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.

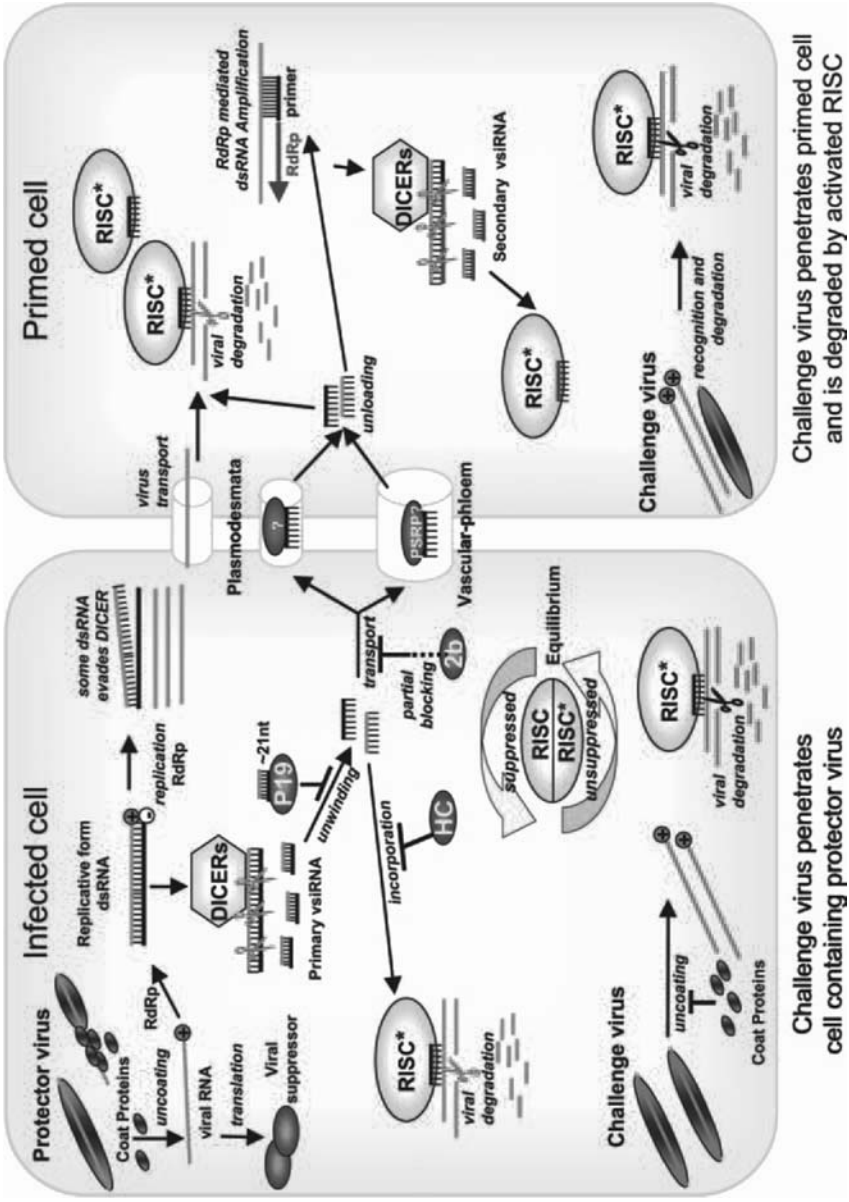
Protector virus
Infected cell
Primed cell
Coat Proteins
uncoating
viral RNA
translation
Viral suppressor
RdRp
Replicative form dsRNA
replication RdRp
some dsRNA evades DICER
DICERs
~21nt
P19
Primary vsiRNA
unwinding
incorporation
HC
transport
partial blocking
2b
Plasmodesmata
?
PSRP?
Vascular-phloem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

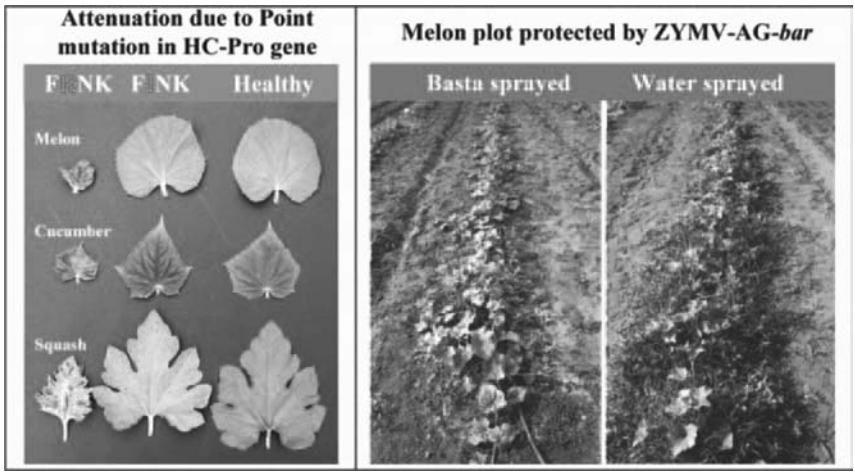

Fig. 2. ZYMV-AGII as an attenuated, aphid non-transmissible, potentially cross-protecting expression vector. A single mutation in the potyviral suppressor gene HC-pro from FRNK to FINK attenuates symptoms in various cucurbits (left). Functional expression of bar via AGII-Bar in cucurbits confers resistance 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)

References

Anandalakshmi, R., Marathe, R., Ge, X., Herr, J.M., Jr., Mau, C., Mallory, A., Pruss, G., Bowman, L. and Vance, V.B. 2000. A calmodulin-related protein that suppresses posttranscriptional gene silencing in plants. *Science* 290: 142-144.

Anandalakshmi, R., Pruss, G.J., Ge, X., Marathe, R., Mallory, A.C., Smith, T.H. and Vance, V.B. 1998. A viral suppressor of gene silencing in plants. Proc *Natl Acad Sci U S A* 95: 13079-13084.

Atkinson, P.H. and Matthews, R.E.F. 1970. On the origin of dark green tissue in tobacco leaves infected with tobacco mosaic virus. *Virology* 40: 344-356.

Beachy, R.N. 1999. Coat-protein-mediated resistance to tobacco mosaic virus: discovery mechanisms and exploitation. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 354: 659-664.

Beachy, R.N., Loesch-Fries, S. and Tumer, N.E. 1990. Coat protein-mediated resistance against virus infection. *Annu. Rev. Phytopathol.* 28: 451-474.

Beclin, C., Boutet, S., Waterhouse, P. and Vaucheret, H. 2002. A branched pathway for transgene-induced RNA silencing in plants. *Curr. Biol*. 12: 684-688.

Bendahmane, M., Fitchen, J.H., Zhang, G. and Beachy, R.N. 1997. Studies of coat protein-mediated resistance to tobacco mosaic tobamovirus: correlation between assembly of mutant coat proteins and resistance. *J. Virol.* 71: 7942-7950.

Bitterlin, M.W. and Gonsalves, D. 1988. Serological grouping of tomato ringspot virus isolates: implications for diagnosis and cross-protection. *Phytopathology* 78: 278-285.

Bourdin, D. and Lecoq, H. 1991. Evidence that heteroencapsidation between two potyviruses is involved in aphid transmission of a non-aphid-transmissible isolate from mixed infections. *Phytopathology* 81: 1459-1464.

Bowman, J.L. 2004. Class III HD-Zip gene regulation, the golden fleece of ARGONAUTE activity? *Bioessays* 26: 938-942.

Brigneti, G., Voinnet, O., Li, *W.X., Ji, L.H., Ding, S.W. and Baulcombe, D.C. 1998. Viral pathogenicity determinants are suppressors of transgene silencing in Nicotiana benthamiana. EMBO J.* 17: 6739-6746.

Broadbent, L. 1976. Epidemiology and control of tomato mosaic virus. *Annu. Rev. Phytopathol*. 14: 75-96.

Cassells, A.C. and Herrick, C.C. 1977. Cross protection between mild and severe strains of tobacco mosaic virus in doubly inoculated tomato plants. *Virology* 78: 253-260.

Chamberlain, E.E., Atkinson, J.D. and Hunter, J.A. 1964. Cross protection between strains of apple mosaic virus. *N. Z. J. Agric. Res.* 7: 480-490.

Chapman, E.J., Prokhnevsky, A.I., Gopinath, K., Dolja, V.V. and Carrington, J.C. 2004. Viral RNA silencing suppressors inhibit the microRNA pathway at an intermediate step. *Genes Dev.* 18: 1179-1186.

Chatchawankanphanich, O., Jamboonsri, W., Kositratana, W., Attathom, S., Orawan, C., Watchareewan, J., Wichai, K. and Supat, A. 2000. Screening of mild strains of papaya ringspot virus for cross protection. *J. Agric. Sci*. 33: 3-4.

Chellappan, P., Vanitharani, R. and Fauquet, C.M. 2004. Short interfering RNA accumulation correlates with host recovery in DNA virus-infected hosts, and gene silencing targets specific viral sequences. *J. Virol.* 78: 7465-7477.

Costa, A.S. and Muller, G.W. 1980. Tristeza control by cross protection: a U.S.-Brazil cooperative success. *Plant Dis.* 64: 538-541.

Covey, S., Al-Kaff, N., Langara, A. and Turner, D. 1997. Plants combat infection by gene silencing. *Nature* 385: 781-782.

Culver, J.N. 1996. Tobamovirus cross protection using a potexvirus vector. *Virology* 226: 228-235.

Desbiez, C., Gal-On, A., Raccah, B. and Lecoq, H. 1997. Characterization of epitopes on zucchini yellow mosaic potyvirus coat protein permits studies on the interactions between strains. *J. Gen. Virol*. 78: 2073-2076.

Desbiez, C. and Lecoq, H. 1997. Zucchini yellow mosaic virus. Plant Pathol. 46: 809-829.

Dias, P.R.P. and Rezende, J.A.M. 2000. Cross protection of hybrid squash Tetsukabuto for the control of papaya ringspot virus type W. *Summa Phytopathologica* 26: 390-398.

Dietrich, C. and Maiss, E. 2003. Fluorescent labelling reveals spatial separation of potyvirus populations in mixed infected Nicotiana benthamiana plants. *J. Gen. Virol*. 84: 2871-2876.

Dodds, J.A. 1982. Cross-protection and interference between electrophoretically distinct strains of cucumber mosaic virus in tomato. *Virology* 118: 235-240.

Dodds, J.A., Lee, S.Q. and Tiffany, M. 1985. Cross protection between strains of cucumber mosaic virus: effect of host and type of inoculum on accumulation of virions and double-stranded RNA of the challenge strain. *Virology* 144: 301-309.

Dugas, D.V. and Bartel, B. 2004. MicroRNA regulation of gene expression in plants. *Curr. Opin. Plant. Biol.* 7: 512-520.

Dunoyer, P., Lecellier, C.H., Parizotto, E.A., Himber, C. and Voinnet, O. 2004. Probing the microRNA and small interfering RNA pathways with virus-encoded suppressors of RNA silencing. *Plant Cell* 16: 1235-1250.

Fraser, R.S.S. 1998. Introduction to classical cross protection. In *Plant Virology Protocols.* (eds. G.D. Foster, and S.C. Taylor), pp. 13-44. Humana Press, Totowa, N. J.

Fulton, R.W. 1951. Superinfection by strains of tobacco mosaic virus. Phytopathology 41: 579-592.

Fulton, R.W. 1986. Practices and precautions in the use of cross protection for plant virus disease control. *Annu. Rev. Phytopathol*. 24: 67-81.

Gal-On, A., Antignus, Y., Rosner, A. and Raccah, B. 1992. A zucchini yellow mosaic virus coat protein gene mutation restores aphid transmissibility but has no effect on multiplication. *J. Gen. Virol.* 73: 2183-2187.

Gal-On, A. and Raccah, B. 2000. A point mutation in the FRNK motif of the potyvirus helper component-protease gene alters symptom expression in cucurbits and elicits protection against the severe homologous virus. *Phytopathology* 90: 467-473.

Gerber, M. and Sarkar, S. 1989. The coat protein of tobacco mosaic virus does not play a significant role for cross-protection. *J. Phytopath*. 124: 1-4.

Gonsalves, D. 1998. Control of papaya ringspot virus in papaya: a case study. *Annu. Rev. Phytopathol.* 36: 415-437.

Gonsalves, D. and Garnsey, S.M. 1989. Cross-protection techniques for control of plant virus diseases in the Tropics. Plant Dis. 73: 592-597.

Goto, T., Iizuka, N. and Komochi, S. 1984. Selection and utilization of an attenuated isolate of pepper strain of tobacco mosaic virus. *Ann. Phytopathol. Soc. Jpn* 50: 221-228.

Grant, T.J. and Costa, A.S. 1951. A mild strain of the tristeza virus of. citrus. Phytopathology 41: 114-122.

Hall, J.S., French, R., Hein, G.L., Morris, T.J. and Stenger, D.C. 2001. Three distinct mechanisms facilitate genetic isolation of sympatric wheat streak mosaic virus lineages. *Virology* 282: 230-236.

Hamilton, R.I. 1985. Using plant viruses for disease control. *Hort. Science* 20: 838-852.

Harrison, B.D. 1958. Ability of single aphids to transmit both avirulent and virulent strains of potato leaf roll virus. *Virology* 6: 278-286.

Himber, C., Dunoyer, P., Moissiard, G., Ritzenthaler, C. and Voinnet, O. 2003. Transitivity-dependent and -independent cell-to-cell movement of RNA silencing. *EMBO. J.* 22: 4523-4533.

Hughes, J.d.A. and Ollenu, A.A. 1994. Mild strain protection of cocoa in Ghana against cocoa swollen shoot virus - a review. *Plant Pathol.* 43: 442-457.

Hull, R. and Plaskitt, A. 1970. Electron microscopy on the behavior of two strains of alfalfa mosaic virus in mixed infections. *Virology* 42: 773-776.

Huss, B., Walter, B. and Fuchs, M. 1989. Cross-protection between arabis mosaic virus and grapevine fanleaf virus isolates in Chenopodium quinoa. *Ann. appl. Biol*. 114: 45-60.

Jacquemond, M. and Tepfer, M. 1998. Satellite-madiated resistance to plant virus: are ecological risks well assessed? In: *Plant Virus Disease Control*. (eds. A. Hadidi, R.K. Kheterpal, and H. Koganezawa), pp. 94-120. APS Press, St. Paul, Minisota.

Jedlinski, H. and Brown, C.M. 1965. Cross protection and mutual exclusion by three strains of barley yellow dwarf virus in Avena sativa L. *Virology* 26: 613-621.

Kameya Iwaki, M., Tochihara, H., Hanada, K. and Torigoe, H. 1992. Attenuated isolate of watermelon mosaic virus (WMV-2) and its cross protection against virulent isolate. *Ann. Phytopathol. Soc. Japan* 58: 491-494.

Karyeija, R.F., Kreuze, J.F., Gibson, R.W. and Valkonen, J.P. 2000. Synergistic interactions of a potyvirus and a phloem-limited crinivirus in sweet potato plants. *Virology* 269: 26-36.

Kasschau, K.D., Xie, Z., Allen, E., Llave, C., Chapman, E.J., Krizan, K.A. and Carrington, J.C. 2003. P1/HC-Pro, a viral suppressor of RNA silencing, interferes with Arabidopsis development and miRNA unction. *Dev. Cell* 4: 205-217.

Koo, J.C., Asurmendi, S., Bick, J., Woodford-Thomas, T. and Beachy, R.N. 2004. Ecdysone agonist-inducible expression of a coat protein gene from tobacco mosaic virus confers viral resistance in transgenic Arabidopsis. *Plant J.* 37: 439-448.

Kosaka, Y. and Fukunishi, T. 1993. Attenuated isolates of soybean mosaic virus derived at a low temperature. *Plant Dis*. 77: 882-886.

Kubota, K., Tsuda, S., Tamai, A. and Meshi, T. 2003. Tomato mosaic virus replication protein suppresses virus-targeted posttranscriptional gene silencing. *J. Virol.* 77: 11016-11026.

Kurath, G. and Dodds, J.A. 1994. Satellite tobacco mosaic virus sequence variants with only five nucleotide differences can interfere with each other in a cross protection-like phenomenon in plants. *Virology* 202: 1065-1069.

Kurihara, Y. and Watanabe, Y. 2003. Cross-protection in Arabidopsis against crucifer tobamovirus Cg by an attenuated strain of the virus. *Mol. Plant Pathol.* 4: 259-269.

Kuti, J.O. and Moline, H.E. 1986. Effects of inoculation with a mild strain of tomato aspermy virus on the growth and yield of tomatoes and the potential for cross protection. *J. Phytopathol.* 115: 56-60.

Lakatos, L., Szittya, G., Silhavy, D. and Burgyan, J. 2004. Molecular mechanism of RNA silencing suppression mediated by p19 protein of tombusviruses. *Embo* J 23: 876-884.

Latorre, B.A. and Flores, V. 1985. Strain identification and cross-protection of potato virus Y affecting tobacco in Chile. *Plant Dis*. 69: 930-932.

Lecoq, H., Lemaire, J.M. and Wipf-Scheibel, C. 1991. Control of zucchini yellow mosaic virus in squash by cross protection. *Plant Dis*. 75: 208-211.

Lecoq, H. and Raccah, B. 2001. Cross-protection: interactions between strains exploited to control plant virus diseases. In *Biotic Interactions in Plant Pathogen Associations.* (eds. M.J. Jeger, and N.J. Spence), pp. 177-192. CAB International.

Liefting, L.W., Pearson, M.N. and Pone, S.P. 1992. The isolation and evaluation of two naturally occurring mild strains of vanilla necrosis potyvirus for control by cross-protection. *J. Phytopath* 136: 9-15.

Loebenstein, G., Cohen, J., Shabtai, S., Coutts, R.H.A. and Wood, K.R. 1977. Distribution of cucumber mosaic virus in systemically infected tobacco leaves. *Virology* 81: 117-125.

Lomonossoff, G.P. 1995. Pathogen-Derived resistance to plant viruses. *Annu. Rev. Phytopathol*. 33: 323-343.

Lu, B., Stubbs, G. and Culver, J.N. 1998. Coat protein interactions involved in tobacco mosaic tobamovirus cross-protection. *Virology* 248: 188-198.

Mahmood, T. and Rush, C.M. 1999. Evidence of cross-protection between beet soilborne mosaic virus and beet necrotic yellow vein virus in sugar beet. *Plant Dis*. 83: 521-526.

Mallory, A.C., Reinhart, B.J., Bartel, D., Vance, V.B. and Bowman, L.H. 2002. A viral suppressor of RNA silencing differentially regulates the accumulation of short interfering RNAs and micro-RNAs in tobacco. *Proc. Natl. Acad. Sci. USA* 99: 15228-15233.

Matthews, R.E.F. 1949. Stadies on potato virus Xx. II. Criteria of relatonships between strains. *Ann. appl. Biol*. 36: 460-474.

McKinney, H.H. 1929. Mosaic diseases in the Canary Islands, West Africa and Gibraltar. *J. Agric. Res*. 39: 557-578.

Montasser, M.S., Tousignant, M.E. and Kaper, J.M. 1998. Virus satellite RNAs for the prevention of cucumber mosaic virus (CMV) disease in field-grown pepper and melon plants. *Plant Dis.* 82: 1298-1303.

Moore, C.J., Sutherland, P.W., Forster, R.L., Gardner, R.C. and MacDiarmid, R.M. 2001. Dark green islands in plant virus infection are the result of posttranscriptional gene silencing. *Mol. Plant-Microbe Interact*. 14: 939-946.

Mourrain, P., Beclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.B., Jouette, D., Lacombe, A.M., Nikic, S., Picault, N., et al. 2000. Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell* 101: 533-542.

Murphy, P.A. 1938. A discussion on new aspects of virus disease. *Proc. R. Soc. London Ser*. B 125: 307-308.

Niblett, C.L., Dickson, E., Fernow, K.H., Horst, R.K. and Zaitlin, M. 1978. Cross protection among four viroids. *Virology* 91: 198-203.

Oshima, N. 1975. The control of tomato mosaic virus disease with attenuated virus of tomato strain of TMV. *Rev. Plant Protec. Res*. 8: 126-135.

Owor, B., Legg, J.P., Okao Okuja, G., Obonyo, R., Kyamanywa, S. and Ogenga Latigo, M.W. 2004. Field studies of cross protection with cassava mosaic geminiviruses in Uganda. *J. Phytopathol*. 152: 243-249.

Palukaitis, P. and Zaitlin, M. 1984. A model to explain the "cross-protection" phenomenon shown by plant viruses and viroids. In *Plant-Microbe Interactions: Molecular and Genetics Perspectives*. (eds. T. Kosuge, and E.W. Nester), pp. 420-429. Macmillan, New York.

Posnette, A.F. and Todd, J.M.A. 1955. Virus diseases of cacao in west Africa. IX. Strain variation and interference in virus 1A. *Ann. appl. Biol* 43: 433-453.

Powell, P.A., Sanders, P.R., Tumer, N., Fraley, R.T. and Beachy, R.N. 1990. Protection against tobacco mosaic virus infection in transgenic plants requires accumulation of coat protein rather than coat protein RNA sequences. *Virology* 175: 124-130.

Rankovic, M. and Paunovic, S. 1989. Further studies on the resistance of plums to sharka (plum pox) virus. *Acta Hortic.* 235: 283-290.

Rast, A.T.B. 1972. MII-16, an artificial symptomless mutant of tobacco mosaic virus for seedling inoculation of tomato crops. *Neth. J.Plant Pathol.* 78: 110-112.

Ratcliff, F.G., MacFarlane, S.A. and Baulcombe, D.C. 1999. Gene silencing without DNA: RNA-mediated cross-protection between viruses. *Plant Cell* 11: 1207-1215.

Roth, B.M., Pruss, G.J. and Vance, V.B. 2004. Plant viral suppressors of RNA silencing. *Virus Res*. 102: 97-108.

Salaman, R.N. 1933. Protective inoculatiion against plant virus. *Nature* 131: 468.

Sherwood, J.L. 1987. *Mechanisms of Cross-Protection between Plant Virus Strains*. Wiley, Chichester (UK), pp. 136-150.

Sherwood, J.L. and Fulton, R.W. 1982. The specific involvement of coat protein in tobacco mosaic virus cross protection. *Virology* 119: 150-158.

Shiboleth, Y.M., Arazi, T., Wang, Y. and Gal-On, A. 2001. A new approach for weed control in a cucurbit field employing an attenuated potyvirus-vector for herbicide resistance. *J. Biotechnol.* 92: 37-46.

Szittya, G., Silhavy, D., Molnar, A., Havelda, Z., Lovas, A., Lakatos, L., Banfalvi, Z. and Burgyan, J. 2003. Low temperature inhibits RNA silencing-mediated defence by the control of siRNA generation. *ENBO J.* 22: 633-640.

Tanzi, M., Betti, L. and Canova, A. 1988. Protection of pepper crops under glass against PepMV artificial infection with the mild mutant M-1: analysis of its influence on growth and yield. *Adv. Horti. Sci.* 1: 19-22.

Tennant, P., Fermin, G., Fitch, M.M., Manshardt, R.M., Slightom, J.L. and Gonsalves, D. 2001. Papaya ringspot virus resistance of transgenic Rainbow and SunUp is affected by gene dosage, plant development, and coat protein homology. *Euro. J. Plant Pathol.* 107: 645-653.

Tennant, P.F., Gonsalves, C., Ling, K.S., Fitch, M., Manshardt, R.M., Slightom, L.J. and Gonsalves, D. 1994. Differential protection against papaya ringspot virus isolates in coat protein gene transgenic papaya and classically cross-protected papaya. *Phytopathology* 84: 1359-1366.

Tien, P. and Wu, G. 1991. Satellite RNA for the biocontrol of plant diseases. *Adv. Virus Res.* 39: 321-339.

Tomlinson, J.A. and Shepherd, R.J. 1978. Studies on mutagenesis and cross-protection of cauliflower mosaic virus. *Ann. appl. Biol*. 90: 223-231.

Urban, L.A., Sherwood, J.L., Rezende, J.A.M. and Melcher, U. 1990. Examination of mechanisms of cross-protection with non-transgenic plants. In *Recognition and Response in Plant-Virus Interactions.* (ed. R.S.S. Fraser), pp. 415-426. Springer-Verlag, Berlin.

Urcuqui-Inchima, S., Maia, I.G., Arruda, P., Haenni, A.L. and Bernardi, F. 2000. Deletion mapping of the potyviral helper component-proteinase reveals two regions involved in RNA binding. *Virology* 268: 104-111.

Valkonen, J.P., Rajamaki, M.L. and Kekarainen, T. 2002. Mapping of viral genomic regions important in cross-protection between strains of a potyvirus. *Mol. Plant Microbe Interact.* 15: 683-692.

Vance, V.B. 1991. Replication of potato virus X RNA is altered in coinfections with potato virus Y. *Virology* 182: 486-494.

Vaucheret, H., Vazquez, F., Crete, P. and Bartel, D.P. 2004. The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. *Genes Dev* 18: 1187-1197.

Walkey, D.G.A., Lecoq, H., Collier, R. and Dobson, S. 1992. Studies on the control of zucchini yellow mosaic virus in courgettes by mild strain protection. *Plant Pathol.* 41: 762-771.

Wang, H.L., Gonsalves, D., Provvidenti, R. and Lecoq, H.L. 1991. Effectiveness of cross protection by a mild strain of zucchini yellow mosaic virus in cucumber, melon, and squash. *Plant Dis.* 75: 203-207.

Wang, M. and Gonsalves, D. 1992. Artificial induction and evaluation of a mild isolate of tomato spotted wilt virus. *J. Phytopathol* 135: 233-244.

Wang, Y., Gaba, V., Yang, J., Palukaitis, P. and Gal-On, A. 2002. Characterization of synergy between Cucumber mosaic virus and potyviruses in cucurbit hosts. *Phytopathology* 92: 51-58.

Wang, Y., Lee, K.C., Gaba, V., Wong, S.M., Palukaitis, P. and Gal-On, A. 2004. Breakage of resistance to Cucumber mosaic virus by co-infection with Zucchini yellow mosaic virus: enhancement of CMV accumulation independent of symptom expression. *Arch. Virol*. 149: 379-396.

Watts, J.W. and Dawson, J.R.O. 1980. Double infection of tobacco protoplasts with brome mosaic virus and cowpea chlorotic mottle virus. Virology 105: 501-507.

Webb, R.E., Larson, R.H. and Walker, J.C. 1952. Relationships of potato leaf roll virus strains. *Res. Bull. Wisconsin Agric. Exp. Stat.* 178: 1-38.

Wen, F., Lister, R.M. and Fattouh, F.A. 1991. Cross-protection among strains of barley yellow dwarf virus. *J. Gen. Virol.* 72: 791-799.

Xie, Z., Johansen, L.K., Gustafson, A.M., Kasschau, K.D., Lellis, A.D., Zilberman, D., Jacobsen, S.E. and Carrington, J.C. 2004. Genetic and functional diversification of small RNA pathways in plants. *PLoS Biol.* 2: E104.

Yang, S.J., Carter, S.A., Cole, A.B., Cheng, N.H. and Nelson, R.S. 2004. A natural variant of a host RNA-dependent RNA polymerase is associated with increased susceptibility to viruses by Nicotiana benthamiana. *Proc. Natl. Acad. Sci. USA* 101: 6297-6302.

Yarden, G., Hemo, R., Livne, H., Maoz, E., Lev, E., Lecoq, H. and Raccah, B. 2000. Cross-protection of Cucurbitaceae from zucchini yellow mosaic virus. In Proceedings of 7th EUCARPIA meeting on cucurbits genetics and breeding, *Acta Hortic*.. (eds. N. Katzir, and H.S. Paris), pp. 349-356.

Ye, K., Malinina, L. and Patel, D.J. 2003. Recognition of small interfering RNA by a viral suppressor of RNA silencing. *Nature* 426: 874-878.

Yeh, S.D. and Gonsalves, D. 1984. Evaluation of induced mutants of papaya ringspot virus for control by cross protection. *Phytopathology* 74: 1086-1091.

Yeh, S.D., Gonsalves, D., Wang, H.L., Namba, R. and Chiu, R.J. 1988. Control of papaya ringspot virus by cross protection. *Plant Dis.* 72: 375-380.

Yoo, B.C., Kragler, F., Varkonyi-Gasic, E., Haywood, V., Archer-Evans, S., Lee, Y.M., Lough, T.J. and Lucas, W.J. 2004. A systemic small RNA signaling system in plants. *Plant Cell* 16: 1979-2000.

Yoshida, K., Goto, T. and Iizuka, N. 1985. Attenuated isolates of cucumber mosaic virus produced by satellite RNA and cross protection between attenuated isolates and virulent ones. *Ann. Phytopathol. Soc. Japan* 51: 238-242.

Zhang, X.S. and Melcher, U. 1989. Competition between isolates and variants of cauliflower mosaic virus infected turnip plants. *J. Gen. Virol.* 70: 3427-3437.