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PERSPECTIVES ON PROGRESS IN PLANT VIROLOGY

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INTRODUCTION

The editors asked that I write a chapter on landmarks in plant virology, a topic that has been covered by several authors, for example by Henderson Smith (89), Bawden (9), Holmes (51), Markham (62), Harrison (44), Black (12), and Matthews (64). In 1938, the first of these authors, Henderson Smith (89), divided his presidential address to the Society for Applied Biology between the control of plant-virus diseases and the nature of plant viruses. Progress since then has been much more rapid in the latter than in the former area. Henderson Smith could easily understand today's literature on losses, control, breeding for resistance, and vector relations. He would find new virus diseases and vectors, but the concepts would be familiar. However, he would be completely lost trying to read about the nature of virus particles. There he would find references to ssRNA, dsRNA, translation, transcription, reading frames, site-directed mutagenesis, subgenomic RNAs, genome-linked proteins, and many other terms and concepts that have appeared in the past few decades. Nor would he recognize the experimental techniques, for most of those now commonly used have been developed since 1938.

As interpreted by previous authors, landmarks are discoveries in plant virology that significantly affect subsequent research in the field. Each author has a somewhat different view of the landmarks, depending on interests and background. I had no formal training in plant pathology or virology. Educated

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as a biochemist, my first contact with plant virology was in 1947 when I worked with Dr. L. M. Black at the Brooklyn Botanic Garden on wound-tumor virus. I learned what virology I know from Dr. Black and from experience, reading, and listening to various scientists as they gave papers or chatted informally. This type of education leaves one with gaps in his knowledge and nonstandard conceptions of ideas that are "generally recognized as true" (GRAT). Once an idea is placed in the GRAT category, it can be difficult to remove it. My views of the changes in these GRAT ideas and of important discoveries are necessarily colored by my background. I agree fully that the accomplishments cited by previous authors are important landmarks. However, rather than repeat what has been said before, I will discuss some accomplishments or landmarks in related sciences that have influenced plant virology, some relatively neglected landmarks, and recent results that may be landmarks for the future.

LANDMARKS FROM RELATED SCIENCES

Virology is not an isolated science. Virologists formulate hypotheses within the framework of biological theories and test them with techniques of biochemistry, molecular biology, and biology as well as of plant pathology. Virology and the rest of biology are interlocked and must develop together. Many of the landmark discoveries influential in virology were not made in virology proper, but in biology or biochemistry. In turn, advances in virology have often set the pace in biology.

The advances in biology and biochemistry include not only techniques and identifiable theories, but also a decrease in the mysticism that once limited our ability to think about questions in virology. The mysteries included not only the nature of genes, but also the nature of enzymes, mechanisms of protein synthesis and denaturation, the structure of nucleic acids, and much else.

In this section, I discuss a few examples of how the decrease in mysticism in biochemistry and biology has had a major effect on plant virology.

Proteins

Forty years ago, proteins were just emerging from the clouds of mystery. Most biochemists accepted the theory that they were polypeptides. However, the known amino-acid composition did not add up to 100% for a single protein. The possibility still existed that unknown chemical constituents would account for some of proteins' unusual properties. Consider denaturation, for example. The ability to be denatured almost defined proteins. The original meaning of denaturation was a change in properties, usually a decrease in solubility or loss of enzymatic activity. The reason for the change was unknown. Few good techniques for studying native proteins existed, and

none for denatured ones. The denatured protein was pelleted out, discarded and, of necessity, forgotten.

It slowly became accepted in the 1940s and 1950s that denaturation was the unfolding of the peptide chain (57). In many respects, denatured proteins are easier to work with than are native ones. In contrast to native proteins, most denatured proteins have similar solubilities. Native proteins have a great tendency to associate with other proteins, and their properties depend on which other proteins are present. When dissolved in denaturing solvents, denatured proteins usually do not aggregate with one another. Therefore, analysis of denatured proteins in denaturing solvents can be relatively unambiguous. The importance of SDS-PAGE (polacrylamide gel electrophoresis of sodium dodecyl sulfate derivatives of denatured proteins) is perhaps best appreciated by thosc of us that did research without it. One of the most useful solvents for denatured proteins, sodium dodecyl sulfate (SDS), is a contribution of plant virology. It was introduced into biochemistry as an agent to disrupt TMV (78).

We now think of proteins and nucleic acids as well-defined entities with a structure that can be determined. Forty years ago not everyone believed that proteins were discrete entities (22). It seemed unlikely that a molecule as large as a protein could have a structure as precisely defined as, say, benzene. A number of protein enzymes had been crystallized, but many proteins could not be purified to homogeneity. For example, seed storage proteins always seemed to be mixtures whose composition depended on the method of purification. This heterogeneity was compatible with the idea that biological systems were variable, whereas chemical compounds were invariable in composition. Proteins belonged in the biological sphere. So did viruses, and the question of their homogeneity arose. Pirie (77) carefully considered the available evidence and concluded that infectious particles of TMV might vary in size and properties. The elucidation of the role of DNA both in genetics and in prccisely specifying the sequcnce of amino acids in a protein established as GRAT the proposition that proteins have a discrete structure.

Even with modern methods, purified viruses have particles of a range of sizes and properties. But sequencing studies of proteins and nucleic acids have shown that most have identical sequences (37) and furthermore that a single well-defined sequence is sufficient for infection and disease (4, 23, 70). The heteogenous structures are confidently disregarded as errors of synthesis or assembly, or mutants, which can be ignored, unless, of course, one is interested in assembly or mutants.

Nucleic Acids

Our knowledge of nucleic acids (apart from the chemistry of nucleotides, nucleosides, and the bases) has evolved in the last 50 years. This knowledge

has been very important for progress in plant virology in particular and, of course, for the rest of plant-, animal-, and microbial-biology as well. Research in plant virology has contributed to these discoveries, e.g. the fact that TMW RNA was infectious (36) was the third line of direct experimental evidence that nucleic acids by themselves carried genetic information, and the first evidence that RNA could do so. Previous evidence indicated that plant virus-infectivity required RNA, but not that it was sufficient. The various strategies by which plant viruses express their genetic information within the confines of the plant cell reveal much about plant-cell biology. Thus, to adapt to the preference of the plant cell for translating monocistronic messenger RNAs and still produce the multiple proteins they need, plant viruses have developed several strategies: They have subgenomic messenger RNAs, multiple component genetic RNAs that can serve as monocistronic messengers, overlapping reading frames, and production of polyproteins and proteases to cleave them after translation (6).

Genetic concepts were abstract before the elucidation of nucleic-acid chemistry and the genetic code. In my informal education in biology, I learned that mutation was a heritable change, almost always recessive. When I asked about the source of natural mutations, I was told that no one knew for sure, but that they probably were caused by cosmic radiations. I realized rather slowly the full implications of McClintock's claims for genetic or biological mechanisms for mutations (65).

Mutations have been studied and used in plant virology since McKinney reported them in 1935 (66). But the experimental use we make of them and the conclusions we draw have been changed in a major way by the advances in nucleic-acid chemistry. It was easy to conceive of information being lost owing to the impact of ionizing radiation or the action of a chemical mutagen. But it was difficult to conceive of information being gained by such events. Surely genetic information had to come from somewhere, it could not be created de novo. As understood on the basis of nucleic-acid chemistry, "mutation" covers a multitude of events. Common causes are rearrangements and insertions. Insertions of nucleic-acid segments into the coding region can produce a larger gene, an apparent gain in information. As an example, consider the RNA gamma of barley stripe-mosaic virus, which in the Type strain is larger than in the ND18 strain. Nucleic-acid sequencing showed a 366-base direct tandem repeat in RNA from the Type strain but not in ND18 (41). At one time, I would not have seriously considered that Type could have arisen from ND18, because I could not conceive how genetic information could be created from nothing. Now it seems obvious that either strain could have arisen from the other and probable that Type arose from ND18 through duplication of a RNA segment.

Serology

Early virologists recognized the value of antibodies that animals produce when foreign proteins are injected into them (104). Our current understanding of the complex animal immune system is one of the triumphs of molecular biology. In the early days, serology was as mysterious as the immune system. Animals, usually rabbits, were injected with purified virus and bled after several weeks. The serum from the blood would precipitate purified virus. The potency of the serum varied from animal to animal, with time after injection, and according to the skill of the experimenter. A negative test could be due to too much virus or antibody as well as too little. None of these variations could be explained with certitude. Negative tests were generally interpreted as evidence that no reaction had occurred between the antigen and antibody. There was no explanation as to why the antigen and antibody would react at lower concentrations in the presence, e.g. of latex particles, than in their absence. Realization that the reactions between antigen and antibody occurred at very low concentrations, but that the reactions could not be readily detected, stimulated a search for sensitive methods to detect the reactions resulting in such tests as ELISA, radioimmunoassays, and electron microscope assisted assays.

Development of monoclonal antibodies not only improved specificity of virus detection, but also explained the differences that had been observed among antisera from different animals and from different bleedings from the same animal, as well as lack of agreement of reciprocal cross reactions between heterologous antisera and heterologous antigens. Apparently complex aspects of serology became simple when it was realized that ordinary antisera are polyclonal, a complex mixture of antibodies.

These developments in immunology along with our improved understanding of proteins and the nature of antigenic groups have changed serology from an art to a science (104). We now can logically decide to use monoclonal (42) or polyclonal antisera, which may be to virions or to capsid proteins, denatured capsid proteins, other virus-coded proteins, or to synthetic peptides whose sequence is deduced from the nucleotide sequence.

UNMARKED LANDMARKS

Many important plant-virological findings have, nevertheless, stimulated less research than I expected. There are various reasons for this. Sometimes the techniques are difficult, and experiments time consuming. Researchers may accept the results without repeating them. Or the experiments may involve unfamiliar viruses or procedures. I discuss a few examples of this research

because I think the results are important, or simply because the results changed my idea of what is GRAT.

Inclusion Bodies

Inclusion bodies were associated with virus diseases long before infectious particles were identified. The association of inclusions with virus diseases has never been questioned, but their nature has never been entirely clear. By light microscopy, they resembled protozoa or cell organelles. At first it was uncertain if they were a response of the plant to the infection, a product of the virus, or the infectious virus itself. Were they cause or effect? Steere & Williams (94) were the first to definitely identify one type of inclusion body when they showed that the crystalline plates seen in TMV-infected cells were composed of virus particles. Some other inclusions, but not all, are composed of virions. The nuclear inclusion bodies of tobacco etch virus have two proteins. One is the protease that cleaves the polyprotein that is the primary translation product (19). Some inclusions, the viroplasms, are thought to be sites of virion assembly or synthesis. The function of others, such as the pinwheel inclusions, remains speculative.

The potential usefulness of inclusion bodies for identification of viruses has been repeatedly pointed out, and repeatedly ignored by most of us. Edwardson (29) classified Potyviruses partly on the basis of the ultrastructure of inclusion bodies. Jensen (56) further showed that pinwheel-inclusion body proteins were useful to classify strains of maize dwarf mosaic virus. Perhaps plant virologists will use inclusion bodies when they have antisera to the constituent proteins and can use the ELISA test.

Plant virus-inclusion bodies have been reviewed by McWhorter (68) and Martelli & Russo (63).

Replication of Plant Viruses in Vectors

For some reason, leafhopper-transmitted plant viruses were unknown in Europe and Great Britian before 1950, though they had been reported from North America, Japan, and Africa. Many of the leafhopper-transmitted viruses could not be transmitted to another plant by the leafhopper until after a latent period of days or weeks after the start of the acquisition feeding. With aphid-transmitted viruses, the latent period, if present, was only a matter of hours or a day. Multiplication of the virus in the leafhopper was one possible explanation of the long incubation period. Two approaches showed that this was indeed the case (13). Rice dwarf virus passes through the egg to the next generation. Fukushi (32) carefully removed freshly hatched nymphs before they could feed and placed them on healthy plants. The nymphs were transferred to fresh healthy rice plants daily to be sure they were always feeding on

uninfected plants. Even after seven generations, the leafhoppers transmitted virus to the same proportion of plants as the original insects had.

In the second type of experiment, the virus was transferred from one generation of insects to another by injection with extracts from infected insects. Black and I (14) transmitted wound-tumor virus through seven generations of insects. The insects were raised on a cultivar of alfalfa immune to the virus. Extracts from the seventh generation of leafhoppers were as infectious as those from the first. Similar experiments showed that wheat striate mosaic virus (87) and oat blue dwarf virus (7) multiplied in their leafhopper vectors. Sylvester & Richardson (95) showed that sowthistle yellow vein virus multiplied in its aphid vector. In addition, pea enation mosaic virus multiplies in cultured cells of its aphid vector (2), and potato yellow dwarf virus in cells of its leafhopper vector (21). These findings lead to the conclusion that the viruses multiplied in the insect vector as well as in the plant (13).

This conclusion was initially greeted with skepticism by Bawden, perhaps the most influential plant virologist in England, though eventually he was also convinced (13, 32). At first glance, it is surprising that a virus such as wound-tumor virus can multiply in such widely different organisms as leafhoppers and sweet clover but can fail to multiply in alfalfa. The cellular environment that the virus requires must be common to many cells, but at the same time, there must be specific features of certain cells that make them unsuitable for virus multiplication.

Insect Tissue Cultures as a Tool for Studying Plant Viruses

Monolayer cultures of animal cells have provided an invaluable tool for the assay and propagation of animal viruses. Plant cells do not grow as monolayers in tissue culture but as callus or as dispersed cells. Protoplasts of plant cells are good for certain experiments, but not for plaque assays. They cannot be maintained in culture long enough to serve for propagation of virus. This lack of a monolayer tissue-culture system has prevented plant virologists from adopting standard animal- and bacterial-virus technology.

This drawback was rectified for viruses that multiply in vectors by the development of monolayer tissue cultures of leafhopper and aphid cells (11). The leafhopper cells are obtained from embryonated eggs (50). The culture media and the use of high-quality components are very important. With proper attention to these points, Black (11) maintained some leafhopper cell lines through more than 300 passages.

Vector monolayer cell cultures provide the most sensitive bioassay available for viruses of flowering plants. Hsu & Black (52) found that the dilution end point of potato yellow dwarf virus was 300–600 times greater on vector cell monolayers than on leaves. On the basis of infections per number of cells

inoculated, the monolayer assay was 5000 times more sensitive than the leaf assay. This is the only virus of the group for which a direct comparison between leaves and monolayers is possible because it is the only plant virus that multiplies in vectors and can be mechanically inoculated to plants.

Vector monolayer cell cultures provide a powerful tool for the study of the viruses that multiply in vectors, especially since most of them cannot be mechanically transmitted. Before the advent of the monolayers, wound-tumor virus was assayed by injecting leafhoppers which were then fed on plants that eventually developed symptoms if the original extract contained virus particles (61). Completion of the assay took 3 months, compared to about 2 days for assays on vector cell monolayers. Furthermore the assays on vector cell monolayers have a lower coefficient of variation than local lesions or insect injection assays.

Virus-Induced Mutations in Maize

H. H. McKinney was a virologist of great originality, an independent thinker with his own set of GRAT concepts. He was cautious in his conclusions, and his accomplishments have not been appreciated fully. As a graduate student in the early 1920s, he investigated the yellow spots in TMV-infected tobacco and soon concluded they were due to mutations of the virus. Convinced that his professors would not believe him, and at the same time fearful that they would take the problem from him and assign it to others, he left the University of Wisconsin without his Ph D and went to work on soilborne wheat mosaic virus for the US Department of Agriculture. He eventually showed that the soilborne wheat mosaic virus had a living soil organism for a vector (67). He and Linford probably would have been the first to identify a fungus *(Polymyxa graminis)* as a plant-virus vector if they had not lost the use of borrowed space in a cold temperature growth room. In addition to his assigned research on wheat viruses, McKinney continued research on tobacco viruses and mutants (66). He separated strains for every virus he worked on and showed that one strain could block (or interfere with) the infection of a second, related strain. He consistently refused to separate strains by local lesions, preferring to isolate them from areas of the leaf showing unique symptoms. He observed and frequently discoursed on the similarity between symptoms of virus diseases and mutant phenotypes of the host plant. From time to time, he tried to find a relation between virus diseases and mutant plants. These attempts were unsuccessful until, after he had retired in 1959, he collaborated with George Sprague, a maize geneticist.

Sprague and McKinney showed that progeny of maize plants infected with certain viruses had a higher frequency of mutation than those from uninfected maize plants (90–92). Three viruses (barley stripe mosaic, wheat streak mosaic, and corn lily fleck) could induce the effect (the aberrant ratio

phenomenon) if the corn was infected when the tassel and pollen were differentiating. The higher mutation rate is apparently due to activation of transposon systems by the virus disease (17, 72, 76).

This unusual symptom is of little practical importance, but it has interested geneticists as an example of plants responding to stress by increasing mutation rates and variability (65). It would be a long-term response in contrast to the production of toxic secondary metabolites (such as phytoalexins), which are a short-term response to stress. While experimental evidence of the influence of virus infection on plant genes is still limited to a few hosts and viruses, such effects cannot be casually observed. Hence, they may be more common than present reports indicate.

Why Some Viruses Are Not Mechanically Transmissible

Most of the viruses with leafhopper vectors and many of those with aphid vectors cannot be transmitted mechanically. These viruses also proved difficult to purify, and none was purified for nearly 30 years after TMV was purified. At one time, there were two explanations for the lack of mechanical transmission and the resistance to purification. One was that these viruses were present in low concentration, and the other that these viruses were in some way fundamentally different than TMV and the other mechanically transmissible viruses. Both answers proved to be partly correct; and in addition there was a third answer: tissue localization.

Luteoviruses are localized in the phloem, and this is probably one reason they are difficult to purify and impossible to transmit mechanically (45, 55). The usual methods of mechanical transmission do not put these viruses into the same cells as the aphids do. The phloem tissue, being difficult to grind, is a poor source for virus purification. Some of these nonmechanically transmissible viruses are present in very low concentration, but others are present in low to moderate concentration. Wound-tumor virus is present in up to 10^{12} particles per gram of clover tumor-tissue—a relatively high concentration (58). Wound-tumor virus is limited to phloem or protophloem tissue in the sweet-clover tumors (73). Maize chlorotic dwarf virus, which is not mechanically transmissible, is present in about the same concentration as maize dwarf mosaic virus, strain A, which is mechanically transmissible (39).

Finally, some of these "viruses" did prove to be fundamentally different from other viruses. Aster yellows virus was considered the archetype of the yellows viruses, the largest group of nonmechanically transmissible, aphid- or leafhopper-transmitted viruses. Aster yellows and many of the other yellows "viruses" were later shown to be mycoplasma-like organisms, a type of bacteria (15, 28). They are fastidious in their nutrient requirements and cannot be cultured as easily as typical bacteria. One subgroup, the spiroplasmas, has been cultured in vitro (31, 83).

Concerning Experimental Hosts

Forty years ago when I started working on plant viruses, it was GRAT that that the best experimental host was the natural host or a closely related plant. Viruses of tobacco and potato were studied in tobacco, potato, and other solanaceous plants. That caused no problems, but when viruses of fruit trees were studied just in fruit trees, there was a problem: little progress. The viruses could not be sap-transmitted from tree to tree. They could only be transmitted by grafting. Many of the source trees were infected with mixtures, and the tester trees might or might not be infected with a symptomless virus. There was no sure way to tell. In the mid 1950s, I sat through a North Central regional meeting of stone-fruit virologists and listened to numerous reports of attempts to identify viruses by grafting to tester trees. To a novice such as myself, there seemed to be as many viruses, strains, and/or mixtures as the product of sick trees by tester trees.

Though Hildebrand recovered tomato ringspot virus from currants in 1942 (49), general use of herbaceous hosts for the study of viruses of fruit trees really dates from 1948 when Moore et al (71) showed that a virus from stone fruit trees could be sap-transmitted to cucumber. Subsequent progress was rapid as these viruses were purified and characterized, and antisera were prepared. Fulton was an important contributor and has reviewed the subject (33).

Noncapsid Viral Proteins

In the beginning, it was difficult to identify the particles causing virus diseases. After TMV was purified and scientists knew what to look for, and with the advent of the electron microscope, good centrifugal methods, the analytical ultracentrifuge, and sucrose density gradient centrifugation (16), it was relatively easy to identify virions. With further characterization of virions, identification of the RNA as the genetic component, discovery of the 3-base genetic code and estimation of the size of the RNA, it was apparent that the viruses could code for two or more proteins, in addition to the capsid protein. It was postulated that these extra proteins would prove to be enzymes involved in RNA replication. The first evidence that these proteins were produced in infected plants was the double labeling experiments of Zaitlin & Hariharasubramanian in 1972 (111). Infected plants were labeled with one isotope and healthy plants with another. Extracts of the plants were mixed, and the proteins were separated by gel electrophoresis. The ratio of the two isotopes differed from the average in slices containing proteins synthesized only in the infected plants. These proteins were assumed to be viral-coded, although they could have been host proteins produced in increased amounts as a result of infection.

These experiments indicated to me that noncapsid viral-coded proteins were produced in infected plants, but at low concentrations, and could only be detected by sensitive procedures. This low concentration was consistent with the idea that these proteins were enzymatic. However, the conclusion that these proteins are invariably present only in low concentration is incorrect. Inclusion body proteins are produced in about as high a concentration as capsid proteins in plants infected with Potyviruses and other viruses with long, flexuous virions such as wheat streak mosaic virus and wheat spindle streak mosaic virus (18, 19, 48, 56). These proteins are as easy to purify by centrifugation and gel electrophoresis as virions. Specific antisera can be produced and could be used to identify the viruses, much as antisera to virions are used. Barley stripe mosaic infection also produces high concentrations of a noncapsid viral protein, but only in leaves with an acute stage of infection (54). However, this is not an inclusion body protein.

Why is there such a high concentration of these proteins? Suggested functions—to facilitate transport or to replicate RNA—would not need such high concentrations because these are basically enzymatic or catalytic functions. The protease of tobacco etch virus appears to be present in great excess over the amount needed for its enzymatic function (19). Do these viruses lack a regulatory mechanism and keep producing because they cannot stop? Or am I again a victim of *my* GRAT concepts?

RECENT LANDMARKS

Viruses Infecting Eukaryotic Chlorella-like Green Algae

Plant virologist have studied viruses of crop plants almost exclusively. These viruses and hosts have drawbacks as experimental systems. They usually are not ideal model systems for basic investigations of relations of viruses and eukaryotic plant cells. Recently discovered viruses of unicellular, eukaryotic *Chlorella*-like algae offer such a model system. They have also raised fascinating questions about the possible role of viruses in symbiotic systems.

Hydrae are simple animals, related to jellyfish, and mostly plankton-eaters. Those of one species, *Hydra viridis,* obtain food from symbiotic, intracellular, *Chlorella*-like green algae. A hydra can be freed of the alga and will grow if supplied with an energy source. However, it has been difficult to grow the alga after separating it from the hydra. Meints et al (69) found that the alga succumbed to a lytic virus infection soon after separation from the hydra. Morphologically similar viruses were isolated from symbiotic Chlorella in *Paramecium bursaria* and four additional sources of Hydrae (99). These viruses are large polyhedra with about 50 proteins, 5–10% lipid, and a dsDNA genome of about 300 kbp (88, 98, 103).

Research on the virus (PBCV-1) from *Chlorella* symbiotic in *P. bursaria*

has been facilitated by the discovery that it will infect a *Chlorella* (NC64a) that had been isolated from a symbiotic association with *P. bursaria* (therefore, "exsymbiotic") (100). In addition to PBCV-1, Van Etten et al (102) found viruses infectious to NC64a in 37% of 35 water samples collected from various sites in the United States. Similar viruses infectious to NC64a have since been found in surface waters from China and Japan (J. L. Van Etten, personal communication.). Many European surface waters contain viruses that infect a *Chlorella* exsymbiotic from an European strain of *P. bursaria* (80).

The algae exsymbiotic from *P. bursaria* can be grown on a lawn, which gives plaques after infection with virus (100). This highly sensitive assay and other properties of the system resemble the bacteriophage-bacteria system and make these large dsDNA algal viruses an excellent experimental model system (103).

The DNAs of the viruses that infect NC64a show a wide range of methylation, from no m^6dA and 0.1% m^5dC at the low end of the range to 37% m^6dA and 45% m^5dC at the high end (101); the result of these is variable sensitivity to restriction endonucleases (85). The viruses induce formation of DNA-restriction endonucleases and DNA methyltransferases in infected algae (107–110). The variation in the extent of methylation from virus to virus suggests that the methylation and restriction endonucleases are viral-coded. This hypothesis has been confirmed by the cloning of the viral gene encoding one methyltransferase (74). This is the first restriction-methylation system to be found outside of prokaryotic organisms.

As expected, these algal viruses are susceptible to genetic manipulation in the pattern of the DNA bacteriophages (96).

The ecological role of these viruses is unknown. Their complexity and widespread occurrence indicate that they are evolutionarily ancient (103).

Application of Recombinant DNA Techniques to Plant Viruses

There have been tremendous advances in understanding the translation strategy and genome structure of plant viruses in the last decade, primarily because of powerful new techniques in molecular biology. The most powerful of these are the recombinant DNA techniques.

VIROIDS, VIRUSOIDS, AND SATELLITE VIRUSES Because of their small size, plant virus genomes are relatively easy targets for sequencers, but viroids and satellite RNAs are even easier. In most aspects, application of recombinant-DNA techniques to viroids is ahead of the application to RNA viruses, despite the fact that viroids were discovered relatively recently (25–27, 81, 86). cDNA probes are routinely used for identification of potato spindle tuber viroid (53, 75). Plants can be infected with cDNA copies of

viroids inserted in Ti plasmids used to transform the plant. Numerous sequences of viroids and satellite RNAs have been reported, as well as site-directed mutagenesis. Comparisons of sequences have revealed portions that are conserved, others that are variable, and similarities to small nuclear RNAs (U1–U7) of higher organisms (24). This latter trait has lead to the hypothesis that viroids cause disease by interfering with RNA splicing.

VIRUSES The genomes of more than a dozen plant viruses have been sequenced, including ssRNA, dsDNA, and ssDNA genomes. Reports of additional sequences are appearing with increasing frequency. Knowledge of the nucleic-acid sequence allows the prediction of the number of viral proteins and their amino-acid sequences. Comparison with sequences of proteins of known function permits guesses about the function of the proteins. The presumed RNA replicases of several plant viruses have sequence similarities, even though the viruses are not obviously related (5, 47). Taxonomic relations between animal and plant viruses with ssRNA genomes have been suggested on the basis of sequence comparisons (38). The nucleic-acid sequences also allow the selection or construction of hybridization probes for identification that are either highly selective (based on unique sequences), or that will detect related viruses (based on conserved sequences).

Plasmids containing complete genomes of the DNA viruses have often been infectious (34, 43, 93). The plasmids of the cDNA of ssRNA viruses appear not to be infectious. However, RNAs transcribed from the cloned cDNA of brome mosaic virus (BMV) (4) and TMV (23, 70) are infectious. This makes it possible to apply the techniques for modifying DNA to genomes of RNA plant viruses and to test the result on the biological activity of the virus. Saito et al (84) have already shown that the coat-protein gene of TMV is responsible for the necrotic response to the N' resistance gene of *Nicotiana sylvestris*. Knorr & Dawson (59) further traced this response to the substitution of uridine for cytosine at position 6157 of the RNA. This results in phenylalanine instead of serine at position 148 of the coat protein. It is not known if the change in the protein or in the RNA is responsible for the phenotype change.

Properties of infectious BMV transcripts have been reviewed (3). The genes for RNA replication are carried by RNAs I and II of BMV. Even after rather extensive modifications, RNA III is still replicated in the presence of RNA I and II. The gene for chloramphenicol acetyltransferase was inserted into RNA III in place of the coat-protein gene. Significant amounts of chloramphenicol acetyltransferase were produced in barley protoplasts infected with the altered virus (30), which illustrates the use of this virus as a vector for introducing new genetic information into plant cells. However, viral sequences did not insert into nuclear DNA.

In another experiment, in vivo genetic recombination for this RNA plant

virus was proven by inoculating a plant with a strain having nucleotides deleted from positions 80–100 from the 3' end of RNA III. The mutant RNA III replicated more slowly than wild type. After prolonged replication, wild type RNA III was recovered. The only known source for the recovered wild type RNA III is recombination between either RNA I or RNA II and the mutant RNA III, which results in an exchange of at least 100 bases near the 3' end (3). Except for a few bases, the sequences near the 3' ends are the same for all 3 genomic RNAs. The few differences serve as markers to confirm the recombination. Evidence for such genetic recombination had long been sought for RNA plant viruses (10, 105), but interpretation of previous results had been equivocal because of possible reassociation of multicomponent genomes, mutations, or inadequate evidence for purity of original strains. Thirty years ago, the evidence for recombination of BMV RNA would have been viewed as important evidence that RNA could be as competent a genetic material as DNA, and fifty years ago as evidence that viruses were alive. Now, the results may be viewed as just more evidence of the versatility of RNA.

Another potential method to obtain infections from a cloned genome of a plant virus is to transform plants with an *Agrobacterium* Ti plasmid containing a copy of the cloned DNA or cDNA (for an RNA virus). The procedure has been used with satellite viruses (35, 46) and cauliflower mosaic virus (40). More importantly, this technique can be used to introduce a single gene from a plant virus into the host and have it expressed. Abel et al (1) introduced the TMV coat-protein gene into tobacco plants. The transgenic plants expressing the coat-protein gene were partially resistant to infection by inoculation with TMV. Van Dun et al (97) reported a similar experiment with alfalfa mosaic virus. Their transgenic plants expressing the coat protein were resistant to infection when inoculated with virions but became infected when inoculated with a mixture of RNAs I, II, and III (a mixture that is infectious only if coat protein is present).

The goal of introducing the coat protein gene was partly a practical one of producing resistant plants. The Ti plasmid transformation is also a potentially very powerful technique for studying the function of virus genes. Introduction of the coat protein gene tells something about mechanisms of cross protection. Baughman et al (8) have introduced gene VI of cauliflower mosaic virus into tobacco plants that then developed mosaic-like symptoms. This result not only tells which virus gene is responsible for symptoms, it also suggests that the mosaic pattern is not always due simply to patterns of cells with different concentrations of virus.

Another approach to introducing resistance or tolerance into the host plant is to use the Ti plasmid to introduce a symptom-modulating satellite virus into the host genome. Harrison et al (46) thus introduced a cucumber mosaic virus

satellite RNA into tobacco, which was then partly resistant to infection with cucumber mosaic virus. Gerlach et al (35) introduced a satellite of tobacco ringspot virus into tobacco to produce a resistant plant.

The Versatile RNA

The dogma of molecular biology long held that the primary role of RNA was information transfer, with subsidiary structural roles in ribosomes and genetic roles in some viruses. However, the GRAT view of the role of RNA has drastically changed with the discovery by Cech and associates that RNA has enzymatic activity in RNA processing and might catalyze its own synthesis (20, 60, 112). RNA is now viewed as the one material that can perform all the essential functions of life, and as the best candidate for the original "living" material. RNA viruses are at center stage in this speculation as possible remnants of the "RNA world" (106).

The potential of RNA to perform many functions and the speculation it has spawned will strongly influence future plant-virus research. Already, it has been reported that satellite virus RNA has enzymatic activity and can self-splice (79). A low level of self-splicing (1–5%) was reported for viroids (82). Perhaps these theories will stimulate a new approach to the study of the replication of plant-virus RNA. One reason so little progress has been made in the search may be that we have been looking for the wrong thing in searching for a traditional protein enzyme as the RNA replicase.

SOME FUTURE PROSPECTS

Henderson Smith divided his remarks half a century ago between the nature of viruses and the control of virus diseases (89). He said nothing about how viruses cause disease, perhaps because of lack of information. Equally likely, the subject was in the realm of mysticism. Neither the ideas nor the language were available to discuss it.

The situation has not changed much. We still know little about the mechanism of symptom production, and seldom talk about it. But this will soon change. The few examples above illustrate the potential of "genetic engineering" techniques. We will continue to learn more about viruses as additional nucleic acids are sequenced. Moreover, as functions of individual virus genes are elucidated, we will learn of their importance in symptom production.

A major difficulty in studying how viruses cause symptoms in plants has been the lack of knowledge about plants. Many plant virus symptoms appear to result from interference with plant development. For example, chloroplasts develop abnormally in leaves with mosaic. Stunted plants may have fewer and smaller cells per leaf. The development of extra axial buds gives proliferation of shoots.

Our knowledge about control of regulation of gene expression in plant development has been superficial, but this, too, is changing. Techniques are now available to measure the expression of the three plant genomes. This, coupled with the ability to insert single viral genes into plants (8), and to make precise mutations in viral genes (59, 84), promises an exciting future in plant virology as questions on the mechanisms by which viruses cause symptoms and other mysteries are answered.

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