CURRENT REVIEW

Interactions Between Tobamovirus Replication Proteins and Cellular Factors: Their Impacts on Virus Multiplication

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Most viral gene products function inside cells in the presence of various host proteins, nucleic acids, and lipids. Thus, viral gene products come into direct contact with these molecules. The replication proteins of tobamovirus participate not only in viral genome replication but also in counterdefense mechanisms against RNA silencing and other plant defense systems. Accumulating evidence indicates that these functions are carried out through interactions with specific host components. Interactions with some cellular factors, however, are inhibitory to virus multiplication and contribute to host range restriction of tobamovirus. The interactions that have positive and negative impacts on virus multiplication should have been maintained and lost, respectively, during adaptation of the viruses to their respective natural hosts. This review lists the host factors that interact with the replication proteins of tobamovirus and discusses how they influence multiplication of the virus.

All viral gene products are primarily synthesized in the cytoplasm by the host translation machinery, and most function inside the host cells. Therefore, virus-related molecules can make direct contact with numerous cellular components. Some of the interactions between viral and host factors are required for efficient viral multiplication or spread. Plant positive-strand RNA viruses *Brome mosaic virus* (BMV; genus *Bromovirus*) and *Tomato bushy stunt virus* (TBSV; genus *Tombusvirus*) can replicate in yeast *Saccharomyces cerevisiae* (Janda and Ahlquist 1993; Panavas and Nagy 2003). Genome-wide screening using the yeast single-gene-knockout library identified 58 genes whose deletion inhibited BMV RNA-mediated reporter gene expression by threefold or more, and 90 genes whose deletion inhibited accumulation of TBSV defective-interfering RNA by twofold or more (Kushner et al. 2003; Panavas et al. 2005). These results indicate that many host factors are required for efficient multiplication of BMV or TBSV RNA, although some of these factors may indirectly influence virus multiplication. Importantly, there are only three genes whose deletion impairs RNA replication of both BMV and TBSV, suggesting that viruses use different sets of host factors for multiplication (Panavas et al. 2005).

Not only do host organisms provide factors necessary for virus multiplication, they also defend themselves against viruses by RNA silencing and other resistance systems. Viruses often evolve counterdefense mechanisms to these host defense systems; for example, many viruses encode a suppressor of RNA

silencing with diverse structure and function (Díaz-Pendón and Ding 2008). Interactions between viral and host factors also include those important for elicitation or suppression of host defense responses. Theoretically, interactions maintained in natural hosts throughout the evolution of viruses should be beneficial to the survival of viruses.

The genus *Tobamovirus* is a group of plant positive-strand RNA viruses that has long served as a model to investigate the mechanisms of multiplication and interactions with host organisms (Scholthof 2004). The genome of a tobamovirus encodes at least four proteins (Goelet et al. 1982). After infection, the genomic RNA is translated in the cytoplasm of the host cell to produce proteins of approximately 130 kDa (130K protein) and its read-through product of 180 kDa (180K protein). These proteins harbor the methyltransferase (MT) domain that plays a role in 5' capping of viral RNAs and the helicase (Hel) domain. The 180K protein has the polymerase domain in the read-through region. The 130K and 180K proteins are collectively designated here as “replication proteins.” In addition to its functions in RNA replication, the 130K protein suppresses posttranscriptional gene silencing (PTGS) (Kubota et al. 2003; Ding et al. 2004). The replication proteins are also involved in viral cell-to-cell movement (Hirashima and Watanabe 2001). The other two tobamoviral proteins, the 30-kDa cell-to-cell movement protein and the coat protein, are encoded downstream (3') of the replication proteins and are expressed from respective subgenomic mRNAs synthesized during viral RNA replication.

To date, more than 10 host proteins have been shown to interact with the replication proteins: most of these do so with the Hel domain (Table 1). In this review, we discuss the roles of these host factors in tobamovirus multiplication and pathogenicity.

Host factors that are recruited for tobamovirus multiplication.

Tobamovirus multiplication 1. Tobamovirus multiplication 1 (TOM1) is a plant seven-pass transmembrane protein localized on both vacuolar membranes and unidentified membranes of higher density (Yamanaka et al. 2000; Hagiwara et al. 2003). In *Arabidopsis thaliana*, simultaneous knockout of *TOM1* and its homolog *TOM3* completely inhibits tobamovirus multiplication (Yamanaka et al. 2002; Fujisaki et al. 2006). In *Nicotiana tabacum*, simultaneous knockdown of the orthologs of *TOM1* and *TOM3* strongly inhibits tobamovirus multiplication (Asano et al. 2005), indicating that TOM1 and TOM3 are required for tobamovirus multiplication in plants.

TOM2A, a four-pass transmembrane protein, was also shown to be involved in tobamovirus multiplication. Knockout

of the *TOM2A* gene and some of its homologs in *A. thaliana* partially inhibits the multiplication of a crucifer-infecting tobamovirus (*Tobacco mosaic virus* [TMV]-Cg) but has little effect on the multiplication of *Tomato mosaic virus* (ToMV; genus *Tobamovirus*) (Tsujimoto et al. 2003; Fujisaki et al. 2008). Thus, *TOM2A* is most likely not an essential factor of tobamovirus multiplication but functions as an enhancer of replication complex formation of some tobamoviruses. The results of a split ubiquitin-based two-hybrid assay suggested that *TOM2A* interacts with *TOM1* and with itself (Tsujimoto et al. 2003).

Replication of all known eukaryotic positive-strand RNA viruses takes place on the host membrane (Mackenzie 2005; Dennison 2008). Although obvious amino acid sequences that function as membrane-targeting signals or membrane-spanning regions are not found in tobamovirus replication proteins, a fraction of the replication proteins binds tightly to the membranes of infected cells. Similar to other positive-strand RNA viruses, the tobamovirus RNA-synthesizing activity is bound to the membranes (Watanabe and Okada 1986; Young and Zaitlin 1986; Osman and Buck 1996). The subcellular fractionation pattern of the activity is quite similar not only to that of membrane-bound tobamovirus replication proteins but also to that of *TOM1* (Hagiwara et al. 2003). In addition, a yeast SOS-recruitment assay suggested that *TOM1* interacts with the Hel domain of tobamovirus replication proteins (Yamanaka et al. 2000, 2002). The binding was confirmed by copurification of *TOM1* with the active tobamovirus replication proteins in detergent-solubilized membranes prepared from infected tobacco cells (Nishikiori et al. 2006). These observations are consistent with the suggestion that *TOM1* is an integral component of tobamovirus replication complexes and helps tether tobamovirus replication proteins on membranes.

Consistent with the above suggestion, overexpression of *TOM1* in tobacco leads to increased accumulation of the membrane-bound forms of the replication proteins and decreased accumulation of the soluble (nonmembrane-bound) ones. Despite increased amounts of membrane-bound replication proteins, overexpression of *TOM1* inhibits tobamovirus multiplication, suggesting an important role for the soluble replication proteins (Hagiwara-Komoda et al. 2008). As described above, tobamovirus replication proteins participate in both viral RNA synthesis and suppression of PTGS. Agroinfiltration and other experiments demonstrated that *TOM1* overexpression decreases the activity of tobamovirus replication proteins to suppress PTGS (Hagiwara-Komoda et al. 2008).

Another line of experiments also suggested a link between soluble replication proteins and PTGS suppression. A ToMV mutant, TLJ, which possesses an amino acid substitution

(C349Y) in the replication proteins, shows attenuated pathogenicity and reduced ability to suppress PTGS (Kubota et al. 2003). Hagiwara-Komoda and associates (2008) reported that the level of soluble replication protein accumulation in TLJ is lower than that in wild-type ToMV in infected cells. Thus, both *TOM1* overexpression and TLJ mutation affect accumulation of the soluble replication proteins and the ability to suppress PTGS. Based on these results, it is likely that the soluble forms of replication proteins function in PTGS suppression (Hagiwara-Komoda et al. 2008).

Note that, although wild-type ToMV does not multiply efficiently in *TOM1*-overexpressing and -silenced plants, TLJ multiplies more efficiently in *TOM1*-silenced plants than in nontransgenic plants, perhaps due to increased levels of the soluble forms of the replication proteins (Hagiwara-Komoda et al. 2008). These results suggest that balanced accumulation of the soluble and membrane-bound replication proteins is important for efficient multiplication of tobamoviruses (Fig. 1). To achieve this balance, each tobamovirus may have evolved to have an optimal affinity of the replication proteins to *TOM1* expressed in their respective natural hosts.

Eukaryotic translation elongation factor 1A. Eukaryotic translation elongation factor (eEF1A) binds to aminoacyl-transfer RNAs and brings them to the A sites of translating ribosomes. Plant eEF1A interacts with the 3' untranslated region of tobamovirus RNA as well as the MT domain of tobamovirus replication proteins (Zeenko et al. 2002; Yamaji et al. 2006). Knockdown of the *eEF1A* genes results in reduced multiplication of tobamoviruses (Yamaji et al. 2010), suggesting the involvement of eEF1A in tobamovirus multiplication. Currently, however, the roles of eEF1A in tobamovirus RNA replication remain elusive.

Interactions of eEF1A or its homologs with the genomic RNA or replication-related proteins and the involvement of these proteins in viral RNA replication have also been reported for many other positive-strand RNA viruses, including bacteriophage Q β , *West Nile virus*, *Turnip yellow mosaic virus* (TYMV; genus *Tymovirus*), and TBSV (Blumenthal and Carmichael 1979; Matsuda and Dreher 2004; Davis et al. 2007; Li et al. 2009). For bacteriophage Q β , EF-Tu, the bacterial homolog of eEF1A, is one of the four subunits of viral replicase. EF-Tu has been proposed to bind to Q β RNA, place its 3' terminus at a proper position on the replicase, and facilitate initiation of negative-strand synthesis (Brown and Gold 1996).

General control derepressed 10. ToMV RNA polymerase purified from tomato leaves contains a protein that reacts with antibodies against wheat eukaryotic translation initiation factor (eIF) 3 and yeast general control derepressed (GCD) 10 (Osman and Buck 1997). GCD10 was initially believed to be one of the

Table 1. Cellular proteins that interact with tobamovirus replication proteins

Protein	Domain ^a	Method ^b	Source	Reference
AAA-ATPase	Hel	Y2H	Tobacco	Abbink et al. 2002
ADRC	Hel	Y2H, far-Western	Tobacco	Shimizu et al. 2004
ATAF2	Hel	Y2H, IP	<i>Arabidopsis thaliana</i>	Wang et al. 2009
eEF1a	MT	Y2H, IP	Tobacco	Yamaji et al. 2006
GCD10	MT	Y2H	Yeast	Taylor and Carr 2000
IAA26	Hel	Y2H, dot-blot	<i>A. thaliana</i> , tomato	Padmanabhan et al. 2005, 2008
N	Hel	Y2H, IP	<i>Nicotiana glutinosa</i>	Ueda et al. 2006
NRIP1	Hel	Y2H, IP	<i>N. benthamiana</i>	Caplan et al. 2008
Oxygen-evolving complex subunit	Hel	Y2H	Tobacco	Abbink et al. 2002
P58 ^{IPK}	Hel	Y2H, IP	<i>N. benthamiana</i> , bovine	Bilgin et al. 2003
Tm-1/tm-1	Hel ^c	IP	<i>Solanum habrochaites</i> , tomato	Ishibashi et al. 2007, 2009
TOM1/TOM3	Hel	Y2H	<i>A. thaliana</i>	Yamanaka et al. 2000, 2002
14-3-3 proteins	Hel	Far-Western	Tobacco	Konagaya et al. 2004

^a Hel = helicase and MT = methyltransferase.

^b Y2H = yeast two-hybrid system and IP = immunoprecipitation.

^c K. Ishibashi and M. Ishikawa, unpublished result.

eIF3 subunits but was later found to play a role in the modification of tRNA (Anderson et al. 1998). Antibodies to the yeast GCD10 protein inhibit exogenous template-dependent RNA synthesis by ToMV RNA polymerase, and the inhibition is reversed by prior addition of exogenous yeast GCD10 (Osman and Buck 1997). Taylor and Carr (2000) reported that yeast GCD10 interacts with the MT domain of ToMV replication proteins in a two-hybrid assay. Further investigations are needed to clarify the functions of GCD10/eIF3 subunits in tobamovirus RNA replication.

Host factors that elicit or modulate host defense against tobamoviruses.

ATAF2. Plants employ a basal defense system which is activated by pathogen infection. One of the key molecules in this basal defense response is salicylic acid (SA) (Vlot et al. 2009). Overexpression of the NAC domain transcription factor

ATAF2 of *A. thaliana* activates the expression of SA-induced defense genes, including *PR1*, and ATAF2 knockout plants express the defense genes at low levels on SA treatment, indicating that ATAF2 is a positive regulator of the SA-dependent basal defense responses (Wang et al. 2009). ATAF2 was found to interact with the Hel domain of the TMV replication proteins (Wang et al. 2009). In response to TMV infection, expression of the ATAF2 mRNA is upregulated but the ATAF2 protein is destabilized in a proteasome-dependent manner (Wang et al. 2009). Thus, TMV may suppress SA-mediated host defense through degradation of ATAF2 triggered by the interaction with the replication proteins to promote systemic infection. How TMV replication proteins destabilize ATAF2 is an interesting question that remains to be answered.

P58^{IPK}. In animals, virus infection activates double-stranded RNA-dependent protein kinase (PKR). PKR induces phosphorylation of the α subunit of eIF2 (eIF2 α), which results in

Fig. 1. Possible mechanism of the inhibition of tobamovirus multiplication by knockdown or overexpression of TOM1.

translational suppression, activation of the type I interferon response, and apoptosis (Gil and Esteban 2000). P58^{IPK}, a cellular inhibitor of PKR, is activated upon influenza virus infection via its dissociation from hsp40 (Lee et al. 1992; Melville et al. 1999). Activation of P58^{IPK} is thought to increase the production of influenza virus progeny by preventing PKR-mediated translational suppression and apoptosis (Goodman et al. 2009).

A plant ortholog of P58^{IPK} was found to interact with the Hel domain of TMV and *Tobacco etch virus* (TEV; genus *Potyvirus*) replication proteins (Bilgin et al. 2003), and the function of P58^{IPK} in plants has been investigated in depth. When P58^{IPK}-silenced *N. benthamiana* plants are infected with TMV or TEV, or when P58^{IPK}-knockout *A. thaliana* plants are infected with *Turnip mosaic virus* (genus *Potyvirus*) or *Turnip vein clearing virus* (genus *Tobamovirus*), severe cell death occurs (Bilgin et al. 2003). In P58^{IPK}-silenced *N. benthamiana* plants, eIF2 α is phosphorylated upon TMV infection, and the expression of nonphosphorylatable eIF2 α mutant protein suppresses TMV-induced cell death (Bilgin et al. 2003). These results suggest that P58^{IPK} prevents cell death via inhibition of eIF2 α phosphorylation. As a consequence, TMV and TEV accumulate at lower levels in P58^{IPK}-silenced *N. benthamiana* plants than in nonsilenced plants. Thus, TMV and TEV may activate P58^{IPK} similarly to influenza virus, possibly through the interaction with the replication proteins.

A PKR-like activity (i.e., a double-stranded RNA-dependent protein kinase activity that phosphorylates eIF2 α) has been found in plants, which is upregulated upon infection of TMV or *Potato spindle tuber viroid* (Crum et al. 1988; Hiddinga et al. 1988; Langland et al. 1995, 1996; Chang et al. 1999). These results collectively suggest that the PKR pathway contributes to antiviral defense in plants. However, the genes responsible for the plant PKR-like activity have not yet been identified. Although *A. thaliana* GCN2 protein is known to phosphorylate eIF2 α (Lageix et al. 2008; Zhang et al. 2008), the relationship of this protein with the previously characterized PKR-like activity is still unclear.

Auxin/indole-3-acetic acid proteins. Auxin/indole-3-acetic acid (AUX/IAA) family proteins PAP1/IAA26, IAA27, and IAA18 of *A. thaliana* and IAA26 of tomato interact with the Hel domain of TMV replication proteins (Padmanabhan et al. 2005, 2006, 2008). AUX/IAA family proteins are transcription factors that negatively regulate the expression of auxin-responsive genes. These proteins are localized in the nucleus and degraded at high auxin concentrations. In TMV-infected cells, these IAA proteins move from the nucleus to co-localize with the 130K protein in the cytoplasm (Padmanabhan et al. 2006). This relocalization predicts the loss of IAA protein functions. Silencing of IAA26 causes the TMV-induced disease-like developmental abnormality in both *A. thaliana* and tomato, suggesting that the cytoplasmic sequestration of IAA26 by replication proteins accounts, at least in part, for the TMV-induced disease symptoms (Padmanabhan et al. 2005, 2008).

An amino acid substitution (V1087I) in the replication proteins of TMV disrupts the interaction with IAA26 (Padmanabhan et al. 2005). A TMV mutant that possesses a mutation causing this amino acid change normally multiplies in young leaves and shows mild disease symptoms (Padmanabhan et al. 2005, 2008). However, in old leaves in which IAA26 accumulates to higher levels than in young tissues, the mutant TMV multiplies less efficiently than wild-type TMV (Padmanabhan et al. 2008). In addition, overexpression of a proteolysis-resistant IAA26 mutant (P108L) protein results in reduced accumulation of TMV-V1087I, even in young leaves (Padmanabhan et al. 2008). Thus, AUX/IAA proteins are involved in age-related resistance, by which old plants and tissues are less susceptible to viruses; TMV replication proteins bind to and suppress the

function of the AUX/IAA proteins to avoid the age-related resistance and, consequently, achieve high levels of multiplication. Padmanabhan and associates (2008) proposed that the symptoms associated with the loss of AUX/IAA functions are the inevitable result of overcoming the age-related resistance, because no evident advantage exists for virus survival gained from damaging the host.

N gene. In tobacco plants carrying the *N* gene, a hypersensitive response that accompanies cell death takes place upon TMV infection to result in the inhibition of systemic spread of the virus (Holmes 1938). *N* was bred into tobacco from the wild tobacco species *N. glutinosa* and encodes a nucleotide-binding site and leucine-rich repeat-containing protein (Whitham et al. 1994). The Hel domain of the TMV replication protein (p50) is the elicitor of the *N*-mediated resistance (Padgett et al. 1997; Abbink et al. 1998; Erickson et al. 1999). *N* protein interacts directly with p50 in an ATP-dependent manner (Ueda et al. 2006). In addition, tobacco 14-3-3 proteins and the chloroplastic protein NRIP1 bind both *N* and p50 (Konagaya et al. 2004; Caplan et al. 2008). NRIP1 is required for the resistance to TMV, and relocalizes in the presence of p50 from the chloroplast to the cytoplasm and nucleus, where *N* exists (Caplan et al. 2008). More details regarding the action of *N* are discussed in another review in this issue (Padmanabhan and Dinesh-Kumar 2010).

A cellular factor that binds to and inhibits the functioning of tobamovirus replication proteins.

Tm-1/tm-1. *Tm-1* is a semidominant trait of tomato (*Solanum lycopersicum*) that confers resistance to tobamoviruses (Porte et al. 1939; Pelham 1966; Fraser and Loughlin 1980). It was originally introgressed from the wild tomato species *S. habrochaites*. *Tm-1* prevents ToMV multiplication in protoplasts (Motoyoshi and Ohshima 1977) and, unlike most other disease resistance genes, does not induce a hypersensitive reaction (Yamafuji et al. 1991). *Tm-1*-breaking ToMV mutants have amino acid substitutions in the Hel domain of replication proteins, suggesting that Tm-1 recognizes ToMV replication proteins (Meshi et al. 1988; Hamamoto et al. 1997). Ishibashi and associates (2007) reported that the extract of *Tm-1* tomato cells inhibits ToMV RNA replication in vitro. The inhibitory activity was purified by fractionation of the cell extract to identify a protein responsible for the activity. The protein is composed of two uncharacterized domains and encoded by the *Tm-1* gene itself. Tm-1 binds to ToMV replication proteins but not to those of the resistance-breaking ToMV mutant, suggesting that binding of the Tm-1 protein causes inhibition of the functioning of ToMV replication proteins and that resistance-breaking mutants emerge by escaping from the inhibitory interaction (Ishibashi et al. 2007).

ToMV-susceptible tomato cultivars have a *Tm-1* allele, *tm-1*. Although the amino acid sequence of the tm-1 protein shows 97% identity with that of Tm-1, tm-1 neither binds to ToMV replication proteins nor inhibits RNA replication (Ishibashi et al. 2007). *Tobacco mild green mosaic virus* (TMGMV) and *Pepper mild mottle virus* (PMMoV) are members of the genus *Tobamovirus* that can infect tobacco but not any cultivars of tomato (i.e., tomato is a nonhost of TMGMV and PMMoV). The multiplication of TMGMV and PMMoV is inhibited in transgenic tobacco expressing tm-1, and the tm-1 protein binds to the replication proteins of TMGMV and PMMoV (Ishibashi et al. 2009). Thus, tm-1 is also an inhibitor of RNA replication and accounts, in part, for the inability of these tobamoviruses to multiply in tomato.

The homologs of the *Tm-1* gene are conserved in many plants, fungi, archaea, and bacteria, suggesting that Tm-1/tm-1 has a primary although as-yet-undetermined function other than

tobamoviral resistance and, incidentally, has the potential to bind to tobamovirus replication proteins and cause inhibition (Ishibashi et al. 2009). Several other findings (Zhu et al. 2007; Fujisaki and Ishikawa 2008; Mehle and Doudna 2008; Cheng et al. 2009; Li et al. 2009; Mahon et al. 2009; Sasaki et al. 2009) also suggest the presence of inhibitory factors for virus multiplication in organisms to which the virus is not well adapted. Thus, inhibitory interactions between viral and cellular factors may often occur in infected cells, although they are rarely found in the natural hosts because viruses must have escaped from such inhibitory factors. The lack of an interaction between the replication proteins of ToMV and tm-1 may represent such an evolutionary outcome.

Concluding remarks.

As described above, tobamovirus replication proteins interact with many cellular proteins. Although not discussed here due to the space limitation, they also interact with other host proteins (Abbink et al. 2002; Shimizu et al. 2004; Liu et al. 2005; Harris et al. 2010). In addition to interacting with host proteins, tobamovirus replication proteins interact with themselves (Goregaoker et al. 2001), with the tRNA-like structure of the viral genomic RNA (Osman and Buck 2003), and with small RNA duplexes (Mérat et al. 2006; Csorba et al. 2007; Kurihara et al. 2007; Hagiwara-Komoda et al. 2008).

Many interaction partners play distinct roles to facilitate virus multiplication. In such a situation, as TOM1 overexpression lowers the accumulation of soluble ToMV replication proteins that function as a PTGS suppressor, an excessive dominance of the binding of a particular host factor to the replication proteins can be inhibitory to overall virus multiplication because it may interfere with the function mediated by other interaction partners. Mutations in viral or host factors that increase the affinity to the partner can also lead to similar results, as found in the TLJ mutant case. Thus, to fulfill the multiple functions, tobamovirus replication proteins have likely evolved to have optimal (i.e., not too strong but not too weak) affinity to each interaction partner in their natural host cells that harbor given concentration of the host factors of given amino acid sequences.

On the other hand, some interactions that occurred in nonadapted organisms may be inhibitory, and indeed, tm-1 binds to TMGMV replication proteins to inhibit viral RNA replication in tomato. Such interactions should have been nullified in the natural hosts through adaptation and, thus, the ubiquitousness of inhibitory interactions may be underestimated. In addition to its role in host range restriction, negative interactions between viral and cellular factors may also play a role in avoidance of serious damage to the hosts due to overmultiplication (Mahajan et al. 2009).

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