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REVIEW: PART OF A SPECIAL ISSUE ON PLANT IMMUNITY

Modulation of host plant immunity by Tobamovirus proteins

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• **Background** To establish successful infection, plant viruses produce profound alterations of host physiology, disturbing unrelated endogenous processes and contributing to the development of disease. In tobamoviruses, emerging evidence suggests that viral-encoded proteins display a great variety of functions beyond the canonical roles required for virus structure and replication. Among these, their modulation of host immunity appears to be relevant in infection progression.
• **Scope** In this review, some recently described effects on host plant physiology of *Tobacco mosaic virus* (TMV)-encoded proteins, namely replicase, movement protein (MP) and coat protein (CP), are summarized. The discussion is focused on the effects of each viral component on the modulation of host defense responses, through mechanisms involving hormonal imbalance, innate immunity modulation and antiviral RNA silencing. These effects are described taking into consideration the differential spatial distribution and temporality of viral proteins during the dynamic process of replication and spread of the virus.
• **Conclusion** In discussion of these mechanisms, it is shown that both individual and combined effects of viral-encoded proteins contribute to the development of the pathogenesis process, with the host plant's ability to control infection to some extent potentially advantageous to the invading virus.

Key words: *Tobacco mosaic virus*, replicase, coat protein, movement protein, immune response, salicylic acid, reactive oxygen species, DELLA proteins, RNA silencing.

INTRODUCTION

An important aspect of plant–virus interaction which is currently insufficiently understood concerns the mechanisms by which viruses dynamically modulate host physiology to successfully replicate. It is well established that an effective infection only occurs when the virus finds a suitable environment supplied by host factors and succeeds in the evasion of host defence responses. The progression of infection is a dynamic process in which viral factors play different roles according to their relative amounts and temporal distributions within the host cells and distal tissues. Consideration of events on a temporal scale may shed new light on the complex interaction, in which it is typically difficult to distinguish factors associated with viral requirements for replication from those related to host defense responses or host damage.

In this review, we propose to expand the understanding on the process of pathogenicity establishment by extensively discussing some evidences from Tobamoviruses. We address the topic by initially summarizing the dynamic process of *Tobacco mosaic virus* (TMV) replication and subsequently by giving a brief overview of the different antiviral host defence strategies. We mainly focus the discussion on recently described non-canonical roles of individual viral proteins in host physiology which result in the modulation of host defence responses. We also attempt to model globally the interaction taking into consideration that the individual impacts of viral proteins act in a concerted manner according to their spatial distribution and temporality in the dynamics of viral replication and movement.

TMV STRUCTURE AND DYNAMIC OVERVIEW OF VIRAL REPLICATION PROCESS

Tobacco mosaic virus is an emblematic member of the Tobamovirus genus and the most studied plant virus since its discovery a hundred years ago (K. G. Scholthof *et al*., 2011). The genome of TMV is composed of single-stranded RNA (ssRNA) of 6395 nucleotides, containing four open reading frames (ORFs). The genomic RNA is directly translated to produce the replicase protein which is composed of two subunits of 126 and 183 kDa, and is involved in TMV replication and also in the suppression of silencing the counter-defence mechanism described later. The movement protein (MP) (30 kDa) and the coat protein (CP) (17·5 kDa) are translated from sub-genomic mRNAs produced during replication (for a review, see Dawson, 1992; Klug, 1999). The 30 kDa protein is required for the virus to move from infected to adjacent cells. The process occurs through plasmodesmata and requires the interaction of the MP with viral RNAs (vRNAs) and host factors to modulate the transport (see Beachy and Heinlein, 2000; Heinlein, 2015). The CP is a multifunctional viral protein with a structural role in the formation of viral particles. It is also required for stability of the genomic RNA in infected cells and permits the transmission of the virus from one plant to another. The CP is also a component of long-distance movement through the phloem, allowing the virus to reach systemic tissues (for reviews, see Callaway *et al*., 2001; Culver, 2002; Bol, 2008; Makarov and Kalinina, 2016).

Considering tobamoviral replication as a dynamic temporal–spatial process (Fig. 1), it is usually assumed that physiological changes in the host that occur during the initial phases of

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progression of the infection are immediate or early responses, then there is an intermediate stage, followed by late responses (reviewed in Maule *et al*., 2002). During late stages, the disease symptoms are usually observed.

The first TMV infection event occurs when the virus enters the symplast after mechanical damage of the cell wall and plasma membrane. Within 3 min after entry, the TMV CP begins to disassemble from the capsid. Genomic 5′ ORFs are immediately translated to form the replicase protein, which initiates the replication of the viral genome (Shaw, 1999). Considering that the replication of the vRNAs produces double-stranded RNAs (dsRNAs), the induction of antiviral gene silencing is the first defence response to become active. Here, the replicase protein, apart from its role during the replication of vRNA, is the suppressor of RNA silencing [viral suppressor of RNA silencing (VSR)] which provides the first round of counter-defence against the host. Viral replication takes place in the proximity of the endoplasmic reticulum (ER) membranes, into the so-called 'viral replication factories' of which the MP is a pivotal component (Beachy and Heinlein, 2000; Asurmendi *et al*., 2004). The MP directs the vRNA for its passage through the plasmodesmata (Peña and Heinlein, 2012), becoming the next viral component required to initiate the intermediate stage of the dynamic viral replication. This protein is located predominantly at the leading front of infection, controlling plasmodesmata gating in a temporal manner (Oparka *et al*., 1997). During late stages of the infection, the CP becomes the more abundant protein and the last viral protein to be produced following the leading front of infection. When the virus local spread reaches the vascular tissues, the CP is required for systemic movement across the phloem to invade distal parts of the host plant. The cells which complete the infection 'cycle' do not die but accumulate huge amounts of virions (mostly CP), while the infection spreads to adjacent tissues. Finally, the outcome of the progressive spread of the virus to systemic tissues is the appearance of symptoms. These symptoms represent an accumulation of subcellular physiological and structural alterations associated with defective growth and abnormal whole-plant development.

DEFENCE MECHANISMS INVOLVED IN ANTIVIRAL IMMUNITY

Several mechanisms are involved in plant defence against viruses. Generally, plant–virus interactions are grouped into incompatible and compatible interactions depending on the outcome of the interaction. During the former, plant viruses are recognized by resistance genes that activate a series of physiological changes which subsequently arrest the development of virus infection at a site near the site of inoculation. However, in compatible interactions, plant viruses are able to spread to systemic tissues and develop symptoms. In this review, we focus on defence mechanisms involved during compatible interactions, those that produce the greatest losses in agriculture due to the development of disease symptoms.

RNA interference-mediated resistance

When an RNA viral pathogen enters the symplast and decapsidates its vRNA, the post-transcriptional gene

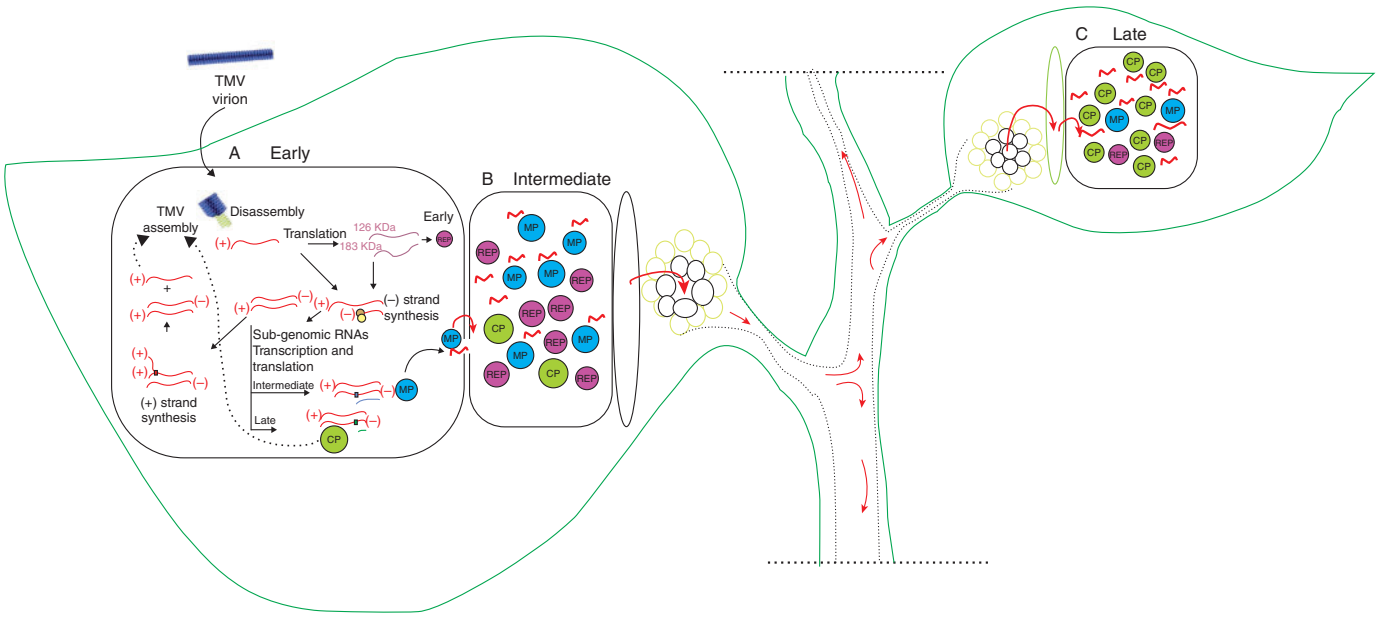

FIG. 1. Schematic view of the proposed events associated with the replication and movement of TMV. (A) The early stage of the infection is determined by the entry of a viral particle into the symplast of a single cell, the disassembly of the virions and translation of the 126 and 183 kDa subunits of the replicase. Subsequently, negative strands and sub-genomic RNAs are synthesized to produce first the MP and then the CP. The accumulation of positive stranded viral RNAs and CPs permit the assembly of new viral particles. (B) The intermediate stages of infection are determined by the local spread of the viral RNAs between adjacent cells. The MP is implicated in facilitating the transport due to its ability to interact with plasmodesmata and bind RNA. (C) The late stages of infection are initiated when the virus reaches distant parts of the plants through vascular tissues and the infection becomes systemic. The systemic virus movement is facilitated by the CP by an incompletely understood mechanism. At this stage, the newly assembled viral particles can invade other plants by direct transmission.

silencing (PTGS) pathway evolved in plants as a first line of defence, which directly recognizes and degrades the invading nucleic acids. The PTGS process is triggered by the presence of dsRNAs. The dsRNA degradation mechanism is based on RNase-mediated cutting of dsRNAs [by Dicer-like proteins (DCLs)] into small interfering RNAs (siRNAs) that range from 20 to 25 nucleotides in length (for a review, see Bologna and Voinnet, 2014). PTGS controls gene expression in three different ways: by degradation of transcripts; by inhibition of mRNA translation; or by promoting directed DNA methylation (transcriptional gene silencing) (Bäurle *et al*., 2007; Brodersen *et al*., 2008). Virus replication and the secondary structures displayed by vRNAs are both a source of dsRNAs. The viral siRNAs (vsiRNAs) produced by DCL processing recognize and degrade complementary viral nucleic acids by binding to the RISC (RNA-induced silencing complex) (reviewed in Wang and Metzlaff, 2005; Ding and Voinnet, 2009; Llave, 2010)

The antiviral RNA silencing is amplified by the action of endogenous RNA-dependent RNA polymerases (RDRs). These enzymes synthesize dsRNA by using ssRNA as template. Then, the new dsRNAs are cleaved by different DCLs, producing secondary siRNAs that are able to move systemically and amplify the silencing signal (for reviews, see Voinnet, 2008; Bologna and Voinnet, 2014). In *Arabidopsis thaliana*, six different RDRs have already been identified (RDR1–RDR6) (reviewed in Willmann *et al*., 2011). RDR1 and RDR6 are the two main antiviral RDRs (Wang *et al*., 2010). It was proposed that RDRs display specific sensitivities to different viruses, and small RNA deep sequencing analysis has shown that both RDR1 and RDR6 play a role in the biogenesis of TMV-Cg vsiRNAs (Qi *et al*., 2009). Moreover, it was shown that transgenic NtRDR1 antisense tobacco plants are more susceptible to TMV infection than wild-type plants (Xie *et al*., 2001). There are many reports demonstrating the role played by RDR1 and RDR6 in the defence against viruses (Pandey and Baldwin, 2007; Liu *et al*., 2009; Rakhshandehroo *et al*., 2009; Qin *et al*., 2012; Liao *et al*., 2014; Lee *et al*., 2016). Thus, there are several reports which support the RDR1 contribution to the antiviral defence mechanism during Tobamovirus infections.

Conversely, viruses are not defenceless; they evolved, in parallel, a wide range of multifunctional proteins that efficiently counteract gene silencing. These viral factors are known as VSRs. Several of these proteins bind to silencing machinery components and interfere with their function (Voinnet *et al*., 1999; Burgyán and Havelda, 2011; Shimura and Pantaleo, 2011; Jiang *et al*., 2012; Wieczorek and Obrępalska-Stęplowska, 2014). In the context of plant–virus interactions, it is assumed that the interplay between antiviral RNA silencing and viral suppression of RNA silencing constitutes the first round of defence and counter-defence responses displayed.

Plant hormones and reactive oxygen species in antiviral-mediated resistance

Plant hormones and reactive oxygen species (ROS) play important roles in regulating transcriptomic changes during stress responses. During compatible plant–virus interactions, besides RNA silencing, the plant defence system is also activated by additional mechanisms which profoundly alter gene expression profiles (Golem and Culver, 2003; Huang *et al*., 2005). It was demonstrated that the majority of defence-related genes whose expression is increased during viral infections are responsive to salicylic acid (SA) (Huang *et al*., 2005). In addition to this, it was shown that SA plays a prominent role during plant–virus interactions (Nicaise, 2014; Alazem and Lin, 2015) and together with ROS act in a concerted manner to regulate the transcriptional responses to stress (Herrera-Vasquez *et al*., 2015). Considering the relevance of SA during viral infections and the cross-talk with ROS signalling pathways, in this section we summarize the most relevant findings that support the role of both signalling molecules during plant–viral interactions.

The role of SA. The hormone SA is the most widely mentioned hormone in viral defence immunity. SA is involved in the plant defence response to a broad spectrum of pathogens. The role of SA-mediated resistance was extensively studied in resistant hosts (Yalpani *et al*., 1991; Kachroo *et al*., 2000; Park *et al*., 2007). Apart from being implicated in incompatible interactions, SA also plays a role during infections in susceptible tobacco and *A. thaliana* plants (Chivasa *et al*., 1997; Wang *et al*., 2009). SA treatment of tobacco leaf tissues reduced TMV RNA accumulation by interfering with TMV replication in mesophyll cells (Chivasa *et al*., 1997; Murphy and Carr, 2002). Exogenous SA can also inhibit cell to cell movement in epidermal cells (Murphy and Carr, 2002). Moreover, it was found that *Plum pox virus* (PPV; defective in systemic movement) was able to move to distal tissues in transgenic tobacco plants expressing bacterial salicylate hydroxylase (NahG) which degrades SA (Alamillo *et al*., 2006). Based on this evidence, it was proposed that SA plays a positive role in antiviral resistance by interfering with different steps of the viral cycle (Chivasa *et al*., 1997; Murphy and Carr, 2002; Alamillo *et al*., 2006). In addition, recent findings showed that SA treatment delays systemic movement of *Potato virus X* (PVX) (SCP1 strain) and ameliorates induced symptoms in tomato plants (Cueto-Ginzo *et al*., 2016). Thus, SA seems to play different roles in antiviral defence depending on the plant–viral pathosystem.

In seeking players involved in the SA signalling pathway against viral pathogens, it was found that the mitochondrial alternative oxidase (AOX), induced by SA, was involved in the SA-induced resistance (Chivasa *et al*., 1997). Initially, the action of AOX in the SA-induced resistance was characterized in tobacco tissues employing salicyl-hydroxamic acid (SHAM), an inhibitor of AOX activity. It was shown that SHAM antagonized SA-induced resistance to TMV, in both susceptible and resistant cultivars (Chivasa *et al*., 1997). Although the mechanism by which AOX reduces virus accumulation is not well understood, it was proposed that ROS generated in the mitochondria may be involved in defensive signalling (Singh *et al*., 2004).

As previously stated, RDR1 was extensively implicated in antiviral immunity by allowing the production of secondary vsiRNAs (Xie *et al*., 2001; Garcia-Ruiz *et al*., 2010). RDR1 expression is also induced by SA and in response to infection by Tobamovirus (Xie *et al*., 2001; Yu *et al*., 2003). In *Nicotiana*

tabacum, NtRDR1 activity was found to increase transcriptional levels following SA treatment (Xie *et al*., 2001). Orthologous genes of NtRDR1 have also been characterized in other species, such as *Nicotiana glutinosa* (NgRDR1), *Nicotiana benthamiana* (NbRDR1m), *A. thaliana* (AtRDR1), *Medicago truncatula* (MtRDR1) and rice (OsRDR1), all of them being induced by both viral infection and SA treatment (Yu *et al*., 2003; Yang *et al*., 2004; Quilis *et al*., 2008; Liu *et al*., 2009). Several findings indicate that RDR1 could be involved in the SA-induced resistance against viruses (Alamillo *et al*., 2006; Lee *et al*., 2016). The impact of SA on the modulation of RDR1 action was demonstrated in tobacco NahG transgenic plants infected with PPV. These plants accumulated lower levels of vsiRNAs than wild-type tobacco, and this reduced accumulation is associated with the pattern of expression of RDR1 (Alamillo *et al*., 2006). The involvement of RDR1 in antiviral SA-mediated resistance was also explored in transgenic *N. benthamiana* plants constitutively expressing MtRDR1 (Lee *et al*., 2016). MtRDR1 expression did not restrict TMV movement into non-inoculated tissues, but its expression was able to inhibit the extent of spread of TMV into the tissues adjacent to the apical meristem. This restriction of viral movement becomes more pronounced following treatment of MtRDR1-transgenic plants with SA. Moreover, SA treatment enhanced the recovery from severe TMV disease observed in MtRDR1 transgenic plants. Based on these findings, these authors suggested that the severity of virus-induced symptoms is ameliorated by the delay of viral entry into the apical meristem.

Non-expressor of pathogenesis-related protein 1 (NPR1) is a key regulator component of the SA-dependent pathway against microbial pathogens (Vlot *et al*., 2009). Given its central position in SA-mediated resistance, the role of NPR1 in defence against viral pathogens deserves a particular mention. First, the role of NPR1 was analysed during the SA-induced resistance to *Turnip vein clearing virus* (TVCV) in *A. thaliana*. This study showed that SA-induced resistance is independent of the action of NPR1. Moreover, the induction of AOX by SA was not impaired in *npr*1 mutant plants. Based on these findings, it was proposed that NPR1 was not involved in the SA-induced resistance against TVCV (Wong *et al*., 2002). The role of NPR1 during compatible virus interactions was also investigated in *A. thaliana* plants infected with *Oilseed rape mosaic virus* (ORMV) (Huang *et al*., 2005). Infection of *npr*1 mutants with ORMV has shown the ability of NPR1 to modulate the expression of some defence genes that are upregulated during ORMV infection. However, the authors could not detect enhanced susceptibility to ORMV in *npr*1 mutants compared with wild-type plants (Huang *et al*., 2005). More recent findings demonstrated that *Cauliflower mosaic virus* (CaMV) P6 alters the expression and sub-cellular localization of NPR1 and inhibits the SA-dependent defence responses (Love *et al*., 2012). Moreover, another recent report showed that SA-induced RDR1 expression is dependent on NPR1 (Hunter *et al*., 2013). Thus, although NPR1 does not seem to be involved in SA-mediated resistance, its role during compatible viral interactions cannot be ruled out at this stage.

The role of reactive oxygen species. The outcome of the plant defence response is also modulated by the accumulation of ROS. Accumulation of ROS in the apoplast/cell wall compartments takes place early after the activation of the hypersensitive response (HR) (Sagi and Fluhr, 2001). Accumulation of ROS was also observed during viral compatible interactions (Riedle-Bauer, 2000; Love *et al*., 2005; Inaba *et al*., 2011; Manacorda *et al*., 2013). Moreover, in some plant–virus interactions, necrotic symptoms are associated with an increase of hydrogen peroxide (H_2O_2) production (Inaba *et al*., 2011; Manacorda *et al*., 2013). Following this line of evidence, it was observed that a TMV-Cg mutated strain, carrying a truncated version of CgCP, induced reactive oxidative bursts that led to necrotic phenotypes in arabidopsis and tobacco plants (Kurihara and Watanabe, 2004). Based on these findings, the authors proposed that the expression of this mutated CgCP is responsible for the disease symptoms observed.

Altered ROS levels were also observed in tobacco transgenic plants expressing the MP and in double-transgenic lines expressing CP^{T42W} (a mutated version of CP) and MP (MP × CP^{T42W}) (Conti *et al*., 2012). The MP line and the MP × CP^{T42W} double-transgenic line accumulated elevated levels of H_2O_2 and O_2 compared with wild-type plants. Also, it was reported that CP triggered a rapid oxidative burst when added to the apoplast of tobacco epidermal cells (Allan *et al*., 2001). The elicitor seems to be the virus CP, which stimulates a plant NAD(P)H oxidase-like activity via an active signal transduction pathway. In contrast, treatment of tobacco epidermal cells with *Cucumber mosaic virus* (CMV), a virus that infects tobacco but has a dissimilar structure, did not elicit a fast oxidative burst. Based on these results, the authors proposed that the induction of ROS by CP was specific to Tobamoviruses (Allan *et al*., 2001).

In parallel to ROS overaccumulation, the expression of genes involved in ROS-scavenging systems is frequently increased during viral infections (Espinoza *et al*., 2007; Conti *et al*., 2012; Rodriguez *et al*., 2014). In particular, the expression of superoxide dismutases (CSD2), GDP-mannose pyrophosphorylase 1 (GMP1) and ascorbate peroxidase (APX1) was enhanced in the systemic tissues of infected plants at late stages of Tobamovirus infections (Conti *et al*., 2012; Rodriguez *et al*., 2014). These responses were also observed in transgenic tobacco expressing both the CP^{T42W} and MP (Conti *et al*., 2012). To assess the impact of ROS-scavenging enzyme imbalances during viral infections, virus- induced gene silencing (VIGS) of GMP1 transcript in *N. bethamiana* and subsequent infection with TMV was studied by Conti *et al*. (2012). *gmp*1 silenced plants produced enhanced levels of ROS and reduced TMV accumulation. In agreement with this, previous reports had shown that reduced levels of GMP1 were associated with augmented basal defences mediated by SA and pathogenesis-related (PR) protein expression in *A. thaliana* (Barth *et al*., 2004; Pavet *et al*., 2005). Conti *et al*. (2012) demonstrated that *gmp*1 silenced *N. benthamiana* plants also displayed an increased accumulation of PR proteins. These results showed that alteration of ROS-scavenging systems can modulate the defence response against viral pathogens.

Overall, the results obtained by different groups indicate that Tobamovirus proteins can modulate ROS signalling pathways and suggest that the modulation of ROS by viral proteins may affect the outcome of defence responses and symptom severity during Tobamovirus–plant interactions.

IMPACT OF VIRAL PROTEINS ON THE MODULATION OF ANTIVIRAL DEFENCE MECHANISMS

Given the fact that viral pathogens display small genomes, viral-encoded proteins are usually multifunctional and can act in different stages of the viral replication cycle, inducing the modulation of host defence responses. Here we summarize several lines of evidence that show that Tobamovirus proteins can affect multiple defence pathways.

Effects of TMV replicase protein

As was previously mentioned, the TMV replicase complex, translated immediately after viral disassembly, is composed of two 5′ ORFs which encode 183 and 126 kDa proteins. The replicase complex is associated with virus replication and, in particular, the 126 kDa component has been identified as the TMV VSR.

A large number of VSRs from different viruses have been identified from both RNA and DNA strands. The action of VSRs is based on different strategies: inhibiting DCL activity, binding to the viral dsRNAs and protecting them from the subsequent DCL processing, sequestering and/or degrading vsiRNAs, inactivating RISCs and also inhibiting the amplification and/or spread of the silencing signal (Mallory *et al*., 2002; Qiu *et al*., 2002; Vargason *et al*., 2003; Soitamo *et al*., 2011; Várallyay *et al*., 2014). In particular, the TMV replicase suppressor of silencing activity is mediated by the protection of viral transcripts from enzymes of the RNA silencing pathway rather than defeating them (Ding *et al*., 2004; Kurihara *et al*., 2007). The silencing suppressing activity is located in the viral 126 kDa small replicase subunit by interfering with HEN1-mediated methylation of small RNAs. This interference is closely associated with the formation of disease symptoms (Vogler *et al*., 2007). It was also reported that the transgenic expression of the 126 kDa replicase subunit in *N. tabacum* produced enhanced susceptibility to several viruses (Harries *et al*., 2008).

The counter-defence activities of several VSRs generate side effects on endogenous mechanisms of RNA silencing such as interference with the microRNA (miRNA) biogenesis pathway. Several studies demonstrated that transgenic expression of VSRs leads to the alteration of miRNA accumulation and, consequently, numerous developmental abnormalities are produced (Kasschau *et al*., 2003; Chapman *et al*., 2004). These alterations underlie many disease symptoms typically observed during plant viral infections (Siddiqui *et al*., 2008; Bazzini *et al*., 2011; Shimura and Pantaleo, 2011). Thus, the production of symptoms due to the effect of VSRs, including TMV replicase, has been associated with side effects over off-target components shared between pathways involved in silencing mediated by miRNA/trans-acting siRNAs (tasiRNAs) and vsiRNAs (Yu *et al*., 2006; Vogler *et al*., 2007).

Beside its reported role in the suppression of RNA silencing, there is plenty of evidence which demonstrate the interaction of TMV replicase with host factors that mediate the activation of host defences. The most studied evidence is the interaction of the helicase domain p50 with the tobacco N gene, which triggers host programmed cell death, resulting in an incompatible interaction (Caplan *et al*., 2008). In contrast, during compatible interactions, the transgenic expression of the 126 kDa replicase subunit in *N. tabacum* produces enhanced susceptibility to several viruses (Liu *et al*., 2009). It was reported that TMV replicase interacts and interferes with the activity of ATAF2, a NAC domain transcription factor whose overexpression inhibits TMV accumulation (Wang *et al*., 2009). The TMV replicase complex also interacts with a specific auxin/indole acetic acid (Aux/IAA) host transcriptional regulator. This interaction disrupts the control of host genes implicated in virus movement, plasmodesmata gating and defence in the companion cells, enhancing TMV phloem loading (Padmanabhan *et al*., 2008; Collum *et al*., 2016).

Considering all these data together, it could be suggested that the TMV replicase protein is a pathogenicity determinant that enhances plant susceptibility to viral infections by modulating the host defences in diversified range of strategies.

Effects of TMV movement protein

As was previously discussed in this review, the TMV 30 kDa MP is required for cell to cell movement by allowing the virus to enter and initiate replication in adjacent cells. The passage is possible due to the interaction of MP with plasmodesmata, by modifying their size exclusion limit and also by the ability of MP to bind RNAs non-specifically (for a review, see Heinlein, 2015). The implication of both the cytoskeleton and the ER in the interaction of TMV MP and plasmodesmata was reported (Hofmann *et al*., 2007). Numerous host factors involved have also been identified. For example, the targeting and anchorage of MPs to plasmodesmata require the action of a cell wall-associated pectin-methylesterase (PME) protein (Chen *et al*., 2000; Lionetti *et al*., 2014). An MP-binding protein 2C (MPB2C) was shown to co-localize with MP on microtubules (Kragler *et al*., 2003). Plasmodesmata-associated kinase (PAPK) specifically phosphorylates TMV MP at its C-terminus *in vitro* (Lee *et al*., 2005). Calreticulin, a calcium-sequestering ER-resident protein, binds MP *in vitro* and interferes with TMV movement in overexpressing transgenic plants (Chen *et al*., 2005). Actin elements (Hofmann *et al*., 2007) and myosins (Amari *et al*., 2014) are required for MP–plasmodesmata interaction. Regardless of all the studies in the field, the detailed interaction between MPs and host cells to facilitate cell to cell movement is not fully understood.

Other aspects of tobamovirus MP effects are considered in this review, such as the influence of these viral proteins on host physiology and in the modulation of the different host defence responses. Besides the replicase, other mechanisms acting simultaneously are also involved in the modulation of host defences. In a previous work from our group, we demonstrated that transgenic expression of TMV MP in *N. tabacum* was sufficient to produce a stress-like response. This phenotype was characterized by ROS accumulation, reduction of total ascorbate, expression of ROS-scavenging genes, increased levels of SA and induction of SA-responsive genes including PR1, PR2 and PR5. These results suggest that MP could be seen as a defence elicitor inducing defence signalling mediated by SA and ROS (Conti *et al*., 2012).

The RNA silencing pathway, usually targeted by VSRs, can also be modulated by other mechanisms. For example, it was reported that hormone imbalances triggered in response to viral infections are also implicated in the modulation of RNA silencing. There are several pieces of evidence that point out connections between RNA-silencing pathways and the defence signalling mediated by SA (reviewed in Carr *et al*., 2010). Both the RNA-silencing and SA-dependent responses were suggested to play key roles in limiting *Tomato ringspot virus* spread in tobacco (Jovel *et al*., 2011). SA has already been proposed to enhance antiviral RNA silencing against PPV in tobacco and, accordingly, suppressors of gene silencing such as P1/HC-Pro would interfere with SA-mediated defence (Alamillo *et al*., 2006). In line with these findings, there are several studies demonstrating that some MPs from certain viruses behave as viral enhancers of RNA silencing (VERs) by facilitating the propagation of vsiRNAs from cell to cell (Vogler *et al*., 2008; Zhou *et al*., 2008; Lacombe *et al*., 2010). It is unclear whether the increased spread of the silencing signal induced by MPs could be mediated by the sequence-unspecific MP–RNA binding activity (Citovsky *et al*., 1990) or by an increase of the size exclusion limit of the plasmodesmata (reviewed in Burch-Smith and Zambryski, 2016) and/or by altering the components that mediate RNA silencing pathways (for a review, see Amari *et al*., 2012).

The transgenic expression of MP in *N. tabacum* triggered a particular response against TMV infection (inoculation with naked genomic vRNAs) characterized by an initial phase of enhanced susceptibility, with rapid systemic spread of the virus and visible symptoms. Then, a recovery from infection was observed, with a significant reduction of viral accumulation and production of symptoms in the growing tissues (Conti *et al*., 2012). We hypothesized that at early stages, the movement of viral RNA between adjacent cells was facilitated by MP expression as was previously reported (Guenoune-Gelbart *et al*., 2008; Niehl and Heinlein, 2011), and that was the reason why the virus achieved a systemic infection faster that wild-type infected plants. It could also be argued that the enhanced expression of β 1–3 glucanases (PR-2) in MP-expressing lines might facilitate virus movement due to degradation of callose in plasmodesmata (Baebler *et al*., 2011). At late stages, the recovery phase observed in MP-expressing lines could arise from the combined effect of enhanced transport of vsiRNAs and also be due to the defence elicitation induced by ROS and SA. Both impacts of MP on host defences give rise to the activation of a strong immune response and antiviral RNA silencing (reviewed in Link and Sonnewald, 2016).

In conclusion, the MP from Tobamoviruses is able to interact with numerous components of the host, producing a wide spectrum of physiological effects, including modification of plasmodesmata size exclusion, SA and ROS induction and activation of RNA silencing. The combined effects result in the induction of host susceptibility at early stages and defence elicitation at late stages of infection.

Effects of TMV coat protein

The multifunctional role of CPs during the viral cycle is widely reported (Callaway *et al*., 2001; Makarov and Kalinina, 2016). Apart from their structural role in encapsidation, they are also involved in viral translation, replication, cell to cell movement and systemic movement (Callaway *et al*., 2001; Makarov and Kalinina, 2016). In particular, Tobamovirus CPs are required for long-distance movement, but are not necessary to promote cell to cell movement (Callaway *et al*., 2001). Recently, Tobamovirus CP has also been implicated in the negative modulation of SA-responsive gene expression. The constitutive transgenic expression of a mutated version of the CP from TMV (CP^{T42W}) in *N. tabacum* and also the inducible expression of the TMV CgCP in *A. thaliana* (Conti *et al*., 2012; Rodriguez *et al*., 2014) downregulated the expression of a set of SA-responsive genes including PR1 and RDR1 in *N. tabacum*, and WRKY70, AOX1A and RDR1 in arabidopsis. However, none of the CPs altered the endogenous SA hormone levels. It was also observed that in arabidopsis seedlings and adult plants, CgCP altered normal development when induced during early stages of development. These data suggest that the CP of Tobamoviruses could have a non-canonical role as a negative modulator of plant defence by altering antiviral defence mechanisms mediated by RDR1 and others genes regulated by SA (reviewed in Makarov and Kalinina, 2016).

Having observed that CgCP alters the normal development of arabidopsis seedlings and adult plants, and based on the findings that several viral proteins altered hormone cross-talk (Garcıa and Pallás, 2015), the role of DELLA proteins during CgCP expression was explored (Rodriguez *et al*., 2014). DELLA proteins are growth repressors, which are also involved in the alteration of ROS-scavenging enzymes and in the modulation of multiple hormone signalling pathways including defence pathways (Grant and Jones, 2009). Navarro *et al*. (2008) first reported the impact of DELLA proteins on defence signalling pathways. This report showed that arabidopsis plants, in which four out of the five DELLA genes were absent, displayed increased levels of SA and strong induction of SA-dependent genes when infected with *Pseudomonas syringae*. Later, it was observed that DELLAs can also modulate the jasmonic acid (JA) signalling pathway by interacting with JAZ proteins, which lead to inhibition of AtMYC2 repression and thereby activate the JA signalling (Yang *et al*., 2012). Findings by our group showed that CgCP delayed the gibbberellic acid (GA)-mediated degradation of green fluoprescent protein (GFP)–RGA fusion protein (one of the five DELLA proteins). Moreover, the expression of DELLA target genes was increased in plants expressing CgCP. Interestingly; we observed that the expression of RDR1 was downregulated in mutant plants carrying a 17 amino acid deletion within the *DELLA* domain of GAI which are insensitive to GA treatment. Given that SA-induced RDR1 expression is dependent on NPR1 protein and is enhanced by H_2O_2 (Hunter *et al*., 2013; Liao *et al*., 2013), it was proposed that DELLA could be attenuating RDR1 expression by its impact on SA signalling and ROS pathways (Rodriguez *et al*., 2014). Moreover, when viral accumulation was analysed in plants carrying a quadruple DELLA mutation, a reduced viral accumulation was observed in the quadruple DELLA mutants with respect to wild-type plants. These findings suggest that the stabilization of DELLA proteins during viral infection may be enhancing the susceptibility of plants to viruses. Kurihara and Watanabe (2004) showed the influence of Cg on ROS induction of the HR. These authors showed that a

viral strain of TMV-Cg carrying a mutation in the CgCP region induced a necrotic phenotype in *A. thaliana*. This finding led the authors to propose that a toxic effect triggered by the expression of the mutated CP was responsible for the necrotic phenotype observed. Following this line of thought, stabilization of DELLA proteins (involved in the modulation of ROS levels) by wild-type CgCP may allow the virus to evade the development of a necrotic phenotype.

Tobamovirus CPs have also been implicated in long-distance movement. However, the mechanism by which CP contributes to Tobamovirus systemic movement is unknown (Hilf and Dawson, 1993; Callaway *et al*., 2001). It has been suggested that TMV CP could be repressing a defence response and, in this way, it could assist in Tobamovirus systemic movement (Callaway *et al*., 2001). Considering the recent findings of our group, it could be speculated that the downregulation of antiviral defences by CP may be facilitating virus movement to systemic tissues.

OUTLOOK

To provide an integrative view of the different functionalities of TMV proteins on the modulation of host immunity during the progression of the infection, we proposed a model to summarize the whole scenario. Overall, the individual effects observed for TMV proteins on the RNA silencing pathways and the SA-mediated defence responses may reflect the ability of virus to balance the progression of tissue invasion. For successful plant infection, it appears that the host plant's control of infection is, to some extent, advantageous to the virus. A rapid depletion of host resources could be detrimental to propagation of the infectious agent. On the other hand, the negative modulation of the defence system may allow the virus to be able to move systemically, although there are several strategies of antiviral defence that can restrict the viral infection.

In Fig. 2, we schematize different stages of viral infection progression to represent plant–virus interaction process where viral proteins trigger responses in the host plant. In the first instance, the virus breaks into the cell and the replication initiates, supported by the replicase protein. This first step triggers a host response: RNA silencing. The intermediate dsRNAs produced during the replication processes are recognized by the silencing machinery and trigger the production of vsiRNAs that target the RISC to the viral genome. In this context, the dsRNA acts as a regular PAMP (pathogen-associated molecular pattern) and triggers the activation of silencing as a PTI (PAMP-triggered immunity) antiviral response. In a recently published study by Niehl *et al*. (2016), the treatment of arabidopsis plants with dsRNAs induced an antiviral PTI response mediated by a signalling cascade dependent on SERK1 and independent from DCLs, suggesting that dsRNAs represent genuine PAMPs.

In the case of TMV, the first round of viral counter-defense involves the replicase protein, which antagonizes silencing by VSR activity and induces a viral ETS (effector-triggered susceptibility) response. The replicase, the first viral transcript to be translated, keeps the system in equilibrium to allow the virus to pursue the infection process even with the silencing immunity active. The MP is the second protein to accumulate in infected cells and is required for cell to cell movement. This activity marks the next stage of the infection, the intermediate stage where the virus initiates localized spread. MP accumulation has an effect on virulence by stimulating host defence responses in the leading front of infection. MP induces SA and ROS overaccumulation, increases the expression levels of SA-responsive transcripts and downregulates ROS-scavenging genes. MP is also implicated in the local enhancement of antiviral silencing by facilitating the movement of vsiRNAs across plasmodesmata. After the cell-to-cell movement to reach the main veins and enter the vascular tissues, a subsequent process is initiated, the systemic spread of the virus. The movement of the virus in the phloem requires the participation of the CP (Hilf and Dawson, 1993; Callaway *et al*., 2001). The accumulation of this viral protein reaches its highest levels in late stages of the infection, with it becoming the most abundant viral component translated in the host tissues. On the other hand, in this late stage of infection, the entire machinery of antiviral defence is active in the whole host. Hence, a new wave of counter-defence response is required by the virus to continue replication and to spread the infection further. The multifunctional CP is associated with negative modulation of host defences, acting as a virulence enhancer. The rational thinking to explain this negative modulation of defences triggered by CP at late stages of infection is probably a result of the proportion of viral components in the systemic infection. We hypothesize that the ratios of the viral components influence the equilibrium between the different forces (defence and counter-defence responses). Robust evidence of this effect is provided by the modulation of a group of SA-regulated genes, which show increased levels of accumulation at early and intermediate stages, but decreased levels at late stages of infection, in agreement with the demonstrated effect of the transgenically expressed CgCP in *A. thaliana* (Rodriguez *et al*., 2014).

The second round of counter-defence activity displayed by CP may also be required to allow viral systemic movement. Rodriguez *et al*. (2014) demonstrated that CP attenuation of SA-induced defence responses is directed by DELLA protein stabilization, that uncouples the SA accumulation and SA-mediated responses, modifying the required signaling.

The chronology of the events proposed is in agreement with the zig-zag model of the plant–pathogen interaction which is represented at the base of Figure 2 (Jones and Dangl, 2006; Zvereva and Pooggin, 2012). In this, the deployment of consecutive rounds of defense and counter-defense will determine whether infection occurs or not. We hypothesize that there are multiple effectors, each of low impact, that can act together and influence the outcome in the arms race between virus and host. As a final remark, we conclude that to fully understand the process of viral infection in plant hosts, both virus and plant immunity perspectives must be considered. Recent advances in technology development and bioinformatics will undoubtedly stimulate considerable progress in this direction. The tight balance between defence activation and plant growth and development is of crucial importance for the two components of the interaction. Bally *et al*. (2015) showed that the reconstitution of Rdr1 activity in *N. benthamiana* plants provided protection against viral infections with, more interestingly, the silencing of a functional allele in wild strains resulting in hyper-susceptibility to viral

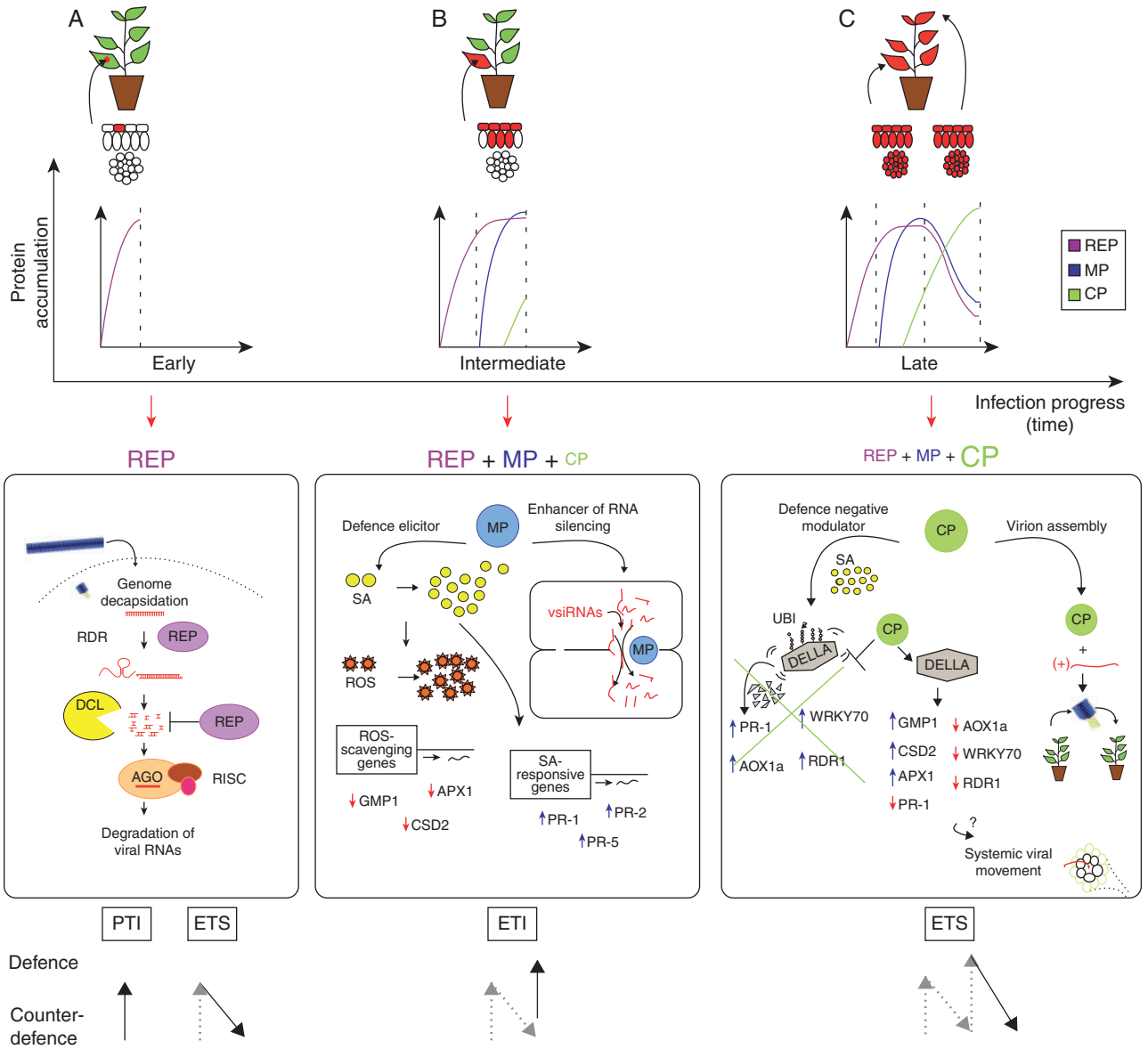

FIG. 2. Dynamic overview of plant immunity modulation mechanisms mediated by the different TMV proteins. (A) In the single-cell early stages of the infection, the uncoating of the viral RNA (viral PAMP) triggers the activation of RNA silencing as a first barrier of antiviral defence (antiviral PTI). The replicase is the first viral protein to be translated and presents silencing suppression activity (viral ETS). In the bottom of this panel, a representative zig–zag model is shown. (B) At intermediate stages of the infection, the MP initiates accumulation and allows the virus to invade adjacent cells by facilitating passage through plasmodesmata. The MP is detected by the host and activates immune defences, thus triggering an antiviral ETI response. It acts as a defence elicitor mediated by induction of ROS and SA accumulation, induction of SA-responsive genes and downregulation of ROS-scavenging genes. It also acts as an enhancer of RNA silencing, mediating the facilitation of vsiRNAs transport across plasmodesmata. In the bottom of the figure, a representative zig–zag model is shown. (C) During late stages of infection, the CP becomes the more abundant viral protein accumulated in the infected cells. The CP is involved in the systemic movement, permits the assembly of new viral particles and is implicated in the negative modulation of host immunity, giving rise to a new round of viral ETS. The suppression of SA-mediated defence responses by the CP is triggered by the downregulation of SA-responsive genes via the stabilization of DELLA proteins. In the bottom of the figure, a representative zig–zag model is shown.

infection, together with the doubling of seed size and enhanced early growth. The activation of defence mechanisms mentioned in this review are required for the host to respond to infection. Activation of such defense responses may, however, result in undesired side-effects detrimental to normal developmental processes and/or programmed plant growth. Such side-effects, which can be the central cause of disease symptom development, may originate through direct processes such as hormone cross-talk to redirect gene expression from growth to defense responses, and/or by indirect processes, such as the double use of RNA silencing machinery in antiviral RNA silencing and miRNA pathways. They could originate by expected processes (such as the hormone cross-talk required for redirecting the transcriptome from growth to defence) and/or by indirect

mechanisms, such as alteration of the the miRNAs by means of the double use of the RNA silencing machinery in different pathways (antiviral RNA silencing and the miRNA pathway).

As a final remark, we conclude that to fully understand the process of viral infection in plant hosts, both virus and plant immunity perspectives must be considered. Recent advances in technology development and bioinformatics will undoubtedly stimulate considerable progress in this direction.

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