THE ROLE OF VIRAL EFFECTOR PROTEINS IN SUPPRESSION OF PLANT ANTIVIRAL DEFENSES BASED ON RNA SILENCING AND INNATE IMMUNITY

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SUMMARY

Plant viruses are widespread and economically important pathogens. Currently, there are more than one thousand viruses that are known to be potentially capable of infecting plants and new viruses are being discovered every day. Many of them could cause important diseases of various cultivated plants that humans grow for food, fiber, feed, construction material and biofuel. Therefore understanding the biology of plant viruses is important for development and improvement of cultivated plant resistance to viral pathogens.

A major role in plant resistance against viruses belongs to the process called RNA silencing, that targets both RNA and DNA viruses through the small RNA-directed RNA degradation and DNA methylation pathways. In addition, plants respond to virus infection using an innate immune system that recognizes microbe-associated molecular patterns (MAMPs) of potential pathogens and elicits both local and systemic defense responses. However, in order to be succesfull and break the host resistance, plant viruses have evolved a variety of counter-defense mechanisms such as expressing effector proteins, which are used to downregulate plant antiviral responses. Here, we performed comparative investigation of viral effector proteins from two distanly-related pararetroviruses, *Cauliflower mosaic virus* (CaMV) and *Rice tungro bacilliform virus* (RTBV), to understand their role in the suppression of plant antiviral defenses based on RNA silencing and innate immunity. The CaMV P6 protein has previously been shown to serve as a silencing suppressor, while the function of RTBV P4 protein was unknown. Through the use of a classical transient assay in leaves of the *N. benthamiana* transgenic line 16c we show that RTBV P4 can suppress cell-to-cell spread of transgene silencing, but enhance cell autonomous transgene silencing, which correlates with reduced accumulation of 21-nt siRNAs and increased accumulation of 22-nt siRNAs, respectively. Furthermore, we demonstrate that CaMV P6 from strain CM1841 and RTBV P4 proteins are able to suppress the early plant innate immunity responses, such as oxidative burst. In contrast, CaMV P6 from strain D4 failed to suppress innate immunity, but was capable of suppressing RNA silencing as P6 protein from strain CM1841.

We also elucidated the role of P4 F-box-like motif and N-terminal domain that are required for RTBV P4 effector functions and protein stability, respectively.

Finally, through the use of agroinoculation of *Oryza sativa* plants with RTBV infectious clone we tested if the P4 F-box motif is required for infectivity and our preliminary results show that the F-box mutant virus exhibts drastically reduced infectivity. Furthermore, we found that RTBV circular double-stranded DNA evades siRNA-directed cytosine methylation in infected rice plants and that rice plants overexpressing an OsAGO18 protein are resistant to RTBV infection.

1. GENERAL INTRODUCTION

1.1. PLANT VIRUSES

The history of viruses has begun in 1892 with the discovery of *Tobacco mosaic virus* (TMV), causing mosaic disease in tobacco plants. Since that time, many plant, animal, fungal and bacterial viruses were discovered, which are currently classified into 7 orders, 111 families, 609 genera and 3704 species (ICTV Virus Taxonomy 2015). The 1019 species of plant viruses are found in three orders, 22 families and 108 genera (Balique *et al.,* 2015) and their hosts include angiosperms (flowering plants), gymnosperms (conifers), pteridophytes (ferns), bryophytes (mosses and liverworts) and green algae (Cooper, 1993; Mascia *et al.,* 2014; Hull 2014 Plant Virology).

All viruses infecting plants contain one of the four types of nucleic acid molecules in their viral particles as genetic material. These molecules are single-stranded (ssRNA) (about 75% of plant viruses), double-stranded RNA (dsRNA), single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) (Bustamante *et al.,* 1998; Hull 2014 Plant Virology).

Although the majority of scientifically or economically important plant viruses have single stranded, positive-sense RNA genome packaged in viral particles (virions), viruses that contain another molecules as their genomic material are also of huge importance for scientists studying molecular plant pathology (Scholthof, *et al.,* 2011). Particularly, in the following sections I will describe two dsDNA viruses of the *Caulimoviridae* family, *Rice tungro baciliform* virus (RTBV) and *Cauliflower mosaic virus* (CaMV), which served as model systems in my thesis project to investigate the role of viral effector proteins in suppression of plant antiviral defenses based on RNA silencing and innate immunity.

1.2. FAMILY *CAULIMOVIRIDAE*

The family *Caulimoviridae* contains plant viruses using a reverse transcription step in their replication cycle that together with the *Hepadnaviridae* family of vertebrate viruses form the pararetrovirus group, whose members are similar to plant and animal retrotransposons (former retroviruses) as well as animal retroviruses (true retroviruses) sharing the mechanism of genome replication by reverse transcription and functionally conserved gag-pol core that encodes structural proteins (gag) and a polyprotein (pol) consisting of protease (PR), reverse transcriptase (RT) and RNAse H (RH) domains. Pararetroviruses lack an integrase domain encoded by the retroviral pol in order to integrate the viral DNA into the host genome (Haas *et al.,* 2002; Hohn and Rothnie, 2013). As opposed to true retroviruses, in which single-stranded genomic RNA is packaged in the virion and reverse-transcribed proviral DNA integrates into the host genome, pararetroviruses encapsidate into the virion a double-stranded genomic DNA that also accumulates as thousands of episomal copies (so called minichromosomes) in the host cell nucleus after reverse

transcription of viral pregenomic RNA (Haas *et al.,* 2002). One possible explanation for the lack of the integration step in the replication cycle of plant pararetroviruses is to avoid the repressive action of RNA-directed DNA methylation (RdDM), which results in transcriptional gene silencing of plant genome-integrated transposons and transgenes: this is likely the reason why true retroviruses with host genome-integrated proviral DNA don't exist in plants (Pooggin, 2013).

The *Caulimoviridae* family comprises eight genera, which are distinguished from each other by their genome organization. Depending on the genus the viral genome can vary in size between 7.2-9.2 kb and in number of ORFs between one lagre ORF encoding a polyprotein (*Petuvirus*) to eight smaller ORFs (*Soymovirus*) (Fig. 1)(Bhat *et al.,* 2016). All members of the family are non-enveloped viruses that could be divided in two subgroups based on the structure of their protein-coated virions. The first subgroup including *Rosadnavirus*, *Cavemovirus*, *Petuvirus*, *Caulimovirus*, *Soymovirus*, and *Solendovirus* genera, has isometric particles that are usually found in cytoplasmic inclusion bodies. The members of the second subgroup including *Badnavirus* and *Tungrovirus* genera have bacilliform particles and are not found to be associated with cytoplasmic inclusion bodies (Geering, 2014; Hull, 2007).

As mentioned above, replication of pararetroviruses does not involve compulsory integration into the host genome. Nonetheless, several pararetrovirus species within four genera (*Badnavirus*, *Petuvirus*, *Solendovirus* and *Caulimovirus*) were found to be integrated in their host plant nuclear genomes. These endogenous viral elements (EVEs) are the result of illegitimate recombination events showing varying levels of fragmentation, duplication, and rearrangements (Geering, 2014). Interestingly, there are a few examples of endogenous pararetroviral sequences (EPRVs) that can be released from their host genome and become infective (Gayral *et al.,* 2010).

The replication cycle of plant pararetroviruses includes two main steps in the nucleus and the cytoplasm. (1) Following entry into the plant cell and disassembly of the capsid proteins, the pararetroviral dsDNA is imported into the nucleus, where it associates with histones to form minichromosomes that are used as templates for transcription by the host DNA-dependent RNA polymerase II (Pol II) producing a capped and polyadenylated pregenomic RNA (pgRNA) and, in some genera, subgenomic RNAs. (2) The pgRNA migrates to the cytoplasm, where its translation and reverse transcription processes take place. The newly synthesized dsDNA is packaged into the virion to move from cell to cell and to be transmitted from plant to plant. Interestingly, the pararetroviral dsDNA encapsidated into virions is characterized by at least one discontinuity located at specific sites of each DNA strand: one in the negative strand at the biding site for Met-tRNA primer initiating reverse transcription and one to three in the positive strand at the polypurine site(s) priming the positive strand DNA synthesis (Geering, 2014).

Most of the virus species in the *Caulimoviridae* family have narrow host ranges and could infect only either dicotyledonous or monocotyledonous host plants. For instance, the members of the genera *Caulimovirus*, *Soymovirus*, *Cavemovirus*, *Solendovirus* and *Petuvirus* infect dicotyledonous

plants, while the single member of the genus *Tungrovirus* RTBV could infect only monocotyledonous rice plants (Geering, 2014; Hull, 2007).

The pararetroviruses in the *Caulimovirus*-like subgroup are transmitted mostly by aphids, while the members of the *Badnavirus*-like subgroup are transmitted by mealybugs, except for RTBV which is transmitted by leafhoppers in the semipersistent manner (Geering, 2014).

The members of *Caulimoviridae* family induce a variety of symptoms on the leaves, ranging from mosaic or streak chlorosis to necrotic lesions, and deformation of leaf surfaces, as well as stunted growth and other developmental abnormalities, which altogether can be extremely detrimental to commercial crops. By understanding the mechanisms of plant–virus–vector interactions we may be able to minimize crop losses due to these pathogens (Geering, 2014; Hull 2014 Plant Virology).

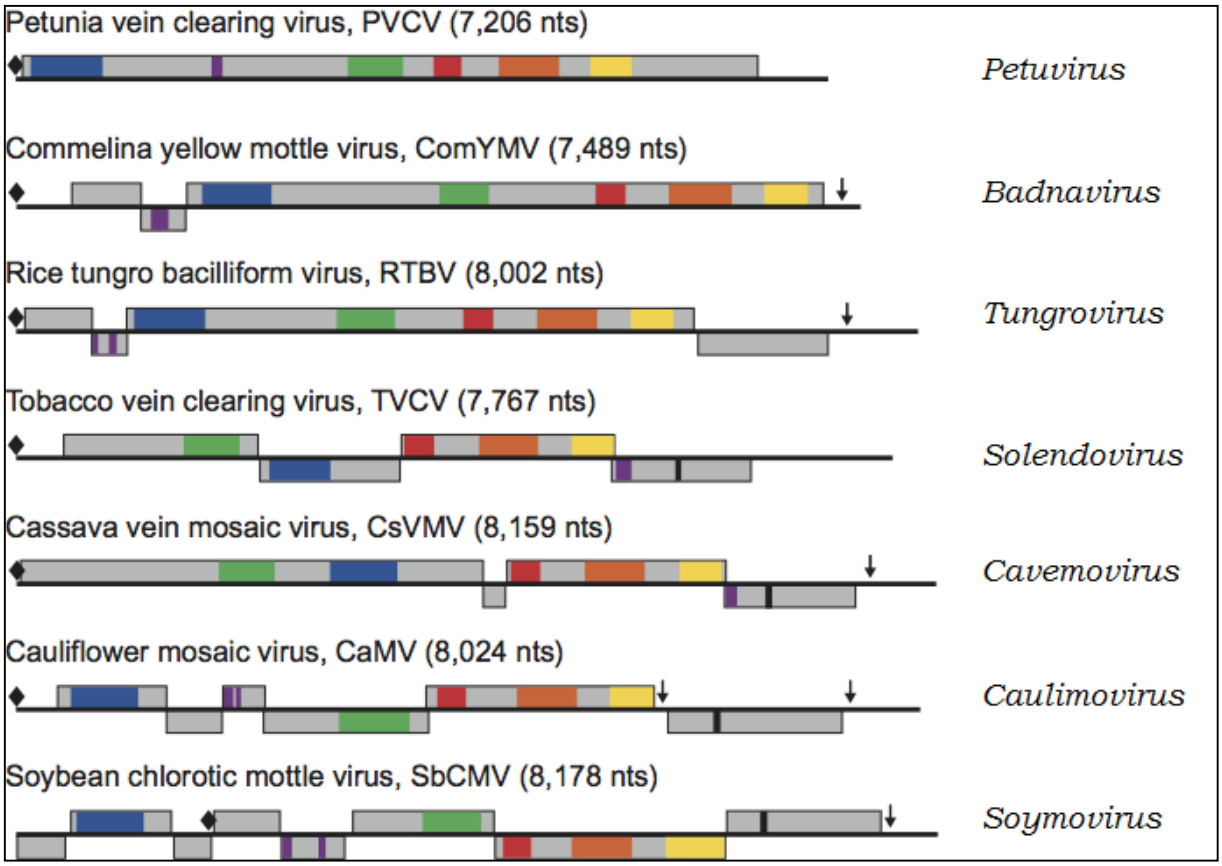


Figure 1. Genome organisation of the members of *Caulimoviridae* family (adopted from the website https://talk.ictvonline.org)

1.3. *RICE TUNGRO BACILLIFORM VIRUS*

1.3.1 INTRODUCTION

Rice tungro bacilliform virus (RTBV) is a type (and the only) member of the *Tungrovirus* genus in the family *Caulimoviridae*. Unlike other pararetroviruses from a closely related genus (*Badnavirus*) or other genera of the family *Caulimoviridae* the most distal open reading frame (*ORF IV*) of RTBV genome is expressed by a unique mechanism using a spliced RNA as a messenger

(Futterer *et al.,* 1994). Moreover, none of the members of *Caulimoviridae* family possess any *ORF IV*-related gene, the product of which is a protein P4 with previously unknown function that was investigated in this PhD thesis.

1.3.2. STRUCTURE OF RTBV PARTICLES

Similar to badnaviruses, RTBV has non-enveloped bacilliform particles of about 130 X 30 nm that contain a single circular dsDNA molecule with two discontinuities, one on each strand. The structure of these particles is based on icosahedral (T=3) symmetry (Fig. 2)(Hull, 1996).

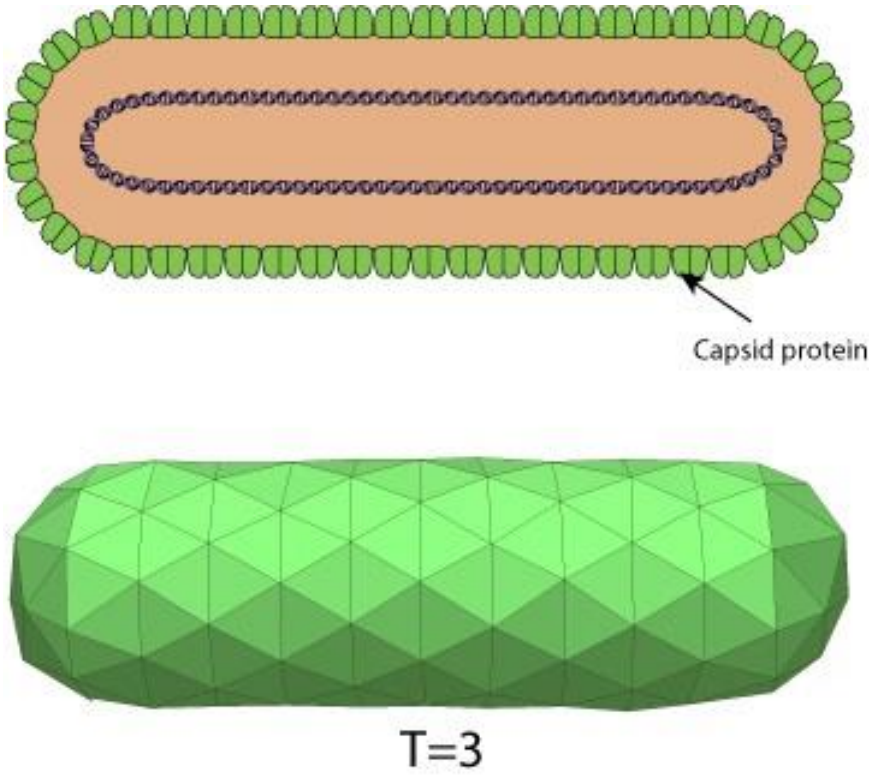


Figure 2. Structure of RTBV particles (taken from the website http://viralzone.expasy.org)

1.3.3. RTBV GENOME ORGANIZATION AND PROTEIN EXPRESSION

Unlike badnaviruses having three ORFs, the RTBV genome has four ORFs (Fig. 3). The first three ORFs (*ORF I*, *ORF II* and *ORF III*) are consecutive with overlapping stop and start codons, whereas *ORF IV* is separated from *ORF III* by a short noncoding region. There is also a large intergenic region between *ORF IV* and *ORF I*, containing Pol II promoter elements, a transcription start site and a 697-nt leader sequence with a poly(A) signal, several short ORFs (sORF) and stable secondary structure, which is a common feature within family of plant pararetroviruses (Pooggin et

al. 1999). All the pgRNA transcripts of plant pararetroviruses have this highly structured leader forming a large stem-loop secondary structure brings the first sORF into close spatial proximity to the first long ORF (*ORF I*) that is necessary for initiation of pgRNA translation (Futterer et al. 1993; Pooggin et al. 2008). Similar to CaMV, RTBV pgRNA translation is initiated by a ribosome shunt mechanism. Following the recognition of 5′ cap structure, and scanning process, a majority of the scanning 40S ribosomes assemble at the first AUG that is the start codon of the 5′-proximal sORF (sORF1) into complete 80S ribosomes to initiate translation. After translation of sORF1 and the product release, the 80S ribosomes disassemble at the stop codon UAG of sORF1 (a take-off site) and a fraction of the released 40S subunits shunt over an extensive downstream stem-loop structure and land to an unstrucrured AU-rich sequence (a shunt landing site). Finally, the shunting ribosomes resume scanning and reinitiate translation at a non-AUG start codon (AUU) of *ORF I* located at the 3′-end of the pgRNA leader (Futterer *et al.* 1996; Pooggin *et al.,* 2006). Given a suboptimal nature of the AUU start codon, only small fraction (about 10%) of the shunting ribosomes initiates translation of *ORF I*, while the majority thereof continues the scanning process to reach the start codons of *ORF II* and eventually *ORF III* by a so-called leaky scanning mechanism (Futterer *et al*. 1997) (Fig. 3).

Thus, the first three RTBV ORFs are expressed from pgRNA by leaky scanning mechanism, while the *ORF IV* is expressed from a subgenomic RNA formed by splicing of pgRNA. The splicing of the RTBV pgRNA brings together in frame the leader-based sORF1 with the 5' end of *ORF IV* and releases a large intron of 6.3 kb (Futterer et al. 1994).

As was mentioned above plant pararetroviruses replicate by reverse transcription that requires the activity of two virus-encoded enzymes: reverse transcriptase and ribonuclease H (RNAse H) (Hohn *et al.,* 1997). In RTBV, both enzymes are translated from *ORF III* encoding a large polyprotein (P3) of 196 kDa that contains five domains corresponding to the movement protein (MP), coat protein (CP), as well as PR, RT, and RH enzymes. The viral PR of 13.5 kDa that shows homologies with retroviral proteases is at least partly involved in the processing of P3 in order to release the products from the N-terminal and C-terminal portions of polyprotein (Hull, 1996). The N-terminal part of P3 is processed to give MP of 40 kDa and CP of 37 kDa. MP was identified by sequence similarities with cell-to-cell proteins found in other plant viruses, while the function of this protein has not been confirmed as RTBV replicates only in phloem cells and has not been detected in mesophyll tissue, where a majority of viral MP are known to operate. CP is characterized by the presence of two nuclear localization signals (NLS) and is used to facilitate the import of viral virions into the nucleus through its interaction with a nuclear import factor importin-alpha (Guerra-Peraza *et al.,* 2005). Interestingly, the 12 kDa product of *ORF II* (P2), which possesses the nucleic acid binding activity, was shown to interact with CP, suggesting its involvement in particle assembly. Although this interaction is required for RTBV infectivity (Herzog *et al.,* 2000), the function of P2 remains to be investigated. The C-terminal portion of P3 contains a

self-releasing PR of about 20 kDa possessing a conserved DSGS catalytic motif of the retroviral aspartic proteases, as well as RT of 62 kDa and RH of 55 kDa, released by the PR activity and involved in reverse transcription (Hull, 1996).

The RTBV *ORF IV* product of 46 kDa (P4) is a unique protein of unknown function, which has not been found in other pararetroviruses.

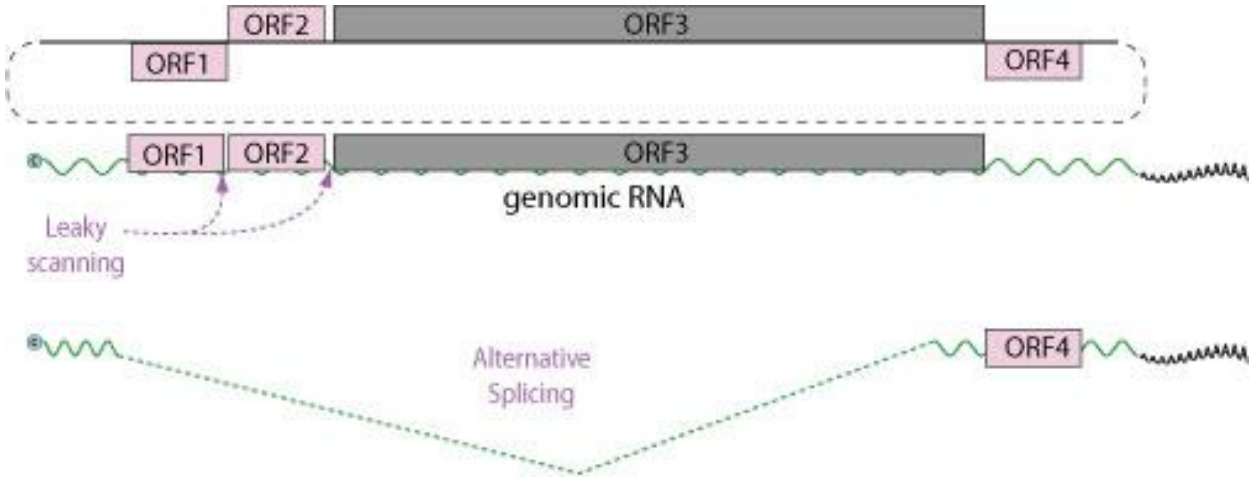


Figure 3. RTBV genome organisation (taken from the website http://viralzone.expasy.org)

1.3.4. RTBV GENOME TRANSCRIPTION

Like other members of the *Caulimoviridae* family the RTBV genome is transcribed asymmetrically having all its coding capacity on one strand, the positive (+) strand. Similar to CaMV, there are several elements that control the RTBV transcription process, including Pol II promoter and terminator elements. The RTBV promoter consists of the conserved TATA-box, transcription start site (TSS) and other promoter-specific regions directly upstream and downstream of TSS. Although RTBV is considered to be phloem-limited, its promoter was found to be active in all vascular, epidermal and, albeit weakly, in leaf mesophyll cells and additionally is strongly stimulated by promoter-specific sequences downstream of the TSS (Klöti *et al.,* 1999). The RTBV terminator elements include the classical polyadenylation signal AAUAAA based in the pgRNA leader, which is bypassed by Pol II on its first encounter and is recognized on the second passage around the circular genome, and the upstream UUUGUA repeats found to considerably enhance pgRNA processing and polyadenylation (Rothnie *et al.,* 2001).

1.3.5. RTBV $_{PG}$RNA REVERSE TRANSCRIPTION

RTBV pgRNA is reverse transcribed in the cytoplasm by the viral RT. Methionine initiator tRNA (Met-tRNA) is used as a primer for the (-) DNA strand synthesis and its binding site is located within the leader sequence, 600 nts downstream of the 5'-cap. At the first step a short minus-strand

DNA molecule that is covalently linked to the tRNA primer and called strong-stop DNA is produced on the leader sequence template. Upon degradation of the RNA template by RH, the strong-stop DNA of about 600 nt is switched to the 3' end of the pgRNA, due to the terminal repeat in order to continue the synthesis of (-) DNA strand (Hull, 1996). Subsequently, the rest of pgRNA template is digested by RH, except for one resistant poly-purine stretch, which constitutes the initiation site of the (+) DNA strand transcription. This RNA oligonucleotide remains annealed to the (-) strand and serves as a primer for the transcription of (+) strand. The newly synthesized pararetroviral dsDNAs have two discontinuities with small RNA overhangs, one on each strand, that are matched to the RT priming sites. These discontinuities, upon the infection of new plant cells and release of viral dsDNA into the nucleus, are repaired by the host nuclear enzymes to yield a covalently-closed supercoiled dsDNA molecule, which associates with histones in order to form a minichromosome and become transcribed by Pol II (Hull, 1996).

1.3.6. RICE TUNGRO DISEASE

Rice tungro disease (RTD) is considered as the most important of the 14 recognized rice viral diseases that affect a majority of South and Southeast Asian countries (India, Indonesia, Thailand, Malaysia and Philippines) and is characterized by its virulence, high annual losses that it causes and enormous difficulty of controlling it. The «tungro» that is translated as retarded growth from the Filipino dialect has a long history with it being first recognized as the cause of a rice disease outbreak in 1859 in Indonesia. More recently, in the 1960s and 1970s, due to the production of new varieties of rice in combination with intensive rice cultivation, the transmission of viruses infecting rice by various vectors was extremely facilitated. This led to a dip in rice production in South and Southeast Asian countries and stimulated the conduct of epidemiological studies in order to develop efficient tungro management strategies (Azzam *et al.,* 2002).

In the late 1970s it was confirmed that RTD is caused by a complex of two viruses, the above-described RTBV and an RNA virus, *Rice tungro spherical virus* (RTSV). Notably, RTBV could be transmitted only in the presence of RTSV by the rice green leafhopper vector (*Nephotettix virescens*) in a semipersistent manner. Symptoms of RTD in rice plants could vary from mild or even indistinct when the plant is infected with RTSV alone to severe including stunting and yellow to orange discoloration of the leaves in plants infected with RTBV (Fig. 4). However, the most conspicuous symptoms could be observed when the rice plants are infected with both viruses. Interestingly, that RTSV is largely susceptible to control measures including generation of transgenic plants and thought to have evolved with the rice plant. On the other hand, RTBV is thought to be a relatively new virus infecting rice plants as it is more resistant to antiviral measures (Azzam *et al.,* 2002).

Figure 4. Rice tungro disease and green leafhopper vector (*Nephotettix virescens*) (taken from the websited https://microbewiki.kenyon.edu and http://www.knowledgebank.irri.org)

1.4. *CAULIFLOWER MOSAIC VIRUS*

1.4.1. INTRODUCTION

Cauliflower mosaic virus (CaMV) is the most extensively studied member of the *Caulimovirus* genus, first plant virus group whose genome was shown to consist of dsDNA instead of RNA (Shepherd *et al.,* 1970). As was mentioned above, *Caulimovirus* and the other seven plant virus genera replicating their genomic DNA via the reverse transcription of a pgRNA intermediate comprise the *Caulimoviridae* family of plant viruses (Hull 2014 Plant Virology).

1.4.2. CAMV VIRION STRUCTURE AND TRANSMISSION

The CaMV viral particle has spherical 520 Å diameter shape, icosahedral T7 symmetry and a structure with a large 250 Å inner cavity surrounded by three concentric shells built from 420 capsid subunits (Hoh *et al.,* 2010). Among the seven proteins coded by the CaMV genome, P3 (15 kDa) has been demonstrated to be associated to the viral particles and is therefore often referred to as Vap (virion-associated protein). The N-terminal ectodomains of P3 form an antiparallel α-helical coiled-coil network at the surface and the C-terminal ends interact with the coat protein and penetrate the virus particle. Presumably, the C-terminus binds the DNA genome, packed between the intermediate and inner shells (Leh *et al.,* 1999, 2001; Leclerc *et al.,* 2001; Drucker *et al.,* 2002). CaMV P4 (56 kDa, also known as Gag) that is the precursor of the capsid protein, consisting of two very acidic terminal domains and a basic region between amino acids (aa) 327 and 410. This region, containing a nucleic acid binding domain and a conserved Cys/His Zn-finger motif, interacts with a purine-rich region in the leader of the pgRNA upon cleavage of the acidic domains of the pre-protein by the viral aspartic protease. The mature capsid protein possesses a nuclear localization

signal close to its N-terminus, which targets the virus particles for their docking at the host cell nuclear pore (Leclerc *et al.,* 1999; Karsies *et al.,* 2002).

Although host plants can be mechanically inoculated with the CaMV under laboratory conditions, the only transmission mode documented in nature is the non-circulative transmission by several aphid species, such as *Myzus persicae* (Hull 2014 Plant Virology; Ng and Falk, 2006). The virion is retained in the mouthparts of the aphid vector after its acquisition from an infected host by the N-terminus of the viral helper protein P2 (aphid transmission factor), which specifically recognizes a cuticular receptor on the tip of the maxillary stylets (Moreno *et al.,* 2005; Uzest *et al.,* 2007), while the C-terminus α-helix of this protein forms coiled-coil structures binding the ectodomain of the P3 protein attached to the surface of the virion. Thus both P2 and P3 are required for the aphid-mediated transmission of the virus (Leh *et al.,* 1999; Hébrard *et al.,* 2001; Plisson *et al.,* 2005).

The CaMV host range is mostly restricted to plants of the *Cruciferae* family, although some of virus strains could be distinguished by their ability to infect members of the *Solanaceae* (Pagan *et al.,* 2010). While D4 and W260 can infect systemically species such as *Nicotiana bigelovii* or *Datura stramonium*, CM1841 does not produce any systemic symptoms in either host inducing local chlorotic lesions in *N*. *bigelovii* and hypersensitive response (HR) in *D*. *stramonium* (Schoelz *et al.,* 1986; Qiu and Schoelz, 1992). W260 also produces mosaic symptoms followed by cell death in *N*. *clevelandii* and elicits a hypersensitive response in *N*. *edwarsonii* (Palanichelvam *et al.,* 2000; Palanichelvam and Schoelz, 2002). Systemic symptoms include mild to severe chlorosis, mosaic, vein clearing and stunting, depending on the strain, host ecotype and environmental conditions (Melcher, 1989; Wintermantel *et al.,* 1993). Chimeric viruses constructed between D4 and CM1841 demonstrated the role of CaMV P6 protein in determining systemic infection of these two solanaceous species (Daubert *et al.,* 1984; Schoelz *et al.,* 1986). The transgenic expression of P6 in *Arabidopsis thaliana* elicited CaMV infection symptoms and altered the expression pattern of more than 30 genes (Zijlstra *et al.,* 1996; Cecchini *et al.,* 1997). Thus, P6 protein is an important determinant of symptom expression which functions as a virulence/avirulence factor upon CaMV infection (Geri *et al.,* 1999; Zvereva and Pooggin, 2012).

1.4.3. CAMV GENOME ORGANIZATION

CaMV has been extensively studied as a type member of the caulimoviruses in order to understand the genome organization of this genus. It consists of a dsDNA molecule of approximately 8 kbp (Cheng *et al.,* 1992). Due to the presence of single-stranded interruptions in both DNA strands, whose number and position varies depending on the virus strain, the genome exists in an open circular form inside the viral particle. The triple-stranded sequence discontinuities are repaired by host nuclear enzymes to yield a supercoiled DNA molecule, which

associates with histones in order to form a minichromosome harbouring 42 + 1 nucleosomes (Haas *et al.,* 2002).

The complete genome sequencing of three CaMV isolates (Franck *et al.,* 1980) revealed the presence of seven ORFs located on the (+) strand and two intergenic regions of approximately 700 and 150 bp respectively, containing regulatory sequences. Except for *ORF VI*, which lies between the two intergenic regions, all the ORFs are separated or overlap by a few nucleotides (Haas *et al.,* 2002). The large intergenic region contains a Pol II promoter driving transcription pf pgRNA (35S RNA), while the short intergenic region a Pol II promoter for subgenomic RNA (19S RNA), an mRNA for the viral multifunctional protein P6. The constitutive nature and high efficiency of the 35S promoter has made it a biotechnological tool that is extensively used in the construction of vectors for gene overexpression in most cell types and developmental stages (Tani *et al.,* 2004). In addition to the core promoter containing the TATA-box, regions A (-90 to -46) and B (-343 to -90) have been described as enhancer domains controlling expression in leaves and roots respectively (Benfey *et al.,* 1990).

1.4.4. CAMV TRANSCRIPTION AND REPLICATION

Once the viral particles have penetrated the nucleus of the host plant cell, the CaMV minichromosome is transcribed unidirectionally by the Pol II into two capped and poly-adenylated transcripts, 35S pgRNA and 19S subgenomic RNA. The 35S RNA containing all seven viral ORFs as a template for revese transcription is a polycistronic mRNA for translation of *ORF VII*, *I*, and *II*, whereas 19S RNA is a monocistronic RNA contaning *ORF VI* (P6/TAV) (Covey *et al.,* 1981; Driesen *et al.,* 1993; Hohn and Rothnie, 2013). A fraction of the 35S RNA undergoes splicing that gives rise to several spliced RNAs one of which serves as an mRNA for *ORFs III* (VAP), *IV* (GAG) and *V* (POL). The pgRNA is terminally redundant due to the fact that the Pol II ignores the polyadenylation signal, located approximately 180 nts downstream from the transcription start site, at its first passage (Sanfaçon and Hohn, 1990). This signal consists of the classical AAUAAA sequence determining the cleavage of the transcripts 13 nts downstream and cis-acting upstream elements that increase the efficiency of the 3' processing. A repeated UUUGUA motif was also identified as an important upstream accessory element (Sanfaçon *et al.,* 1991; Rothnie *et al.,* 2001).

The splicing of 35S RNA is essential for infectivity. The four splice donors, one located in the leader region of the 35S RNA and the other three in the 3' terminus of *ORF II*, use the same acceptor within *ORF II*. Splicing between the leader and *ORF II* produces mRNA for *ORFs III, IV and V*, while the three other splicing events lead to the production of *ORF I-II* in-frame fusions, whose functions remain unknown. By reducing the translation of *ORF II* protein, the splicing prevents the toxic effects derived from its overaccumulation (Kiss-László *et al.,* 1995; Froissart *et al.,* 2004).

The CaMV pgRNA is reverse transcribed to dsDNA by the RT, encoded by the C-terminal region of *ORF V*. Like in the case of RTBV, Met-tRNA is used as a primer for the (-) DNA strand

synthesis and its binding site is located within the leader sequence. Simultaneously, the RNA template is digested by RNase H, except at one or more resistant poly-purine stretches (RRRRR), which constitute the initiation site of the (+) DNA strand transcription. Oligoribonucleotides remain annealed to the (-) strand at this site and serve as primers for the transcription of (+) strand (Fig. 5) (Hohn and Rothnie, 2013).

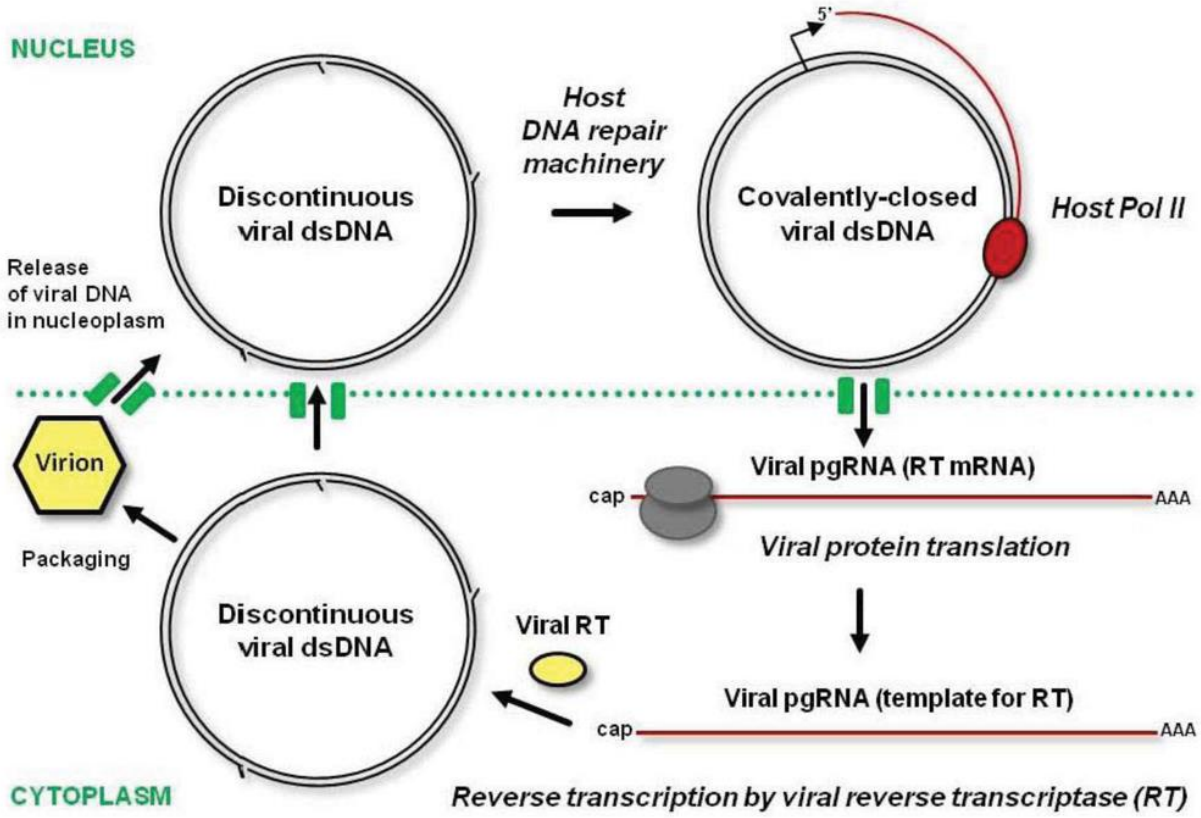


Figure 5. Model of pararetrovirus replication (Pooggin 2013)

1.4.5. CAMV PROTEIN TRANSLATION AND FUNCTION

The transcribed 19S RNA, 35S RNA and its spliced variants are transported to the cytoplasm for their subsequent translation. The 35S RNA has a long 5'-UTR (over 600 nts) containing a stable stem-loop secondary structure, several sORFs, and signals for polyadenylation and packaging. In order to overcome ribosome scanning inhibition at such a long and structured leader, CaMV has developed a shunt mechanism where the 40S ribosome initiation complex bypasses the stable secondary structure after translation of the 5'-proximal small *ORF A*, which ends a few bases before the structure, and lands at the 3' end of the structure to resume translation at *ORF VII* (Fütterer *et al.,* 1993; Hohn *et al.,* 2001). If either the sORF or the secondary structure is mutated, infectivity is delayed producing the first and the second site reversions that restore the sORF and the structure (Pooggin *et al.,* 1998).

After shunt-mediated translation initiation at *ORF VII*, a viral protein P6, also known as tarsactivator/viroplasmin (TAV) activated translation reinitiation mechanism allows the translation of the further downstream ORFs encoded by 35S RNA (Fig. 6). While TAV has only a stimulatory effect on ribosomal shunting, it plays an essential role for translation of *ORF I* and *II* (as well as further downstram ORFs from spliced 35S RNAs) through its association with polysomes and translation initiation factors, leading to the reprogramming of the ribosome machinery to translate the polycistronic 35S RNA (Bonneville *et al.,* 1989; Pooggin *et al.,* 2000). To transactivate translation reinitiation, TAV physically interacts with the subunit g of the eukaryotic initiation factor eIF3 (in competition with eIF4B), the 60S ribosome subunit proteins L18, L24 and L13, and a reinitiation-supporting protein (RISP). (Leh *et al.,* 2000; Park *et al.,* 2001, 2004; Bureau *et al.,* 2004; Thiébeauld *et al.,* 2009). The reinitiation mechanism also depends on the hyperactivation of target-of-rapamycin (TOR) through binding with TAV, which triggers the phosphorylation cascade involving TOR, S6K1, RISP and eIF3, ultimately allowing the reuse of the two latter components to regenerate reinitiation-competent ribosomal complexes (Schepetilnikov *et al.,* 2011).

Kinetic studies performed *in planta* and in turnip protoplasts have revealed a differential regulation of the CaMV protein expression throughout the infection cycle, in which P1, P5 and P6/TAV are expressed earlier than P2, P3, and P4 (Maule *et al.,* 1989; Kobayashi *et al.,* 1998).

ORF I encodes a 40 kDa movement protein P1 that formes tubules extending from the surface of infected cells and projecting through the plasmodesmata (Perbal *et al.,* 1993). The protein contains a central domain targeting it to the cell periphery, which is partially overlapping with an RNA-binding domain. Except for the C-terminal region exposed at the lumen, most of the protein constitutes the tubular structure (Citovsky *et al.,* 1991; Thomas and Maule, 1995, 1999; Huang *et al.,* 2001). Through a C-terminally-located coiled-coil domain, it self-assembles as a trimer and binds the virion-associated P3 in order to mediate cell-to-cell movement of the virions (Stavolone *et al.,* 2005). P1 has also been demonstrated to interact with plant host proteins, such as the *Arabidopsis* movement protein AtMPI7 and the tobacco cell wall-associated pectin methylesterase (Chen *et al.,* 2000; Huang *et al.,* 2001). Interestingly, yeast two-hybrid assays demonstrated the interaction of P1 and P6, suggesting a role for P6 in assisting P1 in the movement process (Hapiak *et al.,* 2008).

ORF II encodes a 18 kDa aphid transmission factor (P2) that self-assembles into paracrystalline filaments by forming coiled-coil structures involving the C-terminal region, which can also interact with P3 (Blanc *et al.,* 1996). In infected cells, P2 was found in a large cytoplasmic electron-lucent inclusion body called “transmission body” (TB) in co-aggregation with virion-free P3 and few virions (Woolston *et al.,* 1987; Espinoza *et al.,* 1991; Drucker *et al.,* 2002). CaMV transmission by aphids requires the formation of a complex composed of the virion, the virion-associated protein P3 and the helper transmission factor P2, which mediates the binding of the

virions to the aphid stylet by acting as a bridge (Leh *et al.,* 1999; Hébrard *et al.,* 2001; Moreno *et al.,* 2005; Plisson *et al.,* 2005; Uzest *et al.,* 2007).

ORF III encodes a 15 kDa protein P3 that frequently undergoes cleavage by a cysteine proteinase to form an 11 kDa virion-associated protein (Guidasci *et al.,* 1992; Dautel *et al.,* 1994). The protein is physically associated to virions, with their N-terminal ectodomains forming an antiparallel α-helical coiled-coil network at the surface and the C-terminal domains interacting with the coat protein P4 and reaching inside the viral particle (Mougeot *et al.,* 1993; Leclerc *et al.,* 2001; Drucker *et al.,* 2002). Although its essential role in aphid transmission and infection has been proved, P3 is dispensable for viral replication in single cells (Daubert *et al.,* 1983; Jacquot *et al.,* 1998; Leh *et al.,* 1999; Kobayashi *et al.,* 2002).

The CaMV capsid protein (CP) is translated from *ORF IV* of spliced pgRNA as a 57 kDa precursor (pre-CP, P4) which is further processed by the virus-encoded protease PR (within P5) into three subspecies: p44, p39 and p37. All three of them lack the C - and N-terminal domains of pre-CP that are shown to inhibit CP interaction with the leader of the viral pgRNA and CP targeting to the nucleus (Torruella *et al.,* 1989; Karsies *et al.,* 2001; Champagne *et al.,* 2004). Interestingly, phosphorylation of the C- and N-termini of the pre-CP by host casein kinase II (CKII) stimulates its cleavage by PR and plays an important role in the infectivity of the virus (Champagne *et al.,* 2007). CaMV CP is involved in virion assembly, packaging of the viral RNA and delivery of the viral dsDNA to the nucleus (Chapdelaine and Hohn, 1998; Leclerc *et al.,* 1999; Guerra-Peraza *et al.,* 2000; Karsies *et al.,* 2002).

The polyprotein P5 (78 kDa) translated from *ORF V* of spliced pgRNA is homologous to the Pol gene product of retroviruses and harbors a reverse transcriptase/RNAse H and an aspartic proteinase/PR (18 kDa) that is released by self-cleavage (Torruella *et al.,* 1989; Haas *et al.,* 2002).

The multifunctional P6 (62 kDa), translated from 19S RNA, was initially identified as the main component of the numerous electron-dense inclusion bodies, also known as virus factories (VFs) (Shockey *et al.,* 1980; Covey and Hull, 1981). These are non-membranous structures of 2-10 μm of diameter depending on the viral cycle stage, CaMV strain and host species, which contain a matrix of P6, 95% of the viral particles and the virion-associated P3 protein (Shalla *et al.,* 1980; Covey *et al.,* 1981; Mazzolini *et al.,* 1985; Plisson *et al.,* 2005). VFs constitute the site of protein synthesis, genome replication and virion assembly during the early stages of the infection cycle and serve as virion reservoir once replication has ceased (Rothnie *et al.,* 1994).

As described above, P6/TAV is also essential for the transactivation of translation of the other viral proteins from polycistronic 35S pgRNA and its spliced variants (Bonneville *et al.,* 1989; Pooggin *et al.,* 2000) Its interaction with ribosomal proteins L13, L18 and L24, the initiation factor eIF3g, the initiator protein RISP and the protein kinase TOR, reprograms host cell ribosomes to initiate polycistronic translation (Leh *et al.,* 2000; Park *et al.,* 2001; Bureau *et al.,* 2004; Park *et al.,* 2004; Thiébeauld *et al.,* 2009; Schepetilnikov *et al.,* 2011). The use of P6 deletion mutants proved

the key role of the central mini-TAV domain in the translational transactivation process (Kobayashi and Hohn, 2003).

The above-mentioned interaction of P6 with P1 as well as association of this protein with microfilaments, microtubules and the ER observed through its fusion to green fluorescent protein (GFP), have suggested the role of this protein in the movement of VFs along the host cytoskeleton (Haas *et al.,* 2005; Hapiak *et al.,* 2008; Harries *et al.,* 2009). Subsequent studies have revealed the interaction of P6 with CHUP1, a protein anchoring chloroplasts to microfilaments, and plasmodesmata-localized proteins PDLP1 and AtSRC2.2, as well as the role of these interactions in viral cell-to-cell movement (Schoelz *et al.,* 2015).

The function of P7 encoded in *ORF VII* remains unknown. The majority of the *ORF VII* can be deleted without noticeable effects on viral infection, although mutations in the initiation codon delay viral symptoms and viruses harboring this mutation revert frequently. The impossibility to detect P7 in virus-infected plants suggested the instability of this protein, which is supported by its P5-mediated cleavage observed *in vitro* (Dixon *et al.,* 1986; Wurch *et al.,* 1990; Guidasci *et al.,* 1992). Intriguingly, P6 interacted with this protein in yeast two-hybrid and maltose-binding protein pull-down assays (Lutz *et al.,* 2012).

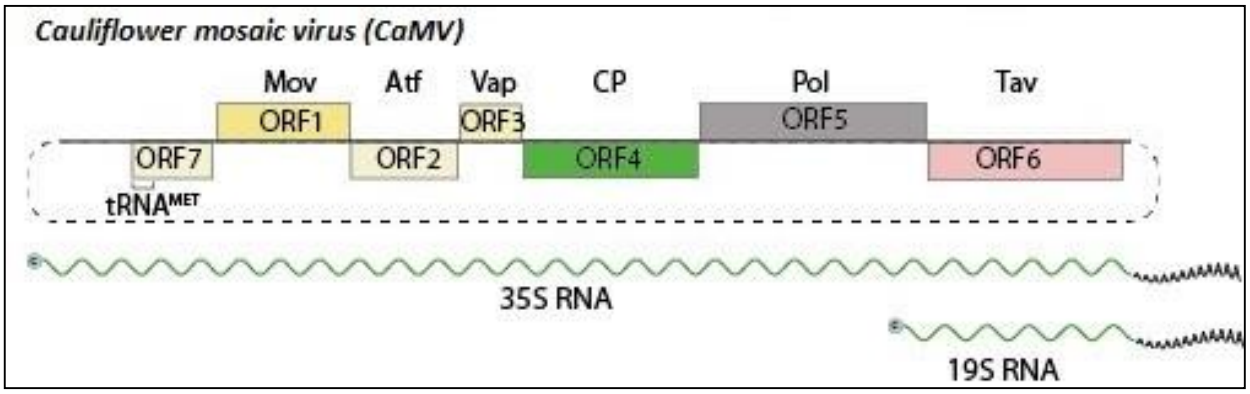


Figure 6. Genome organisation of CaMV (taken from the website http://viralzone.expasy.org)

1.4.6. P6 INTERACTIONS WITH HOST PLANT DEFENCE SYSTEM

The key function of P6 in eliciting plant defenses and infection symptoms in resistant and sensitive hosts respectively was discovered short after the mapping of its sequence on the CaMV genome (Bonneville *et al.,* 1989). P6 is responsible for triggering a non-necrotic defense response in *Arabidopsis thaliana* ecotype Tsu-0, *Nicotiana bigelovii* and *Nicotiana glutinosa*, while inducing hypersensitive response (HR) in *Datura stramonium* and *Nicotiana edwardsonii*. The latter suggests its role as an avirulence (Avr) factor in particular combinations of plant species and virus strains, which was subsequently proved by agroinfiltration assays (Palanichelvam *et al.,* 2000). The use of P6 deletion mutants allowed the mapping of the Avr domain and proved its dispensability in CaMV replication, while having a role in the efficient spread of the virus throughout the plant. In addition,

the regions responsible for the hypersensitive response were mapped at the C- and N-termini and the interaction of this protein with the gene product *ccd1* (*CaMV cell death 1*) was shown to induce systemic cell death symptoms (Palanichelvam and Schoelz, 2002; Kobayashi and Hohn, 2004). Analysis of chimeric CaMV strains W260 and CM1841 localized the resistance-breaking determinant to the region of gene *VI* encoding the 184 N-terminal amino acid residues (Schoelz *et al.,* 1986; Schoelz and Sheperd, 1988; Cole *et al.,* 2001). Additionally, P6 is capable of eliciting chlorosis independently of other viral proteins, as observed in *Arabidopsis* plants expressing the protein from strains CM1841, W260 or Cabb B-JI. However, the symptoms are strain-specific and plants transformed with P6 from strain D4 remained symptomless. In *Nicotiana clevelandii*, P6 from strain W260 induces systemic cell death (Daubert *et al.,* 1984; Cecchini *et al.,* 1997; Király *et al.,* 1999; Palanichelvam *et al.,* 2000; Yu *et al.,* 2003). In addition, this protein induces alterations in the salicylic acid (SA) and jasmonic acid (JA) pathways, as well as in the accumulation of H_2O_2. Transgenic *Arabidopsis* and *Nicotiana benthamiana* plants expressing P6 showed suppressed and enhanced expression of SA- and JA-responsive genes respectively (Geri *et al.,* 2004; Love *et al.,* 2005, 2007a). NPR1, important regulator of the crosstalk between both hormone signal transduction pathways, was upregulated and mislocalized to the nucleus upon expression of P6, which also altered the ethylene and auxin pathways (Smith, 2007; Love *et al.,* 2012)..

The presence of a Leu-rich sequence in the N-terminus α-helix that is required for the self-assembly of P6 allows the targeting of a small fraction of this protein to the nucleus, where it was found to suppress silencing of GFP in *Arabidopsis* transgenic plants (Haas *et al.,* 2005; Love *et al.,* 2007b). By using a combination of cell biology, genetics and biochemistry, the import of monomeric P6 into the nucleus through two importin-α-dependent signals, which is carried out independently of the translational transactivation and viroplasm formation, was demonstrated to be essential for CaMV infectivity. P6 was found to act as a viral silencing suppresor that physically interacts with DRB4, preventing it from activating the antiviral enzyme DCL4, which ultimately processes the RDR6-dependent dsRNA precursors of small interfering RNAs (siRNAs) (Haas *et al.,* 2008; Shivaprasad *et al.,* 2008). The antisilencing activity of P6 does not appear to determine the host range or pathogenicity of CaMV strains, since CM1841 and D4 cause severe and mild disease symptoms respectively in *Arabidopsis* Col-0 plants despite expressing protein versions with similar antisilencing activities (Blevins *et al.,* 2006; Shivaprasad *et al.,* 2008). The use of deletion mutants identified the region responsible for RNA silencing suppresion at the distal end of subdomain D-1b (aa 80-110) (Laird *et al.,* 2013).

1.5. RNA SILENCING IN PLANTS

1.5.1. INTRODUCTION

RNA silencing is a highly conserved sequence-specific RNA degradation system of eukaryotes that links developmental programs, physiological processes and environmental responses to changes in gene expression (Horiguchi, 2004; Brodersen and Voinnet, 2006). In plants, it is involved in regulation of growth, development, genome stability, abiotic stress responses as well as defense against viral and non-viral pathogens (Sunkar *et al.,* 2012; Pumplin and Voinnet, 2013).

RNA silencing was first observed in transgenic tobacco plants transformed with CP gene of TMV that showed delayed and less severe viral disease symptoms upon infection with TMV (Powell *et al.,* 1986). However, the mechanism of so-called 'co-suppression' was described later when introduction of extra copies of the flower pigmentation chalcone synthase (*CHS*) gene into petunia plants resulted in suppression of the transgene and the endogenous *CHS* RNA producing flowers with paler colors compared to wild type plants (Napoli *et al.,* 1990; Jorgensen, 1990). The real breakthrough in this field came with the discovery that injection of double-stranded RNA (dsRNA) in *Caenorhabditis elegans* resulted in degradation of cognate endogenous mRNA (Fire *et al.,* 1998) and continued with the uncovering of small RNA species associated with transgene silencing and virus infection in plants (Hamilton and Baulcombe, 1999), which consider now as a hallmark of RNA silencing.

The mechanism of RNA silencing is based on the regulatory activity of 21-24 nucleotide (nt) small RNAs that are broadly classified into microRNAs (miRNAs) and small interfering RNAs (siRNAs), being proccessed from hairpin-like stuctures and dsRNA precursors, respectively. Small RNAs act as guides for inactivation of homologous sequences by promoting mRNA cleavage/degradation or translational repression, DNA/chromatin modifications and transcrptional gene silencing (Brodersen and Voinnet, 2006). The production of siRNAs depends on the activity of two key enzymes: Dicer-like (DCL) nucleases, which belong to the RNase III family of dsRNA-specific endoribonucleases that process long dsRNA precursors into 21-24-nt primary siRNAs, and RNA dependent RNA polymerases (RDR), that convert single-stranded (ss)RNA into dsRNA precursors of secondary siRNAs. There are four distinct DCLs and six RDRs (Wassenegger and Krczal, 2006) encoded by *Arabidopsis thaliana* genome. DCL1 plays a major role in processing of 21-22-nt miRNAs from hairpin-like precursors of MIR gene transcripts, while both DCL4 and DCL2 are responsible for production of endogenous, viral and transgene-derived siRNAs, generating 21-nt and 22-nt siRNAs, respectively. In addition, DCL3-dependent 24-nt siRNAs, mostly derived from repetitive DNA loci (repeat-associated siRNAs; ra-siRNA), likely control genome stability through RNA-dependent DNA methylation and histone modification (Brodersen and Voinnet, 2006).

Another key component of the RNA silencing machinery is an Argonaute (AGO) protein, which preferentially binds sRNAs to form an RNA-induced silencing complex (RISC). RISC mediates

sequence-specific cleavage of complementary mRNA or its translation inhibition, resulting in post-transcriptional gene silencing (PTGS), and/or cytosine DNA methylation, resulting transcriptional gene silencing (TGS). In *Arabidopsis*, there are ten distinct AGO protein-coding genes, which can be classified into three major phylogenetic clades, comprised by AGO1/AGO5/AGO10; AGO2/AGO3/AGO7 and AGO4/ AGO6/AGO8/AGO9 (Morel *et al.,* 2002; Mallory and Vaucheret, 2010). Different AGO proteins sort sRNAs mostly based on their size and 5'-nucleotide identity. For instance, AGO4, -6 and -9 preferentially bind 24-nt sRNAs, while AGO1, -2, -5, -7 and -10 associate with 21-22-nt sRNAs. AGO1 and -5 bind 21-22-nt sRNAs with 5'-uridine or cytosine, respectively, whereas AGO2, -4, -6 and -9 associate with 5'-adenosine sRNAs (Mi *et al.,* 2008). Although, slicing activity has been demonstrated only for AGO1, AGO2, AGO4, AGO7 and AGO10, most of the AGO proteins are involved either in TGS or PTGS (Martinez de Alba *et al.,* 2013). Among others, AGO1 is the founding member of AGO proteins and plays an essential role in miRNA-mediated regulation, trans-acting siRNA (tasiRNAs) production and antiviral silencing (Mi *et al.,* 2008; Rogers and Chen, 2013; Carbonell and Carrington, 2015). Interestingly, AGO10, showing the closest homology to AGO1, promotes establishment of shoot apical meristem by sequestering members of the miR165/166 family and preventing their loading onto AGO1 (Zhu *et al.,* 2011). The third member of the AGO1/AGO5/AGO10 clade, AGO5 is expressed in and around megaspores during the transition to megagametogenesis and in the sperm cell cytoplasm of mature pollen (Tucker *et al.,* 2012). The member of another clade, AGO2, displays some additive and redundant functions with AGO1, such as the miR408-mediated plantacyanin mRNA regulation, siRNA-mediated silencing of intergenic regions, pseudogenes and transposons, while the role in DNA repair and antiviral defense has also been demonstrated for this protein (Maunoury and Vaucheret, 2011; Wei *et al.,* 2012). Although AGO3 is very similar to AGO2 in sequence and only 3 kb apart in a direct tandem repeat, no biological role has been reported for it so far (Bologna and Voinnet, 2014). AGO7 binds almost exclusively to miR390 and triggers biogenesis of AUXIN-RESPONSE FACTOR 3 (ARF3) and ARF4-regulator tasiRNAs, ensuring juvenile-to-adult transition and adaxial-abaxial patterning (Axtell *et al.,* 2006; Montgomery *et al.,* 2008). The members of AGO4/AGO6/AGO8/AGO9 clade preferentially bind 24-nt siRNAs and function in nuclear RNA-directed DNA methylation (RdDM) and TGS pathways (Qi *et al.,* 2006; Olmedo-Monfil *et al.,* 2010), except AGO8 that has been proposed to be a pseudogene (Vaucheret, 2008; Mallory and Vaucheret, 2010).

1.5.2. RNA SILENCING PATHWAYS

RNA silencing machinery consists of several mechanistically related pathways, which regulate gene expression either transcriptionally (TGS) through cytosine methylation and histone modifications or post-transcriptionally (PTGS) by cleavage or translation repression of mRNA targets (Fig. 7)(Hohn and Vazquez, 2011; Parent *et al.,* 2012; Ghoshal and Sanfaçon, 2015).

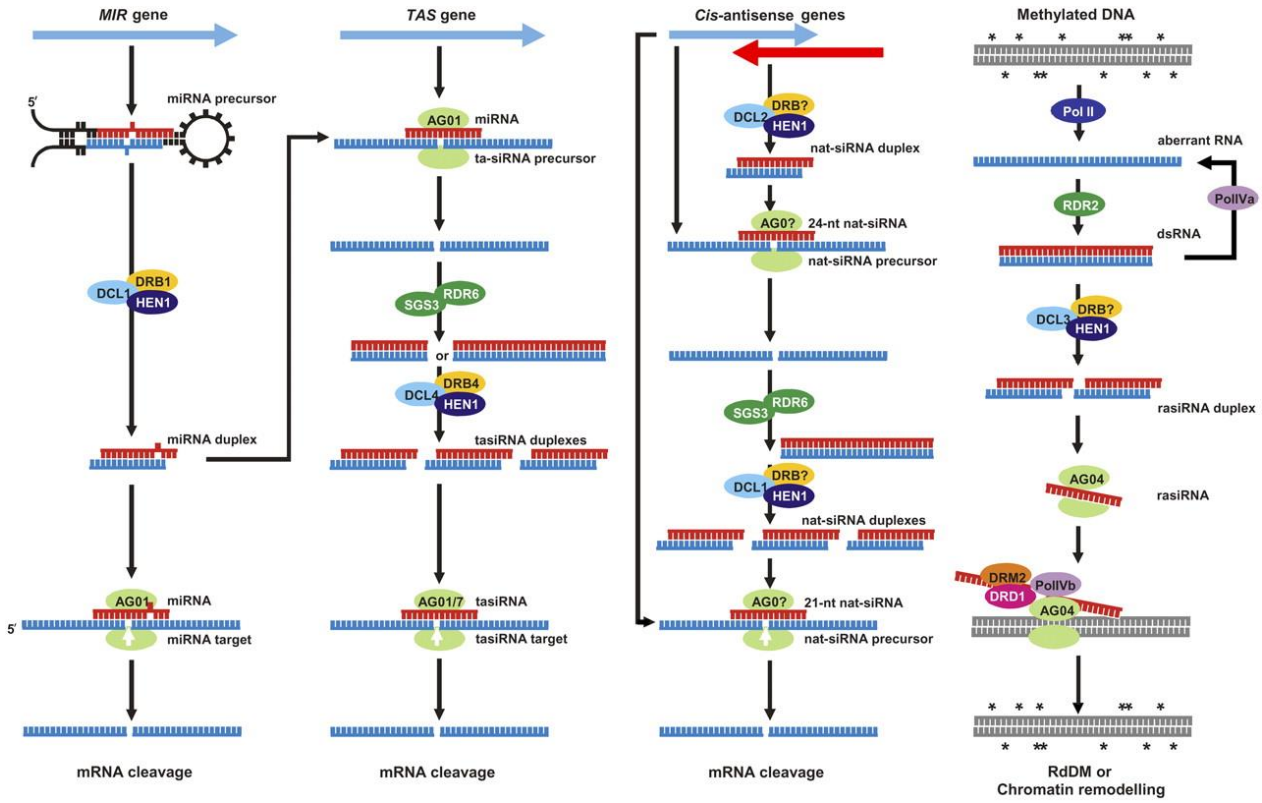


Figure 7. RNA silencing pathways (Eamens *et al.,* 2008)

1.5.2.1 POST-TRANSCRIPTIONAL GENE SILENCING

1.5.2.1.1. MIRNA PATHWAY

miRNAs are small, endogenous RNAs that are derived from long single-stranded transcripts with a distinctive RNA stem-loop secondary structure and function as in trans regulators of gene expression in plants and animals. In plants, these are 21-24-nt sRNAs processed from the primary transcripts, termed pri-miRNAs, which are the products of Pol II-mediated transcription of MIR genes. Initially, the newly synthesized pri-miRNA undergoes the DCL-dependent cleavage that occurs near the base of its hairpin-like tructrure and excises the stem-loop structures contained precursor miRNA (pre-miRNA), which are subsequently processed into the mature miRNAs. miRNA processing is a result of the reaction that in *Arabidopsis thaliana* plants occurs in the nucleus and includes two cleavage events operated by DCL1 in association with dsRNA-binding proteins HYPONASTIC LEAVES 1 (HYL1), TOUGH (TGH) and SERRATE (SE). The first cleavage site constitutes a key determinant of the miRNA specificity that defines its sequence, while the second one is usually at a fixed distance of 21-nt and releases short dsRNAs consisting of mature miRNA guide and passenger (miRNA*) strands with 2-nucleotide 3' overhangs (Jones-Rhoades *et al.,* 2006). In the case of non-conserved miRNAs derived from young MIR genes, the DCL4-DOUBLE-STRANDED RNA BINDING PROTEN 4 (DRB4) pair substitutes DCL1-HYL1 for the pri-miRNA processing (Jones-Rhoades *et al.,* 2006). Upon its release, the miRNA/miRNA* duplexes are 2'-O-methylated by HEN1 that most likely protects them from degradation and exported from the

nucleus into the cytoplasm by the exportin-5 homolog HASTY (HST). In the cytoplasm, miRNAs are loaded onto AGO1 to guide their mRNA transcript cleavage, resulting in target gene repression (Jones-Rhoades *et al.,* 2006). Although most miRNAs are loaded onto AGO1, specific associations with AGO2 (miR408, miR393*), AGO7 (miR390) and AGO10 (miR165/miR166) have been also reported (Mi *et al.,* 2008; Montgomery *et al.,* 2008; Zhu *et al.,* 2011).

1.5.2.1.2. siRNA PATHWAYS

In siRNA pathways, aberrant RNAs derived from transgenes, viruses or some endogenous genes are converted into dsRNA structures by RDR6 with the involvement of SUPPRESSOR OF GENE SILENCING 3 (SGS3), SILENCING DEFECTIVE 3 (SDE3) and KU70 (Brodersen and Voinnet, 2006). These dsRNAs are processed into siRNA duplexes by DLC4 and/or DCL2. These duplexes get methylated at the 2'-hydroxyl group of their 3'-terminal nucleotides by the methyltransferase HEN1 in order to avoid degradation and the siRNAs guide strands are loaded onto AGO-containing RISC complexes, where they bind to their target mRNA through base-pair complementarity and direct AGO-mediated cleavage. Once the target RNA is cleaved, the 5' and 3' products are degraded by the exosome and specific nucleases. The cytoplasmic AtXRN4 displays 5' to 3' exonucleolytic activity and presumably competes with RDR for substrates, while XRN2 and XRN3 are involved in processing of 3'-end cleaved RNAs in the nucleus. Mutant *xrn2* plants overaccumulate miRNA precursors, while loss of *xrn3* function causes embryo lethality. The nucleotidase/phosphatase FIERY 1 (FRY1) suppresses endogenous PTGS by co-repressing these three nucleases. In the *fry1* mutant, *xrn2*, *-3* and *-4* mutations are recapitulated and resistance against CMV is enhanced, probably through an increment of RDR substrate for siRNA signal amplification (van Hoof and Parker, 1999; Gazzani *et al.,* 2004; Souret *et al.,* 2004; Gy *et al.,* 2007).

Based on the origins, biogenesis pathways and functions of the siRNA species, they can be subdivided into four groups: direct or inverted repeat associated or heterochromatic siRNAs, natural antisense siRNAs (nat-siRNAs) and trans-acting siRNAs (ta-siRNAs)(Fig. 7) (Bologna and Voinnet, 2014).

1.5.2.1.3. NAT-SIRNA PATHWAY

NAT-siRNAs are formed by the annealing of two complementary RNA strands of natural antisense transcripts (NAT) into dsRNAs, which could be subsequently processed into cis- or trans-nat-siRNAs, respectively, depending on whether both strands arise from the same genomic locus or not. The biogenesis pathways observed so far differ from one case to another depending on the DCL protein involved, while they all require the action of Pol IV, RDR6 and SGS3 for the dsRNA generation and methylation of the mature siRNAs by HEN1. For example, while DCL2 produces 24-

nt siRNAs involved in tolerance to salt stress, DCL1-generated 22-nt siRNAs and 39-41-nt long-siRNAs formed by both DCL1 and DCL4 are important for resistance against bacterial pathogens (Borsani *et al.,* 2005; Jen *et al.,* 2005; Katiyar-Agarwal *et al.,* 2006, 2007; Zhang *et al.,* 2013b).

1.5.2.1.4. TA-SIRNA PATHWAY

The genome of *Arabidopsis* encodes eight trans-acting (*TAS*) loci: *TAS1a-c*, *TAS2*, *TAS3a-c* and *TAS4* transcribed by Pol II as long non-coding TAS primary transcripts (pri-TAS) with a 5' cap and a 3' poly(A) tail, which are subsequently processed by DCL4 to release mature ta-siRNAs. Initially, pri-TAS is cleaved by particular miRNAs at one or two specific target sites depending on the TAS family. While the primary transcripts derived from TAS1/TAS2 and TAS4 families are recognized at one target site by 22-nt miR173 and miR828, respectively, which direct their AGO1-mediated cleavage, TAS3 transcripts contain two target sites (3'-cleavable and 5'-noncleavable) recognized by miR390, which must be loaded onto AGO7. After cleavage, the products derived from 5'-end of TAS3 and 3'-end of TAS1/TAS2/TAS4 transcripts are converted into dsRNAs by the action of RDR6 and SGS3 (Suppressor of gene silencing 3) with the following processing by DCL4 in collaboration with its interacting partner DRB4 into 21-nt tasiRNAs which regulate gene expression by guiding cleavage of their target RNA. According to the recent data, tasiRNAs generated from TAS1 and TAS2 loci mainly regulate the expression of pentatricopeptide mRNAs, while those from TAS3 and TAS4 control mRNAs encoded auxin-response and MYB transcription factors, respectively (Hohn and Vazquez, 2011; Allen *et al.,* 2005; Yoshikawa *et al.,* 2005; Rajeswaran and Pooggin 2012b; Rajeswaran et al. 2012). Interestingly, tasiRNAs derived from the TAC1c gene were found to be master regulators of tasiRNA biogenesis of TAS1a-c and TAS2 genes (Rajeswaran et al. 2012).

1.5.2.2. TRANSCRIPTIONAL GENE SILENCING

TGS refers to the stable repression of homologous DNA transcription that occurs in the nucleus through sequence-specific RNA-directed DNA methylation (RdDM) or chromatin modification. The key component of TGS is Pol IV, which transcribes heterochromatic regions and DNA repeats with the assistance of SNF2-domain-containing CLASSY1 (CLSY1) and SAWADEE HOMEODOMAIN HOMOLOG 1 (SHH1) to produce single-stranded RNAs (ssRNAs), which are converted into dsRNAs by RDR2 in partnership with INVOLVED IN DE NOVO 2 (IDN2)/RNA-DIRECTED DNA METHYLATION 12 (RDM12) and subsequently cleaved by DCL3 into 24-nt siRNA duplexes. Methylation of the 2'-OH group by methyltransferase HEN1 is followed by degradation of the siRNA passenger strand and loading of the guide strand onto AGO4, AGO6 or AGO9 depending on the loci and tissues. Pol V forms then a scaffold transcript that is recognized by the AGO protein

bound to the sRNA through a link made by KOW DOMAIN-CONTAINING TRANSCRIPTION FACTOR 1 (KTF1). The resulting complex recruits DOMAIN REARRANGED METHYLASE (DRM)1 and -2, which trigger the RdDM reaction with the help of the donor of the methyl group S-adenosyl-L-methionine (SAM), and other methylases such as histone H3K9 methylase (KYP), chromomethylase (CMT3) and chromoproteins (Li *et al.,* 2006; Zilberman *et al.,* 2006; Zheng *et al.,* 2007; Wierzbicki *et al.,* 2008; Matzcke *et al.,* 2009; Blevins et al. 2015; review from Pikaard or Jacobsen groups). Following de novo DNA methylation via RdDM, methyltransferases DNA METHYLTRANSFERASE 1 (MET1), CHROMOMETHYLTRANSFRASE (CMT)3 and -2 are required for the maintenance of the CG and CHG methylation pattern, respectively, while DECREASED DNA METHYLATION 1 (DDM1) facilitates DNA methyltransferase's access to heterochromatin (Kankel *et al.,* 2003; Zemach *et al.,* 2013). The actions of the methylases are counter-balanced by demethylating DNA glycosylases DEMETER (DME), DEMETER-LIKE 2 (DML2) and -3, and REPRESSOR OF SILENCING 1 (ROS1) or ROS4/INCREASED DNA METHYLATION 1 (IDM1) (Penterman *et al.,* 2007; Ortega-Galisteo *et al.,* 2008; Zheng *et al.,* 2008; Qian *et al.,* 2012). In an independent way, Pol V is assisted by the putative chromatin-interacting ATPase DEFECTIVE IN RNA-DIRECTED DNA METHYLATION (DRD1), the hinge-domain protein DEFECTIVE IN MERISTEM SILENCING 3 (DMS3) and the ssDNA-binding protein RNA-DIRECTED DNA METHYLASE 1 (RDM1) in generating transcripts at RdDM target loci. AGO4 can be bound to Pol V transcripts in an interaction that is believed to serve as scaffold. The AGO4-siRNA complex has also been suggested to be stabilized through the interaction of AGO4 with the C-terminal domain of the largest Pol V subunit, using RDM1 as a bridge connecting it to DRM2 (Law *et al.,* 2010; Law and Jacobsen, 2010; Wierzbicki *et al.,* 2012; Zhong *et al.,* 2012). Additionally, Pol II recruits the former two polymerases at intergenic, low copy number loci and facilitates the amplification of Pol IV-dependent siRNAs at the DNA target site, contributing thus to RdDM. The activity of the three polymerases is regulated by the conserved transcription factor INTERACT WITH RNA POL II (IWR1)/RDM4/DMS4 and MORPHEUS' MOLECULE 1 (MOM1) (Zheng *et al.,* 2009; Kanno *et al.,* 2010; Yokthongwattana *et al.,* 2010; You *et al.,* 2013). Transcriptionally silent heterochromatin can also be generated through methylation of histone H3 lysine 9 and histone H3 lysine 27 residues with the help of HISTONE DEACETYLASE 6 (HDA6), histone methyltransferses SU_(VAR) 3-9 HOMOLOG (SUVH)2, -4, -5 and -6, chromatin remodeling factors DDM1 and DRD1, and the UBIQUITIN PROTEASE 26 (UBP26) (Aufsatz *et al.,* 2002; Jackson *et al.,* 2002; Ebbs *et al.,* 2005; Ebbs and Bender, 2006; Johnson *et al.,* 2007; Sridhar *et al.,* 2007).

1.5.3. RNA SILENCING IN RICE

While *Arabidopsis* genome encodes four DCL (AtDCL) proteins, 8 DCL genes have been identified in rice (Kapoor *et al.,* 2008). Like in *Arabidopsis*, rice DCLs (OsDCLs) can be divided into four clades, DCL1-4. The first clade comprises OsDCL1a, -b and –c, which are highly related to

AtDCL1. Like AtDCL1, loss of function of OsDCL1a resulted in reduced miRNA accumulation and pleiotropic developmental defects, therefore both proteins are considered to be orthologues. Its expression is downregulated upon infection with *Magnaporthe oryzae* (*M. oryzae*), thereby perturbing miRNA biogenesis and activating the constitutive expression of defense genes (Liu *et al.,* 2005; Zhang *et al.,* 2015). The osDCL2 clade members OsDCL2a and -b, closely related to AtDCL2, have been barely studied and only known to be highly expressed in the egg cell, together with OsDCL4 and HEN1 (Takanashi *et al.,* 2012). OsDCL3 clade members OsDCL3a and -b shows the highest similarity with AtDCL3. OsDCL3b is required for the processing of 24-nt phased small RNAs, while OsDCL3a processes the miniature inverted-repeat transposable element (MITE)-derived precursors of repeat-associated small interfering RNAs (ra-siRNAs) (Yan *et al.,* 2011; Song *et al.,* 2012). The only member of the rice DCL4 clade, OsDCL4/SHO1, is responsible for the processing of 21-nt siRNAs, including those associated with inverted repeats, tasiRNAs and over 1000 phased small RNA loci (Liu *et al.,* 2007; Song *et al.,* 2012). In contrast to *Arabidopsis*, the genome of rice encodes five RDRs, termed OsRDR1, OsRDR2, OsRDR3a, OsRDR3b and OsRDR6 (Kapoor *et al.,* 2008). By characterizing the loss of function *rdr1* mutant, Wang and collaborators (2014) revealed the participation of this polymerase in regulating numerous endogenous genes through small RNA-mediated pathways involving DNA methylation. The role of OsRDR2 in the processing of OsDCL3-depending nat-siRNAs has been demonstrated through sRNA deep sequencing experiments (Yan *et al.,* 2011; Zhang *et al.,* 2012). OsRDR6, also referred to as SHOOTLESS 2 (SHL2), is required for the correct embryo development and its expression is regulated by the ABA signaling pathway. Deep sequencing data showed that the rice *rdr6* mutant accumulated reduced levels of virus-derived siRNAs, indicating the role of OsRDR6 in antiviral defense (Yang *et al.,* 2008; Jiang *et al.,* 2012).

As mentioned above, the genome of *Arabidopsis* encodes 10 AGO proteins, which can be classified into three major clades (Morel *et al.,* 2002; Mallory and Vaucheret, 2010). In rice, 19 members of this family have been identified and phylogenetically clustered into four clades: MEL1, AGO1, AGO4 and AGO7 (Kapoor *et al.,* 2008). The MEL1 clade shows the highest homology with AtAGO5 and includes OsMEL1 and OsAGO11-14. Proteins in this clade show 25-60% overall identity among each other, with their PIWI domains sharing 75-94% sequence similarity. Among them, only MEL1 (MEIOSIS ARRESTED AT LEPTOTENE 1) has been characterized so far. The protein is specifically expressed in germ cells and regulates the cell division of premeiotic germ cells, the modification of meiotic chromosomes, the progression of meiosis and the epigenetic large-scale meiotic chromosome reprogramming by binding to 21-nt phased siRNAs (phasiRNAs) generated from over 700 large intergenic non-coding RNAs (lincRNAs) through miR2118- and DCL4-dependent pathways. The AGO1 subgroup includes PINHEAD 1 (PNH1), sharing high similarity with AtAGO10, and the four homologues to AtAGO1, OsAGO1a-d (Kapoor *et al.,* 2008). Based on its expression pattern in developing tissues of leaf primordia and the malformed leaves observed in antisense-mediated knockdown plants, OsPNH1 was proposed to function in both

shoot apical meristem maintenance and leaf formation through vascular development (Nishimura *et al.,* 2002). By employing RNAi lines for all the other members of this subgroup and purifying the complexes formed by OsAGO1a, -b and -c, Wu and collaborators (2009) determined their slicer activity and preference for small RNAs with 5' U. Co-expression relationships were observed between OsAGO1b, OsAGO1c, OsAGO1d, OsAGO4a, OsAGO4b, OsAGO7, OsAGO16, OsAGO17 and MEL1, PNH1 and MADS5, and OsAGO1a, OsAGO2 and MADS15, implying the involvement of these genes in flower development (Yang *et al.,* 2013). In the AGO4 clade, OsAGO4a and –b are highly homologous to AtAGO4, while OsAGO16 is more closely related to AtAGO6. Their role in repeat silencing, as seen for their *Arabidopsis* counterparts, remains to be tested (Kapoor *et al.,* 2008). The OsAGO7 clade contains three members: OsAGO2, OsAGO3 and OsSHL4. The latter has been proven to participate in the tasiRNA pathway, together with OsDCL4/SHO1 (Nagasaki *et al.,* 2007). Together with that of OsAGO1d, OsRDR1 and OsRDR6, the expression of OsAGO2 has recently been found to be upregulated upon infection with *Southern rice black-streaked dwarf virus* (SRBSDV) (Xu and Zhou, 2017). Although the biological function of OsAGO3 remains unknown, its expression is known to be panicle-specific (Sharma *et al.,* 2012b). Finally, the two remaining AGO proteins, OsAGO17 and OsAGO18, cannot be clustered into any of these clades. Through a comparative microarray analysis, OsAGO17, in addition to OsAGO12 and -13, was found to be male gametophyte-specific (Peng *et al.,* 2012). Upon infection with the two taxonomically different RNA viruses *Rice stripe Tenuivirus* (RSV) and *Rice dwarf Phytoreovirus* (RDV), the expression of OsAGO18 was upregulated. Because it competes with OsAGO1 for binding miR168, OsAGO18 alleviates OsAGO1 repression by this miRNA, thereby promoting antiviral defense (Wu *et al.,* 2015).

The process of TGS has not been as extensively studied in rice as in *Arabidopsis*. Although cytosine methylation in CpG, CpHpG and CpHpH contexts has been demonstrated at rice endogenous genes through targeting of siRNAs to their promoters, TGS was not always observed. The analysis of the epigenetic modifications revealed that the gene-specific effects of siRNA tend to induce higher de novo methylation of CpG dinucleotides than of other cytosines (Okano *et al.,* 2008; Miki and Shimamoto, 2008). The genome of rice encodes 10 proteins with methyltransferase activity. The employment of homologous recombination-mediated knock-in targeting led to the identification of two methyltransferases involved in TGS in rice: OsMET1 and OsDRM2 (Sharma *et al.,* 2009; Moritoh *et al.,* 2012). While *Arabidopsis* carries only one copy of the MET1 gene, two alternative splicing forms, OsMET1a/OsMET1-1 and OsMET1b/OsMET1-2 have been found in rice, being the latter more abundantly accumulated. Long micro RNAs (lmiRNAs) are 24-nt miRNAs identified in both rice and *Arabidopsis*. RNAi approaches demonstrated that OsDCL3a is responsible for their production in rice, which is followed by their loading onto OsAGO4. Interestingly, lmiRNAs were proven to direct DNA methylation at both the loci from which they were produced and target genes in order to regulate gene expression (Zhu *et al.,* 2008; Wu *et al.,* 2009, 2010).

siRNAs were first identified in rice through the generation of small RNA cDNA libraries. Most of them originated from intergenic regions, while 22% derived from gene introns and exons. Based on sequence homology, a total of 25 transposons and 21 protein-coding genes were predicted to be cis-targeted by siRNAs. Alternatively, 111 putative trans-targets were predicted for 44 of the siRNAs. Further studies employing sRNA deep sequencing approaches allowed for the identification of novel miRNAs and miRNA candidates (Sunkar *et al.,* 2005, 2008; Barrera-Figueroa *et al.,* 2012). Small RNA sequencing combined with degradome sequencing analysis led to the identification of miRNAs regulated by treatment with *M. oryzae* and its elicitors in rice. Among them, miR162 and miR168 target OsDCL1 and OsAGO1 clade members, respectively, pointing to a pathogen-regulation of the miRNA machinery (Campo *et al.,* 2013; Baldrich *et al.,* 2015). Interestingly, the perturbed miRNA biogenesis caused by silencing of OsDCL1 activated basal defenses against this pathogen (Zhang *et al.,* 2015). Overexpression of miR160a and miR398b caused transgenic rice plants to increase H_2O_2 production and upregulate the expression of defensive genes, resulting in an enhanced resistance to *M. oryzae* (Li *et al.,* 2013). For example, miR444 has recently been discovered to target the OsRDR1-repressing MIKCC-type MADS-box genes OsMADS23, OsMAS27a and OsMADS57 in order to allow the expression of this polymerase, thereby conferring resistance to RSV (Wang *et al.,* 2016). Components of the miRNA biogenesis pathway are additional key players of viral resistance. Infection with RVS upregulates OsDCL2 and downregulates OsDCL3a and -b. Silencing of OsRDR6 enhances susceptibility to this virus in rice plants. Knock-down lines for OsDCL2 and OsRDR4 showed instability of the *Oryza sativa endornavirus* (OsEV)-derived double-stranded RNA (dsRNA). As described above, OsAGO18 confers broad spectrum virus resistance in rice by sequestering miR168 and preventing its negative regulation of OsAGO1 (Urayama *et al.,* 2010; Du *et al.,* 2011; Jiang *et al.,* 2012; Wu *et al.,* 2015).

1.5.4. ANTIVIRAL RNA SILENCING IN ARABIDOPSIS

The majority of known plant viruses have RNA genomes and replicate through dsRNA intermediates. The recognition of these and other types of viral RNAs such as imperfect hairpins of ssRNA or overlapping sense/antisense transcripts from ssDNA viruses by DCL proteins triggers the RNA silencing response (Ruiz-Ferrer and Voinnet, 2009). All four *Arabidopsis* DCL proteins show a coordinated and hierarchical action in viral siRNA (vsiRNA) biogenesis (Fig. 8)(Deleris et al. 2006; Blevins et al. 2006). In wild type plants, DCL4-generated 21-nt vsiRNAs are primarily accumulated upon infection with RNA viruses, as seen in the cases of *Turnip crinkle virus* (TCV) or *Cucumber mosaic virus* (CMV), while DCL2-produced 22-nt vsiRNAs are barely detectable in the presence of a functional DCL4 and only play an important role in *dcl4* mutants. In addition, DCL3 can produce 24-nt vsiRNAs in *dcl2 dcl4* double mutants, but they are insufficient to trigger the antiviral response. However, DCL3 plays an important role upon infection with DNA viruses (Akbergenov 2006;

Blevins *et al.,* 2006). Although, DCL1 only plays indirect role as negative regulator of vsiRNA biogenesis, it contributes to the synthesis of 21-nt siRNAs from DNA viruses (Blevins et al. 2006; Blevins et al. 2011; Aregger et al. 2012). Simultaneously, the expression of DCL4 and DCL3 is upregulated in *dcl1*, resulting in a reduced accumulation of viruses. A possible explanation for this phenomenon is the induction of these genes by transcription factors that are negatively regulated by miRNAs produced by DCL1. Likewise, expression of both AGO1 and AGO2, involved in plant antiviral response in *Arabidopsis*, is downregulated by DCL1-generated miRNAs, miR168 and miR403, respectively (Vaucheret *et al.,* 2004; Allen *et al.,* 2005)

DCL-generated vsiRNA duplexes are then sorted by AGOs to create AGO-RISC complexes targeting viral transcripts. In *Arabidopsis*, AGO1 and AGO2 are the major AGOs involved in the host plant response against RNA viruses (Fig. 8)(Carbonell and Carrington, 2015). Several pull-down experiments have shown association of *Arabidopsis* AGO1 with vsiRNAs derived from TCV and *Turnip yellow mosaic virus* (TYMV), while AGO2 was confirmed to be essential for antiviral resistance to *Turnip mosaic virus* (TuMV) and PVX (Qu *et al.,* 2008; Garcia-Ruiz *et al.,* 2015; Carbonell and Carrington, 2015). Accordingly, *ago1* and *ago2* mutants exhibit hypersusceptibility to wild type CMV infection (Morel *et al.,* 2002). Although, it was initially reported that AGO2 could function only as a second defense layer against viruses that suppress AGO1 (e.g. CMV or TCV), more recent observations have confirmed the major role of AGO2 in antiviral response against viruses that are not known to target AGO1 such as *Tobacco rattle virus* (TRV) and *Turnip mosaic virus* (TuMV) (Garcia-Ruiz *et al.,* 2015). Alternatively, antiviral activity has also been assigned to AGO4, AGO5 and AGO7. While AGO5 was shown to be associated with CMV-derived vsiRNA in *Arabidopsis*, AGO7 has been proposed as a surrogate slicer in the absence of a functional AGO1 protein (Mi *et al.,* 2008; Qu *et al.,* 2008; Takeda *et al.,* 2008; Duan *et al.,* 2012). Additionally, despite little is known on involvement of AGOs in defense against DNA virises, AGO4, functioning in TGS, was shown to play an antiviral role against both RNA (Hamera *et al.,* 2012; Bhattacharjee *et al.,* 2009) and DNA viruses in *Arabidopsis* (Raja *et al.,* 2008). In the case of DNA pararetrovirus CaMV, AGO1 was shown to bind 21-nt vsiRNAs, while AGO4 failed to do so despite massive production of 24-nt siRNAs from the CaMV leader region by all four DCLs (Blevins et al. 2011)

In plants, virus-derived siRNAs can act as signals to induce a systemic response in order to restrict viral infection to certain types of cells or tissues. These signals consist of secondary siRNAs, which, as opposed to the primary siRNAs, are generated by DCL-mediated processing of RDR-dependent dsRNA precursors (Fig. 8). As it has been shown for the biogenesis of endogenous plant secondary siRNAs, the mechanism that involves the conglomerate action of RDR6, SGS3, and DCL4 is required for the production of virus-derived secondary siRNAs (Voinnet, 2005; Ding and Voinnet, 2007). The other RDRs seem to be dispensable for this process, however, tomato *Ty-1* and *Ty-3* alleles that encode *Arabidopsis* RDR3 orthologs confer a resistance against DNA geminivirus TYLCV, while *Arabidopsis rdr1* mutants were shown to be defective in vsiRNA production in

response to TuMV (Verlaan, 2013; García-Ruiz *et al.,* 2010). Accordingly, both RDR1 and RDR6 were found to be required for amplification of CMV-derived sRNAs (Wang *et al.,* 2010).

The recovery phenotype observed in new leaves of virus infected plants is a sign of movement of the viral silencing signals beyond their sites of synthesis. At early stages of the infection when virus titers are high, the plant shows severe symptoms in both infected and systemically invaded tissues. However, upon recovery, symptoms on new leaves are attenuated, virus accumulation in upper non-inoculated leaves is reduced and tissues become resistant to re-inoculation. Similarly, movement-defective PVX containing fragments of the gene coding for the small subunit of RUBISCO induced a systemic silencing of the host gene despite being confined to a single leaf (Ratcliff *et al.,* 1997, 1999; Himber *et al.,* 2003). There are two types of RNA silencing signal movement in plants (Himber *et al.,* 2003; Voinnet, 2005). Short-range movement, that is dependent on DCL4, but RDR-independent, occurs via plasmodesmata (PD), spreading within the first 10–15 cells from the initiating silencing cells, while long range movement requires RDR activity and occurs via plant vasculature system. Despite specific RNA-binding proteins have been suggested to function in cell-to-cell movement of viral silencing signals, the RNA species and underlying mechanism remain to be investigated, as does the question of whether plasmodesmata are the sole channels for this movement (Mlotshwa *et al.,* 2002; Dunoyer *et al.,* 2009). In grafting experiments, the long-distance signal was successfully transmitted from rootstocks to scions, supporting the idea of sRNAs as part of the RNA silencing signal, which accordingly were identified in the phloem sap of *Cucumber yellows closterovirus* (CuYV)-infected pumpkin (Yoo *et al.,* 2004; Molnar *et al.,* 2011).

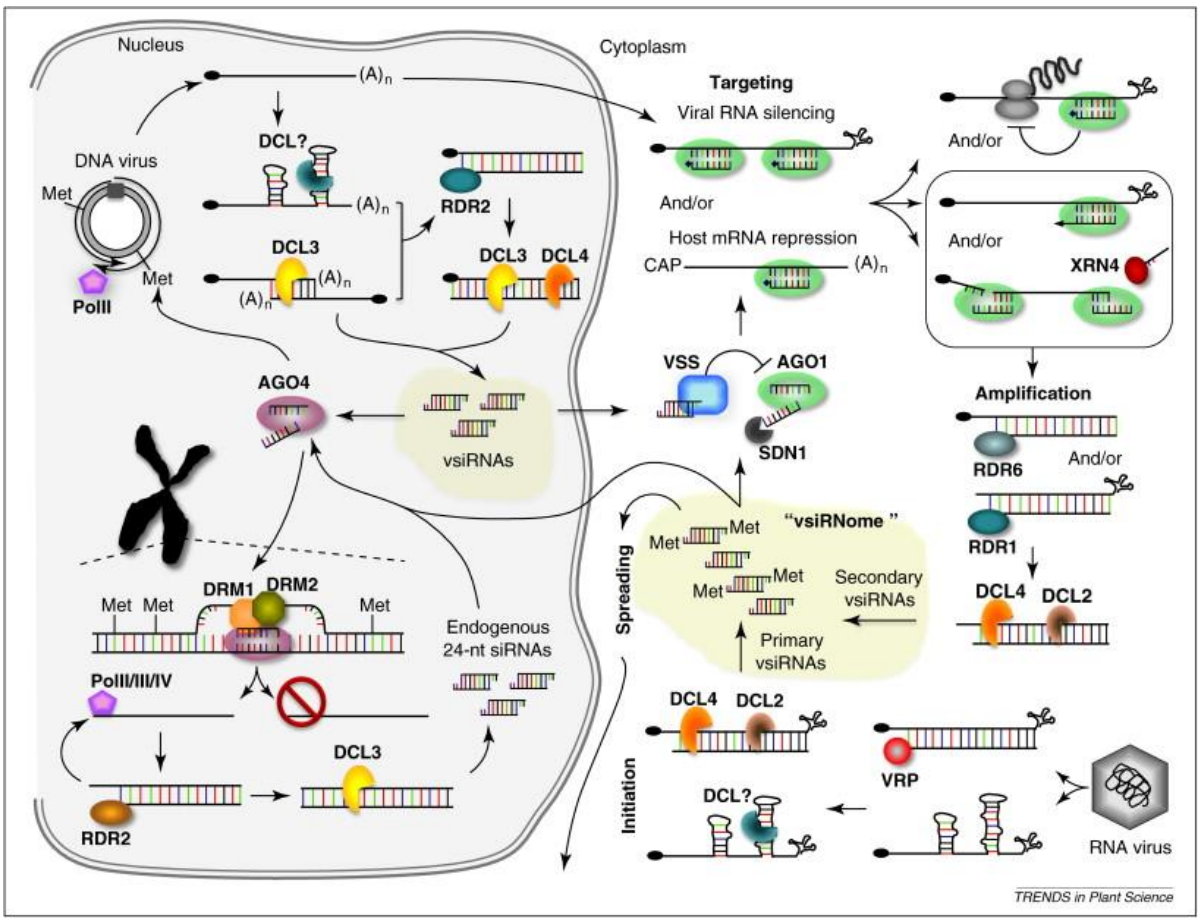


Figure 8. Antiviral RNA silencing (taken from Llave 2010)

1.5.5. VIRAL SUPPRESSORS OF RNA SILENCING

As a counter-defensive mechanism against RNA silencing, viruses have evolved a diverse range of suppressor proteins targeting different components of the antiviral silencing pathways, which are responsible for viral RNA recognition, dicing, RISC assembly, RNA targeting and amplification (Fig. 9). These viral suppressors of RNA silencing (VSRs) are often multifunctional proteins, which operate as coat proteins, replicases, movement proteins, helper components of viral transmission, proteases or transcriptional regulators that cause difficulties to investigate their functions as suppressors of RNA silencing responses (Burgyán and Havelda, 2011).

Although this strategy is relatively infrequent, some viral proteins have been proven to inhibit viral RNA recognition and dicing (Fig. 9). For instance, both P14 of *Pothos latent aureusvirus* (PoLV) and P38 of TCV bind dsRNAs in a size-independent manner and inhibit their processing into siRNAs. The action of P38 has been demonstrated to occur through AGO1 binding, resulting in the interference of its homeostatic network and ultimately the inhibition of DCL proteins, including vsiRNA-generating DCL4 (Déléris *et al.,* 2006; Mérai *et al.,* 2006; Azevedo *et al.,* 2010). Alternatively, P6 of CaMV interferes with vsiRNA processing by interacting with DRB4, which is required for the production of DCL4-dependent 21-nt siRNAs derived from endogenous TAS loci,

exogenous transgenes and viral RNAs (Love *et al.,* 2007; Haas *et al.,* 2008; Shibvaprasad et al. 2008). *Red clover necrotic mosaic virus* (RCNMV) recruits DCL enzymes into its replication complex, depriving them from the silencing machinery. Similarly, CaMV produces massive amounts of vsiRNAs from its 35S leader sequence, which serve as a decoy sequestering the silencing machinery effectors (Blevins *et al.,* 2011).

Another group of viral suppressors is known to prevent RISC assembly by siRNA sequestration (Fig. 9). As an example, the 2b proteins encoded by *Tomato aspermy virus* (TAV) and CMV have the ability to bind to 20-30 bp dsRNAs *in vitro*. (Masuta *et al.,* 2007; Chen *et al.,* 2008; Rashid *et al.,* 2008). Similarly, crystal studies demonstrated that p19 of *Carnation Italian ringspot virus* (CIRV) and *Tomato bushy stunt virus* (TBSV) form homodimers that specifically bind siRNAs in a sequence-independent manner, showing high affinity for 19-bp dsRNA with blunt ends or a 2'-nucleotide 3' overhang. Although the binding of p19 to vsiRNAs has been confirmed in immunoprecipitation assays, it does not prevent their loading onto RISC owing to the similar accumulation of wild type and p19-deficient CymRSV in protoplasts and inoculated leaves. This suppressor was also shown to sequester DCL4-dependent 21-nt siRNAs, preventing the transmission of the silencing signal (Silhavy *et al.,* 2002; Havelda *et al.,* 2003; Vargason *et al.,* 2003; Patel *et al.,* 2003; Lakatos *et al.,* 2004, 2006). P21 of *Beet yellow virus* (BYV) and p122 of TMV bind preferentially to siRNAs/miRNAs with a 2'-nt 3' overhang in a size-dependent manner and inhibit their incorporation into RISC *in vitro*. Additionaly, p122/p126 protein of tobamoviruses is a competitive inhibitor of HEN1-mediated 3' methylation of siRNAs and miRNAs, which compromises their stabilization and prevents their loading onto RISC (Ebhardt *et al.,* 2005; Csorba *et al.,* 2007; Lakatos *et al.,* 2006; Lózsa *et al.,* 2008; Burgyán et al.). The RNase III encoded by *Sweet potato chlorotic stunt virus* (SPCSV) cleaves the 21-24 bp siRNAs derived from the host, but not those produced by the virus itself, into 14-bp fragments (Kreuze *et al.,* 2005; Cuellar *et al*. 2009). The NS3 protein of *Rice hoja blanca virus* (RHBV) and NSs of *Tomato spotted wilt virus* (TSWV) can bind dsRNAs *in vitro* and show high affinity for 21-nt siRNAs regardless of 2-nt overhangs and, to a lesser extent, for 26-nt siRNAs. Given its ability to bind both ss- and ds-siRNA/miRNA, P10 of *Grapevine virus A* (GVA) has also been suggested to act through RNA sequestration (Bucher *et al.,* 2003; Chellappan *et al.,* 2005; Zhou *et al.,* 2006; Hemmes *et al.,* 2007; Xiong *et al.,* 2009). Potyviral HELPER COMPONENT-PROTEASE (Hc-Pro), which is a multifunctional protein involved in many aspects of the viral cycle, acts as a silencing suppressor through vsiRNA sequestration and interference with their methylation (Lakatos *et al.,* 2006; Lózsa *et al.,* 2008). The HC-Pro protein from *Zucchini yellow mosaic virus* (ZYMV) interacts with HEN1 and the host RNA silencing suppressor rgsCAM, although the latter has been proposed to prevent HC-Pro dsRNA binding and promote its autophagy-mediated degradation. The *Papaya ringspot virus* (PRSV) version of HC-Pro modulates host defense to virus infection through calreticulin-triggered calcium signaling. The highly variable region (HVR) of this protein in *Potato virus A* (PVA), *Potato virus Y* (PVY) and TEV

allows it to interact with the microtubule-assocaited protein HIP2, thereby promoting virus accumulation and negatively regulating host pathogen-related signaling pathways (Anandalakshmi *et al.,* 2000; Gy *et al.,* 2007; Endres *et al.,* 2010; Shen *et al.,* 2010a, 2010b; Nakahara *et al.,* 2012; Haikonen *et al.,* 2013a, 2013b; Li *et al.,* 2014).

The 2b protein encoded by the Fny strain of CMV (Fny-CMV) colocalizes in the nucleolus and cytoplasmic foci with AGO1, inhibiting thus its slicing activity through an interaction with the PAZ and PIWI domains that phenocopies the *ago1-27* mutation. Furthermore, 2b interacts with AGO4 in the nucleolus and competes with this protein for 24-nt long repeat-associated siRNAs binding, which suppresses AGO4-mediated DNA methylation (Mayers *et al.,* 2000; Guo and Ding, 2002; Zhang *et al.,* 2006; Díaz-Pendón *et al.,* 2007; Hamera *et al.,* 2011). P0 from poleroviruses, which is essential for infection by *Beet western yellow virus* (BWYV) and *Potato leafroll virus* (PLRV), promotes AGO degradation by interacting with the SCF family of E3-ligase S-phase kinase-related protein-1 (ASK1) and -2 components through its minimal F-box motif. The degradation is insensitive to proteasome inhibitors, ruling out the role of P0 in targeting AGO1 for ubiquitination and subsequent proteasome-dependent degradation. While it cannot interfere with the slicing activity of AGO1 bound to siRNAs/miRNAs, it does prevent the *de novo* loading of sRNAs onto RISC (Mayo and Ziegler-Graff, 1996; Pazhouhandeh *et al.,* 2006; Baumberger *et al.,* 2007; Bortolamiol *et al.,* 2007; Csorba *et al.,* 2010). The GW/WG peptide motif, identified in the NRPD1b subunit of Pol V, functions as a "hook" facilitating its interaction with AGO proteins. P38 of TCV contains two GW repeats that mimic the AGO-hook and facilitate its interaction with AGO1 and AGO4, preventing RISC complex formation. Similarly, the coat protein P37 of *Pelargonium line pattern virus* (PLPV) contains GW motifs that are essential for its localization, interaction with AGO1 and sRNA-binding capacity, while P1 of *Sweet potato mild mottle ipomovirus* (SPMMV) contains three GW/WG motifs on its N-terminus and inhibits both existing sRNA-loaded and *de novo* formed RISC through its binding to AGO1 (Xie *et al.,* 2003; Mérai *et al.,* 2006; El-Shami *et al.,* 2007; Pérez-Cañamás and Hernández, 2015; Azevedo *et al.,* 2010; Giner *et al.,* 2010; Szabo *et al.,* 2012). By binding to AGO1, the CP suppressor of *Tomato ringspot virus* (ToRSV) both blocks its translational inhibitory activity and promotes its degradation through autophagy (Várallyay *et al.,* 2010; Burgyán and Havelda, 2011; Karran and Sanfaçon, 2014). P25 encoded by PVX has been shown through co-IP assays to interact with AGO1, -2, -3 and -4, but not -5 and -7. This supressor inhibits sense transgene or dsRNA-induced RNA silencing by reducing the accumulation of primary and secondary siRNAs, without affecting levels of endogenous miRNAs and siRNAs. The reduction of *AGO1* expression mediated by this protein is blocked by the proteasome inhibitor MG132, which proves the role of P25 in promoting the proteasome-dependent degradation of AGO1. Additionally, P25 facilitates virus movement and could inhibit the transmission of the silencing signal in an AGO1-dependent manner (Bayne *et al.,* 2005; Moissiard *et al.,* 2007; Chiu *et al.,* 2010; Voinnet *et al.,* 2016a).

The role of CMV 2b in inhibiting the transmission of the silencing signal, together with observations in the *rdr6* mutant and the 2b-defective CMV strain, established a model in which 2b promotes systemic viral infection by inhibiting the amplification of RDR6-depedent secondary vsiRNAs (Fig. 9)(Ding and Guo, 2002; Schwach *et al.,* 2005; Sunpapao *et al.,* 2009; Wu *et al.,* 2010b; Wang *et al.,* 2011b). V2 from *Tomato yellow leaf curl virus* (TYLCV) is known to suppress viral silencing and has been proposed to interact with SGS3, involved in the RDR6-mediated signaling amplification pathway. In *in vitro* assays, V2 was shown to compete with SGS3 for binding a dsRNA with 5' ssRNA overhangs. In *N*. *tabacum*, RDR1 antagonizes RDR6-mediated antiviral RNA silencing, functioning thus as a silencing suppressor (Kumakura *et al.,* 2009; Jauvion *et al.,* 2010; Ying *et al.,* 2010). The beta satellite TYLCCNB of *Tomato yellow leaf curl China virus* (TYLCCV) encodes the beta-C1 protein, which in *N*. *benthamiana* represses RDR6 expression and therefore secondary siRNA production by interacting with the endogenous suppressor of silencing calmodulin-like protein rgsCAM. Antiviral silencing blocking through RDR6 repression is a widespread strategy that is also employed by HC-Pro from *Sugarcane mosaic virus* (SCMV), 2b from TAV and Pns10 from *Rice dwarf phytoreovirus* (RDV) (Zhang *et al.,* 2008; Ren *et al.,* 2010; Li *et al.,* 2014).

Finally, some viruses have evolved VSRs that are able to interfere with repressive action of TGS. The geminiviruses *Tomato golden mosaic virus* (TGMV) and *Beet curly top virus* (BCTV) encode the closely-related AL2 and L2 VSRs, respectively, which interact with and inactivate ADENOSINE KINASE (ADK), thereby evading the repressive action of DNA methylation (Moffatt *et al.,* 2002; Wang *et al.,* 2003, 2005; Bisaro, 2006; Buchmann *et al.,* 2009). AC2 from *Mungbean yellow mosaic virus* (MYMV) and *African cassava mosaic virus* (ACMV) induces the expression of a host gene network controlling silencing in a transcription-dependent manner (Trinks *et al.,* 2005). C2 protein encoded by *Beet severe curly top virus* (BSCTV) interacts with SAM decarboxylase (SAMDC) and interferes with its proteasome-mediated degradation, thereby interfering with the host plant antiviral mechanism (Zhang *et al.,* 2011). Finally, the βC1 protein of TYLCCV satellite DNA interacts with and inhibits S-adenosyl-L-homocysteine hydrolase (SAHH), causing reduced methylation of both the viral and host plant genomes and the reversal of TGS (Yang *et al.,* 2011).

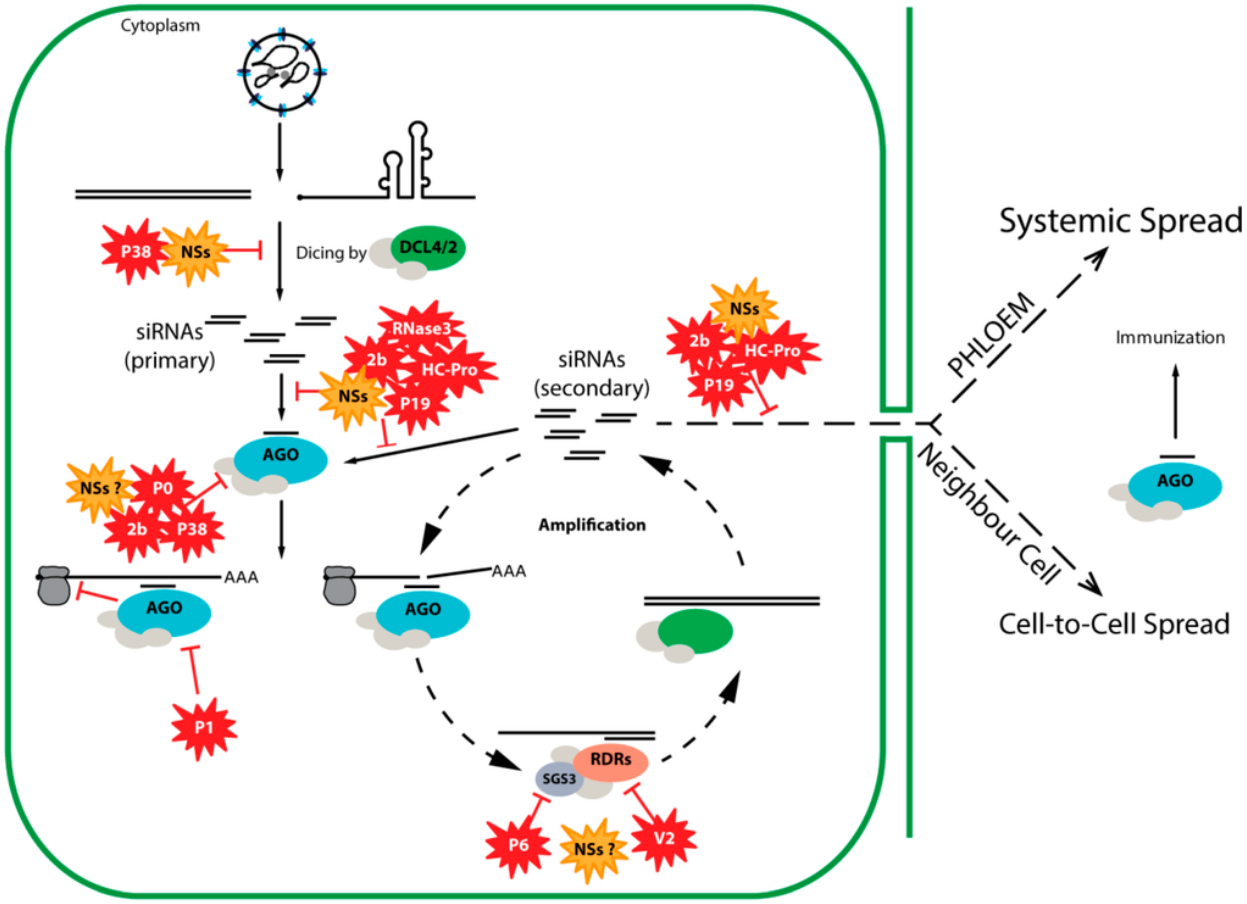


Figure 9. Viral suppressors of RNA silencing (taken from Hedil and Kormelink, 2016)

1.6. PLANT INNATE IMMUNITY
1.6.1. INTRODUCTION

In order to counteract with pathogens, plants rely on the presence of numerous surveillance-type receptors that could perceive and transmit the message of invasion to elicit both local and systemic defense responses, termed innate immunity, in contrast to the adaptive immune system comprising specialized cells that move through the circulatory system and is specific only for animals (Kumar *et al.,* 2011).

The innate immune system of plants is composed of two main branches: pattern- and effector-triggered immunity (Jones and Dangl, 2006). Pattern-triggered immunity (PTI) constitutes the initial recognition of pathogens by pattern recognition receptors (PRR) located on the cell surface, which detect the presence of small motifs of large molecules essential for microbial survival, such as lipopolysaccharides, chitin or flagellin, called pathogen- or microbe-associated molecular patterns (PAMP or MAMP). As a result, a myriad of processes, including the production of reactive oxygen species (ROS), hormone signaling pathways, mitogen-activated protein kinase (MAPK) cascades and responsive gene expression are activated (Fig. 10) (Janeway, 1989; Felix *et al.,* 1999; Schwessinger and Zipfel, 2008). In order to be successful plant pathogens overcome this front-line system using effector proteins to inactivate PTI signaling. Simultaneously, plants have evolved the resistance (*R*) proteins that recognize these specific effectors in order to directly or indirectly activate the second layer of defense called effector-triggered immunity (ETI). ETI triggers salicylic acid (SA) synthesis and signaling, leading to the induction of local cell death and systemic acquired resistance (SAR), which results in a hypersensitive response against biotrophic pathogens (Metraux *et al.,* 1990; Delaney *et al.,* 1994; Jones and Dangl, 2006), as well as ethylene (ET) and jasmonic acid (JA) signaling that synergistically activate defenses against necrotrophic pathogens (Niki *et al.,* 1998; Thomma *et al.,* 1998).

In mammals and invertebrates, innate immunity forms the first line of defense that constitutively functions to respond to pathogens before the sophisticated and specific adaptive immune system takes over (Hoebe *et al.,* 2004; Sansonetti, 2006). Despite individual components of the plant and animal PTI and ETI share some common features including defined receptors for microbe-associated molecules, conserved MAPK signaling cascades and the production of antimicrobial peptides, numerous differences can be observed (Ausubel, 2005). While the family of conserved toll-like receptors (TLR), formed by an extracellular leucine rich repeat (LRR) and a cytoplasmic toll-interleukin (IL-1) receptor (TIR) domain, functions as PRR for microbe-associated molecules in animals (Aderem and Ulevitch, 2000; Medzhitov and Janeway, 2000), plant transmembrane receptor-like kinases conserve the LRR extracellular region, but contain a cytoplasmic kinase domain (Song *et al.,* 1995; Gómez-Gómez and Boller, 2000; Shiu and Bleecker, 2001). Similarly, intracellular receptors for bacterial effectors share an overall structure with C-terminal LRR and central nucleotide-binding site (NBS) regions in both kingdoms, but N-terminal

domains are usually TIR or coiled-coiled in plants and caspase activation/recruitment (in Nod1 and 2) or pyrin and NACHT (in NALP proteins) in animals, respectively (Chamaillard *et al.,* 2003; Holt *et al.,* 2003; Nimchuk *et al.,* 2003; Tschopp *et al.,* 2003; Ting and Davies, 2005). Perception of pathogens by animal TLRs leads to the activation of cascades where the NF-kB-like transcription factors results in the production of antimicrobial peptides and signaling molecules (Georgel *et al.,* 2001). Plants do not have transcription factors homologous to NF-kB and activate instead WRKY transcritption factors downstream of PRRs (Gómez-Gómez and Boller, 2000, 2002; Asai *et al.,* 2002). As main strategic difference, plants contain a wide variety of pathogen-specific PRRs, whereas those of animals only recognize very highly conserved microbe-associated molecules (Ausubel, 2005).

1.6.2. PATTERN-TRIGGERED IMMUNITY

The first line of active defense against plant pathogens consists of the recognition of PAMPs by PRRs, leading to the PTI response. PAMPs are defined as invariant epitopes within molecules that are essential to the pathogen's survival, widely distributed among microbes, absent in the plant and recognized by a wide range of potential hosts. Plant PRRs are either receptor-like kinases (RLKs), composed of a ligand-binding ectodomain, a single-pass transmembrane region and an intracellular kinase domain, or receptor-like proteins (RLPs), where the kinase domain is absent. LRR-type ectodomains bind proteins or peptides, while other types of domain are involved in the recognition of carbohydrate-containing molecules (Schwessinger and Zipfel, 2008).

The PTI component is triggered through the recognition of PAMPs by the PRR (Jones and Dangl, 2006). The most studied case is that of FLAGELLIN-SENSING 2 (FLS2), a leucine-rich repeat receptor kinase that recognizes the N-terminal immunogenic epitope of 22 amino acids in bacterial flagellin (flg22) and induces the recruitment of the co-receptor BRASSINOSTEROID-INSENSITIVE1-ASSOCIATED KINASE (BAK1), necessary for full activation of flg22-triggered immunity (Felix *et al.,* 1999; Nürnberger and Kemmerling, 2006). Other examples of PAMPs include the bacterial elongation factor EF-TU, fungal chitin and cell wall polysaccharides, the sulfated peptide Ax21, peptidoglycan (PGN) and oomycete glucans (Dow *et al.,* 2000; Erbs *et al.,* 2008; Liu *et al.,* 2013).

PAMP perception induces rapid PRR receptor complex formation at the plasma membrane that leads to different auto- and trans-phosphorylation reactions and initiation of downstream signaling. A common event in early PTI responses is the rapid influx of Ca^{2+} in the cytosol (Blume *et al.,* 2000; Ranf *et al.,* 2011), which occurs at ~30 s to 2 min after MAMP perception and leads to opening of other membrane transporters (influx of H^+, efflux of K^+, Cl^- and NO_3^-), extracellular alkalinization and plasma membrane depolarization (Jeworutzki *et al.,* 2010). Followed by Ca^{2+} influx, rapid and transient accumulation of phosphatidic acid (PA) was observed in tomato cells treated with flg22, xylanase and chitin, as well as in tobacco cells upon treatment with *Cladosporium fulvium* elicitor AVR4 (van der Luit *et al.,* 2000; de Jong *et al.,* 2004; Bargmann *et al.,*

2006). PA is a key intermediate of phospholipid biosynthesis with signaling function in plants and likely a prerequisite for ROS production (Testerink and Munnik, 2005), which rapidly occurs at ~2–3 min, and predominantly generated by plasma membrane NADPH oxidases. In addition to strengthening the cell wall, it induces intracellular signaling pathways such as activation of MAPK cascades, which mediate plant immunity through up-regulation of defense-related genes via phosphorylation of WRKY and ERF transcription factors (Meng and Zhang, 2013). Besides Ca^{2+} influx, production of PA, reactive oxygen and nitrogen species, PTI signalling induces production of the classical immunity hormones such as SA, JA and ET. While SA signaling is generally important for immunity against biotrophs (e.g. *Hyaloperonospora arabidopsidis*) or hemibiotrophs (e.g. *Pseudomonas syringae*), JA and ET normally mediate immunity against necrotrophs such as *Alternaria brassicicola* (Glazebrook, 2005).

1.6.3. EFFECTOR-TRIGGERED IMMUNITY

Several plant pathogens such as fungi, oomycetes and bacteria have evolved protein effectors that can be delivered into host cells to suppress pattern-triggered defenses and break resistance of the host plants. For example, AvrPto and AvrPtoB from *P. syringae pv. tomato* are potent suppressors of PAMP-triggered early defense and MAPK signaling in *Arabidopsis*. They have been shown to target receptors FLS2, EFR and CRK1, as well as coreceptor BAK1 (Gohre *et al.,* 2008; Xiang *et al.,* 2008; Giménez-Ibáñez *et al.,* 2009; Macho *et al.,* 2014). In turn, plants have counter-evolved *R* genes that could specifically recognize some of these effector proteins, called Avr factors, leading to a rapid programmed cell death (PCD) in the local tissue, called the hypersensitive response (HR). This second layer of defense, that is referred to as ETI, also induces the production of the hormone SA and activates a broad-spectrum, long-lasting resistance in uninfected tissues of the whole plant called systemic acquired resistance (SAR) (Dangl *et al.,* 1996; Durrant and Dong, 2004; Katagiri, 2004).

For the activation of ETI, Avr effectors must be recognized directly or indirectly by the R receptors, that are intracellular nucleotide-binding leucine-rich repeat proteins (NB-LRR) with a variable N-terminal region classified into the coiled-coil (CC)-NB-LRR and toll/interleukin 1 receptor-like (TIR)-NB-LRR protein families (Wu *et al.,* 2014). The direct interaction between plant R proteins and pathogen Avr effectors has been demonstrated for several R/Avr combinations, such as *Arabidopsis* RRS1-R/*Ralstonia solanacearum* PopP2, rice Pi-ta/*Magnaporthe grisea* AvrPita or rice RGA5/*Magnaporthe oryzae* Avr1-CO39 and Avr1Pia (Jia *et al.,* 2000; Deslandes *et al.,* 2003; Cesari *et al.,* 2013). At the same time, according to the guard model, Avr effectors could be indirectly recognized by R proteins, which detect the perturbation caused by pathogen effectors in host plant target proteins upon infection (Dangl and Jones, 2001). As an example, RPM1-INTERACTING PROTEIN 4 (RIN4) plays an intermediary role in the recognition of AvRpm1 and AvRpt2 by RPM1 and RPS2, since these R proteins sense its phosphorylation and disappearance,

respectively (Mackey *et al.,* 2002, 2003). Some target proteins evolved into decoys and maintain their role as targets for effectors and guardees for R proteins despite having lost their biological function. The *Arabidopsis* non-functional kinase ZED1 is acetylated by the *Pseudomonas syringae* type III effector HopZ1a, which is recognized by ZAR1 to induce a robust immune response limiting the bacterial growth (Lewis *et al.,* 2013).

Activated R proteins trigger an array of immune responses including Ca^{2+} spikes, ROS burst, MAPK cascades, transcriptional reprogramming and production of SA, JA and ET. Signal transduction can involve direct transcriptional regulation by these receptors or long-distance control of nuclear transcriptional reprogramming (Buscaill and Rivas, 2014; Cui *et al.,* 2015). In order to kill biotrophic pathogens, ETI signaling rapidly causes localized PCD, which is referred to as autolytic if it involves the release of hydrolases from the vacuole to clear the cytoplasm. PCD is regulated by increases in SA concentration upon infection by an avirulent pathogen. High SA levels at the center of the infection site cause cell death, while its intermediate concentration in neighboring cells allows the interaction with transcription factors and activation of plant defenses (Enyedi *et al.,* 1992; van Doorn, 2011).

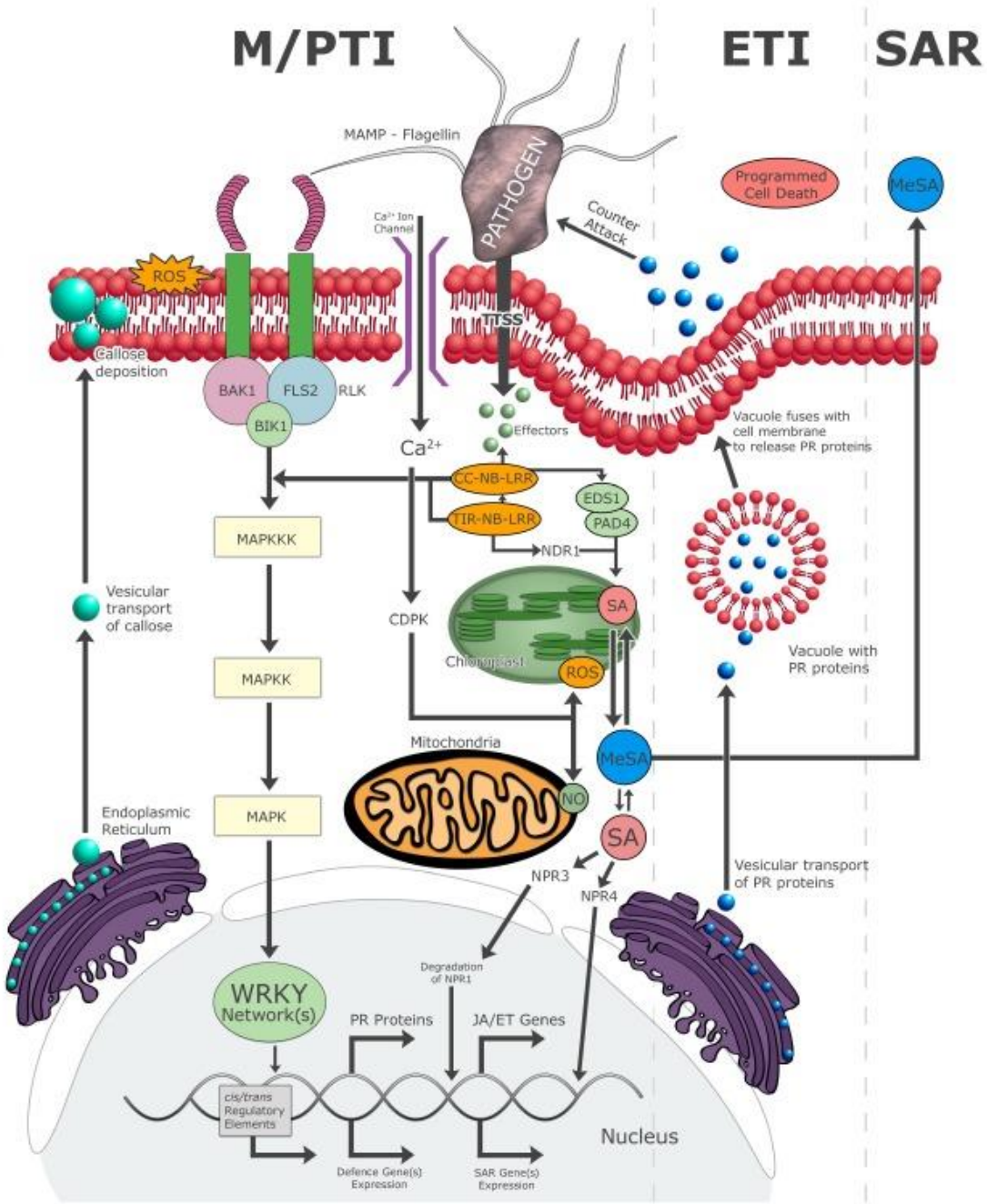


Figure 9. Model of plant innate immunity (taken from Klemptner *et al.,* 2014)

1.6.4. INNATE IMMUNITY IN RICE

1.6.4.1. PATTERN-TRIGGERED IMMUNITY IN RICE

Rice FLAGELLIN-SENSING 2 (OsFLS2) can recognize the N-terminal immunogenic epitope of 22 amino acids in bacterial flagellin (flg22) from incompatible strains of *Pseudomonas avenae* and *Acidovorax avenae* triggering immunity response against these pathogens (Che *et al.,* 2000; Tanaka *et al.,* 2003; Qu le and Takaiwa, 2004; Takai *et al.,* 2008). The XA21-associated kinase OsSERK2 was shown to regulate immunity mediated by OsFSL2 among other receptors. OsFSL2 has

also been found to interact with the PTI-involved guanine nucleotide exchange factor OsRac1GEF1 (Akamatsu *et al.,* 2013; Chen *et al.,* 2014). Recently, Katsuragi and collaborators (2015) revealed that the CD2-1 and CD2-0 domains of the flagellin carboxy-terminal region induce much stronger PTI responses than flg22 in rice, suggesting that an alternative protein to OsFSL2 might be the main flagellin receptor in this species.

Chitin is a major component of fungal cell walls known to trigger defense responses in plants and animals (Wan *et al.,* 2008). The LysM-containing RLP CHITIN ELICITOR BINDING PROTEIN (CEBiP) of rice was the first PRR found to recognize chitin. Because the protein lacks an intracellular kinase domain, CEBiP homodimers require the formation of hetero-oligomeric complexes with CHITIN ELICITOR RECEPTOR KINASE 1 (OsCERK1) upon chitin binding, constituting a sandwich-type receptor system (Kaku *et al.,* 2006; Miya *et al.,* 2007; Shimizu *et al.,* 2010; Hayafune *et al.,* 2014). LYSIN MOTIF-CONTAINING PROTEIN 4 (OsLYP4) and 6 (OSLYP6) also bind chitin and participate in its responsiveness. Knockout mutants for CEBiP, OsCERK1, OsLYP4 and OsLYP6 show reduced chitin-triggered immunity response, which leads to hypersensitivity to *M. oryzae* (Kaku *et al.,* 2006). In addition to chitin, OsLYP4 an OsLYP6 can sense the chitin structurally-related bacterial cell wall component peptidoglycan (PGN) in rice cells. The receptor-like cytoplasmic kinases OsRLCK176 and -185 function downstream of OsCERK1 in the chitin and PGN signaling pathways. Silencing of these proteins results in increased susceptibility to *Xanthomonas oryzae pv. oryzae* (Xoo) in transgenic rice plants (Liu *et al.,* 2012a; Yamaguchi *et al.,* 2013; Ao *et al.,* 2014). Upon chitin recognition, CEBiP forms a complex with FSL2, which phosphorylates OsRac1GEF1 leading to activation of OsRAC1, a central protein of the rice defensome. Downstream of OsRAC1, the kinase RACK1 enhances ROS production and interacts with the NADPH oxidase, as well as with the key regulators of plant disease resistance RAR1 and SGT1 (Nakashima *et al.,* 2008; Yamaguchi *et al.,* 2012; Akamatsu *et al.,* 2013). CEBiP competes with the *M. oryzae* effector protein Slp1 for binding chitin. By sequestering this polysaccharide, Slp1 prevents PAMP-triggered immunity and facilitates the spread of *M. oryzae* within the plant. N-glycosylation by ALG3 is necessary for Slp1 to evade host innate immunity (Mentlak *et al.,* 2012).

The Xa21 gene confers resistance to several strains of Xoo and codes for a receptor kinase with extracellular leucine rich repeats (LRR), together with transmembrane, juxtamembrane and intracellular kinase domains (Song *et al.,* 1995; Dardick and Ronald, 2006). Previous efforts to identify the bacterial ligand had revealed an operon required for XA21 activation constituted by the tyrosine sulfotransferase RaxST and the type I secretion system components RaxA, -B and -C. The hypothesis that the ligand was a tyrosine-sulfated, type I-secreted protein finally led to the uncovering of the protein RaxX as the XA21-mediated immunity activator (da Silva *et al.,* 2004; Lee *et al.,* 2009, 2013; Pruitt *et al.,* 2015). Additionally, numerous components of the XA21 signaling pathway have been identified. The E3 ubiquitin ligase XB3 binds to XA21 through its ankyrin repeat domain and is necessary for the full accumulation of this protein, thereby allowing for the onset of

the immunity response (Wang *et al.,* 2006). The ATPase activity of XB24 promotes the autophosphorylation of XA21, which keeps it in an inactivated state. In turn, the PP2C phosphatase XB15 physically interacts with and dephosphorylates autophosphorylated XA21, also resulting in reduced immune response (Park *et al.,* 2008; Chen *et al.,* 2010). Transiently overexpressed XA21-GFP is cleaved and translocated to the nucleus, where it can interact with the negative regulator OsWRKY62 (Peng *et al.,* 2008; Park and Ronald, 2012). The reduction of XA21-mediated immunity to Xoo, XA21 stability and proteolytic cleavage in BiP3-overexpressing plants indicates a role for this protein as an upstreams regulator of XA21 (Park *et al.,* 2010). Similarly, the Xoo-responsive gene XIK1 is required in XA21-mediated disease resistance, as demonstrated through RNAi silencing assays in rice plants. XB25 is also required to maintain XA21-mediated disease resistance in planta (Jiang *et al.,* 2013).

1.6.4.2. EFFECTOR-TRIGGERED IMMUNITY IN RICE

The genome of rice encodes approximately 100 R genes conferring resistance to *M. oryzae*, from which 23 have been cloned. Most of them constitute dominant NB-LRR genes, except for dominant Pi-d2, coding for an RLK protein, and recessive pi21, encoding a proline-rich protein. However, only five *M. oryzae* Avr effectors have been shown to correspond to these R genes (Chen *et al.,* 2006; Fukuoka *et al.,* 2009; Sharma *et al.,* 2012a). The Pita/AvrPita was the first case of R/Avr recognition reported. Another example of recognition of a single *M. oryzae* Avr gene product by a dominant rice R gene leading to effector-triggered immunity is that formed by the Piz-t/AvrPiz-t pair, although they do not interact directly and instead use the E3 ligase APIP10 as an intermediary connecting both proteins. The bZIP-type transcription factor APIP5 interacts with Piz-t in the cytoplasm and suppresses its transcriptional activity and protein accumulation, thereby preventing effector-triggered necrosis. In turn, AvrPiz-t suppresses the ubiquitin ligase activity of APIP6, which prevents the onset of flg22- and chitin-induced PTI (Jia *et al.,* 2000; Li *et al.,* 2009; Park *et al.,* 2012, 2016; Wang *et al.,* 2016). Some interactions require the recognition of a single Avr effector by two R genes simultaneously, as in the case for the pairs Pi5-1/Pi5-2 and RGA4/RGA5, in which all single mutants are susceptible to infection by *M. oryzae* (Lee *et al.,* 2009; Okuyama *et al.,* 2011; Cesari *et al.,* 2013). The Pik locus is highly polymorphic and has at least six alleles (Pik, Pikm, Pikp, Piks, Pikh and Pi1), conferring different degrees of tolerance to *M. oryzae* infection. The molecular characterization of Pik, Pikm, Pikp and Pik1 loci revealed the requirement for the Pik-1/Pik-2, Pikm1-TS/Pikm2-TS, Pikp-1/Pikp-2 and Pi1-5C/Pi1-6C highly related gene pairs for their resistance function (Ashikawa *et al.,* 2008; Yuan *et al.,* 2011; Zhai *et al.,* 2011; Hua *et al.,* 2012). Similarly, the Avr-Pik effector encodes five different alleles, Avr-PIKA to -E (Kanzaki *et al.,* 2012). The adjacent RGA4 and RGA5 genes cause rapid cell death upon co-expression with Avr-Pia, indicating specific recognition that leads to blight resistance. Interestingly, they both confer resistance to Avr1-CO39 (Okuyama *et al.,* 2011; Ribot *et al.,* 2012; Cesari *et al.,* 2013). Pi21 encodes

a cytoplasmic proline-rich protein that delays the host defenses. The recessive deletion of the proline-rich motif confers durable resistance to *M. oryzae* in mutant rice plants (Fukuoka *et al.,* 2009). Through overexpression experiments, the protein OsGF14e was shown to play a positive role in resistance to *M. oryzae*. The gene is regulated by WRKY71 and its way of action involves activation of SA-dependent and repression of JA-dependent pathways (Liu *et al.,* 2016). In contrast, 14 of the 37 R genes identified against Xoo are inherited recessively and only Xa1, showing dominant inheritance, codes for an NB-LRR protein (Yoshimura *et al.,* 1998; Wang *et al.,* 2014). Although AvrXa3 has been identified as a transcription activator-like (TAL) effector, direct interaction with Xa3/Xa26 has not been demonstrated and this protein is believed to activate Xoo resistance by upregulating Xa3/Xa26 (Schornack *et al.,* 2013). Transcription of Xa27 is initiated upon binding of the TAL effector AvrXa27. Other dominant R genes suspected to be activated by Avr effectors include Xa7, Xa10 and Xa23 (Gu *et al.,* 2009; Bogdanove *et al.,* 2010; Hummel *et al.,* 2012). In contrast to its role against *M. oryzae*, gene silencing experiments showed that GF14e negatively affects the induction of Xoo- and *Rhizoctonia solani*-mediated ETI response. Similarly, silencing of OsDR10 mediated enhanced resistance to a broad spectrum of Xoo strains, increased production of SA, suppressed accumulation of JA and modified expression of defense-responsive genes (Xiao *et al.,* 2009; Manosalva *et al.,* 2011). The R gene OsDR8 positively regulates resistance to Xoo and *M. oryzae*, presumably by upregulating defensive genes (Wang *et al.,* 2005).

1.6.5. PLANT VIRUSES AND INNATE IMMUNITY

Plant viruses are not generally viewed as encoding PAMPs or effector proteins according to the current definitions of these concepts that commonly exclude the antiviral immune response from plant innate immunity models (Jones and Dangl, 2006; Boller and Felix, 2009; Spoel and Dong, 2012). However, growing evidence suggests that plant viruses, which are thought to be targeted mostly by host plant RNA silencing machinery, could be also recognized by plant intracellular innate immune receptors leading to the activation of downstream resistance (Padmanabhan and Dinesh-Kumar., 2014).

During the last decades, several *R* genes encoding NBS-LRR proteins and conferring resistance to plant viruses have been cloned and characterized (Whitham *et al.,* 1994; Collier and Moffett, 2009; Moffett, 2009; Gururani *et al.,* 2012). The Avr determinants for these genes can correspond to the coat (e.g., locus *L* from *Capsicum* against Tobamoviruses), replicase (e.g., *Tm-1* from tomato against Tobamoviruses) or silencing suppressor proteins (e.g., *HRT* from Arabidopsis against *Turnip crinkle virus*) (Meshi *et al.,* 1989; Ishibashi *et al.,* 2012; Moury and Verdin, 2012). Although the first recognition mechanism to be proposed was a simple receptor-ligand model, no viral R-Avr pair fitting it has been identified. Instead, the “guard hypothesis” formulated by van der Biezen and Jones (1998) is one of the most commonly accepted and has been demonstrated for the

pair formed by the coat protein of TCV and HRT, where TCV interacts with the transcription factor TIP (TCV-Interacting Protein) and inhibits its nuclear location as a previous step for the triggering of the HRT-elicited defense responses (Ren *et al.,* 2000, 2005). More recently, the "bait and switch" model proposed that the inactivated *R* gene product forms a complex with the guardee/decoy protein, which leads to a conformational change allowing the activation of downstream resistance upon interaction with the Avr effector (Collier and Moffett, 2009). Indeed, the resistance gene *Rx* from potato is maintained in an inactivate state through intramolecular interactions until interaction with the PVX effector protein releases them and triggers the defense signaling cascade (Bendahmane *et al.,* 2002; Lukasik and Takken, 2009).

As it was described above for bacterial and fungal pathogens, plant viruses induce hypersensitive response (HR) involving PCD upon recognition by R proteins. This leads to metabolic changes in hormone levels (SA, JA, ET), accumulation of NO, Ca^{2+} and production of ROS that trigger downstream signaling cascades followed by upregulation of genes coding for glucanases, chitinases or defensins among others (Mur *et al.,* 2008; Loebenstein, 2009; Carr *et al.,* 2010). The first module functioning in early HR signaling against viruses and bacteria alike comprises the adaptor protein SGT1, which physically interacts with REQUIRED FOR MLA12 RESISTANCE 1 (RAR1), Hsp90 and the R proteins. This complex mediates downstream MAPK activation, regulates defense gene and hormone levels and ensures correct folding and stability of R proteins, which facilitates recognition of pathogen elicitors (Austin *et al.,* 2002; Takahashi *et al.,* 2003; Bieri *et al.,* 2004). SGT1 also interacts with multiple E3-ubiquitin ligases, as well as with CSN3 and CSN8, together with RAR1, in order to mediate *N* gene resistance to TMV (Azevedo *et al.,* 2002; Liu *et al.,* 2002; Shirasu, 2009). Hsp90 is also a key player of *N* gene resistance through its physical interaction with the N protein (Liu *et al.,* 2004). The other module capable of mediating HR against viral pathogens is that formed by lipases ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) and PHYTOALEXIN DEFICIENT 4 (PAD4) interacting with SENESCENCE-ASSOCIATED GENE 101 (SAG101) (Falk *et al.,* 1999; Feys *et al.,* 2005). This complex regulates HRT-mediated resistance against TMV in *Arabidopsis*, which requires a functional SA-mediated signaling pathway (Chandra-Shekara *et al.,* 2004; Zhu *et al.,* 2011). During compatible Avr-R interactions, the resistance is also transduced to non-infected distant tissues and causes the accumulation of hormones such as SA and JA, which ultimately leads to the onset of SAR (Vlot *et al.,* 2008). SAR can be sustained for long time periods (e.g., 3 weeks for TMV-triggered SAR), being epigenetic changes critical and responsible for its transmission to the next generation (Ross, 1961; Luna *et al.,* 2012; Spoel and Dong, 2012). The NON-EXPRESSOR OF PR1 (NPR1) mediates changes in expression of defense genes by functioning downstreams of SA and interacting with the JA signaling. A functional version of this protein is required for the transgenerational stability of SAR (Dong, 2004; Luna *et al.,* 2012). An increased homologous recombination rate could be observed in tobacco plants treated with TMV and *Oilseed rape mosaic virus* in both infected and non-inoculated leaves. In the case of TMV, this phenomenon

persisted in the progeny, which exhibited broad-spectrum tolerance to the virus, *P. syringae* and *Phytophtora infestans* (Kovalchuck *et al.,* 2003). The nature of the signal mediating SAR in non-infected tissues remains unknown and probably involves crosstalk among multiple molecules and environmental factors (Vlot *et al.,* 2008).

In resistant (or non-compatible) Avr-R interactions, HR is not triggered and local necrotic lesions are not produced. Instead, a systemic necrosis response is manifested. The symptoms are primarily observed in the upper non-inoculated tissues at much later infection stages than HR. This phenomenon is a lethal response that does not preclude virus multiplication or systemic movement throughout the plant. However, both systemic necrosis and local necrotic lesions triggered by HR share similarities at the molecular and biochemical level, since they involve PCD, altered expression of similar genes and accumulation of ROS (Kim *et al.,* 2008; Komatsu *et al.,* 2010; Xu *et al.,* 2012). Indeed, the study of the transcriptional changes caused by systemic necrosis in *Nicotiana benthamiana* infected with the recombinant PVX vector expressing the potyviral helper component-proteinase (HC-Pro) revealed striking similarities with those observed in HR-associated necrosis (González-Jara *et al.,* 2004; Pacheco *et al.,* 2012).

Similarly to non-viral pathogens, plant viruses have evolved proteins, which in addition to their primary role in suppression of RNA silencing, are used to counteract with host plant innate immunity responses. For instance, the CaMV *ORF VI* product (P6/TAV) was shown to be a multifunctional protein (see chapter 1.4.) and counteract with both RNA silencing through its interaction with the dsRNA-binding protein DRB4 (Haas *et al.,* 2008) and innate immunity responses supressing oxidative burst and SA-dependent signalling (see the Results below Love *et al.,* 2012). In some cases, RNA silencing suppressor activity of viral proteins requires the interaction with the components of plant defense system. For example, RAV2, which is the ethylene-inducible transcription factor as well as HC-Pro-interacting protein, is required for RNA silencing suppression by potyvirus HC-Pro and carmovirus P38 (Endres *et al.,* 2010). In contrast, the interaction between HC-Pro and the plant calmodulin-like protein rgs-CaM leads to degradation of viral protein by autophagy, interfering with HC-Pro silencing suppressor activity (Pruss *et al.,* 2004). Finally, several viral suppressors of RNA silencing, such as 2b of cucumoviruses, potyviral HC-Pro and CP of TMV were found to interfere with SA-mediated responses, while Plum pox virus (PPV) capsid protein has been shown to act as a PTI suppressor, impairing early immune responses such as the oxidative burst and enhancing expression of PTI-associated marker genes during infection in *Arabidopsis* (Alamillo *et al.,* 2006; Lewsey *et al.,* 2010a; Nicaise and Candresse, 2016). Interestingly, PPV CP displays both virulence and avirulence functions acting as a PTI suppressor and recognized by antiviral R proteins during elicitation of ETI, respectively, confirming that plant viruses also fit into the zigzag model of co-evolving pathogenic virulence strategies and plant defense responses (Jones and Dangl, 2006; Zvereva and Pooggin 2012).

1.7. AIMS OF THE THESIS

In spite of the significant progress in the identification and further characterization of the plant viral proteins exhibiting RNA silencing suppressor activity (VRSs), the functions of some potential plant VRSs still remain to be investigated.

To further understand the role of CaMV P6 in suppression of RNA silencing and explore if RTBV P4 can aslo serve as silencing suppressor the following objectives have been set:

1. Comparative analysis of the CaMV P6 and RTBV P4 activities in suppression of plant RNA silencing pathways
2. Identification of the functional motifs of RTBV P4 that might interact with plant RNA silencing

On the other hand, besides their primary role as supressors of RNA silencing, VRSs could be involved in the suppression of host innate immunity responses. Hence, further objectives of this work were:

3. Investigation of the CaMV P6 and RTBV P4 activities in suppression of plant innate immunity
4. Identification of effector motifs of RTBV P4 that might interact with plant innate immunity

Finally, to undertand the mechanisms of plant-virus interaction, we aimed at studying RTBV interactions with the plant defense systems based on RNA silencing and innate immunity in the context of viral infection in the host plant *Oryza sativa*, and more specifically to elucidate the role of P4 in the interaction with the rice defense pathways. To this end the following objectives were set:

5. Construction and test of a P4-deficient RTBV mutant virus for infectivity in rice plants
6. Investigation of methylation status of RTBV dsDNA in rice plants infected with wild type and P4-deficient virus
7. Test of the rice plants overexpressing the putative antiviral osAGO18 gene for susceptibility to RTBV infection

3. MATERIALS AND METHODS

3.1. PLANT MATERIAL AND GROWTH CONDITIONS

Arabidopsis thaliana (L.) Heynh wild-type (Col-0) and transgenic (all in Col-0 background) P6-CM1841 and P6-D4 (described in Yu *et al.,* 2003) line plants were grown in phytochambers (Sanyo, Gunma, Japan) at 20–22°C and 12h photoperiod.

Nicotiana benthamiana wild type and transgenic 16c line plants (supplied by Prof. D.C. Baulcombe, Uni Cambridge, UK) were grown in soil in an open glasshouse at 24-25°C under natural light. Four to five weeks old plants were used for agroinfiltration.

Oryza sativa japonica wild type plants of two different ecotypes Taipei 309 and Nipponbare as well as transgenic lines PUBI and PGX6 of ecotype Nipponbare (kindly provided by Dr. J-B Morel, INRA, Montpellier) were grown in soil in an open glasshouse at 25°C under natural light and high humidity conditions. Three to four weeks old plants were used for RTBV infection.

The transgenic line PGX6 was generated from Nipponbare wild type plants by agro-mediated trasformation with a transgene harboring OsAGO18 gene under control of the rice UBI promoter, while PUBI was transformed with the empty UBI vector and used as a control for PGX6.

3.2. RTBV INFECTION

Following germination in soil, three to four weeks old *Oryza sativa* plants were inoculated with an infectious clone of RTBV isolate Philippines (GenBank accession X57924) (Hay et al. 1991) using *Agrobacterium tumefaciens* GV3859 harboring pRTRB1162 (or the empty vector pBin19 for mock inoculation). At 50 days postinoculation, rice plants showing the characteristic disease symptoms (slight stunting of the plant and weak yellowing of the leaves) were taken for further analysis. All the samples from RTBV-infected plants were checked by PCR for the presence of RTBV DNA using primers listed in Table 1.

3.3. DNA MANIPULATIONS AND MOLECULAR CLONING

3.3.1. DNA ISOLATION

High quality rice plant DNA was isolated using CTAB (cetyltrimethylammonium bromide) method, as follows. Approximately 0.5 g of rice tissue was ground in a mortar with liquid nitrogen. 0.05 g of the finely ground tissue sample was mixed in 2 ml microtubes with 500 µL preheated (65^{o} C) 2x CTAB buffer (0.1 M Tris-HCl pH 8.0, 0,02 M EDTA pH 8.0, 1.4 M NaCl and 2% CTAB) containing 2 µL/mL β-mercapthoethanol. The mixture was vortexed vigorously for a few seconds and incubated at 65^{o}C for 1 hour. During incubation the tube was shaken lightly for a few seconds. After cooling at room temperature, 500 µL of chloroform-isoamyl alcohol (24:1) was added. The

tube was shaken gently using a rotor for 20 minutes at room temperature to form an emulsion. It was then centrifuged at 5000 rpm for 15 minutes to pellet the debris. The upper phase was transferred into a clean 2 mL tube and 1 volume of 2-propanol was added. The tube was immediately inverted, gently and repeatedly, and incubated at -20°C for 30 minutes until DNA precipitation occurred. After DNA precipitation was observed, the mixture was centrifuged at 5000 rpm for 10 minutes to pellet the DNA. The pellet was washed with 70% ethanol, dried drain and re-suspended with 100 µl TE buffer. RNAse (10 mg/ml) was added and mixture was incubated at 37°C for 30 minutes. After incubation, 10 µl of sodium acetate and 200 µl of absolute ethanol were added and the tube was placed into -20°C for 1 hour. The mixture was then centrifuged at 5000 rpm for 15 minutes and dried drain. The pellet was rinsed with 70% ethanol, air dried and dissolved in 100 µl TE buffer.

Plasmid DNA from *E. coli* was extracted and purified with the GenElute™ HP Plasmid Maxiprep and GenElute™ PCR Clean-Up Kits (Sigma-Aldrich), respectively, according to the manuals.

DNA concentrations were estimated by measuring the absorbance at 260 nm using NanoDrop spectrophotometer (NanoDrop Technologies).

3.3.2. TRANSFORMATION INTO *E. COLI*

For transformation 100 µl of competent *E. coli* cells and 1 µl of plasmid DNA were added into a sterile tube, which was then incubated on ice for 15 minutes, followed by a heat shock at 42 ºC for 2 minutes and further incubation on ice for 5 minutes. Then 0.5 ml of LB medium was added and the mixture was incubated at 37 ºC with shaking at 250 rpm for 1 h. The bacterial solution was then poured on the solid LB medium with antibiotics (kanamycin 50 mg/ml for binary vectors or carbenicillin 50 mg/ml for other plasmids) for selection of transformed cells at 37ºC until single colonies appeared. Randomly chosen colonies were then analysed by PCR.

3.3.3. PCR

The PCRs for amplifying the RTBV genomic regions and high fidelity cloning PCRs were performed according to the requirements for Taq and Vent DNA polymerases (New England Biolabs), respectively, as described in the manufacturer's manuals.

Cloning sequences and RTBV DNA fragments were amplified using primers listed in Table 1.

Table 1. List of PCR primers and probes for blot hybridyzation

Primer name	Sequence 5'-3'
AttB1_Rtbv4_s	ACAAGTTTGTACAAAAAAGCAGGCTACACCATGGCTCAGGGACAAGCTTCTTCCTCTAGTCG
AttB2_Rtbv4_as	GGGGACCACTTTGTACAAGAAAGCTGGGTTTTAAGCATTGTCCATACGATGGATCC
AttB1_Rtbv4_ delN _s	GGGGACAAGTTTGTACAAAAAAGCAGGCTACACCATGAATATAGAGTACCCGTACTCAATCCAC
Rtbv7970_s	AGCAACGAGAAAAGTTAGGGGGGTGCCTAGAAA
Rtbv7488_s	GCGATCAATGGCTCAGGTCAGTGA
Rtbv7722_as	TCCCTTGCCATAACACGGCCTGAC
pRTBVwt_s	CGACCAAGGTTCCTGAAGGATT
pRTBVwt_as	GCATTGTCCATACGATGGATCC
mGFP5_ NOSterm_as	CGCAAGACCGGCAACAGGATTCAATCTTAAGAAACTTTATTG
NbmiR482a_as	TAGGAATGGGTGGAATTGGAAA
siR255_as	TACGCTATGTTGGACTTAGAA
Nb_Ago1_as	CAGATGTCTCTGGCTCCATGTAAAACCGAG
Nb_Ago2_as	GCACGGCCCATCTTCAGCCCGTACCATTTC
Met-tRNA_as	TGGTATCAGAGCCAGGTTTCGATCC
18S rRNA	ATCATTCAATCGGTAGGAGCGACG
pRTBVwt_s	CGACCAAGGTTCCTGAAGGATT
pRTBVwt_as	GCATTGTCCATACGATGGATCC
pRTBVmut_s	GACCAAGGTTCCTGAAGGAGC

3.3.4. DNA CONSTRUCTION AND CLONING

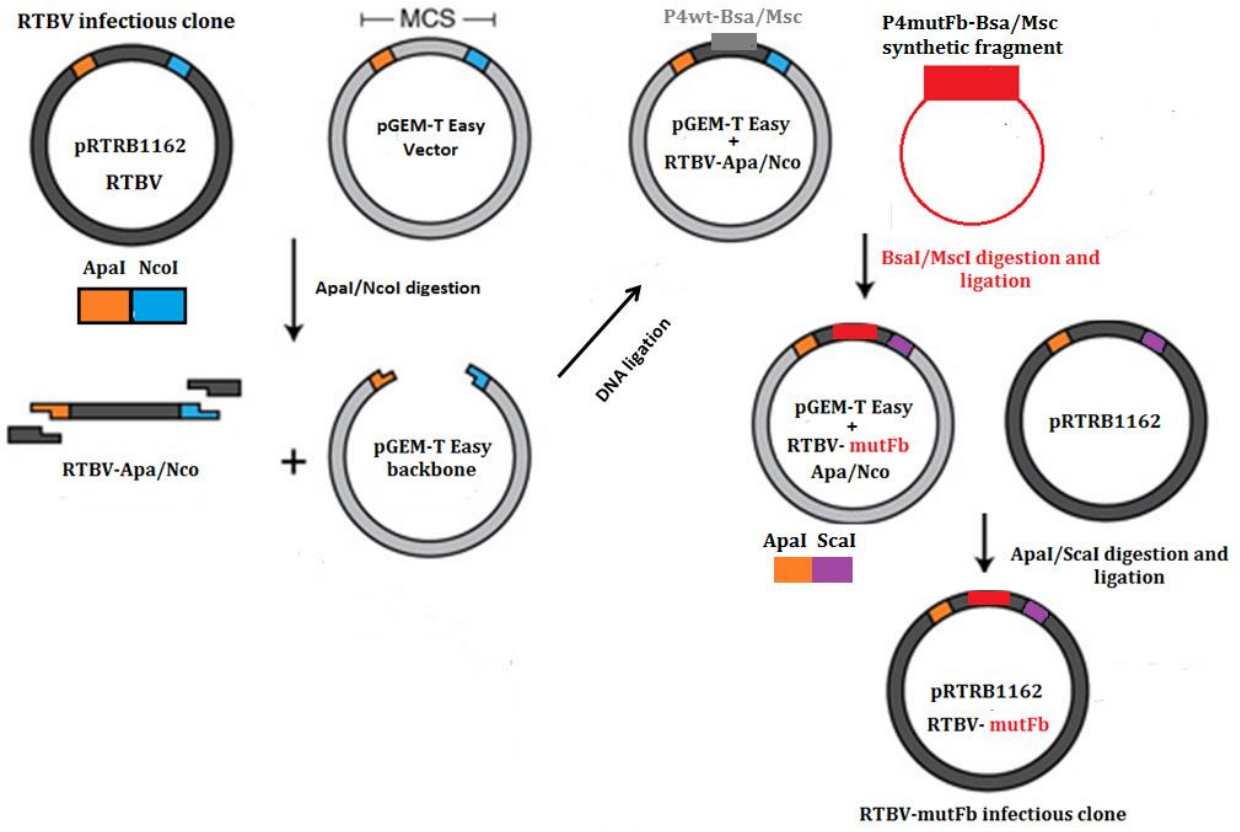
In order to produce the RTBV P4-mutFb expressing construct, we introduced four point mutations into RTBV P4 wild type ORF ligated into pGEM-Teasy vector (P4wt-vector) using manufacturer's protocol (Promega). For that, we ordered the synthetic fragment containing these four mutations and two unique restriction sites (P4mutFb-Bsa/Msc)(Fig. 10), which were used to excise the fragment from supplier's vector using standard restriction protocol. Simulteneously, two fragments P4wt-Bsa/Msc and P4wt-Msc/Bsa were excised from P4wt-vector. The P4wt-vector backbone was then treated with Shrimp Alkaline Phosphatase (SAP) in order to remove the 5' phosphate groups. As the last step, both P4mutFb-Bsa/Msc and P4wt-Msc/Bsa fragments were ligated with P4wt-vector backbone using standard ligation protocol.

Then, the fragment, containing all four point mutations, was cloned into RTBV-expressing contruct pRTRB1162 according to the Scheme 1. The presense of the mutation in the F-box motif of

RTBV-expressing contruct (RTBV-mutFb) was checked by PCR analysis using pRTBVmut_s and pRTBVwt_as primers (see Table 1).

```
                + BsaI                                                                  ** *
RTBV_P4wt   : AAGGTTTATTGGAGACCTTTATGCTCATGGTTTTATTAAACAAATAAACTTCACGACCAAGGTTCCTGAAGGATTGCCGCCAATCATAGCGGA
RTBV_P4-mu  : AAGGTTTATTGGAGACCTTTATGCTCATGGTTTTATTAAACAAATAAACTTCACGACCAAGGTTCCTGAAGGAGCGGCGCCAATCATAGCGGA
              AAGGTTTATTGGAGACCTTTATGCTCATGGTTTTATTAAACAAATAAACTTCACGACCAAGGTTCCTGAAGGA  G CGCCAATCATAGCGGA

                                    *
RTBV_P4wt   : AAAACTTCAAGACTACAAGTTCCCTGGATCAAACACCGTCTTAATAGAACGAGAAATTCCTCGCTGGAACTTTAATGAAATGAAGAGAGAGAC
RTBV_P4-mu  : AAAACTTCAAGACTACAAGTTCGCTGGATCAAACACCGTCTTAATAGAACGAGAAATTCCTCGCTGGAACTTTAATGAAATGAAGAGAGAGAC
              AAAACTTCAAGACTACAAGTTC CTGGATCAAACACCGTCTTAATAGAACGAGAAATTCCTCGCTGGAACTTTAATGAAATGAAGAGAGAGAC


RTBV_P4wt   : ACAGATGAGGACCAACTTATATATCTTCAAGAATTATCGCTGTTTCTATGGTTATTCACCATTAAGGCCATACGAACCTATAACTCCTGAAGA
RTBV_P4-mu  : ACAGATGAGGACCAACTTATATATCTTCAAGAATTATCGCTGTTTCTATGGTTATTCACCATTAAGGCCATACGAACCTATAACTCCTGAAGA
              ACAGATGAGGACCAACTTATATATCTTCAAGAATTATCGCTGTTTCTATGGTTATTCACCATTAAGGCCATACGAACCTATAACTCCTGAAGA


RTBV_P4wt   : ATTTGGGTTTGATTACTACAGTTGGGAAAATATGGTTGATGAAGACGAAGGAGAAGTTGTATACATCTCCAAGTATACTAAGATTATCAAAGT
RTBV_P4-mu  : ATTTGGGTTTGATTACTACAGTTGGGAAAATATGGTTGATGAAGACGAAGGAGAAGTTGTATACATCTCCAAGTATACTAAGATTATCAAAGT
              ATTTGGGTTTGATTACTACAGTTGGGAAAATATGGTTGATGAAGACGAAGGAGAAGTTGTATACATCTCCAAGTATACTAAGATTATCAAAGT

                                    + MscI
RTBV_P4wt   : CACTAAAGAGCATGCATGGGCTTGGCCA  :  400
RTBV_P4-mu  : CACTAAAGAGCATGCATGGGCTTGGCCA  :  400
              CACTAAAGAGCATGCATGGGCTTGGCCA
```

Figure 10. Synthetic fragment P4mutFb-Bsa/Msc containing four mutations in the F-box-like domain of RTBV protein P4, indicated (*), and two unique restriction sites BsaI and MscI, which were used for cloning of this fragment into P4-mutFb-expressing construct.



Scheme 1. P4-mutFb-expressing construct cloning strategy.

3.3.4.1 RESTRICTION ANALYSIS

Digestion of plasmid DNAs were perfomed as shown in Table 2. Samples were incubated at 37°C for 2 hours. The size of digested products was confirmed by agarose gel electrophoresis. The appropriately sized DNA fragments were excised from the gel using a clean scalpel following by purification using the gel purification kit (Qiagen) in accordance with the manufacturer's instructions.

Table 2.

Component	Volume, µl
Nuclease-free water	54
Restriction buffer (10x)	10
BSA (100x)	10
BsaI (10U/µl)	5.5
MscI (5U/µl)	5.5
plasmid DNA (180ng/µl)	15

3.3.4.2. DNA LIGATION

A 10 µl ligation reaction was prepared using an approximate 5:5:1 molar ratio of the insert and vector as shown in Table 3. The reaction was incubated at 4°C for at least 12 hours. 5 µl of ligation reaction was then used for transformation into DH5α *E.coli* strain.

Table 3.

Component	Volume, µl
T4 DNA Ligase Buffer (10x)	1.5
Vector DNA (50 ng/µl)	1
Insert DNA1 (40 ng/µl)	2.6
Insert DNA2 (7 ng/µl)	7.4
T4 DNA Ligase (Promega, 1U/µl)	2.5

3.3.5. GATEWAY CLONING

Clonings were performed according to the Gateway manual (Thermo Fisher Scientific), using donor plasmid pDONR™/Zeo (Thermo Fisher Scientific), pEarlyGate vectors (100 (no tag) and 201 (HA tag)) and PCR fragments amplified with Gateway compatible primers listed in Table 1.

The RTBV P4, RTBV P4-mutFb, RTBV P4-delN and CaMV P6-CM1841 (P6-CM) ORFs were subcloned from the RTBV and CaMV infectious clones, respectively, into the pEarlyGate vectors 100 (no tag) and 201 (HA tag) using two pairs of PCR primers listed in Table 1. To account for the pgRNA splicing that brings together in frame a short open reading frame (sORF 1) in the RTBV leader sequence with the 5' end of *ORF IV* (Fütterer et al. 1994), the sequence of sORF 1, which contains the P4 start codon, was imbedded in the forward primer AttB1_Rtbv4_s. The resulting plasmids, which carry the CaMV 35S promoter-driven P4 and P6 protein expression cassettes (designated RTBV P4, RTBV P4-HA and CaMV P6-CM), were mobilized to *Agrobacterium tumefaciens* strain C58C1 for agro-infiltration assays.

3.3.6. TRANSFORMATION INTO *AGROBACTERIUM TUMEFACIENS*

For transformation 500 µl of fresh culture of the *A. tumefaciens* strains C58C1 (used for *N. benthamiana* transent assays) and GV3859 (used for rice inoculation with RTBV constructs) grown overnight at 28°C in 5 ml of liquid LB medium supplemented with 50 mg/mL rifampicin was mixed with 0.5-1 µg of plasmid DNA followed by incubation on ice for 10 min and a heat shock at 37°C for 15 minutes. Afterwards 0.5 ml of liquid LB medium was added and cells were incubated for 3 h at 28°C with shaking at 3000 rpm. The bacterial solutions were then poured on solid LB medium with 50 mg/mL rifampicin and 50 mg/mL kanamycin for selection of transformed cells at 28°C for 2 days until single colonies appeared. Three single colonies from the plate were chosen for further analysis by PCR.

3.3.7. AGAROSE GEL ELECTROPHORESIS

For DNA gel electrophoresis, 1% (w/v) agarose gel was made in 1x TAE (Sambrook and Russel 2001) and supplemented with EtBr (1 mg/ml) for nucleic acid visualization under UV. DNA samples (PCR or restriction fragments) were mixed with 6x DNA-loading buffer (6x TAE, 30% (v/v) glycerol, 0.125% (w/v) bromophenol blue, 0.125% (w/v) xylene cyanol), loaded on the gel and run using the MUPID-exU Horizontal Electrophoresis System (Helixx) at 100 V. The GeneRuler 1kb+ DNA Ladder (Fermentas) was used as a size marker. The GenElute™ Gel Extraction and GenElute™ PCR Clean-Up Kits (Sigma-Aldrich) were used to purify DNA bands from agarose gel, if needed.

3.4. RNA ISOLATION AND BLOT HYBRIDIZATION

Total RNA was isolated from 0.5-1 g of *N. benthamiana* or rice plant tissue ground in liquid nitrogen using GHCL buffer according to the manufacturer's protocol using TRIzol reagent (Sigma-

Aldrich). For analysis of small RNAs, 10 µg of total RNA was resuspended in 10 µl loading buffer (95% formamide, 20 mM EDTA, pH 8.0, 0.05% bromophenol blue and 0.05% xylene cyanol), heated at 95°C for 2 min and separated on 15% polyacrylamide gel (a 19:1 ratio of acrylamide to bis-acrylamide, 8 M urea). The gel was run using the SE 600 electrophoresis machine (Hoefer) at 300 V for 4 h. For analysis of long RNAs, 10 µg of total RNA was re-suspended in 10 µl 2x RNA loading buffer (Thermo Fisher Scientific), heated at 95°C for 2 min and separated on 1% agarose gel, containing 2.2 M formaldehyde and 1× MOPS. The gel was run using the MUPID-exU Horizontal Electrophoresis System (Helixx) at 100 V for 3 h. Ethidium bromide (EtBr) staining of the gels was used for loading control. RNAs were transferred to Hybond N+membrane (Amersham) by electroblotting in 1x TBE buffer at 10 V overnight and crosslinked to the membrane in an UV Stratalinker 1800 (Stratagene) using the 'autocrosslink' function. The membrane was sequentially hybridized with ^{32}P-ATP-labeled DNA oligonucleotide probes (Table 1).

The blot hybridization was performed at 37°C overnight in an UltraHyb-oligo buffer (Ambion) using, as a probe, one or several short DNA oligos (Table 1) end-labeled with ^{32}P by T4 polynucleotide kinase (Roche) and purified through MicroSpin™ G-25 columns (Amersham) according to the manufacturers' recommendations. The blot was washed three times with 2× SSC, 0.5% SDS for 30 min at 37°C. The signal was detected after 1–5 days exposure to a phosphor screen using a GE Typhoon 8600 imager (GE Healthcare Life Sciences). For repeated hybridization the membrane was stripped with 0.5× SSC, 0.5% SDS for 40 min at 80°C and then with 0.1× SSC, 0.5% SDS for 40 min at 80°C.

3.5. P4 AND P6 TRANSIENT EXPRESSION IN *N. BENTHAMIANA*

For transient expression experiments using *N. benthamiana* transgenic 16c line plants, the agro-strains carrying the 35S-P4, 35S-P4-HA, 35S-P4-mutFb, 35S-P4-mutFb-HA, 35S-P4-delN, 35S-P4-delN-HA, 35S-GFP (GFP silencing trigger) and the 35S-TBSV p19 silencing suppressor (positive control for suppression of both cell-autonomous GFP silencing and cell-to-cell spread of GFP silencing) cassettes were inoculated into 2 mL of LB media supplemented with 50 mg/mL kanamycin and 10 mg/mL rifampicin and grown at 28°C for 16 hrs. Cells were precipitated, resuspended to a final optical density at 600 nm (OD600) of 0.3-0.4 in agroinfiltration buffer (10 mM MES pH 5.6, 10 mM MgCl2, 100 µM acetosyringone) and, before infiltration, mixed in equal proportions. The GFP fluorescence was monitored under UV light at 3 and 8 days post-infiltration (dpi) (Figure 11, F). Samples of the infiltrated tissues were taken at 8 dpi and used for the molecular analysis.

For transient expression experiments using *N. benthamiana* wt plants, the agro-strains carrying the 35S-P4, 35S-P4-HA, 35S-P4-mutFb, 35S-P4-mutFb-HA, 35S-P4-delN, 35S-P4-delN-HA, 35S-P6-CM and 35S-P6-D4 were prepared for agroinfiltration as described above. Depending on the

experiment, samples of the infiltrated tissues were taken at 2, 3 or 4 dpi and used for the molecular analysis.

3.6. WESTERN BLOT ANALYSIS

10-20 mg of collected *N. benthamiana* leaf tissue samples was ground in liquid nitrogen and solubilized in 100-200 µl of concentrated 6x SDS sample buffer (0.35 M Tris, pH 6.8, 22.4% glycerol, 10% SDS, 0.6 M DTT, bromophenol blue), heated to 95°C for 5 min, and centrifuged at 12000 g for 5 min. Obtaining supernatant was loaded onto 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for separation of P4 and GFP. The proteins were transfered onto polyvinylidene fluoride membrane (GE Healthcare, Europe GmbH, Glattbrugg, Switzerland), and blocked with 2% BSA w/v (Sigma) in TBS-tween (0.1%) for 1.5 h. The primary antibodies were diluted to the following concentrations in TBS-tween (0.1%) and incubated with the membrane overnight at +4°C: anti-GFP (1:1000, Sigma-Aldrich); anti-P4 (1:1000, see below); anti-HA (1:1000, Roche). Secondary anti-mouse (1:10000; SouthernBiotech), anti-rabbit (1:10000, Sigma-Aldrich) or anti-rat (1:10000, Sigma-Aldrich) HRP antibodies were diluted in TBS-tween (0.1%) and incubated with the membrane for 1 h at room temperature. Bands were visualized using ECL Prime Western Blot Detection Reagent (GE Healthcare), followed by the membrane staining with amidoblack or ponceau for loading control.

In order to detect RTBV P4, P4-mutFb and P4-delN, a primary anti-peptide serum (Eurogentec SA) was raised in rabbits by immunization with a synthetic peptide (PLRPYEPITPEEFGF) shared by all three proteins. After purification, the antibodies were used at the suggested dilution (1:1000).

3.7. SOUTHERN BLOT HYBRIDIZATION

For methylation-dependent enzymatic treatment and subsequent Southern blot hybridization, 2 µg total plant DNA from RTBV-infected and mock-inoculated rice plants was taken and digested with 30 Units of McrBC enzyme (New England BioLabs) overnight at 37°C as recommended by the manufacturer. As a positive control for McrBC analysis, 0.5 µg of methylated plasmid (with one McrBC site; supplied by the manufacturer) was used. The nontreated total DNA samples were incubated in parallel under the same conditions as the McrBC-treated total DNA samples but without the McrBC enzyme.

Following the treatment with or without McrBC, the DNA of each total reaction mixture was separated in one 1% agarose gel in 1× TNE buffer (40 mM Tris-acetate, 20 mM sodium acetate, 2 mM EDTA, pH 7.5), stained with EtBr (Fig. 22, 26), and then transferred onto a Hybond N+ membrane (Amersham). The membrane was hybridized overnight at 45°C in UltraHyb-oligo buffer (Ambion) with a mixture of RTBV specific probes (Table 1), which were pooled and end labeled

with P32 by the use of polynucleotide kinase for hybridization. After 16 h of hybridization, the blot was washed two times with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.5% SDS solution for 30 min at 45°C and the signal was detected after 20 h to 5 days of exposure to a phosphor screen using a Molecular Imager (Typhoon FLA 8600; GE Healthcare Life Sciences). For repeated hybridizations, the membrane was stripped with 0.5× SSC–0.5% SDS for 30 min at 80°C and then with 0.1× SSC–0.5% SDS for 30 min at 80°C.

3.8. ROS BURST MEASUREMENT

The production of reactive oxygen species was measured upon treatment with bacterial elicitors flg22 and elf18 in 4 week-old *N. benthamiana* wild type and *A. thaliana* transgenic P6-CM and P6-D4 plants. Leaf discs of diameter 4 mm were placed into 96-well LIA plate and incubated overnight in 100 µL of ddH_2O in the darkness at room temperature. The next day water was replaced with a solution of 10 µg/ml horseradish peroxidase (Sigma-Aldrich) and 100µM luminol (Sigma-Aldrich). The plate was placed into a MicroLumat LB96P reader (Berthold Technologies) for 10 min to assess the basal level of luminescence. Upon treatment with bacterial elicitor luminescence was measured immediately after addition of flg22 or elf18 to a final concentration of 1 µM for 30min, and plotted in all figures as the peak of luminescence achi eved during the 30 min of measurements.

4. RESULTS

4.1. RTBV P4 CAN SUPPRESS CELL-TO-CELL SPREAD OF RNA SILENCING

As described in the introduction, RTBV encodes a unique protein P4 with previously unknown function, which is not possessed by any member of closely-related genus *Badnavirus* or other genera of the family *Caulimoviridae*. Based on the fact that this protein does not have any structural similarities with other plant viral proteins involved in replication, assembly, or movement of the virus, we hypothesized that it could be acquired to counteract the host plant defenses based on RNA silencing and/or innate immunity. In order to determine whether or not P4 possesses silencing suppressor activity we employed a classical transient assay in leaves of the *N. benthamiana* transgenic line 16c expressing green fluorescent protein (GFP). In this system, suppression of cell-autonomous and mobile silencing of the GFP transgene that is triggered by the agrobacterium-mediated inoculation of *N. benthamiana* line 16c leaves with GFP-expressing construct (sGFP) could be observed when sGFP is co-infiltrated with a construct expressing a suppressor protein and results in the maintenance of relatively high levels of GFP protein accompanied with increased green fluorescence signal. In contrast, co-infiltration of sGFP and proteins without silencing suppression activity leads at latter time points to the reduction of both GFP protein accumulation and green fluorescence signal in the infiltrated tissue, which is initially strongly green fluorescent due to superimposed expression of the ectopic and stably integrated GFP transgenes. In addition, activation of cell-autonomous GFP silencing causes sequence-specific degradation of GFP transcripts in tissues located outside from the infiltration zone defined as a short-distance movement process, which is initiated from a small group of cells, spread over a nearly constant number of 10–15 cells and indicated as characteristic red ring around the infiltrated spot (Himber et al. 2003).

For our experiment we used P4 protein-expressing cassete (or its HA-tagged version), which were co-delivered with sGFP construct expressing an endoplasmic reticulum targeted GFP variant known as mGFP5 in the leaves of *N. benthamiana* line 16c via infiltration with agrobacterial strains carring the sGFP and the putative suppressor protein expression cassettes. Simultaneously, empty agrobacteria were co-infiltrated with sGFP and used as a negative control for GFP silencing suppression, while an agrobacterial strain carrying the strong silencing suppressor TBSV p19-expressing cassette was used as a positive control (Fig. 11, A).

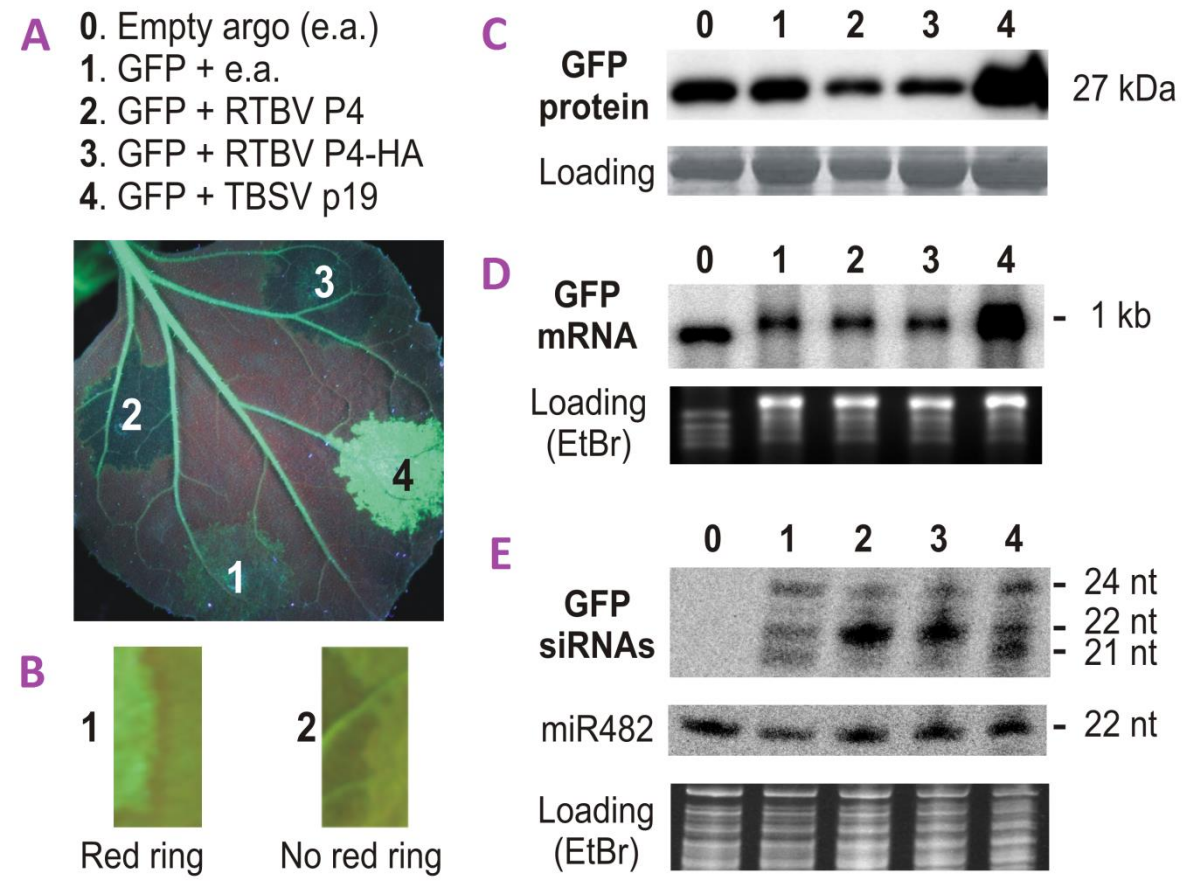
Suprisingly, unlike other supressors of GFP silencing, P4 or P4-HA co-expression led to the reduced level of GFP fluorescence signal at 3 dpi, compared to empty agro and p19 controls (Fig. 11, F), whereas the accumulation of GFP protein was comparable in all analysed samples (data not shown). Accordingly, at 8 dpi P4- and P4-HA-infiltrated *Nicotiana benthamiana* 16c tissues showed

even more significant decrease of GFP fluorescence signal, compared to empty agro and p19 controls, that was confirmed by western blot to be accompanied with the reduced accumulation of GFP protein (Fig. 11, A, C).

Nonetheless, similar to p19, P4 and P4-HA protein expression abolished the formation of characteristic red ring around the infiltrated patch at 8 dpi, confirming the involvement of these proteins in the suppression of GFP silencing movement (Fig. 11, B).

Thus, in spite of our suggestion of RTBV protein P4 as a potential suppressor of RNA silencing, we demonstrated that the co-expression of P4 (or its HA-tagged version) with sGFP in the leaves of *N. benthamiana* line 16c led to the significant reduction in both GFP fluorescence and GFP protein accumulation that could be interpreted as an enhancement of cell-autonomous GFP silencing by P4. At the same time, we could support our initial idea showing that the short-range cell-to-cell movement of GFP silencing represented by the characteristic red ring around the infiltrated spot was abolished by the activity of P4, meaning that, in fact, this protein could be involved in the suppression of cell-to-cell spread of silencing likely mediated by mobile 21-nt sRNAs.

To examine a role of RTBV P4 in the suppression of short-distance mobile silencing, that was shown in this transient assay system to be dependent on the action of 21-nt siRNAs, but not 24-nt siRNAs (Hamilton et al. 2002; Himber et al. 2003), we performed a Northern blot analysis of GFP siRNAs extracted from the infiltrated leaf tissues of *N. benthamiana* line 16c at 8 dpi (Fig. 11, E). Indeed, the accumulation of 21-nt siRNAs was strongly reduced by P4, while accumulation of 24-nt siRNAs was only slightly affected, confirming RTBV P4 protein ability to suppress short-distance GFP mobile silencing by interfering with 21-nt siRNAs biogenesis (Fig. 11, E). However, the reduced accumulation of 21-nt siRNAs was not associated with the suppression of cell-autonomous GFP silencing, but on the contrary with its enhancement. The P4-mediated enhancement of GFP silencing in the infiltrated patch can only be explained by the increased accumulation of 22-nt siRNAs (Fig. 11, A, E). Surprisingly, P4-mediated enhancement of cell-autonomous GFP silencing was correlated with reduced accumulation of GFP protein, compared to control, while GFP mRNA accumulation was equal between P4 and empty agro control, meaning that P4 acts as an enhancer of cell-autonomous GFP silencing at the level of GFP protein translation (Fig. 11, A, C, D). Thus, based on our results, we could confirm the RTBV P4 ability to suppress short-range mobile silencing signals probably by interfering with *N. benthamiana* DCL4 (NbDCL4) activity that leads to the reduced accumulation of 21-nt siRNAs, and provokes enhancement of cell-autonomous GFP silencing due to the increased production of 22-nt siRNAs by NbDCL2, which is in agreement with the previous data in *Arabidopsis* (Deleris *et al.,* 2006; Bouche et al. 2006) demonstrating the antagonism between DCL4 and DCL2 activities.



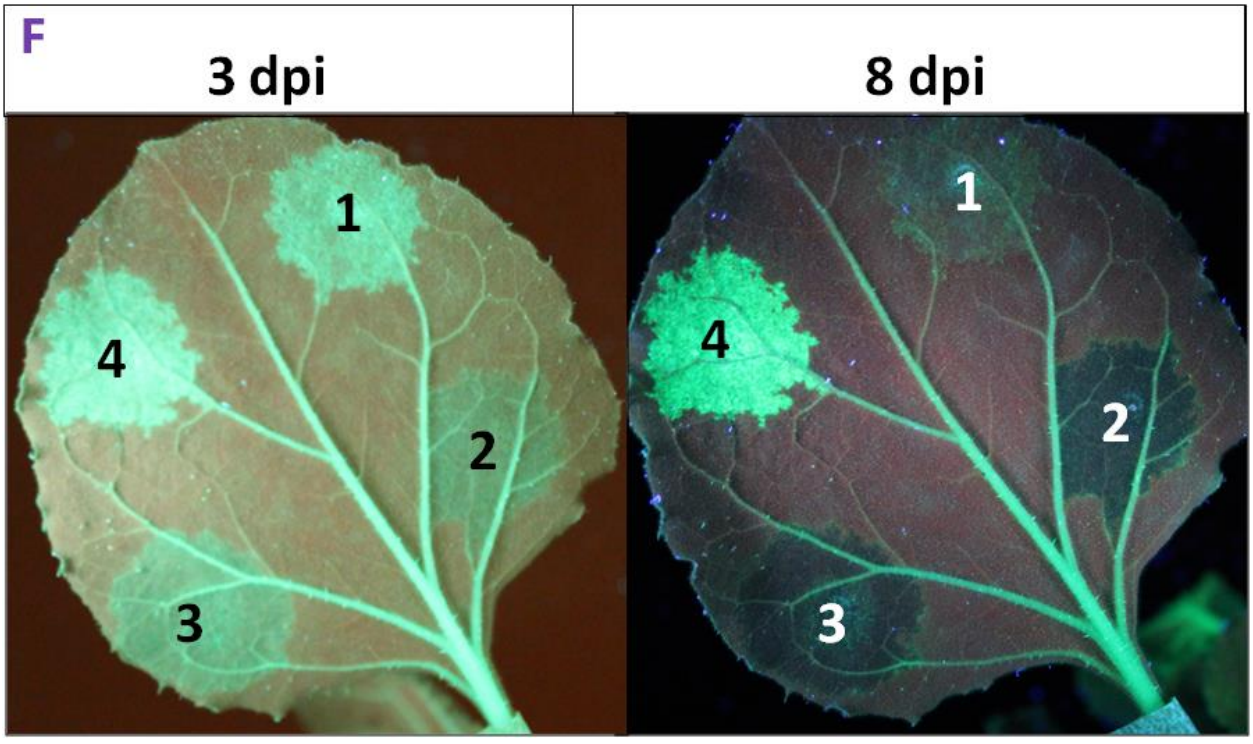


Figure 11. RTBV P4 suppresses cell-to-cell spread of green fluorescent protein (GFP) silencing, but enhances cell-autonomous GFP silencing in *Nicotiana benthamiana* 16c plants. **(A)** Analysis of Agrobacterium-infiltrated leaf tissues in line 16c under UV light at 8 days post-infiltration (dpi). Four leaf tissue patches shown in the image were co-infiltrated with the agro-strain carrying the GFP expression cassette (GFP) in combination with the agro-strain carrying no vector (patch 1), the

RTBV P4 (patch 2), the RTBV P4-HA (patch 3), or the TBSV p19 (patch 4) expression cassettes, and the picture was taken at 8 days post-infiltration. **(B)** The cropped images of the leaf patches co-infiltrated with GFP + empty agro (patch 1) and GFP + RTBV P4 (patch 2) are enlarged. A thin border of red tissue (red ring) is visible in the absence of P4, but not in its presence. **(C, D, E)** Molecular analysis of the agroinfiltrated tissues from **(A)** (lane numbers correspond to the patch numbers) by Western **(C)**, Northern **(D),** and sRNA blot hybridization **(E).** The Western blot membrane was probed with GFP protein-specific antibody and then stained with amidoblack for loading control (Rubisco). The Northern blot membrane was probed with the GFP mRNA specific probe; ethidium bromide (EtBr) staining of the agarose gel before blotting was used as a loading control. The sRNA blot membrane was successively hybridized with DNA oligonucleotide probes specific for the GFP 3′ untranslated region sequence-derived siRNAs and the *N. benthamiana* miRNA miR482; EtBr staining of the gel before blotting was used as a loading control. **(F)** Comparison of Agrobacterium-infiltrated leaf tissues in line 16c under UV light at 3 dpi and 8 dpi. Four leaf tissue patches shown in the image were co-infiltrated with the agro-strain carrying the GFP expression cassette (GFP) in combination with the agro-strain carrying no vector (patch 1), the RTBV P4 (patch 2), the RTBV P4-HA (patch 3), or the TBSV p19 (patch 4) expression cassettes, and the pictures of the same leaf were taken at 3 and 8 days post-infiltration.

4.2. RTBV P4 AND CAMV P6 SUPPRESS OXIDATIVE BURST

It is now clear that the majority of plant pathogens, including bacteria, fungi, oomycetes as well as viruses are recognized by innate immunity system of host plant leading to the activation of defense mechanisms, such as PTI and ETI that restrict pathogen infection at a particular site. However, both viral and non-viral pathogens have evolved proteins that are used to counteract with innate immunity responses and break resistance of the host plants (Zvereva and Pooggin 2012).

As described in the Introduction, the oxidative burst, that includes the production of large amounts of ROS at the plant cell surface, is one of the earliest plant responses to invasion of both viral and non-viral phytopathogenic microorganisms as well as to challenges by various elicitor molecules. For instance, flg22, the 22 amino acid active epitope of bacterial flagellin, is one of the most commonly used elicitors, which is perceived by the majority of plant species mediating rapid production of ROS (Felix *et al.,* 1999, Gomez-Gomez and Boller, 2000; Chinchilla et al, 2006).

Hence, to examine a role of two viral silencing suppressor proteins RTBV P4 and CaMV P6 (from strain CM1841, designated P6-CM) in the inhibition of early plant innate immunity responses, we measured flg22-triggered ROS burst in the leaves of *N. benthamiana* wild type plants transiently expressing RTBV P4 or CaMV P6-CM proteins. For that, we inoculated leaves of *N. benthamiana* plants with agrobacteria carrying P4- or P6-CM-expression constructs as well as with

empty agrobacteria as a control with the following collection of infiltrated leaf tissue at 2 dpi (Fig. 12, 13). The collected tissues were incubated in a solution of horseradish peroxidase and luminol, following treatement with bacterial flg22. As a result, the level of the extracellular ROS production in the *N. benthamiana* leaf tissues expressing RTBV P4 and CaMV P6-CM, estimated as the peak of luminescence exhibited by oxidized luminol and achieved during the 30 min of measurements, was significantly reduced by both proteins upon flg22 treatment, compared to empty agro control (Fig. 12, 13). Thus, we conclude that RTBV P4 and CaMV P6-CM in addition to their primary role in suppression of RNA silencing could be responsible for inhibition of the early plant innate immunity responses to viral infection. As stated above, no viral PAMP was conclusively identified so far except for dsRNA, which can trigger both RNA silencing and innate immunity responses (Niehl *et al.,* 2016).

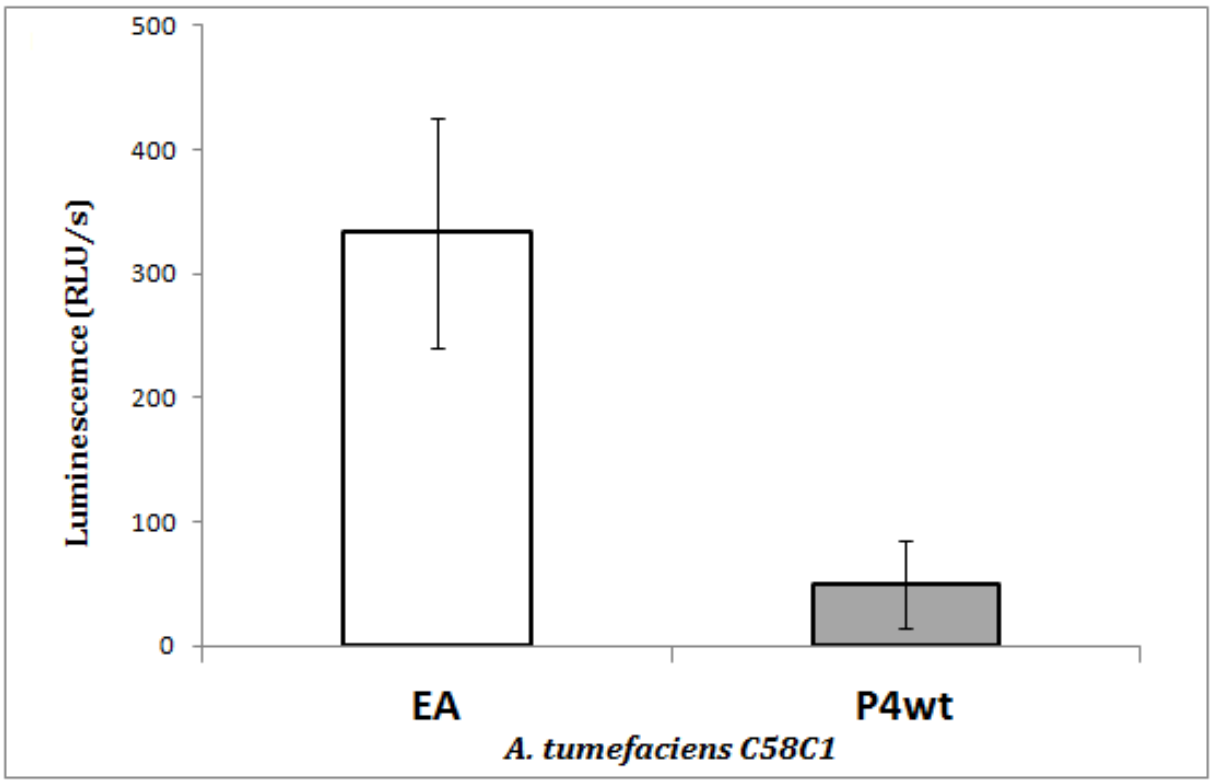


Figure 12. ROS burst triggered by flg22 in *Nicotiana benthamiana* leaves infiltrated with an empty *Agrobacterium tumefaciens* strain C58C1 (EA) or the C58C1 carrying a binary vector with the 35S promoter-driven RTBV P4 expression cassette (P4wt), plotted as RLU/s following the addition of 1μM flg22 peptide.

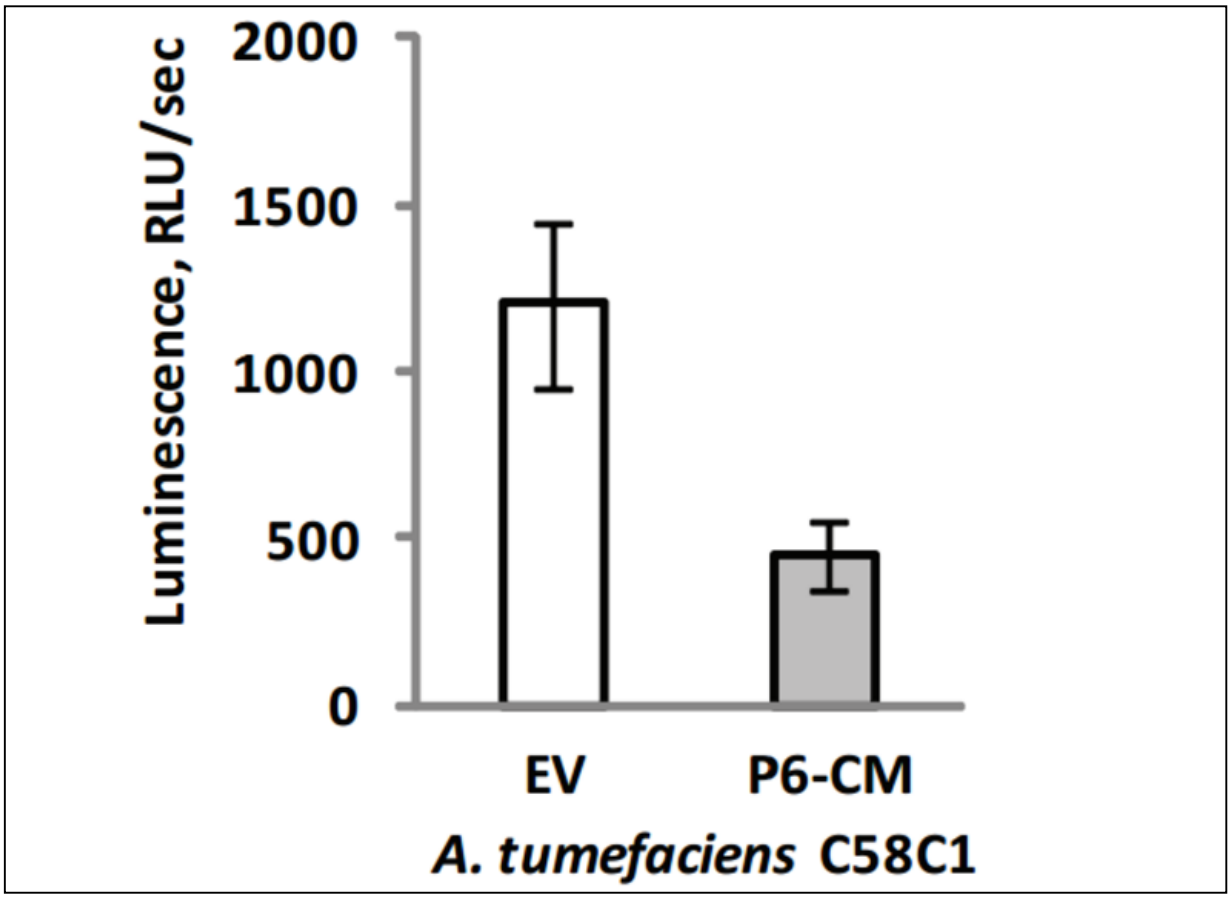


Figure 13. ROS burst triggered by flg22 in *Nicotiana benthamiana* leaves infiltrated with an empty *Agrobacterium tumefaciens* strain C58C1 (EV) or the C58C1 carrying a binary vector with the 35S promoter-driven CaMV P6-CM expression cassette (P6-CM), plotted as RLU/s following the addition of 1μM flg22 peptide.

4.3. RTBV P4 COUNTERACTS TBSV P19-MEDIATED SUPPRESSION OF CELL-AUTONOMOUS RNA SILENCING

p19 proteins of TBSV and CymRSV are strong suppressors of RNA silencing having the ability to sequester siRNAs duplexes and thus inactivate the formation of silencing effector complexes. In *N. benthamiana* 16c plants, TBSV p19 suppresses GFP RNA silencing through its ability to bind GFP siRNAs that results in a prolonged green fluorescence of GFP as well as high levels of GFP mRNA and GFP protein accumulation (Ye *et al.,* 2003).

Given the higher affinity of TBSV p19 to bind DCL4 dependent 21-nt siRNAs duplexes rather than DCL2 dependent 22-nt duplexes (Vargason *et al.,* 2003), we co-expressed it with both RTBV P4 and sGFP constructs in the leaves of *N. benthamiana* line 16c in order to test the ability of RTBV P4 to counteract TBSV p19-mediated suppression of GFP silencing by promoting the production of 22-nt siRNAs (Fig. 14, A). Indeed, co-expression of RTBV P4 with TBSV p19 substantially reduced both GFP mRNA and protein accumulation at 8 dpi, compared to p19 alone, which was accompanied

with increased accumulation of 22-nt GFP siRNAs (Fig. 14, C-E). Accordingly, cell-to-cell spread of GFP silencing manifested by red ring development was still abolished by co-expression of P4 and p19 both having ability to block the biogenesis of 21-nt siRNAs duplexes (Fig. 14, B, E). Taken together, we can conclude that RTBV P4 counteracts TBSV p19-mediated suppression of cell autonomous GFP silencing by promoting the accumulation of 22-nt GFP siRNAs, which may not be as efficiently sequestered by TBSV p19 as 21-nt siRNAs and, consistent with our findings, mediate cell-autonomous GFP silencing in the absence of 21-nt siRNAs, but not its short-range cell-to-cell spread.

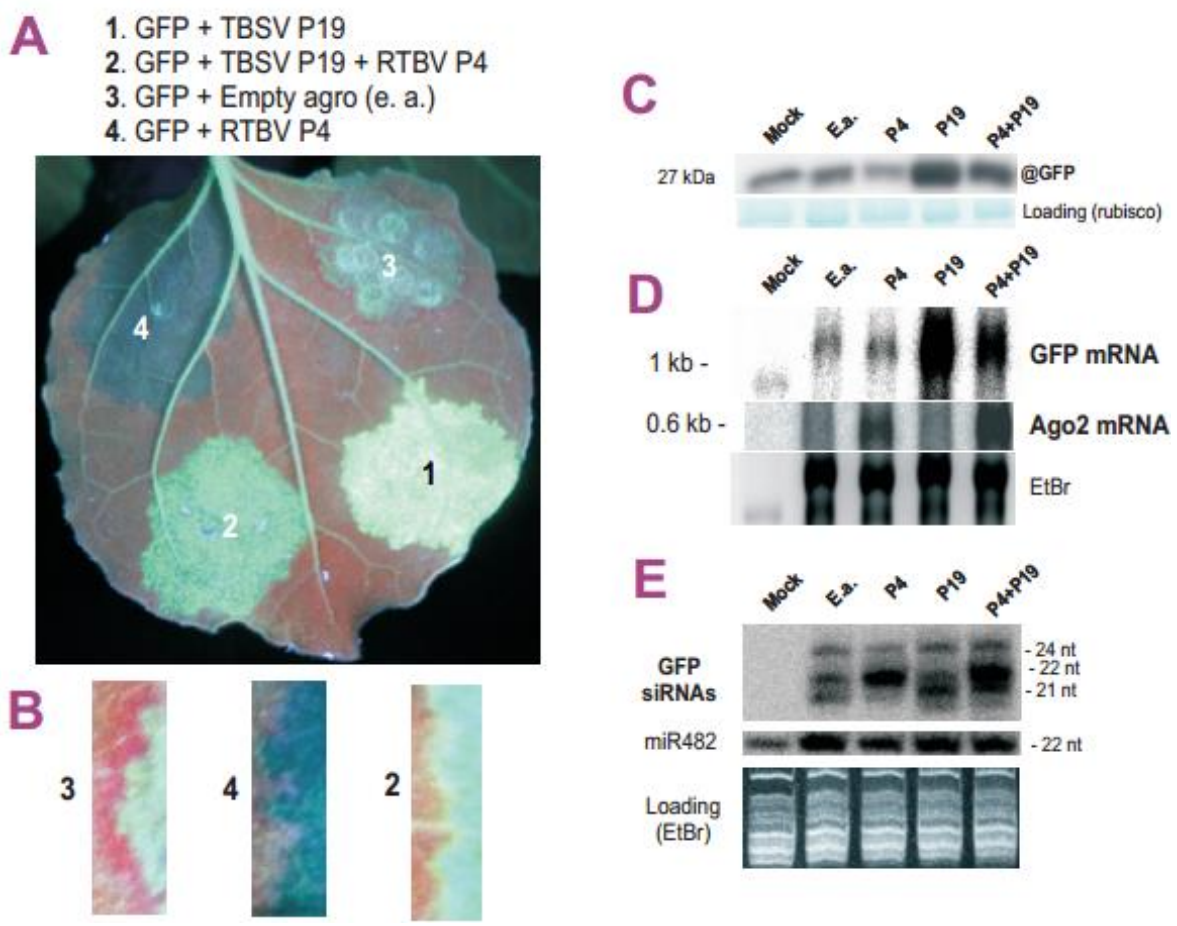


Figure 14. Co-infiltration of RTBV P4 with TBSV p19 protein. **(A)** Analysis of Agrobacterium-infiltrated leaf tissues in line 16c under UV light at 8 dpi. Four leaf tissue patches shown in the image were co-infiltrated with the agro-strain carrying the GFP expression cassette (GFP) in combination with the TBSV p19 alone (patch 1), the RTBV P4 + TBSV p19 together (patch 2), the agro-strain carrying no vector (patch 3), or the RTBV P4 alone (patch 4) expression cassettes, and the picture was taken at 8 days post-infiltration. **(B)** The cropped images show the boarder between non-infiltrated (on the left) and infiltrated (on the right) tissues of the leaf patches co-infiltrated with GFP + empty agro (patch 3), GFP + RTBV P4 (patch 4) and GFP + RTBV P4 + TBSV p19 (patch 2) are enlarged. **(C, D, E)** Molecular analysis of the agroinfiltrated tissues from **(A)** (lane numbers correspond to the patch numbers) by Western **(C)**, Northern **(D),** and sRNA blot hybridization **(E).** The Western blot membrane was probed with GFP protein-specific antibody

and then stained with amidoblack for loading control (Rubisco). The Northern blot membrane was probed with the GFP mRNA- and then Ago2 mRNA-specific probes; Ethidium bromide (EtBr) staining of the agarose gel before blotting was used as a loading control. The sRNA blot membrane was successively hybridized with DNA oligonucleotide probes specific for the GFP mRNA 3′ untranslated region sequence-derived siRNAs and the *N. benthamiana* miRNA miR482; EtBr staining of the gel before blotting was used as a loading control.

4.4. MUTATION OF THE P4 F-BOX MOTIF INHIBITS SUPPRESSION OF CELL-TO-CELL SPREAD OF RNA SILENCING AND OXIDATIVE BURST

Some plant viruses have evolved proteins which are used to target components of host plant RNA silencing machinery for degradation. For instance, P0 proteins from *Pea enation mosaic virus-1* (PEMV-1) and *Cucurbit aphid-borne yellows virus* (CABYV) were reported to cause the 26S proteasome-dependent degradation of AGO1 probably by interaction with the ASK1 and ASK2 components of ubiquitinating SCF complexes through the minimal F-box-like motif ((**LP**xx(**L/I**)x$^{10-13}$**P**)) (Fusaro *et al.,* 2012; Bortolamiol *et al.,* 2007). As a similar motif was found in the sequence of RTBV P4 (**LP**PI**I**x^{9}**P**) (Fig. 15), we suggested that it could be essential for the silencing or/and innate immunity suppressor activities of P4.

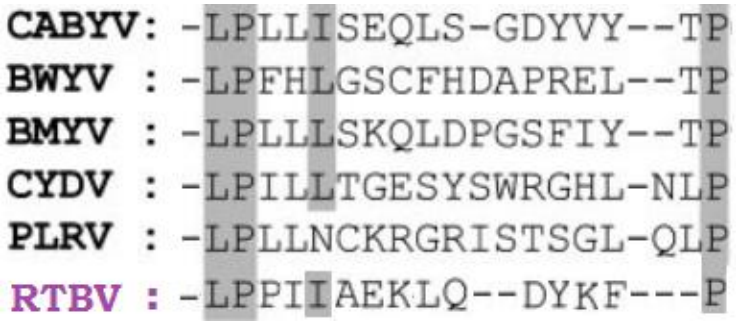


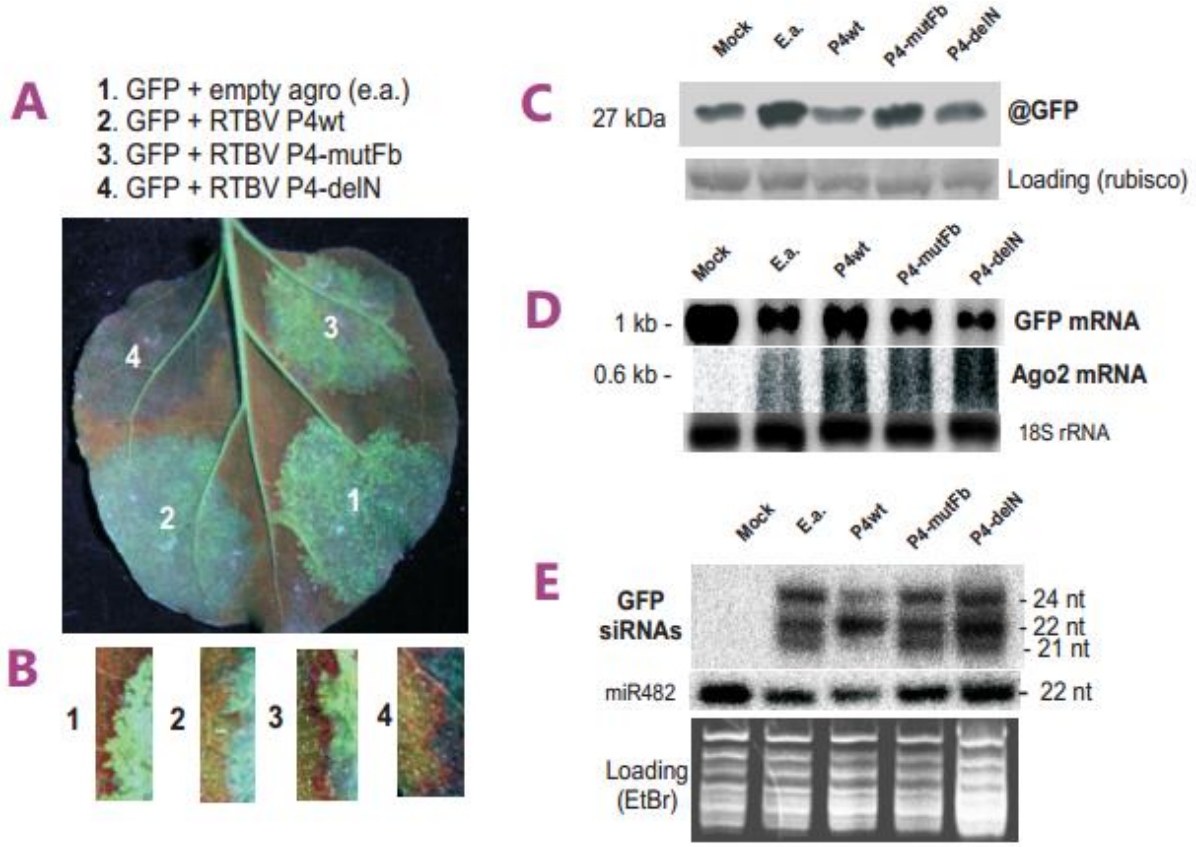
Figure 15. F-box motif sequences of RTBV P4 and P0 of *Cucurbit aphid-borne yellows virus* (CABYV), *Beet western yellow virus* (BWYV), *Beet mild yellowing virus* (BMYV), *Cereal yellow dwarf virus* (CYDV), *Potato leafroll virus* (PLRV).

To test our hypothesis we introduced triple amino acid substitution, changing leucine-283, proline-284 and proline-297 to alanine residues, (**AA**PI**I**x^{9}**A)** into the F-box-like motif of RTBV P4 (Fig. 16), which were shown to be essential for the suppressor silencing activity of P0 protein from PEMV-1, and used the obtained mutant protein (P4-mutFb) in the transient assay in leaves of the *N. benthamiana* transgenic line 16c.

```
                 * *                 *
P4wt      : LPPIIAEKLQDYKFP
P4_mutFb  : AAPIIAEKLQDYKFA
              PIIAEKLQDYKF
```

Figure 16. Mutation of the F-box-like motif in the RTBV protein P4. The amino acid changes are indicated with stars.

In support of our hypothesis, P4-mutFb transiently co-expressed with sGFP construct in leaves of the *N. benthamiana* line 16c did not enhance cell-autonomous GFP silencing compared to wild type P4 (P4-wt), and was not able to interfere with the appearance of red ring around the infiltrated patch at 8 dpi (Fig. 17, A, B, E). Moreover, unlike P4-wt, P4-mutFb was not able to suppress the accumulation of 21-nt GFP siRNAs and did not affect the accumulation of 22-nt GFP siRNAs. In addition, the GFP mRNA and protein levels were shown to be comparable with those of the control tissue containing empty agrobacteria, at 8 dpi (Fig. 17, C-E).



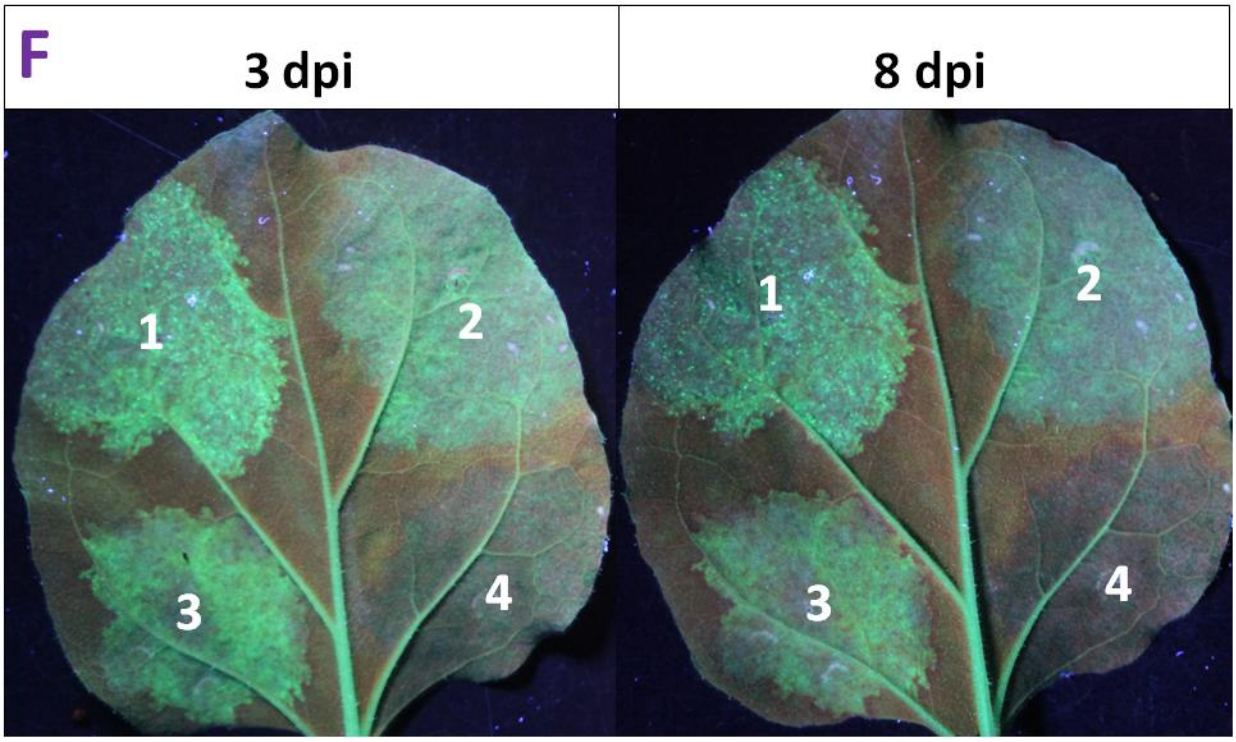


Figure 17. Effects of the mutations in RTBV P4 protein on its ability to interfere with RNA silencing in *N. benthamiana* 16 leaves. **(A)** Analysis of Agrobacterium-infiltrated leaf tissues in line 16c under UV light at 8 dpi. Four leaf tissue patches shown in the image were co-infiltrated with the agro-strain carrying the GFP expression cassette (GFP) in combination with the agro-strain carrying no vector (patch 1), the RTBV P4 (patch 2), the RTBV P4-mutFb (patch 3), or the RTBV P4-delN (patch 4) expression cassettes, and the picture was taken at 8 days post-infiltration. **(B)** The cropped images of the leaf patches co-infiltrated with GFP + empty agro (patch 1), GFP + RTBV P4 (patch 2) and GFP, RTBV P4-mutFb (patch 3), RTBV P4-delN (patch 4) are enlarged. **(C, D, E)** Molecular analysis of the agroinfiltrated tissues from **(A)** (lane numbers correspond to the patch numbers) by Western **(C)**, Northern **(D),** and sRNA blot hybridization **(E).** The Western blot membrane was probed with GFP protein-specific antibody and then stained with amidoblack for loading control (Rubisco). The Northern blot membrane was probed with the GFP mRNA and Ago2 specific probes, as well as 18s rRNA probe, which is used as a control. The sRNA blot membrane was successively hybridized with DNA oligonucleotide probes specific for the GFP 3′ untranslated region sequence-derived siRNAs and the *N. benthamiana* miRNA miR482; EtBr staining of the gel before blotting was used as a loading control. **(F)** Comparison of Agrobacterium-infiltrated leaf tissues in line 16c under UV light at 3 dpi vs 8 dpi. Four leaf tissue patches shown in the image were co-infiltrated with the agro-strain carrying the GFP expression cassette (GFP) in combination with the agro-strain carrying no vector (patch 1), the RTBV P4 (patch 2), the RTBV P4-mutFb (patch 3), or the RTBV P4-delN (patch 4) expression cassettes, and the pictures were taken at 3 and 8 days post-infiltration.

To verify if the introduced mutation did not affect the stability of P4 protein, we measured the accumulation of P4-mutFb and P4-wt by Western blotting, using P4-specific antibodies raised with the P4 peptide not affected by the F-box or delN mutations (see Materials and Methods). The levels of P4-mutFb and P4 wild type proteins were comparable at 3 dpi, while P4-mutFb protein accumulated even at higher level than P4-wt at 4 dpi, indicating the increased stability of the mutant protein in this transient expression system (Fig. 18). Note that at 8 dpi P4-wt and P4-mutFb were below detection with this antibody (data not shown). Thus, we concluded that the F-box-like motif of RTBV P4 is required for P4-mediated suppression of cell-to-cell spread of GFP silencing as well as for P4-mediated enhancement of cell-autonomous GFP silencing.

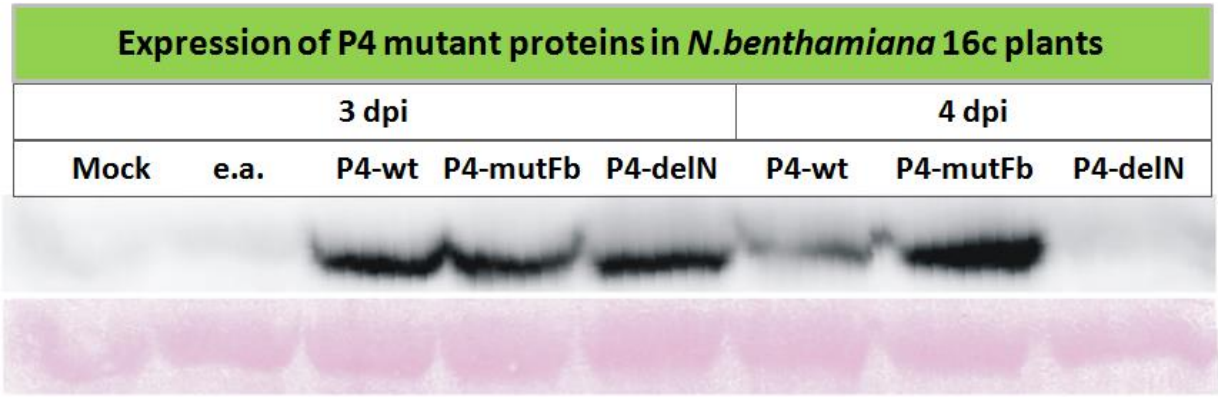


Figure 18. Western blot analysis of P4 wild type (P4-wt) and P4 mutant proteins (P4-mutFb and P4-delN) accumulation in the *Nicotiana benthamiana* 16c plants. Ponceau staining of the blot membrane is shown as loading control.

To test if P4 F-box motif is required for suppression of the early plant innate immunity responses, such as oxidative burst, we compared flg22-triggered ROS production in leaves of *N. benthamiana* wild type plants transiently expressing P4 wild type and P4-mutFb proteins. Unlike wild type P4, expression of P4-mutFb did not significantly affect the accumulation of extracellular ROS upon flg22 treatment at 2 dpi, which was comparable with the control (Fig. 19), bringing us to the conclusion that F-box-like motif, being essential for P4 anti-silencing activity, is also required for P4-mediated suppression of innate immunity, particularly oxidative burst.

In addition, we found that transient expression of P4-wt (but not P4-mutFb) protein lead to increased accumulation of *N. benthamiana AGO2* mRNA (Figure 17, C). Interestingly, this mRNA was pre-induced by the empty agrobacteria and its level was further elevated by P4-wt (but not P4-mutFb-expressing bacteria) (Figure 17, C). This suggests that the P4 activity in suppression of silencing cell-to-cell movement and/or innate immunity responses as well as the agrobacterial infection are both monitored by the plant defence system involving AGO2. Indeed, AGO2 has been implicated not only in antiviral defence based on RNA silencing (as described above), but also in innate immunity-based defense against bacterial pathogenes in *Arabidopsis* (Zhang *et al.,* 2011).

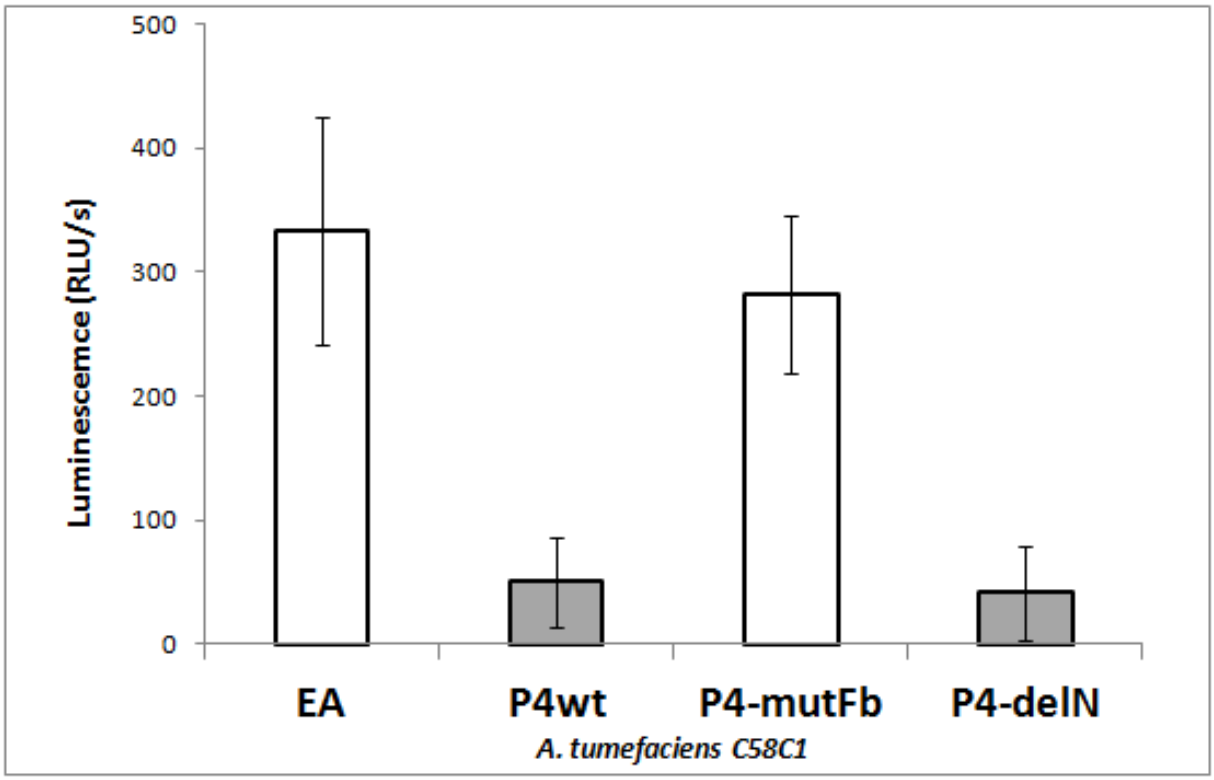


Figure 19. ROS burst triggered by flg22 in *Nicotiana benthamiana* leaves at 2 days post-infiltration with an empty *Agrobacterium tumefaciens* strain C58C1 (EA) or the C58C1 carrying a binary vector with the 35S promoter-driven RTBV P4 (P4wt), P4-mutFb and P4-delN expression cassettes, plotted as RLU/s following the addition of 1μM flg22 peptide.

4.5. THE N-TERMINAL RTBV P4 REGION IS REQUIRED FOR P4 ANTI-SILENCING ACTIVITY, BUT DISPENSIBLE FOR P4-MEDIATED SUPPRESSION OF INNATE IMMUNITY

Like other plant and animal viruses, plant pararetroviruses evolved different strategies in order to express downstream proteins. For example, CaMV encodes P6 protein, which functions as a transactivator of the CaMV pgRNA polycistronic translation. Alternatively, RTBV evolved several mechanisms, such as leaky scanning, proteolytic processing of polyprotein and expression from spliced mRNA. The letter mechanism, which has not been found in other pararetroviruses, is used for expression of downstream *ORF IV* gene from sgRNA formed by splicing of pgRNA that brings together in frame the leader-based sORF1 with the 5' end of *ORF IV* and releases a large intron of 6.3 kb (Futterer *et al.,* 1994) (Fig. 20). The resulting ORF codes for the P4 protein with N-terminal extension of 26 amino acids with respect of the methyonine encoded by the *ORF IV* ATG start codon. Since splicing is essential for RTBV infectivity (Futterer *et al.,* 1994), it is assumed that P4 is

translated from the spliced RNA. However, it cannot be excluded that internal initiation of tranlation at the *ORF IV* start codon may result in an N-terminally truncated variant of the P4 protein.

To examine an importance of the first 26 amino acids of RTBV P4 for P4-mediated silencing and innate immunity supression activities, we made a truncated P4 expression construct (P4-delN) and expressed it in leaves of *N. benthamiana* transgenic line 16c (Fig. 17). The expression of P4-delN protein resuted in reduced levels of both GFP fluorescence and GFP protein at 8 dpi, compared to empty agro control (Fig. 17, A, C, E). Interestingly, the reduction of GFP fluorescence was more pronounced for P4-delN than P4-wt, while GFP protein accumulation was comparable between P4-wt and P4-delN. Northern blot analysis showed that GFP mRNA accumulation in the presence of P4-delN was lower than that in the presence of P4-wt, suggesting that P4-delN protein did not repress GFP translation as efficiently as P4-wt. Furthermore, the appearance of the red ring was not fully abolished by P4-delN, compared to P4-wt. Small RNA blot hybridization analysis revealed that the shift from 21-nt to 22-nt GFP siRNA production was less pronounced in the case of P4-delN, compared to P4-wt and the accumulation of 21-nt siRNA was not abolished by P4-delN. These findings explain the differences in red ring phenotype and GFP mRNA accumulation suggesting that the N-terminal extension is required for full activity of the P4 protein in suppression of cell-to-cell movement of silencing mediated by 21-nt siRNAs and in concomitant enhancement of cell-automomous silencing through translational repression mediated by 22-nt siRNAs (Fig. 17, B, E). To verify if stability of P4 protein was affected by the deletion we compared the accumulation of P4-delN and P4-wt by Western blot analysis. Interestingly the accumulation of both proteins was comparable at 3 dpi, while P4-delN was barely detectable at 4 dpi, compared to P4-wt (Fig. 18), suggesting that in this transient assay system the N-terminal extension stabilized the P4 protein. This may explain incomplete abolishment of the red ring by 8 dpi, when P4-delN accumulation might be much lower than that of P4-wt. At the same time, higher activity of P4-delN in inducing 22-nt siRNA levels at earlier time points when it accumulates at levels compatable to P4-wt, may explain stronger enhancement of GFP silencing as manifested by GFP fluorescence (Fig. 17, F).

We then tested if the N-terminal extension is required for suppression of the early innate immunity responses. Similar to P4-wt, P4-delN was able to suppress ROS burst in *N. benthamiana* wild type plants upon treatment with bacterial flg22 (Fig. 19). Note that the ROS burst was measured at 2 dpi when both P4-wt and P4-delN accumulated at comparable levels (data not shown). Based on these results, we concluded that the N-terminal extention of RTBV P4 is dispensible for P4-mediated suppression of innate immunity.

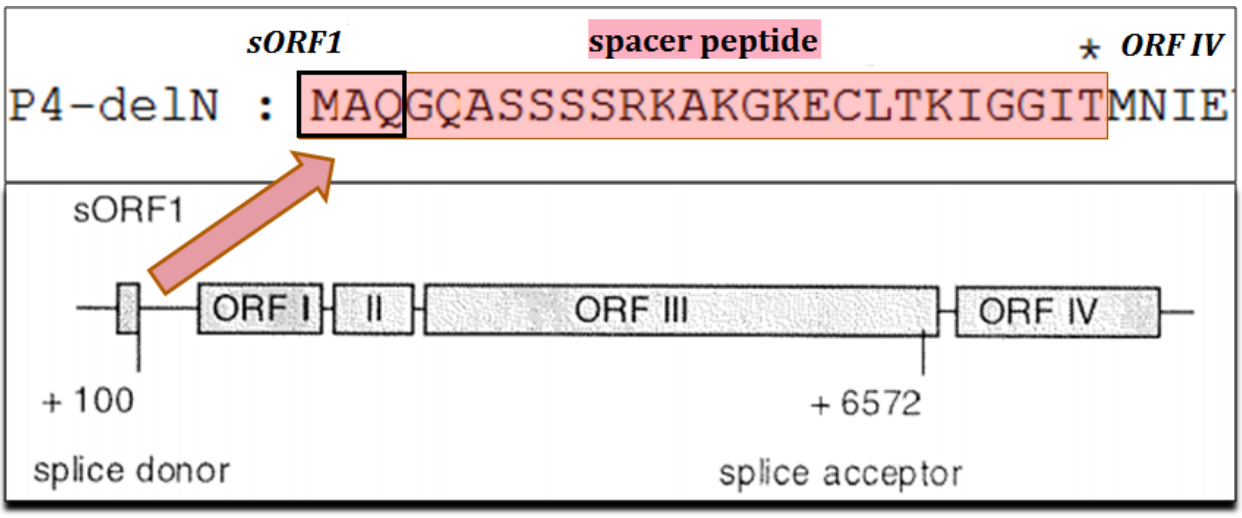


Figure 20. RTBV P4-delN mutant, which lacks 26 N-terminal amino acids of P4-wt,including the first three amio acids encoded by a 7-codon sORF1 (boxed) and the spacer peptide of 23 amino acids.

4.6. P6 FROM STRAIN D4 FAILED TO SUPPRESS OXIDATIVE BURST, BUT NOT RNA SILENCING

As was mentioned above, *Cauliflower mosaic virus* P6 protein plays a key role in several essential activities of viral infection cycle including translation of the 35S RNA, formation of inclusion bodies, viral movement as well as suppression of host plant antiviral responses based on RNA silencing and innate immunity. Besides, P6 was confirmed to be associated with viral pathogenicity inducing virus-like symptoms upon transformation into several species of plants. For example, P6 from CM1841 CaMV strain (P6-CM) induces strong chlorotic symptoms and stunting in transgenic *Arabidopsis thaliana* plants. However, these symptoms are strain-specific and *Arabidopsis* plants transformed with P6 from CaMV D4 strain, which, unlike CM1841, develops severe systemic symptoms in Solanaceous hosts, remained symptomless, showing that in addition to its role in virulence, P6 also functions as a main determinant of the host range (Schoelz *et al.,* 1986; Yu *et al.,* 2003).

Interestingly, both P6-D4 and P6-CM proteins exhibited strong antisilencing activity when expressed in Col-0 transgenic plants (Shivaprasad *et al.,* 2008). Therefore we decided to investigate whether P6-D4 and P6-CM are able to suppress host plant innate immunity responses in the transgenic plants, since the pathogenicity and the host range of different CaMV strains might be determined by P6-mediated suppression of innate immunity, in addition to its antisilencing activity.

To test the ability of P6 proteins from strains CM1841 and D4 to suppress the early plant innate immunity responses, such as oxidative burst, we compared flg22-triggered ROS production in *Arabidopsis thaliana* transgenic line plants expressed P6-D4 and P6-CM proteins. For that, we collected leaf tissue samples of D4 and CM transgenic lines, treated them with the bacterial PAMPs

(flg22 and elf18 peptides) and then incubated in a solution of horseradish peroxidase and luminol. As a result, the level of the extracellular ROS production in *Arabidopsis thaliana* leaf tissues upon flg22 and elf18 treatments, estimated as the peak of luminescence exhibited by oxidized luminol and achieved during the 30 min of measurements, was significantly reduced only by P6-CM protein, compared to Col-0 control, while P6-D4 was not able to suppress ROS burst (Fig. 21, B). In addition, these results were confirmed by the evidence that transgenic expression of the P4-CM, but not P6-D4 protein, activated the plant TOR kinase, which resulted in down-regulation of cellular autophagy and suppression of bacterial pattern-triggered immunity in *Arabidopsis thaliana* plants (see the results in the publication Zvereva, Golyaev *et al.,* 2016 in Annex). Thus, we show that, unlike CaMV P6-CM, P6-D4 protein is not able to interfere with PTI-based responses in *A. thaliana* plants, confirming that the antisilencing activity of P6-D4 is not sufficient for its effector function in PTI suppression.

Consistent with the previous findings (Shivaprasad *et al.,* 2008), both P6-D4 and P6-CM proteins exhibited antisilencing activities as they could interfere with DCL4-mediated processing of dsRNA precursors of tasiRNAs (Fig. 21, A). Indeed, RNA blot hybridization analysis of total plant RNA using the siR255-specific probe indicated that both transgenic lines accumulate long RNA precursors of siR255 tasiRNAs. These long siR255 precursors ranging in size from ~35 to ~600 nt were detected in both D4 and CM lines, but not in control Col-0 plants (Fig. 21, A). Furthermore, siR255 precursors were found to be more abundant in the D4 transgenic line, probably because of the higher level of P6-D4 protein accumulation measured by Western blot analysis (Fig. 21, B). Accordingly, significant reduction in siR255 accumulation level was observed only in the D4 line (Fig. 21, A). Thus, we confirmed that both P6-D4 and P6-CM proteins implicated in RNA silencing suppression interfering with DCL4-mediated tasiRNA processing in *Arabidopsis thaliana* transgenic line plants, while P6-D4 exhibited stronger antisilencing activity than P6-CM.

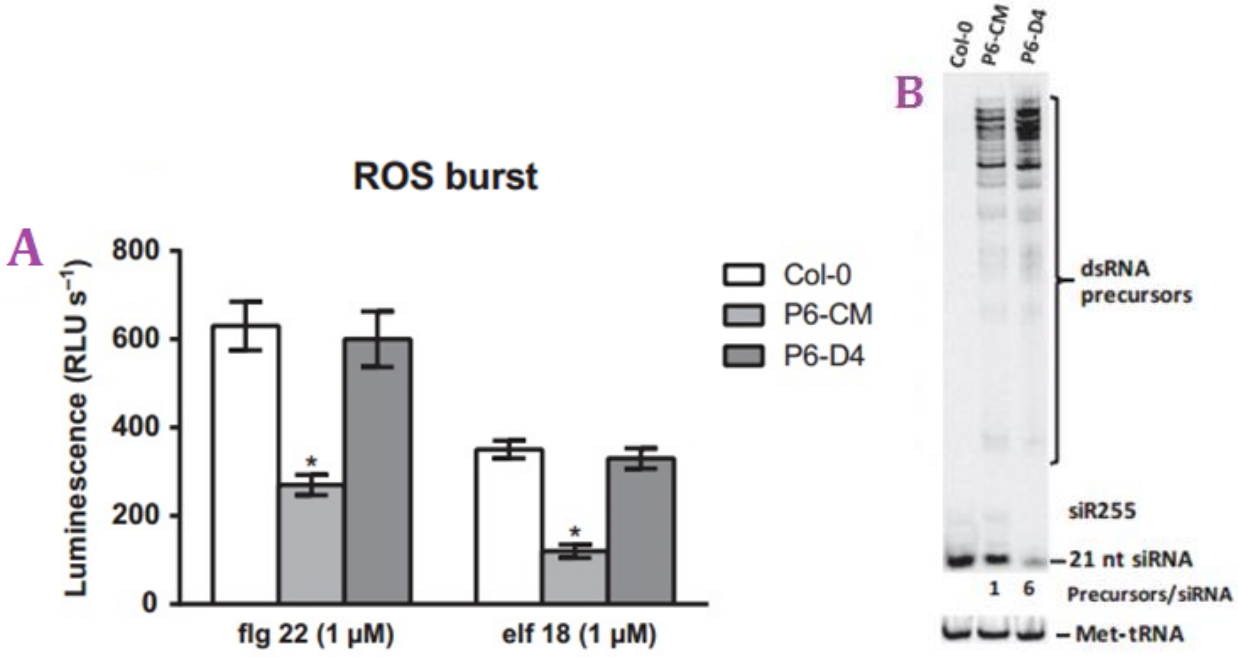


Figure 21. Effects of P6 homologs from *Cauliflower mosaic virus* (CaMV) strains CM1841 and D4 on bacterial reactive oxygen species (ROS) burst and double-stranded dsRNA processing in *Arabidopsis thaliana*. **(A)** ROS burst triggered by the bacterial microbe-associated molecular patterns (MAMPs) flg22 and elf18 on the P6-transgenic and control plants, plotted as the peak of relative luminescence units (RLU)/s during 30 min of measurements following the addition of 1μM MAMP peptide. **(B)** Blot hybridization analysis of total RNA from the P6-transgenic and control plants. The blot membranes were successively hybridized with short DNA probes specific for plant 21-nt transacting short interfering RNA (siRNA; siR255) and Methyonine transfer-RNA (Met-tRNA). Positions of the siRNA and its long dsRNA precursors are indicated and the precursor/siRNA relative ratios are shown under the respective scan, with the ratio for P6-CM set to 1.

4.7. RTBV CIRCULAR DSDNA EVADES CYTOSINE METHYLATION IN INFECTED RICE PLANTS

In parallel with my experiments on RTBV P4 activities in suppression of RNA silencing in *N. benthamiana*, Dr. Rajeshwaran, a postdoctoral fellow in our group, found out that rice plants infected with RTBV accumulate massive quantities of 21-, 22-and 24-nt viral siRNAs from the RTBV pgRNA leader region, which are likely produced by multiple OsDCLs, including OsDCL3 (see the publication Rajeswaran, Golyaev *et al.,* 2014a in the Annex). We therefore decided to examine whether or not these siRNAs accumulating in RTBV-infected rice plants direct methylation of RTBV dsDNA. To address this question, we exploited the cleavage activity of the McrBC methylation-dependent enzyme, which recognizes 5'-methylcytosines in an R^mC (R = A or G) context and cleaves between two recognition sites (Rajeswaran *et al.,* 2014b). As a plant material for this experiment, we used two different ecotypes of rice plants Taipei 309 and Nipponbare JB33, which were previously shown to be susceptible to RTBV infection. For inoculation of rice plants we used *Agrobacterium tumefaciens* strain GV3859 harboring the infectious clone of RTBV isolate Philippines or the empty vector pBin19. The agro-strains were inoculated into the stem of 4-week old rice plants and at 50 dpi systemic leaf tissues of the rice plants were evaluated for RTBV symptomes and harvested for molecular analysis. As a control, non-inoculated leaf tissue was harvested along with inoculated samples.

As any circular viral dsDNA with at least one 5′ methylcytosine in an R^mC context should be digested by McrBC, we isolated total DNA from RTBV infected and control samples, treated with McrBc and then loaded on a 1% agarose gel together with total DNA aliquots of the same samples treated under the same conditions but without McrBC. EtBr staining revealed that the rice genomic DNA contained in all McrBC-treated samples was almost fully digested by the enzyme, indicating that it was extensively methylated (Fig. 22). As a control, a methylated plasmid subjected to McrBC treatment was digested, yielding expected fragments (Fig. 23).

To evaluate the methylation status of RTBV DNA we perfomed Southern blotting hybridization using a mixture of RTBV forward and reverse strand-specific probes that allowed us to detect all major forms of viral DNA and measured their relative levels of accumulation. The results revealed two major forms of circular viral dsDNA of expected sizes, the more abundant open circular dsDNA and the less abundant covalently closed (supercoiled) dsDNA, both appeared to be resistant to McrBC (Fig. 23, 24). Thus, we concluded that the major fraction of viral genomic DNA (i.e. the supercoild dsDNA) accumulating in the nucleus for Pol II-mediated transcription of pgRNA is not methylated in RTBV-infected rice plants Taipei 309 (T309) and Nipponbare JB33. In addition, using a strand-specific probe we detected RTBV strong-stop DNA, which is a common feature of pararetroviruses produced at the first step of reverse transcription of pgRNA, where viral RT primed with plant Met-tRNA transcribes the pgRNA leader sequence and stops at the 5'-end of pgRNA (followed by the template switch step and resumption of reverse transcription at the 3'-end of pgRNA). Unexpectedly, despite this viral DNA form is single-stranded (not detectable with RTBV forward (sense) strand-specific probe Rtbv7722_as probe, see Fig. 24), it appeared to be sensitive to McrBC treatment in two of the three samples (Fig. 23), possibly as a result of unspecific activity of McrBC.

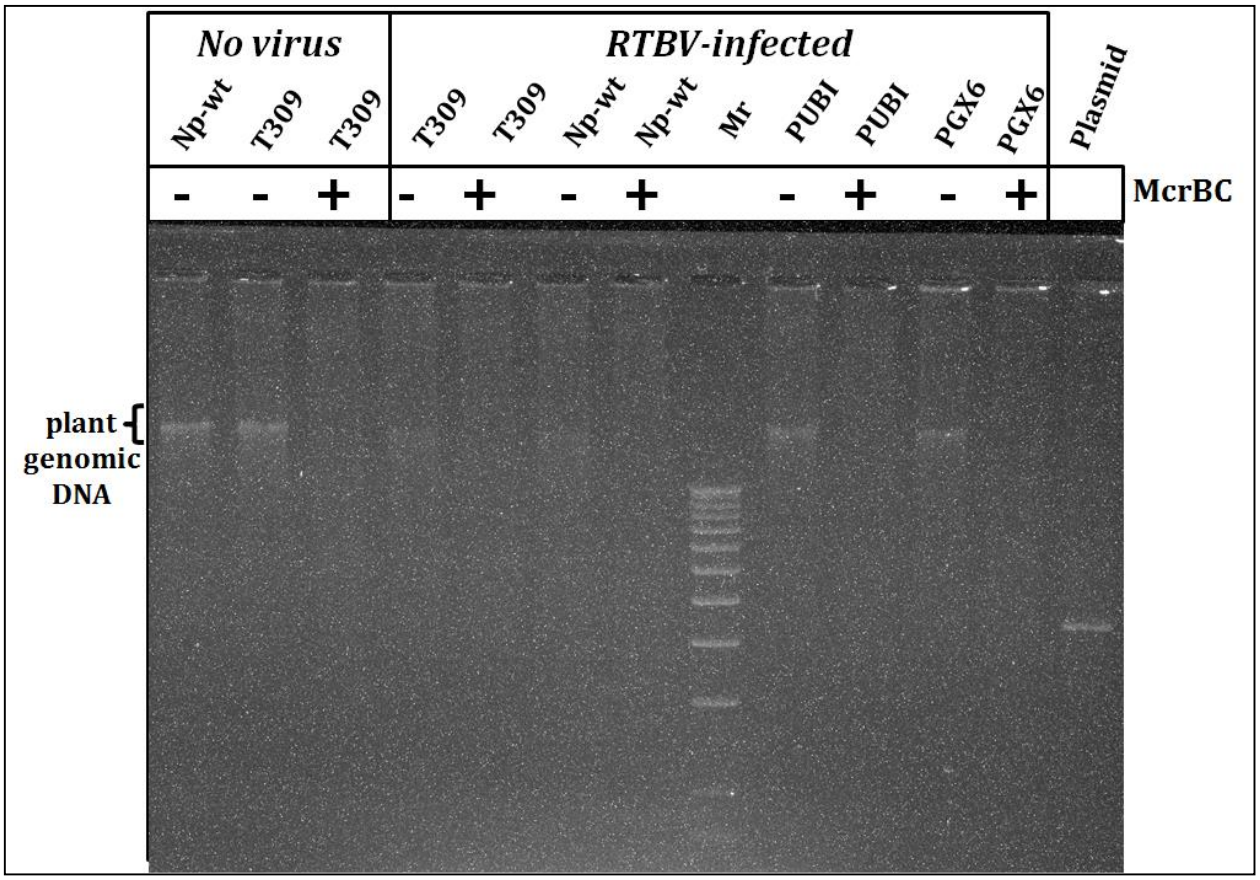


Figure 22. EtBr staining of the gel containing RTBV-infected and control rice samples. As a positive control (plasmid), a methylated plasmid DNA was subjected to McrBC treatment. As a DNA size marker, a 1-Kb+ ladder was used.

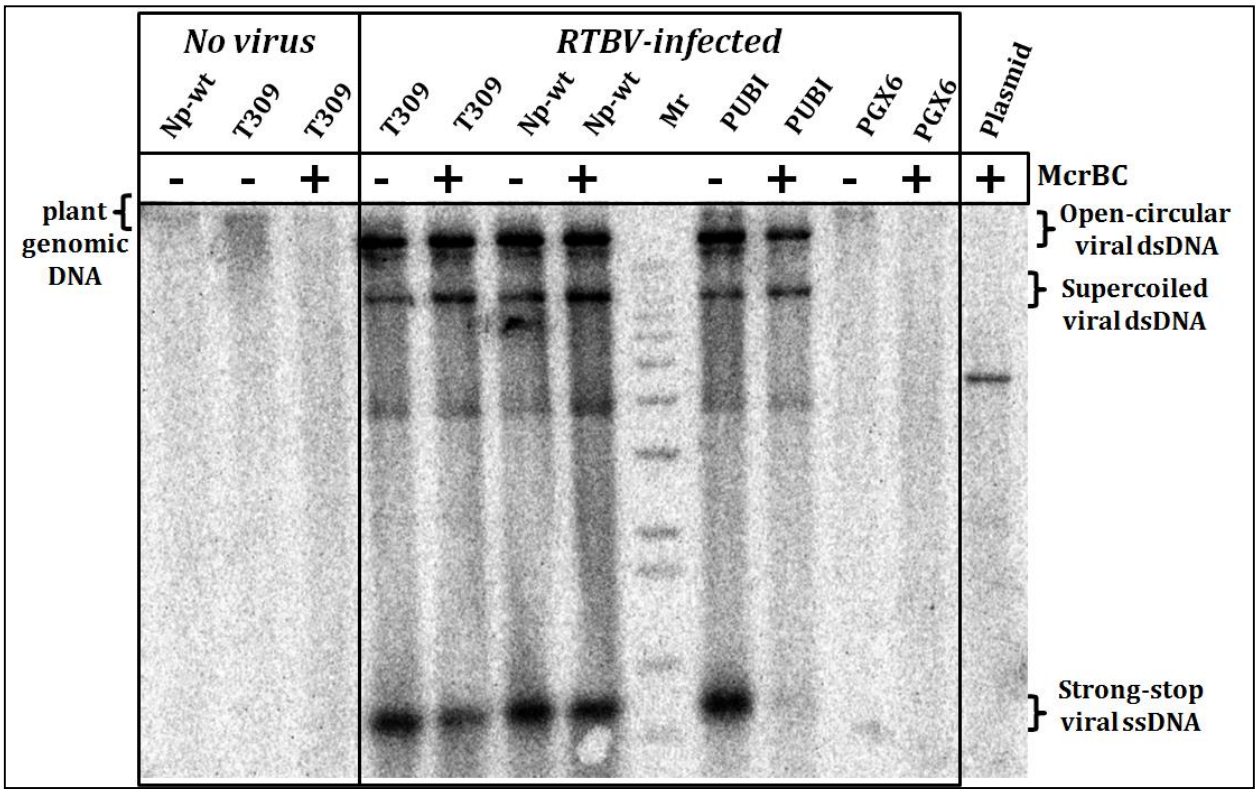


Figure 23. Analysis of relative accumulations of viral DNA and methylation statuses of the supercoiled and open circular forms of RTBV dsDNA using Southern blot hybridization. Hybridization was done using a mixture of probes specific for RTBV viral reverse (Rtbv7970_s, Rtbv7488_s) and forward (Rtbv7722_as) strands (see Table 1 for probe sequences). As a positive control (plasmid), a methylated plasmid DNA was subjected to McrBC treatment. As a DNA size marker, a 1-Kb+ ladder was used.

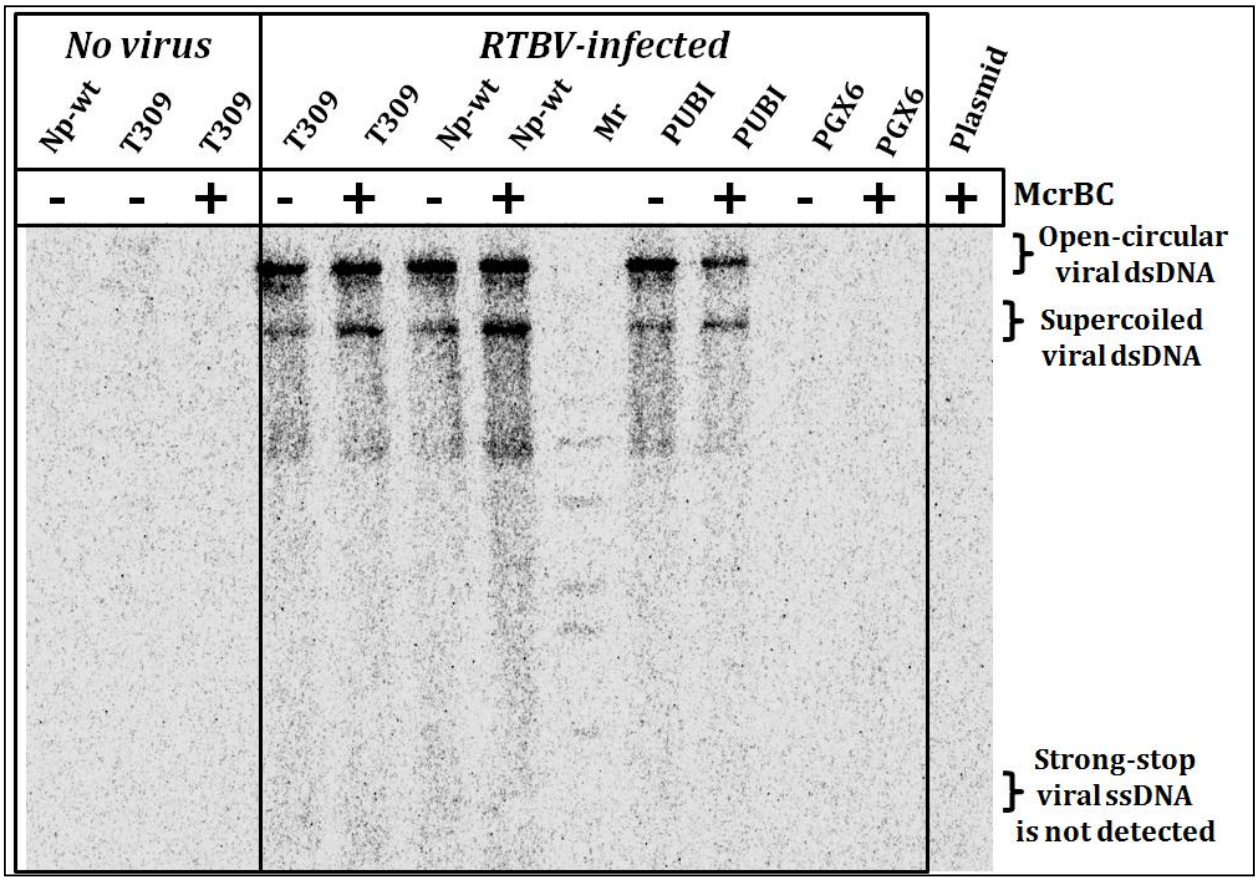


Figure 24. Analysis of relative accumulations of viral DNA and methylation statuses of the supercoiled and open circular forms of RTBV dsDNA using Southern blot hybridization. Hybridization was done using RTBV viral forward (sense) strand-specific probe (Rtbv7722_as, see Table 1). As a positive control (plasmid), a methylated plasmid DNA was subjected to McrBC treatment. As a DNA size marker, a 1-Kb+ ladder was used.

4.8. RICE PLANTS OVEREXPRESSED OSAGO18 PROTEIN ARE RESISTANT TO RTBV INFECTION

One of the multiple rice AGOs, OsAGO18, was shown to confer resistance to two different RNA viruses in rice plants (Urayama *et al.,* 2010; Du *et al.,* 2011; Jiang *et al.,* 2012; Wu *et al.,* 2015). We were interested to test whether or not transgenic rice plants overexpressing OsAGO18 under the constitutive UBI promoter (Nipponbare PGX6 line gerenated in the lab of Dr. Morel, Montpellier) is resistant to the DNA pararetrovirus RTBV. As a control for PGX6, we used Nipponbare transgenic line PUBI trasformed with the empty UBI vector.

To test the resistance of PGX6 to RTBV infection and the status of the virus methylation in these plants, we inoculated the transgenic plants with RTBV at 50 dpi, harvested the leaves for total DNA extraction and MrcBC-Southern analysis as described above for wild type plants. Suprisingly, we were not able to detect any forms of viral DNA in the PGX samples neither treated nor untreated with McrBC (Fig. 23, 24). In contrast, all the major forms of viral DNA were detected in RTBV-

infected Nipponbare wild type and the PUBI empty vector plants (Fig. 23, 24). Thus, we concluded that Nipponbare rice plants overexpressed OsAGO18 protein are immune to RTBV infection.

4.9. THE P4 F-BOX IS LIKELY REQUIRED FOR RTBV INFECTIVITY

To examine the importance of the P4 F-box motif for RTBV infectivity, we inoculated 3-week old Taipei 309 rice plants with agrobacteria (GV3859), carrying empty vector (pBin19), RTBV wild type (RTBV-wt) or RTBV F-box mutant (RTBV-mutFb) infectious clones (four plants per construct). At 50 dpi, systemic leaf tissues of RTBV-infected and control rice plants were harvested and tested by PCR for the presence of the wild type and the mutant viruses (using diagnostic primers pRTBVwt_s, pRTBVmut_s and pRTBVwt_as, Table 1). Two of the three PCR positive plants carrying the mutant virus and one representative plant infected with the wild-type virus (Fig. 25) were used for analysis of the relative accumulation and methylation status of RTBV-wt and RTBV-mutFb DNAs by McrBC-Southern as described above (see chapter 4.7).

For that, we extracted total DNA from RTBV-infected and control (EV) rice plants, treated with McrBC, and then loaded on a 1% agarose gel together with total DNA aliquots of the same samples treated under the same conditions but without McrBC. Staining with EtBr revealed that the rice gDNA contained in all McrBC-treated samples was almost fully digested by the enzyme, indicating that it was extensively methylated. As a control, a methylated plasmid subjected to McrBC treatment was digested producing several expected fragments between approximately 700 bp and 2.3 kb in size (Fig. 22).

The results revealed strongly reduced accumulation of two major forms (open circular and supercoiled) of circular RTBV-mutFb viral dsDNA, compared to RTBV-wt, while both forms appeared to be resistant to McrBC (Fig. 23). Notably, of the two rice plants shown to be PCR-positive, only one plant was clearly Southern-positive (Fig 23, RTBV-mutFb1). Thus, we can conclude that RTBV P4 F-box motif mutation drastically reduced RTBV infectivity and viral DNA accumulation in systemic rice leaf tissues, while it doesn't appear to affect the non-methylated status of the major fraction of viral genomic DNA. It should be noted, however, that the supercoild form of the viral dsDNA accumulates at a very low level to be absolutely sure about the proportion of it resistant to McrBC.

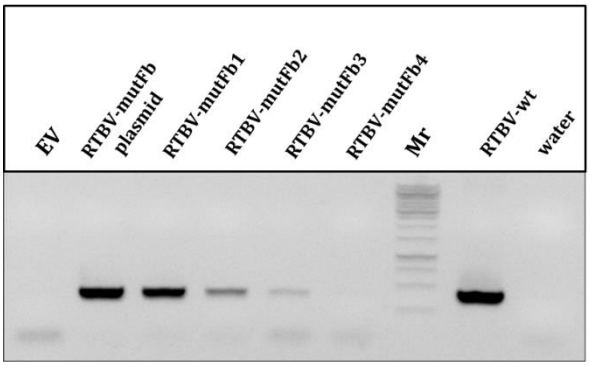


Figure 25. PCR of RTBV-infected and control (EV) rice samples using diagnostic primers to detect RTBV-wt (pRTBVwt_s and pRTBVwt_as) and RTBV-mutFb (pRTBVmut_s and pRTBVwt_as) viral DNAs. As a positive control and a DNA size marker (Mr), RTBV-mutFb-expressing plasmid and a 1-Kb+ ladder were used, respectively.

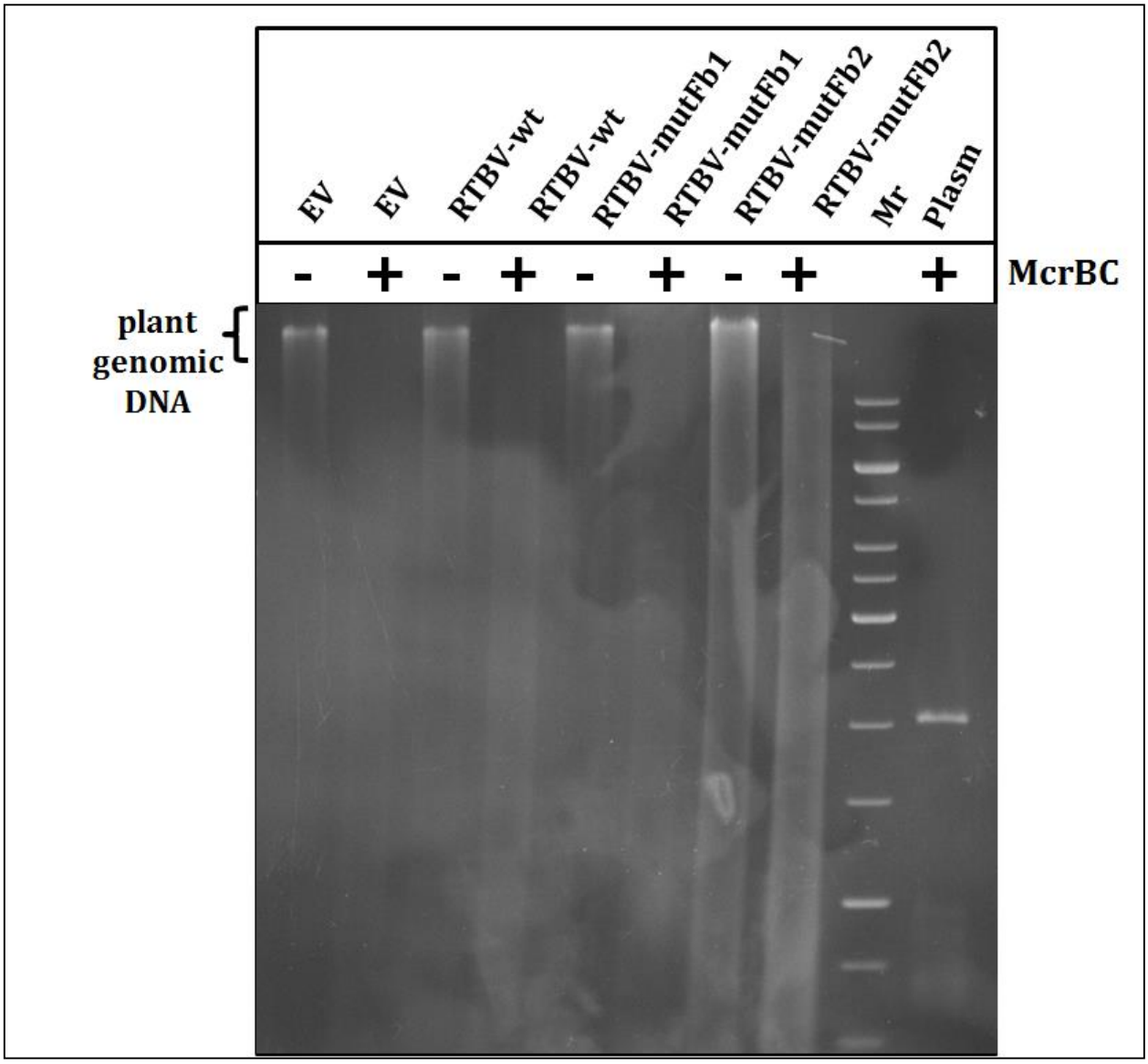


Figure 26. EtBr staining of the gel containing RTBV-infected and control (EV) rice samples. As a positive control (plasm), a methylated plasmid DNA was subjected to McrBC treatment. As a DNA size marker, a 1-Kb+ ladder was used.

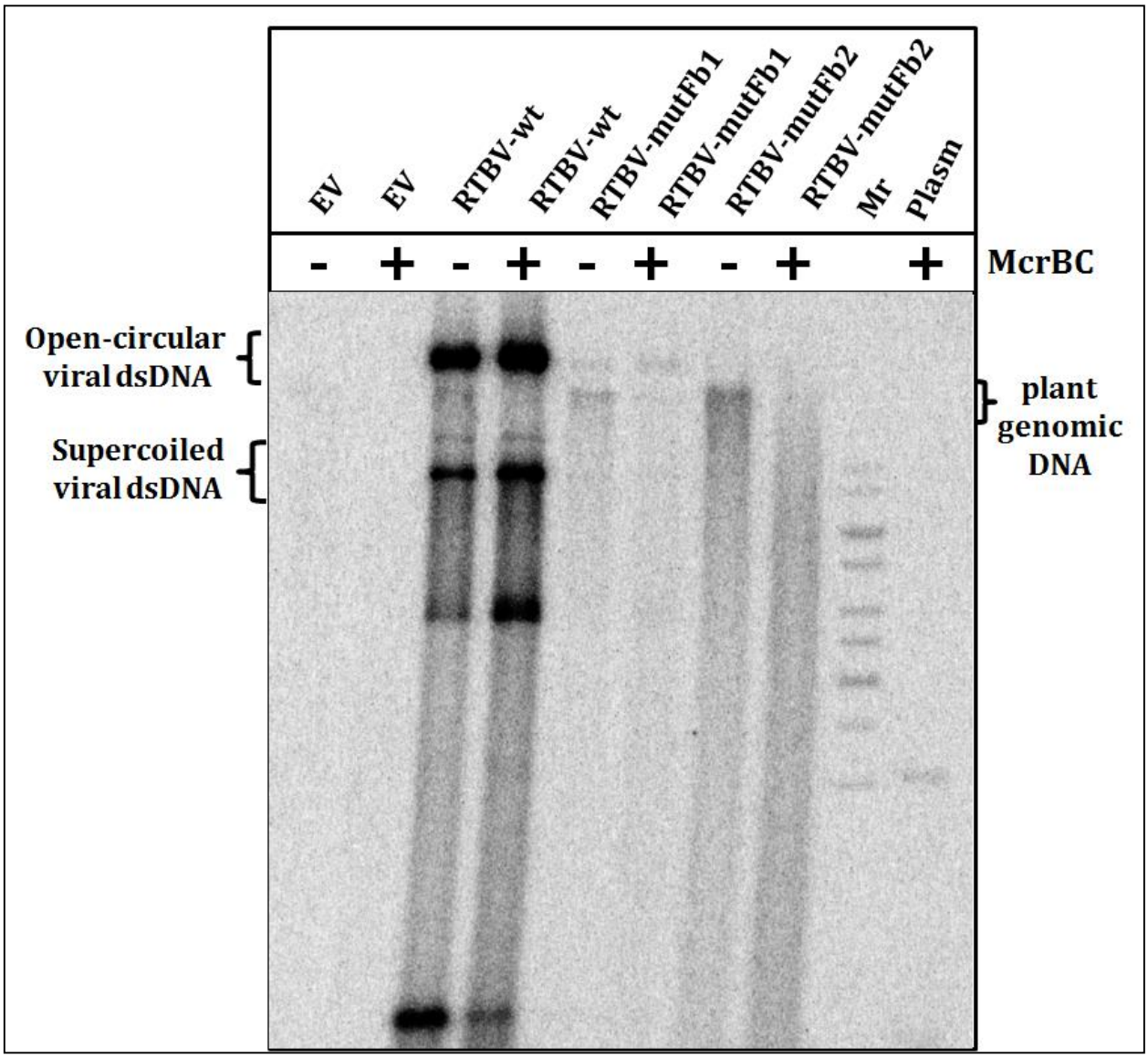


Figure 27. Analysis of relative accumulations of viral DNA and methylation statuses of the supercoiled and open circular forms of RTBV dsDNA using Southern blot hybridization. Hybridization was done using a mixture of RTBV viral sense and antisense probes (Rtbv7970_s, Rtbv7488_s and Rtbv7722_as, see table 1). As a positive control (plasm), a methylated plasmid DNA was subjected to McrBC treatment. As a DNA size marker, a 1-Kb+ ladder was used.

5. DISCUSSION

5.1. RTBV P4 IS A SUPRESSOR OF HOST PLANT ANTIVIRAL RESPONSES

In most eukaryotes, RNA silencing is a central mechanism that regulates gene expression, genome stability, abiotic stress responses acting both at the transcriptional level through DNA methylation and the post-transcriptional level through direct mRNA interference mediated by siRNAs. In plants and invertebrates, the same mechanism is also used in host defence against viral and non-viral pathogens by targeting «foreign» RNAs for degradation. In addition the majority of plant pathogens, including viruses are recognized by innate immunity system of host plant leading to the activation of defense mechanisms, such as PTI and ETI that restrict pathogen infection at a particular site. However, successful pathogens have consequently evolved diverse mechanisms to avoid, actively suppress or even hijack host defence pathways commonly through the expression of effector proteins, which function as suppressors of host plant antiviral responses based on RNA silencing and innate immunity.

Here we demonstrate that the RTBV protein P4, of previously unknown function, has the properties of viral effector protein, which is involved in suppression of host plant antiviral responses. Particularly, RTBV P4 interferes with the biogenesis of transgene-derived 21-nt siRNAs in *N. benthamiana* and blocks cell-to-cell spread of transgene silencing likely mediated by 21-nt siRNAs. Recently, DCL4 was shown to restrict systemic (but not local) infection of an RNA virus in *N. benthamiana* (Cordero *et al.,* 2017). Based on this finding and our results we propose that RTBV P4 most likely interfere with DCL4 activity generating 21-nt viral siRNAs that mediate cell-to-cell spread of RNA silencing. When DCL4 is missing or is inhibited by viruses, DCL2 can substitute DCL4 activity producing 22-nt viral siRNAs as was shown in *Arabidopsis* (Bouche *et al.,* 2006). Our results in *N. benthamiana* indicate that 22-nt siRNAs can direct cell-automomous silencing, but cannot serve as a mobile signal spreading silencing from cell to cell. P4-mediated suppression of DCL4 activity might be relevant at the early stages of RTBV replication and cell-to-cell movement, when 21-nt siRNAs generated by DCL4 could move from cell to cell ahead of the virus and immunize the cells against the incoming virus. The concomitant enhancement of 22-nt siRNA production by DCL2 might be tolerated by the replicating virus within a cell by a different mechanism. Indeed in the couse of my PhD project, in collaboration with Dr. Rajeswaran, we demonstrated that RTBV evades antiviral silencing by producing a dsRNA decoy from the highly-structured leader region, which engages all the DCLs in massive production of viral siRNAs and thereby protects other regions of the viral genome from repressive siRNAs (see Rajeswaran, Golyaev et al. 2014 in the Annex). It remains to be investigated whether or not these suppressor/enhancer P4 properties that we dicovered in *N. benthamiana* are relevant in the context of RTBV infection in rice plants. Viral 21-nt and 22-nt viral siRNAs accumulate at comparable levels in RTBV-infected plants and the biogenesis of 21-nt viral siRNAs does not appear to be affected at the late stages of RTBV infection (see

Rajeswaran, Golyaev et al. 2014 in the Annex). We assume that the RTBV P4 gene is expressed only during early stages of viral infection, because P4 protein is translated from the spliced pgRNA (Futterer *et al.,* 1994). The splicing is likely repressed at the late stages of infection to promote production of the full-length pgRNA for reverse transcription. Therefore, analysis of the P4 protein activities at the early stages of viral infection would be important to further investigate its interactions with the rice defense system. Our results in *N. benthamiana* also suggest that P4 protein is an intrinsically unstable protein, which may not persist in the virus-infected cell for a long time. Based on the findings for the human homolog of Slimb (HOS) F-box protein (Li *et al.,* 2004), the intrinsic instability of RTBV P4 is likely due to its F-box motif that may function through the protein degradation pathway, in which P4 may target some component of the plant antiviral defences for co-degradation in proteosomes (see more discussion below). Consistent with the findings of Ying Li and colleagues (Li *et al.,* 2004), mutation of the F-box motif stabilizes P4 protein transiently expressed in *N. benthamiana*.

In addition to its role in suppression of RNA silencing, we found that RTBV P4 can interfere with host plant innate immunity responses. Particularly, we demonstrate that P4 suppresses the production of ROS in *N. benthamiana* plants in response to bacterial PAMP. ROS play a central role in plant defense against various pathogens. The rapid accumulation of plant ROS at the site of infection, a phenomenon called oxidative burst is toxic to pathogens directly. Moreover, it could lead to a hypersensitive response involving programmed cell death that restricts biotrophic pathogen infection at a particular site (Liu *et al.,* 2010). Given that oxidative burst is one of the earliest plant innate immunity responses to biotrophic pathogen attack elicited by the majority of plant species, we propose that RTBV P4 protein is solely required for the virus to overcome the rice plant defense at the early stage of infection. Since no viral PAMP was identified so far, except dsRNA (Niehl *et al.,* 2016), we could suggest that RTBV dsRNAs accumulating during viral replication (Rajeshwaran, Golyaev, et al. 2014 see it in the Annex) or other not yet identified RTBV PAMP(s) are perceived in host rice plants eliciting innate immunity responses that could be coped by RTBV P4 for successful virus infection. Interestingly, the F-box motif was equally required for P4-mediated suppression of cell-to-cell spread of silencing as well as oxidative burst, suggesting that RTBV P4 may have a common target in the antiviral silencing and innate immunity pathways. The cross-talk between RNA silencing and innate immunity in plant-pathogen interations is well documented (Zvereva and Pooggin 2012; Pumplin and Voinnet 2013). It is also conceivable that the plant ETI system may recognize the activities of RTBV P4 in suppressing PTI responses and/or silencing and restrict RTBV infection in non-host plants. Indeed, our results obtained using the transient assays in *N. benthamiana* (which cannot support RTBV infection; Rajeswaran and Pooggin, unpublished) point at a strong response of the plant cells on P4 expression, manifested not only as enhanced cell-autonomus transgene silencing as discussed above, but also as chlorosis of the P4-expressing tissues (data not shown). Accordingly, the plant response observed as chlorosis

was less pronounced and drastically reduced in the cases of P4-delN and P4-mutFb-expressing tissues, respectively, compared with P4-wt (data not shown). Further supporting this hypothesis is our finding that transient expression of P4 (but not its F-box mutant version) in *N. benthamiana* is upregulating mRNA levels for NbAGO2 gene (Fig. 14, 17, C). Indeed, AGO2 has been implicated in ETI-based response to a bacterial pathogen in *Arabidopsis* (Zhang *et al.,* 2011)

5.2. THE IMPORTANCE OF RTBV P4 F-BOX-LIKE AND N-TERMINAL MOTIFS FOR P4-MEDIATED SUPRESSION OF HOST PLANT ANTIVIRAL RESPONSES

As was mentioned above, majority of viral pathogens have evolved diverse mechanisms to avoid, actively suppress or hijack host defence pathways in order to establish successful infection. One of the potential targets used by several virus families to complete their infection cycle is ubiquitin–proteasome system (UPS), which mediates ubiquitination of proteins targeted for degradation by the proteasome. Since the UPS plays a critical role in the regulation of many cellular processes, such as cell division, development, hormone signaling and others, it is not surprising that several unrelated viruses have evolved convergent strategies to exploit this mechanism. For instance, several families of the plant and animal viruses use the mechanisms that are adopted for the de-regulation of the host's ubiquitin–proteasome system through degradation or mimicking of the components of the SCF (SKp1, Cullin, F-box protein) E3 ubiquitin–ligase complex that participates in the recognition and recruitment of target proteins for ubiquitination and degradation by the ubiquitin 26S proteasome system. These viruses typically act at the ubiquitination step, either by expressing their own E3 ligase with appropriate properties or by altering the specificity of the host E3 ubiquitin–ligase complex. The latter strategy is exploited by the members of *Enamovirus* (*Pea enation mosaic virus-1*) and *Nanovirus* (*Faba bean necrotic yellows virus)* genera encoding F-box proteins (FBPs), the main components of host E3 ubiquitin–ligase complex mediating ubiquitination of proteins targeted for degradation by the proteasome, which are used by the virus to target essential components of the host antiviral defense system for degradation by the ubiquitin 26S proteasome system (Correa *et al.,* 2013).

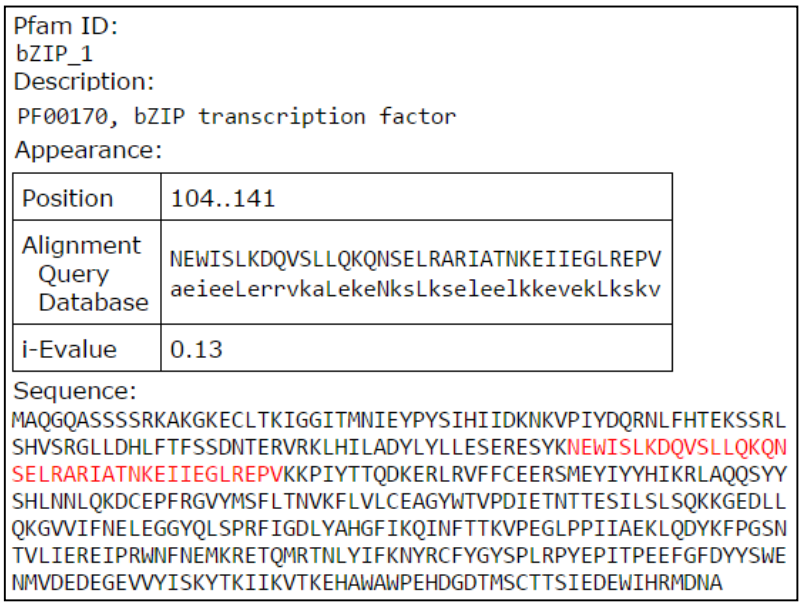
Plant FBPs are structurally and functionally diverse proteins, which are used for selection of target proteins that will be degraded by the SCF E3 ubiquitin–ligase complex, interacting with the core members of this complex through the conventional F-box domain, consisted of a short conserved sequence of about 50 amino acids. In contrast, plant viruses encode F-box-like proteins with the non-conventional F-box motif (**LP**xx(**L/I**)x$^{10–13}$**P**), which matches the start of the plant F-box consensus sequence (**LP**xx**L/I**), the most highly conserved part of the domain in plant F-box proteins (Zhuo *et al.,* 2013). As the similar motif (**LP**PI**I**x^{9}**P**) was found in the sequence of RTBV P4 protein, we hypothesized that it could be essential for the silencing or/and innate immunity suppressor activities of P4.

Here, we demonstrate that F-box-like domain is required for RNA silencing and innate immunity suppressor functions of RTBV protein P4. Particularly, we show that RTBV P4 abilities to interfere with the biogenesis of transgene-derived 21-nt siRNAs in *N. benthamiana* and block cell-to-cell spread of transgene silencing were diminished when P4 mutant protein (P4-mutFb) with triple amino acid mutation in the F-box-like domain was expressed in *N. benthamiana* 16c line plants. This evidence corresponds with the presense of the red ring around the leaf zone infiltrated with P4-mutFb, which is an indicator of short cell-to-cell spread of mobile silencing signals. In addition, unlike P4 wild type, the P4-mutFb co-expression is not associated with enhancement of cell-autonomous GFP silencing. The most straightforward interpretation of our findings is that RTBV P4 acts as an F-box protein that targets an essential component(s) of the host RNA silencing machinery for degradation mediating the suppression of cell-to-cell spread of mobile silencing signals. Furthermore, given that cell-to-cell spread of transgene silencing is likely mediated by DCL4-generated 21-nt siRNAs we could suggest that DCL4 protein is one of the potential targets for P4-mediated protein degradation.

In addition, we demonstrate that F-box-like domain of RTBV P4 is required for its innate immunity suppressor activity. Particularly, we show that, unlike wild type P4, P4-mutFb doesn't suppress oxidative burst in *N. benthamiana*, meaning that, besides targeting the components of the host RNA silencing machinery, it could target for degradation the components of host plant innate immunity system. Thus, F-box-like domain is definitely essential for RTBV P4-mediated suppression of plant antiviral responses, which in the context of viral infection could be used to overcome the rice plant defense system.

In addition, we demonstrate that the N-terminal domain of RTBV P4 is required for the protein stability, while it is dispensible for the suppression of innate immunity by P4 in *N. benthamiana*. Although, in our silencing assay P4-delN mutant protein did not exhibit full activity in suppressing the production of 21-nt GFP siRNA and cell-to-cell spread of GFP silencing, this compromised activity can be explained by lower stability of P4-delN protein, compared to P4-wt at the latter time points, while in the oxidative burst assay in *N. benthamiana* the measurements were taken at the earlier time point when both proteins accumulated at the comparable levels. Based on these results, we could hypothesize that N-terminal domain of RTBV P4 can modulate its activity as the F-box protein in the proteosome degradation pathway.

Besides the F-box domain, FBPs contain other domains and motifs related to protein–protein interactions, such as leucine rich repeats (LRR), WD40 repeats (WD), Kelch, which are usually present in the C-terminal region of FBPs repeats and used to interact with their targets. Interestingly, the basic Leucine Zipper Domain (bZIP domain) was identified at the N-terminus of RTBV P4 protein (Fig. 28) and could be used to analyze potential targets of P4.


Pfam ID:
bZIP_1
Description:
PF00170, bZIP transcription factor
Appearance:

Position	104..141
Alignment Query Database	NEWISLKDQVSLLQKQNSELRARIATNKEIIEGLREPV aeieeLerrvkaLekeNkslkseleelkkevekLkskv
i-Evalue	0.13

Sequence:
MAQGQASSSSRKAKGKECLTKIGGITMNIEYPYSIHIIDKNKVPIYDQRNLFHTEKSSRL
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SELRARIATNKEIIEGLREPVKKPIYTTQDKERLRVFFCEERSMEYIYYHIKRLAQQSYY
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TVLIEREIPRWNFNEMKRETQMRTNLYIFKNYRCFYGYSPLRPYEPITPEEFGFDYYSWE
NMVDEDEGEVVYISKYTKIIKVTKEHAWAWPEHDGDTMSCTTSIEDEWIHRMDNA

Figure 28. Identification of the basic leucine zipper domain (bzip domain) of P4 by motif finder software (http://www.genome.jp/tools-bin/search_motif_lib)

Therefore, future studies should address two questions: 1) which component(s) of the host RNA silencing and/or innate immunity machinery could be targeted by RTBV P4 protein, 2) role of the bZIP domain in RTBV P4 interaction with its target protein(s).

5.3. PATHOGENICITY AND THE HOST RANGE OF DIFFERENT CAMV STRAINS IS DETERMINED BY P6-MEDIATED SUPPRESSION OF INNATE IMMUNITY

As was already described above, CaMV genome encodes a multifunctional P6 protein, which, besides its role in the translation of viral 35S RNA and formation of inclusion bodies, exerts both RNA silencing and innate immunity suppressor activities. Moreover, P6 is the major genetic determinant of virus pathogenicity and the host range (Baughman *et al.,* 1988; Kobayashi & Hohn, 2004; Schoelz *et al.,* 1986; Stratford & Covey, 1989).

Here we demonstrate that pathogenicity and the host range of different CaMV strains is determined by P6-mediated suppression of host plant innate immunity responses, in addition to its antisilencing activity. Particularly, we show that CaMV P6 proteins from both CM1841 and D4 strains, causing severe and mild symptoms in *A. thaliana*, respectively, could supress RNA silencing in transgenic *A. thaliana* plants, while only P6 protein from CM1841 mediates the suppression of ROS burst, SA-dependent autophagy and make *A. thaliana* plants more susceptible to infection with *P. syringae* (see Zvereva, Golyaev *et al.,* 2016 in the Annex). The main difference between two strains is that, unlike CM1841, strain D4 exhibits only very mild symptoms in *A. thaliana* Col-0, while it induces severe systemic symptoms in *Datura stramonium*, *Nicotiana edwardsonii* and *Nicotiana bigelovii*, compared to CM1841, which is unable to systemically infect any solanaceous species (Schoelz *et al.,* 1986). The mild symptoms induced by P6-D4 in transgenic and CaMV-

infected *A. thaliana* plants could be related to weak expression of P6-D4, compared to P6-CM, or structural differences between two proteins. To test that, we analyzed the accumulation of both proteins in transgenic *A. thaliana* plants and concluded that P6-D4 protein accumulated even at higher level than P6-CM (Fig. 29). In addition, we found that, dsRNA-binding (dsR) domain of P6 is required for P6-mediated suppression of innate immunity in *A. thaliana* Col-0 transgenic lines expressing P6 protein from CaMV strain JI (see Zvereva, Golyaev *et al.,* 2016 in the Annex). Interestingly this domain varies in P6-CM and P6-D4 proteins, suggesting that it could be essential for the pathogenicity and the host range of different CaMV strains. The importance of amino acid variations in the P6 dsRNA domain remains to be further investigated.

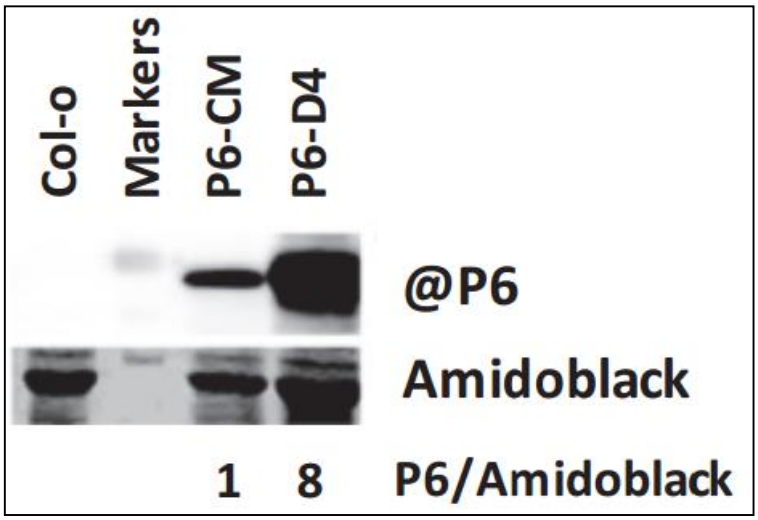

Figure 29. Western blot analysis of P6 protein accumulation in the P6-transgenic and control plants using anti-P6 antibody. Amidoblack staining of the blot membranes is shown as loading control. The normalized densities (P6/amidoblack) are shown under the scans, with the value for P6-CM set to 1.

5.4. RTBV EVADES SIRNA-DIRECTED DNA METHYLATION IN INFECTED RICE PLANTS

Plant DNA viruses accumulate in the nuclei of infected plant cells as multiple circular minichromosomes, which resemble the host plant chromosomes and are transcribed by the host Pol II generating capped and polyadenylated viral RNAs. However, plant could recognize and repress the replication of these «foreign» minichromosomes in the nucleus using pathways that regulate host gene expression and chromatin states, such RNA-directed DNA methylation (RdDM).

RdDM is a nuclear pathway of the plant RNA silencing machinery that is responsible for the regulation of gene expression and defence against invasive nucleic acids such as transposons, transgenes and viruses. Upon viral infection, the plant RNA silencing machinery generates 21, 22 and 24-nt virus-derived siRNAs, which serve as guide molecules for the silencing complexes that promote viral RNA cleavage/degradation or translational repression through posttranscrptional gene silencing (PTGS), and viral DNA methylation through transcrptional gene silencing (TGS). The TGS through de novo DNA methylation is directed by 24-nt siRNAs, the most diverse and abundant class of plant small RNAs (Pooggin, 2013).

In contrast to RNA viruses, plant DNA viruses were shown to spawn massive quantities of virus-derived 24-nt siRNAs, which can potentially direct viral DNA methylation and transcriptional silencing. However, growing evidence indicates that DNA viruses most likely evade or suppress siRNA-directed DNA methylation. For example, the cytoplasmic step of pararetrovirus replication through pgRNA should effectively protect viral DNA from repressive action of RdDM. However, covalently-closed circular dsDNA, which is transcribed in the nucleus, can potentially be methylated de novo by the RdDM machinery charged with viral 24-nt siRNAs (Pooggin, 2013).

Here we show that the most of the circular covalently closed viral dsDNA in RTBV-infected rice plants is non-methylated. Thus, multiple RTBV minichromosomes appear to evade siRNA-directed DNA methylation in the nucleus and thereby retain the potential for active Pol II transcription. The molecular details of how viruses avoid the repressive action of host plant RdDM have not yet been fully understood. However, we could hypothesize that plant pararetroviruses, including RTBV, exploit the cytoplasmic step of their replication cycle to create new copies of dsDNA molecules which avoid methylation and could be transmitted to the nucleus for the next round of replication. Furthermore, some DNA viruses evolve effector proteins, which could be used to interfere with 24-nt siRNAs biogenesis. As was described earlier, RTBV genome encodes P4 protein, which though was shown to suppress the accumulation of 21-nt, but not 24-nt siRNAs. Moreover, it doesn't suppress the systemic silencing of GFP in *Nicotiana benthamiana*, which was shown to be associated with the long distance movement of 24-nt siRNAs (data not shown). Thus, we can conclude that P4 protein most likely is not involved in the suppression of host plant RNA-directed DNA methylation machinery. The evasion of 24-nt siRNA-directed DNA methylation in RTBV-infected rice plants is likely mediated by the viral dsRNA decoy mechanism as was first proposed for the distantly related pararetovirus in *Arabidopsis* (Blevins *et al.,* 2011) and confirmed during these PhD studies for RTBV in rice (Rajeswaran, Golyaev *et al.,* 2014 see in the Annex). Nonetheless, it has also been found in our lab that the pararetroviruses from genus *Badnavirus*, which may potentialy express only a very short decoy dsRNA, are also able to evade siRNA-directed DNA methylation in banana plants (Rajeswaran et al. 2014b)

5.5. OSAGO18 TRANSGENIC RICE PLANTS ARE MORE RESISTANT TO RTBV INFECTION

OsAGO18 is a member of the new rice AGO clade conserved in monocots, which is specifically induced by the infection of two taxonomically different viruses, *Rice stripe Tenuivirus* (RSV) and *Rice dwarf Phytoreovirus* (RDV) and required for the antiviral function of AGO1. As it has been shown, OsAGO1 antiviral activity was abolished in loss-of-function *ago18* mutant rice plants, whereas transgenic OsAGO18-overexpressing rice plants were more resistant to the infection with both viruses (Wu *et al.,* 2015).

Here we demonstrate that the independently-generated transgenic rice plants overexpressing OsAGO18 protein are immune to RTBV infection (as no replicative forms of RTBV viral DNA were detected) compared to wild type rice plants, thus extending the previous findings and implicating OsAGO18 in a broader-specrum resistance to both RNA and DNA viruses Since previous findings indicate that OsAGO18 counteracts OsAGO1 activity, it would be interesting to examine whether or not rice *ago18* mutant plants are more susceptible to RTBV infection.

6. CONCLUDING REMARKS

During the course of my PhD work, we characterized two viral effector proteins, RTBV P4 and CaMV P6, which possess the ability to suppress host plant antiviral responses based on RNA silencing and innate immunity. Particularly, we showed that RTBV protein P4, of previously unknown function, is able to suppress cell-to-cell spread of mobile silencing signals and oxidative burst in *Nicotiana benthamiana*, while CaMV P6 being the main determinant of virus host range mediates the suppression of plant innate immunity responses, such as ROS burst and SA-dependent autophagy. In addition we determined that F-box-like motif is required for RTBV P4 anti-silencing activity and suppression of oxidative burst, while the N-terminal domain modulates the P4 activity and stability. Finally, we studied RTBV infection and the role of P4 F-box motif in rice plants, and showed that RTBV virus evades siRNA-directed DNA methylation in infected rice plants and that OsAgo18 transgenic rice plants are more resistant to RTBV infection.

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LIST OF ABBREVIATIONS

AGO - Argonaute

Avr – avirulence

BAK1 - BRASSINOSTEROID-INSENSITIVE1-ASSOCIATED KINASE

bZIP - basic Leucine Zipper Domain

CaMV - *Cauliflower mosaic virus*

CP - coat protein

CTAB - Cetyltrimethylammonium bromide

dsR - dsRNA-binding

dsDNA - Double-stranded DNA

dsRNA - Double-stranded RNA

DTT - Dithiothreitol

ECL - Enhanced chemiluminescence

EDTA - Ethylenediaminetetraacetic acid

elf18 - bacterial elongation factor Tu peptide

ET - ethylene

EtBr - Ethidium bromide

ETI - Effector-triggered immunity

FBP - F-box protein

flg22 - bacterial flagellin

FLS2 - FLAGELLIN-SENSING 2

GFP - Green fluorescent protein

HA-tag - Human influenza hemagglutinin

Hc-Pro - HELPER COMPONENT-PROTEASE

HR - Hypersensitive response

HRP - Horseradish Peroxidase

JA - Jasmonic acid

LB - Lysogeny broth medium

LRR - Leucine rich repeat

MAMP - Microbe-associated molecular pattern

MAPK - Mitogen-activated protein kinase

MES - 2-(N-morpholino)ethanesulfonic acid

miRNA – microRNA

MOPS - 3-(N-morpholino)propanesulfonic acid

MP - movement protein

NB-LRR - nucleotide-binding leucine-rich repeat proteins

NLS - Nuclear localization signal

nt - Nucleotide

OD - Optical Density

ORF - Open reading frame

PAMP - Pathogen-associated molecular pattern

PCD - Programmed cell death

PCR - polymerase chain reaction

pgRNA - pregenomic RNA

Pol - RNA polymerase

pre-miRNA - precursor miRNA

PTGS - Post-transcriptional gene silencing

PTI - Pattern-triggered immunity

PRR - Pattern recognition receptor

RdDM - RNA-directed DNA methylation

RDR - RNA-dependent RNA polymerases

RISC – RNA-induced silencing complex

RH - ribonuclease H

RLK - Receptor-like kinase

RLP - Receptor-like protein

RNAi - RNA interference

ROS - Reactive oxygen species

RT – Reverse transcriptase

RTBV – *Rice tungro baciliform virus*

Rubisco - Ribulose-1,5-bisphosphate carboxylase

SA - Salicylic acid

SAR - Systemic acquired resistance

SDS - Sodium dodecyl sulfate

SDS-PAGE - SDS-polyacrylamide gel electrophoresis

siRNA - small interfering RNA

ssDNA - single-stranded DNA

ssRNA - single-stranded RNA

SSC - Saline-sodium citrate

sORF - short open reading frame

ta-siRNA - trans-acting siRNA

TBE - Tris/Borate/EDTA buffer

TGS - Transcriptional gene silencing

TRIS - 2-Amino-2-hydroxymethyl-propane-1,3-diol

UPS - Ubiquitin–proteasome system

VRS - viral proteins exhibiting RNA silencing suppressor activity