TRANSGENIC VIRUS-RESISTANCE PLANTS AND NEW PLANT VIRUSES

Many genes derived from plant RNA viruses expressed in transgenic plants confer resistance against infection by viruses that were the original source of the genes and in some cases, by other viruses as well. As of March 17, 1995, over 240 field tests of virus-resistant transgenic plants had been conducted in the United States. Recently, the first virus-resistant transgenic plant (ZW 20 squash developed by Asgrow Seed Company) was approved by the U.S. Department of Agriculture (USDA) for widespread commercial production. Other virus-resistant plants are likely to be commercialized by the turn of the century. Although the use of viral genes for resistance to plant viruses has many potential benefits, some risk issues associated with the use of these genes have been raised.

The principal issues are associated with the possibility that recombination between an infecting plant RNA virus and a viral RNA produced from a transgene will result in a new, problematic virus. Additional issues include potential synergism, transcapsidation, and other interactions that might lead to new or more severe disease problems.

To address these issues, the USDA's Animal and Plant Health Inspection Service (APHIS) and the American Institute of Biological Sciences (AIBS) convened a two-day work-shop (appendix A) on April 20-21, 1995, in Beltsville, Maryland. Workshop participants included virologists and others with expertise in molecular biology and plant pathology (appendix B). Support was provided by APHIS and by the Biotechnology Industry Organization.

With assistance from the program committee (appendix C), APHIS developed a list of 18 questions related to the potential risks of transgenic virus resistance. The questions were categorized as follows: Recombination and Mixed Infections; Transgenes; Experiments; and Benefits and Post-Commercialization Monitoring. To provide a context for answering the questions, formal presentations were given by leading virologists on resistance mediated by viral coat proteins, movement proteins, replicases, and RNA, as well as presentations on transgenic comple-mentation, recombination, synergism, and replication. Issues concerning the potential effect of gene flow of transgenes to wild plant populations were not addressed.

After the formal presentations, the workshop participants broke into five groups according to their interests. There were two groups for coat protein, one group for replicase, one group for movement protein, and one group to cover other viral genes. Each group was asked to address the 18 questions in terms of their group's gene assignment.

SUMMARY REPORT OF BREAKOUT SESSION DISCUSSIONS

Even though each group had a specific gene assignment, discussions tended to focus on the likelihood that a recombination event would result in a new, viable, and

competitive virus. Since many of the principles of recombination are not gene specific, much of the groups' discussions were not specific to any particular gene. Therefore, the following summary is not organized on a gene-specific basis. If the groups identified information related to particular viral genes, this infor-mation was included in the summary text.

Unfortunately, this summary does not comprise a comprehensive record of all the ideas discussed during the workshop. Rather, it reflects the discussion of the final plenary session and attempts to document key points made during the breakout group discussion periods.

RECOMBINATION AND MIXED INFECTIONS

1) Based on current data, do certain viral taxa appear to have the propensity to recombine? List them in order from least likely to most likely. What is the basis of the order (analysis of related sequences, RNA structural motifs, etc.)?

For the purposes of this summary, a viral taxon is defined as a virus group, such as the tobamoviruses, which shares common particle morphology, size, and genome organization and expression. Most workshop participants stressed that our base of knowledge on recombination and the generation of new plant viruses is limited, but growing rapidly.

Recombination between Viral Taxa

Current theories of modular evolution (i.e., specific nucleic acid sequences or functional domains within genes can recombine to form active genes) indicate that recombination between and within viral taxa has occurred over the course of evolutionary time to shape the genomes of modern plant RNA viruses. However, sequence comparisons of viral genomes, as well as current experimental data, indicate that recombination resulting in competitive viruses does not readily (e.g., growing season to growing season or year to year) occur between viral RNAs of different taxa or between viruses and RNAs of either plant or viral transgene origin.

Computer-assisted sequence comparisons indicate that RNA recombination between members of different viral taxa might have played a role in shaping the genomes of luteo, tobra, and potyviruses. However, considering the large number of plant viral RNA sequences that have been analyzed over the past several years, recognizable inter-taxa recombination events that resulted in new viruses are thought to be very rare. This argues for the integrity of viral taxa; viral genes from different taxa are not readily exchanged.

Even though certain gene products, such as movement proteins, can complement across viral taxa, attempts to construct viable recombinant viruses with chimeric movement protein genes from different taxa have been unsuccessful. However, viable recombinant viruses have been produced when a movement gene from one taxon is substituted with a movement gene from a different taxon (e.g., the 3a gene from cowpea chlorotic mottle virus substituted with the 30-kDa gene from sunn-hemp mosaic virus). Recombination involving viral replicase genes is unlikely to produce viable virus, since replicases appear to be

highly template specific for their homologous virus sequence. Coat protein from one viral taxon has been shown to encapsidate the nucleic acid of another viral taxon. However, the resulting chimeric virions generally are less stable structurally than the native virions and therefore less able to withstand the rigors of infection and pathogenesis.

Therefore, the importance of recombination between different viral taxa must be placed in the proper perspective. There is no evidence to support the notion that frequent recombination events occur between taxa resulting in viable virus from growing season to growing season. However, limited evidence does suggest that in an evolutionary time frame, recombination events between viral taxa have resulted in the generation of new plant viruses and virus strains.

Recombination between Viruses of the Same Taxon

While the data indicate a lack of recombination between viral taxa, a growing body of evidence indicates that recombination between viruses in the same taxon may be common. Under both field and laboratory conditions, and with varying degrees of selection pressure, virologists have been able to detect recombination between members of the bromo, carmo, diantho, tombus, tobra, and alfalfa mosaic viruses. Since the scientific literature only focuses on a few viral taxa, virologists are at the very beginning of recombination studies. The lack of literature documenting recombination in a particular taxon does not mean that it does not occur. Indeed, recombination may be occurring but going undetected because the recombinants may be noncompetitive (relative to the wild-type or parent virus) or nonviable. Some taxa show little genetic diversity; others are very diverse. It is unknown whether this diversity is due to recombination or some other mechanism of genetic diversification, such as nucleotide mutations and deletions.

2) Based on current data from virology and molecular evolution, what are the characteristics of RNA sequences that recombine?

There is no evidence to support RNA/RNA recombination through nucleic acid cleavage and ligation events. Rather, data support the hypothesis that recombinants arise through replication events that result in the viral replicase switching from one template to another, forming a hybrid nucleic acid molecule. This is termed copy-choice switching.

It is not yet known what characteristics of viral RNAs (including RNAs produced by transgenes) affect the rates of recombination through copy-choice switching. However, some evidence indicates that several factors may be involved. For the bromo-viruses and turnip crinkle virus, recombination appears to be associated with RNA replication signals either in the form of unique sequences or secondary structure, which might promote copy-choice template switching. Other characteristics might include sequence similarity, sequence complementarity (local), and sequence length. In particular, shared sequences (e.g., the 5' and 3' untranslated regions of some multipartite viruses) or shared structural motifs may be sites for recombination. In fact, specific sequences or regions may be

hot spots for recombination, but these are not well characterized.

The concentration of RNA or its rate of replication in the cell also may play a role in the frequency of recombination. For example, the greater the concentration of RNA, the greater the probability that it can serve as a template in copy-choice switching. Similarly, the higher the rate of replication, the higher the probability of a replicase undergoing copy- choice switching. Host plants also exert pressure on the rate of recombination. For example, some plants tend to promote the development of defective interfering particles, whereas others plants apparently do not.

3) What data are available on the frequency of mixed viral infections in crops? What sources are available to determine the frequency of mixed viral infections in commercial crops? How reliable are these sources?

Mixed infections are common and can be expected at many field sites. This is especially true in areas where a diversity of vectors is common (aphids, beetles, thrips, etc.) or where a single vector may carry many different types of viruses. Virologists in California frequently have observed multiple viruses naturally infecting vegetable crops; there are cases where five or more viruses have been observed in a single plant. Mixed infections are very common in perennial crops. Fruit trees, such as apple, pear, and cherry, are routinely infected with multiple viruses. All in all, the scientific literature is replete with reports of mixed infections, but this information has not been collected into a single database.

The various techniques field virologists use to identify viruses include serology (ELISA, RIA, etc.); electron microscopy (SSEM, leaf dips, and identification of inclusion bodies); nucleic acid homology (dot blots and PCR); and host range determinations. All of these techniques are reliable when used properly with suitable controls.

TRANSGENES

4) Is there a difference in the rate of recombination in virus-resistant plants expressing a virus transgene compared to plants that express a virus transgene but are not virus resistant or compared to plants infected with multiple viruses?

There are not many data available to answer this question with certainty. However, a few predictions can be made.

Recombination between Transgene Trans-cript and Viral RNA to Which the Transgene Confers Resistance

The likelihood of recombination between a transgene transcript and an infecting virus will not be great if the plants truly resist infection. There would likely not be enough RNA from the infecting virus present in the cell to make recombination with the transgene transcript a probable event. Recombination becomes even more unlikely if the size of the transcript is reduced and its sequence modified to eliminate signals or hot spots for recombination. Nevertheless, resistance-breaking strains of the virus might be able to establish successful infections and produce sufficient levels of RNA to increase the probability of recombination.

Recombination between Transgene

Trans-cript and Viral RNA to Which the Transgene Does Not Confer Resistance

The rate of recombination between a transgene transcript and viral RNA that can successfully replicate in the plant will depend on many factors, including the concentration of the two RNAs and other characteristics (e.g., the structure of the viral genome, nature of the viral replicases, etc.) that enhance the frequency of recombination. Currently there are no data to predict at what recombination frequency the probability of generating a new plant virus or a viable recombinant becomes sufficiently high to make the risk associated with the use of a transgenic plant unacceptable.

Comparison of Recombination Frequencies in Plants Having Mixed Infections with Those Expressing a Viral Transgene

Is the frequency of recombination between viruses in a mixed infection different from the frequency expected between a virus and a transgene transcript? This question is often asked in relation to the relative risk of using viral transgene-induced resistance. Unfor-tunately, the answer remains unknown, because the recombination rate between viruses in mixed infections is unknown. Therefore, there is currently no way of determining how this rate compares with recombination rates in transgenic plants.

Experiments are needed to answer a number of questions that relate to the spatial and temporal characteristics of mixed infections, including the following: Are all cells infected with both viruses? Are the replication cycles of the viruses synchronized in such a way that copy-choice switching is likely? Are replicating viral RNAs compartmentalized in an infected cell? (In other words, if two viruses do infect the same cell, do they interact sufficiently to make recombination likely?) Do recombination rates differ between viruses in the same taxon compared with viruses in different taxa? Is the rate of recombination that results in a viable virus higher between viruses in the same taxon compared with viruses in different taxa? Sequence information obtained from viral RNA genomes indicates that inter-taxa recombination that results in a viable competitive virus is rare in anything other than an evolutionary time frame.

5) How do plant mechanisms, such as co-suppression, that alter the expression of transgenes affect the risk of recombination between an infecting virus and viral transgene?

Co-(or sense) suppression is a phenomenon observed in plants in which the expression of an exogenous transgene results in the suppression of both the endogenous and exogenous gene. This suppression appears to be a post-transcriptional event that likely occurs in the cytoplasm. It has been suggested that RNA-mediated resistance is a form of sense suppression. Since this process is thought to result in decreased levels of transgene transcripts, the probability of recombination should also be reduced.

6) Is the expression level of both the protein and the RNA of transgenes important with respect to recombination or transcap-sidation? Is the expression level or accum-

ulation more important? Why? Should the expression of protein and RNA be determined in transgenic crops before their com-mercialization?

Compared with plant virus infections, transgenes are expressed at very low levels. The implications of these low expression levels for recombination are not clear. Even assuming that the higher the concentration of transgene RNA the greater the chance for recombination, we do not yet know what comprises a meaningful range; what are low and high concentrations of transgene transcript relative to unacceptable recombination rates? Even though most workshop participants believed that determining the concentration of transgene RNA is important to the overall characterization of the transgenic plant, the group was split on whether this information should be required before commercialization can proceed. Similarly, workshop participants were split on whether commercialization should await the determination of concentrations of transgene-derived proteins, such as coat protein. Currently, this information is of no use to regulatory agencies because there in no way to factor con-centrations of RNA or protein into risk determination in a meaningful manner. However, good scientific practice and curiosity would demand the information.

Certain viral gene products produced from transgenes, such as coat proteins and movement proteins, might have the potential to alter the pathology of an invading virus within the infected transgenic plant (altered insect transmission due to transcapsidation, altered cell-to-cell or long-distance movement within the plant) without altering the genomic make-up of the invading virus. Such possibilities are a concern to seed producers and growers because these viral gene constructs would result in an unsuccessful cultivar, if released. Since the genome of the invading virus is not changed, long-term environmental impacts would not be likely. Therefore, even though the evaluation of likely *trans* complementation interactions should be done by developers of new transgenic cultivars, regulatory agencies should not require these types of data before commercialization is approved.

7) Is type of cell or subcellular location of transgene expression important? Is it a concern if the transgene is expressed in a cell that it is not expressed in during viral infection?

In cases where a plant is a systemic host (virus can be found in all cell types) for both the challenge virus and the virus from which the transgene was derived, the cellular location of the transgene is not an issue. The use of transgenic resistance opens the possibility of new interactions between tissue-specific viruses and other viruses. For example, if a coat protein transgene from a phloem-restricted virus is used for resistance, this would increase the probability of new interactions between the transgene transcript or its gene product and other viruses that replicate only in nonphloem tissues (e.g., subliminally infecting viruses). This new interaction might result in modified movement properties, symptoms, or insect transmission of the infecting virus. However, unless a recombination event between the transgene transcript and the virus were to occur and result in a viable competitive recombinant (see above for discussion of the probability

of recombination and selection), the effect would be temporary and restricted to the given plant, field, or crop expressing the transgene.

8) Are there ways in which transgenes can be manipulated to minimize their ability to recombine?

There are two potentially effective strategies to minimize the risk of recombination. The first strategy would be to design transgenes that express RNA that has a reduced ability to recombine by altering or deleting the *cis* elements required for replication. This might be done by avoiding transgenes that contain 5' and 3' ends of viral RNAs (genomic and subgenomic RNAs). However, it is important to note that all the rules governing this type of recombination are not well defined. The second strategy would be to select transgenic plants that express the desired level of viral resistance and contain minimal amounts of the transgene RNA. Lowering the concentration of the RNA should reduce the probability of recombination. Even though exact threshold levels for RNA concentration are not defined, one may assume that the lower the concentration, the lower the probability of recombination.

In resistance strategies not requiring an active viral protein, such as RNA-mediated resistance, risks associated with recombination can also be reduced if the transgene is constructed to contain several translational stop codons, which would render the transcript untranslatable. In protein-based resistance strategies, mutating the transgene to produce a defective protein that still imparts resistance might be another approach to reduce the risks of recombination. However, using altered forms of viral proteins may trigger Food and Drug Administration regulations under the Federal Food Drug and Cosmetic Act, since this protein might be considered new to the diet of consumers, although this should not be a problem with translocations or minor alterations of the gene sequence.

9) Some scientists have said that interactions between two viruses are rare because the replication complexes are compartmentalized. However, it is unknown whether the RNA of viral transgenes is similarly compart-mentalized. Is compartmentalization of trans-gene RNA important with respect to recombination? Can the compartment-alization of transgene RNA be determined? What is known about compartmentalization of two similar viruses or two dissimilar viruses in the same plant?

Very little is known about the compart-mentalization of replication complexes with most virus infections. Still, evidence exists that compartmentalization does occur with some viruses, including tombusviruses, tobraviruses, and turnip yellow mosaic virus. However, evidence does not indicate that compartmentalized replication sites exclude host RNA or RNA from other viruses. The assumption is that the propensity for a transgene RNA to recombine with another RNA depends on the two RNA molecules occupying the same subcellular location. Therefore, the subcellular location of the RNA is important. If the risks associated with recombination of a particular transgene RNA and another RNA are viewed as sufficiently high, it might be possible in the future to direct the subcellular location of the transgenic RNA. However, current data do

not indicate a pressing need to make the development of this technology for subcellular RNA localization a priority for risk reduction purposes. Cell biologists and virologists currently have the capability to study RNA trafficking in virus-infected cells, but these experiments are very demanding technically.

10) Will wide-scale deployment of transgenic virus-resistant plants increase the potential risk of formation of a new virus? The standard risk assessment equation states risk = hazard X frequency (or exposure). Some scientists have said that the frequency value will increase with the use of transgenic plants because all plants will contain and express the transgene every year. With virus-infected plants, the number of plants infected may vary widely from year to year. If a new virus appears via recombination, how likely is it that recombination results in a virus with increased virulence or expanded host range?

There was no clear consensus among workshop participants on whether wide-scale use of transgenic plants expressing viral transgenes would increase the potential risk for formation of new viruses. Variables such as type of crop, cropping system, and environment might need to be considered. For example, assuming that the widely prevalent potato leaf roll virus (PLRV) undergoes recombination in naturally infected plants, recombination frequencies between PLRV transgene RNAs and other viral RNAs may actually be reduced in transgenic plants compared with recombination frequencies in PLRV-susceptible nontransgenic plants. The overall amount of PLRV RNA available for recombination might be less in transgenic plants compared with the amount found in a field of PLRV-infected potatoes.

Since every plant in a field of transgenic plants would uniformly produce viral sequences, the recombination frequency between viral transgene RNA and viral RNA from a nonrelated virus would probably increase with wide-scale use of transgenic plants compared with the recombination frequency between the chance occurrence of two unrelated viruses in a mixed infection. However, based on obser-vations of natural mixed infections, RNA sequence data, and laboratory experiments designed to select for recombinants, the likelihood that a viable, competitive virus with altered properties, such as host range, movement, or virulence, will result from this type of recombination is thought to be low even if the frequency of the event is increased greatly. Because transgenic plants resist infection, the frequency of recombination events between viral transgene RNA and viral RNA from the homologous virus or closely related viruses may actually decrease with the use of transgenic plants compared with nontransgenic plants. This should lower the probability of sufficient numbers of RNA/RNA interactions to generate a viable new virus.

Many workshop participants pointed out that this is actually a population biology question. As the level of use of viral transgenes increases in plants, the greater the probability of a recombination event that might result in a new, deleterious virus also increases. Therefore, the question that needs to be asked is: At what level of use does the risk associated with recombination in transgenic plants outweigh the agricultural and environmental benefits of resistance?

11) Is genomic masking/phenotypic mixing important biologically for certain taxa? Assume that the coat protein gene engineered into a transgenic plant is from a virus strain that is widely prevalent in that crop in the United States. Does this minimize the risks associated with genomic masking/phenotypic mixing? Are there additional risks if the coat protein gene was derived from a virus not present in the United States, but from the same host plant? Or what if the coat protein gene was from a viral strain commonly isolated from different host plants?

Coat proteins produced by one virus in an infected plant or by translation of coat protein transgene RNA can partially (phenotypic mixing) or entirely (genome masking) encapsidate the RNA of a second virus that occurs in the same plant. Both of these phenomena are referred to as trans-capsidation. With transcapsidation, any biological property that can be attributed to the coat protein of the first virus, such as insect and seed transmission, might temporarily be imparted to the second virus; however, since the genome has not been changed, these new properties are not transmitted to subsequent generations of the second virus. Genome masking/phenotypic mixing has been documented to be important in the biology of some viruses, such as the luteoviruses. However, it is generally seen as an artifact of mixed infections rather than a biologically significant phenomenon. This is true even if the engineered coat protein is new to the United States or to the crop.

The temporary nature of these events indicates that they pose little, if any, long-term risk. However, the relative impact of trans-capsidation in transgenic plants might be greater than in nontransgenic plants. A uniform field of plants producing coat protein increases the probability that a virus that is new to that crop (not a new virus) can replicate and spread from plant to plant or field to field. The chances then become greater that the virus will encounter another plant containing the necessary coat protein that can transcapsidate the viral genome, allowing the virus to continue to be transmitted. However, for transcapsidation to have an impact on the spread of a new viral disease, the only factor limiting the successful infection of the new host with the virus must be the lack of an appropriate insect vector or another characteristic that can be complemented by the coat protein being generated by the transgene RNA. Most likely, however, numerous other factors limit the ability of a virus to successfully infect a new host (e.g., lack of proper movement functions, etc.).

Alternatively, the potential short-term risks associated with transcapsidation might be lower with large-scale use of transgenic plants compared with the risks associated with transcapsidation as a result of natural mixed infections. The concentration of transgene-derived coat protein in transgenic plants is much lower than in plants infected naturally by viruses. Therefore, in transgenic plants there might be less chance of transcapsidation than would occur in mixed infections.

12) If viral synergism has been documented between two viruses in a crop plant and a transgenic plant expressing a gene from one of them is developed, should the interaction of the transgene with the other virus be evaluated? How?

It was the consensus of the workshop participants that if synergy is known to occur between two viruses and one of them has sequences expressed in transgenic plants, the transgenic plant should be challenged with the second virus to determine if a synergistic-like interaction occurs. However, most parti-cipants felt that this was a quality issue that should be a concern only to plant breeders and seed producers, since there is no long-term agricultural impact because the plant would not be commercially competitive. The broader environmental impacts of synergism were also thought to be minimal by most participants, although they merit further evaluation. For example, if a viral transgene was introgressed into a population of wild relatives (e.g., from sunflowers to wild relatives), would potential synergistic interactions between the transgene and viruses that infect the wild relative have a significant impact (negative or positive) on the population of wild relatives? Gene flow issues were not addressed by the workshop participants.

EXPERIMENTS

13) Experiments in which recombination has been detected between a transgene and a virus have been performed under some level of selection pressure. Define high, moderate, and low selection pressure as it relates to these types of experiments. How do "natural" conditions relate to these levels? How often does recombination happen between a virus and a transgene? In what time frame?

For all breakout groups except coat protein: If in your opinion recombination is unlikely to occur in the above situation in a small, contained field test (less than 10 acres), would you support the addition of noncapsid viral genes to the notification process for field testing under APHIS oversight?

The definition of levels of selection pressure and comparison with natural levels is an important concept, because this will allow for better extrapolation from laboratory recombination studies to what might happen in the field. High selection pressure is defined as conditions that favor the recombinant virus, for example, a situation where the virus is not viable unless a recombination event occurs. Low selection pressure would be a situation where the novel phenotype does not confer a competitive advantage to the recombinant under the conditions of the experiment. Natural conditions can encompass both high and low selection pressures; however, in nature selection will normally be against the recombinant (assuming the recombinant is viable) since it is often less adapted to the environment. Although the frequency of such recombination resulting in a viable virus is unknown, it appears to be low based on anecdotal evidence and studies that have been conducted to date.

The time frame for recombination between a virus and a transgene RNA, if it occurs, is not fixed. Recombination might occur in the first cell the virus infects, or it might occur after several weeks of infection, if at all. Moreover, because the probability of generating viable competitive virus through recombination in nature is subject to all the evolutionary forces that shape all living organisms, a more suitable time frame for meaningful recombination events might be years, decades, or centuries.

The workshop participants agreed that APHIS should expand the list of viral genes that should be covered under their notification procedures for field tests under 10 acres. The participants agreed that the risk considerations for coat protein (currently on the list for notification) are the same as those for other known viral genes, and therefore the list should be expanded. The one qualification to this was that native movement proteins should not be added to the list because there is a high probability that these proteins will be active in *trans* and affect the distribution of other plant viruses within the plant.

14) Design an experiment where the recombination rate between two viruses (or two strains) is to be determined under low selection pressure? Under moderate selection? Under high selection pressure? Could this experiment be performed in growth chambers, in greenhouses, or in the field? How many plants might be needed? Which viruses, strains, or taxa might be candidates for evaluation?

Experiments Under High Selection Pressure

An example of an experiment to determine the frequency of recombination under high selection pressure could be carried out using tomato mosaic virus (ToMV) and ToMV-resistant tomatoes (those containing the *Tm1* resistance gene). In this system, a mutation in the 126K open reading frame of the ToMV genome allows this virus to overcome *Tm1* resistance. Therefore, *Tm1* tomato plants expressing the mutant form of the 126K gene could be challenge-inoculated with wild-type ToMV. If the plants become systemically infected, this would indicate that the mutated form of the 126K transgene RNA recombined with the wild-type gene contained in the infecting virus. In order for this experiment to be successful, the mutated 126K transgene must not confer resistance to ToMV infection and the 126K transgene must not act in *trans* to overcome the *Tm1* resistance gene. If *trans* complementation is a problem with the intact gene, attempts can be made to only use that portion of the 126K gene that overcomes *Tm1* resistance.

Experiments Under Low Selection Pressure

Experiments conducted under low selection pressure are more relevant than those conducted under high selection pressure for the assessment of risk associated with recombination of transgene RNA and viral RNA. This is because low selection pressure experiments more closely reflect field conditions. However, these experiments are more difficult technically because the recombinants will rarely have a competitive advantage over the infecting wild-type virus. Therefore, recombinants must be detected through the use of molecular markers such as RNA sequence, or biological markers such as infection of indicator plants.

Virologists can also use the same ToMV/*Tm1* system described above to conduct low selection pressure experiments. For example, use of tomato plants that do not have the *Tm1* resistance phenotype and that express the 126K mutant would allow for wild-type ToMV infection. At various intervals (days, weeks, months, etc.) progeny virus can be harvested from these plants and used to inoculate tomato plants containing the *Tm1* resistance gene. Only recombinants containing the 126K mutation could

successfully infect these plants. However, proper controls will be crucial for this experiment to be meaningful; for example, determining the natural rate of formation of *Tm1* resistance-breaking strains under these experimental conditions.

An important question is the relative rate of recombination in mixed infections compared with recombination between viral transgenes and intact replicating viral RNA. To assess the potential recombination rates between two viruses (or strains) in a mixed infection, suitable biological markers, such as divergent host ranges, could be identified that would allow for the selection of recombinants after a set period of time of infection in a common host. For example, progeny of a mixed infection of any two viruses could be inoculated to a wide range of indicator plants to look for phenotypic variants (host range/symptoms) that might indicate recombination between the two original viruses. Variants can be isolated and sequenced to determine if recombination occurred.

Workshop participants proposed field experiments that would assess the rate of mutation, transcapsidation, or recombination between two isolates of papaya ringspot virus (PRV) with the selectable markers being aphid transmissibility and host range. These experiments would use two isolates of PRV: PRV-w, which infects cucumber and not papaya; and PRV-p, which infects both cucumber and papaya. Both the w and p forms of the virus are aphid transmissible. PRV-HA 5-1 (a coat protein mutant) is a non-aphid transmissible isolate of PRV-p. First, to assess background mutation rate from PRV-w to PRV-p, PRV-w-infected cucumber would be co-planted with papaya. Viruses from infected papaya would be sequenced to determine if PRV-w mutated to a p type and jumped host range from cucumber to papaya. Second, to assess the potential impact of transcapsidation or recombination, PRV-HA 5-1-infected transgenic cucumber expressing the coat protein gene of PRV-w would be co-planted with uninfected papaya. Papaya plants could then be monitored to determine if non-aphid transmissible PRV-HA 5-1 was transmitted to papaya and whether its transmission was mediated through mutation, transcapsidation of PRV-HA 5-1 RNA with PRV-w coat protein, or recombination between PRV-HA 5-1 RNA and PRV-w transgene RNA. With appropriate controls, these experiments should provide an assessment of frequency, and therefore potential risk, associated with mutation, transcapsidation, and recombination.

A physical approach that is independent of virus phenotype would be to simply perform PCR on viral RNAs from mixed infections or from infected plants containing viral transgenes. PCR primers would be specific to each viral sequence: primers to each virus in a mixed infection or primers to the virus and to the transgene sequence. PCR products would be obtained only if recombinants were present. To quantify this, additional primer pairs would be used in which each pair amplifies a nonrecombinant form of one of the parent viruses or transgene RNA sequence. The ratio of the products that require recombination for PCR to work to those that do not would give an estimate of the recombination frequency. One can also sequence hundreds or even thousands of independent cDNA clones for viruses from a mixed infection or from transgenic plants infected with a virus. The

ratio of recombinants to nonrecombinants would provide a direct measure of the recombination frequency.

15) Given that funding for risk assessment research is limited and assuming that the experiments described are equally feasible, which one do you think would be most informative and relevant to the issue of commercialization of virus-resistant transgenic crops?

There was a consensus that experiments that assess recombination frequencies under low or zero selection pressure would be the most relevant to what might be expected under field conditions. Experiments should be conducted with mixed infections as well as with transgenic plants that exhibit virus resistance.

Caution was raised at the meeting that even though these experiments might be scientifically interesting, they might not truly reflect the risks (or lack of risks) associated with large-scale plantings of transgenic crops. When the probability of new virus formation is assessed in terms of population biology and ecology rather than molecular biology, recombination frequencies only present one small part of the risk assessment picture. Factors such as viability, stability, fitness, competition (both within the plant and from cropping season to cropping season), and virulence play dominant roles in assessing potential risk.

While experiments conducted at low selection pressure are important, experiments that provide a clearer picture of how recombination occurs between viruses and between a virus and a transgene RNA are also important. Data gained through these experiments will provide important clues to how the risks associated with recombination can be minimized, if necessary.

A few workshop participants suggested that the best option for the USDA Risk Assessment Program would be to focus on recombination risks associated only with large-scale (commercial-scale) use of transgenic plants, since experiments conducted on a smaller scale or in the laboratory are difficult to relate to large-scale use. Small-scale experiments will either overestimate or underestimate the level of risk associated with recombination. It was suggested that USDA support a major 2- to 3-year effort on a comprehensive survey of transgenic plants once they are commercially deployed. This should be an exhaustive study with proper controls (nontransgenic plants). It should be undertaken by a team of scientists led by a plant pathologist and including molecular biologists, statisticians, population biologists, ecologists, and epidemiologists.

BENEFITS AND POST-COMMERCIALIZATION MONITORING

16) What are the expected potential benefits of using transgenic plants?

Since the expected potential benefits of transgenic virus resistance have been discussed in other reports, they are not addressed in detail here. However, the following potential benefits were identified by the workshop participants: increased yield, reduced pesticide use to control vectors, improved crop quality, increased potential for multiple virus resistance traits, and decreased seed certification costs. In addition, viral transgenes may be the sole

source of resistance for some viruses.

17) When a transgenic virus-resistant plant becomes available on the market, would monitoring of the plants for the generation of a new virus derived from the transgene be feasible? Is current disease monitoring for new viruses adequate to detect a new virus? Are there effective measures currently available to minimize the impact of the occurrence of a new virus on agriculture?

Regular monitoring of all transgenic plants for the production of new plant viruses would not be feasible. Only recombinants that result in new diseases or more severe forms of existing diseases are important from an economic perspective, and these would be readily detected by growers and seed producers. Recombination events that result in new viruses that produce no new symptoms or disease (in economically important plants or native plants) are only important from an evolutionary perspective. However, data suggest that the frequency of occurrence of these new viruses resulting from recombination events in transgenic plants will be low and may not be significantly different from the frequency experienced in mixed infections or genetic variability that occurs naturally within a given population of virions in an infected plant or field of infected plants. However, more research will provide a better understanding of the significance of recombination events in transgenic plants and in mixed infections. With or without the use of transgenic plants, new plant virus diseases will develop that will require attention. No technology is risk free; a determination will need to be made whether the benefits associated with the use of transgenic virus resistance are greater than the risks.

18) Given that many different types of genes may be used as sources of resistance, are certain genes of more concern than others? Certain viruses? Why?

In general, insufficient data exist to support generic statements about the relative level of concern associated with the use of any particular viral transgene in wide-scale commercial plantings. More study is needed; each gene/plant/environment combination will present a different set of issues requiring varying degrees of analysis. However, the use of transgenes for wild-type movement proteins to confer resistance is of greater concern than other viral genes products. Data indicate that

movement proteins have a high probability of acting in *trans* and thereby altering the movement patterns and, potentially, the host range of infecting viruses. In addition, transgenes from exotic viruses (viruses not known to occur in a given geographical area) also present a heightened level of potential risk. Although it is equally unlikely that a viable virus might result from recombination between an indigenous virus RNA and an exotic or nonexotic viral transgene RNA, the progeny containing the exotic sequences might represent a greater hazard as those viruses may not have had previous opportunities to recombine during mixed infections.

ABSTRACTS OF PRESENTATIONS

Rebecca Grumet
Michigan State University

Coat-Protein-Mediated Virus Resistance in Plants

In 1986, Powell-Abel et al. (30) produced transgenic tobacco plants expressing the coat protein (CP) gene of tobacco mosaic virus (TMV) and found that they were more resistant to infection by TMV than were the nontransgenic controls. This initial demonstration of the feasibility of CP-mediated protection was followed by a host of examples; by the end of 1994, there were more than 50 published reports of genetically engineered CP-mediated plant virus resistance in various systems. The different viruses for which resistance has been demonstrated represent at least 13 different groups and include positive sense, negative sense, single- and double-stranded RNA viruses and at least one DNA virus.

The type and extent of protection conferred by a given coat protein gene is variable depending on the type of virus and the individual transgenic line. There even can be variation among different individuals or families derived from the same line and possessing the same gene at the same location in the genome (e.g., 34). In general, upon infection with the virus from which the gene was derived, the inoculated leaves of the transgenic plants show fewer viral lesions that do control plants, and/or systemic spread of infection is prevented, delayed, or reduced. There are also examples where plants transformed with a coat protein gene initially become infected and then later recover (e.g, 7, 16). In the majority of cases, virus accumulation is reduced or absent.

The theoretical basis for the use of coat protein genes as possible virus resistance genes originally came form two directions, classical cross protection and pathogen-derived resistance. In classical cross protection, it is possible to protect a plant from the effects of infection by a severe virus by preinoculating the plant with a mild strain or mutant of the virus (5, 29). Although the mechanism of cross protection is still not understood clearly, one hypothesis suggests that the coat protein of the first virus interferes with an early stage in the life cycle of the second virus, such as attachment, entry, or uncoating. Pathogen-derived resistance, states that it should be possible to disrupt the normal pathogenic cycle by causing the host to express a pathogen gene at the wrong time, in the wrong amount, or in a counterfunctional form (33). Native or altered viral-derived genes might be used to interfere with various stages in the viral life cycle such as uncoating, translation, replication, cell-to-cell or long-distance movement, or vector-mediated transmission.

In the case of coat proteins, which have many roles in the life cycle of the virus, there is good evidence to indicate that the mechanism of protection is not the same in every virus-CP-host combination. One feature of CP-mediated resistance that might reflect mechanism is the relationship between the extent of protection and the level of CP expression. One might predict that higher levels of CP would result in greater protection, and in several experiments the level of protection observed was directly correlated to the amount of CP present [e.g., alfalfa mosaic virus (AlMV) 10, 17; potato virus X (PVX) 9, 11; rice stripe virus, 8]. For AlMV and TMV,

transformation with translationally defective CP genes that could produce RNA but not protein, showed that it was the coat protein molecule and not the coat protein mRNA that conferred resistance (31, 43). In several systems, however, particularly for members of the potyvirus, luteovirus, and tospovirus groups, the protection conferred by the CP gene appears to be partially or completely due to the viral-derived RNA rather than the CP. This type of resistance is not within the scope of this summary and will be discussed in a later section by Dr. W. Dougherty. In some cases, such as tomato spotted wilt virus (TSWV) both RNA and protein may be involved in conferring protection (27, 39).

For TMV and AlMV there is good evidence to suggest that interference with uncoating is a key component to the protection. If transgenic TMV- or AlMV-CP expressing plants or TMV CP expressing protoplasts are inoculated with whole virions they are protected against infection, but if they are inoculated with naked viral RNA, they are not protected (24, 42). On the other hand, transgenic plants expressing the coat proteins of some potex-, carla-, and nepoviruses (1, 2, 9, 19) were protected against infection even when inoculated with viral RNA. In these cases some step other than, or in addition to, interference with uncoating must be affected. In the case of cucumber mosaic virus (CMV), whole plants were protected against systemic infection by both virions and RNA, but protoplasts were protected only against virions and not against RNA (25). These observations suggest that more than one mechanism may be operating, one at the cellular level likely to involve interference with uncoating, and another at the cell-to-cell movement or whole plant level. Similarly, Wisniewski et al. (44) found limited spread of TMV after initial infection with RNA. Interestingly, with AlMV there are transgenic lines that are not protected against RNA, but there are other lines that are protected against RNA (36), again suggesting that more than one mechanism may be involved.

Several hypotheses for the mechanism of CP action, such as interference with uncoating, translation, or replication, depend on interaction between the transgene-expressed CP and the viral RNA. There are some instances, however, where the ability to interact with the RNA may not be sufficient to confer resistance. Although the CPs of two strains of tobacco rattle virus (TRV) are capable of reciprocal encapsidation, the CPs only confer protection against the homologous strain (41). Other experiments suggest that an amino terminal portion of the protein that is not essential for viral assembly is critical for CP-mediated protection; possibly via interaction with a host factor. Although changing the second amino acid of the AlMV CP did not alter the ability of the CP to bind AlMV RNA in vitro, the mutant CP no longer conferred resistance to infection (38). Further, although binding and assembly functions of potyviruses are thought to reside in the trypsin-resistant core portion of the CP, transgenic melon plants expressing only the core of the zucchini yellow mosaic virus (ZYMV) CP gene were only partially resistant to ZYMV infection, whereas those expressing the full length CP were completely resistant (4). Similarly, removal of the amino terminus of the CP of the potexvirus potato acuba mosaic virus eliminated the ability to confer protection against virus infection, while on the other hand, mutation in a domain thought to be essential for viral assembly did not reduce the level of resistance observed relative to the full length CP (15).

It should be noted than in many cases it has been possible to overcome the CP-mediated resistance with increasing concentrations of viral inocula [e.g., TMV, 30; AlMV, 37; soybean mosaic virus, 35; TSWV, 18; watermelon mosaic virus (WMV) 22]. On the other hand, comparable levels of resistance were observed over a range of inoculum levels for several viruses including CMV (3), potato leaf roll virus (12), potato virus S (19, 20), and arabis mosaic virus (1). From an applied point of view, greater levels of protection would be most valuable, but perhaps the most critical factor is whether the levels of resistance are sufficient for inoculum concentrations likely to be encountered in the field via vector mediated transmission.

Where it has been tested, CP-mediated protection has been effective against insect-vectored [aphid (6, 12, 14, 32, 40), leafhopper (8) and whitefly (13)] transmission. In contrast, the CP of TRV conferred protection against rub inoculation, but not against nematode transmission, possibly due to the large number of virus particles injected during nematode feeding (28).

As with naturally occurring virus resistance genes, strain specificity and breadth of protection are important questions, and different genes vary in their specificity. As a general rule, the plants are best protected against the virus (or strain) from which the CP gene was derived, but in many cases, the transgenic plants also were protected against additional virus strains and/or related heterologous viruses. Although percent homology is clearly not the only factor involved, there is often a general correlation between the extent of protection and the relatedness between the challenge virus and the virus from which the CP gene was derived (21, 23, 26, 41). In other cases specific strains are capable of overcoming resistance. Interestingly, the ZYMV strains that have been found to overcome ZYMV-CP mediated resistance in melon also can systemically infect *Nicotiana benthamiana*, a species that is not normally a systemic host for this virus (Grumet et al. unpublished).

As a final comment, a critical question--and one that makes this workshop relevant--is what is the performance of these materials in the field? The possible ecological implications of viral recombination only become a serious issue if we are dealing with large-scale agricultural production of transgenic viral gene-expressing plants. The majority of the field experiments (ca. 75% of the USDA permits issued from 1987-1993) have been performed by industry. Although little has been published regarding field trial performance, those reports that have been published have been encouraging. Perhaps more importantly, Asgrow Seed currently has transgenic CP-expressing ZYMV- and WMV-resistant squash cultivars approved and ready for market. Other virus-resistant crops are likely to become available in the near future. From here, only time will determine the real effectiveness and long-term stability of this strategy for conferring commercially valuable levels of resistance in large-scale production systems.

References

1. Bertioli, D.J. et al. (1992) Ann Appl. Biol. 120:147
2. Brault, V., et al. (1993) Plant Mol. Biol. 21:89

3. Cuozzo, M., et al. (1988) Bio/Technology 6:549
4. Fang, G., Grumet R. (1993) Mol. Plant-Microbe Interact 6:358
5. Fulton, R.W. (1986) Ann Rev. Phytopathol. 24:67
6. Gonsalves, D., et al. (1992) Bio/Technology 10:1562
7. Hammond, J., Kamo, K.K. (1993) Acta Horticulture 336:171
8. Hayakawa, T., et al. (1992) PNAS USA 89:9865
9. Hemenway, C., et al. (1988) EMBO J 7:1273
10. Hill, K.K., et al. (1991) Bio/Technology 9:373
11. Hoekema, A., et al. (1989) Bio/Technology 7:273
12. Kawchuk, L., Martin, R.R., McPherson, J. (1990) Mol Plant-Microbe Interact 3:301
13. Kunik, T., et al. (1994) Bio/Technology 12:500
14. Lawson, C., et al. (1990) Bio/Technology 8:127
15. Leclerc, D., AbouHaidar, MG. (1995) Mol. Plant-Microbe Interact 8:58
16. Lindbo, J.A., Dougherty, W.G. (1992) Mol. Plant-Microbe Interact 5:144
17. Loesch-Fries, L.S., et al. (1987) EMBO J 6:1845
18. MacKenzie, D.J., Ellis, P.J. (1992) Mol. Plant-Microbe Interact 5:34
19. MacKenzie, D.J., Tremaine, J.H. (1990) J. Gen. Virol. 71:2167
20. MacKenzie, D.J., Tremaine, J.H., McPherson, J. (1991) Mol. Plant-Microbe Interact 4:95
21. Namba, S., et al. (1991) Gene 107:181
22. Namba, S., et al. (1992) Phytopathology 82:940
23. Nejidat, A., Beachy, R.N. (1990) Mol. Plant-Microbe Interact 3:247
24. Nelson, R.S., Powell-Abel, P., Beachy, R.N. (1987) Virology 158:126
25. Okuno, T., et al. (1993) Phytopatholoogy 83:542
26. Pang, S.Z., et al. (1992) Phytopathol. 82:1223
27. Pang, S.Z., et al. (1993) Phytopathol. 84:243
28. Ploeg, A.T., et al. (1993) J. Gen. Virol. 74:2709
29. Ponz, F., Bruening, G. (1986) Ann. Rev. Phytopathol. 24:355
30. Powell-Abel, P., et al. (1986) Science 232-738
31. Powell, P.A., et al. (1990) Virology 175:124
32. Quemada, H.D., Gonsalves, D., Slightom, J.L. (1991) Phytopathol. 81:749
33. Sanford, J.C., Johnston, S.A. (1985) J. Theor. Biol. 113:395
34. Smith, H.A., et al. (1994) Plant Cell 6:1441
35. Stark, D.M., Beachy, R.N. (1989) Bio/Technology 7:1257
36. Taschner, P.E.M., et al. (1994) Virology 203:269
37. Tumer, N.E. et al. (1987) EMBO J 6:1181
38. Tumer, N.E., et al. (1991) PNAS USA 88:2331-2335
39. Vaira, A.M. et al. (1995) Mol. Plant Microbe Interact 8:66
40. van der Wilk, F., et al. (1991) Plant Mol. Biol. 17:431
41. van Dun CMP, Bol, J.F. (1988) Virology 167:649
42. van Dun CMP, Bol, J.F., van Vloten Doting L. (1987) Virology 159:299
43. van Dun CMP, et al. (1988) Virology 164:383
44. Wisniewski, L.A., et al. (1990) Plant Cell 2:559

Roger N. Beachy
The Scripps Research Institute

Movement Protein-Mediated Resistance

The potential use of virus movement proteins as sources of genes for resistance to virus infection was predicted following research in the late 1980s that identified viral genes that are responsible for local virus spread (3). The first example of such resistance was demonstrated through the work of Malyshenko et al. in 1993 (5). These workers described transgenic plants that carry a temperature sensitive (ts) mutation in the movement protein (MP) of tobacco mosaic virus (TMV). Transgenic plants that carry this tsMP, when held at the nonpermissive temperature for 14-days prior to inoculation, reduced the rate of infection caused by TMV compared with control plants. Furthermore, plants that carried the nonmodified 3a gene of brome mosaic comovirus were resistant to infection by TMV.

Lapidot et. al. (4) described a mutation in the movement protein of TMV caused by a 3-amino acid deletion that resulted in a dysfunctional movement protein (dMP). Transgenic plants that carried the dMP were resistant to TMV and a number of other tobamoviruses. Resistance, although not complete, caused a restriction in numbers of multicellular infection sites, and a reduction in the rate of local and systemic spread of virus infection.

Cooper et al. (2) used these transgenic tobacco plants to demonstrate that the dMP of TMV conferred resistance against a number of nontobamoviruses, including alfalfa mosaic ilarvirus, peanut chlorotic streak caulimovirus, tobacco rattle tobravirus, tobacco ringspot nepovirus, and potato virus Y potyvirus. Resistance against nontobamoviruses was, however, different from the resistance against tobamovirus in these transgenic plants. Whereas the dMP restricted the local spread of the tobamoviruses, it apparently did not do so in the case of the nontobamoviruses. The heterologous viruses tested were capable of infection and local spread but were restricted in movement from the inoculated leaf to upper noninoculated leaves; with the result that there was reduced accumulation of virus in upper leaves, and significant reduction in the development of systemic disease symptoms.

Beck et al. (1) reported that transgenic tobacco plants that produce a mutant of the p13 protein of white clover mosaic potexvirus (WCMV) strain O (p13 is one of the triple block proteins implicated in local spread of potexviruses) exhibited resistance to the O, M, and J strains of WCMV. Some transgenic lines were also resistant to potato virus X and narcissus mosaic potexvirus, and potato virus S carlavirus; there was no resistance to TMV.

The cellular and molecular mechanisms of resistance conferred by the dMP of TMV are currently under study. It is proposed that the dysfunctional MP prevents the accumulation of the movement protein of the challenge virus in such a way that the MP of the challenge virus is unable to complete its function. Interference of function could be at the level of multiprotein interactions, of the targeting of the protein to the cell wall or to plasmodesmata, or interference with association of the MP with nucleic acids, the modification of the plasmodesmata structure

and/or function, or other mechanisms.

A prominent feature of studies with the dMP of TMV is that the activity of the dysfunctional movement protein as a resistance gene is correlated with the finding that transgenic plants that carry the nonmodified (wild-type) MP accelerate the local and systemic spread of the heterologous viruses for which resistance by the dMP is effected. This supports the suggestion that the dysfunctional MP interferes with a function that is common amongst a number of plant viruses. Furthermore, this observation supports the conclusion that it would be unwise to use a wild type, functional movement protein as a gene for resistance, since plants that carry such a gene are likely to have increased susceptibility to certain other viruses.

Since the dMP does not interfere with infection but interferes with the spread of the disease agent, its application in agriculture will likely rely on the development of *trans* genes whose dysfunctional movement protein produces a phenotype that results in a strong interference of the homologous virus as well as heterologous viruses. It is unclear whether or not highly resistant or immune plants can result from the application of this technology; additional research is necessary to address this issue.

When considering the likelihood that a dMP could be acquired by a challenge virus in transgenic plants, one considers the likelihood that a dMP could be acquired through homologous or nonhomologous recombination. It is possible that such an event could occur, but the likelihood of creating a new virus is considered to be highly remote since there are no known examples in which nonhomologous and cellular mRNA sequences have been acquired through plant virus infection and retained as a functional element of a replicating RNA virus. There also is little evidence of exchange of sequences related to movement proteins amongst virus groups as a result of mixed infections. Although there may be sequence domains or motifs that are recognizable amongst movement proteins of different viruses, whether they evolved from common ancestral genes or were acquired through exchange through the evolutionary time of virus development is not clear. Furthermore, the use of dMPs as resistance genes would seem to have certain advantages, because, in order to cause a functional change in a MP, it would be essential that the domain acquired fit with sequences of the challenge virus to alter its host range or pathogenicity. While the remote possibility of this event is acknowledged, the likelihood of its occurrence is viewed as extremely low.

References

1. Beck, D.A., Van Dullard, C. J., Laugh, T.V., Balmier, E., Volt, D.A., Anderson, M.A., O'Brien, E.W., and Forster, L.S. (1994) PNAS USA 91:10310-10314.
2. Cooper, B. Lapidot, M., Heick, J.A., Dodds, J.A. and Beachy, R.N. (1995) Virology 206:307-313.
3. Deom, C.M., Lapidot, M., and Beachy, R.N. (1992) Cell 69:221-224.
4. Lapidot, M., Gafny, R., Ding. B., Wolf, S., Lucas, W.J. and Beachy, R.N. (1993) The

Plant Journal 2:959-970.
5. Malyshenko, S.L., Kondakowva, O.A., Nazarova, J.V., Kaplan, I.B., Taliansky, M.E. and
Atabekov, J.G. (1993) J. of Gen. Virol. 74:1149-1156.

Milton Zaitlin
Cornell University

Replicase-Mediated Resistance

Transformation of plants with plant viral replicase sequences, resulting in induction of resistance was first reported in 1990, employing the 54-kDa read-through region of the TMV replicase gene transformed into *Nicotiana tabacum* (11). This concept has now been applied to a number of other viruses (enumerated below) and other plants; it has been reviewed by Carr and Zaitlin (8) and by Baulcombe (3).

The characteristic features of "replicase-mediated resistance" are the high degree of resistance shown both to viral and to RNA inocula, and the narrowness of the specificity (i.e., resistance is shown only to the virus from which the replicase gene was derived and to very closely related strains or mutants). In most cases examined, there is a substantial inhibition of virus replication in initially inoculated cells and some limited cell-to-cell movement, but the infection does not spread from the inoculated leaf and no systemic disease develops. To all practical purposes, the plants are immune from disease.

What viral sequences are required for resistance induction? A precise answer to this question is not possible. As seen in the following table, a number of different constructs have been utilized, ranging from full-length genes to mutated or truncated versions. In most cases constructs have been engineered to ensure that a protein can be translated from the construct, but its translation may or may not be required. Furthermore, transformation with full-length and functional replicase genes does not always lead to resistance (10,11,19).

All of the answers as to mechanism are not in hand; there is strong evidence that there is no universal answer to replicase-mediated resistance. Indeed, in my laboratory we have made the case for several mechanisms being involved in a given system (i.e., CMV in tobacco). We have shown that, in addition to a substantial suppression of virus replication, there is an inhibition of long-distance transport postulated to result from inhibition of entry into the vascular system (9) and a shut-down of the plasmodesmata resulting in a lack of cell-to-cell movement (unpublished study in collaboration with W. Lucas).

A case has been made for an RNA-mediated mechanism for replicase mediated-resistance of potato virus X in tobacco. In essence, those plants that express the highest level of transgene mRNA show the least resistance and vice versa (3). On the other hand, with another virus in another replicase gene-transformed host, namely, alfalfa mosaic virus in alfalfa, those plants expressing the highest level of replicase protein showed the highest level of resistance (6). We have presented evidence from in vitro studies that the TMV 54-kDa resistance requires a functional protein (7), implicating a protein-mediated mechanism in that system.

The only conceivable scenario in which the replicase could pose a supposition of risk would be if a replicase-resistant plant was to become virus infected, and the replicase gene in that

virus would recombine with the replicase sequence from the transgenic plant. Recombination between an infecting virus and coat protein-encoding transgene from a virus has been demonstrated (12), under conditions where a strong selection had to be introduced for the recombinant to be expressed. Although analogous recombination could occur between the transgenic replicase sequence, in my opinion it would be very unlikely that a recombinant virus would contain a functional replicase, or that the recombinant virus would have any selective advantage over the infecting virus. Replicases by their nature are very specific, replicating only the specific virus from which they are derived and closely related viruses. They will not replicate host mRNAs or RNAs of unrelated viruses. Additionally, exchanges between replicase sequences from unrelated viruses will in all likelihood be nonfunctional; replicase genes frequently overlap with other viral genes or they contain sequences for subgenomic promoters. Thus, sequence exchanges between replicases could perturb other viral functions, resulting in loss of viability.

Table. Summary of viral gene constructs used to engender replicase-mediated resistance.

	Replicase Read-through ORF's	References
Tobacco mosaic tobamovirus	Unmodified 54-kDa	11
Pea early browning tobravirus	Unmodified 54-kDa	15
Pepper mild mottle tobamovirus	Unmodified 54-kDa	4
	Viruses Where Gene Is Translated Directly from a Subgenomic or the Genomic RNA	
Potato virus X potexvirus	Unmodified full-length, truncated, or mutated GDD	5,14
Cymbidium ringspot tombusvirus	Unmodified full-length	17
Cucumber mosaic cucumovirus	Truncated or full-length	1
Tobacco mosaic tobamovirus	Modified 183-kDa	10
Alfalfa mosaic alfamovirus	Mutated GDD	6
	Viruses Where Genes Are Derived from Polyprotein(s)	
Potato virus Y potyvirus	Unmodified full-length or truncated	2,13
Cowpea mosaic comovirus	Unmodified full-length	18
	Viruses with Overlapping Genes	
Potato leaf roll luteovirus	Unmodified full-length	13

References

1. Anderson, J.M. et al. (1992). PNAS USA 89:8759-8763
2. Audy, P. et al. (1994). Molecular Plant-Microbe Interactions 7: 15-22.
3. Baulcombe, D. (1994). Trends in Microbiology 2:60-62.
4. Berzal-Herranz et al. (1995). Virology (In Press).
5. Braun, C.J., and C.L. Hemenway (1992). The Plant Cell 4: 735-744.
6. Brederode, F.T., et al. (1995). Virology 207:467-474.
7. Carr et al. (1992). Molecular Plant-Microbe Interactions 5:397-404.
8. Carr, J.P., and M. Zaitlin (1993). Seminars in Virology 4: 339-404.
9. Carr, et al. (1994). Virology 199: 439-447.
10. Donson, J. et al. (1993). Molecular Plant-Microbe Interactions 6: 635-642.
11. Golemboski, D.B., et al. (1990). PNAS USA 87:6311-6315.
12. Greene, A.E., and R.F. Allison (1994). Science 263: 1423-1425.
13. Kaniewski, W. (1995). Personal communication.
14. Longstaff, M. et al. (1993). The EMBO Journal 12: 379-396.
15. MacFarlane, S.A., and J.W. Davies (1992). PNAS USA 89:5829-5833.
16. Mori, M. et al. (1992). Journal of General Virology 73:169-172.
17. Rubino, L. et al. (1993). Molecular Plant-Microbe Interactions 6:729-734.
18. Sijen, T. et al. (1995). Molecular Plant-Microbe Interactions (In Press).
19. Taschner, P.M. et al. (1991). Virology 181:45-450.

William G. Dougherty
Oregon State University

RNA-Mediated Transgenic Virus Resistance

Virus resistance can be engineered in plants by expressing transgenes that code for RNA species that are not translated into proteins. Four RNA-based strategies have been examined that result in the expression of either a sense RNA, an antisense RNA, a defective interfering (DI) RNA, or a satellite RNA. Plants expressing transgenes that encode an antisense RNA (1,2,3,4,5), and DI RNA (6), or a satellite RNA (7) have been constructed and a minimal level of protection from virus-induced disease symptoms has been observed. Current results would not support the development and deployment of these transgenes as effective antiviral strategies. Surprising results have been achieved with sense RNA-mediated resistance (8,9,10,20), and this strategy is the focus of this section.

Sense RNAs, expressed from transgenes, represent RNA sequences that are identical (or nearly identical) to host or virus coding sequences. It has been observed with plant genes, that the expression of exogenous transgenes can, in some instances, suppress the expression of the endogenous host gene homologue (11,12,13). This effect on plant gene expression has been coined sense (or co-) suppression. A similar effect also appears to be functioning during virus resistance, and the expression of a sense RNA can result in the dramatic suppression of virus replication (9,10). Sense RNAs can be either translatable, and code for a protein, or untranslatable, and not code for a protein. Untranslatable sense RNAs are generated in two ways: The AUG initiation cordon can be deleted (9) or changed to a sense codon, or termination stop codons can be positioned just downstream of the initiation codon (8). Translatable and untranslatable sense RNAs are able to induce an extreme level of virus resistance suggesting the protein product derived from the translatable sense RNA transgene is not involved in the process (10). Only the untranslatable sense RNA strategy that induces an extreme level of virus resistance will be discussed as there are inherent benefits to this approach in its simplicity (only RNA no protein) and in possible risks posed.

Sense RNA-mediated resistance (10,14) functions in a manner quite distinct from pathogen-derived resistance (15) or dominant negative mutations (16,17), the underlying rational upon which the other antiviral approaches discussed at this meeting are based. For these other strategies, the expectation is that the transgene-derived product competes or interferes with the viral genome-derived homologue during replication. This competition results in an aborted or altered replication cycle and a plant phenotype in which disease symptoms are attenuated. In the cases of sense RNA-mediated resistance, the expectation is that a cellular system is activated which specifically eliminates particular RNA sequences (10,11,14). The system can be "pre-programmed" by over expressing a transgene that contains viral sequences. Quantitative and qualitative elements of the transgene transcript are important in programming the response (11,18). Once the cellular system is activated, it rapidly eliminates the RNA species being targeted. In the case of a transgene expressing a sense RNA comprised of viral sequences, this results in the low steady state level of the transcript even though the gene is being actively

transcribed at a relatively high rate (10,18). If the cell is infected with a viral genome from which the transgene was derived, the viral sequence contained in the genomic RNA will also be targeted and eliminated, resulting in a highly resistant state in which no virus or viral RNA can be detected (19). The cellular system appears to be quite specific, and preliminary data suggests RNAs that share ~ >90% homology will be recognized.

Sense RNA-mediated virus resistance has been extensively tested in greenhouse studies (8,10,18) and in field settings (20). Highly resistant germplasm, identified in greenhouse tests has functioned as expected during 3 years of field tests. Highly resistant tobacco etch virus (TEV) or potato virus Y (PVY) transgenic lines remained free of TEV or PVY delivered via mechanical inoculation or aphid vectors (20; Dougherty, Wernsman, and Whitty, unpublished observations).

The brief summary above describes the current status of RNA-mediated resistance and attempts to define the fundamental differences between sense RNA-mediated resistance and pathogen-derived resistance schemes. The benefits of this and other antiviral technologies in the near term will be the increased production and quality of fruits and vegetables of annual plants. However, the real benefit of this technology will be realized when fruit and nut trees and caneberries and other perennial plant species are protected from virus infection.

There are risks associated with antiviral strategies (21,22,23). The real questions center on the significance of these risks (28). Are transgenic plants different from virus-resistant hybrid lines established via classical breeding methods, and do transgenic plants provide a new environment not currently available that will increase the chance of selecting virus variants?

One can argue that an untranslatable sense RNA strategy minimizes the risks (real or perceived) while maximizing the effectiveness of this technology. Many of the perceived risks associated with antiviral strategies focus on the protein product or the genetic material of the transgene being incorporated into or temporarily used by another viral pathogen to alter host range or pathogenicity (21,22). Transcomplementation (24,25), transencapsidation (26), and recombination (27) have all been cited as potential problems. The untranslatable sense RNA strategy negates concerns about transencapsidation and transcomplementation as no protein product is produced. RNA recombination between a viral genome and an untranslatable transgene transcript also should not pose a problem as it should lead to a lethal recombination event. Additional stop codons can also be strategically placed in the untranslatable sense RNA to ensure protein can not be made and that a viable recombinant will not form.

Another consideration is that a transgene produces a level of viral protein sufficient for an allergic response. This is not an issue with the untranslatable sense RNA resistance, as no protein is produced. In strategies that produce a protein it also should not be an important concern. Naturally occurring virus infections produce protein levels that far exceed transgene-derived protein levels, and surveys of most vegetables in the marketplace would test positive for plant viruses.

Finally, there is concern that viral transgenes will genetically move to weed species (29,30,31). There are two components to this scenario. The genetic material must not only move into the germinal tissue of the weed species but must also provide a selective advantage to that species. Companies that develop transgenic lines will likely deploy these traits in varieties that they can control using a male sterile background, thereby minimizing concerns of gene flow in these crop species. In cases where a male sterile containment strategy is not practical, gene flow may be possible. However, I am unable to document examples of classically bred virus resistance that has moved into related species to create a more fit species. Usually, weedy relatives of domesticated crop species are inherently better able to deal with pathogens. Examples of endogenous plant species being held in check by virus infection are also lacking. If the transgene expressing the untranslatable sense RNA were to move, it may be possible to prevent the establishment of virus resistance. Key in establishing the antiviral state is transgene copy number and the level of transgene transcription. We are just beginning to appreciate the nuclear requirements to establish virus resistance; however, it should be possible to select for transgene configurations that, when outcrossed, no longer induce the resistant state.

Acknowledgments

The author thanks Dawn Parks for her comments. Research in our laboratory is supported by grants from the US Department of Agriculture NRGI Program and the Oregon-Massachusetts Biotechnology Partnership.

References

1. Nellen, W., and Lichtenstein, C. (1993) TIBS 18:419-423.
2. Takayama, K.M., and Inouye, M. (1990) Biochem. and Mol. Biol. 25:155-184.
3. Bejarano, E.R., and Lichtenstein, C.P. (1994) Plant Mol. Biol. 24:241-248.
4. Rezaian, M.A., Skene, K.G.M., and Ellis, J.G. (1988) Plant Mol. Biol. 11:463-471.
5. Powell, P.A., Stark, D.M., Sanders, P.R., and Beachy, R.N. (1989) PNAS USA 86:6949-6952.
6. Kollar, A., Dalmay, T., and Burgyan, J. (1993) Virology 193:313-318.
7. Harrison, B.D., Mayo, M.A., and Baulcombe, D.C. (1987) Nature 328:799-802.
8. Lindbo, J.L., and Dougherty, W.G. (1992) Mol. Plant-Microbe Interact. 5:144-153.
9. van der Vlugt, R.A.A., Ruiter, R.K., and Goldbach, R. (1992) Plant Mol. Biol. 20:631-639.
10. Smith, H.A., Swaney, S.L., Parks, T.D., Wernsman, E.A., and Dougherty, W.G. (1994) The Plant Cell 6:1441-1453.
11. Doughterty, W.G., and Parks, T.D. (1995) Current Opinion in Cell Biology 7, in press.
12. Flavell, R.B. (1994) PNAS USA 91:3490-3496.
13. Finnegan, J., and D. McElroy. (1994) Bio/Technology 12:883-888.
14. Lindbo, J.L., Silva-Rosales, L., Proebsting, W.M., and Dougherty, W.G. (1993) Plant Cell 5:1749-1759.
15. Sanford, J.C., and Johnston, S.A. (1985) J. Theor. Biol. 113:395-405.
16. Herskowitz, I. (1987) Nature 329:219-222.

17. Szybalski, W. (1991) Gene 107:177-179.
18. Dougherty, W.G., Lindbo, J.L., Smith, H.A., Parks, T.D., Swaney, S., and Proebsting, W.M. (1994) Mol. Plant-Microbe Interact. 7:544-552.
19. Lindbo, J.A., and Dougherty, W.G. (1992) Virology 189:725-733.
20. Whitty, E.B., Hill, R.A., Christie, R., Young, J.B., Lindbo, J.A., and Dougherty, W.G. (1994) Tobacco Science 38:30-34.
21. de Zoeten, G. (1991) Phytopathol. 81:858-586.
22. Tepfer, M. (1993) Bio/Technology 11:1125-1130.
23. Rissler, J., and Mellon, M. (1993) Union of Concerned Scientists. Washington, D.C. 92pp.
24. Dolja, V.V., Haldeman, R. Robertson, J.L., Dougherty, W.G., and Carrington, J. C. (1994) The EMBO J. 13:1482-1491.
25. Li, X.H., and Carrington, J.C. (1995) PNAS USA 92:457-461.
26. Farinelli, L., Malnoë, P., and Collet, G.F. (1992) Bio/Technology 10:1020-102.
27. Green, A.E., and Allison, R.F. (1994) Science 263:1423-1425.
28. Falk, B.W., and Bruening, G. (1994) Science 263:1395-1396.
29. Regal, P.J. (1994) Mol. Ecol. 3:5-13.
30. Kareiva, P., Morris, W. and Jacobi, C. M. (1994) Mol. Ecol. **3:**15-21.
31. Crawley, M.J., Hails, R.S., Rees, M., Kohn, D., and Buxton, J. (1993) Nature 363:620-623.

James C. Carrington
Texas A&M University

Transgenic Complementation

Genetic complementation is a powerful tool to dissect relationships between genes and their protein products. Complementation can provide information regarding the location of mutations, domains within proteins affected by mutations, and cis/trans relationships among proteins. Complementation between viruses typically involves coinfection of cells with two mutants and scoring a significant increase in virus activity compared to either mutant individually. Despite the relative ease with which genomes can be manipulated, however, genetic complementation has not been used widely as a means to study plant RNA viruses. We have been using a transgenic complementation strategy to investigate functions of proteins encoded by tobacco etch virus (TEV), a representative member of the large and diverse *Potyviridae* family.

The TEV genome is approximately 9.5 kb in length, and it encodes a large polyprotein that undergoes co- and post-translational processing (9). Polyprotein cleavage is catalyzed by three TEV-encoded proteinases (P1, HC-Pro, and NIa), yielding at least 10 distinct products (6). Most of the viral proteins participate in either catalytic or accessory roles during viral RNA replication. At least two proteins [HC-Pro and capsid protein (CP)] are required for cell-to-cell and systemic movement through plants, as well as transmissibility by aphids. A single protein, CP, provides the encapsidation function. Given that all viral proteins arise through proteolytic processing, it is possible that some proteins possess unique function within the context of a polyprotein intermediate. This would increase the complexity of activities that TEV proteins may perform. Further, some TEV proteins may function a soluble, trans-acting factors, whereas others may function only within an immobile complex or in association with the specific RNA molecule from which they are encoded.

To identify and understand potential cis and trans interactions among TEV proteins, transgenic complementation has been pursued. This strategy involves production of transgenic plants that express the coding sequence for either mature or polyprotein forms of TEV proteins and testing the ability of these to complement TEV mutants possessing defects in the same coding sequences. These plants can also serve as sources of protoplasts to quantitatively assay for complementation of mutants in a cell culture system. All of these studies have employed a TEV variant that expressed the reporter protein, β-glucuronidase (GUS). Genome amplication, cell-to-cell movement, and systemic movement can be quantitated quickly using colorimetric or fluorometric assays for GUS (5).

Several RNA replication functions can be provided in trans by transgenic hosts. Nonviable variants with either point or deletion mutations within the NIb (polymerase) coding sequence can be complemented in transgenic plants or protoplasts expressing the NIb protein (8). Similarly, replication-debilitated mutants lacking the P1 coding sequence are stimulated in transgenic plants expressing the P1 proteins (10). However, several mutants containing defects

affecting other TEV replication-associated proteins or proteinases cannot be complemented in transgenic plants (7; M.C. Schaad and J.C. Carrington, unpublished observations). These data suggest that some viral functions involved in RNA replication can function in trans, whereas others may have specific polyprotein or cis requirements.

Movement functions performed by CP and HC-Pro can be supplied by transgenic hosts in trans. Mutations within the core region of CP debilitate encapsidation and all intercellular movement. Capsid protein expressed in transgenic plants can complement both cell-to-cell and long-distance movement defects of these mutants (3, 4). Mutants with alterations in the N- or C-terminal regions of CP or the central region of HC-Pro are cell-to-cell movement competent but fail to move systemically. These mutants can be complemented in transgenic lines expressing CP or HC-Pro, respectively, although the efficiency of complementation varies depending on the specific mutation (2, 4).

The relevance of these genetic studies to transgenic risk issues is not yet resolved. A trans-active viral function supplied by a transgenic host could conceivably stimulate replication or movement of a heterologous virus. It has been demonstrated that expressing the 5'-terminal region of the potyvirus genome in transgenic plants is sufficient to stimulate potato virus X (PVX) in a manner that mimics the classic synergism response upon potyvirus/PVX coinfection (1). The potential trans-activation risks should be minimized, however, in transgenic strategies involving expression of defective or nontranslatable viral genes.

References

1. Bowman-Vance, V., P. H. Berger, J. C. Carrington, A. Hunt, and X. M. Shi (1995) Virology 206:583-590.
2. Cronin, S., J. Verchot, R. Haldeman-Cahill, M. C. Schaad, and J.C. Carrington (1995) The Plant Cell 7: in press.
3. Dolja, V. V., R. Haldeman, N. L. Robertson, W. G. Dougherty, and J.C. Carrington (1994) EMBO J. 13:1482-1491.
4. Dolja, V. V., R. Haldeman-Cahill, A. E. Montgomery, K. A. VandenBosch, and J. C. Carrington (1995) Virology 207:1007-1016.
5. Dolja, V. V., H. J. McBride, and J. C. Carrington (1992) PNAS USA 89:10208-10212.
6. Dougherty, W. G. and B. L. Semler (1993) Microbiol. Rev. 57:781-822.
7. Kasschau, K. D. and J. C. Carrington (1995) Virology 209: in press.
8. Li, X. H. and J. C. Carrington (1995) PNAS USA 92:457-461.
9. Riechmann, J. L., S. Laín, and J. A. García (1992) J. Gen. Virol. 73:1-16.
10. Verchot, J. and J. C. Carrington (1995) J. Virol. 69:in press.

Richard Allison
Michigan State University

RNA Plant Virus Recombination

RNA recombination, a process that unites two previously distinct RNAs, relies on the ability of the viral replicase to switch templates during the synthesis of viral RNA. The process was first studied in animal RNA viruses and phage (reviewed in 1) and more recently in plant RNA viruses (2-4). The importance of RNA recombination to virus-resistant transgenic plants is that the sequence of viral RNA expressed in a transgenic plant may be available for recombination into a challenging virus. The evaluation of this concept is the focus of this meeting and research in my laboratory. We have sought to determine if a transgenic viral RNA expressed by a transgenic plant is available to a replicating RNA virus for recombination.

Our bioassay for recombinant virus is based on the requirement of cowpea chlorotic mottle virus (CCMV) for a functional capsid gene to support systemic movement (5). *Nicotiana benthamiana* was transformed with the 3' two thirds of the CCMV capsid gene. Transcripts from this truncated gene were insufficient to provide the transformants with resistance. These plants were challenged with a CCMV deletion mutant lacked the 3' one third of the capsid gene and was incapable of systemic infection. Thus a systemic viral infection could occur only if a functional capsid gene was restored through recombination between the transgenic insert and the challenging deletion mutant. Three percent of the inoculated plants became infected systemically. Recombinants were confirmed by identification of markers in the recovered viral RNA that were present originally only in the transgenic insert. Recovery of viable virus indicated that in our system viral RNA transcribed by the transgenic plant was available to the replicating virus in quantities adequate to support RNA recombination.

Analysis of the recombinant virus indicated that each was derived from a unique aberrant homologous recombination event. Several recombinant viruses provided symptoms on cowpeas that distinguished them from wild-type CCMV. All mutants mapped to a distinct area of the capsid gene, which apparently codes for a region of the protein that is tolerant of modification.

Transgenic *N. benthamiana* expressing the same CCMV insert as described above were challenged with a second bromovirus, brome mosaic virus (BMV), which also was denied systemic movement by a deletion introduced into the 3' region of the capsid gene. Systemic movement of BMV in several transgenic plants suggested restoration of the BMV capsid gene. Sequences of several PCR-amplified cDNAs from these plants indicated that numerous recombination events had occurred. However, the initial sequences did not reveal a functional capsid gene. Thus the RNA sequences identified were likely from a collection of heterogeneous molecules generated by RNA replication. The chimeric nature of the recombinants suggested that RNA recombination incorporated RNA from various sources into the viral genome.

Our data suggests that the viral RNA in virus-resistant transgenic plants may also be available to replicating RNA viruses that challenge transgenic plants. Theoretically, chimeric

viruses generated by such recombination events will provide material for the process of natural selection.

While virus-resistant transgenic plants are resistant to a limited number of closely related viruses, these plants continue to be susceptible to other viruses. Additionally, numerous viruses that are incapable of causing systemic infections replicate in initially inoculated cells. Thus we anticipate that in the field many viruses will have an opportunity to recombine with the transgenic insert.

Multiple viral infections of a single plant are thought to have provided opportunities for RNA virus recombination. Although there are numerous descriptions of more than one virus present in a single plant, there is little data to support the hypothesis that two RNA viruses replicate simultaneously in the same plant cell. Exceptions appear to be infections where transencapsidation has been observed and perhaps synergistic infections.

During a systemic infection the virus moves from cell to cell initiating and completing the replication process as it proceeds; therefore in infected plants, the virus is not replicating continuously in each cell. Thus multiple infections may represent several distinct viral infections that have proceeded through the plant at different times. In this situation, opportunities for RNA recombination may be limited. In contrast, in virus-resistant transgenic plants expression of the viral insert is driven by a constitutive promoter, which ensures the presence of the viral insert in each cell that may be challenged. Thus the potential for RNA recombination in virus-resistant transgenic plants may be greater than in plants naturally infected by several viruses.

RNA recombination apparently occurs more often in some plant virus groups than others. Conserved among the plant RNA virus groups is a small amino acid motif in the viral-encoded polymerase that suggests that the viral replication mechanism is conserved. If the replication mechanism is conserved, recombination differences may reflect the affinity of the replicase complex for its template rather than fundamental differences in replication. Although it may appear that recombination is less likely if the transgene is from a virus that recombines infrequently, the frequency at which transgenic inserts are involved in recombination will more likely reflect the recombination frequency of the challenging virus rather than the frequency of the virus from which the transgene was derived.

References

1. Lai, M.C.M. (1992) Microbiol. Rev. 56:61-79.
2. Bujarski, J.J. and Kaesberg, P., (1986) Nature 321:528-531.
3. Allison, R.F., Thompson, C., and Ahlquist, P. (1990) PNAS 87:1820-1824.
4. Simon, A.E. and Bujarski, J.J. (1994) Ann. Rev. Phytopath. 32:337-362.
5. Greene, A.E. and Allison, R.F. (1994) Science 263:1423-1425.

Vicki Bowman Vance
University of South Carolina

Viral Synergism

Plants are commonly exposed to multiple viral infections and interactions between co-infecting pathogens that may be antagonistic or synergistic. Antagonistic reactions may render a host plant infected with one virus resistant to subsequent infection with a related virus (cross protection). The mechanisms involved in such antagonistic interactions are currently the focus of intense research in a variety of host/virus systems. Using a transgenic plant approach, investigators have determined that the expression of individual virus genes can result in resistance to subsequent virus infection. This line of experimentation has yielded valuable clues to the basic mechanism of cross protection, as well as being of practical significance. However, since the mechanisms by which virally encoded resistance genes mediate protection are not completely understood, the question of the safety of this biotechnological breakthrough has been raised.

One potential risk in the use of virus-encoded genes to confer protection involves another class of viral interactions that occurs very commonly in plants, viral synergism. In contrast to cross protection, the importance of the synergistic-type viral interactions in understanding viral disease has not been extensively studied and is just beginning to come to light. Many plant diseases are caused by the interaction of two unrelated viruses in the same host plant, and more examples are reported each year. Thus, although synergism has been known since 1925, we may be just beginning to understand the extent and complexity of such interactions in nature. Our recent work indicates that at least one of these viral synergistic diseases, the interaction of potato virus (PVX) with any of a number of viruses in the potyviral group of plant viruses, can be mimicked by expressing a subset of the potyviral genome in transgenic plants and then infecting the plant singly with the second virus of the synergistic pair, PVX. Thus viral genes expressed in plants for the purpose of cross protection may interact with an unrelated virus to produce a synergistic disease instead of the expected resistance. It is not known if other viral synergisms are also mediated by such "synergism genes" or if there are different classes of synergism that are induced by different classes of viral genes. Since viral synergisms are common in plants and most are completely unstudied, basic research into the mechanism of plant viral synergism is warranted, and should include a number of model viral systems.

William O. Dawson
University of Florida

Virus Replication

With the imminent use of transgenes derived from viral sequences used to protect plants from diseases caused by specific viruses, there is some question about the probability of creation of conditions that would cause the development of new viruses that could cause diseases. One question is whether there are characteristics of the replication strategies of some viruses that would favor or decrease this probability. However, there appear to be other questions that perhaps are more prominent in this process. It has been shown that recombination between viruses and transgenes can occur as can recombination between viruses in multiple infections. It appears that with enough opportunity recombination between different viruses and transgenes will occur. The more important questions are first whether the resulting recombinant would participate in the replication process and systemic infection process within the plant and become a factor in nature, and secondly whether the new virus would be a disease-causing agent.

The first question is a population biology question. The vast majority of progeny virus do not function in the replication process. Most progeny viral RNAs are encapsidated into virions and remain in the cell with no further function and are finally degraded as the leaf is degraded. They are not copied in the replication process within the cell, they do not move to adjacent cells to establish an infection, they do not move to newly developing areas of the plant, and they are not transmitted by a vector to another plant. The probability of a new recombinant becoming a factor in progeny populations is affected by its competitiveness, by selective forces, and by its competition. A new recombinant in a cell containing a million competing RNAs is likely not to produce progeny, even if it is much more competitive than its competition. An effective combination of selective forces and lack of competition is needed for recombinants to effectively survive.

If a new hybrid virus was developed that effectively survived in nature, the probability would be low that it would cause disease in plants. When one considers all species of plants and all plant viruses, the likelihood that a randomly chosen virus would infect any randomly chosen plant is minute. Systemic infection of a plant by a virus generally is required for production of disease. There is a low probability that a newly created virus could systemically infect any new plants. Also, most viruses that can systemically infect a plant do not cause disease in that plant. Thus, even though a new virus was created that was able to systemically infect a new plant, it is unlikely that it would cause disease in that plant.

Thus the prediction of the probability of development of new virus diseases resulting from recombination of between existing viruses and viral sequences in transgenes would be largely dependent on a better understanding of the population biology of viruses in cells and movement within plants and a better understanding of how viruses cause diseases.

APPENDICES

Appendix A

AGENDA

USDA-APHIS Workshop
Transgenic Virus-Resistant Plants and New Plant Viruses
Holiday Inn (Grand Ballroom B, C, D)
10,000 Baltimore Boulevard
College Park, Maryland

FRIDAY, APRIL 21, 1995

8:15 a.m.	Welcome	**Dr. Sue Loesch-Fries**, *Chair* Purdue University **Dr. Clifford Gabriel** American Institute of Biological Sciences
8:30 a.m.	Moving into the Future: Questions, Reasoning, and Probabilities	**Dr. Sally McCammon** Science Advisor USDA-APHIS
8:50 a.m.	Regulatory Issues	**Dr. James White** USDA-APHIS
9:10 a.m.	Coat Protein-mediated Resistance	**Dr. Rebecca Grumet** Michigan State University
9:30 a.m.	Movement Protein-mediated Resistance	**Dr. Roger Beachy** Scripps Research Institute
9:50 a.m.	BREAK	
10:15 a.m.	Replicase-mediated Resistance	**Dr. Milton Zaitlin** Cornell University
10:35 a.m.	RNA-mediated Resistance	**Dr. William Dougherty** Oregon State University
10:55 a.m.	Transgenic Complementation	**Dr. James Carrington** Texas A&M University
11:15 a.m.	Virus Recombination	**Dr. Richard Allison** Michigan State University
11:35 a.m.-1:15 p.m.	LUNCH (on your own)	
1:20 p.m.	Viral Synergism	**Dr. Vicki Vance** University of South Carolina

1:50 p.m.	Viral Replication	**Dr. William Dawson** University of Florida
2:10 p.m.	BREAK	
2:30 p.m.	Break-Out Sessions Meet	
	a. Coat protein (Group 1) Coat protein (Group 2)	**Grand Ballroom B, C, & D** **Prince George Rooms A & B**
	b. Movement proteins	**Maryland Room B**
	c. Replicase proteins	**Maryland Room A**
	d. Other viral genes (VPg, protease, helper component, cylindrical inclusion, nucleocapsid)	**Baltimore Room**
6:00-7:30 p.m.	DINNER* (Sponsored in part by the Biotechnology Industry Organization)	**Grand Ballroom A**
8:00 p.m.	Break-Out Sessions (continued)	

*During dinner, there will be a presentation by **Dr. Bernice Slutsky**, U.S. Environmental Protection Agency entitled "Oversight of Plant Pesticides."

SATURDAY, APRIL 22, 1995

8:00 a.m.	Break-Out Sessions	**Same meeting rooms as Friday**
Noon	LUNCH (on your own)	
1:30 -2:45 p.m.	Break-Out Sessions (continued)	
2:45 p.m.	BREAK	
3:00-5:00 p.m.	5:00 p.m.	Reports of Break-Out Sessions General Discussion

Adjourn **Grand Ballroom B, C, & D**

Appendix B

Workshop Participants

Ms. Marti Asner
U.S. Department of Agriculture
14th & Independence Ave, S.W.
Room 1001, RP-E
Washington, DC 20250-2200

Dr. David Bays
U.S. Environmental Protection Agency
Office of Pesticide Programs/BPPD
2805 Crystal Drive
Crystal Station, 5th Floor
Arlington, VA 22202

Dr. John Beringer
University of Bristol
School of Biological Sciences
Woodland Road
Bristol B58 1UG, UNITED KINGDOM

Dr. David Bisaro
Ohio State University
Plant Biotechnology Center
201 Rightmire Hall
1060 Carmack Road
Columbus, OH 43210

Dr. Dag-Ragnar Blystad
Norwegian Crop Research Institute
Plant Protection Centre
Fellesbygget, N-1432 As, NORWAY

Dr. John Bol
Leiden University
Gorlaeus Laboratories
Einsteinweg 55
2333 CC Leiden, THE NETHERLANDS

Dr. Charles Brown
USDA-ARS
Route 2
Box 2953A
Prosser, WA 99350

Dr. Judith Brown
University of Arizona
Department of Plant Sciences
Forbes Building 303
Tucson, AZ 85721

Dr. Jozef Bujarski
Northern Illinois University
Plant Molecular Biology Center
Montgomery Hall, Room 325
DeKalb, IL 60115

Dr. Brandt Cassidy
Samuel Roberts Noble Foundation
P.O. Box 2180
2510 Sam Noble Parkway
Ardmore, OK 73402

Ms. Pat Cimino
U.S. Environmental Protection Agency
Office of Pesticide Programs/BPPD
401 M Street, S.W. (7501W)
Washington, DC 20460

Dr. Candace Collmer
Wells College
Department of Biology
Aurora, NY 13026

Dr. J. I. Cooper
Natural Environment Research Council
Institute of Virology
Mansfield Road
Oxford, UNITED KINGDOM OX1 3SR

Dr. James Culver
University of Maryland
Center for Agricultural Biotechnology
UMBI
College Park, MD 20742

Dr. Vern Damsteegt
USDA-ARS

Foreign Disease
Weed Sci Res Unit, Building 1301
Fort Detrick, MD 21702

Dr. Valerian Dolja
Oregon State University
Department of Botany
Cordley Hall 2082
Corvallis, OR 97331

Dr. Bryce Falk
University of California, Davis
Department of Plant Pathology
Davis, CA 95616

Mr. Vicente Febres
University of Florida
Plant Pathology Department
1453 Fifield Hall
Gainesville, FL 32611

Dr. Richard Ford
University of Illinois
Department of Plant Pathology
1102 South Goodwin Avenue
Urbana, IL 61801

Mr. Claus Frier
Danish Environmental Protection Agency
Strandgade 29
DK-1401
Copenhagen K, DENMARK

Dr. Marc Fuchs
Cornell University
Department of Plant Pathology
Geneva, NY 14456

Ms. Jeanette Glover Glew
Food and Drug Administration
200 C Street, S.W.
HFS-246
Washington, DC 20204

Ms. Carol Gonsalves
Cornell University
Department of Plant Pathology
Geneva, NY 14456

Dr. Dennis Gonsalves
Cornell University
Department of Plant Pathology
Geneva, NY 14456

Dr. Donald Gordon
The Ohio State University
OARDC, Department of Plant Pathology
1680 Madison Avenue
Wooster, OH 44691

Dr. Subhash Gupta
USDA-APHIS-BBEP
4700 River Road
Unit 147
Riverdale, MD 20737-1237

Dr. John Hammond
USDA-ARS
FNPRU, Room 208, B-004
10300 Baltimore Avenue
Beltsville, MD 20705

Dr. Rosemarie Hammond
USDA-ARS
MPPL, Room 252, B-011A
BARC-West
Beltsville, MD 20705

Dr. Gary Hellmann
R. J. Reynolds Tobacco Company
Bowman Gray Technical Center
P.O. Box 1487, Agric. Sci. R&D
Winston-Salem, NC 27102-1487

Dr. Christine Henry
Central Science Laboratory Maff
Matching Green
Harrguden
Hertfordshire, UNITED KINGDOM

Mr. David Heron
USDA-APHIS
4700 River Road
Unit 147
Riverdale, MD 20737

Dr. Hei-ti Hsu
USDA-ARS
Beltsville Agric Research Ctr
Beltsville, MD 20705

Dr. John Hu
University of Hawaii
3190 Maile Way
Department of Plant Pathology
Honolulu, HI 96822

Ms. Suzanne Hurtt
USDA-ARS
PSI-Natl Germplasm Res Lab
Bldg 580, BARC-East
Beltsville, MD 20705-2350

Dr. Ramon Jordan
USDA-ARS
U.S. National Arboretum, FNPRU
Building 004, Room 208
10300 Baltimore Avenue
Beltsville, MD 20705

Dr. Wojciech Kaniewski
Monsanto
700 Chesterfield Village Parkway
Chesterfield, MO 63198

Dr. Susan Koehler
USDA-APHIS
BBEP, BP
4700 River Road, Unit 147
Riverdale, MD 20737

Dr. John Kough
U.S. Environmental Protection Agency
Office of Pesticide Programs/BPPD
2805 Crystal Drive
Crystal Station, 5th Floor
Arlington, VA 22202

Dr. Quentin Kubicek
USDA-APHIS
Room 1128
South Agriculture Building
Washington, DC 20250

Dr. Molly Kyle
Cornell University
310 Bradfield Hall
Department of Plant Breeding
Ithaca, NY 14853

Dr. Paul Lavrik
Monsanto Company
Produce Division
700 Chesterfield Parkway North
Chesterfield, MO 63198

Dr. Marc Law
Ciba-Geigy
3054 Cornwallis Road
Research Triangle Park, NC 27709

Mr. E. Clifford Lawson
Monsanto
GG4D
700 Chesterfield Village Pkwy
Chesterfield, MO 63198

Dr. Richard Lee
University of Florida
700 Experiment Station Road
Lake Alfred, FL 33850

Dr. Laurene Levy
USDA-APHIS
PPO, B-580, BARC-East
Powder Mill Road
Beltsville, MD 20705

Dr. Gabriel Macaya
Universidad de Costa Rica
Ciudad Universitaria
Rodrigo Facio
San Jose, COSTA RICA

Dr. Ved Malik
USDA-APHIS
BBEP-BP
4700 River Road
Unit 147
Riverdale, MD 20737

Dr. Richard Manshardt
University of Hawaii
Department of Horticulture
3190 Maile WaYes
Honolulu, HI 96822

Dr. Robert Martin
Agriculture & Agri-Food Canada
6660 N.W. Marine Drive
Vancouver, British Columbia, CANADA V6T 1X2

Dr. James Maryanski
Food and Drug Administration
Center for Food Safety and
Applied Nutrition (HFS-13)
200 C Street, S.W.
Washington, DC 20204

Dr. Larry McDaniel
American Type Culture Collection
Molecular Biology & Virology
12301 Parklawn Drive
Rockville, MD 20852

Dr. Ulrich Melcher
Oklahoma State University
Dept of Biochemistry & Molecular Biology
NRC 246
Stillwater, OK 74078-0454

Dr. W. Allen Miller
Iowa State University
Plant Pathology Department
351 Bessey Hall
Ames, IA 50011

Ms. Norunn Myklebust
Directorate for Nature Management
Tungasletta 2
7005 Trondheim, NORWAY

Dr. Richard Nelson
Samuel Roberts Noble Foundation
Plant Biology Division
P.O. Box 2180
Ardmore, OK 73402

Dr. M. Nishiguchi
National Institute for Agrobiological Resources
2-1-1 Kan-nondai
Tsukuba, JAPAN

Dr. Donald Nuss
Roche Institute of Molecular Biology
340 Kingsland Street
Nutley, NJ 07110-1199

Mr. Michael Omunyin
Iowa State University
Department of Plant Pathology
Ames, IA 50011

Dr. Robert Owens
USDA-ARS
Room 252, Bldg 011A
Beltsville Agricultural Research Center
Beltsville, MD 20705

Dr. John Payne
USDA-APHIS
BBEP
4700 River Road
Riverdale, MD

Mr. Keith Perry
Purdue University
Department of Botany
1155 Lilly Hall
West Lafayette, IN 47907

Dr. Edward Podleckis
USDA-APHIS
PPQ, BATS
4700 River Road, Unit 133
Riverdale, MD 20787-1236

Dr. Mary Price
University of Florida
700 Experiment Station Road
Lake Alfred, FL 33850

Dr. Francois Pythoud
Federal Office of Environment
Hallwylstr. 4
CH-3003
Bern, SWITZERLAND

Dr. Richard Reich
R. J. Reynolds Tobacco Company
Bowman Gray Technical Center
P.O. Box 1487, Agric. Sci. R&D
Winston-Salem, NC 27102-1487

Dr. Jane Rissler
Union of Concerned Scientists
1616 P Street, N.W.
Washington, DC 20036

Dr. D'Ann Rochon
Agriculture and Agri-Food Canada
6660 N.W. Marine Drive
Vancouver, BC, CANADA V6T 1X2

Dr. Robert Rose
U.S. Environmental Protection Agency
Biopesticide & Pollution Prevention Div
Office of Pesticide Programs (H7501 W)
401 M Street, S.W.
Washington, DC 20460

Ms. Ileana Salazar
Agribiotecnologia de Costa Rica
P.O. Box 25-4001
Alajuela, COSTA RICA

Dr. Mary Schaad
Texas A&M University
Department of Biology
College Station, TX 77843

Dr. Michael Schechtman
USDA-APHIS
BBEP-BCTA
4700 River Road, Unit 146
Riverdale, MD 20737

Dr. James Schoelz
University of Missouri
Department of Plant Pathology
108 Waters Hall
Columbia, MO 65211

Dr. Sivramiah Shantharam
USDA-APHIS
BBEP-BP
4700 River Road, Unit 147
Riverdale, MD 20737-1228

Dr. Laura Silva-Rosales
CINVESTAV-IPN, U. Irapuato
Km 6.8 Libramiento Nte. Autopista Irapuato-Leon
Apdo. Postal 629 I
Irapuato, Gto. 36500, MEXICO

Dr. John Stommel
USDA-ARS
Vegetable Lab, B-004, Room 220
BARC-West, 10300 Baltimore Avenue
Beltsville, MD 20705

Mr. Terry Stone
Monsanto Company
700 Chesterfield Village Parkway North
Chesterfield, MO 63198

Mr. Hans Erik Svart
National Forest and Nature Agency
Haraldsgade 53
DK-2100
Copenhagen O, DENMARK

Dr. Mark Tepfer
Inst. National de la Recherche Agronomique
Laboratoire de Biologic Cellulaire
INRA
78026 Versailles Cedex, FRANCE

Dr. Peter Thomas
USDA-ARS
Prosser IARES
Route 2
Box 2953-A
Prosser, WA 99350

Dr. Sue Tolin
Virginia Tech University
Dept of Plant Pathology
Plant Molecular Bldg, Rm 102
Blacksburg, VA 24061-0330

Mr. David Tricoli
Asgrow Seed Company
2605 East Kilgore Road
Kalamazoo, MI 49002-1782

Dr. Nilgun Tumer
Rutgers University
AgBiotech Center
Cook College
P.O. Box 231
New Brunswick, NJ 08903-0231

Dr. Rodrigo Valverde
Louisiana State University
Department of Plant Pathology
and Crop Physiology

302 Life Science Building
Baton Rouge, LA 70803

Dr. Sally Van Wert
Agro Evo USA Co.
Little Falls Centre One
2711 Centerville Road
Wilmington, DE 19808

Dr. Thanda Wai
USDA-ARS
Rm 252, Bldg 011A, MPPL, PSI
BARC-West
Beltsville, MD 20906

Dr. Michael Watson
USDA-ARS
Plant Pathology Laboratory
Bldg. 011A, Rm 252, BARC-W
Beltsville, MD 20745

Mr. Harry Wells, Jr.
U.S. Environmental Protection Agency
Ofc of Pesticide Programs/BPPD
401 M Street, S.W., 7501W
Washington, DC 20460

Dr. T. Michael A. Wilson
Scottish Crop Research Institute
Invergowrie
Dundee, UNITED KINGDOM DD2 6DA

Dr. Gail Wisler
USDA-ARS
1636 East Alisel Street
Salinas, CA 93905

Dr. H. Alan Wood
Boyce Thompson Institute for Plant Research
Tower Road
Ithaca, NY 14853-1801

Dr. Zhongguo Xiong
University of Arizona
Department of Plant Pathology
Forbes 204
Tucson, AZ 85721

Dr. Stephen Yarrow
Agriculture and Agri-Food Canada
59 Camelot Drive
Nepean, Ontario, CANADA K1A 0Y9

Dr. Luz Marcela Yepes
Cornell University
Department of Plant Pathology
Geneva, NY 14456

Dr. James Zalewski
NatureMark Potatoes
300 East Mallard Drive
Boise, ID 83706

Dr. Paul Zubkoff
U.S. Environmental Protection Agency
Ofc of Pesticide Pgms/Biopesticides
401 M Street, S.W.
Washington, DC 20460

Appendix C

Program Committee and Speakers

Dr. Sue Loesch-Fries (Chair)
Dept. of Botany and Plant Path.
Purdue University
West Lafayette, IN 47907-1155

Dr. Steve Lommel
Dept. of Plant Path.
North Carolina State University
Raleigh, NC 27695-7616

Dr. Mark Young*
Dept. of Plant Path.
Montana State University
Bozeman, MT 59717

Dr. James White
USDA-APHIS
4700 River Road
Riverdale, MD 20737

Dr. William Dougherty
Dept. of Microbiology
Oregon State University
Corvallis, OR 97331

Dr. James Carrington*
Dept. of Biology
Texas A&M University
College Station, TX 77843

Dr. Richard Allison
Dept. of Botany and Plant Path.
Michigan State University
East Lansing, MI 48824

Dr. Vicki Vance
Dept. of Biology
University of South Carolina
Columbia, SC 29208

Dr. William Dawson
Citrus REC
University of Florida
Lake Alfred, FL 33850-2299

Dr. Bernice Slutsky
OPPTS
U.S. EPA
Washington, DC 20460

Speakers

Dr. Sally McCammon
USDA-APHIS
Administrator's Office
Washington, DC 20090

Dr. Rebecca Grumet*
Dept. of Horticulture
Michigan State University
East Lansing, MI 48824

Dr. Roger Beachy*
Scripps Research Institute
10280 N. Torrey Pine Road
La Jolla, CA 92037

Dr. Milton Zaitlin*
Dept. of Plant Path.
Cornell University
Ithaca, NY 14853

AIBS Staff

Dr. Clifford Gabriel
Ms. Donna Haegele
Ms. Kathy Marshall
American Institute of Biological Sciences
730 11th Street, NW
Washington, DC 20001-4521

*Breakout session chair