THE ROYAL SOCIETY

Tobacco mosaic virus and the study of early events in virus infections

John G. Shaw

Department of Plant Pathology, University of Kentucky, Lexington, KY 40546, USA

In order to establish infections, viruses must be delivered to the cells of potential hosts and must then engage in activities that enable their genomes to be expressed and replicated. With most viruses, the events that precede the onset of production of progeny virus particles are referred to as the early events and, in the case of positive-strand RNA viruses, they include the initial interaction with and entry of host cells and the release (uncoating) of the genome from the virus particles. Though the early events remain one of the more poorly understood areas of plant virology, the virus with which most of the relevant research has been performed is tobacco mosaic virus (TMV). In spite of this effort, there remains much uncertainty about the form or constituent of the virus that actually enters the initially invaded cell in a plant and about the mechanism(s) that trigger the subsequent uncoating (virion disassembly) reactions. A variety of approaches have been used in attempts to determine the fate of TMV particles that are involved in the establishment of an infection and these are briefly described in this review. In some recent work, it has been proposed that the uncoating process involves the bidirectional release of coat protein subunits from the viral RNA and that these activities may be mediated by cotranslational and coreplicational disassembly mechanisms.

Keywords: uncoating; disassembly; cotranslational disassembly; infection; early events

1. INTRODUCTION

Most historians who have described the founding of the science of virology in 1898 have also acknowledged the earlier work of Professor Adolf Mayer, who demonstrated that the mosaic disease of tobacco was caused by an infectious agent that could be transferred from diseased to healthy plants (Mayer 1886). With his experiments, performed during the period of 1882–1885 at the Agricultural Experiment Station in Wageningen, Mayer may be considered to have conducted the first investigation of the early events of a virus infection. The purpose of this article is to provide a brief review of what is known and not known about the initial events in the establishment of infections by tobacco mosaic virus (TMV). As TMV has been at the forefront of virology since the founding of the science, it will be appropriate to mention, in addition to the most recent contributions, some of the efforts to investigate this subject during the periods when the very nature of viruses was either uncertain or just beginning to be understood. In the spirit of the centennial celebration, the article will be restricted to studies with TMV, but it should be appreciated that the subject has been explored with other plant viruses and with many animal and bacterial viruses.

2. THE EARLY EVENTS

It has been the custom for many years to consider the initial events in virus infections to involve three stages: attachment, entry and uncoating. The process has been thought to begin at the moment virus particles have been delivered to some extra- or intracellular site in the potential victim, to include the passage of virus particles or parts thereof into one or more cells of the invaded organism, and to end when the genomes of these particles have been released (uncoated) in a state that permits their expression and replication. This has been a convenient way to designate the early events but, as will be noted below, discrimination between some of the stages, and between these and later stages, is becoming increasingly less obvious.

3. DELIVERY OF VIRUS PARTICLES TO PLANTS

In natural settings, many plant virus infections begin because some agent, in the process of satisfying its own nutritional or reproductive requirements, delivers virus particles to a plant where it has established temporary or permanent residence. Reports that insects and other arthropods were involved in such a role appeared during the first third of this century and, about 40 years ago, it was shown that nematodes and fungi could also transmit viruses from plant to plant. However, to our knowledge the first virus to be deliberately transferred from diseased to healthy plants was TMV, which is not transmitted by such organisms but rather by physical contact between plant tissue and virus-contaminated implements or surfaces. From a historian's perspective, this is an important feature of the virus, since plant virologists might this year be in a less celebratory mood if, instead of working with a disease caused by a virus as stable and infectious as TMV, Mayer (1886) and Beijerinck (1898) had been conducting their inoculation and filtration experiments

with a disease caused by one of the plant viruses that is not transmitted by manual inoculation. In the sense that its mode of delivery to plants is not that of so many other plant viruses, TMV might be considered a poorly representative subject for the study of the early events of infection. However, the ability of investigators to initiate infections by means of manual inoculation procedures has been essential in attempts to conduct such studies because the prospects for examining the fate of the minute number of virus particles delivered to plants by non-human agents under natural conditions are truly daunting.

4. MANUAL (MECHANICAL) INOCULATION OF PLANTS

As a result of the procedures used by medical researchers, Mayer probably expected that inoculation of a plant with a potentially infectious agent in a tissue extract would require that the material be injected into the internal parts of the plant. The technique he chose and the use by others of needles and other instruments with which to scratch parts of leaves to which droplets of virus-containing extracts had been applied, were both tedious and inefficient, but they did give rise to the notion that wounding of the tissues was required for success in establishing infections in plants. Wiping or rubbing the surfaces of leaves with extracts of infected plants was less time-consuming and more efficient and, with the presence of a powdered abrasive during the process (Fajardo 1930; Rawlins & Tompkins 1936), this became and remains the method of choice for the manual inoculation of plants with many viruses. For several decades, much effort was given to the establishment of conditions that would increase the efficiency of the inoculation procedure, but none of it provided an adequate explanation of the fate of the virus particles during the process or of the factors essential for the initiation of infection.

Most of the experiments in the past century that involved the manual inoculation of plants with viruses have been conducted for purposes other than investigation of the early events in the infection process. Assessments of viral viability and phenotype, quantitative estimations of virus content, host range studies, identification of viruses and purification of virus particles have been much more common reasons for manually inoculating plants. It is therefore understandable that, in many such applications, there should have been little thought given to the mechanisms involved in the establishment of the infections that were being generated, but an unfortunate consequence of this inattention has been that few virologists have registered much concern with this situation.

Approximately 30 years ago, a system that would prove to be very useful for the study of some of the early events of infection was developed (Takebe & Otsuki 1969). Protoplasts isolated from leaves of healthy plants were shown to be able to be inoculated with TMV particles or viral RNA if the inocula contained a polycationic substance such as poly-L-ornithine or a cell-fusing agent such as polyethylene glycol, or if they were electroporated in the presence of the virus. The proportion of cells in which primary infections could be established was much greater than could ever be achieved by the manual inoculation of intact plant tissues.

5. VIRUS ENTRY

Many years ago, it was proposed that the manual inoculation process involves the creation of small wounds in epidermal cells that underlie the formidable barrier of epicuticular waxes, cuticle and cell-wall materials in plant tissues. These wounds were considered to be sufficient to 'pierce leaf cells and allow virus particles to enter but in certain cases [they] do not injure the pierced cells sufficiently to prevent virus multiplication' (Rawlins & Tompkins 1936, p. 586). Later investigations favoured the idea that the attachment and entry processes occur very rapidly, perhaps simultaneously, and are irreversible (reviewed by Wildman 1959). These assumptions have been accepted by many plant virologists and experiments in support of them have occasionally been reported (e.g. Plaskitt *et al.* 1988). However, there have been reports (reviewed by Shaw 1985) in which some less direct methods of entry have been proposed (figure 1). The observation of TMV particles that had become fixed end-on to cuticular or outer cell-wall surfaces during manual inoculation prompted the suggestion of an extracellular attachment site with either the particles or uncoated viral RNA entering cells via channels (ectodesmata) or temporarily hydrated areas ('blebs') in the cell walls (Gerola *et al.* 1969; Gaard & De Zoeten 1979). Pinocytotic uptake of virus particles after their attachment to the cell membrane has also been mentioned as an entry mechanism. Unfortunately, there remain many questions about where the virus particles that will actually initiate infections are deposited during inoculation and how, where and in what form the virus becomes initially situated in the invaded cell. One of the main obstacles to the resolution of these questions is the presence in inocula of very large numbers of virus particles and the problem of determining which of these particles actually participate in the infection and which do not. Until the use of minimal doses of particles and more sensitive detection techniques are applied to tackle this problem, the uncertainties will remain.

In the years following the development of the protoplast system for plant virus research, there were several attempts to determine whether virus particles entered protoplasts by pinocytosis, by passage through wounds in the protoplast membrane or by some other mechanism. Again, the issue has not yet been resolved; most of the studies involving protoplasts have been undertaken with the objective of examining replication or some other activity that occurs after the early events in infection have been completed.

Another question that occasionally has been considered is whether TMV and other positive-strand RNA viruses of plants, like animal viruses, become attached to specific cell-surface receptors during inoculation. Attempts have been made to isolate substances from membranous fractions of plants that bind TMV particles and that might be such a receptor (Kiho 1974; Kiho *et al.* 1979), but these have not been fruitful. Because the inoculation of plant cells or protoplasts requires a physical treatment that perturbs or wounds cell surface structures, it seems

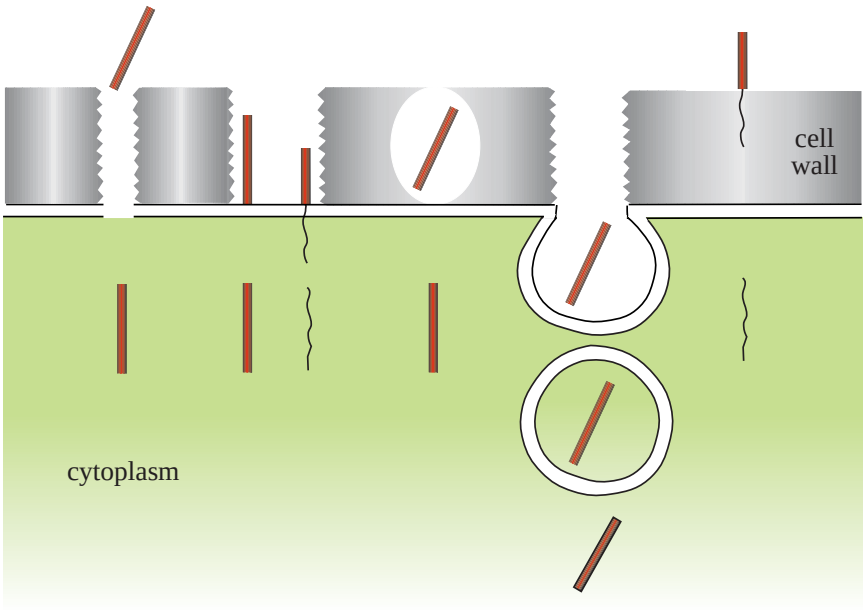

Figure 1. Proposed routes of entry of TMV particles during manual inoculation of leaves. None of these routes has been demonstrated directly and all remain unproven. Left to right: direct entry of virus particle through wound; attachment of virus particle to cell membrane and passage of virus particle or viral RNA into cell; passage of virus particle through cell wall via ectodesma or 'bleb'; attachment of virus particle to cell membrane and entry after invagination of membrane and formation of endocytotic vesicle; attachment of virus particle to outer cell wall and passage of viral RNA through wall into cell.

more likely that entry is accomplished by brute force rather than a receptor-mediated endocytotic mechanism. Of course, if inoculation does result in the deposition of intact plant virus particles in the cytoplasm of epidermal cells, there may well be substances or subcellular structures that play a receptor-like role in the post-entry events required for establishing an infection.

Once a single cell in a plant has become infected, the entry process can be considered to be completed since the virus is able to move to other cells and tissues via the symplastic continuum of the plant. Breaching of extracellular barriers of the type found in the surface layers of leaves should not again be necessary.

6. EARLY *IN VIVO* UNCOATING EXPERIMENTS

During the 20-year period from the mid-1930s, when the structure and chemical composition of viruses were being vigorously investigated, it was demonstrated that the infectious component of a virus particle is its nucleic acid and that this component is enclosed in a coat of protein molecules (subunits). With this information, it became apparent that the nucleic acid would be unable to perform its functions while confined to its physical state in the virus particle. Thus, it has been obvious for many decades that, once a positive-strand RNA virus particle has been delivered to some part of a potential host in which an infection may be initiated, it is essential that its genome be released so that translation of viral genes and replication of the viral RNA can occur. This release is referred to as uncoating of the viral genome or disassembly of the virus particle. If the process does not occur, there will be no infection and no disease.

Many of the investigations of TMV that began to appear shortly after the purification of the virus had been achieved dealt with the stability of purified virions and with the conditions under which they could be disassembled *in vitro*. Quite drastic treatments were shown to be required for the release of coat protein subunits and it has remained a fascinating feature of the virus that a nucleoprotein particle can be so extraordinarily stable in its isolated form but that it can come apart so readily in the seemingly benign environment within a living cell.

In the 1950s and 1960s, many experiments designed to characterize the infection process with TMV were reported and some of these were claimed to provide indirect evidence of virus particle disassembly after manual inoculation of leaves. 'Infective centres' in leaves inoculated with TMV RNA became resistant to subsequent inactivating treatments with ultraviolet irradiation (Siegel *et al.* 1957; Bawden & Kleczkowski 1960), acids (Semal 1962) or heat (Kassanis 1959) sooner than those in leaves inoculated with TMV particles. Leaves infiltrated with ribonuclease within a few hours of inoculation did not develop infections while those treated with the enzyme at later times became infected (Hamers-Casterman & Jeener 1957). Progeny virus was detected sooner or maximal numbers of lesions were produced sooner when leaves were inoculated with TMV RNA than with intact virions (Fraenkel-Conrat *et al.* 1958; Schramm & Engler 1958; Wu & Rappaport 1962). Some investigators concluded that the differences in the intervals observed in their experiments with the two types of inocula represented the stage during which virus particles were being disassembled. These differences ranged from one to a few hours which, even at the time, must have seemed rather lengthy periods for virus particle disassembly. Other investigators took a more sceptical view of the conclusions drawn from these types of experiments (e.g. Bawden 1964).

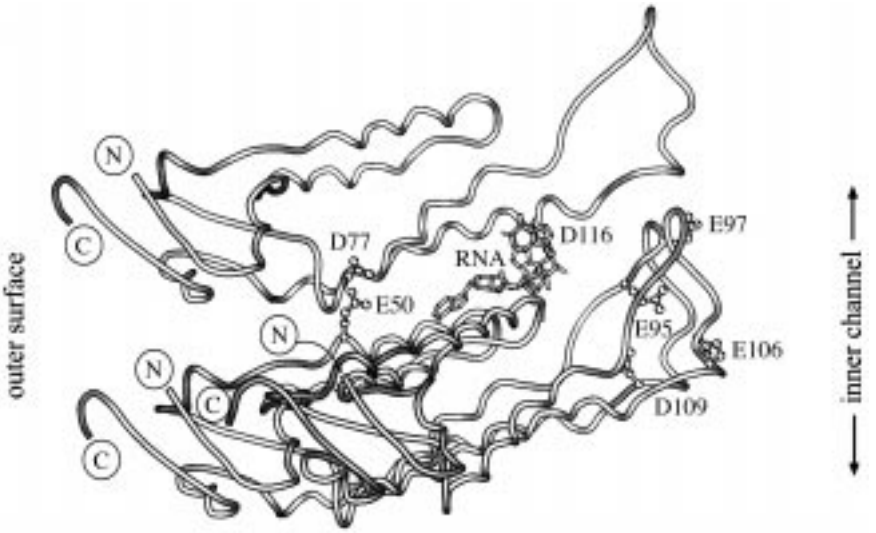

Figure 2. Ribbon diagram of three TMV coat protein subunits showing carboxylates involved in protein–protein and protein–RNA interactions. Two adjacent subunits in the same layer (bottom) and one in the next layer (top) of the helix, arranged as they would be in a virus particle in a vertical position, are shown. The outer surface of the virus particle is at the left. The viral RNA is marked as sticks; the side chains of amino acids the carboxylates of which are thought to be involved in virion disassembly are marked as ball-and-stick figures. D77 and E50 are axial interactions and the E95–E97–E106–D109 cluster is involved in lateral interactions. D116 carboxylates interact with RNA phosphates. Figure was kindly provided by G. Stubbs, Vanderbilt University.

It became clear that a more persuasive case for virus particle disassembly *in vivo* would require direct evidence of structural alterations of the particles at the earliest stages of the infection process. The most obvious way to address the problem seemed to be to perform inoculations with radioactive virus particles and reports involving this approach began to appear in the 1960s. Extracts of leaves inoculated with ^{32}P-labelled TMV particles and collected at various times thereafter were treated with ribonucleases and the amount of radioactive material in an acid-soluble form was taken as a measure of the extent of uncoating of the viral RNA (Reddi 1966; Kurtz-Fritsch & Hirth 1967; Hirashima & Hirai 1969*a*). Estimates of the time required before uncoating was initiated ranged from a few minutes to a few hours, but in these types of experiments the lengths of the uncoated RNA molecules could not be determined since they had been destroyed. In attempts to obtain such information, density-gradient centrifugation analyses of extracts of leaves inoculated with radioactive TMV particles were performed (Shaw 1967, 1969, 1970; Hirashima & Hirai 1969*b*). In these experiments, it was shown that, a few minutes after inoculation, some coat-protein subunits had been removed from the particles and uncoated viral RNA, a small proportion of it apparently of full length, could be detected. A large problem with these experiments was the lack of sensitivity of detection of virus particles and their components; in spite of the potential hazards involved, the 'hottest' preparations that could be produced in this laboratory were of the order of 50 000 particles per disintegrations per minute (dpm) ^{32}P. Large doses of virus had to be applied to leaves and this resulted in the presence in extracts of large numbers of particles that would not be involved in the establishment of infection but that would obscure the activities of those that were involved. What was gained from these types of experiments was an indication that disassembly of some TMV particles began very soon after inoculation, but details of the initiation, rate, extent and direction of uncoating were not obtained. More worrisome, there remained the possibility that the observed disassembly had actually been nothing more than the disintegration of particles that had remained on leaf surfaces rather than one of the first steps in the infection process (Kassanis & Kenten 1978).

Some attempts were made to determine the part of the TMV particle in which disassembly begins after manual inoculation. Various answers, including the end bearing the 5′-terminus of the RNA, were obtained (Kiho 1972; Shaw 1973; Hayashi 1974) but these experiments suffered from the dosage problem mentioned above and from the use of samples collected too long after inoculation.

During this period, prompted by the suggestion that protein–protein and protein–RNA interactions in TMV particles might be destabilized in lipid-containing structures in the cell (Caspar 1963), efforts were being made to determine the site at which disassembly occurred. One suggestion, derived from analyses of cation binding by virus particles, was that the virion is disassembled while penetrating the cell membrane as a result of release from the particle of calcium ions and the consequent decrease in stability of intersubunit interactions (Durham 1978). The energy for this process was claimed to be the calcium-ion gradient across the cell membrane. This model was embraced by some virologists but has not been adequately tested. Another view held that the virus particle is initially destabilized by contact with the cuticular surface of the plant and that the uncoated viral RNA passes through the cell wall and penetrates the plasmalemma of an epidermal cell (De Zoeten 1995). In this model, the released coat protein subunits were thought to play a role in a membrane-altering process that permits entry of the viral genome. The requirement for wounding was thought to be more important for subsequent replication than for the entry of the viral

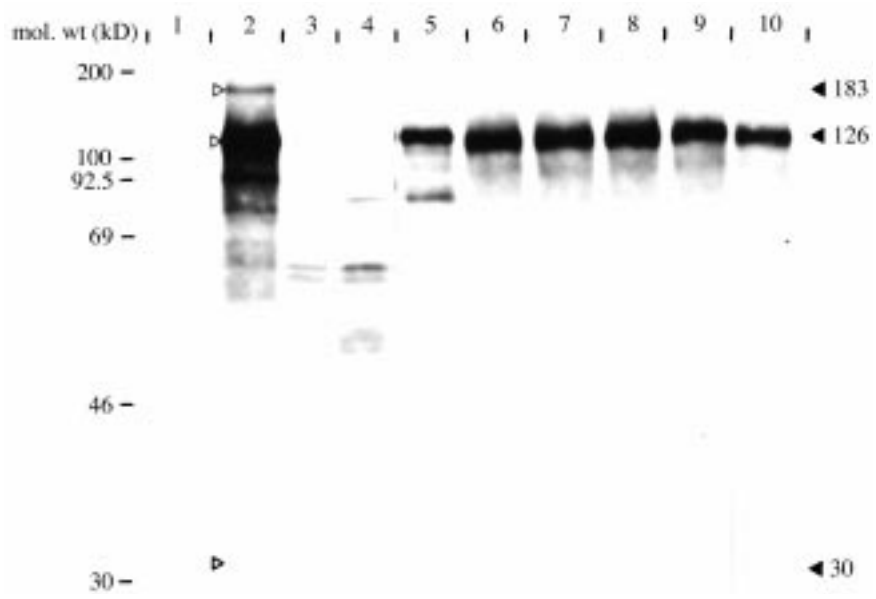

Figure 3. *In vitro* cotranslational disassembly of TMV. Electrophoretic resolution of the products of cell-free translation reactions programmed with TMV RNA (lane 2) or purified TMV particles that had been pretreated at pH 8.0–8.2 (lanes 5–10). Numbers at left show positions of markers; those at right are positions of TMV proteins. The appearance of the 126 kDa product provided evidence of a cotranslational disassembly mechanism. From Wilson (1984). Reproduced with the permission of Academic Press.

RNA. Like any of the other models of the early events, this one left unexplained important details, one of which is how the partially or completely uncoated viral RNA molecules make their way, and avoid degradation during their passage, from the cuticle or outer edge of the epidermal cell wall to the plasmalemma and then into the cytoplasm.

7. INTRA-PARTICLE INTERACTIONS INVOLVED IN VIRION DISASSEMBLY

Structural investigations of TMV began some 60 years ago and for many years the virus served as a model system for the study of protein–nucleic acid interactions and macromolecular assembly mechanisms. Even before a high-resolution X-ray diffraction analysis of viral coat protein assemblies had been achieved, it had been suggested that disassembly might be the result of the close proximity in the particles of carboxylate groups with abnormal pK values (Caspar 1963). The importance of these groups was noted in subsequent X-ray diffraction investigations of the coat protein and virus particles and, in an analysis of the virus at 2.9 Å resolution, three sites where carboxylate groups might promote electrostatic repulsion between subunits were identified (Namba *et al.* 1989). Two of these involved carboxyl–carboxylate interactions between axially or laterally adjacent subunits and another involved a carboxylatephosphate interaction between coat protein and viral RNA (figure 2). At least two of these sites were considered to be calcium-binding sites.

It has been proposed that *in vivo* disassembly occurs because the virus particle experiences a lowering of calcium-ion concentration and a raised pH when it enters the cell; the removal of calcium ions and protons from the carboxyl–carboxylate and carboxyl–phosphate sites then creates the electrostatic repulsion of proximal negative charges that destabilizes the virion (Namba *et al.* 1989). Subsequent mutational analyses of amino acid residues in the TMV coat protein have provided evidence that inter-subunit repulsion is involved in particle disassembly, though one of these interactions has been found to be more complex than originally predicted (Lu *et al.* 1996).

8. COTRANSLATIONAL DISASSEMBLY

One of the shortcomings of the earliest efforts to examine the *in vivo* disassembly of TMV particles was the lack of demonstration of the involvement of newly uncoated RNA molecules in an activity essential for continuation of the infection process. The issue was first addressed when an association of radioactive viral RNA with polyribosomes was reported in extracts of leaves inoculated with ^{32}P-labelled TMV particles (Kiho 1970) and it was suggested that translation of the RNA might begin as soon as the initiation codon had been uncoated. Since the initial activity in which the genomes of positive-strand RNA viruses must be engaged is translation of the first open reading frame, these experiments raised the possibility that uncoating and translation might be coupled activities. Unfortunately, a major effort to explore this idea was not undertaken and the study of this aspect of virus particle disassembly languished for over a decade.

A breakthrough in early events research finally came when Wilson (1984) reported the results of some *in vitro* translation experiments in which the reaction mixtures contained purified TMV particles rather than viral RNA. The virus particles had been briefly incubated in a buffer of pH 8 prior to addition to the cell-free system. Leaving aside the question of what might have prompted him to conduct such experiments with particles of a virus of such remarkable stability, the results must have been viewed

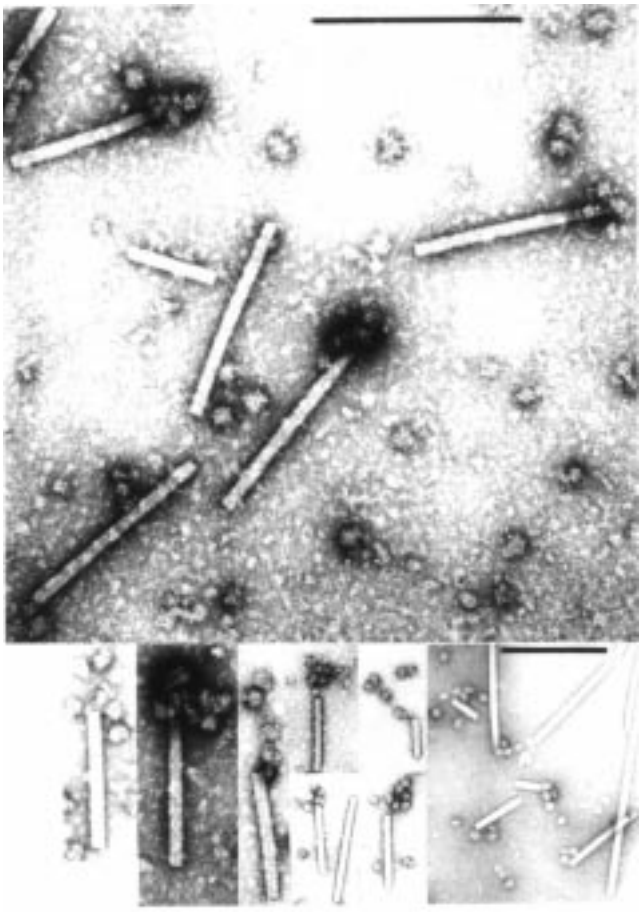

Figure 4. 'Striposome' complexes. Electron microscopic examination of the products of *in vitro* translation reactions programmed with TMV particles. One end of some of the particles is associated with structures thought to be ribosomes. The complexes are thought to be intermediates in the cotranslational disassembly process. From Wilson (1984). Reproduced with the permission of Academic Press.

with astonishment by Wilson—efficient synthesis of the entire 126 kDa protein encoded by the first open reading frame in the viral genome (figure 3). The same report included electron micrographs of complexes that had been sedimented from the reaction mixtures and that appeared to consist of ribosomes attached to one end of less-than-full-length virus particles (figure 4). It was concluded that the mildly alkaline pretreatment had in some way altered the stability of the end of the particle containing the 5′-terminus of the RNA such that 40S ribosomal subunits could begin scanning and ribosomes could dislodge coat protein subunits while the first open reading frame was being translated. The process was referred to as cotranslational disassembly and the complexes containing partially disassembled particles, ribosomes and, presumably, nascent polypeptide chains as 'striposomes'. It was later estimated that as many as 24 ribosomes could be detected in association with uncoated viral RNA in these complexes (J. W. Roenhorst, unpublished results in Verduin 1992).

Even though Wilson's experiments had been conducted with cell-free translation systems, the cotranslational disassembly hypothesis was immediately recognized as providing a very attractive mechanism for one of the key activities in the early events of the infection process. If *in vivo* disassembly involved this mechanism, the viral RNA would remain protected by coat protein subunits until the particle had reached a subcellular site in which translation could be initiated, i.e. uncoated TMV RNA molecules would not be swimming about the cell in a precarious state. In addition to its appeal, the hypothesis also opened two very important questions. What triggers the process? How does it continue beyond the first stop codon?

The question of how the virus particle is destabilized so that ribosome scanning can begin and the disassembly mechanism be triggered has been explored. Exposure of purified TMV particles to mild alkali or detergent treatment resulted in the rapid exposure of *ca.* 200 nucleotides at the 5′-end of the viral RNA in a subpopulation of the particles (Mundry *et al.* 1991). It was concluded that, at least in these particles, the 68-nucleotide 5′-leader sequence, which lacks G residues, interacts more weakly with coat protein subunits than do other regions of the genome and that the pretreatment results in uncoating of the leader sequence and the first AUG codon. It would seem logical to assume that, if cotranslational disassembly is involved in uncoating of TMV RNA *in vivo*, the virus particle must undergo a similar type of partial destabilization. What would trigger such a process and where it might occur in or on the inoculated cell are not known. The answer may be as simple as an encounter by the virus particle of pH and ionic strength conditions in the cytoplasm of the invaded cell that have the same effect as the particle pretreatments used by Mundry *et al.* (1991). However, it should be noted that this possibility may be based on a false analogy, since there is evidence that encapsidated derivatives of TMV RNA that are not destabilized by such treatments and cannot undergo cotranslational disassembly *in vitro* are nevertheless capable of being disassembled *in vivo* (Gallie *et al.* 1987; Wilson *et al.* 1990). Another possible triggering mechanism could involve a receptor-like subcellular component to which the incoming virus particle becomes attached and that somehow causes the degree of instability required for uncoating of the leader sequence. There is at present no evidence to support either of these suggestions or any alternative explanation of the triggering event, but the question is critically important if the cotranslational disassembly model operates *in vivo* and is to be explained fully.

Another puzzle arising from the initial *in vitro* cotranslational disassembly experiments was the expectation that ribosomes would be disengaged after reaching the termination codons of the 126 kDa or 183 kDa protein open reading frames thus leaving a quarter to half of the genome still encapsidated. How would the uncoating process be completed in the inoculated cell? An intriguing possibility suggested by Wilson (1985) was that the newly synthesized 126 kDa and/or 183 kDa polymerase protein(s) might seek the encapsidated end of the same or another virus particle and initiate the simultaneous uncoating of the RNA in the 3′→5′ direction and synthesis of progeny negative-strand RNA molecules, i.e. a coreplicational disassembly mechanism. The 3′-terminus of TMV RNA folds into a tRNA-like structure which specifically binds histidine to the 3′-OH via the cognate cellular aminoacyl-tRNA synthase and it was

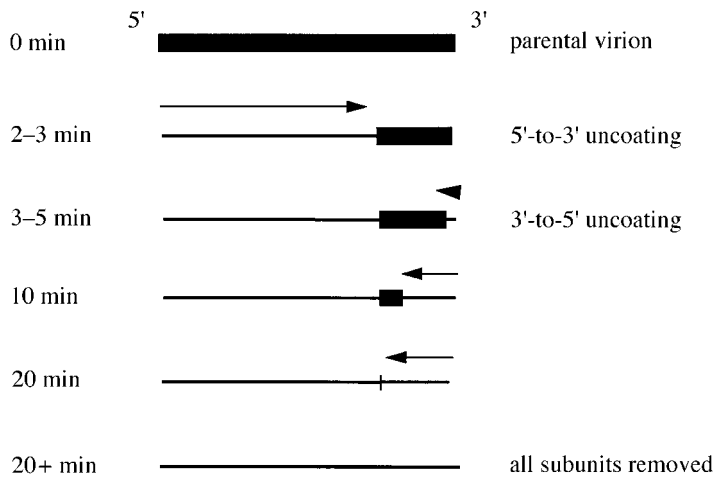

Figure 5. Bidirectional disassembly of TMV particles *in vivo*. Coat-protein subunits are removed in the 5′→3′ direction from *ca*. 75% of the viral RNA in the first 2–3 min after inoculation of protoplasts. Uncoating of the 3′-end of the RNA begins shortly thereafter and is completed by removal of subunits in the 3′→5′ direction. From Wu *et al*. (1994) and Wu & Shaw (1996).

suggested that the formation of such a complex and its recognition by the viral polymerase (or an associated host component) may be an early step in such a disassembly mechanism (Wilson 1988). Attempts to address the coreplicational disassembly hypothesis were not reported until several years later.

Shortly after the first report of the cotranslational disassembly of TMV particles *in vitro*, an attempt was made to determine whether such a mechanism might exist *in vivo*. Extracts of epidermal cells collected from leaves a few minutes after inoculation with radioactive TMV particles contained complexes that were similar to *in vitro*-generated striposomes in their appearance in the electron microscope and in their sedimentation behaviour in isopycnic gradients (Shaw *et al*. 1986). These observations provided preliminary evidence of the occurrence of a cotranslational disassembly mechanism during the early stages of infection. However, there seemed little chance that experiments of this type with intact plant tissues would be useful in attempts to investigate disassembly in further detail and attention eventually turned to an examination of the efficacy of the virus–protoplast system for such purposes.

9. DISASSEMBLY OF TMV PARTICLES IN PROTOPLASTS

Two major problems continued to hinder attempts to learn more about the fate of TMV particles at the onset of the infection process. One was the need for a system with which a much larger proportion of the cells in a sample could be inoculated with a minimal dose of virus particles. The other was the lack of methods that were sensitive enough for the detection and characterization of the particles that were undergoing disassembly.

The plant protoplast system offered the most promising approach with which to deal with the first of these problems. Samples in which significant numbers of the protoplasts would become infected could be collected easily at any time after inoculation. Moreover, much simpler and more efficient methods could be used for extraction and processing of the protoplast samples than with leaf tissue samples. Although the earliest methods for inoculation of tobacco protoplasts with TMV particles required the use of additives to the inoculum that had undesirable side effects, the advent of electroporation as a method of inoculation provided a much less complicated process.

While studies with radioactive virus particles or particles in which the RNA consisted of viral genome sequences and various types of reporter constructs had provided some useful information, the need for methods with which minimal doses of virus particles could be used for inoculation had become increasingly apparent. This would require techniques that would not simply reveal that virus particles had undergone some degree of disassembly, but that would also permit additional features of the process, such as the rate, extent and direction of uncoating, to be examined.

In the past five years, experiments with improved methods for investigating the disassembly of TMV particles *in vivo* have been reported. Extracts of protoplasts that had been collected at various times shortly after inoculation were incubated under conditions in which uncoated parts of the viral RNA would be destroyed by nucleases but the parts still enclosed in coat protein subunits would not (Wu *et al*. 1994). The protected parts of the RNA were then isolated and identified by RT-PCR analysis with primers representing various regions in the viral genome. The 'missing' parts of the viral RNA represented those that had been uncoated prior to collection of the sample of protoplasts. The results indicated that, within the first 2–3 min after electroporation of the protoplasts, approximately 75% of the RNA, including the leader sequence and most or all of the 183 kDa ORF, had been uncoated in what was assumed to be the 5′→3′ direction. However, the data indicated that coat protein subunits were not removed from the region near the origin-of-assembly sequence until several minutes later. No information was obtained about uncoating of the 3′-end of the viral RNA. There was some evidence of the appearance of progeny virus particles in samples collected 45 min after inoculation.

These experiments did not address the question of whether disassembly of TMV in inoculated protoplasts involves a cotranslational mechanism. In recent years, there has been an ever-increasing view that positive-strand RNA viruses do undergo cotranslational disassembly during initiation of the infection process. However, direct and conclusive evidence in support of the hypothesis has yet to be provided and it remains critically important that the involvement of such a mechanism be put to the test.

A later study (Wu & Shaw 1996), in which similar methods of sample preparation and RT-PCR analysis were used, dealt with uncoating of the 3′-end of the viral RNA. The results indicated that disassembly at this end of the virus particle began between 2 and 5 min after inoculation and that subunits were removed more slowly than in the initial disassembly reaction and in the 3′→5′ direction. It was concluded that disassembly of TMV particles *in vivo* is a bidirectional process with a region at or near the origin-of-assembly sequence being the last to be uncoated (figure 5). Many years ago, in a report describing experiments with extracts of leaves inoculated with radioactive TMV particles, it was suggested that *in vivo* disassembly of TMV particles might be a two-stage process (Shaw 1969). This suggestion was met with a few statements of unquestioned acceptance and several others of well-deserved scepticism. It is now mildly amusing to consider that there may have been a thread of validity to this rather insignificant piece of conjecture.

Evidence of a bidirectional uncoating process immediately renewed interest in the suggestion (Wilson 1985) that the second phase might involve a coreplicational disassembly mechanism. Experiments involving RT-PCR analysis of total RNA in protoplasts inoculated with TMV particles indicated that progeny negative-strand viral RNA begins to be produced at the same time as 3′→5′ disassembly is initiated and this suggested that disassembly and the initiation of replication might be coupled processes (Wu & Shaw 1997).

More persuasive evidence of coreplicational disassembly was obtained in experiments in which protoplasts were inoculated with particles containing replication-defective mutants of the viral RNA (Wu & Shaw 1997). Versions of TMV RNA in which large deletions in the 183 kDa ORF (open reading frame) or small deletions in conserved sequences in the 126 kDa ORF were made and packaged in coat protein subunits. Particles containing the mutant RNAs were unable to undergo 3′→5′ disassembly in electroporated protoplasts. However, when the inoculum contained a mixture of these particles and wild-type TMV RNA, translation of which could provide the 126 kDa and 183 kDa proteins, 3′→5′ disassembly of the particles proceeded as with wild-type virus particles. It was concluded that one or both of the polymerase proteins encoded by the viral RNA is involved in the completion of disassembly of TMV particles *in vivo*.

More work is needed before the coreplicational disassembly hypothesis can be accepted but one appealing feature of such a process would be protection of the 3′-terminus of the viral RNA until it can become engaged in the initiation of synthesis of progeny viral RNA. However, the existence of a mechanism of this type would also raise some interesting questions. Where in the cell does it occur? How is the newly synthesized polymerase targeted to the end of the virus particle containing the 3′-terminus of the RNA? What mechanism is responsible for the removal of a sufficient number of coat protein subunits to expose a polymerase binding site? How and where does the polymerase molecule bind? By what mechanism does the polymerase (or some other factor recruited by the polymerase) dislodge coat protein subunits? Does the 126 kDa or 183 kDa protein contain an uncoating domain? What happens when the polymerase arrives at the part of the RNA where uncoating in the 5′→3′ direction ceased? Are ribosomes still associated with this and upstream parts of the uncoated RNA? Is there a collision between polymerase molecules and ribosomes? Or is it conceivable that cotranslational disassembly serves only to provide the polymerase protein(s) but remains an incomplete process and that it is other, intact particles that will be fully disassembled by an *in trans* coreplicational mechanism? (The results of Wu *et al*. (1994) argue against but do not disprove such a possibility.) Unfortunately, it will not be a simple matter to devise experiments with which to seek answers to most of these questions.

10. CONCLUDING REMARKS

As is evident from this review, there remains much to be done before a full and adequate understanding of the early events in TMV and other plant virus infections can be attained. There may be members of the virology community who feel that the newly emerging plant disease control strategies will only be effective if they are targeted to later events in the infection process and who therefore consider determination of the mechanisms involved in the early events to be of secondary importance. Hopefully, most virologists will continue to appreciate the need for an understanding of the entire spectrum of events in virus infections. If so, it is a good bet that a number of them will be conducting their experiments with the virus that is the subject of this centennial celebration.

REFERENCES

Bawden, F. C. 1964 *Plant Virology* (ed. M. K. Corbett & H. D. Sisler), pp. 365–385. Gainesville, FL: University of Florida Press.

Bawden, F. C. & Kleczkowski, A. 1960 Some effects of ultra-violet radiation on the infection of *Nicotiana glutinosa* leaves by tobacco mosaic virus. *Virology* **10**, 163–181.

Beijerinck, M. W. 1898 Uber ein contagium vivum fluidum als Ursache der Fleckenkrankheit der Tabaksblatter. *Verhandelingen der Koninklijke Akademie van Wettenschappen te Amsterdam* **65**, 3–21.

Caspar, D. L. D. 1963 Assembly and stability of the tobacco mosaic virus particle. *Adv. Protein Chem.* **18**, 37–121.

De Zoeten, G. A. 1995 Plant virus infection: another point of view. *Adv. Bot. Res.* **21**, 105–124.

Durham, A. C. H. 1978 The roles of small ions especially calcium in virus disassembly, takeover, and transformation. *Biomedicine* **28**, 307–313.

Fajardo, T. G. 1930 Studies on the mosaic disease of the bean (*Phaseolus vulgaris* L.). *Phytopathology* **20**, 469–494.

Fraenkel-Conrat, H., Singer, B. & Veldee, S. 1958 The mechanism of plant virus infection. *Biochim. Biophys. Acta* **29**, 639–640.

Gaard, G. & De Zoeten, G. A. 1979 Plant virus uncoating as a result of virus–cell wall interactions. *Virology* **96**, 21–31.

Gallie, D. R., Sleat, D. E., Watts, J. W., Turner, P. C. & Wilson, T. M. A. 1987 *In vivo* uncoating and efficient expression of foreign mRNAs packaged in TMV-like particles. *Science* **236**, 1122–1124.

Gerola, F. M., Bassi, M., Favali, M. A. & Betto, E. 1969 An electron microscopy study of the penetration of tobacco mosaic virus into leaves following experimental inoculation. *Virology* **38**, 380–386.

Hamers-Casterman, C. & Jeener, R. 1957 An initial ribonuclease-sensitive phase in the multiplication of tobacco mosaic virus. *Virology* **3**, 197–206.

Hayashi, T. 1974 Fate of tobacco mosaic virus after entering the host cell. *Jap. J. Microbiol.* **18**, 279–286.

Hirashima, A. & Hirai, T. 1969*a* The fate of tobacco mosaic virus introduced into tobacco leaves. *Ann. Phytopath. Soc. Japan* **35**, 21–24.

Hirashima, A. & Hirai, T. 1969*b* Dissociation of tobacco mosaic virus particles immediately after infection of tobacco leaves. *Ann. Phytopath. Soc. Japan* **35**, 25–28.

Kassanis, B. 1959 Comparison of the early stages of infection by tobacco mosaic virus and its nucleic acid. *J. Gen. Microbiol.* **20**, 704–711.

Kassanis, B. & Kenten, R. H. 1978 Inactivation and uncoating of TMV on the surface and in the intercellular spaces of leaves. *Phytopath. Z.* **91**, 329–339.

Kiho, Y. 1970 Polysomes containing infecting viral genome in tobacco leaves infected with tobacco mosaic virus. *Jap. J. Microbiol.* **14**, 291–302.

Kiho, Y. 1972 Polycistronic translation of plant viral ribonucleic acid. *Jap. J. Microbiol.* **16**, 259–267.

Kiho, Y. 1974 Model system for the adsorption of tobacco mosaic virus. *Jap. J. Microbiol.* **18**, 313–320.

Kiho, Y., Abe, T. & Ohashi, Y. 1979 Disassembly of tobacco mosaic virus by membrane lipid isolated from tobacco leaves and polyornithine. *Microbiol. Immunol.* **23**, 1067–1076.

Kurtz-Fritsch, C. & Hirth, L. 1967 Premiers événements de l'infection virale chez les plantes. *C. R. Acad. Sci.* (*Paris*) **265**, 220–223.

Lu, B., Stubbs, G. J. & Culver, J. N. 1996 Carboxylate interactions involved in the disassembly of tobacco mosaic tobamovirus. *Virology* **225**, 11–20.

Mayer, A. 1886 Uber die Mosaikkrankheit des Tabaks. *Die Landwirtschaftlichen Versuchs-Stationen.* **32**, 451–467.

Mundry, K. W., Watkins, P. A. C., Ashfield, T., Plaskitt, K. A., Eisele-Walter, S. & Wilson, T. M. A. 1991 Complete uncoating of the 5′ leader sequence of tobacco mosaic virus RNA occurs rapidly and is required to initiate cotranslational virus disassembly *in vitro*. *J. Gen. Virol.* **72**, 769–777.

Namba, K., Pattanayek, R. & Stubbs, G. 1989 Visualization of protein–nucleic acid interactions in a virus. Refined structure of intact tobacco mosaic virus at 2.9 Å resolution by X-ray fiber diffraction. *J. Mol. Biol.* **208**, 307–325.

Plaskitt, K. A., Watkins, P. A. C., Sleat, D. E., Gallie, D. R., Shaw, J. G. & Wilson, T. M. A. 1988 Immunogold labelling locates the site of disassembly and transient gene expression of tobacco mosaic virus-like pseudovirus particles *in vivo*. *Mol. Plant–Microbe Interact.* **1**, 10–16.

Rawlins, T. E. & Tompkins, C. M. 1936 Studies on the effect of carborundum as an abrasive in plant virus inoculations. *Phytopathology* **26**, 578–587.

Reddi, K. K. 1966 Studies on the formation of tobacco mosaic virus nucleic acid. VII. Fate of tobacco mosaic virus after entering the host cell. *Proc. Natl Acad. Sci. USA* **55**, 593–598.

Schramm, G. & Engler, R. 1958 The latent period after infection with tobacco mosaic virus and virus nucleic acid. *Nature* **181**, 916.

Semal, J. 1962 Action of hydrochloric acid on the infection of *Nicotiana glutinosa* leaves by tobacco mosaic virus and its nucleic acid. *Virology* **16**, 230–235.

Shaw, J. G. 1967 *In vivo* removal of protein from tobacco mosaic virus after inoculation of tobacco leaves. *Virology* **31**, 665–675.

Shaw, J. G. 1969 *In vivo* removal of protein from tobacco mosaic virus after inoculation of tobacco leaves. II. Some characteristics of the reaction. *Virology* **37**, 109–116.

Shaw, J. G. 1970 Uncoating of tobacco mosaic virus ribonucleic acid after inoculation of tobacco leaves. *Virology* **42**, 41–48.

Shaw, J. G. 1973 *In vivo* removal of protein from tobacco mosaic virus after inoculation of tobacco leaves. III. Studies on the location on virus particles for the initial removal of protein. *Virology* **53**, 337–342.

Shaw, J. G. 1985 Early events in plant virus infections. In *Molecular plant virology. II. Replication and gene expression* (ed. J. W. Davies), pp. 1–12. Boca Raton, FL: CRC Press.

Shaw, J. G., Plaskitt, K. A. & Wilson, T. M. A. 1986 Evidence that tobacco mosaic virus particles disassemble cotranslationally *in vivo*. *Virology* **148**, 326–336.

Siegel, A., Ginoza, W. & Wildman, S. G. 1957 The early events of infection with tobacco mosaic virus nucleic acid. *Virology* **3**, 554–559.

Takebe, I. & Otsuki, Y. 1969 Infection of tobacco mesophyll protoplasts by tobacco mosaic virus. *Proc. Natl Acad. Sci. USA* **64**, 843–848.

Verduin, B. J. M. 1992 Early interactions between viruses and plants. *Semin. Virol.* **3**, 423–431.

Wildman, S. G. 1959 The process of infection and virus synthesis with tobacco mosaic virus and other plant viruses. In *The viruses* (ed. F. M. Burnet & W. M. Stanley), pp. 1–31. New York: Academic Press.

Wilson, T. M. A. 1984 Cotranslational disassembly of tobacco mosaic virus *in vitro*. *Virology* **137**, 255–265.

Wilson, T. M. A. 1985 Nucleocapsid disassembly and early gene expression by positive-strand RNA viruses. *J. Gen. Virol.* **66**, 1201–1207.

Wilson, T. M. A. 1988 Structural interactions between plant RNA viruses and cells. *Oxf. Surv. Pl. Mol. Cell Biol.* **5**, 89–144.

Wilson, T. M. A., Plaskitt, K. A., Watts, J. W., Osbourn, J. K. & Watkins, P. A. C. 1990 *NATO ASI series. Recognition and response in plant–virus interactions*, vol. H41 (ed. R. S. S. Fraser), pp. 125–145. Berlin: Springer.

Wu, J.-H. & Rappaport, I. 1962 Competition between infectious tobacco mosaic virus nucleic acid and intact virus on *N. glutinosa*. *Nature* **193**, 908.

Wu, X. & Shaw, J. G. 1996 Bidirectional uncoating of the genomic RNA of a helical virus. *Proc. Natl Acad. Sci. USA* **93**, 2981–2984.

Wu, X. J. & Shaw, J. G. 1997 Evidence that a viral replicase protein is involved in the disassembly of tobacco mosaic virus particles *in vivo*. *Virology* **239**, 426–434.

Wu, X., Xu, Z. & Shaw, J. G. 1994 Uncoating of tobacco mosaic virus RNA in protoplasts. *Virology* **200**, 256–262.