Micro-Review

Tobraviruses—plant pathogens and tools for biotechnology

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SUMMARY

The tobnaviruses, *Tobacco rattle virus* (TRV), *Pea early-browning virus* (PEBV) and *Pepper ringspot virus* (PepRSV), are positive-strand RNA viruses with rod-shaped virus particles that are transmitted between plants by trichodoriid nematodes. As a group, these viruses infect many plant species, with TRV having the widest host range. Recent studies have begun to dissect the interaction of TRV with potato, currently the most commercially important crop disease caused by any of the tobnaviruses. As well as being successful plant pathogens, these viruses have become widely used as vectors for expression in plants of nonviral proteins or, more frequently, as initiators of virus-induced gene silencing (VIGS). Precisely why tobnaviruses should be so effective as VIGS vectors is not known; however, molecular studies of the mode of action of the tobnavirus silencing suppressor protein are shedding some light on this process.

INTRODUCTION

The study of plant viruses can be motivated by different but often overlapping interests. Initially, a particular virus may become noticed because it emerges as the cause of disease affecting a valued crop or wild plant. The virus may then be studied as an organism in its own right, focusing on, for example, the genome organization and structural features of the virus. The virus might also be studied as an example of a plant pathogen, with the interactions between virus and plant host as the focus. More recently, plant viruses have been adapted as biotechnological tools, useful for studying aspects of plant biology completely separate from their original roles as plant pathogens. The tobnaviruses have passed through all of these stages in their research history, with one of their number, *Tobacco rattle virus* (TRV), probably now being more widely known as a tool for molecular genetic research than as a disease agent.

This review aims to build on an earlier article in this series that described the molecular details of the transmission of tobnaviruses by soil-inhabiting nematodes (MacFarlane, 2003), and presents more recent research findings for these viruses.

THE GENOME STRUCTURE OF TOBRAVIRUSES

The genus *Tobravirus* comprises three viruses, TRV, *Pea early-browning virus* (PEBV) and *Pepper ringspot virus* (PepRSV), which, in the early literature, was referred to as the CAM strain of TRV (Harrison and Robinson, 1986; MacFarlane, 1999; Robinson, 2005). Tobnaviruses have two rod-shaped particles of different sizes and, characteristically, are transmitted between plants by trichodoriid nematodes. Each of these viruses has a genome of two positive-sense, single-stranded RNAs. The larger RNA (RNA1) is about 6.8 kb in size and has a large 5' proximal open reading frame (ORF) encoding a 134–141-kDa molecular mass protein with methyltransferase and helicase amino acid motifs. Readthrough translation of the stop codon of this ORF produces a 194–201-K protein with RNA-dependent RNA polymerase (RdRp) motifs in its C-terminal portion. Further downstream in RNA1 is the *1a* ORF encoding a 29–30-K movement protein, followed by the *1b* ORF encoding a 12–16-K cysteine-rich silencing suppressor protein. In TRV, there is an ORF located in a different reading frame within the *1b* gene, potentially encoding a 13-K protein. The helicase and RdRp proteins are translated directly from RNA1, whereas the *1a* and *1b* genes are translated from subgenomic RNAs (sgRNAs).

The second, smaller genomic RNA (RNA2) encodes the virus coat protein (CP) as well as one or more additional proteins (2b and 2c) that are involved in the transmission of tobnaviruses by nematodes. RNA2 varies considerably (1.8–3.9 kb) between different tobnavirus isolates, where one or both of the *2b* and *2c* genes may be missing, and where part or all of the 3' region of RNA2 that encodes these genes and the 3' noncoding region may be replaced by recombination with 3' portions of RNA1. Isolates that lack the *2b* gene are expected not to be nematode transmissible, and would be confined to the plant in which they were found, although TRV and PEBV are seed transmissible in some plant species, which may allow the spread of some of these

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deletion-containing isolates. All the genes on RNA2, including the 5' proximal CP gene, are translated from sgRNAs. Recently, RNA2 of the SYM isolate of TRV has been found to encode several novel genes upstream of the CP gene, the only example in which the tobnavirus CP gene is not located at the 5' end of RNA2 (S. MacFarlane, unpublished).

AREAS OF RECENT TOBRAVIRUS RESEARCH

PepRSV has not appeared in the literature since the completion of sequencing studies (Bergh and Siegel, 1989), a structural study of the CP (Brierley *et al.*, 1993) and the modification of PepRSV RNA2 as a vector for the expression of nonviral proteins (MacFarlane and Popovich, 2000). Molecular aspects of the nematode and seed transmission of PEBV have been described previously (Escaler *et al.* 2000; MacFarlane, 2003; Wang *et al.*, 1997). More recent work on PEBV has focused on its use in virus-induced gene silencing (VIGS) studies (see below). No recent reports have been made describing infection by PepRSV or PEBV in cultivated crops or wild plants.

TRV AS A POTATO PATHOGEN

TRV continues to be a significant pathogen of cultivated potato, producing arcs and flecks of corky material (referred to as spraing or corky ringspot) in tubers of some cultivars. These disease symptoms can render an entire crop unmarketable, which has stimulated research into the production of diagnostics, crop production techniques and breeding for resistance.

There is very high nucleotide sequence homology (more than 95% identity) between RNA1 species from different TRV isolates, whereas RNA2 species may have very little sequence identity. Thus, polymerase chain reaction (PCR) tests for TRV are usually based on RNA1 sequences (Robinson, 1992). PCR tests have been used to detect TRV in vector nematodes, also using RNA2-specific tests when defined TRV isolates are under study (Holeva *et al.*, 2006). Increased sensitivity has been achieved using capture hybridization prior to reverse transcriptase-polymerase chain reaction (RT-PCR) (Miglino *et al.*, 2007) or modifications to sample preparation (Martin *et al.*, 2009).

Potato cultivars can be divided into three groups depending on their reaction to TRV. One group, which includes Bintje, Saturna and Record, is fully resistant to most isolates of TRV. A second group, which includes Pentland Dell and Russet Burbank, is not resistant to TRV, but responds by the formation of spraing symptoms, which can increase during the storage of the tubers after harvest (Ryden *et al.*, 1994). Plants showing spraing symptoms may have virus that is restricted to only a few stems or tubers, and frequently the virus may consist of RNA1 only, a so-called nonmultiplying (NM) infection in which virus particles are not formed (as CP is encoded by RNA2, which is absent in

these plants). Nevertheless, such infections can be passed on to daughter tubers. The third group, which includes Wilja, King Edward, Saxon and Rocket, is fully susceptible to TRV, with virus particles (containing RNA1 and RNA2) present in tubers and foliage. These cultivars, however, do not show any symptoms of infection, except that continued propagation of infected tubers reduces tuber size and overall yield (Dale *et al.*, 2000, 2004). Different isolates of TRV not only have different gene sequences in RNA2, but may also have different vector nematodes (species of the genera *Trichodorus* or *Paratrichodorus*). Several studies have reported that different TRV isolates have different symptom effects on a range of field-grown cultivars (Hoek *et al.*, 2006; Mojtahedi *et al.*, 2001). The latter study reported that potato cultivars showed a large difference in susceptibility to TRV, but that, generally, all cultivars were more susceptible to a TRV isolate transmitted by *Paratrichodorus pachydermus* and less susceptible to an isolate transmitted by *Trichodorus primitivus*. Another study did not find differences in symptoms caused by virus isolates with different virulence, but suggested that environmental conditions could cause differences in the population numbers and activity of nematodes at different locations (Robinson *et al.*, 2004).

The demonstration that variation in the reaction of potato cultivars can be caused by TRV isolate type resulted from the analysis of the TRV isolate Pp085M (Robinson, 2004). As noted above, Bintje is considered to be resistant to TRV infection; however, an isolate of TRV was recovered from a *P. pachydermus* nematode collected in soil in the Netherlands, where Bintje potatoes with spraing symptoms were prevalent. By isolating RNA1 from this isolate (Pp085M) and combining it with RNA2 from another isolate (PpK20) not able to infect Bintje, it was confirmed that unidentified sequences in Pp085M RNA1 were responsible for overcoming resistance to TRV in Bintje. TRV Pp085M was not able to cause spraing in Record, Climax or Saturna (all considered to be resistant to TRV), but did cause spraing in Arran Pilot (also considered to be resistant to TRV). Thus, in these cultivars, there are at least two different sources of resistance to TRV.

Ghazala and Varrelmann (2007) examined the resistance responses of different potato cultivars to TRV derived from infectious clones of isolate PpK20 in which RNA2 was modified to remove the 2b and 2c genes and replaced with a gene encoding the fluorescent protein DsRed. These experiments involved mechanical inoculation (or infection by agroinfiltration) to leaves, which may not mirror the reactions to root inoculation that operate in the field. In Russet Burbank, which produces spraing in tubers, TRV caused a spreading necrosis and death of inoculated leaves. However, upper, noninoculated leaves became infected with both RNA1 and RNA2, but without necrosis, a situation which differs from the usual development of NM infection from spraing-containing tubers. When the resistant cultivar

Bintje was inoculated, RNA2 was restricted to within necrotic lesions that formed on the inoculated leaves, whereas RNA1 moved outside the lesions but did not become systemic. A different resistance operated in the cultivar Saturna, where no host reaction was apparent and no viral RNAs were detected in inoculated or systemic leaves, suggesting an extreme resistance operating at the single-cell level.

Expression of the TRV 29-K movement protein, from either an *Agrobacterium* binary plasmid or a *Potato virus X* vector, induced necrosis in Bintje and Saturna, showing that this protein is an elicitor of resistance. Expression of the 29-K protein from TRV Pp085M, which overcomes Bintje resistance, did not induce necrosis in Bintje, suggesting that sequence changes in this protein are responsible for the resistance-breaking capability of this isolate. Interestingly, the Pp085M 29-K protein induced necrosis in Saturna, again pointing to a difference in the resistance mechanisms operating in Bintje and Saturna.

Several studies have examined the utility of transgenic resistance to tobnaviruses, in which, for example, incorporation of the readthrough portion of the PEBV *RdRp* gene in *Nicotiana benthamiana* plants made them resistant to infection by manual inoculation of the leaves with PEBV (MacFarlane and Davies, 1992). This resistance, subsequently, was demonstrated to operate by an RNA silencing mechanism (Van den Boogaart *et al.*, 2001). More recently, similar studies have been performed with TRV, in which the 57-k *RdRp* gene sequence was transformed into potato (Melander, 2006) and tobacco (Vassilakos *et al.*, 2008). Some transgenic potato lines exhibited reduced disease symptoms (tuber necrosis) in a limited number of glasshouse trials in which viruliferous nematodes were added to the soil in which the plants were grown. For tobacco, different transgenic lines showed different levels of resistance to TRV when inoculated manually to leaves. When plants were challenged by growth in soil containing viruliferous nematodes, the most resistant line showed no infection of aerial parts, but TRV could be detected in the roots, albeit at low levels. Thus, as found in other studies of soil-transmitted viruses, it appears, for reasons that are unknown, that resistance based on RNA silencing is less effective in roots than in leaves (Andika *et al.*, 2005).

VIRUS MOVEMENT

The tobnaviruses are one of a small number of plant viruses that do not require CP for systemic movement. If TRV is inoculated at very high dilution to a local lesion host, such as *Chenopodium amaranticolor*, it will be found that some lesions contain only RNA1. This viral RNA is not encapsidated, and it was found in early studies that it was difficult to initiate infection by mechanical inoculation of buffer extracts from these lesions. This gave rise to the slightly confusing term 'nonmultiplying' or NM infection. Using phenol, however, to extract these lesions protected

the viral RNA from degradation and produced highly infectious material. NM infections have only been described in the field in TRV-infected potato. How they arise is not known, but they possibly could result from the inoculation of limiting amounts of virus particles to tubers by feeding nematodes, or perhaps by a resistance mechanism targeting some aspect of RNA2 replication or gene expression. In early glasshouse studies using *Petunia hybrida*, systemic infection by NM isolates of TRV was reported to be much slower than systemic infection by complete (M-type) isolates, and this was later interpreted to suggest that NM infections probably spread from cell to cell rather than via the vascular system (Harrison and Robinson, 1978). However, using infectious clones of green fluorescent protein (GFP)-tagged TRV and PEBV, it was shown that mutants lacking CP were able to move systemically in *N. benthamiana* and *N. clelandii* at the same speed as unmutated virus, and that the mutant viruses could be shown to exit from the vascular tissue in systemically infected leaves (Swanson *et al.*, 2002). However, it was found that, in the absence of CP, there was a tendency for RNA1 to become separated from RNA2, resulting in the formation of NM infections.

The RNA2-encoded 2b protein is essential for the transmission of tobnaviruses by nematodes, and probably carries out this function by physical interaction with the virus CP (Holeva and MacFarlane, 2006; MacFarlane, 2003). An additional role was discovered for the 2b protein in virus movement, in which GFP-tagged TRV constructs carrying the 2b gene moved more efficiently to noninoculated leaves and to roots of *N. benthamiana* and *Arabidopsis thaliana* plants (Valentine *et al.*, 2004). The 2b-expressing virus was noticeably more efficient at infection of the root meristem, and it was suggested that the 2b protein may antagonize host defences.

THE ROLE OF THE 16-K PROTEIN IN PATHOGENICITY

Several recent studies have concentrated on the role of the TRV 16-K cysteine-rich protein in virus infection. This protein has been shown to suppress RNA silencing in cultured *Drosophila* cells (Reavy *et al.*, 2004) and plants (Ghazala *et al.*, 2008; Martín-Hernández and Baulcombe, 2008; Martínez-Priego *et al.*, 2008). The 16-K protein has two cysteine–histidine motifs, reminiscent of zinc-finger motifs, located in the N-terminal part of the protein. The C-terminal part of the protein is relatively rich in positively charged amino acids (lysine and arginine). In the study of Ghazala *et al.* (2008), pentapeptide insertions were made at various positions across the 16-K protein, revealing that silencing suppression activity was not affected by insertions in the N-terminus or central part of the protein. DsRed-tagged 16-K protein was located primarily in the cytoplasm and, to a small extent, in the nucleus. This compares with a previous electron

microscopy study that found (untagged) 16-K protein to be mainly nuclear, with a smaller fraction also present in the cytoplasm (Liu *et al.*, 1991). Fusing the N-terminal part of the 16-K protein to DsRed produced a cytoplasmic localization, whereas fusing the C-terminal part of the 16-K protein to DsRed produced a nuclear localization. Furthermore, two functional bipartite motifs that caused nuclear localization were identified in the C-terminal part of the protein. Martínez-Priego *et al.* (2008) showed that the 16-K protein could suppress silencing induced by single-stranded and double-stranded RNA (at low concentration) without preventing small RNA production. The 16-K protein was shown to interfere with short-range (cell-to-cell) and long-range (systemic) silencing, but not to affect the production of secondary small interfering RNAs (siRNAs) by cellular RdRps. Expression of the 16-K protein or infection with TRV did not appear to alter the levels of endogenous small RNAs, such as micro-RNAs (miRNAs) or trans-acting siRNAs (ta-siRNAs), which was linked to the usually mild symptoms induced in plants by infection with TRV.

The first study investigating the role of the 16-K protein in TRV infection introduced frameshift mutations or created a partial (73%) deletion in the 16-K gene carried on a full-length infectious cDNA clone of the SYM isolate of TRV (Guilford *et al.*, 1991). In inoculated leaves of *N. tabacum* cv. Samsun NN, the introduction of two frameshift mutations in the 16-K gene had no effect on the local spread and replication of viral RNA, whereas partial deletion of the 16-K gene reduced but did not prevent RNA replication and local spread. A construct in which part of the 16-K gene was replaced with *Tobacco mosaic virus* CP sequences accumulated in inoculated leaves, but was not detected in upper, noninoculated leaves. More recently, mutations of the 16-K gene were introduced into an infectious clone of TRV isolate PpK20 (Martín-Hernández and Baulcombe, 2008; Ratcliff *et al.*, 2001). TRV with partial deletion or frameshift mutation of the 16-K gene was able to replicate in *N. benthamiana* and spread systemically to the same extent as the wild-type virus. However, virus with the 16-K mutation was not found in the apical meristem, whereas wild-type TRV infected the meristem transiently between 7 and 10 days post-inoculation. This result was hypothesized to occur because the 16-K protein is a weakly active silencing suppressor that allows only temporary invasion of the meristem before the plant is able to overcome the virus, whereas, without the 16-K protein, TRV is completely unable to prevent meristem-located defences from excluding the virus.

Somewhat different results were found in another study (Liu *et al.*, 2002a). Here, it was reported that complete deletion or frameshift mutation of the 16-K gene from another infectious cDNA clone of TRV PpK20 RNA1 resulted in very low level virus replication in *N. benthamiana* protoplasts, as well as low levels of viral RNA accumulation in inoculated leaves of *N. benthami-*

ana, and almost no accumulation in upper, uninoculated leaves. Normal levels of virus replication and systemic movement were recovered when the 16-K gene or several other silencing suppressor genes from different viruses were co-expressed from TRV RNA2. Similarly, much reduced replication of TRV with a 16-K deletion in potato and *N. benthamiana* was reported in another study (Ghazala *et al.*, 2008). At the moment, the reasons for these discrepancies in the behaviour of 16-K mutants are not known, but they may be caused by different conditions for plant growth or the fact that the mutants originate from different clones of TRV PpK20 RNA1.

UTILITY AS EXPRESSION OR VIGS VECTORS

Modification of the tobnaviruses as vectors for heterologous gene expression or as viral inducers of gene silencing is made easier by the gene organization of these viruses. RNA1 encodes all the functions necessary for virus replication and spread, and RNA2 in natural isolates is extremely variable in gene content, with only the CP gene being present in all isolates characterized to date. In addition, the genes carried on RNA2 are (probably) all expressed individually from sgRNA promoters, meaning that gene sequences inserted in place of the 2b and 2c genes are likely to be expressed without affecting overall virus infection. Expression vectors were created based on RNA2 of TRV, PEBV and PepRSV (MacFarlane and Popovich, 2000), and have been used to express proteins in leaves (Canto *et al.*, 2004; Carette *et al.*, 2002) as well as in roots, where they have been shown to be taken up by feeding nematodes (Valentine *et al.*, 2007).

Greater use has been made of tobnaviruses, especially TRV, as VIGS vectors, where insertion of a (usually) plant sequence into RNA2 of the virus induces a host silencing response that targets homologous plant mRNA sequences for degradation, thus reducing expression of the host gene. Several groups have constructed VIGS vectors based on TRV (Liu *et al.*, 2002b; Ratcliff *et al.*, 2001) and PEBV (Constantin *et al.*, 2004) that are cloned in *Agrobacterium* binary plasmids and can be delivered into plants by agroinfiltration. There is no infectious clone of PepRSV RNA1, which has prevented the adoption of this virus as a VIGS vector. In addition to the model plant *N. benthamiana*, other species for which TRV-mediated VIGS has been reported include tomato (Fu *et al.*, 2005; Liu *et al.*, 2002c), potato (*Solanum tuberosum* and other *Solanum* species; Brigneti *et al.*, 2004), tobacco and other *Nicotiana* species (Ryu *et al.*, 2004; Senthil-Kumar *et al.*, 2007), pepper (*Capsicum annum*; Chung *et al.*, 2004), deadly nightshade (*Solanum nigrum*; Hard *et al.*, 2008), Californian poppy (Wege *et al.*, 2007), opium poppy (Hileman *et al.*, 2005), *Arabidopsis thaliana* (Burch-Smith *et al.*, 2006), *Aquilegia* sp. (Gould and Kramer, 2007) and petunia (Chen *et al.*, 2004; Spitzer *et al.*, 2007). An extension of this technique is the use of TRV to induce

the production of RNA silencing molecules that are ingested and active in root-feeding nematodes (Dubreuil *et al.*, 2009).

The PEBV vector extends the range of plants in which VIGS can be achieved to a number of important legume species, such as pea (Blein *et al.*, 2008; Wang *et al.*, 2008), *Medicago truncatula* and *Lathyrus odorata* (Grønlund *et al.*, 2008). PEBV was used to study the development of symbiotic root nodules in pea, providing a much more tractable experimental system than was previously available (Constantin *et al.*, 2008).

Why tobnaviruses should be so effective as VIGS vectors is not clear, although TRV, particularly, is successful in part because it has a very wide host range and can induce strong, fairly uniform silencing in tissues throughout the plant. VIGS efficiency is strongly influenced by the plant species being used, with *N. benthamiana* being the most responsive to virus-induced silencing. This may be related to the nonfunctioning of the *N. benthamiana* *RDR1* gene, which plays a role in inducible defence against viruses (Yang *et al.*, 2004). In a quantitative study of TRV-induced gene silencing in *N. benthamiana* and tomato, it was found that TRV spread widely and uniformly in *N. benthamiana*, initially reaching high levels but decreasing in concentration over time (Rotenberg *et al.*, 2006). Although silencing continued during the course of the study, the efficiency of the silencing response decreased as TRV decreased in concentration. For tomato, the spread of TRV was much more patchy, resulting in highly variable silencing, although it was clear that higher levels of virus resulted in stronger silencing.

During infection, TRV is itself targeted by the host silencing system, resulting in the production of TRV-specific siRNAs (Donaire *et al.*, 2008). It is likely that the VIGS ability of TRV reflects a balance between a sufficient degree of suppression of the host silencing system to allow good spread of infection, but an insufficient degree of suppression to irreversibly overwhelm host silencing. In support of this hypothesis, it was demonstrated that the TRV 16-K protein is a weak and transiently acting silencing suppressor protein (Martín-Hernández and Baulcombe, 2008). In a different study, transgenic *N. benthamiana* and *N. tabacum* plants expressing the Tobacco mosaic virus 126-K silencing suppressor protein were infected with TRV carrying a fragment of the host phytoene desaturase (*PDS*) gene (Harries *et al.*, 2008). Firstly, compared with nontransgenic plants, the 126-K transgenic tobacco plants showed stronger infection symptoms and higher levels of virus RNA when inoculated with TRV, indicating the relative weakness of the TRV 16-K silencing suppressor protein. Secondly, individual transgenic plants expressing low levels of 126-K protein showed increased levels of VIGS when infected with TRV *PDS*, whereas transgenic plants expressing a mutant 126-K protein deficient in silencing suppressor activity did not show enhanced VIGS. This demonstrates a clear link between relative silencing suppressor activity and the strength of the VIGS response. Lastly, transgenic plants express-

ing high levels of 126-K protein showed a reduced level of TRV-mediated *PDS* silencing compared with medium- and low-level 126-K expressors, supporting the hypothesis that effective VIGS depends on a balance between control of the virus by the host and suppression of host defences by the virus.

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