruses.

The production of ROIs is biphasic. The first phase is a small burst that happens within minutes of infection. This is induced by an infection with both virulent and avirulent pathogens. For example, the induction of ROIs by the TMV CP is independent of *N* (Allan et al. 2001). The second phase is stronger, more persistent, and is correlated with disease resistance. Doke and Ohashi (1988) were the first to determine that ROIs play an important role during *R* gene-mediated resistance to viruses. They discovered an O_2^- generating system that is activated during *N*-mediated response to TMV and that occurs as a burst (Doke and Ohashi, 1988). Moreover, the generation of O_2^- was dependent on Ca^{2+} and coupled to NADPH, which suggest O_2^- may be produced by an NADPH oxidase.

O_{2^-} is converted to H_2O_2 spontaneously or actively by superoxide dismutase (SOD) (Lamb and Dixon, 1997). Alternatively, H_2O_2 can accumulate when antioxidants such as ascorbate peroxidase, catalase, and carbonic anhydrase are inhibited (Chen et al. 1993; Durner and Klessig, 1995; Slaymaker et al. 2002). A third source of H_2O_2 may come from the extracellular space, also known as the apoplast. Pathogen-induced H_2O_2 moves apoplastically and may be generated by cell wall-localized peroxidases or from polyamine oxidases (Allan and Fluhr, 1997; Yoda et al. 2003). Polyamine oxidases convert polyamines to H_2O_2 by oxidative deamination (Yoda et al. 2003). During TMV-induced HR the polyamine, spermine, is up-regulated 20-fold in the apoplast (Yamakawa et al. 1998).

H_2O_2 is necessary for the regulation of HR. A 2- to 4-fold over-expression of the catalase gene *CTA1* decreases the level of H_2O_2, which subsequently results in larger TMV-induced HR lesions (Talarczyk and Hennig, 2001). However, the targets of H_2O_2 that induce signal transduction are largely unknown. One possibility is H_2O_2 may control Ca^{2+} influx. H_2O_2 induces a dose-dependent increase in cytoplasmic Ca^{2+} that plays an important role during HR (Levine et al. 1994). Alternatively, H_2O_2 may not control HR by directly binding to protein effectors, but rather, may affect

signaling pathways that are sensitive to changes in the cellular redox state. H_2O_2 induces the expression of glutathione-S-transferase and glutathione peroxidase (Levine et al. 1994). These enzymes quickly change the cellular redox state to a more reducing environment. Recently, changes in the cellular redox state were shown to be crucial for mounting a defense response. A reduced cellular environment causes the important signaling component, NPR1, to become monomeric and move into the nucleus (Mou et al. 2003). Once it is there, it binds to TGA transcription factors that induce the expression of defense-related genes (Despres et al. 2003; Mou et al. 2003).

Nitric oxide

The production of nitric oxide (NO) is also biphasic, and occurs at approximately the same time as the production of ROIs (Delledonne et al. 1998). It is thought that NO and ROIs cooperate to signal a HR (Delledonne et al. 1998). NO, like H_2O_2, is an excellent candidate molecule for cell-to-cell signaling. Even though NO is highly reactive with oxygen, it still has a half-life of a few seconds and has the ability to diffuse across membranes (Beligni et al. 2002; Neill et al. 2002).

In mammals, NO signals through a cyclic GMP (cGMP)-dependent pathway. In tobacco, NO and cGMP induce *PAL* and *PR-1*, which are early and late markers of defense respectively (Durner et al. 1998). NO increases the abundance cGMP, which may be formed by guanylate cyclase. The increase in *PAL* expression can be inhibited by LY8358 and ODQ, two inhibitors of mammalian guanylate cyclase. Downstream of cGMP, cyclic ADP ribose (cADPR) functions during Ca^{2+} regulation (Denninger and Marletta, 1999). Addition of cGMP and cADPR to tobacco leaf discs causes a synergistic increase in PAL and PR-1 expression. These data suggest that NO signals partially through a cGMP signaling pathway (Durner et al. 1998). Alternatively, NO may induce disease resistance response through nitrosylation of redox-sensitive amino acids, such as cysteine or tyrosine, or by reacting with transition metal centers (Stamler et al. 2001; Romero-Puertas et al. 2004). However, the role of nitrosylation in plants is currently unclear.

Salicylic acid and the hypersensitive response

In addition to ROIs and NO, SA has been implicated in HRs to both viral and non-viral pathogens. SA is not sufficient for the induction of HR because supplying it exogenously does not cause a HR. However, SA may be necessary to regulate the timing and extent of the HR.

During the HR, SA forms a gradient, with SA accumulating to high levels at the center of the HR lesions, moderate levels at the lesion borders, and low levels in healthy tissue (Enyedi et al. 1992). This accumulation of SA during a TMV-induced HR in *NN* plants is biphasic (Mur et al. 1997). There is a 10-fold increase in the pre-necrotic phase and a 50-fold increase in the necrotic phase. Constitutive expression in transgenic plants of the bacterial *nahG* gene, which encodes the SA-degrading enzyme, salicylate hydroxylase, decreases accumulation of SA in plants. This results in the attenuation of SA-mediated signaling. Thus, in transgenic NN genotype tobacco plants harboring *nahG* constructs driven by CaMV 35S promoters, TMV induces larger sized HR lesions that may eventually lead to a spreading necrosis phenotype (Gaffney et al. 1993; Mur et al. 1997). Interestingly, transgenic *NN*-genotype tobacco plants containing a *nahG sequence* under the tobacco *PR-1a* promoter (which is itself SA-responsive) only lose the second phase of SA accumulation. These plants have similar size lesions to those on plants which do not contain the *N* transgene. A greater increase in lesion size was seen in transgenic plants where the asparagus *AoPR1* promoter was used to drive expression of salicylate hydroxylase (Mur et al. 1997). This promoter is responsive to ROI, not SA, and is active during the pre-necrotic phase of the HR. Therefore, it appears that SA accumulation during the early, pre-necrotic phase of the HR is the most critical for controlling *N*-mediated restriction of TMV spread (Mur et al. 1997).

SA also plays a direct role in resistance to TCV during the *HRT*-mediated HR in Arabidopsis. In *NahG*-transgenic *HRT*-containing plants, resistance to TCV was completely lost in all the plants tested (Kachroo et al. 2000). The cell death associated with HR was undetectable by tryphan blue (Kachroo et al. 2000). Therefore, unlike in the TMV-induced HR in tobacco, during the TCV-induced HR in Arabidopsis both cell death and resistance require an SA-dependent signaling pathway or pathways. This is one of many examples of varying requirements and functions of signaling pathways during resistance to different viruses and/or in different host species.

Varying roles of jasmonic acid and ethylene

Most viruses require SA-dependent signaling pathways. However, the requirement of ethylene (ET) and jasmonic acid (JA) during *R* gene-mediated resistance to viruses is more complex and variable. The crosstalk between ET-, JA- and SA-dependent signaling pathways can have synergistic or antagonistic effects on each other. ET and JA are secondary signaling molecules that function in microbial defense, wounding, and insect attack. During SAR, they induce the expression of specific genes that are not induced by SA. JA induces the expression of *thionin 2.1 (Thi2.1)* and both

JA and ET induce the expression of *Plant Defensin 1.2* (*PDF1.2*) (Kunkel and Brooks, 2002).

Ethylene signalling

Resistance to TMV requires an ET-dependent signaling pathway. The *Arabidopsis* mutant, *ctr1*, undergoes a constitutive triple response to ethylene and functions downstream of ethylene receptors (Kieber et al. 1993). Silencing *NbCTR1* causes a constitutive ethylene response and results in a rapid initiation of TMV-induced HR in *NN*-transgenic *N. benthamiana* plants (Liu et al. 2004a). Analysis of ethylene-insensitive transgenic tobacco (Tet^r) plants indicates ethylene is necessary for SAR and may be necessary for creating or moving the mobile signal necessary for SAR; however, it is unnecessary for sensing the mobile signal (Knoester et al. 1998).

In *Arabidopsis RCY1*-mediated resistance to CMV also requires ET signaling pathways. Only 8% of the *RCY1*-containing ethylene insensitive mutants, *etr1-3* or *ein2-1*, were susceptible to CMV-Y infection (Takahashi et al. 2004). When NahG depleted SA in RCY1 plants, 16% of the plants were susceptible. *RCY1* plants with both *NahG* and *etr1-2*, resulted in 57% susceptibility to CMV-Y (Takahashi et al. 2004). This suggests SA and ET may function synergistically. Furthermore, a third pathway or mechanism must exist to explain why almost half of the plants remained resistant to CMV-Y.

In contrast to *N* and *RCY1*, *HRT*-containing *etr1-1* plants were completely resistant to TCV, and therefore, the ET signaling pathway is not required for resistance mediated by this gene (Kachroo et al. 2000).

Jasmonic acid signalling

Jasmonic acid signaling is required for *N*-mediated resistance to TMV. Silencing of *CORONATINE-INSENSITIVE1* (*COI1)* compromises *N*-mediated resistance to TMV in *NN*-transgenic *N. benthemiana* (Liu et al. 2004a). Furthermore, the levels of JA and its metabolic precursor OPDA (cis-12-oxophytodienoic acid) increase in *NN* tobacco plants infected with TMV (Dhondt et al. 2000).

In contrast, the *coi1-1* mutation in *HRT Arabidopsis* plants did not affect resistance to TCV, suggesting JA is not required (Kachroo et al. 2000). *RCY1*-mediated resistance does not require JA, but rather, JA and SA signaling pathways mutually antagonize each other. JA signaling suppresses the SA-induced expression of *PR-1* and *PR-5* during *RCY1*-mediated resistance to CMV-Y (Takahashi et al. 2002). Conversely, SA signaling suppresses JA-induced expression of *PDF1.2* and *HEL*, two known markers for JA signaling (Takahashi et al. 2002). The varying requirements for ET,

JA, and SA during *RCY1*- and *HRT*-mediated resistance are surprising because these genes are highly similar. One explanation is the requirement for different signaling pathways diverged as the specificity of *RCY1* and *HRT* evolved. Alternatively, all three pathways may be initiated to the same degree by *RCY1* and *HRT*, but the effect of the downstream resistance mechanisms on movement or replication of CMV-Y or TCV may vary.

Signals needed for induction of systemic acquired resistance

SAR confers long-lasting resistance to secondary infections of a wide variety of pathogens, including viruses, bacteria, oomycetes, and fungi (Durrant and Dong, 2004). JA and ET signaling pathways may be necessary for SAR during defense to a variety of pathogens; however, their role during virus-induced SAR is unclear. Many signaling pathways and components have roles during both HR and SAR. For example, SA has varying importance during HR, but has a well-established function during SAR. The ability of SA to induce SAR to viruses was exhibited when exogenously supplied SA in the form of aspirin (acetyl-SA) was shown to reduce the size of HR lesions by 95% during an N-mediated defense response to TMV (White, 1979). One of the hallmarks of SAR is the induction of a set of pathogenesis-related (PR) proteins (Durrant and Dong, 2004). The PR proteins with chitinase and β1,3-glucanase activities have anti-fungal and anti-bacterial properties (Bowles, 1990), but the known PR proteins have not been shown to have anti-viral activities.

SA signaling through the NPR1-dependent pathway

The *Arabidopsis* mutants *npr1* (non-expressor of PR-1), *nim1* (noninducible immunity1), and *sai1(*salicylic acid-insensitive1) are allelic mutations in the *NPR1* gene (Durrant and Dong, 2004). SA or avirulent pathogens fail to induce SAR in *npr1* mutants (Durrant and Dong, 2004). SA induces the expression of PR proteins through an *NPR1*-dependent pathway during both HR and SAR (Durrant and Dong, 2004). SA induces the nuclear localization of NPR1, where it binds to TGA transcription factors that increase the expression of *PR* genes or other defense genes (Durrant and Dong, 2004). The SA-binding protein 2 (SABP2) gene encodes a lipase protein that may be the receptor for SA signaling through the *NPR1*-dependent pathway, because *SABP2*-silenced tobacco plants have a similar phenotype to the Arabidopsis mutant *npr1* mutant. *SABP2*-silenced plants had 41% larger HR lesions, failed to induce SAR, and had a reduced up-regulation of PR-1 expression compared to the wild-type *NN*-genotype tobacco plants (Kumar and Klessig, 2003). The lipase activity of SABP2 is

activated by SA binding and the expression of SABP2 is induced in TMV-infected *NN* plants (Kumar and Klessig, 2003).

R genes for viral recognition have varying requirements for NPR1. NPR1-dependent signaling is necessary for *N*-mediated resistance to TMV because silencing of *NPR1* in *NN*-transgenic *N. benthamiana* plants resulted in a loss of *N*-mediated resistance to TMV (Liu et al. 2002a). *Arabidopsis* plants with the HRT gene and with the *npr1-1* or *npr1-5* mutations had a delayed HR and decreased levels of PR-1, but resistance to TCV is not compromised (Kachroo et al. 2000). Since NahG plants that cannot accumulate SA remained susceptible to TCV, SA must also signal through a pathway that is independent of NPR1. Furthermore, resistance to turnip vein clearing virus (TVCV) can be induced by SA in non-HR responding *Arabidopsis npr1* mutants (Wong et al. 2002).

SA signaling through the SHAM sensitive pathway

An NPR1-independent pathway was recently discovered to be specifically required for resistance to viruses. Salicylhydroxamic acid (SHAM) blocks SA-dependent resistance to TMV in tobacco (Chivasa et al. 1997). Remarkably, SHAM does not inhibit resistance to the bacterial pathogen *Erwinia carotovora*, or the fungal pathogen *Botrytis cinerea*, suggesting that the SHAM sensitive pathway is specific to defense against viruses (Chivasa et al. 1997). The SHAM-sensitive pathway does not induce PR proteins, and consequently, is independent of the *NPR1*-dependent pathway.

SHAM is an inhibitor of alternative oxidase (AOX) as well as an inhibitor of SA-induced resistance to TMV in tobacco (Chivasa et al. 1997). This suggests increases in AOX should induce resistance to TMV. *AOX* transcripts do increase when TMV elicits an *N*-mediated response (Chivasa and Carr, 1998). The metabolic inhibitors antimycin A (AA) and potassium cyanide (KCN) inhibit electron transfer in the cytochrome pathway, which results in an increase in *AOX* transcript levels. This indirect induction of *AOX* correlates with resistance to TMV (Chivasa and Carr, 1998). AA and KCN induce TMV resistance, possibly through AOX, but do not cause an increase in PR-1 (Chivasa and Carr, 1998; Murphy et al. 1999). Additionally, KCN is able to restore the loss-of-resistance caused by a depletion of SA by the SH-L transgene. SHAM, which has the opposite affect of KCN, prevents KCN from restoring resistance (Chivasa and Carr, 1998). Therefore, KCN and SHAM affect the same pathway, possibly through AOX. However, the role of AOX is still unclear because KCN, AA, and SHAM are pharmacological reagents that affect other proteins. Stable over-expression or knockdown lines of AOX will clarify its function during defense (see Chapter 15 by Handford and Carr).

Signaling components and downstream effectors of the SHAM-sensitive pathway are currently unknown. The SHAM-sensitive pathway is necessary for resistance to TMV in tobacco, but it is unclear if it necessary for resistance to other viruses or if it functions in other plant species (see Chapter 6 by Handford and Carr). However, the discovery of the SHAM-sensitive pathway is quite important because it is the first biologically significant virus-specific pathway that defines a new mode of SA-dependent signal transduction.

Spermine-induced signaling pathway

The possible role of polyamines (PAs) during disease resistance to viruses is often overlooked. The most abundant PAs are putrescine (Put), spermidine (Spd) and spermine (Spm) (Janne et al. 2004). They are poly-cationic compounds with a flexible carbon backbone that have the ability to associate with negatively charged compounds, such as nucleic acids, acidic phospholipids, and proteins. In *NN* plants infected with TMV, Spm levels increase by 20 fold suggesting that PAs may play a role in *N*-mediated resistance to TMV (Yamakawa et al. 1998). This increase only occurs in the intercellular spaces and was not detected in whole cellular leaf extracts. The protein abundance levels of PR-1, PR-2, PR-3, and PR-5 increase in response to Spm, SA, and TMV, suggesting Spm may be necessary for SAR. Interestingly, exogenously supplied Spm enhances HR formation in a dose-dependent manner and therefore enhances *N*-mediated resistance to TMV.

SA and Spm may function in separate pathways because SA is unable to cause increases in Spm levels and Spm is unable to cause increases in SA levels. Additionally, the tobacco peroxidase genes, tpoxC1 and tpoxN1, are induced by Spm, but not by SA (Hiraga et al. 2000). Exogenously supplied Spm causes an increase in expression of *HIN1*, *HIN9*, and *HIN18* and this is unaffected in *NahG*-transgenic plants (Yamakawa et al. 1998). Similar upregulation of *HIN1* expression was observed in *NN*-genotype tobacco plants infected with TMV. Spm or TMV specifically induces the increase in HIN1 because other PAs, SA, ET, and JA fail to induce *HIN1* expression. Spm may be important for resistance to other viruses. In Arabidopsis, the up-regulation of *HIN10* during *RCY1*-mediated resistance to CMV-Y is completely independent of SA (Zheng et al. 2004).

Downstream signaling components

Early downstream signaling components

The CC and TIR domains of R genes are structurally different and may signal through different signaling pathways. Many of the TIR-NBS-LRR class of R proteins signal through an *EDS1*-dependent pathway and the CC-NBS-LRR class of R proteins signal through the *NDR1*-dependent pathway (Aarts et al. 1998). *EDS1* is necessary for the function of TIR-NBS-LRR subclass antiviral R protein N (Liu et al. 2002a; Peart et al. 2002a) but not for the CC-NBS-LRR subclass protein Rx1 (Peart et al. 2002a).

The *Arabidopsis* mutant *ndr1-1* has compromised resistance to both bacteria and fungi, but its requirement during virus resistance has not been investigated. *RPP8*, which belongs to the same family as *RCY1* and *HRT* does not require *EDS1* or *NDR1* (McDowell et al. 2000). Therefore, a pathway that is independent of *EDS1* and *NDR1* must exist. *RPP8* has 98% homology to *HRT* and 93% homology to *RCY1* in the CC domain, suggesting that the two antiviral *R* genes products may signal through a third, unknown signaling component. It is possible that *HRT* and *RCY1* do not need *NDR1* but may need one of its homologues. There are 45 *NDR1/HIN1-like* (*NHL*) genes in *Arabidopsis* (Zheng et al. 2004). Interestingly, *NHL10* is highly up-regulated during an RCY1-mediated response to CMV-Y but not during the susceptible response to CMV-B2. Mutants in *EDS1*, *NDR1*, and *NHL10* will have to be analyzed further to determine if *HRT* or *RCY1* signal through one or more of these signaling components.

MAPK cascades

Mitogen-activated protein kinase (MAPK) cascades play roles diverse in plant processes that include cytokinesis, phytohormone signaling, wound responses, osmotic stress, and pathogen resistance (reviewed in Zhang and Klessig, 2001). A MAPK cascade proceeds via a hierarchy of protein kinases in which a MAPK kinase kinase (MAPKKK) activates a MAPK kinase (MAPKK)by phosphorylation, which in turn, activates a MAPK by phosphorylation. During *N*-mediated resistance to TMV two MAPKs, SA-induced protein kinase (SIPK) and wounding-induced protein kinase (WIPK), are activated (Zhang and Klessig, 1998a; Zhang and Klessig, 1998b). Recently it was discovered that a naturally- occurring diterpene, (11E, 13*E*)-lambda-11,13-diene-8α, 15-diol, known as WAF-1 may be the upstream activator of WIPK and SIPK (Seo et al. 2003). Exogenously supplied synthetic and natural WAF-1 activate WIPK and SIPK independently of SA signaling (Seo et al. 2003). WAF-1 levels increase rapidly upon TMV infection in *NN* plants, suggesting WAF-1 may be the endogenous signal for WIPK and possibly SIPK during disease resistance (Seo et al. 2003).

The biological role of SIPK and WIPK function during viral resistance was investigated by over-expression and silencing of *SIPK* and *WIPK*. Silencing *SIPK* and *WIPK* by PVX-based virus-induced gene silencing (VIGS) resulted in an attenuation of *N*-mediated resistance to TMV (Jin et al. 2003). Interestingly, even though both are necessary for defense, over-expression of *SIPK*, but not *WIPK*, leads to HR-like cell death. Silencing of *WIPK* leads to a loss of *N*-mediated resistance but has no effect on the HR. Directly upstream of *SIPK* and *WIPK* is *NtMEK2*, a MAPKK (Yang et al. 2001; Jin et al. 2003). A constitutively active form of NtMEK2, *NtMEK2^{DD}*, causes an activation of SIPK and WIPK and leads to HR-like cell death (Yang et al. 2001). Silencing *NtMEK2* also causes an attenuation of *N*-mediated resistance to TMV (Jin et al. 2003). The MAPKKK upstream of *NtMEK2* and the downstream target of *SIPK* or *WIPK* are currently unknown.

A complete MAPK cascade involving The *NPK1-MEK1-NTF6* has been shown to be necessary for *N*-mediated resistance to TMV (Liu et al. 2004a). Silencing of the MAPKKK, *NPK1*, by VIGS compromises the function of the *N* gene (Jin et al. 2002). Silencing the downstream MAPKK, *MEK1/NQK1*, and the MAPK, *NTF6/NRK1*, by VIGS cause a loss of *N*-mediated resistance to TMV (Liu et al. 2004a).

Transcription factors

TGA, MYB, and WRKY families of transcription factors have been implicated in disease resistance. Activation of TGA by NPR1 is necessary for SA-dependent resistance to non-viral pathogens (Durrant and Dong, 2004). The triple mutant of *tga2-1, tga5-1,* and *tga6-1* was unable to induce the expression of PR genes in response to an SA analog, 2,6 dichloroisonicotinic acid (INA) (Zhang et al. 2003). However, single and double mutants were still responsive to INA suggesting that *TGA2*, *TGA5*, and *TGA6* function redundantly in signaling NPR1-dependent regulation of *PR* genes (Zhang et al. 2003). Silencing of *TGA1a, TGA2.1, TGA2.2,* and *TGA6* by VIGS did not compromise *N*-mediated resistance to TMV (Liu, Y., Schiff, M., and S.P.D-K, unpublished results). However, silencing multiple TGA factors by mixed infection resulted in a partial loss-of-resistance to TMV. Therefore, it is likely that *N*-mediated resistance responses are *NPR1*-dependent and function through TGA transcription factors. Further research must be conducted to conclusively determine the role of TGA transcription factors during viral resistance.

MYB1 expression is induced by SA and during the *N*-mediated response to TMV in tobacco (Yang and Klessig, 1996). The transcription factor MYB1 has been shown to bind to the sequence GTTTGGT in the promoter of *PR-1a*. *MYB1* plays a biologically significant role during *N*-mediated defense because silencing of *NbMYB1* attenuates *N*-mediated resistance to TMV in *N*-transgenic *N. benthamiana* using VIGS (Liu et al. 2004a).

WRKY transcription factors play a crucial role in regulating multiple defense response genes (Eulgem et al. 2000). WRKY transcription factors bind to the W-box ((T)TGAC(C/T)) sequence found in the promoters of various genes including *PR-1*, *PR-2*, and *PR-3* (Eulgem et al. 2000). Overexpression of *WRKY70* induces the constitutive expression of PR proteins (Li et al. 2004). *NtWRKY3* and *NtWRKY4* are highly induced by SA and during the *N*-mediated response to TMV (Chen and Chen, 2000), while the level of *WRKY1* transcript increases to a lesser degree and the level of *WRKY2* transcript does not change (Yang et al. 1999). VIGS of *WRKY1*, *WRKY2*, or *WRKY3* in *NN-transgenic N. benthamiana* plants compromises *N*-mediated resistance to TMV but has no effect on HR (Liu et al. 2004a). This is the first biological evidence that WRKY factors are necessary for viral resistance. However, the function of other members of this numerous family in disease resistance remains to be investigated.

Expression of *WRKY* transcription factors are up-regulated after the activation of SIPK and WIPK. Additionally, SIPK and WIPK induce W-box binding activity of unidentified WRKYs *in vivo* (Kim and Zhang, 2004). This suggests that *WRKYs* are downstream of the *MEK2-SIPK/WIPK* cascade. WRKYs are not phosphorylated, and therefore, there must be additional unknown components between SIPK/WIPK and WRKYs (Kim and Zhang, 2004). Additionally, WRKYs may function by regulating *NPR1* gene expression. The promoter of *NPR1* has three W-box domains that SA-induced WRKY transcription factors specifically bind to (Yu et al. 2001). However, these specific WRKYs have not been identified.

Protein degradation in defensive signaling

Degradation by the 26S proteosome

SGT1 has a second function during disease resistance. In yeast, SGT1 is a conserved component of the ubiquitin ligase SCF (SKP1, Cullin/F-box proteins) complex (Kitagawa et al. 1999). SCF complexes recruit specific proteins and catalyze covalent attachment of ubiquitin. Often, ubiquitinated proteins are targeted for subsequent degradation via the 26S proteasome (Deshaies, 1999). The SGT1-SCF complex may function by targeting regulatory proteins for degradation via the 26S proteosome. For example,

the ubiquitin ligase $SCF^{EBF1/EBF2}$ directs the proteolysis of the EIN3 transcription factor that is both necessary and sufficient for the activity of the ethylene signaling pathway (Guo and Ecker, 2003). The SCF^{COI1} complex regulates JA sensitive genes by controlling protein degradation of histone deacetylases (Devoto et al. 2002). Interestingly, NbSGT1b interacts directly with NbSKP1 of the SCF complex and silencing either of these genes in *N. benthamiana* compromises *N*-mediated resistance to TMV (Liu et al. 2002b).

Recently it was shown that SCF^{COI1} complex, like other plant SCF complexes, is regulated by the COP9 signalosome (CSN) (Feng et al. 2003). The CSN regulates the SCF complex activity by modulating cycles of addition and removal of the ubiquitin-like protein NEDD8 to the SCF subunit, Cullin1 (Deshaies, 1999). Silencing the genes encoding the CSN subunits, *CSN3* and *CSN8*, results in a loss of *N*-mediated resistance to TMV (Liu et al. 2002b). Furthermore, SGT1 associates with the CSN, which provides additional evidence that SGT1 is involved in SCF complex regulation by the CSN (Azevedo et al. 2002; Liu et al. 2002b). In conclusion, silencing of many different components of the ubiquitin-proteosome degradation machinery has resulted in a loss of *N*-mediated resistance to TMV. Since F-box proteins determine the substrate specificity of SCF complexes, it will be important to determine which F-box proteins are necessary for disease resistance and the identity of the F-box's substrates that are subsequently targeted for degradation.

Degradation by caspase-like proteins

The destruction of mammalian cells during PCD is often mediated by caspases that specifically cleave substrates (Zhivotovsky, 2003). There are no known homologues to mammalian caspases in plants but it appears that proteins with caspase-1 or -3 activity play crucial role during the initiation of a TMV-induced HR. Caspase-like protease activity is induced during HR and caspase-1 and -3 inhibitors can prevent TMV-induced HR (del Pozo and Lam, 1998; Chichkova et al. 2004). Both H_2O_2 and NO induced cell death is inhibited by the caspase-1 inhibitor Ac-YVAD-CMK, and therefore, ROI and NO signaling may converge before caspase-1 signaling pathway (Clarke et al. 2000).

Recently, a vacuolar protease (VPE) was shown to have caspase-1 like activity and may trigger HR by aiding in vacuolar collapse (Hatsugai et al. 2004). Silencing of *VPE* suppresses the TMV-induced HR in *NN* plants and increases the protein abundance level of TMV CP (Hatsugai et al. 2004). Further experimentation will have to be conducted to determine if VPE mutants or silencing of VPE causes loss of resistance to TMV. Interestingly, silencing of *VPE* did not increase the protein abundance level of PR-1 and

PR-2. This suggests that VPE belongs to a signaling pathway that regulates HR and possibly *N*-mediated resistance to TMV, but is not necessary for the induction of downstream PR proteins.

Concluding Remarks

In the past decade the inventory of components used during viral recognition and signaling has grown dramatically. The next challenge is to determine how these components form the machinery that drives recognition and signal transduction during disease resistance. Many antiviral *R* genes such as *N, Tm-2*^{2}, *Rx1*, *Rx2*, *Sw-5*, *HRT*, and *RCY1* have been cloned and many of the corresponding viral products that these *R* genes recognize have been discovered. This is an exciting time to study disease resistance signaling to viruses because despite major advances the mechanism for recognition and resistance to viruses is still unclear.

Disease resistance to any pathogen requires a dramatic reprogramming of the cell. Small signaling molecules such as ROIs, SA, JA, ET, and polyamines induce multiple signaling pathways. Many of these pathways function in parallel to one another, while others crosstalk or converge. Consequently, we are faced with a complex signal transduction network whose dissection will require various experimental approaches. For example, powerful genetic approaches have discovered upstream divergence points, such as Rar1, EDS1, and NDR1, as well as downstream convergence points, such as NPR1. Functional genomics have elucidated the interplay between pathways and have implicated gene families, such as the WRKY family of transcription factors. Reverse genetic approaches like VIGS (Burch-Smith et al. 2004) have determined biological roles of JA, ET, and various transcription factors during *N*-mediated resistance to TMV. Thus, as we trek forward, we must carry along these robust, successful approaches and add new tools to our repertoire, such as advanced proteomics and computational biology.

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Chapter A5

The Local Lesion Response

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Introduction

Viruses that cause economically important diseases spread systemically in the plant. However, in several laboratory test or indicator plants, the virus after multiplying in several hundred cells around the point of entry, does not continue to spread and remains in a local infection. Several types of local infections are known (Loebenstein et al. 1982): (a) self-limiting necrotic local lesions such as *Tobacco mosaic virus* (TMV) in *Datura strammonium,* where lesions reach their maximum size three days after inoculation; (b) chlorotic local lesions, such as *Potato virus Y* (PVY) in *Chenopodium amaranticolor,* where infected cells lose chlorophyll; (c) ring-like patterns or ringspots that remain localized, such as *Tetragonia expansa* infected with *Tomato spotted wilt virus* (TSWV); (d) starch lesions, such as TMV in cucumber cotyledons, where no symptoms are observed on the intact leaf, but when it is decolorized with ethanol and stained with iodine, lesions become apparent; (e) microlesions (with a mean size of 1.1 x 10^{-2} mm^2) , such as the U_2 of TMV on Pinto bean leaves; and (f) subliminal symptomless infections not detectable as starch lesions., as in TMV-infected cotton cotyledons, where virus content is 1/200,000 of that produced in a systemic host (Cheo, 1970). The localized infection is an efficient mechanism whereby plants resist viruses, though most viral resistance genes

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are not associated with the hypersensitive response (HR), but affect virus multiplication or movement as a result of incompatible viral and host factors. The local lesion infection is one of the most notable resistance responses and has been used by breeders to obtain resistant cultivars of tobacco and sweet peppers against TMV.

The zone around a TMV lesion on tobacco NN is also resistant to other strains of TMV (tomato aucuba virus and Holmes' ribgrass strain), *Tobacco necrosis virus* (TNV) and *Tomato ringspot virus* (ToRSV), but not to *Turnip mosaic virus* (TuMV). TNV also induced localized resistance to TMV (Ross, 1961). This type of induced resistance was called *localized acquired resistance* (LAR). Apparently in the zone around the lesion virus replication is inhibited.

In *Nicotiana glutinosa* local lesion cells infected with TMV the number of virus particles per cell is about 10^3 (Milne, 1966) or two to four orders of magnitude lower than in a comparable systemic infection, where the number of particles per cell is estimated to be between 10^5 and 6×10^7 (Harrison, 1955). TMV content (as measured by extractable infectivity) in protoplasts of *N. tabacum* Samsun NN, where TMV induces local lesions, was about ¼ - 1/10 of that in protoplasts of *N. tabacum* Samsun, where TMV spreads systemically in the intact plant (Loebenstein et al. 1980. {That in isolated protoplasts from these two cultivars TMV multiplies to the same extent (Otsuki et al. 1972) was due to the presence of 2,4-dichlorophenoxyacetic acid (2,4-D) in the protoplast incubation medium (Loebenstein et.al.1980). 2,4-D has been reported to suppress localization and to enhance virus multiplication in local lesion intact hosts (Simons and Ross, 1965)}. These data indicated that localization is at least partly due to reduced multiplication in the cells of these hosts and not due barrier substances, implicated in early research as possible factors preventing virus movement.

Generally TMV induces local lesions in tobacco containing the *N* gene (NN). The *N* gene originally identified in *N. glutinosa* (Holmes, 1938) is a single locus, dominant gene and a durable source of resistance against tobamoviruses. The gene was transferred from *N. glutinosa* through the amphidiploid *N. digluta* by repeated backcrossing (Holmes, 1954). The genetic history was described by Dunigan et al. (1987). However, the tomato mosaic virus-OB overcomes the *N* gene mediated hypersensitive response (HR) (Tobias et al. 1982) and Padgett and Beachy (1993) showed that the movement protein (see below) alone is not responsible for the resistance breaking character of the OB strain.

The NN-associated resistance affects both cell-to-cell movement and long distance movement of TMV compared to that in tobacco nn. However, in tobacco NN plants kept at temperatures above 28ºC this restriction response is inactive and TMV spreads throughout the plant. Reducing the temperature below 28ºC again allows activation of the N gene, resulting in

necrosis of all the tissues containing TMV and restricting further virus movement. However, movement of a TMV-based vector expressing green fluorescent protein (TMV-GFP) is restricted in tobacco NN, also when these plants were incubated at 33ºC, showing only limited movement. In contrast, TMV-GFP moved efficiently in tobacco that contained the N gene and were transgenic for RNA1 of *Cucumber mosaic virus* (CMV) (Canto and Palukaitis, 2002). These findings indicated a novel temperature-independent resistance to the movement of TMV-GFP, which operates via a pathway independent of salicylic acid (Canto and Palukaitis, 2002).

The L gene in Tabasco and *Capsicum chinense* peppers confers hypersensitivity to infection with TMV. Several alleles occur at a single locus and are partially dominant (Boukema, 1980); and a single dominant gene in beans controls local lesion formation by *Southern bean mosaic virus* (Holmes, 1954).

Cauliflower mosaic virus induces necrotic local lesions in tobacco and *Datura*, *Tomato bushy stunt virus* in tobacco*,* CMV in cowpea, *Potato virus X* in potato cultivars carrying the Nx or Nb genes (Cockerham, 1955) and *Barley stripe mosaic virus* in *Chenopodium amaranticolor.*

In *Arabidopsis*, *Turnip crinkle virus* (TCV) produces an HR 2 to 3 days postinoculation in ecotype Dijon (Simon et al. 1992; Dempsey et al. 1993). Other ecotypes of *Arabidopsis* tested did not give an HR but allow systemic spread of the virus (Li and Simon, 1990). A dominant gene, *HRT,* which confers an HR to TCV, has been identified and mapped in the Di-17 line of Dijon (Dempsey et al. 1997).

Cytopathic changes

In the necrotic lesion the cytopathic cevents leading to collapse and necrosis start with changes in the chloroplast, consisting of swelling and distortion of the chlorophyll lamella and swelling of starch grains, about eight hours after infection (Weintraub and Ragetli, 1964). This led to increased electrolyte leakage, even before local lesions became visible (Weststeijn, 1978), and, for example, the superoxidic radical monohydroascorbate increased markedly when TMV lesions on Xanthi-nc-tobacco leaves developed (Fodor et al. 2001). Incubation of NN tobacco plants to 32^0 or higher inhibits the *N* gene-mediated HR. Transfer back to 20^0 initiates the HR again. Using fluorescent-tagged TMV revealed membrane damage, which preceded visible cell collapse by more than 3 h, and was accompanied by a transient restriction of the xylem within infection sites on *N. edwardsonii.* Following cell collapse and the rapid desiccation of tissue undergoing the HR, isolated, infected cells were detected at the margin of necrotic lesions. These virus-infected cells were able to reinitiate infection on

transfer to 32°C, however, if maintained at 20°C they eventually died. The results indicate that the tobacco mosaic virus-induced HR is a two-phase process with an early stage culminating in rapid cell collapse and tissue desiccation followed by a more extended period during which the remaining infected cells are eliminated (Wright et al. 2000). This programmed cell death (PCD) was accompanied by accumulation of PR1, a protein that is induced during the HR (Linthorst, 1991). Okadaic acid (OA), an inhibitor of type 1 and type 2A serine/threonine protein phosphatases, can block both *N* gene-mediated HR and developmental PCD in plants (Dunigan and Madlener 1995; Lacomme and Santa Cruz, 1999). It was suggested that the TMV-mediated lethal HR in plants requires reversible-protein phosphorylation in a signaling pathway that initiates the cell-death program; however, after entering the execution phase of the program, the process becomes irreversible (Lacomme and Santa Cruz, 1999). There are some parallels between plant HR and animal PCD. One similarity between plant and animal cell-death processes is the apparent role of protein phosphatase(s), which is required for developmental and pathogen Cas-triggered PCD in plants (Dunigan and Madlener 1995) and is also implicated in animal PCD where protein phosphatase 2A activity is specifically up-regulated by a cell-death-related protease (Morana et al. 1996). A key event in animal PCD is the release of cytochrome *c* from mitochondria into the cytosol, initiating the final degradation phase of the cell-death program (Green and Reed, 1998). The ability of the GFP-TM fusion protein to target mitochondria suggests that mitochondrial targeting is also necessary for the plant response (Lacomme and Santa Cruz, 1999). Caspase-like proteases, known to suppress PCD in animals, seem to participate during HR The p^{35} protein from baculovirus is a broad-range caspase inhibitor, and infection of p^{35}– expressing *N* tobacco plants with TMV disrupted *N*-mediated, leading to systemic spreading of the virus (Pozo and Lam, 2003).

The three-dimensional structure of CP is critical to induce the HR response, either directly through specific structural motifs or indirectly via alterations in CP assembly (Culver, 2002).

HR requires cell-to-cell contact and is not expressed in protoplasts. Protoplasts from plants carrying the N gene do not respond to TMV infection with cell death, though TMV multiplication in them is reduced markedly (Loebenstein et al. 1980). Actinomycin D or chloramphenicol when added up to 24 hr after inoculation (but not later) markedly increased TMV replication in protoplasts of tobacco NN, while no increase was observed in protoplasts of tobacco nn (Gera et al. 1983). This indicated that HR and inhibition of virus replication associated with the *N* gene are two different processes, and that this mechanism which presumably requires

DNA-dependent RNA synthesis for its operation, produces a substance that inhibits virus replication (see IVR below).

In leaves of *Nicotiana edwardsonii*, an interspecific hybrid derived from a cross between *N. glutinosa* and *N. clevelandii Cauliflower mosaic virus* strain W260 elicits a hypersensitive response (HR). *N. glutinosa* is resistant to W260, but responds with local chlorotic lesions rather than necrotic lesions. In contrast, *N. clevelandii* responds to W260 with systemic cell death. It was shown that the resistance and cell death that comprise the HR elicited by W260 could be uncoupled. The non-necrotic resistance response of *N. glutinosa* could be converted to HR when these plants were crossed with *N. clevelandii*. Also, cell death and resistance segregated independently in the F2 population of a cross between *N. edwardsonii* and *N. clevelandii*. Cole et al. (2001) concluded that the resistance of *N. edwardsonii* to W260 infection was conditioned by a gene derived from *N. glutinosa*, whereas a gene derived from *N. clevelandii* conditioned cell death.

In chlorotic local lesions, induced by TuMV in *C. quinoa,* disintegration of chloroplasts was not observed, although most palisade and spongy mesophyll cells contained chlorotic chloroplasts forming large aggregates, up to 20 chloroplasts, instead of being uniformly distributed along the cell periphery (Kitajima and Costa, 1973).

In starch lesion hosts, such as TMV in cucumber cotyledons, swelling of chloroplasts are first observed 2 to 2.5 days after inoculation. When viewed 5 days after inoculation these chloroplasts contain large starch grains, but neither they nor the cells disintegrate or die. In the peripheral cells of a starch lesion the number of TMV particles was about 1/10 compared with those in the central part of the starch lesion, with no barriers or ultrastructural changes at the border of the lesion (Cohen and Loebenstein, 1975). In early studies it was observed that when cucumber cotyledons were treated with actinomycin D, chloramphenicol or UV irradiation one day after inoculation with TMV virus concentration increased markedly, indicating that during localization a substance is produced that reduces virus multiplication (Loebenstein et al. 1969;1970; Sela et al. 1969).

Pathogenesis-related proteins (PR-proteins)

Necrotic lesion formation is associated with the induction of a number of PR proteins (Van Loon and Van Kammen, 1970). Tobacco PR proteins consist of at least five families, each of which contains both acidic and basic isoforms (Van Loon et al. 1994). There are 14 families of PR protein (PR1-14), PR-2 and PR-3 having β-1,3-glucanase and chitinase activities, respectively (Kauffmann et al. 1987; Legrand et al. 1987). These proteins have been studied extensively as they can be detected easily by gel-

electrophoresis, but so far no evidence has been provided that they are active in localizing the virus. They are mainly induced in virus infections that do cause necrosis and may therefore be a host response to necrosis (or other stresses). However, PR-proteins were also seen in non-necrotic systemic infections by some viruses as CMV and PVY (Whitham et al. 2002). No antiviral activity of any of the PR-proteins has so far been reported.

Compounds that induce resistance

Various compounds injected into the intercellular spaces of tobacco NN or *Datura stramonium* induced resistance to TMV, resulting in fewer and smaller lesions. Thus, yeast RNA (Gicherman and Loebenstein, 1968), Poly I: Poly C (Stein and Loebenstein, 1970), heat-killed cells of *Pseudomonas syringae* (Loebenstein and Lovrekovich, 1966), polyacrylic acid (Gianinazzi and Kassanis, 1974), several synthetic polyanions (Stahmann and Gothoskar, 1958; Stein and Loebenstein, 1972), root extracts of *Boerhaavia diffusa* - a glycoprotein, applied to the lower leaves (Verma et al. 1979; Verma and Awasthi, 1980), mannan sulphates (Kovalenko et al. 1993), a protein from *Mirabilis jalapa* (Kubo et al. 1990) and other plant extracts (reviewed by Verma et al. 1998) were found to be active. Some of these compounds were also inducers of interferon. Apparently, the mechanism that inhibits virus replication in the local lesion area (and induced resistance) can be activated by various inducing compounds.

Salicylic acid

During the hypersensitive response of *N. tabacum* plants, that possess the *N* gene for resistance, to TMV, salicylic acid (SA) levels rise markedly (Malamy et al. 1990). It was suggested that SA plays a role in localization of the virus, as *NN*-genotype transgenic tobacco plants, which have been transformed with a bacterial salicylate hydroxylase gene and, therefore, cannot accumulate SA, do not limit virus spread. Although the cells of these plants can still undergo HR-type cell death, the plants exhibit a spreading necrosis after TMV inoculation (Mur et al. 1997; Darby et al. 2000), showing that SA accumulation is required to localize TMV. Treatment of susceptible tobacco with aspirin (acetyl-SA) or SA caused a significant reduction in accumulation of TMV in susceptible tobacco cultivars that do not respond hypersensitively to TMV (White et al. 1983; Chivasa et al. 1997). In leaf mesophyll cells of SA-treated plants replication of TMV is greatly decreased, but not in initially inoculated epidermal cells. However, SA induces resistance to movement between epidermal cells, though SA did not inhibit TMV movement by decreasing the plasmodesmatal size exclusion

limit (Murphy and Carr, 2002). SA stimulated formation of callose in *N. glutinosa* infected with TMV, probably affecting the gating capacity of plasmodesmata (Krasavina et al. 2002). Activation of SA-induced protein kinase (SIPK) and wounding-induced protein kinase (WIPK) leads to HR-like cell death (Zhang et al. 2000). WIPK is activated in NN tobacco infected with TMV. *Potato virus X* (PVX)-virus-induced gene silencing attenuated *N* gene mediated resistance (Liu et al. 2003). A WIPK-activating substance was isolated from tobacco leaves and identified as a diterpene. When this compound, natural or chemically synthesized, was applied at nanomolar concentrations to leaves, SA-induced protein kinase was activated and accumulation of transcripts of wound- and pathogen-inducible defense - related genes was enhanced. Treatment of leaves with this diterpene increased resistance to TMV infection (Seo et al. 2003), and in tobacco leaves treated with sulfated fucan oligosaccharides SA accumulated and both local and systemic resistance to TMV was strongly stimulated (Klarzynsky et al. 2003). SA pretreatment primed TMV-infected Xanthi-nc leaves for strong antioxidant induction (Kiraly et al. 2002).

In SA-treated tobacco plants activity of RNA-dependent RNA polymerase (RdRP) increased (Xie et al. 2001). Biologically active SA analogs capable of activating plant defense response also induced the RdRP activity, whereas biologically inactive analogs did not. A tobacco gene, NtRDRP1, was isolated and found to be induced both by virus infection and by treatment with SA, suggesting that inducible RdRP plays a role in plant antiviral defense. Similarly, SA induced in *Arabidopsis* an RdRP gene with a role in antiviral defense (Yu et al. 2003).

Accumulation of PVX is inhibited at the site of inoculation in SA-treated tobacco plants, but not *Cucumber mosaic virus* (CMV), though SA inhibited CMV movement from the inoculated leaf to the rest of the plant (Naylor et al. 1998). Apparently, SA is a component of the signal transduction pathway for induction of resistance, both local and systemic acquired resistance (SAR) (Dempsey et al. 1999; Murphy and Carr, 2002).

Some hypotheses that were raised explaining localization

Death of cells in a necrotic lesion may localize or inactivate the virus. This explanation is not satisfactory even in a necrotic local lesion host and even less so in a chlorotic or starch lesion host, as virus particles are found in apparently viable cells outside the necrotic area (Milne, 1966). Furthermore, studies with green fluorescent protein (GFP)-tagged TMV (TMV.GFP) have shown that live cells around the necrotic area contain TMV for significant periods of time after lesion formation (Wright et al. 2000; Murphy et al. 2001).

Barrier substances that have been observed to surround local lesions include *inter alia* callose deposition around TMV-induced lesions on Pinto beans (Wu and Dimitman, 1970), calcium pectate in the middle lamella of cells surrounding TMV lesions on *N. glutinosa* (Weintraub and Ragetli, 1961), lignin (Favali et al. 1974) and suberin (Faulkner and Kimmins, 1978) (see also Loebenstein, 1972). However, callose depositions were also observed in infections, which produced systemic necrosis, where the virus does not remain localized, as with tobacco infected with TSWV and *Tobacco rattle virus* (Shimomura and Dijkstra, 1975). On the other hand no callose deposition was observed around TMV-induced starch lesions on cucumber cotyledons, where the infection remains localized without necrosis (Cohen and Loebenstein, 1975). It seems, therefore, that the observed barrier substances are a response to necrotization and not necessarily responsible for the localization.

Inactivation of the virus-coded "transport protein" required may also help in the localization of the virus. For TMV, this factor has been identified as a 30kDa non-structural protein (Leonard and Zaitlin, 1982). It was shown that the 30kDa protein accumulated in plasmodesmata, an observation consistent with a role in virus spread (Tommenius et al. 1987). It was also shown that a non-structural protein (P3) of *Alfalfa mosaic virus,* considered to be involved in cell-to-cell spread, is associated with the middle lamella of cell walls (Stussi-Garaud et al. 1987). It was reported that the amount of the 30kDa protein in the cell wall fraction of TMV-infected Samsun NN tobacco plants decreased sharply as soon as necrosis became visible, compared with that in the systemic host Samsun nn. It was suggested that this might explain why TMV infection becomes localized (Moser et al. 1998). In tobacco NN plants the TMV movement protein alters the gating capacity of plasmodesmata and therefore the efficiency of virus movement (Deom et al. 1991). However, the decrease of transport protein alone does not seem to responsible for localization, especially as virus particles are found outside the necrotic area (Milne, 1966), and as mentioned above in starch lesions there is no necrotization. Also, the HR in Samsun NN tobacco can be inhibited without affecting the localization of the virus (Takusari and Takahashi, 1979). Furthermore, infection of cowpea by strains of cucumber mosaic virus involves a local HR and a localization of infection (inhibition of viral RNA synthesis - IR) (Kim and Palukaitis, 1997). Different combinations of specific sequence alterations in the polymerase gene can separate these responses. Kim and Palukaitis (1997) also showed that IR affects viral RNA synthesis in isolated cells, without HR.

Further information on cell-to-cell movement of plant viruses will be presented in chapter A13.

The N' gene

In *N. glutinosa* and *N. tabacum cultivars* that contain the *N* gene, TMV does not spread but remains localized in a lesion of several hundred cells. The *N* gene was originally transferred from *N. glutinosa* to *N. tabacum* via an interspecific hybrid, *N. digluta* (Clausen and Goodspeed, 1925). Holmes (1938) showed that resistance to TMV was controlled by a single dominant gene - which he termed *N*. However, some other genes were also introgressed into tobacco from *N. glutinosa* together with the *N* gene locus (Holmes, 1938). The genetic and breeding history was described by Dunigan et al. (1987). This gene was of major importance in infectivity assays, as the number of necrotic lesions was in a certain proportion to the virus content (Kleczkowsky, 1950). In the early 1990s, the *N* gene was the first plant virus resistance gene to be isolated by transposon tagging, using the maize activator transposon (Whitham et al. 1994; Dinesh-Kumar et al. 1995). The *N* gene encodes a protein of 131.4 kDa, which has three domains: an N-terminal domain similar to that of the cytoplasmic domain of the *Drosophila* Toll protein and the interleukin-1 receptor (TIR) in mammals, a nucleotide-binding (NBS) site and four imperfect leucine-rich regions (LRR). The *N* gene thus belongs to the TIR-NBS-LRR class of *R* genes. The *N* gene encodes two transcripts, N_S and N_L, via alternative splicing of the alternative (AE) exon present in the intron III. The N_S transcript, predicted to encode the full-length N protein containing the Toll-IL-1 homology region, nucleotide binding site, and LRR, is more prevalent before and for 3 hr after TMV infection. The N_L transcript, predicted to encode a truncated N protein (N^{tr}) lacking 13 of the 14 repeats of the LRR, with a deduced molecular weight of 75.3 kDa, is more prevalent 4-8 hr after TMV infection. The ratio of N_s to N_t before and after TMV inoculation is critical to achieve complete resistance. The N^{tr} protein is identical to the amino terminal portion of the N protein, with an additional 36 amino acids at the C-terminus. Plants harboring a cDNA-N_S transgene, capable of encoding an N protein but not an N^{tr} protein, fail to exhibit complete resistance to TMV. Transgenic plants containing a cDNA-N_S-bearing intron III (without introns I, II and IV) and containing 3' *N*-genomic sequences, encoding both N_S and N_L transcripts, exhibit complete resistance to TMV. These results suggest that both *N* transcripts and presumably their encoded protein products are necessary to confer complete resistance to TMV. However, deletion of the 70 bp alternative exon (AE) with flanking splice acceptor-donor sites, resulted in delayed HR upon infection with TMV and the virus continued to spread, resulting in systemic HR (Dinesh-Kumar and Baker, 2000). The presence of TIR, NBS and LRR domains are all necessary for proper *N* function (Dinesh-Kumar et al. 2000). Most in frame deletion mutants in the *N* gene abolished resistance to TMV.

Also, some amino acid substitutions within the TIR domain caused a complete loss of the *N* function, and the plants developed systemic HR. It was concluded that mutations that affect *Drosophila* Toll or human IL-IR signaling also affect the *N*-mediated response to TMV (Dinesh-Kumar et al. 2000). The NBS domain of the *N* gene has amino acid homology with regions of cell death genes (Van der Biezen and Jones, 1998; Arvind et al. 1999). The NBS domain is also found in elongation factors and G-Protein families (Saraste et al. 1990). These serve as molecular switches in growth and differentiation. Point mutation in some subdomains of NBS led to loss of resistance. In some of them timing of HR appearance and size of lesions was normal, but TMV spread systemically through the plant, causing death of the plant within 5-7 days (Dinesh-Kumar et al. 2000). It was shown that introgression of the *N* gene coincided with introgression of DNA-bearing restriction enzyme fragments of *N. glutinosa* origin (Whitham et al. 1994). The *N* protein (to the best of our knowledge) has so far not been purified and its way of function has not been determined. It is possible that the *N* protein may trigger an intracellular signal transduction cascade and induces a variety of defense and signaling proteins, as for example the IVR protein (see below). It may be that the *N* protein activates the HR but activation of resistance requires another gene, similar to the HRT and RRT genes in *Turnip crinkle virus* (TCV) resistant *Arabidopsis* (Kachroo et al. 2000) (see below).

The *N* protein may also function as a receptor that interacts with the gene product of TMV that elicits HR, perhaps by activating a transcription factor that induces the expression of genes responsible for the HR (Whitham et al. 1994). It is suggested that the HR and inhibition of virus replication are two separate processes. Thus in some cases, HR may be activated but fails to restrict virus multiplication or movement resulting in systemic movement.

In addition to the *N* gene, other TIR-NBS-LRR genes, such as L6, RPP5 and RPS4 also encode two or more transcripts. The biological role of these genes is presently unknown (Marathe et al. 2002).

The pathway by which signals from the *N* gene product are transmitted is largely unknown. Yoda et al. (2002) identified seven tobacco genes that are associated with HR upon TMV infection. Transcriptional induction of one of these, which encodes a novel WRKY transcription factor, is independent of SA. Its full-length cDNA of 1346 bp encoded a polypeptide consisting of 258 amino acids. The deduced protein contained a single WRKY domain, a Cys_2His_2 zinc-finger motif and a leucine-zipper motif, showing high similarity to WIZZ, a member of the family of WRKY transcription factors in tobacco. This indicated the presence of salicylic acid-independent pathways for HR signal transduction, in which a novel type of

WRKY protein(s) may play a critical role for the activation of defense (Yoda et al. 2002).

The *N* gene has been transferred to tomato, where it confers resistance to TMV (Whitham et al. 1996).

The N protein, either by itself or in a protein complex, is hypothesized to specifically recognize the 50 kDa C-terminal helicase domain of the TMV replicase protein (Abbink et al. 1998; Padgett et al. 1997) and trigger a signal transduction cascade leading to induction of HR and restriction of virus spread.

In transgenic *Nicotiana benthamiana,* containing the tobacco *N* gene, it was shown that the *Rar1-*, *EDS1-*, and *NPR1/NIM1-* like genes are required for the N-mediated resistance (Liu et al. 2002a). The *Rar1* gene encodes a protein with a zinc finger motif, which is required for the function of *N.* It was shown that *N. benthamiana Rar*1 (NbRar1) protein interacts with NbSGT1 a highly conserved component of an E3 ubiquitin ligase complex involved in protein degradation. It also interacts with the COP9 signalosome, a multiprotein complex involved in protein degradation via the ubiquitine-proteasome pathway. Suppression of NbSGT1 and NbSKP1 (an SCF protein complex component) in *NN* transgenic plants by virus-induced gene silencing (VIGS) resulted in the loss of resistance to TMV. This indicates that NbSGT1 and NbSKP1 are required for *N* function (Liu et al. 2002b). It is interesting to note that genetic studies in barley suggested that *Rar1* functions downstream of pathogen perception and upstream of H_2O_2 accumulation and host cell death (Shirasu et al. 1999).

Infection of resistant tobacco plants that carry the *N* resistance gene with TMV leads to the activation of two tobacco mitogen-activated protein kinases (MAPK), salicylic acid-induced protein kinase (SIPK) and wounding-induced protein kinase (WIPK) (Zhang and Klessig, 1998). WIPK gene transcription is regulated by phosphorylation and de-phosphorylation events and may accelerate the HR cell death (Liu et al. 2003).

The *N* gene shows some structural similarities to a number of other plant genes for disease resistance, as the *RPS*2 and *RPM*1 genes for resistance to *Pseudomonas syringae* pathovars in *Arabidopsis* (Bent at al. 1994) and *Prf* for resistance to *P. syringae* in tomato (Salmeron et al. 1994). There seems therefore to be common structural elements in genes for resistance to different types of pathogens as the NBS and LRR motifs.

A gene with a function similar to that of a resistance gene named NH has been cloned from *N. tabacum* cv. Xanthi nn plants. The coding region of NH is 5.028 base pairs (bp) long and has 82.6% nucleotide identity with the N gene. In contrast to the N gene, the NH gene lacks intron 4 and does not have sites for alternative splicing of intron 3. Analysis of its sequence

revealed that NH belongs to the TIR/NSB/LRR gene class. It was suggested that this gene, homologous to the N gene, plays a role in the HR response of some *Nicotiana* species (Stange et al. 2004).

Other Host-Virus HR Responses

HR in potato to PVX is controlled by genes *Nb* and *Nx,* which have been mapped to a gene cluster in the upper arm of chromosome V (De Jong et al. 1997), and to a region of chromosome IX (Tommiska et al. 1998), respectively. The same region of chromosome IX contains the gene Sw-5 for resistance to tomato spotted wilt tospovirus in tomato. The *Nx*-mediated resistance us elicited by the PVX coat protein gene (Kavanagh et.al. 1992). Most PVX strains induce HR on potato carrying the Rx gene, which depends on the presence of a threonine residue at position 121 of the PVX coat protein. Elicitation of lesions on *Gomphrena globosa* also required presence of the threonine residue at position 121 of the coat protein (Goulden and Baulcombe, 1993). However, the Rx gene confers resistance to PVX in potato by arresting virus accumulation in the initially infected cell without an HR response (Köhm et al. 1993). Rx shows similarities with NBS-LRR class of *R* genes (Bendahmane et al. 1999). Also, many PVY strains induce necrosis (HR) in potato while spreading systemically in the plant (Jones, 1990). Again it is evident that HR and inhibition of virus replication are two separate processes.

The coding region of a potato gene termed Y-1 was found to be structurally similar to the tobacco *N* gene. It is located at the distal end of chromosome XI in potato *Solanum tuberosa subsp. andigena.* This gene also belongs to the TIR-NBS-LRR class and has 57% identity at the amino acid level as predicted by the sequence with that of the *N* gene The *R* gene rich region of chromosome XI is syntenic in potato and tobacco (Vidal et al. 2002). It contains the *N* gene and genes homologous to *N* with unknown functions in potato (Hamäläinen et al. 1998). The coding region of the gene is 6187-bp long. Leaves of transgenic potato plants expressing Y-1 developed necrotic lesions upon infection with PVY, but no resistance was observed, and plants became systemically infected by PVY (Vidal et al. 2002).

The dominant gene *HRT* in TCV-resistant *Arabidopsis* is a member of the class of resistance (*R*) genes that contain a leucine zipper, a nucleotide binding site, and leucine-rich repeats. This gene therefore belongs to the LZ-NBS- LRR class of genes. Inoculation of TCV onto resistant *Arabidopsis* leads to a HR. Other ecotypes of *Arabidopsis* do not give an HR but allow systemic spread of the virus (Li and Simon 1990). *HRT* was cloned and conferred HR TCV in transgenic plants (Cooley et al. 2000). *HRT* shares

extensive sequence similarity with members of the *RPP8* gene family, which confer resistance to the oomycete pathogen *Peronospera parasitica.* Transgenic plants expressing *HRT* developed an HR but generally remained susceptible to TCV because of a second gene, *RRT*, which regulates resistance to TCV (Kachroo et al. 2000). However some of the transgenic lines that were resistant did not develop a normal HR.

Some soybean lines resistant to *Soybean mosaic virus* with the resistant gene *Rsv1* show an HR response to infection (Hill, 2003). This gene is within a cluster with other resistance (R) genes against *Pseudomonas syringae, Phytophthora sojae,* a root-knot nematode and against *Peanut mottle* and *Peanut stripe virus* (mentioned in Penuela et al. 2002). Genes in this cluster code for proteins belonging to the nucleotide binding site (NBS) leucine-rich repeat (LRR) superfamily, with a coiled coil motif (nonTIR) (Penuela et al. 2002). In addition Wang et al. (2003) screened a soybean cDNA library and isolated resistance gene analogs with an NBS domain. A cDNA of 2533 bp in length was obtained that coded for a polypeptide of 636 amino acids with TIR and NBS domain, which was similar to the N gene of tobacco, with sequence identity of 28.1%. The expression of this gene could be induced by exogenous salicylic acid.

The N' gene

The *N'* gene, originating from *N. sylvestris*, controls the HR induced by many strains of TMV, except U1 and OM, which spread systemically and produce mosaic symptoms in *N'* containing plants. Mutants that induce necrosis can easily be isolated from infections causing systemic mosaic symptoms (Culver et al. 1991). This system is a nice one to demonstrate cross protection. Alterations in the structure of the tobacco mosaic virus (TMV) coat protein affect the elicitation of the N' gene hypersensitive response (HR) in N. sylvestris. Two specific amino acid substitutions within the virus coat protein were responsible for host recognition and HR elicitation (Culver and Dawson, 1989). Five amino acid substitutions were identified to elicit HR (Culver et al. 1991). Substitutions eliciting HR were within, or would predictably interfere with, interface regions between adjacent subunits in ordered aggregation of the coat protein. Substitutions that did not elicit the HR were either conservative or located outside the interface region. Radical substitutions that predictably disrupted coat protein tertiary structure prevented HR elicitation (Culver et al. 1994). Transgenic plants expressing elicitor coat proteins developed necrotic patches that eventually coalesced and collapsed entire leaves, demonstrating that

expression of elicitor coat proteins independently of viral replication can induce the HR in *N. sylvestris* (Culver and Dawson, 1991).

Inhibitor of virus replication (IVR)

Localization of TMV in tobacco, containing the N gene, is associated with the presence of a protein with antiviral properties named 'inhibitor of virus replication' (IVR) (Loebenstein and Gera, 1981; Gera and Loebenstein, 1983; Gera et al. 1990). IVR was released into the medium of TMV-infected protoplasts derived from *N. tabacum* cv. Samsun NN (Samsun NN) (in which the infection is localized in the plant). When added to the medium up to18 h after inoculation, IVR inhibited virus replication in protoplasts derived from both local lesions responding resistant Samsun NN and systemically responding susceptible *N. tabacum* cv. Samsun plants (Samsun nn).

IVR inhibited TMV in protoplasts and leaf disks, the effect being dose responsive, reaching 70-80% with 10 units of IVR (Gera et al. 1986). IVR also inhibited PVX, PVY and CMV in leaf disks from different hosts, indicating that IVR is neither host nor virus specific. IVR inhibited TMV replication in intact leaves when applied by spraying to tobacco and tomato plants and CMV in cucumbers. IVR was found to be sensitive to trypsin and chymotrypsin, but not to Rnase. And its ability was abolished by incubation at 60ºC for 10 min (Gera and Loebenstein, 1983).

IVR was also obtained from the intercellular fluid of hypersensitive tobacco leaves infected with TMV (Spiegel et al. 1989). Production of IVR by infected protoplasts and by intact Samsun NN plants was suppressed almost completely when exposed to 35ºC, leading to accumulation of TMV (Gera et al. 1993). Also treatment of Samsun NN protoplasts with actinomycin D and chloramphenicol 5 or 24 h after their inoculation decreased IVR in the incubation medium to close to zero, concomitant with a marked increase of TMV in the protoplasts (Gera et al. 1983). No increase was observed when TMV-infected protoplasts of Samsun (susceptible tp systemic infection by TMV) were incubated in the presence of these antimetabolites. A ca. 23kDa protein band was always associated with samples of crude protoplast IVR, tissue-IVR and IVR purified from induced-resistant tissue; this protein was absent in samples of uninfected plant tissue and protoplasts derived from them. Purification of the 23 kDa protein from SDS-polyacrylamide gels yielded a molecule with antiviral properties in biological tests (Gera et al. 1990). Antibodies against the IVR protein neutralized its antiviral activity and enabled immunodetection of the 23 kDA protein (Gera and Loebenstein, 1989; Gera et al. 1990).

Sequence analysis of clone NC330 indicated that the C-terminus of the deduced protein is highly acidic, rich in aspartic acid and glutamic acid, hydrophobic and with a helical structure (Akad et al. 1999). NC330 Protein (accession CAA08776) motif analysis in silico showed the presence of six sites typical for protein kinase one site for N-glycosylation, two N-merystylation sites and leucine rich repeats (LRR), but it is mainly a tetratricopeptide repeat (TPR) protein. These motifs are known to be involved in protein-protein interactions (Akad et al. in preparation). It is worthwhile to note that TPR motifs are present in many proteins including inducible interferons (Zhang and Gui, 2004; Der et al 1998)).

A direct involvement of TPR motif in plant resistance to pathogens was recently descried with RAR1 interactor protein. The RAR1 is an early convergence point in a signaling pathway engaged by multiple R genes (Azevedo et al. 2002). It was proposed that two TPR proteins RAR1 and SGT1 function with HSP90 in chaperoning roles that are essential for disease resistance (Takahashi et al. 2003; Hubert et al 2003).

When NC330 was compared with other proteins in the GenBank marked identities were observed with two putative proteins from *Arabidopsis* (accessions NP_850309 and A84813). Both proteins had 78% identity with the NC330 protein, indicating that NC330 is a well-preserved protein. The NC330 transcript homolog was found in several Expressed Sequence Tags (EST) from plants, mainly plants at different development stages or stressed plant tissue. This indicates that NC330 is essential for development (as SPY and OGT genes) and in resistance responses (as RAR1, SGT1 and HRT genes).

Transformation of *N. tabacum* cv. Samsun nn, in which TMV spreads systemically, with NC330, encoding an IVR-like protein, resulted in a number of transgenic plants expressing variable resistance to TMV and the fungal pathogen *Botrytis cinerea (*Akad et al. in preparation).

IVR-like compounds associated with other resistance responses

The interspecific hybrid of *N. glutinosa X N. debneyi* is highly resistant to TMV (Ahl and Gianinazzi, 1982). Following inoculation, only a small number of tiny lesions develop from which only an extremely low level of infectivity can be recovered. A specific band corresponding to a 23kDa protein was consistently observed In PAGE of crude hybrid IVR, both from TMV-inoculated and uninoculated hybrid plants. This band reacted specifically in immonoblots with IVR antiserum and crude extracts obtained from inoculated or uninoculated leaves of the hybrid gave positive reactions with IVR antiserum in agar-gel diffusion tests. The precipitation lines fused

without spur formation with the precipitation lines obtained between protoplast IVR and the antiserum. Based on these criteria IVR from the hybrid was indistinguishable from IVR obtained from Samsun NN (Loebenstein et al. 1990).

An IVR-like protein (about 23kDa) is constitutively produced in a resistant pepper cultivar, but not in a susceptible one. This constitutive production of the IVR-like protein in this host may be responsible for its high resistance to TMV (Gera et al. 1994).

A substance(s), which inhibits virus replication (IGI) was released by protoplasts obtained from green island leaf tissue of tobacco, cv. Xanthi-nc, infected with CMV. It was also obtained directly from green island tissue. IGI inhibited both CMV and TMV replication in protoplasts and leaf tissue disks, with the degree of inhibition being dependent upon concentration applied. The IGI was partially purified to yield two active fractions with molecular weights of about 26 kDa and 57 kDa. IGI appeared to be quite similar to IVR since both posses similar serological determinants. Fractions with similar activity were also obtained from green island leaf tissue of tobacco cv. Samsun, infected with CMV. However, these fractions differed serologically from both the IVR and IGI obtained from Xanthi-nc (Gera and Loebenstein, 1988).

Other compounds associated with the local lesion response - AVF

Another putative antiviral factor (AVF) from TMV infected *N. glutinosa* was reported (Sela and Applebaum, 1962), using hydrated calcium phosphate (HCP) to remove TMV from the extract. AVF was characterized as a glycoprotein (Mozes et al. 1978) and was purified by DEAE-cellulose chromatography, and finally by affinity chromatography on a column of concavalin A bound to Sepharose (summarized in Sela, 1981). However, purification resulted in several fractions exhibiting antiviral activity and very little protein. Further purification by immunoaffinity on a column of immobilized monoclonal antibodies to human β-interferon yielded 2 glycoproteins (gps) in pure state (Edelbaum et al. 1990). Computer analysis of partial sequences from them revealed no significant homology to human β-interferon, to each other or any other recorded sequences. However, in a later publication the these two gps to be a basic vacuolar form of beta-1, 3-glucanase while the second is closely related to both the acidic and basic forms of pathogenesis-related protein 5 (Edelbaum et al. 1991). It might be mentioned that so far no antiviral properties have been reported for any of the Pr proteins. This discrepancy may be due to differences in testing. While the Pr proteins were tested mainly as inhibitors of replication, either on virus infected protoplasts or on leaf tissue infected for several hours, AFV was

tested mainly as an inhibitor of infection by mixing the AVF preparation with the virus (Sela, 1981). This, therefore, raises the question if AVF is not one of the many plant extracts that inhibit infection, and not an inhibitor of virus replication as expected for a compound associated with resistance and virus localization.

Concluding thoughts

Major advances in understanding the mechanism of localization and induced resistance have been made since reviewing this subject 30 years ago (Loebenstein, 1972). At that time it became evident that necrotization and ultrastructural changes around the lesion were not the major cause for restricting the virus within the lesion. These predictions that necrotization and localization are two separate processes got support from additional experimental data, as with PVX in Rx potato and TCV in resistant *Arabidopsis.* Since then efforts were made to link Pr proteins as the main factor in the localization of the virus, but so far no evidence has been provided that they are active in localizing the virus. They are not induced in virus infections that do not cause necrosis and may therefore be a host response to necrosis (or other stresses). No antiviral activity of any of the PR-proteins has so far been reported.

A major achievement was in the early 1990s, when the *N* gene was isolated by transposon tagging (Whitham et al. 1994; Dinesh-Kumar et al. 1995) and its transcription strategy determined. The *N* gene encodes a protein of 131.4 kDa, which has three domains: an N-terminal domain similar to that of the *Drosophila* Toll protein and the interleukin-1 receptor (TIR) in mammals, a nucleotide-binding site (NBS) and four imperfect leucine-rich regions (LRR). The *N* gene thus belongs to the TIR-NBS-LRR class of *R* genes. Presence of the TIR-NBS-LRR domains in the predicted *N* protein suggests that it functions in a signal transduction pathway for induction of a cascade and induces a variety of defense and signaling proteins and salicylic acid. It may be that the *N* protein activates the HR but activation of resistance may require another gene, similar to the HRT and RRT genes in *Turnip crinkle virus* resistant *Arabidopsis* (Kachroo et al. 2000).

The *N* protein nay also function as a receptor that interacts with the gene product of TMV that elicits HR, perhaps by activating a transcription factor that induces the expression of genes responsible for the HR (Whitham et al. 1994). It is suggested that the HR and inhibition of virus replication are two separate processes. Thus in some cases, HR may be activated but fails to restrict virus multiplication or movement resulting in systemic movement.

The *N* protein by itself apparently does not inhibit virus replication by itself but induces a cascade of events. We speculate that IVR or an IVR-like protein is produced at the end of the cascade. This protein(s) could be the main factor responsible for inhibiting virus replication resulting in localizing the infection and subsequently in systemic induced resistance (SAR). The IVR protein not only inhibited TMV, but also PVX, PVY and CMV in leaf disks floated on an IVR solution, and may perhaps be a broad-spectrum interferon-like inhibitor.

As to the mode of action of IVR, a speculative possibility is that it binds small RNAs (small interfering RNA or micro RNA), which are involved in transcript turnover, cleavage and translational control (Hutvagner and Zamore, 2002), or part of the RNA-induced silencing complex (RISC) and degrade viral RNA. Recently, it was shown that in several plants small RNA binding proteins were found in the phloem, which bound selectively 25-nucleotide single-stranded RNA species (Yoo et al. 2004). These proteins were in the range of 20.5- 27kD, while IVR was about 23kD. These proteins could mediate the cell-to-cell trafficking of the siRNA's.

Plants transformed with NC330, encoding an IVR-like protein, resulted in a number of transgenic plants expressing variable resistance to TMV and the fungal pathogen *Botrytis cinerea* (Akad et al. in preparation). It will be interesting to see if transformation of different plants with sequences encoding IVR-like proteins will induce resistance to different viruses and perhaps to other pathogens. It might be that resistance of plants to viruses and fungi is associated with siRNA, which recently became known as a more general gene silencing agent, and may be associated with resistance in a variety of organisms.

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Chapter A6

Induced Resistance Mechanisms

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Setting the scene

During the co-evolution of plants and their pathogens, the pathogens developed a wide variety of strategies to infect and exploit their hosts. In response to this pressure, plants countered by deploying a range of defense mechanisms. Some of these are conceptually simple, for example defenses based on physical barriers such as the cell wall or cuticle, or resistance engendered by pre-existing antimicrobial compounds (Osbourn 1996). However, certain resistance mechanisms, most particularly those that are inducible, are complex in nature and have proved to be more difficult to understand, particularly with respect to resistance to viruses.

Inducible resistance mechanisms can be triggered by exposure to pathogenic and non-pathogenic organisms, as well as by certain abiotic stimuli and chemicals. Some of these resistance responses are only local in extent, for example, the synthesis of phytoalexins (weak, broad spectrum antibiotics that affect non-viral pathogens: Kuć, 1995; Hammerschmidt, 1999), or by localized programmed cell death that occurs close to the sites of pathogen penetration (see accompanying Chapter by Loebenstein and Akad). The nomenclature used to describe inducible systemic resistance phenomena can be confusing and is not used in a uniform way throughout the literature. However, most authors refer to systemic resistance induced by a pathogen as "systemic acquired resistance" (SAR) and we shall use this terminology throughout this review. For more information on the history and terminology of induced resistance directed against non-viral pathogens the

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reader is directed to a special issue of the European Journal of Plant Pathology edited by Hammerschmidt et al. (2001).

In general, SAR confers protection against a broad spectrum of pathogens, even though the initial induction of resistance may depend on a highly specific plant-pathogen interaction. Thus, early studies showed SAR induced by one virus was effective against unrelated viruses (Ross, 1966) or even that SAR induced by a fungus could inhibit infection by a virus (Bergstrom et al. 1982). In recent years, it has become clear that there is yet another systemic antiviral resistance phenomenon in plants: RNA silencing. In contrast to SAR, RNA silencing is highly specific with respect both to its induction and activity. RNA silencing is an homology-based RNA degradation mechanism that probably occurs in all eucaryotes, including plants (Moissiard and Voinnet, 2004). One of the functions of RNA silencing is as a defense mechanism against viruses (Waterhouse et al. 1999, 2001; Moissiard and Voinnet, 2004). For further discussion of various aspects of this concept the reader is referred to the accompanying Chapters by Bucher and Prins, Gal-On, Palukaitis and MacFarlane, and by Moore and MacDiarmid.

In this chapter, our aim is to review current knowledge of induced resistance mechanisms against viruses. Viruses pose a distinct challenge to the plant. Unlike the cellular pathogens (fungi, oomycetes, and bacteria) all viruses are non-cellular obligate intracellular parasites that must replicate in intimate association with specific components of the host cell (Hull, 2002). As a consequence, most of the inducible defenses discovered so far, particularly if they are extracellular or targeted against pathogen cell structure or function, have no impact on the virus life cycle. In addition to reviewing our rather scant knowledge of antiviral factors and mechanisms there will also be a discussion of the signal transduction networks that regulate resistance induction and how these might coordinate resistance against diverse pathogens. Additionally, we will address one of the most challenging areas in the study of plant virus resistance, namely, how the "classical" resistance phenomena of SAR may be coordinated with, or overlap with, RNA silencing.

Triggering broad-spectrum induced resistance

Pathogen-induced resistance: The hypersensitive response and SAR

Resistance to a pathogen is often accompanied by a response known as the hypersensitive response (HR): the rapid, localized death of cells at the infection site. The HR can occur in resistant plants in response to viruses, as well as bacteria, fungi or nematodes (Goodman and Novacky, 1994; Kombrink and Schmelzer, 2001). In the best understood systems, the

occurrence of the HR depends upon the possession by the plant and invader of corresponding resistance (*R*) and avirulence (*Avr*) genes respectively, also known as a 'gene-for-gene' interaction (Flor, 1971). According to the modern conceptualization of the gene-for-gene interaction, which is based on recent progress in the isolation and functional analysis of *R* and *Avr* genes, *R* gene products are believed (or in some cases known) to act as receptor molecules that directly or indirectly detect specific elicitors, which are the direct or indirect products of the pathogen's *Avr* gene (Bergelson et al. 2001; Dangl and Jones, 2001). For specific examples and further examples relevant to virus-plant interactions see accompanying Chapters in this volume by Bruening, Caplan and Dinesh-Kumar, Kachroo, Pfitzner and by Schoelz.

In gene-for-gene interactions involving viruses, viral gene products identified as elicitors capable of triggering the HR include replication proteins (Padgett et al. 1997; Abbink et al. 1998; Erickson et al. 1999), viral capsid proteins (Culver and Dawson, 1989; Culver et al. 1994; Bendahmane et al. 1995), and viral movement proteins (Weber and Pfitzner, 1998). Plant *R* genes conferring hypersensitivity to a number of pathogens have been identified and isolated in sufficient numbers to allow their classification into several distinct families (Jones, 2000; Dangl and Jones, 2001). Relatively few virus-specific gene-for gene type *R* genes have been isolated so far. These include the *N* resistance gene from *Nicotiana*, that confers hypersensitivity to *Tobacco mosaic virus* (TMV) and almost all other tobamoviruses (Dinesh-Kumar et al. 1995), the *HRT* gene from *Arabidopsis*, which is required for the HR exhibited by plants of the Dijon ecotype infected with *Turnip crinkle virus* Genus *Carmovirus* (TCV) (Cooley et al. 2000; Kachroo et al. 2000), the potato *Rx* gene for resistance to *Potato virus X* (PVX) (Bendahmane et al. 1997), and the *Tm-2* and *Tm-2*^{2} genes for resistance to *Tomato mosaic virus* (Gerhardts and Pfitzner, 2003 cited in the accompanying chapter by Pfitzner; Lanfermeijer et al. 2003). Further details of the structure and function of *R* and their products (R proteins) are presented in the accompanying chapters by Bruening, Caplan and Dinesh-Kumar, Kachroo, and Pfitzner.

Plant cell death and resistance

The HR is a correlative feature of many, but not all, resistance interactions controlled by *R* genes (reviewed in this volume by Loebenstein and Akad). Conceivably, the cell death reaction seen in the HR may inhibit replication of certain pathogens or deprive them of nutrients. However, investigators now consider this to be a simplistic view and that a more important role for the HR is in the generation of signals that cause local and systemic changes in the plant. Perhaps this is why a local HR is often associated with the onset of systemic resistance (Pennell and Lamb, 1997;

Birch et al. 2000). Of course, cell death as necrosis can also occur in pathogen-infected susceptible plants, but this form of cell death is distinctly different from the HR (see below).

It is generally thought that the HR is a form of programmed cell death (PCD). PCD is defined as cell death resulting from a complex set of genetically controlled physiological and morphological processes. These result in the selective destruction of cells that can be expended (Pennell and Lamb, 1997; Birch et al. 2000). It differs from necrosis, which is caused by microbial toxins or injury, and is not regulated and limited by the plant (Pennell and Lamb, 1997; Birch et al. 2000). It has been suggested that the HR is comparable to the form of animal PCD known as apoptosis. Features shared by the HR and apoptosis include the activation of complex signaling networks, changes in ion fluxes, the generation of reactive oxygen species, and changes in protein phosphorylation (reviewed and discussed in detail by Birch et al. 2000; Heath, 2000; Gilchrist, 1998; Pennell and Lamb, 1997). Some experimental results are consistent with the idea that the HR may be similar to apoptosis. For example, a number of groups have reported cysteine protease activity during the HR which may be indicative of caspase-type activity (reviewed by Birch et al. 2000; Lam and del Pozo, 2000). Furthermore, Bax, an animal PCD effector protein, induced plant cell death when expressed from a viral vector (Lacomme and Santa Cruz, 1999). Nevertheless, there is no evidence that plant genomes encode either caspases or Bax-type proteins. Instead, the reports of PCD-related cysteine protease activity in plants or the effects of animal PCD effectors on plant cell death may be explained by the possibility that in plants entirely novel gene products may have evolved to fulfill the roles of PCD effectors. Most notably, a plant vacuolar processing enzyme (VPE) has a caspase-1 like protease activity and is required for the *N* gene-mediated HR to TMV. Thus, this form of PCD in plants appears to be mediated by VPE and possibly other elements within the cell vacuole (Hatsugai et al. 2004).

Perhaps the nature of plant PCD is a less important puzzle to solve than whether or not host cell death is needed for resistance to viruses to occur during the HR. On one hand, there is often a correlative association between host cell death in the HR and resistance, and recent evidence indicates that certain natural products produced during the HR (e.g. scopoletin: Chong et al. 2002*)* may have antiviral activity. However, there is no direct evidence showing that cell death is an absolute requirement for the limitation of virus multiplication, at least during the period immediately following the appearance of the HR. This point is exemplified by the results of three studies that investigated the interaction of *NN* genotype *Nicotiana* plants with TMV. In the first of these examples, Weststeijn (1981) exploited the temperature-sensitive nature of the HR and TMV localization in tobacco containing the *N* resistance gene to show that increased temperature could be used to facilitate the escape of virus from lesions for up to 12 days after HR

appearance. More recently, Santa Cruz and colleagues (Wright et al. 2000) used genetically engineered TMV expressing the green fluorescent protein (TMV.GFP) to confirm that virus remained in living cells at the periphery of the HR lesion for several days following the appearance of the HR in *NN* genotype *Nicotiana edwardsonii*. Similar evidence for this has also been obtained using TMV.GFP infection of *NN* genotype tobacco (Murphy et al. 2001). In the third example, growing NN genotype tobacco plants in a low oxygen atmosphere inhinbited cell death but TMV remained localized in these plants (Mittler et al. 1996).

Studies in other systems indicate at the genetic level that cell death and resistance can be entirely separate phenomena. For example, an examination of the factors controlling the induction of the HR in cowpea by *Cucumber mosaic virus* (CMV) showed that specific and distinct amino acids within the viral RNA polymerase sequence were responsible for the induction of virus localization and the elicitation of cell death (Kim and Palukaitis, 1997). Furthermore, cell death and resistance induction triggered by *Cauliflower mosaic virus* (CaMV) in *Nicotiana* species are controlled by separate host genes (Cole et al. 2001). Taken with the results of the *N* gene/TMV system, these findings strongly suggest that cell death alone is not responsible for virus localization during or after the HR.

SAR and Salicylic acid

The induction of a HR during a resistance response often results in the induction of SAR. Probably the best known example of this effect in plant-virus interactions is the response of tobacco plants possessing the *N* resistance gene. Ross (1961a, b) showed that inoculation of these plants with TMV resulted in an enhanced degree of resistance to a second inoculation with the virus. This was manifested as the formation of smaller and fewer necrotic lesions not only in tissue close to the primary lesion (Ross, 1961a) but also on un-inoculated parts of the plant (Ross, 1961b). Subsequent work showed that SAR induced by one pathogen could confer resistance to unrelated pathogens (Ross 1966; Bergstrom et al. 1982; Naylor et al. 1998). We now know that the HR and SAR are coordinated and controlled by a complex signal transduction network.

Salicylic acid (SA) plays a central role in the signal transduction pathway that results in SAR. Indeed, SA has repeatedly been shown to accumulate to high levels in both primary inoculated tissues as well as in distal tissue displaying SAR (Malamy et al. 1990; Métraux et al. 1990, and also reviewed by Dempsey et al. 1999). In fact, the signaling pathways leading to SAR are dependent on endogenous accumulation of SA. If SA accumulation is inhibited by engineering plants to express the salicylate-degrading enzyme SA hydroxylase (*nahG*-transgenic plants: Gaffney et al. 1993; Delaney et al. 1994; Mur et al. 1997), or by mutation of SA biosynthetic genes (Nawrath

and Métraux, 1999), plants are not able to express SAR and are more susceptible to virulent and avirulent pathogens. Due, in part, to conflicting results obtained from grafting experiments with different *nahG*-transgenic tobacco lines (Vernooij et al. 1994; Darby et al. 2000) it is not at all clear whether SA is the translocated signal responsible for establishing SAR throughout the plant. Thus, the nature of the mobile SAR-inducing signal remains to be established, although recent work with the Arabidopsis dir-1-1 mutant suggests that a lipid-derived molecule may be the signal (Maldonado et al. 2002).

Inducible gene products associated with the HR and SAR induction

Following on from the work of Ross (1961a, b) several groups attempted to find evidence of changes in host gene expression specifically associated with the HR or SAR induction. Loebenstein and co-workers identified a protein that appeared after a TMV-induced HR. This was released by protoplasts and was present in the intercellular fluid of intact leaves, indicating that it is extracellular. They called this protein inhibitor of virus replication (IVR) since it could inhibit production of several viruses in leaf discs (Loebenstein and Gera, 1981; Spiegel et al. 1989). A cDNA clone encoding IVR was recently isolated by this group (Akad et al. 1999). With this clone now available, it should soon be possible to determine definitively whether IVR has a role in LAR or SAR against viruses (see Loebenstein and Akad in this volume).

However, the most intensively studied inducible gene products associated with the HR and SAR are the pathogenesis-related (PR) proteins. These proteins were discovered independently at around the same time (c. 1969) by van Loon and Gianinazzi, together with their respective colleagues. Both groups found that ‘novel’, host-encoded proteins accumulated in the leaves of NN genotype tobacco plants inoculated with TMV and in leaves expressing SAR (Gianinazzi et al. 1970; van Loon and van Kammen, 1970). Subsequent study in many laboratories has shown that PR proteins are highly diverse and it has been helpful to classify them into a number of families (van Loon and van Strien, 1999). Some PR proteins are induced by SA, while others are regulated by other factors such as ethylene or jasmonic acid, or combinations of factors (Schenk et al. 2000).

Several of the PR proteins have been shown to have direct antimicrobial activity. For example, those with chitinase or β-1,3-glucanase activity can cause the breakdown of fungal cell walls (Mauch et al. 1988; Rauscher et al. 1999; Schlumbaum et al. 1986). Some PR proteins, including the most-studied PR protein, PR1, are known to have antimicrobial properties but their mode of action remains unknown (Alexander et al. 1993; Niderman et al. 1995). So far, none of the PR proteins examined to date have been shown

to have antiviral activity. Paradoxically, over-expression of extracellular β-1,3-glucanase PR proteins (PR2 family; van Loon and van Strien, 1999) can promote viral movement due to the increased breakdown of callose around the plasmodesmata (Bucher et al. 2001). Nevertheless, because PR proteins accumulate so abundantly, they are routinely used as a general marker for the induction of SAR (Ward et al. 1991; Kessmann et al. 1994).

In *Arabidopsis* the NPR1 protein plays a key role downstream of SA in the induction of many *PR* genes and in the establishment of SAR against fungal and bacterial pathogens (Cao et al. 1994; Glazebrook et al. 1996; Delaney et al. 1995; Shah et al. 1997). NPR1 is a 65 kDa protein, containing ankyrin repeats (Cao et al. 1997; Ryals et al. 1997). In non-induced plants NPR1 exists in the cytoplasm as a covalent linked oligomer held together by disulfide bridges. SA induces dissociation and the released NPR1 monomers relocate to the nucleus where they interact directly with members of the TGA/OBF family of transcription factors (Zhou et al. 2000; Després et al. 2003; Mou et al. 2003). Two NPR1-interacting transcription factors are known to bind a crucial element within the PR-1 promoter (Zhou et al. 2000), which had previously been shown to be required for SA-induced PR1 synthesis (Lebel et al. 1998). However, as will be discussed later, SA-induced resistance to viruses does not require PR-protein expression, nor is it dependent on the activity of NPR1.

Chemically-induced resistance

Many different classes of chemicals can induce some form of resistance (Kuć, 2001). However, in searches for agronomically useful molecules that could mimic the action of SA to induce resistance against viruses and other pathogens, two chemicals have been studied in greatest detail: acibenzolar-S-methyl ('Bion' or 'Actigard') and INA (2,6-dichloroisonicotinic acid) (Friedrich et al. 1996; Lawton et al. 1996; Görlach et al. 1996). Of these, Bion has been deployed commercially in several countries as a plant protectant or growth-promoting chemical (Oostendorp et al. 2001). Other chemicals, which are the subject of intense investigation are BABA (ß-aminobutyric acid) and Oryzemate (Probenazole). BABA protects against a wide range of pathogens, including viruses. However, it has not been resolved whether or not it acts *via* a stimulation of the SA pathway (Jakab *et al*. 2001). In contrast, probenazole-induced resistance has been shown to require SA and NPR1 activity (Yoshioka et al. 2001). None of these chemicals show direct antimicrobial activity *in vitro*, but activate resistance to the same range of pathogens as biotic inducers of SAR (Oostendorp et al. 2001; Jakab et al. 2001).

Of course, SA itself or aspirin (acetylsalicylic acid), triggers resistance when applied onto TMV-resistant or susceptible tobacco leaves (White,

1979; White et al. 1983). It was this early work that prompted investigation of SA levels in pathogen-infected plants (Malamy et al., 1990; Métraux et al. 1990) and laid the groundwork for many subsequent studies of signal transduction in SAR induction. Even in otherwise susceptible plants resistance is characterized by a delay in the onset of disease symptoms and by a decreased yield of virus (Chivasa et al. 1997; Naylor et al. 1998; White et al. 1983) and such effects may, in the longer-term, be of practical use. For further discussion regarding the utilization of resistance-inducing chemicals (and beneficial resistance-inducing microbes) to protect plant against virus infection in the field, the reader is referred to the accompanying Chapter by Murphy.

SAR and SA-induced resistance to viruses

Extensive reprogramming of both primary and secondary plant metabolism and gene expression levels is initiated during a resistance response (Hammond-Kosack and Jones, 1996; Kombrink and Schmelzer, 2001; Dixon, 2001). Some of the host protein changes are directly involved in resistance responses, for example production of phytoalexins or biosynthesis of SA (reviewed by Dixon et al. 2002). However, many biochemical and physiological changes in resistant and susceptible plants may only be secondary to the defense response and it is difficult with our present knowledge to distinguish between these roles (see accompanying Chapter by Handford and Carr). In this section we will concentrate on signaling pathways and resistance mechanisms that are known (or thought) to be involved in virus resistance.

Vulnerabilities of plant viruses to induced resistance mechanisms

Since they are dependent on host factors for their replication and movement through the plant (Hull, 2002), the potential targets for plant defense mechanisms are for the most part unique to viruses and distinct from those that could be useful in defense against bacteria and fungi.

Although some plant viruses utilize negative-sense single-stranded or double-stranded RNA, most plant viruses possess genomes consisting of positive-sense (i.e. mRNA sense) single-stranded (ss) RNA. These viruses replicate and in some cases synthesize ‘sub-genomic’ mRNA in the cytoplasm of host cells using an RNA-dependent RNA polymerase (RdRp) complex consisting of proteins encoded by the virus plus factors seconded from the host cell (Buck, 1996; Hull, 2002). There are fewer groups of DNA viruses that infect plants, although some of the diseases they cause can be serious (Hull, 2002). The two best-studied groups of plant DNA viruses are the geminiviruses and the caulimoviruses. Geminiviruses possess circular,

single-stranded DNA genomes and replicate in the host nucleus using a host DNA polymerase (Hanley-Bowdoin et al. 1999; Hull, 2002). The caulimoviruses are double-stranded DNA pararetroviruses; that is, they encode a reverse transcriptase that allows them to replicate *via* an RNA intermediate (Hohn and Futterer, 1997; Hull, 2002).

Viruses such as TMV can enter a plant cell through small wounds caused by abrasion but many other types of virus are introduced into the plant by other organisms acting as vectors. Inside the cell, the virus uncoats, replicates and begins the process of local, cell-to-cell, movement. Most viruses produce one or more movement proteins that mediate transfer of viral RNA (or in some cases entire virus particles) between neighboring cells *via* the plasmodesmata (Carrington, 1999; Heinlein, 2002). Eventually, the virus reaches the host's vascular system and can begin moving systemically. Although the process by which viruses enter the vasculature is poorly understood, it is known that most viruses move in the phloem tissue, the elements of which are responsible for translocation of carbohydrates and other metabolites around the plant (Leisner and Turgeon, 1993; Nelson and van Bel, 1998). Viruses are translocated preferentially towards young leaves, where they unload from the veins and begin to invade the surrounding tissue (Oparka and Santa Cruz, 2000).

Thus, any of the stages in the viral infection process (entry, replication, intercellular movement and systemic movement) could in principle, be the targets of induced resistance mechanisms.

Resistance against viruses in SA-treated and SAR-expressing plants

There is now substantial evidence that some of the antiviral mechanisms triggered by SA and induced in SAR-expressing plants are regulated by redox-mediated signaling in the mitochondria. This branch of the defensive signal transduction pathway is in part regulated by the alternative oxidase (AOX), as indicated by experiments in which *Aox* gene expression has been perturbed in transgenic plants or in plants infected with a viral vector expressing high levels of wild-type or mutant *Aox* sequences (Gilliland et al. 2003; Murphy et al. 2004). The 'virus-specific' signaling pathway can be activated selectively, that is, independently of *PR* gene induction, by non-lethal concentrations of cyanide and antimycin A and, at least in tobacco, its induction can be inhibited to some extent by an inhibitor of AOX, salicylhydroxamic acid (SHAM) (Chivasa et al. 1997; Chivasa and Carr, 1998; Gilliland et al. 2003; Mayers et al. 2005). There is also genetic evidence supporting the existence of a virus-specific pathway or pathways. In *A. thaliana*, SA-induced resistance to *Turnip vein clearing virus* Genus *Tobamovirus*(TVCV) is regulated independently of NPR1 (Wong et al.

2002), as is resistance to TCV mediated by the *HRT* resistance gene (Kachroo et al. 2000).

The biochemical details of this signaling mechanism and the potential role of AOX activity will not be dealt with here in depth since the topic has been reviewed extensively and recently elsewhere (Singh et al. 2004; Gilliland et al. 2005 and in the accompanying chapter by Handford and Carr).

Despite the progress made in understanding some of the signaling involved in SA-induced resistance to viruses, we have not yet identified the host gene products responsible for limiting virus spread. In contrast, a number of the components responsible for limiting the spread of fungal and bacterial pathogens have been identified, which include the PR proteins. However, none of the currently identified PR proteins have been implicated in virus resistance. Transgenic tobacco plants constitutively expressing one or more PR proteins were still susceptible to TMV (Cutt et al. 1989; Linthorst et al. 1989) but showed enhanced resistance against oomycete and fungal pathogens (Alexander et al. 1993).

Recently, an SA-regulated host-encoded RdRp (NtRdRp1) was identified in tobacco and shown to have antiviral properties (Xie et al. 2001*)*. However, it was found that antisense suppression of the gene did not abolish SA-induced resistance to viruses, indicating that it is not essential for induced resistance to viruses, although it could still contribute to resistance, most likely through a mechanism based on RNA silencing (See below).

SA can interfere with virus replication

SA treatment can inhibit the accumulation of certain positive sense ssRNA viruses and at least one DNA virus, CaMV, in directly inoculated tissues and/or protoplasts from tobacco and *Arabidopsis* and cowpea (Chivasa et al. 1997; Hooft van Huijsduijnen et al. 1986; Naylor et al. 1998; Murphy and Carr, 2002; Wong et al. 2002; Gilliland et al. 2005). An early study in cowpea protoplasts demonstrated that SA treatment could interfere with *Alfalfa mosaic virus* (AlMV) replication (Hooft van Huijsduijnen et al. 1986). In TMV-susceptible tobacco leaf tissue, SA caused a dramatic reduction of TMV RNA accumulation (Chivasa et al. 1997). More specifically, it was also found that for this virus the ratio of genomic RNA to coat protein mRNA and the ratio of plus- to minus- sense RNAs were affected by SA, suggesting that SA induces interference with the activity of the TMV RdRp complex (Chivasa et al. 1997; Naylor et al. 1998). Similar effects of SA on TMV RNA accumulation were observed in mesophyll protoplasts generated from SA-treated tobacco plants demonstrating that in this case, SA-induced resistance is operating at the single cell level (Murphy and Carr, 2002). This shows inhibition of replication, rather than cell-to-cell

movement is the principal effect. However, inhibition of virus movement also plays a role in SA-induced resistance to TMV in intact leaf tissue (See below).

SA can inhibit virus long-distance movement

In tobacco and *Arabidopsis* CMV can evade SA-induced interference with replication (Naylor et al. 1998; Ji and Ding, 2001; Mayers et al. 2005). However, SA-treated tobacco plants show a marked delay in CMV symptom development. It was found that although CMV could replicate in directly inoculated SA-treated tobacco leaves, its entry into the phloem cells was delayed (Naylor et al. 1998). Similar results were also observed with AlMV (Naylor, 1999). Presumably, SA affects one or more cell types within the vascular bundle in a way that prevents or slows down phloem loading. CMV inoculated onto *N*-gene tobacco expressing SAR due to prior exposure to TMV was also restricted in long-distance movement (Naylor et al. 1998). In tobacco a mutant of CMV that is unable to express the 2b resistance suppressor protein (CMVΔ2b) appears to be subject to SA-induced interference with CMV replication (Ji and Ding, 2001) and this is discussed later.

SA has cell-specific effects

Whilst work with CMV revealed that SA could target long-distance movement of viruses, further investigation using viruses expressing GFP revealed the fact that SA can have different effects on the same virus in different cell types (Murphy and Carr, 2002). Treatment of susceptible tobacco with SA restricted TMV expressing GFP (TMV.GFP) to single epidermal cell infection sites. The replication of TMV.GFP in single epidermal cells appeared similar in control as well as SA-treated plants, as judged by GFP fluorescence levels, indicating that SA was inhibiting cell-to-cell movement. Recovery of cell-to-cell movement was achieved to some extent when TMV movement protein (MP) was supplied *in trans* in tobacco plants constitutively expressing TMV-MP (Murphy and Carr, 2002). However, even in TMV-MP transgenic plants that had been treated with SA, TMV.GFP was restricted to the epidermal cell layer and did not appear to move into the mesophyll cell layer beneath. This data demonstrated that SA can inhibit cell-to-cell movement of TMV.GFP in the epidermis, but interferes with TMV.GFP replication in the mesophyll cell (Murphy and Carr, 2002).

SA- and cyanide-induced resistance to viruses: Relevance to plants expressing R-gene mediated resistance and SAR

SA-induced resistance to viruses can occur in plants in the absence of any *R*/*Avr* gene interaction or HR-associated cell death. However, SA-induced resistance to viruses in plants lacking an *R* gene is usually expressed only as a delay in the onset of virus spread and disease induction. Nevertheless, investigations of virus spread in SA-treated susceptible plants may shed light on what is happening in resistant plants. For example, TMV.GFP is limited to single–cell infection sites in SA-treated susceptible tobacco (Murphy and Carr, 2002) and this may explain why fewer and/or smaller visible necrotic lesions appear on SAR-expressing, *N*-gene tobacco plants after inoculation with TMV. This finding may be significant since the HR mediated by the *N*-gene, unlike many other pathogen-induced cell death phenomena, cannot occur at the single cell level. For example, TMV does not cause necrosis of TMV-infected protoplasts from *N*-gene tobacco (Otsuki et al. 1972) and a movement-deficient TMV.GFP construct that could only infect single epidermal cells did not elicit cell death in an *N*-gene containing host (Wright et al. 2000).

It has also been demonstrated that the virus-specific signaling pathway is essential for *N* gene-mediated resistance against TMV (Chivasa and Carr, 1998). When *N* gene-containing tobacco was transformed with the bacterial *nahG* gene, it was found that these transgenic plants could no longer restrict the spread of TMV or TMV-induced necrosis (Bi et al. 1995; Ryals et al. 1995; Mur et al. 1997). Thus it was proposed that SA is required for virus localization early on in the HR (Mur et al. 1997). It was then found that cyanide treatment restored *N* gene-mediated resistance to TMV in plants expressing SA hydroxylase (Chivasa and Carr, 1998). Thus, the virus specific defense pathway is required for *N* gene-mediated TMV localization as well as for the subsequent establishment of acquired resistance.

However, other, yet unknown, virus-restricting mechanisms must also be coming into play during the HR, at least in the case of *N* gene-triggered restriction of the spread of TMV. In *N. benthamiana* plants transgenic for the *N* resistance gene it was found that virus-induced silencing of an NPR1-like gene compromised localization of TMV (Liu et al. 2002). Since the virus-specific signaling pathway that we have investigated is not dependent on NPR1 (Wong et al. 2002), this suggests that other signaling pathways are also at work during the HR.

Non-SA-dependent chemically-induced resistance to viruses

So far, only a small number of host gene products have been identified that appear to have any direct role in the inhibiting viral infection. Examples already mentioned in this review include IVR and RdRp1. Ueki and

Citovsky (2002) have added to this short list by identifying the protein mediating an inducible resistance phenomenon that inhibits systemic movement of tobamoviruses in tobacco. In recent years, most studies of inducible resistance to viruses have concentrated on either RNA silencing or on resistance mechanisms regulated by SA. In contrast, Citovsky and co-workers have focused on an inducible resistance phenomenon that is quite distinctive in that it apparently antagonizes the establishment of systemic RNA silencing (Ueki and Citovsky, 2001). Furthermore, it is not reliant on salicylic acid-mediated signaling (Citovsky et al. 1998). This resistance to viruses is induced by treatment of plants with non-toxic concentrations of ions of the heavy metal cadmium (Ghoshroy et al. 1998). The protein that Ueki and Citovsky (2002) identified, CdiGRP, is a glycine-rich protein that promotes the accumulation of callose in the vascular tissue. This callose build up might restrict the unloading of viruses out of the phloem and inhibits systemic virus movement (see accompanying chapter by Ueki and Citovsky).

A possible role for RNA silencing in SA-induced resistance

In the earlier section on resistance against viruses in SA-treated and SAR-expressing plants we proposed that an additional, AOX-independent mechanism may result from increased induction of the gene(s) encoding the RdRP1-type of host RNA-dependent RNA polymerase. RdRPs play important roles in certain pathways leading to the induction of RNA silencing (also known variously as RNA interference, post-transcriptional gene silencing etc.). RNA silencing is a targeted RNA degradation process, affecting all highly homologous sequences in which foreign, over-expressed or aberrant RNA molecules are targeted for destruction in a sequence-specific manner (Ahlquist, 2002; Baulcombe, 2001; Moissiard and Voinnet, 2004). Cellular RdRPs are encoded by gene families and the various family members appear to play roles in different forms of RNA silencing. Thus, the SA-inducible class, RdRP1, does not seem to be absolutely required either for SA-induced resistance or for virus-induced gene silencing in tobacco or *Arabidopsis* (Xie et al. 2001; Yu et al. 2003). However, RdRP1 is an important factor in limiting the extent to which some RNA viruses can accumulate within host tissue. For example, it contributes to a pre-existing or 'basal' resistance to TMV infection in tobacco. But in *N. benthamiana*, in which the *NbRdRP1* gene encodes a non-functional enzyme, this basal resistance to TMV is lacking and the virus accumulates to higher titers than in other *Nicotiana* hosts (Yang et al. 2004). What makes RdRP1 a potential antimycin A-independent contributor to SA-induced resistance is the fact

that, although it is induced by SA, it is not induced by antimycin A (Gilliland et al. 2003; Singh et al. 2004). Currently, our group is attempting to determine whether various elements of the RNA silencing mechanism, for example those features inhibited by the CMV 2b protein and other viral counter defense proteins (Li and Ding, 2001), or RdRP1-type factors really do contribute to SA-induced resistance.

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Chapter A7

Host Gene-mediated Virus Resistance Mechanisms and Signaling in Arabidopsis

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Introduction

Plants resist viral infections either via an active mechanism, involving the participation of resistance (*R*) genes and subsequent signal transduction pathways, or in a passive manner, which entails the absence of essential host factors required for replication or movement of the virus. An active resistance response involves strain-specific recognition of a virus-encoded elicitor, through direct or indirect interaction with the corresponding *R* gene product. This in turn activates downstream signaling, which leads to prevention of viral spread and confers resistance against the pathogen. An *R* gene-mediated recognition of virus often turns on defense responses such as the accumulation of salicylic acid (SA), the expression of pathogenesis-related (*PR*) genes, and the development of a hypersensitive response (HR) on the inoculated leaves. The HR is defined by necrotic lesion formation at the site of infection and is thought to help prevent multiplication and movement by confining the virus to the region immediately surrounding the necrotic lesions.

In comparison to signaling mechanisms required for resistance to bacterial, oomycete and fungal pathogens, the genetic basis of virus-host interactions is poorly understood. One of the likely reasons for the slow advance could be the lack of sufficient incompatible host-viral systems where resistance is induced upon recognition between host- and pathogen-

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encoded products. Although Arabidopsis can serve as a host to a number of viral pathogens (Lee et al. 1994; Lartey et. al. 1998; Yoshii et al. 1998; Martin et al. 1999; Dardick et al. 2000; Chisholm et al. 2000; Whitham et al. 2000; Yamanaka et al. 2000; Kachroo et al. 2000; Dzianott and Bujarski, 2004), most of these interactions are not known to have incompatible outcomes. Much of the recent advances in molecular signaling underlying incompatible host-virus interactions have come from studies on the Arabidopsis-*Turnip crinkle virus* (TCV) and Arabidopsis-*Cucumber mosaic virus*-Y(CMV-Y) systems (Simon et al. 1992; Dempsey et al. 1993; Takahashi et al. 1994; Dempsey et al. 1997; Kachroo et al. 2000; Takahashi et al. 2002; Chandra-Shekara et al. 2004). A comparison of defense pathways required for resistance to TCV and CMV has, for the first time, allowed deciphering of the signaling requirements essential for resistance to viral pathogens and their relationship to resistance pathways against other, non-viral pathogens.

Defense signaling against TCV and CMV

Resistance to TCV and CMV in Arabidopsis is conferred by the *R*-genes *HRT* and *RCY1*, respectively, both of which encode a coiled-coil (CC), nucleotide-binding site (NBS) and leucine-rich repeat (LRR) class of R protein (Cooley et al. 2000; Takahashi et al. 2002). Interestingly, *HRT* and *RCY1* are allelic to one another and the proteins encoded by these genes show ~91% similarity at the amino acid level. The *HRT*/*RCY1* genes are also allelic to *RPP8*, which confers resistance to the oomycete pathogen, *Peronospora parasitica* biotype Emco5 (McDowell et al. 1998; McDowell et al. 2000). Even though HRT, RCY1 and RPP8 share a high level of homology and structural identity, the downstream signaling pathways triggered by these R proteins appear to be unique to each of the pathosystems involved. This is further evident upon comparing the TCV and CMV coat proteins, which are the cognate avirulence (Avr) factors for HRT and RCY1, respectively (Zhao et al. 2000; Takahashi et al. 2001), and which do not show any similarities at the amino acid level. This indicates either that HRT and RCY1 recognize completely different ligands, or that interaction between these R proteins and the Avr factors may be indirect or involve other accessory factors. The latter is likely to be the case, since HRT interacts with the TCV coat protein *in planta*, but not in the yeast two-hybrid system (Cooley et al. 2000; Ren et al. 2000; Wu, H.J. and Klessig D.F., unpublished results). *HRT*-mediated resistance has also been shown to require an additional interaction between the TCV coat protein and a protein belonging to the NAC family of transcription activators (Ren et al. 2000). These observations, and the recent studies conducted with various R and Avr proteins, suggest that interaction between most of these proteins does not

occur directly and requires other accessory proteins/factors (Luderer and Joosten, 2001).

The *HRT*-mediated resistance to TCV is associated with elicitation of necrotic lesions on the inoculated leaf (Fig. 1A), induction of several defense genes, including *PR-1* and *GST1*, accumulation of SA and SA-glucoside (SAG), and induction of the phytoalexin camelexin (Dempsey et al. 1997; Kachroo et al. 2000; Chandra-Shekara et al. 2004). Plants lacking *HRT* do not show HR, exhibit only basal level expression of *PR-1* and *GST1*, and accumulate low levels of SA/SAG and camelexin. In addition, plants lacking

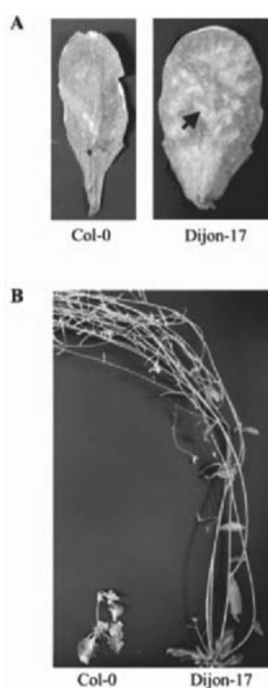

Figure 1. Morphological phenotypes of TCV-infected Col-0 and Dijon-17 plants. (**A**) TCV-inoculated leaves at four days post infection. The small specks seen on the inoculated leaves of Col-0 plants are damaged tissue or dried inoculation buffer. The TCV inoculated resistant plant, Dijon-17, show development of discrete lesion known as hypersensitve response (marked by an arrow). (**B**) The morphological phenotypes of the TCV-inoculated plants at three-weeks post infection. The susceptible plants show severe crinkling of their leaves, remain stunted and show dropping bolts. By comparison, the resistant plants develop normally. (See also, Colorplates, p. xxiv)

HRT allow systemic spread of the virus, which causes crinkling and drooping of the bolt and results in eventual death of the plant (Fig. 1B; Dempsey et al. 1997, Kachroo et al. 2000). Similar to *HRT*-mediated signaling, the *RCY1*-mediated resistance to CMV is also associated with cell death on the inoculated leaf and upregulation of the defense gene *PR-1* (Takahashi et al. 2001; Takahashi et al. 2004).

Hypersensitive response against TCV and CMV

R gene-mediated resistance is often associated with rapid and localized cell death at the site of infection, the HR (Fig. 1A). By comparison, a susceptible response is characterized by spreading chlorosis and necrosis at the infection site. Although the HR is one of the primary manifestations of the resistance response, it remains unclear if the HR is a prerequisite for gene-for-gene-mediated disease resistance. For example, the HR does not develop in potato during *Rx*-mediated resistance against *Potato virus X* (Köhm et al. 1993), or in Arabidopsis plants overexpressing the *R* gene *HRT*, and yet these plants are resistant to their respective viral pathogens (Cooley et al. 2000; Chandra-Shekara et al. 2004). Of profound importance is the finding that, HR and resistance can be uncoupled in the Arabidopsis-TCV pathosystem. Analysis of F2 plants segregating for *HRT* showed that only 25% of HR-forming plants are resistant to TCV (Kachroo et al. 2000; Chandra-Shekar et al. 2004). This indicates that the HR by itself is insufficient to prevent or delay the spread of the virus into uninoculated tissues. Further support for this contention comes from the observation that both HR-forming as well as non-forming susceptible plants show similar levels of virus in the systemically infected tissue.

Transgenic expression of *HRT* in a susceptible background results in induction of HR and *PR-1* gene expression upon TCV inoculation (Cooley et al. 2000). Intriguingly, both HR and the subsequent *PR-1* gene expression were observed only when the transgenic plants expressed low levels of *HRT* and were abolished in lines showing elevated expression (Cooley et al. 2000; Chandra-Shekara et al. 2004). This indicates that a high level of *HRT* transcript somehow suppresses HR. Perhaps increased levels of HRT initiates a rapid signaling response, which restricts the pathogen to the primary infected cell. This in turn would prevent the spread of the viral pathogen into neighboring cells and thereby minimize accumulation of viral coat protein, which acts as an Avr factor and is required to initiate HR. Unlike *HRT*, transgenic expression of *RCY1* in a susceptible background was

unable to produce HR to CMV (Takahashi et al. 2002). In view of the results obtained with HRT overexpressing plants, it is likely that HR to CMV may be suppressed by increased expression of the transgene *RCY1*. Alternatively, *RCY1* may require an additional factor for generation of HR, and this factor may only be present in the resistant background. Nevertheless, both *HRT* and *RCY1* are absolutely required for resistance. This suggests that, in the case of TCV resistance, *HRT* either directly contributes towards the resistance phenotype or the HR conferred by *HRT* is somehow involved in restricting viral movement in a resistant background.

The HR to TCV remains unaffected by mutations that ablate signaling mediated by the defense-related hormone SA or *R*-gene signaling pathways (Kachroo et al. 2000; Chandra-Shekara et al. 2004). Similarly, the levels of *PR-1* gene induced during HR to TCV also remain unaltered by mutations that affect SA levels or signaling pathways triggered by canonical *R*-genes. In contrast, the HR to CMV and *PR-1* gene expression triggered by *RCY1* is compromised by the *eds5* mutation in the SA pathway and by transgene expression of salicylate hydroxylase (*nahG*), which blocks accumulation of SA (Takahashi et al. 2004). These observations support the idea that, although the HR to CMV requires SA, the HR to TCV is either independent of SA, or requires very low levels. Thus, HRs to different viral pathogens are unique events specific to the interactions between the corresponding R and Avr proteins.

HRT- and RCY1-mediated downstream signaling

In addition to its role in HR formation, *HRT* is also required for resistance to TCV. However, *HRT* alone is insufficient to confer resistance to TCV, as ~75% of HR forming F2 plants derived from a cross between resistant and susceptible ecotypes succumb to the viral disease. Since the resistance phenotype segregates in a recessive manner, it was suggested that a recessive locus, designated as *rrt*, regulates resistance to TCV. This possibility was further supported by characterization of Columbia (Col-0) plants containing the *HRT* transgene; over 90% of transgenic plants showed HR but remained susceptible to TCV (Cooley et al. 2000). Strikingly, transgenic expression of *RCY1* transgene in a susceptible ecotype is only able to complement the resistance phenotype in 50% of the plants (Takahashi et al. 2002). The inability of *HRT* and *RCY1* to complement resistance phenotypes in a susceptible background suggests that other cellular factors play an important role in the functioning of these *R* genes. These observations also suggest that functioning of *R* genes may be regulated in several different ways, and that the *R* genes alone may not be sufficient to confer specific or broad-spectrum resistance.

One of the prominent differences between the *HRT*- and *RCY1*-mediated resistance pathways is that while the HRT-TCV pathway has absolute dependence on SA (Fig. 2; Kachroo et al. 2000; Chandra-Shekara et al. 2004), the *RCY1*-triggered resistance to CMV is only partially dependent on

SA (Takahashi et al. 2002; Takahashi et al. 2004). SA is known to be required for resistance against several bacterial and oomycete pathogens, and Arabidopsis mutants that are impaired in SA perception or accumulation show enhanced susceptibility towards these pathogens (Jirage et al. 1999; Falk et al. 1999; Wildermuth et al. 2001; Nawrath et al. 2002). For example, mutations in *eds1* and *pad4* have been shown to compromise SA synthesis and cause increased susceptibility to bacterial and oomycete pathogens. In addition, these mutations block the pathogen-activated expression of *EDS5*, which encodes another component of the SA-signaling pathway. A mutation in *eds5* also prevents accumulation of SA, resulting in enhanced susceptibility to bacterial and oomycete pathogens (Nawrath and Métraux, 1999; Nawrath et al. 2002). Similarly, the *SID2*-encoded isochorismate synthase is required for SA biosynthesis, *PR-1* induction, and local and systemic acquired resistance (SAR) responses in Arabidopsis (Wildermuth et al. 2001). Inoculation of TCV on the resistant ecotype Dijon-17 induces ~10- and 40-fold increases in SA and SAG levels, respectively. By comparison, levels of SA and SAG in susceptible Columbia plants reach levels that are 2- and ~40-fold lower, respectively. *HRT*-mediated resistance is compromised in mutant or transgenic backgrounds that cause a reduction in induced SA levels after TCV inoculation. These include mutations in *eds1*, *eds5*, *pad4* and *sid2* genes and transgenic expression of *nahG*. By comparison, *HRT*-mediated resistance to TCV is independent of *NDR1*, *RAR1* and *SGT1b* genes, which are known to play important roles in the R-protein-mediated resistance response (Aarts et al. 1998; Austin et al. 2002; Azevedo et al. 2002; Liu et al. 2002; Muskett et al. 2002; Tör et al. 2002). Resistance to CMV has so far been tested in the *RCY1 eds5* and *RCY1 nahG* backgrounds, in which it is partially compromised. This indicates that, besides the SA-stimulated pathway, other pathways are also likely to contribute towards *RCY1*-conferred resistance (Fig. 2).

Unlike most CC-NBS-LRR *R* genes, *HRT* requires *EDS1* for its downstream signaling. This finding is unexpected, since *R* genes with a CC-NBS-LRR structure, such as *HRT*, usually require *NDR1* to signal resistance responses, while *R* genes with a Toll-interleukin1-like region (TIR)-NBS-LRR structure utilize *EDS1* (Aarts et al. 1998; Dangl and Jones, 2001). Interestingly, *RPP8*, which is allelic to *HRT*, is independent of both *NDR1* and *EDS1* (McDowell et al. 2000). It is not yet clear if EDS1 plays a signaling role in *HRT*-mediated resistance or merely participates in regulating SA levels, which appears to be critical for a resistance phenotype (Chandra-Shekara et al. 2004). The *HRT*-mediated resistance is also dependent on PAD4. In addition to regulating SA levels, PAD4 also regulates the SA-induced expression of *HRT*. Thus both EDS1 and PAD4 may have regulatory roles other than governing SA levels. It is also possible that EDS1 and PAD4 may be engaged in different roles in the inoculated versus the systemic tissues. This is important because susceptibility to viral

pathogens is highly dependent on their ability to move into systemic tissues. While further characterization of HRT-mediated signaling pathways will be required to elucidate the exact roles of EDS1 and PAD4, a requirement of EDS1/PAD4 for resistance to TCV suggests that resistance signaling against viral pathogens utilizes components that are also required for defense against non-viral pathogens. This is an important finding since, for the first time, overlap is established between signaling mechanisms employed during resistance to viral as well as non-viral pathogens.

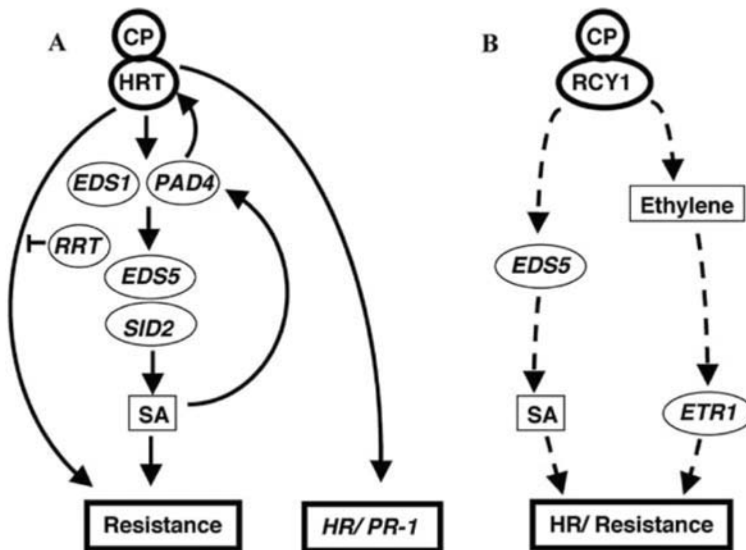

Figure 2. Models for induction of HR and resistance to TCV (**A**) and CMV (**B**). TCV- and CMV-induced defense signaling are initiated upon direct or indirect interaction between the resistance proteins HRT and RCY1, respectively, and the corresponding viral avirulence factor, the coat protein (CP). Upon recognition of TCV, an *HRT*-mediated response leads to the accumulation of SA, which is dependent on the *EDS1*, *PAD4*, *EDS5* and *SID2* genes. In contrast, the HR and *PR-1* gene expression are independent of these genes. *RRT* appears to suppress *HRT*-mediated resistance but not the increase in SA induced by TCV infection and, therefore, is likely to function downstream or independent of the SA pathway.

By comparison to the HRT-TCV interaction, the RCY1-CMV interaction is partially dependent on SA and ethylene (indicated by dashed lines). *RCY1*-conferred HR and *PR-1* expression are partially compromised by the *eds5* mutation. The SA- and ethylene-dependent pathways exhibit synergism, and a *RCY1* plant defective in both of these pathways shows increased susceptibility to CMV.

The EDS1- and PAD4-encoded predicted proteins show homology to triacylglycerol lipases/ esterases. This raises the possibility that lipid/ fatty acid signaling may be involved in resistance to viral pathogens. This possibility is further supported by the recent analysis of the *ssi2* (suppressor of SA insensitivity) mutant, which is defective in a stearoyl-ACP desaturase (a delta-9 desaturase) and consequently produces reduced amounts of oleic acid (Kachroo et al. 2001). The *ssi2* plants are partially resistant to CMV (Sekine et al. 2004). Interestingly, a mutation in the yeast delta-9 desaturase was recently shown to impair the replication of *Brome mosaic virus* (BMV) (Lee et al. 2001). The BMV replication requires unsaturated fatty acids, and viral RNA replication is much more sensitive than yeast growth to reduced unsaturated fatty acid levels (Lee et al. 2001). These results suggest that the increased resistance of *ssi2* to CMV is likely due to the reduced levels of oleic acid, which have also been implicated in regulating defense responses against other pathogens (Kachroo et al. 2003a; Kachroo et al. 2003b; Kachroo et al. 2004). However, *ssi2* plants are susceptible to TCV, which implies that low levels of unsaturated fatty acids may only confer enhanced resistance against certain groups of viruses.

Role of SA in resistance against viral pathogens

Resistance to TCV is restored in both SA-deficient *HRT* plants expressing the *nahG* transgene, as well as in *HRT* plants containing the *eds1*, *eds5* or *sid2* mutations, by exogenous application of SA or the SA analog, benzo(1,2,3)thiadiazole-7-carbothioic acid (BTH). However, exogenous application of SA is unable to confer enhanced resistance in genetic backgrounds lacking *HRT*. SA application upregulates the *HRT* transcript levels by several folds, suggesting that an increase in the *R*-gene levels was responsible for genotype specific SA-mediated enhanced resistance to TCV. Analysis of two different sets of *HRT* transgenic lines generated in the susceptible Col-0 background, as well as of mobilization of *HRT* in a mutant background containing high levels of endogenous SA, further confirmed a positive correlation between the levels of *HRT* and SA-mediated enhanced resistance (Chandra-Shekara et al. 2004). While transgenic lines expressing high levels of *HRT* did not allow any systemic spread of TCV, lines with

basal level expression of *HRT* developed disease symptoms and died three weeks post-infection. High levels of endogenous SA also increased the expression of *HRT* and overcame a requirement for *rrt*, which regulates resistance to TCV. These observations indicate a direct correlation between high *HRT* levels and the resistance response to TCV, and suggest that high levels of *R* gene are required for a stable resistance response. Thus, one of the mechanisms by which SA can confer enhanced resistance to viral pathogens is via the upregulation of *R* genes. Interestingly, the SA-mediated increase in the *HRT* transcript is dependent on *PAD4*; SA-treated *HRT pad4* plants showed basal levels of *HRT* transcript and only a marginal enhancement in resistance upon SA treatment. SA has been shown to upregulate expression of both *EDS1* and *PAD4* and thus participates in a signal-amplification loop involving these genes (Falk et al. 1999; Jirage et al. 1999). The above observations indicate that the SA-PAD4 signal-amplification loop can also condition resistance by upregulating expression of *R* genes. In this regard, it would be valuable to know if PAD4 mediates the upregulation of *HRT* directly, or via some other unknown intermediate component(s).

In addition to a direct or indirect effect on *R* gene expression, SA is also known to trigger resistance to viruses by affecting their replication, cell-to-cell movement, and long distance movement (Wong et al. 2002; Singh et al. 2004). SA was shown to affect the accumulation of *Tobacco mosaic virus* (TMV) coat protein and inhibit viral RNA-dependent RNA polymerase (RdRp) activity (Chivasa et al. 1997; Naylor et al. 1998). However, unlike in the HRT-TCV system, these effects are independent of the function of a specific *R* gene. SA has also been shown to induce expression of tobacco and Arabidopsis RdRp genes, which play an important role in RNA silencing and limit the spread and accumulation of RNA viruses (Xie et al. 2001; Yu et al. 2003). Further, SA has been proposed to enhance viral resistance by inhibiting the respiratory transport chain, leading to an increase in mitochondrial reactive oxygen species (Chivasa and Carr, 1998; Gilliland et al. 2003; Singh et al. 2004). However, it is still not clear how RNA silencing mediated by the host RdRp's and inhibition of mitochondrial respiratory transport ties into the defense gene signaling triggered by *R* genes.

Role of jasmonic acid- and ethylene-dependent pathways in resistance against viral pathogens

In addition to SA, jasmonic acid (JA) and ethylene are two other important signal molecules in plant defense against pathogens (Kunkel and Brooks, 2002). Both the HRT-TCV and RCY1-CMV systems have been assessed for dependence on JA and ethylene-regulated defense pathways. While HRT-mediated HR and resistance are independent of both (Kachroo

et al. 2000), *RCY1*-mediated HR and resistance is partially dependent on ethylene but independent JA (Takahashi et al. 2002). Interestingly, a defect in both SA and ethylene signaling pathways increased the percentage of CMV susceptible plants, suggesting that SA and ethylene-mediated signaling work synergistically. Since removal of both SA and ethylene did not result in complete susceptibility to CMV, other additional signaling pathways/components are likely to be involved in *RCY1*-mediated resistance. One other intriguing aspect of *RCY1*-mediated pathway is that resistance is restored in the *eds5* background by impairing the JA pathway; *RCY1 eds1 coi1* plants are resistant to CMV. This implies that interaction between SA and JA pathways also modulates *RCY1*-conferred resistance to CMV (Takahashi et al. 2004).

Role of NPR1 in resistance against viral pathogens

NPR1 (Non-expressor of *PR-1*), which encodes a positive regulator of SAR is one of the key components of the pathway leading to *PR-1* gene expression and this pathway is required for resistance to several bacterial and oomycete pathogens (Cao et al. 1997; Ryals et al. 1997). However, thus far there is no evidence for the involvement of *NPR1* in viral resistance. The resistance conferred by *HRT* and *RCY1* are both independent of *NPR1* as are the SA-induced resistances to *Turnip vein clearing virus* (TVCV) and *RTM1*-mediated resistance to *Tobacco etch virus* (TEV) (Mahajan et al. 1998; Wong et al. 2002). In addition, work from several laboratories suggests that signaling pathways leading to the induction of *PR-1* gene expression are not required for resistance to viral pathogens (Chivasa et al. 1997; Kachroo et al. 2000; Wong et al. 2002; Chandra-Shekara et al. 2004). These results argue that *NPR1* does not play as important a role in resistance signaling against viruses, as it does against other pathogens. However, it should be noted that recent studies conducted with *N* gene-mediated resistance to TMV indicated a dependence on an *NPR1*-like gene (Liu et al. 2002). This suggests that other members of the NPR1-family may be required for defense against viruses, and their role in resistance signaling against viruses cannot be ruled out at this stage.

Passive resistance to viral pathogens

Since a virus relies on host factors for its multiplication and movement, a loss-of-function mutation in any of these factors should prevent viral multiplication or spread and result in passive resistance towards the virus. Several host components have been identified which are required for multiplication and/or movement of various viral pathogens. These include *CUM1* and *CUM2* (cucumovirus multiplication) genes, which encode

translation initiation factors (Yoshii et al. 2004), *TOM1* and *TOM2* (tobamovirus multiplication) genes, which encode transmembrane proteins (Yamanaka et al. 2000; Tsujimoto et al. 2003) and *RTM2* (restricted TEV movement 2), which encodes a protein with homology to heat-shock proteins (Whitham et al. 2000). Although characterization of components of passive resistance has allowed the acquisition of substantial information about host factors required for viral replication and movement, it has not yet provided a definitive link between active and passive resistance pathways.

It should be borne in mind that in at least one Arabidopsis ecotype, Shahdara, susceptibility and rapid cell-to-cell movement of TMV correlates with the induction of *PR-1* and *PR-5* genes (Dardick et al. 2000). Ecotypes that support slow movement of TMV did not display induction of *PR-1* and *PR-5* genes, suggesting an inverse relationship between mechanisms leading to passive and active resistance to viral pathogens (Dardick et al. 2000). Unlike the Arabidopsis-TMV interaction, resistance conferred by the Arabidopsis cauliflower mosaic virus resistance gene 1 (*CAR1*) against *Cauliflower mosaic virus* (CaMV) does appear to be associated with the activation of host defense responses (Leisner et al. 1993; Callaway et al. 1996). Based on the patterns of accumulation of the wild-type virus and the movement-incompetent CaMV mutant, it was proposed that *CAR1* confers resistance by interfering or failing to support, movement of CaMV. The *CAR1*-containing ecotype Enkheim-2 (En-2) does not show any visible signs of a HR upon CaMV inoculation but does reveal high-level expression of *PR* genes and accumulation of camalexin. Although it is somewhat intriguing that both PR proteins and camalexin accumulate very late in CaMV inoculated En-2 plants (14 days post-inoculation), the finding that mutants upregulated in SAR show enhanced resistance to CaMV indicates that both SA and/or PR proteins may play a role in resistance. A mutation in *npr1* did not enhance susceptibility to CaMV but, since its effect on resistance to CaMV was not tested in mutants upregulated in SAR it is unclear if *PR* gene expression and/ or levels of SA are critical for *CAR1*-mediated resistance to CaMV.

Plants tolerant to viral infections show mild or no symptoms even though they accumulate high levels of pathogen. Recent studies conducted with the nepovirus *Tobacco ringspot virus* (TRSV), and a bromovirus, *Spring beauty latent virus* (SBLV), have identified genetic loci in Arabidospis, which are responsible for conferring tolerance against these viruses (Lee et al. 1996; Fujisaki et al. 2004). Interestingly, tolerance to SBLV was conferred on a sensitive line by removal of SA (achieved by *nahG* expression), suggesting that SA is involved in the development of necrotic symptoms (Fujisaki et al. 2004). Similarly, removal of both SA and ethylene in tomato were shown to lead to tolerance to bacterial pathogens (Lund et al. 1998), indicating that tolerance may involve interaction(s)

between multiple signaling pathways (O'Donnell et al. 2003). Isolation and characterization of genes contributing towards tolerance to viral pathogens will help to determine mechanisms contributing to symptom development and basal resistance.

RTM1-mediated resistance to TEV

Resistance of Arabidopsis to TEV, which is mediated by the dominant gene, RTM1, results from the blockage of long-distance movement of the virus. The RTM1-mediated resistance to TEV does not involve a HR or the induction of marker genes associated with SAR, and remains unaffected by the transgenic expression of nahG. RTM1-mediated resistance is also independent of mutations in *NPR1*, *EDS1*, *PAD4* and *NDR1*, which are critical components of SAR and *R*-gene-mediated resistance against various pathogens (Mahajan et al. 1998). These observations suggest that RTM1-mediated resistance may either be downstream of the various SAR/ R-gene-associated functions or that it functions independently. In addition to RTM1, another dominant locus, RTM2 is also involved in restricting the long-distance movement of TEV. Both RTM1 and RTM2 are specific to TEV and do not function as generalized defense controls against long-distance movement. This suggests that the long-distance movement of viruses may be controlled in several different ways. As described above, *RTM2* encodes a multi-domain protein containing an N-terminal region with high similarity to that of plant small heat-shock proteins (HSP) (Whitham et al. 2000). Although the RTM2 small HSP-like domain is evolutionarily distinct from plant small HSP's, the recent discovery that HSP's can act as molecular chaperones in R-gene signaling pathways (Takahashi et al. 2003; Hubert et al. 2003; Liu et al. 2004) prompts speculation about possible links between RTM-mediated resistance to movement and active resistance conferred by *R* genes. The RTM1 gene encodes a lectin-like protein, and has been suggested to restrict TEV long-distance movement either by physical blockage of viral entry into vascular tissues, or by inhibition of a factor required for long-distance movement. Elucidation of mechanisms underlying RTM1-mediated resistance will help establish if this resistance mechanism is unique to TEV or overlaps with other virus-Arabidopsis signaling pathways. Recently, Arabidopsis was shown to be a host for another potyvirus *Turnip mosaic virus* (Martin et al. 1999) and future studies using this pathogen will also help to establish if RTM1-mediated resistance acts against potyviruses in general or is specific to TEV.

Transcriptome changes during viral infection

The viral infection process can cause alterations in the expression of many of the host genes which, in turn, can influence replication of the virus and/or development of disease. Whole genome analysis of expression profiles is, therefore, very useful in identifying genes that are likely to play roles during compatible or incompatible responses. Comparative analysis of 9000 expressed sequenced tags in CMV inoculated resistant (C24) and susceptible (Col-0) ecotypes showed a change in expression of 909 and 303 genes, respectively (Ishihara et al. 2004). Intriguingly, a greater number of genes were induced during early (6 and 12 hours post-inoculation) stages of infection in Col-0 as against the C24 ecotype, with the reverse being true for later time points (24 and 48 hours post-inoculation). This could be because the virus is able to affect more cells during a compatible response as compared to the incompatible response and thus triggers a more pronounced change in the transcription profile. Induction of fewer genes during an incompatible response should not be interpreted as a passive state, and may very well be suggestive of post-translational changes. Subsequent stages of an incompatible response would result in bolstering the various components of active defense signaling, which directly or indirectly alter the expression of many more genes. Although many of the transcriptome changes during an incompatible interaction may be inconsequential to the actual resistance response, such analyses are still useful in obtaining a global view of how these direct and indirect events relay their information. In this regard, it will be very useful to obtain a transcriptome profile of the Arabidopsis-TCV infection and determine how it compares with the Arabidopsis-CMV transcriptome.

Transcriptome analysis of other compatible interactions involving Arabidopsis and TMV, *Oil seed rape virus*, TVCV and *Potato virus X* have identified a smaller number of genes (68-114) which show altered expression during the infection process (Golem and Culver, 2003; Whitham et al. 2003). Microarray analysis of inoculated and systemic tissues shows a significant overlap in expression profiles and suggests that systemic spread of the virus is not associated with induction of additional genes (Golem and Culver, 2003). Many of the genes showing altered expression during viral infections were stress-related, and included genes such as thioredoxin, glutathione-S-transferase and ß-glucanases (Golem and Culver, 2003; Whitham et al. 2003). These genes were induced during both incompatible and compatible responses, although their expression levels were more elevated during the incompatible response.

Conclusions

The past few years have witnessed a substantial growth in our understanding of the interaction between plants and viruses. In addition to various phytohormones, such as SA and ethylene, increased viral turnover mediated by RdRp's and increased levels of reactive oxygen species in the mitochondria have been implicated in resistance against viral pathogens. It has also become clear that *R*-gene-mediated resistance to viruses requires components of basal defense that are also essential for resistance to many bacterial, fungal and oomycete pathogens. Further analyses of various resistance mechanisms, as well as understanding their coordination and potential links to one another, will be required to gain a mechanistic know-how of the interactions between plants and viral pathogens.

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Chapter A8

Viral Counter-Defense Molecules

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Introduction

The concepts of an RNA surveillance defense operating against plant viruses and plant viruses expressing counter-defense molecules came to a confluence in late 1998, with the publication of three seminal papers (Anandalakshmi et al. 1998; Brigneti et al. 1998; Kasschau and Carrington, 1998). These studies demonstrated that specific viral-encoded proteins, shown to enhance pathogenicity when expressed from viral vectors, could suppress the silencing of a reporter transgene. A fourth paper also published in 1998, demonstrated that infection by *Cucumber mosaic virus* (CMV) could suppress the silencing of reporter transgenes, but did not delimit a specific viral-encoded protein (Béclin et al. 1998). These studies all led to the idea that some viruses are able to counter an inherent defense mechanism in plants based on targeting the viral RNA sequence. Subsequent work has identified many such potential counter-defense molecules in different viruses. These are referred to as silencing suppressors, since it is in such assays that a role for all these proteins in suppressing an RNA surveillance system has been demonstrated. The connection between a role for these proteins in suppressing the silencing of a transgene and inhibition of plant defense mechanisms preventing natural virus infection has only limited direct experimental support. Here we will describe the work that led to the concept of plant viruses expressing counter-defense proteins and the experimental evidence that silencing suppressors are involved in countering some plant defense measures.

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Synergy and counter-defense

The concept of viruses expressing counter-defense proteins arose from work on viral synergism (Pruss et al. 1997). Viral synergy is the observation that double infection by some combinations of viruses induces a worse disease than infection by either virus alone. The observations on synergistic interactions between viruses go back to the 1920's (Vanterpool, 1926; Blood, 1928; Smith, 1928). Numerous examples of synergistic interactions between viruses have since been identified, but only in some cases has there been assessment of the alteration in virus levels as a result of the interaction (Table 1). Many, but not all of these involve double infections where one virus is a potyvirus. In all but one of these situations, the potyvirus did not increase in accumulation, but the co-infecting virus did (Table 1). In the case of sweet potato plants infected with the crinivirus *Sweet potato*

Table 1. Effects of synergy between viruses

Virus 1	Virus 2	Effect on Viruses	References
TMV	PVY*	Increase in TMV	Conover and Fulton, 1953;
PVX	PVY*	Increase in PVX	Rochow and Ross, 1955
TMV	TRSV	Increase in TMV	Garces-Orejuela and Pound, 1957
TMV	CMV	Cyclical incr. in both	Garces-Orejuela and Pound, 1957
PVX	TMV	Increase in PVX	Thomson, 1961
TMV	BMV	Increase in TMV	Hamilton and Nichols, 1977
BPMV	SMV*	Increase in BPMV	Calvert and Ghabrial, 1983
CMV	ZYMV*	Increase in CMV	Poolpol and Inouye, 1986
MCMV	MDV*	Increase in MCMV	Goldberg and Brakke, 1987
PLRV	PVY*	Increase in PLRV	Barker, 1987
MCMV	WSMV†	Incr. in both viruses	Scheets, 1988
CMV	TuMV*	Increase in CMV	Sano and Kojima, 1989
CPMV	SMV*	Increase in CPMV	Anjos et al. 1992
CABYV	ZYMV*	Increase in CABYV	Bourdin and Lecoq, 1994
CMV	BlCMV*	Increase in CMV	Anderson et al. 1996
CMV	PVY*	Increase in CMV	Palukaitis and Kaplan, 1997
SPFMV*	SPCSV	Increase in SPFMV	Karyeija et al. 2000
CMV	WMV*	Increase in CMV	Wang et al. 2002
TVCV	CaMV	Increase in TVCV	Hii et al. 2002
PepGMV	PHV	Increase in PepGMV	Mendez-Lozano et al. 2003

Abbreviations: BPMV, *Bean pod mottle virus*; BlCMV, *Blackeye cowpea mosaic virus*; BMV, *Brome mosaic virus*; CaMV, *Cauliflower mosaic virus*; CPMV, *Cowpea mosaic virus*; CMV, *Cucumber mosaic virus*; CABYV, *Cucurbit aphid-borne yellows virus*; MCMV, *Maize chlorotic mottle virus*; MDV, *Maize dwarf virus*; PepGMV, *Pepper golden mosaic virus*; PHV, *Pepper huasteco virus*; PLRV, *Potato leafroll virus*; PVX, *Potato virus X*; PVY, *Potato virus Y*; SMV, *Soybean mosaic virus*; SPCSV, *Sweet potato chlorotic stunt virus*; SPFMV, *Sweet potato feathery mottle virus*; TMV, *Tobacco mosaic virus*; TRSV, *Tobacco ringspot virus*; TuMV, *Turnip mosaic virus*; TVCV, *Turnip vein-clearing virus*; WMV, *Watermelon mosaic virus*; WSMV, *Wheat streak mosaic virus*; ZYMV, *Zucchini yellow mosaic virus*.

* Potyviruses.

† Rymovirus in the family *Potyviridae*.

chlorotic stunt virus (SPCSV) and the potyvirus *Sweet potato feathery mottle virus* (SPFMV), synergy was accompanied by an increase in the level of accumulation of SPFMV, but not of SPCSV (Kareija et al. 2000).

The classical work on the nature of synergistic interactions between viruses was published by Ross and colleagues between 1950 and 1974. These experiments focussed on synergy between *Potato virus X* (PVX) and *Potato virus Y* (PVY), and showed that PVX levels but not PVY levels increased during the synergy. Moreover, co-infection of PVX and PVY led to a greater increase in PVX levels than when PVX or PVY was inoculated some time before the other virus (Rochow and Ross, 1955; Goodman and Ross, 1974a). However, the data also suggested that for a strong synergistic interaction the cells needed to be actively accumulating PVY when PVX was introduced (Damirdagh and Ross, 1967; Goodman and Ross, 1974a). The increase in disease paralleled the increase in PVX levels, which depended on the growth stage of the inoculated plant, the type of tissue examined and the temperature (Rochow and Ross, 1955). Interestingly, even at this early stage, it was hypothesized that "infection by PVX normally induces reactions that act to limit PVX synthesis but is unable to do so in a cell in which PVY is actively multiplying" (Damirdagh and Ross, 1967), although the nature of the substance limiting infection of PVX was not understood. Subsequent work showed that the enhancement of PVX accumulation occurred in doubly infected cells (Goodman and Ross, 1974b). This also was established for synergy between CMV and the potyvirus *Zucchini yellow mosaic virus* (ZYMV) (Poolpol and Inouye, 1986).

The interactions between PVX and PVY involved in synergy were reinvestigated by Vance (1991), who used techniques not available in the earlier studies to confirm and extend the conclusions of Goodman and Ross (1974a,b) and Goodman (1973); viz., that the level of both PVX capsid protein and viral RNA increased during co-infection with PVY, but the level of PVY did not increase. However, Vance also established that there was a larger proportional increase in the level of accumulation of (-) PVX RNA vs. that of (+) PVX RNA in these doubly infected plants. These changes in either (+) or (-) did not always occur in synergistic interactions resulting in enhanced pathogenicity and may depend on the host as well as the combination of viruses used (Wang et al. 2002; González-Jara et al. 2004).

Subsequently, Vance and colleagues showed that other potyviruses could mediate the synergy of PVX. Moreover, transgenic tobacco plants expressing the P1/HC-Pro/(partial or complete) P3 sequences of *Tobacco*

vein mottling virus (TVMV) and *Tobacco virus etch* (TEV) were able to support a synergistic interaction with PVX, while transgenic plants expressing other sequences of TVMV did not support enhanced disease or increased PVX RNA accumulation (Vance et al. 1995). Further work from this group showed that transgenic tobacco expressing the P1/HC-Pro/(partial) P3 of TEV could also mediate the synergy with CMV and *Tobacco mosaic virus* (TMV). In addition, a PVX vector expressing the HC-Pro protein of TEV was able to mediate both the pathological synergy shown by co-infection of TEV with PXV and an increase in the level of accumulation of PVX (+) and (-) RNA, while expression of P1/HC-Pro enhanced PVX RNA accumulation more than expression of HC-Pro alone (Pruss et al. 1997).

These authors presented a model by which the P1/HC-Pro might function in synergism by interfering with a general host defense mechanism that acts to limit the extent of virus accumulation. They suggested that this defense mechanism could be the same one that had been proposed to be involved in transgene RNA-mediated resistance to viruses referred to by various names: homology-dependent resistance, post-transcriptional gene silencing and more recently RNA silencing or RNA interference (RNAi) (see Chapters 9 and 13). The subsequent studies demonstrated that viral-encoded proteins involved in synergistic interactions could inhibit the RNA silencing of reporter transgenes (Anandalakshmi et al. 1998; Brigneti et al. 1998; Kasschau and Carrington, 1998). As more data became available on the mechanism of RNA silencing of viral and non-viral transgenes as well as native plant genes, a better understanding was developed as to the nature of the host defense system with which these counter-defense molecules interacted.

Defense and counter-defense

The RNA surveillance system now called RNA silencing by plant biologists (described in detail in Chapters 8 and 13) is based on the recognition and targeting of RNAs containing regions that are double-stranded (dsRNA). These dsRNAs are selected for digestion by a ds-specific RNase called Dicer (Bernstein et al. 2001; Cerutti, 2003; Denli and Hannon, 2003). The fragments of digested dsRNAs, consisting of 21-25 bp and referred to as small interfering RNAs (siRNAs) are incorporated into a RNA-induced silencing complex (RISC) which promotes their interaction with complementary sequences on other ssRNA molecules and targets the cleavage of these ssRNAs (Hammond et al. 2000; Zamore et al. 2000 (Martinez and Tuschl, 2004; Schwarz et al. 2004). This RNA surveillance system also degrades putative aberrant RNAs produced by some transgenes,

as well as a few host mRNAs encoding proteins involved in either the synthesis of pigments (Metzlaff et al. 1997; Senda et al. 2004) or seed storage protein (Kusaba et al. 2003), which are transcribed from inverted-repeat structures. It is also believed that this system removes expressed retrotransposons (Flavell, 1994; Wu-Scharf et al. 2000; Hamilton et al. 2002). In addition, this system also targets viruses.

Because ssRNA viruses usually form dsRNA intermediates during replication, the action of a plant RNA-dependent RNA polymerase (RdRp) is not always necessary in the RNA silencing pathway against plant viruses (Dalmay et al. 2000; Mourrain et al. 2000; Voinnet et al. 2000). The Arabidopsis SDE1/SGS2 protein is an RdRp required for transgene-mediated silencing. Mutant plants lacking this protein are no more susceptible than wild type Arabidopsis to TMV, *Tobacco rattle virus* (TRV), or the potyviruses *Turnip mosaic virus* (TuMV) or TVMV. However, these plants are more susceptible to CMV. Arabidopsis has six genes encoding RdRps including SDE1. Another of these genes, *AtRdRP1*, a homologue of the tobacco gene *NtRdRp1*, was shown to be induced by both salicylic acid and virus infection (Xie et al. 2001; Yu et al. 2003). Plants either mutated in this gene or silenced for expression of this gene were more susceptible to TMV, PVX and TRV. *Nicotiana benthamiana* plants are particularly susceptible to virus infection. It was shown recently that this susceptibility might result from a naturally occurring insertion mutation in a salicylic acid-inducible RdRp gene (*NbRdRp1m*) (Yang et al. 2004). Transformation of *N. benthamiana* with a RdRp gene from *Medicago truncatula* produced plants with increased resistance to the tobamoviruses TMV and *Sunn hemp mosaic virus*, as well as the potyvirus TVMV, but with unaltered susceptibility to CMV and PVX.

In order to evade the RNA silencing system, viruses have evolved various counter-defense strategies. In general, these strategies probably involve the inhibition of one or more steps in the RNA silencing mechanism, although in most cases, it is not clear exactly what step is either neutralized or partially inhibited. Most often the silencing suppressors themselves have been so designated by whether they could inhibit RNA silencing of reporter transgenes using a variety of assays that are unrelated to the natural role of the suppressors in virus infection.

Assays for counter-defense molecules

To determine whether a given virus expressed such counter-defense molecules, plants silenced for the expression of a reporter transgene were infected by that virus and the plants were assessed for the suppression of

RNA silencing of the reporter transgene (Béclin et al. 1998; Brigneti et al. 1998; Voinnet et al. 1999). To further delimit which virus-encoded protein(s) function as counter-defense molecules, several other types of assays have been developed. A given suppressor protein may function in one or in some combination of these assays.

A second assay makes use of transgenic plants containing a reporter transgene whose expression is blocked by endogenous silencing. These plants are infected with a PVX vector expressing the protein(s) of interest to determine whether the infection results in silencing suppression and consequent expression of the reporter gene (Anandalakshmi et al. 1998; Brigneti et al. 1998). Not all viral-encoded proteins that induced synergy (i.e., enhanced the pathogenicity of PVX) were able to suppress silencing in this assay. Thus, the PVY HC-Pro and the CMV 2b proteins were able to suppress silencing, while the PVY NIb protein was not able to do so, even though the NIb protein also produced a synergistic reaction when expressed from PVX in *N. benthamiana* (Brigneti et al. 1998). This assay also showed that HC-Pro was able to suppress the silencing of a green fluorescent protein (GFP) reporter gene in the tissue infected by PXV expressing the HC-Pro, but not in uninfected tissues. By contrast, in this assay, the CMV 2b protein was not able to suppress silencing of the GFP transgene in the inoculated tissues, but was able to do so in upper leaves that had not emerged at the time of infection. This led to the proposal that different viruses encoded different suppressor molecules that affected different aspects of RNA silencing. Finally, the assay showed that PVX itself did not suppress the silencing of the transgene, which led to the inaccurate conclusion that PVX did not encode a suppressor and somehow evaded the RNA surveillance system by some unknown means. This was incorrect, but is important because it showed that not all viruses encode silencing suppressors that can be assessed by this assay.

A third assay, which was used by two laboratories to demonstrate that the potyvirus HC-Pro was a silencing suppressor, involves making transgenic plants that express the test protein (Anandalakshmi et al. 1998; Kasschau and Carrington, 1998). In these examples, the transgenic plants expressing HC-Pro were crossed with transgenic plants that were silenced for expression of the β-glucuronidase (GUS) gene. In the presence of the silencing suppressor, the GUS gene was not silencing. This has been used less frequently as an assay for a potential silencing suppressor, since the generation of transgenic plants is quite time consuming. Nevertheless, it has been used to demonstrate silencing suppression activity for several proteins that did not show such activities by other assays. More detailed study of the mechanism of action of HC-Pro and CMV 2b involved grafting experiments in which the rootstock, scion and in some instances a third, spacer section were derived from different combinations of transgenic plants expressing the

suppressor and reporter, either silenced or active (Mallory et al. 2001; Guo and Ding, 2002). This sophisticated and elegant approach has uncovered subtle differences in the action of these suppressors in perception of, movement of and response to the mobile signal that is responsible for systemic silencing.

A fourth system was developed to speed up the testing of putative suppressor proteins and (in part) to obviate the concern that a given suppressor may function at an early stage of RNA silencing and thus not function when using plants that had been pre-silenced prior to expression of the suppressor. In this assay, the suppressor and a reporter gene (encoding GFP in most assays) are present in two different *Agrobacterium tumerifaciens* cultures, which are mixed and co-infiltrated into a leaf of a transgenic plant expressing the same reporter gene (GFP). The agroinfiltrated reporter gene is expressed transiently and induces silencing of the transgene, as well as its own expression from the introduced T-DNA. However, if the co-introduced gene, which also is expressed transiently, encodes a suppressor protein, then this protein might prevent the onset of RNA silencing in the agro-inoculated area. This is sometimes called the agro-patch test. This assay can be used to examine suppression of silencing in both local, i.e., infiltrated tissue, and in systemic, i.e., upper, un-infiltrated leaves. For example, using this assay it was demonstrated that PVX does indeed encode a suppressor, and that this suppressor, the p25 movement protein, acts at a very early stage to prevent the establishment of RNA silencing in systemic tissue (Voinnet et al. 2000).

A variation of the agro-patch test that does not require the use of transgenic plants is to mix three *Agrobacterium* cultures: one expressing the reporter gene; one expressing dsRNA corresponding to the reporter gene (with an intron present between the two arms corresponding to the reporter gene, forming a hairpin in the expressed RNA); and one expressing the putative suppressor (Johansen and Carrington, 2001). The expressed dsRNA activates the RNA silencing mechanism preventing the transient expression of the reporter gene, unless the putative suppressor protein inhibits the initiation or maintenance of the silencing.

A similar system but using cells of *Drosophila melanogaster* instead of plant leaves recently has been described (Reavy et al. 2004). Here *Drosophila* cells growing in tissue culture are simultaneously transfected with three components. One is a plasmid encoding the β-galactosidase (*lacZ*) gene, the second is dsRNA homologous to part of the *lacZ* gene and the third is a plasmid encoding the putative silencing suppressor gene. In the absence of suppression activity transcripts from the *lacZ* gene are targeted by the dsRNA and degraded. In the presence of an active suppressor, the *lacZ* transcripts accumulate, producing β-galactosidase protein, which is

detected in a colorimetric assay. The PVY HC-Pro, TRV 16K and *Groundnut rosette virus* ORF3 (M. Taliansky, personal communication) proteins were able to suppress silencing in this system whereas the CMV 2b protein was not.

In their natural context, expressed as part of the viral genome, many silencing suppressor proteins have been shown to be required for virus multiplication, local and/or systemic spread, and symptom production. Very often heterologous expression of these proteins from a different virus, typically PVX, results in an increase in symptom severity. In related studies, as an indirect assay for "suppressor" function, complementation of a suspected suppressor protein by a known suppressor has been demonstrated. For example, deletion of the 16K gene from TRV prevented systemic movement of the virus, which could be regained by replacement with a known suppressor (CMV 2b) as well as with the *Soilborne wheat mosaic virus* 19K gene and the *Barley stripe mosaic virus* (BSMV) γb gene (Liu et al. 2002). Subsequent studies confirmed that both the TRV 16K and BSMV γb proteins are silencing suppressors (Yelina et al. 2002; Reavy et al, 2004). The BSMV γb protein could itself be complemented by HC-Pro, and suppressed homology-mediated cross-protection (i.e., interference produced by silencing) between TMV and PVX each carrying GFP sequences. In a different study, substitution of the HC-Pro gene of *Wheat streak mosaic virus* (WSMV) with that of other tritimoviruses [various WSMV isolates and *Oat necrotic mottle virus* (ONMV)], as well rymoviruses (*Agropyron mosaic virus* and *Hordeum mosaic virus*) and potyviruses (TEV, TuMV), allowed the modified WSMV to remain infectious on wheat, but only HC-Pro from WSMV/ONMV allowed infection also on oat and maize (Stenger and French, 2004). This showed that the silencing suppressor HC-Pro is a determinant of host range in some instances although the potyviruses TEV and TuMV are not themselves able to infect wheat.

Regulation of silencing suppressors

Details of the regulation of the expression of the various suppressors are not generally known although their synthesis is likely to be tightly controlled by, for example, relative position within the virus genome, promoter strength and codon usage. In many cases it has been found that (uncontrolled) expression of these proteins in *N. benthamiana* using PVX greatly enhances the symptoms of infection caused by this virus (Brigneti et al. 1998; Voinnet et al. 1999). Similarly, moving the gene for the TRV 16K suppressor from its normal position in RNA1 and expressing it from a duplicated coat protein subgenomic RNA promoter located in RNA2 increased the severity of symptoms caused by this virus (Liu et al. 2002). The P0 suppressor that is

encoded by the poleroviruses *Potato leafroll virus* and *Beet western yellows virus* is expressed at very low levels during normal virus infection, in part, because the 5' sequence of the P0 gene is translated with low efficiency (Pfeffer et al. 2002). Attempts to increase P0 synthesis by modification of the translation initiation sequence context were unsuccessful due to the rapid appearance of second-site mutations that dampened down P0 expression.

Several studies have examined the replacement of the suppressor gene from one virus with the homologous gene from a related virus, producing a variety of outcomes. For example, replacement of the HC-Pro gene of TVMV with that of ZYMV produced a viable chimera giving attenuated symptoms (Atreya and Pirone, 1993). Chimeras produced by swapping the HC-Pro genes between several other viruses from the family *Potyviridae* resulted in a range of symptom alterations both attenuated and more severe (Stenger and French, 2004). Similarly, replacing the γb gene of BSMV with that of the related hordeivirus *Poa semilatent virus* greatly increased symptom severity (Yelina et al. 2002), as also did the replacement of the CMV 2b gene with that of the related *Tomato aspermy virus* (Li et al. 1999).

An alternative route to regulation of suppressor activity occurs with the carmovirus *Turnip crinkle virus* (TCV). The suppressor for this virus is the coat protein (CP), and in particular a 25 amino acid region at the N-terminus of the CP (Qu et al. 2003; Thomas et al. 2003). However, this part of the CP is sequestered within the assembled virus particle and only free CP monomers have strong silencing suppression activity. Reducing the amount of virus particle formation, either by mutating the CP or adding a satellite RNA (satC) to the infection, raises the level of free CP, enhances silencing suppression and leads to an increase in symptom severity (Zhang and Simon, 2003).

Silencing suppressor molecules and their proposed targets

Using the various assays described above a variety of plant virus silencing suppressor molecules has been identified (Table 2). Many of these proteins previously had been associated with other viral-encoded functions, especially virus movement. Based on the accumulation of various intermediates in RNA silencing and the particular tissues of infected plants in which silencing occurs, the stages at which various silencing suppressors probably function have been delimited. However, the precise mechanism by which each of these suppressors interferes with silencing has not been determined for most of them. Also, as has been mentioned before, mutating the viral genes that encode these suppressor proteins most often results in multiple observable effects since these proteins are multifunctional.

Table 2. Silencing suppressors of plant viruses

Virus	Suppressor	Biological function(s)	References
TEV, PVY	HC-Pro	Systemic movement, transmission by aphids, genome amplification	Anandalaksmi et al. 1998; Brigneti et al. 1998; Kasschau and Carrington, 1998
CMV, TAV	2b	Systemic and cell-to-cell movement, pathogenicity	Brigneti et al. 1998; Li et al. 1999
ACMV, TGMV, TYLCV	AC2, AL2, C2	Pathogenicity, activation of virus gene expression	Voinnet et al. 1999; Hamilton et al. 2002, van Wezel et al. 2002; Selth et al. 2004
RYMV	P1	Pathogenicity, systemic movement, virus accumulation	Voinnet et al. 1999; Hamilton et al. 2002
TBSV, CymRSV	p19	Pathogencity, cell-to-cell and systemic movement	Voinnet et al. 1999; Qiu et al. 2002; Havelda et al. 2003
PVX	p25	Cell-to-cell movement, egress from veins in systemic leaves, RNA helicase	Voinnet et al. 2000
BSMV, PSLV	γb	Seed transmission, genome amplification, systemic movement	Yelinda et al. 2002
BWYV, CABYV; PLRV	P0	Symptom production, virus accumulation	Pfeffer et al. 2002
PCV	p15	Genome amplification	Dunoyer et al. 2002
TSWV	NS_s	Symptom production, virus movement	Takeda et al. 2002; Bucher et al. 2003
BYV, BYSV, CTV	p21, p20, p23, CP	RNA accumulation, capsid formation	Reed et al. 2003, Lu et al. 2004
RHBV	NS3	Unknown	Bucher et al. 2003
TCV	CP	Capsid formation, virus movement	Qu et al. 2003; Thomas et al. 2003
ToMV, TMV	126 kDa	RNA-dependent RNA polymerase, virus movement	Kubota et al. 2003; Ding, X.S. et al. 2004
CPMV	CP-S	Capsid formation	Liu et al. 2004
TRV	16 kDa	Seed transmission, RNA accumulation	Reavy et al. 2004
TYMV	p69	Virus movement, symptom severity	Chen et al. 2004

Abbreviations: ACMV, *African cassava mosaic virus*; BSMV, *Barley stripe mosaic virus*; BWYV, *Beet western yellows virus;* BYSV, *Beet yellow stunt virus*; BYV, *Beet yellows virus*; CTV, *Citrus tristeza virus*; CPMV, *Cowpea mosaic virus*; CMV, *Cucumber mosaic virus*; CABYV, *Cucurbit aphid-borne yellows virus*; CymRSV, *Cymbidium ringspot virus*; PCV, *Peanut clump virus*; PSLV, *Poa semilatent virus*; PLRV, *Potato leafroll virus*; PVX. *Potato virus X*; PVY, *Potato virus Y*; RHBV, *Rice hoja blanca virus*; RYMV, *Rice yellow mottle virus*; TEV, *Tobacco etch virus*; TMV, *Tobacco mosaic virus*; TRV, *Tobacco rattle virus*; TRSV, *Tobacco ringspot virus*; TAV, *Tomato aspermy virus*; TBSV, *Tomato bushy stunt virus*; ToMV, *Tomato mosaic virus*; TSWV, *Tomato spotted wilt virus*; TYLCV, *Tomato yellow leaf curl virus*; TCV, *Turnip crinkle virus*; TYMV, *Turnip yellow mosaic virus*.

Although it is clear that the role of the HC-Pro protein in aphid transmission of potyviruses is separate from its role in suppression, it is possible that, for example, the seed transmission function of the TRV 16K and BSMV γb proteins might involve silencing suppression. Similarly the known roles of many suppressors in virus multiplication and movement might be related specifically to silencing suppression, although this has not been proven conclusively for any suppressor. It is also the case that a virus might encode more than one suppressor protein. For example, the closterovirus *Citrus tristeza virus* (CTV) encodes three suppressors (Lu et al. 2004). Two of them, p20 and p23, function in the agro-patch assay and also when crossed into a transgenic plant carrying a silenced reporter. However, only p20 and the third suppressor, CP, inhibited systemic movement of the silencing signal in grafted plants. Presumably these three suppressors act in different but overlapping ways to promote CTV infection.

In only one example has a mechanism of suppressor action been determined. For the silencing suppressor protein of *Cymbidium ringspot virus* (CymRSV) is it clear that a dimer of the CymRSV p19 protein actually binds to the siRNAs and apparently prevents them from interacting with target RNA molecules *via* the RISC (Silhavy et al. 2002; Lakatos et al. 2004). The crystal structure of p19 complexed with an siRNA duplex has been determined revealing a mechanism by which p19 interacts preferentially with 20-22ntsiRNAs (Vargason et al. 2003; Ye et al. 2003).

Plant protein-silencing suppressor interactions

One approach to determine how suppressor proteins function is to identify plant proteins with which suppressor proteins interact using the yeast two-hybrid system. Although this strategy has not yet identified known silencing pathway components as targets of viral suppressors, several interesting examples of the alteration of plant gene function by viral suppressor proteins have been discovered.

The TEV HC-Pro was found to interact with a calmodulin domain-containing protein called rgsCaM (Anandalakshmi et al. 2000). Over-expression of this protein also led to suppression of silencing, suggesting that it might be an endogenous suppressor and that the calcium signaling pathway might play a role in silencing. Several other proteins bind to HC-Pro in yeast, although the significance of this is not known (Guo et al. 2003).

The tombusvirus p19 protein binds siRNAs *in vitro* and *in vivo* and is suggested not to require interaction with host proteins for its silencing suppression activity (Lakatos et al. 2004). Nevertheless, p19 interacts with members of the ALY family of RNA-binding proteins, which in animals are

involved in export of RNAs from the nucleus (Park et al. 2004; Uhrig et al. 2004). In plants, expression of p19 leads to re-localization of two of the four ALY proteins from the nucleus to the cytoplasm. Whether this influences RNA silencing or is important for any of the other known roles of p19 is not yet understood.

The CP of TCV suppresses local silencing in the agro-patch assay and prevents the accumulation of siRNAs (Qu et al. 2003; Thomas et al. 2003). A 25 amino acid region at the N-terminus of the protein that is sequestered inside assembled virus capsids was shown to be important for suppression activity, as well as for interaction with the TIP transcription factor (Ren et al. 2000). Furthermore, the CP:TIP interaction is required for a hypersensitive resistance response in Arabidopsis. Recent results show that single amino acid mutations in the N-terminal regions can separate the TIP-binding and suppression activities of the CP, suggesting that TIP may not be involved in the silencing pathway (Choi et al. 2004).

Geminiviruses, which are comprised of single-stranded DNA rather than RNA (in contrast to all the other viruses discussed previously in this review) also encode a silencing suppressor protein (Voinnet et al. 1999). The suppressor protein of *African cassava mosaic virus* is the AC2 protein, which is a transcriptional activator protein involved in CP expression. The homologous protein from *Tomato golden mosaic virus* is called the AL2 protein, and the homologue from *Tomato yellow leaf curl virus* is called the C2 protein (Dong et al. 2003). Transgenic plants expressing AL2 or the positional homologue L2 from *Beet curly top virus* are more susceptible to these viruses and to TMV, an unrelated RNA virus (Sunter et al. 2001). AL2 and L2 interact in plants with SNF1 kinase, which controls the activity of a range of metabolic pathway transcriptional activators and repressors in response to nutritional and environmental stress (Hao et al. 2003). Overexpression of SNF1 causes enhanced resistance to geminivirus infection, and the AL2 and L2 proteins bind SNF1 to inhibit its kinase activity *in vitro* and *in vivo* (in yeast).

Silencing suppressors and plant development

Current work in the area of RNA silencing has revealed a mechanism by which some plant virus-induced disease symptoms can arise. It has been shown that plants contain a wide variety of small RNA species that before the discovery of siRNAs were mostly unknown. One class are called micro-RNAs (miRNAs), which are a similar size to siRNAs (21-25nt) but are formed by processing of a stem-loop-containing pre-miRNA in the nucleus (Carrington and Ambros, 2003; Palatnik et al. 2003; Bonnet et al. 2004). In plants, separate but related Dicer enzymes carry out cleavage of siRNA and

miRNA precursors. Initially, based upon studies in animals, it was suggested that siRNAs are perfectly complementary to their targets and lead to target cleavage, whereas, miRNAs base-pair less perfectly with their targets and lead to translation repression of the target mRNA. This is not necessarily the case in plants where miRNAs can induce cleavage of their targets. miRNAs often target mRNAs encoding proteins such as transcription factors that are involved in the regulation of plant development. Several studies have shown that transgenic plants, which express viral suppressor proteins, have alterations in the pattern of miRNA accumulation, which corresponds with severe disruption of plant growth and development, mimicking some of the symptoms of virus infection (Mallory et al. 2002; Chapman et al. 2004; Chen et al. 2004; Dunoyer et al. 2004). Significantly, perturbation of miRNAs also has been demonstrated in virus-infected plants (Kasschau et al. 2003), showing that this phenomenon does occur in the natural situation.

Animal virus counter-defense proteins

Intense genetic and biochemical analysis of the silencing pathway in plants and other, higher organisms made it clear that, although there are some differences between organisms, in general these systems share many components in common (Xie et al. 2004; Tang et al. 2003; Ding, S-W et al. 2004). This led to experiments to determine whether viral suppressors could function in more than one silencing system. The first study demonstrated that the B2 protein from the nodavirus *Flock house virus* (FHV) could suppress silencing in plants in the agro-patch assay (Li et al. 2002). In nature nodaviruses infect vertebrate and invertebrate hosts and silencing of the gene for AGO2 (which is a component of RISC) in *Drosophila* cells resulted in increased levels of replicating FHV. These results suggested that silencing operates as an anti-viral defense in *Drosophila*, and therefore possibly also in other animals. In a converse approach, several plant virus suppressor proteins were shown to function in *Drosophila* cells (Reavy et al. 2004). By co-injecting live mosquitoes with a GFP-tagged togavirus and dsRNA specific for the *Ago2* gene, it was shown recently that RNA silencing is a natural antiviral defense in these organisms (Keene et al. 2004).

In animal cells viral dsRNA triggers two pathways, both of which also respond to interferons (reviewed in Stark et al. 1998). In one pathway, dsRNA activates PKR, a dsRNA-dependent protein kinase. PKR becomes autophosphorylated, and then functions by phosphorylating and inactivating the translation initiation factor eIF2 leading to shutdown of host mRNA

translation as well as to the initiation of apoptosis. The second pathway stimulates 2'-5'A synthetase to activate RNase L, which degrades single-strand RNA. Initial expectations were that the treatment of animal cells with siRNAs would activate these systems leading to cell death rather than stimulating RNA silencing. Recent studies suggest that animal viruses can be targeted in a sequence-specific manner *via* siRNAs (Karlas et al. 2004; Yoon et al. 2004). However, another study found that siRNAs could stimulate an interferon response mediated by PKR, which may compromise the sequence-specificity of these treatments (Sledz et al. 2003). Nevertheless, the existence in animals of an siRNA-mediated antiviral RNA silencing system was supported by the finding that the influenza virus NS1 protein could suppress RNA silencing in *Drosophila* cells and plants, and could bind siRNAs *in vitro* (Bucher et al. 2004; Delgadillo et al. 2004; Li et al. 2004). Similarly, the E3L protein from vaccinia virus, a double-stranded DNA containing virus, also suppressed silencing in *Drosophila* cells. Interestingly, both influenza virus NS1 and vaccinia virus E3L were already known from other studies to inhibit the innate antiviral response that is mediated by interferon. Whether RNA silencing in animals is part of or separate from this response is not known.

A cellular protein known as $P58^{IPK}$ is recruited by some animal viruses to bind with and inhibit PKR, thus reducing the capacity of the cell to resist the virus. Recently it was found that part of the helicase proteins that are involved in replication of the plant viruses TMV and TEV interact with a plant homologue of $P58^{IPK}$ (Bilgin et al. 2003). Knock-down of expression of the *N. benthamiana* $P58^{IPK}$ gene by PVX-mediated virus-induced gene silencing or knock-out of transcription in T-DNA tagged Arabidopsis resulted in plants that underwent a lethal hypersensitive reaction in response to virus infection. This suggests that a functioning $P58^{IPK}$ protein is required to inhibit a putative plant PKR activity sufficiently to allow virus infection without inducing overwhelming cell death.

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Chapter A9

Dark Greens Islands: the Phenomenon

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Introduction

Dark green islands (DGIs) have been an enigma since they were first documented before the nature of viruses was known (reviewed in Allard, 1914). In 1898 Beijerinck identified the casual agent of tobacco mosaic disease as a contagious solution a "*contagium vivum fluidum"* and described dark green blotches on the upper leaves of infected plants (Goldstein, 1926). When a mosaic virus infects a plant, these discrete regions of dark green tissue occur only on leaves that are systemically infected when immature. Leaves that are fully developed at the time of infection do not develop DGIs. A variety of tools and plant-virus models have been used in the years since Beijerinck's report to compare the dark green tissue with the surrounding yellow tissue. These experiments and observations have been aimed at determining the nature and causes of DGIs.

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There are many basic questions prompted by the appearance of DGIs. Does an active process within the plant establish a DGI? Why can only a part of the leaf develop into a DGI? How do DGIs spread and what limits their spread to prevent them encompassing the entire leaf? What physiological changes in the plant lead to chlorotic or DGI tissue? What aspect of a mosaic virus means that the host plant can form a DGI? Is there a benefit to the virus by forming DGIs in plants? Are DGIs a characteristic of a long established and balanced relationship between a pathogen and host?

This review attempts to summarise the investigations into DGIs and describe the current knowledge of DGIs. Despite recent research and the molecular tools available today many of these questions remain unanswered, providing a rich area of research and discovery for plant virologists.

Composition of dark green islands

DGIs are distinct areas of dark green contrasting against the yellow chlorotic virus-infected tissue. They can be very small or cover a large proportion of the leaf. DGIs appear on the upper side of the leaf and some but not all are visible when viewed from the underside of the leaf. DGIs may form raised 'blisters' of green tissue on the otherwise chlorotic leaf. The DGIs can encompass one or many cell layers (Matthews, 1991), originating from different cell lineages, and may spread in any direction throughout the leaf.

Goldstein (1926) carried out the first detailed cytological study *"of tobacco mosaic* [virus] *with the hope of adding to our knowledge of the effects of the casual agent, whatever it is, on the structure and function of the diseased cells"*. These studies were undertaken on *Nicotiana tabacum* grown from the then current commercial variety Connecticut Seed Leaf. These plants were highly susceptible to the *Tobamovirus Tobacco mosaic virus* (TMV) and produced very uniform plant growth and virus symptom development. She found that DGIs were cytologically similar to uninfected healthy leaves, whereas the yellow-green tissue was altered and less differentiated with noticeable chloroplast and developmental abnormalities (Goldstein, 1926).

Histology of DGIs compared to chlorotic tissue

In detailed cytological studies, Goldstein (1926) noted correlations between the symptom patterning, the leaf formation at the time of inoculation, the position of leaf with reference to the inoculated leaf, the position of the leaf on the plant, and the time the disease became evident in the leaf. She observed that deep green areas appear first in leaves that

develop immediately after the initial virus symptoms appear, and in those leaves that grow subsequently. Such dark green areas were noted to contain normal plastids and all stages of histogenic development, according to the size and age of the leaf. Dark green areas, like healthy tissue, had six or seven layers of cells, one or two layers of elongated palisade cells, well-distributed plastids and large intercellular spaces between the elongated mesophyll cells. These observations were in contrast to cells within the surrounding yellow tissue that presented altered histological development according to leaf age at time of infection. The yellow diseased leaf parts were reduced in cross-sectional diameter when compared to both healthy and the dark green areas (Fig. 1). Such differences in leaf thickness may lead to blisters of raised DGIs in many mosaic infections. Yellow areas had palisade cells that often failed to elongate completely, with few plastids, large swollen nuclei and few intracellular spaces, and these leaf areas never possessed more than six cell layers. Thus, "*the time at which infection has taken place in a leaf of a diseased plant can be ascertained by a study of its anatomical structure*" (Goldstein, 1926). The chlorophyll content and thus intensity of colour in yellow tissue is greatly decreased. In this tissue chloroplasts often clump together in a manner foreign to healthy cell structures (Goldstein, 1926). In the mildest form of clumping individual chloroplasts may be arranged in rows in contact with each other. In more severely infected tissue chloroplasts are grouped in irregular clumps in which the outlines of the individual chloroplasts are barely discernible. Thus, the histology within DGIs and uninfected tissue are indistinguishable while the histology within yellow tissue surrounding the DGIs is distinct.

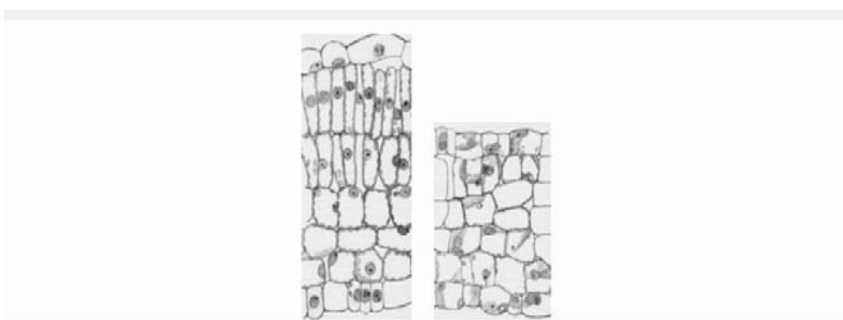

Fig. 1. Cross-section of a *Tobacco mosaic virus*-infected leaf comparing dark green islands (DGIs) and yellow tissue. Two of the original figures from Bessie Goldstein's (1926) paper showing the differences in overall cell shape, chloroplast distribution, and laminal thickness that she observed in cross-sections of induced DGIs (left) or the surrounding yellow tissue (right) in TMV-infected *Nicotiana tabacum*.

Symptoms of virus infection in yellow tissue

The chlorosis that can be observed in a virus-infected plant is due to a variety of physiological changes, some of which derive directly from the deleterious effects the presence of the virus has upon the plant. Others are a result of the various steps that the plant takes to counteract the viral attack (Maule *et al.* 2002). The common yellowing or chlorosis of leaves in virus infection is typically associated with a loss of photosynthetic capacity (Bedbrook and Matthews, 1972; Bedbrook and Matthews, 1973; Hodgson *et al.* 1989; Clover *et al.* 1999; Herbers *et al.* 2000), altered starch accumulation (Ushiyama and Matthews, 1970; Shalitin and Wolf, 2000), an increase in respiration and a change in carbohydrate partitioning with significant elevation in the proportion of sucrose in source leaves (Herbers *et al.* 2000; Shalitin and Wolf, 2000). These yellow areas of infected tissue also have a relatively high virus titre. Interestingly, both TMV RNA and coat protein (CP) are capable of entering chloroplasts where the CP has a direct affect on photosynthetic capacity (Schoelz and Zaitlin, 1989; Banerjee and Zaitlin, 1992).

DGIs contain few viral particles

Using a variety of methods, several groups have demonstrated that DGIs contain less infectious virus than the surrounding yellow tissue. Some early work was performed using fine enamel insect pins (number 00) to transmit TMV by puncturing leaves containing virus and then repeating the puncture into healthy leaves (Holmes, 1928). With such sharp pins this technique required no handling of the plants and since new pins were used for each experiment, this technique led to little cross contamination. The dose lifted on the pins was uniform and the inoculations were sufficiently rapid to process 500 inoculations per hour with reproducible results. Using this technique, Holmes generated a dilution chart that correlated concentration of virus to number of test plants infected. Green and yellow areas of mottled

leaves were then tested for virus titre. By reference to the dilution chart Holmes calculated that green tissue contained no more than 1/28 (4%) the virus concentration of yellow tissue.

Solberg and Bald (1962) used local lesion bioassays of TMV on *N. glutinosa* and virus particle counts from electron micrographs to demonstrate that dark green areas of leaves consistently showed no or little virus (0 -7 % of that found in adjacent yellow areas). In an alternative approach, ^{32}P-labeled plants (2 mCi per plant) showing full mosaic symptoms were sampled in yellow or dark green tissues using cork borers to ensure that equal amounts of tissue were obtained. Analysis of the amount of radiolabel incorporated into the viral RNA revealed that DGIs had a virus content 3.6 % of that found in chlorotic tissue samples (Reid and Matthews, 1966). Using local lesion hosts to assess infection load, virus titre from virus preparations in analytical ultracentrifuges, and also serological precipitation end points, Atkinson and Matthews (1970) calculated that dark green tissue contained between 0-9 % of the amount of virus found in yellow tissue. In *N. tabacum* cv Xanthi-nc infected with the *Cucumovirus Cucumber mosaic virus* (CMV), passaging to a local lesion host showed that DGIs contained less than 5 % of infectivity found in yellow tissue (Loebenstein et al. 1977). Furthermore, no antigen could be detected from CMV-induced DGIs and only 2-7 % of protoplast derived from DGIs showed the presence of antigen. These tests all revealed an inverse relationship between the intensity of green pigmentation and the virus content of a leaf.

An inhibitor of virus replication (IGI) was purified by zinc acetate precipitation from both DGI tissue from leaves or from the medium of DGI-derived protoplasts (Gera and Loebenstein, 1988). When purified and added exogenously the IGI activity inhibited replication of both CMV and TMV in either protoplasts or in leaf discs. The inhibitory activity reduced replication to less than 15% of untreated controls as determined by local lesion assays. The IGI activity was found to comprise of two inhibitory components of 26 kDa and 57 kDa that were susceptible to inactivation by proteases, however these presumed proteins have not been further identified.

Molecular tools have also been used to study virus content of DGIs (Moore et al. 2001; Moore, 2003). Northern analysis confirmed that the level of viral RNA was lower in DGIs than in the surrounding yellow tissue. This work was performed with DGIs induced by TMV or CMV in *N. tabacum* and by the *Potyvirus Potato virus A* (PVA: isolate *Tamarillo mosaic virus*, TamMV) in both *N. benthamiana* and *Cyphomandra betacea* (tamarillo) (Fig. 2).

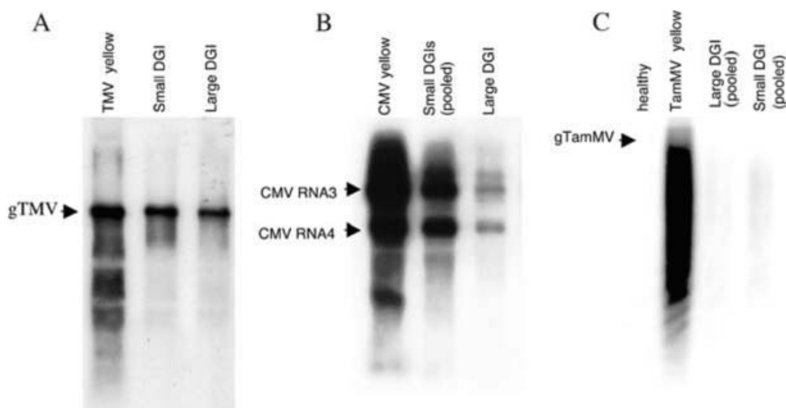

Fig. 2. Northern analysis of DGI and surrounding yellow tissue infected with TMV, *Cucumber mosaic virus*, or *Tamarillo mosaic virus* reveals less virus content in DGIs. Total RNA was extracted from yellow or DGI tissue of *N. tabacum* infected with TMV (A), or CMV (B), or from *N. benthamiana* infected with TamMV (C) and run on a formaldehyde-agarose electrophoresis gel before transfer to nylon. Each RNA blot was probed with a virus-specific DNA probe corresponding to the 30 kDa gene of TMV (A), the CP gene of CMV (B), or the CP gene of TamMV (C). For each virus infection the DGI had less virus content than the surrounding yellow tissue. Figure from Moore (2002).

In a large survey of 93 DGIs induced by TamMV in *N. benthamiana,* the ratio of viral RNA in DGIs to that in yellow tissue was assessed. This was done by excision of the target tissue, followed by RNA extraction, northern analysis using virus-specific probes, and phophorimager quantitation (Moore et al. 2001). This revealed a difference of at least a hundred-fold in virus RNA accumulation between yellow tissue in most (55 %) of the DGIs. Another 33% of DGIs contained between 1-10% of the viral RNA content present in surrounding tissue. The remaining 12% of DGIs accumulated 10-50% the viral RNA content of adjacent yellow tissue. In no samples analyzed did the accumulation of virus RNA in DGIs reach levels equivalent to those found in adjacent yellow tissue. This technique may overestimate

the virus RNA content of very small DGIs as it required excision of the DGIs from the surrounding yellow tissue and might conceivably include infected yellow tissue in the DGI sample as the margins of the islands are often abrupt but jagged through the leaf section.

Transition at the edge of DGIs

The transition between dark green tissue and yellow tissue occurs at a distinct interface that is visible with the naked eye on the leaf surface. At the cellular level, Iwanowski first showed a sharp histological differentiation of as few as two or three rows of cells between yellow and green areas using light microscopy (Iwanowski, 1903). Atkinson and Matthews (1970) analyzed electron micrographs of large sections cut across these stable sharp junctions between yellow tissue and green islands and observed that dark green tissues had no crystalline arrays of TMV rods. Even individual TMV rods were rarely observed in DGIs. The few rods observed formed a gradient of concentration, decreasing with distance from the yellow cells over a zone of 1-6 cells wide. However, no difference was observed in the frequency of plasmodesmata suggesting normal connections between green and yellow neighboring cells.

The distribution of TamMV within the cells of a leaf section was determined by blotting tissue from the cut edge across a DGI onto nylon membrane followed by northern or western analysis (Moore et al. 2001). This technique detected the TamMV RNA and coat protein (CP) respectively, and matched the DGI boundary that was observed with the naked eye. Both TamMV RNA and protein were detected primarily in yellow infected areas rather than in adjacent dark green tissue (Fig. 3).

Patterns of DGIs in leaves

DGIs can be of varying size and position on systemically infected leaves. Atkinson and Matthews (1970) followed closely the pattern of yellow infected areas and DGIs formed in tobacco plants (*N. tabacum* L. 'White Burley') inoculated with TMV. They noted that "*for most leaves, the islands of dark green tissue persist for the life of the leaf, or at least until yellowing due to senescence obscures them.*" Dark green areas of tissue were noted to increase in size during the development of the leaf while retaining their initial shape. During senescence, the borders of some DGIs were impinged with yellow green indicating breakdown of resistance to the virus and some showed areas of yellow, local lesion-like spots from an early age. However once most DGIs were formed they remained stable through out the life of the leaf unless exposed to extremes of temperature (Johnson, 1922). DGIs (or

their equivalent) have not been observed on root or stem tissue, but this may be due to a lack of a conveniently visible indicator such as chlorophyll.

The patterns of mosaic formation in systemically infected leaves are one of the most common features of virus diseases and are extremely variable. Factors such as virus strain, leaf age, season of the year, and time of infection can influence the amount of dark green tissue that develops. This unpredictable occurrence of DGIs has made them difficult subjects to study. Ferguson and Matthews (1993) described an interaction between the European strain of the *Tymovirus Turnip yellow mosaic virus* (TYMV) and Chinese cabbage (*Brassica pekinsis*, Rupr 'Wong Bok'). Under glasshouse conditions (21 ^{o}C ± 3 ^{o}C with supplementary lighting during winter) this virus-plant interaction gave a commonly occurring sequence of patterns of dark green and infected tissue in successive expanded leaves 4 weeks post-inoculation. This patterning appeared to be related to active cell division at the time when the virus entered the leaf. With this plant-virus interaction about 80 % of the fifth leaves of small seedlings developed a large border of dark green tissue with a central infected zone. This pattern of DGI formation is predictable and consistent and therefore provides a potential model system for future investigations into the processes that initiate and delimit DGIs.

Resistance of DGIs to superinfection

Cells within DGIs are not only relatively free of viral RNA and proteins but also demonstrate resistance to superinfection by the original and closely related viruses but remain susceptible to infection by unrelated viruses (summarized in Matthews, 1991).

Through successive passaging of the *Potexvirus Potato virus X* (PVX) through tobacco plants over a long period of time, strains of the virus that exhibit distinct symptoms can be generated. Using such a pair of viruses (which exhibited either severe or mild symptoms) Salaman (1933) investigated the effects of double-inoculation either concomitantly or successively. He demonstrated that initial inoculation with a mild form of PVX could provide, in symptomatic tissue, protection to successive infection or superinfection with the severe strain. Salaman (1933) concluded that once a virus had established a symbiotic relationship with a plant, then the plant was not able to enter a relationship with another virus of the same type. Using a similar system but with PVY Salaman then described how DGIs induced by the mild strain of PVY were resistant to superinfection by both the mild strain and the severe strains.

The agent providing the protection in the DGIs was unclear to Salaman (1933) as neither the mild or severe strain could infect the virus-resistant

islands in the first instance. The lack of an initial infection in DGIs led him to comment that, "*How such green areas, surrounded by a solid mass of heavily infected cells, retain their freedom from virus, is a problem distinct from that of the corresponding areas in the X-infected plant*".

Many subsequent examples have demonstrated the resistance of DGIs to superinfection. Tobacco plants reinoculated with the same strain of TMV gave no increase in the virus concentration in DGIs (Fulton, 1951). *N. tabacum* cv Xanthi-nc infected with CMV form DGIs that when challenged with either of several strains of CMV remain resistant to superinfection (Loebenstein et al 1977). However, this resistance to superinfection was overcome by challenge with the unrelated TMV. The DGIs in *B. pekinsis* infected with TYMV developed no further infection or symptoms when challenged with TYMV (Ferguson and Matthews, 1993). These and other examples with various plant and virus combinations reinforce those results from Salaman (1933) and earlier work by Thung (reviewed in Fulton, 1951).

True DGIs versus pseudo-DGIs

Atkinson and Matthews (1970) drew a distinction between true DGIs and what they termed "pseudo dark green tissue". Pseudo dark green tissue is typified by early breakdown of resistance. They noted that pseudo-DGIs have a propensity to form in leaves 5 and 6 above the TMV-inoculated *N. tabacum* leaf between 12-17 days post inoculation (dpi). Breakdown of DGIs in areas described as pseudo dark green tissue was shown to be due to virus replication rather than virus movement into the dark green area (Atkinson and Matthews, 1970). These experiments were performed on excised tissue disks from yellow or pseudo green areas that were maintained on ^{32}P-labeled orthophosphate and then harvested 1, 2 or 3 days later. Incorporation of ^{32}P into TMV was measured after sucrose density gradient analysis relative to the amounts of ^{32}P taken up by each disk of tissue. These experiments demonstrated a rapid incorporation of radiolabel into TMV over 2-3 days in pseudo islands compared to yellow tissue. By contrast, true dark green islands were shown to be resistant to mechanical inoculation whereby post-inoculation virus replication was assessed again by ^{32}P incorporation and sucrose density gradient analysis. Understanding the molecular differences between pseudo and true DGIs will provide insight into the mechanisms that form and maintain them.

Formation of dark green islands

It is not clear what the causal agent for DGIs is, or what defines the final size of a DGI. As described earlier, the microscopy studies of Goldstein (1926) addressed how DGI formation correlates with the histology of the leaf at time of infection. Atkinson and Matthews (1970) used synchronized infections of TMV and leaf analysis or symptom development. TMV was inoculated onto the fifth true leaf of *N. tabacum* followed by a regime of leaf measuring, excision and maintenance under conditions that would support virus replication. Virus titre was then assessed by inoculation on to a local lesion host (*N. glutinosa*). This data was compared to symptom development on synchronously inoculated matched plants on which leaves were measured but not excised. Tobacco leaves of 0.9-1.2 cm or less upon infection most reliably yielded DGIs 12 days or more after inoculation.

With this reliable system in place, the increase in the number of cells in a leaf between age of infection and mature size was compared to the number of cells in a DGI. These data were used to assess the possibility that DGIs arise from a single cell. Most DGIs in a leaf that had been infected for 17 days were between 0.3-8.0 mm^2 and during this time the total leaf cell count had undergone seven rounds of cell doubling. A single cell doubling seven times would result in 128 cells with the area of only 0.05 mm^2. Based on this calculation a DGI is unlikely to arise through replication of a single cell. Moreover, the probability that DGIs may arise by chance from adjacent cells is less than the observed incidence of DGIs greater than 0.05 mm^2. Thus, DGIs must arise by some active process (Atkinson and Matthews, 1970). One possibility is that strains of extremely mild strains of TMV cause DGIs. This was eliminated as those few infectious units isolated from DGIs did not result in dark green symptoms following local lesion purification. Atkinson and Matthews concluded that some "*dark green agent*" must spread from cell to cell to delimit DGIs at, or near, the time of infection by virus. They also concluded that the nature of the agent must be strain specific as Fulton (1951) had demonstrated that DGIs were resistant to superinfection by similar but not distinct strains of TMV.

In *N. benthamiana*, DGIs are appreciably round in shape, which supports the concept of cell-to-cell spread of a DGI silencing signal from a single point of initiation. In contrast to the situation in *N. benthamiana*, DGIs in other plants are frequently rectangular and bordered by veins; this pattern suggests that there is some impedance of DGI spread. It is not surprising that plants vary in their manifestation of DGIs if a diffusible signal is involved, as there is known to be variation in the parameters for cell-to-cell trafficking in different plants (Lee et al. 2002).

Post-transcriptional gene silencing maintains DGIs

As reviewed, DGIs have some common characteristics:

1. DGIs appear healthy and have been shown to contain little or no viral RNA and protein.
2. DGIs demonstrate resistance to superinfection by the original and closely related viruses, but are susceptible to infection by unrelated viruses.
3. The development of DGIs is related to the developmental stage at which a leaf is invaded by virus.
4. DGIs contain more cells than can be accounted for if a DGI was the product of a single cell's division. A 'diffusible factor' was postulated to be responsible for the formation and maintenance of the islands (Atkinson and Matthews, 1970).
5. This unknown 'diffusible factor' appeared to be induced by, and dependent upon, viral infection (Atkinson and Matthews, 1970).

This set of characteristics is reminiscent of a family of phenomena that includes cross protection, recovery, and a specific form of pathogen-derived resistance based on post-transcriptional RNA silencing (PTGS- also known as RNAi in animals, or quelling in fungi: reviewed in Ding et al. 2004). These phenomena are mediated through the sequence-specific recognition and degradation of target RNA.

PTGS can be induced by the introduction of transgenes that result in the production of double-stranded (dsRNA) in cells. This has been facilitated through the use of transgenic plant technologies. One such example is transgenic plants containing portions of the viral genome sequences either to express proteins or to only express the RNA transcript. When inoculated with the virus of the same sequence it was noted that some transgenic plants exhibit a recovery phenotype in virus-infected *N. benthamiana* plants transgenic for the CP (or only the CP RNA) of the cognate virus (Lindbo and Dougherty, 1992; Eagles, 1994). This "recovery" is characterised by the development of virus-free, healthy tissue in the youngest leaves of a

"systemically infected" and otherwise symptomatic transgenic plant. The recovered region of the leaf appears to correspond to that portion of the leaf that is still a metabolic "sink". The first recovered leaf is often bizonal but subsequent leaves are typically completely symptom-free as well as virus-free. In addition, the virus-free tissues are resistant to superinfection by the same or closely related viruses.

Recovery can also occur naturally in infections of non-transgenic plants (including *N. benthamiana*) with some RNA viruses, especially in infections by members of the nepovirus group (Ratcliff *et al.* 1997). As in DGIs, recovered tissue is relatively free of viral RNA and proteins and is resistant to superinfection by the same or closely related viruses, but is susceptible to infection by other viruses. Like DGIs, the boundary between infected and recovered cells in leaves is abrupt and crosses all cell layers in the leaf (Fig. 3). Plants that display the recovery phenotype also often produce DGIs in leaves before recovery sets in (Eagles, 1994) or contain DGIs amidst the non-recovered tissue of a partially recovered leaf. With these similarities in mind, and the growing evidence that recovery is a result of PTGS, Moore and co-workers (2001) hypothesized that like recovered tissues, DGIs are maintained by a PTGS mechanism.

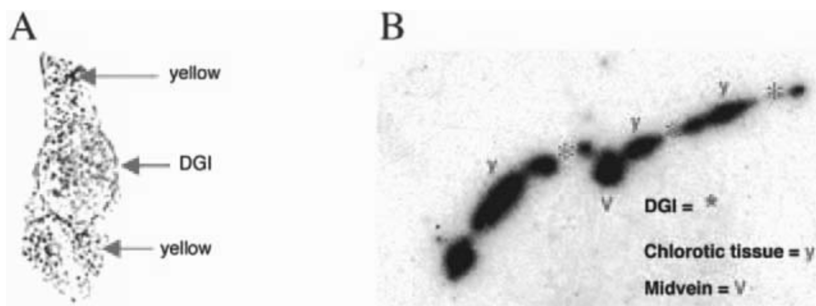

Fig. 3. Tissue prints of TamMV-infected leaves containing DGIs shows viral protein and RNA exclusively in yellow tissue. Both viral protein and RNA are present in the yellow, chlorotic tissue surrounding DGIs and are not detected or are present at low levels within DGI tissue. (A) Detection of TamMV CP in a *Cyphomandra betacea* (tamarillo) leaf with anti-PVA (TamMV) antibody results in a purple color whereas those regions not positive for the presence of CP remained green (chlorophyll). The single visible DGI is indicated with a dotted line, the yellow surrounding tissue was chlorotic in the sampled leaf and corresponds to the purple hue. (B) Detection of TamMV CP RNA in a section of TamMV-infected N. benthamiana leaf cut traverse to three DGIs and the mid-vein. The probe detects the TamMV CP RNA,which is part of the full-length TamMV RNA genome (black = positive for TaMV CP RNA). Figure from Moore (2002). (See also Colorplates, p. xvii)

Moore et al. (2001) used three viruses to examine the mechanism of DGI maintenance. Firstly they used the *Potyvirus* TamMV, to generate DGIs on *N. benthamiana*. Secondly, as a unrelated virus they used an infectious transcript of the *Potexvirus White clover mosaic virus* (WClMV) that is asymptomatic on *N. benthamiana* and can infect DGIs induced by TamMV. Finally, to deliver only part of the TaMV genome they built an infectious chimeric virus, WTam, comprised of WClMV and additional sequences for the replication but not for the translation of the TamMV CP RNA. They then determined whether WTam could infect DGIs that had been induced by TamMV. By testing the ability of these viruses to replicate on either wild type or transgenic *N. benthamiana* that expressed the RNA of the TamMV CP gene from a transgene, Moore and her co-researchers were able to distinguish between PTGS or some other form of active resistance against the chimeric virus. They found that WTam RNA was present at a very low level or absent from TamMV-induced DGIs. They also discovered that the transcript of the TamMV CP expressed from the transgene in plants was also absent from DGIs. This transcript could be discriminated from the CP RNA derived from WTam by a unique 3' untranslated region. This experimental system demonstrated that an RNA sequence-specific mechanism could target the potexvirus only if it contained the sequences of the potyvirus, which had initially induced the DGIs. It was also demonstrated that only a part of the DGI-forming virus, the *CP* gene, was sufficient to render the chimeric virus susceptible to degradation within DGI tissue. This established that in DGIs viral RNA is actively degraded on the basis of RNA sequence.

In an investigation of the function of the γB protein of the *Hordeivirus Barley stripe mosaic virus* (BSMV) Yelina and co-workers (2002) used a chimera termed BγP. In this construct the wild type γB gene of BSMV is substituted by the γB of the *Hordeivirus Poa semilatent virus*. Compared with wild-type BSMV, the chimeric BγP virus gave earlier and more severe symptoms, including large DGIs, on systemically-infected *N. benthamiana* leaves 12-40 days post-inoculation. In contrast to plants infected with BSMV, the plants infected with the chimeric BγP virus fully recovered from infection, showing no symptoms on young leaves. RNA isolated from DGIs and surrounding yellow tissue was analyzed for small RNAs that might indicate active and sequence-specific RNA degradation occurring *via* the PTGS pathway. Such small RNAs between 22-25 nucleotides (nt) in length were present at 16-fold higher concentration in DGIs compared to adjacent chlorotic tissue, and were also present in younger recovered leaves. These results provide further support that DGIs, like recovered tissue, are actively maintained by PTGS (Moore et al. 2001) and reveal the characteristic small RNAs, termed short interfering RNAs (siRNAs), that provide sequence specificity to this RNA degradation mechanism. Interestingly, small dsRNAs of viral origin were detected in TYMV-infected tissue that also produces

DGIs (Bedbrook et al.. 1974). These RNAs were not investigated further due to the paucity of molecular tools available at the time.

Further extending the work with BSMV, the chimeric BγP virus was inoculated onto transgenic *N. benthamiana* expressing the HC-Pro protein from the *Potyvirus* PVA (Yelina et al. 2002). HC-Pro is a potyvirus-encoded protein that is known to suppress the maintenance of PTGS, although its mechanism of action is unclear (Mallory et al., 2001; Hamilton et al. 2002; reviewed in Roth et al. 2004). The *N. benthamiana* expressing HC-Pro gave either no, or smaller and fewer DGIs. In addition, the HC-Pro plants did not recover from virus infection and no or few small RNAs were observed (Fig. 3). This work demonstrates that when the PTGS activity is suppressed DGIs do not occur and likewise that recovery does not proceed.

Consistent with this idea, it was found that in transgenic *N. tabacum* plants that were silenced for a plant-encoded RNA-dependent RNA polymerase (RdRp), *NtRdRP1*, systemic TMV symptoms did not include DGIs (Xie et al. 2001). This suggests a role of the plant-encoded RdRp in the virus defense mechanism that results in DGI formation.

Together these experiments demonstrate that DGIs are maintained by PTGS. Although there exist strong similarities between DGIs and recovered tissue there are also clear differences in the size, timing and spread of DGIs and recovered tissues. Thus, DGIs are small and seemingly randomly located within individual leaves, whereas recovered tissue develops from the base of a leaf and progresses further along the lamina in each leaf that developes subsequently.

Theoretical frameworks

DGIs are formed during the processes of leaf development, virus infection, and plant defense against virus infection. Each of these processes is complex, changes over time, interacts with the other processes, and is understood to some greater or lesser extent. For ease of analysis, we propose that the process of DGI formation is divided into five theoretical stages: initiation; spread; delimitation; amplification, and maintenance.

There is compelling evidence that PTGS is involved in the maintenance of DGIs (Moore et al. 2001; Yelina et al. 2002; Xie et al. 2001). The remaining four stages are less well understood but we propose that initiation, spread, delimitation and amplification institute the PTGS mechanism in the cells that will form DGIs. The larger size-range of siRNA (24-26 nt) is likely to be involved in long distance, systemic signal translocation in the phloem, whereas short siRNAs (21-23 nt) are more likely to be intrinsic to the local cell-to-cell spread of the DGI signal (Mallory et al. 2001, 2003; Vance and Vaucheret, 2001; Hamilton et al. 2002; Klahre et al. 2002; Mlotshwa et al. 2002; Himber et al. 2003).

Initiation of DGIs

There is evidence for a signal that initiates PTGS, which moves systemically through the phloem of plants. A potential candidate for the signal that initiates a DGI near the shoot apex is the longer size-class of siRNAs that have been shown to be long-distance silencing signals (Hamilton et al. 2002, Himber et al. 2003). It is likely that these siRNA signals translocate in association with phloem-specific RNA binding proteins such as PSRP1 identified in pumpkin by Yoo et al. (2004). Evidence for this includes the finding that in grafting experiments siRNAs derived from transgenic rootstocks capable of conferring silencing to a wild type scion have been identified in the phloem of those scions (Yoo et al. 2004). Similarly, siRNAs directed against sequences along the entire length of the virus genome were also detected in the phloem of virus-infected plants (Yoo et al. 2004). However, DGIs develop after systemic infection of the DGI-forming virus has already taken place. Both the DGI-forming virus and a cognate PTGS signal move throughout the plant during infection and likely reach the shoot apex in a similar timeframe. Therefore, it is difficult to determine which of these is the DGI-initiating signal near the shoot apex.

Within plants that are systemically infected with a mosaic-inducing virus DGIs appear in leaves that are still undergoing active cell division. However, the precise importance of leaf development and/or cell cycle in the formation of DGIs is unclear. The development of DGIs in these tissues may be associated with the fact that the young tissues are sinks for photoassimilates and by default receive phloem-translocating PTGS signals and virus from distant, virus-infected cells. However, older leaves also receive these and do not form DGIs. Young dividing cells seem to possess a necessary factor for the DGI-initiation process. This may be the ability to produce, perceive, and respond to a unique DGI-initiating, secondary signal. If we assume that in DGIs the initiating event occurs stochastically in single leaf cells during cell division one possibility is that the breakdown and reformation of the nuclear membrane could provide a window of opportunity for RNA sequences to enter the nucleus and trigger the production of the silencing signal there (Moore 2003). The 2b inhibitor of PTGS initiation, encoded by CMV, localises to the nucleus and loses its effectiveness if the nuclear targeting sequence is deactivated (Lucy *et al.*, 2000). Thus, although the degradation event occurs in the cytoplasm, the initiation of PTGS may well be nuclear-based.

The initiation of DGIs in dividing leaf cells may also involve a component of pre-existing PTGS signalling from the infection process occurring lower in the plant. A systemic PTGS signal appears to be generated in the initial stages of virus infection and to spread throughout the

plant via the phloem (reviewed in Fagard and Vaucheret, 2000). DGIs may be initiated in dividing cells in which that signal arrives before the infecting virus.

A constant threat to genetic integrity is the movement of transposons. Since PTGS acts to inhibit transposon movement and also virus infection, the initiation of DGIs that are resistant to virus infection may reflect an essential mechanism that is established to maintain genetic integrity of dividing cells. A "red alert" status during cell replication may switch on an extra level of protection against dsRNA resulting in silencing signals derived through cell-cycle-specific expression of silencing-pathway genes (Foster et al. 2002).

Different DICER-related proteins are believed to generate functionally different siRNAs (Xie et al. 2004) and the expression of these, and thereby the generation of a DGI-spreading signal, may be specific to a particular cell replication stage. Specific ARGONAUTE (AGO) nucleases that "slice" the target RNA (Liu et al. 2004; Song et al. 2004) may also be differentially expressed (reviewed in Bowman, 2004) resulting in unique PTGS activities. Other processes involved in the reception (e.g. entry of the DGI-signal) or response to the silencing signal (e.g. spread or amplification of the DGI-signal (Xie et al. 2004) may also be upregulated in dividing cells. Candidates for upregulation also include the inducible *NtRDRP1* that is required for DGI formation (Xie et al. 2001) or other RdRp genes encoded by plants. DGI initiation could thus potentially coincide with heightened protection of the cell against foreign nucleic acids.

Local spread of a DGI signal: the "dark green agent"

A diffusible signal, the "dark green agent", has been proposed to be involved in the spread of a DGIs across cell layers (Atkinson and Matthews, 1970). Although this signal has not been conclusively identified to date, several of its qualities are known. It is sequence specific, and induces DGI formation locally as opposed to systemically. The signal is susceptible to some suppressors of silencing and not others as illustrated by the experiments of Yelina and co-workers (2002). The production of or the response to, the diffusible signal is dependent on the cell cycle: cells dividing at the time of infection are competent to form DGIs, whereas mature cells are not.

The most likely signals for the spread of PTGS between cells that form DGIs are the short siRNA species. Such short siRNA species have been shown to move from cell to cell from experiments on the systemic delivery of silencing by virus induced gene silencing, by phloem delivered dsRNA (Himber et al. 2003), or from a local silencing source delivered by *Agrobacterium*-mediated transient expression. The short siRNAs appear to

consistently spread for a distance of 10-to-15 cells and induce silencing against RNAs containing homologous sequences. Short siRNAs may be generated upon virus entry of the cell and/or in response to phloem-delivered long siRNAs and initiate PTGS. The short siRNA species has not been identified in DGIs to date but the very tiny quantities likely to be present may require considerable effort to detect in cells during the DGI initiation phase . Those siRNAs that have been identified in developed DGIs were of the longer size class (Yelina et al. 2002) that appear to be characteristic of systemic transmission of the silencing signal (Hamilton et al., 2002 and Himber et al. 2003).

Delimitation of DGIs

The inability of DGIs to spread and encompass an entire leaf or to send a successful systemic silencing signal to new leaves is intriguing. What delimits the extent of the spread and thus determines the final size of a DGI? Five mechanisms of DGI delimitation may occur: spread of a limited supply of a silencing signal; the capacity of cells to respond to signal reducing with the age of the cell; limited distance of cell-to-cell movement without co-ordinate signal amplification; cell multiplication; and a race between silenced and virus infected cells. Not all of these mechanisms are exclusive; some may act in concert with others.

The number of cells to which the local DGI-forming signal is distributed before it reaches a critical dilution end-point may limit the size of the DGI. The size of the DGI would then depend on the amount of the signal available from the originating cell, assuming that amplification in neighbouring cells does not occur during DGI-delimitation.

The size of a DGI may also be determined by the responsiveness of neighbouring cells to the local signal, perhaps the ability of the cell to perceive a signal or the ability to regenerate the signal. Perhaps amplification of the signal occurs in specific circumstances, (e.g. requiring an RdRp or other components of the PTGS machinery to generate further short siRNAs). It is thus intriguing to consider the influence of cell replication in the determination of DGI size.

Limited cell-to-cell movement of silencing signals from the originating cell could limit DGIs to a particular size in each particular plant-virus system. For instance, if the short siRNAs are the local signal, and this signal can spread without amplification to 10-15 cells in a single direction (Himber et al. 2003) then the total number of cells containing the signal from a single originating cell would be approximately 706 cells ($\Pi\ 15^2$, this assumes equal spread in all directions). After seven cell division cycles, as calculated by

Atkinson and Matthews (1970), this would account for 90,432 cells, and using the approximate calculation for *Nicotiana* species that 128 cells are within 0.05mm^2 of leaf tissue then this would account for a DGI of 35mm^2. The observed norm of DGI size in a leaf that had been infected for 17 days and undergone seven rounds of replication was between 0.3-8.0 mm^2 (Atkinson and Matthews, 1970). Thus, the short siRNA signal could theoretically (and with the current assumptions) account for DGIs of substantial size. This is a high approximation as *Nicotiana* leaf tissue is generally not more that seven cells thick.

Cell division may contribute to the numerical increase of cells that have established PTGS against the DGI-inducing virus, though it seems that cellular multiplication is not sufficient to account for the size of DGIs (Atkinson and Matthews, 1970). Areas of a leaf that are not undergoing significant cell division may establish a DGI-state in individual cells but not contain sufficient cells or amplification capacity to establish a visible DGI. Mature leaf cells are capable of mounting the PTGS mechanism as demonstrated by transient expression experiments and are capable of cell-to-cell movement of a silencing signal (Ryabov et al. 2004 and Himber et al. 2003).

The size of a DGI may also be restricted to cells within the leaf which the silencing signal reaches ahead of the virus or prior to the translation of sufficient virus-encoded suppressor of silencing proteins. Thus, DGIs would always neighbour infected cells. Such infected cells would have established virus protein expression including suppressor of silencing activities, thereby inhibiting spread of the silencing signal and establishment of an additional silenced cell. Such a competition model could act in concert with other models to further restrict the size of DGIs.

Amplification of PTGS in DGIs

Two phases of amplification are considered. The first is during the spread and delimitation stages of DGI formation. The second is during the maintenance of DGIs. During DGI initiation, spread, and delimitation it is difficult to reconcile the spread of such a dilute signal across an entire DGI without some form of amplification. Since the virus sequence is not present in the plant genome it would seem apparent that the signal is amplified from the challenging virus genome or parts thereof. By contrast, if each DGI were initiated from a single siRNA alone, then one would predict resistance of each DGI to be targeted against a specific subset of the virus genome. In the single study using the chimeric virus WTam, Moore et al. (2001) demonstrated that DGIs induced by TamMV commonly confer resistance to superinfection with a chimeric potexvirus carrying only the TamMV CP. If

each DGI was initiated by a single siRNA species, and calculating the CP as one-tenth the genome of a potyvirus one would predict the same proportion of DGIs would be resistance to WTam. Since resistance to WTam is common, it seems unlikely that the resistance of each DGI is targeted to a distinct sub-sequence of the original infecting virus, but rather that each DGI is resistant to the entire DGI-inducing virus. If correct (and it requires testing) then this poses the following question. How does a systemic silencing signal of only ~25 nt generate resistance against all sequences in the virus? Intriguingly, DGIs are not larger when formed in the presence of a transgene transcript of homologous sequence to the DGI-initiating virus (Moore et al. 2001 and unpublished data).

There seems to be no dilution effect where less resistant cells exist on the perimeter of the DGI and most resistant DGIs at the centre. This suggests that there may not simply be a dilution end point but rather implies an additional amplification step to provide potent resistance throughout the established DGI. Likewise, once PTGS has been established in DGIs it is extremely potent and stable, often lasting up to the lifetime of the leaf. This defence mechanism provides strong protection against superinfection of related viruses, even to those related viruses that encode suppressors of silencing. How does the DGI maintain such an active, long-lasting, and potent defence? Are the siRNAs amplified once DGIs have been established? Perhaps amplification does indeed occur and uses systemically infecting, DGI-inducing virus transcript as template for amplification resulting in the siRNAs that have been identified in DGIs (Yelina et al. 2002). Alternatively, a suite of siRNAs against the DGI-forming virus may selectively enter the DGI cells. Either of these possibilities could account for the presence the potent defence mechanism mounted against homologous viruses throughout these seemingly healthy tissues.

Maintenance of DGIs

Experiments performed by Moore et al. (2001) and Yelina et al. (2002) have demonstrated that DGIs once formed are maintained by PTGS. In both these studies the silencing occurring in DGIs degrades RNA of the same sequence as the DGI-initiating virus. This PTGS mechanism generates siRNAs, hallmarks of the PTGS mechanism (Yelina et al. 2002). DGI formation by BSMV is inhibited by HC-Pro (Yelina et al. 2002), a potyvirus-encoded suppressor of silencing that affects PTGS maintenance (Llave et al. 2000) and perhaps other stages of PTGS (reviewed in Roth et al. 2004). In a separate study, TMV inoculated onto *N. benthamiana* compromised for inducible RdRp activity did not produce the characteristic TMV-induced DGIs (Xie et al. 2001). RdRp activity has previously been

implicated in PTGS (Schiebel et al. 1998). Together this evidence strongly supports the concept that DGIs are maintained by PTGS.

Future perspective

Mosaic disease, including the presence of DGIs, is one of the most common symptoms in virus-infected plants (Hull, 2002). However, within an infected plant and with some notable exceptions (such as the Chinese cabbage interaction with TYMV) DGI formation is a relatively infrequent event and results in a seemingly stochastic random distribution of islands of dark tissue within the context of systemically infected leaves. Thus, the events that are required for DGIs formation: initiation; spread; delimitation; amplification; and maintenance, must seldom sufficiently exist together in order to form DGIs.

Particular tools will be of use to further investigate the formation of DGIs: predictable interactions that form DGIs such as the interaction between Chinese cabbage and TYMV; plants that carry mutations for genes involved in the initiation, spread, delimitation, amplification or maintenance of PTGS; suppressors of PTGS that can interfere with these processes in predictable ways, viruses that are altered in their ability to form DGIs, and molecular analysis of the PTGS signals, especially the ability to clone and sequence small RNAs (Llave *et al.*, 2002). Together these will aid the fine dissection of the processes that ultimately manifest as DGIs.

Increasing our understanding of the cellular machinery involved in PTGS and the mechanisms of action of the suppressors of silencing in plants (Roth et al. 2004) may reveal plausible mechanisms for the proposed five stages of DGI formation. Once these basic stages are elucidated, other questions remain to tantalise us. Are DGIs a plant victory in battle or do they perform some function that assists the virus in its long-term war strategy? Could it be that plants with DGIs are more attractive to feeding insects and thus provide increased spread of the infecting virus? Do DGIs permit the infected plant to survive longer and therefore provide more opportunity for virus transmission? Historically the study of viruses has revealed much about the processes of the cells that they infect. The study of the phenomenon of DGIs has been no exception and may yet prove to be a model system with both the control and the experimental regions within the same leaf and environment.

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Chapter A10

Resistance to Infection

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Introduction

Without the equivalent of a copper or fungicide spray, virtually all realistic control measures for plant virus diseases in the field fall into two categories (Fraser, 1990), (i) preventing the virus and the plant from coming in contact in a manner that can initiate an infection (Chapter 14), and (ii) biologically based interference with virus replication, spread or symptom induction. The intimate interaction of virus and cell is demanding on any potential anti-viral and, thus far, none are inexpensive. "Curing" a plant virus disease usually is economical only for propagation stock, accomplished by long term plant culture at elevated temperature ("thermotherapy"), chemotherapy or plant micropropagation. Preventing virus-plant contact may involve clean stock programs and/or at least minimal applications of pesticides to control virus vectors (Jones, 2004). Biologically based interference is considered in Chapters 1, 6 and 13 and here.

Table 1. Virus abbreviations

ACMV *African cassava mosaic virus*	PLRV *Potato leafroll virus*
AlMV *Alfalfa mosaic virus*	PSbMV *Pea soilborne mosaic virus*
BCMNV *Bean common mosaic necrosis virus*	PVA *Potato virus A*
BCMV *Bean common mosaic virus*	PVM *Potato virus M*
BCPMV *Blackeye cowpea mosaic virus*	PVX *Potato virus X*
BCTV *Beet curly top virus*	PVY *Potato virus Y*

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BaMMV *Barley mild mosaic virus*
BeYMV *Bean yellow mosaic virus*
BGMV *Bean golden mosaic virus*
BSMV *Barley stripe mosaic virus*
BYMV *Barley yellow mosaic virus*
CABNV *Cowpea aphid borne mosaic virus*
CaMV *Cauliflower mosaic virus*
CCMV *Cowpea chlorotic mottle virus*
CelMV *Celery mosaic virus*
ClYVV *Clover yellow vein virus*
CMV *Cucumber mosaic virus*
CPMV *Cowpea mosaic virus*
CPSMV *Cowpea severe mosaic virus*
CTV *Citrus tristeza virus*
LIYV *Lettuce infectious yellows*
LMV *Lettuce mosaic virus*
MBYMV Mungbean yellow mosaic *virus*
MNSV *Melon necrotic spot virus*
PeMV *Pepper mottle virus*
PStV *Peanut stripe virus*
PWV *Passionfruit woodiness virus*
RCVMV *Red clover vein mosaic virus*
RTSV *Rice tungro spherical virus*
RYMV *Rice yellow mottle virus*
SCMoV *Subterranean clover mottle virus*
SCPMV *Southern cowpea mosaic virus*
SHMV *Sunn-hemp mosaic virus*
SMV *Soybean mosaic virus*
SqMV *Squash mosaic virus*
SugMV *Sugarcane mosaic virus*
TCV *Turnip crinkle virus*
TEV *Tobacco etch virus*
TMV *Tobacco mosaic virus*
TRSV *Tobacco ringspot virus*
TSWV *Tomato spotted wilt*
TuMV *Turnip mosaic virus*
TYLCV *Tomato yellow leaf curl virus*
WMV *Watermelon mosaic virus*
ZYMV *Zucchini yellow mosaic virus*

The most common approach to controlling virus replication or spread or symptom induction is the deployment of resistance (*R*) genes. Deployment of *R* genes requires no special equipment and is readily accomplished because the seed or other propagation material is itself the package for delivering the control measure. Thus, *R* genes, when available, usually present the most laborsaving, economical and environmentally sound approach to control of virus diseases. One or more *R* genes often provide the only effective method for managing a virus-induced disease. The availability of genes conferring resistance to specific viruses has been markedly enhanced by the development of transgene approaches in which sequences or mutated sequences derived from the virus genome (pathogen-derived resistance) or elsewhere are expressed in the plant. However, the topic of this review, in conformance with the thrust of this book, is naturally occurring plant *R* genes effective in controlling virus infection. R.S.S. Fraser (e.g. Fraser, 1992, 1998a, b, 2000) and others (Carrington and Whitham, 1998; Hammond-Kosack and Jones, 1997; Harrison, 2002; Hull, 2002a; Kelly, 1997; Khetarpal et al. 1998; Martin et al. 2003; Murphy et al. 2001; Parker and Coleman, 1997; Pennazio et al. 1999; Solomon-Blackburn and

Barker, 2001; Strange, 1998; Takken and Joosten, 2000) have chronicled the discovery and characteristics of genes conferring resistance to plant viruses and models for their action.

Resistance and Tolerance

"Resistance" and "tolerance" occasionally are used as synonyms. However, it is more usual to relate resistance to reductions in virus titre and tolerance to amelioration of symptom development. To recognize a 'reduction' or 'amelioration' requires a reference state of susceptibility. A typical and economically relevant reference state of susceptibility is a symptom inducing, systemic infection. Tolerance as used here refers to systems in which virus-induced symptoms are greatly reduced in intensity or are absent, but the virus titre is unreduced or only slightly reduced relative to the reference infection. Examples of virus-host systems exhibiting genotypic tolerance are: BYDV and *Yd2*-bearing barley (Ranieri et al. 1993), BCPMV and cowpea (Anderson et al. 1996), BCTV and Arabidopsis (Park et al. 2002), PRSV and cucumber (Wai and Grumet, 1995), TRSV and Arabidopsis (Lee et al. 1996), and TYLCV and *Lycopersicon pimpinellifolium* (Fargette et al. 1996). The tomato *Tm-1* gene confers tolerance to TMV at 33°C but resistance at 20°C (Fraser and Loughlin, 1982).

Operationally, the effect of a *R* gene is to reduce the titre of the virus, i.e., the accumulation of viable virions or other efficiently propagated form, below the accumulation observed for a reference state of susceptibility, ideally comparing nearly isogenic resistant and susceptible lines. Excluded from this short review are variation due to genetic inhomogeneity or stochastic processes, since uniform results are obtained in many systems when homogeneous stocks of host plant and virus and appropriate inoculum concentrations are employed. Also ignored here are a generalized resistance against viruses (Akad et al. 1999), the influence of developmental state on resistance (Leisner et al. 1993; Ullah and Grumet, 2002), and the role of gene silencing in resistance (Chapters 3, 8 and 12). Non-host (non-genotypic) resistance, in which all lines of a plant species or higher taxonomic order resist a given virus, is considered only tangentially.

Degree and locale of resistance

Each of the following five resistance state descriptions, modified from (Dawson and Hilf, 1992), is considered relative to a successful systemic infection. Descriptions four and fife correspond to extreme resistance. The descriptions are listed order of expected decrease in virus titre and spread.

Systemic infection with reduced virus titre

Infections fitting the description of systemic infection with reduced virus titre most commonly are observed after graft inoculation of virus. For the system TYLCV and tomato (*Lycopersicon esculentum*), graft-inoculation of TYLCV to cultivars Tyking and Fiona resulted in reduced severity of symptoms and in TYLCV titres that were 5% to 10% of the titres achieved in graft-inoculated reference cultivar Moneymaker. A line of *L. chilense*, which is compatible with *L. esculentum* in wide crosses, supported development of a TYLCV titre only 1% of that found for Moneymaker. The distribution of TYLCV antigen was very similar in the resistant lines and in Moneymaker, e.g., with similar partitioning between internal and external phloem (Fargette et al. 1996; Friedmann et al. 1998). The accumulation of virus in the systemically infected plant may be reduced and uneven, compared to susceptible reference plants, due to apparent differential susceptibility of specific tissues or organs. When plants from a line of *Solanum phurega* were exposed to PLRV-bearing aphids, PLRV was detected in leaves but at greater titres in the petioles and stems (Franco-Lara and Barker, 1999).

Restricted inter-organ movement of the virus

These phenomena result in lack of systemic infection and often in reduced titre in the infected organ(s). Reference cowpea (*Vigna unguiculata*) lines developed necrotic etching on unifoliate leaves inoculated with CCMV and a chlorotic mottle on systemically infected, trifoliate leaves. Resistant cowpea line PI186465 developed no symptoms and CCMV was not detected in the trifoliate leaves. CCMV steadily increased in the inoculated unifoliate leaves but at a rate less than 10% of the rate that CCMV achieved in inoculated leaves of a reference line (Wyatt and Kuhn, 1979). Among the other examples of virus restriction to inoculated leaves are:

CaMV in Arabidopsis (Tang and Leisner, 1997)
CMV and pepper (Caranta et al. 2002)
CMV and *Cucumis figarei* (Kobori et al. 2000)
CMV and several potato lines (Celebi et al. 1998)
LMV and Arabidopsis (Revers et al. 2003)
PVA strain M and *Nicandra physaloides* (Rajamaki and Valkonen, 2004)
PVY and common bean (Kyle and Provvidenti, 1987)
TEV and Arabidopsis (Chisholm et al. 2001)

PeMV on the Avelar line of pepper showed, compared to infections of other reference pepper lines, reduced accumulation in inoculated leaves and spread limited to stem just below and above the leaf attachment site, with no infection of uninoculated leaves (Murphy and Kyle, 1995). In other systems, genotypic virus restriction from other plant organs has been noted (Franco-Lara and Barker, 1999; Johansen et al. 1994).

Impaired virus spread in the inoculated organ

These phenomena are often associated with the appearance of local lesions. The *N* gene of tobacco, conferring resistance to most strains of TMV, provides the most extensively studied example of impaired accumulation and spread by local lesion formation. The local lesions are necrotic, the result of a hypersensitive reaction (HR) due to programmed cell death (PCD) (del Pozo and Lam, 2003). TMV is not constrained to the necrotic area but accumulates also in a region of 1-2 mm outside the necrotic area, but not beyond (Weststeijn, 1981; Wright et al. 2000). *N* gene resistance and other genotypic resistance associated with necrotic lesions are intimately associated with PCD, and PCD may be necessary for robust resistance. However, PCD and HR, though possibly observed, are not essential to inhibiting virus accumulation and spread (Bendahmane et al. 1999; Kim and Palukaitis, 1997; Mittler et al. 1996). For some systems, impaired spread is not associated with necrosis or even with symptom appearance, e.g., Gibb et al. (1989); Gunduz et al. (2004); Njeru et al. (1995).

Infection limited to the inoculated cells

In several instances of non-host resistance, inoculated cells accumulate virus, but the infections are limited to the inoculated cells (Bak et al. 1998; Mise et al. 1993; Sulzinski et al. 1994; Sulzinski and Zaitlin, 1982; Wang et al. 1999). For genotypic resistance in which no virus increase was detected in the inoculated leaf but protoplasts derived from the resistant plant supported virus increase after *in vitro* inoculation (see section on virus infection of protoplasts, below), limitation of the virus to the individual inoculated leaf cell often is postulated but only rarely has been verified by cytological examination (Kobori et al. 2000; Nishiguchi and Motoyoshi, 1987).

Operational immunity: no accumulation of virions detected

Extreme resistance will result from infection that is limited to the

inoculated cell and from inoculations that are not productive, even in cells receiving the virus inoculum. Negative results from the most sensitive methods of virus detection, polymerase chain reaction (PCR) and reverse-transcription-PCR, suggest operational immunity (Parrella et al. 2004). However, detection of residual inoculum may obscure interpretation of results unless quantitative PCR is applied to samples taken at various times after inoculation to distinguish an increase in virion concentration (suggestive of infection limited to the inoculated cells) from the (likely declining with time) concentration of residual inoculum (Balaji et al. 2003). A quantitative PCR time course also has limitations. Although inoculation of *Southern cowpea mosaic virus* (SCPMV, *Sobemovirus*) to its non-host common bean results in synthesis of SCPMV genomic RNA and capsid protein, new virions are not formed and bean must be considered to be operationally immune to SCPMV (Hacker and Fowler, 2000). Recombinant viruses expressing green fluorescent protein (GFP) provide a tool for direct assessment of virus replication in inoculated cells. Sato et al. (2003) detected recombinant ClYVV expressing GFP in single cells shortly after ClYVV inoculation of susceptible common bean plants but failed to observe fluorescent cells after inoculation of resistant bean line Jolanda.

Relevant infection phenomena

For mechanically transmissible viruses, screening of lines for resistance usually employs rub inoculation of leaves. Other modes of inoculation and co-inoculation may reveal general information on resistance mechanisms.

Virus infections of protoplasts

The susceptibility, or reduced susceptibility, of protoplasts from *R* gene-bearing plants, relative to protoplasts from susceptible lines, often is considered to be revealing of the possible mode of action of the gene in the intact plant. The host range of a virus typically is broader in the protoplasts of plants than it is in the corresponding intact plants and often includes protoplasts from non-hosts (Bak et al. 1998; Dawson and Hilf, 1992). Viruses subject to genotypic resistance evidenced by local lesion formation, other localized symptoms or no obvious symptoms may infect leaf protoplasts from the same line *in vitro* and achieve an increase in titre comparable to what is seen in protoplasts from nearly isogenic, susceptible lines (Arroyo et al. 1996; Barker and Harrison, 1984; Nasu et al. 1996; Ndjiondjop et al. 2001; Njeru et al. 1995).

Protoplasts must be regarded as seriously injured and likely de-differentiated cells which differ greatly in physiology from the

corresponding cell in the intact leaf. Protoplasts may fail to express one or more components of a resistance mechanism that restricts virus replication in the intact leaf cell. Protoplasts from a plant line exhibiting extreme resistance and protoplasts from a corresponding susceptible line, both inoculated *in vitro*, may support virus accumulation to similar titres. The common interpretation is that the resistance is based on restricted cell-to-cell movement of the virus. However, this conclusion is not justified in the absence of other information, such as cytological evidence of single-cell infections (Kobori et al. 2000; Nishiguchi and Motoyoshi, 1987). Even where there is substantial accumulation of virus, in the inoculated cells only, it is difficult to disentangle slowed replication and inhibited cell-to-cell spread. Slowed replication alone may give the plant defence time to be activated, greatly slowing virus spread. The interpretation is more straightforward when protoplasts from a resistant line fail to support virus increase (no detected accumulation of virions) after *in vitro* inoculation under conditions resulting in virus accumulation in protoplasts from a corresponding susceptible line. Examples are CMV and cucumber (Coutts and Wood, 1977), MNSV and melon (Diaz et al. 2004), PSbMV and pea (Keller et al. 1998), PeMV or TEV and Capsicum chinense (Murphy et al. 1998), PVX and potato (Bendahmane et al. 1995), PVY and potato (Barker and Harrison, 1984), TEV and pepper (Deom et al. 1997), and TMV L and homozygous *Tm-1*/*Tm-1* gene tomato (Motoyoshi and Oshima, 1979).

Of the cited works, the last provides the earliest report of a satisfying approach: a quadratic check for resistant and susceptible plant line versus wild-type and virulent virus, with the same outcome: resistance observed for both the intact plant and *in vitro*-inoculated protoplasts but only for the wild-type TMV-L-*Tm-1*-tomato pair. *Tm-1* is regarded as incompletely dominant gene and may be, for example, a dominant negative allele of a gene whose product is required for TMV-L replication (Ishikawa and Okada, 2004; Ohmori et al. 1996).

The SCPMV-common bean system (Fuentes and Hamilton, 1993; Hacker and Fowler, 2000) presents an unusual relationship between non-host resistance and protoplast insusceptibility because both virus RNA and coat protein accumulate in the *in vitro*-inoculated protoplasts but no virions form.

Graft inoculation

Graft inoculation often is the most reproducible method for inoculating vector-transmitted viruses that do not infect by rub inoculation. For viruses in general, graft inoculation is considered to be a severe test to which all but extreme resistance usually succumbs. In some instances of graft inoculation, virions can be detected in targeted, resistant tissue but disappear after

separation from the grafted source of the virus (Hassan and Thomas, 1988), indicating that even when graft inoculation brought virions continuously into the target tissue, new infection was not initiated. Examples of systems showing extreme resistance to virus that is not overcome by graft inoculation to systemically virus-infected stock are CMV and several lines of rough-seeded lupin (Jones and Latham, 1997), PVA and *Solanum tuberosum* subsp. *andigena* (Hamalainen et al. 2000), PLRV and an accession of *Solanum chacoense* (Brown and Thomas, 1993), PVM and *Solanum gourlayi* (Dziewonska and Ostrowska, 1978), and PVY and *Ry* gene potato (Barker, 1996). Resistance to graft inoculation provides presumptive evidence that cells in the intact plant are not susceptible to the virus or what susceptible cells are present are not accessible to the virus. In other systems, graft inoculation overcomes extreme resistance (Abad et al. 2000) or restrictions to infection (Njeru et al. 1995) that were observed for rub-inoculated virus.

Concurrent protection

Ponz and Bruening (1986) proposed the term “concurrent protection” to describe a reduction in challenging virus infection frequency and/or titre due to co-inoculation (but not sequential inoculation) with a protecting virus that is subject to extreme resistance in the host plant (Hull, 2002b). Extreme resistance to CPMV is reflected by resistance to graft inoculation and no development of symptoms and no virion accumulation after inoculation with CPMV at a concentration 10,000-fold greater than a concentration that uniformly infected susceptible seedlings. Co-inoculation, but not sequential inoculation, of CPMV as protecting virus and another comovirus, CPSMV, as the challenging virus, resulted in diminution of CPSMV-induced local lesions and reduced CPSMV virion accumulation. CPMV-mediated concurrent protection also was observed against two other challenging viruses, SBMV and CLRV, which are not comoviruses, showing that concurrent protection is not correlated with the degree of relatedness between the protecting and challenging viruses. Extreme resistance to CPMV and CPMV-mediated concurrent protection were co-inherited as a dominant locus (Bruening et al. 2000).

Comoviruses have two genomic RNAs. The RNA1-encapsidating CPMV virion alone mediated concurrent protection, suggesting that cell-to-cell movement, a function encoded by RNA2, is unnecessary for concurrent protection in the CPMV system and that concurrent protection is a phenomenon of the inoculated cell. However, simply co-inoculating a virus that is confined to the inoculated cell, TMV (Sulzinski and Zaitlin, 1982), with a challenging virus, CPSMV, did not result in protection against the challenging virus (Bruening et al. 2000). These results are consistent with a

mechanism for concurrent protection in which the inoculated plant cell recognizes the presence of the protecting virus, e.g., a protein encoded on RNA1 of CPMV, and initiates a defense mechanism that is effective against both the protecting virus and the co-inoculated challenging virus. The requirement for co-inoculation, rather than sequential inoculation, in achieving protection is consistent with a phenomenon that is limited to the inoculated cell. Rapid healing occurs after cells are wounded (Shackel et al. 1991), which would prevent sequentially inoculated viruses from reaching the same cell.

Concurrent protection also has been demonstrated for inoculated protoplasts. The amino acid change Q-979-E in the 126K replicase protein of TMV-L is one of the two changes responsible for conversion of TMV-L to the virulence mutant TMV-Lta1. TMV-Lta1 overcomes the tomato resistance gene *Tm-1*. According to one interpretation, this mutation may alter the interaction of the 126K or 183K protein of TMV-L with a resistance gene product capable of inactivating the wild type replicase (Hamamoto et al. 1997). Alternatively, the Q-979-E mutation may prevent recognition of the replicase protein in *Tm-1*-bearing tomato cells. Evidence for the latter possibility is provided by experiments of Yamafuji et al. (1991) in which TMV-Lta1 was co-inoculated to protoplasts with a TMV-derived RNA replicon expressing the 126K/183K sequence of TMV-L. A substantial decrease in TMV-Lta1 genomic RNA accumulation (i.e., concurrent protection) was observed compared to the result for protoplasts inoculated with TMV-Lta1 alone, although no accumulation of the RNA replicon was detected.

Rx is a potato gene conferring extreme resistance against PVX. *Rx*-bearing potato does not become infected when grafted to PVX-infected, PVX-susceptible potato (Bendahmane et al. 1999). Protoplasts from *Rx*-bearing potato line Cara accumulated only trace amounts of PVX RNAs, demonstrating that the extreme resistance of the intact plant is exhibited to a significant extent in protoplasts. Kohm et al. (1993) showed that co-inoculation of protoplasts from *Rx* potato with PVX and CMV, or PVX and a PVX strain (PVX-KH2) that overcomes *Rx*, resulted in a drastic decrease in CMV or PVX-KH2 accumulation compared to the results obtained from inoculating protoplasts with CMV or PVX-KH2 alone. Although Kohm et al. (1993) did not compare co-inoculation with sequential inoculation of the protecting and challenging viruses, the PVX-*Rx* system appears to show concurrent protection.

Amplification of virus infection by co-infection

Some double inoculations of viruses result in the enhancement of an

otherwise highly limited virus infection. Several phloem-limited members of the *Geminiviridae* and *Luteoviridae* increase substantially above the control level in hosts infected by a taxonomically distinct virus (Atabekov and Taliansky, 1990; Barker, 1989). Gene silencing (Chapter 3) has been implicated in phloem limitation (Barker et al. 2001), which suggests an anti-silencing role for the systemically infecting helper virus. A systemically infecting virus also can facilitate the increase of another virus in the second virus' non-host. Dodds and Hamilton (1972) observed accumulation of TMV to the level of several g per kg of tissue in upper leaves of the TMV non-host barley when the barley had been inoculated with TMV and BSMV. The TMV yields were comparable those achieved in some authentic TMV hosts. More than 20 examples have been documented of virus infection of a non-host that was infected by a specific, taxonomically distinct, systemically moving helper virus (Atabekov and Taliansky, 1990; Ryang et al. 2004; Takeshita and Takanami, 2000), including helper viruses with movement proteins of different general classes (Waigmann et al. 2004). When the cell-to-cell movement protein of the helped virus was deleted or inactivated, the enhancement of accumulation and/or spread nevertheless was observed, whereas an intact movement protein gene of the helper virus was required (Malyshenko et al. 1989; Ryang et al. 2004; Taliansky et al. 1982a; Taliansky et al. 1982b). Replacing in *cis* or in *trans* the endogenous virus cell-to-cell movement protein gene (Dejong and Ahlquist, 1992; Rao et al. 1998; Solovyev et al. 1996; Tamai et al. 2003) or gene conferring long distance movement (Ryabov et al. 1999; Spitsin et al. 1999) with a corresponding protein from a taxonomically distinct virus has in several instances created a movement competent virus, and even virus capable of invading a non-host (Huppert et al. 2002).

Given the above findings, examples of genotypic resistance being overcome by co-infection with a taxonomically distinct virus are expected: CMV helped by ZYMV in zucchini squash (Choi et al. 2002), PeMV helped in pepper by CMV (Guerini and Murphy, 1999; Murphy and Kyle, 1995), PeMV helped by TMV-P in pepper (Pieczarka and Zitter, 1981), PVY(0) helped by TMV in *Solanum brevidens* (Valkonen, 1992), and TMV helped by PVX in Tm-2 tomato but not in Tm-1 tomato (Taliansky et al. 1982a). The taxonomic disparity between the helper virus and the assisted challenging virus in the examples of alleviated non-host and genotypic resistance cited above suggests that the helper virus does not contribute to the replication of the helped virus. Indeed, CMV, which overcomes a genotypic resistance of pepper to PeMV, did not stimulate PeMV accumulation in co-inoculated PeMV-resistant pepper protoplasts (Guerini and Murphy, 1999). Therefore, when resistance is overcome by co-infection, it is reasonable to suggest that the restriction imposed by that resistance does

not function *per se* against replication of the challenging virus but acts at some other level.

General genetics of resistance

A resistance derived from *Nicotiana glutinosa*, encoded by the *N* gene locus, which has been transferred into many tobacco species and lines. This system provided the first demonstration of inherited resistance to a plant virus (Holmes, 1938). The *N* gene is inherited as a simple, dominant character. However, many examples of resistance to specific viruses are polygenic, requiring additional effort in cultivar breeding compared to resistance controlled by a single locus. Unraveling the biochemical contributions of individual gene products to any multigenic virus resistance remains to be accomplished, though progress is being made (Chisholm et al. 2001). Therefore, the focus here is on monogenic resistance in which a single genetic locus is sufficient to confer resistance.

Many examples of monogenic inheritance are considered to be unambiguously dominant or recessive or incompletely dominant. For example, accessions of *Solanum chacoense* accumulated PLRV to a titre that could be detected by enzyme-linked immunosorbent assay (ELISA) after being exposed to viruliferous aphids or grafting to systemically infected PLRV stock. Crosses of homozygous resistant to homozygous susceptible lines gave F1 progeny that, after inoculation with PLRV, were symptom free and ELISA negative. Crosses of F1 progeny to a homozygous susceptible line gave progeny segregating 1:1 for resistance and susceptibility. The *Solanum chacoense*-derived resistance to PLRV is thus considered to be an example of extreme resistance inherited as a simple dominant character (Brown and Thomas, 1993).

The G1 strain of SMV was inoculated to a resistant soybean accession and to progeny from its crosses with susceptible lines. The resistant phenotype was local lesions on inoculated unifoliate leaves but without symptoms elsewhere. The susceptible phenotype was characterized by vein clearing on trifoliate leaves within 7 days of inoculation, developing into a systemic mosaic. F1 progeny developed symptoms distinct from those seen for either of the parents: no symptoms until at least 14 days after inoculation, with transitory chlorotic islands on the trifoliate leaves. F2 progeny segregated 1:2:1, for resistant:late developing symptoms:susceptibility, consistent with incomplete dominance of resistance (Gunduz et al. 2004).

After inoculation of rice cultivar Gigante with RYMV, no virions or virus RNA accumulated to a detected level. A cross of Gigante to susceptible rice cultivar IR64 resulted in all F1 progeny being susceptible to RYMV. Segregation in the F2 and F3 lines was consistent with resistance to

RYMV being under the control of a single recessive locus (Ndjiondjop et al. 2001).

Other examples of inherited resistance are conditional or ambiguous, unlike the 'clean' and consistent phenotypes described above. The distinctions between dominant, incompletely dominant and recessive traits may be murky. Collmer et al. (2000) demonstrated that the *I* gene of common bean, for resistance to BCMV, confers a dominant extreme resistance at 23°C. That is, both *I/I* and F1 *I/i* genotypes did not support accumulation of BCMV in inoculated unifoliate or uninoculated trifoliate leaves. At 34°C, plants of *I/I* lines continued to prevent accumulation of BCMV in the inoculated unifoliate leaves, but BCMV was detected in the uninoculated trifoliate leaves. F1 bean plants supported BSMV increase in both unifoliate and trifoliate leaves at 34°C, but to a lower titre than was achieved in BCMV susceptible lines. That is, the BCMV-common bean *I* gene system exhibits dominant resistance at 23°C but incomplete dominance at 34°C. The soybean *Rsv4* gene for resistance to SMV was dominant when homozygotes and heterozygotes were challenged with SMV strain G7 but incompletely dominant when challenged with SMV strain G1 (Gunduz et al. 2004).

Recessive and incompletely dominant resistance may be difficult to distinguish. A transgene derived from the coat protein gene of SqMV provided a strong resistance to SqMV, which is reflected in a failure of SqMV to move out of infected cotyledons or leaves. Surprisingly, the inheritance of this trait was found to be recessive (Provvidenti and Tricoli, 2002). A transgene has no susceptible allele in the usual sense, and the active agent in the resistance mechanism presumably is either SqMV coat protein interfering with long distance spread of the virus or silencing of SqMV genomic sequences initiated by SqMV coat protein messenger RNA sequences. Therefore, transgenic resistance to SqMV is expected to be dominant or incompletely dominant, not recessive. Progeny tests were performed by inoculating each seedling twice with a 1:15 dilution of extract from a SqMV infected leaf, first to the expanded cotyledon and later to the first leaf. SqMV is a high titre virus, so the described inoculation procedure must be considered intensive. A possibility, which is consistent with the association of the NPTII antibiotic resistance marker with susceptible and resistant phenotypes and recovery from symptoms of approximately half of the population [Table 2 of Provvidenti and Tricoli, (2002)], is that the coat protein gene-derived transgenic resistance to SqMV actually is incompletely dominant rather than recessive. However, the strong inoculum of SqMV may have caused incomplete dominance to be scored as susceptibility, resulting a segregation consistent with a recessive trait.

Kheterpal et al. (1998) assembled tables reporting 139 examples of

monogenic resistance to plant viruses. The list is composed of 81 dominant genes, 15 incompletely dominant genes, and 43 recessive genes. Tables 2 and 3 extend the (Khetarpal et al. 1998) tables with more recent examples. Table 2 combines monogenic dominant and monogenic incompletely dominant genes because of the uncertainties indicated above. Table 3 identifies references describing recessive resistance.

Recessive resistance

Diaz-Pendon et al. (2004) review crop plant recessive resistance to viruses in relationship to model plant loss-of-susceptibility mutants and non-host resistance. They examine the generally accepted broad mechanistic explanations of recessive resistance. Firstly, recessive resistance is due to loss or modification of a host factor that participates in virus replication, RNA translation, movement or pathogenicity (Whitham and Wang, 2004). Secondly, and so far not demonstrated for a virus, a dominant factor suppresses resistance. Recessive resistance based on loss of a host factor is supported by genetic fine mapping results showing an extensive deletion associated with various alleles of the tobacco *va* gene, which confers resistance to PVY (Noguchi et al. 1999). Co-inoculation of wildtype PVY and a PVY variant that overcomes *va*-mediated resistance resulted in an infection that generated the PVY variant only (Nicolas et al. 1997). That is, wild-type PVY did not initiate concurrent protection against the PVY variant. If concurrent protection had been observed, a mechanism dependent on recognition of the invading wild-type virus would have been suspected.

Recessive resistance, based on modification of a host factor, has been confirmed by the molecular cloning of genes for resistance to specific potyviruses and groups of potyviruses. These recessive resistance genes are alleles of eIF4E, a eukaryotic translation initiation factor and RNA-binding protein. The observed examples of eIF4E-mediated recessive resistance vary from an asymptomatic systemic infection to a more an extreme type in which virus was not detected in inoculated protoplasts (Keller et al. 1998) or epidermal cells of the intact leaf (Sato et al. 2003). These results have been connected (reviewed by (Gao et al. 2004)) to known functions of eIF4E and to documented interactions between eIF4E and the virus VPg (protein covalently linked 5’ to the genomic RNA) or VPg precursor.

Recessive resistance to Melon necrotic spot virus (MNSV, *Tombusviridae*) conferred by the melon gene *nsv* has unusual characteristics (Diaz et al. 2004). MNSV strain 264 overcomes *nsv* resistance. Host range

Table 2. Monogenic, dominant or incompletely dominant resistance[a,c]

Virus	Host	References
AlMV	*Lycopersicon hirsutum*	(Parrella et al. 2004)
ACMV	*Manihot esculenta*	(Akano et al. 2002)
BaMMV	*Hordeum vulgare*	(Ruge et al. 2003)
BCMNV	*Phaseolus vulgaris*	(Provvidenti, 2001)
BCMV	*Phaseolus vulgaris*	(Collmer et al. 2000) [b]
CTV	*Poncirus trifoliate*	(Deng et al. 2001; Mestre et al. 1997; Yang et al. 2003)
CABNV	*Vigna unguiculata*	(Bashir et al. 2002)
CPSMV	*Vigna unguiculata*	(Ouedraogo et al. 2002) [b]
CMV	*Arabidopsis thaliana*	(Takahashi et al. 2002)
	Cucurbita pepo	(Brown et al. 2003)
	Lupinus luteus	(Jones and Latham, 1997)
LIYV	*Cucumis melo*	(McCreight, 2000)
LMV	*Arabidopsis thaliana*	(Revers et al. 2003)
MNSV	*Cucumis melo*	(Mallor Gimenez et al. 2003)
PStV	*Glycine max*	(Choi et al. 1989) [b]
PeMV	*Capsicum chinense*	(Grube et al. 2000)
RCVMV	*Trifolium pratense*	(Khan et al. 1978)
SugMV	*Zea mays*	(Melchinger et al. 1998)
TEV	*Arabidopsis thaliana*	(Chisholm et al. 2001)
TYLCV	*L. pimpinellifolium*	(Kasrawi, 1989)
TCV	*Arabidopsis thaliana*	(Cooley et al. 2000)

[a] Extending the sets of 81 dominant and 15 incompletely dominant virus resistance loci compiled by (Khetarpal et al. 1998); [b] incompletely dominant; [c] additional references for dominant and incompletely dominant *R* genes listed by (Khetarpal et al. 1998): BCMV (Miklas et al. 2000), BNYVV (Amiri et al. 2003), CMV (Nasu et al. 1996; Stamova and Chetelat, 2000), PRSV (Wai and Grumet, 1995), PWV (Provvidenti, 2000), PLRV, (Barker et al. 1994), PVS (Marczewski et al. 2002), PVX (Marano et al. 2002), PVY (Boiteux et al. 1996), SBMV (Lee and Anderson, 1998), SMV (Gunduz et al. 2004; Hayes et al. 2004), SugMV (Quint et al. 2003), TSWV (Gordillo et al. 2003; Jahn et al. 2000; Spassova et al. 2001), TuMV (Hughes et al. 2003; Jenner et al. 2003)

analysis for recombinants between MNSV-264 and wild-type MNSV allowed MNSV-264 virulence to be traced to the 3'-untranslated region of the MNSV genomic RNA, suggesting that the viral RNA, rather than a virus-encoded protein, is the virulence factor acting in this system. MNSV-264 infects not only *nsv*-melon but also *Nicotiana benthamiana* and *Gomphrena globosa*, two non-hosts for wild-type MNSV.

The largest class of resistance genes

Given the intimate integration of virus functions into the infected cell, it is logical to postulate that the product of a plant *R* gene could act directly on the product of a virus gene to interfere with virus replication or cell-to-cell movement. However, this hypothesis is not consistent with the known similarities among various *R* genes effective against non-viral pathogens, nematodes and sucking insects, suggesting that plants have evolved general mechanisms to accomplish resistance. Members of the largest class of *R* genes do not act against the pathogen directly but instead mediate recognition of an elicitor molecule (avirulence factor) of the pathogen or pest, consistent with gene-for-gene interactions (Flor, 1971). Recognition results in a cascade of events that culminate in resistance that is effective against the target pathogen (Martin et al. 2003). Often, protection against some other pathogens is achieved as well. If recognition is so critical to resistance gene action, a question arises: why is it that a pathogen should retain its avirulence gene in a form that has the effect of reducing the host range of the pathogen? Several avirulence factors have been demonstrated to be virulence factors that make the pathogen more effective on host plants that lack the cognate *R* gene (Goulden et al. 1993; Kjemtrup et al. 2000).

Based on advances in molecular cloning technologies, including map-based cloning and transposon tagging, about 35 plant *R* genes that are associated with gene-for-gene interactions have been isolated and sequenced. *R* gene isolation usually is confirmed by complementation, e.g., by transformation of a susceptible plant line to resistance. Deduced *R* gene amino acid sequences revealed two common motifs: a nucleotide binding site (NBS) motif with a characteristic internal hydrophobic domain and, to the carboxyl side of the NBS, a leucine-rich repeat (LRR) motif (Belkhadir et al. 2004; Hammond-Kosack and Jones, 1997; Martin et al. 2003). NBS-LRR resistance genes are further subdivided into TIR and non-TIR subclasses. TIR *R* genes possess a domain with homology to the *Drosophila* Toll and mammalian interleukin-1 receptors (Hulbert et al. 2001). LRR sequences apparently have evolved to bind to specific proteins (Jones and Jones, 1997). Many NBS-LRR genes have encoded proteins that are predicted to reside entirely in the cytoplasm (Martin et al. 2003) and therefore potentially to be in contact with products of virus infection. Genes containing NBS and LRR motifs, and possibly other motifs characteristic of *R* genes, are referred to as *R* gene homologues (RGHs). The RGHs constitute a large, diverse and apparently rapidly evolving family of plant genes frequently occurring in clusters and clusters of clusters (Cannon et al. 2002; Grube et al. 2000; Quint et al. 2003). An indicator of NBS-LRR gene diversity and evolution is the 88% identical amino acid sequences of the potato Rx1 and Gpa2 genes, conferring resistance to PVX and the potato

cyst nematode, respectively (Bakker et al. 2003). Similarly, three allelic Arabidopsis genes, each acting through a different signalling cascade, confer resistance to an oomycete fungus, TCV and CMV (Takahashi et al. 2002). There are about 150 members of the RGH family in *Arabidopsis thaliana* (Meyers et al. 1999), about 160 in the model legume *Medicago truncatula* (D. R. Cook, personal communication) and over 500 members in rice (Koczyk and Chelkowski, 2003).

Table 3. Monogenic, recessive resistance to specific viruses[a]

Virus	Host	References
BGMV	*Phaseolus vulgaris*	(Urrea et al. 1996; Velez et al. 1998)
CelMV	*Apium graveolens*	(D'Antonio et al. 2001 ; Ruiz et al. 2001)
CABMV	*Vigna unguiculata*	(Bashir et al. 2002)
LMV	*Arabidopsis thaliana*	(Revers et al. 2003)
MNSV	*Cucumis melo*	(Mallor Gimenez et al. 2003)
PRSV	*Cucurbita moschata*	(Brown et al. 2003)
PVA	*Solanum tuberosum*	(Hamalainen et al. 2000)
PVY	*Capsicum Annuum*	(Arroyo et al. 1996; Ruffel et al. 2002)
RYMoV	*Oryza sativa* *O. glaberrima*	(Albar et al. 2003; Ndjiondjop et al. 2001)
WMV	*Cucumis sativus*	(Wai and Grument, 1995)

[a] extending the set of 43 recessive virus resistance loci compiled by (Khetarpal et al. 1998); [b] additional references for recessive *R* genes listed by (Khetarpal et al. 1998): BaMMV (Le Gouis et al. 2004; Okada et al. 2003), BYMV (Okada et al. 2003), BeYMV (Kasimor et al. 1997), MBYMV (Sirohi et al. 2002), PSbMV (Frew et al. 2002; Kasimor et al. 1997; Keller et al. 1998), PVY (Boiteux et al. 1996), RTSV (Habibuddin et al. 1997), TEV (Deom et al. 1997), ZYMV (Kabelka and Grumet, 1997)

Cloned NBS-LRR genes conferring resistance against viruses are listed in Table 4, with the corresponding virus elicitors. *Rx2* cloning was accomplished by a functional screen and without recourse to map-based cloning or transposon tagging. An *Agrobacterium*-mediated transient expression system presented members of a potato cDNA library in leaves of plants expressing the PVX coat protein elicitor, revealing a necrotic reaction for library members encoding a candidate Rx2 gene (Bendahmane et al. 2000).

Following the identification of the TMV 126K replicase protein as the elicitor for the tomato gene Tm-1, a variety of virus proteins have been identified as elicitors/avirulence factors for other *R* genes (e.g., (Malcuit et al. 1999)). An avirulence factor has been mapped to a segment of a virus

genomic RNA that is not expected to be translated into protein, implicating the RNA segment *per se* as a possible elicitor (Szittya and Burgyan, 2001). Although many RGHs have been characterized, the cascade of reactions that follows *R* gene-mediated recognition remain unclear even for the most intensely investigated systems (Chapter 4 and (Belkhadir et al. 2004)).

Two of three genes identified as contributing to preventing TEV long distance movement in Arabidopsis leaf, *RTM1* and *RTM2*, were cloned by a map-based approach, and their identities were confirmed by transgenic complementation. *RTM1* and *RTM2* are not NBS-LRR genes (Chisholm et al. 2001). RTM1 has lectin-related amino acid sequences, and RMT2 has sequences related to heat shock proteins. The distribution of RTM1 and RTM2 proteins were deduced from the distribution of GUS reporter expressed from transgenic fusion constructions. RTM1 appears to be targeted to sieve elements, whereas the RTM2 fusion accumulated in sieve elements and companion cells, consistent with the role of these proteins in preventing long distance movement.

Durability and yield penalties

In practical terms, tolerance or resistance is genuinely durable if it provides agronomically significant protection against the adverse effects of the pathogen for the commercial life of the cultivar. Durability is a critical consideration in plant breeding because of the high cost of creating a new cultivar. It is generally recognized that genes conferring resistance to plant viruses are more durable, on the average, than genes conferring resistance to other plant pathogens (Garcia-Arenal and McDonald, 2003; Harrison, 2002; Khetarpal et al. 1998). Durability is favoured if the *R* gene is effective against the full range of variants of the virus occurring in the area of cultivation (Garcia-Arenal and McDonald, 2003) and when virulent strains are at a competitive disadvantage in the absence of the cognate resistance gene(s). Greater durability also is likely when existing virus strains must accumulate multiple mutations in order to overcome resistance, particularly if less than the full set of mutations that is needed for virulence results in reduced fitness relative to the wild-type virus population (Goulden et al. 1993; Harrison, 2002; Lecoq et al. 2004). Durability is correlated with examples of resistance that are maintained when protoplasts are inoculated *in vitro* with the virus (Adams et al. 1986; Barker and Harrison, 1984; Murphy et al. 1998). Polygenic, quantitative resistance and recessive resistance are regarded as likely to be more durable than monogenic resistance (Fraser, 1992; Harrison, 2002; Lindhout, 2002), and some observers consider tolerance to be typically more durable than resistance (Salomon, 1999; Singh et al. 1993). Applying DNA marker-assisted

selection to pyramid several *R* genes offers opportunities for achieving durability with possibly less effort than would be required to breed for polygenic, quantitative resistance.

The agronomic value of a *R* gene is depreciated if incorporation of the gene results in a yield penalty or some other adverse effect. For a *R* gene introduced by a conventional genetic cross, particularly from a wild relative of the crop species, the adverse effect may result from gene or genes carried with the *R* gene by linkage drag rather than to any function of the *R* gene itself (Brown, 2002; Sharp et al. 2002).

Table 4. Cloned NBS-LRR genes **conferring resistance to viruses**

Resistance gene	Target	Virus elicitor	References
Tobacco *N*	TMV	126K protein	(Marathe et al. 2002)
Potato *Rx1*	PVX	Coat protein	(Bendahmane et al. 1999)
Potato *Rx2*	PVX	Coat protein	(Bendahmane et al. 2000)
Tomato *Sw-5*	TSWV	Glycoprotein M	(Brommonschenkel et al. 2000; Hoffmann et al. 2001; Spassova et al. 2001)
Tomato *Tm-2*^{2}	TMV	Movement protein	(Lanferrneijer et al. 2004; Weber and Pfitzner, 1998)
Arabidopsis *HRT*	TCV	Coat protein amino end region	(Cooley et al. 2000; Kachroo et al. 2000; Ren et al. 2000)
Arabidopsis *RCY1*	CMV	Coat protein	(Takahashi et al. 2002)

Conclusions

The plant *R* gene, when available, usually presents the most labour-saving, economical and environmentally sound approach to the control of a plant virus or other pathogen and its disease. Depending on the virus, the *R* gene, and environmental conditions, resistance demonstrates itself in various degrees of reduced virus titre and/or restricted invasion of the plant relative to the reference point of a systemic, and usually damaging, infection. Several relatively easily observed characteristics of the interaction between the virus and the *R* gene bearing plant can provide insight into the resistance mechanism. Resistance that is exhibited by protoplasts from the resistant line may be taken to reflect a mechanism that acts at the replication phase and is likely to be durable. Resistance that is not overcome by graft inoculation is taken as an indicator of insusceptibility and/or inaccessibility of potential host cells. Co-inoculation of the plant with a virus that is subject to the *R* gene and another virus that is not may result in increased accumulation of

the subject virus, suggesting *R* gene interference with movement of the subject virus when it is inoculated alone. A co-inoculation that results in decreased accumulation of the non-subject virus (concurrent protection) suggests a *R* gene mechanism that, once activated by the subject virus, is effective against other viruses. Resistance inherited as a simple recessive character has been associated with production of a variant form of a host protein required by the virus, or with a loss of such a protein. Dominant resistance is correlated with *R* genes of the large NBS-LRR class and gene-for-gene interactions. Several *R* genes effective against specific viruses have been isolated and sequenced, revealing resistance mechanisms that depend on recognition of the invading virus and a subsequent cascade of events resulting in establishment of defence. Other cloned *R* genes are effective because their product(s) interfere with virus movement.

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Chapter A11

Reducing Virus Associated Crop Loss Through Resistance to Insect Vectors

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Introduction

The development of sustainable, environmentally-benign methods of crop protection is an important priority in agricultural research. A variety of insects attack crops, causing damage and reducing yields and crop quality. Insects cause crop loss directly through feeding on leaves, flowers, fruit or seed. A subset of insects damages crops indirectly, through transmission of plant viruses, resulting in reduced yield and crop quality. Breeding for disease resistance has been an important strategy for protection of crops against fungal, bacterial or viral diseases; however, resistances have not yet been identified or transferred for many major diseases. Although integrated pest management (IPM) strategies have been implemented with noted success, insect control has more often relied on the use of pesticides, leading

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to the evolution of pesticide-resistant insects and to increasing health and environmental concerns. The development of pest resistant plants is an attractive alternative strategy for the control of insects and the direct damage they cause. For a target pest that is also the vector of a plant pathogenic virus, the question arises as to whether an effective insect resistance could also serve as a component in an integrated control strategy for insect vectored viruses.

Use of insect control to reduce losses due to viral disease is not a new concept. There are several instances in which crops are sprayed with pesticides for protection from vectors and the viruses they transmit. Similarly, systemic insecticides such as Imidocloprid are applied to the root zone with irrigation water to reduce vector populations. While effective in controlling insect populations, both methods have met with varying degrees of success in reducing viral infection (Perring et al. 1999). Another method used for vector control is the application of insecticide to non-crop plants that harbor virus and/or vectors, to reduce vector populations before they have an opportunity to transmit viruses to nearby crops. In California, insecticide sprays targeting weeds have been used since the mid-20th century to control *Beet curly top virus* (BCTV; Genus *Curtovirus*). The insecticide applications are directed at the overwintering breeding hosts (annual and perennial weeds) of the beet leafhopper (*Circulifer tennellus*) to decrease the spring populations of the vector (Cook, 1943). Growers pay over $1.25 million annually for spraying 80,000-200,000 acres of uncultivated land on the west side of the San Joaquin Valley with insecticide (Clark, 1995). Although it is somewhat difficult to measure the efficacy of the insecticide treatments, this control measure is thought to work well in certain years and locations, and be inadequate in others (Cook, 1943; Morrison, 1969). The use of insect resistant plants for reduction of losses due to viral disease would be a logical extension of these existing strategies. This chapter examines the biological features of the interactions among virus, vector and host that would determine the potential success of using insect resistance as a component of an integrated control strategy for insect transmitted viruses.

Goals of insect control vs. control of viral diseases

A review of the practical concerns for crop protection is necessary before considering how the interactions of virus, vector, and host plant could impact the efficacy of crop protection strategies. There are fundamental similarities and differences between protecting crops against economic loss caused by the direct attack of pests vs. that caused by viral disease. In either case, the concern is to

minimize the economic loss, not to achieve the absence of the pests or the virus in the field, although absence of the pest/virus could engender the least damage. The differences between losses based on direct pest damage vs. viral disease are based on the probable thresholds for economic losses in these two cases. It is possible to sustain some direct pest damage yet suffer little economic loss, provided that the damage does not seriously impact the yield or quality of the crop. For example, foliar pests such as leafminer can cause foliar damage in tomato, but economic damage could be minimal if the leafminer populations are low enough to avoid significant defoliation. Greater economic loss would result if the pest damaged the harvested portion of the plant, resulting in lower acceptable threshold levels for such pests. There is a greater opportunity for limiting direct damage after initial infestation with insects than indirect damage due to viral infection. If insect pest levels rise above acceptable thresholds during a growing season, it is possible to reduce pest levels by deployment of control strategies to prevent or limit economic loss. In contrast, a plant can become infected by a virus after it is visited by as few as one viruliferous vector. If the plant is not resistant to the virus, the virus spreads throughout the plant, causing damage ranging from minimal to complete economic loss. In addition, the presence of infected plants in a field increases the risk of infection and damage to other plants at that location, since the infected plants provide a local source of virus. Therefore, the tolerance for the presence of some level of the insect could be much higher if the goal is control of direct damage caused by the pest rather than the reduction of infection by a viral pathogen vectored by the insect.

Viruses transmitted by insect vectors and the nature of virus transmission

Most insect transmitted viruses are vectored by insects with piercing-sucking mouthparts such as aphids, whiteflies, leafhoppers, or thrips. We will chiefly focus on these four major vectors, although some plant viruses are also transmitted by beetles, mites or other types of chewing insects. One characteristic common to insects with piercing-sucking mouthparts is the use of stylets (hollow tube-like structures that can puncture cell walls) for feeding (Pollard, 1977; Backus, 1985; Hunter and Ullman, 1992). Vector transmitted viruses have a specific association with the vector insect that is required for efficient transmission. Some types of viruses associate with the stylet itself, while others associate with other areas of the insect digestive tract, including the foregut, midgut and hindgut. Others allow the virus to pass into the hemocoel (body cavity) where it circulates in the hemolymph (the equivalent

of blood in the insect) and can pass to other parts of the insect body (for reviews see Gray and Banerjee, 1999; Ng and Perry, 2004).

The specific relationship between vector and virus that determines transmission is a complex relationship involving not only the virus and vector, but also host plant and environmental influences. In addition, the nature of virus acquisition and association with the vector, other actions including landing and probing the food source, as well as feeding patterns may influence efficiency of virus transmission. Acquisition period refers to the time necessary for a vector to obtain virus from an infected plant during feeding. Transmission period refers to the length of time following acquisition during which a vector remains capable of transmitting the virus to a new host. Viruses transmitted by biological vectors are classified as nonpersistent, semipersistent, or persistent based on the nature of the transmission event. Nonpersistent viruses are acquired rapidly by vectors as the insects probe different plants with their stylets while seeking suitable food sources (known as test probing), or during the feeding process itself. Nonpersistent viruses remain associated with insect mouthparts, and can be transmitted for only a few minutes to a few hours (Gray and Banerjee, 1999; Pirone and Perry, 2002). In stark contrast, persistent viruses, once acquired by the vector, are usually retained for the life of the insect. Acquisition and transmission periods are much longer for persistent viruses, ranging from a few hours to several days, and often involve lengthy latent periods during which the virus cannot be transmitted. Between these two extremes are the semipersistent viruses. These viruses are also acquired quickly by vectors, but unlike nonpersistent viruses, semipersistent viruses are generally retained by the vector for periods of days to a few weeks.

Nonpersistent and semipersistent viruses have been shown to be specifically associated with the epicuticular lining of insect mouthparts, specifically the stylet or foregut. This lining is shed when the insect molts, and any virus associated with it is lost at that time (Ammar et al. 1994; Gray and Banerjee, 1999; Martin et al. 1997; Wang et al. 1996a). Nonpersistent and semipersistent viruses, which associate with insect mouthparts and do not cross membranes within the vector, are known collectively as noncirculative viruses.

Persistent viruses require virus particles to be fully ingested by the insect and transported to the insect hemocoel and ultimately into the salivary glands from which they can be transmitted to new plants during feeding (Gray and Banerjee, 1999). This type of transmission is referred to as circulative, because the virus must circulate through the body of the insect. Circulative transmission requires movement across cell membranes within the vector.

There are two types of circulative viruses; those that simply move through the body of an insect, and those that actually replicate inside the insect. Those that do not replicate in the insect vector are known as circulative nonpropagative. Those that replicate in the vector are known as circulative propagative viruses.

Some viruses are transmitted by chewing insects, such as beetles and eriophyid mites. Beetle-transmitted viruses are generally believed to be transmitted through regurgitant. Virus is acquired during insect feeding, and is transmitted to new plants through regurgitant produced by viruliferous beetles. Some beetle-transmitted viruses, like persistent circulative viruses transmitted by piercing-sucking insects, can be transmitted for very long periods of time. In some, but clearly not all cases, virus becomes circulative in the hemolymph of the vector. *Southern bean mosaic virus* (SBMV; genus *Sobemovirus*) is not circulative in the Mexican bean beetle, but is circulative in two other beetle species (Wang et al. 1992). Other viruses, such as *Bean pod mottle virus* (BPMV; genus *Comovirus*) have long transmission periods, however, BPMV is not detected in the hemolymph of the bean leaf beetle vector (Wang et al. 1992). This suggests that circulation of virus may not be critical for beetle transmission, at least for some beetle transmitted viruses.

Mite transmitted viruses include *Wheat streak mosaic virus* and numerous other viruses in the genera *Rymovirus* and *Tritimovirus* (family *Potyviridae*). While mechanisms of mite transmission are not well known, indications are that these viruses, like aphid transmitted potyviruses, can in some cases be acquired with very short feeding periods of a few hours (Thresh, 1971).

Dynamics of vector feeding and effect on transmission

The mechanics of virus transmission differ dramatically between circulative and noncirculative viruses, and within these, between nonpersistent and semipersistent (all noncirculative), and between persistent viruses (circulative-propagative and circulative-nonpropagative). Nonpersistent viruses are associated with the stylets of the vector and are retained for only a few hours. These stylet-borne viruses are acquired rapidly by their vectors, predominantly aphids, and are readily lost during feeding or probing. Interestingly, nonpersistent viruses are transmitted most efficiently when acquisition feeding periods are short. Transmission efficiency decreases with prolonged acquisition feeding, suggesting that bound virus may be easily dislodged during extended feeding, and cannot be reacquired immediately (Gray and Banerjee, 1999). Many insect vectors conduct test

probes on different tissues to identify desirable feeding sites. Test probing is likely the predominant means by which most nonpersistent viruses are transmitted. Although a number of differing theories exist on how transmission of nonpersistent viruses occurs, the process is clearly a specific relationship involving interactions between one or more virus proteins and proteins or other factors associated with the cuticular lining of the stylets (Pirone and Blanc, 1996). Some of the best-known examples of the nonpersistent viruses are the members of the *Potyviridae*, including *Potato virus Y*, *Tobacco etch virus, Turnip mosaic virus* and others.

Semipersistent viruses are generally associated with the insect foregut, rather than stylets. These viruses are usually retained for periods ranging from a few hours to several days (Perring et al. 1999). Efficiency of transmission increases with longer acquisition feeding periods. This suggests that unlike nonpersistent viruses, semipersistent viruses can continue to accumulate until all binding sites become saturated (Gray and Banerjee, 1999). Examples of semipersistent viruses are found in the *Caulimoviridae*, *Closteroviridae* and other virus families.

Transmission of persistent circulative viruses and circulative propagative viruses involves movement of virus across cell membranes within the digestive tract of the insect. Following ingestion, virus is actively taken up by epithelial cells of the midgut or hindgut of the insect, and is translocated across the gut membrane to the hemocoel. The virus moves through the hemocoel, and sometimes other tissues, ultimately reaching the salivary glands from which it is secreted with saliva and transmitted to new plants through probing or feeding (Gray and Banerjee, 1999). Circulative nonpropagative viruses are found in the *Luteoviridae* and *Geminiviridae*. During whitefly feeding these viruses are ingested by the vector and become circulative in the hemocoel of the whitefly vector prior to transmission. Once acquired, circulative nonpropagative viruses can be transmitted for extended periods ranging from weeks to the life of the insect (Gray and Banerjee, 1999).

Circulative propagative viruses are similar in many respects to circulative nonpropagative viruses, but differ in that propagative viruses can replicate inside the vector. Circulative propagative viruses are found in a number of families, but can be represented by *Tomato spotted wilt virus* (TSWV; genus *Tospovirus)*. TSWV is transmitted by both larval and adult thrips of numerous *Frankliniella* and *Thrips* species (Nagata and Peters, 2001), although plant-to-plant spread occurs by adult transmission. Acquisition of sufficient quantities of virus for transmission was as short as 5 minutes, with maximum efficiency by 21 hours, although the mean was 1 hour (Wijkamp et

al., 1996; Nagata and Peters, 2001). Similarly, inoculation access periods of 5 minutes resulted in 6% transmission to *Petunia hybrida*, and 17% to *Datura stramonium* (Nagata and Peters, 2001). Consequently, any method that would be effective in controlling transmission of TSWV or other circulative propagative viruses would need to essentially prevent feeding altogether.

Under what circumstances could vector control effectively reduce virus transmission?

It is clear that insect-transmitted viruses are extremely variable with regard to the many factors associated with transmission. Clearly many could not be controlled effectively by efforts at reducing vector feeding or vector numbers. This may not be universally true, however, and numerous examples exist to support this possibility. It is true that the best form of resistance is against the virus itself, since this will not only prevent damage to the crop exhibiting the resistance, but will also reduce the pool of available virus, thus reducing spread to additional crops. In many cases, however, resistance to virus infection is not available, or is not easily incorporated into commercial varieties. This can result from interspecific sexual barriers between the crop species and the wild relative that is the source of the resistance, the multigenic nature of the resistance trait, or association of the resistance trait or gene(s) with deleterious effects. Chemical control of vectors, while reducing populations, is becoming less desirable through efforts to use more environmentally friendly production methods. While virus control based on reducing vector population or feeding may not be a universal solution to all virus problems, it may be a valuable and effective tool for many. Review of the application, to date, of strategies to control the damage caused by plant viruses through genetic control of vectors has indicated a steady increase in interest for this type of control, ranging from as few as eight cases in 1976 (Kennedy, 1976) to over 20 in 1987 (Jones, 1987, 1998).

The factors that will determine efficacy of vector control for control of plant viruses are many and varied. Of paramount importance is the mode of transmission. Nonpersistent viruses are unlikely to be controlled through any type of vector management that allows significant levels of probing or feeding on the tissue. Additionally, controls that will ultimately kill the insect over a period of time will also be ineffective, as nonpersistent viruses

can be transmitted quickly by test probing in a matter of seconds (Perring et al., 1999).

Control of persistent circulative viruses through methods that reduce or prevent vector feeding may offer more potential, however, effectiveness will also be influenced by the nature of transmission. Circulative viruses, once acquired, move throughout the body of the insect. Consequently, ingestion will lead to uptake, and sequential ingestion will likely lead to more and more virus accumulation in the vector. The begomovirus, *Tomato yellow leaf curl virus* (TYLCV; family *Geminiviridae*) is transmitted by the silverleaf whitefly, *Bemisia tabaci* biotype B. The virus can be acquired by individual whiteflies with acquisition access periods and inoculation access periods as short as 5 minutes each (Atzmon et al. 1998; Czosnek et al. 2001), although efficiency of virus acquisition improves with longer feeding periods. Czosnek et al. (2001) also demonstrated that all individual whiteflies were able to transmit with inoculation access periods of 30 minutes. TYLCV can be acquired and transmitted with very short feeding periods on susceptible host plants, yet can be retained by the vector for long periods. Similar results are found with other members of the *Geminiviridae* as well (Duffus, 1987). Since the virus only needs to be ingested, it is simply a matter of sufficient virus being acquired for some of it to progress through the insect and reach the salivary glands in an infectious state.

One of the more promising virus genera for which vector based control may be effective is the genus *Crinivirus* (family *Closteroviridae*). These semipersistent viruses require longer feeding periods for efficient virus acquisition and transmission than many other plant viruses (Wisler and Duffus, 2001). In addition, efficient transmission of criniviruses usually requires several whiteflies feeding for extended periods. For example, *Beet pseudo yellows virus* (BPYV) can be transmitted with 10 percent efficiency by individual viruliferous greenhouse whiteflies (*Trialeurodes vaporariorum*), and *Cucurbit yellow stunting disorder virus* (CYSDV) can be transmitted with 3 percent efficiency by individual silverleaf whiteflies (*B. tabaci*, biotype B also known as *B. argentifolii*). These viruses can both be transmitted with approximately 85% percent efficiency when 40 and 60 vector whiteflies are used in single plant transmissions of BPYV and CYSDV, respectively. Consequently, limiting the amount of feeding by whitefly vectors can in some instances dramatically reduce the rate of plant infection by these criniviruses, although it is not known how universal this is among semipersistent viruses in general.

Studies by Wisler and Duffus (2001) compared numerous factors associated with vector acquisition and transmission among eight crinivirus

species and four vector species in two whitefly genera. Results were widely variable. Most criniviruses were transmitted by a single genus or in some cases a single species of whitefly (Wisler and Duffus, 2001). *Lettuce infectious yellows virus* is transmitted with high efficiency by the sweet potato whitefly (*Bemisia tabaci* biotype A), but with very low efficiency by the silverleaf whitefly. One crinivirus, *Tomato chlorosis virus* (ToCV), is the only known virus to be transmitted by 4 different species of whitefly in two different genera (Wisler et al. 1998). Interestingly, there were clear differences in ToCV transmission efficiency between each of the vector species. *B. tabaci* biotype B transmitted ToCV most efficiently, followed by *T. abutilonea*, *B. tabaci* biotype A, and *T. vaporariorum* in order of decreasing efficiency (Wisler and Duffus, 2001). This variability in transmission characteristics among virus species must be considered when evaluating the potential of vector-based reduction of virus infection.

Criniviruses can be vectored by whiteflies in both *Bemisia* and *Trialeurodes* genera (Wisler et al. 1998; Wintermantel, 2004). The specific relationship between virus and vector differs for each virus-vector combination with respect to acquisition period, transmission period and virus retention time in the vector. While ToCV was only retained by *B. tabaci* biotype B for 24 hours, *Cucurbit yellow stunting disorder virus* was retained for up to 9 days in the same vector (Wisler and Duffus, 2001). Most criniviruses also have extensive latent periods in their hosts ranging from three to five weeks after transmission before disease symptoms become apparent on plants. It is clear from comparisons even within the genus *Crinivirus* that a number of semipersistent viruses exhibit vastly different traits with regard to insect transmission. In spite of this, semipersistent viruses overall are probably better suited for vector-mediated control than many other types of viruses, simply by the nature of transmission.

Insect resistance mechanisms in plants

Host/insect interactions for plant protection were originally classified as being due to antibiosis, non-preference, or tolerance (Painter, 1958; Beck, 1965), although the term "antixenosis" was suggested as a more accurate term than non-preference (Kogan and Ortman, 1978). Under antibiosis a resistant plant exerts an adverse effect on the growth and survival of the insect. Antibiosis can be due to physical characteristics of the plant or due to secondary metabolites such as toxins. Under antixenosis (non-preference), a plant exerts influences on insect behavior, deterring the insect from using the plant as a host (Painter, 1958; Beck, 1965), hence the use of the term "deterrence" in some references. "Tolerance"

indicates that the pest is neither deterred from the host plant nor adversely affected by the host plant, but the damage resulting from the pest infestation is reduced compared to that suffered by susceptible varieties of the crop (Painter, 1958; Beck, 1965; Reese et al., 1994). These systems of insect resistance may not be mutually exclusive. It is possible that a resistance mechanism could have aspects of both antibiosis and deterrence.

Breeding for insect resistance has a long history, although insect resistance has been used less than disease resistance in most crops. The wheat variety "Underhill" was reported to have Hessian fly resistance in 1782. Despite resistance breakdown over the years in a number of Hessian fly resistance sources, many wheat varieties have been bred to include this trait (Panda and Khush, 1995; Everson and Gallun, 1980). Another historical example is grape phylloxera (*Daktulosphaira vitifoliae*), a North American aphid that was inadvertently transferred to France *ca.* 1860. Grape phylloxera feeds on grape roots, resulting in decreased productivity and vine death. Wild North American grape possessed natural resistance to the pest. This resistance was transferred to develop phylloxera resistant rootstocks that saved the French wine industry. Rootstocks with similar resistance are still in use (Granett et al., 2001).

There are too many examples of pest resistances and mechanisms to cover in this chapter but some examples can be cited to illustrate the differences in mechanisms and their potential utility. Some systems of natural insect resistance are based upon physical structures or characteristics. A resistance to potato leafhopper (*Empoasca fabae)* in bean (*Phaseolus vulgaris*) is due to a high density of hooked nonglandular trichomes. These trichomes act as physical barriers, entrapping nymphs as their hooks became imbedded in the nymphs' bodies (Pillemer and Tingey, 1976, 1978). The waxy surface of plants has also been implicated in reducing insect infestation. "Glossy" mutants, lacking the normal waxy layer or "bloom" of non-mutant plants, have been found in a number of crop species. Sadasivan and Thayumanavan (2003) list instances in *Brassica,* raspberry, castor, sorghum, wheat, sugarcane, and onion in which the glossy plants are more susceptible to a variety of insect pests than the normal waxy plants. This could be due to adverse effects of the waxy layer on the ability of insects to adhere, move, or feed on the plant. Differences in wax layer may also affect the choice of the plant as for feeding or oviposition. Consequently such waxy surfaces may confer either antibiosis or antixenosis depending on their mode of action against different pests.

A number of insect resistance systems are based upon secondary metabolites that are toxic or otherwise detrimental or noxious to pests. Secondary

metabolites are a very diverse array of compounds that are produced by plants but which are not considered essential for basic metabolic function or processes. There are too many secondary metabolites to describe in any detail here (see Hadacek, 2002; Singer et al. 2003; Sadasivan and Thayumanavan, 2003), but a few well-known examples are 2-tridecanone, cucurbitacins, and glycoalkaloids.

The 2-tridecanone, a methyl ketone, is a secondary metabolite in glandular trichomes that is the basis of insect resistance in *Lycopersicon hirsutum* var. *glabratum* (Williams et al. 1980; Fery and Kennedy, 1987). 2-tridecanone has been implicated in the resistance of *L. hirsutum* to tobacco hornworm (*Manduca sexta*), spider mite species *(Tetranychus spp.),* Colorado potato beetle (*Leptinotarsa decemlineata*), tomato pinworm (*Keiferia lycopersicella*) and beet armyworm (*Spodoptera exigua)* (Kennedy, 1976; Gonçalves et al. 1998; Farrar and Kennedy, 1991; Lin et al. 1987; Maluf et al. 1997). This compound is quite toxic, and also acts as an oviposition and/or feeding deterrent.

Some compounds provide resistance to one pest, but increase the damage caused by another pest. An example of this is found with the cucurbitacins. These tetracyclic triterpenoids confer resistance to spider mites in cucumbers through feeding deterrence (antixenosis). However, cucurbitacins are also feeding stimulants for cucumber beetles, thereby increasing damage caused by the latter pest (DaCosta and Jones, 1971). Problems also arise if the control compound is detrimental to humans. Foliar glycoalkaloids of potato are associated with Colorado potato beetle resistance due to the toxicity of the glycoalkaloids toward the pest (antibiosis). However, glycoalkaloids are also toxic to humans, and high foliar glycoalkaloid levels can be correlated with high glycoalkaloid levels in tubers. Consequently, this means of resistance must be used with care (Tingey, 1984).

An increasing number of crops are protected against various pests through expression of foreign genes in plants. Such plants are referred to here as genetically modified organisms (GMOs). A group of delta-endotoxins, known as Bt, derived from *Bacillus thuringiensis*, are used to protect an increasing number of crop plants from insect pests. This method has been so widely used in important crops that Bt GMO crops are the second most utilized GMO crops (James, 2003). GMO crops with a transgene other than Bt delta-endotoxins are also being tested for efficacy against target insects (reviewed in Ferry et al., 2004). The compounds included in this work include: biotin-binding proteins (Burgess et al., 2002, Kramer et al., 2000); chitinases (Wang et al., 1996a); spider venom peptides (Penaforte et al., 2000), enzyme inhibitors and lectins (Ceci et al., 2003, Rahbé et al., 2003); toxins from bacterial symbionts of entomopathogenic

nematodes (Kramer et al., 2001); enhancins from insects (Cao et al., 2002); and even plant hormones (Smigocki and Neal, 1998). Many of these transgenic pest resistance mechanisms are based on a toxin or other compound(s) that are detrimental to pest health and survival. For example, an insect feeds on a Bt GMO plant until it ingests sufficient toxin to be killed. Therefore, many of these GMO systems for insect resistance may be classified as examples of antibiosis.

Several of the natural and GMO systems of antibiosis for insect resistance control insect pests and their direct damage quite well. If the target pest were also a virus vector, would the resulting insect resistance also be expected to reduce crop loss due to insect transmitted viruses? In these resistance systems, insect feeding on the plant is usually required for acquisition of the toxin or to trigger either natural genes or transgenes involved in a response to herbivore activity. If this feeding is as long as or longer than the transmission period for a particular virus, the system would probably allow sufficient time and opportunity for virus transfer before the resistance mechanism against the insect effectively eliminated it as a vector. Therefore, the likelihood that this type of a pest resistance would significantly affect viral disease transmission is minimal. Similarly, natural pest resistance that is based upon antibiosis can reduce pest population growth and pest use of plants, thereby reducing crop loss caused directly by pests. However, in most of these virus-host systems the interaction of the insect with the host plant would be of sufficient length such that virus transmission would not likely be reduced by antibiosis.

Would resistance based upon antixenosis be any more likely to affect virus transmission or reduce economic loss due to insect vectored viruses than antibiosis? Insect resistance based on antixenosis could be of benefit if the deterrence were sufficiently strong and rapid that insect feeding was prevented or delayed enough to reduce or slow transmission rate, infection and symptom development. The first case of this may be the antixenosis found in some Solanaceous species due to the production of acylsugars.

Acylsugar mediated pest resistance and its possible effects on insect vectored viruses

One system of pest resistance that is largely due to deterrence is the resistance in various species in the *Solanaceae* that is based upon the production of acylsugars. Acylsugars are secondary metabolites that are produced by and exuded from type IV glandular trichomes. The wild tomato

L. pennellii has high densities of type IV trichomes on all aboveground green tissues of the plant (Lemke and Mutschler, 1984) and acylsugars comprise *ca*. 90% of the exudates of these trichomes (Burke et al. 1987; Fobes et al. 1985). Structurally, these acylsugars include 2, 3, 4-tri-*O*-acylglucoses, 3', 3, 4-tri-*O*-acylsucroses and 3', 3, 4, 6-tetra-*O*-acylsucroses, with a range of odd and even short- to medium-chain length fatty acid constituents (Burke et al., 1987; Fobes et al., 1985; Shapiro et al., 1994). The fatty acid constituents are present in different combinations and proportions on acylsugars across an array of *L. pennellii* accessions (Shapiro et al., 1994). These acylsugars mediate the resistance of *L. pennellii* to many pests of tomato including: fruitworm (*Helicoverpa,* formerly *Heliothis zea*); tomato pinworm (*Keiferia lycopersicella*); beet armyworm (*Spodoptera exigua)*; silverleaf whitefly (*B. tabaci* biotype B); leafminer (*Liriomyza* spp); potato aphid (*Macrosiphum euphorbiae*), and green peach aphid (*Myzus persicae*) (Goffreda et al., 1988; Rodriguez et al., 1993; Liedl et al., 1995; Hawthorne et al., 1992; Juvick et al., 1994.) Acylsugars also mediate pest resistance in other genera in the Solanaceae, including *Nicotiana, Solanum*, *Petunia*, *Datura,* as well as other *Lycopersicon* species (Gibson, 1976c; Gibson and Valencia, 1978; King et al., 1987, 1990; Severson et al., 1985; Holley et al., 1987; Neal et al., 1989, 1990; Kennedy et al., 1992; Cutler et al., 1986; Buta et al., 1993).

Experiments using acylsugars purified from *L. pennellii* LA716 demonstrated that acylsugar-mediated resistance is largely due to deterrence of the affected pests. Appropriate application of the pure acylsugars reduces feeding of aphids *Myzus persicae* and *Macrosiphum euphorbiae* (Rodriguez et al., 1993; Goffreda et al., 1988, 1989), and sharply reduces oviposition and feeding of leafminer *Liriomyza trifolii* (Hawthorne et al*.,* 1992) and whitefly *Bemisia tabaci* (Liedl et al*.,* 1995). In a study using pure acylsugars, neonate fruitworm (*Helicoverpa zea*) and beet armyworm (*Spodoptera exigua*) larvae, the resistance to these pests was expressed as reduction of larval feeding, which led to a decline in larval development and survival when alternative food supplies were not available (Juvik et al., 1994). This deterrence is very strong. In a potato aphid study that used electronic feeding monitoring (EFM), 35% of aphids placed on *L. pennellii* plants failed to probe over a 45 minute period, and the remaining pests showed a delay of over 20 minutes in the time to first probe, as well as highly significant reductions in the number of probes, and percentage of time spent probing over the test period. Similarly, 22% of aphids placed on the interspecific hybrid *L. esculentum* x *L. pennellii* failed to probe over a 45 minute period, and the remaining pests showed a delay of over 13 minutes in the time to first probe, as well as highly significant reductions in the number of probes and percentage of time spent

probing over the test period (Goffreda et al., 1988). A subsequent EFM study on green peach aphid produced essentially the same results. That is, highly significant reductions in percentage of insects that probed, significant delays in the time to first probe, as well as highly significant reductions in the number of probes and the percentage of time spent probing on the plants that produced acylsugars (Rodriguez et al., 1993). Considering the dynamics of insect-vectored virus transmission, this strong alteration in aphid behavior could have significant impact on the likelihood and efficacy of virus transmission by this vector.

Testing insects with pure acylsugars revealed several unique advantages of acylsugar-mediated pest control. First, the system mediates strong resistance to a broad spectrum of both chewing and sucking insects. In comparison, transgenic insect resistant plants have utilized the Bt toxin for control of chewing pests, and such toxins are generally not active against phloem-feeders (Gasser and Fraley, 1989; Gill et al., 1992; Meeusen and Warren, 1989). Second, the unusual mode of action associated with acylsugar-mediated resistance has advantageous consequences. Antibiosis-based systems producing toxins such as Bt impose strong selection pressure, that can favor the generation of resistant pest biotypes. Thus, it may be possible for an extremely strong deterrence-based mechanism to impose substantial pressure toward generation of resistant biotypes, as well. Pests known to be sensitive to acylsugars, however, are not limited to feeding on tomato. Indeed most of these pests have a wide range of acceptable host species. Consequently, the deterred insects are likely to find alternative hosts, thus reducing the selection of resistant pest biotypes. Another disadvantage of toxin-based antibiosis systems of resistance is the problem of tritrophic relationships, in which the presence of the toxin in the insect pest is detrimental to a beneficial predator of the pest (Kennedy, 2003), although this is rather unlikely in the case of Bt-mediated protection since the delta-endotoxin is specific to a relatively narrow range of insect species. A deterrence system, such as the acylsugar system, will not result in toxic pests, and so should not have this affect, although the reduction of pest populations in a field would probably also result in reduced levels of predator populations that can be supported in that location. Acylsugars do not affect bee visitation, since the acylsugars are not present on the petals or anthers within the flowers.

The goal for the development of acylsugar-producing tomato lines has been insect control, and data to date indicate that this goal should be attainable once the lines are brought to fully acceptable horticultural type. Field tests showed that the two acylsugar-accumulating breeding lines,

produced by transfer of the trait from *L. pennellii* to tomato after five backcrosses to tomato, substantially reduced *B. tabaci* eggs and nymphs (Mutschler et al. in prep). Considering that the acylsugar-mediated deterrence discourages insects from feeding on these plants, might acylsugars also provide protection against some insect transmitted viruses? This must be tested directly against different vector/virus combinations under a variety of typical field conditions and environments. As discussed above, crinivirus transmission may be reduced and development of disease symptoms delayed by external treatments that limit whitefly feeding periods on hosts to very short time periods, making this an attractive virus/vector/host combination to test. Preliminary tests indicated that tomato hybrids producing acylsugars significantly reduced the rate of *Tomato infectious chlorosis virus* symptom development on the plants over a season with heavy whitefly pressure. Plants that did not produce acylsugars developed virus symptoms up to a month earlier than those that produced acylsugars. In fact, many acylsugar-expressing lines never became infected, while most non-acylsugar expressing plants did. This illustrates the potential for this type of vector-based resistance in controlling semipersistent viruses affecting tomato (Wintermantel and Mutschler, unpublished data). The results of one season, with one virus/vector combination is encouraging but does not indicate the efficacy of the resistance across virus/pest combinations, with different levels of pest pressure, or in different environments. The recent production of new acylsugar tomato lines will facilitate the trials needed to assess the potential of acylsugar-mediated resistance for control of both insect vectors and the viruses they transmit, and how antixenosis can be used as part of an integrated strategy for the control of losses due to viral diseases.

Conclusions

Insect resistance has considerable value for control of pests and the direct damage they cause to crops. Although there is no practical example currently in use of indirect control of viral disease through plant resistance to insect vectors, we believe that there is real potential for such control with some combinations of virus/vector/crop/resistance mechanisms. The virus/vector combinations most likely to be controlled for a specific crop would be those that involve semipersistent viruses and/or viruses that require relatively long feeding periods for efficient virus acquisition and transmission. The most effective host plant resistance systems could be those that are rapid acting, perhaps constitutive, and thus have the potential for preventing or delaying vector feeding, rather than killing the vector after feeding. Use of pest

resistance to decrease losses due to viral diseases is unlikely to be the sole control utilized, but could be a valuable component of an integrated control strategy when coupled with other measures to decrease the exposure of the crop to viruliferous vectors. The combination of vector resistance with genetic resistance to viruses would be complementary, and perhaps help reduce the speed or likelihood of selecting virus strains that overcome sources of virus resistance. Cooperative work is needed to complete development and utilization of some of the more promising of these pest resistance systems. Efforts should focus on using this vector control material in coordinated field trials to determine its value against direct losses caused by insects, and on losses due to insect vectored viruses. These studies could determine the utility of vector control strategies, the conditions for their effective use, and the best means to deploy such resistances within a coordinated strategy of integrated pest management.

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Chapter A12

Cross-Protection

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Introduction

Description of the phenomenon and its history

Cross-protection is a natural phenomenon whereby tolerance or resistance of a plant to one virus strain is induced by systemic infection with a second. Eighty years have passed since the phenomenon was first demonstrated by McKinney (1929), who observed that in tobacco plants systemically infected with a "light green strain" of *Tobacco mosaic virus* (TMV: Genus *Tobamovirus*), the appearance of yellow symptoms after re-inoculation with a TMV "yellow mosaic strain" was repressed. In contrast, a "mild dark green" strain did not repress these yellow symptoms upon challenge. Later Salaman (1933) demonstrated that an avirulent strain of *Potato virus X* (PVX: Genus *Potexvirus*) provided protection against superinfection with a virulent strain of PVX in potato. Webb et al. (1952) showed that cross protection against the phloem-limited virus, *Potato leafroll virus* (PLRV: Genus *Polerovirus*) could be achieved by infection with the aphid vector and not only by sap inoculation. The first demonstrations of virus-disease control by mild strains were done with *Citrus tristeza virus* (CTV: Genus *Closterovirus*) (Grant and Costa, 1951), and Cacao swollen shoot disease (Posnette and Todd, 1955). For many years

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serological and cross-protection tests were used as routine methods to determine strain interrelationships in plant viruses (Latorre and Flores, 1985). Apparently, cross-protection seemed to be a general phenomenon with viruses for which distinct strains could be found (Fulton, 1986; Sherwood, 1987; Fraser, 1998). Various terms were used to describe this phenomenon, including "acquired immunity", "antagonism", "cross immunization", "induced immunity", "interference", "preimmunity" "protection inoculation". The term "cross-protection" (Matthews, 1949) seemed most appropriate, since it described an aspect of the phenomenon concerned with disease control in crops (Fulton, 1986; Lecoq, 1998).

In this review we propose a model for cross protection in which the terms from the 1940s – "pre-immunity" and "induced resistance" – seem to be best to address the mechanism of the phenomenon. Thus, the first inoculative virus, usually a mild strain, is the "protector" against the "challenge" virus, but may also be the "inducer" that initiates resistance based on the gene-silencing mechanism. We view this chapter as an opportunity to present the accumulated data on a silencing mechanism that incorporates a new RNA-based model that was first proposed by Ratcliff et al. (1999). We feel that a model based on a combination of RNA silencing and coat-protein-mediated resistance can explain the cross-protection phenomenon in a relatively complete manner for RNA and DNA viruses, as well as for viroids.

Cross-protection mechanisms

Over the years, ever since the first demonstration by McKinney (1929), several models have been proposed to explain cross-protection. Recently, because of an explosion of new data on plant-virus interactions and gene-silencing mechanisms new ideas have been proposed. Cross-protection is complicated by the fact that each plant-virus interaction is multifaceted, and that different viruses can have a number of patterns of interaction within an infected plant. However, with the introduction of virus-resistant transgenic plants it was possible to develop a model to examine the mechanism of cross-protection. Transgenic plants can be seen as a simplified model of cross-protection, with the protector being reduced to one or more genes (or defined sequences) that are constitutively expressed in the plant (Beachy et al. 1990; Lomonossoff, 1995). In parallel with this, since the mid-1980s data on viral genome organization and viral sequences have expanded markedly. This has allowed additional studies to be conducted with viral vectors (Culver, 1996) and *Agrobacterium*-infiltration mediated transient expression (Ratcliff et al. 1999). These methods produce transient over-expression of a foreign sequence, in contrast to the permanent (stable) expression of a gene in a transgenic plant (Lomonossoff, 1995). Though these serve as important tools for the elucidation of virus-resistance mechanisms in plants, it should

be kept in mind that these model systems do not take into account all of the factors involved in a plant-virus interaction. For example, although gene shutoff in transgenic plants by DNA and histone methylation is related to gene-silencing and is guided by short interfering RNAs (siRNAs), it is probably irrelevant to cross protection between RNA viruses. In the present review we will focus on the mechanism of cross-protection, by comparison with, and extrapolation from, the analogous systems mentioned above.

Previously suggested mechanisms of cross-protection

Two major virus-resistance mechanisms have been described with regard to both transgenic plants and cross-protection: Coat-protein (CP)-mediated resistance and RNA-mediated resistance.

Coat-protein-mediated resistance

CP-mediated resistance has been shown to be involved in cross-protection between strains of TMV (Sherwood and Fulton, 1982) and *Cucumber mosaic virus* (CMV: Genus *Cucumovirus*) (Dodds et al. 1985). Transgenic plants expressing CP may show features in common with cross-protected plants (Beachy et al. 1990; Lomonossoff, 1995; Beachy, 1999). CP-mediated resistance in transgenic plants depends on the expression level of the transgene CP, and a higher level of transgene expression elicits better protection. In general, CP-mediated resistance is broken by a high level of challenge virus (Powell et al. 1990). CP-mediated resistance and cross-protection are both less sequence/strain specific than RNA-mediated resistance, and can protect against a broader diversity of virus strains (Lomonossoff, 1995). CP-mediated resistance does not usually confer immunity, and the resistance can be overcome in different circumstances (Beachy, 1999).

The most commonly proposed model for CP-mediated resistance is based on prevention of the uncoating of the challenge virus as it enters the plant cell, which interferes with the translation and replication processes (Culver, 1996; Lu et al. 1998). In an experiment in which resistance to TMV is provided by the virus vector PVX expressing TMV-CP, mutant "TMV CPs that were incapable of helical aggregation or unable to bind viral RNA did not delay the accumulation of TMV" (Culver, 1996; Lu et al. 1998). The action of such a mechanism in conferring transgenic plant resistance can be proven by negative conjecture, whereby out-of-frame TMV CP (Powell et al. 1990) or non-assemblable TMV CP (Bendahmane et al. 1997) do not confer resistance. CP is detectable by immunological methods in resistant plants,

which indicates that it is not targeted by siRNA. By using an inducible promoter for expression of TMV CP, (Koo et al. 2004) provided direct evidence that expression of the CP is a requirement for resistance.

However, this is not the only potential mechanism of cross-protection since CP-defective viruses and viroids can confer cross-protection (Niblett et al. 1978; Gerber and Sarkar, 1989). Also, in many cases virus symptoms on leaves are not uniform, and the appearance of “dark green islands” (see chapter A 9) and mosaics is very common. It was well established that the virus titer was either low or undetectable. These islands were protected against closely related viruses (Fulton, 1951; Atkinson and Matthews, 1970; Loebenstein et al. 1977). As CP subunits do not move from cell to cell, it is clear that this protection cannot be attributed to a CP-mediated mechanism. Therefore, CP-mediated resistance probably participates in cross-protection in many cases, but is restricted to virus-infected cells.

Resistance mediated by RNA hybridization

An RNA-mediated resistance mechanism for cross-protection was first proposed by (Palukaitis and Zaitlin, 1984). In their model the protector virus produces excess progeny positive-sense RNA, which hybridizes to the first minus-strand RNA of the challenge virus, thereby blocking further replication and translation of the incoming virus. This model, too, is restricted to protection of virus-infected cells, and has never been directly tested. In retrospect, a model that incorporates RNA silencing can be seen as a logical extension of the above model.

Cross-protection by RNA silencing

Ratcliff et al. (1999) demonstrated that in plants co-infected with two viruses, one virus can overwhelm the other through RNA-mediated cross-protection if both viruses share a nucleotide sequence. Thus, when the unrelated viruses PVX and TMV, which can normally co-infect the plant, were modified to express the same GFP reporter gene, only PVX-GFP was found in systemically infected leaves (Ratcliff et al. 1999). Nucleotide homology-derived cross-protection seems to be the best explanation for these results.

RNA silencing is a general term for an ancient host defense mechanism that is targeted against invasive viruses, viroids or mobile RNA-transposable elements, and leads to sequence-specific RNA degradation. In plants this general mechanism is known as post-transcriptional gene silencing (PTGS). The PTGS process is initially triggered by long dsRNA such as the commonly found replicative intermediate form of RNA-genome viruses and

viroids. Structured regions of single stranded RNA such as stem loops can also induce PTGS. This enables potent induction of RNA silencing early in replication. PTGS can also be induced against DNA viruses, as they too make dsRNA by transcribing overlapping genes with opposite polarities, as predicted in the case of ssDNA Geminivirus (Chellappan et al. 2004).This may explain the early observation that post-transcriptional gene silencing underlies the recovery of Kholrabi infected with the dsDNA virus CaMV, first made by (Covey et al. 1997).

RNA-silencing mechanism

The RNA-silencing mechanism is being rapidly unraveled and new elements in this system are constantly being discovered. Currently, we know that dsRNA is initially cleaved by a ribonuclease III (RNAse III)-like enzyme family termed DICER. This cleavage produces 21–25 nt double-stranded minihelix molecules with distinctive 5' phosphate and 3' overhangs of two nucleotides termed siRNAs (Xie et al. 2004). The siRNA minihelixes are unwound and the resulting single-strand molecules are individually, incorporated into a multi-component nuclease-silencing complex called RNA-induced silencing complex (RISC).

RISC is the effector complex, which contains an ARGONAUTE (AGO) nuclease, also known as "Slicer" (Vaucheret et al. 2004). *Arabidopsis*, for example, contains 10 distinct AGO-like proteins that could possibly assemble to form RISCs that have differing or redundant functions (Bowman, 2004). Within the activated RISC (RISC*), the incorporated strand of the siRNA can act as a guide to bring the complex into contact with complementary target RNAs, thereby causing their cleavage and subsequent degradation (or translation inhibition in some cases). The degradation of mRNA or pathogen RNA occurs only when there is perfect or near-perfect base pairing with the siRNA. RISC* can potentially degrade any incoming single-stranded viral RNA. The non-encapsidated minus strand of the virus is degraded in the same fashion. Plants contain several populations of small RNAs that result from cleavage by (in the case of *Arabidopsis*) the dicer-like (DCL) enzymes DCL1, DCL2 and DCL3. DCL1 cleaves microRNA (miRNA) precursors to ~21 nt miRNAs, DCL3 cleaves transposons and other endogenous dsRNAs into ~24 nts siRNAs, and presumably, both DCL2 and DCL3 cleave viral RNA to ~21 and ~24 nts siRNA (here designated vsiRNA), respectively (Xie et al. 2004). In *dcl1*, *dcl2* and *dcl3* mutants both CMV and *Turnip mosaic virus* (TuMV) titers, symptoms and vsiRNA levels were unchanged from those in the parent plant, which indicates that various dicer functions can be redundant (Xie et al. 2004). However, in *dcl2* plants infected with *Turnip crinkle virus* (TCV) viral

symptoms were more severe and siRNA level accumulation was delayed (Xie et al. 2004).

As a result of co-evolution plant viruses possess a counter-defense mechanism against the plant's RNA-mediated defense system. Thus, they have acquired genes that encode suppressors of RNA silencing and which are important for combating the host plant defenses (Roth et al. 2004). Plant viral suppressors of RNA silencing have been identified in various virus families, of both RNA and DNA genomes. Known suppressors from different virus families share no obvious similarities at either the nucleic acid or the protein level, reflecting differences at the mechanistic level as well. Viral suppressors such as the HC-Pro of potyviruses (Anandalakshmi et al. 1998), may indirectly interfere with some dicer activities, such as duplex unwinding (Chapman et al. 2004) or altering the composition of accumulated small RNAs (Mallory et al. 2002), thereby suppressing the activation of RISC. Others, such as the P19, P25 and 2b proteins of the tombusvirus, potexvirus and cucumovirus genera, respectively, may block systemic silencing. P19 is a unique suppressor that binds double-stranded siRNAs directly, thereby blocking their function (Lakatos et al. 2004).

The mobile silencing signal

RNA silencing is non-cell-autonomous, and a silencing signal may move directly from the induced cell to neighbouring cells or through the vascular system (Himber et al. 2003). Both signal and virus may be transported actively from cell to cell *via* plasmodesmata and through the vascular system, and both include an amplification process (Fig. 1). Amplification allows the plant to be prepared with sufficient pre-activated RISC against virus invasion in cells distant from virus infected tissue. The short-range signal is limited to 10-15 cells because the signal is serially diluted in the absence of template-virus (Himber et al. 2003). The initial cell-to-cell signal is probably a primary single-stranded viral fragment (vsiRNA) produced by DCL2 or DCL3 from replicative-form viral dsRNA in RNA viruses or an overlapping bidirectional transcript in the ssDNA geminiviruses. This may be followed by a reiterative wave of secondary siRNAs that are produced when the primary vsiRNA primes a dsRNA elongation reaction, using virus RNA as a template (Fig. 1, in "primed cell"). In *Arabidopsis* this amplification process utilizes an RdRp such as SDE1 (SGS2/RDR6) and the helicase SDE3.

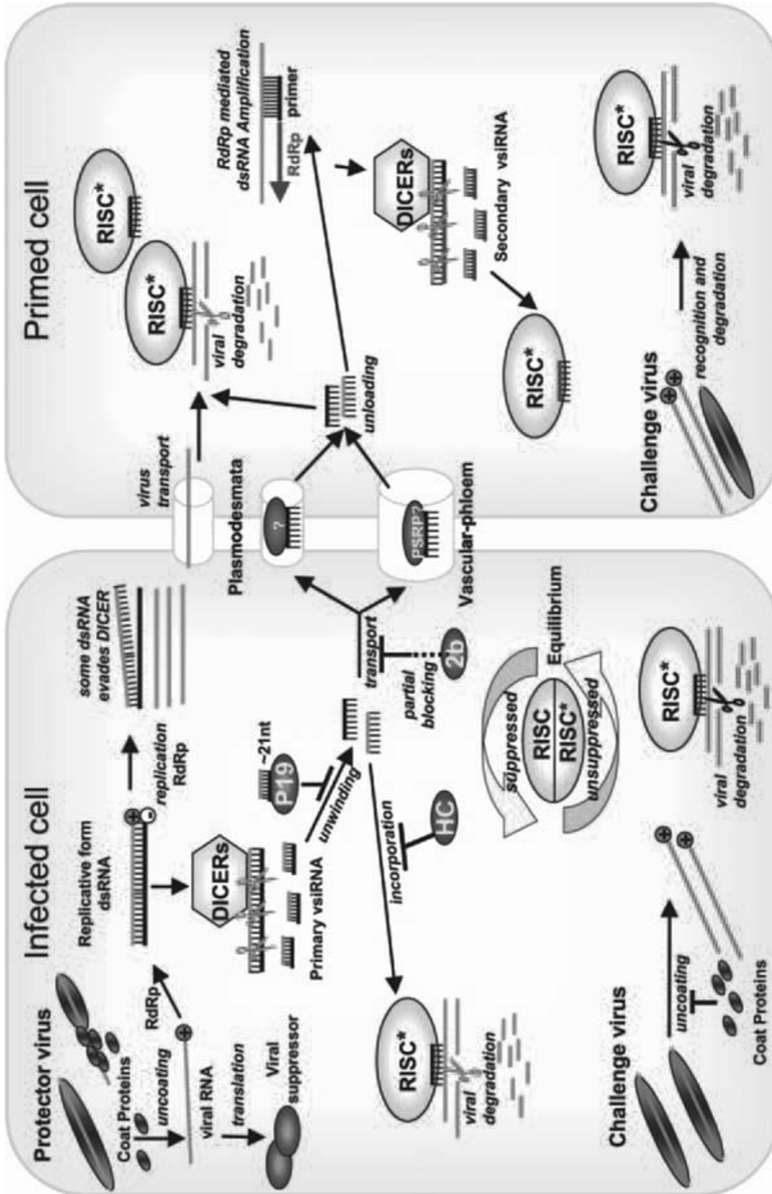
Infected cell
Protector virus
Coat Proteins
uncoating
viral RNA
RdRp
translation
Viral suppressor
Replicative form dsRNA
replication RdRp
some dsRNA evades DICER
DICERs
Primary vsiRNA
P19
~21nt
unwinding
partial blocking
transport
2b
incorporation
HC
RISC*
viral degradation
RISC
suppressed
unsuppressed
Equilibrium
Challenge virus
uncoating
Coat Proteins
Plasmodesmata
?
Vascular-phloem
PSRP?
virus transport
Primed cell
unloading
RdRp mediated dsRNA Amplification
RdRp
primer
Secondary vsiRNA
recognition and degradation
Challenge virus penetrates cell containing protector virus
Challenge virus penetrates primed cell and is degraded by activated RISC

Fig. 1. Model of RNA-mediated viral cross-protection in RNA viruses. Initially the cell is infected with the protector virus (green) (**left**). The protector virus uncoats and its coding sense strand is translated, producing, among other things, a suppressor of silencing. Viral RNA-dependent RNA polymerase (RdRp) makes replicative form dsRNA which is cleaved by Dicer RNAse III type enzymes into ~21 and ~25 class viral siRNAs (vsiRNAs). Some dsRNAs must evade Dicer cleavage for virus establishment to take place. vsiRNA is unwound by a helicase and its separate strands are incorporated into the RNA-Induced Silencing Complexes (RISC), to form "activated RISCs" (RISC*s). RISC*s can now recognize and cleave any complementary viral RNA, of either strand, in the infected cell. A race now takes place between active transport of viral RNA, and transport of vsiRNA through the plasmodesmata and the vascular phloem [with a putative Phloem Small RNA-binding Protein (PSRP)]. If a cell has been reached first by vsiRNA, (**right**) then this cell will have been "primed" for viral degradation through the direct activation of RISC. Upon entry of viral RNA a priming reaction is performed on any complementary viral template that enters, causing the production of secondary vsiRNAs. Secondary vsiRNAs are Dicer-cleaved amplification products of plant RdRps based on viral templates. This secondary amplification occurs in the infected cell also (not drawn). These primed cells could be a possible explanation for "green islands", mosaics and the recovery phenomenon. In the infected cell (**left**) the viral suppressors of different viruses (blue ovals) act at different stages; for example, *Tomato bushy stunt virus* P19 binds ~21nt dsRNA, thus blocking transport, CMV 2b blocks transport, and HC-Pro of potyviruses might interfere with the activation of RISC. Suppression is partial, not total, and attains a state of equilibrium (see text). On the entry of a challenge virus (red) into the infected cell, RISC that has been activated with the related sequence of the protector can cause its degradation. Additionally a CP-mediated protection mechanism may operate when challenge virus enters a virus-infected cell. On the entry of a challenge virus into a primed cell (right) immediate degradation by pre-activated RISC will occur. (See also Colorplates, p. xxii)

Both short-range and long-range silencing through the phloem may be dependent on single-stranded RNA molecules trafficked by proteins such as the phloem small RNA binding protein 1 (PSRP1) of *Cucurbita maxima* (Yoo et al. 2004). Yoo et al. (2004) showed that the phloem naturally contains ~21nt and ~25nt class small RNAs, and no dsRNA. PSRP, though expressed only in phloem-associated cells of *C. maxima,* was shown to traffic small ssRNA also through plasmodesmata of non-phloem tissue when it was co-injected into *N. benthamiana* leaves. In phloem from *C. maxima* infected with *Cucumber yellows virus* (CYV: Genus *Closterovirus*) 57% of all small RNAs were of viral origin, principally belonging to the ~21nt class and comprising both strands. Thus, for long-range movement, vsiRNA enters the vascular system (Yoo et al. 2004) and probably primes secondary amplification by means of an endogenous RdRp in the sink tissue.

Endogenous RdRp may be required for defense against certain viruses (Mourrain et al. 2000). A striking example of this requirement was shown in *N. benthamiana*, which naturally lacks a functional salicylic acid-inducible RdRp (Yang et al. 2004) and is hypersusceptible to many viruses. Complementation of transgenic *N. benthamiana* with a functional RdRp1 from *Medicago trunculata* led to improved resistance to TMV and to several other tobamoviruses but not to CMV or PVX (Yang et al. 2004). However, cross-protection does not have a compelling requirement for host RdRp or a systemic signal, because the protector virus could theoretically move from cell to cell and in the phloem, and re-induce each group of infected cells. Recently it has been demonstrated that the RdRp SDE1 associated with PTGS in *Arabidopsis* was not essential for cross-protection between crucifer tobamoviruses (Kurihara and Watanabe, 2003). Consequently, the plant and the virus wage a classic struggle between host and parasite. If the virus enters distant cells before the signal, or impedes the transported signal in some way, infection can be established. However, if the mobile silencing signal reaches the distant cell first, the virus will enter, only to find itself targeted by pre-activated RISC, and the infection will fail to become systemic. The outcome can be affected by the physiological status of the plant and by environmental conditions.

Several lines of evidence support a model whereby adjacent cells are protected by a silencing mechanism. In the case of the "green islands", and in the "recovery" phenomenon whereby young leaves of an infected plant show fewer symptoms than older leaves, or even no symptoms, it has been demonstrated that the viral RNA level is either low or undetectable (Atkinson and Matthews, 1970). It was demonstrated in *N. benthamiana* infected with *Tamarillo mosaic virus* (Genus *Potyvirus*) that the resulting "green islands" are a recovery-related phenomenon caused by PTGS (Moore et al. 2001). It has been shown that the recovery phenomenon caused by various virus families is associated with RNA-mediated cross-protection against secondary infection (Ratcliff et al. 1999). Recovery was also found in cassava infected with a ssDNA geminivirus. In this case, symptom remission was correlated with the accumulation of vsiRNA, because of the processing of dsRNA from overlapping bi-directional transcription (Chellappan et al. 2004). On the other hand, recovery from CaMV, while associated with PTGS (Covey et al. 1997), is not known to include bi-directional transcription.

Green islands, mosaics and recovery may all be manifestations of the competition between the mobile signal RNA and the viral RNA that encodes the suppressor protein (see chapter A 9). These phenomena relate to cross-protection, since it is the outcome of this competition, which determines which of these processes becomes established, that will decide the fate of the invading virus in each cell. It is clear that the protecting virus, too, does not establish itself in all cells of the plant, but the green islands or recovered organs (termed "primed cell" in Fig. 1) are nonetheless resistant to the challenge virus. It is proposed that in the green islands a viral-sequence-derived memory RNA molecule, probably already in its effector form of activated RISC, lies in wait for an intruding ssRNA viral target (Fig. 1, right). vsiRNA has been shown to accumulate in plants infected with members of at least five genera of viruses: TuMV (Genus *Potyvirus*), TCV (Genus *Carmovirus*) and CMV (Genus *Cucumovirus*) (Xie et al. 2004); CYV (Genus *Closterovirus*) (Yoo et al. 2004); *Cymbidium ringspot virus* (CyRSV: Genus *Tombusvirus*) (Szittya et al. 2003), and *Tomato mosaic virus* (ToMV: Genus *Tobamovirus*) (Kubota et al. 2003). This indicates that the silencing process is initiated in at least some of the cells of the plant despite the presence of viral suppressors. Nevertheless, silencing and viral

replication may also occur concurrently and reach a state of equilibrium inside each infected cell.

Feedback inhibition could be attained, for example, in the following scenario: virus levels rise → more dsRNA, +strand RNA and suppressor are made→ more secondary amplification on viral template → Dicer makes more primary and secondary vsiRNA → more RISC is activated → viral RNA is cleaved → less virus can be replicated and translated to make suppressor → virus levels fall → less template for plant RdRp and less primary and secondary target dsRNAs for Dicer → less new RISC is activated→ and so on. This type of equilibrium is probably necessary for RNA-mediated cross-protection to take place when an already infected cell is challenged. Indirect evidence that plant anti-viral mechanisms may still be active in infected cells lies in the finding that in such cells virus titres reach lower levels than their full potential, and are thus at equilibrium. Proof that the silencing mechanism is involved in restriction of viral accumulation at least in some viruses is that the *Arabidopsis* mutants *sde1* and *sde3* (mutants in the RdRp and helicase genes responsible for the generation of secondary vsiRNA) are highly sensitive to CMV and accumulate a fivefold excess of viral RNA (Mourrain et al. 2000; Beclin et al. 2002).

Virus synergism may be another example of loss of equilibrium. For example, in cucurbits CMV RNA levels can be synergistically boosted at the cellular (protoplast) level by superinfection with *Zucchini yellow* mosaic virus (ZYMV: Genus *Potyvirus*) (Wang et al. 2002; Wang et al. 2004) This suggests that RISC might be a possible limiting step for CMV proliferation, since potyviral HC-Pro may interfere with RISC activation. A classic example of synergy is PVY and PVX co-infection of tobacco protoplasts, in which PVX levels rise dramatically whereas PVY levels remain unchanged (Vance, 1991). Conversely, *Sweet potato feathery mottle virus* (Genus *Potyvirus*) levels increase markedly in the presence of the phloem-limited *Sweet potato chlorotic stunt virus* Genus *Crinivirus* whose levels and localization remain unchanged (Karyeija et al. 2000). It has been shown that temperature can drastically modulate the amount of vsiRNA found in CyRSV in *N. benthamiana* protoplasts (Szittya et al. 2003). Thus, the greatest amount of vsiRNA was correlated with the highest temperature, whereas CyRSV levels peaked at a more moderate temperature. These findings would fit a model in which equilibrium is reached in each cell, as opposed to total shutdown of defence mechanisms by viral suppressors. Thus, equilibrium between suppressed and activated RISC (*Potyvirus* infection) or between inactivated and active signal (*Cucumovirus* infection) or sequestered vs free minihelix dsRNA (*Tombusvirus* infection) could be maintained in the cell.

Challenge-virus entry

There are three scenarios that can be envisaged to explain cross-protection against challenge-virus entry.

a. The challenge virus enters a cell that is infected with the protector virus. Here it is possible that all cross-protection mechanisms may be able to function: over-expressed CP may prevent uncoating of the challenge virus (Beachy et al. 1990); uncoated RNA may be degraded by RISC* (Fig. 1), and the minus RNA strand of the protector virus may hybridize to the challenge virus RNA. The dsRNA hybrids produced by this third mechanism might now be susceptible to degradation by Dicer. It is not clear which of each of these different processes contribute to defense in this case.

b. The challenge virus enters primed cells that contain vsiRNA but are not infected with the protector virus, as in "green islands" (Fig. 1). In such a situation we assume that the RISC* targets the challenge viral RNA and degrades it since other models do not explain cross protection in this type of phenomena.

c. The challenge virus infects primed cells remote from protector-virus infected cells, as in the "recovery phenomenon". In such a case, the vsiRNA is amplified by the endogenous RdRp, having travelled through the vascular system, and will be able to activate RISC and degrade the challenge-virus RNA.

In conclusion, the protector (i.e. the first virus to enter the plant) induces a certain level of the vsiRNA in various cells and tissues, including those that the protector virus has not invaded. The challenge virus (i.e., the second virus to enter the plant) enters a few cells and is now exposed to plant cells, which already produce or host RISC*, or both RISC* and protector virus.

Features of the silencing model to explain cross protection

a. Strain specificity

Classical cross-protection can be obtained only between closely related strains of the same virus. For example, it was demonstrated that the mild ZYMV-WK strain was effective in protecting against serologically related strains but not against divergent strains of ZYMV (Wang et al. 1991; Desbiez and Lecoq, 1997). This was also observed in *Papaya ringspot virus* (PRSV: Genus *Potyvirus*), (Chatchawankanphanich et al. 2000) and *Barley yellow dwarf virus* (Genus *Luteovirus*) (Wen et al. 1991). Serological divergence between strains represents amino acid sequence differences at the N'-terminus of the CP. In potyviruses much of the N' is unconserved as opposed to the CP core. These in turn reflect divergence also at the nucleotide level, and coincide with silent mutations in conserved domains. As trans-encapsidation can occur between serologically unrelated viruses of

the same family (Bourdin and Lecoq, 1991), it seems that strain specificity must be effected at the RNA level.

According to the silencing model (Fig. 1), a plant infected with the protector virus activates RISC* at the cellular level. Since RISC* can efficiently degrade the challenge-virus RNA only when the siRNA complementation is nearly perfect this may explain why ZYMV-WK was not efficient in controlling all the ZYMV isolates (Lecoq and Raccah, 2001).

b. Interval between inoculations

The interval between inoculations of the protector and the challenge virus is important. This interval is often the one required for the full establishment of protector virus in the plant, usually between 1-2 weeks. Shorter intervals can be observed at the cellular level: less than eight hours in protoplasts protected with the *Bromovirus Brome mosaic virus* against the *Bromovirus Cowpea chlorotic mottle virus* (Watts and Dawson, 1980).The two viruses are distinct viruses that share a high homology in RNA2. It has been shown in the laboratory that an interval of 48 hrs is sufficient to achieve cross-protection between ZYMV mutants that differ in only five nucleotides located in the CP N-terminus (Desbiez et al. 1997). In a field test, 14 days were required between the mild ZYMV-WK and the severe ZYMV (Walkey et al. 1992). The differences between these intervals could be due to physiological differences caused by field conditions, or they could be because the homology between the mild (WK) and the severe ZYMV was lower than that between the highly similar ZYMV mutants. We speculate that the interval could depend on the time necessary for accumulation of RISC* and that a higher titre of the protective virus might be required when homology between the strains is lower. The same phenomenon was demonstrated between two non-coding TMV satellite strains, where inoculation within three days of either type prior to the other, resulted in a mixed infection. The first-inoculated strain predominated as a function of time until at three days when cross-protection was fully established (Kurath and Dodds, 1994).

Co-inoculation of two *Plum pox virus* (PPV: Genus Potyvirus) clones expressing GFP and DsRed results in distinct patches of cells infected with only one of the viruses (Dietrich and Maiss, 2003). Similar results were obtained with attenuated ZYMV-AG expressing these same reporters, but with a twist (Gal-On; unpublished results): the GFP-expressing virus (ZYMV AG-GFP) was more viable and accumulated to higher levels in the plant. Each construct completely protected against the other when challenged by mechanical inoculation after two weeks, but in bombardment

with both of these constructs, each on a separate cotyledon, ZYMV AG-GFP dominated, and infected many more areas than ZYMV AG-DsRed.

c. Challenge titer

A higher inculum concentration of the challenge virus can break the protection in some cases (Cassells and Herrick, 1977). Presumably, if the level of challenge viral RNA penetrating the cells exceeds the molar ratio of available RISC* then resistance might be broken.

d. Late breakage of protection and co-existence

Breakage of resistance can occur after initial establishment of cross-protection. In several of the cross-protection breakdown phenomena the challenge virus becomes dominant. Greater viability of the challenge strain (possibly due to faster movement or replication) could permit it to dominate, so that the symptoms could become those of the challenge virus. This can happen in a certain percentage of plants in the field such as with PRSV in papaya where breakdown occurred in 25% of protected trees after 6 months (Gonsalves and Garnsey, 1989). If the sequence match between the RISC* and the challenge virus is not optimal then not all of the challenge virus RNA will be degraded and the "escaped" virus will start to replicate in parallel to the protector virus.

Viral symptoms and cross-protection

Practical cross-protection requires mild or attenuated virus strains. Virus symptoms may develop as a consequence of the direct or indirect action of viral proteins. Many (but not all) viral symptoms have been associated with viral suppressors of gene silencing (Brigneti et al. 1998; Kasschau et al. 2003; Roth et al. 2004). Viral symptoms can mimic developmental abnormalities. Many of the viruses that cause such symptoms have suppressors that are known pathogenicity factors (Chapman et al. 2004), and different suppressors can cause remarkably similar symptoms (Dunoyer et al. 2004), similar also to DICER-LIKE-1 (*dcl1*) mutants of *Arabidopsis* which affects development through biogenesis of miRNA (Kasschau et al. 2003). Many, but not all, elements of the siRNA and miRNA biosynthetic and effector system are shared. It has recently become clear that many developmental events are negatively regulated by miRNAs through posttranscriptional regulation of target mRNAs, of which many are transcription factors (Dugas and Bartel, 2004). Some of these viral suppressors of siRNA may cause symptoms by interfering with the shared

stages of the miRNA synthetic or effector processes as a "bystander" effect, preventing proper downstream mRNA target cleavage. Transgenic *Arabidopsis* expressing tombusviral P19, potyviral P1-HC-Pro and *Peanut clump virus* (PCV: Genus *Furovirus*) P15 all had such symptoms and all suppressed silencing upon crossing with a chalcone synthase silenced line (Dunoyer et al. 2004). Three different routes may lead to this same outcome:

a. Plants that express PCV P15 do not have altered levels of miRNA.

b. Plants that express tombusviral P19 have altered levels of miRNA. P19 probably directly binds and sequesters both the minihelix formed from the siRNA duplex and that formed from the miRNA/miRNA* couple cleaved by DCL-1 (Ye et al. 2003; Dunoyer et al. 2004) and might cause the loss of their 3' overhangs. miRNA* is the opposite strand by-product of miRNA production.

c. Plants that express potyvirus P1-HC-Pro have altered levels of miRNA and especially accumulate miRNA* whose levels are normally nearly undetectable (Chapman et al. 2004). P1-HC-Pro does not bind dsRNA itself (Urcuqui-Inchima et al. 2000) but is known to bind plant proteins such as rgs-Cam that have suppressor activities of their own (Anandalakshmi et al. 2000). This aberrancy in miRNA* might be an effect of indirect duplex stabilisation causing inhibition of target cleavage (or translational repression).

Selection of mild strains for cross-protection

Natural selection

Natural selection of mild strains of plant viruses may arise through evolution, as a result of the actions of direct and indirect forces. Direct selective forces against aggressive strains include reduction of the host pool. The cross-protection mechanism may be an indirect force through which plants develop resistance to aggressive virus infection. Thus, a plant population that allows the systemic infection by and transmission of a mild strain might have an advantage when a new aggressive form appears. Indeed most vegetatively cultivated plants contain viruses that do not cause severe disease or significantly affect reproduction. The extensive cultivation, breeding and transport of crop plants during the recent centuries have interrupted the plant-virus equilibrium, causing cultivars to become more susceptible (as is common with annual crops) and creating opportunities for new virus-plant combinations. Attempts to isolate mild strains from non-cultivar plant species were unsatisfactory for cross protection applications, since they significantly affected the yield. Consequently, the need for control

of epidemic viral diseases brought scientists to search for alternative ways to obtain mild strains (Lecoq and Raccah, 2001).

Artificial selection

Three methods have been used for selection of mild strains.

a. Selection from naturally occurring strains. This method is based on surveying mild symptoms in the field, and subculturing the isolated putative mild virus. Such a method is more often applied to viruses of orchard crops such as *Cocoa swollen shoot virus* (Genus *Badnavirus)* in cocoa (Gonsalves and Garnsey, 1989), CTV from citrus (Costa and Muller, 1980), and *Arabis mosaic virus* (Genus *Nepovirus*) from grapevine (Huss et al. 1989).

b. Selection from a mixed population. This method is based on inoculation of an isolated virus population on host plants, which react to virus infection with a local-lesion phenotype. Each local lesion represents one or several particles from the mixed population, and is amplified by several passages on local lesion hosts prior to inoculation on a systemic host. The ZYMV-WK mild strain was isolated in such a manner (Lecoq et al. 1991).

c. Selection of mild mutants induced under artificial conditions. It has been shown that growing plants infected by viruses at high or low temperatures could induce the formation of mild strains of TMV in tomato and of *Soybean mosaic virus* (Genus *Potyvirus*) in soybean (Oshima, 1975; Kosaka and Fukunishi, 1993). In addition, exposure of a virus preparation to mutagenic nitrous acid was successfully used to generate mild strains of ToMV and PRSV (Rast, 1972; Yeh and Gonsalves, 1984). The artificial treatments were followed by single-local-lesion selection to generate the mild strain.

The potential of genetic engineering for producing mild strains

In contrast to the empirical methods for mild strain selection, the ability to generate an infectious clone of many agriculturally important viruses and accumulated data on molecular determinants of virus pathogenicity potentially facilitate the engineering of new attenuated viruses.

Random mutations throughout the virus genome created mild strains of several viruses. However, such attenuated mutants were usually defective in replication or movement compared with the wild type. For such reasons no artificial attenuated viruses were successfully produced. The engineered ZYMV-AG is a mild virus, which is accumulated and systemically spreads similarly to the wild type ZYMV (Gal-On and Raccah, 2000). The AG strain is a unique mild cloned virus, which contains two mutations. The first mutation alters the symptoms from severe to attenuated; it is located in the

HC-Pro gene in the conserved FRNK motif (Fig. 2) (Gal-On and Raccah, 2000). This mutation was found in the two wild-type strains of ZYMV (WK and NAT). The second mutation is located in the N'-terminus of the coat protein in the conserved DAG motif, and eliminates aphid transmissibility (Gal-On et al. 1992).

The use of an engineered clone offers several advantages over empirical selection methods. A clone is a contamination-free, homogenous and identifiable source of inoculum. It is possible to add extra mutations, such as eliminating the possibility of insect transmission (Gal-On et al. 1992). Vector non-transmissibility isolates the field in which the clone is used from those nearby, as the virus cannot leave the inoculated crop. Industrially it is easier and probably cheaper to employ an engineered clone (cDNA) for mass inoculation than growing infected plants as a source of inoculation. The greatest advantage, however, is that another gene can be added to the clone to provide plant protection in the field, in addition to cross-protection. We recently showed a unique example of such a system, when we demonstrated the expression of a herbicide resistance gene (bar) that was successfully tested in the field with several cucurbit crops (Fig. 2) (Shiboleth et al. 2001). This AG-*bar* vector can potentially also be used for viral cross-protection.

Cross-protection as a practical method for virus control

Cross-protection has been demonstrated with many viruses belonging to different families. Most of the published cross-protection experiments are summarized in Table 1. These were performed either in the laboratory or in the field, and include both RNA and DNA viruses. Although many scientific publications have shown the effectiveness of viral cross-protection against many different viruses, its actual use as a bio-control agent has been relatively limited and today it is almost unused. In general, cross-protection might be common and effective in perennial crops (e.g., trees), in which the protector virus (a natural mild strain) is spread naturally by an insect vector, since many stone-fruit and citrus trees are persistently infected. Examples are presented in a review by Fulton (1986).

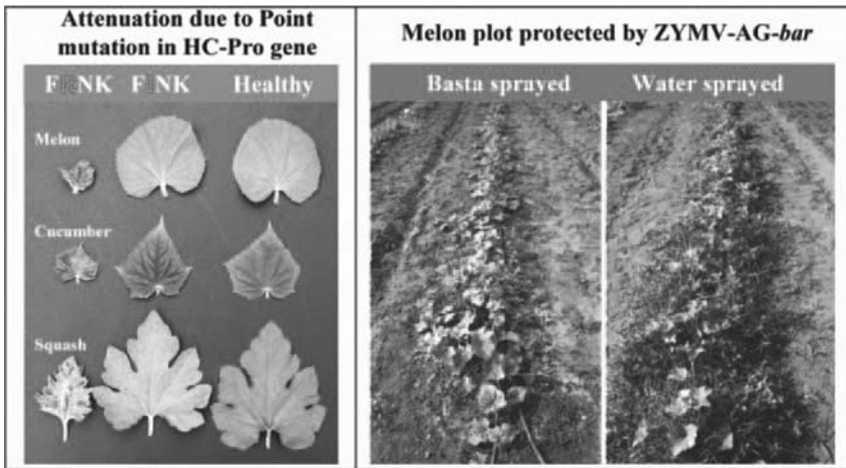

Fig. 2. ZYMV-AGII as an attenuated, aphid non-transmissible, potentially cross-protecting expression vector. A single mutation in the potyviral suppressor gene HC-pro from FRNK to FINK attenuates symptoms in various cucurbits (left). Functional expression of bar via AGII-Bar in cucurbits confers resistance to glufosinate ammonium herbicide (Basta®) (right). Melons were sprayed with 0.5% Basta 14 days after planting and photographed 5 days later. (See also Colorplates, p. xvii)

Current uses of cross-protection

Protection of orchard crops. There are only two examples of cross-protection of major economic importance in orchards that are in use today: against CTV and PRSV.

CTV. In several regions of the world CTV was the most disastrous epidemic disease in citrus orchards, and protection by graft inoculation (budding) with mild naturally occurring strains provided good protection

(Hamilton, 1985). A total of 50 million orange trees have been protected with a mild strain in Brazil (Costa and Muller, 1980; Urban et al. 1990). Successful control of CTV had a great impact, with a natural mild virus strain in Australia, India, Israel, Japan, South Africa and the USA (Hamilton, 1985). CTV is a phloem-limited virus transmitted by aphids in semi-persistent manner; therefore control of infection of the challenge (severe) virus is restricted to phloem cells, which are infected (or not) with the protector virus.

PRSV. This virus, identified about 30 years ago, is the most destructive papaya disease worldwide, and has become a limiting factor in production (Gonsalves, 1998). Damage can reach 100%, and no naturally resistant cultivars are available. An attenuated PRSV mutant has been produced by nitrous acid mutagenesis of the HA strain, since there was no natural mild strain (Yeh and Gonsalves, 1984). Cross-protection against PRSV has been achieved successfully and is widely used in Taiwan, Thailand, Mexico, Florida and Hawaii, with several different mutated mild viruses. It was demonstrated that the mild mutant from Hawaii was unable to protect papaya in Taiwan and *vice versa*, indicating insufficient sequence homology between the protector and the challenge wild-type strains of PRSV in those areas (Yeh et al. 1988; Tennant et al. 1994). Sequence homology between the PRSV isolates from Hawaii and the Far East were 84-90%, which may explain the low protection level, based on RNA-mediated resistance rather then CP-mediated resistance. Similar strain-specific resistance was observed with transgenic papaya (harboring the PRSV-HA CP gene). This resistance was shown to be based on an RNA-silencing mechanism and therefore is restricted to the local isolates (Tennant et al. 2001).

Protection of annual crops. Cross-protection in annual cultivars has been demonstrated with many viruses (Table 1). However, commercial applications are currently of lesser significance and are restricted to a few examples including CMV, ToMV and ZYMV.

CMV. Cross-protection was applied in China against wild-type CMV strains, with or without a necrogenic satellite (Tien and Wu, 1991) but is no longer deployed. It has also been successfully tested in Europe and the USA (Jacquemond and Tepfer, 1998).

ToMV. This virus is very common in field and glasshouse tomato crops, in which it causes severe symptoms on the fruit and dramatically reduces yields. Succesful protection was reported under commercial conditions from 1972-1983, using the MII-16 mild strain obtained by nitrous acid mutagenesis of ToMV (Rast, 1972).

ZYMV. This is one of the most important pathogens in cucurbits worldwide. The virus can cause a devastating disease and can cause total loss (Desbiez and Lecoq, 1997). Currently the WK mild strain (a naturally

occurring mutant) is being used commercially in Israel, mainly in watermelon and squash. Inoculation is performed with a mechanized spray in the nursery, with leaves of greenhouse-grown source plants used as inoculum (Yarden et al. 2000).

Limitations in cross-protection use

There are a number of reasons for the currently limited use and application of classical cross-protection. These include practical and safety concerns (Fulton, 1986; Lecoq and Raccah, 2001). These include the following:

a. Loss of yields as a result of the mild strain infection in certain physiological instances.
b. Incomplete protection and breakdown of protection.
c. A strain that is mild in one crop might be severe in another.
d. Difficulty in restricting the protector virus to the treated field, because of natural vectors.
e. Unavailability of mild strains of practical value.
f. Synergism and ruinous interactions with other viruses.
g. Genetic instability of the protector virus because of mutation or recombination.
h. Farmers' reluctance to use live viruses.
i. Availability of alternative technologies such as transgenic plants and introgression of natural resistance traits.
j. Difficulties and cost of practical inoculum preparation and crop inoculation.

Summary

Viral cross-protection as a practical method is strictly limited to cases where no other solution is available, such as during epidemics when no natural or transgenic resistance is available. Also, transgenic plants do not always provide a solution to viral infections. A major advantage of cross-protection with mild strains is its versatility in terms of plant genotype and cultivar. Currently, in Israel ZYMV-WK is being used to protect a variety of cultivars of watermelon and squash. In the future, "smart viruses" which will be mild, vector non-transmissible and cloned, and which will protect against several viruses may be a feasible transitional solution until transgenic resistant plants are produced. These viruses could have added traits such as herbicide resistance (Fig. 2) or other traits to provide additional benefits to consumers and growers.

Table 1. Cross-protection experiments effective in the field or laboratory

Protecting virus	Challenging virus	Host plant	Test site	Reference
Alfamovirus				
Alfalfa mosaic virus mild strain	AlMV Severe strain	Bean	lab	(Hull and Plaskitt, 1970)
Badnavirus				
Cocoa swollen shoot virus	CSSV wild type	Cocoa	field	(Hughes and Ollenu, 1994)
Caulimovirus				
Cauliflower mosaic virus UN130 strain	CaMV Cabb S strain	Turnip, Brussels sprout	lab	(Tomlinson and Shepherd, 1978; Zhang and Melcher, 1989)
Closterovirus				
Citrus tristeza virus mild strains	CTV severe strain	Citrus	field	(Costa and Muller, 1980)
Cucumovirus				
Cucumber mosaic virus (S) mild strain	CMV(P) severe strain	Tomato, Tobacco Squash	lab	(Dodds, 1982; Dodds et al. 1985)
CMV with satellite		Pepper, Melon	lab, field	(Yoshida et al. 1985; Montasser et al. 1998)
Tomato aspermy virus	virulent TAV strains	Tomato	lab	(Kuti and Moline, 1986)
Furovirus				
Beet soilborne mosaic virus	*Beet necrotic yellow vein virus*	Sugarbeet	lab	(Mahmood and Rush, 1999)
Geminivirus				
African cassava mosaic virus-Uganda	Virulent ACMV strains	Cassava	field	(Owor et al. 2004)
Ilarvirus				
Apple mosaic virus	Virulent ApMV strains	Apple	field	(Chamberlain et al. 1964)
Luteovirus				
Barley yellow dwarf virus- MAV	BYDV PAV	Cereal, Oat	lab	(Jedlinski and Brown, 1965; Wen et al. 1991)
Potato leaf roll virus mild strain	Severe strain of PLRV	Potato	lab	(Webb et al. 1952; Harrison, 1958)
Nepovirus				
Arabis mosaic virus	*Grapevine fanleaf virus*	*C. quinoa*	lab	(Huss et al. 1989)
Tomato ringspot virus	virulent ToRSV strains	Peach	lab	(Bitterlin and Gonsalves, 1988)

Protecting virus	Challenging virus	Host plant	Test site	Reference
Potexvirus				
Potato virus X mild strain	PVX severe strain	Tobacco	lab	(Salaman, 1933; Murphy, 1938)
Potyvirus				
Papaya ringspot virus – mutated mild strain	Virulent PRSV-P wild type strain	Papaya	field	(Yeh and Gonsalves, 1984; Gonsalves, 1998)
PRSV-W	Virulent PRSV-W wild type strains	Squash, Watermelon	field	(Dias and Rezende, 2000)
Pepper severe mosaic virus (M-1)	Virulent PeSMV strains	Pepper	lab	(Tanzi et al. 1988)
Plum pox virus	Virulent PPV strains	Plum	lab	(Rankovic and Paunovic, 1989)
Potato virus Y non-necrotic	PVY necrotic	Tobacco	lab	(Latorre and Flores, 1985)
Potato virus A -tobacco strains	PVA -potato strains	Tobacco	lab	(Valkonen et al. 2002)
Soybean mosaic virus – Aa15-M2	Virulent SMV strains	soybean	lab field	(Kosaka and Fukunishi, 1993)
Vanilla necrosis virus Mild strain	Virulent VNV strains	*N. benthamiana*	lab	(Liefting et al. 1992)
Water melon mosaic virus MV-2 (W1-9)	Virulent WMV strains	Cucurbits	lab	(Kameya Iwaki et al. 1992)
Zucchini yellow mosaic virus-WK	Virulent ZYMV wild type strains	Squash, Melon, Watermelon,	field lab	(Lecoq et al. 1991; Yarden et al. 2000)
Clone of ZYMV- AG	Virulent ZYMV wild type strains	Squash, Melon, Watermelon, Cucumber	field lab	(Gal-On and Raccah, 2000; Shiboleth et al. 2001)
Rymovirus				
Wheat streak mosaic virus US strain	Virulent WSMV strains	Wheat	lab	(Hall et al. 2001)
Tobamovirus				
Tobacco mosaic virus – Light green mosaic	TMV-Yellow mosaic strain	Tobacco	lab	(McKinney, 1929; Broadbent, 1976)
TMV (MII-16)	TMV type O	Tomato	lab field	(Cassells and Herrick, 1977)
TMV mild strain	TMV	Pepper	lab	(Goto et al. 1984)
Satellite STMV (T5)	Satellite STMV (T5)	Tobacco	lab	(Kurath and Dodds, 1994)
Crucifer TMV-Cg (engineered)	Virulent CTMV-CgYD strain	*Arabidopsis*	lab	(Kurihara and Watanabe, 2003)
Tospovirus				
Tomato spotted wilt virus mild strain	TSWV-BL severe strain	Datura	lab	(Wang and Gonsalves, 1992)

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Chapter A13

Arrest in Viral Transport as the Basis for Plant Resistance to Infection

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Overview of virus movement in host plants

After initial inoculation, most viruses spread in host plants via two mechanisms: local, cell-to-cell movement and systemic movement. Cell-to-cell movement occurs through intercellular connections, plasmodesmata (PD), between epidermal (EP) cells and mesophyll (MS) cells, or MS cells and MS cells. Systemic movement is more complex, comprising three distinct stages: viral entry into vascular system from MS cells in the inoculated leaf, long distance transport through the vasculature, and viral egress from the vascular tissues into MS cells within uninoculated, systemic organs. Generally, local movement is a relatively slow process (e.g., 5-15 μm/hr, see Gibbs, 1976), which, in some hosts, may be further restricted by limitations in the viral replication rate. On the other hand, long distance movement through the vascular system is rather rapid (e.g., 50-80 mm/hr, see Gibbs, 1976), occurring with the flow of photoassimilates and, in many if not all cases, not requiring viral replication (Wintermantel et al. 1997; Susi et al. 1999). Studies to date show that these two processes are mediated by different sets of viral proteins, implying that cellular machineries, especially those for the PD transport that viruses utilize in their two modes of movement are quite different from each other.

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Local movement

Viral local movement consists of two sequential steps, intracellular targeting of the virus (or viral transport intermediate) to PD within the host cell wall, and its intercellular transport by translocation through PD to neighboring cells. The first step requires replication of the viral genome, which in different viruses may occur either in the host cell cytoplasm or in its nucleus. Different viruses may utilize different cellular machineries to deliver their genomes to PD. For example, viral RNA genomes that usually replicate in the cytoplasm are likely transported directly to PD, whereas genomes of some DNA viruses that replicate in the nucleus must first be exported into the cytoplasm. In virtually all plant viruses, however, the transport to and through PD requires one or more specialized viral proteins called movement proteins (MPs). Viral MPs, in turn, interact with cellular factors and structures, such as endoplasmic reticulum (ER) and actin and microtubule network, suggesting their involvement in the viral intracellular translocation pathway(s) (reviewed in Lazarowitz and Beachy, 1999; Tzfira et al. 2000; Heinlein, 2002a; Waigmann et al. 2004).

Once a virus reaches the PD it has to enter and cross this channel connecting neighboring cells. The size exclusion limit (SEL) of PD varies depending on the type of tissue and its developmental stage but in some cases, it can be as small as ~1 kDa (Barclay et al. 1982; Tucker, 1982; Goodwin, 1983; Terry and Robards, 1987; Wolf et al. 1989), which is obviously too small to allow transport of viruses or even free viral genomes (Gibbs, 1976). Thus, viruses have evolved the ability to increase the SEL of PD to allow their movement through the channel (reviewed in Maule, 1991; Citovsky and Zambryski, 1993; Zambryski, 1995; Citovsky, 1999; Lazarowitz and Beachy, 1999; Tzfira et al. 2000; Zambryski and Crawford, 2000; Ueki and Citovsky, 2001b; Heinlein, 2002b; Lucas and Lee, 2004; Waigmann et al. 2004). Viral strategies for the cell-to-cell movement can be classified into two main groups, "PD gating" and "tubule formation". In the gating strategy, which is non-destructive, MP associates with the viral genome into a movement (M) complex and reversibly increases PD permeability to allow the passage of the M-complex into the adjacent cell, after which the PD SEL reverts to its default value. This general movement mechanism is probably utilized by the majority of plant viruses, some of which (e.g., tobamoviruses) encode a single MP, usually with a size of 30 kDa, while others (e.g., potyviruses, hordeivirus, geminiviruses, and potexviruses) produce several proteins with a movement function. In the tubule formation strategy, employed by several specific viruses, such as *Cauliflower mosaic virus* (CaMV) (Kasteel et al. 1996), *Cowpea mosaic virus* (CPMV) (Kasteel et al. 1993; Kasteel et al. 1996; Kasteel et al. 1997),

Grapevine fanleaf virus (GFLV) (Ritzenthaler et al. 1995), *Tomato spotted wilt virus* (TSWV) (Storms et al. 1995), and *Alfalfa mosaic virus* (AlMV) (Kasteel et al. 1997; Huang and Zhang, 1999; Huang et al. 2000), viral MPs promote formation of large tubules that span PD, irreversibly eliminating their inner structure and allowing transport of the entire viral particles or partially encapsidated viral genomes.

Whichever strategy is used, the invading virions continue to spread locally, from cell to cell until they reach the host vascular system, which they then utilize for systemic movement.

Systemic movement

Having reached the host vasculature, the virus insinuates into this conduit and spreads to most (but not all) parts of the plant. The vast majority of viruses move through the phloem component of the vascular system, although some, such as *Rice yellow mottle virus* (RYMV) (Opalka et al. 1998) and *Cucumber green mottle mosaic virus* (CGMMV) (Moreno et al. 2004), have been reported to move through xylem parenchyma and xylem, respectively. To enter vasculature from MS cells, virus must traverse PD between several different types of tissues. The spreading virus will encounter PD in cell walls at the boundaries between: (1) ME and bundle sheath (BS); (2) BS and vascular parenchyma (VP), and (3) VP and the phloem companion cell (CC)/sieve element (SE) complex or VP and xylem. Studies using various viruses and their mutants demonstrated that viral systemic movement could be blocked at most of these intercellular boundaries, suggesting that PD at each boundary possess different and specific structural and biochemical features. Once within phloem, the virus moves rather rapidly to reach the uninfected tissues. In this process, most viruses, except for umbraviruses that do not posses capsid protein (CP), are thought to move in either an encapsidated form or otherwise associated with CP (Waigmann et al. 2004, and references therein). Tracking systemically moving viruses, such as CaMV (Leisner et al. 1993) or GFP-expressing recombinant tobamoviruses and PVX (Santa Cruz et al. 1998; Cheng et al. 2000), confirmed that these viruses, and presumably other viruses that move through the phloem, follow the route that the host plant uses for trafficking photoassimilates from its source leaves to the sink tissues. Thus, sink tissues represent the major and preferential targets for viral systemic movement. Having entered SE, viruses move in two opposite directions: upward to the sink leaves and downward to the roots. Interestingly, the upward movement occurs significantly faster than the downward spread. That viruses utilize structurally different types of phloem, i.e., internal and external, for their upward and downward movement, respectively, may underlie this difference

in the rate of movement (for details, see Andrianifahanana et al. 1997; Guerini and Murphy, 1999; Cheng et al. 2000).

In the sink leaves, virus exits the vascular tissue into ME cells. The mechanism by which virions exit the vascular conduit is likely different from that by which they enter this transport system. For example, some abiotic factors (e.g. cadmium ions: Citovsky et al. 1998; Ghoshroy et al. 1998) and cellular proteins (e.g. cadmium-induced glycine rich protein (cdiGRP): Ueki and Citovsky, 2002) inhibit only viral egress from, but not entry into, the host phloem. In addition, GFP-expressing viruses load into the vascular tissue via both minor and major veins in the inoculated leaves, but unload only from major veins in uninoculated leaves, but not from minor veins (Santa Cruz et al. 1998; Cheng et al. 2000). Furthermore, transport of numerous cellular proteins in the plant vasculature often is also polar; specifically, proteins synthesized within CC move into SE but fail to be transported into the surrounding VP, BS, or ME cells (reviewed in Lucas and Gilbertson, 1994). Collectively, these observations suggest that macromolecular transport into the vasculature may be more promiscuous, i.e occurring by diffusion or by a loosely regulated process, whereas transport out of the vasculature may be selective and/or tightly regulated by host factors. Potentially, at least some of these as yet unidentified regulatory mechanisms/pathways may also contribute to the arrest of virus movement at specific intercellular boundaries in some hosts, resulting in resistance to viral systemic infection.

Host resistance to viral infection based on restriction of virus movement

Ideally, resistance to viral infection can be attributed to restriction of virus movement *per se* if: (1) virus replication and accumulation still occurs in the initially inoculated cell, (2) infection does not spread or spreads very slowly to uninoculated areas, local or systemic, and (3) the lack of viral spread does not involve mechanisms, such as systemic acquired resistance (SAR), the hypersensitive response (HR), or post transcriptional gene silencing (PTGS), that do not target viral transport directly. The third criterion is particularly important because the involvement of SAR, HR or PTGS can produce a lack of infection symptoms that may be interpreted as arrest in viral movement. For example, in *Nicotiana tabacum* cv. Xanthi nn, Tobacco mosaic virus (TMV) Holmes' masked strain (TMV-M) accumulates only at low levels in vascular tissues of the inoculated and uninoculated, systemic leaves whereas TMV-U1 strain accumulates to high levels in both types of leaves of the same host (Nelson et al. 1993; Ding et al. 1995). Initially, these attenuated symptoms were attributed to a combination of low replication efficiency and suppression of virus systemic movement, and one of the TMV

components, the 126 kDa protein, was implicated in these effects on viral spread (Nelson et al. 1993; Ding et al. 1995). Recently, however, the 126-kDa protein has been shown to suppress RNA silencing in *N. tabacum* and *N. benthamiana* plants, indicating that the lack of TMV-M movement is most likely due to the weaker ability of this virus strain to suppress RNA silencing (Ding et al. 2004). Here, we focus on the examples of host resistance to viral movement that fulfill the first two criteria, leaving the effects of SAR, the HR or PTGS beyond the scope of this discussion.

The host-virus combinations that develop only limited viral movement are summarized in Table 1. Importantly, these combinations are usually very specific, and a host that restricts movement of a certain virus strain remains susceptible to other strains of the same viral species. Often, this restriction is determined by a viral factor that has only few amino acid changes compared to the unrestricted virus strains, suggesting that this viral factor plays a crucial role in the movement process.

Restriction of systemic movement of tobamoviruses

Systemic movement of several strains of TMV is hindered in specific plant hosts. For example, when inoculated on spinach plants (*Spinacia oleracea*) TMV accumulates only in the inoculated, but not in the non-inoculated leaves (Spitsin et al. 1999). This restriction of systemic movement is overcome when TMV CP is substituted with CP of AlMV, which infects the host systemically. Thus, it is TMV CP that is incompatible with the systemic transport machinery of spinach.

Table 1. Host resistance to viral infection based on restriction of virus movement

Virus/host combination	Host genetic trait	Description	Viral movement determinant	Reference
TMV / Spinach	N.D.[1]	The virus is restricted to the ılated tissues.	CP	(Spitsin, et al. 1999)
PVY, TVMV, TEV / Tobacco lines carrying the *va* mutation	Recessive *va* mutation induced by X-ray irradiation	Virus is restricted to a few epidermal cells after inoculation and does not spread.	VPg	(Miller, 1987, Gibbs, et al. 1989, Nicolas, et al. 1997)
PSbMV-NY / *Chenopodium quinoa*	N.D.	The virus is restricted to the inoculated tissues.	CP	(Andersen and Johansen, 1998)
PSbMV P1/P4 pathotypes / Pea	Recessive. *sbm-1* and *sbm-4* *(eIF4E)*	*Pisum sativum* lines PI193835 and JI1405 are resistant to PSbMV pathotypes P1 and P4 most likely due to suppression of viral cell-to-cell movement.	VPg	(Keller, et al. 1998, Borgstrom and Johansen, 2001, Gao, et al. 2004b)

Virus/host combination	Host genetic trait	Description	Viral movement determinant	Reference
TEV-HAT / *N. tabacum* cv. V20	Two non-linked, recessive loci	The virus is restricted to the inoculated tissues.	VPg	(Schaad and Carrington, 1996, Schaad, et al. 1997)
PVA-M / *Nicandra physaloide*	N.D.	The virus is restricted to the inoculated tissues.	6K2 and VPg	(Rajamaki and Valkonen, 1999)
PVA-Ali and B11 / Potato	Recessive *ra* gene	The virus is restricted to the inoculated tissues.	VPg	(Hamalainen, et al. 2000, Rajamaki and Valkonen, 2002)
PPV / *N. tabacum* Xanthi-nc	N.D.	The virus is restricted to the inoculated tissues.	HC-Pro	(Saenz, et al. 2002)
TEV / *Arabidopsis thaliana* ecotypes Col-0, Col-3, Ws-2, and others.	Dominant *RTM1* locus, jacaline-like protein	The virus is restricted to the inoculated tissues.	N.D.	(Mahajan, et al. 1998, Chisholm, et al. 2000)
PepMoV-FL / *C. annuum* cv. Avelar	Single, recessive locus	Upward movement of the virus through the phloem is suppressed.	N.D.	(Guerini and Murphy, 1999)
CaMV CM4-184 / *A. thaliana* ecotype Ws-0	N.D.	Developmental stage-dependent restriction in systemic movement.	N.D.	(Leisner and Turgeon, 1993, Leisner, et al. 1993)
CaMV CM4-184 / *A. thaliana* ecotype En-2	Single, dominant locus	Restricted to inoculated tissues, regardless of developmental stage.	N.D.	(Leisner and Turgeon, 1993, Leisner, et al. 1993)
PLRV / Potato	N.D.	Phloem limited, but co-inoculation with a helper virus, such as PVY, increases PLRV levels in MS cells 10-fold[2]	N.D.	(Barker, 1987)
PLRV / Potato lines, such as Petland Crown, G7032(5), etc.	N.D.	Viral accumulation is suppressed in external phloem.	N.D.	(Barker and Harrison, 1986, Derrick and Barker, 1992, 1997)
BCTV-Logan / *A. thaliana* ecotypes Ms-0 and Pr-0	Single recessive locus	Asymptomatic infection.	N.D.	(Lee, et al. 1994)
TAV / Cucumber	N.D.	Virus loading into vasculature is arrested at the BS/phloem boundary.	N.D.	(Taliansky and Garcia-Arenal, 1995)
CCMV / Soybean line PI346304	N.D.	Virus loading into vasculature is arrested at the BS/phloem boundary.	N.D.	(Goodrick, et al. 1991b)

[1] Not determined

[2] Helper virus may suppress host defense reaction against PRLV.

Studies of arrested movement of tobamoviruses helped to define intercellular boundaries, which the virus must cross to establish systemic infection. For example, when a CP-deficient TMV mutant strain is inoculated on *N. tabacum* cv. Xanthi-nn, the mutant virus accumulates in VP, but does not enter CC, demonstrating that the VP/CC boundary can impede virus transport (Ding et al. 1996). The existence of intercellular boundaries for tobamoviral systemic transport is also indicated by the observations that, when wild-type tobamoviruses, such as TMV or *Turnip vein clearing virus* (TVCV), are inoculated onto tobacco plants treated with sub-toxic concentration of cadmium ions or onto transgenic tobacco plants over-expressing cdiGRP, the virus is unable to unload from the vasculature in systemic leaves, but the viral cell-to-cell movement remains intact (Citovsky et al. 1998; Ghoshroy et al. 1998; Ueki and Citovsky, 2002). That blocking systemic transport of tobamoviruses at these cellular interfaces may also to underlie a naturally occurring host resistance, remains to be demonstrated.

Interestingly, host plant mutants or cultivars/ecotypes that specifically restrict tobamoviral local movement have not been reported to date. This could be because, to move between cells, tobamoviruses utilize the cellular machinery that is vital for the host plant life cycle, and impairing this cell-to-cell transport ability would be lethal for the host. Indeed, in all known cases, extensive genetic screens failed to identify mutants of *Arabidopsis thaliana*, a choice plant for genetic research (Meyerowitz, 1987; Pang and Meyerowitz, 1987; Simon, 1994), that are compromised in local movement of tobamoviruses (Ishikawa et al. 1991; Ishikawa et al. 1993; Lartey et al. 1998; Sheng et al. 1998; Yamanaka et al. 2002).

Restriction of local and systemic movement of potyviruses

(a) Resistance of commercial tobacco plants to potyviruses mediated by the host *va* gene

Strictly speaking, the resistance conferred by the *va* gene is not an example of “natural resistance”, because the *va* gene is originally derived from a mutant tobacco line “Virgin A Mutante” generated by X-ray irradiation (Koelle, 1961). It is a single recessive gene which has been transferred into many commercial tobacco lines to produce resistance against

three potyviruses, *Potato virus Y* (PVY), *Tobacco vein mottling virus* (TVMV) and *Tobacco etch virus* (TEV) (Miller, 1987). When a tobacco plant homozygous for the *va* gene, such as cultivar TN86, is infected by one of these potyviruses, the virus is restricted to epidermal cells of the inoculated leaves (Miller, 1987). The detailed molecular mechanism by which the *va* gene confers potyviral resistance is unknown. That a TVMV strain, TVMV-WT, is restricted to only few isolated cells or a group of cells when mechanically inoculated onto TN86 leaves, but retains its replication capacity in TN86 protoplasts suggests that initial replication is not the step impaired in the host plant (Gibbs et al. 1989). Also, co-inoculation of the resistance-breaking strain TVMV-S with the attenuated strain TVMV-WT does not restore TVMV-WT infection in the TN86 plant, demonstrating that the ability of TVMV-S to move in the TN86 plants is not due to suppression of the host defense reaction. Moreover, systemic accumulation of TVMV-S is not impaired when co-inoculated with TVMV-WT, suggesting that TVMV-WT does not elicit anti-TVMV defense reaction, such as SAR. Collectively, these observations suggest that tobacco plants homozygous for the *va* mutation do not support cell-to-cell movement of potyviruses (Nicolas et al. 1997).

While the mechanism by which the *va* mutation affects viral movement is still obscure, the studies using chimeric virions consisting of TVMV-WT and TVMV-S sequences revealed that a domain within the viral genome-linked protein VPg of TVMV-S is responsible for overcoming the *va*-gene resistance (Nicolas et al. 1997). In addition, an extensive genetic study demonstrated that the resistance conferred by *va* is due to deletion of a large fragment of a genomic sequence at the *Va* locus which governs susceptibility to potyvirus infection (Noguchi et al. 1999).

(b) Resistance of *Chenopodium quinoa* to *Pea seed-borne mosaic virus* (PSbMV)

When two isolates of PSbMV, DPD1 and NY, are inoculated on *Chenopodium quinoa,* PSbMV-NY does not move long distance and accumulates only in the inoculated leaves whereas PSbMV-DPD1 spreads systemically (Andersen and Johansen, 1998). Importantly, PSbMV-NY is not detected in the stem internodes above or below the inoculated leaf even at 6 weeks post inoculation, suggesting impairment of viral transport through the host vascular system (Andersen and Johansen, 1998). Also, co-inoculation with PSbMV-NY does not block the systemic spread of PSbMV-DPD1, showing that the inability of PSbMV-NY to establish systemic infection in *Chenopodium quinoa* is not due to SAR induction (Andersen and Johansen, 1998). Thus, *Chenopodium quinoa* most likely directly restricts systemic transport of PSbMV-NY (Andersen and Johansen, 1998).

Mutational analysis of the PSbMV genome reveals that, among 12 changes in the amino acid composition found between N-terminal 130 amino acids of CPs of the DPD1 and NY isolates, the serine-to-proline change at position 47 (Ser47Pro) is responsible for the differences in systemic movement between these PSbMV isolates. Specifically, replacing the Ser-47 residue of the PSbMV-NY CP with proline is sufficient to restore systemic spread in *Chenopodium quinoa* while replacing Pro-47 with serine in the CP of PSbMV-DPD1 restricts the systemic spread of this virus (Andersen and Johansen, 1998). This role of CP Ser-47 residue in restricting PSbMV systemic movement is likely specific for the NY and DPD1 isolates because other isolates of this virus, such as s6 and nep-1, that contain Ser-47 in their CPs still exhibit systemic movement in the same host (Andersen and Johansen, 1998).

(c) Resistance of pea to PSbMV

Pea (*Pisum sativum*) lines PI193835 and JI1405 are resistant to PSbMV pathotypes P1 and P4, such as PSbMV-DPD1 and PSbMV-NY isolates, respectively, whereas PI269818 is resistant to PSbMV P1, but susceptible to P4 (Keller, et al. 1998; Borgstrom and Johansen, 2001; Gao et al. 2004a; Gao et al. 2004b). The recessive genes that confer the resistance to pathotypes P1 and P4 of PSbMV are designated *sbm1* and *sbm4*, respectively. In the case of *sbm1*, the viral VPg protein was identified as the virulence determinant of PSbMV which may reduce viral amplification due to a weaker interaction with a putative PI269818 host factor encoded by *sbm1* (Keller et al. 1998; Borgstrom and Johansen, 2001). The eukaryotic translation initiation factor eIF4E from various plant species has been shown to interact with the potyviral VPg protein or its proteolytic precursor VPg-Pro (NIa) in yeast two-hybrid system or *in vitro* pull-down assays, suggesting that eIF4E may represent a host factor involved in potyviral infection (Wittmann et al. 1997; Leonard et al. 2000; Schaad et al. 2000; Leonard et al. 2004). Also, a genetic study showed that an eIF4E allele is tightly linked with *sbm-1* (Gao et al. 2004a). Moreover, when GUS- or GFP-expressing PSbMV P1 and P4 infectious clones are co-bombarded with an expression vector carrying eIF4E from a susceptible plant line into the resistant line JI1405, the virus accumulates and spreads in the plant of the resistant genetic background (Gao et al. 2004b). Thus, eIF4E from a susceptible line is sufficient to rescue not only the initial amplification of the virus but also its subsequent local movement (Gao et al. 2004b). Sequence comparison of the eIF4E proteins from pea lines resistant (JI1405 and PI193835) and susceptible (JI2009 and 744) to PSbMV pathotypes P1 and P4 shows 5 amino acids differences: Leu62Trp, Asp73Ala, Asp74Ala, Arg107Gly, and Lys169Asn. Interestingly, line PI269818, which is resistant to PSbMV pathotype P1 but susceptible to pathotype P4, shows only 3

amino acid substitutions, Pro73Ala, Asp74Ala, and deletion77Ser, demonstrating that subtle changes in amino acid composition of eIF4E are sufficient to change the resistance spectrum of the host (Gao et al. 2004b). Thus, mutations in the *eIF4E* gene underly the *sbm-1* and *sbm-4* phenotypes, and the eIF4E protein is involved both in potyviral replication process as well as viral cell-to-cell movement (Gao et al. 2004b).

Additional reports substantiate plant resistance to potyviruses that involves viral VPg and cellular eIF4E sequences (Ruffel et al. 2002; Nicaise et al. 2003). While the detailed mechanisms of this resistance are yet to be elucidated, they may involve a restriction in viral local movement due to incompatibility between the viral VPg and the plant eIF4E proteins.

(d) Resistance of tobacco to TEV

Two TEV strains, TEV-HAT and TEV-Oxnard, replicate and move from cell-to-cell in *N. tabacum* cv. V20, but TEV-HAT does not spread systemically in this host (Schaad and Carrington, 1996; Schaad, et al. 1997). The TEV infection process was traced using recombinant TEV-HAT and TEV-Oxnard strains expressing the β-glucuronidase (GUS) reporter gene (Schaad and Carrington, 1996). When protoplasts from the resistant host V20 and susceptible host Havana 425 are infected with GUS-expressing TEV-HAT, they exhibit comparable levels of GUS activity, indicating that the resistance is not based on the suppression of the initial infection and replication of the virus (Schaad and Carrington, 1996). Also, determination of the size of infection foci in both hosts detected no significant differences in the rate of cell-to-cell movement rate between both hosts (Schaad and Carrington, 1996). On the other hand, viral accumulation in systemic tissues of these hosts shows striking differences. In the V20 cultivar, the systemic tissue remains almost completely free of GUS activity associated with the recombinant virus even two weeks post inoculation whereas the systemic tissue of Havana 425 shows increasing GUS activity starting from day five after inoculation (Schaad and Carrington, 1996). Histochemical staining revealed that in the resistant strain V20 the virus remains confined to the initial infection foci. At 14 days post inoculation the infection foci coalesce, and an apparent tracking of infection along primary veins is observed. When examined at microscopic level, strong GUS activity is observed in MS, BS, and phloem cells of the inoculated leaf, suggesting that the GUS-expressing TEV-HAT is able to load into phloem cells from the surrounding MS cells (Schaad and Carrington, 1996). Thus, suppression of systemic movement of TEV-HAT occurs after entering the vasculature or at the boundary between vasculature and MS in uninoculated leaves. The Havana 425 and V20 lines were crossed and the progeny plants analyzed for susceptibility to TEV-

HAT. The results suggested that the resistant V20 phenotype is due to two non-linked, recessive loci (Schaad and Carrington, 1996).

Comparison of systemic movement of chimeric viruses assembled from TEV-HAT and TEV-Oxnard revealed that the TEV-HAT VPg is the limiting component for restriction of systemic movement in *N. tabacum* cv. V20 (Schaad et al. 1997). Within the VPg coding sequence, a 67-nucleotide segment containing 10 nucleotide differences, but only five amino acid differences, between TEV-HAT and TEV-Oxnard is responsible for controlling the viral systemic infection phenotype in the tobacco cultivar V20 (Schaad et al. 1997).

Besides VPg, another TEV protein, HC-Pro, may be involved in the systemic movement of this virus in tobacco. For example, when the amino acid motif ^{293}CCC^{295} is substituted with RPA within HC-Pro in a GUS-expressing TEV, the mutant virus exhibits only minor debilitation in replication, accumulates in BS and CC, but does not appear in uninoculated MS, indicating that the mutant virus is unable either to load from CC into SE or to unload from SE into uninoculated tissues (Cronin et al. 1995). Thus, HC-Pro may function in later steps of long-distance transport at a specific intercellular boundary within the plant vasculature or between the vascular and non-vascular tissues. Although the mechanism(s) by which HC-Pro affects systemic movement is still obscure, its indirect function in the transport process is suggested by the observations that HC-Pro is an efficient suppressor of the anti-viral PTGS defense reaction of the host plant (Kasschau and Carrington, 1998; Mallory et al. 2001; Kasschau et al. 2003).

(e) Resistance of *Nicandra physaloides* and potato to *Potato virus A* (PVA)

In *N. physaloides*, the M strain of PVA is restricted within the inoculated leaves whereas the B11 strain spreads systemically(Rajamaki and Valkonen, 1999). When the portion of the genomic sequence of PVA-B11 encoding the C-terminal parts of CI, VPg, and 6K2 proteins and the N-terminal part of the NIa-Pro protein is replaced with the corresponding region of PVA-M, the resulting chimeric strain, B11-M, loses its ability to move systemically. The replaced sequence contains four amino acid differences between the two isolates: one in the 6K2 protein and three in the VPg protein, suggesting that both proteins may function in the systemic movement of PVA (Rajamaki and Valkonen, 1999). Because 6K2 exists as polyprotein with VPg and NIa in the infected cells (Restrepo-Hartwig and Carrington, 1994), it may function synergistically and in *cis* with VPg during the process of viral systemic movement. The step of systemic movement that is restricted remains to be determined.

In the case of a different host, *Solanum commersonii*, PVA-Ali and B11 are restricted within the inoculated leaf whereas other strains, such as PVA-M, PVA-U, and PVA-TamMV, infect the host systemically, penetrating

upper systemic leaves and tubers (Rajamaki and Valkonen, 2002). When viral accumulation within the inoculated leaves is compared between these strains, the restricted strains PVA-Ali an PVA-B accumulate to levels 2-10 fold higher than those of the unrestricted strains, demonstrating that the restriction in systemic movement is not due to suppression of replication (Rajamaki and Valkonen, 2002). When PVA-U or B11 is inoculated on a potato leaf grafted onto a tobacco plant (*N. tabacum* cv. Samsun nn), which is susceptible to both of the PVA isolates, the tobacco root stock is infected by PVA-U, but not by PVA-B11, suggesting that PVA-B11 is unable to penetrate the potato vascular system for systemic spread into tobacco. Also, *in situ* hybridization as well as immunohistochemistry revealed that B11 is rarely observed in vascular tissues of the inoculated leaves, suggesting that the impaired loading of B11 into phloem cells from MS cells may underlie the restriction of PVA-B movement in potato (Rajamaki and Valkonen, 2002). A single amino acid substitution, His118Tyr, in the central domain of the PVA-B11 VPg protein restores the systemic movement of PVA-B11 in potato (Rajamaki and Valkonen, 2002). Additional amino acid substitutions in the central (residue 116) and C-terminal domains of VPg (residue 185) and in the N-terminus of the 6K2 protein (residue 5) alter virus accumulation and the rate of systemic infection, but are not sufficient, if introduced by themselves, to restore the systemic infection by PVA (Rajamaki and Valkonen, 2002). Thus, the central domain of PVA VPg is important for specific virus-host interactions that allow invasion of phloem cells by PVA (Rajamaki and Valkonen, 2002).

As for host factors involved in the restriction of PVA movement, a recessive gene *ra*, that may be linked with or allelic to the extreme resistance locus Ry_{adg}, has been shown to cause a complete blockage of vascular transport of PVA in *S. tuberosum* cv. andigena (Hamalainen et al. 2000).

(f) Resistance of *N. tabacum* cv. Xanthi-nc to *Plum pox virus* (PPV)

PPV systemically infects several species of the *Nicotiana* genus including *N. clevelandii* and *N. benthamiana*. PPV also replicates in the inoculated leaves of *N. tabacum* cv. Xanthi-nc, but it fails to infect this host systemically (Saenz et al. 2002). However, when PPV is inoculated on transgenic *N. tabacum* cv. Xanthi-nc plants that express the HC-Pro, P1, and P3 proteins encoded by the 5'-terminal part of the genome of TEV, which moves systemically in this host species, systemic movement of PPV also occurs. The ability of these transgenic tobacco plants to allow PPV systemic infection is abolished by mutating the HC-Pro part of the transgene (Saenz et al. 2002), indicating that it is the HC-Pro protein that determines the host specificity of PPV and TEV systemic infection, and that HC-Pro protein may represent a limiting factor for systemic infection of *N. tabacum* by PPV (Saenz et al. 2002). Because HC-Pro suppresses PTGS (Kasschau and

Carrington, 1998; Mallory et al. 2001; Kasschau et al. 2003), it may also function in the systemic movement indirectly, by suppressing silencing of the potyviral genomes by the host (Ratcliff et al. 1997; Kasschau and Carrington, 1998; Li and Ding, 2001; Baulcombe, 2002; Moissiard and Voinnet, 2004).

(g) Resistance of *A. thaliana* cultivars to TEV

When inoculated with TEV, some *A. thaliana* ecotypes, such as C24 and Landsberg *erecta* (La-*er*), support both local and long-distance spread of TEV, whereas other ecotypes, such as Columbia-0 (Col-0), Col-3, Wassilewskija-2 (Ws-2) and several others, allow only local, cell-to-cell movement (Mahajan et al. 1998; Whitham et al. 1999). The latter group appears to restrict TEV systemic movement by a mechanism different from the classic HR, because of the absence of local lesions in the inoculated leaves. The SAR pathways are also not involved in this resistance because Col-0 plants transgenic for the *NahG* gene, which do not develop SAR due to conversion of salicylic acid to catechol by the *NahG*-encoded salicyalte hydroxylase (Gaffney et al. 1993), are still unable to support TEV systemic infection. Moreover, TEV does not move systemically in Col-0 plants carrying *npr1* alleles that are unable to activate SAR and in Col-0 plants with *ndr1* and *pad4* alleles that do not develop R-gene-mediated resistance (Mahajan et al. 1998). Therefore, the lack of systemic infection in those cultivars is likely due to the restricted systemic movement *per se* rather than to SAR or HR.

Genetic crosses between the susceptible and resistant *A. thaliana* ecotypes identified and mapped a dominant *RTM1* locus in Col-3 plants which restricts TEV systemic movement (Mahajan et al. 1998). A later study revealed that *RTM1* encodes a protein similar to a lectin jacalin, suggesting its involvement in plant defense (Chisholm et al. 2000). However, jacalin-like proteins are known to function in plant defense pathways distinct from virus resistance. Thus, RTM1 may inhibit TEV systemic movement in resistant plants by a novel, albeit still not elucidated, mechanism (Chisholm et al. 2000).

(h) Resistance of pepper (*Capsicum annuum*) cv. Avelar to the *Potyvirus Pepper mottle virus* (PepMoV)

The Florida isolate of PepMoV (PepMoV-FL) causes attenuated systemic symptoms in *C. annuum* cv. Avelar (Guerini and Murphy, 1999). The virus replicates in protoplasts from this plant species, suggesting that impaired viral replication does not represents the cause of the restricted infection (Guerini and Murphy, 1999). When inoculated onto intact plants, however, the upward movement of PepMoV-FL through the phloem is suppressed. Tissue immunoblots probed with anti-PepMoV-FL CP antibody showed

that, 21 days after inoculation, the virus accumulates in one or two stem internodes above the inoculation leaf, but not in the internal phloem of the stem segments beyond this point (Guerini and Murphy, 1999). On the other hand, over time, PepMoV-FL moves downward through the external phloem and accumulates to detectable levels in the uninoculated organs located below the inoculated leaves. These observations suggests that it is the upward movement of the virus that is suppressed in *C. annuum* cv. Avelar, confining the infection to the inoculated leaf and plant parts below the inoculated leaf. The restriction on the PepMoV-FL upward movement in *C. annuum* cv. Avelar is released when the KM strain of CMV (CMV-KM) is co-inoculated with PepMoV-FL (Murphy and Kyle, 1995). Most likely, this alleviation of the restriction is not due to suppression of the host defense pathways by CMV-KM because co-infection of plant protoplasts with both viruses does not increase the accumulation levels of PepMoV-FL. This observation supports the notion that the limited systemic movement of PepMoV-FL in *C. annuum* cv. Avelar is based on impaired systemic movement through internal phloem (Guerini and Murphy, 1999).

Genetic analyses suggested that a single recessive *pvr3* locus underlies the resistance of *C. annuum* cv. Avelar to PepMoV-FL but the identity or function of the protein product of this locus remains unknown (Murphy and Kyle, 1995; Murphy et al. 1998). The resistance of *C. annuum* cv. Avelar is virus-specific, as this host remains susceptible to another potyvirus, TEV.

Differential susceptibilities of A. thaliana ecotypes to an isolate of Cauliflower mosaic virus (CaMV)

Several *A. thaliana* ecotypes are resistant to systemic infection by the CaMV isolate CM4-184 (Leisner et al. 1993). When CaMV is inoculated on plants 14 days post germination and the infection is analyzed 35 days after inoculation, the early flowering ecotype Ws-0 shows viral accumulation levels comparable to those observed in the infected ecotype Col-1 in flower stalks, cauline leaves and siliques, but low or no virus accumulation in rosette leaves. In contrast, in the late flowering ecotypes, Frankfurt-2 (Fr-2) and Finland-3 (FL-3), viral infection is more extensive, spreading also to rosette leaves. This difference in the spread of the virus is presumably due to differences in the timing of sink-to-source transition in different tissue of each ecotype. Since flower stems, flowers, siliques, and, presumably, cauline leaves are the permanent sink tissues, they are expected always to receive the virus from infected source tissue, i.e. rosette leaves. On the other hand, rosette leaves are destined to transform from sink to source at a certain developmental stage, and the timing of this transition appears to correlate with the emergence of the flower stem (bolting) (Leisner et al. 1993). Tracing ^{14}C-labeled photoassimilates generated in a rosette leaf reveals that

they translocate into the adjacent rosette leaves when the plant is at the developmental stage before bolting. Once the flower stem has emerged, however, the photoassimilates move into the upper parts of the plant, including the flower stem and cauline leaves, but not into rosette leaves (Leisner et al. 1993).

That CaMV moves throughout the host plant with the flow of photoassymilates makes this virus a sensitive and specific “marker” of the sink/source profiles of different tissues in *A. thaliana* ecotypes. For example, when the Col-1 ecotype is inoculated with CaMV at 9 and 11 days after germination, the virus spreads within the inoculated rosette leaf as well as into uninfected rosette leaves. However, when plants are inoculated with the virus at later developmental stages, i.e., 15, 17 and 27 days after germination, the virus spreads within the inoculated rosette leaf and into the flower stem, cauline leaves, flowers and siliques, but it does not enter into uninoculated rosette leaves. In the case of the Ws-0 ecotype, the correlation between CaMV resistance and developmental stage is supported by the observations that the plants show virus accumulation in rosette leaves when the plants are maintained at conditions that delay flowering (Leisner et al. 1993). Thus, CaMV resistance of rosette leaves in early flowering ecotypes and their susceptibility to the virus in late flowering ecotypes are actually determined by the source/sink status of these leaves at the time of inoculation (Leisner and Turgeon, 1993; Leisner et al. 1993).

In contrast to these ecotypes, in which the susceptibility to CaMV infection is developmentally controlled, another *A. thaliana* ecotype Enkheim-2 (En-2) sho*ws bona fide* resistance to CaMV. When rosette leaves of this ecotype are inoculated with CaMV, the virus accumulates in and around the inoculation site, but does not spread into systemic tissues regardless of their developmental stage (Leisner and Turgeon, 1993). These data suggest that En-2 supports CaMV replication, but does not allow long distance transport of the virus. Analysis of the F2 progeny of genetic crosses between En-2 (resistant ecotype) and Col-0 (susceptible ecotype) indicated that the restriction of CaMV systemic movement in En-2 may be determined by a dominant trait in a single locus (Leisner and Turgeon, 1993).

Resistance of A. thaliana ecotypes to the Geminivirus Beet curly top virus (BCTV)

Two strains of BCTV, BCTV-Logan and BCTV-CFH, exhibit different infectivity in eight *A. thaliana* ecotypes among 46 tested (Lee et al. 1994). Both virus strains infect most of *A. thaliana* ecotypes, for example Col-0, systemically, and induced severe symptoms, such as leaf curling, inflorescence curling, and stunting (Lee et al. 1994). On the other hand, in some *A. thaliana* ecotypes, such as Ms-0 and Pr-0, only BCTV-CFH induces

systemic symptoms while BCTV does not. Viral DNA accumulation levels parallel the severity of the symptoms, indicating that mild or no symptoms are likely due to low levels of viral accumulation rather than asymptomatic systemic infection. That BCTV replicates in excised inflorescence pieces from the resistant ecotypes suggests that the resistance is due to a block in viral movement rather than replication. Genetic crosses of the susceptible ecotype Col-0 to the resistant ecotypes Ms-0 and Pr-0 showed that the resistance is specified by a single recessive locus (Lee et al. 1994).

Resistance of potato to the Luterovirus Potato leafroll virus (PLRV)

In PLRV-infected plants, the virus remains largely limited to the SE/CC complex and only very few ME cells adjacent to minor veins become infected by the virus. This phloem limitation is relieved by infection with several “helper” viruses, such as PVY, increasing the proportion of PLRV-infected ME cells by 10-fold (Barker and Harrison, 1986; Barker, 1987). Thus, PLRV most likely lacks an effective cell-to-cell movement function required for viral movement into and between ME cells. Alternatively, helper viruses may suppress the host defenses against PLRV, allowing PLRV to accumulate in ME cells.

Suppression of vascular movement of PLRV has been characterized in several potato cultivars. Because potato plants possess tubers, large underground sink tissues, they exhibit—in addition to the viral spread to young apical tissues—a pronounced downward virus movement from the above-ground sink tissues to tubers. In some resistant cultivars, such as the Bismark cultivar, the impeded virus translocation is accompanied by severe phloem necrosis (Wilson and Jones, 1992, and references therein). However, in other cultivars the arrest in viral infection and movement occurs without visible necrosis, presumably by blockage of the movement process *per se* (Barker and Harrison, 1986; Derrick and Barker, 1992, 1997). When the resistant potato cultivars, such as Pentland Crown, G7032(5), G8176(1), G8107(1), and G7445(1), are grafted to an infected susceptible cultivar Maris Piper, almost no PLRV-infected cells are detected in the external phloem bundles, and PLRV infection is almost entirely restricted to the internal phloem bundles in young stems (Barker and Harrison, 1986; Derrick and Barker, 1992, 1997).

The rate of PLRV vascular infection can be tested using a “sandwich grafting” system, in which the tested stem piece is grafted between the apical scion and rootstock from a susceptible host, either infected or uninfected. In this system, the downward viral movement is assessed from PLRV levels within the uninoculated rootstock, which are due to the viral transport from the infected apical tissue through the tested stem piece. Which are due to the viral transport from the infected rootstock through the tested stem piece not

clear. These experiments demonstrated that the rate of the PLRV vascular transport, irrespective of its direction, is similar in susceptible and resistant plants (Derrick and Barker, 1997). Also, the time required for acquisition of functional phloem continuity after grafting, as analyzed by monitoring translocation of 6(5)-carboxyfluorescein, is similar to that required for virus movement, suggesting that virus moves systemically right after the phloem continuity is recovered, and that the translocation through the phloem is a rapid and passive process (Derrick and Barker, 1997). Both rootstock- and scion-inoculated plants carrying the virus-resistant graft contained fewer infected cells associated with external phloem as compared to plants with PLRV-susceptible graft, suggesting that the suppression of infection of the external phloem bundle underlies the resistance. The sandwich grafting experiments indicate that the viral short distance transport, such as movement between SE and CC or VP, is impeded in the resistant hosts while the passive transport of the virus through SE is not affected (Derrick and Barker, 1997). The molecular mechanism of this resistance, however, remains unknown.

Resistance of cucumber to the Cucumovirus Tomato aspermy virus (TAV)

Both TAV and CMV replicate and spread cell-to-cell within inoculated cotyledons or leaves of cucumber (*Cucumis sativus*). However, while CMV can infect the host systemically, TAV infects only the inoculated leaves (Taliansky and Garcia-Arenal, 1995). CMV CP is required for both local and systemic movement of the virus, and it also functions as a determinant of the host-virus compatibility (Taliansky and Garcia-Arenal, 1995). When complemented with CMV CP, TAV is able to move long distance and infect the normally restricted host systemically (Taliansky and Garcia-Arenal, 1995). On the other hand, when a chimeric CMV carrying TAV RNA3, that encodes the 3a protein and coat protein, is inoculated on a cucumber plant, the virus is unable move long distance, demonstrating that TAV CP is likely the limiting factor for TAV systemic movement in the resistant plants (Thompson and Garcia-Arenal, 1998). A detailed immunohistochemistry analysis showed that, in the inoculated leaves, the CMV/TAV RNA 3 chimeric virus accumulates in BS but not in phloem cells, indicating that the movement is arrested at the BS-phloem interface (Thompson and Garcia-Arenal, 1998). This intercellular boundary is known to represent a blockage site for systemic movement of several other viruses (Goodrick et al. 1991b; Ding et al. 1995; Wintermantel et al. 1997), suggesting that PD at the BS-phloem interface possess functional characteristics distinct from those of PD connecting ME cells.

Resistance of soybean cultivars to the Bromovirus Cowpea chlorotic mottle virus (CCMV)

CCMV infects cowpea (*Vigna unguiculata walp.unguiculata*) as well as many cultivars of soybean (*Glycine max*), whereas the soybean cultivar PI346304 is resistant to the CCMV systemic infection (Goodrick et al. 1991b). When CCMV is mechanically inoculated, a susceptible soybean cultivar Davis shows systemic symptoms, such as chlorotic mosaic, stunting, and distortion of leaves, whereas the cultivar PI346304 remains largely asymptomatic, with only <5% of the infected plants developing localized chlorosis around a few veins (Goodrick et al. 1991b). Both cultivars develop local chlorosis and accumulate CCMV virions in the inoculated leaves to similar levels, suggesting that replication and cell-to-cell movement of the virus occurs at comparable levels in both cultivars (Goodrick et al. 1991b). The resistance of PI346304 is not due to SAR, because secondary infection allows higher accumulation of CCMV in the inoculated leaves of previously infected PI346304. Genetic crosses between the resistant host PI346304 and the susceptible host Davis followed by analysis of the F2 and F3 progeny revealed that the PI346304 resistance to CCMV is determined by two recessive loci (Goodrick et al. 1991a).

Immunochemical staining shows that CCMV accumulates within epidermal cells, ME, and BS cells of the inoculated leaves of both Davis and PI346304 whereas vascular tissues in the inoculated leaves of PI346304 remain largely free from CCMV. Thus, PI346304 most likely resists CCMV infection by blocking the viral movement at the interface between BS and VP cells. Furthermore, systemic spread of another member of the *Bromoviridae*, the Fny strain of CMV, in transgenic tobacco plants expressing a mutated CMV-Fny 2a replicase protein gene sequence in inhibited at the same cellular interface (Wintermantel et al. 1997), suggesting that entry into the phloem from BS may represent the major limiting step for *Bromoviridae* systemic movement. Taken together with the block of the systemic movement of TAV at the BS-phloem interface (Thompson and Garcia-Arenal, 1998), these observations indicate that PD connecting BS to VP function as a specific transport boundary the crossing of which initiates viral systemic movement.

Concluding remarks

In this chapter, we reviewed our current knowledge about host-virus resistance based on restriction of viral movement. The discussed above examples of such resistance suggest that it represents one of the major

strategies for plant defense against viral infection. It is interesting to note that this type of host resistance is very specific since in most cases the host is resistant to few specific isolates of virus, but not to other closely related isolates. One explanation of such specificity is that the host plant cannot tolerate alterations in its intercellular transport machinery dramatic enough to impede movement of a wide spectrum of viruses. In other words, viruses may have evolved to "pirate" for their own spread those host transport pathways that are essential for the physiology of the host plant itself.

In some cases, involvement of other defense pathways, such as HR, SAR and PTGS, are possibly involved in the restriction of virus infection and movement. However, these distinctive mechanisms may not be mutually exclusive, but instead may utilize similar or overlapping cellular processes. For example, recent studies suggest that SAR signals may be produced in the inoculated leaves and then translocated using systemic transport pathways, including the transport through phloem, to uninoculated leaves (Kiefer and Slusarenko, 2003; Durrant and Dong, 2004). Moreover, local and systemic movement of PTGS signals most likely occurs *via* intercellular transport pathways, i.e., cell-to-cell through PD and long distance through phloem, utilized by plant viruses (Voinnet and Baulcombe, 1997; Voinnet et al. 1998; Palauqui and Balzergue, 1999; Fagard and Vaucheret, 2000; Ueki and Citovsky, 2001a; b; c). Thus, because at least some defense signals likely share, at least partially, transport pathways with plant viruses, activation of the defense pathways may inherently affect viral movement activity.

Also, in some cases, such as resistance of some pea cultivars to PSbMV isolates (Gao et al. 2004a), virus resistance is based on disruption of both replication and cell-to-cell movement. However, because viral movement between cells obviously requires replication, a defect in replication may be interpreted as a lack of cell-to-cell movement. Thus, in every case of restricted local movement of a virus, it is essential to determine whether or not this virus can replicate in the initially inoculated cells or protoplasts derived from the resistant host.

Although many examples of virus resistance based on limitation of movement are known, the nature and identity of the host factors involved in this process of restricting viral spread remain obscure, potentially due to the lack of genomic information for many of the host species. Thus, *A. thaliana*, for which the full genome sequence and a wealth of genetic and molecular tools and information are available (Meinke et al. 1998; The *Arabidopsis* Genome Initiative, 2000; Ausubel and Benfey, 2002; Wortman et al. 2003; Zhang et al. 2003; Østergaard and Yanofsky, 2004) and various ecotypes of which are differentially infected by diverse viruses (Simon, 1994), represents an especially attractive system for identification of the host components involved in natural mechanisms of restriction of viral movement and host/virus compatibility.

To date, the use of different combinations of host species and cultivars with various wild type viruses and genetically engineered virus strains has identified several intercellular boundaries that are critical for viral cell-to-cell and systemic movement. In different plant species, these boundaries represent "check points" or "road blocks" at which viral spread can be restricted or even arrested. Thus, the studies of viral systemic movement and its restriction in different hosts shed important and novel light on basic biological processes of macromolecular trafficking in plants. In addition, this knowledge will likely contribute to development of new strategies to produce virus-resistant crops.

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Chapter A14

Plant Metabolism Associated with Resistance and Susceptibility

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Introduction

Viruses are subcellular parasites that replicate within a host cell with no intervening membrane to insulate host and viral gene products from each other (Hull, 2002). The highly intimate nature of this relationship suggests that the biochemical and physiological processes occurring in the various host cell types through which a virus must propagate will significantly affect the outcome of the infection. In plants, drastic alterations in, and re-direction of, host metabolism have been observed in many studies of both incompatible and compatible host-virus interactions. However, is it safe to suggest that these changes in plant metabolism influence whether a plant is resistant or susceptible to the virus infection? The answer to this question is important for a number of reasons. Firstly, it will lead to a better general understanding of the plant-virus interaction. Secondly, it may reveal mechanisms underlying induced resistance phenomena. Finally, it may allow us to identify targets for novel, artificial methods of inducing resistance to plant viruses.

In this review we will examine how certain aspects of plant photosynthetic and respiratory metabolism are altered by infection by viruses while others may play role(s) in counteracting it.

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Virus-induced changes in carbon metabolism in susceptible plants

The effects of virus infection on the metabolism of a susceptible plant are frequently profound, influencing multiple pathways such as respiration, carbohydrate partitioning and photosynthesis in both directly- inoculated and systemically -infected tissue (Hull, 2002). Some of these effects are due to virus-induced changes in gene expression controlled at the transcriptional level (Wang and Maule, 1995). Others may be influenced post-transcriptionally through perturbation of levels of microRNAs (miRNAs) by certain viral gene products such as the potyviral HC-Pro (reviewed by Bartel, 2004). Other virus-induced effects on metabolism can occur through a more direct physical perturbation of subcellular structures, for example the disruption of photosynthetic activity by the interaction of viral gene products with components of the photosynthetic apparatus (see **Photosynthesis** below), or the deregulation of carbohydrate partitioning by viral movement proteins (Herbers et al. 1996a;b). Metabolic disturbances are also observed in plants undergoing an incompatible (resistance) reaction to virus infection and some of these are discussed in the section 2 of this review as well as in the accompanying chapter by Loebenstein and Akad.

Virus-infected plants do not respond uniformly because not all of the host cells become infected and those that are may not have become infected at the same time. Even with the most concentrated of virus inocula, only about 0.1% of the cells in directly inoculated leaves actually become infected (Matthews, 1991). Therefore, the results of the many studies that have examined the physiological responses of a mixed population of virus-infected and non-infected cells, which for example, occurs in systemically, infected tissue, need to be interpreted cautiously. Thus, whenever possible, virus-induced metabolic alterations are best studied in the earliest phases of infection of directly inoculated tissues since these show the greatest degree of synchrony with respect to the changes caused by the infection. However, even in directly inoculated tissue virus-induced metabolic changes in plants are not uniform (Doke and Hirai, 1970; Técsi et al. 1994a, 1994b, 1996; Wang and Maule, 1995; also discussed in Hull, 2002).

Starch. Starch is the major carbohydrate store in most plants and, compared to most other plant metabolites, it is relatively easy to detect and assay using iodine staining. Thus, starch accumulation has been, historically, one of the most commonly used indicators for virus-induced alterations in plant metabolism during both compatible and incompatible interactions. Abnormal accumulation or disappearance of starch is diagnostic for net alterations in the balance between those processes

responsible for creation and utilization of carbohydrate namely, photosynthesis and respiration.

It has been known for many decades that changes in the accumulation of starch frequently precede the appearance of virus symptoms (Bolas and Bewley, 1930; Holmes, 1931). For example, Holmes (1931) noted discrete regions of the tobacco leaf retained starch at the end of the night after inoculation with *Tobacco mosaic virus* (TMV), whilst the rest of the leaf was starch-less. At four days post-inoculation, the starch-rich region had expanded into a ring, surrounding a zone of starch-less cells termed a starch ringspot. Holmes (1931) also found that lesions contained less starch than the surrounding, uninfected tissue if staining was carried out at the end of the day. This important elementary work demonstrated that early in the infection process, even before symptoms are discernible, virus infection alters both the starch production during the day, and its degradation and/or mobilization at night. Starch ringspots have also been seen in other plant-virus systems (eg Técsi et al. 1994a; Roberts and Wood, 1982) including *Arabidopsis thaliana* leaves (Fig.1). Subsequently, chloroplasts containing enlarged starch grains were observed using TEM (e.g. Zechmann et al. 2003). For example, two zones containing chloroplasts with altered structure were discernible in tobacco leaves inoculated with *Cucumber mosaic virus* (CMV) (Cohen and Loebenstein, 1975). In cells at the center of the lesion, CMV particles were visible and all chloroplasts contained enlarged starch grains. In contrast, cells at the outer edge of the lesion contained fewer virus particles and only about half the cells contained chloroplasts with large starch grains. The development of starch ringspots in inoculated tissue has been examined in greatest detail in marrow (Técsi et al. 1994a, 1994b, 1996, see below).

Changes in starch level are even more apparent once the virus starts to spread from the initial inoculation site. In point-inoculated leaves stained at the end of the night, the path taken by the virus through veins towards the midrib was seen as a zone of starch accumulation (Holmes, 1931). Conversely, regions of starch-less cells reveal the path of virus movement in leaves stained during the day (Bolas and Bewley, 1930; Samuel, 1934).

Partitioning of carbohydrate. Many studies carried out over the last 40 years, on a variety of host-virus systems, have shown that partitioning of newly fixed carbon between soluble sugars (sucrose, fructose and glucose) and organic and amino acids is perturbed (reviewed by Porter, 1959; Goodman et al. 1986). For example, decreased soluble sugar content in infected tissue has been seen in Chinese cabbage infected with *Turnip yellow mosaic virus* (TYMV; Bedbrook and Matthews, 1973) and *Squash mosaic*

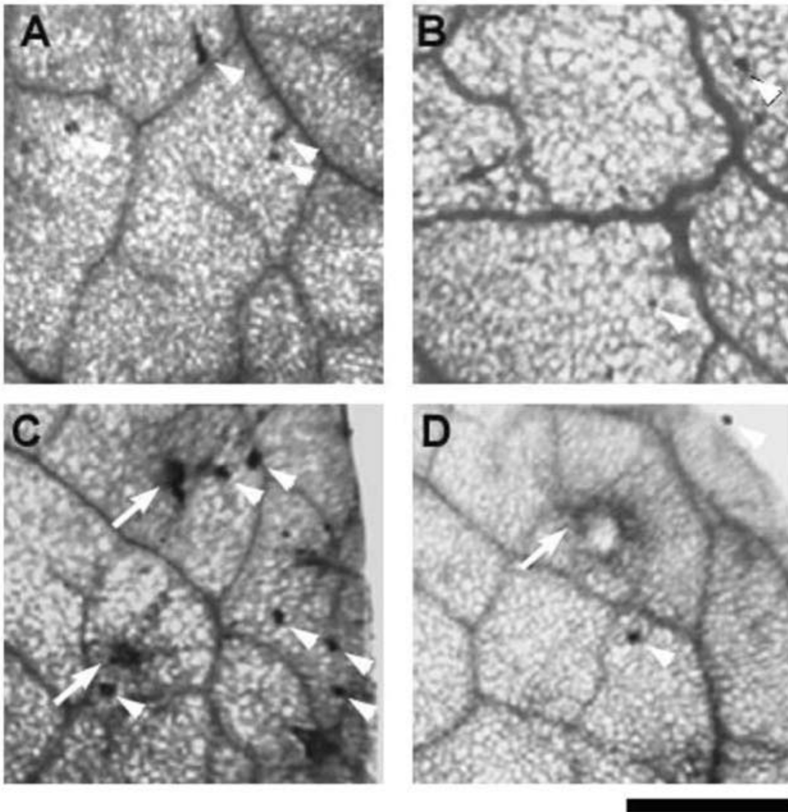

Fig. 1. Virus-induced starch lesions in TMV UI-inoculated *Arabidopsis* leaves *Arabidopsis thaliana* (Col-0) plants (17 days post-seeding) were dusted with carborundum and mock- (panels A and B) or TMV U1-inoculated (10 µg/ml: panels C and D). Two (panels A and C) and three (B an d D) days post-inoculation (dpi), the inoculated leaves were removed two hours into the light period, decolorized in boiling ethanol, stained for starch in I_2/KI solution and imaged. Starch lesions (arrows) and carborundum particles (arrowheads) are mark ed. At 1 dpi, both mock- and virus-inoculated leaves had no zones of altered starch content (data not shown). Discrete regions of elevated starch became visible 2 dpi for leaves inoculated with TMV, expanding into a ring surrounding a region of starch-less cells by 3 dpi. Size bar: 500 µm.

virus (SqMV)-infected squash (Magyarosy et al. 1973). In contrast, sucrose levels in *Zucchini yellow mosaic virus* (ZYMV)-infected marrow plants increased relative to levels in healthy controls (Blua et al. 1994). Organic acid levels rose in CMV-infected tobacco (Porter and Weinstein, 1957) while amino acid content rose in ZYMV-infected marrow (Blua et al. 1994) and *Tomato spotted wilt virus* (TSWV)-infected tomato (Selman et al. 1961). Increases in both fractions were found in TYMV-infected Chinese cabbage (Bedbrook and Matthews, 1973), SqMV-infected squash (Magyarosy et al. 1973) and CMV-infected cowpea (Welkie et al. 1967).

Due to the limitations of biochemical analytical methods available at the time most of these early studies focused on a small number of metabolic changes. One of us (Handford, 2000) carried out a 'metabolomic' study of how virus infection alters carbohydrate partitioning in the model plant *A. thaliana*. Plants (ecotype Col-0) were fed with $^{13}CO_2$ 17 days after inoculation with two strains of TMV known to cause mild or severe symptoms in tobacco (TMV strains U1 and YSI/1, respectively; Banerjee et al. 1995) and which accumulate to significant levels in Arabidopsis (Handford, 2000). The methanol-extractable components from these plants were analyzed by 2-dimensional ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy. As shown in Table 1, incorporation of ^{13}C-label into carbohydrates, amino acids and organic acids was 2-4 times higher in virus-inoculated plants compared to the mock-inoculated control, and was proportionally greater after inoculation with the U1 strain of TMV. Such changes could reflect a greater pool size of the metabolites in TMV-infected plants, similar to the findings of, for example, Blua et al. (1994) and Bedbrook and Matthews (1973). Alternatively, the rise could reflect a decrease in turnover of the metabolites, a phenomenon seen by Técsi et al. (1994b) where photosynthetically fixed ^{14}C was lost at a lower rate in CMV-infected marrow cotyledons compared to healthy controls. To conclude, in a wide variety of plant-virus interactions there is a shift in carbon partitioning away from soluble sugars towards organic and amino acids, although this is not necessarily the case in every system analyzed.

Respiration. In by far the majority of cases, the respiration rate (broadly defined as net O_2 uptake) is increased in virus-infected plants. Pennazio (1996) reviewed reports of respiration rates in virus-infected and healthy plants and documented a rise in 17 cases, a fall in one and no change in three compatible interactions. For example, the respiration rate of tomato tissue systemically infected with *Tomato yellow mosaic virus* (ToYMV) was 80-100% higher than in healthy plants (Leal and Lastra, 1984). Similarly, in barley leaves inoculated with *Barley yellow dwarf virus* (BYDV), respiration rate per gram of fresh tissue increased, whilst that of the healthy leaf decreased over the course of the experiment (Jensen, 1967).

Table 1. Distribution of ^{13}C in aerial tissues of mock- and TMV-inoculated Arabidopsis

Fraction	n	^{13}C per fraction as % of ^{13}C fixed by mock			P		
		Mock	U1	YSI/1	Mock v U1	Mock v YSI/1	U1 v YSI/1
Carbohydrates	10	100	363 ± 9	181 ± 8	0.000	0.000	0.000
Amino acids	8	100	242 ± 18	177 ± 19	0.001	0.005	0.009
Organic acids	6	100	470 ± 129	256 ± 53	0.035	0.032	0.041

Arabidopsis plants were mock-, TMV U1- or TMV YSI/1-inoculated and after 17 days were incubated in a $^{13}CO_2$-rich atmosphere for 24 h. Following incubation, shoots were excised, weighed and extracted twice in methanol. The methanol-soluble components were analysed by 2D ^{1}H- and ^{13}C-NMR, and peaks were identified by comparing to standards. The labelling intensity of compounds in each fraction from mock-inoculated plants was assigned as 100%. The labelling intensity of compounds in each fraction from TMV-inoculated samples is expressed relative to mock-inoculated plants of the same line and corrected for shoot biomass. Each value is the mean labelling intensity per fraction ± SEM (n). Values were compared by a paired two sample, two-tailed Student's t-test and the probability (P) of the two samples being from the same population is shown.

Photosynthesis. Many reports have suggested that photosynthetic rate, determined by $^{14}CO_2$ incorporation, is decreased by virus infection (reviewed by Diener, 1963; Zaitlin and Hull, 1987). For example, Leal and Lastra (1984), Jensen (1967) and Naidu et al. (1984) observed falls in photosynthetic rate and chlorophyll amount in ToYMV-infected tomato, BYDV-infected barley and peanut infected with *Peanut green mosaic virus* (genus Potyvirus), respectively. Changes in chloroplast ultrastructure are also associated with virus infection in systemically infected tissue. These chloroplasts typically have disrupted integrity of the grana and stromal lamellae as well as a reduced number of thylakoids (Hršel, 1962; Honda and Matsui, 1974; Ehara and Misawa, 1975; Izaguirre-Mayoral et al. 1990; Zechmann et al. 2003).

In plants infected with TMV, damage to chloroplast structure and function is induced by uptake of viral coat protein (CP) into chloroplasts *via* an apparently unique mechanism involving aggregated forms of the CP (Gunasinghe and Berger, 1991; Naderi and Berger, 1997; Banerjee and Zaitlin, 1992; Banerjee et al. 1995; Carr, 2004). In tobacco plants infected with TMV strains causing severe chlorotic symptoms (e.g. YSI/1, *flavum* or PV230) there is a greater proportion of CP inside the chloroplasts than in plants infected with strains causing milder symptoms, e.g. TMV-U1 and TMV-PV42 (Reinero and Beachy, 1986; Banerjee et al. 1995; Lehto et al. 2003). In addition, the ratio of CP associated with thylakoids to that associated with the stromal fraction is greater in infections by severe strains than for mild strains (Reinero and Beachy, 1989; Banerjee et al. 1995). TMV CP is not loosely attached to the thylakoid membrane surface but rather it is embedded within it (Reinero and Beachy, 1986).

TMV CP embedded in the thylakoids hinders the rate of photosynthetic electron transfer through photosystem (PS) II, but not PSI (Reinero and Beachy, 1989; Hodgson et al. 1989; Naidu et al. 1984; Seaton *et al.* 1996; Funayama et al. 1997). Moreover, the decrease in PSII activity seen in chloroplasts isolated from plants infected with a severe TMV strain, but not a mild strain, and correlates with the proportion of CP taken up into the thylakoids (Reinero and Beachy, 1989; Barón et al. 1995; Banerjee et al. 1995). But how could CP inhibit electron transfer through PSII? Recent results from Lehto et al. (2003) suggest that the CP promotes an increase in the breakdown of specific proteins within the PSII core complexes leading to increased light-induced free radical accumulation and consequent break-down of the chloroplasts' protective pigments. Eventually this results in damage to chloroplast structure and the visible symptom of chlorosis.

Early metabolic events in the inoculated tissue appear to be coordinated and highly organized

Doke and Hirai (1970) analyzed changes in host physiology in space and time around the virus entry site by autoradiography of mock- and TMV-inoculated tobacco leaves after a brief exposure to $^{14}CO_2$. At one day post-inoculation, small, heavily labeled areas of tissue were visible only in virus-inoculated samples. By two days post-inoculation the lesions had expanded, forming a ring of heavily labeled tissue surrounding a region without labeling. This heavily labeled ring enlarged further, and by about 5 days post-inoculation the labeled areas were diffuse. Doke and Hirai (1970) interpreted these discrete, heavily labeled regions as infected tissue with an elevated photosynthesis rate. The idea that virus-induced changes in metabolism are highly localized and coordinated was later explored using

CMV-inoculated marrow cotyledons by Maule, Leegood, Técsi and colleagues. In a series of important papers these workers used a variety of immunohistochemical and cytological techniques, to explore the highly dynamic nature of the viral infection site (Técsi et al. 1994a, 1994b, 1995, 1996).

Localized changes in starch levels and photosynthetic rate in CMV-inoculated marrow cotyledons. At two days post-inoculation, iodine staining of CMV-inoculated cotyledons revealed localized accumulation of starch. By four days post-inoculation, the infection zone had expanded producing a ring of starch-rich cells, surrounding a zone of starch-less cells with a small group of starch-rich cells at the centre. By 6 days post-inoculation, the lesion was diffuse, due to adjacent infection sites coalescing. Analysis of serially sectioned lesions alongside iodine staining and immunocytochemistry revealed that at four days post-inoculation, the starch ring lay just inside the expanding infection front. Incorporation of $^{14}CO_2$ demonstrated that maximal photosynthesis roughly coincided with the starch-rich cells in the central dot and peripheral ring, and that in the zone of starchless cells, $^{14}CO_2$ fixation was below levels in the surrounding uninfected cotyledon (Técsi et al. 1994a). These findings parallel those of Doke and Hirai (1970) and it was concluded that the outer starch ring was a consequence of raised photosynthetic rates induced in some fashion by CMV replication.

Chlorophyll *a* fluorescence quenching measurements for photosynthetic activity were consistent with these conclusions. These studies showed that the rate of fluorescence quenching was elevated in a ring-dot pattern like that observed for starch accumulation. However, superimposition of the starch and fluorescence quenching data revealed that the cells affected were not coincident. There was roughly a 24-h time delay with cells showing enhanced chlorophyll *a* fluorescence quenching preceding those with enhanced starch content (Técsi et al. 1994a).

Local changes in other biochemical processes in CMV-inoculated marrow cotyledons. Subsequently, Técsi et al. (1996) examined the metabolic processes responsible for the fluctuations in starch, photosynthetic $^{14}CO_2$ fixation and chlorophyll *a* quenching. Their hypothesis was that in infected cells, carbohydrate reserves might be diverted into the synthesis of CMV RNA and proteins. Using *in situ* assays, activities of specific enzymes involved in carbohydrate metabolism and CMV replication were analyzed across the viral lesion. Enzyme activities were also correlated with starch accumulation.

Experiments were carried out at three days post-inoculation, when the starch-rich ring and starchless central zone had developed but before the appearance of the central starch-rich dot. Although all cells in the lesion contained virions, replication only occurred at the leading edge of the expanding infection front, a phenomenon also seen in pea cotyledons point-inoculated with a potyvirus *Pea seed-borne mosaic vir*us (PSbMV: Wang and Maule, 1995; Aranda et al. 1996). Behind the virus replication zone was the band of cells with raised photosynthetic capacity. Protein synthesis in general, monitored by ^{35}S-methionine incorporation, and viral CP synthesis in particular, was raised in this zone. In the region of starchless cells, several metabolic changes were noted. Firstly, the activities of enzymes of the oxidative pentose phosphate pathway, and of glycolysis were raised. Secondly, no reductive pentose phosphate pathway (Calvin cycle) activity was detected in regions coinciding with the decreased rates of photosynthetic $^{14}CO_2$ fixation and lower chlorophyll *a* fluorescence. Finally, total starch hydrolase activity was raised. It was concluded that these complex changes in photosynthetic capacity and starch catabolic activities across a viral lesion could account for the development of starch rings (Técsi et al. 1996).

Localized changes in metabolism are probably controlled through changes in host gene expression. The precise cause(s) of these biochemical alterations in virus-infected tissues is unclear. However, virus infection does alter host gene expression in a complex fashion in and around the infected cells. This is best exemplified by work on the PSbMV-pea embryo interaction (Wang and Maule, 1995; Aranda et al. 1996).

In situ hybridization for minus-strand viral RNA showed that PSbMV replication in the pea seed was limited to a narrow band of cells, as in the CMV-marrow cotyledon system (Técsi et al. 1996). Meanwhile, *in situ* hybridization for transcripts of nine pea genes, revealed a severe reduction in the level of host gene expression for all genes studied only at the replication front (Wang and Maule, 1995). Previous to this study, virus-induced host gene shut-off had not been seen in plants only studied in detail in animal cell cultures (Aranda and Maule, 1998). Behind the replication front, host transcripts for many 'housekeeping' proteins were found to be elevated over the levels seen in uninfected tissue (Wang and Maule, 1995; Aranda et al. 1996). Similar effects were seen in other tissues infected with other viruses and in addition it was shown that levels of certain transcripts, notably those for heat shock protein 70-type proteins are elevated in cells supporting active virus replication (Aranda et al. 1999; Escaler et al. 2000). At the infection front, a concomitant reduction in the levels of product of eight of the genes was seen, although no effect was seen on the level of expression of the ADP glucose pyrophosphorylase protein, implying that virus infection may

perturb control of transcription and translation differentially between genes (Wang and Maule, 1995). Recent findings on the effect(s) of the potyviral HC-Pro protein and other viral counter-silencing factors on miRNA levels support the concept that some of these effects may be mediated at post-transcriptional levels (see Bartel, 2004).

However, the approaches taken by these workers could not allow any determination to be made of how essential these perturbations in host gene expression and consequent metabolic changes are in the establishment of infection.

Metabolic changes in the inoculated tissues: how important are they? In this and other systems, at least during the early stages of infection, the virus is probably not a general sink for host metabolites. For example, CMV accounts for about 1% of total protein in infected marrow tissues, insufficient by itself to explain the large-scale redirection of metabolism (Técsi et al. 1994b). However, at the very local level, that is, in the narrow band of cells in which the virus is replicating, the virus may be a very strong sink. This demand could be met by the localized changes observed in biosynthetic and respiratory pathways. It seems likely that the temporary carbon source for the enhanced biosynthetic pathways is the elevated level of starch found in the starch-ring as a consequence of enhanced photosynthesis. This starch is made from carbon fixed *in situ* rather than being manufactured from photosynthates imported from surrounding cells, because direct illumination of these cells was needed for starch levels to be raised (Técsi et al. 1994a).

However, it is questionable if localized starch build up is a prerequisite *per se* to support the demand for carbon-containing compounds. The rate of CMV accumulation was the same in marrow plants kept in a diurnal light regime or if kept covered for two days after inoculation (Técsi et al. 1994a). This was also true if black grids were placed on the inoculated leaf for two days. Virus accumulation was identical under the black bars and in the open spaces, yet starch only accumulated in those regions exposed to the light (Técsi et al. 1994a). Additionally, experiments with mutant lines of *A. thaliana* with specific mutations in starch metabolism showed that there is no simple relationship between the starch build-up in the inoculated tissue and susceptibility to virus infection (Handford, 2000). For example, the TC75 mutant is unable to accumulate starch (Caspar et al. 1985). In TC75 plants the development of starch rings in response to virus infection does not occur, yet several viruses, including CMV, the *Cauliflower mosaic virus* (CaMV), a tobamovirus, *Turnip vein clearing virus* (TVCV), and TMV U1 all accumulate to the same levels as in wild-type plants (Handford, 2000).

Thus, we are still left with an important question. Which of the complex changes in host metabolism seen in directly infected tissue are truly necessary to ensure a positive outcome for the virus, i.e. the successful establishment of viral infection within the inoculated tissue followed by its propagation throughout the host?

Salicylic acid-induced resistance to viruses: A possible role for alternative respiration in defensive signaling.

Potential relationships between salicylic acid-induced resistance, carbohydrate metabolism, and respiration. Salicylic acid (SA) is an important signal involved in the establishment of induced resistance (systemic acquired resistance: SAR) against viruses and other types of pathogen, as well as playing less well-understood roles in development and responses to abiotic stresses (Loebenstein and Akad, this volume; Martinez et al. 2004). In most, but not all, plants it is present at a low basal level and accumulates dramatically following an appropriate biotic or abiotic stimuli, such as the hypersensitive response (HR) or exposure to UV, respectively (Malamy et al. 1990; Métraux et al. 1990; Surplus et al. 1998). SA is a product of secondary metabolism and is made either from phenylalanine via cinnamic and benzoic acids, or from isochorismic acid in the plastid, the latter pathway likely to be the more important for establishment of SAR (Wildermuth et al. 2002; reviewed by Métraux, 2002).

The synthesis of SA and consequent stimulation of defense-related gene expression can be stimulated by perturbations in carbohydrate metabolism. This was shown by Herbers et al. (1996a) who disrupted the partitioning of photosynthates by constitutive expression of invertase in the apoplast or vacuole in transgenic tobacco plants. This engendered a lesion mimic phenotype in the transgenic plants, together with higher than normal basal SA levels, induction of pathogenesis-related (PR) proteins (see Loebenstein and Akad, this volume), and a decrease in susceptibility to *Potato virus Y*. Interestingly, transgenic plants expressing invertase in the cytosol did not exhibit these effects, leading to the suggestion that hexose-sensing signaling mechanisms associated with the secretory apparatus are able to cross-talk with the defensive signaling pathway (Herbers et al. 1996a). This data and other work from the same group on sugar-induced PR protein synthesis (Herbers et al. 1996b) provides further support for the idea that carbohydrate metabolism affects the resistance/susceptibility status of plants.

Our group became interested in the possibility of a relationship between respiratory metabolism and SAR because of the known connection between SA and a respiratory enzyme, the alternative oxidase (AOX).

The alternative respiratory pathway. The mitochondria of plant cells can utilize two respiratory electron transport chains. One of these, the cytochrome pathway, is also present in animal cell mitochondria, while the other, which is called the alternative or cyanide-insensitive respiratory pathway, is not. The alternative respiratory pathway branches from the conventional pathway at ubiquinol/ubiquinone (UQ). The alternative respiratory pathway consists of only one enzyme, AOX, which 'siphons' electrons out of UQ pool, reducing oxygen to water (see Murphy et al. 1999). Since this reaction is not coupled chemiosmotically to ATP synthesis it generates heat (Laties, 1982; Siedow and Moore, 1993; Affourtit et al. 2002). Indeed, for many years the only firmly established role for AOX was in thermogenesis in a specialized floral structure, the spadix, found in the inflorescences of plants belonging to the Araceae, such as the voodoo lily, *Sauromatum guttatum* (Raskin et al. 1987, 1989; Meeuse and Raskin, 1988), and the smaller 'cuckoo pints' or *Arum* lilies (ap Rees et al. 1976).

However, in most plants the major physiological role for AOX lies in the maintenance of mitochondrial homeostasis by maintaining a steady flow of reducing power into the respiratory chain (and indirectly regulate processes supplying the reducing power, such as the Krebs' cycle), and preventing reactive oxygen species (ROS) generation by components of the respiratory chain (Affourtit et al. 2001, 2002; Maxwell et al. 1999; Moore et al. 2002; Sakano, 2001; Yip and Vanlerberghe, 2001). AOX is encoded by a small family of nuclear genes, some of which are inducible. The AOX polypeptide (c. 35 kDa) is synthesised in the cytoplasm and translocated into the mitochondrion (Vanlerberghe and McIntosh, 1997). Once it reaches the inner mitochondrial membrane, AOX can form non-covalently bound enzymatically-active homodimers that can inter-convert to less active covalently linked homodimers held together by a disulfide bridge (Umbach and Siedow, 1993; Affourtit et al. 2001, 2002).

In thermogenic plants such as *S. guttatum* SA is the natural trigger for AOX-mediated thermogenesis (Raskin et al. 1987), and in both thermogenic and non-thermogenic plants SA stimulates AOX activity and *Aox* gene expression (Rhoads and McIntosh, 1992). Further underlining the potential connection between AOX and SA-mediated defensive signalling, it was found that a synthetic SAR-inducing chemical, 2,6-dichloroisonicotinic acid (INA), triggers thermogenesis in *Arum italicum*, and *Aox* gene expression in non-thermogenic plants (Chivasa and Carr, 1998; Chivasa et al. 1999).

Correlative evidence has also suggested an involvement of AOX in the HR. Thus, several groups have observed that *Aox* gene expression and AOX protein accumulation are elevated in plant tissue expressing the HR, further suggesting an association between AOX and pathogen resistance (Lennon et al. 1997; Chivasa and Carr, 1998; Lacomme and Roby, 1999; Simons et al. 1999). In a *Nicotiana sylvestris* cytoplasmic male sterility mutant with a defect in electron transport complex I there is an elevation in the basal level of AOX. Interestingly, when these plants were crossed with *NN* genotype tobacco, the progeny exhibited fewer lesions, suggesting they had an enhanced ability to localize TMV (Dutilleul et al. 2003).

Pharmacological and genetic modifications of the alternative respiratory pathway affect virus infection.

Pharmacological studies. We decided to test the apparent correlations between AOX SA and resistance. Initial experiments took a pharmacological approach, utilizing chemicals known to induce AOX activity, such as the cytochrome pathway inhibitors cyanide and antimycin A (AA), and an inhibitor of AOX, salicylhydroxamic acid (SHAM). Early on in this work it became apparent that there seemed to be no relationship between AOX and SA-induced resistance to bacterial and fungal pathogens (Chivasa et al. 1997; Simons et al. 1999). However, there did appear to be a relationship with induced resistance to viruses (Chivasa et al. 1997; Chivasa and Carr, 1998; Wong et al. 2002).

In tobacco, SA-induced resistance to the accumulation of TMV and PVX in inoculated tissue and to the systemic movement of CMV was inhibited by salicylhydroxyamic acid (SHAM) (Chivasa et al. 1997; Naylor et al. 1998). However, SHAM did not prevent SA-induced synthesis of PR proteins or prevent SA-induced resistance to fungal or bacterial pathogens (Chivasa et al. 1997). In later experiments, non-lethal concentrations of AA or cyanide were found to induce resistance to TMV in susceptible tobacco without inducing *PR1* gene expression (Chivasa and Carr, 1998). In *A. thaliana* these chemicals also induced resistance to TVCV and the DNA virus CaMV without any concomitant activation of PR gene expression (Wong, 2001; Wong et al. 2002). Based on this evidence, a model was proposed in which the signal transduction pathways involved in virus resistance separate downstream of SA: one branch (activated by SA or by AA or cyanide) leads to resistance to viruses, the other (activated by SA but not AA or cyanide) to the induction of PR proteins and to bacterial and fungal resistance (Murphy et al. 1999, 2001).

Work by our group, and that of Klessig and co-workers, using *Arabidopsis npr1* mutants provided support for this model. The product of the wild-type *NPR1* gene regulates induction of several PR proteins, is an important regulator of induced resistance to bacteria and fungi, and is considered to be an element of central importance in the establishment of SAR and certain other forms of induced resistance (see Durrant and Dong, 2004). It may also be important in restriction of TMV spread during the *N* gene mediated HR in Nicotiana species (see accompanying chapter by Dinesh-Kumar and colleagues). However, Kachroo et al. (2000) showed that *HRT* gene-mediated resistance to the *Turnip crinkle virus* (TCV), which is SA-dependent, could still occur in *npr1* mutants. Similarly, Wong et al. (2002) found that *npr1* mutant plants were uncompromised in their ability to express SA- or AA-mediated resistance to TVCV. Thus, SA-induced resistance to viruses in *A. thaliana* does not require NPR1 activity.

The pharmacological evidence, backed up by the work with *npr1* mutants, showed convincingly that regulation of PR gene induction and activation of resistance to viruses were activated by separate branches of the defensive signal transduction pathway. But since some of the chemicals used, particularly cyanide and SHAM, affect several enzymes other than AOX, this evidence could not show definitively that AOX is involved in signaling (Murphy et al. 1999, 2001; Singh et al. 2004). This provided the impetus to test the putative relationship between AOX and virus resistance using transgenic plants in which *Aox* gene expression has been modified.

Genetic modification of *Aox* gene expression. *Aox* gene expression can be altered in plants using constitutively expressed sense or antisense *Aox* cDNA sequences. However, some specific challenges are encountered when analysing the characteristics of *Aox*-transgenic plants.

Firstly, the best method for direct measurement of AOX activity *in vivo* uses specialized mass spectrometry equipment to determine the relative uptake of the ^{16}O and ^{18}O isotopes of oxygen during respiration (Robinson et al. 1992). But this is impractical to carry out on chemically treated, virus-infected tissue. Furthermore, it would not give valid results if additional oxygen consuming reactions were induced by exposure to viruses or chemicals, for example as during the HR, since the selective utilization of the two oxygen isotopes by AOX and cytochrome oxidase would be obscured (J. N. Siedow, personal communication). Therefore, lines of *Aox*-transgenic plants have been characterized in terms of their alternative respiratory pathway capacity (APC). APC is a measure of the maximum potential activity of AOX (Moore and Siedow, 1991) and is relatively straightforward to measure in plant cells and tissues using oxygen electrodes to measure oxygen consumption in the presence or absence of inhibitors of the cytochrome and alternative respiratory pathways.

A second challenge encountered with *Aox*-transgenic plants is that the changes in APC that can be achieved by constitutive expression of *Aox*-derived constructs are relatively modest (two to threefold above or below wild-type levels). This is probably because alterations of APC to any greater extent would interfere drastically with mitochondrial homeostasis and render such transgenic plants unviable. Support for this idea was obtained by Murphy et al. (2004) who used a TMV-derived vector (TMV.AOX) to drive AOX protein synthesis up to levels ten-fold or greater than those seen in unmodified plants. They found that in the highly susceptible host *N. benthamiana* infection with TMV.AOX resulted in systemic necrosis.

In initial experiments it was found that increasing or decreasing *Aox* gene expression and APC to the extent possible in *Aox*-transgenic plants did not alter either the overall susceptibility of plants to TMV-induced systemic disease, or their ability to resist the systemic spread of the virus following treatment with SA (Gilliland et al. 2003; Ordog et al, 2002). However, when accumulation of the virus was examined in the directly inoculated tissues of these *Aox*-transgenic plants, it was found that in plants with increased APC, AA-induced resistance to TMV was compromised. Meanwhile, in plants with decreased APC, SA or AA-induced resistance was transiently enhanced (Gilliland et al. 2003). We have suggested that the differential effect of altering *Aox* gene expression and APC on SA- vs. AA-induced resistance to TMV may be explained if SA, but not AA, can trigger more than one signalling pathway leading to the induction of mechanisms that limit virus accumulation (Gilliland et al, 2003; Singh et al. 2004). In this model, AA and SA can both induce signaling via the mitochondrion, which is influenced by AOX and can be to some extent disrupted in *Aox*-transgenic plants, while SA can trigger an additional antiviral mechanism that is not affected by AOX (below).

The degree to which *Aox* gene expression or APC can be altered in transgenic plants is, as noted above, rather limited. More recently, our group has found that by using TMV-derived transient expression vectors to express *Aox* sequences, far higher levels of expression of wild type or mutant AOX protein can be produced *in planta* (Murphy et al. 2004). When TMV vectors were used to drive very high levels of expression of either AOX, or AOX mutated in its active site (AOX-E), virus spread was enhanced and thereby induced larger HR lesions than those produced by the 'empty' viral vector after inoculation onto *NN*-genotype tobacco. Consistent with this, in the highly susceptible host *N. benthamiana,* systemic movement of TMV vectors expressing AOX or AOX-E was at least as fast as that of the empty vector and faster than that of TMV constructs bearing sequences of comparable length (the green fluorescent protein sequence or antisense *Aox*).

These results suggest that expression of either AOX or AOX-E is allowing the viral vector to overcome, at least to some extent, a pre-existing or basal resistance to the spread of TMV. This contrasts with some of our own earlier results and conclusions (Gilliland et al. 2003) in which we found that although altering *Aox* gene expression in stably transformed tobacco could affect certain aspects of induced resistance it did not affect the basal resistance or susceptibility to infection with TMV. We have suggested that the far higher levels of *Aox* expression (achievable with the viral vectors) are negatively affecting the operation of basal resistance to the spread of TMV and that this basal resistance is at least partly controlled by mitochondrial signaling mechanisms (Murphy et al. 2004).

Interestingly, we recently found that although TMV.AOX can readily spread and form lesions in the *NN*-genotype Xanthi-nc tobacco, it does not form HR lesions on plants of another *NN*-genotype cultivar, Samsun NN, unless they are transgenic for the bacterial salicylate hydroxylase encoding gene, *nahG,* and therefore cannot accumulate normal levels of SA (unpublished data). This suggests that Samsun NN tobacco possesses a basal resistance to the spread of TMV that is stronger than that in Xanthi-nc. Although this basal resistance must be, in part, dependent on SA, it cannot be overcome by high-level expression of *Aox* sequences from the virus. The result suggests that tobacco varieties vary widely in their basal resistance to virus infection independently of whether they possess a major single gene resistance such as that controlled by the *N* gene.

Salicylic acid-induced resistance to viruses: Induction and modes of action.

How does AOX function in resistance induction? As mentioned above, one of the functions of AOX is to negatively regulate the accumulation of ROS within the mitochondrion. These ROS are generated as a by-product of the activity of the respiratory electron transport chain, particularly when flow through the chain is constricted by, for example, the presence of metabolic inhibitors like cyanide or by other stresses.

We recently suggested a model in which changes in ROS levels in the mitochondrion act as signals controlling a subset of the antiviral resistance mechanisms induced by SA (Gilliland et al. 2003; Singh et al. 2004). Similar mechanisms have also been proposed for the coordination of defensive and stress-induced signaling via the mitochondrion (Dutilleul et al. 2003; Maxwell et al. 2002; Norman et al. 2004). One element of the mechanism put forward by Dutilleul et al. (2003) is that redox-responsive proteins in the

mitochondrion would detect these variations in ROS level. We suggest that these could transmit information into the cytosol and then, probably *via* further intermediates, to the nucleus to regulate changes in gene expression such as those described by Maxwell and colleagues (2002). We have suggested that some of the genes affected in this way may encode host factors affecting virus replication and movement (Singh et al. 2004).

If defensive signaling in the mitochondrion were transduced *via* alterations in ROS, this would offer an explanation for the modified responses of the *Aox*-transgenic plants to SA and AA. In non-transgenic plants AA and SA can, even at low concentrations, constrict electron flow through the respiratory chain, which will lead to a transient increase in ROS (Maxwell et al. 1999; Xie and Chen, 1999; Norman et al. 2004). However, in transgenic plants with an increased APC the mitochondrion would be ‘buffered’ against the build up of ROS, thus ‘damping’ the signal. This can explain why AA-induced resistance is seen to be inhibited in these transgenic lines but not why SA-induced resistance to TMV can still be detected (Gilliland et al. 2003). This has led us to propose that there is at least one additional mechanism, most likely involving gene silencing that contributes to SA-inducible resistance but which is not regulated *via* the mitochondrion and inducible by AA (the evidence for this is reviewed in Singh et al. 2004 and in the accompanying chapter by Gilliland et al.).

The nature of SA-induced virus resistance mechanisms is not the same for all viruses, plant cells or plant species. One of the problems with many models for defensive signal transduction pathways in plants is that they can give the misleading impression that a single signaling pathway regulates all resistance processes. In fact, SA-induced resistance appears to manifest itself in different ways in different cell types and involves resistance to at least three phases of the viral infection process: replication, cell-to-cell movement, and long distance movement.

Recent studies with TMV revealed that SA induces inhibition of both replication and cell-to-cell movement of this virus but these effects were found to be cell-specific (Murphy and Carr, 2002; Carr, 2004). When SA-treated tobacco plants were inoculated with TMV engineered to express the jellyfish green fluorescent protein (TMV.GFP), virus cell-to-cell movement was inhibited in the cells of the epidermis. Meanwhile, in protoplasts derived from mesophyll cells accumulation of the virus was dramatically decreased. In studies of directly inoculated leaf tissue it was found that the ratio of genomic RNA to coat protein mRNA and the ratio of plus- to minus- sense RNAs were affected by SA. Taken together, these results suggested that SA induces interference with the activity of the TMV RdRp complex (Chivasa

et al. 1997; Naylor et al. 1998; Murphy and Carr, 2002; Carr, 2004). However, taking into account the possible role of RdRP1 mentioned above, the decrease in TMV accumulation in these cells may also be due in part to an increase in the rate of viral RNA turnover (Gilliland et al. 2003).

Another reason that it is difficult to generalize about SA-induced virus resistance mechanisms is that not all viruses are affected in the same way in all plants. Thus, in tobacco SA does not inhibit the replication or movement of CMV in directly inoculated tissue but it does inhibit the systemic movement of this virus (Naylor et al. 1998; Murphy and Carr, 2002). In tobacco, SA-induced resistance to CMV is antagonized by SHAM, indicating that the resistance mechanism is controlled via the AOX-influenced mitochondrial-signaling pathway (Naylor et al. 1998). A similar situation occurs in *A. thaliana* where inhibition of CMV systemic movement is also induced by SA as well as by AA (Mayers et al. 2005). These results indicate that the mechanisms underlying induced resistance to CMV in tobacco and *A. thaliana* are very similar. However, not all plants combat CMV in this way.

In squash (*Cucurbita pepo*) SA-induced resistance to CMV results from inhibition of virus accumulation in directly inoculated tissue and this is due predominantly to inhibition of cell-to-cell movement. Furthermore, neither of the AOX inducers AA or KCN induced resistance to CMV in squash and AOX inhibitors, which can inhibit SA-induced resistance to CMV in tobacco, did not inhibit SA-induced resistance to the virus in this plant (Mayers et al. 2005). In *Nicotiana*, the ability of CMV to evade SA-induced resistance to movement and replication is conditioned by the 2b counter-defense protein (Ji and Ding, 2001; Palukaitis and García-Arenal, 2003). Evidently, the 2b protein is not able to subvert this type of resistance in this cucurbit host, possibly because the AOX-regulated signaling pathway is not involved in resistance induction.

We have suggested that the evolution of different resistance mechanisms to CMV in cucurbits *versus Nicotiana* and *A. thaliana* may have been driven to some extent by differences in plant anatomy and the photosynthate translocation mechanisms utilized by these hosts (Mayers et al. 2005). Broadly speaking, virus systemic movement follows the translocation of photosynthates, predominantly sucrose, from carbon source to carbon sink tissues (Nelson and van Bel 1997). However, the sucrose and virus may briefly part company during loading from the mesophyll cells into the phloem. This is because for viruses like CMV the entire route from the leaf mesophyll cells to the sieve element–companion cell complex must occur

via plasmodesmata, i.e. symplastically. However, the route taken by sucrose can be symplastic or apoplastic, depending upon the plant species (ap Rees 1994; Truernit 2001). Whether the transfer of sucrose occurs predominantly via the symplastic or apoplastic route depends upon the abundance of plasmodesmata linking the phloem tissue (sieve elements and companion cells) and the surrounding mesophyll cells (Truernit 2001). Detailed electron microscopic examination has shown, that in squash, which is a symplastic loader, these connections are abundant (Gamalei 1989). In contrast, the number of plasmodesmal connections per μm^2 of this interface is about 600-fold less in tobacco, and in *A. thaliana* there are between 37 and 10-fold fewer connections, depending upon the correction factor used (Gamalei 1991; Haritatos et al. 2000).

In tobacco and *A. thaliana*, where there are relatively few plasmodesmal connections between the mesophyll and phloem tissue, any inhibition of virus movement through the plasmodesmata at this interface will significantly compromise the ability of a virus to spread out of the primary inoculated leaf. But in squash the large number of plasmodesmal connections between the mesophyll and phloem cells may render any inhibition of virus movement at this interface to be less effective in preventing systemic virus movement from occurring. This may have placed a selective pressure on squash, and probably other cucurbits, for SA-induced resistance to CMV to target an earlier stage in virus invasion than we see in tobacco or *A. thaliana*.

These comparative studies of SA-induced resistance to CMV in different plant species show that different host species may use significantly different approaches to resist infection by the same virus. They also imply that caution may be required when attempting to apply findings on plant-virus interactions from model systems to a wider range of host species.

Summing up: Can plant biochemical studies help us to a better understanding of virus resistance and susceptibility?

At the beginning of this review we put forward the idea that the intimacy of the host-virus relationship could make the biochemical activity of the host a decisive factor in determining the outcome of the interaction in plants: resistance *versus* susceptibility. In the first section we examined the situation with regard to primary carbon metabolism in compatible interactions between viruses and plants. This has been an extensively studied area and a vast and complex array of metabolic changes occurring in time and space have been linked to virus infection. However, at the present time no direct causal relationship has been demonstrated between any virus-induced biochemical symptom and the success of the virus infection in a

susceptible host. This is unfortunate since if a biochemical change was identified as being required for successful virus replication and spread, the host enzymes involved could be targeted for chemical or genetic manipulation to induce resistance to the virus. In the future, increasingly powerful methods of large scale metabolic analysis combined with improved means of high-throughput genetic manipulation, such as virus-induced gene silencing, hairpin-mediated RNAi or the screening of libraries of T-DNA knockout plants, may allow us to identify unambiguously those virus-induced metabolic changes which are required for successful infection.

With respect to induced resistance, it does seem that certain elements of primary metabolism may affect the outcome of the virus-plant encounter. These elements include sugar sensing, which appears to play a role in regulation of SAR-related gene expression (Herbers et al. 1996a,b), and alternative respiration, which through its role as a negative regulator of ROS in the mitochondrion, modulates induction of some antiviral mechanisms in tobacco and *A. thaliana* (Singh et al. 2004). Thus, metabolic pathways are participating in resistance through signaling, rather than through, for example, redirecting metabolism to decrease levels of substrates needed for viral replication.

In conclusion, our understanding of biochemical changes in the virus-infected plant is still, for the most part, at the descriptive level. Nevertheless, studies have revealed roles for plant metabolic pathways in defensive signaling, and new approaches for the analysis and controlled perturbation of plant biochemical pathways hold the promise of yielding information useful in the design of future protection strategies.

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This review is dedicated to the memory of Professor Tom ap Rees.

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Chapter B1

Resistance to Viruses in Potato

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Introduction to potato and potato viruses

The European cultivated potato (*Solanum tuberosum* ssp *tuberosum*) is a self-compatible outbreeding tetraploid species (2n = 4x = 48) and ranks fourth after wheat, rice and maize in terms of importance to human nutrition. It was introduced into Europe in the late 16th century from the Andes of South America and later transported to the rest of the world. By the end of the 18th century, it had been adapted to long-day conditions through selection by its early cultivators for early-tubering and high yields. Potato is susceptible to a wide range of fungal, bacterial and virus diseases as well as various insect and nematode pests. As its importance as a staple food crop increased, so did problems associated with its clonal means of multiplication, notably caused by various virus diseases, described as a degeneration of seed tubers due to 'the curl' (reviewed by Salaman, 1926). In time it was realized that some of the viruses were transmitted by aphids. This led to the development of seed industries in many countries where high-grade virus-free seed tubers were produced in areas that are climatically and geographically suitable with regards to isolation from sources of infection and reduced numbers of virus vectors.

Viruses are important pathogens that can substantially decrease yield and quality of the potato crop. Potatoes are susceptible to about 40 viruses and two viroids (Jeffries et al. 2005). Of these, eight viruses and one viroid have significant economic impact on a world-wide basis, while others are either of unknown or low importance in potato or are only important in localised

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areas. These eight viruses and the viroid (Table 1) can cause yield losses, which vary between cultivars, and can also cause tuber defects that may render the tubers unsaleable. However, although some of these (and some of the other viruses that infect potato) cause few if any symptoms, in combination as mixed infections they can cause much more severe symptoms. Effects on yield are common, but in some situations the effects on tuber quality are more important and these include ‘spraing’ (necrotic arcs or rings in or on the tubers) resulting from *Tobacco rattle virus* (TRV) or *Potato mop-top virus* (PMTV); ‘potato tuber necrotic ringspot disease’ resulting from *Potato virus Y* ^{NTN} (PVY^{NTN}); ‘net necrosis’ resulting from *Potato leafroll virus* (PLRV) in a few cultivars and deformed tubers resulting from *Potato spindle tuber viroid* (PSTVd) infection. Symptom severity also depends on whether the infection is primary (current season infection) or secondary (tuber-borne), with symptoms generally being more severe, certainly in economic effects, when it is tuber-borne.

Viruses such as PLRV and PVY can affect yield quite substantially and cause higher losses (up to 80%) than caused by viruses producing mild or latent symptoms such as PVX (10-15%) and PVS (10-20%) (Burton, 1989). However, the above losses are based on 100% infection, but in a crop yield loss is reduced by the compensatory effect of adjacent healthy plants, which yield more due to reduced competition from the diseased plants (Reestman, 1970).

Yield losses due to PLRV infections worldwide have been estimated at 20 million tonnes per year (Kojima and Lapierre, 1988). Losses resulting from PLRV, PVY and PVX infections in the UK were estimated, using 1982 prices, at £30-50 million during a year of average infection (Hull, 1984). The potential yield increase from using cultivars resistant to these three important viruses was estimated at 22% in Mexico (Quaim, 1998). Direct losses to the grower include reduced yields, downgrading of seed crops, and/or tuber blemishes but, in most countries, the hidden substantial costs must also include expensive control measures.

PLRV is probably the most damaging and widespread virus of potato and is found wherever potato crops are grown. PVY is next in importance and although it tends to cause less damage than PLRV in some cultivars, it can cause severe damage in others (Beemster and Rozendaal, 1972), and isolates of the PVY^{NTN} subgroup cause severe tuber symptoms. PVY is also widespread, and in some parts of Europe it is more common than PLRV.

Table 1. The main potato viruses

Virus species (acronym)	Genus	Means of transmission	Distribution
Potato leafroll virus (PLRV)	*Polerovirus*	Aphid - persistent	Worldwide
Potato virus Y (PVY)	*Potyvirus*	Aphid - non-persistent	Worldwide
Potato virus A (PVA)	*Potyvirus*	Aphid - non-persistent	Worldwide
Potato virus X (PVX)	*Potexvirus*	Contact	Worldwide
Potato mop-top virus (PMTV)	*Pomovirus*	*Spongospora subterranea*	Andes, North America, China, Japan, Russia, Northern Europe
Tobacco rattle virus (TRV)	*Tobravirus*	Trichodorid nematodes	Europe, China, Japan, New Zealand, North/Central/South America
Potato virus S (PVS)	Carlavirus	Contact (aphid - non-persistent*)	Worldwide
Potato virus M (PVM)	Carlavirus	Aphid - non-persistent (contact†)	Worldwide
Potato spindle-tuber viroid (PSTVd)	Pospiviroid	Contact (aphid‡)	Worldwide

* Some isolates of PVS are transmitted by aphids.
† Some isolates are transmitted by contact.
‡ PSTVd can be transmitted by aphids if encapsidated by PLRV (Querci et al. 1997).
Note: PVY, PVA and PVV can also be transmitted by contact, but much less readily than PVX and PVS.

PVA, which is related to PVY but less commonly reported, occurs worldwide (except for the Andes) and causes symptoms that range from very mild to fairly severe. PVX occurs worldwide and is also important, although its symptoms tend to be mild. However, mixed infections with PVX and other viruses are very damaging. Other viruses that we shall consider, such as PVS, PVM, TRV and PMTV, tend to be locally important within some regions. For example, PMTV appears to be restricted to areas with cooler climates such as the Andean region of South America, Canada, China, Japan and Northern Europe. TRV is prevalent in light sandy soils in more temperate areas that favour the trichodorid nematode vectors. PSTVd occurs in North and South America, China and parts of Eastern Europe but is considered to be non-indigenous in most of Western Europe.

The eight viruses and viroid in Table 1 belong to seven genera and have different modes of transmission. All are transmitted through seed tubers, although PMTV and TRV are to a certain extent self-eliminating from tuber stocks of some cultivars because the virus does not move into all tubers of infected plants. However, only PSTVd is transmitted through true potato seed.

Types of resistance

Resistance to infection

In describing the responses of plants to viruses, plants that do not become infected after challenge inoculations applied in conditions typical for the crop are sometimes referred to as 'field immune'. This concept of immunity or resistance in field conditions (sometimes called 'field resistance') has been recognised for many years. An alternative and less confusing way of describing such resistance is as 'resistance to infection'. This term describes the situation where the likelihood of infection (by natural means) is reduced in resistant plants, e.g. quantitative resistance to PLRV whereby fewer plants of resistant clones or cultivars become infected by aphids in field conditions (Davidson, 1973; Barker 1987). It can be determined by field exposure trials where the virus is spread from infector plants by aphids (predominantly *Myzus persicae*) and secondary infection in daughter plants is assessed by symptoms, ELISA or by a combination of the two, in comparison with standard control cultivars (Davidson, 1973; Solomon-Blackburn and Barker, 1993). Resistance to infection with PLRV can clearly be the result of several different mechanisms that affect the vector and virus separately, and such resistance appears to be inherited polygenically (Ross, 1958; Baerecke, 1961; Davidson, 1973), probably as a result of the combination of several different mechanisms that contribute to the trait. Resistance to PLRV infection in some German cvs appears to have been derived from introgression of genes from *Solanum demissum* (Ross 1966; Davidson 1980).

Quantitative resistance to PVY infection, from *S. phureja*, has been reported (Davidson, 1980), but it dissipated in outcrossing and would be difficult to assess accurately enough for selection. It had been thought that this quantitative resistance to PVY might be more durable than major gene resistance, but extreme resistance (ER) and hypersensitive resistance (HR) conferred by single dominant genes have proved quite durable and offer a higher degree of protection. The phenomena of ER and HR are described in greater detail in a subsequent section of this review.

Resistance to virus accumulation

Plants with resistance to virus accumulation can be infected but the virus reaches only a relatively low concentration in the plant. This type of resistance has been shown for PLRV in a range of *S. tuberosum* breeding lines and other potato material (Barker and Harrison, 1985; Gase et al. 1988; Swieżyński et al. 1988; Wilson and Jones, 1993; Van den Heuvel et al. 1993). The most resistant tetraploid genotypes had 1-5% of the PLRV concentration found in susceptible clones. The advantage conferred by this type of resistance is that virus is less likely to be acquired and spread to other plants by aphids (Barker and Woodford, 1992). To assess resistance to PLRV accumulation, plants can be graft-inoculated and the virus concentration determined by quantitative ELISA (Barker and Harrison, 1985). It was found that the most reliable and consistent results were obtained by testing tuber-progeny plants (i.e. with secondary infection).

A high level of resistance to PLRV accumulation in *S. brevidens* was identified by Jones (1979). Gibson et al. (1990) also found a high level of resistance to accumulation of PVX and PVY, as well to PLRV in *S. brevidens*. In attempts to transfer this resistance into a *S. tuberosum* background by somatic hybridization, it was apparent that the gene controlling resistance to PLRV is different from those controlling resistance to PVY and PVX, and that genes conferring resistance to PVY and PVX are linked (Valkonen, 1994). The resistance to PVY and PVX in *S. brevidens* is thought to be associated with slow cell-to-cell spread of the viruses (Valkonen et al. 1991).

Resistance to virus movement in plants

Resistance to virus movement occurs where some kind of movement of virus through the plant is impeded, for example where a lower percentage of the tuber progeny of an infected plant are infected. This type of resistance has long been recognised in certain potato clones with respect to PLRV (Hutton and Brock, 1953; Barker, 1987). In some cases it is associated with phloem necrosis as in cv. Bismark (Hutton and Brock, 1953) and cv. Apta (Golinowski et al. 1987). Resistance to phloem transport has been demonstrated in cv. Bismark by Wilson and Jones (1992), who found it to be separate from resistance to accumulation and resistance to infection. Phloem transport was not impeded in a number of clones studied by Derrick and Barker (1997), but virus accumulation was largely restricted to the internal phloem bundles (whereas in susceptible clones virus accumulated in internal and external phloem tissue). Swieżyński et al. (1989) found a high level of

PLRV resistance associated with limited virus spread in four diploid potato clones.

HR could be regarded as a kind of resistance to virus movement, because movement is arrested or impeded by cell death (see below).

Mature plant resistance

The more advanced the crop growth at the time of inoculation, the less likely it is that the daughter tubers will become infected. This is because virus replication at the site of inoculation and translocation to the tubers may be slower in plants showing mature plant resistance than in plants not showing such resistance. Long before crop maturity, virus ceases to move from the inoculated leaf (Beemster, 1987). Mature plant resistance has been demonstrated for PLRV, PVM, PVS, PVX and PVY^{O}, but for some viruses (e.g. PVY^{N}) such resistance develops later (Beemster, 1987) and may explain why PVY^{N} is more difficult to control than PVY^{O}. Mature plant resistance starts to develop at around the time of tuber initiation and can be complete 4 weeks later. It occurs in most cultivars, but its particular characteristics are cultivar specific and it differs according to virus, the virus strain and environmental conditions (Beemster, 1987). However, although it is important to recognize this type of resistance exists, it is unlikely that it could be easily exploited and manipulated in a breeding program.

Tolerance

Although tolerance is not a form of virus resistance, this trait has been either deliberately or inadvertently selected in many breeding programs. Tolerant cultivars can be defined as those that show no symptoms or few obvious symptoms when plants are infected. Although tolerance may be seen as a valuable trait, it has a major disadvantage since tolerant cultivars are in essence susceptible genotypes that exhibit less damage when infected than other susceptible genotypes, and importantly, they can act as virus reservoirs. The underlying causes of ‘tolerance’ are difficult to identify and may be related to many plant factors that influence the replication and movement of virus particles and the expression of disease symptoms. However, it is increasingly recognized that there are dangers in breeding for tolerance because of the risk of virus spread from infected symptomless stocks grown in proximity to healthy material, or of introducing a soil-borne virus to sites that were previously uncontaminated.

Resistance to virus vectors

Resistance to aphid vectors has been sought in *Solanum* species and should be a promising trait for breeding because it should help diminish virus spread, and also because aphids can themselves cause significant crop damage. A promising type of resistance is found in wild Bolivian potato species *Solanum berthaultii*. Plants of some accessions of this species possess two types of glandular hairs: A-type hairs, which when ruptured physically entrap aphids with their contents, and B-type hairs, which entangle aphids, making them struggle more and so rupture more A-type hairs (Tingey and Laubengayer, 1981). In addition, B-type hairs are a source of (E)-β-farnescene, which is the main component of the alarm pheromone for most aphids and the activity of this chemical can act to repel *M. persicae* and to induce rapid dispersal from the leaf (Gibson and Pickett, 1983). Rizvi and Raman (1983) investigated two accessions of *S. berthaultii*, one with type A and B hairs and one with type A only. Both accessions were susceptible to PVY and PLRV. In a field trial, both accessions were exposed to aphid-borne infection by the viruses and both were equally infected with PVY, indicating that type A and B hairs had no effect on PVY spread. However, the spread of PLRV was reduced significantly by B type hairs (22% spread in the accession with A- and B-type hairs and 84% in the accession with A-type hairs only). Unfortunately it has proved to be difficult to incorporate the genes for the B-type hairs without also introducing undesirable characteristics. Thus, Kalazich and Plaisted (1991) found that in plants from backcrosses between *S. bertaultii* and *S. tuberosum*, there was a strong association between the presence of the B-type trichomes and undesirable characteristics such as lower yields, fewer tubers and later maturing plants.

There are no sources of resistance or tolerance to PMTV that have been deliberately used in breeding programs, but resistance to *Spongospora subterranea* (the vector of PMTV) has been found in *Solanum* species and certain *S. tuberosum* cvs, but whether such resistance could be developed to a level that would confer resistance to infection with PMTV is not known.

HR/ER resistance

The HR prevents spread of the virus throughout the plant. Plants with HR show either local necrotic lesions, which prevent the infection from spreading further, or systemic necrosis. Virus can almost always be detected in affected leaves. HR is often strain-specific. It can also be affected by environmental conditions or by the physiology of the host plant (e.g. maturity). ER and HR to PVX and potyviruses can be simply determined by sap-inoculation or graft-inoculation, observing the response and testing for

infection. Plants with ER to a virus show no symptoms, or limited necrosis (e.g. pinpoint lesions, flecks, or localized stem necrosis), when inoculated with virus. Only extremely low amounts of virus, if any, can be detected by sensitive techniques. ER can be comprehensive, conferring resistance to several strains or even two or three viruses, and for these reasons is frequently regarded as the best type of resistance to breed into potato. ER is often regarded as an immune response, i.e. plants cannot become infected no matter how intense the inoculum pressure and can be regarded as a 'non-host' of the virus. However, as indicated above, this may not always be strictly true. The underlying mechanisms of HR resistance in potato are not yet clear, but some indications can be gathered from work done in tobacco. The *N* gene of tobacco mediates resistance to *Tobacco mosaic virus* (TMV). The *N* gene product is a cytoplasmically localized protein with a protein sequence motif known as '*leucine-rich repeats*' (LRR) (Whitham et al. 1994). The N protein appears to recognize the presence of virus by binding, probably indirectly, to the TMV *replicase* protein. Subsequently, a series of steps is triggered that results in a hypersensitive response (HR) in the host plant - (See Chapter A4). During the HR the cells containing virus die, resulting in a small necrotic lesion in the leaf. Goldbach et al. (2003) suggested that R genes might have evolved from ancient gene families by duplication, with subsequent mutation and recombination. Valkonen (1994) and Goldbach et al. (2003) present full reviews of the possible mechanisms of virus resistance in such a fast moving field.

A connection between ER and HR has been suggested, because necrosis can sometimes occur in plants with ER genes (Ross, 1958; Cockerham, 1970; Delhey, 1974). Hinrichs *et al.* (1998) reported that PVY replicated in initially infected leaf cells of inoculated plants of cultivars with the ER gene Ry_{sto}, and was transported into neighboring cells, prior to a limited necrotic reaction after which the infection ceased. The ER gene Rx_{adg} in cv. Cara has now been found to control separate virus resistance and cell death responses (Bendahmane et al. 1999); cell death does not normally occur when plants carrying Rx_{adg} are inoculated with PVX because the ER is epistatic over the HR. When studying *S. stoloniferum* genes conferring resistance to PVY and PVA, Cockerham (1970) found genes for ER to be dominant or epistatic over genes for HR. Valkonen (1994) also found the ER gene Ry_{adg} to be epistatic to the HR gene Ny_{adg} in an Andigena-derived genotype.

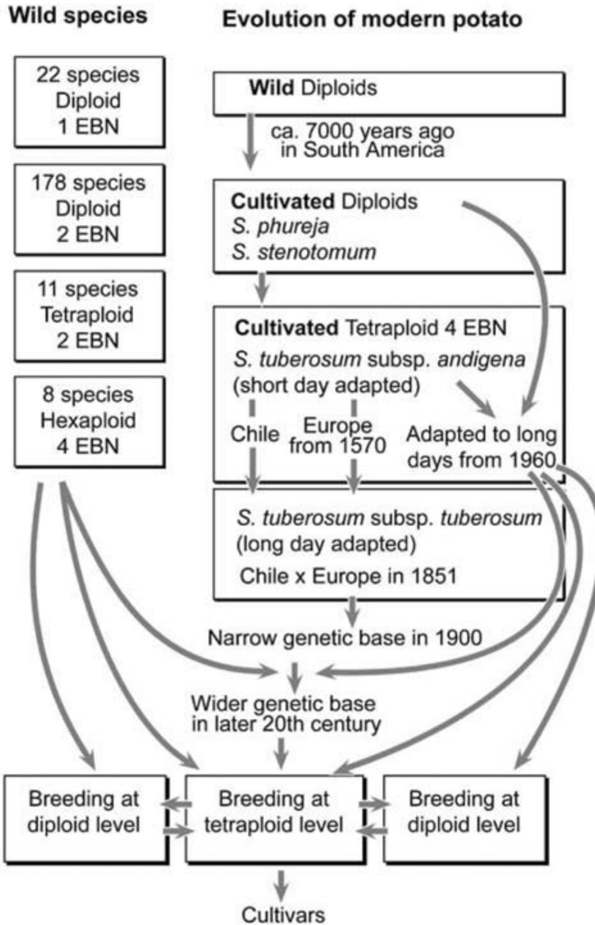
Breeding techniques and strategies

Historical aspects to virus resistance breeding

Potato breeding in the modern sense began in 1807 in England, when Knight made deliberate hybridizations between different varieties by artificial pollination (Knight, 1807), and expanded during the second half of the 19th century when many new cultivars were produced by farmers and hobby breeders. Potato breeding progress in the 20th century, compared to some other crops, initially appears to have been somewhat limited following the rapid progress made during the 19th century. This has been attributed by some to a narrow genetic base (Simmonds, 1969; Mendoza and Hayes, 1974), which led to the pan-European devastation of the potato crop during the late blight (*Phytopthora infestans*) epidemics of 1845 and the following years. These epidemics stimulated intensified breeding efforts to produce new varieties, though with a reduced range of germplasm. During the 20th century, following the rediscovery of Mendel's research in 1900 and the development and application of modern genetics to potato breeding, significant progress has been made in spite of the complex inheritance patterns associated with tetrasomic inheritance.

Sources of resistance in wild species and germplasm collections

At the beginning of the 20th century, potato breeding appeared to be restricted by a narrow genetic base tracing back to the few original introductions of *S. tuberosum* ssp. *andigena* from South America to Europe in the latter part of the 16th century and limited further casual introductions in the 17th and 18th centuries. A single cross with a Chilean *S. tuberosum* ssp. *tuberosum* accession (Rough Purple Chili) in the 19th century was introduced into the USA in 1851 (Goodrich, 1863), and its descendents were widely used as parents in crosses with European Tuberosum by the end of the 19th century (Hawkes, 1990). It is therefore probable that Chilean Tuberosum cytoplasm is a significant contributor in modern cultivars (Fig. 1). Furthermore, it is believed that relatively few of the 228 wild tuber-bearing taxonomic species of the genus *Solanum* were involved in the early domestication process in the Andes - probably just several closely related and inter-fertile members of the series Tuberosa. During the 20th century the genetic base of *S. tuberosum* ssp. *tuberosum* has been significantly widened as demonstrated in Figure 1, with resistance to cyst nematodes, late blight as well as to a number of potato viruses being introgresssed.



From the 1920's many germplasm-collecting expeditions to Mexico and South America, the centers of origin and diversity of potato, led to the collection and taxonomic description of over 200 wild and 8 cultivated tuber-bearing Solanum species (Hawkes, 1990). There are a number of genebanks which maintain extensive collections of wild and cultivated *Solanum* species, including the International Potato Centre (CIP, Lima, Peru), the Dutch-German collection (CGN, Wageningen, The Netherlands), the Groß Lüsewitz Potato Collection (GLKS, IPK, Groß Lűsewitz, Germany), The US Potato Genebank (NRSP-6, Sturgeon Bay, USA), the Potato Collection of the Vavilov Institute (VIR, St. Petersburg, Russia) and the Commonwealth Potato Collection (CPC, Scottish Crop Research Institute, UK, www.scri.sari.ac.uk/cpc). These genebanks are important

sources of new traits, including resistance to the many viruses that affect the potato. Resistance has been identified in many accessions of wild and cultivated potatoes (Hawkes, 1990) and examples of wild species with known resistance to viruses are given in Table 2. However, compared with the huge natural genetic diversity available in the wild relatives of the potato, only a small proportion has actually been used for introgression of resistance traits into cultivars. Often the introduction of detrimental "wild" traits occurs together with the resistance trait, and it requires several generations of backcrossing and recurrent selection before acceptable cultivars can be obtained from such germplasm. Nevertheless, most genes for resistance to viruses, fungi, and nematodes present in modern potato varieties and breeding materials have been deliberately introgressed from closely related tuber-bearing *Solanum* species. Further details on the history of resistance breeding can be found in Ross (1986) and Hawkes (1990). Resistance genes have been introduced into *S. tuberosum* from various wild *Solanum* species (Davidson, 1980; Ross, 1986; Bradshaw and Mackay, 1994), mostly from diploids or allopolyploids in which inheritance is disomic (Cockerham, 1970; Ross, 1986). Resistance to PLRV is not mentioned in Table 2 because, although there are several wild species with known resistance to PLRV (see section on PLRV), few have been deliberately used in breeding programs for that trait. Brown and Thomas (1994) reported transferring major gene resistance from *S. chacoense* into a diploid *S. tuberosum*.

Table 2. Examples of sources of some virus resistance genes commonly utilized in potato breeding programs.

Wild and cultivated Solanum species	Resistance to:	Types of resistance
S. stoloniferum	PVY PVA PVV	ER, HR
S. tuberosum ssp. *andigena*	PVA PVY PVX	ER, HR
S. demissum	PVY PVA	HR
S. hougasii	PVY PVA	ER
S. chacoense	PVY PVA PVX	HR
S. microdontum	PVY PVA	HR
S. tuberosum	PVY PVA PVV PVX	HR, ER
S. acaule	PVX	HR, ER
S. sparsipilum	PVX	HR

Methods of screening for resistance including marker-assisted selection

For any breeding program to achieve its objectives, it is imperative that there are robust and proven selection procedures for the traits under selection. The degree of phenotypic resistance to viruses is usually confirmed in the final stages of selection by use of a field exposure trial, particularly for resistance to PLRV infection. Tests have been developed that can be applied at the earlier stages of selection in a glasshouse environment, such as testing for major gene resistance to the manually transmitted viruses PVX and PVY. However, with an increasing number of molecular markers associated with different virus resistance genes being identified, the potential to use these (genotypic selection) instead of glasshouse or field resistance tests (phenotypic selection) is becoming a reality. In future, if the price of such tests is reasonable they could have a major impact on selection procedures.

Molecular markers are based on polymorphisms that occur in the DNA sequences (e.g. deletions, additions, substitutions) and rely upon the establishment of a linkage between a molecular marker and the trait to be selected. There are various methods to detect and amplify these polymorhisms including simple sequence repeats (SSRs), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP). The application of such molecular markers within breeding programs has a number of advantages and is a powerful tool to directly select genotypes with the desired traits. Some of their important advantages when compared to morphological, physiological or isozyme traits is that they are unlimited in number, are independent from environmental influences and can generally be detected at any stage of plant development. Breeders can select the desired genotype at an early stage without field-testing. This approach is particularly useful for traits that are difficult to measure or require substantial resources including glasshouse space, labour and time, e.g. testing over several years.

The application of marker-assisted selection within breeding programs for virus resistance is, as yet, limited principally by the lack of good markers associated with known resistance genes. The successful application of marker-assisted selection for virus resistance will be dependent on access to easily applied markers with close linkage to the resistance genes. Such markers have been developed for *Ry* (Hamalainen et al. 1997; Kasai et al. 2000), and for PVS major gene resistance (Marczewski et al., 2002) and for the *Rx* PVX resistance gene (Bendahmane et al. 1997; De Jong et al. 1997) amongst others. However, most work with markers has been to construct genetic maps and they have yet to be applied in earnest within breeding

programs. The practical application of molecular markers to aid selection will progress in a number of areas as more markers are developed, notably to speed the introgression of novel virus resistance from wild species into simultaneously within breeding programs.

Breeding and the introgression of virus resistance

Selection for resistance to viruses is a major component of many breeding programs and is generally regarded as the most sustainable and environmentally sustainable strategy for virus control. This occurs despite the positive impact of the use of healthy tubers *via* regulated certified seed programs in isolated pathogen-free regions and the use of appropriate agrochemicals to control virus vector problems. Thus, the numbers of cultivars available to growers is considerable, although it should be noted that many virus resistant cultivars may not be widely grown because they may have an agronomic defect or susceptibility to another pathogen, and furthermore, few lines have resistance to all the major viruses. Information on the European database (http://www.europotato.org/) (Table 3) gives an indication of the extent and range of available cultivars and breeding lines and their resistance to viruses. However, it should be noted this data was obtained from a variety of sources and could not be derived from tests in a common environment so exact comparisons between individual cultivars may not be possible.

Development of parents with multiplex resistance genes

There are a number of important resistances to potato pathogens governed by major dominant genes. The most well documented example is

Table 3. Numbers of cultivars and breeding lines with high levels of resistance to viruses (as at November 2004).

	Total no. of	Level of resistance		
Virus	cvs	High (7)	High - very high (8)	Very high (9)
PVY	1812	446	240	244
PVX	1226	350	69	232
PLRV	1785	424	113	65
PVA	1260	388	163	452

Assessed on a 1 (very susceptible) to 9 (very high) resistance scale, see http://www.europotato.org/ for details.

probably the *H1* gene originally discovered in CPC clone 1673(1), which provides qualitative resistance to pathotype RO1 of PCN (*Globodera rostochiensis*), now present in most modern cultivars. There are also genes such as Ry_{sto} and Rx_{adg} , which convey extreme resistances to viruses PVY and PVX respectively and also numerous '*R*' genes which confer resistance to specific races of *Phytophthora infestans*. However, the nature of tetraploid genetics of *S. tuberosum* ssp. *tuberosum* complicates the use of such valuable sources of resistance and most parental material tends to be simplex at their resistance gene loci. The consequences of tetrasomic inheritance are such that an allele at any given genetic locus can occur in four allelic states: simplex (present once at the locus), duplex (present two times), triplex (present three times) and quadruplex (present four times, i.e. homozygous). In crosses with susceptible clones, a parental genotype simplex for one such gene will produce a progeny with 50% of the offspring that inherit the resistance, and half the progeny will be susceptible. However, a susceptibleparent crossed to a parent duplex for such a gene will produce more than 80% resistant progeny, and crosses with triplex or quadruplex parents guarantees resistance in all progeny. By deliberately intercrossing clones with these genes then test crossing their progeny, it is possible to selectively breed parental clones duplex at their resistance gene loci. Examples of some of the expected ratios derived from multiplex parents are given in Table 4.

Table 4. Ratios of individuals within progenies derived from hybridising multiplex parents.

Crosses between: Male parent	Crosses between: Susceptible parent	Ratio of phenotypes in progeny	Ratio of genotypes in progeny
Simplex (1 *Ry* gene)	Nulliplex (0 *Ry* genes)	1/2 Susceptible 1/2 Resistant	1/2 Susceptible 1/2 Simplex
Duplex (2 *Ry* genes)	Nulliplex (0 *Ry* genes)	1/6 Susceptible 5/6 Resistant	1/6 Duplex 2/3 Simplex 1/6 Susceptible
Triplex (3 *Ry* genes)	Nulliplex (0 *Ry* genes)	All resistant	1/2 Duplex 1/2 Simplex
Quadruplex (4 *Ry* genes)	Nulliplex (0 *Ry* genes)	All resistant	All Duplex

N.B. These are examples of some of ratios of individuals within progenies derived from hybridising multiplex parents with susceptible parents, assuming Mendelian chromosomal (not chromatid) segregation. Examples are based on a gene of major effect, e.g. PVY Ry_{sto} gene.

Despite the apparent complexity and work involved with this breeding method, the SCRI cv. Spey is triplex for the *H1* gene and demonstrates tha or quality. The most obvious advantage of this resistance breeding method is that the routine use of triplex or quadruplex parents obviates the need to test progeny for the desired resistance and releases resources for screening other traits.

Studying isolated resistance genes

When investigating the molecular mechanisms underlying resistance, it should be possible to make rapid progress once genes have been isolated and cloned. To date, only a few plant genes encoding resistance to plant viruses have been isolated, although work aimed at isolating many others is in progress. Other cloned virus-specific *R* genes include HRT for TCV resistance in Arabidopsis and *Tm2* and $Tm2^2$ for TMV in tomato (See chapters A4 and B4). One of these genes is the well-known *N* gene, specifying a hypersensitive resistance to TMV originating from *Nicotiana glutinosa*. The other is the *Rx* gene from potato originating from *Solanum andigena*, and which encodes extreme comprehensive resistance to PVX. This gene has been expressed as a transgene in two *Nicotiana* species and potato, where it has been shown to induce extreme resistance to PVX (Bendahmane et al. 1999). The transgenic *Rx*-mediated resistance was indistinguishable from the *Rx*-mediated phenotype in cultivar Cara. The elicitor for this gene was previously mapped to the capsid protein (Bendahmane et al. 1995), and the extreme resistance was shown to be due to the elicitation of a response that was not viral target sequence specific (Köhm et al. 1993). Whether the PXV capsid protein interacts with the *Rx* gene product, or whether the *Rx*-encoded protein is responsible for the generation of another protein that interacts with the PVX capsid protein is not known. Both the *N* and *Rx* genes have similar organizations to a number of plant resistance genes from other types of pathogens (Jones and Jones, 1997). They contain certain conserved elements, such as leucine-rich repeats, and several kinase domains referred to as the nucleotide binding site (Bendahmane et al. 1999; Whitham et al. 1994). However, unlike resistance genes that are specific to bacteria, fungi, or insects, the two plant virus resistance genes do not encode a membrane anchoring domain, indicating that these gene products interact with the virus once it is inside the cell. Whether this will be the case for virus resistance genes that do not encode an extreme or hypersensitive form of resistance is not known.

Specific examples of resistance against the major potato viruses

- **PLRV**

PLRV is a major disease of potatoes worldwide. The virus is transmitted by aphids, in particular the green peach aphid (*Myzus persicae*). An aphid usually requires 24 to 48 hours feeding on an infected plant before it can transmit this virus. This relatively long acquisition phase provides the opportunity to control potential spread of the virus by use of systemic insecticides. Once the aphid has acquired the virus it remains infective for life. Primary infection symptoms include upright, rolled leaves and slight yellowing that appears mainly in the young leaves. Leaf rolling may only be evident at the base of the leaflet rather than the whole leaflet, and may eventually spread to the lower leaves. Plants infected early in the season may also be dwarfed. Crops planted with these tubers can be severely stunted, resulting in significant yield reduction. PLRV also causes net necrosis in infected tubers, rendering them unmarketable. Internal net necrosis is visible when the tuber is cut and this is particularly marked in certain cultivars. The distribution of the virus within infected plants is generally restricted to the phloem cells.

Despite causing the major virus disease of potato, remarkably few cultivars exhibit very strong resistance. Pentland Crown is the only resistant cultivar that has been grown widely in the UK. Its resistance proved to be valuable in 1975 and 1976 when there was considerable spread of PLRV in seed growing areas but less than 1% of the acreage of Pentland Crown crops was rejected from the seed-potato classification scheme, whereas a much larger acreage of crops of susceptible cvs (up to 20%) were rejected. (Barker, 1987). The resistance in Pentland Crown was the result of at least two forms of resistance, resistance to infection and resistance to virus accumulation. Breeding for resistance to PLRV is limited by a lack of highly heritable sources of complete resistance, and also by cumbersome screening procedures that depend on consistent vector pressure and several vegetative growth cycles to assess resistance.

Although there is a source of hypersensitivity to PLRV controlled by a single gene, it has not been widely used. The plants with this type of resistance die when infected, making the risk of total crop failure a possibility. The most common form of resistance to PLRV is partial field resistance (e.g. as described in Pentland Crown above). The genetic control is polygenic in nature producing small percentages of highly resistant breeding clones (Davidson, 1973), whereas resistance to PLRV accumulation may be controlled by a single dominant gene or a major heritable factor (Barker and Solomon, 1990; Brown and Thomas, 1994; Solomon-Blackburn et al. 2005).

Although several very good sources of resistance have been identified in wild *Solanum* germplasm, including *S. brevidens, S. etuberosum, S chacoense* and *S. phureja* (reviewed by Solomon-Blackburn and Barker, 2001), none of these have yet been deployed in commercial cultivars. Immunity to PLRV has been detected in *S. chacoense*. Marczewski et al. (2001) identified a major quantitative trait loci (QTL) and two minor QTL's accounting for between 50 and 60% of the phenotypic resistance to PLRV in an F_1 population. The major QTL mapped to chromosome XI, while the two minor ones mapped to chromosomes V and VI. Clearly there is a significant opportunity to develop PLRV resistant lines, probably with the aid of marker-assisted selection.

- **PVY and PVA**

PVY and PVA are potyviruses and are the second most important group of potato viruses, although PVY is probably more widely known. Infection can reduce yields by up to 80% (Hooker, 1981). Potyviruses are transmitted by aphid vectors, but are also easily mechanically transmitted. They can be particularly difficult to control by use of insecticides because the virus is transmitted in a non-persistent manner by many aphid species, and prophylactic applications of insecticide are only effective in controlling aphid populations rather than preventing virus acquisition and transmission that occurs after very brief probing activity. There are many cultivars that contain HR genes that confer a useful degree of resistance to the potyviruses. These genes arise largely because of selection for virus resistance within the Tuberosum gene pool in early breeding programs by selecting cultivars that withstood virus degeneration better than others.

Two principal groups of PVY have been recognised: PVY^{O}, or the common strain, which is severe in potato, but produces a mild mosaic in tobacco; and 2) PVY^{N} ('necrotic' strain), which is mild in potato but is severely and systemically necrotic in tobacco, and from which the 'necrotic' name is derived. It is rare in certain countries and this has created a special sensitivity to trade and quarantine issues. There are other strain groups, of which the most notable is PVY^{NTN}, which causes potato tuber necrotic ringspot disease (severe necrosis) in the tuber flesh of some cultivars. PVY^{NTN} appears to be the result of recombination between the genomes of PVY^{O} and PVY^{N}. Fortunately two genes are available that have been shown to be effective against all strains of PVY, namely Ry_{sto} and Ry_{adg}, derived from *S. stoloniferum* and *S. tuberosum* ssp *andigena*, respectively. These genes control ER to virus infection. Attempts to introgress ER to PVY and PVA have been made since the 1940s. However, relatively few cultivars have ER to PVY, probably because ER genes were generally much more recent introductions to the *S. tuberosum* gene pool, were introduced from

relatively few sources and *S. stoloniferum* (the source of genes Ry_{sto} and Ry_{stona}) does not intercross freely with *S. tuberosum*. No U.K.-bred cultivars are known to have a *Ry* gene, although several *Ry*-containing cultivars have been produced in Germany, Holland, Poland and Hungary, probably because of the higher priority of obtaining PVY resistance there.

- **PVX**

PVX is a widespread virus infecting many commercial stocks throughout the world and although infection tends to produce mild symptoms, yield losses of up to 15% in some varieties are known. PVX is mechanically transmitted through plant-to-plant contact, machinery contact in the field, seed graders and foliage cutters. PVX is often a latent virus i.e. the symptoms are not visible to the naked eye. The virus may show symptoms ranging from a mild mottling of the leaf to a severe mosaic, with a dwarfing of the plant and reduced leaflet size. When it occurs in multiple infections with other viruses, the disease symptoms are more severe and can cause substantial yield reductions.

As with HR genes for potyviruses, there are many cultivars that contain genes for PVX resistance including Nc_{tbr}, Nx_{tbr} and Nb_{tbr}. Many of these arose as a result of breeding programs incorporating wild species from South America that had been used since 1851. One such example is the hexaploid *S. demissum* used as a source of blight resistance in the UK by Salaman in 1909 (Bradshaw and Mackay, 1994). Although genes Nx_{tbr} for PVX (Cadman, 1942; Cockerham, 1970) and Ny_{tbr} for PVY (Davidson, 1980; Jones, 1990) have been widely used for many years, they still confer very useful resistance, though neither offers resistance to all strains. Although some evolution of resistance-breaking strains has occurred (Jones, 1982, 1985), it is a relatively slow process and resistance-breaking strains often do not become prevalent (Harrison, 1981). Attempts to introgress ER to PVX have been made since the early 1950s. Cultivars with Rx_{tbr}, Rx_{adg} or Rx_{acl} that confer ER have been produced in several countries including USA, Germany, Argentina (Ross, 1986), UK and Ireland.

- **PMTV**

PMTV is transmitted by the motile zoospores of *Spongospora subterranea* (the causal agent of powdery scab on tubers). Infection with PMTV can cause damage known as 'spraing' that occurs as brown arcs and circles on the tuber surface and in the tuber flesh of susceptible cultivars. There are no sources of resistance or tolerance to PMTV that have been deliberately used in breeding programs and there is no reliable method to screen for tolerance or field resistance (Solomon and Wastie, 1988).

However, there are differences in susceptibility among cultivars. For example, cv. Saturna, widely used in the Scandinavian potato-processing industry, is very sensitive (Sandgren, 1995; Nielsen and Mølgaard, 1997). Cultivars Appell and Desirée, on the other hand, are more resistant to PMTV infection. Cultivars Bintje, King Edward, Maris Peer, and Record are tolerant and infected tubers are symptomless (Kurppa, 1990; Arli-Sokmen et al. 1998). Resistance to powdery scab has been found in *Solanum* species and certain *S. tuberosum* cvs, but whether such resistance could be developed to a level that would confer resistance to infection with PMTV is not known.

- **TRV**

TRV, which is transmitted by several species of *Paratrichodorus* and *Trichodorus* nematodes, is the other virus that can cause spraing symptoms in potato and directly affect tuber quality (Harrison and Robinson, 1981). Spraing symptoms induced by TRV appear as arcs and lines of corky necrotic tissue in the tubers. Hence, the disease is sometimes called 'corky ringspot', and may render entire crops unmarketable at relatively low levels of symptom expression (Brown and Sykes, 1973). TRV has a wide host range, infecting over 100 plant species in nature, including a number of important crop plants, and a further 400 species under laboratory conditions (Harrison and Robinson, 1978).

Recent work has demonstrated that some potato cultivars can become systemically infected with TRV while exhibiting few, if any, spraing symptoms in the tuber flesh (Xenophontos et al. 1998). The virus isolates in such infections were of the M-type, i.e. they contained RNA-1 and RNA-2, produce nucleoprotein particles and can be maintained through several generations of vegetative propagation. Dale et al. (2000) demonstrated that infections of this type in the cv. Wilja severely affected overall yield and yield components such as tuber size and tuber number, also quality traits such as dry matter, after-cooking blackening and the chemical composition of the tubers despite the lack of obvious symptoms.

The appearance of distinctive consecutive arcs of necrotic cells often observed within the tuber flesh of spraing susceptible cultivars is not attributed to repeated feeding opportunities by the nematode vector. It is probable that the plant initiates an HR-like response on recognition of the virus at the cellular level resulting in a layer of necrotic 'corky' cells that partially obstructs further progress of the virus into the tuber. However, the virus would appear to penetrate beyond this initial response further into the flesh, thereby triggering further necrotic 'corky' layer(s).

There are several varieties that are immune to TRV, including Record, Hermes and Lady Rosetta and several cvs in which an HR-type response

occurs including Pentland Dell and Russet Burbank. Unlike the situation with PVY and PVX where HR can provide a useful level of resistance, with TRV although the HR-type response can provide a certain measure of infection resistance, it is undesirable because of the detrimental effect of corky ringspot (spraing) on tuber flesh quality. Recent work at the Scottish Crop Research Institute indicates that resistance in cv. Record appears to be conferred by a single resistance gene. AFLP markers linked to this resistance have been identified. The HR-type response in Pentland Dell has been analysed using AFLP markers and a major heritable factor identified. The results from these two populations indicate that different and separate major genes are involved in the processes of resistance and the development of spraing symptoms in the different tetraploid populations. The recognition of such major genetic factors and the identification of usable markers associated with them will greatly enhance the efficiency of breeding for improved resistance (immunity rather than an HR-type response) to TRV which at present relies on field assessments which are slow, laborious and at times unreliable depending upon environmental conditions.

- **PVS and PVM**

PVS and PVM are common viruses of potatoes and are virtually symptomless in most of the widely grown potato varieties. The existence of PVS was only detected during efforts to produce an antiserum to PVA. Controversy exists as to whether PVS alone consistently reduces yield, but losses of 10-20% have been reported, and similar losses are reported with PVM. PVS is transmitted mechanically by infected sap, for example between seed tubers on equipment such as seed graders and seed cutters, and by plant-to-plant (*via* foliage) contact through machinery movements in the field. PVM can also be transmitted mechanically. Some isolates of PVS and PVM are transmitted by aphids in a non-persistent manner.

There seems to be much less effort devoted to breeding for resistance to PVS and PVM, probably because they cause relatively less damage than most other viruses, and also because there has been less work to identify sources of resistance. Ross (1986) mentioned *S. megistracrolobum* as a source of the major dominant gene (*Nm*) for hypersensitivity. Dziewonska and Ostrowska (1978) reported that *S. gourlayi* contains a dominant major gene (*Gm*) that confers infection resistance to PVM, which is effective even after graft inoculation. The related virus PVS has a few sources of resistance, including resistance to infection that has been identified in cvs Adretta and Saco bred in Germany and the USA respectively (Ross, 1986). In addition, there is a source of localised hypersensitivity that originally came from *S. tuberosum* ssp. *andigena* and is inherited by the dominant gene

Ns. A few cultivars have been bred with gene *Ns* including Szignal and Fantasia, bred in Hungary and Germany respectively (Ross, 1986).

- **PSTVd**

PSTVd represents a particular problem to breeders because it is transmitted through true seed. This means that breeders must remain vigilant in case it becomes established in breeding parents. Another danger is that PSTVd can be transmitted by aphids if encapsidated by PLRV in co-infected plants (Querci et al. 1997). There are few reports of natural resistance to PSTVd and as far as is known, no reports of resistance in commercial cultivars. The Commonwealth Potato Collection (CPC) (www.scri.sari.ac.uk/cpc) lists *S. acaule, S. guerreroens* and *S. berthaultii* as having some resistance and it seems likely that effort put into screening accessions of these species would be productive. Resistance to PLRV would also be a desirable characteristic in some regions to ensure that aphid-transmitted PSTVd does not become a problem.

Conclusions

In comparison to viruses infecting most other crops, potato viruses have been extensively studied, partly because of the importance of the crop and also because of the early breeding efforts that revealed many of the basic facts about the viruses and the related resistance genes. Most potato viruses are well characterised and for most, detection methods are well established. Moreover, over a long period of time many measures have been developed to aid the production of healthy seed tubers and to shorten the number of generations of seed tuber production. However, despite the widespread use of healthy seed in most temperate agronomic systems, problems of virus control in crops remain. In developed agricultural systems these largely concern the difficulties in controlling virus vectors, particularly the increasing number of insecticide resistant clones, and lack of epidemiological knowledge concerning these vectors. In developing countries, problems are largely ones of providing the detection and control methods that can be harnessed to the production of clean seed at a realistic cost. In both situations, improved plant resistance through use of host genes or genetic modification (GM) in breeding programs, may offer a solution, particularly if there is pressure to reduce pesticide use on food crops. There is undoubtedly much promise in the GM route, but with recent developments in genomics, the development of molecular marker technology and bioinformatics, the more precise selection and manipulation of host

resistance genes looks to be a realistic prospect in the near future. We think that increased effort in this area will prove to be valuable.

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Chapter B2

Common Beans

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Introduction

The common bean (*Phaseolus vulgaris* L.) is one of the most widely cultivated legumes in the world, occupying over 27 million hectares of tropical and temperate agricultural land in the Americas, Europe, Africa and Asia (FAO, 2003). The genus *Phaseolus* is of American origin and comprises over 30 species (Debouck, 1999). *P. vulgaris* is the most widely grown legume, occupying almost 90% of the area planted to *Phaseolus* species in the world. The centre of origin and domestication of common bean includes the Andean region of South America and Middle America, from Chile up to approximately the Tropic of Cancer in Mexico (Singh, 2001). Genetic diversity in common bean is represented by large-seeded Andean, and small- and medium-seeded Middle American gene pools (Evans, 1980). There are two major commercial classes of common bean: snap and dry beans. In the case of snap beans, the green pods are harvested, whereas for dry beans, the seed is extracted from mature pods. The dry bean is the preferred form of consumption, with over 70% of the total common bean production area corresponding to this commercial class. In general terms, the genetic base of common bean cultivars is narrow, because only a small proportion of wild common bean populations were domesticated (Gepts et al. 1986).

Legumes in general can be infected by over 140 different plant viruses (Edwardson and Christie, 1991), and *P. vulgaris* is probably the most 'infectible' plant species in the *Leguminosae*. Yet, only about 20 different

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plant viruses are mentioned as important natural pathogens of common bean around the world (APS, 1991), and no more than half a dozen viruses affect common bean production in any given agricultural region of the world. This observation suggests that the existing common bean cultivars possess a broad spectrum of genetic resistance to many different plant viruses found in common-bean production areas around the world.

Due to the early adoption of common bean as a popular food crop in Europe and North America, the first viral disease of this legume was already observed towards the end of the XIX century, coinciding with the first report of a 'virus' (*Tobacco mosaic virus*) by Iwanowski (1894). However, it was not until 1917, that Stewart and Reddick (1917) called the causal agent 'bean mosaic virus'. This name was later modified (Pierce, 1934) by adding the epithet 'common', in order to distiguish it from a second virus found to induce mosaic in common bean: *Bean yellow mosaic virus* (BYMV). The 'mosaic' symptoms induced by *Bean common mosaic virus* (BCMV) vary considerably according to the bean genotype infected, the time of infection in relation to plant development, the environmental conditions, and the strain of BCMV that infects a common bean plant. Most BCMV-susceptible common bean genotypes express noticeable mosaic and leaf malformation symptoms when infected by this virus, but mild mosaic symptoms are also characteristic of infections in certain common bean genotypes. However, even symptomless BCMV infections can induce significant (>50%) yield losses in susceptible common bean genotypes (Zaumeyer and Thomas, 1957; Morales and Bos, 1988).

The search for BCMV-resistant common bean genotypes started in 1918, but a decade later, only one common bean genotype, 'Robust', had shown to be resistant to bean common mosaic (Reddick and Stewart, 1918; Rands and Brotherton, 1925). In 1935, Pierce (1935) pointed out that the BCMV resistance present in cultivar 'Robust', was conditioned by recessive genes. It would take four more decades before the painstaking and elaborate genetic study of Drijfhout (1978) demonstrated that resistance to BCMV in 'Robust' and other mosaic-resistant common bean cultivars developed in the early 1930s (Pierce, 1933, 1934) was determined by a strain-unspecific gene and at least one strain-specific recessive gene effective against the pathogenic BCMV strain inoculated. These recessive genes prevented the chronic systemic infection (common mosaic) of the resistant common bean genotype. Four recessive resistance genes in the host, some of which are allelic, interact with corresponding pathogenicity genes in seven BCMV strains, according to the gene-for-gene model of Flor (1956). On the basis of their susceptibility or resistance to the existing BCMV strains, Drijfhout (1978) created six groups of common bean cultivars, which may or may not be infected by at least one BCMV strain.

Bean common mosaic resistance had also been found in the early 1990s, in the common bean cultivar 'Corbett Refugee'. However, unlike the case of the common mosaic-resistant cultivar 'Robust', the resistance of 'Corbett Refugee' was shown by Pierce (1935) to be inherited dominantly. A decade later, Grogan and Walker (1948) demonstrated that common bean cultivars possessing this type of dominant common mosaic resistance, reacted to some BCMV strains with systemic vascular necrosis but not mosaic. This symptom, referred to as 'black root', had been previously described by Jenkins (1940) as a new disease of common bean. The 'black root' symptoms initially appear as local pin-point lesions, which later enlarge and give rise to vein necrosis originating in the local lesions. The youngest trifoliate leaves develop a net-like vein necrosis that extends down the stem in the form of necrotic streaks, eventually affecting the entire vascular system, including the roots (hence its name) and the pods if they are already formed. Affected plants wilt and die within a few days after the onset of systemic necrosis. Drijfhout (1978) confirmed earlier studies showing that 'black root' was a necrotic systemic reaction induced by the presence of a dominant gene (*I*), which Drijfhout called the 'necrosis' gene. This systemic necrosis reaction is only induced by 'necrosis-inducing' strains of BCMV (NL2 and NL6) and by all strains of *Bean common mosaic necrosis virus* (BCMNV-NL3, NL5 and NL8). The latter strains were previously considered as pathogenic variants of BCMV, until their molecular characterization showed them to be strains of a related but distinct virus species named BCMNV (Berger, et al. 1997). Another difference between the necrosis-inducing strains of BCMV and BCMNV is their different behaviour in relation to temperature (Drijfhout, 1978). BCMV NL2 and NL6 require high (> 26º C) temperatures to induce necrosis in *II* gene common bean genotypes (temperature-dependent), whereas BCMNV strains can cause 'black root' at lower temperatures (temperature-independent).

'Black root' is often considered a fatal disease of common bean, sometimes causing total yield losses in regions where bean cultivars possessing monogenic dominant resistance are attacked by necrosis-inducing BCMV or BCMNV strains, particularly BCMNV-NL3. However, 'black root' is a systemic hypersensitive response (HR) to the inoculation of necrosis-inducing strains of BCMV or BCMNV, and, therefore, it should be considered as a 'resistance mechanism'. The dominant necrosis gene (*I*) prevents the chronic systemic infection (mosaic) of common bean genotypes by BCMV or BCMNV strains, and blocks the primary mechanism of BCMV/BCMNV dispersal: their transmission via infected seed (Morales and Bos, 1988). One could also argue that HR prevents the second most important mode of BCMV/BCMNV dissemination: its transmission by several aphid species in a non-persistent manner (Morales and Bos, 1988).

However, as reported in the case of other viral pathosystems (Valkonen, 2002), HR does not prevent virus multiplication, but rather the movement and establishment of the virus into susceptible cells. Thus, although the virus can be recovered from non-inoculated trifoliate leaves of common bean plants inoculated on their primary leaves with BCMNV (F.J. Morales, *unpublished data*), the rapid development of 'black root' symptoms would limit the usefulness of necrotic common bean plants as virus sources for aphid vectors.

The dominant HR also confers 'broad spectrum resistance' in common bean to other legume potyviruses related to BCMV and BCMNV. In 1979, Tamayo et al. (1980) observed the typical hypersensitive reaction induced by necrotic strains of BCMV/BCMNV in dominant *I* gene bean genotypes, using a strain of *Soybean mosaic virus* (SMV) from the Cauca Valley, Colombia, to inoculate the primary leaves of the common bean cultivar 'Jubila' (*I bc-1*). Kyle and Provvidenti (1987) observed that a single allele at the *I* locus confers hypersensitivity to SMV, *Blackeye cowpea mosaic virus* (BlCMV), Cowpea aphid-borne mosaic virus, and watermelon mosaic virus. The latter two viruses are currently considered strains of BCMV and SMV, respectively (Berger et al. 1997). The HR can also be elicited by strains of *Peanut mottle virus*, sometimes resulting in very high incidences of 'black root' (F.J. Morales, *unpublished data*).

However, the incorporation of broad spectrum dominant resistance in common bean is not devoid of adverse side-effects. In certain common bean-producing regions of the world, particularly in East Africa, Europe and some temperate countries of the Americas, the frequency of BCMNV can be high enough to cause occasional but severe 'black root' outbreaks. Also, in Central America, Morales and Castaño (1992) reported on the occurrence of a systemic hypersensitive reactions in *I*-gene common bean cultivars infected by a comovirus related to *Cowpea severe mosaic virus*. This disease, referred to as 'severe mosaic', does not kill diseased plants as rapidly as 'black root', but it may eventually lead to plant death in common bean genotypes possessing dominant resistance to BCMV. Some *P. vulgaris* genotypes react with apical necrosis to the severe mosaic comovirus, but this reaction is induced by a different dominant gene (*Anv*) that is epistatic over the dominant gene (*Lnv*) that conditions the localized necrosis reaction known as 'severe mosaic' (Morales and Singh, 1997).

As mentioned before, one of the reasons for the global importance of BCMV and BCMNV as viral pathogens of common bean, is their ability to infect a high proportion (30-50%) of the plants derived from seed produced by systemically infected bean plants. Even a 1% level of seed infection, accepted in seed certification programs for BCMV, represents 2,500 BCMV-infected plants per hectare, as initial virus sources for aphid vectors. It was

also mentioned that common bean genotypes possessing the dominant *I* gene do not transmit either BCMV or BCMNV through their seed. Thus, genetic 'resistance to infection of the seed embryo or gametophyte tissue' can be considered a natural mechanism of resistance to plant viruses in certain plant species or plant genotypes. Morales and Castaño (1987) demonstrated that the incidence of BCMV/BCMNV transmission in common bean depends not only on viral determinants but also on host genetic determinants. In this investigation, five common bean cultivars transmitted four BCMV strains and one BCMNV strain to 40-54% of the progeny, whereas common bean genotypes Imuna, and the Great Northern lines 31 and 123, showed seed transmission incidences below 1%. Pinto 114 did not transmit BCMV-NY 15 in tests involving over 1,000 seeds collected from infected bean plants.

Viruses also have genetic determinants responsible for their transmission in the seed of their hosts. *Cucumber mosaic virus* (CMV) is one of the most ubiquitous viral pathogens in the world, due to its extremely broad host range, including *P. vulgaris*. Some strains of CMV can be seed-borne in common bean, and an investigation by Hampton and Francki (1992) using a pseudo-recombinant virus obtained by combining RNA components of a seed-transmitted and a non-seed-transmitted strains of CMV, showed that the genetic determinant for seed transmission was in RNA1. This component is primarily involved in viral replication and movement in the case of CMV. Perhaps, viruses uncapable of fast movement and replication in meristematic tissue cannot be seed transmitted.

Among the natural defence mechanisms displayed by *P. vulgaris* against plant viruses, it is worth mentioning the production of a diverse array of 'local lesions'. These localized reactions may appear as rings, spots, vein necrosis, discoloration, or the typical pin-point local lesions (Drijfhout, 1978). Most of these reaction are elicited by plants inoculated artificially with selected strains of BCMV, BCMNV or other related legume potyviruses, but common bean plants may exhibit some of these localized reactions under natural conditions. The expression of local lesions is generally associated with the presence of the dominant *I* gene in *P. vulgaris*, but ring-shaped lesions and localized vascular discolorations are characteristic of some common bean genotypes, such as 'Monroe', devoid of dominant resistance genes (Drijfhout, 1978). The expression of these localized reactions in non-*I*-gene common bean genotypes, does not necessarily preclude systemic invasion by the eliciting virus.

The expression of pin-point and similar localized lesions in *I* gene common bean genotypes, on the other hand, is extremely useful for the identification of genotypes possessing multiple resistance to both mosaic- and necrosis-inducing strains of BCMV and BCMNV. As mentioned before,

even though 'black root' is a HR, poor farmers in East Africa cannot afford to lose their common bean plantings to BCMNV or other related legume potyviruses that can elicit this systemic necrosis reaction in bean cultivars possessing monogenic dominant resistance conditioned by the *I* gene. To protect this gene, Drijfhout (1978) showed that the combination of strain-specific recessive genes, namely *bc-2*^{2}, which cannot be challenged by any known necrosis-inducing BCMV or BCMNV strain, an the dominant *I* gene, protected common bean genotypes from 'black root'. Common bean plants possessing the *Ibc-2*^{2} genotype, react with typical pin-point local lesions on artificially inoculated primary (cotyledonary) leaves.

'Immunity' to all known BCMV and BCMNV strains in common bean was found by Drijfhout (1978) in a line selected in Holland from a common bean accession maintained in New York State, U.S.A. The immunity of the Dutch line IVT 7214 was conferred by a single recessive gene (*bc-3*) for which no matching pathogenicity gene has been found to date in any BCMV or BCMNV strain.

BCMV probably co-evolved for centuries with *P. vulgaris* in its Latin American centers of origin before this legume was taken to other continents. However, the main BCMV strain found in Latin America is the type strain, the least pathogenic of all BCMV strains. In fact, most of the 30,000 accessions of *P. vulgaris* maintained in the main common bean germplasm collection at the International Centre for Tropical Agriculture (CIAT), Cali, Colombia, do not have any genes for resistance to BCMV. Yet, most common bean landraces throughout Latin America yield acceptably (over 500kg/ha), considering BCMV incidences between 60-100% under field conditions. Some common bean landraces planted year after year in the field despite a 100% incidence of BCMV, are so severely malformed that farmers believe that is their natural phenotype. Thus, 'tolerance' is probably the main mechanism of resistance to BCMV found in the main landraces still under cultivation in developing countries.

P. vulgaris is also commonly infected by some viruses that do not cause noticeable symptoms or yield losses. Bean southern mosaic and bean mild mosaic viruses are highly infectious viruses readily transmitted to common bean by mechanical means and by chrysomelid beetles (Morales and Gámez, 1989). The absence of noticeable symptoms, at least to the untrained eye, suggests that *P. vulgaris* can 'tolerate' these viruses. However, bean southern mosaic can cause significant yield losses in some sensitive common bean genotypes, and even in genotypes that express only mild symptoms. The number and weight of seed produced by infected plants can be significantly (>50%) reduced in the case of bean southern mosaic (Morales and Castaño, 1985). Hence, the term 'tolerance' as a natural mechanism of virus resistance has to be used with caution in these cases, because some of these mild

infections can significantly reduce yield, and neither the term 'resistant' nor 'susceptible', apply to these viruses that can reach very high titers in infected common bean plants despite the absence of noticeable symptoms. An alternative explanation may be that breeders have been unconciously selecting against common bean genotypes that show mild but appreciable virus-induced symptoms (leaf curling, chlorosis, etc), favouring the selection of genotypes with inapparent virus infections. The absence of noticeable symptoms characteristic of bean southern mosaic or bean mild mosaic, does not necessarily imply that their incidence is low. A few years ago, the author witnessed the complete destruction of several breeding materials obtained from interspecific crosses made between *P. vulgaris* and *P. acutifolius* at CIAT, Palmira, Colombia. Artificial inoculation of the parental tepary bean (*P. acutifolius*) accessions with *Bean southern mosaic virus*, showed this species to be highly sensitive to the virus.

As mentioned above, different legume potyviruses attack *P. vulgaris* under natural conditions, particularly in temperate countries. *Bean yellow mosaic virus* (BYMV) is the third most important potyvirus attacking *P. vulgaris* in the Americas (i.e. southern cone of South America and North America), Europe and Asia. The importance of BYMV as a common bean pathogen lies in its wider host range and greater pathogenic variability, compared to BCMV and BCMNV. Even 'mild' BYMV strains have an extended pathogenicity range and greater virulence in common bean genotypes used to differentiate BCMV/BCMNV strains; inducing mosaic, epinasty, dwarfing, vein and top necrosis, plant malformation, and even plant death. These symptoms occur both in *I* gene and non-*I* gene common bean genotypes, but bean genotypes possessing the dominant necrosis gene (*I*) usually display more severe symptoms, including systemic and top necrosis (F. Morales and M. Castaño, *unpublished data*). In Chile, South America, two Great Northern genotypes (31 and 123), Pinto 114, and Imuna, showed adequate levels of resistance to BYMV A simple cross between Great Northern 31 and a susceptible local black-seeded cultivar, led to the development of a BYMV-resistant cultivar (Cafati et al. 1976). Great Northern has also been used as a source of resistance to BYMV since the early 1950's (Thomas and Zaumeyer, 1953). Although some BYMV strains can overcome the apparently recessive resistance genes present in Great Northern genotypes (Tatchell et al. 1985), usually weak or incompatible reactions are observed between the virus and the host, leading to attenuated symptom development. This recessive resistance mechanism has been observed in other plant-virus interactions (Valkonen, 2002). Resistance to BYMV has also been identified in *P. coccineus* (Bagget, 1956). A single dominant gene, *By-2,* conditions this resistance (Dickson and Natti, 1968).

Another group of plant viruses that exhibit considerable pathogenic variability in *P. vulgaris*, is the cucumovirus genus. Fortunately, the use of molecular techniques to characterise different cucumovirus strains isolated from common bean (F.J. Morales, *unpublished data*), has shown that the majority of cucumovirus isolates belong to two different species: *Cucumber mosaic virus* (CMV), and *Peanut stunt virus* (PSV). Of the two cucumoviruses, PSV tends to predominate and cause more damage to susceptible common bean cultivars in temperate agricultural regions, where different legume species are inter-cropped. In areas planted with both cucurbits and common bean, CMV can easily jump from cucurbits to common bean plants. Resistance to mild strains of PSV have been identified in Great Northern, Red Mexican, Pinto and some navy bean cultivars (F. J. Morales, *unpublished data*). The main natural mechanism of resistance to both PSV and CMV is a localised hypersensitive response, although the HR does not preclude the systemic invasion of the plant by these viruses in many susceptible common bean genotypes. The author found a very aggressive natural PSV/CMV recombinant virus affecting common bean in Chile, South America (White et al. 1995).

Whitefly-transmitted geminiviruses (begomoviruses) are currently the main threat to common bean production in the lowlands and mid-altitude valleys of tropical and subtropical South America, Mexico, Central America and the Caribbean region (Morales and Anderson, 2000). These single-stranded DNA viruses replicate in the nuclei of infected cells, and cause severe plant damage and total yield losses in most common bean cultivars grown to date. Despite the evaluation of thousands of common bean genotypes available commercially and in gene banks, no immune genotypes have been identified so far against the main begomoviruses that affect common bean production in the region (Morales and Niessen, 1988).

Bean golden mosaic virus (BGMV) was first observed in 1961, in Brazil (Costa, 1965). Within the following two decades, this virus had become the most limiting problem of common bean production in eastern Brazil, northwestern Argentina and south-eastern Bolivia, due to the rapid expansion of soybean as a non-traditional export crop. Soybean is a suitable breeding host for *Bemisia tabaci*, the whitefly vector of begomoviruses. A closely relatedbegomovirus, *Bean golden yellow mosaic virus* (BGYMV), originally thought to be BGMV, infects common bean in southern Mexico, Central America and the Caribbean region (Morales and Anderson, 2000). *Bean dwarf mosaic virus* (BDMV), first described in Brazil (Costa, 1965), became economically important in the early 1980s, when it totally destroyed over 40,000 hectares of common bean plantings in northwestern Argentina (Morales et al. 1990). *Bean calico mosaic virus* (BCaMV) is a geminivirus transmitted by *B. tabaci* to common bean in northwestern Mexico (Brown

and Bird, 1992). This begomovirus was initially thought to be BGMV, but it was later shown to be a distinct virus species related to *Squash leaf curl virus* (Loniello et al. 1992). BCaMV causes widespread epidemics in common bean plantings in the states of Sonora, Sinaloa and Nayarit, Mexico (Morales and Anderson, 2000).

Initial attempts in Brazil, to breed common bean for BGMV resistance were disappointing. Pompeu and Krantz (1977) made individual plant selections of symptomless common bean plants found in BGMV-affected plantings of three common bean cultivars: Rosinha G2, Aetê 1, and Carioca 99. These symptomless plants were shown to be fully susceptible to the virus in subsequent evaluations (Costa, 1987). The presence of 'escapes' in common bean fields showing high BGMV incidence, is a common phenomenon associated with begomovirus attacks.

The failure to identify natural sources of BGMV resistance at that time, led Brazilian scientists to create atomic mutants (Tulman-Neto, 1979). One of these common bean mutants named TMD-1, showed partial resistance to the virus but its use in conventional breeding programs did not produce any outstanding progenies.

Another breeding project was initiated in 1974, to solve the bean golden yellow mosaic problem in Central America, Mexico and the Caribbean. Approximately, 7,000 germplasm bank accessions of common bean were evaluated under natural disease pressure in southeastern Guatemala, but no immune genotypes could be identified. However, a group of black-seeded genotypes, namely Turrialba 1, Porrillo 70, Porrillo Sintetico, ICA-Pijao and ICA-Tui, exhibited moderate levels of resistance to BGYMV (Yoshii et al. 1980). The natural resistance mechanism of these genotypes was thought to be tolerance (acceptable yield despite noticeable foliar yellowing), but systemically infected plants showed mosaic symptoms and distorted pods. A careful analysis of these black-seeded genotypes showed that yield was the result of low to moderate disease incidence in populations of these genotypes. Porrillo Sintetico and ICA-Pijao were ultimately selected, together with Turrialba 1, as potential parental materials. The best lines derived from different crossed between the selected parental genotypes: DOR 41 (Porrillo Sintético X ICA-Pijao), DOR 42 (ICA-Pijao X Turrialba 1) and DOR 44 (sister line from the cross ICA-Pijao X Turrialba 1), were soon released in Guatemala as cultivars ICTA-Quetzal, ICTA-Jutiapan and ICTA-Tamazulapa, respectively (Yoshii *et al.* 1980). In the absence of pesticide applications, ICTA-Jutiapan, ICA-Pijao and the local black-seeded land-race, Rabia de Gato, sustained yield losses of 38%, 53% and 86%, respectively. An artificial inoculation test using the best source of resistance, Porrillo Sintético, revealed that early (7-8 days after sowing) inoculation of this genotype resulted in 100% disease incidence, whereas inoculation 9-11

days after sowing, reduced disease incidence by 50%. This mechanism of resistance seems to be related to the considerable 'plant vigor' (rapid rate of vegetative growth) characteristic of these Mesoamerican, black-seeded genotypes. Additionally, most common bean genotypes show 'mature plant resistance' to begomoviruses, and, thus, virus inoculation during the reproductive phase of the plant often results in disease escape or moderate yield reduction.

Despite initial successes in developing BGYMV-resistant common bean genotypes, two constraints remained. First, yield losses fluctuated proportionally with viruliferous *B. tabaci* populations, and, secondly, there was no progress in breeding for BGYMV resistance in non-black-seeded common bean cultivars. This situation persisted for some years, with only some agronomic improvements to the first generation of black-seeded DOR lines, such as the recovery of 'earliness' (short life cycle) from the BGYMV-susceptible landraces. This physiological trait also contributed to lower disease incidence because early common bean genotypes reached maturity sooner and grew faster, resulting in 'disease escape'.

In the mid 1980s, a common bean line (A 429) developed at CIAT, Colombia, for superior upright architecture, showed an unexpected high level of BGYMV resistance under field conditions in Central America. An evaluation of its parental genotypes for their reaction to BGYMV, did not reveal genotypes possessing a high level of resistance. However, one parent, a common bean genotype belonging to the Mexican Durango race (Singh et al. 1991), did not react with the characteristic yellowing when inoculated with BGYMV, despite being systemically affected by plant malformation and severe flower abortion caused by the virus (Morales and Niessen, 1988). This common bean genotype, called 'Garrapato', soon became one of the most widely used sources of begomovirus resistance in common bean-breeding programs in Latin America (Singh et al. 2000). The gene *bgm-1* was shown to condition mosaic resistance in Garrapato (Morales and Niessen, 1988; Blair and Beaver, 1993). A new natural mechanism of resistance to disruption of the photosynthetic capacity (mosaic) induced by mosaic-inducing begomoviruses, had thus been identified in *P. vulgaris* (Morales and Niessen, 1988).

Later on, a red kidney line (DOR 303) was also selected for its high level of BGYMV resistance under field conditions in Central America. An evaluation of the parental materials selected to produce this line, revealed the presence of a red kidney genotype, Red Kloud, of Andean (race Nueva Granada) origin (Singh et al. 1991), besides the traditional black-seeded source of resistance, Porrillo Sintetico. Red Kloud was shown to exhibit 'tolerance' to BGYMV, producing flowers and pods despite the presence of striking mosaic/yellowing foliar symptoms (Morales and Niessen, 1988).

Based on this finding, several other common bean genotypes of Andean origin have been selected as sources of tolerance to whitefly-transmitted viruses. The retention of the flowering capacity of several Andean common bean genotypes infected by begomoviruses, is another natural mechanism of 'resistance to flower abortion' induced by begomoviruses in other races of common bean, such as the Mesoamerican and Mexican (Durango) races.

The interracial recombination of Mesoamerican and Andean genes produced a red-seeded common bean genotype possessing high levels of BGYMV resistance. The BGYMV-resistance gene in DOR 303 was later identified as *bgm-2* (Velez et al. 1998). Some Andean common bean genotypes also possess genes for 'resistance to the pod malformation', characteristically induced by BGYMV in susceptible bean cultivars (Morales and Niessen, 1988). Molina and Beaver (1998) reported on the existence of a dominant gene, *Bgp*, responsible for this trait, which apparently requires the presence of *bgm-1* for complete expression. Pod malformation affects critical yield components, such as the number of pods per plant and number of seeds per pod.

Based on the above findings, an extensive search for new sources of resistance was launched using the common bean collection maintained at CIAT. A selection of diverse grain types was evaluated in different countries of Latin America, from Argentina to northern Mexico, to identify different mechanisms of virus resistance and sources of resistance to begomoviruses infecting common bean in this region. At least 10 new sources of resistance were identified in the *P. vulgaris* accessions possessing grain colors different than black. The most interesting bean begomovirus-resistance mechanisms were: disease escape, low mosaic expression, hypersensitivity, reduced flower abortion, and resistance to pod malformation (Morales and Niessen, 1988).

The general combining ability of these traits was highly significant ($P<0.01$) and greater than values for specific combining abilities, suggesting that selection for the various traits was possible in true breeding lines, due to significant additive genetic variance (Morales and Singh, 1991). In subsequent studies, 83 recombinant inbred lines (RIL) selected from a population generated from the cross between a Mexican (Pinto UI 114) and a Mesoamerican (ICA-Pijao) common bean genotype, were evaluated for their reaction to BGYMV. Of these lines, 11 did not show symptoms, 24 lines had mean disease incidence of 8%, 28 lines had a disease incidence of 26.6% and developed intermediate mosaic symptoms, and 20 lines were more susceptible than either of the parents. Thus, values for the 83 RILs transgressed the reactions observed for the two parents, showing both higher and lower levels of disease incidence and mosaic expression ('transgressive segregation'). These results suggested that the BGYMV-resistance genes in

the two parental genotypes were different and complementary to each other and, consequently, that gene pyramiding may be a viable breeding strategy.

Subsequent interracial crosses produced highly resistant lines, which have become cultivars in different countries of Central America. Begomovirus replication in these improved genotypes was highly restricted during the initial stages of infection (up to 15 days after inoculation) according to nucleic acid hybridization tests performed on these lines (Morales, 2000). This type of resistance has also been associated to quantitative traits (QTLs), which reduce symptom expression (Miklas et al. 1996). On the contrary, common bean genotypes derived from intra-racial populations (*e.g.* DOR 41, DOR 390, DOR 500), usually behave as moderately resistant under severe whitefly/virus pressure (Singh, et al. 2000).

The 'hypersensitive response' (HR) is not a common natural resistance mechanism against begomoviruses, but it has been observed in a bean genotype of Mexican (race Durango) origin, Red Mexican 35, following its manual inoculation with BGYMV (Morales and Niessen, 1988).

Some of the sources of resistance to BGMV and BGYMV identified in *P. vulgaris* are also effective against the other begomoviruses that attack common bean. For instance, Azufrado Higuera is a new cultivar developed from Nueva Granada (Andean) sources of resistance (originally identified in South America) released in north-western Mexico to control *Bean calico mosaic virus* (Morales, 2000). The resistance mechanisms found in race Nueva Granada, are not only effective in controlling flower abortion due to virus infection, but also flower abortion caused by high temperatures during certain months of the year. Some common bean genotypes, such as Pinto 114 and Red Mexican 35, known sources of resistance to BGMV and BGYMV, are immune to *Bean dwarf mosaic virus* (Morales et al. 1990).

It is interesting to note that the above-mentioned common bean genotypes belonging to race Durango of *P. vulgaris*, have resistance to different whitefly-transmitted viruses. In fact, these and other bean genotypes from the central plateau of Mexico, show resistance to several other common bean viruses, including BCMV, BYMV, and CMV (F.J. Morales, *unpublished data*). However, only BCMV has been observed by the author to occur in their semi-arid region of origin in central Mexico. It is possible that these genotypes possess 'generalized stress tolerance', also referred to as 'rusticity' (White and Izquierdo, 1991), rather than virus-specific genes for resistance. These 'rustic' genotypes also possess other traits, such as 'earliness' and 'vigour', which have been observed to help plants escape virus infection.

The natural resistance mechanisms described here can be efficiently exploited in crop improvement programs, to prevent or reduce yield losses

caused by plant viruses known to attack common bean. Unfortunately, some of these resistance mechanisms are conditioned by multiple genes, which are not readily identified by molecular markers. In the absence of effective multiple gene tagging techniques and conventional breeding work under field conditions, some of these valuable natural resistance mechanisms are being under-utilised in current common bean improvement projects.

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Chapter B3

Virus Susceptibility and Resistance in Lettuce

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Introduction

Lettuce (*Lactuca sativa*) is a widely cultivated crop. Historically, the ancient Egyptians cultivated lettuce for its seed oil, which they believed had relaxing and aphrodisiac properties. Later, the Victorian English and others used its latex as a substitute for opium ("lactucarium"). Although stem lettuce is still cultivated in some Asian countries, lettuce is nowadays best known as a leafy vegetable and a raw ingredient in salads (Ryder, 1999; Maisonneuve, 2003). Lettuce is a member of the family *Asteraceae* in the subfamily *Cichorioideae* and the tribe *Lactuceae*. The family *Asteraceae* also contains such crops as endive, chicory, artichoke, sunflower, safflower and many ornamental plants such as *Chrysanthemum*, *Gazania*, *Osteospermum*, etc. Lettuce shows a broad phenotypic diversity with several distinct horticultural types identified such as crisphead (or iceberg lettuce), romaine (cos lettuce), leaf lettuce, Batavia and butterhead lettuce (Ryder, 1999; Maisonneuve, 2003). *L. sativa* is closely related to its common relative *L. serriola* L. (wild or prickly lettuce) and, more distantly, to two other wild species, *L. saligna*. and *L. virosa.* Lettuce is a naturally self-pollinating species so that the principal breeding strategies used with this species are pedigree breeding and back-crossing. Because it is possible to produce

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interspecific crosses between *L. sativa* and the three other species of the compatibility group (*L. serriola*, *L. saligna* and *L. virosa*), these have sometimes been used in lettuce breeding programs, in particular as sources of resistance to pathogens and pests.

Lettuce has been reported to be a natural host for a broad range of over 30 different plant virus species (Davies et al. 1997; Blancard et al. 2003). However, many of these viruses do not seem to have a significant impact on the crop outside of specific circumstances. This explains why germplasm screening and resistance breeding efforts have only been carried out (and sometimes in a very limited fashion) for about half of these agents (Robinson and Provvidenti, 1993; Davies et al. 1997; Blancard et al. 2003). Significant efforts have been invested in studies concerning infection of lettuce with a wide range of viruses. These include: *Lettuce mosaic virus* (LMV) and, to a lesser extent, other potyviruses such as *Turnip mosaic virus* (TuMV), *Bidens mottle virus* (BiMoV) and *Endive necrotic mosaic virus*; the viruses responsible for the lettuce big vein disease, *Mirafiori lettuce virus* and *Lettuce big vein virus*; the viruses inducing yellowing diseases, *Beet western yellows virus* (BWYV) and members of the family *Closteroviridae* such as *lettuce infectious yellows virus* (LIYV); *Tomato spotted wilt virus* (TSWV); *Cucumber mosaic virus* (CMV); *Broad bean wilt virus* (BBWV); *Dandelion yellow mosaic virus*, and *Tomato bushy stunt virus* (and closely related *Tombusviridae*) (Blancard et al. 2003).

For many of these viruses, screening and breeding efforts have met with only partial success as no complete resistance has been identified in the *L. sativa* species. Although breeders have sometimes identified better resistance sources in the wild species and are trying to introgress these into cultivated lettuce (Maisonneuve, 2003), most efforts have concentrated on the selection and breeding of less susceptible or partially resistant varieties. Such situations include breeding for resistance to the big vein disease (Bos and Huijberts, 1990; Ryder and Robinson, 1995; Fujii et al. 2003) or resistance towards viruses such as CMV (Provvidenti et al. 1980; Walkey et al. 1985), BWYV (Walkey and Pink, 1990; Pink et al. 1991; Maisonneuve et al. 1991), BBWV (Provvidenti et al. 1984), LIYV (Mc Creight et al. 1986; Mc Creight, 1987), and TSWV (O'Malley and Hartmann, 1989; Hartmann, 1991; Wang et al. 1992). These efforts have in many cases been at least partially successful. However, the genetic factors and mechanisms involved in these resistance phenomena remain completely unknown.

The situation with the potyviruses and in particular with LMV is somewhat different. On a worldwide basis, LMV is the most detrimental virus in lettuce crops in the absence of control measures (Dinant and Lot, 1992; Davies et al. 1997; Blancard et al. 2003; Le Gall, 2003). LMV may cause severe symptoms; it is seed-borne and highly transmissible by aphid

vectors (Dinant and Lot, 1992; Le Gall, 2003). As a consequence, extensive efforts have been made to breed LMV-resistant lettuce and some resistance determinants have been identified.

LMV and other Potyvirus resistance determinants in lettuce

Both recessive (Von der Pahlen and Crnko, 1965; Bannerot et al. 1969; Ryder, 1970) and dominant (Pink et al. 1992) resistance genes against LMV have been identified in lettuce. The same situation applies to two other potyviruses, TuMV and BiMoV. For TuMV a dominant gene (*Tu*), genetically linked to the downy mildew resistance gene *Dm5/8* on the second major cluster of resistance genes of lettuce, has been identified (Duffus and Zink, 1969; Zink and Duffus, 1973, 1975; Robbins et al. 1994). In the case of BiMoV the resistance found in the variety Valmaine has been shown to be recessive and to be conditioned by a single gene (*bi*) (Zitter and Guzman, 1977). The resistance afforded by *Tu* appears to be complete (no virus accumulation detectable) and effective against a broad range of TuMV isolates. This resistance is present in many varieties, but since it is linked with downy mildew (*Bremia lactucae* Regel) susceptibility alleles at the *Dm5/8* locus, efforts towards *Bremia* resistance have in some cases (mostly in Batavia or Iceberg-type lettuce) resulted in the elimination of the *Tu* gene and therefore in TuMV susceptibility (Blancard et al. 2003). Much less information is available on the *bi* gene but unpublished results from our groups indicate that it is different from the recessive LMV resistance genes *mo1*^{1} and *mo1*^{2} (see below).

In the case of LMV, three dominant resistance genes have been identified. One of them, *Mo2*, was observed in *L. sativa* germplasm (Pink et al. 1992a), while the two others, *Mo3* and *Mo4*, have been identified in *L. virosa* (Maisonneuve et al. 1999; Le Gall et al. 1999). *Mo2* has not been extensively studied or used since it is readily overcome by many LMV isolates, so that its usefulness for breeding efforts appears to be extremely limited (Dinant and Lot, 1992; Bos et al. 1994; Revers et al. 1997). *Mo3*, on the other hand, has a broad spectrum of activity against LMV isolates (Maisonneuve et al. 1999) and is associated with a very strong level of resistance or even to immunity (Le Gall et al. 1999). However, difficulties were encountered when trying to introgress it into *L. sativa* (Maisonneuve, 2003) and doubts about its potential field durability have so far hampered progress on its use for the protection of lettuce crops. *Mo4*, which has not been well characterized, conditions a hypersensitive reaction that blocks the spread of LMV in the inoculated plant (Fig. 1B). However, *Mo4* is overcome

in a significant proportion of the infected plants after a single passage (Le Gall et al. 1999).

The two known recessive LMV resistance genes, named $mo1^{1}$ and $mo1^{2}$, have been used widely and successfully by breeders and are the target of intensive research efforts (Dinant and Lot, 1992; Candresse et al. 2002; Le Gall, 2003). The $mo1^{1}$ gene was initially named *g* and identified in the variety "Gallega de Invierno" (Von der Pahlen and Crnko, 1965; Bannerot et al. 1969). It was used mostly by European breeders and introduced into many lettuce varietal types, including butterhead, Batavia, looseleaf, crisphead and cos lettuces (Pink et al. 1992b). The $mo1^{2}$ gene was first identified in PI251245 and a few other accessions of primitive cultivated *L. sativa* from Egypt (Ryder, 1970a and b). Originally named *mo*, the $mo1^{2}$ gene was subsequently introgressed into cultivated lettuce and mostly deployed by North American breeders in crisphead and cos lettuce. Confusion initially prevailed concerning the relationship between the $mo1^{1}$ and $mo1^{2}$ genes, which were at one time considered to be identical (Ryder, 1970b). They were later shown to have different protection specificities and recent evidence (see below) has demonstrated them to be allelic (Dinant and Lot, 1992; Pink et al. 1992b).

The resistance phenotypes of the $mo1^{1}$ and $mo1^{2}$ genes

LMV causes a variety of symptoms in lettuce including leaf deformation, dwarfing, vein clearing, mosaic, mottling or necrosis of the leaves, and defective heading (Figs. 1A, 1D, 1E) (Dinant and Lot, 1992). Symptoms show substantial variability and depend on the cultivar, the developmental stage at which the plant becomes infected and the environmental conditions. Although symptoms on leaves are usually conspicuous, in some horticultural types such as red cultivars or light green Batavia-type cultivars with savoyed and irregular leaves, they may be much harder to observe (Dinant and Lot, 1992), requiring either field experiments (Walkey et al.. 1985a) or repeated observations under controlled conditions in growth chambers (Bos et al. 1994; Revers et al. 1997).

The variability and complexity of LMV infection symptoms is further modified by the resistance phenotypes of the $mo1^{1}$ and $mo1^{2}$ genes. For most LMV isolates, such as those of the LMV-Common group (Krause-Sakate et al. 2002), the *mo1* genes generally afford only tolerance, and symptomless virus accumulation is usually observed in varieties bearing these genes (Fig. 1C) (Walkey et al. 1985a; Dinant and Lot, 1992; Bos et al.. 1994; Montesclaros et al.. 1997; Irwin et al. 1999). However, in some varieties a complete absence of virus accumulation can be observed in part of the inoculated plants (Revers et al. 1997). In most, but not all, virus-cultivar combinations where viral accumulation is observed, the viral concentrations

reached in *mo1*-carrying varieties are generally lower than those observed in varieties lacking the *mo1* genes (Walkey et al. 1985a; Bos et al.. 1994; Pink et al. 1992a; Pink et al. 1992b; Revers et al. 1997). In addition, experiments with GUS or GFP-tagged recombinant viruses have indicated that viral accumulation is always detected in the inoculated leaves of resistant plants (Fig. 1C) (German-Retana et al. 2000) so that failure to detect systemic viral accumulation corresponds to a blockage in viral long distance movement. Precise measurements also seem to indicate that the cell-to-cell spread of

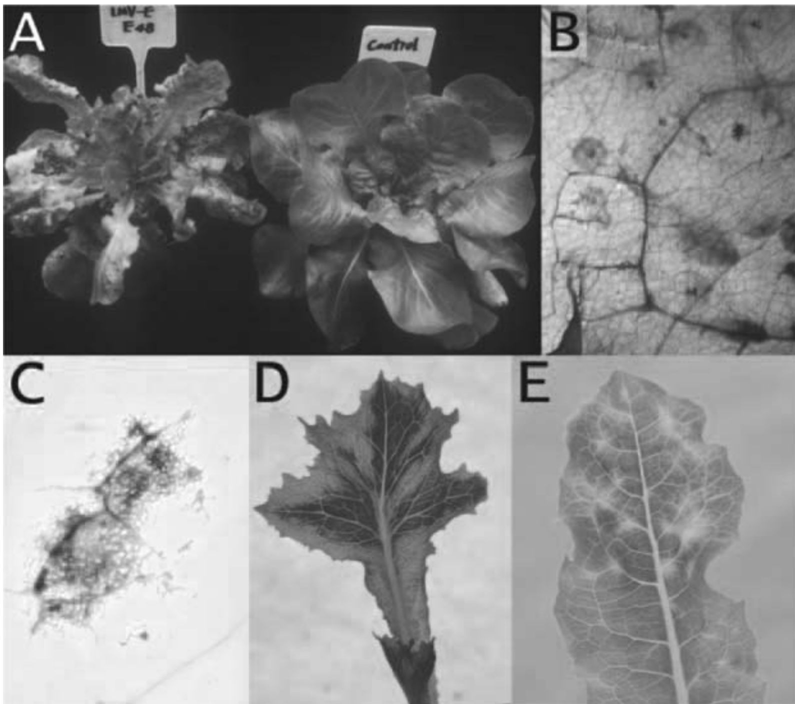

Fig. 1. Phenotypes of LMV infection in lettuce. A: typical mosaic symptoms induced by LMV-E in a susceptible butterhead lettuce (Trocadéro). **B**: necrotic local lesions limit the spread of a GUS-tagged LMV in rub-inoculated leaves of *Lactuca virosa* containing the *Mo4* gene. **C**: accumulation of a GUS-tagged LMV-Common (non-resistance-breaking) isolate in an inoculated leaf of a $mo1^2$ crisphead variety (Vanguard 75). **D**: mosaic symptoms induced by LMV-Most (resistance-breaking) in a $mo1^2$ crisphead lettuce (Vanguard 75). **E**: vein clearing and "star" symptoms induced by LMV-E (resistance-breaking) in a *mo1* crisphead breeding line. (See also Colorplates, p. xviii)

these recombinant viruses is slowed in resistant varieties as compared to susceptible varieties (German-Retana et al. 2000).

With a few LMV isolates that overcome only the *mo1*^{1} allele, such as LMV-1 or LMV-9, the *mo1*^{2} allele confers a true resistance with no detectable virus accumulation in the resistant cultivars (Dinant and Lot, 1992; Bos et al. 1994; Revers et al. 1997). Such isolates, with their differential behaviour towards *mo1*^{1} and *mo1*^{2}, provided the first evidence that these genes are not identical (Dinant and Lot, 1992).

Even when virus is detected in the resistant plants, the *mo1* genes afford protection against the expression of the typical LMV symptoms. The level of this protection is variable and depends, in particular, on the genetic background of the plants. In some cases, no symptoms are observed while in other varieties residual symptoms can be observed, in the form of more or less pronounced pinpoint or "star" chlorotic lesions (Walkey et al. 1985a; Dinant and Lot, 1992; Pink et al. 1992a; Bos et al. 1994; Revers et al. 1997; Irwin et al. 1999). Additionally, the presence of the *mo1* genes has been shown to abolish completely or to inhibit markedly seed transmission of LMV-Common isolates (Marrou et al. 1969; Ryder, 1973; Falk and Guzman, 1984; Dinant and Lot, 1992).

The *mo1* genes can, therefore, be considered to be both resistance and tolerance genes, depending on the viral isolate with which they are confronted, and probably the genetic background of each individual variety (Dinant and Lot, 1992; Bos et al. 1994). One consequence of these unusual properties is that evaluation of resistance to LMV has sometimes posed significant problems and led to confusing results since in a number of situations both viral detection (such as by ELISA assays) or the visual assessment of symptoms may be unreliable to determine the "resistance" status of plants. For example, using some of the same mapping populations, Irwin et al. (1999) unambiguously mapped the *mo1*^{2} resistance to a single locus while in an earlier study Montesclaros et al. (1997) had concluded that resistance was probably controlled by more than one gene.

Recently, Mazier et al. (2004) have shown that reliable tests to evaluate the LMV resistance status can be carried out on *in vitro* cultivated lettuce plantlets. Although not widely used yet, such an approach can provide consistent results within 2-3 weeks after inoculation as well as appropriate containment when working with genetically modified plants.

Identification of mo1^1 *and mo1*^{2}

Nicaise et al. (2003) recently identified the *mo1*^{1} and *mo1*^{2} genes and showed that they encode variants of the cellular translation initiation factor eIF4E. This identification was based on three converging lines of evidence:

(1) allelic sequence co-variation between the eIF4E gene and the *mo1¹* and *mo1²* resistance status of the plants; (2) co-segregation of mutations in the eIF4E gene and the *mo1¹* and *mo1²* resistance status and finally, (3) functional complementation using a viral transient expression vector to restore LMV susceptibility in *mo1¹*- or *mo1²*-carrying lettuce plants using the eIF4E allele from susceptible plants. These results led to the final demonstration of the allelic nature of *mo1¹* and *mo1²* and to the identification of the differences separating the susceptibility alleles from the resistance allele (Nicaise et al. 2003). The *mo1¹* allele is characterized by a deletion of three amino acids (Glutamine-Glycine-Alanine) at positions 108-110 that are replaced by a single Histidine residue and by a single nucleotide polymorphism (SNP) changing an Alanine to a Serine at position 186. The

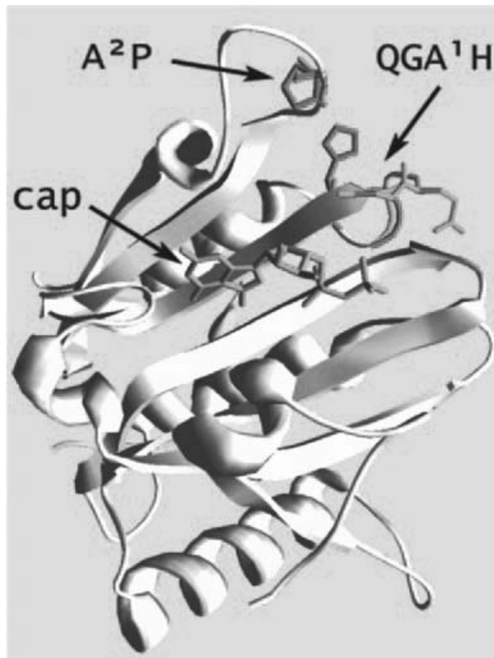

Fig. 2. 3D modelling of lettuce eIF4E. The structure is predicted based on that of its mouse homologue (see Nicaise et al. 2003). The predicted differences between the *mo1¹* ("QGA¹H") or *mo1²* ("A²P") forms and the susceptibility allele are shown in red versus green. The cap analogue ("cap") is shown bound in the cap-binding pocket. The yellow helix in the background is the portion of the eIF4G protein known to interact with eIF4E for translation initiation. (See also Colorplates, p. xxi)

mo1² allele corresponds to a SNP changing an Alanine to a Proline at position 70. X-ray crystallography data describing the structure of the mouse eIF4E has made possible the 3-D modelling of the lettuce protein. Remarkably, although they affect distant portions of the primary sequence of the lettuce eIF4E protein, the mutations present in the *mo1¹* and *mo1²* alleles alter portions of the protein that are located close together when considering the three dimensional structure of the lettuce eIF4E molecule (see Fig. 2). These two mutations were predicted to be located in exposed loops near the cap-recognition pocket of the eIF4E protein.

It appears, therefore, that despite the fact that the phenotypes of the two *mo1* genes may differ, they both encode eIF4E variants, providing final proof of their allelic nature. Similarly, recent findings concerning *Potyvirus* resistance mediated by recessive genes in other plants such as tomato, pepper (Ruffel et al. 2002) and pea (Gao et al. 2004) have demonstrated that alleles of the gene encoding eIF4E can also confer resistance. In addition, inactivation by point mutations or by T-DNA insertion of a gene encoding an isoform of eIF4E, eIF(iso)4E, in *Arabidopsis thaliana* resulted in loss of susceptibility to a range of potyviruses including *Tobacco etch virus*, TuMV and LMV (Lellis et al. 2002; Duprat et al. 2002). These latter results demonstrate that mutant alleles of eIF(iso)4E can confer resistance to infection by several potyviruses and, therefore, correspond to recessive resistance genes.

The best explanation for these results is that eIF4E or its isoform(s) is (are) recruited by potyviruses to act as host factors to aid plant infection. Thus, unavailability (knock-out mutants) or unsuitability (point mutants) of these factors disrupts the normal plant invasion processes and results in resistance or, more correctly, lack of susceptibility to the virus. Although several hypotheses have been proposed as to what the role of eIF4E in the infection processes might be (Lellis et al. 2002) there are currently no precise data to support these ideas. Further studies are clearly needed in order to reach an understanding of the mechanism(s) underlying this seemingly crucial and possibly general interaction phenomenon between plants and potyviruses. Current research (MM) is directed towards extending our understanding through the study of transgenic lettuce lines constitutively over-expressing several eIF4E genes. In addition to the expected functional complementation obtained with susceptibility alleles from lettuce (R. Sanjuan et al. manuscript in preparation), some phenotypic modifications in the reaction to LMV inoculation have been observed with heterologous eIF4E alleles (M. Mazier et al. manuscript in preparation).

Breaking of resistance mediated by mo1 genes

The *mo1* resistance genes have proven thus far highly durable. Although

breeders in most parts of the world have extensively deployed them, they have generally retained a high level of efficacy (Dinant and Lot, 1992; Blancard et al. 2003). However, the existence of both *mo1*[1] and *mo1*[2] resistance-breaking isolates was described relatively early on (Pink et al. 1992a, b; Dinant and Lot, 1992). A large scale analysis of LMV isolates from all over the world (Krause-Sakate et al. 2002) showed that resistance breaking can be observed in at least three types of LMV isolates. Firstly, in unusual, geographically limited and non-seed-borne isolates such as those isolated in the Yemen Arab Republic or those of the minor phylogenetic group occurring in Greece; secondly, in rare isolates such as E, 1, and 9 belonging to the major phylogenetic group of LMV isolates, and which appear not to be seed-borne and thirdly, in a frequently observed group of very closely related, seed-borne isolates that form a coherent cluster within the major phylogenetic group of LMV isolates (Krause-Sakate et al. 2002). Isolates of this third cluster, which have been collectively named MOST isolates (**mo**-breaking, **s**eed-**t**ransmitted: Krause-Sakate et al. 2002), appear to have recently been widely distributed in many parts of the world through the trading of contaminated seed lots. This also provided opportunities for the appearance of natural genetic shuffling with the LMV-Common type (Krause-Sakate et al. 2004). Fortunately, the efficient seed control measures applied in the major lettuce growing areas in Europe and North America has thus far limited the impact of these isolates. To date, only the *Mo3* gene identified in *L. virosa* appears to provide an efficient protection against all these *mo1*-breaking isolates (Maisonneuve et al. 1999).

Reverse genetic analysis of the viral resistance-breaking determinants using full-length infectious clones of LMV (Yang et al. 1998) have demonstrated that a part of the viral polyprotein encompassing the last 52 C-terminal amino-acids of the cylindrical inclusion (CI) gene, the 6K2 gene and the first 345 N-terminal amino-acids of the viral protein covalently linked to the 5’ end of the genome (VPg) contain the resistance-breaking determinant(s) (Redondo et al. 2001). These results point to a significant role for the VPg in controlling resistance-breaking. This is similar to what has been reported in several other plant-*Potyvirus* systems involving the breaking of resistance mediated by recessive genes (Nicolas et al. 1997; Schaad et al. 1997; Masuta et al. 1999; Borgstrom and Johansen, 2001; Moury et al. 2004). Interestingly, a physical interaction between the VPg and the eIF4E or eIF(iso)4E has been demonstrated by various methods for several plant-*Potyvirus* systems (Wittmann et al. 1997; Schaad et al. 2000; Leonard et al. 2000, 2004; our own unpublished results). However, the detailed analysis in these systems of the interactions between the VPg from normal or from resistance-breaking viral isolates with the susceptibility or resistance eIF4E alleles has not so far demonstrated a direct link between the

existence or strength of the VPg-eIF4E interaction and the ability of a particular viral isolate to infect a plant carrying a particular allele, raising questions about the significance of this interaction.

More recent results from our laboratory (T. Guiraud et al., manuscript in preparation) do, however, demonstrate that in the case of the LMV-E resistance-breaking isolate, different viral determinants are involved in the overcoming of *mo1*^{1} *versus* *mo1*^{2} resistance. In addition, these results surprisingly show that resistance-breaking determinants are found both within the CI and within the VPg regions. These last results suggest that although there are common factors shared between the *mo1*-LMV interactions and other plant-*Potyvirus* interactions involving recessive resistance genes, there may also be subtle but significant differences between them.

Genes identified as modifiers of the LMV-lettuce interaction

As indicated earlier, both the symptoms of LMV infection in susceptible varieties and the LMV infection phenotype occurring in plants that possess the *mo1* resistance genes can be substantially affected by additional genes carried by the lettuce variety being analyzed. The gene(s) behind these modulations of the LMV-lettuce interaction are largely uncharacterized. However, in recent years breeders have made some progress towards the identification of such background genetic factors.

For example, in the case of symptom expression in susceptible cultivars, Ryder (2002a) identified a dominant gene, named *Ne*, which controls the appearance of a necrotic reaction upon LMV inoculation instead of the mosaic/mottling symptoms, which develop in *ne* plants. Meanwhile, in plants possessing the *mo1* resistance gene, two different phenotype modifiers have been characterized, which when present in a variety that contains one of the *mo1* alleles further increase the resistance level and leads to a so-called “highly resistant” or HRes phenotype. In such plants, LMV accumulation is usually not observed, nor is the minor “star” symptom that sometimes occurs in plants containing only the *mo1* gene. Genetic analysis has been carried out for at least two sources of *mo1* phenotype modifiers, an accession from Egypt, “Balady Aswan Green” (BAG) (Ryder, 2002b), and the PI226514 accession (Hayes et al. 2004). Surprisingly, the results obtained show that very different modifier genes for the HRes phenotype occur in the two accessions. A dominant gene (which has been named *Mi*) is responsible in BAG, while in PI226514 the HRes phenotype appears to be conferred by a recessive gene.

None of these “modifier” genes has thus far been mapped on the LMV genome or identified and the infection phenotypes as modified by these genes are still rather poorly described. However, it appears that *Mi* alone in a

susceptible background significantly reduces the accumulation of non-resistance-breaking LMV isolates (unpublished observations, TC). Clearly, the identification of such genes would further our understanding of lettuce-LMV interactions and could potentially shed light on the mode of action of the *mo1* genes.

Conclusion

Although breeding for resistance against many different viruses in lettuce has been going on for many years, and in many cases allowed the development of resistant or less susceptible lettuce varieties, there appears to still be little knowledge concerning the genetic and molecular bases of the resistance manipulated by the breeders. In the case of potyviruses in general, and of LMV in particular, more extensive efforts have led to the genetic characterization of both dominant (*Tu* resistance to TuMV, *Mo2* to *Mo4* resistances to LMV) and recessive (*bi* resistance to BiMoV, *mo1* resistance alleles to LMV.) resistance. It has also led to the characterization of genetic factors, which modulate the LMV-lettuce interaction (*Ne*, *Mi*, recessive gene of PI226514). The recent identification of the cellular translation initiation factor 4E (eIF4E) as the product of the *mo1* resistance gene (Nicaise et al. 2003) has provided a new framework for understanding the LMV-lettuce interactions. Of particular interest is the observation that eIF4E or its isoform(s) appear(s) to play a key role in a number of *Potyvirus*-plant interactions. This significance of eIF4E is further strengthened by recent results involving a completely unrelated plant virus, *Cucumber mosaic virus* (CMV), which demonstrated that two genes controlling the level of CMV accumulation in *Arabidopsis* corresponded to the plant eIF4E and eIF4G genes (Yoshii et al. 2004). A clear understanding of the role(s) played by these proteins in the infection cycle of these viruses should quickly emerge from work undertaken in a variety of laboratories. Conceivably, genes that are identified as modifiers of the phenotypes observed in *mo1* plants could encode host proteins involved, together with eIF4E in these still unknown mechanisms in the viral the infection cycle.

Whatever the underlying mechanisms, the *mo1* genes have demonstrated an excellent durability in practical terms, with the possible exception of the problems presented by the LMV-MOST isolates (Krause-Sakate et al., 2002). These seed-borne resistance-breaking isolates have already been widely dispersed by the movement of contaminated seed lots and unless specific control measures are enforced to avoid their further spread, these isolates present a clear threat to the long-term use of the *mo1* resistance, for which no alternative is currently available.

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Chapter B4

Resistance to *Tobacco Mosaic Virus* and *Tomato Mosaic Virus* in Tomato

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Introduction

Tobacco mosaic virus (TMV) and *Tomato mosaic virus* (ToMV) cause a serious disease in tomato, with systemic mosaic symptoms and losses in fruit yield and quality. Both viruses are closely related tobamoviruses, plus stranded RNA viruses with a rod like particle structure. The genomic structure of TMV and ToMV has been well characterized, as a positive-sense single-stranded RNA genome that encodes at least four proteins (Goelet et al. 1982; Ohno et al. 1984; Canto et al. 2004). The 130 kDa methyltransferase/helicase and the 180 kDa RNA dependent RNA polymerase are translated directly from the genomic RNA using the same first initiation codon, the latter is synthesised by the read-through of the amber termination codon of the 130 kDa protein gene. The movement protein (MP) and the coat protein (CP) are translated from their respective subgenomic mRNAs, which are synthesised during the replication cycle. Involvement of the 130 kDa and 180 kDa proteins in intracellular replication has been demonstrated by deletion or substitution mutants of each protein (Ishikawa et al. 1986). It has also been shown that the MP is involved in cell-to-cell transport (Meshi et al. 1987), and that the CP is involved in long-distance movement (Saito et al. 1990; Hilf and Dawson, 1993). In tomato, TMV infection is more or less a rare event because the virus is soon competed out in tomato populations by ToMV, which is more adapted to

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this host plant. Both viruses are readily sap transmitted but can also be transmitted through root infection from contaminated soil. ToMV is present in the external mucilage, testa and sometimes endosperm of tomato seeds, but was not proved to be within the embryo (Broadbent, 1976). The percentage of contaminated seeds varies greatly in different fruits; up to 94% of the seeds may contain the virus (Broadbent, 1976). Because ToMV infection is aggressive and highly contagious, many breeding programs were started to find sources of resistance against this virus. So far, three dominant resistance genes have been found in wild *Lycopersicon* species and introduced into commercial tomato lines: ***Tm-1*** (from *Lycopersicon hirsutum*), ***Tm-2*** and ***Tm-2^2*** (both from *Lycopersicon peruvianum*) (Pelham, 1966; Pelham, 1972).

Tm-1

Breeding of ToMV resistance based on the Tm-1 gene

The first report of any form of resistance to ToMV in the genus *Lycopersicon* came from infection experiments with plants of *L. hirsutum* grown from seeds collected in South America. Infected plants were symptomless even though low levels of virus were detected in their tissues (Porte et al. 1939). In the following decades many plant breeders tried to transfer this resistance gene from *L. hirsutum* into *L. esculentum* (for a review see Pelham, 1966). A work of major importance was started in 1941 in Hawaii (Frazier et al. 1946) with the aim to transfer resistance from *L. chilense* and *L. hirusutum* to *L. esculentum*. This breeding program yielded useful breeding stock for a number of different attempts to obtain new resistance sources. Holmes (1954) used the back cross method for transferring resistance from the Hawaii material into a susceptible variety of *L. esculentum*. The gene was later given the symbol *Tm-1*. In 1960 a back cross breeding programme was started at the Glasshouse Crops Research Institute in Sussex, England aiming to incorporate ToMV resistance into commercial tomato varieties. *Tm-1* was included in this breeding programme. A homozygous line was generated and the resistance gene was mapped on chromosome 5 (Pelham, 1972).

Characterisation of the resistance mechanism of Tm-1

Major contributions concerning the understanding of the resistance mechanism of *Tm-1* were made by Motoyoshi and Oshima (1977, 1979) and Fraser and coworkers (Fraser and Loughlin, 1980; Fraser et al. 1980). Motoyoshi and Oshima isolated protoplasts from Pelham's breeding lines, which contained the *Tm-1* resistance gene in a homozygous form in the genetic background of the tomato line "Craigella". By infecting the protoplasts with ToMV they showed, that the *Tm-1* resistance is not only found in differentiated tomato plants but also in isolated protoplasts. This was surprising, because many classical resistance genes like N' are not active in protoplasts but rather depend on differentiated tissue to induce a defence reaction (Pfitzner and Pfitzner, 1990). Furthermore, Motoyoshi and Oshima could show by inoculation of tomato plants homozygous for *Tm-1* with ToMV RNA, that this resistance is also effective against infection with RNA inocula. This indicated that the *Tm-1* resistance somehow interferes with ToMV RNA replication rather than virus uncoating. These experiments were confirmed by Fraser and co-workers (1980) who performed infection experiments with tomato plants without any resistance gene (GCR 26, +/+), plants heterozygous for *Tm-1* (J484, *Tm-1*/+), or tomato plants homozygous for *Tm-1* (GCR 237, *Tm-1*/*Tm-1*) with ToMV. Furthermore, they could show that inhibition of virus replication by the *Tm-1* gene is gene dose dependent. Thus, while in homozygous *Tm-1* tomato plants, virus RNA accumulation was reduced down to 1% in comparison to susceptible tomato lines, heterozygous plants only show a reduction to 10%. This is in good agreement with the data from tomato protoplast infections obtained by Okada and co-workers (Yamafuji et al. 1991). In addition, Fraser and co-workers (1980) showed, that the virus responsible for the low level ToMV replication in *Tm-1* plants is not a resistance breaking virus strain, but that the low background replication rate is still possible in the presence of the *Tm-1* gene. Furthermore, they demonstrated that the *Tm-1* resistance also suppresses symptoms.. *Tm-1*/*Tm-1* and *Tm-1*/+ tomato plants show no viral symptoms like leaf mosaic or malformation of tomato fruits, although a considerable amount of ToMV (up to 10 % of the wild type rate) was found in the heterozygous plants. This feature made *Tm-1* even more attractive for plant breeders.

Tm-1 resistance breaking ToMV strains

Soon after the introduction of *Tm-1* into commercial breeding lines ToMV strains were observed which were able to overcome the *Tm-1* gene. In fact, it turned out that *Tm-1* breaking ToMV strains occur quite frequently and could be isolated from many different locations where tomato plants are

grown (for a review see Pelham, 1972). These ToMV strains caused severe mosaic symptoms on *Tm-1*/*Tm-1* plants (Pecaut, 1966), and could replicate in protoplasts of tomato plants homozygous for the *Tm-1* gene to even higher levels as in wild type tomato plants (Motoyoshi and Oshima, 1979). After the cDNA cloning and sequencing of the RNA genome of TMV (Goelet et al. 1982) and ToMV (Ohno et al. 1984), the tools were available to determine the molecular basis of the *Tm-1* resistance-breaking phenotype. Several *Tm-1* breaking ToMV strains were sequenced by Okada and co-workers (Meshi et al. 1988) and Pfitzner and co-workers (Strasser, 2002). Comparison of the nucleotide sequence of all these virus strains and also of the deduced amino acid sequence of the respective viral proteins revealed that all *Tm-1* breaking ToMV strains contained amino acid exchanges in the overlapping open reading frames of the 130 kDa /180 kDa replication proteins (Fig. 1). In fact, all amino acid exchanges are found in a small region of about 150 amino acids at the C-terminus of the 130 kDa methyltransferase/helicase protein. Mutation analysis of these ToMV strains revealed that at least two amino acid (aa) exchanges (aa 979 Gln > Glu and aa 984 His > Tyr) are necessary to overcome the *Tm-1* resistance. Single substitutions resulted in secondary, compensatory amino acid exchanges in this area of the 130 kDa / 180 kDa proteins (Meshi et al., 1988). However, amino acid 979 (Gln) can not only be replaced by glutamic acid, but also by lysine or aspartic acid and to a minor extent by arginine and asparagine (Hamamoto et al. 1997). The resulting virus is a viable ToMV strain, which is able to overcome the *Tm-1* resistance gene. These results indicate that the region between amino acid 900 – 1100 is not important for the function of these proteins in the replication complex but is important for the interaction with the putative *Tm-1* gene product. However, the *Tm-1* gene product must be an integral part of each replication complex, because a *trans*-complementation is not possible. *Tm-1* breaking ToMV strains are not able to rescue the replication of wild type ToMV in protoplasts from tomato plants containing the *Tm-1* gene (Yamafuji et al. 1991).

```
aa-Pos.    940                 970 971                 990
130.0      INRVTGFPYPA--//--RCP ADVTHFLNQRYEGHVMCTSS
130.1      INRVIGSPYPA--//--RCP ADVTHFLNERYEGYVMCTSS
130.Lta1   INRVTGFPYPA--//--RCP ADVTHFLNERYEGYVMCTSS
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Fig. 1. Amino acid sequence of the C-terminus of the 130 kDa protien of wild type ToMV (130.0, Ohno et al.1984) , and of two *Tm-1* breaking ToMV strains (130.1, Strasser, 2002 and 130.Lta1, Meshi et al. 1988). Amino acid exchanges are depicted in bold letters.

Tm-2 and Tm-2^2

Breeding of ToMV resistance based on Tm-2 and Tm-2^2

A second dominant gene for resistance to ToMV was isolated from the Hawaiian breeding lines by Soost (1958, 1959, 1963). It was reported to confer a higher level of ToMV resistance than *Tm-1* and was allocated to chromosome 9. It was given the symbol *Tm-2*. Unfortunately, an undesirable recessive gene, "netted-virescent" (*nv*), which caused stunting and yellowing in the homozygous condition was found to be tightly linked to it (Clayberg, 1959). Attempts to break this linkage were unsuccessful but a new source of *Tm-2* was located in *L. peruvianum* by Laterrot and Pecaut (1969), which did not contain the *nv* gene.

An additional gene for resistance to ToMV infection was selected from a cross with *L. peruvianum* P.I. 128650 (Alexander, 1963). The resistance was found to be due to a single dominant gene. Pecaut (1965, 1966) and Schroeder et al. (1967) studied the allelic relationship of the gene *Tm-2* and the new resistance factor. Both were shown to be on chromosome 9, either at the same locus or extremely closely linked. Because of the apparent allelism with *Tm-2,* the gene symbol *Tm-2^2* was proposed.

Characterisation of the mechanism of Tm-2 and Tm-2^2

The first information on the possible resistance mechanism of *Tm-2* and *Tm-2^2* came from infection experiments done by Pelham (1964). He found that both resistance genes are effective against ToMV. Sometimes, however, a necrotic reaction occurred in both genotypes in response to infection by common strains of the virus. This reaction was of two types, either local necrotic lesions or systemic necrosis. The former usually appeared on inoculated leaves within four days of inoculation and is regarded by most breeders as a hypersensitive reaction - an expression of resistance. The other form of necrosis, the systemic reaction, is considered to be a consequence of incomplete dominance. It is shown slightly by *Tm-2* and particularly by *Tm-2^2* genotypes at higher temperatures. Many plant breeders also refer to this type of necrotic reaction as systemic hypersensitivity, even though the term hypersensitivity normally refers to systems where pathogens are localised in necrotic lesions. The development of the necrotic phenotype also depends on the gene dose. ToMV produces no necrosis at any temperature on *Tm-2/Tm-2* plants but it does on *Tm-2*/+ plants at 30°C. Systemic necrosis appears on *Tm-2^2* /+ plants at 25°C and on *Tm-2^2/Tm-2^2* tomatoes at 30°C.

Another line of information concerning the mode of action of the *Tm-2* alleles came from protoplast infection experiments. Motoyoshi and Oshima

(1975, 1977) found that leaf mesophyll protoplasts from isogenic lines of *Lycopersicon esculentum* cv. Craigella carrying *Tm-2/Tm-2* or *Tm-2*^{2}/*Tm-2*^{2} were as readily infected by ToMV as those without any *Tm* gene. ToMV growth and final yield of the virus between the three types were not significantly different. However, virus multiplication in leaf discs was limited by the presence of *Tm-2* and *Tm-2*^{2}. These results suggested that the genes might be acting to prevent cell-to-cell movement of virus.

Resistance breaking ToMV strains for Tm-2 and Tm-2^2

McRitchie and Alexander (1963) in Ohio, USA identified four tomato strains of TMV (ToMV), designated I, II, III and IV, by the reactions produced by one resistant line of cultivated tomato and three accessions of *L. peruvianum*. Further characterisation of these naturally occurring ToMV strains by Pelham (1972) revealed that Alexander strains I and II cannot overcome *Tm-1* or *Tm-2* and were classified as ToMV-0. Alexander strain III is able to overcome *Tm-1* and was named ToMV-1. Alexander strain IV was able to infect T*m-2* containing tomato plants and was designated ToMV-2. Several other *Tm-2* breaking ToMV strains were recognized and characterized in detail (Pelham 1972; Hall, 1980).

To analyse the molecular interaction between ToMV and the *Tm-2* resistance genes, different *Tm-2* breaking viruses from Italy (Strasser, 2002), from Japan (Meshi et al. 1989) and from the Netherlands (Calder and Palukaitis, 1992) were sequenced. All these ToMV strains contained nucleotide exchanges in the ORF coding for the 30 kDa MP in comparison to ToMV-0, which resulted in amino acid substitutions (Fig. 2). Interestingly, there is one amino acid exchange (aa 133 Glu > Lys) found in all *Tm-2* breaking virus strains, indicating that this region of the 30 kDa MP is important for the recognition of the *Tm-2* gene. As observed for the *Tm-1* overcoming virus isolates, there is always a second amino acid substitution in the *Tm-2* breaking MP, probably to compensate for the structural changes caused by the first amino acid substitution.

aa-Pos.	36 — 55	56 — 75
30.0	VSKVDKIMVHENESLSEVNL	LKGVKLIEGGYVCLVGLVVS
30.2	VSKVDKIMVHENESLSEVNL	LKGVKLIEGGYVCLVGLVVS
30.Ltb1	VSKVDKIMVHENESLSEVNL	LKGVKLIEGGYV**F**LVGLVVS
	76 — 126	127 — 146
30.0	GEWNL----//----VPNYG	ITTKDAEKNIWQVLVNIKNV
30.2	GEWNL----//----VPNYG	ITTKDA**K**K**S**IWQVLVNIKNV
30.Ltb1	GEWNL----//----VPNYG	ITTKDA**K**KNIWQVLVNIKNV

Fig. 2. Partial amino acid sequence of the 30 kDa protein of wild type ToMV (30.0, Ohno et al. 1984), and of two *Tm-2* breaking ToMV strains (30.2, Strasser, 2002 and 30.Ltb1, Meshi et al. 1989). Amino acid exchanges are depicted in bold letters.

In contrast to *Tm-1* and *Tm-2*, *Tm-2*^{2} remained in commercial use for several years. Only two isolations of *Tm-2*^{2} overcoming virus strains have been reported (Hall, 1980; Rast, 1975). These ToMV strains were less virulent than wild type isolates (Fraser et al. 1989) and were therefore not capable of becoming a threat to the durability of the *Tm-2*^{2} resistance. Two ToMV-*2*^{2} strains have been cloned and sequenced (Calder and Palukaitis, 1992; Weber et al. 1993). Both strains contain amino acid exchanges in the 30 kDa MP. This was not unexpected because, as an allele of *Tm-2*, the *Tm-2*^{2} gene was supposed to interact with the same viral gene product. However, the amino acid exchanges are different from the substitution of the *Tm-2* resistance breaking virus strains. Four amino acid alterations were found for both ToMV-*2*^{2} movement proteins, three of which are identical (aa 130 Lys > Glu , aa 238 Ser > Arg, aa 244 Lys > Glu). These results already indicate that the evolution of *Tm-2*^{2} resistance breaking viruses requires much more drastic changes in the viral sequence than are required for breakage of *Tm-2* or *Tm-1* resistance.

Molecular analysis of the interaction between the 30 kDa movement protein of ToMV and the resistance genes Tm-2 and Tm-2^{2}

From the sequence analysis of *Tm-2* and *Tm-2*^{2} breaking ToMV strains it was clear that both resistance genes somehow interact with the 30 kDa movement protein. On the other hand, although being alleles, both resistance genes behave quite differently. *Tm-2* can be overcome by naturally occurring virus strains, while *Tm-2*^{2} has been stable for decades and so far only few, very ineffective *Tm-2*^{2} breaking virus strains have been isolated. The first series of experiments to study this phenomenon was conducted by generating recombinant viruses with different numbers of the amino acid substitutions originally found in the resistance breaking movement protein. Meshi et al. (1989) showed that a single amino acid exchange at position 133 (Glu > Lys) or at position 68 (Cys > Phe) yielded virus mutants which could partially overcome the *Tm-2* resistance. Both substitutions are needed for a full virulent phenotype in *Tm-2* tomatoes. Weber et al. (1998) showed that *Tm-2*^{2} recognition requires the C-terminus of the ToMV MP and that both amino acid substitutions in this region (aa 238 Ser > Arg, aa 244 Lys > Glu) are needed for overcoming the resistance gene.

Virus mutants can give only limited information on the molecular interaction between a viral gene product and a plant resistance gene because the number of mutants that can be generated is restricted by the functional requirements of the particular gene product. In addition, the MP mutants analysed could not distinguish between the two potential modes of action for the resistance genes: restriction of virus movement mediated directly by *Tm-*

2 and *Tm-2*^{2} , or interaction of *Tm-2* and *Tm-2*^{2} with the ToMV MP eliciting a hypersensitive defence reaction. To distinguish between these possibilities, Weber et al. (2004) generated transgenic tomato lines with constructs comprising the MPs of wild type or resistance breaking ToMV strains in the cultivar *Moneymaker*, which does not contain a resistance gene against ToMV. To investigate the effect of different domains of the movement protein in the resistance reaction, deletion mutants of the ToMV movement protein as well as N- and C- terminal fusions of the 30 kDa protein to the β-glucuronidase (GUS) coding region were constructed and transferred to *Moneymaker* tomatoes. The transgenic lines were crossed to isogenic lines of the tomato cultivar *Craigella,* containing either *Tm-2*, *Tm-2*^{2} or no resistance gene. The phenotypes of the germinated progeny were scored and the results are summarised in Figure 3.

In the *Tm-2* genetic background the MPs of ToMV-0 (pTA.30-L) and ToMV-2^2 (pTA30. 2^2), but not ToMV-2 (pTA30. 2), were able to elicit a necrotic reaction, indicating that *Tm-2* resistance involves a hypersensitive reaction. Fusion of the 30 kDa protein to β-glucuronidase (pTA30G, pTAG.30) and C-terminal deletions (pTA30.5) did not abolish the necrotic response. These results indicate that *Tm-2* recognizes a well-defined domain in the N-terminal part of the movement protein.

construct	response *Tm-2*	response *Tm-2*^{2}
pTA.30-L	HR	HR
pTA.30-2 (Tm-2)	-	HR
pTA.30-2^2 (Tm-2^2)	HR	-
pTA.30.5	HR	HR
pTA.30.3	-	-
pTA.30G (GUS)	HR	-
pTA.G30 (GUS)	HR	HR

Fig. 3. Responses of tomato plants containing the *Tm-2* or the *Tm-2*^{2} gene upon expression of different ToMV movement protein constructs. Expression of construct TA.30G, although containing resistance inducing sequences for both resistance genes, elicits differential reactions in tomato plants carrying *Tm-2* or *Tm-2*^{2}, thus suggesting that the native three dimensional structure of the ToMV movement protein is crucial for interaction with the *Tm-2*^{2} gene. HR = hypersensitive reaction.

Similar results were obtained for the full length ToMV MP in the *Tm-2*^{2} genetic background. The MP of ToMV-0 (pTA.30-L)and ToMV-2 (pTA30.2), but not the resistance breaking ToMV-2^2 (pTA30. 2^2), induced a necrotic reaction in the progeny seedlings. These results support the hypothesis that the *Tm-2*^{2} resistance gene also induces a hypersensitive reaction. However, the deletion mutants and the fusion constructs gave a completely different picture of the interaction of *Tm-2*^{2} with the 30 kDa protein in comparison to *Tm-2*. Modification of the C-terminus of the 30 kDa protein (pTA30.G) completely abolishes the elicitor activity of the protein. On the other hand, expression of the 78 amino acid C-terminal peptide (pTA30.3) was not sufficient on its own to induce a necrotic reaction. The N-terminal part of the 30 kDa MP (pTA30.5) which is inactive in the context of the full length protein, leads to a delayed necrotic response if expressed together with the *Tm-2*^{2} resistance gene. These results show, that the interaction of *Tm-2*^{2} with the 30 kDa protein is complex. It involves at least two different binding sites, one at the C-terminus and a second one at the amino terminal part of the movement protein (Fig. 4).

Cloning of the Tm-2 and Tm-2^2 the resistance genes

Many laboratories have tried to isolate the *Tm-2* genes because of the durability of the *Tm-2*^{2} gene. Molecular cloning of these genes via the map based approach turned out to be difficult, especially due to the lack of recombination in the centromeric region of chromosome 9 (Tanksley et al. 1992; Sobir et al. 2000). Two groups independently designed a transposon tagging approach (Silber, 2001; Lanfermeijer et al. 2003), which resulted in the isolation of the *Tm-2* (Gerhardts and Pfitzner, 2003) and the *Tm-2*^{2} (Lanfermeijer et al. 2003) resistance genes. The observation of Weber and Pfitzner (1998), who showed that the cross between tomato plants containing the *Tm-2*^{2} gene or the *Tm-2* gene and transgenic tomato plants expressing the ToMV MP gene results in a progeny which dies after germination, was used to develop a selection method.

Plants with the lethal combination of the *Tm-2* or the *Tm-2*^{2} gene and the MP will survive if the resistance gene is inactivated by the insertion of a transposable element. For both resistance genes, tagging lines were developed which contained a Ds-element closely linked to the *Tm-2* locus (Knapp et al. 1994). The Ds-elements were activated by an immobilised Ac-element (sAc: Jones et al. 1992) and the mutagenized tomato lines were crossed with the screening lines, which contained the ToMV MP transgene. The progeny were germinated and surviving plants were obtained. However,

further analysis revealed, that most of the surviving tomato plants had either lost the transgene (Silber, 2001) or the resistance gene (Lanfermeijer et al. 2003) by recombination. Finally, plants were identified, which contained a transposon inserted in the respective resistance gene.

These genes were isolated, their sequences analysed and the corresponding alleles were amplified by PCR (Gerhardts and Pfitzner, 2003; Lanfermeijer et al. 2003). The genes for *Tm-2* and *Tm-2*^{2} both contain one open reading frame of 2586 bp, which translates into a protein of 861 amino acids. Alignment of the predicted proteins with the data bases revealed that the *Tm-2* genes belong to the CC-NBS-LRR class of resistance genes. The highest homology was found with the RPP13 gene from *Arabidopsis thaliana*, which confers resistance to infection with *Peronospora parasitica* (Bittner-Eddy et al. 2000). In the first 100 N-terminal amino acids 12 putative leucine zipper motifs (CC) could be recognized. A NB-ARC (nucleotide binding site, apoptosis, resistance gene products, CED4) (Hammond-Kosack and Jones, 1997) region between amino acid 145 – 441 was predicted using the BLAST-P program. The carboxy-terminal part of the *Tm-2* protein contains 16 LRR (leucine rich region) domains, typical for most resistance genes. The *Tm-2* gene product contains 40 amino acid substitution in comparison to the susceptible allele (*tm-2*), two exchanges in the CC domain, 6 exchanges in the NB-ARC domain and 32 in the LRR domain, which is consistent with the hypothesis that the LRR region of the resistance genes is mainly responsible for the recognition of the pathogen (Ellis et al. 2000). Interestingly, *Tm-2* and *Tm-2*^{2} are highly homologous, with only four different amino acids in the putative protein sequence. The differences in the amino acid sequence are in the NB-ARC region at aa 257 Phe (*Tm-2*) > Ile (*Tm-2*^{2}) and aa 286 Ile (*Tm-2*) > Met (*Tm-2*^{2}) and in the LRR region at aa 767 Asn (*Tm-2*) > Tyr (*Tm-2*^{2}) and aa 769 Thr (*Tm-2*) > Ser (*Tm-2*^{2}).

The differences in the amino acid sequence between *Tm-2* and *Tm-2*^{2} are significant for two reasons. Firstly, deletion analysis had revealed that, in contrast to *Tm-2,* there are at least two different sites of interaction between the *Tm-2*^{2} resistance gene and the ToMV 30 kDa protein (Weber et al. 2004). Therefore, alterations at two domains of the respective gene product of *Tm-2*^{2} in comparison to *Tm-2* would have been predicted. This is in good agreement with the results of Baulcombe and co-workers (Moffet et al. 2002), who showed that the Rx resistance gene, a CC-NBS-LRR resistance gene against *Potato virus X*, also requires two protein domains, the NB-ARC region and the LRR region for the recognition of the viral coat protein. The results of the molecular interaction experiments and the sequence comparisons are summarised in Figure 4.

The second, interesting aspect, which comes from the comparison of the sequences of *tm-2, Tm-2* and *Tm-2²*, contributes to the question of the origin of *Tm-2* and *Tm-2²*. Although originally obtained from different sources

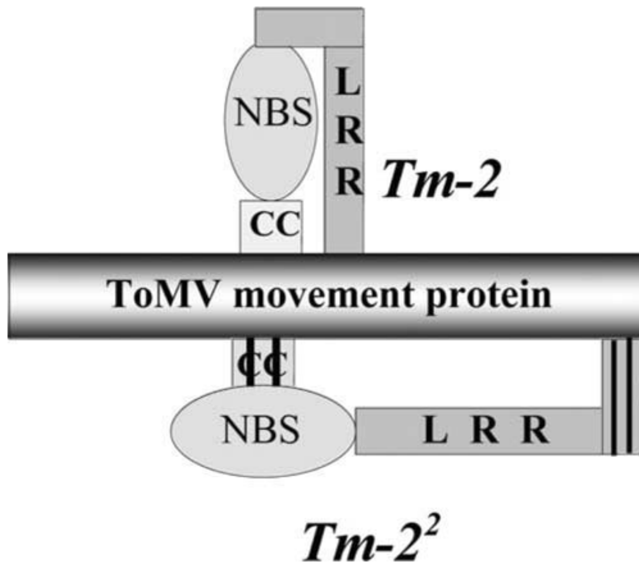

Fig. 4. Hypothetical model for the interaction of the *Tm-2* or the *Tm-2²* gene products with the ToMV movement protein. The four amino acid differences between the *Tm-2* and the *Tm-2²* gene are indicated by black bars.

(Soost, 1958, Alexander, 1963) both genes behave like alleles. Thus, they ended up integrated at the same region of chromosome 9, both give a similar defence response against ToMV, and both show a necrotic reaction if they are heterozygous. Therefore, it was argued by many breeders that *Tm-2²* might have evolved from *Tm-2*. The argument against this common origin was that if *Tm-2²* is only an optimised form of *Tm-2*, all *Tm-2²* breaking virus strains should be able to overcome *Tm-2*. This is not the case. If we compare the amino acid sequence of the *Tm-2* and the *Tm-2²* gene products it turns out, that the two amino acid exchanges in the LRR region between *Tm-2* and *Tm-2²* are different for *tm-2*, *Tm-2* and *Tm-2²*. For the two amino acid exchanges in the NB-ARC region, the situation is different. While *Tm-2* has different amino acids at position 257 and 286 in comparison to the

susceptible allele *tm-2,* the amino acid sequence at this position is identical between *tm-2* and *Tm-2*^{2}. Therefore, *Tm-2*^{2} has only 38 amino acid exchanges compared to *tm-2*. Since it is highly unlikely that the amino acid sequence of the *Tm-2*^{2} protein was reverted to the wild type sequence at two positions during the course of the evolution from *Tm-2* to *Tm-2* ^{2}, it seems more reasonable to assume that *Tm-2* and *Tm-2*^{2} developed independently from a common ancestor gene.

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Chapter B5

Resistance to *Turnip mosaic virus* in the *Brassicaceae*

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Introduction

Turnip mosaic virus (TuMV) is a member of the *Potyviridae*, the largest group of plant viruses. It is a particularly interesting member of the family as it has a very broad host range including monocots and dicots and shows differing degrees of adaptation to different plant groups. In some respects e.g. interactions with plant resistance genes (Walsh and Jenner, 2002), ecology in wild plants (Raybould et al. 2003) and genetic diversity (Tomimura et al. in press) it is the best characterised potyvirus. As the potyvirus best adapted to the model, fully sequenced dicot plant *Arabidopsis thaliana*, it represents an excellent model with which to gain fundamental insights into plant – virus interactions.

In addition to being an excellent model, TuMV is also economically important. It was stated to be one of the two most important viruses affecting field-grown vegetables worldwide (Tomlinson, 1987). Figure 1 shows a cabbage plant (*Brassica oleracea*) with a severe TuMV infection. It is also an important pathogen of arable crops in some parts of the world. In certain countries, for instance parts of China, it is the most important pathogen of some crops, being more important than fungal or bacterial pathogens.

An excellent comprehensive review of the biology, epidemiology and control of TuMV has been written by Shattuck (1992), its ecology in wild *Brassica* populations has been reviewed (Raybould et al. 2003) and a recent review has dealt with molecular aspects, diversity, ecology and interactions with brassicas and *Arabidopsis* (Walsh and Jenner, 2002).

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Fig. 1. A *Turnip mosaic virus* infected cabbage plant in the field showing severe necrotic symptoms. (See also Colorplates, p. xix)

Diversity of the pathogen TuMV

Being a virus that is distributed worldwide, infecting a large variety of plant families, and having an RNA polymerase-based mode of replication, it is not surprising that TuMV as a species shows a high degree of variability. The publication of the first complete genome sequence (Nicolas and Laliberté, 1992) has been followed by many more (Ohshima et al. 1996; Jenner et al. 2000; Tomimura et al. 2003), so now there are more than 40 complete sequences (and many more incomplete) deposited in the GenBank database. A comparison of the complete genomes of isolates reveals over 88% identity in amino acid sequences.

Strains can be defined by coat protein (CP) sequence diversity, directly (Chen et al. 2002) or via reactions with monoclonal antibodies raised to the CP (Jenner et al. 1999). Similar groupings are also identified when whole genomes are examined (Tomimura et al. 2003). Recombination events appear to be frequent between strains (Tan et al. 2004; Tomimura et al. in press).

The genetic diversity is reflected in the ability to infect certain hosts. In particular, several authors have noted gross genetic differences (verging on being sub-species) between isolates able to infect *Brassica* spp. and those infecting *Raphanus* spp. (Lehmann et al. 1997; Ohshima et al. 2002; Sánchez et al. 2003; Tomimura et al. 2003; Tomimura et al. in press). The underlying genetic basis for these host range differences has been investigated using viral chimeras, and differences in the P3 protein have been found to be of particular importance (Suheiro et al. 2004).

The genetic basis underlying other historical "strains" has not been studied, e.g. "ordinary" and "cabbage" (*B. oleracea*) strains (Yoshii, 1963), strains defined by symptom severity using *B. rapa* lines (Choi et al. 1980), or those defined by reactions on a combination of *B. rapa* and *B. oleracea* lines (Liu et al. 1990). However, it is known that the viral P3 protein also has a role in symptom severity in *B. napus* (Jenner et al. 2003).

Both *B. rapa* (Provvidenti, 1980; Green and Deng, 1985) and *B. napus* (Walsh, 1989; Jenner and Walsh, 1996) have been used to generate sets of differential lines able to split TuMV isolates into five or at least 12 pathotypes (Hughes et al. 2003) respectively. Interactions range from immunity, through limited local infection and/or necrotic reactions, to full infection, dependent on the combination of isolate and host line. There is a degree of overlap between the two differential series; strain C1 isolates, characterised by an inability to infect *B. rapa* Tropical Delight (Provvidenti, 1980), also appear to be pathotype 1 isolates, unable to infect *B. napus* line R4 (Jenner and Walsh, 1996). The genetics, of both host and pathogen, underlying these reactions is being investigated (see below), and is consistent with classical gene-for-gene models of plant pathogen resistance.

Sources of resistance, genetics and mapping of resistance genes

We do not intend to review all types and sources of resistance to TuMV but will focus on plant resistance to viral infection / replication mostly in the *Brassicaceae*.

A surprising proportion (40%) of the resistance genes to potyviruses are recessive (Provvidenti and Hampton, 1992), much higher than for other virus groups. A range of dominant and recessive resistances to TuMV has been reported. Sources of resistance to TuMV have been identified in a diverse range of plant species, ranging from *Brassica* species through radish (*Raphanus*) and stock (*Matthiola*) to lettuce (*Lactuca*). Many resistances involve single dominant genes, but there are examples of polygenic and recessive resistances. The dominant *Tu* gene in lettuce confers resistance to all isolates of TuMV so far tested (Zink and Duffus, 1970), has been mapped in the lettuce genome (Robbins et al. 1994) and to date has been extremely durable.

A wide range of resistances has been identified in *Brassica* spp. The inter-relationship of the different *Brassica* species has been described by U (1935). Much screening of the diploid A (*B. rapa*; Chinese cabbage, turnip etc.) and C (*B. oleracea*; cabbage, cauliflower, broccoli, Brussels sprout and kohlrabi etc.) genome species and the amphidiploid AACC (*B. napus*; swede/rutabaga and oilseed rape) species for resistance to TuMV has been carried out. Tests on a broad range of 114 accessions of C genome types selected to represent the diversity of *B. oleracea* from a genebank collection revealed no major sources of resistance (Walsh and Jenner, 2002). Similarly, screening of cultivated *B. oleracea* types failed to identify any sources of extreme resistance (Walkey, 1982; Pink et al. 1986; Pink and Walkey, 1988; Walkey and Pink, 1988). Genes controlling quantitative resistance have been postulated in *B. oleracea* (Pink et al. 1986). Quantitative trait loci (QTL) (*TuRB02*) controlling weak quantitative resistance to an Asian isolate of TuMV have been mapped in the C genome of *B. napus* (Walsh et al. 1999).

Different extreme forms of resistance to TuMV have been found in *B. rapa* and *B. napus*. Most are dominant and effective against specific TuMV isolates/genotypes and confer immunity, where no virus replication is detectable. However, more recently, recessive sources of broad-spectrum resistance have been found and characterised in *B. rapa*. The resistance genes and their specificities are summarised in Table 1.

The first *Brassica* resistance gene to be mapped was *TuMV RESISTANCE IN BRASSICA 01* (*TuRB01*, Walsh et al. 1999) in a line derived from the spring oilseed rape cultivar Westar. This dominant gene confers an extreme form of resistance (possibly immunity where no symptoms are seen and no virus is detected) to all pathotype 1 isolates of TuMV and is located on chromosome N6 of the *B. napus* A genome (Walsh et al., 1999). The specificity of the resistance conferred by *TuRB01* (Walsh et al. 1999) and the mechanism of its interaction with TuMV isolates (Jenner et al. 2000) are identical to those of the differential line R4 of the European pathotyping scheme (Jenner and Walsh, 1996). Resistance derived from the cultivar of Chinese cabbage (*B. rapa*), Tropical Delight has been found to have a virtually identical specificity and mechanism to *TuRB01* (Walsh et al. 2002). The dominant gene controlling the resistance maps to the same interval on chromosome R6 as *TuRB01* does on N6 and hence has been named *TuRB01b* (Rusholme, 2000). This explains why C1 strains defined by the inability to infect Tropical Delight also appear to be pathotype 1 isolates unable to infect *B. napus* line R4 (Walsh et al. 2002). Another single dominant gene, *TuRB03* conferring an extreme form of resistance (possibly immunity) to the pathotype 4 isolate of TuMV CDN 1 has been mapped in the A genome of oilseed rape, *B. napus* (Hughes et al. 2003) and maps very

Table 1. Resistance genes to *Turnip mosaic virus* (TuMV) in *Brassica* spp. and their specificities.

Resistance gene	*Brassica* sp. (genome[1])	Effective against pathotype(s)[2]/isolate	Reference
TuRB01	*B. napus* (A)	1	Walsh et al. (1999)
TuRB01b	*B. rapa* (A)	1	Rusholme (2000)
TuRB02[3]	*B. napus* (C)	CHN 1, JPN 1	Walsh et al. (1999)
TuRB03	*B. napus* (A)	CDN 1	Hughes et al. (2003)
TuRB04[4]	*B. napus* (A)	1, 3	Jenner et al. (2002a)
TuRB05[5]	*B. napus* (A)	1, 3	Jenner et al. (2002a)
retr01[6]	*B. rapa* (A)	1, 3, 4, 7, 8, 9, 12	Rusholme (2000)
ConTR01[7]	*B. rapa* (A)	1, 3, 4, 7, 8, 9, 12	Rusholme (2000)

[1]Genome where resistance gene is located; A genome derived from *B. rapa* and C genome derived from *B. oleracea*; [2]Pathotypes defined by Jenner and Walsh (1996); [3]Quantitative trait locus controlling the degree of susceptibility to TuMV isolates in a quantitative manner; [4]Epistatic to *TuRB05* and in conjunction with this gene confers resistance to pathotypes 1 and 3; [5]Controls a necrotic response (HR) to TuMV that limits systemic spread and in conjunction with *TuRB04* confers resistance to pathotypes 1 and 3; [6]Recessive gene which in combination with *ConTR01* controls resistance to systemic spread of TuMV; [7]Dominant gene which in combination with *retr01* controls resistance to systemic spread of TuMV.

close to *TuRB01*. Further single dominant genes *TuRB04* and *TuRB05* have been identified in the swede line 165 (Jenner et al. 2002a) and have been mapped in the *B. napus* A genome (Walsh, Higgins, Hughes and Lydiate, unpublished). *TuRB04* controls an extreme form of resistance to some TuMV isolates. *TuRB05* controls a necrotic hypersensitive (HR) response to some isolates of TuMV that limits systemic spread. *TuRB04* is epistatic to *TuRB05* and together, the two genes confer extreme resistance (possibly immunity) to pathotype 1 and pathotype 3 isolates of TuMV. More pathotype/isolate specific resistances are being characterised and the genes controlling them mapped (Walsh, Bambridge, Higgins and Lydiate, unpublished).

A number of broad-spectrum sources of resistance to TuMV were described by Provvidenti (1980) in *B. rapa*, including one line with resistance to all four isolates of TuMV tested. However, isolates capable of overcoming the latter source of resistance were subsequently found (Green and Deng, 1985). Further sources of broad-spectrum resistance have recently been described in *B. rapa* (Liu et al. 1996; Hughes et al. 2002; Walsh et al. 2002) and genetic characterisation carried out (Yoon et al. 1993; Suh et al. 1995; Rusholme, 2000; Hughes et al. 2002). Resistance effective

against strains C1-C5 of TuMV has been characterised in the line '0-2' and reported to be controlled by two recessive genes (Yoon et al. 1993). Subsequently, when monoclonal antibodies were used to determine the susceptibility of plants to isolates of TuMV strains C1-C5, resistance in the line '0-2' was reported to be controlled by a single dominant gene, or double dominant genes. Such differences in the number and type of genes thought to be involved seemed to depend upon sensitivity of TuMV detection, the TuMV strain used to challenge plants or which susceptible plant line the resistant plants were crossed with. Resistance to all TuMV isolates so far tested, including representatives of pathotypes 1, 3, 4, 7, 8, 9 and 12 defined by Jenner and Walsh (1996) and strain C5 (Provvidenti, 1980) has been identified in the Chinese cabbage lines BP079 and BP058 (Walsh et al. 2002). Our crosses show that these broad-spectrum sources of resistance are controlled by recessive gene(s) and are not of the extreme type seen for the pathotype-specific resistances described above. Symptoms were seen and virus was detected in mechanically inoculated leaves of line BP079, but there was no systemic spread (Walsh et al. 2002). The resistance in line BP058 appeared to be slightly more effective. No clear symptoms were seen in leaves of plants inoculated with any of the isolates, plate-trapped antigen ELISA detected TuMV in leaves of plants inoculated with one isolate (UK 4), more sensitive detection methods detected other isolates in inoculated leaves of other plants but not all plants (Walsh et al. 2002). From crosses carried out elsewhere, it was claimed that the resistance to TuMV isolates representing strains C1 and C4 in BP058 was controlled by one or two dominant genes (Suh et al. 1996). The resistance in one line we are working on (RLR22) that shows broad-spectrum resistance appears to be controlled by the combined action of a recessive (*retr01*) and a dominant (*ConTR01*) gene (Rusholme, 2000). These broad-spectrum resistances are undergoing further evaluation and characterisation.

The completion of the sequencing of the *Arabidopsis* genome (Arabidopsis Genome Initiative, 2000), the model dicot plant for research, may provide opportunities for studying and understanding plant resistance. *Arabidopsis* is readily infected by TuMV and as the only potyvirus regularly found naturally infecting cruciferous plants, TuMV is probably the best adapted potyvirus to *Arabidopsis* and may have had an impact on *Arabidopsis* evolution/adaptation.

The suitability of *Arabidopsis* for studies on plant – virus interactions was described by Martinez-Herrera et al. (1994). TuMV was shown to induce severe infections in *Arabidopsis* ecotype Col-0. In a detailed study of the interaction between the UK 1 isolate (Walsh, 1989) of TuMV and a collection of 106 ecotypes of *Arabidopsis*, 33 ecotypes were uniformly susceptible, 69 showed a range of responses and 4 ecotypes showed no symptoms and no virus detected in them (Martín Martín et al. 1999). Three

of these four ecotypes showed a mixture of responses, with some plants showing no infection at all, while others showed infection of inoculated but not uninoculated leaves. The other ecotype, Bay-0 was uniformly resistant with no detection of TuMV in inoculated or uninoculated leaves by ELISA. A collection of 16 different *Arabidopsis* lines have been tested to assess their interaction with the UK 1 isolate of TuMV (Hughes, 2001). No resistance to TuMV was found. Subsequently a subset of 7 of these lines plus 5 new lines were assessed for their interaction with TuMV isolates UK 1, CZE 1, CHN 5, and CDN 1, representing pathotypes 1, 3, 3 and 4 respectively (Hughes, 2001). Again no resistance was found although there were differences in the types of symptoms seen in some ecotypes for the different TuMV isolates. For example, the UK 1 isolate of TuMV had a much more severe effect on some ecotypes than the CZE 1 isolate. Considering the large number of *R* loci or 'resistance' genes to bacterial, fungal and viral pathogens described in *Arabidopsis*, the relative dearth of resistance to TuMV in *Arabidopsis* could be considered surprising.

A screen has been carried out on 159,600 M2 plants derived from 14,000 ethyl methanesulfonate-mutagenised plants to identify *Arabidopsis* mutants with altered susceptibilities to TuMV (Lellis et al. 2001). Eleven mutants showing reduced levels of virus infection were recovered. The mutants have been designated *loss-of-susceptibility to potyviruses (lsp)*. Further genetic characterisation of the mutants has identified two independent complementation groups that map to different regions of chromosome 5. Three independent mutants that conferred immunity to TuMV were isolated and assigned to the same complementation group. The locus was named *lsp1*. *LSP1* was isolated by map-based cloning and identified as the gene encoding translation initiation factor eIF(iso) 4E (Lellis et al. 2002). The eukaryotic initiation factor eIF4E has been shown to be involved in natural recessive resistance to the potyvirus *Potato virus Y* (PVY) (Ruffel et al. 2002). It has also been shown independently that a transposon insertion in the gene coding for the isozyme form of the eukaryotic initiation factor eIF(iso) 4E of *A. thaliana* conferred resistance to TuMV (Duprat et al. 2002).

Viral genetics of interactions with resistance genes

The construction of infectious clones of TuMV (Sánchez et al. 1998; Jenner et al. 2003; Suheiro et al. 2004) has facilitated the study of viral genetics. A variety of chimeric and mutated versions of TuMV have been examined on a range of host plants, revealing the various viral proteins involved in both susceptible and resistant plant interactions.

The first TuMV protein to be implicated in a resistance response was the cytoplasmic (or cylindrical) inclusion protein (CI) (Jenner et al. 2000). This

was the first record of a potyvirus CI being the determinant for a plant resistance gene. Many isolates of TuMV are unable to infect *B. napus* lines possessing the resistance gene *TuRB01* (i.e. are avirulent on this line). Either of two naturally-occurring mutations (CI H630R or CI N459D) enable avirulent isolates to infect, producing high viral titres with necrotic symptoms (Jenner et al. 2000). The same mutations enable *TuRB01b*, a resistance gene in *B. rapa*, to be overcome (Walsh et al. 2002).

The CI protein is also involved in the interaction with the *B. napus* resistance gene *TuRB05,* a gene that normally controls a hypersensitive (necrotic) reaction. Possession of the mutation CI M589T enables TuMV to infect plants possessing *TuRB05* without triggering host necrosis (Jenner et al. 2002a). To date no other potyvirus CI has been found to be the determinant for a plant resistance gene. Similar situations have been found for the TuMV P3 protein. The mutations P3 F312L and P3 I153F allow TuMV isolates to overcome the *B. napus* resistance genes *TuRB04* (Jenner et al. 2002a) and *TuRB03* (Jenner et al. 2003) respectively. Normally, both of these resistance genes completely prevent viral replication without any symptoms.

Viral resistance-breaking ability often appears to come at a price. The viral mutations to virulence against *TuRB04* and *TuRB05* are unstable in the absence of resistance gene selection pressure and viral populations rapidly revert to wild-type sequences (Jenner et al. 2002a). The CI H630R mutation (virulent on *TuRB01*-possessing plants) is more stable, but is nonetheless gradually out-grown by the wild-type in mixed populations (Jenner et al. 2002b). The inability to infect *B. napus* lines possessing these three resistance genes is the defining characteristic of pathotype 1 isolates. As pathotype 1 is one of the commonest pathotypes found in a large worldwide survey (Jenner and Walsh, 1996), this is further evidence suggesting that resistance-breaking comes at a cost.

Most of the resistance genes studied in *Brassica* spp. to date have been dominant genes. Viral components interacting with recessive genes of *Brassica* spp. have not yet been studied, but some interesting data is emerging from *Arabidopsis*. TuMV generally infects *A. thaliana* but infection does not occur when the mutation VPg D77N, affecting the interaction of the viral genome-linked protein (VPg) with the *A. thaliana* translation initiation factor eIF(iso)4E is introduced into TuMV (Wittmann et al. 1997; Léonard et al. 2000). The known TuMV determinants for plant genes conferring resistance to the virus are shown in Table 2.

Resistance mechanisms

Introduction

General information on the mechanisms of resistance has already been

Table 2. *Turnip mosaic virus* determinants for plant genes conferring resistance to the virus

Resistance gene	Viral determinant	Reference
TuRB01	CI	Jenner et al. 2000
TuRB01b	CI	Walsh et al. 2002
TuRB03	P3	Jenner et al. 2003
TuRB04	P3	Jenner et al. 2002a
TuRB05	CI	Jenner et al. 2002a
lsp1	VPg	Léonard et al. 2000
pvip1	VPg	Dunoyer et al. 2004
pvip2	VPg	Dunoyer et al. 2004

given in earlier chapters of this book. Most current knowledge about resistance mechanisms in *Brassicaceae* has been obtained from *Arabidopsis*. Information will be harder to come by in *Brassica* species due to the greater size and complexities of the genomes. Microarrays are now being used on *Arabidopsis* to study the overall picture of plant responses during susceptible interactions (Whitham et al. 2003), but similar studies during resistant interactions have not yet been published.

Resistance in *Brassica* spp. may be broad-spectrum or effective against only a few isolates. It manifests itself in many guises, including phenotypes such as immunity (no symptoms and no viral replication) and hypersensitive responses (HR, necrotic symptoms: Niu et al. 1983; Walsh, 1989; Jenner and Walsh, 1996; Walsh et al. 2002). When viral replication does occur, this may be limited in the plant by reduced cell-to-cell movement or by preventing long distance spread from one leaf to another (Walsh et al. 2002). Similar phenotypes have been observed in *A. thaliana* challenged with TuMV (Martín Martín et al. 1999).

General resistance

Some general antiviral defences in plants rely on the recognition of dsRNA intermediates produced by a replicating RNA virus, followed by the triggering of gene-silencing (see earlier chapters). Potyviruses, including TuMV, have a counter-defence strategy to this. The viral helper component protease (HC-Pro) has several functions including a role in suppressing host gene silencing (Anandalakshmi et al. 1998; Brigneti et al. 1998; Kasschau and Carrington, 1998). In *Arabidopsis*, the P1 + HC-Pro interferes with the activity of miR171, which directs cleavage of several mRNAs coding for Scarecrow-like transcription factors. Thus, not only is the antiviral RNA-silencing pathway affected, there is also interference with miRNA-controlled

developmental pathways. This is a possible explanation of how some symptoms in infected plants arise (Kasschau et al. 2003).

R *gene resistance*

There are clear examples of resistance in *Brassica* spp. with narrow spectra of specificity. These all appear to be under the control of single dominant genes, for example those involving *TuRB01*, *TuRB01b, TuRB03*, *TuRB04* and *TuRB05*. *TuRB01*, *TuRB01b* and *TuRB04* appear to confer immunity, whereas *TuRB05* is involved in a necrotic (HR) response. Currently, we do not know whether *TuRB03* confers immunity, a HR response, or both. We have identified the virulence determinants for all five genes (see above) and all involve gene-for-gene interactions typical of other gene-for-gene systems. Some necrotic (HR) responses are reasonably effective at limiting the degree of TuMV replication e.g. *TuRB05* (Jenner et al. 2002a) but other necrotic symptoms are accompanied by high levels of viral replication e.g. when some isolates overcome *TuRB01*. This reflects a general finding that cell death (the hypersensitive response, HR) and virus resistance are not necessarily one and the same phenomenon (Bendahmane et al. 1999). Rather, the apparent effectiveness of HR may depend on the speed with which *other* additional host resistance pathways are activated. There is much literature on the *R* genes involved in HR and mechanisms of action have been postulated, however much less is known about genes conferring immunity. The *Rx* gene of potato confers an extreme resistance against *Potato virus X.* However, the *Rx* protein is structurally similar to products of disease resistance genes conferring the hypersensitive response (Bendahmane et al. 1999) and perhaps this suggests the mechanism underlying immunity may be similar to that involved in the HR response. Although we have identified four critical gene pairs above, it is not yet known whether the host resistance gene and pathogen proteins interact directly or indirectly to trigger a resistance response.

We have limited data suggesting some TuMV resistance genes (*TuRB01* and *TuRB03*) may be clustered or allelic in the *Brassica* genome. Clustering of *R* genes seems to be common in *A. thaliana* (Meyers et al. 2003) and a number of other plant species. The distribution and diversity of resistance genes in *Arabidopsis* is said to have been generated by extensive duplication and ectopic rearrangements that involved segmental duplications as well as microscale events (Meyers et al. 2003). The observed diversity of the R gene proteins may also reflect the variety of recognition molecules available.

Studies on the Bay-0 source of resistance to TuMV in *A. thaliana* involving Northern blot hybridisation analysis and RT-PCR suggested that there was a very low level of virus replication and accumulation in inoculated leaves. Studies on protoplasts prepared from inoculated leaves of

Bay-0 and a susceptible ecotype showed that a small proportion of protoplasts from Bay-0 (1%) appeared to be infected by TuMV UK 1. The results suggested that cell-to-cell movement of TuMV UK 1 was affected in ecotype Bay-0 (Martín Martín et al. 1999).

Although there are examples of single dominant *R* genes that have provided durable resistance to viruses in general (the *Tu* gene of lettuce is an example of a single dominant gene conferring durable resistance to TuMV), to date no such genes have been found in *Brassica*. We have shown that for the resistance gene *TuRB01*, resistance breaking isolates of TuMV are less fit than the wild-type avirulent isolate (Jenner et al. 2002b). This suggests that although most of the single dominant resistance genes we have identified appear not to be completely durable, they may be quite effective in the field. It has been suggested that the evolutionary potential of pathogens is an important determinant of the durability of resistance genes against plant viruses (García-Arenal and McDonald, 2003). The wide host range of TuMV, inferred recombination rates (Tan et al. 2004) and ability to mutate to overcome known *Brassica* resistance genes suggests that TuMV has a relatively high evolutionary potential in the context of brassica resistance genes. Hence, durable single dominant gene resistance to this virus may never be found in brassicas.

Broad-spectrum resistance

The genetic control of broad-spectrum resistance in brassicas is currently not well understood although from our work it appears to be predominantly recessive (Rusholme, 2000; Hughes, 2001; Hughes et al. 2003). The resistance to TuMV in mutated *A. thaliana* lines is recessive and involves lack of function of the plant eIF4E gene. Yeast-2-hybrid experiments have shown that the TuMV viral protein genome-linked (VPg) interacted with the *A. thaliana* eIF(iso)4E protein (Wittmann et al. 1997). Subsequently it was shown that TuMV $6K_2$–VPg-Pro/VPg-Pro and eIF(iso)4E interacted *in planta* (in *B. perviridis*), that VPg-Pro interacted with the poly(A)-binding protein (PABP) and $6K_2$–VPg-Pro/VPg-Pro polyproteins were associated with endoplasmic reticulum membranes (Léonard et al. 2004). As eIF(iso)4E is a translation initiation factor it has been proposed that it functions in translation initiation on viral RNA like a typical 5’ cap structure to recruit translation initiation factors and 40S ribosomal subunits (Lellis et al. 2002). Although *Arabidopsis* mutants that lack eIF(iso)4E possess the functional isoform eIF4, TuMV will still not replicate in such mutants, showing that the resistance mechanism relates to eIF(iso)4E. It remains to be seen whether any of the recessive *Brassica* resistances are due to the inability of TuMV VPg to interact with the equivalent brassica translation initiation factor.

VPg also interacts with *Potyvirus* VPg-interacting protein (PVIP), a protein present in *Arabidopsis* and many other plants. Mutation of the VPg to interrupt this interaction also reduces viral cell-to-cell movement and affects systemic symptoms (Dunoyer et al. 2004).

As described above, some strains of TuMV are so distinct that their host range has become more specialised to exclude *Raphanus* spp. (Ohshima et al. 2002). The alternative point of view is that *Raphanus* possesses broad-spectrum resistance against many isolates. The viral P3 protein is known to be critical to this host range dichotomy (Suheiro et al. 2004). A possible hypothesis is that the P3 protein needs to interact with particular host proteins (or variants of these) that are present in *Brassica* but absent from *Raphanus* in order to complete the viral life-cycle. Such a resistance would probably be genetically recessive, and be analogous in its mode of action to the VPg/*lsp1* resistance already described. It can therefore be seen that a lot remains to be understood about the mechanisms of resistance to TuMV in brassicas.

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Chapter B6

Virus Resistance in Rice

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Introduction

The genus *Oryza* of the Family Gramineae comprises 18 tropical and subtropical species, of which two species are cultivated as rice: *O. sativa* and *O. glaberrima.* Rice provides the staple food of 60% of mankind, and is cultivated in all tropical and subtropical countries. *Oryza sativa* is thought to have been domesticated in China before 6500 BC, in India between 2000 and 1500 BC and even earlier than 5000 BC in northeast India (Chauvet, 2004). Molecular markers strongly suggested that the asian rice has been domesticated twice independently to give rise to the so-called *japonica* and *indica* groups of varieties similar to subspecies in China and South-India, respectively (Second, 1982). A third domestication took place in West Africa probably around 1500 BC from the wild relative *O. brevigulata* (syn. *O. barthii*) to give the african cultivated rice species *O. glaberrima* which is isolated from *O. sativa* by reproductive barriers (Oka, 1958; Second, 1982). Rice is a natural host for 20 viruses and an experimental host for 17. About 16 viruses may seriously affect rice yield (Lapierre and Signoret, 2004). The distribution of each virus is generally restricted to only one of the continents in which rice is grown. Host plant resistance has been reported for several viruses. Intensive breeding programs have been carried out to obtain resistance to the main virus diseases: rice tungro viruses and *Rice stripe*

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virus in Asia, *Rice hoja blanca virus* in South-America, and *Rice yellow mottle virus* in Africa. In this chapter, the main biological and molecular properties of these viruses, and the general features of the diseases they induce are summarised. Then, characteristics of the natural and transgenic resistances are described and attempts to incorporate them into cultivated lines in order to reduce the disease impact are considered. Special attention is paid to rice yellow mottle resistance whose phenotype, inheritance, mechanism and durability have been studied in particular detail.

Rice yellow mottle virus

The virus and the disease

Rice yellow mottle virus (RYMV) is present only in Africa. It was reported first in Kenya in 1966 and subsequently in West Africa in 1976. Since the early 1990s, RYMV has become present nearly everywhere where rice is grown throughout sub-Saharan Africa and in Madagascar. RYMV causes severe yield losses to rice production ranging from 25 to 100% and is considered the most important disease of rice in Africa. The natural host range is restricted to a few members of the Gramineae family. The principal vectors are chrysomelidae beetles. Transmission through plant residues, irrigation water, contact between plants and by infected agricultural tools is suspected. Seed transmission does not occur. RYMV is an emergent disease. It is thought that the virus originated in wild Gramineae and only recently infected cultivated rice. The rapid and intense spread of the virus was associated with changes of agricultural practices with the introduction of high performing but susceptible *indica* cultivars from Asia. Phytosanitary measures are sometimes advised even though their effect in reducing virus spread and economic impact are unknown. They include protection of seedbeds by nets, disinfection of the tools used at replanting, and destruction of volunteers and rice residues (Calvert et al. 2003).

The genome of RYMV is a 4450 nt long, single stranded, linear, positive-sense, non-polyadenylated RNA. It belongs to the sobemovirus genus and has a genomic organisation similar to *Cocksfoot mottle virus* (Fargette et al. 2004). The coding sequences are as follows: ORF1 encodes a protein P1 involved in movement and which is an inhibitor of gene silencing. ORF2 encodes a protein with a serine protease-like domain, a virus protein genome linked (VPg), and a RNA-dependent RNA polymerase. The ORF3 encodes the capsid protein via a sub-genomic RNA. A satellite RNA, not involved in the pathogenicity, is often associated with the virus. Several strains have been distinguished on the basis of their immunological properties and nucleotide sequences. They were related to the geographic origins of the

isolates, with different strains occurring in East, Central and West Africa. Phylogeographic studies suggested an earlier diversification in East Africa and a latter radiation in West-Central Africa (Traoré et al. 2005).

Extensive studies are in progress on the interaction between rice and RYMV. This work is motivated both by the economic importance of the disease and by the characteristics of this interaction which could serve as a model for studying various mechanisms of virus resistance in monocotyledons. Most rice cultivars are susceptible to RYMV and very few sources of high resistance have been identified. Moreover, no variety with both a high level of resistance and adaptation to irrigated cultivation has yet been released. So several breeding programs to obtain acceptable resistant cultivars are in progress. Recently, two kinds of natural resistance have been characterised at the genotype and phenotype level: a partial resistance phenotype and a high resistance phenotype. Furthermore, transgenic resistance to RYMV has also been achieved. However, resistance breakdown to all three types of resistance has been observed (Sorho et al. 2005).

The partial resistance phenotype

Partial resistance to RYMV is generally expressed by delay in symptom expression and virus accumulation (Ioannidou et al. 2000; 2003). Varieties with a significant partial resistance to RYMV are *japonica* varieties adapted to upland cultivation. The genetic determinism of this resistance is polygenic. Quantitative trait loci (QTL) for resistance have been mapped in a double-haploid population resulting from a cross between a susceptible irrigated *indica* variety, IR64, and a resistant upland *japonica* variety, Azucena (Ghesquière et al. 1997; Albar et al. 1998). Fifteen QTLs involved in resistance have been identified on seven chromosomal fragments. For 14 of them, the resistant parent Azucena provided the favourable allele. Significant phenotypic correlations and colocalisations of QTLs were observed between resistance and traits related to plant architecture and development: tillering ability, plant height, and cycle duration (Albar et al. 1998). In particular, the semi-dwarfing gene *sd-1* on chromosome 1, provided by the susceptible parent IR64, may have a direct or indirect role in RYMV susceptibility since it is mapped in a region where a major resistance QTL has been detected. In contrast, the resistance QTL, mapped on chromosome 12 (QTL12), had an important effect on leaf virus content but did not co-locate with any plant-aerial part morphology QTL (Albar et al. 1998). The search for interactions between this QTL and the rest of the genome provided evidence that a complementary epistasis between QTL12 and a region of chromosome 7 (QTL7) could be the major genetic component explaining the differences in virus content in this progeny

resistance (Pressoir et al. 1998). QTL12 is located in the centromeric part of chromosome 12 where resistance genes to blast have been found and cloned (Sallaud et al. 2003). This may explain why varieties such as Moroberekan, 63-83, OS6, Lac23 and Dourado Precoce often used as donors of partial resistance to RYMV were also found to be blast resistant under African growing conditions. Moreover, a gene for resistance to *Rice hoja blanca virus* (RHBV) has been tentatively mapped on chromosome 12, and an *indica* variety resistant to RHBV has also shown partial resistance to RYMV. Marker-assisted introgression was applied to fix the favourable alleles of QTLs of chromosome 12 and 7 in a near isogenic line of IR64. Both the additive effect of QTL12 and the interaction between QTL12 and chromosome 7 were confirmed in the IR64 genetic background (Ahmadi et al. 2001).

The NIL approach was used to refine the analysis of the QTL12 effect independently of other resistance components provided by Azucena. Then, the response of the NIL for QTL12 was followed over time at the leaf and tissue levels and compared to Azucena and the recurrent susceptible parent IR64 (Ioannidou et al. 2003). It was concluded that Azucena combined (i) a tolerance observed long after inoculation and characterised by mild symptoms despite generalised distribution and accumulation of the virus and (ii) a partial resistance which was transient and consisted in a one week delay in virus accumulation and symptom expression when compared to IR64. Phenotype of the NIL suggested that the partial resistance could be attributed to the presence of the Azucena allele at QTL12 independently of the *indica* or *japonica* genetic background and any morpho-physiological related traits, whereas tolerance is probably associated with other QTL or morphological traits. A similar delay in virus detection was observed at tissue levels in the vascular bundle-sheath layers of Azucena and NIL for QTL12. The bundle sheath-phloem interface is critical for virus trafficking into, through and from vascular tissues and can be an efficient barrier to virus movement. Impaired virus movement through vascular bundle sheaths was proposed to explain this partial resistance (Ioannidou et al. 2003).

The high resistance phenotype

High resistance to RYMV is very rare. Among the thousands of lines tested over the past decades, it was observed only in five *O. glaberrima* accessions including Tog5672 and Tog5681 (Thottappilly and Rossel, 1993; Coulibaly, 1999) and in one *O. sativa* cultivar named Gigante (Ndjiondjop et al. 1999). These varieties expressed no symptoms after mechanical inoculation, and the virus content could not be detected by ELISA. This high resistance proved to be effective against a large range of RYMV

isolates both in greenhouse and field conditions (Ndjiondjop et al. 1999; N'Guessan et al. 2001). This promising resistance was characterised genetically and phenotypically and was introgressed by classical and marker-assisted selection at IRD into some representative lowland varieties of West-Africa.

The genetic basis of this resistance has been studied in IR64 x Gigante and IR64 x Tog5681 crosses. The resistance is recessive and under the control of a single recessive gene. The same locus is involved in the two varieties (Ndjiondjop et al. 1999). The high resistance gene has been mapped on the long arm of chromosome 4 between microsatellites markers RM252 and RM273 (Albar et al. 2003) and localised within a region of 160 Kb (Boisnard et al. 2004). Further physical mapping and cloning of this gene are in progress. The genetic diversity of microsatellite markers spanning the resistance locus excluded the hypothesis of a recent introgression from *O. glaberrima* into the *indica* cultivar Gigante and vice-versa. Laboratory experiments based on serial inoculations of Gigante and Tog5681 demonstrated that different strains of the virus overcome one or the other of the resistant accessions which suggested that different alleles of resistance were present in Gigante and Tog5681 (Fargette et al. 2002; Konaté et al. 1997). Cultivar Tog5672 was reported to be immune to most RYMV isolates (Konaté et al. 1997) and to suffer less from infection by virulent isolates than Tog5681, suggesting that another allele of resistance existed.

Trangenic resistance

A transgenic approach using widely grown RYMV-susceptible cultivars of rice and a transgene encoding the RNA-dependent RNA polymerase of RYMV was applied (Pinto et al. 1999). Transformed lines showed resistance to RYMV strains from different African locations. The resistance derived from an RNA-based mechanism associated with postranscriptional gene silencing (PTGS). However, compared to the naturally occurring high resistance phenotype described earlier, the transgenic resistance was found to be only partial and temporary (Sorho et al. 2005). The P1 protein of RYMV is an inhibitor of gene silencing in heterologous (Voinnet et al. 1999) and in homologous systems (Siré et al. 2004). This possibly explains why PTGS-mediated transgenic resistance in rice is not high or durable.

Resistance breakdown

Partial and high resistance to RYMV were challenged in host passage experiments. Pronounced changes in pathogenicity occurred over serial passages of virus isolates inoculated to partially or highly resistant cultivars (Fargette et al. 2002). The changes encompassed the known existing pathogenic variability of field isolates. Ultimately, the high resistance of Gigante or Tog5681 and the partial resistance of Azucena broke down. There was no loss of fitness of resistance-breaking isolates as they were not counter-selected, impaired or outperformed after serial passages in susceptible cultivars, even in mixture with avirulent isogenic wild type (Sorho et al. 2005). In the fields, isolates able to break the high resistance phenotype has been found in each strain and in all regions of Africa, in both cultivated rice and wild grass species (Konaté et al. 1997; Sorho et al. 2005). However, the frequency of the different virulent pathotypes varied among ecological zones (O. Traoré and G. Konaté, personal communication). However, such resistance breakdown was often partial: only a low proportion of plants is infected and symptom expression and/or virus multiplication was localised to a few leaves. Only in rare instances were virulent field isolates able to induce a systemic infection with pronounced symptoms and high virus accumulation directly after inoculation in Gigante or Tog5681. Resistance breakage was highly dependent on the virus inoculum concentration and on the mode of transmission (Sorho et al. 2005). Experiments are being conducted to assess whether an increase in pathogenicity comparable to that observed by serial passages in laboratory experiments could occur in the fields. This work on the ecology of the durability is critical to assess the field durability of resistant cultivars and their importance to control the disease.

A high resistance breaking specific mutation was identified by comparison of the sequence of the avirulent wild-type isolate with that of a virulent isolate derived by serial passaging. Introduction of this mutation into an infectious clone of the avirulent RYMV by site-directed mutagenesis conferred the ability to break the high resistance. This mutation was located in the VPg (E. Hébrard, A. Pinel and D. Fargette, unpublished results). VPg is involved in resistance breaking of several potyviruses (Diaz-Pendon et al. 2004). Then, the breakdown of resistance to RYMV associated to VPg mutation converged with potyvirus examples that identified the eukaryotic translation factors as the genetic determinant of recessive high resistance. The potyviral VPg is considered as the viral factor that directly interact with them to promote virus multiplication (Diaz-Pendon et al. 2004).

Rice tungro disease

The viruses and the disease

Tungro disease is caused by dual infection with two viruses, *Rice tungro bacilliform virus* (RTBV) and *Rice tungro spherical virus* (RTSV) (see Calvert et al. 2003 and Hibino, 1996 for reviews). RTSV confers leafhopper transmissibility to the complex and RTBV contributes to most of the symptoms. The disease is restricted to South and Southeast Asia. Tungro is particularly important in highly intensive irrigated rice ecosystems and was first reported and attracted attention in the early years of the 'green revolution' following the introduction and use of improved high-yielding varieties, artificial fertilizer and intensive systems of cultivation. Because of their potential injury and unpredictability, tungro outbreaks can cause up to 80% yield loss. TRBV and RTSV are not mechanically transmissible. Transmission is vector-dependent and occurs via six species of green leafhoppers of which *Nephottetix virescens* is by far the most important. The mode of transmission is semi-persistent and the vectors remain viruliferous for 4-5 days. Most RTBV-infected plants show yellow or yellow-orange leaf discoloration and stunted growth.

RTSV has isometric particles of 30 nm in diameter. The genome is positive-sense, single-stranded RNA of *c*. 12200 nucleotides, which encodes a single polyprotein of 393 kDa that is cleaved into functional virus proteins. The sequence of the coat protein suggests that there are two groups, one from South East Asia and the other from the Indian subcontinent. RTSV is the type species of the genus *Waikavirus* of the family *Sequiviridae*. RTBV particles are bacilliform, 100-300 nm long and 30-35 nm wide. The genome consists of an 8 kb circular double-stranded DNA, with two discontinuities, one on each strand. Similar to other retroviruses and pararetroviruses, RTBV is transmitted asymmetrically with all the coding capacity on the negative strand. Spatial and temporal variants have been distinguished. The genomic structure of RTBV is mostly similar to that of other members of the *Badnavirus* genus. These viruses have been classified as pararetroviruses because they package DNA into the virion but replicate in the cell on an RNA template by reverse transcription. This is a process used also by retroviruses whereby they package RNA in the virion but replicate on a DNA template. RTBV isolates mainly fall into two groups, those from the Indian subcontinent and those from Southeast Asian countries. The genomes of isolates from the Indian subcontinent contain a deletion of 64 bp when compared with those from Southeast Asia. Although RTSV variations were

not so conclusive as those of RTBV because of the high degree of micro-variation in RNA genomes, three genomic variants of RTSV have been identified. The geographic distribution of these RTSV variants does not correlate with that of strains of RTBV (see Azzam, 2004; Hull, 1996, 2004 for reviews)

Resistance

Modern rice varieties with high yield potential were adopted in the late 1960s and the early 1970s to help avert the impending food shortages in developing countries of Asia. Because some of these early modern varieties were susceptible to tungro and its vectors, the epidemic outbreaks caused big production losses in many parts of South and Southeast Asia. Extensive cultivation of susceptible varieties in continuous sequence, asynchrony of planting, and increased vector populations were identified as factors influencing the disease epidemics (Cabanunagan et al. 2001). The need to produce rice varieties with resistance to tungro and its main vector *N. virescens* was recognised in the mid-1960s as the full scale of the problem became apparent. Conventional assessment of tungro incidence is based on visual scoring of symptoms. Early breeding programs were hampered by the absence of a suitable technique for discriminating between resistance to vector and to virus.

For the past three decades, the introduction of vector-resistant varieties was the major strategy of controlling tungro (Azzam and Chancellor, 2002; Koganezawa, 1998). Most of the resistant varieties released by the International Rice Research Institute (IRRI) and by national breeding programs in the region had resistance to the vector and not to the viruses. Most IRRI crosses made after 1969 had at least one parent with resistance to *N. virescens*. Of the seven known genes for vector resistance, four have been incorporated into improved varieties. Such rice cultivars resistant to the vector adequately escape tungro infection in the field under light to moderate tungro and vector pressure. These varieties made a major contribution to reducing the incidence of tungro, but in many cases the resistance was not durable and new varieties had to be introduced as the old ones became ineffective. The breakdown of vector resistance usually occurred after 4-5 consecutive seasons of intense cultivation of initially resistant cultivars (Dahal et al. 1990, 1992).

Consequently, although several additional sources of leafhopper resistance have been identified in recent years, the focus has shifted to identifying and utilising tungro virus resistance (Hibino et al. 1990; Azzam and Chancellor, 2002). However, it was not until the late 1970s that it was confirmed that two distinct viruses were associated with the disease. Agroinoculation allowed the screening of rice for resistance to *Tungro*

bacilliform virus (Sta Cruz et al. 1999). A serological assay was then developed for each of the viruses, and serology remains the most widely used diagnostic method in breeding work and epidemiology (Sta Cruz et al. 2003). Many accessions are resistant to the most common strain of RSTV-A and several are resistant to both RSTV strain A and strain 6. No sources of true resistance to RTBV have yet been identified, but a few accessions show tolerance to infection. Advanced breeding lines with resistance to TRSV and tolerances to RTBV were shown to have infection in multilocal field trials. However, farmers prefer to grow rice with better eating quality but without tungro resistance even in tungro endemic areas. Furthermore, virulent virus strains were found in the Philippines (Koganezawa, 1998) indicating that resistance to virus may not be durable. A combination of vector and virus resistance may confer greater durability to rice varieties. Overall, although many papers about varietal reaction to tungro have been published, consistent results have not always been obtained. Effective and sustainable tungro management requires a combination of resistant varieties and adoption of synchronous planting. Breeding of resistant varieties will continue to be the main component in tungro management research. Genetic studies have also been conducted to understand the inheritance of TSTV resistance. In some crosses, a single recessive gene is involved (Azzam et al. 2001).

Additional sources of resistance are being sought through the use of transgenic plants. Transgenic *japonica* rice plants were produced containing the RTSV replicase gene in the sense and antisense orientation (Huet et al. 1999). Plants producing antisense sequences exhibited moderate but nevertheless significant resistance to RTSV. Plants expressing the full-length Rep gene as well as a truncated Rep gene in the (+) sense orientation were totally resistant to RTSV, even when challenged with high levels of inoculum and against geographically distinct RTSV isolates. Moreover, RTSV-resistant transgenic rice plants were unable to assist transmission of RTBV. The coat protein genes of RTSV were introduced individually or together to *indica* and/or *japonica* rice cells. A moderate level of resistance was observed (Sivamani et al. 1999). In contrast, transgenic plants transformed with RTBV genes did not show any protection against RTBV.

Rice stripe tenuivirus

The virus and the disease

Rice stripe virus (RSV), which causes severe damage to rice in Japan, Korea, China and Taiwan is the type member of the genus *Tenuivirus* (see Toriyama, 2004 for review). It is transmitted by the small brown

planthopper *Laodelphax striatellus* in a persistent manner. It has the typical genomic organisation of a tenuivirus as described for RHBV (see below). Early infection of rice plants causes significant loss of yield; late infection also reduces yield by retarding ear emergence and ripening. Between 1960 and 1970, stripe disease incidence was greatest in Japan, Korea and Taiwan and the damage to rice production was severe.

Resistance

Most Japanese paddy varieties are highly susceptible to RSV, but Japanese upland varieties and *indica*-type rice varieties are resistant. Therefore, RSV-resistant hybrids of paddy and upland or *indica* varieties were bred. In the field, RSV infections on the resistant cultivars were suppressed to insignificant levels even when RSV was epidemic in the locality. So far, no virulent RSV strains have arisen, even in areas where more than 80% of the total rice fields are planted with resistant cultivars. However, it may be a problem that most of the released RSV-resistant cultivars originate from a single or closely related gene source. Therefore, breeding of resistant cultivars having different resistance genes is therefore needed to increase genetic diversity (Kisimoto and Yamada, 1998).

The coat protein gene of RSV was introduced into two *japonica* varieties of rice by electroporation of protoplasts (Hayakawa et al. 1992). The resultant transgenic plants expressed the CP at high levels and exhibited a significant level of resistance to virus infection. However, resistance of these transgenic lines has not been assessed under natural conditions, during severe RSV outbreaks.

Rice hoja blanca

The virus and the disease

Rice hoja blanca virus was first reported from Colombia, South America in 1935. The virus is found predominantly in northern South America, but also occurs sporadically in Brazil, Central America and the Caribbeans. Virions appear in electron microscopy as fine filaments, *c*. 3 nm in diameter and of variable length. Some particles have a tightly spiralled configuration, whereas other virions form circles or dissociated aggregates. The genome of RHBV consists of four RNA segments. The terminal sequences of each RNA segment are complementary and the genome has an ambisense coding strategy. The genomic RNA is not capped at its 5' terminus and the 3' terminus is not polyadenylated. RHBV belongs to the genus *Tenuivirus*, members of which infect only Gramineae (see Ramirez and Haenni, 1994; Morales, 2004 for reviews).

RHBV is restricted to species of *Poaceae* and specifically to *O. sativa* under natural conditions. The virus is transmitted preferentially by the planthopper *Tagosodes orizicolus* in a persistent, circulative and propagative manner. RHBV is transovarially transmitted but is not seed-borne. RHBV-infected rice plants exhibit characteristic chlorotic stripes and yellow stippling on the leaf blade. Rice infected at an early stage of development is stunted and may develop necrosis. Plants infected late may produce panicles, but with few or no seeds. Yield losses vary with age of plant at infection, number of viruliferous insects per plant, and plant genotype. During the peak of RHBV epidemics, yield losses have been estimated to be between 25% and 50%. RHBV epidemics recur cyclically, apparently following the development of RHBV-resistant (low disease incidence) and susceptible (high disease pressure) generations of the planthopper, in which the virus is also damaging (see Calvert et al. 2003 for review).

Resistance

While resistant plant genetic resources have been identified, screening for resistance to RHBV depends on maintaining highly viruliferous colonies of *T. orizicolus* which has proved to be time consuming. A field method to screen for resistance to RHBV was developed and thousands of lines were tested each year at the International Center for Tropical Agriculture (CIAT) (Zeigler et al. 1988). This method is effective in eliminating the most susceptible lines, but not sufficient to select highly resistant varieties. Additional screening using different disease pressures and age of infestation have led to the development of varieties that are more resistant than the parents used in the crosses (Zeigler et al. 1994). Varieties and advanced lines are classified as susceptible, intermediate resistant and highly resistant. No rice varieties in Latin America are immune to the virus, and resistance can be overcome under high disease pressure. Nevertheless, even varieties that are classified as intermediate confer adequate field resistance.

New sources of resistance that complement the conventional resistances were sought. Rice transformed with the RHBV nucleocapsid protein (N) gene had a significant reduction in disease development (Lentini et al. 2003). Several reactions were observed that ranged from susceptible to completely resistant plants. These transgenic RHBV-resistant lines expressed the N gene RNA at low levels and the nucleocapsid could not be detected. This suggests that the resistance encoded by the N gene is RNA mediated by post-transcriptional gene silencing.

Conclusions

Rice is particularly prone to infection with viruses and other pathogens because of the way it is cultivated over very large areas. Moreover, much of the crop is grown in monoculture and in overlapping sequence throughout all or much of the year. This explains the prevalence of infection and the need for control measures.

In developing control measures much attention has been given to breeding for resistance to the four main virus diseases of rice, as discussed in the foregoing sections. The emphasis on resistance breeding is understandable because the approach has obvious advantages compared with other possible measures. For example, the use of insecticides to control insect vectors creates an additional expense and hazards to human health, natural enemies and the environment. Moreover, insecticide and spraying equipment are not always available and at prices farmers can afford. Even if available the experience has been that insects soon become resistant to insecticides and this can soon become a problem. There are also difficulties with other approaches to control by manipulating sowing date or by an appropriate disposition of plantings. This is because fields are often small and labour may be scarce, especially during the main planting season. The choice of site is often limited and farmers have little or no control on the actions and decision-making of their immediate neighbours.

The use of resistant varieties avoids many of these difficulties and involves no additional expense once suitable varieties have been developed and made available. Moreover, there are no undesirable side effects and the approach is easy for extension services to promote in rural farming communities that often lack basic education and expertise. Nevertheless, problems have arisen in identifying adequate sources of virus resistance that can be combined with other desirable attributes to produce varieties that are fully acceptable to farmers and consumers concerned with flavour, texture and other quality traits. There is also concern that the virus resistance may not be durable, although the threat of breakdown seems to be less than with resistance to insect vectors. Concerns about durability of resistance in rice to viruses are similar to those occurring in different plant/virus pathosystems (see Harrison, 2002 and Lecoq et al. 2003).

From the experience gained with the four main virus diseases of rice it is clear that the use of resistant varieties has brought considerable benefit. However, the varieties already available are unlikely to provide a complete or lasting solution and there is a need for continuing research to identify and exploit new sources of resistance and to deploy the resistant varieties that are available more effectively than in the past. This is necessary to meet the changing demands of farmers and consumers and on the nature of the virus disease problem. Changes occur with the emergence of new virus strains or

vector biotypes or the adoption of new cropping practises. Indeed, rice viruses provide a striking example of the continuing 'battle of the genes' that is such a marked feature of other pathosystems (Thresh, 1989).

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Chapter B7

Cassava

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Introduction

Cassava (*Manihot esculenta*, Euphorbiaceae) is one of the main tuber crops and the staple or subsistence food for about one fifth of the world's population (Edison, 2000). Demand for cassava is expected to rise in the next 20 years due to human population growth (Scott et al. 2000).

Resistance to viruses is particularly important in vegetatively propagated crops like cassava since they become virus-infected during year-on-year propagation. Eighteen different viruses have been reported from cassava (Calvert and Thresh, 2002; Thottappilly et al. 2003).

The Situation in Africa

Prior to 2003 nine cassava-infecting viruses had been reported in Africa (Thottappilly et al. 2003). With the recent addition of three new *Begomovirus*

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species affecting cassava in Africa, viz. *East African cassava mosaic Cameroon virus*, *East African cassava mosaic Malawi virus* and *East African cassava mosaic Zanzibar virus* (Fauquet et al. 2003), the total number of viruses rises to 12. Cassava mosaic disease (CMD) is the main biotic constraint in cassava production and the most important threat to food security in sub-Saharan Africa (Thottappilly, 1992). Originally, one geminivirus was regarded as the causal agent of CMD. However, recent studies have shown that several similar, but distinct, whitefly-transmitted geminiviruses cause CMD in Africa (Fauquet et al. 2003) and they occur singly or in combinations. At least six different cassava-infecting *Begomovirus* species are reported from Africa. Consequently, if not otherwise specified, the viruses causing CMD in Africa are referred to here as cassava mosaic begomoviruses (CMBs). From 1988 to present, a major pandemic of an unusually severe form of CMD has been spreading throughout East and Central Africa, causing massive losses and affecting the region's food security (Otim-Nape et al. 1997; Legg et al. 2001).

Breeding for resistance has been considered a feasible strategy for the control of CMD (Thresh et al. 1994; Calvert and Thresh, 2002; Thottappilly et al. 2003). However, good performance of resistant varieties can only be guaranteed under certain conditions. Increased inoculum pressure, agroecological changes, vector population explosion or immigration of a new vector biotype can have a significant impact on the expression of resistance in cassava.

Use of resistant genotypes of cassava

The search for resistance to CMD started in the 1920s, but the most rewarding programs began at Amani in Tanzania during the late 1930s (Jennings, 1994) and later in Madagascar, where all local varieties and many diverse cassava accessions were screened (Cours-Darne, 1968). Varieties such as "Bouquet de la Reunion", "Java 12/28", and "Criolina" were identified and released to farmers. However, since more effective resistance was needed, these were crossed with several wild species including the tree cassava species, *M. glaziovii* (Nichols, 1947; Cours, 1951; Jennings, 1957, 1994). The hybrids had non-tuberous roots but some plants, though infected by CMD, showed only mild and transient symptoms of CMD. At Amani, three backcrosses to cassava were made to restore root quality and maintain resistance to CMD (Nichols, 1947). Intercrosses between third backcross selections produced hybrids that combined good quality roots and effective virus resistance (Jennings, 1994). Open-pollinated seeds from these hybrids were sent to many African countries including Nigeria for evaluation, with clone 58308 coming up as a superior one (Ekandem, 1970; Hahn et al. 1980, 1989; Jennings, 1994). This clone

had poor root yield but high resistance to CMD. It was used extensively at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria (Hahn et al. 1989) to develop resistant genotypes. The IITA program combined high levels of resistance to CMD, good root yield and root quality to produce the Tropical *Manihot* Selection (TMS) cassava lines which are currently the most widely deployed source of CMD resistance in Africa, and until recently the best sources of CMD resistance. Seeds of CMD-resistant genotypes and *in-vitro* virus-free clones were sent by IITA to over 30 national programmes in Africa for evaluation and selection under specific agroecologies (Ng et al. 1992; Mahungu et al. 1994).

The genetics of currently deployed CMD resistance in the TMS lines derived from *M. glaziovii* is polygenic, and this involves recessive genes that are additively inherited, with a heritability of over 60% (Hahn et al. 1989; Jennings, 1994; Mahungu et al. 1994; Mba and Dixon, 1997). CMD incidence on the resistant genotypes is usually low: 20% incidence in genotype 30395 as against 100% on susceptible Isunikankiyan after 6 months of growth under high disease pressure. Similar results are reported from Côte d'Ivoire between resistant Garimoshi (30% incidence) and moderately resistant CB (90%) within six months (Fargette and Vié, 1995). In Uganda, the percent incidence on improved genotypes such as TMS 30572, TMS 60142, TMS 30337 and TMS 30395 was less than 30% during ten months of growth. In contrast, the local susceptible genotypes such as Ebwanateraka and Senyonjo had 100% incidence (Otim-Nape et al. 1998).

The first resistant varieties retained the broad-based polygenic resistance derived from *M. glaziovii*. Resistant cassava genotypes show certain features that distinguish them from susceptible ones. They were largely tolerant to CMD infection, incurring little or no yield loss. Epidemiological studies and field evaluation of the resistant genotypes reveal that they are not readily infected (Hahn et al.. 1980), and if infected, show mild symptoms, which may be restricted to some shoots (Jennings, 1960; Fargette et al. 1996). Some of the resistant genotypes are characterised by transient or mild symptoms when infected by CMBs (Jennings, 1994; Thresh et al. 1994), while others develop conspicuous symptoms that are restricted to a few leaves or shoots (Thresh et al. 1998b). Virus concentration in resistant genotypes was reported to be low and a significant correlation was shown between symptom severity and CMD titre among resistant genotypes (Fargette et al. 1996). However, the severity of symptoms expressed was not necessarily a reflection of virus concentration in some of the genotypes (Ogbe et al. 2003).

In the field TMS resistant cassava lines could be infected with CMD and the first few leaves show good symptoms, especially during the rainy season. Then the plant produced several leaves without symptoms. After this one or two leaves will show symptoms, probably due to new infection. Again the plant would produce several leaves without symptoms. In the susceptible

varieties, once the plant is infected almost all leaves would show symptoms. Based on this observation, it could be speculated that this is due to inhibition of the long distance transport function. Once a leaf becomes infected, the virus spreads effectively from cell to cell but not to other leaves. Therefore, it appears that cell-to-cell movement is not inhibited and the recessive gene affects long distance transport of the virus.

The resistance has been effective for more than 40 years in East and West Africa and there is no breakdown till now, due to the emergence of resistance-breaking strains of CMBs. Interestingly the use of resistant varieties contributed in overcoming the recent pandemic of EACMV-UG in Uganda, where IITA genotypes have been widely used (Otim-Nape et al. 1994).

More recently, classical genetic analysis and molecular mapping confirmed the polygenic nature of the *M. glaziovii* source of resistance to CMD (Akano et al. 2002). Bulk segregant analysis (BSA) (Michelmore et al. 1991) of a BC_1 population derived from TMS 30572 identified a simple sequence repeat marker SSRY40 on linkage group D of the TMS30572-derived genetic map of cassava that explains 48% of the phenotypic variance of CMD resistance at $P<0.001$ (Fregene, 2000). The resistance gene(s) associated with SSRY40 was also shown to be recessive. The linkage group D of the TMS30572-derived genetic map shows reduced recombination and a high number of markers, evidence of inter-specific introgression. The gene controlling resistance is within an introgression from *M. glaziovii* and has been designated CMD1. Although highly resistant varieties of this type are available in many countries, they are not always widely grown due to the lack of adequate quantities of planting material, and in many countries farmers continue to grow local varieties including some that may have little or no resistance to CMD. This explains why the disease is widespread in many areas, causing serious losses as during the current pandemic in East Africa.

CMB infections are not always fully systemic and uninfected cuttings can be obtained from some branches of infected plants, especially those of resistant varieties not expressing symptoms. The resistant/tolerant genotypes often have two categories of plants: symptomless and symptomatic plants (Jennings, 1994; Fargette et al. 1996). Among the latter, at least some branches of infected, symptomatic plants of resistant varieties spontaneously become free from virus under natural conditions through ‘reversion’, a form of recovery (Storey and Nichols, 1938; Njock et al.. 1996; Fargette et al. 1994). The extent of reversion depends on the genotype, and is regarded as a component of the resistance of cassava to CMBs (Fargette et al. 1996; Thresh et al.1998a,b; Fondong et al. 2000). Reversion has been exploited to select and produce healthy cuttings for CMD epidemiological studies in Côte d’lvoire (Fargette et al. 1985, 1988). In resistant genotypes, cuttings obtained from the lower portions of the main stem are more likely to grow into virus-

infected plants than cuttings from the upper portion of the main stem and stem branches (Cours, 1951; Njock et al. 1994).

The partial systemicity of CMBs is probably associated with the reversion phenomenon (ability to provide uninfected cuttings even when infected). Reversion has probably prevented total infection of the vegetative stock of resistant genotypes. For example, 96.0, 95.7, and 23.7% symptomless plants were recorded for resistant genotypes TMS 30001, TMS 30395, and TMS 30572, respectively, after 6 years of vegetative propagation (Hahn et al. 1980). Recent studies showed that restriction of virus movement into axillary buds is an important aspect of resistance in CMD (Ogbe et al. 2002). This probably explains the reversion phenomenon in which infected stems from resistant genotypes could sprout into healthy plants in subsequent generations and why reversion of resistant genotypes is a feature of the resistance gene.

Resistance to virus infection differs from resistance to the whitefly vector. Fargette et al. (1996) found that cassava genotypes differed widely in whitefly infestation. In contrast, Hahn et al. (1980) observed similar numbers of whiteflies on resistant and susceptible genotypes and thus inferred that resistance to the vector was unlikely. Nevertheless, variation in the suitability of cassava as a host for *B. tabaci* has been noted (Legg, 1994). This variation could be exploited in breeding for resistance to the vector, since several studies have shown a correlation between whitefly population and disease incidence (Leuschner, 1977; Otim-Nape et al. 1998). More recently, a cassava genotype, MECU72, showing resistance to the whitefly vector has been identified (A.C. Bellotti, personal communication). It would be interesting to see if this genotype can become infected with CMBs under natural conditions in the field.

As IITA was deploying these resistant materials, it took into account the need to diversify resistance and expand the available gene pool (Dixon et al. 2001). A novel source of resistance was recently identified in a Nigerian cassava landrace (TME-3) that confers immunity to CMD (Dixon et al. 2001; Fregene et al. 2001a). AFLP analysis of the CMD resistant land races and the TMS lines reveal significant genetic dis-similarity between them, reducing the possibility that the land races are escapes from the back cross derivatives with the *M. glaziovii* source of CMD resistance (Fregene et al. 2000).

Genetic analysis of two crosses involving the CMD resistant local Nigerian varieties TME 3 and TME4 revealed a major dominant gene control for the new extreme resistance source (Akano et al. 2002). The dominant CMD resistance gene has been designated *CMD2* and it is flanked by SSRY28 and GY1 at 9 and 8cM respectively. Since then 2 additional markers, NS158 (SSR marker) and RME-1 (SCAR marker) at <2cM and <1cM to the gene, respectively have been identified, explaining more than

90% of the phenotypic variation for resistance. Considerable progress has been made in developing a comprehensive molecular genetic map (Fregene et al. 1997, 2001b) and a clustering of cassava accessions into groups having differential resistance has been achieved (Fregene et al. 2000). Furthermore, progress has been reported in localizing resistance genes (Akano et al. 2002). This provides opportunity to apply marker-assisted breeding for efficient selection of this trait. The advantage of marker assisted selection (MAS) is that it enables the breeder to eliminate at an early stage CMD susceptible genotypes, which in the case of the heterozygous CMD resistant land races is 50%, reducing the costs of disease evaluation by half.

The new source of resistance is controlled by a single dominant gene, CMD2 (Akano et al. 2002), which is reminiscent of plant *R* genes that condition a hypersensitive response (HR), i.e. resistance associated with a localized cell death and tissue necrosis at the site of pathogen ingress. Other conspicuous features of the new source of CMD resistance are the lack of observable disease symptoms and the suppression of virus accumulation in infected cells of cassava leaves (Rossel et al. 1994; F.Ogbe, personal communication). Several improved lines have been developed using this new source of resistance. Since then, several additional land races from all over West Africa, with possible additional sources of resistance to CMD, have been identified (Dixon, 2004). Putatively, they represent seven different resistance genes, but this needs to be confirmed by further studies.

To test the use of CMD2 for resistance breeding, a pilot experiment was set up in 2000. Six crosses, and reciprocals, over 2,400 genotypes, were made between two cassava land races from Nigeria that carry CMD2, and a susceptible Nigerian land race and 2 elite cassava varieties from IITA, one tolerant and the other susceptible to CMD. The crosses were evaluated in Ibadan (Nigeria), at high CMD pressure area and molecular marker analysis conducted with SSR marker NS158 tightly associated with CMD2. Marker analysis alone was able to predict CMD resistance with 95% accuracy. Based on this result, a molecular marker-assisted breeding (MAB) of resistance to CMD was initiated at CIAT using the markers NS158 and RME1. The MAB project is a pre-emptive measure in case the disease is accidentally introduced into Latin America, as cassava germplasms are very susceptible to CMD (Okogbenin et al. 1998). The MAB scheme at CIAT currently involves crossing CMD resistant parents to CIAT's elite cassava parents and the embryo rescue of seeds followed by multiplication *in vitro* as well as molecular analysis. Genotypes shown to be resistant are transferred to the screen house for hardening and to the regular breeding program. Samples of resistant genotypes are also shipped as *in vitro* plants to collaborators in Africa and India. In 2003, 2315 genotypes were processed but the capacity for MAB at the moment is 5000 seeds, the current cost of MAB per genotype is US$0.5 (Fregene et al. 2004).

MAB of CMD and the cassava green mites (CGM) have also recently been initiated in Tanzania to transfer the concept to National programs. Tanzania is the fourth largest producer of cassava with average yields of 8 tons/ha compared to 10 tons/ha for the rest of Africa. The breeding project employs elite cassava parents that have been bred for resistance to CMD, the TME3 source, using molecular markers at CIAT, and a source of resistance to CGM from the wild species, *M. esculenta* sub spp *flabellifolia*. The improved introductions will be crossed to farmer preferred local varieties and molecular markers associated with CMD and CGM will be used to eliminate progeny that do not carry the resistance genes, leaving a largely reduced breeding population for more careful agronomic evaluation under typical farmer's conditions.

Mechanisms of the resistance of CMD2 have been studied at the Donald Danforth Center for Plant Science, St Louis (USA) and the DSMZ-Plant Virus Division at Braunschweig (Germany). Protoplast cultures of TME3 and TMS117 were transformed, by electroporation, with infectious virus clones and grown for 24h, after which the protoplast cultures were harvested and subjected to Southern analysis using an infectious virus DNA clone as probe. The results reveal that ACMV could replicate equally in both clones discarding interference with replication as the resistance mechanism (C. Fauquet, personal communication). However when infectious virus clones were introduced into resistant and susceptible varieties via microprojectile bombardment, both groups of varieties became infected but infection in the resistant genotypes did not become systemic, suggesting that interference with movement of the virus is the principal mode of resistance in the TME3 source (Winter et al. 2004).

Serial analysis of gene expression (SAGE) was used to analyze the gene expression pattern in a bulk of 40 genotypes each of CMD resistant and susceptible genotypes drawn from a gene mapping progeny (Fregene et al. 2004). Messenger RNA used for the SAGE analysis came from plants that have been exposed to heavy disease pressure over a period of two years in the field. One hundred and seventy five transcripts were expressed 3 to 12 times more in the resistant bulk compared 94 transcripts found 3-5 times in the susceptible bulk implying that many more genes have been switched on in the resistant bulk in response to virus infection. SAGE analysis of bulks of CMD resistant and susceptible cassava genotypes identified genes known to be involved in the systemic acquired resistance (SAR) response to disease in plants.

There is also interest in cloning CMD2 for use in genetic transformation (Fregene et al. 2001a; Fregene and Puonti-Kaerlas, 2002). The gene could be incorporated into desirable local/improved cultivars to enhance CMD resistance through genetic engineering. A high-resolution map of the region of the cassava genome bearing the CMD2 gene was developed towards positional cloning of CMD2 using a full-sib population of 1690 individuals

(Moreno et al. 2004). A bacterial artificial chromosome (BAC) library with more than 70,000 clones, with an average size of 110kb, and a more than 10X coverage of the cassava genome was also developed. The BAC library was screened with the two markers closest to CMD2, RME1 and NS158, and positive clones were used to construct contigs. The end of the contigs were sequenced and then mapped, as single nucleotide polymorphisms (SNPs), in the recombinants, 112 in total of the fine mapping population. BAC clones that flank CMD2 have been identified and sub-cloned for use in genetic complementary experiments to identify the clones that carry the gene.

Asia and the Pacific Region

A cassava mosaic disease similar to CMD is present in India and Sri Lanka. In order to differentiate screening and resistance breeding work against begomoviruses in Africa from that in India, we prefer to describe CMD in India as Indian cassava mosaic disease (ICMD), although symptoms are identical between CMD in Africa and ICMD in India. Two distinct begomoviruses, viz. *Indian cassava mosaic virus* (Hong et al.1993) and *Sri Lankan cassava mosaic virus* (Saunders et al. 2002) cause ICMD in Asia.

ICMD is a severe constraint to cassava production in India. This disease is widespread in South India mainly in Kerala, Tamil Nadu and Andhra Pradesh (Narasimhan and Arjunan, 1976). However due to the systemic nature of the disease, and frequent and common use of infected cuttings, this disease spread to other areas of the country.

Yield losses of up to 88 per cent in highly susceptible cultivar 'Kalikalan' and 17 to 36 per cent in improved varieties released by Central Tuber Crops Research Institute (CTCRI), Thiruvananthapuram, were reported (Malathi et al. 1985). ICMV is transmitted by the whitefly *Bemisia tabaci* and the extent of spread by this vector in the field varies (Palaniswami et al. 1996). Transmission by the vector is reported to be very low in improved cultivars (Chacko and Thankappan, 1973; Hrishi et al. 1977). Whether this low rate of transmission is due to the field resistance of these varieties or due to the inefficiency of the vector is not clearly understood. Recently it was reported that only the cassava biotype of *B. tabaci* transmitted ICMV (Palaniswami et al. 2004).

Resistance Breeding in India

In India cassava breeding is mainly carried out at CTCRI, Thiruvananthapuram, as well as in the State Agricultural Universities through the All India Co-ordinating centres (Abraham et al. 2000; Unnikrishnan et al. 2002). During the last four decades of research at CTCRI, a large number of

cassava varieties with varying reaction to ICMV have been released (Nair et al. 1998).

CTCRI has a rich collection (1638) of indigenous (854) and exotic (784) cassava germplasm (Pillai et al. 2004a) of which only 113 accessions were found to be free from ICMD. Genetic diversity among these ICMD free accessions were assessed in terms of esterase isozyme polymorphism which showed ten distinct clusters; with the number of accession in each group ranging from one to 27, and similarity between different clusters ranging from 15-50% (Pillai et al. 1995,1999). Screening of land races of cassava in India showed that most of them were susceptible to ICMD (Pillai et al. 2004b). Graft transmission studies showed that four germplasm accessions had a high degree of resistance when compared to the highly susceptible cultivar 'Kalikalan' (Rajendran et al. 1995).

Recently, cassava variety MNga-1 and *M. caerulescence* were identified as resistant to ICMV (Unnikrishnan et al. 2002; Sheela et al. 2004). MNga-1 is a breeding line from IITA, designated as TMS 3001 there, received via CIAT in 1994 has been continuously evaluated for CMD for the past 10 years and showed 0 to 1% infection at field level, while other lines exhibited 3 to 67% infection (Unnikrishnan et al. 2002). In intervarietal hybridization programs MNga-1 was used as the ICMV resistance donor parent and crosses were made with released varieties and promising selections from indigenous germplasm. Evaluation of seedling population for ICMV showed that open pollinated populations showed lesser ICMV incidence (15-34%) than the crosses (37%) (Unnikrishnan et al. 2002). However, hybrid progenies, obtained from crosses with Ambakadan X MNga-1, showed resistance to ICMV at seedling and first clonal stage (Rajendran et al. 2004).

Reactions of Indian cassava cultivars to ICMV

Since 1963 nearly 20 varieties have been released from CTCRI, Kerala Agricutlural University and Tamil Nadu Agricultural University. The response of these cultivars to ICMV varies from susceptible to field tolerant (Shanmugavelu et al. 1987; Thamburaj, 1990, Joseph et al. 1990; Nair et al. 1998). Although large collections of germplasm are available, no immune or highly field resistant cultivars were released, though five cultivars had field tolerance. A number of cultivars like Kalikalan, Ariyan and Burmah were found 100 per cent infected by the virus, while clones CE-9 (1310), CE-14 (1315), CE-92 (2171) and CE-101 (2350) showed a high degree of resistance (Jos and Sreekumari, 1994).

Wild genetic resources

The genus *Manihot* comprises 98 species and all are natives of the New World tropics. Presently 8 species are maintained at CTCRI of which *M.*

glaziovii, showed resistance to CMD in Africa (Jennings 1972, 1977; Doughty, 1958).

In interspecific breeding programs, *M. glaziovii, M. caerulescence, M. tristis, M. flasellifolia, M. peruviana* and *M. pseudoglaziovii* were used for development of ICMV resistant lines. Among them, accessions of *M. caerulescence* exhibited high level of resistance and were used as donor parents for transferring resistance to elite Indian cultivars (Sheela et al. 2002, 2004). Among the progeny resulting from these crosses, one interspecific hybrid of cassava with *M. caerulescence* (CMC-1) has shown complete resistance to ICMV for the past twelve years of evaluation, while others were field tolerant, showing varying degrees of the disease. This hybrid is now being backcrossed with elite cassava cultivars to recover resistant plants with the necessary quality attributes for agricultural use (Unnikrishnan et al. 2002; Sheela et al. 2002, 2004).

In order to identify new sources of ICMD resistance, 44 wild *Manihot* accessions were tested, of which 14 accessions belonging to *M. caerulescence, M. carthaginensis, M. dichotoma* and *M. pseudoglaziovii* were resistant to ICMV (Unnikrishnan et al. 2004). Similarly evaluations of back cross hybrids, three BC hybrids showed no ICMD symptom (Unnikrishnan et al. 2004).

Resistance against Vectors

Evaluation of 20 varieties of cassava having high degree of variation on leaf characters against the preference of whitefly showed that, varieties with green petioles and soft leaves were preferred to those with red or red-green petioles and coarse leaves and erect leaf orientation had double the number of whiteflies than horizontal or downward ones (Nair and Daniel, 1983). However the preference of whitefly to cassava varieties and their reaction to ICMV were shown not to be related. Spread of ICMV by the vector is reported to be low in improved cultivars (Hrishi et al. 1977). Leuschner (1977) reported from Nigeria a close relationship between population density of *Bemisia* sp., and the development of CMD. The extent of spread of ICMD in the field in different varieties of cassava was found to vary (Chacko and Thankappan, 1973). It was 30 and 15 per cent respectively in hybrids H 43 and H 226 and less than 5 per cent in H 97, H 165, Sree Visakham, Sree Sahya and M4. However infection reached 52 per cent in Kalikalan. Nair (1981) achieved a reduction in whitefly population through insecticidal sprays, but the field spread of ICMD was not reduced.

Physiological basis of Resistance to ICMD

Varying levels of susceptibility and tolerance to ICMD have been identified among cassava cultivars, based on a disease severity index, disease spread and yield loss (Malathi et al. 1985). The factors contributing to this variation in susceptibility are not known. Neither the inoculum source nor the whitefly population harboured in the cultivars was found to have correlation with susceptibility (Nair and Daniel, 1983). Cassava has high concentration of cyanoglucosides (linamarin and lotaustralin) present in all plant tissues, especially in leaves. The physiological role of these compounds is not clear, although they are believed to be involved in repelling or inhibiting pathogens and pests (Conn, 1980). The cyanoglucosides in leaves and the activity of cyanide metabolizing enzymes were studied in ICMV– susceptible and tolerant cassava cultivars. The results showed that cyanoglucosides do not have any role in resistance to ICMV. Neither leaf cyanoglucoside content nor cyanide metabolism in leaf was related to ICMV tolerance. Mild disease symptoms were associated with decrease in cyanide levels and severe symptoms with an increase in cyanide content in all cultivars (Balanambisan and Malathi, 1993). Studies on the role of virus–induced proteins in the pathogenesis of ICMV were done for soluble and total protein profiles of healthy and diseased cassava leaves and the results show that higher amounts of soluble protein were found in diseased leaves than in healthy ones. However, whether these proteins are host specific, found in response to virus infection or are associated with the virus has yet to be established (Balanambisan, 1996).

South and Central America

Although seven viruses are reported from South and Central America, cassava frogskin disease (CFSD) is economically the most important. The causal agent of CFSD is not known, but a virus is suspected. CFSD was first reported in 1971 from southern Andean region of Colombia (Lozano and Nolt, 1989). The range of CFSD is increasing and it is becoming more frequent in areas of Colombia, Costa Rica, Venezuela and Brazil.

In the Amazon regions of Brazil and Colombia, differences in the reaction of varieties to CFSD were observed. Some varieties developed typical root symptoms. Other varieties or landraces often in the same field did not develop symptoms. This led to the idea that some cassava landraces may be resistant to CFSD.

In CFSD affected cassava, the root periderm and corky layers enlarge to form raised lip shaped fissures. Severely affected roots do not fill with starch, and yield losses can be 100% (Lozano and Nolt, 1989). In some cassava landraces including Secundina, CFSD affected plants are stunted and the leaves develop mosaic symptoms.

Cassava is propagated vegetatively, and all the plants grown from affected plants will have CFSD. The symptom severity is affected by temperature. As the temperatures increase there tends to be a decrease in symptoms. For example, CFSD affected Secundina grown at constant temperature of 30C will not develop the mosaic leaf symptoms. Because of the inhibition of transmission by high temperatures, thermo-therapy followed by *in vitro* meristem culture can be used to eliminate the disease from infected plants (Maffla et al. 1984).

Evaluation of cassava for resistance to CFSD

The Centro Internacional de Agricultura Tropical (CIAT) core collection, which consists of 640 cassava lines, representative of the CIAT cassava collection of over 6000 lines, were tested for their reaction to CFSD. Five plants from each line were inoculated by grafting them with CFSD affected stem cuttings. In the subsequent years, 10-20 plants per line were grown for 12 months and evaluated visually for root symptoms. Those cassava lines with either moderate or severe symptoms were eliminated from the experiment. After five years, 121 cassava lines were still showing a good level of resistance to CFSD. Although the origins of cassava lines were from many countries throughout the world, 70% of the resistant landrace were from Brazil (31), Peru (18) and Colombia (18) and Paraguay (16).

After five years, the 66 lines that were resistant to CFSD and had good agronomic characteristics were grown at the CIAT experiment stations . Only very mild or no symptoms were present on any of the lines during the next 3 years of testing. Representative plants from 66 lines were assayed for CFSD by grafting stem cuttings (rootstock) to Secundina (scion), and the new leaves were examined for mosaic symptoms. Plants from all of the lines were positive for CFSD. This confirmed that the cassava lines were tolerant to the disease even though they were affected with CFSD.

The large percentage of accessions in the CIAT core collection that have tolerance to cassava frogskin disease was surprising because they had never been selected for that trait. After eight years of field trials, some lines never had any visible symptoms. Since these plants were still infected by CFSD, it appears that the tolerance is stable. More than 55% of the lines selected for their resistance to CFSD came from Brazil, Colombia and Peru, countries where CFSD is endemic. Tolerance usually occurs only after a long association between pathogen and host. The earliest known cultivation of cassava is from the Brazilian Amazon region, which is also thought to be the origin of CFSD, implying a long association of this pathogen with its host.

Concluding remarks

Use of resistant cultivars, production and distribution of healthy planting material, improved cultural practices and eventually strategic use of transgenic crops could provide more sustainable solutions to cassava virus problems. Host plant resistance to viruses and their vectors, eventually remain one of the most important means of disease control.

Yield losses due to CMBs may increase if virulent strain(s) reach new areas by natural spread or through the movement of infected planting material. The occurrence of different viruses or virus combinations in different regions undermines the effectiveness of resistance breeding programmes. Germplasm exchange is important for breeding purposes. However, it is essential to follow strict quarantine regulations when disseminating vegetative material. So far, there is no report of any virus of cassava being transmitted through seeds. Hence, currently this may be the safest way to exchange germplasm.

The CMBs that differ from each other in sequence identity are considered to be of independent origin. It is obvious that whitefly-transmitted geminiviruses are becoming increasingly important with novel geminiviruses and new whitefly biotypes. For CMBs, this has been shown by many reports on the emergence of new virus types through recombination between and among virus species contributing to a high genetic diversity of begomoviruses (Padidam et al. 1999).

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Chapter B8

Natural Resistance Mechanisms to Viruses in Barley

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Introduction

More than fifty viruses are known to be able to establish experimental infections in barley (*Hordeum vulgare*), but only about 25 viruses naturally infect this cereal (Mathre, 1997). Among these, only the *Hordeivirus*, *Barley stripe mosaic virus* (BSMV), members of the *Barley yellow dwarf virus* (BYDV) (Genus *Luteovirus*) family complex, and two *Bymoviruses*, *Barley mild mosaic virus* (BaMMV) and *Barley yellow mosaic virus* (BaYMV), cause widespread diseases or substantial yield losses. For this chapter, we will focus on only these viruses, since information about resistance to viruses of lesser economic importance is limited.

BSMV is primarily restricted to barley and can cause appreciable economic losses when infected seed are planted (see Carroll, 1986). However, because BSMV overwinters solely in infested seed and has no known biological vectors, spread in the field is directly related to the rate of mechanical transmission from infected seedlings. Therefore, effective control can be achieved by eliminating the virus from seed stocks (Carroll, 1986). Although the virus is still present in some barley growing areas of

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the world, it is no longer a major problem in the barley producing areas of the USA.

In contrast, members of the BYDV complex are difficult to control and collectively cause the most significant world-wide losses on small grain cereals of any virus. Since the initial discovery of barley yellow dwarf in California (Oswald and Houston, 1951), advances in understanding the specificity of aphid transmission of virus subgroups and their genome organization have revealed that the BYDV complex consists of at least two distinct genera (*Luteovirus* and *Polerovirus*) within the *Luteoviridae* family (D'Arcy et al. 2000). The most important of these members are the MAV and PAV strains of BYDV and the *Polerovirus, Cereal yellow dwarf virus* (CYDV-RPV), which previously was known as the BYDV-RPV strain. In addition, substantially different serotypes, and possibly even different viruses, may cause yellow dwarf symptoms (Miller et al. 2002). Widespread dispersal of all members of the complex depends on the ecology of viruliferous aphid species, timing of crop planting, selective application of insecticides, and susceptibility of cereal hosts and grasses within the vicinity. Although the use of insecticides (eg. Plumb and Johnstone, 1995) and genetic resistance (eg. Sip et al. 2004) has proven useful for control of BYD, substantial yield problems persist.

Members of the *Bymoviruses*, the third group of viruses to be discussed, are transmitted to the roots by the plasmodiophorid, *Polymyxa graminis* (Gray and Rochon, 1999). Although hints of a soil-borne disease of barley had been available for more than 40 years in northern Japan, shifts in cropping to Fall planting schedules first revealed the presence of BaYMV and later BaMMV in Europe (Plumb, 2002). It is now known that both viruses cause economically important diseases in the colder climates of North-West Europe and East Asia. Because the appearance of disease symptoms is largely dependent on cool soils, changing sowing dates to the Spring can circumvent disease when this is practical agronomically. Although chemical treatments of the soil or other agronomic measures have been unsuccessful at controlling these viruses, resistance genes can provide high levels of protection against both BaYMV and BaMMV (McGrann and Adams, 2004).

Because of the destructive potential of barley viruses, particularly the barley yellow dwarf and the BaYMV/BaMMV complexes, significant resources have been invested in identification and characterization of resistance genes, the development of resistant cultivars, and the molecular genetics and analysis of pathogenicity. Yet detailed knowledge of the mechanism of natural resistance is still lacking for viruses infecting barley as well as other hosts. This chapter will summarize what is known about the biological and molecular properties of these viruses, and will discuss the natural resistance genes in barley and present a prospectus for the future.

Biological, molecular and genetic properties of Barley stripe mosaic virus

BSMV was probably the first cereal virus to be recognized as causing a disease problem. A distinct disease syndrome was noticed as early as 1910 in North America and leaves collected in 1913 showed clear signs of a disease named false stripe by Mr. Ibra Conners in 1924. Conners believed that an infectious agent caused the syndrome (Hagborg, 1954; Conners, I.L., personal communication to AOJ), but it was not until the early1950's that a viral etiology was firmly demonstrated by mechanical transmission (McKinney, 1951) and the detection of rod-shaped particles in infected plants (Gold et al. 1954). In nature, BSMV is primarily restricted to barley, although the virus occasionally has been isolated from wheat and wild oats (Mathre, 1997; Slykhuis, 1967; Jackson and Lane, 1981). Losses result from reduced seed set, shriveled seeds, and decreased vigor of seedlings. A large number of strains were initially described based on symptom variability (Fig. 1) and physicochemical properties (McKinney and Greeley, 1965; for review, see Jackson and Lane, 1981), and these observations were subsequently confirmed by molecular analysis of the viral RNAs (Jackson et al. 1989) and genetic tests with selected virus strains (Jackson et al. 1991). These studies clearly show that symptoms in plants depend on a plethora of interacting factors, including the virus strain, the host genotype, the stage of infection, and the environmental conditions. Because BSMV has no known biological vectors and requires a combination of seed transmission and efficient mechanical transmission from infected seedlings for maintenance and spread, planting virus-free seed provides an effective method of control. In North America this practice has significantly reduced yield losses due to BSMV infection of barley (Carroll, 1986). Certified seed production is now facilitated by sensitive detection methods developed over the past 25 years, virtually eliminating BSMV from common seed stocks. For this reason, the need for genetic control measures has not been as urgent for BSMV as for other barley viruses.

BSMV infected plants contain characteristic rigid rod-shaped particles that are ~25 nm in diameter, with lengths ranging from ~100 to 160 nm depending on the sizes of the encapsidated RNAs. Although all BSMV strains are composed of three genomic RNAs designated α, β, and γ (Fig. 2), agarose gel analysis reveals considerable strain-specific complexity in the apparent number of RNAs (Jackson et al. 1989). The 3.8 kb α and 3.2 kb β RNAs of the sequenced strains are similar in size, but strains differ in the sizes of RNAγ. The γRNA of the ND18 strain is 2.8 kb in size, whereas the Type strain RNAγ is 3.2 kb, and the Argentina Mild strain contains mixtures of RNAγ species of 3.2, 2.8, and 2.6 kb. The 2.6 kb Argentina Mild γRNA is defective as the result of a deletion in the 3' terminus of the γa gene. Each of the RNAs has a 7-methylguanosine cap at the 5' terminus and a conserved

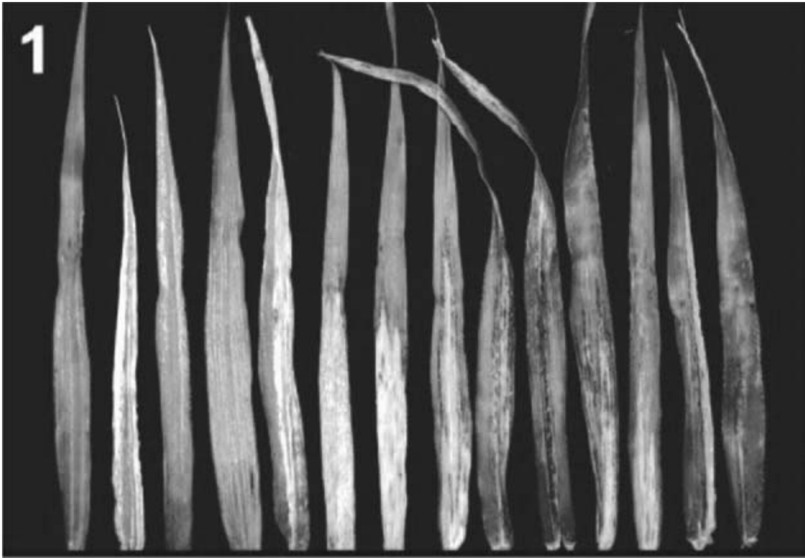

Fig. 1. Typical leaf symptoms induced by *Barley stripe mosaic virus* on 'Dickson' barley on leaves of plants in a field planted with infected seed. The symptoms range from mild mosaic to severe necrosis, often form a V-shaped pattern or chevron. (See also Colorplates, p. xix)

238 nt 3' terminal non-translated sequence. The 3' terminal sequences are capable of forming tRNA-like structures that can bind tyrosine *in vitro* and *in vivo*. An internal poly(A) sequence of varying length is located directly upstream of the tRNA-like structure and separates the coding region of each of the genomic RNAs from the 3' terminus.

The RNA-dependent RNA polymerase (RdRp) of BSMV is encoded by proteins residing at the 5' proximal ends of RNAs α and γ (Fig. 2). The αRNA encodes a single protein (αa) that forms a subunit of the RdRp and contains a methyltransferase domain presumably involved in RNA capping, and a helicase domain characteristic of proteins involved in nucleic acid unwinding. The γRNA encodes a second viral-encoded RdRp protein subunit (γa) and also encodes a cysteine-rich protein, designated γb, that is expressed from a subgenomic mRNA. The γa protein contains the conserved GDD motif found in the RdRp polymerase subunit of RNA viruses, so γa appears to be critical for processive activities during polymerization. Thus, host resistance targeted against the replicase proteins could potentially provide a potent defence mechanism.

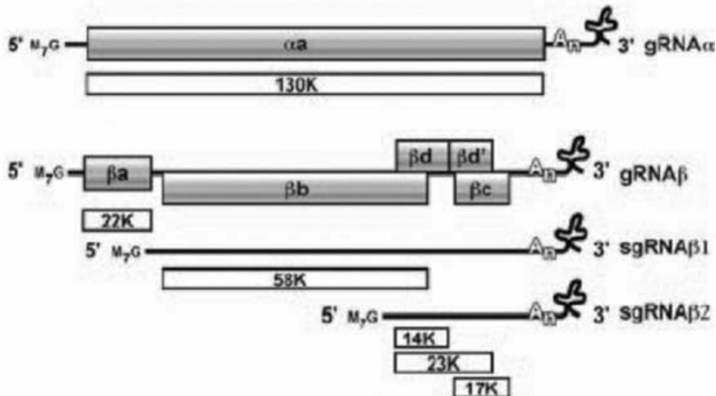

Fig. 2. Genomic organization and proteins encoded by *Barley stripe mosaic virus.* Black lines represent single-stranded, positive-sense genomic and subgenomic BSMV RNAs (gRNAs and sgRNAs, respectively). Open reading frames (ORFs) are drawn as shaded boxes on the gRNAs (designated α, β, and γ) and a 5' cap structure present on all RNAs is represented by M7G. Each 3' proximal ORF terminates with a UAA codon followed by an internal 4 to 40 nt polyadenylate sequence (An) that precedes the 238 nt tRNA-like 3' terminus. White boxes represent the protein products with molecular weights in kilodaltons (K) and are illustrated below the gRNA or sgRNAs from which they are expressed. The αa protein is required for replication and contains helicase (Hel) and methyltransferase (Mt) domains, forming the "helicase subunit" of the viral replicase complex. The gRNAβ contains five open reading frames. The coat protein, βa, is translated directly from the gRNA. The overlapping triple gene block proteins, βb, βc, and βd, are each required for virus movement and are expressed from two sgRNAs, sgRNAβ1 and sgRNAβ2. TGB1 contains a helicase (Hel) domain and RNA binding activity, whereas TGB2 and TGB3 are small hydrophobic proteins that target TGB1 to plasmodesmata. TGB2' is a minor translational readthrough protein that is dispensable for infection. The gRNAγ is bicistronic and functions in translation of the γa protein, which contains the GDD domain and is the polymerase subunit of the replicase. The cysteine-rich γb protein, which is expressed from sgRNAγ, is involved in pathogenesis.

The γb protein appears to have important roles in disease development and shares some structural similarities to a number of cysteine-rich proteins encoded by other small RNA viruses, including the tobra- and carlaviruses, but has no obvious amino acid sequence homology with these proteins. The wild-type γb protein appears to be cytoplasmically localized as assessed by subcellular fractionation studies (Donald et al. 1993) and appearance of a γb-GFP (green fluorescent protein) fusion protein in infected cells (Lawrence and Jackson, 2001a, b). The protein has been expressed and purified from *Escherichia coli* and shown to bind ssRNAs *in vitro* (Donald and Jackson, 1996) and the RNA binding domain has been mapped to a basic region separating the cysteine-rich motifs. We (Bragg et al. 2004) have recently shown that both cysteine-rich motifs and the basic motif within the amino terminus of the γb protein each have zinc binding activities. We have also demonstrated that the carboxyterminal half of the γb protein has a coiled-coil motif that participates in protein-protein interactions required for function of the protein (Bragg and Jackson, 2004). In addition, the ability of the γb protein to suppress gene silencing in various analyses and the reduced levels of viral RNAs in plants infected with γb mutants (Petty et al. 1994; Donald et al. 1994) suggest that a major role of the γb protein is to interfere with the innate antiviral defences that have evolved in plants.

RNAβ is essential for formation of virions and for local and systemic movement in plants. The first open reading frame (ORF) encodes the 22 kD coat protein (βa), which is expressed by direct translation of the genomic RNA (Fig. 2). The βa ORF is followed by a series of overlapping genes termed the "triple gene block" that are also present in Carla-, Furo-, and Potexviruses. As outlined below, genetic analyses have shown that the coat protein is dispensable for cell-to-cell movement and is not required for systemic invasion of plants. These results thus show that BSMV is unusual in that vascular movement does not require a coat protein function or the formation of virions.

The second ORF of RNAβ encodes the triple gene block βb protein, which is translated from a subgenomic mRNA (sgRNAβ1) that is present in reasonably high abundance in infected cells (Fig. 2). The βb protein contains a conserved NTPase/helicase domain with seven characteristic motifs similar to the helicase domain present in the αa protein. A recombinant protein has been purified from infected plants and bacteria, shown to have a high binding affinity for single-stranded and double-stranded RNAs, and nucleotide triphosphates, and to exhibit ATPase activity *in vitro* (Donald et al. 1997). Helicase activity has also been detected during *in vitro* assays with the purified protein (Kalinina et.al., 2002) and a GFP fusion protein forms intense foci at the plasmodesmata (Lawrence and Jackson, 2001a). These results are consistent with genetic analyses showing

that βb is essential for cell-to-cell movement and that the protein appears to function in part by RNA binding activities. Mutations in any one of the six conserved helicase motifs prohibit the ability to function in movement, possibly because of a failure to mediate appropriate conformation of viral RNAs transported by the movement complexes or because of disruption of protein:protein interactions that may be controlled by the helicase domain. The βb protein appears to associate with membranes in infected protoplasts and cells, as assessed by biochemical assays and visualization of the GFP:βb fusion protein. Viral RNA also associates with βb *in vivo*, because a βb:RNA complex can be recovered from infected tissue (Lawrence et al. unpublished). The RNAs in this complex are BSMV-specific and plus sense in nature, so cell-to-cell movement is predicted to involve plus sense RNAs rather than dsRNA replicating intermediates.

The remaining two ORFs of the triple gene block encode the small hydrophobic proteins, βc and βd, that are strictly required for cell-to-cell movement of BSMV. The proteins are expressed from a small subgenomic mRNA designated sgRNAβ2 that is present in very low abundance in infected plants (Fig. 2). The βd protein is present in low abundance and is membrane-associated, but βc has not been detected in infected plants. This is thought to be a consequence of low antigenicity and low abundance *in vivo*. Each protein contains two hydrophobic regions separated by a hydrophilic stretch suggestive of transmembrane domains. The βc and βd proteins are able to form heterologous interactions in yeast two-hybrid assays (Lawrence and Jackson, unpublished), and ectopic expression experiments suggest that the two proteins function to target βb-viral RNA complexes to the plasma membrane and presumably to plasmodesmata (Lawrence and Jackson, 2001b).

Genetic analysis of BSMV pathogenicity

The availability of full-length cDNA clones and the subsequent generation of infectious transcripts has permitted functional analyses of pathogenicity factors encoded by the BSMV genome. Natural and *in vitro* mutants in both the noncoding and coding regions of RNAγ have been shown to have a number of strain-specific effects on replication and pathogenesis (Petty et al. 1990). The early results showed that γb is not strictly required for infectivity in plants, but that it has important effects on pathogenicity and on the expression of genes encoded by RNAβ. For example, null mutations in γb inhibit infections of the Type strain of BSMV, but the ND18 strain is able to establish systemic infections albeit with an altered symptom phenotype. The failure of the Type strain to move in the

absence of γb can, however, be reversed by mutations that increase the abundance of the γa replicase or that remove an amino terminal extension of the γa protein that may reduce the function of the protein. These results suggest that complex interactions may affect the disease phenotype elicited by various mutants, and that the levels of replication may have important effects on virulence requirements. In addition, biochemical examination of plants infected with several site-specific γb mutants shows that some of these have secondary effects that culminate in greatly reduced abundance of RNAβ and the coat protein, as well as sgRNAβ1, which directs the synthesis of the βb protein (Donald and Jackson, 1994; Zhou and Jackson, 1996). Genetic analyses also reveal several distinct phenotypes in the dicot hosts, *Chenopodium amaranticolor* and *Nicotiana benthamiana*, as well as in barley (Petty et al. 1994).

BSMV is unusual in that the coat protein is not required for infectivity in barley and *N. benthamiana* (systemic hosts) or *Chenopodium* species (local lesion hosts), suggesting that the coat protein is not a major determinant in either cell-to-cell or systemic movement. Moreover, the infections elicited by coat protein-deficient BSMV mutants are more aggressive, and the phenotype is more severe and more protracted in barley than in the presence of the coat protein. These results suggest that the coat protein may have a critical role in regulating kinetics of replication late in infection, as well as being essential for formation of virions.

Natural phenotypic variation among BSMV strains has facilitated identification of the determinants of seed transmission (Edwards, 1995) and host range (Weiland and Edwards, 1994, 1996; Santoso and Edwards, 2003). Primary determinants of seed transmission reside on RNAγ, with sequences of both the 5' untranslated leader of RNAγ and the γb gene influencing efficiency of seed transmission and symptom expression. Considering the trans effects of the γb gene on RNAβ gene expression noted above, phenotypic effects attributed to γb could result from complex interactions with RNA β and γ gene products. In addition, an approximately 370 nt repeat present in the γa gene of some strains reduces seed transmissibility, perhaps by reducing the efficiency of replication and/or indirectly movement via reduced activity of the γa gene product.

Although there is no evidence in favor of a role for RNAα in seed transmission, the αa gene plays a key role in host range. Sequence analysis and recombination studies suggested that nucleotide changes in the αa gene determine pathogenicity to both barley and oats (Weiland and Edwards, 1994; Santoso and Edwards, 2003). Subsequent mutation analysis demonstrated that a single nucleotide substitution, resulting in a single amino acid change, can confer oat pathogenicity to a strain otherwise nonpathogenic to oat (Weiland and Edwards, 1996). Several other nearby nucleotide substitutions resulting in amino acid changes can confer

pathogenicity to resistant barley. Since all of the αa sequence variants tested are viable in at least one host, sequence changes within the αa gene must affect the ability of the virus to interact with host-encoded components required for virus accumulation. The sequences thus far identified as crucial to seed transmission and host range can all be envisioned to influence both replication and movement, even if only indirectly.

In summary, each of the proteins encoded by BSMV have defined roles in replication, formation of virions, movement, or pathogenicity; hence each may be a potent target for host resistance responses.

Resistance to BSMV

BSMV resistance in barley has been known for many years and the inheritance of that resistance has been the subject of several studies. 'Modjo' (CI 3212) and 'Modjo-1' (CI 14048) barley were first reported to possess a single recessive gene mediating resistance to the California "E" isolate of BSMV after seedling tests, although the authors also suggested that a second gene was involved at later stages of plant growth (Sisler and Timian, 1956; Timian and Sisler, 1955). A single recessive gene also was reported to mediate resistance to BSMV strain ND1 in 'Traill' (CI 9538), 'Modjo-1', and 'Moreval' (CI 5724) barley by Timian (1975), while a multiple allelic series was suggested to control reaction to the Type strain. Timian and Franckowiak (1987) also identified a single recessive gene conferring resistance to BSMV strain CV42 and found that it was linked to the Lk2 locus (controlling awn length) on chromosome 7H in 'Modjo-1', 'Moreval', and 'CI 4197' barley. More recently, resistance to strain CV42 was mapped to the centromeric region of chromosome 7H based on its linkage to molecular markers on the Steptoe/Morex map (Edwards and Steffenson, 1996). This gene (rsm1) cosegregated with the RFLP marker ABC455, and the nearest flanking markers were at least 6 cM away. Although differences among virus strains and barley genotypes in these studies prevent definitive identification of relationships among these genes, it appears reasonable to suggest that alleles for the same recessive gene were identified in several of the studies. Unfortunately, the location of rsm1 near the centromere in a region of fairly low recombination reduces the likelihood of finding adequate markers to pursue positional cloning to isolate and characterize this gene.

The recessive nature of the BSMV resistance in these barley varieties suggests that resistance may be due to the absence of a required function for virus replication and/or movement, rather than due to an active response to BSMV infection. This lack of function could result from the complete absence of a required factor or, as is more likely, a functional absence resulting from mutation of an essential host replicase protein. The latter

possibility is supported by the fact that other strains (e.g. ND18) can successfully establish infection in the resistant varieties (Edwards and Timian, 1986). The required factor must also be involved in virus replication, because resistance to CV42 is expressed in protoplasts of Modjo-1, Moreval, CI 4197, and Morex barley (Zheng and Edwards, 1990; Santoso and Edwards, unpublished).

Further evidence for the involvement of the susceptibility/resistance factor in virus replication comes from mapping of viral pathogenicity determinants. Pathogenicity to resistant barley (Modjo-1, Morex) maps to an approximately 200 nt region within the αa gene, which encodes the helicase subunit of the RdRp. The nonpathogenic CV42 strain differs from the pathogenic ND18 strain by six amino acids within this region, but recent evidence shows that a three amino acid cluster ($Q^{733}S^{734}Q^{736}$ in ND18 vs. $K^{733}T^{734}K^{736}$ in CV42) determines pathogenicity (Santoso and Edwards, 2003 and unpublished).

It is intriguing to note that the same region of the αa gene was previously identified as critical to the oat pathogenicity of strain CV42, but the specific amino acid substitutions required are not identical (Weiland and Edwards, 1994; Weiland and Edwards 1996). In this case, a single nucleotide substitution, resulting in the amino acid substitution of P^{724} with T^{724}, is adequate to confer oat pathogenicity to the nonpathogenic ND18. Despite the fact that the same region of BSMV αa is involved in pathogenicity to both hosts, resistance in oats appears to be due to inhibition of virus movement because ND18 can replicate in oat protoplasts. Weiland and Edwards (1994) speculated that replicase-associated genes such as αa may be somehow involved in virus movement, because subtle host specific alterations in the levels of replication are known to tip the balance toward or against infection, as is the case with mutations in RNAγ that differentially affect movement of BSMV strains (Petty et al. 1990; Jackson et al. 1991a, b). In the case of either barley or oat pathogenicity, amino acid substitutions in αa could result in conformational changes that influence the efficiency as well as functionality of the αa protein in replication. Alternatively, resistance to ND18 in oats may be due to the reduced ability or inability of the virus to replicate in some cell or tissue type through which the virus must pass in order to establish systemic infection. Such effects obviously would be construed as blocks to cell-to-cell movement.

Complexity of Barley Yellow Dwarf Isolates

Barley yellow dwarf has long been considered to be the most widespread and economically significant virus disease of small grains, and a number of viruses have been demonstrated to be causal agents of this disease since its viral etiology was first described in 1951 (Oswald and Houston,

1951). Members of the BYDV complex infect many grass species and all major cereal crops, including barley, oats and wheat, where collectively they cause a characteristic yellowing syndrome (Fig. 3), stunting, low seed set, and reduced grain weight. Aphid movement can quickly result in BYDV infection of cereal seedlings from grass reservoirs, as well as secondary spread within an affected field. Therefore, losses can be quite substantial and 11 to 33% yield reductions have been reported in previous reviews (Lister and Ranieri, 1995). However, outbreaks of BYDV are notoriously difficult to forecast due to the complexities of aphid transmission, availability of seasonal bridging hosts, and annual environmental variables that affect accurate yield loss projections. The world-wide distribution of barley yellow dwarf and its economic importance have led to the accumulation of a large volume of literature, much of which has been compiled into the excellent overview "BYDV: Forty Years of Progress" (edited by D'Arcy and Burnett, 1995).

BYDV infected plants contain 25 to 30 nm particles that are primarily localized to the phloem, in which they reach high concentrations. BYDV is not transmissible using manual inoculation techniques, and natural plant to plant passage requires circulative aphid transmission. Among the 25 or more known aphid vector species, transmission specificity and efficiency exhibit enormous variation, and even biotypes of a given aphid species may vary significantly in their transmission ability. Classical studies initiated by Rochow (1970) to explore such variability in New York first revealed the presence of different BYDV isolates. These isolates were separated into five serologically distinct BYDV subtypes whose designation and aphid vectors are: RPV (*Rhopalosiphum padi*), RMV (*R. maidis*), MAV (*Sitobion avenae*), SGV (*Schizaphis graminum*) and PAV (*R. padi*, *S. avenae* and several other aphids). Although some variation between serotype and aphid transmission characteristics became evident in subsequent studies, this classification scheme provided a useful framework until more refined molecular analyses could be conducted.

Nucleotide sequence analyses have recently resulted in the separation of BYDV subtypes into two distinct genera, *Luteovirus* and *Polerovirus*, within the *Luteoviridae* family. The International Committee on the Taxonomy of Viruses (ICTV) has assigned the MAV and PAV serotypes as the sole members of the *Luteovirus* genus, and RPV has been renamed *Cereal yellow dwarf virus*-RPV (CYDV-RPV) and placed in the *Polerovirus* genus (D'Arcy et al. 2000). The BYDV GPV, RMV and SGV subtypes are not currently assigned to a genus.

Fig. 3. Barley yellow dwarf focus in a field near Purdue University photographed by Richard Lister in 1968. Note the yellowing plants and the circular ring of infection that probably resulted from movement of apterous aphids from plant to plant. (See also Colorplates, p. xx)

BYDV/CYDV Structure and Molecular Biology

All members of the *Luteoviridae* have nonenveloped T=3 icosahedral particles with diameters between 25 to 30 nm. The two *Luteovirus* BYDV subtypes and the *Polerovirus* CYDV-RPV particles are composed of 180 protein subunits consisting of a mixture of a predominant 22 kD population and a small number of 50 to 55 kD readthrough proteins (D'Arcy et al. 2000). The positive sense viral RNAs represent about 28% of the mass of the virion, range in size from 5.6 to 5.8 kb, and lack a cap and a poly A tail (Fig. 4). Among other distinguishing characteristics described below, the *Polerovirus* genus contains a genome-linked protein (VPg) and a 5' ORF, designated ORF 0, both of which are lacking in members of the *Luteovirus* genus. The *Luteovirus* and *Polerovirus* RdRp genes also exhibit striking differences, and the two genera differ in sequences at their 3' termini. These characteristics are summarized below and have recently been the subjects of an excellent overview by Miller et al. (2002).

The *Luteovirus* subtypes are very closely related except for the sequences of their coat proteins, so they will be discussed together. The *Luteovirus* genome (Fig. 4) consists of five major ORFs that appear to be expressed in

infected plants. A minor ORF 6 can be translated from viral RNAs *in vitro*, but has not been detected *in vivo*. The "cap independent" translation of the RdRp subunits requires interactions of a 3' translation element for initiation at the ORF 1 AUG, while production of the low abundance ORF 2 polymerase fusion subunit of the RdRp requires a low frequency –1 translational frame shift at a secondary structural element residing between ORF's 1 and 2 (see Miller et al. 2002 for review). Molecular genetic evidence has shown that the remaining 3' proximal ORFs are dispensable for BYDV RNA replication in protoplasts.

ORFs 1 and 2 are expressed directly from the genomic RNA and ORFs 3, 4 and 5 are translated from sgRNA 1 via unconventional mechanisms (Miller et al. 2002). The first of the sgRNA mechanisms requires the interaction of a 3' translation element with the 5' terminus to facilitate translation of the high abundance ORF 3 coat protein at the first AUG. A second feature is a minor translational read-through that produces a low abundance ORF 5 coat protein fusion product that is essential for aphid transmission but dispensable for virion assembly (Chay et al. 1996). The third strategy involves a leaky scanning mechanism for expression of the ORF 4 nested gene, which encodes a protein essential for cell-to-cell transmission through phloem plasmodesmata. A high abundance sgRNA 2 that can be detected in plants directs synthesis of an ORF 6 gene product *in vitro*. However, all available evidence indicates that the ORF 6 protein is not translated in protoplasts, so an attractive hypothesis is that sgRNA 2 may regulate translation of the genomic RNA and sgRNA 1 in trans (Miller, et al. 2002). Late plant infections also contain an additional, high abundance, small sgRNA 3 (Kelly et al. 1994); however, its significance is obscure because some natural isolates causing severe symptoms fail to produce detectable amounts of sgRNA 3 (W. Allen Miller, personal communication). Irrespective of the functions of sgRNAs 2 and 3, transcription of all three of the sgRNAs is regulated by negative strand promoters with different structural properties (Koev and Miller, 2000).

The 5600 nt CYDV-RPV *Polerovirus* differs from the BYDV *Luteoviruses* by having a 5' terminal ORF 0 of no known function. ORF 1 of CYDV-RPV overlaps both ORF 0 and ORF 2, but the CYDV ORF 1 is much larger and has a considerably different sequence than the *Luteovirus* ORF 1 (Fig. 3). ORFs 0, 1 and 2 are translated from the genomic RNA. ORF1 is presumably translated via a leaky scanning mechanism using ribosomes that fail to initiate translation at the ORF 0 initiation codon. ORF 1 encodes the major RdRp subunit protein, which differs from the *Luteovirus* ORF 1 by containing a cysteine protease domain that serves to cleave the 17 kD VPg during RNA replication. The minor polymerase RdRp subunit

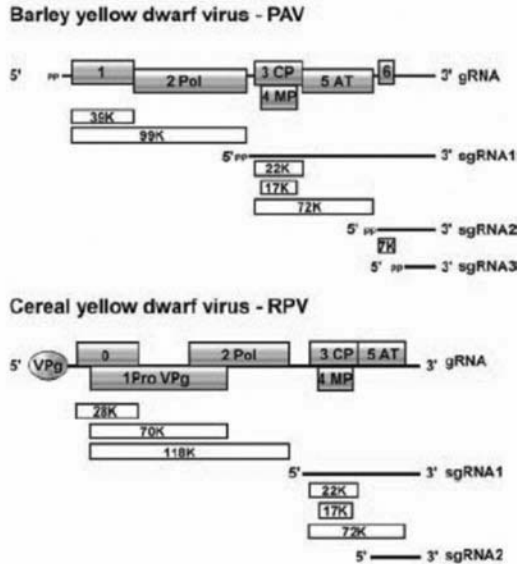

Fig. 4. Organization and proteins encoded by the *Barley yellow dwarf virus* (PAV) and cereal yellow dwarf *Polerovirus* (CYDV) genomes. Black lines represent single-stranded, positive-sense genomic (gRNA) and subgenomic (sgRNA) RNAs with open reading frames (ORFs) indicated as shaded boxes on the gRNAs. White boxes illustrated below the gRNA or sgRNA molecules represent the protein products and their molecular weights in kilodaltons (K). The protein functions are indicated where known: Pol (polymerase), CP (coat protein), MP (movement protein), and VPg (genome-linked protein). The designation AT represents a CP readthrough domain that probably is required for aphid transmission. The function of the 7K product encoded by BYDV-PAV is not known.

encoded by ORF 2 is expressed as a result of a low abundance translational frameshift from ORF 1. As indicated above, a translational frameshift mechanism also accounts for synthesis of the *Luteovirus* genus ORF 2 polymerase subunit, but the two genera have substantial differences in the sequences of the frameshifting motifs (Miller et al. 2002). These differences and the differences in sequences of the RdRp proteins suggest that the *Polerovirus* and the Sobemovirus genera have a common origin, whereas the *Luteovirus* RdRp's are more similar to those of members of the Tombusviridae (Miller et al. 2002). In contrast to the lack of homology between their 5' genes, ORFs 3, 4 and 5 of the *Luteoviruses* and the *Poleroviruses* share homology, have similar functions, and are translated by similar mechanisms from sgRNA 1. Hence, an attractive hypothesis is that

the 5' encoded sequences of BYDV and CYDV evolved from Tombusviridae and Sobemovirus progenitors, respectively, and that their 3' sequences (ORFs 3, 4 and 5) form a "*Luteovirus* block" with a common origin (Miller et al. 2002).

BYDV has been known to be transmitted in a circulative nonpropagative fashion by a large number of aphids for nearly 50 years. Subsequently, a large body of evidence has resulted in a detailed model for strain-specific transmission by aphid species (for detailed reviews see Gray and Rochon, 1999; Miller, 1999; Miller et al, 2002; Miller and Rasochova, 1997; Young and Filichkin, 1999). A rudimentary understanding of the specificity of transmission of BYDV by different aphid species was first obtained by Rochow's (1969) studies, revealing a remarkable specificity that resulted in the first reliable BYDV classification system. His experiments also showed that heterologous transcapsidation could readily occur in plants infected with different BYDV mixtures, and that transmission resulted from highly specific interactions between the viral capsid proteins and the aphid vector (Rochow, 1970).

An emerging model for transmission, based on work by Gildow and colleagues (Gildow, 1999), indicates that three barriers in the aphid must be circumvented for transmission to occur. The first event following virus ingestion requires specific binding to epithelial cells in the hindgut, followed by membrane transport into the haemocoel. This event is not highly specific, but once inside the haemocoel, the coat protein readthrough domain is thought to interact with symbionin, an endosymbiotic bacterial protein (Young and Filichkin, 1999). Although the interaction is not highly specific, symbionin is thought to protect the virus particle from degradation and permit its survival for substantial periods in the aphid. For specific transmission to occur, the virus is thought to require interactions with the readthrough domain of the coat protein for movement from haemocoel into the basal lamina of the accessory salivary glands and subsequent receptor-mediated endocytosis across the plasmalemma. After movement into the salivary canal, virions are able to enter the phloem during feeding by the viruliferous aphids. This model only highlights the many exquisite molecular events that are critical for transmission and maintenance of the virus; hence, a more detailed understanding of these events might lead to novel strategies to interdict transmission and provide effective control of barley yellow dwarf disease.

Resistance to BYDV/CYDV

Breeding for resistance to barley yellow dwarf has been practiced for over 50 years in some countries (Burnett et al. 1995), yet adoption of alternative control measures precluded resistance breeding from ever really

being a priority in other countries such as the UK (Plumb, 2002). Effective breeding requires evaluation and selection for strain-specific resistance under field conditions. Unfortunately, such screening is notoriously variable because it generally has been based on symptom expression and yield assessment rather than by quantitative assays that account for the particular virus strain and virus titer. Thus, the conclusion of Burnett et al. (1995), as well as other researchers, is that tolerance, rather than resistance per se better describes what has been traditionally measured in field studies.

The most commonly used gene in breeding for BYD tolerance is *Ryd*2, or as it is more commonly known, *Yd*2. This semidominant gene was first identified in barleys of Ethiopian origin (Rasmusson and Schaller, 1959), and is located on chromosome 3H (Schaller et al. 1964). A lower level of tolerance is conferred by *yd*1, a recessive gene discovered in Rojo barley by Suneson (1955), but the level of protection is apparently so low that the gene has not been used or studied extensively. Recently, Niks et al. (2004) identified a novel gene near the centromere of chromosome 6H in the Ethiopian barley line L94 and proposed the name *Ryd*3. The degree of dominance is not yet known, but the resistance conferred appeared comparable to that of *Ryd*2. The search for other sources of resistance and/or tolerance within the genus *Hordeum* has been fairly extensive, so it is not clear how a gene with the major effect of *Ryd*3 has gone undetected until now. Nevertheless, the resistance conferred by *Ryd*2 remains the most commonly available at this time.

More than 7,000 entries from the USDA world collection of barley were screened by Schaller and colleagues (Schaller et al. 1963; Qualset and Schaller, 1969), who found 189 entries with some measure of tolerance. One entry was from China, but the vast majority of those with tolerance were introductions from Ethiopia. The remaining tolerant entries were either hybrids with Ethiopian parentage or of likely Ethiopian origin, thus the majority of those with tolerance may have possessed *Ryd*2 or possibly even *Ryd*3 from North Africa. Since these early screening successes, organizations around the world (eg. ICARDA, CIMMYT) have continued screening programs. A number of “somewhat BYDV-tolerant” barley cultivars have been released that have no relation to the Ethiopian barleys and do not contain *Ryd*2. ‘Post’ winter barley is an example (Grafton et al. 1982). However, defined genes present in these cultivars have not been specifically identified and characterized, and therefore the remainder of our discussion will focus on *Ryd*2.

There is some evidence that the effectiveness of *Ryd*2 varies with genetic background (eg. Catherall et al. 1970). Some breeders have found that *Ryd*2 is less effective in later maturing germplasm (Catherall and Hayes, 1966;

Jones and Catherall, 1970), but this is not the case in other germplasm (Schaller, 1984). Where *Ryd*2 is effective, the level of virus accumulation in the host plant is reduced as a result of resistance targeting some aspect of virus accumulation (Ranieri et al. 1993; Larkin et al. 1991; Skaria et al. 1985), suggesting that *Ryd*2 is truly a "resistance" gene. This reduction in virus accumulation is generally effective only against BYDV (eg. MAV, PAV, genus *Luteovirus*), and not against CYDV (originally BYDV-RPV). An exception is that Larkin et al. (1991) found that *Ryd*2 conferred resistance to an Australian RPV, although the effectiveness varied with the genetic background. Larkin et al. (1991) also showed that *Ryd*2 does not function in leaf protoplasts, leaving open the possibility that replication itself may not be restricted substantially. Cell-to-cell movement might be impaired, or *Ryd*2 may only be expressed in phloem cells, which are not well-represented in protoplast populations. Given the data to date, it is probably more accurate to say that this gene restricts virus accumulation, rather than virus replication or spread.

The *Ryd*2 gene has been precisely mapped by Collins et al. (1996) as a prelude to map-based cloning strategies. These studies demonstrated that *Ryd*2 cosegregated with RFLP markers Xwg889 and XYlp on the long arm of chromosome 3, about 0.5 cM from the centromere. Sequence data reveals that the Ylp alleles differ by a single nucleotide in barley with and without *Ryd*2, and the tight linkage between Ylp and *Ryd*2 provided an opportunity to develop a robust marker for *Ryd*2 selection. Subsequently, a codominant PCR-based marker designated YLM was developed (Paltridge et al. 1998).

The precise mechanism by which *Ryd*2 functions is not known. It is known that the tightly-linked Ylp encodes a vacuolar H^+-translocating ATPase subunit E (Ford et al. 1998; Dietz et al. 1995), but a role for this peptide in resistance remains speculative.

Barley yellow mosaic complex

Barley yellow mosaic is a serious disease of winter barley in East Asia and Europe (Mathre, 1997; Plumb, 2002). Although the disease was originally thought to be caused exclusively by BaYMV, it is now known to be caused also by BaMMV, and diseased plants may have either individual or mixed infections. While barley is susceptible to both viruses, wheat (*Triticum aestivum*), oats (*Avena sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), and corn (*Zea mays*) are not susceptible (Brunt et al. 1996). Both viruses elicit similar symptoms in barley, including longitudinal streaks on unfurling leaves (Fig. 5), some necrosis, and possible leaf rolling conveying a spiky appearance to the plants. Therefore, these species must be distinguished serologically (Brunt et al. 1996), which becomes particularly important when screening for virus-specific resistance. A number of

BaYMV strains have been identified on the basis of cultivar reactions in Asia and Europe (Kashiwazaki et al. 1989; Adams, 1991). Although BaMMV is now known to be distinct (Huth and Adams, 1990), it was initially thought to be a BaYMV strain that was more amenable to mechanical transmission than previously studied BaYMV strains that were mechanically transmitted only with difficulty. Both viruses are soilborne and are transmitted in nature by the plasmodiophorid *Polymyxa graminis*, an obligate parasite of plant roots that causes little direct damage to plants but is a significant factor in transmission of several viruses (Kanyuka et al. 2003). The resting spores of *P. graminis* provide a key niche for virus survival and infection of subsequent crops, and the persistence of these spores for many years in infected fields precludes efficient disease control through chemical or cultural methods. Therefore, the use of resistant varieties has been the principal means of control in both East Asia and Europe.

Molecular biology of BaYMV and BaMMV

BaYMV and BaMMV are members of the *Bymovirus* genus within the family *Potyviridae*, and much of the molecular information attributed to these two viruses is inferred from studies of the aphid transmitted members of the monopartite *Potyvirus* genus (Lopez-Moya and Garcia, 1999; van Regenmortel et al. 2000). The *Bymovirus* genus is unique within the *Potyviridae* because its genome is divided between two single-stranded, positive-sense RNA molecules. The size of RNA1 ranges from 7.5 to 8.0 kb, and RNA2 varies from 3.5 to 4.0 kb. Like other members of the *Potyviridae,* the genomic RNAs contain a covalently linked 5' VPg and a 3' terminal poly(A) sequence. Aside from their 5' noncoding region and the poly(A) tails, *Bymovirus* RNAs 1 and 2 share little sequence similarity. The RNA1 sequence corresponds to the 3' three-fourths of the monopartite *Potyvirus* genome, while the 5' third of RNA2 encoding P1 corresponds to the remaining portion of the genome. The remaining RNA2 sequence encoding the P2 protein is not analogous to sequences found in potyviruses, and this protein is thought to be involved in fungal transmission. As is the case for all members of the *Potyviridae*, each RNA molecule contains a single open reading frame (ORF) encoding a polyprotein that becomes proteolytically processed into functional, mature products.

Fig. 5. Symptoms induced by *Barley yellow mosaic virus* on barley in Japan. Yellow-green spots and short streaks are typically produced on leaves, and as yellowing increases, large yellow patches appear in affected fields. Courtesy B. Steffenson, University of Minnesota. (See also Colorplates, p. xx)

BaYMV and BaMMV replicate in the cytoplasm, and reflecting their bipartite genomes, two distinct 13 mm wide particles with modal lengths of 250-300 nm and 500-600 nm are observed in virus preparations. These flexuous, filamentous particles comprise 5% genomic RNA and 95% coat protein by weight. The polyprotein strategy of gene expression implies that all of the viral proteins from a single genomic RNA are produced in equimolar amounts. Another similarity to the *Potyvirus* genus is that *Bymovirus*-infected cells contain cytoplasmic pinwheel-like inclusions and membranous network structures that are composed of virus-encoded proteins, but they lack the nuclear inclusions seen in some potyvirus infections. Although these structures do not contain virions per se, virus particles may be attached to the pinwheel inclusions.

Seven of the eight major proteins encoded by BaYMV and BaMMV RNAs 1 and 2 (Fig. 6) are analogous to *Potyvirus*-encoded proteins and are predicted to have equivalent functions to their counterparts. RNA1, which has been shown to carry the pathogenic determinants of BaMMV, is translated as an ~270 kDa polyprotein. This polyprotein is proteolytically processed into six major proteins as well as two small, hydrophobic 6 kDa and 8 kDa peptides that modify the activity of adjacently encoded proteins.

Table 1. Virus resistance genes in barley (*rym1-13* data adapted from that of Ordon et al. [2004] and Werner et al. [2003a]).

Resistance Gene	**Chromosome**	**Source**	**Resistance to:**[a]	**References**[d]
rsm1	7H	Morex	BSMV CV42	3, 24
ryd1		Rojo	BYDV	22
Ryd2	3HL	various Ethiopian landraces	BYDV (eg. MAV, PAV)	2, 19, 21
Ryd3	6H	L94	BYDV (eg. MAV, PAV)	15
rym1	4HL	Mokusekko 3	BaMMV, BaYMV, BaYMV-2 (Germany) BaYMV-I, BaYMV-II, BaYMV-III (partial),	4, 12, 17, 23
		Y4	BaMMV-Ka1, BaMMV-Na1 (Japan)	16
rym2	7HL	Mihori Hadaka 3	BaMMV, BaYMV, BaYMV-2	4, 17, 23
rym3	5HS	Ea 52, Ishuku Shirazu	BaYMV, BaYMV-2	4, 17, 20
rym4	3HL	Ragusa, Franka	BaMMV, BaYMV	4, 5, 17, 18
rym5	3HL	Mokusekko 3, Resistant Ym No.1, W122/37.1	BaMMV, BaYMV, BaYMV-2	4, 6, 8, 12, 17
rym6	3HL	Prior, Amagi Nijo	susceptible (Germany) BaYMV-II (Japan)	10, 11, 13
rym7	1HS	HHor 3365	BaMMV	9
rym8	4HL	10247	BaMMV, BaYMV	1, 4, 9, 17
rym9	4HL	Bulgarian 347	BaMMV	1, 4, 17
rym10	3HL	Hiberna	BaYMV, BaYMV-2	6, 8
rym11	4HL	Russia 57	BaMMV, BaYMV, BaYMV-2	1,4, 17
rym12	4HL	Muju covered 2	BaMMV, BaYMV, BaYMV-2	4, 7, 17
rym13	4HL	Taihoku A	BaMMV, BaYMV, BaYMV-2	4, 17, 25
rym15	6HS	Chikurin Ibaraki 1[b]	BaMMV	14

[a]For bymovirus resistance genes, the resistance is that of the donor germplasm as tested in Germany unless otherwise noted.

[b]Chikurin Ibaraki 1 has been shown to be resistant in Germany to BaYMV and BaYMV-2 as well as BaMMV. However, the BaYMV/BaYMV-2 resistance of this cultivar is due to a resistance locus on chromosome 5HS rather than to *rym15* (Werner et al. 2003a).

[c]The dominant gene *Rym14*^{Hb} was introgressed into barley chromosome 6HS from *H. bulbosum* and confers complete resistance to all known European isolates of BaYMV and BaMMV (Ruge et al., 2003).

[d]References: [1]Bauer et al., 1997; [2]Collins et al. 1996; [3]Edwards and Steffenson 1996; [4]Götz and Friedt, 1993; [5]Graner and Bauer, 1993; [6]Graner et al., 1995; [7]Graner et al., 1996; [8]Graner et al., 1999a; [9]Graner et al., 1999b; [10]Iida and Konishi, 1994; [11]Iida et al., 1999; [12]Konishi et al., 1997; [13]Konishi et al., 2002; [14]Le Gouis et al. 2004; [15]Niks et al. 2004; [16]Okada et al. 2003; [17]Ordon et al., 1993; [18]Ordon et al., 1995; [19]Rasmusson and Schaller 1959; [20]Saeki et al., 1999; [21]Schaller et al. 1964; [22]Suneson, 1955; [23]Takahashi et al., 1973; [24]Timian and Franckowiak, 1987; [25]Werner et al., 2003b.

The 5' proximal protein on RNA1 is homologous to the potyvirus P3 protein. The information available about the function of P3 is scant, but the protein is known to be associated with cylindrical inclusions in *Bymoviruses* and nuclear inclusions in some monopartite *Potyviruses*. One possibility is that the P3+6K fusion protein is the functional form of the protein and that cleavage to remove the 6K fragment (which contains hydrophobic domains and is potentially membrane-associated) modulates P3 activity.

The gene adjacent to P3 is designated HC (helper component), even though this protein does not appear to function in fungal transmission of the *Bymoviruses*. In contrast, HC of the aphid-vectored potyviruses is required for insect transmission. HC has a papain-like cysteine protease that cleaves at its C-terminus, and by analogy to the TEV HC, may also function as a suppressor of RNAi (Kasschau and Carrington, 1998). Furthermore, the HC protein often forms amorphous cytoplasmic inclusions. The 8K protein fragment of *Bymoviruses* is believed to anchor replication complexes to membranes, whereas the VPg, which is covalently linked to the 5' end of the genomic RNAs, is proposed to function as a primer for the initiation of viral replication. Additional cleavage events yielding mature viral proteins are mediated by the protease (Pro) located directly 3' of the *Bymovirus* VPg. Sequence similarities suggest that Pro is analogous to the NIa protease (a serine-like protease containing a cysteine residue in the active site) of the monopartite potyviruses. NIa has RNA binding activity, which is also probably a feature of Pro, but the nuclear inclusion bodies formed by NIa are absent in the *Bymoviruses*. The replication protein (Rep) is an RNA-dependent RNA-polymerase and contains the conserved GDD polymerase motif. The *Bymovirus* Rep protein is homologous to potyvirus NIb and is proposed to recruit replication complexes through interactions with NIa. The final product of the RNA1 ORF is the coat protein (CP). In *Bymoviruses*, the CP ranges in size from 28.5 to 33 kDa, and the N-terminal region varies in length when compared with the CPs of the monopartite potyviruses. However, the internal cores of these proteins (approximately 220 amino acids) are more highly conserved in order to fulfill the structural requirements of virion formation. The BaYMV CP exhibits nonspecific RNA binding activity, and like other *Potyvirus* CPs, is predicted to have a role in cell-to-cell and long distance movement.

RNA2 of the *Bymoviruses* encodes two proteins that are processed from an ~ 98 kDa polypeptide (Fig. 6). The first, P1, is a chymotrypsin-like serine

protease. Homologues of this protein are highly variable between potyviruses, and they do not yet have an established function, although they undergo autolytic cleavage and have RNA binding activity. In addition, P1 is postulated to participate in trans during genome amplification. The P2 protein is unique to the *Bymoviridae* and is proposed to function in fungal transmission. Indeed, repeated mechanical transmission of *Bymovirus*es can lead to the loss of fungal transmission and this loss has been shown to correspond to an approximately 1 kb deletion in the P2 region of RNA2 (Jacobi et al. 1995; Peerenboom et al. 1996). Furthermore, P2 contains an amino acid combination (ER or QR) that is also found in the CP readthrough derivatives that are implicated in fungal transmission of *Benyviruses*, *Furoviruses*, *Pomoviruses*, and *Pecluviruses* (Peerenboom et al. 1996).

Resistance to BaYMV and BaMMV

At least fifteen genes are known to confer resistance to BaYMV and/or BaMMV (Table 1 and references therein), and extensive surveys have demonstrated that many barley landraces, eg. Mokusekko 3, possess more than one resistance gene (Le Gouis et al. 2004). The 15 resistance genes are distributed over six different chromosomes (1, 3, 4, 5, 6, and 7), and all but one are recessive. Fourteen of these genes were identified in *Hordeum vulgare*, while one was introduced from *H. bulbosum.* The latter gene, *Rym*14^{Hb}, is the only dominantly inherited resistance gene identified to date, and it confers complete resistance to all known European isolates of BaYMV and BaMMV (Ruge et al. 2003). Chromosomal locations have been determined for all 15 of the named genes (reviewed in Werner et al. 2003a; Ordon et al. 2004). The resistance gene *rym*1 has been mapped to the centromeric region of chromosome 4HL (Takahashi et al. 1973; Konishi et al. 1997), *rym*2 to chromosome 7HL (Takahashi et al. 1973), *rym*3 to chromosome 5HS (Saiki et al. 1999), *rym*4, *rym*5, and *rym*10 to the telomeric region of chromosome 3HL (Graner et al. 1995; Graner et al. 1999a; Konishi et al. 1997), *rym*6 to the telomeric region of chromosome 3HL (Iida and Konishi, 1994; Iida et al. 1999), *rym*7 to the centromeric region of chromosome 1HS (Graner et al. 1999b), *rym*8, *rym*9 (Bauer et al. 1997), *rym*12, *rym*13 (Graner et al. 1996; Werner et al. 2003b) to the telomeric region of chromosome 4HL, *rym*11 to the centromeric region of 4HL (Bauer et al. 1997), and *rym*15 near the centromeric region of chromosome 6HS (Le Gouis et al. 2004). Closely linked, PCR-based

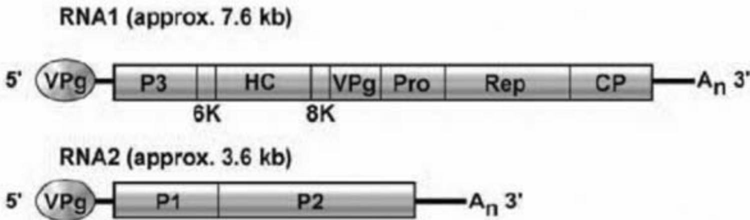

Fig. 6. Genome organization and proteins encoded by *Barley yellow mosaic virus*. The bipartite genomes of BaYMV and BaYVV have similar single-stranded, positive-sense RNA genomes that contain a genome-linked protein (VPg) at the 5' end and a polyadenylate (A_n) tract at the 3' end. Each RNA encodes a single open reading frame (ORF) illustrated as a shaded box that is translated as a polyprotein and is proteolytically processed into mature gene products. The known protein functions are: HC (helper component), VPg (genome-linked protein), Pro (protease), Rep (replicase), CP (coat protein), P1 (protease), P2 (putative fungal transmission factor). The function of P3 protein is unknown, and the 6K and 8K protein fragments are postulated to modify the activity of the P3 and VPg proteins, respectively.

molecular markers (eg. microsatellite, STS markers) have been developed for a number of the resistance genes, including *rym*4 (Graner et al. 1999), *rym*5 (Graner et al. 1999), *rym*9 (Werner et al. 2000), *rym*11 (Bauer et al. 1997), *rym* 13 (Werner et al. 2003b), *Rym*14^{Hb} (Ruge et al. 2003), *rym*15 (Le Gouis et al. 2004), and the BaYMV resistance gene of 'Chikurin Ibaraki 1' (Werner et al. 2003a).

The most widely deployed and effective gene in Europe is *rym*4, a recessive gene that confers immunity to both BaYMV and BaMMV (Graner and Bauer, 1993). In the late 1980's, a new BaYMV strain pathogenic to cultivars with *rym*4 was discovered and subsequently designated BaYMV-2 (Adams, 1991; Kühne et al. 2003; Steyer et al. 1995). An extensive search of germplasm for new sources of resistance led to the identification of *rym*5 (Graner et al. 1999; Konishi et al. 1997), a gene conferring resistance to BaYMV and BaMMV as well as BaYMV-2. Although *rym*5 is not as widely used as *rym*4, it has been successfully incorporated into some modern cultivars in both Europe and Japan.

Recently, a new BaMMV variant (BaMMV-Sil) that is pathogenic to cultivars possessing *rym*5 was described in France (Hariri et al. 2003). Other pathotypes of BaYMV and BaMMV have also been identified. Characterization of BaYMV and BaMMV isolates in France uncovered variants of both viruses capable of overcoming at least seven of the known resistance genes (*rym*3, *rym*4, *rym*6, *rym*8, *rym*9, *rym*10, *rym*11), as well as the resistance of a few varieties with unidentified resistance genes (Hariri et al. 2000). Seven strains of BaYMV and two strains of BaMMV have been described in Japan (Kashiwazaki et al. 1989; Nomura et al. 1996), and still other variants have been identified in China (Chen et al. 1996).

Studies of the nature of resistance to these viruses have not been extensive, although the reported immunity to BaYMV and BaMMV conferred by *rym*4 appears well founded. No infection of leaves occurs following mechanical inoculation, and *P. graminis* zoospores are normally unable to acquire and transmit virus from roots of *rym*4 plants (Adams et al. 1987; Schenk et al. 1995). The nature of resistance to the common strain of BaMMV, conferred by *rym*4 and most other *rym* genes, was investigated by McGrann and Adams (2004), using both mechanical inoculation and *P. graminis* transmission at two different temperatures. They found that *rym*1, *rym*2, *rym*5, and *rym*11 seemed to confer immunity to BaMMV, since virus could not be detected in any plant tissues, nor were any bioassays successful. Resistance conferred by *rym*7, *rym*8, and *rym*10 was only partial and was temperature sensitive, breaking down at higher temperatures (20° C). From these results, it appears that there may be some form of 'translocation resistance' blocking movement of the virus from root to shoot in *rym*8, *rym*9, and *rym*10 plants. Whereas *rym*8 and *rym*10 did not block infection via mechanical inoculation of leaves, resistance derived from *rym*9 seemed to be tissue-specific in that it precluded leaf, but not root, infection. No resistance to BaMMV was conferred by *rym*3 or *rym*6 in these experiments. When plants carrying the *rym*7 or *rym*10 genes were inoculated with BaMMV-Sil (pathogenic to barley with *rym*5), they also showed partial resistance with delayed virus accumulation (Kanyuka et al. 2004). This isolate was unable to infect plants carrying *rym*1, *rym*4, *rym*8, *rym*9, or *rym*11, but it is not clear whether these genes confer immunity to this virus.

Clearly, there are degrees of resistance conferred by known *Bymovirus* resistance genes, but details of the actual mechanism(s) of resistance are not known for any case. It is intriguing to speculate that translation initiation factor eIF4E or its isoforms may mediate resistance in at least some instances. Mounting evidence shows that eIF4E (or an isoform) plays an essential role in susceptibility to potyviruses (Duprat et al. 2002; Lellis et al.

2002) and that it corresponds to a recessive resistance gene in a number of *Potyvirus*-plant interactions (Ruffel et al. 2002; Nicaise et al. 2003). Furthermore, a role for eIF4E in assisting pea seedborne mosaic *Potyvirus* cell-to-cell movement, as well as replication, has been demonstrated (Gao et al. 2004). Such an evolutionarily conserved host factor could conceivably be involved in any of the forms of resistance to *Bymoviruses* thus far exhibited, given the other similarities in the molecular biology of *Bymoviruses* and potyviruses. If so, it would be reasonable to expect a role for VPg as a pathogenicity determinant, given that several *Potyvirus* VPg genes have been identified as pathogenicity determinants in overcoming recessive resistance genes (Nicolas et al. 1997; Schaad et al. 1997; Keller et al. 1998; Moury et al. 2004). A possible *Bymovirus* resistance mechanism involving eIF4E is thus further supported by the recent finding that pathogenicity of BaYMV-2 to plants possessing *rym*4 correlates with a codon change in the central VPg coding region (Kühne et al. 2003). Changes in the VPg of BaMMV-Sil were also implicated by sequence analysis as being responsible for its pathogenicity to plants with *rym*5, and pathogenicity of another BaMMV isolate pathogenic to *rym*5 in Japan has been attributed to its RNA1, which encodes VPg (Kashiwazaki and Hibino, 1996).

No other pathogenicity determinants for *Bymoviruses* have been found to date. In characterizing French resistance-breaking isolates of BaYMV, no strain-specific amino acid differences in the N-terminal region of the coat protein were evident that could explain resistance breaking properties of the BaYMV isolates tested (Hariri et al. 2000), nor were any strain-specific differences found in the coat proteins of English BaYMV or BaYMV-2 isolates in earlier studies (Shi et al. 1995).

Conclusions and Prospectus

Advances in the molecular genetics of virus pathogenicity and host resistance have provided a solid foundation for further exploration of resistance phenomena. Despite these advances and the relative importance of virus disease problems in barley, a great deal remains to be learned regarding the molecular interactions underlying resistance and susceptibility. At this time one can only speculate on the specific mechanisms of resistance to these viruses or whether these mechanisms are active or passive. The recessive nature of many of the resistance genes certainly supports the existence of passive mechanisms that may depend on the functional absence of a factor crucial for virus accumulation. On the other hand, dominant

genes such as *Ryd*2 and *Rym*14^{Hb} are expected to confer an active defence response to viral infection. Unfortunately, none of the virus resistance genes thus far identified in barley have been cloned, so their precise nature remains enigmatic. Significant interest in the isolation of these genes exists among several groups, and recently a candidate gene for *Bymovirus* resistance at the *rym* 4/5 locus was identified through chromosome walking (Stein et al. 2004).

The development of more highly saturated genetic maps in the vicinity of *Ryd*2 and *rym*4/5 should enable positional cloning of the genes and the eventual investigation of gene function. Identification of closely linked molecular markers has already facilitated marker-assisted selection and helped bring the tools of modern genomics technology into breeding programs (Michelmore, 2000). Unfortunately, the presence of a number of virus resistance genes in the vicinity of the centromere could impede progress toward cloning due to the tendency toward low recombination frequency in these regions (Künzel et al. 2000).

The cloning of various resistance genes and subsequent investigations into their function will provide valuable insight into the mechanism(s) of resistance in barley and probably also mechanisms of virus replication and movement. The viruses described here present quite diverse targets for host resistance responses; hence, it is conceivable that characterization of the resistance genes could reveal novel virus resistance mechanisms. It will also be of interest to determine potential relationships between the numerous *Bymovirus* resistance genes, or whether any significance should be attributed to the location of several resistance genes in the vicinity of the centromere. Comparison of barley virus resistance genes with their counterparts from other crops will reveal whether any of these genes have common functions or whether they represent unique gene classes. Certainly the recessive resistance genes of barley may differ markedly from previously cloned virus resistance genes such as *N* or *Rx*. Hopefully advances in our understanding of these underlying mechanisms will lead to strategic improvements in the efficacy and durability of virus resistance in barley and other crops.

In addition to understanding the fundamental mechanisms of disease resistance pathways in cereals, having cloned disease resistance genes in hand, particularly for BYDV, could provide attractive future resources for disease control in transgenic oats, wheat and rye. No natural BYDV disease resistance has yet been discovered in wheat, although some varieties have some tolerance and several grass species exhibit various levels of resistance (Francki et al. 2001). Indeed, the only effective BYDV resistance currently being used in breeding programs was introgressed into wheat from the wheatgrass, *Thinopyrum intermedium*, to produce Bdv2 (Banks et al. 1995;

Sharma et al, 1995). However, although Bdv2 provides resistance to some BYDV serotypes, it does not provide broad-spectrum resistance, and recent studies show that *Thinopyrum* species harbor a number of genes providing partial resistance (Francki, et al. 2001). Therefore, transfer of genes from this species into wheat and selection of translocation lines by use of molecular markers may provide breeding lines with additional resistance to other BYDV strains. Thus, it is important to understand the basis of the multigenic resistance to various BYDV strains in *Thinopyrum* and other potential grass sources of resistance to obtain lines that can be used in traditional breeding programs.

Because of advances in cereal transformation, including barley (eg. Bregitzer, et al. 2002), the availability of cloned genes could also provide important resources for cross species transfer of existing disease resistance from barley to other cereal crops. One of the important questions critical for such transfers is whether or not the signal transduction machinery will function effectively with resistance genes across species and family boundaries. Encouraging results obtained over the past decade have indicated that transfers to related species within the *Solanaceae* are functional, although most resistance genes that have been studied appear not to confer resistance outside the family from which they were isolated (Hulbert et al. 2001). This suggests that components of the resistance-signaling pathway may vary in their ability to interact with various host genes. Nevertheless, alien chromosomal fragment introgressions and substitution lines generated from wheat grasses and more distantly related species are encouraging, and suggest considerable cross species compatibility within the *Gramineae*. Thus, as the ability to clone disease resistance genes advances, more extensive searches for monogenic sources of resistance from undomesticated grass species may provide additional genetic resources for species in which function is maintained. Clearly, transgenic transfers of monogenic sources of resistance such as the *Ryd*2 or *Ryd*3 genes could provide a less complex alternative to the technical difficulties (cell culture, wide species crosses and irradiation) currently faced in generating the chromosomal translocations necessary for incorporation into breeding programs. These technologies combined with modern approaches suitable for analysis and stacking of polygenic resistance traits (Michelmore, 2000) are expected to contribute to highly resistant cultivars as appropriate cloned disease resistance genes become available.

Although a detailed discussion of synthetic resistance using recombinant DNA approaches is beyond the scope of the current review, we feel that it is appropriate to conclude by stressing the potential applications of gene silencing techniques to elicit adaptive resistance against barley viruses. Use of portions of virus genes to provide virus disease control in genetically

modified plants has proven to be remarkably simple and effective across a broad spectrum of viruses (Waterhouse et al. 2001). Although many of the details of this elegant natural defence system have yet to be clarified, practical applications arising from current findings offer a flexible and relatively rapid approach for specific control of different viruses. It is becoming increasingly evident that this adaptive defence system can be activated reproducibly and simply by expression of double stranded (ds) or hairpin (hp) RNAs that result in targeted degradation of portions of the viral genome to produce plants that are highly resistant to a virus or a particular strain of the virus (Smith et al. 2000; Waterhouse et al. 2001). Applications of this approach to BYDV in barley have been shown to elicit immunity or extreme resistance directed specifically against the PAV strain of BYDV without affecting susceptibility to CYDV-RPV (Wang et al. 2000). In these experiments, transgenic barley lines were designed to express a single copy hpRNA construct consisting of a 1.6 kb dsRNA derived from the ORF 1 and a portion of the ORF 2 sequence linked with an 0.86 kb connecting loop consisting of the remainder of ORF 2. Upon challenge by aphid transmitted PAV and CYDV, lines expressing the polymerase derived hpRNA were apparently immune to PAV infection as assessed by the absence of detectible amounts of virus, by enzyme-linked immunoabsorbent assays, and by failure to transmit the virus during subsequent aphid recovery experiments. In contrast, the plants were susceptible to CYDV and, in aphid cotransmission experiments with both viruses, CYDV replicated readily, whereas the plants remained resistant to PAV. The resistance was inherited in a simple Mendelian manner suggesting that strategies utilizing RNA interference have enormous potential for incorporation into cereal breeding programs. We anticipate that this relatively simple methodology can be extended broadly to elicit specific resistance to all members of the BYDV complex and to other barley viruses. These results thus provide optimism that a combination of classical and innovative molecular breeding strategies will result in future protection of barley and other cereals against yield losses resulting from virus infection.

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Chapter B9

Resistance to *Tomato yellow leaf curl virus* in Tomato

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Introduction

Tomato yellow leaf curl virus (TYLCV) is one of the most devastating viruses of cultivated tomatoes in tropical and subtropical regions. TYLCV is a monopartite begomovirus, first described in Israel (Cohen and Nitzany, 1966). Although originally found only in the eastern Mediterranean, it is now a problem in the western Mediterranean, the Caribbean, Japan, and the southern U.S. (Polston and Anderson, 1997; Polston et al. 1999). Infection of susceptible tomato plants results in cupping of leaves, chlorosis, prominent stunting of the growing point, and flower abscission. Depending on the timing of infection, yield losses can reach 100%. In many tomato-growing areas, TYLCV has become the limiting factor for production of tomatoes in both open field and protected cultivation systems (Lapidot and Friedmann, 2002).

TYLCV is a monopartite begomovirus transmitted by the tobacco whitefly, *Bemisia tabaci* (Gennadius). The only known vectors of TYLCV are in the *B. tabaci* species complex (Brown et al. 1995), which includes *B. tabaci* and *B. argentifolii* (Bellows et al. 1994). TYLCV transmission by

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whiteflies has been characterized as being persistent and circulative in nature, that is the virus is retained through the life of the adult insect after acquisition, and moves through the insect body to the salivary glands where it can leave the body of the whitefly in the saliva (Cohen and Harpaz, 1964; Cohen and Nitzany, 1966; Nault, 1997). However, it has been shown for TYLCV that transmission efficiency declines with time (Cohen and Harpaz, 1964)

TYLCV has a small genome (2.8 kb) with 6 open reading frames that are organized bidirectionally (Fig. 1) (Gutierrez, 1999). The *Rep* (replication associated protein) gene is a multi-functional gene, essential for viral DNA replication, and is involved in transcriptional regulation (Fontes et al. 1994; Lazarowitz, 1992). In other begomoviruses, *TrAP* (transactivation of transcription) gene has been shown to play an important role in the systemic viral infection of *Nicotiana benthamiana*, enhance the expression of the coat protein and play a role in the suppression of host defense responses (Bisaro et al. 1999; Brough et al. 1992; Etessami et al. 1991). The *REn* (replication enhancer protein) gene of other begomoviruses has been shown to enhance replication and mutations in this gene were shown to attenuate plant disease symptoms (Etessami et al. 1991; Sunter et al. 1990). *REn* is not virus specific and is able to interact with the *Rep* of other geminiviruses (Sunter et al. 1994). C4 has been implicated to play a role in pathogenicity (Krake, 1998) and *V1* has been shown to play a role in virus movement (Wartig et al. 1997). The TYLCV coat protein gene is the most abundant protein produced by TYLCV (Timmermans et al. 1994). This protein is required for whitefly transmission, binds to viral single stranded DNA (ssDNA), may play a role in systemic movement, and contains a nuclear targeting signal, which mediates movement of viral nucleic acid into the host cell nucleus (Azzam et al. 1994; Briddon et al. 1990; Kunik et al. 1998; Palanichelvam et al. 1998). Therefore, its DNA replication cycle, like other begomoviruses, relies largely on the use of host cellular DNA replication proteins. Only the *Rep* gene is essential for begomoviral DNA replication. The replication strategy used by TYLCV consists of a first stage, the conversion of ssDNA into double-stranded DNA (dsDNA) intermediate, followed by the second stage in which the dsDNA is used as a template to produce ssDNA genomes by a rolling-circle replication mechanism (Gutierrez, 1999; Hanley-Bowdoin et al. 1999).

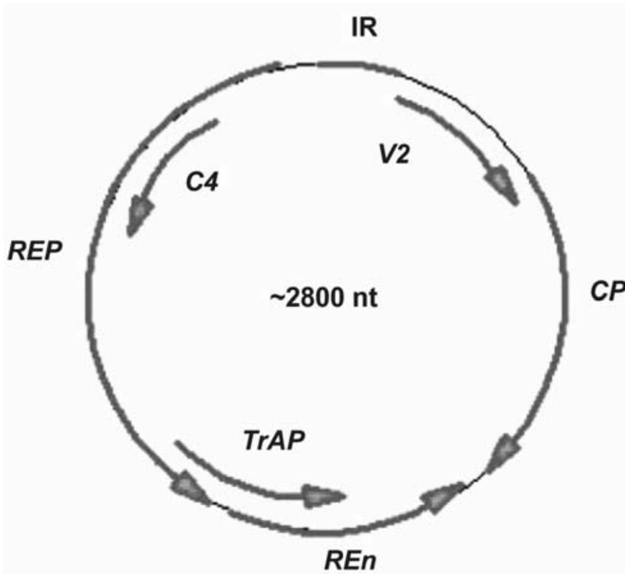

Figure 1. Genome organization of *Tomato yellow leaf curl virus*. The non-coding region is the IR (intergenic region). The encoded proteins are: MP (movement protein), CP (coat protein), Rep (replication initiation protein), TrAP (transcriptional activator protein), REn (replication enhancer protein), and C4 (a determinant of symptom expression). The arrows refer to the direction of transcription. The MP and CP are encoded on the virion (genomic) strand, while Rep, TrAP, REn and C4 are encoded on the complimentary DNA strand.

Taxonomy

In the past there was some confusion regarding the taxonomy of TYLCV. Several begomoviruses that induce symptoms in tomato similar to those elicited by TYLCV were initially named TYLCV. Later analyses of the sequences of these viruses showed them to be unique begomoviruses and not closely related to TYLCV. This confusion was addressed by a committee of the ICTV and a clarification published (Fauquet et al. 2003). A brief summary of changes with respect to viruses called TYLCV is presented

(Table 1). The problems that can arise due to an ambiguous viral nomenclature is manifested in a work that was published only 4 years ago regarding the mapping of TYLCV resistance originated from the wild tomato *Lycopersicon hirsutum* (Hanson et al. 2000). The authors screened resistant plants using three different isolates of TYLCV – or so they thought. Today we know that these viral isolates were in fact three isolates of *Tomato leaf curl virus* (ToLCV) and not TYLCV.

Two strains of TYLCV have been reported in Israel, TYLCV and TYLCV-Mld (Antignus and Cohen, 1994; Navot et al. 1991). TYLCV-Mld produces symptoms in tomato indistinguishable from those of TYLCV. TYLCV-Mld was recognized due to its ability to infect and induce disease symptoms milder than those of TYLCV on TY-20, a tomato cultivar resistant to TYLCV (Antignus and Cohen, 1994; Antignus, pers. comm.). TYLCV occurs much more commonly in tomato fields in Israel than TYLCV-Mld (M. Lapidot, unpub.). TYLCV was reported to be a recombinant virus between TYLCV-Mld and an ancestor of a second begomovirus, ToLCV, as described from India (Harrison and Robinson, 1999; Navas-Castillo et al. 2000). TYLCV possesses a portion (N-terminal region) of the *Rep* and intergenic region (IR) of ToLCV and the rest of the genome is very similar to that of TYLCV-Mld.

Geographic distribution

Until about 1990, TYLCV was recognized as a pathogen of tomato in the eastern Mediterranean, and was reported from Cyprus, Egypt, Jordan, Israel, Lebanon, Syria, and Turkey (Czosnek and Laterrot, 1997). However, over the last decade or so the geographic range of TYLCV has greatly expanded to include Japan, the western Mediterranean, the Caribbean, and the south-eastern U.S. (reviewed by Moriones and Navas-Castillo, 2000). TYLCV appeared in the eastern Caribbean in the late 1980's, and was found for the first time in tomato in Cuba in 1989, the Dominican Republic in 1992, Jamaica in 1993, The Bahamas in 1996, and Puerto Rico in 2001 (Bird et al. 2001; Martinez-Zubiaur et al. 1996; McGlashan et al. 1994; Nakhla et al. 1994; Polston et al. 1994; Ramos et al. 1996; Sinisterra et al. 2000). In the western Caribbean it has been found in Yucatan, Mexico in 1997 (Ascecio-Ibanez et al. 1999). TYLCV was detected for the first time in the United States in Florida in 1997, followed by Georgia in 1998, Louisiana in 2000, and Mississippi and North Carolina in 2001 (Ingram and Henn, 2001; Momol et al. 1999; Pappu et al. 2000; Polston et al. 1999; Polston et al. 2002; Valverde et al. 2001).

Table 1. List of begomoviruses with names similar to *Tomato yellow leaf curl virus*

Currently Accepted virus Nomenclature	Acronym	Previous Nomenclature	Accession No.
Tomato yellow leaf curl virus	TYLCV	TYLCV, TYLCV-Il	X15656
Tomato yellow leaf curl China virus	TYLCCV	TYLCV-China, TYLCV-CN	AF311734
Tomato yellow leaf curl Gezira virus	TYLCGV		AY044137-9
Tomato yellow leaf curl Malaga virus	TYLCMalV		AF271234
Tomato yellow leaf curl Sardinia virus	TYLCSV	TYLCV-Sardinia, TYLCV-Sar	X61153
Tomato yellow leaf curl Thailand virus	TYLCTHV	TYLCV-Thailand, TYLCV-TH	Various accession numbers
Tomato yellow leaf curl – Mild	TYLCV-Mld	TYLCV-IL[Mld]	X76319

The need for resistance

The management of TYLCV is difficult, expensive, and with limited options. In many regions traditional control measures for TYLCV emphasize vector control (Cohen and Antignus, 1994; Hilje et al. 2001; Palumbo et al. 2001; Polston and Anderson, 1997), mainly through multiple applications of insecticides or physical barriers. Chemical control methods have been only partially effective, since whitefly populations can reach very high numbers, leading to intensive pesticide use (sometimes twice daily) in attempts to eliminate the vector before it transmits the virus. Furthermore, there are concerns that the vector may develop pesticide resistance and the intense application of pesticides may have deleterious effects on the environment (Palumbo et al. 2001; Pico et al. 1996). Physical barriers such as fine-mesh screens have been used in the Mediterranean Basin to protect crops (Cohen and Antignus, 1994; Hilje et al. 2001; Palumbo et al. 2001; Polston and Anderson, 1997). Recently, UV-absorbing plastic sheets and screens have been shown to inhibit penetration of whiteflies into greenhouses (Antignus et al. 2001; Antignus et al. 1996). Furthermore, filtration of UV light was shown to hinder the whiteflies' dispersal activity, and consequently reduce virus spread (Antignus et al. 2001). However, adoption of physical barriers adds to production costs and these screens create problems of shading, overheating, and high relative humidity. Therefore, the best way to reduce yield losses due to TYLCV is by breeding crops resistant or tolerant to the virus (Lapidot and Friedmann, 2002; Morales, 2001; Pico et al. 1996).

Definition of resistance

A common problem for researchers interested in resistance is the lack of a standard terminology used by both plant breeders and plant pathologists. Breeders are mainly interested in improving the overall performance of a plant variety under field conditions. Thus, yield and fruit quality (as well as fruit color and shape) are paramount. In contrast, plant pathologists place an emphasis on the fate of the virus in the plant. A similar cause of confusion lies in whether or not a researcher makes the distinction between resistances to the pathogen *versus* resistance to the effects of the pathogen (i.e. symptoms of the disease). Another frequent source of confusion occurs when the resistance level in question is mediocre or unsatisfactory and is described with the terms "tolerance" or "field resistance" in an undefined manner. The definitions of resistance proposed by Cooper and Jones (1983) are used in this manuscript and are summarized below.

Resistance – A host plant is resistant if it can suppress the multiplication of a virus, and consequently suppress the development of disease symptoms.

Regardless of the mechanism of resistance (the host may be resistant to establishment of infection, viral replication or viral spread within the plant), the final outcome is the same – fewer virions accumulate in the resistant host. Resistance can range from very high (up to immunity – no virus accumulates in the host and the plant is, in fact, a non-host), to moderate, or low. However, even for low resistance, the resistant plant will accumulate fewer virions than the susceptible host, and may express milder disease symptoms and/or a delay in the onset of symptoms.

Tolerance – This is a unique instance where in response to virus infection, the host expresses negligible or mild disease symptoms, but supports normal levels of virus multiplication. Thus, the plant, rather than being resistant to the virus, "tolerates" the pathogen and, despite its presence, expresses milder symptoms and produces a good yield (Cooper and Jones, 1983; Walkey, 1985). Hence, tolerance is not a code name for low-level forms of resistance but is, rather, a specific plant response: milder symptoms despite a normal level of virus accumulation.

Inheritance of TYLCV resistance

There have been focused and prolonged efforts to breed cultivars resistant to TYLCV. Since all cultivars of tomato (*Lycopersicon esculentum*) are extremely susceptible to TYLCV, wild *Lycopersicon* species were screened for their response to the virus in order to identify and introgress genes for resistance (reviewed in Laterrot, 1992; Nakhla and Maxwell, 1998; Pico et al. 1996; Pico et al. 1999; Pilowsky and Cohen, 2000). Thus, breeding programs have been based on the transfer of resistance genes from accessions of wild origin into the cultivated tomato. Progress in breeding for TYLCV resistance has been slow, primarily because of the complex genetics of the resistance, the interspecific cross ability barriers between the wild and domesticated tomato species, and the need to set up a reliable screen for resistance to the virus, which is dependent on the availability of viruliferous whiteflies (Lapidot and Friedmann, 2002; Lapidot et al. 1997; Vidavsky et al. 1998).

This chapter will review some of the work done on different resistance sources to TYLCV with an emphasis on the inheritance of the resistance. For a list of resistant wild *Lycopersicon* species see previous reviews by (Laterrot, 1992; Nakhla and Maxwell, 1998; Pico et al. 1996; Pico et al. 1999; Pilowsky and Cohen, 2000). The inheritance of resistance to TYLCV from a number of the different resistant sources has been identified and a summary is presented in Table 2.

Table 2. Inheritance of different sources of TYLCV resistance.

Source of resistance			
Species	Accession No.	Inheritance	Reference
L. pimpinellifolium	LA 121	Monogenic, partial dominance	Pilowsky and Cohen, 1974
	Hirsute-INRA; LA 1478	Monogenic, dominant	Kasrawi, 1989
L. peruvianum	PI 126935	Recessive, controlled by five genes	Pilowsky and Cohen, 1990
	PI 126926 & PI 126930 & PI 390681 & LA 441	Three interacting genes, one with partial dominance the others recessive	Friedmann *et al*., 1998
L. chilense	LA 1969	A major gene (*TY-1*) with partial dominance and two modifier genes	Zamir *et al*., 1994
L. hirsutum	LA 386	Dominant polygenic	Hassan *et al*., 1984
	LA 1777 & LA 386	Two mechanisms: (1) Resistance controlled by two to three additive recessive genes (2) Tolerance controlled by a dominant major gene	Vidavsky and Czosnek, 1998
L. cheesmanii	LA 1401	Recessive	Hassan *et al*., 1984

Breeding for TYLCV resistance was initiated in Israel in the late 1960's using accessions of the wild tomato *L. pimpinellifolium* (Pilowsky and Cohen, 1974). It was found that the TYLCV resistance derived from accession LA121 was monogenic with partial dominance (Table 2). Other studies with different accessions of *L. pimpinellifolium* such as hirsute INRA found the resistance to be mediated by a single dominant gene (Table 2). In a later study, bulked segregant analysis was employed to identify random amplified polymorphic DNA (RAPD) markers that were linked to the TYLCV resistance derived from *L. pimpinellifolium* hirsute INRA (Chague et al. 1997). Four RAPD markers were identified which were linked to a quantitative trait locus (QTL) responsible for up to 27.7% of the resistance. This differs from earlier results in which the resistance was reported to be mediated by a single dominant gene (Kasrawi, 1989). Interestingly, this QTL was mapped to chromosome 6 (Chague et al. 1997) as was the TYLCV resistance gene, *TY-1* (see below). However, the level of resistance from accessions of *L. pimpinellifolium* was found to be insufficient – while resistant plants derived from LA121 showed moderate disease symptoms following infection, these plants suffered from markedly reduced growth and yield (Pilowsky and Cohen, 1990). Thus other sources of resistance were sought.

The first commercial resistant hybrid, 'TY-20', was released in 1988 (Pilowsky et al. 1989). 'TY-20' carried resistance derived from *L. peruvianum* (accession PI 126935) that was later determined to be mediated by five recessive genes (Pilowsky and Cohen, 1990). The resistance in 'TY-20' induced a delay in the development of disease symptoms upon infection but, despite this, infected 'TY-20' plants were still able to produce an acceptable yield. The resistance in *L. chilense* (accession LA 1969) is controlled by a major partially dominant gene termed *TY-1* and at least two more modifier genes (Zamir et al. 1994). *TY-1* was mapped to chromosome 6 using restriction fragment length polymorphism (RFLP), while the two modifier genes were mapped to chromosomes 3 and 7 (Zamir et al. 1994). Since it is relatively easy to introgress a single dominant gene, a number of commercial hybrids have been released carrying *TY-1* resistance. Currently, all the commercial TYLCV-resistant tomato hybrids, including those carrying *TY-1,* out-yield susceptible hybrids in the presence of TYLCV. However, since all these hybrids display disease symptoms after infection, higher levels of TYLCV resistance were sought.

One approach being used to increase levels of resistance is to combine different resistance genes into a single cultivars (i.e., pyramiding resistances) (Kelly et al. 1995). An example of this is line TY-172, which exhibited the highest level of resistance during a field trial, in which the yield components of various resistant cultivars and lines, which had been inoculated with TYLCV, were evaluated and compared (Lapidot et al. 1997). TY-172 had

been derived from four different accessions of *L. peruvianum* (Friedmann et al. 1998). These four accessions were crossed with L. *esculentum*, and the resulting F_1 interspecific hybrids were backcrossed to the susceptible parent until a BC_3F_3 generation was secured. At this stage crosses were made between the four different lines, and F_2 and F_3 generations were produced and screened for resistance. A highly resistant F_3 line was selected, and its F_4 offspring were bulked and designated TY-172 (Friedmann et al. 1998). TY-172 is a symptomless host of TYLCV, which contains very low levels of viral DNA. Either when infected in the greenhouse with viruliferous whiteflies or when grown in the field under conditions of natural infection, TY-172 shows no symptoms of TYLCV infection. Attempts to produce disease symptoms on TY-172 plants by grafting with a susceptible infected donor were unsuccessful. Thus, even when exposed continuously to very high levels of viral inoculum, line TY-172 did not develop disease symptoms (Friedmann et al. 1998). When TY-172 was crossed with susceptible lines, the resulting hybrids exhibited milder symptoms and although they had a lower viral content than the susceptible parent, it was nevertheless much higher than that of TY-172, suggesting partial dominance of the resistance. Analysis of F_2 populations, suggested that the resistance in line TY-172 is controlled by at least three interacting genes (Friedmann et al. 1998).

Two other examples of improved resistance through the combination of different resistance sources are lines 902 and 908, which express high levels of resistance to TYLCV. The resistance in these lines was derived from the cross between *L. hirsutum* accessions LA 1777 and LA 386. The resulting F_1 plants were crossed with *L. esculentum* followed by selfing of resistant, symptomless individuals, which resulted in two stable BC_1F_4 lines, designated 902 and 908 (Vidavsky and Czosnek, 1998). Line 902 does not produce disease symptoms and does not support viral accumulation following whitefly-mediated inoculation with TYLCV. However, virus accumulation was detected in line 902 following grafting with an infected susceptible donor. Segregation analysis indicated that two to three additive recessive genes control the resistance to TYLCV in line 902 (Vidavsky and Czosnek, 1998). Also, line 908 does not show any disease symptoms following whitefly-mediated inoculation but, unlike line 902, TYLCV does accumulate in the plants. Segregation analysis indicated that a single dominant major gene controls the resistance in 908 (Vidavsky and Czosnek, 1998).

Mechanism of resistance

Despite the considerable efforts devoted to the development of TYLCV-resistant cultivars, very little is known about the mechanisms of the

introgressed viral resistances. The levels of TYLCV DNA accumulation in TY-20 and four other TYLCV-resistant lines were compared with that in a susceptible line (Rom et al. 1993). Following whitefly-mediated inoculation and for a period of approximately 40 days, samples were taken from the plant apex of inoculated plants and analyzed using dot-blot hybridization. It was found that at all time points, the resistant cultivars accumulated significantly less viral DNA compared to the susceptible line. The authors concluded that viral DNA accumulation was positively correlated with symptom severity, and suggested the monitoring of viral DNA level as a tool for the selection of TYLCV-resistant genotypes (Rom et al. 1993). These results were consistent with those of another study in which different *Lycopersicon* accessions were screened for resistance to TYLCV using the amount of viral DNA present in inoculated plants as an indicator of resistance (Zakay et al. 1991). Another study used serological assays to rank the level of resistance of tomato lines to three different tomato begomoviruses (including *Tomato yellow leaf curl Sardinia virus*) and found a positive correlation between the level of resistance and amount of virus detected in the plant (Fargette et al. 1996). The authors concluded by suggesting that viral resistance should be assessed using serological assays.

A more recent study indicated that there was not always a good correlation between severity of disease symptoms and levels of TYLCV DNA accumulation with the effects on yield (Lapidot et al. 1997). The effects of TYLCV on total yield and yield components of four resistant F_1 tomato cultivars and two breeding lines were evaluated in the field. Plants of resistant and susceptible cultivars were infected with TYLCV at the first-leaf stage by whitefly-mediated inoculation. After a short recovery period, the plants were transplanted to the field. Inoculated plants of each cultivar or line were compared with their respective control, non-inoculated plants, in terms of total yield, average fruit weight and number, and plant fresh weight. Disease symptom severity and level of viral DNA accumulation in the inoculated plants were monitored throughout the growing season (approximately 90 days). There were substantial differences among the entries with respect to the amount of yield loss caused by TYLCV as well as the amounts of viral DNA accumulated. All the resistant cultivars showed milder symptoms, expressed lower yield losses, and accumulated lower amounts of viral DNA when compared to the susceptible variety. Hence, a positive correlation was observed between disease resistance and amounts of viral DNA when resistant plants were compared to susceptible controls. However, there was not a strong correlation between lower amounts of viral DNA with higher crop yields. Plants of the highly resistant breeding lines TY-172 and TY-197 suffered the least relative yield loss and showed the lowest level of viral DNA. However, while TY-172 and TY-197 plants accumulated viral DNA to the same level, TY-172 plants expressed a higher level of resistance to the virus than TY-197 plants as determined by the

effects on yield, suggesting that reduction in virus titer is not the only factor that determines resistance level. Thus, although the accumulation of TYLCV DNA can serve as an indicator for resistance level, it is best that this is not used as the sole indicator (Lapidot et al. 1997).

The first attempt to understand the mechanism underlying a TYLCV resistance at the molecular level was using the resistance to TYLCV derived from *L. chilense*, which contains the resistance locus *TY-1* (Michelson et al. 1994). Two nearly isogenic tomato lines, which differed only in the presence or absence of the *L. chilense* chromosome segment associated with resistance to TYLCV, were developed by RFLP-assisted selection. Plants from line 50, which did not contain the *TY-1* allele from *L. chilense*, were susceptible and showed disease symptoms after whitefly-mediated inoculation with TYLCV under field conditions. In contrast, plants from line 52, due to the presence of the *TY-1* allele from *L. chilense*, were resistant to TYLCV and remained symptomless after whitefly-mediated inoculation with TYLCV under field conditions. The effect of the *TY-1* gene on TYLCV accumulation and translocation was studied by comparing viral DNA accumulation in lines 50 and 52. TYLCV DNA accumulation in plants of the line 52 was found to be a function of the amount of inoculum. When the inoculum titer was low (three whiteflies per plant), TYLCV DNA accumulated to a low level in the resistant line. When the inoculum was high (50 whiteflies per plant) similar amounts of viral DNA accumulated in both the resistant and susceptible lines 28 days after inoculation. However, the rate of DNA accumulation was slower in the resistant line than in the susceptible line. When the movement of viral DNA from the inoculated leaf (youngest leaf of each plant) was followed, it was found that in the susceptible plants viral DNA moved to the upper leaves and to the roots, the same route as followed by photoassimilates. In contrast, viral DNA movement was restricted to the second leaf and to the shoot apex in the resistant plants. The authors concluded that the *TY-1* gene is associated with inhibition of disease symptoms through two mechanisms: by reducing viral DNA accumulation in inoculated tissue exposed to low inoculum titers and at higher titres of virus inoculum by limiting viral long-distance movement (Michelson et al. 1994).

Recently, the first step was made to elucidate the resistance mechanism shown by TY-172 (derived from *L. peruvianum*) under conditions of high inoculum pressure (Segev et al. 2004). The resistance mechanism was addressed by inoculating selected leaves on intact TY-172 and susceptible tomato plants with TYLCV and comparing the amount of viral ssDNA and dsDNA produced at the inoculation site over time. The plants were inoculated with whiteflies using clip cages, thus, a clear inoculation site was created on the inoculated leaf. Moreover, the use of clip cages allowed control over the number of whiteflies used to inoculate each plant, thus

reducing variation due to varying amounts of inoculum and enabling comparisons between different inoculated plants.

When the amount of TYLCV DNA at the site of inoculation was evaluated over time, it was found that at each time point, the amount of new viral ssDNA in the resistant host was much lower than that of the susceptible host. However, the changes observed in viral ssDNA detected over time were not reflected by parallel changes in the amounts of TYLCV dsDNA detected in the same tissues. Viral dsDNA accumulated to the same level in both the resistant and susceptible hosts at all time points examined. Moreover, the amount of viral dsDNA detected was much lower than the amount of viral ssDNA detected in both resistant and susceptible hosts, which is consistent with the role of viral dsDNA as an intermediate form of DNA in begomovirus replication. It is well established, that upon begomovirus entry to the plant cell, the viral ssDNA serves as a template for the synthesis of a dsDNA intermediate replicating form. In the second stage of the replication cycle, the dsDNA replicating form serves as template for the production of new viral ssDNA, via a rolling circle mechanism (Gutierrez, 1999; Hanley-Bowdoin et al. 1999).

To test whether TY-172 resistance also affects long-distance movement of the virus, the appearance of viral DNA at the plant apex was monitored following inoculation of the third leaf from the top. Viral DNA was detected in the plant apex two days after inoculation in both the susceptible and resistant plants. Viral DNA accumulation at the plant apex was the same in both hosts until seven days after inoculation, after which a greater amount of viral DNA was found in the susceptible host. Overall, these results suggest that TY-172 interferes with the accumulation of viral ssDNA but not with viral long distance movement (Segev et al. 2004).

Concluding Remarks

Substantial progress has been made in the development of TYLCV-resistant tomatoes since efforts began nearly 40 years ago. Although no resistance was found in the cultivated tomato (*L. esculentum*), several sources of resistance have been found in various wild tomato species. These resistances vary in their mode of inheritance and, for the few that have been studied, are based on different resistance mechanisms. Since these individual sources provide only a limited level of resistance, improved resistance has been obtained by combining different resistances into single cultivars. However, for this approach to be successful, distinct virus resistance genes must be brought together, i.e. combining the same resistance genes (or alleles), even those originating from different resistant wild sources will probably not result in improved resistance. In order to do this, one must be able readily to distinguish different resistance genes.

Resistance genes can be distinguished by developing linked molecular markers, using these markers to map the different resistance genes, leading ultimately to the identification and isolation of the resistance genes. However, development of linked molecular markers could be very difficult when resistance is controlled by complex genetics, as seems to be the case with most of the resistances to TYLCV. Instead of following the genes that mediate the resistance, another approach would be to identify the mechanism by which the resistance interferes with viral infection. Combining different resistance genes which operate *via* different mechanisms and which are able to operate simultaneously, may potentially lead to the development of tomato plants with superior and long-lasting resistance to TYLCV.

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