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VIRAL INTERACTIONS IN VIRAL COAT PROTEIN TRANSGENIC PLANTS: A LITERATURE REVIEW

Virus infection is a serious problem in agricultural production. Virtually every plant species is susceptible to infection by at least one of more than 500 known plant viruses (Waterhouse et al. 2001). Plant viruses create economic losses for a vast variety of crops by reducing yields and negatively affecting the quality of the crop (Tolin 1991). Growers may need to use several control methods during a crop season in an attempt to prevent viral infection and dissemination, primarily by planting virus-free material for mechanically transmitted viruses. For vector-transmitted viruses, control measures have often focused on chemical insecticides, fungicides, and nematicides to reduce the population of vectors that transmit viruses from plant to plant. However, control of vectors is not always feasible or effective as a way to control virus transmission (OECD Environment Directorate 1996). In another common control strategy, plants are infected with a mild form of a virus to confer resistance to a more virulent form. This method has serious limitations as well. In some cases, the development of resistant cultivars can be the only viable means of virus control. Plants developed through conventional breeding techniques offer some degree of virus resistance. However, breeding for resistance has not been successful for the majority of field crops that are severely affected by viruses (Tolin 1991). More recent transgenic methods for creating resistant varieties incorporate viral DNA into the plant's genome to confer effective resistance to infection by that virus as well as others of similar sequence.

Many different types of transgenes have been used to confer resistance to viral infection including viral replicase, movement protein, and nuclear inclusion genes as well as nonviral sequences from a variety of species (Tepfer 2002). However, the most common type of transgenes used to confer virus resistance are viral coat protein (VCP) genes (White 2000). VCPs encapsidate the viral nucleic acid and are thought to be important in nearly every stage of viral infection including replication, movement throughout an infected plant, and transport from plant to plant (Callaway et al. 2001). Given their central role in viral infection, the interaction of a VCP transgene with the genome of an invading virus could potentially exacerbate viral disease through a change in the disease characteristics or transmission properties of that virus. Continued development and testing of new VCP-transgenic plants has facilitated evaluation of such potential impacts in the 18 years since the first one was created (Abel et al. 1986). This review evaluates the current scientific understanding of the potential risks associated with viral interactions in VCP-transgenic plants.

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Viral Interactions

Mixed viral infections can be extremely common in crops and other plants (for review see Hammond et al. 1999). In natural, mixed infections, viral genomes from different strains and/or different species simultaneously infect the same plant and thus have opportunities to interact. In spite of many opportunities for interaction in nature, such events rarely lead to any detectable adverse outcome (Falk & Bruening 1994). However, such *in planta* interactions do have the potential to result in a virus that causes increased agricultural or other environmental damage. For example, the epidemic of severe cassava mosaic disease in Uganda is thought to be due to the combination and/or sequential occurrence of several phenomena including recombination, psuedorecombination, and/or synergy among cassava geminiviruses (Pita et al. 2001). VCP-transgenic plants potentially raise concerns because every virus infection is essentially a mixed infection with respect to the CP gene (de Zoeton 1991). The pertinent question is whether the risks associated with VCP-transgenic plants may be either greater in degree or different in kind than are presented by natural, mixed infections (Tepfer 2002).

Recombination, heterologous encapsidation, and synergy are discussed individually to evaluate each viral interaction as it occurs under natural conditions, its potential to occur in VCP-transgenic plants, and ways that the frequency of its occurrence could be reduced if warranted. Then the limited number of field evaluations of viral interactions in VCP-transgenic plants are discussed. Finally, the ecological significance of these studies is put into context by considering whether viral interactions in transgenic plants could occur at an increased frequency or be unlike those that occur in natural, mixed infections.

Recombination

Recombination under natural conditions

Recombination plays a significant role in virus evolution. Evidence of past recombination having led to the creation of new DNA and RNA viruses has been found in a number of different groups including bromoviruses (Allison et al. 1989), caulimoviruses (Chenault & Melcher 1994), luteoviruses (Gibbs & Cooper 1995), nepoviruses (Le Gall et al. 1995), cucumoviruses (Masuta et al. 1998), and geminiviruses (Pita et al. 2001; Zhou et al. 1997). Sequence analysis of viruses from the family Luteoviridae indicated that this family has evolved via both intra- and interfamilial recombination (Moonan et al. 2000).

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The propensity to recombine and thus the significance of recombination varies within and across virus groups (Worobey & Holmes 1999), possibly due to the degree of dissimilarity the viral replicase can tolerate (Hammond et al. 1999). Sequence analysis of the CP and 3′ untranslated region (UTR) of 109 potyvirus isolates revealed intraspecies recombination led to viable recombinants in four out of the eight species represented (Revers et al. 1996).

Recombination is more likely to occur and thus more likely to result in viable recombinants that have the appropriate transcription recognition signals the more closely related the viruses are. However, experiments suggest that recombination between even unrelated viruses may prove significant. A pseudorecombinant strain created by experimentally combining regions of the cucumber mosaic virus (CMV) and tomato aspermy cucumovirus (TAV) genomes was found to have more severe symptoms than either of the parentals, although the recombinant wasn't able to move beyond infection of the initially infected cells (Salánki et al. 1997). Experiments have also shown interspecific recombination between CMV and TAV under conditions in which recombinants would not be expected to have any particular fitness advantage (Aaziz & Tepfer 1999). In another example, alteration of the host range of tobacco mosaic virus (TMV) occurred when a chimeric virus expressed the CP from alfalfa mosaic virus (AMV) instead of its own (Spitsin et al. 1999).

In addition to virus-virus recombination, recombination has also been found to occur between virus and plant host RNA. Sequence analysis of the 5′ terminal sequence of potato leafroll virus (PLRV) suggests that it arose via recombination with host mRNA (Mayo & Jolly 1991). Evidence suggests that such recombination events can affect virus virulence (for review see Rubio et al. 1999).

Recombination only rarely leads to the persistence of a new, viable virus. Whether a recombinant virus survives in the field depends on how well it is able to compete with other viruses at all stages of the infective cycle: transmission, gene expression, replication, and assembly of new virions (Falk & Bruening 1994). An analysis of CMV isolates in natural populations showed that while mixed infections are common, viable recombinants were very rarely recovered. Such results suggest that recombinants are infrequent or are at a selective disadvantage relative to parental viruses (Fraile et al. 1997). However, a virus that has altered transmission patterns from parental varieties might be able to invade a new niche and thus escape such intense competition (Gibbs 1994). Laboratory experiments have demonstrated that some RNA-RNA recombinant viruses are potentially able to have increased fitness, possibly due to increased

replication efficiency or increased stability of the viral RNA (Fernández-Cuartero et al. 1994).

Laboratory experiments of recombination in transgenic plants with viral transgenes

Like a plant host genome, viral transgenes would be available for recombination with infecting viruses, and portions of the transgene could thus be incorporated into the replicating virus. Laboratory experiments with pseudorecombinant transcripts of papaya ringspot virus (PRSV) have shown that recombinant viruses that theoretically could be produced in field-grown transgenic papaya would be able to affect the virulence of the infecting strains (Chiang et al. 2001). Viruses that are pathogens of the host plant would be available for recombination with transgenes. In addition, other viruses that are generally not pathogens may also be available if the plant resists infection by that virus by restricting its movement while still allowing its replication (Greene & Allison 1994). The theoretical possibility exists that recombination between a replication-competent but nonpathogenic virus and a transgene could convert the virus to a pathogen (Allison et al. 1996).

Several laboratory experiments have investigated the potential for recombination between the RNA of viral genes in a transgenic plant and an infecting RNA virus of the same type:

- Transgenic *Nicotiana benthamiana* containing the red clover necrotic mosaic virus cell-to-cell movement protein transgene were inoculated with infectious transcripts of the virus. Sequence analysis of virus found in noninoculated, systemically infected leaves showed that the infecting virus had recombined with the cell-to-cell movement protein transgene to yield a virus capable of systemic infection (Lommel & Xiong 1991).
- Transgenic *Nicotiana benthamiana* containing the 3′ 2/3 of the cowpea chlorotic mottle virus (CCMV) CP gene were inoculated with a CCMV deletion mutant lacking the 3′ 1/3 of the gene such that recombination within the central 1/3 region of the gene would allow restoration of a virus capable of systemic infection. Three percent of inoculated plants produced viable recombinants, each from a separate aberrant homologous recombination event, in spite of experimental conditions designed to favor precise homologous recombination (Greene & Allison 1994).
- Transgenic *Nicotiana benthamiana* containing the CP coding sequence from tomato bushy stunt virus (TBSV) were inoculated with CP mutants of TBSV. Double

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recombination events restored wild-type virus phenotype in up to 20% of the plants (Borja et al. 1999).

- Transgenic *Nicotiana benthamiana* containing the CP coding region of TMV was inoculated with a CP deletion mutant. Recombinant RNA was detected in 32% of the infected plants, but encapsidated recombinant virions were not detected. No recombinant RNA was detected when the region investigated was a gene nonessential for infection rather than the CP gene (Adair & Kearney 2000).
- Transgenic *Nicotiana benthamiana* containing the CP coding region of plum pox virus (PPV) with either a complete or a partially deleted 3′ UTR were inoculated with CP mutants of an infectious full-length clone of PPV. Recombination produced viable PPV, but only when the transgenic plants contained the complete 3′ UTR (Varrelmann et al. 2000).

Similar experiments show that recombination between the RNA of viral transgenes and infecting DNA viruses can also occur. The transgenic plants used in these experiments (detailed below) actually show no resistance to the DNA virus whose coat protein they contain, and attempts to develop transgenic DNA virus-resistant plants in general have had little success (Hammond et al. 1999). Thus, the relevance of the recombination events observed in these plants as an indicator of environmental concern might be questioned.

- Transgenic *Brassica* were infected with a defective CaMV genome. A gene that complements the defective region was inserted into the plants such that recombination between virus and transgene could produce a functional copy of the virus genome that would enable virus replication and spread. Although CaMV is a DNA virus, it replicates through an RNA intermediate. Both RNA and DNA recombination events appeared to be responsible for generation of viable recombinants in four out of 12 inoculated plants (Gal et al. 1992).
- Transgenic *Nicotiana bigelovii* containing a transgene from an infective strain of CaMV were inoculated with isolates of a CaMV strain that do not infect *N. bigelovii* systemically. Complementation but not recombination occurred with one isolate, and recombination but not complementation occurred in eight out of ten plants of another isolate. Recombination between the CaMV transgene and infecting viruses was able to alter the symptomatology and expand the host range of the infecting virus strain (Schoelz & Wintermantel 1993).
- Transgenic *Nicotiana bigelovii* containing a transgene from CaMV were inoculated with CaMV strains that could infect *N. bigelovii* systemically, so recombinant viruses would be expected to have little selective advantage over the parentals.

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Recombinant viruses were recovered in three out of 23 transgenic plants inoculated with one isolate and three out of 32 plants inoculated with another. Under strong selection pressure for recombination, recombinants were recovered from all 24 transgenic plants tested (Wintermantel & Schoelz 1996).

- Several transgenic *Nicotiana benthamiana* lines containing the CP coding sequence from African cassava mosaic virus (ACMV; a geminivirus) were inoculated with a CP deletion mutant of ACMV. Recombinant progeny were recovered from transgenic lines only when they contained geminiviral noncoding sequence on both sides of the CP transgene. The frequency of recombinants varied from <1% to 67% of plants in a given line (Frischmuth & Stanley 1998).

Experimental evidence thus demonstrates that recombination can occur between viral transgenes and invading viruses. However, to facilitate the detection of recombinants, most of these experiments were conducted under conditions of high selective pressure, i.e., the virus was not viable unless a recombination event occurred. The selective pressure is usually likely to be much weaker under normal field conditions. Parental viruses will outnumber the new recombinant and will be competent in all of the functions needed for propagation. The relevance of experiments demonstrating recombination under conditions of strong selection is thus unclear beyond documenting that recombination between viral genomes and transgenes can occur.

Although the laboratory experiments outlined above artificially establish a high selection pressure for recombinants that is unlikely to occur in nature, such selection might arise in transgenic plants when viruses partially overcome the engineered resistance, as they have been known occasionally to do (Carrington et al. 2001). If the VCP transgene only partially eliminated an infecting virus, recombinant virions that acquired a CP gene from a different infecting virus would be able completely to evade the resistance conferred by the VCP transgene. Such recombination events among different infecting viruses could occur in the absence of the transgene. However, the transgene could create a selective environment that would favor persistence of recombinants and thus increase their potential to be epidemiologically significant (Jakab et al. 1997).

The potential environmental impact of any recombinant viruses that might arise in VCP-transgenic plants and the likelihood of their arising need to be evaluated in the context of the events that occur in non-transgenic plants. The recombinants that arise in transgenic plants are theoretically unlikely to be qualitatively different from those that could arise in natural, mixed infections if the transgene is from a virus that normally infects the plant and is expressed in the same cells that normally are infected. Under

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such circumstances the potential new viruses that could be created through recombination are expected in theory to be the same in transgenic and nontransgenic plants (Roossinck 1997).

Reducing the frequency of recombination in transgenic plants

All evidence suggests that recombination among RNA viruses occurs via template switching by the viral replicase during replication such that a hybrid molecule is formed (AIBS 1995). Modifications of CP transgenes may reduce the possibility of their participation in recombination. A number of ways have been suggested, including:

- Remove the 3′ untranslated region (UTR) in the CP mRNA transcript (Teycheney et al. 2000). Inclusion of this region may enable replication to begin on the mRNA transcript and then switch to the RNA of the invading virus. Removal of this region would necessitate two separate template-switching events to form a successful recombinant and thus reduce its likelihood of occurrence (Greene & Allison 1994). Experiments with CCMV demonstrated that deletions in the 3′ UTR did indeed reduce the recovery of recombinant viruses (Greene & Allison 1996). Since functional resistance is still conferred by constructs containing a CP lacking the 3′ UTR, this region may not be necessary.
- Reduce the extent of shared sequence similarity between the infecting virus and the transgene to reduce the opportunities for homologous recombination (Nagy et al. 1999).
- Exclude any sequences containing replicase recognition sites that are potential sites of recombination and any sequences known or thought to be recombination hotspots, e.g., promoters for genomic and subgenomic RNA synthesis (Miller et al. 2000).
- Avoid potential hairpin structures in the transgene that might function as acceptor structures for the replicase complex (Nagy et al. 1998).
- Insert GC-rich sequences downstream of any AU-rich region thought to be important for conferring resistance (Hammond et al. 1999). AU-rich regions were found to be associated with imprecise homologous recombination in brome mosaic virus (BMV; Nagy & Bujarski 1996), and insertion of GC-rich sequences in BMV was found to reduce the incidence of recombination in spite of increasing the total amount of sequence identity (Nagy & Bujarski 1998). The applicability of these results to other viruses has yet to be demonstrated.

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- Use the smallest viral fragment that confers effective resistance, as longer fragments provide larger recombination targets (Nagy et al. 1999). In addition, longer segments are more likely to encode functional modules that could be incorporated into another virus as a single unit, thus increasing the chance that recombinants would be viable.

Heterologous encapsidation

Heterologous encapsidation under natural conditions

For many viruses, transmission from plant to plant occurs by insect vectors and each virus tends to be transmitted by only one type of insect (Callaway et al. 2001). The CP, possibly in conjunction with other viral factors, is essential for transmission and responsible for conferring the high degree of specificity. Heterologous encapsidation occurs when the capsid protein subunits of one virus surround the nucleic acid of a different virus, thus potentially changing its vector specificity.

Most evidence of heterologous encapsidation is derived from laboratory or greenhouse studies. The high frequency of mixed infections suggests the potential for heterologous encapsidation to occur in nature is great, but most mixed infections do not lead to heterologous encapsidation, and those virus interactions that do occur are very specific (Falk et al. 1995). Heterologous encapsidation is however known to be a regular occurrence among some plant viruses. Its frequency depends on the viruses involved and is more likely to occur among close relatives (Tepfer 1993). An expansion of aphid vector specificity due to heterologous encapsidation was first observed in plants infected with two different isolates of barley yellow dwarf luteovirus (BYDV; Rochow 1970) and was later shown to be a general phenomenon among these viruses in natural populations of several plant species (Creamer & Falk 1990). Heterologous encapsidation was also shown to occur in potyviruses. An isolate of zucchini yellow mosaic virus (ZYMV) that is normally non-aphid transmissible due to a transmission-deficient CP was found to be aphid transmissible due to heterologous encapsidation when in a mixed infection with another potyvirus, papaya ringspot virus (Bourdin & Lecoq 1991). Heterologous encapsidation may be an important route of disease transmission for viruses that have no CP. For example, potato spindle tuber viroid is transmitted only by foliar contact or botanical seed unless encapsidated by the coat protein of PLRV which renders it aphid transmissible (Querci et al. 1997).

Heterologous encapsidation is considered a possible environmental concern because if the new vector has a host range different from the original vector, the virus may be

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spread to new plant varieties that can support its replication but that it would not have infected if not for the heterologous encapsidation (de Zoeton 1991). Such concerns are largely mitigated by several factors. If replication is possible in the new host, it would cause the virus to be encapsidated by its own CP, thus limiting the epidemiological consequences of heterologous encapsidation. Secondly, the vector transmission of the encapsidating virus may require regions of the genome other than the CP for effective transmission, so heterologous encapsidation could not lead to a change in vector specificity (Robinson 1996). Thirdly, the vector may have a limited host range in the area where the crop is to be grown such that it would be unlikely to transmit a heterologous virus to a novel host. Rather, the vector would transmit virus only to the same plant that the virus is already able to infect (Robinson 1996).

However, under certain limited circumstances heterologous encapsidation theoretically might still have environmental or agricultural impacts. For one, a virus may become available for transmission by new potential vectors that feed on the new host but not the virus' original host (Hammond et al. 1999). Through such a mechanism, theoretically both the host and vector range of a virus may be expanded, and the change of both plant host and vector could make detection of heterologous encapsidation difficult. In addition, with a high enough frequency of vector transmission to a new host due to heterologous encapsidation, secondary spread among new plant hosts might not be required for the phenomenon to affect them. Due to the lack of a proofreading mechanism during replication of RNA viruses, they are thought to exist as "quasispecies" in which each viral genome differs by a few nucleotides from a consensus sequence. After expansion to a new host, rapid selection of variants best adapted to the new environment might lead to the evolution of a new virus (Hammond et al. 1999). No direct evidence of such events exists, but their occurrence is suggested by the appearance of "new" viruses in areas that have had recent agricultural expansions. When previously wild areas are cultivated, novel interactions may occur among potential vectors, viruses in the local natural vegetation, and crops previously unexposed to these viruses (Hammond et al. 1999).

Laboratory experiments of heterologous encapsidation in transgenic plants with viral transgenes

Experimental studies have shown that the protein from VCP genes in transgenic plants has the ability to encapsidate even unrelated infecting viruses:

- Transgenic tobacco plants engineered with the CP of TMV were infected with strains of TMV defective for the CP gene. The transgenic CP was able to complement the defective TMV and enable long-distance transport (Osbourn et al. 1990).

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- Transgenic tobacco plants engineered with the CP gene of AMV were infected with CMV. Heterologous encapsidation was observed to occur between these two unrelated viruses in one third of plants at a frequency of about 5×10^{-7} (Candelier-Harvey & Hull 1993).
- Transgenic tobacco plants engineered with the CP of PPV were infected with a strain of ZYMV that is non-aphid transmissible due to the lack of a functional CP gene. Aphid transmission of ZYMV was found to occur, most likely by heterologous encapsidation in the PPV CP (Lecoq et al. 1993).
- Transgenic tobacco plants engineered with the CP gene of PPV were infected with various potyviruses and with viruses from other groups. Newly formed potyvirus particles were found to contain PPV CP, but no other viral particles did (Maiss et al. 1994).

Heterologous encapsidation can only occur in transgenic plants if the coat protein is expressed. Therefore, in transgenic VCP plants that express very little CP (i.e., those relying on post-transcriptional gene silencing to effect resistance), any effects associated with heterologous encapsidation would be minor except in cases of resistance breakdown. In addition, as with recombination, as long as the VCP inserted in the transgenic plant is from a virus that normally infects the plant in the area where it is planted, the outcome of any heterologous encapsidation that may occur is expected to be qualitatively the same in transgenic plants as in natural, mixed infections.

Reducing the frequency and/or impact of heterologous encapsidation in transgenic plants

Steps can be taken to reduce the likelihood of heterologous encapsidation and/or vector transmission occurring. Specific locations within the CP gene of several viruses have been shown to affect aphid transmission:

- Amino acid changes in the CP of CMV differentially reduce the efficiency of transmission by two different aphid species (Perry et al. 1998).
- The readthrough domain (RTD) of the CP plays a key role in determining aphid transmission specificity in PLRV (Jolly & Mayo 1994), BYDV (Chay et al. 1996), and beet western yellows luteovirus (BWYV; Brault et al. 1995; Reinbold et al. 2001; Bruyère et al. 1997; Brault et al. 2000).
- Point mutations in the major capsid protein (P3) of BWYV affect aphid transmission (Brault et al. 2003).

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- Amino acid changes in a conserved loop structure of the CP of CMV affect aphid transmission (Liu et al. 2002).
- A three amino acid sequence, asp-ala-gly (DAG) is conserved in aphid-transmissible strains of potyviruses including tobacco vein mottling virus (Atreya et al. 1991) and ZYMV (Gal-On et al. 1992). Mutations in this region in either virus render it non-aphid-transmissible. The particular context in which this amino acid triplet is found also appears to be important in determining aphid transmissibility in TVMV (López-Moya et al. 1999).

Experiments suggest that VCP gene modifications could reduce the frequency of heterologous encapsidation and/or vector transmission. Mutations in the two assembly motifs (RQ and D) of the CP of PPV were found to suppress heterologous encapsidation, particle assembly, and complementation without affecting resistance to viral infection (Varrelmann & Maiss 2000). Likewise, the CP gene of PPV was modified or truncated in order to reduce any potential impacts of heterologous encapsidation: either the DAG triplet was deleted or the first 420 nucleotides of the PPV CP gene were removed (Jacquet et al. 1998b). Both transgenic lines were resistant to PPV infection, indicating that the full-length CP region is not needed for resistance. Further experiments confirmed that such changes in the CP did effectively mitigate the potential for heterologous encapsidation to have any effect (Jacquet et al. 1998a). However, investigation of the DAG motif in begomoviruses revealed that it is not the precise determinant of whitefly transmission of this virus. Rather, amino acids 123 to 149 of the CP are minimally required for transmission, and amino acids 149 to 174 contribute to efficient transmission (Höhnle et al. 2001).

If changes that resulted in loss of aphid transmissibility were to be incorporated into the VCP genes used in transgenic plants, then heterologous encapsidation would be extremely unlikely to change vector specificity, since the CP would no longer confer any specificity at all. Experiments with various potyviruses showed that the degree of heterologous encapsidation failed to correlate with the degree of resistance conferred by different CP constructs (Hammond & Dienelt 1997), suggesting that such strategies could be deployed without compromising product efficacy. However, while particular changes in the CP are effective at reducing any potential impacts associated with heterologous encapsidation, variation among viruses may preclude a single generic solution that could be used for all.

Synergy

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Synergy under natural conditions

In addition to the creation of a novel virus through recombination and the alteration of transmission properties of an existing virus through heterologous encapsidation, viral interactions may cause increased disease severity through alteration of the replication and/or movement efficiency of a virus. In a synergistic disease the severity of two viruses together is greater than expected based on the severity of each alone. When potato virus X (PVX) is coinfected with a number of potyviruses including TVMV, TEV, and pepper mottle virus, the disease symptoms are considerably worsened and PVX accumulates to a greater concentration (Vance et al. 1995). CMV and TAV cause no synergistic disease in double infections, but an interspecies hybrid was significantly more virulent than either parent in all plant species tested (Ding et al. 1996). The increased severity was most likely due to a synergistic interaction between TAV and the protein produced from the CMV portion of the hybrid. A listing of reported viral synergisms has been compiled (OECD Environment Directorate 1996).

Synergistic interactions are extremely common among the luteoviruses, but the CP is considered much less likely to be responsible for synergism than other regions of the viral genome, for example the polymerase gene (Miller et al. 1997). Within potyviruses, the 5′ proximal 1/3 of the viral genome is thought to contain the factors that mediate synergism, including the 5′ UTR and the coding region for the 5′ N-terminal portion of the viral polyprotein (Pruss et al. 1997).

Synergy in transgenic plants with viral transgenes

Synergy in transgenic plants is generally an agroeconomic rather than environmental concern. Any negative effects are expected primarily to affect the transgenic crop itself which would be quickly abandoned once such effects were detected. Temporarily increased viral loads caused by synergistic disease could result in limited damage to nearby conventional crops if the transgenic crop were already deployed in large field acreages when synergistic disease was discovered (Miller et al. 1997). However, synergistic interactions can be evaluated in transgenic plants before deployment by experimental inoculation with all of the viruses likely to be encountered in the field (Robinson 1996). Developers have a strong incentive to undertake such efforts to ensure the efficacy of their product after deployment.

Reducing the frequency of synergy in transgenic plants

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As with heterologous encapsidation, constructs can be engineered to reduce the likelihood of synergy. Particular transgenes known to participate in synergistic interactions could be avoided or defective copies of genes could be used. Stacking multiple resistances within the same plant such that it will have reduced viral loads for all of its normal pathogens has also been suggested (Palukaitis 2000). The benefits of this approach may however be outweighed by the concerns associated with stacking in VCP-transgenic plants (discussed below).

Field evaluations of viral interactions in transgenic plants with viral transgenes

The many laboratory experiments outlined above that investigated the potential viral interactions in VCP-transgenic plants are only one part of evaluating potential environmental impacts of such events. Consideration of the potential impact under natural, field conditions evaluated in the context of the likelihood of any such event occurring is equally important (García-Arenal et al. 2000). Relatively few field studies have been conducted to address these questions, but those that have been done have found no significant impact associated with deployment of VCP-transgenic plants beyond natural background events. A six-year experiment searched for and failed to find evidence of interactions involving viral transgenes in 25,000 transgenic potato plants transformed with various PLRV CP constructs. Both greenhouse and field tests failed to show any change in the type or severity of disease symptoms, and all viruses isolated were previously known to infect the plants and had the expected transmission characteristics (Thomas et al. 1998).

An experiment with transgenic melon and squash expressing CP genes of an aphid-transmissible strain of CMV failed to find evidence that either recombination or heterologous encapsidation enabled spread of an aphid non-transmissible strain of CMV in the field (Fuchs et al. 1998). A similar experiment with transgenic squash expressing CP genes of an aphid-transmissible strain of watermelon mosaic virus (WMV) showed that an aphid non-transmissible strain of ZYMV was not detected in nontransgenic fields but was transmitted to 2% of plants in transgenic fields, likely due to heterologous encapsidation. However, this rate of transmission failed to lead to the development of an epidemic of ZYMV in transgenic squash fields (Fuchs et al. 1999).

In an experiment to assess the biological and genetic diversity of California CMV isolates before and after deployment of transgenic melon containing the CMV CP gene showed that the only CMV isolate to show significant sequence changes after infecting the transgenic squash was not the result of recombination (Lin et al. 2003). The only field experiment to directly assess the effect of recombination in a VCP-transgenic plant

found no detectable grapevine fanleaf virus (GFLV) recombinants containing the inserted CP sequence over the course of a four-year study (Vigne et al. 2004).

The limited number of field evaluations thus indicate that the likely environmental consequences of viral interactions in VCP-transgenic plants are minimal. However, large acreages of VCP-transgenic plants grown over many years may provide increased opportunity for rare events to occur that are unlikely to be detected in experimental studies (Miller et al. 1997), so consideration of theoretical outcomes and their likelihood of occurrence may be necessary to facilitate a complete evaluation of the risk concerns.

Is the frequency of viral interactions in transgenic plants different than in natural, mixed infections?

Few experimental studies have addressed the relative degree of risk directly, mostly because it is hard under any circumstances to determine the frequency of viral interactions that lead to a measurable effect given how rare these events are. Some characteristics of VCP-transgenic plants suggest that the frequency of interactions may be lower than in natural, mixed infections. Other characteristics suggest that the frequency may be higher (see below).

One factor that may *decrease* the frequency of interactions in transgenic systems is that the cellular concentration of viral RNA transcripts expressed from transgenes will be orders of magnitude lower than the concentration of viral RNA commonly found in natural, mixed infections (Allison et al. 1996). The concentration of infecting viral RNA from the target virus will also be considerably reduced, particularly when the mechanism of resistance relies on post-transcriptional gene silencing to remove all viral RNA transcripts with homology to the transgene (Rovere et al. 2002). However, the significance of these observations is difficult to interpret. While it is known that greater concentrations of RNA will provide greater opportunity for interactions, meaningful values for high or low concentrations are unavailable (AIBS 1995).

The frequency of interactions in transgenic systems may be *increased* because promoters currently used in VCP-transgenic plants cause constitutive expression of transgenes at developmental stages that might otherwise be unaffected by viral infection and often in tissues that the virus does not normally infect (Allison et al. 2000). For example, luteoviruses are normally expressed only in phloem tissue, but the cauliflower mosaic virus (CaMV) promoter drives expression of luteoviral CP in all plant cells. Some evidence suggests that in natural infections different viruses have different temporal or spatial expression patterns that would limit their interactions (Gibbs 1994; Hull 1994;

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Aaziz & Tepfer 1999). Viruses must simultaneously replicate in the same cellular compartment for their RNA to be able to interact. However, when a virus invades a cell, it often replicates and then moves to other cells within the plant. The RNA remaining in the initially infected cell becomes encapsidated and is no longer available for interactions with another invading virus (Allison et al. 2000).

How these competing factors balance out to affect the relative frequency of viral interactions in transgenic versus nontransgenic plants is thus likely to vary with the virus, the plant, and the mechanism of resistance. Whether the nature of the interactions that do occur may be different in VCP-transgenic plants than expected in natural, mixed infections must also be considered.

When could viral interactions in transgenic plants be unlike those likely to occur in natural, mixed infections?

Another key question for evaluating the significance of the above studies is whether the viral interactions in transgenic plants may be unlike those likely to occur in non-transgenic plants. The potential risks associated with viral interactions in VCP transgenic plants are similar to those that arise with natural, mixed infections. Recombination, heterologous encapsidation, and synergy may occur in both cases, although a comparison of frequencies is difficult. Theoretically, the hazards associated with these phenomena are expected to be similar whether these events occur in transgenic or nontransgenic plants. However, under certain circumstances, transgenic plants may in theory provide opportunities for unique interactions that would not be expected to occur in a natural, mixed infection:

- Transgenic multiresistances: A plant may be engineered to resist infection from multiple viruses by incorporation of several CPs into the same plant. While generally, a transgenic plant that is resistant to a particular virus will be planted only where the virus is a problem, with stacked resistances, the likelihood of doing otherwise increases. For example, a cucurbit resistant to CMV, WMV2, and ZYMV has been created (Fuchs et al. 1997). Such a plant would probably be used in all areas where ZYMV is prevalent, including tropical areas where WMV2 never or only rarely occurs (Hammond et al. 1999). WMV2 could then interact with a local strain that would otherwise have had no opportunity for interaction with it.
- Heterologous resistance: A plant may be resistant to infection by a particular virus in spite of having the CP of another virus incorporated into its genome. For example, VCP genes from LMV were used to provide resistance to PVY in tobacco which is

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not infected by LMV (Dinant et al. 1993). In such plants, LMV might have a new opportunity to interact with viruses that infect tobacco.

- Plants may be engineered with VCP genes from an exotic strain of a virus that may be more virulent or have other properties different from endemic isolates. Under certain circumstances, the desire that transgenes be from an endemic isolate may be balanced by other concerns. For example, when the costs of an exotic viral invasion would be particularly devastating, incorporation of a viral transgene intended to prevent such establishment might be warranted (Hammond et al. 1999). In addition, identification of local virus isolates may not always be possible, especially given that the spectrum of isolates in a given area will change over time. Even the definition of what an endemic isolate is can be complicated by international trade in seed and vegetative propagating material. Nevertheless, thorough evaluation of a VCP-transgenic plant requires knowledge of the plant viruses that are present in the release environment and that naturally infect the host (Robinson 1996).
- Plants may express VCP genes in cells and/or tissues that the virus does not normally infect (as discussed above).
- Plants may be engineered with VCP genes that have been altered such that they do not resemble any that exist in nature. Any interactions involving such VCP genes would thus be novel.

Such situations may present the opportunity for novel viral interactions in VCP-transgenic plants, i.e., interactions between portions of two or more different viruses not expected to occur in a mixed infection found in nature. Such situations may be avoided in designing and deploying VCP-transgenic plants. However, if avoidance is impossible or undesirable, strategies such as those discussed above to prevent the occurrence of viral interactions would greatly reduce the frequency of all interactions, whether novel or not.

Conclusion

While the nature of the potential risks posed by novel interactions among viral genomes in transgenic plants is relatively well understood, data to evaluate the actual impact and likelihood of such events under a wide range of natural conditions for different VCP-transgenic plants are sparse. Further experimental data may help to provide a more complete picture that would allow a careful evaluation of the likelihood of adverse impacts and the subsequent evaluation of the risks and benefits of deploying VCP-transgenic plants. Although current evidence suggests that the risks are unlikely to be

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much greater than those found in natural, mixed infections (Hammond et al. 1999; Bruening & Falk 1994), steps may be taken when designing products to minimize any potential impacts.

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