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Tobacco mosaic virus virulence and avirulence

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In celebration of a century of research on tobacco mosaic virus that initiated the science of virology, I review recent progress relative to earlier contributions concerning how viruses cause diseases of plants and how plants defend themselves from viruses.

Keywords: tobacco mosaic virus; avirulence genes; elicitor; Beijerinck; mosaic; vein clearing

1. INTRODUCTION

As we celebrate the one-hundredth year since M. W. Beijerinck reported that the agent causing 'the spot disease (mottle or mosaic) of tobacco leaves' was 'a contagium vivum fluidum', it is equally remarkable to consider his other observations and conclusions in that same paper (Beijerinck 1898). Beijerinck carefully described the process of symptom development, the progressive development of green islands on a background of yellowed or light-green tissues. He understood that the disease resulted from abnormal development—that these symptoms were produced only in the leaves that developed subsequent to viral infection. In fact, he even realized that the major effect of the virus in stunting plants was on overall photosynthesis, referring to it as 'a disease of the chlorophyll particles'. By observing disease development from different areas of inoculation, he reasoned that the primary mode of movement throughout the plant was in the phloem and that the virus moved in parallel with photoassimilates in the plant. He also observed that a stage of local infection (cell-to-cell movement) preceded systemic infection (long-distance movement). Additionally, he demonstrated that the virus could overwinter in soil from around an infected plant and that new seedlings planted in that soil could become infected. This defined one of the methods by which plants become infected in the field.

Although presently there is a tendency for scientists in this field to believe that good science began with the advent of recombinant DNA technology and the related technologies it spawned, such as reverse genetics and the production of transgenic plants, and that publications older than your car have no value, Beijerinck gives us pause to think. In this paper, I present my perspective of our current understanding of virus–plant interactions that result in disease or resistant responses in the context of some of the original contributions by pioneers of this field.

2. VIRAL HOST RANGE

When we think of virus–plant interactions, we tend to think of active responses in which the virus begins replicating and spreading, then either continues to infect the plant causing systemic disease or is recognized by the plant in such a way that it responds with an active defence mechanism that confines the virus infection and prevents further damage. However, both of these processes are extraordinarily rare. Most plants fail to be infected by most viruses. Most viruses can infect and cause disease in only a relatively few species of plants. To infect a plant systemically, the virus must (i) be introduced into appropriate tissues; (ii) replicate; (iii) move to adjacent cells; (iv) enter; and (v) exit the phloem and then repeat steps (ii) and (iii). Each of these processes appears to require precise interactions between viral gene products and plant gene products (see Dawson 1992). It is essentially a 'rare miracle' when all of the interactions are sufficient for a particular virus to invade a particular plant systemically (see Dawson & Hilf 1992). And often when the miracle happens, there is no visible response in the plant.

However, as plant pathologists, we examine diseases, even though they might represent the atypical situation. So a recurrent question has been 'How do viruses cause diseases in plants?' How far have we progressed? There were several hypotheses described in Bawden's *Plant viruses and virus diseases* (1964) based on 'direct metabolic fatigue from host materials being diverted into virus particles'. However, as pointed out by Bawden, 'some hosts can accommodate virus multiplication without noticeable inconvenience' and other viruses 'can cripple with one-thousandth the concentration.' Additionally, some tobamoviruses have satellite viruses that are replicated to levels as high as the helper virus, essentially doubling the amount of virus made, with no additional effects on symptomology (Rodríguez-Alvarado *et al.* 1994). So, how do viruses cause disease?

3. INDUCTION OF DISEASES

Virologists from Beijerinck's time onwards continued to examine the TMV–tobacco interaction. Bawden (1964) describes the infection in three phases. The first phase is the local infection that usually occurs after inoculation of mature leaves. These leaves usually exhibit no visible effects or only a transient yellowing, depending on the virus strain and the environmental conditions. In the second phase, the virus moves from cell to cell until it

reaches a vein and vascular phloem in which it moves rapidly into a set of young leaves. These upper leaves are beyond cell division, but are still expanding and are not yet photosynthetically independent, continuing to import carbohydrates from the phloem. At the beginning of new viral replication, this area exhibits 'vein clearing', which is a translucence around tertiary and quaternary veins. The photosynthetically independent leaves between these two sets of leaves generally develop no disease symptoms. The third phase of symptomology results when a set of even smaller leaves, still undergoing cell division, grows and differentiates after infection. These leaves develop an uneven pattern of dark-green patches on a light-green background, often with considerable leaf deformity. Disease symptoms induced in each of the phases probably result from different mechanisms.

4. DISEASE SYMPTOMS IN MATURE LEAVES

Many strains of TMV and other tobamoviruses induce chlorosis as the virus multiplies in inoculated tobacco leaves. The leaves that normally are selected for inoculation are almost fully grown with fully developed chloroplasts. Some mutants of TMV induce dramatic yellowing of inoculated leaves. Our laboratory (Dawson *et al*. 1988) and Okada's laboratory (Saito *et al*. 1989) produced such mutants by making deletions in the coat protein gene. Amino-acid substitution mutants (Banerjee *et al*. 1995) or assembly-competent insertion mutants (Turpen *et al*. 1995) can induce similar symptoms. Electron microscopic examination of the chlorotic tissues demonstrated that the chloroplasts within the affected areas were rapidly disrupted in contrast to the normal chloroplasts in adjacent non-infected areas (Lindbeck *et al*. 1991). A direct correlation was found between the degree of chloroplast disruption and the amount of cytoplasmic aggregates of non-assembled coat protein. Even though coat protein of some mutants has been found within chloroplasts (Banerjee *et al*. 1995), we were able to find none of the deleted coat protein within chloroplasts (Lindbeck *et al*. 1991). Since these chloroplasts were mature prior to the cell becoming infected by the virus, the chloroplasts appear to have become disorganized after the cell became infected. Since chloroplasts are dynamic organelles that require constant maintenance, the accumulation of the excess coat protein in the cytoplasm appeared to interfere with their maintenance. However, we do not know how altered coat protein molecules, perhaps from outside the chloroplast, interfere with its maintenance and integrity.

In 1931, Holmes showed that TMV infections interfere with carbohydrate transport. Iodine, which was used to assay starch in cleared leaves, was found to be useful for monitoring or quantifying viral infections in inoculated leaves. If the starch-iodine test was conducted on leaves in early evening after a day of light, starch staining was lighter in infected areas, but if the assay was conducted in the early morning, infected areas stained more darkly. Lucas and co-workers have found that the TMV movement protein affects photoassimilate partitioning between various organs in plants (Balachandran *et al*. 1995; Almon *et al*. 1997). Constitutive expression of the TMV movement protein in transgenic plants inhibits sucrose export and causes increased accumulation of carbohydrates in source leaves during the day. It also causes significant reductions in root growth, resulting in plants with a lower root-to-shoot ratio. Does the virus-produced movement protein, moving essentially as a wave through the plant, affect carbohydrate partitioning as does constitutive expression of this protein in transgenic plants?

5. VEIN CLEARING OF UPPER SMALL LEAVES

Experimentally inoculated plants, in which the lower mature leaves are heavily inoculated, produce one large flush of virus that moves through the phloem into the set of small upper leaves (*ca*. 1–4 cm long) and the basal region of the adjacent larger leaves (up to *ca*. 7 cm) that still are phloem sinks. Zech (1952) reported that most cells in these leaves appeared to be near the same stage of infection, based on the even distribution and development of viral inclusions. Nilsson-Tillgren *et al*. (1969) later re-examined this system by electron microscopy and showed that progeny virions accumulated in most cells at the same time. These observations led to our development of the 'magic box', in which differential temperatures were used to further synchronize this systemic inoculation process (Dawson & Schlegel 1973; Dawson *et al*. 1975). The lower inoculated leaves of tobacco plants were maintained at a temperature (25 °C) that was optimal for replication and movement, while the upper (phloem-sink) leaves were maintained at a temperature restrictive for replication (3 °C). After the virus had time to move into the upper leaves, they were shifted to a permissive temperature (25 °C) to allow initiation of replication. These upper leaves developed the vein-clearing symptoms.

For a time, W. W. Thomson (University of California, Riverside) and I collaborated to examine the development of vein-clearing symptoms induced by TMV in these leaves. As differential temperatures could be used to help synchronize TMV infections in the upper leaves of tobacco plants, they also synchronized the vein-clearing process. After shifting the small leaves from the restrictive to the permissive temperature, vein clearing occurred at 28 h (±30 min). This was about the time that replication in these leaves switched from the exponential to the linear phase of virus accumulation (Dawson & Schlegel 1976). Vein clearing only occurred at temperatures above 25 °C and the intensity of the vein-clearing symptoms increased with increasing temperatures up to *ca*. 40 °C. Vein clearing at 25 °C requires a trained eye for detection, but vein clearing that occurred at 40 °C was so visible that it could be seen from a distance of 20 m. Vein clearing was light-dependent and did not occur in plants incubated in darkness. Not only did vein clearing precede most of the viral replication in the leaves, but it could occur during a period in which there was no ongoing viral replication. TMV replication stops at 40 °C (Dawson 1976). If leaves were shifted from 25 °C to 40 °C to stop replication at 20 or 24 h, vein clearing still occurred at 28 h. The most remarkable observation was that the full manifestation of vein clearing could occur within a 5 min period (W. O. Dawson, unpublished data).

Unfortunately, we found that defining the conditions for production of vein-clearing symptoms was much easier than describing the physical effects of vein clearing within the leaf. Bill Thompson and Tony Endress

(University of California, Riverside) examined the ultrastructure of vein-cleared leaves. Although we expected that vascular chloroplasts would be degraded or abnormal, they found no abnormalities after looking at hundreds of micrographs. In fact, looking at vein clearing was like looking at clouds: the closer we looked, the less we saw. Experts in chloroplast biology suggested that the rapid time (*ca.* 5 min) of development of visual vein clearing was too short for chloroplasts to degrade and that the translucence might be some type of optical illusion. We were so discouraged by our abortive attempts to detect vein-clearing ultrastructure that we never published any of the data regarding this phenomenon.

6. DEVELOPMENT OF MOSAIC OR MOTTLE SYMPTOMS

The definitive work on development of mosaics was from R. E. F Matthews's laboratory, a quarter of a century ago, largely done by examining turnip yellow mosaic virus in Chinese cabbage (see Matthews 1973, 1981). Enough work was done with TMV in tobacco to demonstrate the similarity of the two virus–plant systems. Green islands consist of clusters of cells that are resistant to infection. Cells from a chlorotic sector, which are full of virus, can be adjacent to green-island cells that contain no virus. Plants regenerated from green-island-derived protoplasts can be virus-free and resistant to viral infection for a period of time (Murakishi & Carlson 1976). Matthews and co-workers showed that green islands result as the cells in small leaves (a few millimetres in length) divide and develop after infection of the leaf. The green islands are clones of cells that originate from a single cell or a cluster of a few cells. Most of the photosynthesis in an infected plant comes from the lower, non-symptomatic leaves and the green islands. Little photosynthesis occurs in the chlorotic tissues. The stunting of infected plants is due to the deficit in net photosynthesis. This was demonstrated by removing the non-symptomatic leaves from infected plants and their equivalents from healthy plants. The subsequent growth of the infected plants was less than 10% of that of the corresponding healthy plants. Since this seminal work was done between 1965 and 1975 by the Matthews group, there has been little further information concerning the development of this basic type of virus-induced symptom.

The precise induction of clones of cells that are resistant to viral infection might parallel the 'recovery' phase that occurs in other types of virus diseases. In these plants, the upper developing leaves progressively develop fewer symptoms. Recent experiments have shown this phenomenon to be due to expression of 'RNA-mediated resistance' or 'gene silencing' (Ratcliff *et al.* 1997). This also parallels the 'recovery' phenomenon, which has been shown to be a form of RNA-mediated resistance in transgenic plants that is activated by viral infection (Lindbo *et al.* 1993). It would not be surprising if the formation of dark-green islands is found to be a manifestation of this same process.

After the virus moves near the shoot apex, either by long-distance movement from lower leaves, by cell-to-cell movement up the plant stem or by directly inoculating sites near the shoot apex, only cell-to-cell movement is needed for infection of newly developing leaves and induction of mosaic symptoms. This portion of the systemic infection is not due to phloem-associated long-distance movement. TMV mutants with defective or totally deleted coat protein genes induce normal mosaic symptoms if they are able to reach this area (Culver & Dawson 1989; Lindbeck *et al.* 1992). Additionally, this demonstrates that even though the coat protein can be involved in disorganization of mature chloroplasts, the coat protein is not involved in induction of mosaic symptoms. However, the mutant coat proteins that cause mature chloroplast disorganization can modify the mosaic symptoms by adding a bright yellowing component to the light and dark green of the mosaics (Lindbeck *et al.* 1992).

Several tobamovirus mutants fail to induce mosaic symptoms, even though they infect the plant systemically. Most of these mutations were mapped to the viral replicase genes. An attenuated strain of tomato mosaic virus, L11A, which produces very mild symptoms in tobacco, has ten nucleotide changes in the 126/183 kDa ORF compared to the wild-type virus (Nishigushi *et al.* 1985). Similarly, the reduced severity of mosaic symptoms of Holmes's masked strain of TMV was mapped to the replicase gene (Holt *et al.* 1990), in which a minimum of eight nucleotides is required for the phenotypic change (Shintaku *et al.* 1996). This mutant has been shown to be slightly delayed in long-distance movement. We isolated a mutant of TMV with a single amino-acid change in the replicase protein that induces no visible symptoms in fully infected tobacco plants. This mutation results in reduced levels of replication but allows normal movement (Lewandowski & Dawson 1993). These and other data suggest that the timing of virus movement into these leaves and thus the stage of development that cells or their neighbours become infected is the critical determinant of the induction of the pattern of chlorotic and resistant clones of cells that develop into a mosaic symptom (see Dawson 1990). Yet, we still do not know how the virus affects the development of the chloroplasts.

Although the 'type plant virus disease' is TMV in tobacco, one of the failures of my career came from trying to get TMV to cause disease in tobacco tissue cultures. With the idea that plant mutants could lose their ability to support a function needed by the virus, we wanted to develop a method for selecting such a plant from cells in culture (Dawson 1985). Infected callus cultures, initiated from infected plants, maintain the virus during proliferation and regenerate into new plants in culture that have a recognizable mosaic before the time the plant is 1 cm in height. Professor T. Murashigi, Carol Boyd and I examined a large number of optimal, and purposefully suboptimal, conditions (temperatures, light, hormones, sugars, antagonists, etc.) during growth or regeneration in an effort to give healthy cultures an advantage over TMV-infected cultures. We never found anything encouraging. Although the virus greatly reduces the growth of tobacco plants, we could not find any negative effect of TMV in tobacco tissue cultures.

Recent studies from Maule's laboratory (Aranda *et al.* 1996; Técsi *et al.* 1996) demonstrated that the polyviral infection causes a transient inhibition or interruption of host metabolism. This phenomenon only occurred at the

leading edge of the infection as it spread as a wave through tissues. There are numerous 'leading-edge' phenomena associated with viral symptomologies. Different types of 'ring-spot' symptoms are the result of diurnal patterns of spread of the viral infection. More complex symptom patterns, such as herringbone-like designs (see Dawson *et al*. 1988), might result from related interactions. We found that manipulation of temperature and light regimes can be used to create elaborate symptom patterns in which white or yellow lines demarcate the borders between healthy and infected cells (Lozoya-Saldana & Dawson 1982).

A characteristic of many systemic symptoms induced by strains of TMV and other tobamoviruses is necrosis, often mixed within the mosaic. This is strikingly evident when reading symptom descriptions of TMV strains and related tobamoviruses in Smith's (1957) *A textbook of plant virus diseases*. Many strains of tobamoviruses induce a pattern of mosaics or mottles of light green and dark green mixed with necrotic spots. In fact, the isolate of virus used by Beijerinck (1898) induced necrotic spots mixed in the mosaic. An extreme example is the internal-browning disease of tomatoes, characterized by death of the interior of fruit with the exterior appearing normal. In the next section I discuss the hypersensitive response (HR) that confines the virus within or near a necrotic area. Although this can be a resistance reaction, the response clearly is a race between the speed with which the virus can replicate and move within the plant and how fast the plant defence response can confine the virus. If the virus wins, the plant dies. One can artificially manipulate this process to cause small necrotic spots that quickly confine the virus, or to allow the virus to induce full systemic necrosis. Although the HR might be responsible for some of the necrosis mixed with the mosaic pattern, HR is thought to result from a specific reaction in which one plant gene specifically recognizes a viral component. Some other types of necrotic responses are less specific. Several coat protein deletion mutants induced necrosis, but apparently not in a gene-for-gene manner, because they induced similar necrosis in essentially all plants infected (Dawson *et al*. 1988; Dawson & Bubrick 1989). Likewise, a substantial proportion of all coat protein-fusion mutants, including an array of peptides attached to different parts of the TMV or other viral coat protein, have induced a similar necrosis (W. O. Dawson, unpublished data). Since many tobamovirus isolates induce mixed necrotic and mosaic symptoms in a range of plant species, the necrotic component of these diseases might be due to this non-specific type of necrosis.

7. PLANT RESISTANCE RESPONSES AND VIRAL AVIRULENCE GENES

The HR is an important resistance response of plants against pathogens. It is an active response in which the plant recognizes the pathogen and turns on a cascade of events that leads to confinement of the pathogen to the initial infection area. The HR normally results in localized cell death. With the recent isolation and description of HR-type resistance genes and pathogen avirulence genes, the consensus appears to be that the trigger of the HR is a specific interaction between a receptor domain of the plant resistance gene product and a specific pathogen elicitor, which is the product of the avirulence gene. Different forms of the elicitor can interact differently with the resistance gene. Strong elicitors are recognized and induce the resistance response quickly, with the pathogen then being confined to a small area surrounding the initial infection site. Weak elicitors induce the HR slowly, allowing the pathogen to spread further before it is confined, if at all. These differences are probably due to the availability or affinity of the elicitor to the receptor.

The most-studied genes for resistance to tobamoviruses are the *N* and *N′*-genes in tobacco. One of the most important discoveries of plant virology was that local lesions could be used to quantify the infectivity of virus preparations (Holmes 1929). This was the assay that was needed for the initial purifications and characterizations of TMV, and it has been an important part of the foundation of TMV biology. The *N* gene from *Nicotiana glutinosa* has been bred into tobacco to confer resistance to TMV. It is a dominant gene that provides resistance to essentially all tobamoviruses. Weber (1951) showed that *N′*, which occurs in *N. sylvestris* and several varieties of tobacco, localizes most tobamoviruses and some TMV mutants, but not wild-type TMV. This gene is semi-dominant.

(a) *The* N′ *gene elicitor*

One of the first avirulence genes and elicitors defined for pathogen–host systems was the tobamovirus coat protein that triggers the *N′* gene-mediated resistance. Since the TMV coat protein was one of the first proteins sequenced and TMV mutants that induce the *N′* HR are easy to isolate, the amino-acid changes in the coat protein had long been associated with the induction of the HR (Mundry & Gierer 1958; Funatsu & Fraenkel-Conrat 1964). However, development of reverse-genetic systems allowed the definitive demonstration that the coat protein gene was the avirulence gene (Saito *et al*. 1987; Knorr & Dawson 1988). Although the *N′* gene is not characterized, substantial information is known about the elicitor (see Culver *et al*. 1991). This is largely due to the fact that we developed a genetic system for a protein whose structure was already solved; this was begun about 50 years ago by Watson (1954) and Franklin (1956) and later greatly refined (Stubbs *et al*. 1977; Bloomer *et al*. 1978; Namba *et al*. 1989). Essentially, the entire structure of the coat protein is required for recognition by the *N′* product (Saito *et al*. 1989). Mutations of elicitor coat proteins that cause incorrect folding render them non-elicitors (Culver *et al*. 1994). The wild-type TMV coat protein is not recognized, but specific structural alterations cause the proteins to become recognized. The wild-type protein is able to overcome recognition by hiding the recognition site. Mutants that fail to be recognized form tight coat protein aggregates, weak elicitors form looser aggregates, whereas strong elicitors form the loosest aggregates. Mutations that cause the coat protein to become recognized are those that weaken protein–protein interactions. Amino acids that appear to have a direct interaction with the resistance gene product are internal in protein aggregates along the right-hand face of the helical bundle (Taraporewala & Culver 1996, 1997).

(b) *The* N *gene elicitor*

The *N* gene has been isolated and characterized (Whitham *et al*. 1994; Baker, this issue) and the corresponding avirulence gene has been mapped to the helicase domain of the 126/183 kDa replicase gene (Padgett & Beachy 1993; Padgett *et al*. 1997). However, based on several observations, there are probably specific forms of replicase that induce this reaction. First, the induction of the HR is temperature-sensitive for the specific tobamovirus. For example, our isolate of TMV-Ob only induces necrotic local lesions at temperatures below 20 °C, whereas, TMV and several other tobamoviruses induce lesions below *ca*. 28 °C, and TMV strains U5 and U2 (tobacco mild green mosaic virus) induce lesions below 30 °C (D. D. Dunigan and W. O. Dawson, unpublished data). Also, necrosis is induced only in areas of the leaf in which there is active virus replication. If leaves are shifted from the restrictive to the permissive temperature at ten days after inoculation, a time when the viral infection has spread to approach the margins of the leaf and replication has stopped in the earlier infected areas, necrosis only occurs in a ring near the advancing edge of the infected areas. If the leaves are maintained at the restrictive temperature for 14 days and then shifted to the permissive temperature, no necrosis occurs in that leaf. It is easy to isolate mutants of TMV that are altered in this phenotype (see Lewandowski & Dawson 1993).

Among different *Nicotiana* species, there are numerous *N* and *N′* phenotypes that probably are due to different, perhaps related, genes. Although reacting specifically to certain tobamoviruses, these responses tend to vary in expression. For example, both *N* and *N′* responses are stronger and faster in the more mature leaves of the plant, whereas in other species the HR is often stronger in the younger leaves. Another phenotypic variation is random necrosis in which only a portion of the infected areas becomes necrotic. One HR phenotype in the tobacco variety, Yellow Prior, was almost indistinguishable from the *N* phenotype, yet the response appeared to be recessive (D. J. Lewandowski and W. O. Dawson, unpublished data). An eventual understanding of how these resistance gene products interact with viruses will probably give us a much better understanding of how plants evolved resistance against viruses and other pathogens.

(c) *Other avirulence genes*

Avirulence genes corresponding to resistance genes in other plants also have been characterized. In tomato, the *Tm-2* and *Tm-2²* genes provide an HR-type resistance to tobamoviruses. The avirulence gene for this reaction is the movement protein (Meshi *et* al. 1989; Calder & Palukaitis 1992; Weber *et al*. 1993). In pepper, two resistance genes, *L2* and *L3*, recognize the pepper mild mottle tobamovirus coat protein (Berzal-Herranz *et al*. 1995; Tsuda *et al*. 1998; de la Cruz 1997). In eggplant, the tobamovirus coat protein also is the elicitor (Dardick & Culver 1997). However, these interactions appear to be different from the *N′* interaction. Since all of the tobamoviral gene products have been shown to act as an elicitor in some plant, viral elicitors appear to be any viral product that the plant can evolve a way to recognize, and different plants can recognize the same viral gene product differently.

8. CONCLUSIONS

It is important to understand that there are two different levels at which a virus interacts with a plant. The primary functions of viral gene products are those functions that are necessary for the virus to survive: replication, movement and encapsidation. These gene products must interact precisely with a complementary set of host plant gene products to accomplish these functions. For the complementary plant gene products, their virus-related functions are secondary. Viral gene products function at a secondary level by interfering with some host processes, causing disease or being recognized by a plant resistance gene to trigger a resistant response. These secondary interactions are probably accidental. One goal for the next hundred years is to define these interactions.

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