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Historical overview of research on the tobacco mosaic virus genome: genome organization, infectivity and gene manipulation

Y. Okada

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THE ROYAL SOCIETY

Historical overview of research on the tobacco mosaic virus genome: genome organization, infectivity and gene manipulation

Y. Okada

Department of Bioscience, Teikyo University, Utsunomiya, Japan

Early in the development of molecular biology, TMV RNA was widely used as a mitochondrial RNA that could be purified easily, and it contributed much to research on protein synthesis. Also, in the early stages of elucidation of the genetic code, artificially produced TMV mutants were widely used and provided the first proof that the genetic code was non-overlapping. In 1982, Goelet *et al.* determined the complete TMV RNA base sequence of 6395 nucleotides. The four genes (130K, 180K, 30K and coat protein) could then be mapped at precise locations in the TMV genome. Furthermore it had become clear, a little earlier, that genes located internally in the genome were expressed via subgenomic mRNAs. The initiation site for assembly of TMV particles was also determined.

However, although TMV contributed so much at the beginning of the development of molecular biology, its influence was replaced by that of *Escherichia coli* and its phages in the next phase. As recombinant DNA technology developed in the 1980s, RNA virus research became more detached from the frontier of molecular biology. To recover from this setback, a gene-manipulation system was needed for RNA viruses. In 1986, two such systems were developed for TMV, using full-length cDNA clones, by Dawson's group and by Okada's group. Thus, reverse genetics could be used to elucidate the basic functions of all proteins encoded by the TMV genome. Identification of the function of the 30K protein was especially important because it was the first evidence that a plant virus possesses a cell-to-cell movement function. Many other plant viruses have since been found to encode comparable 'movement proteins'. TMV thus became the first plant virus for which structures and functions were known for all its genes. At the birth of molecular plant pathology, TMV became a leader again.

TMV has also played pioneering roles in many other fields. TMV was the first virus for which the amino acid sequence of the coat protein was determined and first virus for which cotranslational disassembly was demonstrated both *in vivo* and *in vitro*. It was the first virus for which activation of a resistance gene in a host plant was related to the molecular specificity of a product of a viral gene. Also, in the field of plant biotechnology, TMV vectors are among the most promising. Thus, for the 100 years since Beijerinck's work, TMV research has consistently played a leading role in opening up new areas of study, not only in plant pathology, but also in virology, biochemistry, molecular biology, RNA genetics and biotechnology.

Keywords: tobacco mosaic virus; gene manipulation of TMV; RNA virus; infectious cDNA clone; TMV vector; movement protein

1. GENOMIC NUCLEIC ACID OF TOBACCO MOSAIC VIRUS (TMV)

(a) *Ribonucleic acid in purified TMV particles*

To isolate and purify a virus, it is necessary first to establish an experimental system for assaying the virus. Before 1929, there were few methods that could be used to measure TMV quantitatively, but the necrotic local lesion assay method, developed by Holmes (1929) using *Nicotiana glutinosa*, was the simplest and the most accurate method of its day. This method became an important aid for researchers attempting to purify TMV particles.

At about the same time, methods for isolating and purifying proteins had made progress, and Sumner (1926) succeeded in crystallizing purified urease. This triggered, in the early 1930s, a flurry of attempts by many researchers in various countries to isolate and purify plant viruses. At that time, various biochemical studies suggested that plant viruses might be proteins. For example, Stanley (1934) showed that the infectivity of TMV was largely destroyed by pepsin at a pH at which the virus was otherwise stable. This was followed by his purification of TMV by a method similar to that used for proteins, resulting in crystallized TMV preparations. These crystals were infectious in aqueous solution even at 10^{-9} g ml^{-1}. Stanley's (1935) chemical studies led him to conclude that TMV was an autocatalytic protein which required the presence of living cells for its production.

In the following year, however, Stanley's description of the chemical constitution of TMV had to be revised, when Bawden *et al.* (1936) reported that they had isolated liquid crystalline preparations of TMV from TMV-infected

plants, and that these TMV preparations contained nucleic acid of the ribose type. Soon after, Stanley (1937) and Best (1937) both confirmed the nucleoprotein nature of TMV. At that time, however, they did not recognize that the RNA is the element essential for infectivity.

The discovery of the chemical components of TMV particles had a tremendous impact on biological science. It was a revolutionary idea to think of an infectious disease agent as a crystallizable material. Once the idea was accepted, however, investigations on the chemical and physical properties of viruses began in earnest.

(b) *Infectious TMV RNA and its reconstitution with TMV coat protein*

Until about 1948, most attention was focused on the protein part of viruses. Although it was known that enzymes, which carried out important biological functions in cells, were proteins, the function of the nucleic acid was not known. However, Markham & Smith (1949) showed that purified turnip yellow mosaic virus preparations contained two classes of particles, one an infectious nucleoprotein and the other a non-infectious particle which apparently consisted of an identical protein but lacked RNA. This suggested that the viral RNA was essential for infectivity. Harris & Knight (1952) reported that the C-terminal threonine of TMV coat protein (CP) could be removed by carboxypeptidase without altering the infectivity of the virus, and that inoculation with such dethreoninized TMV gave rise to normal TMV again possessing C-terminal threonine. This result supported the idea that the viral RNA, not the viral protein, controlled the specificity of the viral protein. These pioneering experiments were published in the same year that the well-known Hershey–Chase (1952) experiment was reported.

However, doubts as to whether the RNA in TMV was really a genetic material or not persisted until 1956. Fraenkel-Conrat & Williams (1955) had shown that on incubating a solution containing a mixture of a non-infectious disassembled TMV protein and purified, apparently non-infectious, TMV RNA at neutral pH, normal-looking infectious TMV particles were formed. This experiment made a big impact because it appeared that a 'living molecule' had been put together *in vitro* from 'non-living' constitutents. The next year, the pioneering experiments of Gierer & Schramm (1956) demonstrated that the free TMV RNA alone was capable of initiating virus replication, and this result was confirmed by Fraenkel-Conrat (1956). Thus it became clear that the infectivity observed a year before in the TMV reconstitution experiment was a result of labile infectivity of TMV RNA being protected by the CP and not of the creation of infectivity.

Fraenkel-Conrat & Singer (1957) began *in vitro* studies on reconstitution of the CP of common strain TMV and RNAs from other TMV strains, and Holoubeck (1962) further extended their studies. In these experiments, the reconstituted virus had the host range specificity characteristic of the virus from which the RNA was obtained.

Many years after the first reconstitution of TMV, studies on its mechanism were taken up in laboratories in England, France and Japan (Butler & Klug 1971; Lebeurier *et al*. 1977; Otsuki *et al*. 1977).

2. TMV MUTANTS AND THE GENETIC CODE

By 1966, the genetic code was known in almost full detail (Crick 1966). Much of this knowledge came from *in vitro* studies using synthetic polyribonucleotides of known composition and sequence as messenger RNA in various cell-free translation systems of *Escherichia coli*. However, to prove that the postulated genetic code was correct in living cells as well, it was necessary to perform experiments with proteins synthesized *in vivo*. For such experiments, the CP of plant viruses was useful.

Gierer & Mundry (1958) first demonstrated the high efficiency of nitrous acid as an *in vitro* mutagen for TMV RNA. The mutagenic action was considered to be through deamination of cytosine to give uracil and deamination of adenine to give hypoxanthine, which behaved chemically like guanine. Nitrous acid produced many TMV mutants with altered biological characteristics. Hundreds of TMV mutants were prepared at Berkeley, Tübingen and elsewhere to analyse the amino acid constitution of the CP of the TMV mutants and determine the amino acid replacements (Tsugita & Fraenkel-Conrat 1960, 1962; Funatsu & Fraenkel-Conrat 1964; Wittmann & Wittmann-Liebold 1966). Almost all the amino acid exchanges thus observed could be explained by the transition A→G or C→U in a single codon in the TMV CP gene. This would not have been possible if the codes postulated from *in vitro* studies were incorrect *in vivo*, or if the codes were not universal among bacterial and plant cells. Furthermore, it was observed that only one amino acid residue was replaced in most of the mutants and that, when two residues were changed, they were not neighbours, thus providing the first direct proof of the long-held assumption that the genetic code is non-overlapping. These experiments made an important contribution to our understanding of the genetic code.

3. PRIMARY STRUCTURE OF TMV RNA AND GENOME ORGANIZATION

(a) *Terminal sequences of TMV RNA*

TMV RNA was the first pure RNA molecule that became available for various studies. Therefore, methods for determining end-groups and terminal sequences were developed using TMV RNA. Sugiyama & Fraenkel-Conrat (1961) determined that the 3′-end of TMV RNA was an unphosphorylated adenosine, a result confirmed by Stein-Schneider & Fraenkel-Conrat (1966). A little later, the 3′-end sequence of about 70 nucleotides was established for several strains of TMV (Guilley *et al*. 1975; Lamy *et al*. 1975).

It was believed for a long time that the 5′-end of TMV RNA was also an unphosphorylated adenosine (Sugiyama & Fraenkel-Conrat 1963). Later, however, the presence of a typical 5′-terminal cap structure (m^7GpppG) was discovered in TMV RNA, although, unlike eukaryotic cellular mRNAs, the one or two bases adjacent to the m^7GpppG in TMV RNA were not methylated (Zimmern 1975; Keith & Fraenkel-Conrat 1975). The infectivity of TMV RNA was lost on removal of the cap structure by tobacco phosphodiesterase (Ohno *et al*. 1976). Mandeles (1968) isolated several unique oligonucleotides from ribonuclease T1 digests of TMV RNA. The largest of

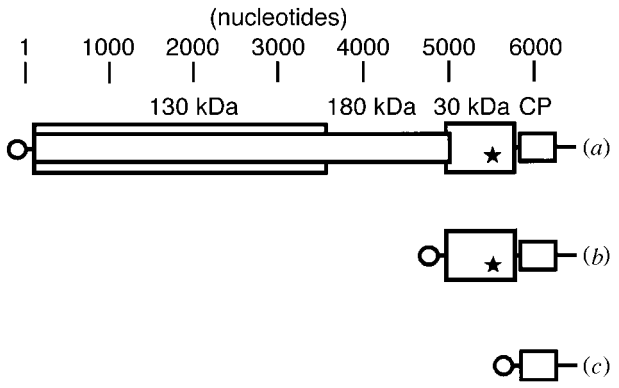

Figure 1. Schematic representation of the organization of the genomic and subgenomic RNAs of the common strain of TMV. (*a*) Genomic RNA; (*b*) subgenomic RNA for the 30K protein; (*c*) subgenomic RNA for the coat protein. Coding and non-coding regions are shown by boxes and lines, respectively. Circles represent the cap structure at the 5′-end. Stars denote the assembly origin.

these was estimated to be about 70 nucleotides long and lacked G. The location of this sequence, called Ω, was first thought to be near the 3′-end, but later it was found to be the 5′-terminal sequence (Richards *et al.* 1978).

(b) *Base sequence determination by RNA sequencing methods*

Although many methods were introduced to determine the base sequence of RNA, earlier analysis was restricted to oligonucleotides obtained after complete or partial digestion of RNA with several ribonucleases. Information obtained in this way was far from being the complete sequence of RNA. One drawback was the difficulty in obtaining pure RNA subfragments spanning the entire region of interest. Guilley *et al.* (1979) solved this problem using RNA from partially stripped TMV. TMV virions disassemble in a polar fashion from the end of the particle containing the 5′-end of the RNA when treated with mild alkali (Perham & Wilson 1976). After nuclease treatment, various discrete size classes of partially stripped rods can be isolated. The RNA in the shortest and most abundant class of rods was approximately 1000 nucleotides in length. Using this RNA, the sequence of the 1000 nucleotides at the 3′-end of TMV RNA was reported (Guilley *et al.* 1979). This provided the base sequence of the CP cistron and predicted that the left-hand portion of the CP cistron was adjacent to the cistron for the 30K protein, separated only by two non-translated nucleotides. This experiment was the first report on the partial genome organization of plant viruses based on nucleotide sequence analysis. However, such RNA sequencing methods still did not provide enough sequence information to reveal the precise genome organization of the remaining parts of TMV RNA.

(c) *Base sequence determination by cDNA sequencing*

In 1970, reverse transcriptase was discovered by Baltimore (1970) and Temin & Mizutani (1970). They showed that RNA could act as the template for transcription of DNA, and opened a way for cloning RNA as cDNA. Furthermore, at about the same time, restriction enzymes were isolated and characterized, so enabling large DNA molecules to be manipulated (Smith & Wilcox 1970). Thus, a little later, new methods for sequencing DNA were developed by Maxam & Gilbert (1977) and Sanger *et al.* (1977). Advances in DNA sequencing techniques had given us the unprecedented ability to determine rapidly the nucleotide sequence of entire virus genomes. Particularly, as sequencing DNA is much easier than RNA, sequencing of cloned cDNAs of RNA genes opened a new era in elucidating genome organization of plant RNA viruses.

For TMV, the 3′-terminal 4000 nucleotides of common strain RNA and 1700 nucleotides of cowpea strain RNA were first successfully cloned as cDNAs and sequenced by Meshi *et al.* (1981, 1982*b*). Soon after, the sequence of about 2000 nucleotides at the 3′-end of the RNA were determined and compared for several tobamovirus strains (Takamatsu *et al.* 1983). For plant viruses, this gave the first classification based on genome structure.

(d) *Genome organization of tobamoviruses*

In 1982, the entire sequence of 6395 nucleotides of the TMV (*vulgare*) genome was completed by Goelet *et al.* (1982). Their sequencing strategy involved cloning TMV cDNA fragments into bacteriophage M13 and sequencing each, then connecting the fragments by finding, with the aid of a computer, short overlapping stretches of nucleotides to piece together the whole sequence. Meanwhile, proteins approximately corresponding in size to the 130K and 180K proteins had already been found in infected tobacco leaves (Scalla *et al.* 1976) and in infected protoplasts (Sakai & Takebe 1974). Besides these, the 30K protein had also been detected in both infected tobacco protoplasts (Beier *et al.* 1980) and leaves (Joshi *et al.* 1983). Goelet et *al.* (1982) were able precisely to locate the corresponding cistrons and the CP cistron on the genome (figure 1).

The cap structure is attached to the first nucleotide. This is followed in the *vulgare* strain by an untranslated leader sequence of 68 nucleotides. The long G-deficient stretch called Ω, extends to the first AUG in this region. After the leader comes an ORF that encodes the 130K protein. The termination codon for the 130K protein is leaky and the read-through protein is of about 180K. The exact molecular weights are 126K and 183K but, as the proteins from different strains of TMV have slightly different numbers of amino acid residues (figure 2), their molecular weights will be slightly different. Therefore, for simplicity, the convention that rounds off the molecular weights to 130K and 180K is adopted here.

The 3′-terminal five codons of the 180K protein gene overlap with the third ORF, coding for the 30K protein. This third ORF terminates two nucleotides before the initiation codon of the fourth ORF, which encodes the 17K CP and is located closest to the 3′ terminus. Later, another ORF for a 54K protein was postulated to exist in-frame in the read-through region of the 180K protein (Sulzinski *et al.* 1985), but this protein has not been detected *in vivo* (Saito *et al.* 1986). Therefore, it is concluded that the TMV genome codes for four gene products. The untranslated 3′-terminal sequence consists of 204 nucleotides. This arrangement, summarized

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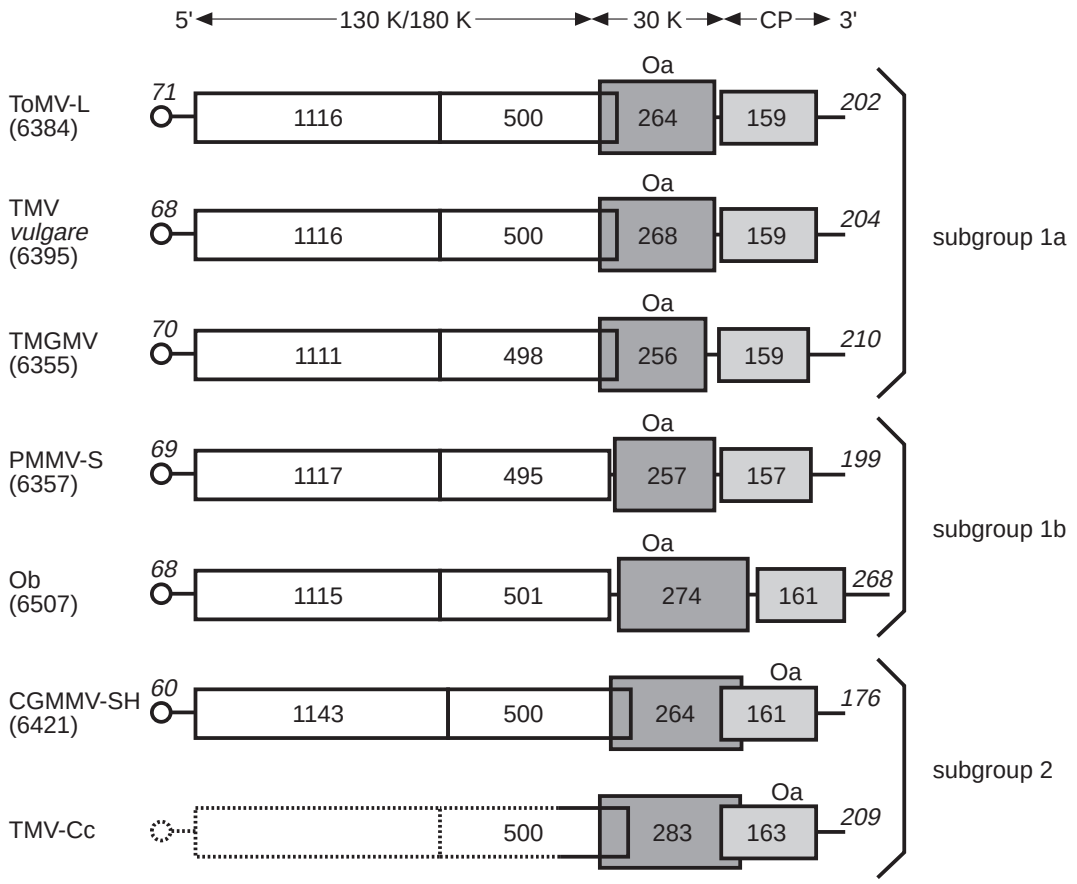

Figure 2. Genome organization of various tobamoviruses (Ikeda *et al.* 1993). Each coding region is shown by a box in which its amino acid length is indicated. Horizontal lines represent 5′ and 3′ non-coding regions with their nucleotide lengths given in italics. The total nucleotide length of each genome is shown in parenthesis below the name of the strain. Oa indicates the predicted position of the assembly origin in each genome. The vertical lines (inside left-hand boxes) indicate the positions of read-through of the 130K protein gene. The region shown by dotted lines in TMV-Cc has not been sequenced.

schematically in figure 1, was the first example of a totally sequenced genome and the genetic map for a plant RNA virus.

Two years later, the complete base sequence of the RNA genome of TMV-L (tomato mosaic virus strain, ToMV) was determined (Ohno *et al.* 1984). Among viruses in the tobamovirus group, ToMV is the only one that can produce necrotic local lesions on tobacco plants with the *N′* gene. Furthermore, TMV-L_{11}A, which is an attenuated strain derived from TMV-L, has been widely used in Japan to prevent TMV infection by cross-protection. We therefore thought the best strain for elucidating plant–virus interactions at the molecular level would be TMV-L. Later, gene engineering systems for TMV-*vulgare* (Dawson *et al.* 1986) and TMV-L (Meshi *et al.* 1986) were established and these two strains became the centre of TMV genome research.

In addition to the *vulgare* and L strains, the complete nucleotide sequences of the RNA of several other tobamoviruses are now known: M strain (an attenuated strain closely related to common strain, Ul; Holt *et al.* 1990); a few tomato mosaic virus strains including L_{11}A (an attenuated strain) (Nishiguchi *et al.* 1985), Ltal and Ltbl (resistance-breaking strains overcoming *Tm-1* and *Tm-2* resistance, respectively; Meshi *et al.* 1988, 1989); tobacco mild green mosaic virus (TMGMV; Solis & Garcia-Arenal 1990); cucumber green mottle mosaic virus (CGMMV SH strain; Ugaki *et al.* 1991); pepper mild mottle virus (PMMV strains including S (Alonso *et al.* 1991) and J (Kirita *et al.* 1997); tobamovirus Ob (Padgett & Beachy 1993; Ikeda *et al.* 1993); rakkyo strain (Chen *et al.* 1996); and a few crucifer-infecting tobamoviruses, including turnip vein clearing virus (Lartey *et al.* 1994), cr-TMV (Dorokhov *et al.* 1994), chinese rape mosaic virus (Aguilar *et al.* 1996) and TMV-Cg (Yamanaka *et al.* 1998).

Several variant types of genome organization were observed among tobamoviruses. Ikeda *et al.* (1993) classified those tobamoviruses for which the sequences were completely determined into three subgroups, as shown in figure 2. TMV *vulgare*, TMV-L and TMGMV have genome structures in which only the 180K and the 30K ORFs overlap. In the genomes of Ob and PMMV-S, there are no overlaps between any adjacent ORFs. In contrast, all adjacent ORFs overlap in the CGMMV-SH and TMV-Cc genomes.

Goelet *et al.* (1982) reported that the 5′-terminal sequence of TMV *vulgare* occurred as two variants, a shorter and a longer one. However, Meshi *et al.* (1983*a*) showed that the longer variant was not *vulgare*, but that it came from a contaminating tomato strain. Dawson *et al.* (1986) confirmed that the 5′-region of TMV RNA is not polymorphic.

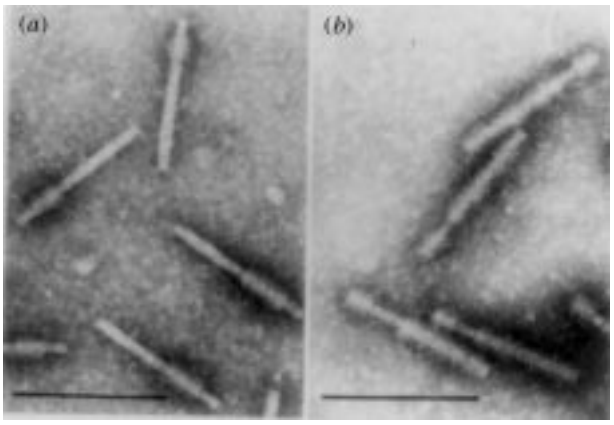

Figure 3. TMV particles after sequential reconstitution from TMV RNA, first with TMV coat protein (CP) and then with ToMV CP. Reconstituted particles were treated with a specific antibody for (*a*) TMV CP or a specific antibody for ToMV (*b*) CP. Bar represents 300 nm.

(e) *Location of the assembly origin*

Since Fraenkel-Conrat & Williams (1955) showed that TMV CP and TMV RNA can assemble *in vitro* to form intact virus particles, many researchers have studied the mechanism of assembly of the TMV rod as a model of macromolecular assembly from protein and RNA. For a long time, it was believed that assembly started at the 5′-end of TMV-RNA (Butler & Klug 1971; Ohno *et al.* 1971). However, this view became doubtful after the finding that the 5′-end of TMV RNA is blocked by the cap structure. Zimmern & Wilson (1976) first located the assembly origin between 900 and 1300 nucleotides from the 3′-end of the RNA. In the following year, by examining many partially reconstituted particles with an electron microscope and by determining the lengths of naked RNAs which were not incorporated into the particle, Lebeurier *et al.* (1977) determined the exact location of the assembly origin.

In the same year, we located the assembly origin by electron microscopic serology using strain specific antibodies (Otsuki *et al.* 1977). Common strain TMV RNA was sequentially reconstituted, first with a small amount of the CP of the common strain, and then with an excess of the CP of a tomato strain. The particles thus reconstituted were treated with antibody specific for each strain and then examined by electron microscopy to locate regions of the rod possessing each kind of CP (figure 3). By this means, the assembly origin was located at about 720 nucleotides from the 3′-end of TMV RNA.

Using this method, combined with base sequences, the nucleotide sequence of the assembly origins for common strain (Zimmern 1977; Jonard *et al.* 1977; Meshi *et al.* 1982*a*), tomato strain (Takamatsu *et al.* 1983), cowpea strain (Meshi *et al.* 1981) and cucumber green mottle mosaic (Meshi *et al.* 1983*b*) tobamoviruses were determined. All the assembly origins had a stable hairpin structure with the looped-out common target sequence, GAXGUUG (Okada 1986). Although internal initiation was the mechanism of TMV assembly for all tobamoviruses studied, the location of the assembly origin differed among strains. Depending on its location, Fukuda *et al.* (1981) classified several tobamoviruses into two subgroups, one subgroup having the assembly origin within the 30K protein cistron and the other within the CP cistron (figure 2).

The confirmation that the assembly origin resides in the internal region of TMV RNA made it inevitable that particle assembly would proceed bidirectionally. Many studies on the mechanism of TMV assembly *in vitro* have been described, and there is a consensus about most of the details of the initial events, but the nature of the elongation process remains controversial (Butler 1984; Okada 1986). In contrast, there has not been much progress in research on TMV assembly in plant cells, but recently the first experiments were reported in which pseudovirus particles were self-assembled in *E. coli* (Hwang *et al.* 1994).

4. FUNCTIONS OF VIRUS-CODED PROTEINS AND UNTRANSLATED REGIONS REVEALED BY GENETICALLY ENGINEERED INFECTIOUS TMV RNA

(a) In vitro *transcription of TMV RNA from full-length cDNA clones*

Although, in 1982, the amino acid sequences of all TMV-coded proteins could be deduced from the nucleotide sequences, the functions of the proteins, other than the CP, were unclear. This is because it was still impossible to isolate the proteins from TMV-infected cells for further study. A solution to this problem was to establish a gene manipulation system by which reverse genetics could be applied. Despite the fact that, by that time, genetic engineering methods for DNA had been established and were used widely in molecular biology research, a system for RNA was lacking. Finally in 1984, Ahlquist *et al.* established an RNA manipulation system using brome mosaic virus (BMV) RNAs. They transcribed infectious BMV RNAs *in vitro* from full-length cDNA clones. Because genes could be manipulated in cDNA, genetically engineered RNAs were now obtainable. This success in manipulating BMV genes encouraged many plant-virus researchers, and the method was applied immediately to TMV.

Infectious TMV RNA has been synthesized successfully *in vitro* from full-length cDNA clones for the common strain (Dawson *et al.* 1986), and the tomato strain (figure 4) (Meshi *et al.* 1986). In the early experiments, infectious TMV RNA was transcribed by *E. coli* RNA polymerase under the direction of the P_M promoter. Recently, however, many researchers have obtained infectious transcripts with T7 RNA polymerase (Holt & Beachy 1991). Using *in vitro* transcripts equivalent to 0.2 to 0.5 μg of authentic TMV RNA, 60–80% of protoplasts derived from suspension-cultured cells of tobacco could be infected by the electroporation method (Watanabe *et al.* 1987*a*).

Today, *in vitro* transcription has become a standard technique in genetic analysis of RNA genomes. Such a system makes it possible to manipulate genomic RNA by modifying biologically active cDNA clones by substitution, deletion or insertion of nucleotides. This has enabled the functions of many virus-coded proteins and untranslated regions to be elucidated by reverse genetics.

(b) *130K and 180K proteins*

The 130K and 180K proteins were thought to be involved in viral RNA replication. For example, their

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

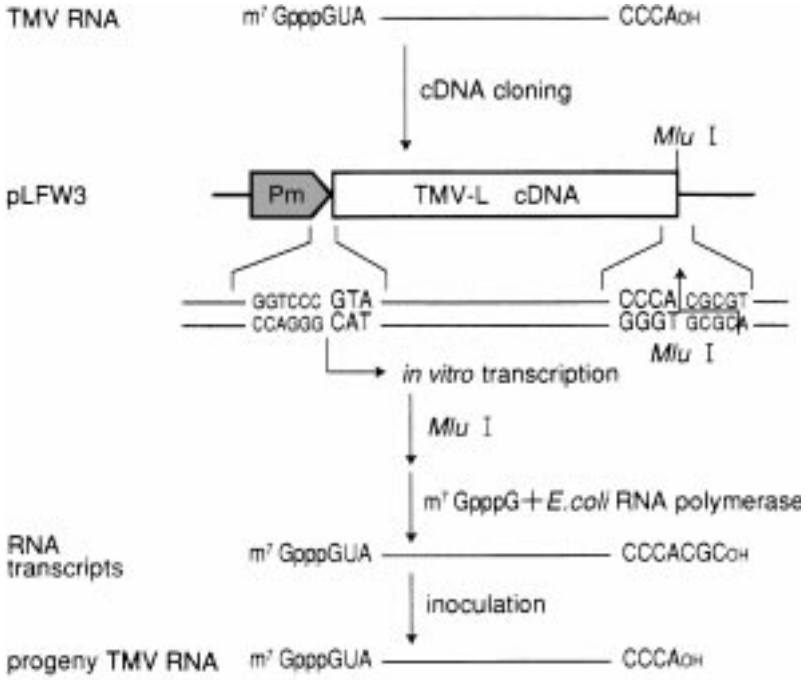

Figure 4. Schematic representation of the method for the *in vitro* synthesis of infectious TMV RNA.

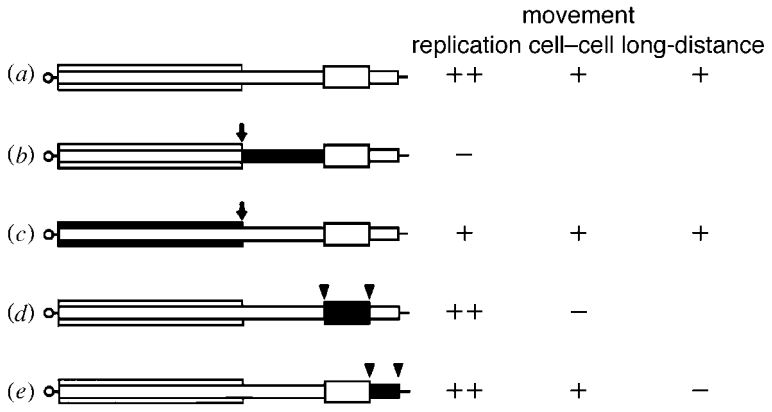

Figure 5. Genetically engineered TMV mutants and their characteristics. The dark areas indicate genes which lost their functions either by base substitution or deletion. (*a*) TMV wild type; (*b*) TMV mutant which produces 130K protein but not 180 K protein; (*c*) TMV mutant which produces 180 K protein but not 130K protein; (*d*) TMV mutant lacking the 30K protein gene; (*e*) TMV mutant lacking the coat protein gene.

amino acid sequences have significant homology with known RNA-dependent RNA polymerases (Kamer & Argos 1984; Haseloff *et al.* 1984). The most convincing evidence that both the 130K and 180K proteins are essential components of the RNA replicase complex was given by a mutant, constructed by Meshi *et al.* (1987), where both the 30K and CP genes were deleted. The mutant replicated in tobacco protoplasts. The time-course of synthesis of progeny viral RNA molecules did not differ significantly from that of wild-type TMV RNA. It was also suggested that the 130K and 180K proteins were involved in synthesis the 30K protein subgenomic mRNA (Watanabe *et al.* 1987*b*).

Ishikawa *et al.* (1986) constructed TMV mutants that had several kinds of mutations at or near the amber termination codon of 130K protein gene. Mutants that did not produce the 180K protein were not infectious (figure 5*b*), but mutants that produced the 180K protein but not the 130K protein (figure 5*c*) retained infectivity although their specific activity was low. Therefore, a balanced expression of the 130K and 180K proteins seems to be necessary for efficient replication of TMV RNA. Recently, Hamamoto *et al.* (1997) reported that a single amino acid substitution (Glu 979 to Ile) in the 130K and 180K proteins of ToMV alters host specificity.

For a long time, the molecular constituents of the TMV replicase complex were not established. Recently, however, Osman & Buck (1997) isolated the TMV RNA polymerase complex. The purified RNA polymerase preparation contained viral 130K and 180K proteins, together with a host 56K protein which is related to the RNA-binding subunits of yeast eIF-3.

(c) *30K protein*

Early genetic studies with *ts* mutants demonstrated the existence of a virus-coded function required for cell-to-cell movement of TMV (Taliansky *et al.* 1982). A TMV *ts* mutant for cell-to-cell movement (Lsl) has greatly contributed to the understanding of this virus-coded function. At 32 °C, Lsl can replicate and assemble normally in leaf cells infected by inoculation, or in protoplasts, but cannot spread from cell to cell in leaves (figure 6), whereas it spreads normally at 20 °C (Nishiguchi *et al.* 1978). Ohno *et al.* (1983) showed that the mutation leading to this *ts* state was a serine to proline change at position 154 in the 30K protein.

The role of the 30K protein was further demonstrated directly by two other kinds of experiment. First, transgenic tobacco that expresses wild-type 30K protein complements the Lsl mutant and allows it to spread from

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

cell to cell and systemically at non-permissive temperatures (Deom *et al.* 1987). Second, various frameshift mutations in the 30K gene gave a defective phenotype for cell-to-cell movement of TMV (figure 5*d*) (Meshi *et al.* 1987). The 30K protein is now called the 'movement protein' (MP).

Tomenius *et al.* (1987) found that MP accumulates in plasmodesmata of TMV-infected tissues. Moreover, Wolf *et al.* (1989) found that the molecular size exclusion limit of MP(+) transgenic tobacco for cell-to-cell transport was about tenfold greater than that of control tobacco. Another property of MP was reported by Citovsky *et al.* (1990) who showed that it binds *in vitro* to single-stranded nucleic acids to form an elongated structure of 1.5–2.0 nm diameter (Citovsky *et al.* 1992). The diameter of the RNA–MP complex was similar to the size of molecules that could pass through the modified plasmodesmata. These observations led to the proposal that tobamovirus genomic RNA forms a complex with MP and passes through the plasmodesmata, which are enlarged by a second activity of MP, resulting in movement of genomic RNA to adjacent cells. Recently, Heinlein *et al.* (1995) and McLean *et al.* (1995) reported that MP is associated with microtubules in TMV-infected protoplasts. They presumed that the MP–microtubule association was a stage on the way to or from plasmodesmata. Watanabe *et al.* (1992) demonstrated that ToMV MP is phosphorylated in infected protoplasts, and Kawakami *et al.* (1998) determined that serine 37 and serine 238 in the MP are sites of phosphorylation.

On infecting tomato protoplasts with ToMV, Takahashi *et al.* (1998) observed a tubular structure on the surface of protoplasts somewhat resembling plasmodesmata (figure 7). These structures result from a function of the 30K protein. There are several other reports that the MP of other plant viruses produced tubular structures similar to the above (Van Lent *et al.* 1991; Kasteel *et al.* 1997; Kikkert *et al.* 1997), but these contain virus particles. It may be that MP not only widens the paths of existing plasmodesmata, but also induces synthesis of new plasmodesmata.

In subsequent studies, MP has been discovered in almost all plant viruses examined. In the process of the evolution of plant viruses, the MP gene must have been acquired to enable them to pass through cell walls. The discovery of this function of the TMV 30K protein was a starting point for attracting many researchers to plasmodesma research. Today, the elucidation of mechanisms of information transmission among cells through plasmodesmata has become a leading topic in plant science.

(d) *The coat protein (CP)*

The CP protects genomic RNA by forming a virus particle. However, reverse genetics experiments showed that the CP is multifunctional.

Some time ago it was observed that mutants with defective CP were not only unable to form virus particles but also unable to spread rapidly to non-inoculated leaves (Siegel *et al.* 1962). Thus, it was suggested that the CP was involved in long-distance virus movement. Takamatsu *et al.* (1987) constructed a TMV mutant, lacking the CP gene (figure 5*e*), which was apparently defective in systemic movement. Involvement of the CP in long-distance movement was further investigated by Saito *et al.* (1990), who made many kinds of mutation in the CP gene. No virus particles were produced *in vivo* by any mutant which had lost the ability to move long distances. Mutants which produced CP retained the ability to form virus particles *in vivo*, spread systemically and caused mosaic symptoms. These results suggested that the ability of TMV CP to assemble into virus particles is crucial for long-distance movement and that TMV particles may play a pivotal role in such movement.

Nicotiana species with the *N′* gene react to the common strain of TMV with systemic mosaic disease, whereas ToMV produces necrotic local lesions. By constructing recombinants between these two virus strains, Saito *et al.* (1987) showed that the CP gene of ToMV encoded a factor responsible for induction of the hypersensitive response (HR) in plants with the *N′* gene. Moreover, Knorr & Dawson (1988) showed that a single point mutation in the CP gene of a common strain of TMV, accompanied by an amino acid change, gave an isolate which induced HR in *N′* plants. Other studies with mutants, in which the CP gene was partially deleted, showed that CP could also influence symptoms in other ways (Dawson *et al.* 1988; Saito *et al.* 1989).

(e) *Untranslated regions*

TMV RNA has a non-coding region 68 nucleotides long at the 5′-end. A long G-deficient stretch here, called Ω (Mandeles 1968), is well conserved among several TMV strains. ToMV mutants which carry several deletions in this sequence were constructed and their multiplication was analysed (Takamatsu *et al.* 1991; Watanabe *et al.* 1996). Several mutants with deletions of about ten nucleotides retained the ability to replicate, but those with long deletions did not.

At the 3′-extremity of the 3′-terminal non-coding region of about 200 nucleotides, a tRNA-like structure can theoretically be folded (Pleij *et al.* 1985; Rietveld *et al.* 1984). The 3′-end of the common strain RNA can be aminoacylated with His (Oberg & Philipson 1972), whereas that of TMV-Cc can be aminoacylated with Val (Beachy *et al.* 1976). Preceding the tRNA-like structure, three consecutive pseudoknot structures are found in all TMV strains sequenced so far (Van Belkum *et al.* 1985; Garcia-Arenal 1988). When the hairpin structure in one pseudoknot was destabilized by base substitutions, infectivity was lost (Takamatsu *et al.* 1990).

5. STRATEGY FOR TMV GENE EXPRESSION

(a) *Expression of the 130K and 180K protein genes*

The genome of TMV encodes at least three non-structural proteins and the CP (figure 1). The genomic RNA has been translated in several cell-free systems. In reticulocyte lysates (Knowland *et al.* 1975; Pelham & Jackson 1976) and in wheat germ extract (Bruening *et al.* 1976), only the two large 130K and 180K proteins, encoded near the 5′-end of the genomic RNA, were translated. Synthesis of the 130K protein predominates over that of 180K protein. Pelham (1978) showed that the synthesis of these two proteins was initiated at the same site, and that the 180K protein was generated by read-through of the termination codon of the 130K

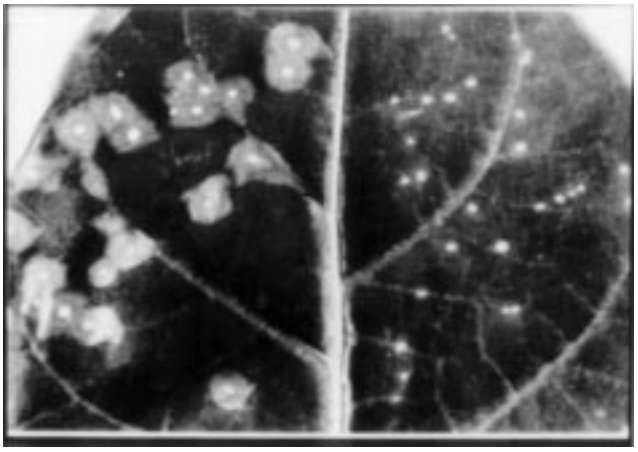

Figure 6. Temperature shift treatment for tobacco half-leaves inoculated with TMV-L or with TMV-Ls1. Necrotic lesions were photographed after treating the infected leaf first at 22 °C for 3 days, then at 32 °C for 2 days, and finally at 22 °C for 1 day. In the area surrounding the necrotic lesions, a collapsed area appeared for TMV-L (left), but not for TMV-Ls1 (right).

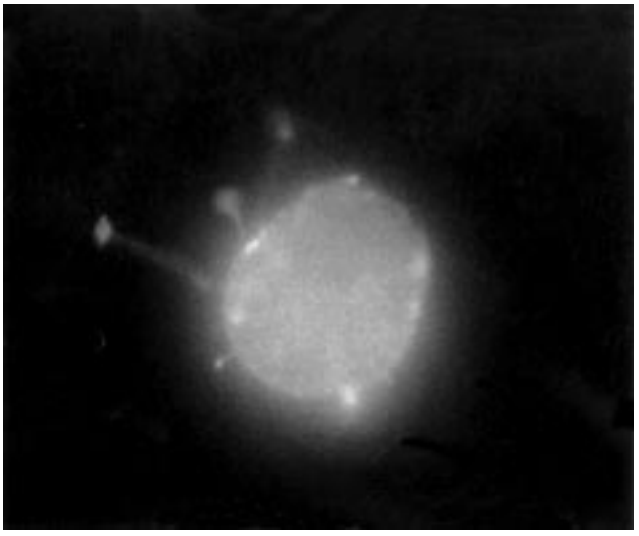

Figure 7. Tubular structures on the surface of a tomato protoplast infected with ToMV carrying a MP-GFP fusion protein gene. Observed under a fluorescence microscope at 18 h after inoculation.

protein gene. The genes located internally in the genome are not expressed from the genomic RNA, but from subgenomic RNAs which are generated during the course of replication (Beachy *et al.* 1976; Hunter *et al.* 1976; Palukaitis *et al.* 1983; Sulzinski *et al.* 1985).

(b) *Expression of the coat protein gene*

Initially Tsugita *et al.* (1962) reported that TMV CP was synthesized under the direction of genomic RNA in a cell-free, protein-synthesizing *E. coli* system. However, a more thorough investigation later revealed that the product synthesized was not a TMV protein (Aach *et al.* 1964; Schwartz 1967). After this revelation, many researchers attempted to produce the CP using genomic RNA in cell-free translation systems: the *E. coli* system (Schwartz 1967), wheat germ system (Hunter *et al.* 1976; Efron & Marcus 1973) and amphibian oocyte system (Knowland 1974). However, all attempts failed and it was concluded that TMV genomic RNA was not an efficient template for CP gene translation.

TMV-induced RNAs smaller than genomic RNA had been found in TMV-infected tobacco tissues and protoplasts, and named LMC-RNA (Jackson *et al.* 1972). This LMC-RNA was shown to be associated with polyribosomes from infected cells, which suggested that it was directly translated *in vivo* (Beachy & Zaitlin 1975). Indeed, in several cell-free systems, LMC-RNA (MW 0.25×10^6) isolated from infected plants could be translated *in vitro* to produce a protein indistinguishable from the authentic CP (Knowland *et al.* 1975; Hunter *et al.* 1976; Siegel *et al.* 1976). Thus, LMC-RNA was confirmed as the subgenomic mRNA for TMV CP (figure 1). The 5′-end of the LMC-RNA is located at residue 693 from the 3′-terminus of the genomic RNA (Browning & Clark 1980; Guilley *et al.* 1979). LMC-RNA was shown to be capped at the 5′-end, just as is the genomic RNA (Zimmern 1975; Keith & Fraenkel-Conrat 1975).

LMC-RNA was absent from RNA extracted from virions of the U1 and U2 strains of TMV (Skotnicki *et al.* 1976; Beachy & Zaitlin 1977). However, the cowpea (Cc) strain produced, in addition to the normal-length TMV rods, other shorter rods containing the CP messenger RNA (Bruening *et al.* 1976; Higgins *et al.* 1976). Later it was shown that cucumber green mottle mosaic tobamovirus, watermelon strain (CGMMV-W) also contained such short rods (Fukuda *et al.* 1981). This is because the particle assembly origin of the Cc and CGMMV-W strains is located in the CP cistron (Fukuda *et al.* 1981).

(c) *Expression of the 30K protein gene*

The RNA isolated from purified TMV particles contains, in addition to the full-length genomic RNA, a heterogeneous population of molecules with molecular weights in the range between 0.6×10^6 and 1.6×10^6. Among these, an RNA of discrete size and molecular weight about 0.68×10^6 was termed intermediate-length RNA-2 (I_2-RNA), and that with molecular weights $0.9–1.6\times10^6$ was called I_1-RNA (Beachy & Zaitlin 1977). These RNAs are derived from the 3′-end of the genomic RNA.

I_2-RNA, isolated from infected tissues, was translated *in vitro* into the 30K protein (Beachy & Zaitlin 1977). Thus, I_2-RNA was confirmed to be the subgenomic mRNA for the 30K protein (figure 1). The 5′-end structure of I_2-RNA is still controversial (Joshi *et al.* 1983; Hunter *et al.* 1983; Beachy & Zaitlin 1977; Watanabe *et al.* 1984*b*). The initiation site for transcription of the 30K protein mRNA was mapped at residue 1558 from the 3′-terminus of genomic RNA (Watanabe *et al.* 1984*b*; Meshi *et al.* 1982*a*). Moreover, homology exists between the sequences around the capping site of the CP mRNA and of the 30K protein mRNA. These homologous areas may represent the promoter for subgenomic RNA synthesis by the viral RNA polymerase (Watanabe *et al.* 1984*b*).

The 30K protein is expressed early and transiently in infection, whereas the CP is expressed later and persistently (Watanabe *et al.* 1984*a*). However, when under the control of the CP subgenomic promoter in a recombinant isolate, expression of the 30K protein became late (Lehto

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

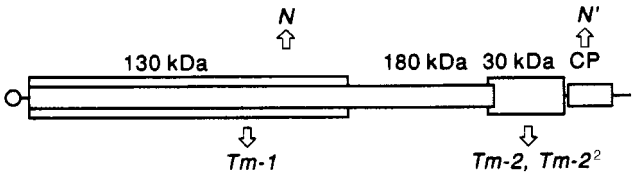

Figure 8. Relationship between host resistance genes and the TMV-coded proteins which activate them.

et al. 1990). This result suggests that the promoter sequence controls the timing of expression.

(d) *Other proteins*

In TMV-infected plants, Goelet & Karn (1982) detected many mRNA species which had the same 3′-terminal sequence as, and hybridized to, cDNA of TMV-RNA. Also, Sulzinski *et al.* (1985) reported that the 54K protein, corresponding to the C-terminal portion of the 180K protein, was translated from I_1-RNA. However, in TMV-infected protoplasts, the genomic RNA, the 30K protein mRNA (I_2) and the coat protein mRNA (LMC) were detected but I_1-RNA (Watanabe *et al.* 1984*a*) and the 54K protein were not (Saito *et al.* 1986). Therefore, only the 130K, 180K, 30K and CPs are expected to play a role in the replication of TMV.

(e) *Uncoating of TMV RNA after inoculation*

For TMV to express its genes after infecting plant cells, the genomic RNA has to be uncoated and made available for translation. Wilson (1984) proposed a cotranslational disassembly hypothesis based on results of experiments *in vitro*. This predicts that CP subunits are released first from the 5′-end of the TMV rod, that ribosomes bind to the exposed part of the RNA and that translation of the first ORF then begins. As the translation progresses, additional CP subunits are released. Mundry *et al.* (1991) have confirmed that the non-coding region at the 5′-end of TMV RNA is uncoated rapidly at the initial stage of translation.

The occurrence of cotranslational disassembly *in vivo* was reported by Shaw *et al.* (1986). Furthermore, Wu *et al.* (1994) showed, using protoplasts, that within the first 3 min of inoculation, a region of at least 4635 nucleotides from the 5′-end was disassembled. This represents about 72% of the TMV particle and roughly equates to the entire 180K protein ORF (78% of the TMV particle).

6. RELATIONSHIP BETWEEN HOST RESISTANCE GENES AND TMV-ENCODED PROTEINS

The resistance genes, *Tm-1*, *Tm-2* and *Tm-2²*, have been identified in tomato and are used in practice to protect tomato plants from TMV infection. In tomato plants with the *Tm-1* gene, multiplication of TMV is inhibited. Meshi *et al.* (1988) examined the nucleotide sequence of TMV Lta1, which is a spontaneous resistance-breaking mutant derived from TMV L. They found two amino acid substitutions in the 130K and 180K proteins. Therefore, the 130K and 180K proteins may be involved in interaction with the putative *Tm-1* gene product.

The resistance genes, *Tm-2* and *Tm-2²*, are expressed only in intact tissues. Wild-type TMV can multiply in protoplasts isolated from tomato plants with either of these genes. Thus these resistance genes operate at the level of cell-to-cell movement. A resistance-breaking strain, TMV Ltb1, which is able to spread systemically in tomato plants that have the *Tm-2* gene, has two amino acid changes in the 30K protein compared with that of the parental isolate (Meshi *et al.* 1989). Similarly, a resistance-breaking strain that can invade tomato plants that have the *Tm-2²* gene also had two amino acids in the 30K protein that were changed from those in the parent virus (Weber *et al.* 1993). The 30K protein may therefore interact with the *Tm-2* and *Tm-2²* gene products to prevent viral cell-to-cell movement.

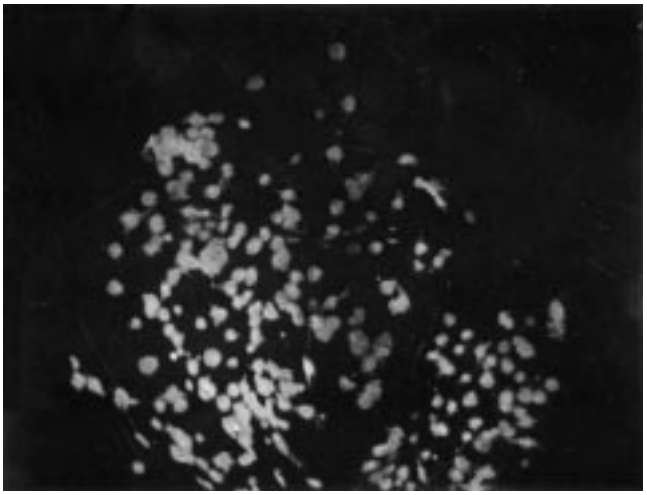

Figure 9. Fluorescence emitted, under UV light, from a tobacco leaf which was infected with TMV whose CP gene was replaced by a GFP gene (seven days after inoculation). The virus spreads from cell to cell only.

Almost all tobamoviruses cause HR in tobacco plants carrying the *N* gene but a tobamovirus uniquely capable of overcoming this resistance has been isolated and designated ToMV Ob (Tobias *et al.* 1982; Csillery *et al.* 1983). Padgett & Beachy (1993) analysed a chemically induced mutant of the cloned Ob virus that induces local lesions on tobacco with the *N* gene. They found that the mutant contained a single amino acid change in the 130K protein, suggesting that the 130K protein is required for HR induction in tobacco with the *N* gene. Recently, the *N* gene was isolated by Whitham *et al.* (1994). The mechanism of how the *N* gene induces HR in tobacco plants by interacting with the 130K protein at the molecular level will doubtless be clarified in the near future.

The relations between TMV encoded proteins and resistance genes in plants are summarized in figure 8. Clearly, plants have evolved diverse mechanisms for preventing TMV infection and invasion of tissues.

7. TMV AS A GENE VECTOR

With the establishment of a genetic engineering system for TMV, it became possible to insert foreign genes into the viral genome and thus to develop TMV as a gene vector. For example, the CP gene could be replaced with the chloramphenicol acetyl transferase (CAT) gene (Takamatsu *et al.* 1987). Alternatively, insertion of a gene

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

for the jellyfish fluorescent protein, GFP, gives a TMV isolate that emits fluorescence under UV illumination, enabling spread of the virus in tissues to be observed (figure 9). However, TMV vectors in which the CP gene is replaced with another gene can multiply only in inoculated leaves.

More recently, many other TMV vectors, which retain the CP gene intact and can infect systemically, have been developed in many research laboratories. These newly developed vectors include those used to express α-trichosanthin (Kumagai *et al.* 1993), various animal virus epitopes (Hamamoto *et al.* 1993; Sugiyama *et al.* 1995), a malarial epitope (Turpen *et al.* 1995) and so on. Recent developments of this kind are summarized by Turpen.

I would like to thank numerous of my staff and students for their many achievements over the years when we were working together on the TMV genome at the University of Tokyo.

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Errata to volume 354

Phil. Trans. R. Soc. Lond. B **354**, 417–433 (February 1999)

Algal morphogenesis: modelling interspecific variation in *Micrasterias* with reaction–diffusion patterned catalysis of cell surface growth

David M. Holloway and Lionel G. Harrison

On page 417, the footnotes indicated by the asterisk and dagger were mistakenly reversed. D. M. Holloway has moved to the British Columbia Institute of Technology and L. G. Harrison is the author for correspondence.
In the abstract (p. 417) it should have read L. G. Harrison and not D. M. Harrison.

Phil. Trans. R. Soc. Lond. B **354**, 569–582 (March 1999)

Historical overview of research on the tobacco mosaic virus genome: genome organization, infectivity and gene manipulation

Y. Okada

In the abstract (p. 569) mitochondrial DNA was incorrectly printed instead of mRNA. The complete, corrected sentence is reproduced below.

Early in the development of molecular biology, TMV RNA was widely used as a mRNA that could be purified easily, and it contributed much to research on protein synthesis.

Phil. Trans. R. Soc. Lond. B **354**, 1325–1346 (July 1999)

Spatial attention and neglect: parietal, frontal and cingulate contributions to the mental representation and attentional targeting of salient extrapersonal events

M.-Marsel Mesulam

In the legend to figure 3 (p. 1332), the words left and right were mistakenly transposed. The complete, corrected sentence is reproduced below.

The arrows depict the directional probability of attentional shifts and representational salience, orange for the right hemisphere, purple for the left hemisphere.

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