Commentary

Recombination with Host Transgenes and Effects on Virus Evolution: An Overview and Opinion

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This commentary relates to the work by M. Borja et al. (M. Borja, T. Rubio, H. B. Scholthof, and A. O. Jackson, MPMI 12:153-162, 1999) that shows that wild-type virus can be restored frequently by double recombination events between a tomato bushy stunt virus mutant with deletions inactivating the coat protein gene and a coat protein transgene. Here, we focus on evidence suggesting that new viruses might evolve via recombination with transgenes used for disease resistance, and discuss the potential effects of widespread use of these sources of resistance on virus evolution. We argue that the benefits arising from using transgenic sources of resistance for virus disease control outweigh potential negative consequences of evolution of novel hybrid viruses with destructive disease potential.

More than a decade ago Beachy and colleagues demonstrated that transgenic plants expressing tobacco mosaic virus (TMV) coat protein exhibited virus resistance (Powell Abel et al. 1986). The subsequent extension of this strategy and other classes of pathogen-mediated resistance to a wide range of other viruses (Beachy 1997) ushered in a new era in plant pathology. Traditionally, disease losses had been particularly problematic with viruses, because resistance genes suitable for control of important diseases often were not available, and a range of expensive and often environmentally deleterious agronomic techniques had proven to be efficacious only in limited circumstances. Although some agronomic strategies enjoyed notable success, most were effective only against specific virus diseases, and, more often than not, modified cropping practices provided only modest and inconsistent increases in yield. Moreover, with limited exceptions, the cost and toxicity of promising chemical applications prevented their widespread use (Barker et al. 1998; Matthews 1991). Therefore, the success of the pioneering genetic engineering experiments raised considerable expectations that engineered resistance could have a substantial role in ameliorating yield losses due to virus infections of food crops.

Several classes of virus genes used in transgenic plants have been shown to induce resistance that normally is restricted to closely related strains of the virus from which the genes are derived. Coat protein-mediated protection has been the most frequently attempted approach to obtain pathogen-mediated protection (Lomonossoff 1995; Beachy 1997), and squash cultivars transformed with coat protein genes have already been approved for commercialization (Fuchs et al. 1997). In addition to coat protein-mediated protection, application of other viral genes, including those encoding dysfunctional replicase or movement proteins, nontranslatable genes, and antisense constructs has been shown to elicit varying degrees of synthetic resistance that may involve different defense mechanisms (Baulcombe 1996; Beachy 1997; de Haan 1998; Lomonossoff 1995). Replication of competent satellite RNAs (Harrison et al. 1987; Gerlach et al. 1987) and defective interfering components (Stanley et al. 1990; Kollar et al. 1993) also provide an additional array of tools that become amplified following infection with their helper or parental viruses.

As the prospects for disease control with viral transgenes have materialized, varying concerns have been raised about safety issues associated with release of genetically engineered plants. The beneficial and potentially detrimental effects of use of pathogen-mediated resistance to control virus diseases have received considerable attention over the past decade, and a number of diverse opinions have been expressed (Chasan 1993; de Zoeten 1991; Falk and Bruening 1994; Hull 1990; Kling 1996; Mikkelsen et al. 1996; Miller et al. 1997; Gray and Raybould 1998; Robinson 1996; Stewart and Prakash 1998; Beachy 1996; Tepfer 1993; Tepfer and Balázs 1997). Some knowledgeable plant scientists have argued that the use of transgenes for synthetic resistance in field crops presents unacceptable risks (de Zoeten 1991), others believe that the potential risks are manageable (Falk and Bruening 1994; Miller et al. 1997), and some have pointed to the growing world crisis in food production and human population as a mandate for applying biotechnology for crop improvement (Vasil 1998). For detailed overviews of strategies being applied to transgenic resistance and synopses of ongoing experiments to evaluate risks, we refer the reader to a series of articles (cf. Tepfer and Balázs 1997) expressing the views of a number of scientists who participated in a conference to address the ecological impact of virus resistance in transgenic

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crops. A general consensus emerging is that some risks are evident, that they merit detailed analysis to determine their probability and potential impact, and that individual decisions on deployment of various genetically engineered sources of resistance should be based on the perceived agronomic and environmental risks versus the benefits of increased crop production needed to nourish the world's growing human population.

Questions concerning whether new viruses with altered virulence, host range, and vector specificities might evolve by recombination with virus transgenes expressed in plants initially arose because of findings implicating recombination in the evolution of a wide range of RNA viruses. A large body of evidence derived from sequence analyses of naturally occurring virus variants is now available (Angenent et al. 1989; Edwards et al. 1992; Goulden et al. 1991; Hahn et al. 1988; Jarvis and Kirkegaard 1992; MacFarlane et al. 1997; Revers et al. 1996; Rumenapf et al. 1995). Consequently, it is generally accepted that RNA recombination together with the high frequency of mutations associated with the replication of RNA viruses plays an important role in genetic variability and virus evolution (see reviews by Nagy and Simon 1997; Gibbs et al. 1997).

In addition to sequence analyses revealing homologous and nonhomologous recombination between viruses, recombination between cellular genes and viral genomes has also been detected (Gorbalenya 1992; Lai 1992; Bujarski 1997; Simon and Bujarski 1994). With plant viruses, potato leafroll isolates have been shown to contain sequences homologous to an exon of tobacco chloroplast DNA (Mayo and Jolly 1991). More substantial documentation with animal viruses also indicates that recombination events between host and virus genes can have profound effects on virulence. Influenza virus derivatives with 28S ribosomal RNA sequence insertions (Khatchikian et al. 1989), and members of the pestivirus group, including bovine viral diarrhea virus and border disease virus with ubiquitin coding sequences inserted in the NS2-3 gene, all have altered cytopathology (Becher et al. 1996; Meyers et al. 1991). Recent experiments also suggest that the hepatitis delta virus may have evolved by recombination between a viroidlike progenitor and a host cellular gene (Brazas and Ganem 1996), and classical studies have shown that numerous oncogenic retroviruses capture host genes via recombination (White and Fenner 1994). These results all support the conclusion that evolution of new virus derivatives with altered virulence potential can occur via a large array of possible recombination events between viruses and the genes expressed by their hosts.

The first extensively documented case of experimental recombination with a transgene involved the cauliflower mosaic virus (CaMV) translational transactivator gene VI, which recombined with a CaMV gene VI deletion mutant (Gal et al. 1992). In an important related study, a CaMV strain (CaMV1841) that is unable to infect solanaceous plants acquired transgenic sequences that altered the virus symptoms and extended the host range to some Solanaceae (Schoelz and Wintermantel 1993). In these cases, nucleotide sequence analysis of the recombinant viruses indicated that integration of transgene sequences into the viral genome involved a two-step, template switching mechanism during the reverse transcription stage of replication. Subsequently, a deletion mutant

of cowpea chlorotic mottle virus was shown to be restored by a single recombination event in transgenic plants expressing the viral coat protein (Greene and Allison 1994). Our experiments (Borja et al. 1999) reveal that wild-type virus regeneration can be readily detected as a consequence of double recombination events between a tomato bushy stunt tombusvirus (TBSV) mutant containing a deletion in the coat protein gene and a coat protein transgene. An earlier study, involving restoration of the cell-to-cell movement function of an RNA virus, red clover necrotic mosaic virus, by recombination with the movement gene expressed in transgenic plants, has been described in an abstract (Lommel and Xiong 1991). Both complementation and recombination between defective potato virus Y strains and transgenic coat proteins have been reported in a meeting (Jakab et al. 1997), but the putative recombinants need to be subjected to more detailed molecular genetic analysis before firm conclusions can be drawn about the biological properties of the recombinants or the nature of the recombination events. In summary, the results above show that recombination can occur between transgenes and defective viruses, and that experimental recovery of recombinants can be detected relatively easily. Nevertheless, we should emphasize that these experimental systems, even those designed with relatively moderate selection pressure (Wintermantel and Schoelz 1996), are far more amenable to recovery of recombinants than would be expected to occur under field conditions with resistant plants that reduce virus replication.

It is also possible that new hybrid viruses might evolve via nonhomologous recombination between invading viruses and transgenes derived from an unrelated virus, but our experiments (Borja et al. 1999) to test this possibility failed to yield recombinants. However, recombination between different viruses has recently been demonstrated in two instances. First, in coinoculations of protoplasts and plants with defective cucumber necrosis and TBSV RNAs, chimeric recombinants emerged that included both DI (defective interfering) RNAs and derivatives that were able to infect plants (White and Morris 1994). Interestingly, neither of the parental RNAs was able to replicate autonomously, but the cucumber necrosis defective RNA was able to direct synthesis of functional replicase. The results suggest that the chimeric recombinants arose via initiation of replication of the nonfunctional TBSV DI RNA, followed either by nonhomologous template switching to the cucumber necrosis virus RNA by the nascent replicase DI transcript complex, or by aberrant homologous strand switching at short regions of homology that are located at noncomparable regions on the genomes. A second example of recombination between two distinct viruses has been detected in coinoculations of tomato aspermy virus (TAV) RNAs 1 and 2 and cucumber mosaic virus (CMV) RNAs 2 and 3 (Masuta et al. 1998). In this instance a novel cucumovirus evolved that encodes a replicase consisting of the TAV helicase subunit and the CMV polymerase subunit. Each of these RNAs was required for systemic infection of *Nicotiana benthamiana*, so it is apparent that the hybrid polymerase is required for maintenance of the chimeric derivative. Interestingly, the chimera was able to support replication of a satellite RNA that can be replicated by the parental CMV but not by TAV. These two examples thus show that functional chimeric viruses can be generated by recombination between distinct viruses, and they provide strong support for the concept that

new virus species could evolve under natural conditions during which plants are coinfecting with two or more viruses.

The finding that viable chimeric viruses can be recovered under experimental conditions naturally raises the question, why are such recombinant viruses not observed frequently in nature? Some insight into resolution of this question can be obtained by consideration of the interactions of synthetic hybrid recombinants. One pertinent example shows that a hybrid virus produced by substitution of the movement protein gene of cowpea chlorotic mottle virus with the 30-kDa movement gene of sunn-hemp tobamovirus is able to facilitate limited movement in cowpea (DeJong and Ahlquist 1992). Other such cases exist, including host-dependent movement effects with barley stripe mosaic virus containing a substitution of the triple gene block movement proteins for the movement protein of red clover necrotic mosaic virus (Solovyev et al. 1997). Some synthetic recombinants between the coat protein and the 3' ends of CMV and TAV RNA 3 are able to systemically infect some infected hosts, but not others (Salánki et al. 1997). However, the results of these and other experiments suggest that most recombinant viruses are compromised in virulence because the heterogeneous gene combinations are not fully compatible. Because of such dysfunctions, the vast majority of hybrid recombinants that might occur in nature should be at a competitive disadvantage, and they would be unlikely to survive. The probability that hybrid viruses with expanded host ranges and/or disease phenotypes will emerge depends on a number of favorable determinants. These include the recombinational ability of the parental viruses, the nature of the recombination event, the ability of mutants to establish systemic infections, the phenotype elicited by the recombinant, and the stringency of negative and positive host selection pressures. For serious disease development to occur, the genes involved in recombination must coalesce to produce hybrids that can compete for replication with their progenitors, that have additional positive interactions between movement and host genes that facilitate systemic invasion, and that can interface with vectors or carry out other functions needed for efficient movement to adjacent hosts. The necessity to accommodate these requirements undoubtedly reduces the frequency of emergence of new viruses from the abundant mixed infections that are present in nature. Similar constraints also would provide powerful negative selection against evolution of viruses with expanded virulence potential that might arise during recombination with host transgenes.

A case can be made that use of virus transgenes for disease control may actually constrain rather than exacerbate evolution of novel virus strains. This view takes into account that the resistance transferred into new crops will reduce the enormous background reservoir of potential sources of recombination that currently exist among complex mixtures of viruses in susceptible crops (Falk and Bruening 1994; Matthews 1991). These include not only mixtures of autonomously replicating viruses, but also a large number of defective viruslike agents present in nature. Some of these defective agents apparently could become autonomous by acquiring only a single critical gene. In some cases, two or more defective agents form synergistic complexes that cause serious plant diseases, so these agents represent enormous potential for natural evolution of new viruses by recombination. We contend that, in addition to amelioration of disease losses, a major ancillary

benefit of widespread use of pathogen-mediated resistance would be to reduce the proportion of multiple and co-dependent infections substantially within cultivated crops. This should provide a great constraint on the possible emergence of viable recombinants.

It is also pertinent to consider that virus replication in highly resistant transgenic plants normally is several-fold lower than the levels of replication occurring in susceptible plants (Baulcombe 1996; Beachy 1993; Lomonosoff 1995; Scholthof et al. 1993; Wilson 1993). This factor alone should result in greatly reduced recombination frequencies compared with those observed in transgenic experiments in which active virus replication occurred. Moreover, pathogen-mediated resistance itself may also play a prominent role in eliminating possible recombinants resulting from capture of host transgenes by nonhomologous invading viruses. One mechanism that could be envisioned would operate through post-transcriptional transgene silencing (Baulcombe 1996; Lindbo et al. 1993; Mueller et al. 1995), which appears to function by activating endogenous host surveillance mechanisms for degradation of specific aberrant RNAs (Baulcombe 1996; Dougherty and Parks 1995; Goodwin et al. 1996; English et al. 1996; Sijen et al. 1996). Therefore, activation of host surveillance mechanisms that specify degradation of transgene sequences could provide a secondary protective benefit by selecting against emergence of novel recombinant virus strains. Indeed, recent evidence has been presented to suggest that transgene silencing contributes to natural host defense responses that attenuate acute virus infections to produce a chronic state during which virus replication is reduced substantially (Covey et al. 1997; Ratcliff et al. 1997; Al-Kaff et al. 1998).

Despite these arguments, a major quandary still exists as to whether and to what extent use of "pathogen-mediated resistance" genes will impact emergence of virulent viruses that could be harmful to agricultural productivity or to native flora. Because of the complexities of the host-pathogen-environmental interactions in nature, it is obvious that the impacts of various deleterious variants that might arise cannot be easily predicted by simple risk analysis experiments, particularly those arising from greenhouse or laboratory experimentation. Clearly, broad field experimentation involving widespread release of transgenic plants is needed to provide realistic assessments about the types of recombinants that might emerge, the frequency of emergence of novel viruses, and what steps can be taken to minimize the appearance of virulent recombinants. Genetically engineered cultivars with resistance against CMV and potyviruses have already been approved for production (Fuchs and Gonsalves 1997) and these plants now provide a resource for conducting such analyses. Other virus-resistant plants also are scheduled for release within the foreseeable future and these releases will provide additional examples to evaluate long-term agronomic or ecological consequences. In fact, considerable attention has already been focused on how to best conduct risk assessment analyses in greenhouse and field trials prior to and following release of cultivars (Fuchs and Gonsalves 1997; Kaniewski and Thomas 1998).

A number of genetic strategies are also being investigated to minimize the risk of transgene recombination. These include coat protein mutants designed to eliminate vector transmission

from recombinants expressing heterologous coat protein genes (Jacquet et al. 1998). Additional studies suggest that it may be possible to reduce transcript size substantially and still maintain effective gene silencing (Sijen et al. 1996; Pang et al. 1997). The results also suggest that the size of the targeted sequence can be reduced even further by constructing fusions of the targeted sequence to foreign genes (Pang et al. 1997). This raises the intriguing possibility that segments derived from several different viruses might function to target broad spectrum resistance against a number of different viruses. Such a strategy would involve transgenes that contain relatively small segments of nontranslatable viral genes fused to nonhomologous sequences in such a way as to minimize the frequency of recombinant viruses. It may also be possible to provide more durable resistance to a single virus by targeting multiple regions of the genome with chimeric transgenes derived by fusing segments from several different portions of the RNA.

In conclusion, the available results suggest that the high levels of recombination observed in our experiments are orders of magnitude higher than would be expected in resistant transgenic plants in which virus replication was curtailed. Nonhomologous recombination between incoming viruses and virus transgenes is also unlikely to provide a substantially increased contribution to virus evolution, particularly when one considers that such events already may occur naturally in plants infected with two or more viruses, or with co-dependent virus complexes. Mechanisms of resistance relying on homology dependent RNA silencing should also mediate active destruction of nonhomologous recombinant viruses that might arise. Moreover, as outlined above, it should be possible to reduce virus evolution resulting from capture of transgenes substantially by innovative design of pathogen-mediated resistance strategies to minimize the probability that recombination events will produce viable virus.

Our opinion is that the potential detrimental effects of accelerated virus evolution will be more than counterbalanced by benefits resulting from use of engineered sources of resistance. These benefits include reduced virus load in crops, decreased spread of viruses from and within resistant crops, reduced pesticide use for control of virus vectors, and general improvements in yield of produce and health of crop plants. Indeed, sources of pathogen-mediated resistance provide a valuable resource that should be particularly useful in conjunction with conventional breeding to pyramid alternative sources of resistance into cultivars. From our perspective, potentially adverse consequences that may arise from exacerbated virus evolution easily fall into an acceptable category, when one considers the positive benefits resulting from reducing virus load in field crops and the increased yield due to lower disease incidence. We further contend that these sources of resistance and numerous other biotechnological improvements affecting yield represent critical innovations that need to be implemented in order to increase food production to levels necessary to ease the transition of the exploding world human population to more sustainable levels.

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