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STUDY OF THE INTERACTIONS BETWEEN BROAD BEAN WILT VIRUS 1 AND ITS HOST PLANTS

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List of abbreviations

Ago	Argonaute protein
Avr	Pathogen avirulence gene product
BBTV	*Banana bunchy top virus*
BBVV	*Broad bean V virus*
BBWV-1	*Broad bean wilt virus 1*
BBWV-2	*Broad bean wilt virus 2*
BCMNV	*Bean common necrotic virus*
BCMV	*Bean common mosaic virus*
BPMV	*Bean pod mottle virus*
BYMV	*Bean yellow mosaic virus*
BYV	*Beet yellows virus*
CaMV	*Cauliflower mosaic virus*
ChLCB	*Chili leaf curl betasatellite*
CMV	*Cucumber mosaic virus*
CP	Capsid protein
CPMV	*Cowpea mosaic virus*
CTV	*Citrus tristeza virus*
CuMMV	*Cucurbit mild mosaic virus*
DCLs	Dicer-like ribonuclease proteins
dsRNA	Double-stranded RNA
FHV	*Flock house virus*
FLUAV	*Human influenza virus*
GeMV	*Gentian mosaic virus*
GFLV	*Grapevine fanleaf virus*
HDV	*Hepatitis delta virus*
HEL	Helicase
HR	Hypersensitive response
LMMV	*Lamium mosaic virus*
MCDV	*Maize chlorotic dwarf virus*
MP	Movement protein
NBT	Nitro blue tetrazolium

PCD	Programmed cell death
PDs	Plasmodesmata
PepMV	*Pepino mosaic virus*
PNRS	*Prunus necrotic ringspot virus*
POL	Polymerase
PPV	*Plum pox virus*
PRO-CO	Protease cofactor
PRs	Pathogenesis-related genes
PTGS	Post-transcriptional gene silencing
PVX	*Potato virus X*
R	Dominant resistance host gene
RdRPs	RNA-dependent RNA polymerases
RISC	RNA-induced silencing complex
RNP	Ribonucleoprotein
ROS	Reactive oxygen species
SAR	Systemic acquired resistance
siRNAs	Short interfering RNA
ssRNA	Single-stranded RNA
TBSV	*Tomato bushy stunt virus*
TCV	*Turnip crinkle virus*
TEV	*Tobacco etch virus*
TGB	Triple gene block
TGS	Transcriptional gene silencing
TMV	*Tobacco mosaic virus*
ToLCNDV	*Tomato leaf curl New Delhi virus*
ToMV	*Tomato mosaic virus*
ToTV	*Tomato torrado virus*
TP	Trypan blue
VACV	*Human Vaccinia virus*
VSRs	Viral suppressors of RNA silencing

Abstract

Broad bean wilt virus 1 (BBWV-1) is the type member of the *Fabavirus* genus, in the *Secoviridae* family. BBWV-1 is worldwide distributed and infects important horticultural and ornamental crops causing considerable economic losses. However, information about the biological and molecular characteristics of BBWV-1 isolates is scarce.

BBWV-1 is composed of two molecules of positive single-stranded RNA (ssRNA+) that are separately encapsidated in virions of icosahedral morphology. Each ssRNA+ encodes polyproteins which are processed by proteolytic cleavage into functional peptides. RNA1 (~ 5.8 kb) encodes for one polyprotein that renders proteins involved in viral replication: a protease cofactor (PRO-CO), an helicase (HEL), a viral genome-linked protein (VPg), a protease (PRO) and an RNA-dependent RNA polymerase (POL). RNA2 (~ 3.4 kb) has two "in frame" successive AUG initiation codons and encodes for two polyproteins (2a and 2b). Both polyproteins render identical large and small coat proteins (LCPs and SCPs, respectively), and putative movement proteins of different sizes that share the same C-terminal amino acid sequence. The longest putative movement protein, named MP, is encoded by polyprotein 2a, whereas the smallest putative movement protein, named VP37, is encoded by polyprotein 2b and lacks the first 90 amino acids present in MP.

To improve the knowledge about BBWV-1, this doctoral thesis was addressed to study the biological properties of different BBWV-1 isolates and the implication of BBWV-1 RNA2-encoded proteins in the plant defence mechanisms and symptom development. For this purpose, four BBWV-1 viral isolates (Ben, PV0548, B41/99 and NSRV) obtained from different countries and clustered in different phylogenetic groups were inoculated in several herbaceous plant species. Each BBWV-1 isolate induced different symptoms on broad bean (*Vicia faba*) and pepper (*Capsicum annum*) whereas no differences of induced symptomatology were observed on *Nicotiana benthamiana* plants. Moreover, tomato (*Solanum lycopersicum*) plants, infected with all four BBWV-1 isolates, remained asymptomatic. No differences of infectivity between BBWV-1 isolates were found in broad bean, pepper, tomato nor in *N. benthamiana* plants. None of these BBWV-1 isolates did infect common bean (*Phaseolus vulgaris*), cucumber (*Cucumis sativus*) and melon (*Cucumis melo*) plants, although these herbaceous species had been previously reported as BBWV-1 or BBWV-2 hosts. RNA accumulation over time of BBWV-1 isolates Ben, B41/99, NSRV and PV0548 on broad bean and pepper plants was not correlated with symptom severity.

To study the functions of BBWV-1 RNA2 encoded proteins, first a BBWV-1 full-length cDNA infectious clone (pBBWV1-Wt) was generated. Then, a mutant construct lacking the VP37 RNA2-encoded protein (pBBWV1-G492C) was engineered. Agroinfiltration assays in different plant species showed that pBBWV1-Wt had the same biological properties than the original BBWV-1 isolate infecting broad bean, tomato, pepper and *N. benthamiana* plants whereas pBBWV1-G492C was unable to infect pepper and tomato plants. Moreover, pBBWV1-G492C induced milder symptoms than pBBWV1-Wt in broad bean and *N. benthamiana* plants. These results suggest that BBWV-1 VP37 protein is related with host specificity and plant symptom development but is not involved in viral multiplication and viral particle formation. Transient expression of BBWV-1 VP37 protein, using a *Potato virus X* (PVX) viral vector, confirmed the VP37 protein as a pathogenicity determinant since it enhanced PVX-related symptoms and induced systemic necrosis associated with programmed cell death (PCD) in *N. benthamiana* plants. Moreover, VP37 protein was characterized as a post-transcriptional gene silencing (PTGS) suppressor by two different assays: transient expression in transgenic *N. benthamiana 16c* plants expressing constitutively the green fluorescent protein (GFP) and movement complementation of a viral construct based on *Turnip crinkle virus* (pTCV-GFP). The same studies were performed on the other RNA2-encoded proteins and showed that BBWV-1 MP and SCP proteins had PTGS suppressor activity. Transient expression of BBWV-1 SCP protein using the PVX viral vector revealed that it was a determinant of pathogenicity, enhancing the PVX-related symptoms and inducing the PCD-associated systemic necrosis in *N. benthamiana* plants.

Chapter 1

Introduction

1.1 Plant viruses

Plant viruses represent an important threat to agriculture worldwide since they are the aetiological agent of many crop diseases and cause considerable economic losses. It was predicted that pre-harvest diseases account the loss of approximately 15% of global crop production and that almost 47% of plant diseases are caused by viruses. Damages caused by plant viruses in horticultural productions cause alterations of quality and aesthetics, diminution of plant fitness, yield reduction and likely lead to a total loss of the harvest (Anderson *et al.*, 2004; Popp & Hantos, 2011).

To date, 23 families and 104 genera of plant-infecting viruses have been described (International Committee on Taxonomy of Viruses, ICTV, 2017, https://talk.ictvonline.org/). Plant viruses are intracellular obligate parasites and rely on the host biochemical machinery for their multiplication, movement and transmission. Viral infectious particles are composed of nucleic acid (single or double-stranded DNA or RNA), enveloped by a protein coat and occasionally surrounded by a cellular derived lipid membrane. The entire infective viral particle is denominated virion, due to its small size, it can only be observed under an electron microscope (Hull, 2009; Islam *et al.*, 2017a).

The main factors that promote viruses as the most common plant pathogens are: (i) the absence of antiviral substances limits control measures on the disease prevention or containment; (ii) the effectiveness of transmission strategies: viruses can rely on vertical transmission through infected seeds and on horizontal transmission though insect vectors; (iii) the high evolution rate: plant viruses, in particular, RNA viruses, are characterized by a high mutational rate that allows their fast evolution and adaptation to new host (Boualem *et al.*, 2016).

1.1.1 Evolution of plant viruses

Plant viral diseases are the result of a complex interaction between host, pathogen and environment (Islam *et al.*, 2017b). Among plant pathogens, viruses are characterized by fast evolution and continuous genetic changes that influence disease severity, host shifting and adaptation. Hence, the knowledge of plant virus evolution is essential for disease prevention, management and eradication. Natural selection and/or genetic drift are the main forces acting on virus variation and driving the genetic structure of virus populations (Acosta-Leal *et al.*, 2011). The variation within virus populations is generated by

recombination between genomic regions, by reassortment between genomic segments within a virus species or between different viral species and by genome mutations that are introduced during viral replication. Recombination events in a RNA virus population permit viral genomes with deleterious mutations to generate functional genomes, and can be distinguished in homologous or heterologous, between viruses that share different levels of RNA complementarity (Roossinck, 1997). Mutation (or misincorporation) consists of the introduction of nucleotide changes during viral RNA or DNA replication. Generally, high mutation rates increase the probability of a virus to generate host-range mutants and emergent variants. RNA viruses are generally characterized by high mutation rates because replication is performed by viral RNA-dependent RNA polymerases (RdRPs) without proofreading activity (Domingo, 1994). Conversely, DNA viruses use cellular DNA replication machinery with high fidelity replication and are presumed to be characterized by lower mutation rates (Duffy *et al.*, 2008). Genetic changes can be maintained (positive selection) and transmitted to the next generations by natural selection, whereas deleterious mutations or changes that do not provide any advantage to the virus are not maintained (negative or purifying selection) (Moya *et al.*, 2004). Additionally, genetic diversity between populations can be reduced by gene flow that is caused by the migration between distant geographical areas (Elena *et al.*, 2011).

1.1.2 Biology of RNA plant viruses

RNA viruses are the major group of plant-infecting viruses and can be composed of positive or negative single or double-stranded RNA molecules. Their genome usually encodes few proteins that are necessary for all the phases of the viral life cycle, which consist in infection, replication, virion assembly, movement, and transmission (Hull, 2009; Islam *et al.*, 2017a). The first step of the viral life cycle consists of the pathogen entry and the infection of the host. Plant wounds allow the virion to entry across the plant cuticle and the cell wall in order to permit the beginning of the viral multiplication. In the case of positive single-stranded RNA viruses, the RdRp catalyses the synthesis of a positive RNA molecule using the viral RNA template, and enables the genome replication and the assembly of the viral infective particle. Viruses move from the replication sites to the distal plant tissues in three different stages. First, they reach the plasmodesmata (PDs), intercellular membranous connections between adjacent cells (intracellular movement). Then, they pass through PDs

to infect neighbour cells (cell to cell movement). Finally, they reach vascular tissues that allow long-distance movement up to the parenchyma of the entire plant (systemic movement) (Hull, 2013). The majority of the plant viruses encodes movement proteins (MPs) that associate to PDs and mediate the transport of the viral particles between cells. Two main transport mechanisms can be distinguished depending on the virus genus. Some MPs associate with the viral RNA and mediate its transport through PDs, forming ribonucleoprotein (RNP) complexes. This mechanism is followed by the triple gene block (TGB) proteins of potexviruses or by the '30K' superfamily of viral MPs of tobamoviruses. Alternatively, comoviruses and caulimoviruses encode for MPs that form tubular structures through PDs, containing the viral particles and enabling their passage across the cells (Melcher, 2000; Lucas, 2006).

1.1.3 Transmission of RNA vector-borne plant viruses

The majority of plant viruses are transmitted by invertebrate vectors, mainly arthropods, which allow their dispersion and long-distance migration. Hemipteran insects, in particular aphids, leafhoppers and whiteflies, transmit the 55% of RNA vector-borne plant viruses. Virus transmission by insect vectors relies on the acquisition of the virus from an infected source, its retention and its final delivery to another site of infection (Ng & Perry, 2004). Generally, viruses belonging to the same genera are transmitted by the same insect vector because of specific interactions between the virus and the vector molecules (Power, 2000). For efficient transmission by the insect vector, some viruses rely only on the coat protein whereas other viruses need specific viral encoded helper proteins that allow the interaction between the viral coat protein and the insects' stylet proteins (Ng & Falk, 2006).

Different types of vector-mediated virus transmission modalities with different retention and acquisition times can be distinguished (Fig. 1.1). In non-circulative transmission, viruses are retained in the stylet or in the foregut of the insect. Depending on the time of retention by the vector, non-circulative transmission can be non-persistent, with very short acquisition and inoculation times, ranging from seconds to minutes, and semi-persistent, with retention times ranging from minutes to days (Nault, 1997; Whitfield *et al.*, 2015). On the other hand, viruses that are transmitted in a circulative manner (persistent) enter the insect foregut, reach its hemolymph, and are transported into the salivary glands and consequently they can be transmitted for longer periods (non-propagative). Finally, in

circulative propagative transmission the viruses are able to replicate inside the insect establishing a permanent association with the vector. For this reason, they can be considered as both plant and insect viruses (Casteel & Falk, 2016).

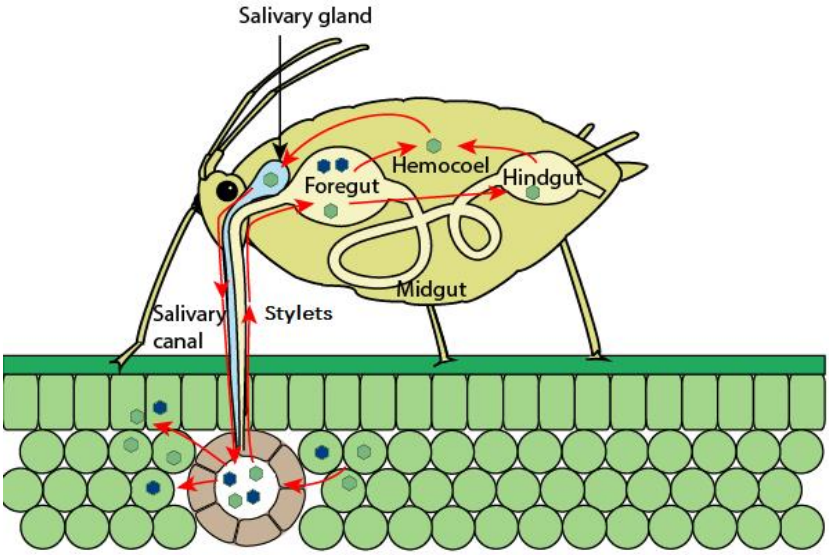

Figure 1.1 Representation of insect-vector mediated transmission of RNA viruses. Non-circulative viruses, represented as blue hexagons, are retained in the stylet or in the foregut of the insect. Circulative viruses, represented as green hexagons, enter in the insect gut, reach hemocoel, are transported into the salivary glands and are further transmitted. The image was readapted from http://viralzone.expasy.org.

1.2 Host defensive responses and pathological process induction

The establishment of plant-virus interactions during the viral life cycle affects host physiology and alters the expression of the host genes that regulate the cellular processes. During plant infection, viruses use the host resources and activate defensive responses that lead to plant disease.

1.2.1 Hypersensitive response and systemic acquired resistance

During viral infection, incompatible plant-virus interactions trigger a defence mechanism known as hypersensitive response (HR), which has the function of limiting the virus locally in the infection site. HR is a primary defence mechanism activated by recognition between a dominant resistance host gene (R) and a pathogen avirulence gene product (Avr). This interaction leads to different signalling pathways that cause plant metabolic changes, such as phosphorylation cascades, deregulation of ion fluxes and alteration of hormone metabolism (Mandadi & Scholthof, 2013). Accumulation of reactive oxygen species (ROS), such as superoxide ion (O^{2-}), hydrogen peroxide (H_2O_2) and nitric oxide (NO), cause cellular oxidative stress, that leads to lipid peroxidation, DNA and protein damage and rapid activation of localized programmed cell death (PCD), to hinder virus movement to other cells (Heath, 2000; Van Breusegem & Dat, 2006).

On the other hand, systemic responses spread throughout the whole plant, such as systemic acquired resistance (SAR) and systemic necrosis. SAR is characterized by the activation of host pathogenesis-related genes (PRs), and by the accumulation of salicylic acid (SA), jasmonic acid (JA), ethylene and nitric oxide (NO). SAR reaches distant non-infected tissues and provides an effective defence also to future viral infections (Fu & Dong, 2013). Otherwise, systemic necrosis manifests later in time in non-inoculated upper leaves and shares some biochemical and physiological properties with HR, such as accumulation of ROS and PCD activation (Komatsu *et al.*, 2010).

1.2.2 RNA silencing

RNA silencing is a gene regulation sequence specific mechanism conserved among almost all eukaryotes. In plants, this mechanism is used for the regulation of endogenous gene expression and as a defence response against virus infection (Csorba *et al.*, 2009). Two types of RNA silencing mechanisms can be distinguished (Liu & Chen, 2016): (i) post-transcriptional gene silencing (PTGS), that regulates the endogenous gene expression and protects from exogenous RNAs through the cleavage and degradation of the homologous sequences of RNAs; (ii) transcriptional gene silencing (TGS), that is based on host DNA methylation and histone modification for the epigenetic control of endogenous transposable elements.

RNA silencing is activated by double-stranded RNA (dsRNA) or highly structured single-stranded RNA (ssRNA). These molecules are recognized by Dicer-like ribonuclease proteins (DCLs) and processed into short interfering RNA molecules (siRNAs) of 21-24 nucleotides in length. SiRNAs are recognized by Argonaute (Ago) proteins and incorporated into the RNA-induced silencing complex (RISC). SiRNAs-RISC complex leads to the degradation of endogenous or exogenous RNAs with complementary sequences to the siRNAs. Alternatively, siRNAs-RISC complex guides the methylation of plant DNA to block the transcription of the corresponding genes (Wang *et al.*, 2012). Another class of small RNAs, named micro RNAs (miRNAs), derives from non-coding plant RNA genes with double-stranded portions. MiRNAs regulate the transcription factors or other regulatory genes that control plant development or signal transduction. Local silencing signal can move through PDs and can reach the vascular system to induce systemic silencing (Molnar *et al.*, 2010). Silencing signal can be amplified by the formation of secondary siRNAs via RdRp synthesis (Baulcombe, 2004).

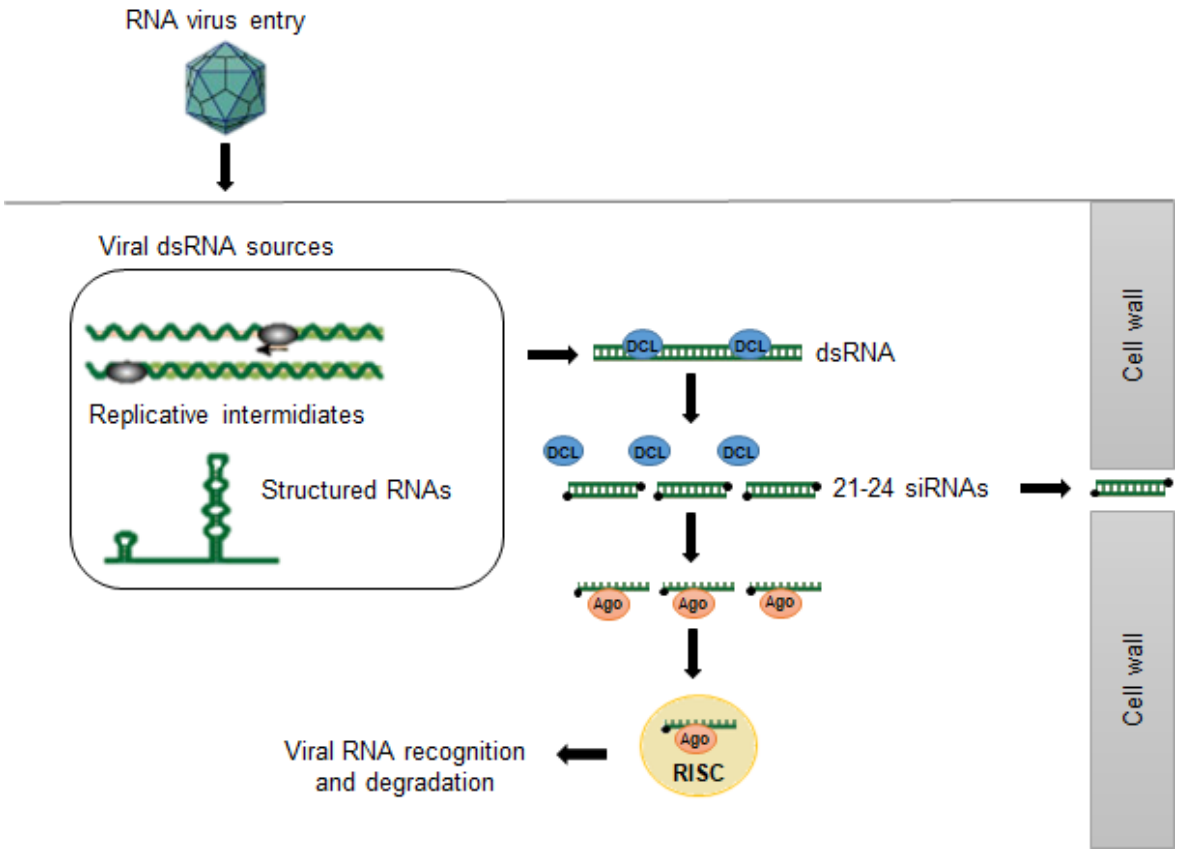

Figure 1.2 Schematic representation of post-transcriptional RNA silencing as defence mechanism against viruses. The image was readapted from http://viralzone.expasy.org.

PTGS is used by plants as a defence mechanism against viral infections (Fig. 1.2). Upon the virus entry inside the plant cell, double-stranded forms of viral RNAs activate PTGS leading to the degradation of the viral RNA to prevent the multiplication and the spread of the virus within the plant. In the case of ssRNA viruses, PTGS is activated by highly structured ssRNA or by dsRNA replicative forms. In the case of DNA viruses, PTGS is only activated by mRNAs with highly structured secondary structures (Wang *et al.*, 2012).

1.2.3 Pathogenicity determinants

Viral pathogenicity determinants are proteins or nucleic acids involved in the induction of the plant symptoms, enhancing virus multiplication and movement in plant tissues, suppressing gene silencing or triggering plant defence responses. Viruses are characterized by small genomes and they encode for few multifunctional proteins that are involved in the key processes of the viral life cycle, such as replication, movement and encapsidation (García & Pallás, 2015). Almost all viruses encode pathogenicity determinants that are able to cause physiologic alterations in the plant and consequent symptom development. Several viral-encoded proteins have been identified as avirulence factors able to elicit plant symptomatology. Among them, viral capsid proteins (CPs) can activate plant resistance by interaction with R genes. For example, CPs of *Tobacco mosaic virus* (TMV, *Tobamovirus* genus) are effectors of HR in tobacco, pepper, and eggplant (Culver, 2002). Moreover, several studies performed on plant RNA viruses belonging to different genera, identified MPs as pathogenicity determinants linked to plant symptom induction and host specificity (Fujita *et al.*, 1996). For example, studies performed with full-length infectious cDNA clones of *Tomato mosaic virus* (ToMV, *Tobamovirus* genus) showed that several mutations in the MP gene were associated to systemic infection and to the development of necrosis in leaves of *Nicotiana tabaccum* plants (Yang *et al.*, 2002). Amino acid substitutions in the MP of *Tomato torrado virus* (ToTV, *Torradovirus* genus) were linked to symptom manifestation and determination of host range infection (Wieczorek & Obrępalska-Stęplowska, 2016b).

1.2.4 Viral suppressors of gene silencing

Plant viruses have evolved to counteract PTGS antiviral plant defence mechanism. Some viral proteins, which participate in the viral life cycle, have also acquired the capacity to suppress PTGS. Viral suppressors of RNA silencing (VSRs) have been identified in almost all genera of plant-infecting viruses, among viral functional proteins such as capsid proteins, movement proteins, replicases and others. VSRs target different steps of PTGS pathway inhibiting this host defence response with different strategies, in order to allow viral multiplication and spread (Burgyán & Havelda, 2011). The major strategy used by viruses to suppress PTGS is based on the sequestration of siRNAs. Viral proteins bind to siRNAs and hinder the formation of the RISC complex. This strategy is adopted by some well-characterized VSRs such as the Hc-Pro protein of *Tobacco etch virus* (TEV, *Potyvirus* genus), the p19 protein of *Tomato bushy stunt virus* (TBSV, *Tombusvirus* genus) and the p122 subunit of TMV (Silhavy *et al.*, 2002; Lakatos *et al.*, 2006; Csorba *et al.*, 2007). Another strategy used by VSRs is based on the interaction with PTGS host effector proteins to block their function: p25 of *Potato virus X* (PVX, *Potexvirus* genus) and P0 of *Poleovirus* interact with the Ago1 protein mediating its degradation (Chiu *et al.*, 2010); P38 of *Turnip crinkle virus* (TCV, *Carmovirus* genus) and P6 of *Cauliflower mosaic virus* (CaMV, *Caulimovirus* genus) block Dicer nuclease activity (Deleris *et al.*, 2006; Haas *et al.*, 2008). Another proposed strategy is that VSRs bind the effector dsRNA to hinder Dicer recognition and processing (Merai *et al.*, 2006).

Viral proteins which act as suppressors of gene silencing can induce plant symptomatology interfering with plant metabolic pathways and/or compromising RNA silencing function in the regulation of host gene expression. Alternatively, VSRs enable viral spread and accumulation increasing virus-induced symptomatology (Senda *et al.*, 2004; Ding & Voinnet, 2007; Wang *et al.*, 2012). For example, p21 of *Beet yellows virus* (BYV, *Closterovirus* genus) and p19 of TBSV were shown to affect siRNA and miRNA biogenesis (Chapman *et al.*, 2004); 2b protein of *Cucumber mosaic virus* (CMV, *Cucumovirus* genus) is a well-characterized VSR that enhances virus-related symptoms in combination with other CMV products in *Arabidopsis thaliana*, *Nicotiana benthamiana* and tobacco plants (Zhang *et al.*, 2006; Lewsey *et al.*, 2009) and p23 protein of *Citrus tristeza virus* (CTV, *Closterovirus* genus) was found to be a multifunctional protein related to PTGS suppression and plants symptoms manifestation (Ruiz-Ruiz *et al.*, 2013).

1.3 Control of plant viruses

Nowadays no antiviral products exist to cure infected plants, and disease control measures are based on prevention, eradication or reduction of viral outbreaks. Therefore, early diagnosis of the viral aetiological agents is essential to allow an immediate application of the adequate measures of disease control. Prophylactic measures are based on the use of virus-free plant material based on certification and quarantine programs to hinder the propagation of viruses through infected seeds and plants (Van den Bosch *et al.*, 2007). Moreover, appropriate agronomic and cultivation practices, such as correct decontamination of working tools, cultivation of specific crops to interrupt the virus transmission cycle and eradication of infected plants, are essential to hinder virus spread. Other control measures of viral diseases are based on the control of vectors by chemicals or using predators (biological control). Moreover, cross-protection, based on the inoculation of plants with mild viral isolates to induce resistance and protect them from further infection of more severe isolates, is a controversial strategy used with success in some horticultural crops (Lecoq, 1998). Tomato plants pre-inoculated with mild strains of *Pepino mosaic virus* (PepMV, *Potexvirus* genus) are currently used to prevent the infection by severe strains of the virus in some production areas (Gomez *et al.*, 2009). Finally, the use of resistant plants obtained by breeding or by genetic engineering represents one of the most effective methods to combat viral diseases. Genetic engineering allows to introduce in the plant specific genes, which confer resistance to a particular virus, or genetic viral fragments to trigger RNA silencing (Kang *et al.*, 2005; Prins *et al.*, 2008; Simón-Mateo & García, 2011).

1.4 RNA viral infectious clones

The progress of genetic engineering allowed the development of new strategies to improve the knowledge of viruses and their hosts. Full-length cDNA infectious clones of plant viruses represent an important biotechnological tool in molecular virology and are used to study viral molecular and cellular biology and host-virus interactions. Viral genomes can be manipulated *in vitro* to perform reverse genetic studies and to evaluate viral replication, gene expression, movement, pathogenic determinants, and recombination. Viral full-length cDNA infectious clones can be used for the construction of viral vectors for transient expression of exogenous proteins in plants with pharmaceutical or industrial applications.

Finally, viral vectors can be used to silence host genes to study their function and to engineer transgenic plants (Boyer & Haenni, 1994).

For the development of full-length cDNA clones, the viral genome is cloned into a binary plasmid that allows its multiplication and storage in bacterial cells. For viruses with an RNA genome, the RNA must be first transcribed *in vitro* into complementary cDNA and then inserted into the binary plasmid (Hull & Davies, 1983). For RNA viruses, two types of infectious clones can be distinguished depending on the site of transcription (Fig. 1.3): (i) infectious RNAs which are transcribed *in vitro* prior to inoculation and (ii) infectious cDNAs which are transcribed *in vivo* in the plant inoculated cells (Nagyová & Subr, 2007).

To obtain infectious RNA transcripts, the viral genome is cloned under a strong bacteriophage promoter (SP6, T3 and T7) into a binary plasmid. After RNA transcription *in vitro*, RNA transcripts are delivered by mechanical inoculation, electroporation or particle bombardment into the recipient plant or protoplasts. RNA transcripts are then used as mRNA and traduced by cellular machinery to produce the final viral particles. Infectivity of infectious RNA transcripts depends on the heterogeneity of the transcript population, the presence of mutations that affect the replication, the presence of Cap structures that are essential for their stability and the presence of a polyA tail. Moreover, the size of the genome, the inoculation method and the host can also affect the infectivity of the RNA transcripts (Nagyová & Subr, 2007).

On the other hand, to obtain infectious cDNAs, the viral genome is cloned under the CaMV 35S promoter in a binary plasmid that contains the T-DNA, derived from the tumour-inducing (Ti) plasmid. The recombinant construct is then introduced into the plant through *Agrobacterium tumefaciens* mediated gene transfer and the viral cDNA is randomly integrated into the plant genome. The viral genome transcription occurs in the plant cell nucleus and produces viral particles that spread into the plant (Peyret & Lomonossoff, 2015). Infectious cDNA clones present some advantages in comparison to infectious RNA transcripts because the infectivity is less prone to RNA degradation and they do not need *in vitro* transcription. Moreover, infectious cDNA clones are characterized by an increased expression efficiency and they can be used also for viruses that are not mechanically transmissible (Nagyová & Subr, 2007).

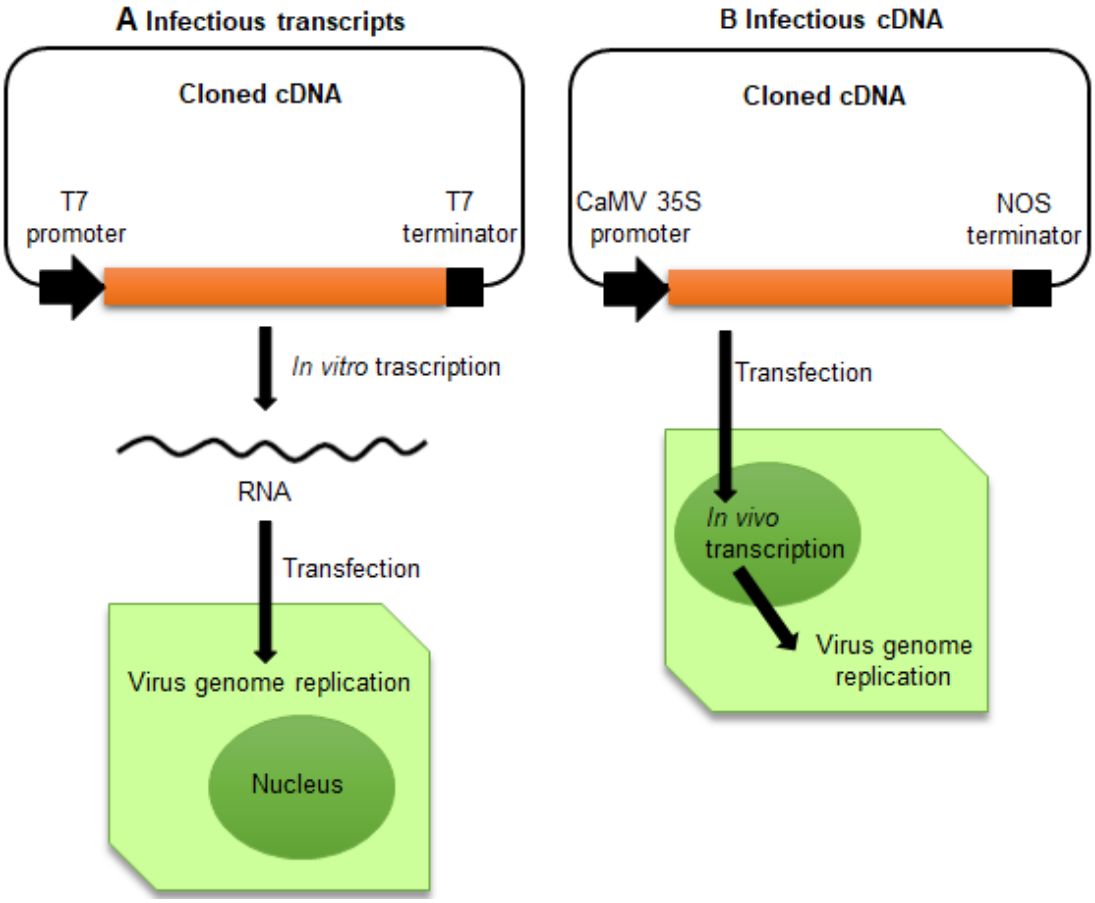

Figure 1.3 Schematic representation of RNA viral infectious clones. Orange bars represent cloned cDNA genome of the virus. **(A)** Infectious RNA transcripts, T7 driven. **(B)** Infectious cDNA clones, 35S driven. Images were readapted from Nagyová & Subr, 2007.

1.5 Plant virus used in this study: *Broad bean wilt virus 1*

Broad bean wilt virus 1 (BBWV-1) is the type member of the *Fabavirus* genus from the *Secoviridae* family. The name of the *Secoviridae* family derives from the amalgamation of the former families *Sequiviridae* and *Comoviridae* (Ninth report of the ICTV, 2009, https://talk.ictvonline.org/). *Secoviridae* family groups all plant-infecting picorna-like viruses and comprises the genera *Cheravirus*, *Sadwavirus*, *Sequivirus*, *Torradovirus*, *Waikavirus* and the sub-family *Comovirinae* (that includes the genera *Comovirus*, *Nepovirus* and *Fabavirus*) (Sanfaçon, 2015). Genus classification of the *Comovirinae* sub-family has been mainly based on the biological transmission: comoviruses are transmitted by beetles, nepoviruses by nematodes and fabaviruses by aphids (Fauquet *et al.*, 2005). BBWV-1 belongs to *Fabavirus* genus that includes other four virus species: *Broad bean wilt virus 2*

(BBWV-2), *Lamium mosaic virus* (LMMV), *Gentian mosaic virus* (GeMV) and *Cucurbit mild mosaic virus* (CuMMV).

1.5.1 Distribution, host range and transmission of fabaviruses

Fabaviruses are worldwide distributed and are characterized by a wide host range. Epidemic outbreaks were recorded during the last 40 years in different world regions causing considerable harvest losses (Lisa & Boccardo, 1996). Fabaviruses have been reported in Oceania (Stubbs, 1947; Shukla *et al.*, 1980), in North America (Schroeder & Provvidenti, 1970; Lockhart & Betzold, 1982; Provvidenti *et al.*, 1984; Scott & Barnett, 1984; Rist & Lorbeer, 1989), in South America (Gracia & Gutierrez, 1982), in Africa (Castrovilli *et al.*, 1985; Makkouk *et al.*, 1990) and in Asia (Mali *et al.*, 1977; Makkouk *et al.*, 2001). In Europe they were identified in Britain (Frowd & Tomlinson, 1970), in Slovenia (Mehle *et al.*, 2008) in Bulgaria (Yankulova & Kaitazova, 1979), in Germany (Weidemann *et al.*, 1975), in southern France (Marrou *et al.*, 1976), in Spain (Rubio *et al.*, 2002) and in Italy (Conti & Masenga, 1977; Rosciglione & Cannizzaro, 1977; Davino *et al.*, 1989).

Fabaviruses host range comprises more than 400 plant species and includes horticultural crops such as pepper (*Capsicum annuum*), tomato (*Solanum lycopersicum*), broad bean (*Vicia faba*), common bean (*Phaseolus vulgaris*), artichoke (*Cynara scolymus*), carrot (*Daucus carota*), lettuce (*Lactuca sativa*), eggplant (*Solanum melongena*), spinach (*Spinacia oleracea*), pea (*Pisum sativum*), and ornamental plants such as daffodil (*Narcissus*) and begonia (*Begonia semperflorens*). Depending on the infected plant species, the virus induced symptomatology ranges from mosaic, foliar distortion, ringspot, wilting, apical necrosis, and chlorosis, but also symptomless infections were recorded (Lisa & Boccardo, 1996).

Fabaviruses are transmitted by more than 20 aphid species in a non-persistent manner. Acquisition time ranges from 15 seconds to 10 minutes, whereas retention time between 30 seconds to 24 hours. Fabaviruses can be transmitted mechanically but not vertically by seed (Lisa & Boccardo, 1996). Besides visual and microscopic observation, several diagnostics methods for viruses belonging to *Fabavirus* genus are available. In the case of BBWV-1, diagnosis can be performed by ELISA, RT-PCR (Ferrer *et al.*, 2007; Panno *et al.*, 2014), quantitative RT-PCR (Ferriol *et al.*, 2011), and molecular hybridization (Ferrer *et al.*, 2008).

1.5.2 Virion and genome organization of *Broad bean wilt virus 1*

BBWV-1 is composed of two molecules of positive ssRNA that are separately encapsidated in non-enveloped virions with icosahedral morphology of 25-30 nm in diameter. The capsid is constituted of two different capsid proteins (Fig. 1.4). Genomic RNAs present a viral genome-linked protein (VPg) at their 5' ends and a polyA tail at their 3' ends. Each genomic RNA encodes for a polyprotein that is processed by a viral-encoded protease in functional peptides (Sanfaçon, 2015). RNA1 (~ 5.8 kb) encodes for a polyprotein which renders, after proteolytic cleavage, a protease cofactor (PRO-CO), a helicase (HEL), a viral genome-linked protein (VPg), a protease (PRO) and an RNA-dependent RNA polymerase (POL). RNA2 (~ 3.4 kb), like other viruses belonging to the genera *Fabavirus* and *Comovirus*, has two "in frame" successive translation initiation codons encoding for alternative polyproteins which differ only in their N-terminal extension length. Both polyproteins render by proteolytic cleavage identical large and small coat proteins (LCPs and SCPs, respectively) but putative MPs of different sizes with overlapping C-terminal regions: one protein with molecular weight of 47 kDa (MP) and one protein with a molecular weight of 37 kDa (VP37) lacking the first 90 amino acids. However, there are no experimental evidences of in vivo accumulation of VP37, whereas studies performed with BBWV-2, member of the *Fabavirus* genus, or with *Bean pod mottle virus* (BPMV) and *Cowpea mosaic virus* (CPMV), members of the *Comovirus* genus, demonstrated the synthesis *in vivo* of the two putative MPs of different size with overlapping C-terminal regions (Chen & Bruening, 1992; Lekkerkerker *et al.*, 1996; Qi *et al.*, 2002; Lin *et al.*, 2014). It was demonstrated that this protein was associated with the formation of tubular structures involved in cell to cell virus movement through PDs (Van Lent *et al.*, 1990; Liu *et al.*, 2009; Liu *et al.*, 2011; Xie *et al.*, 2016).

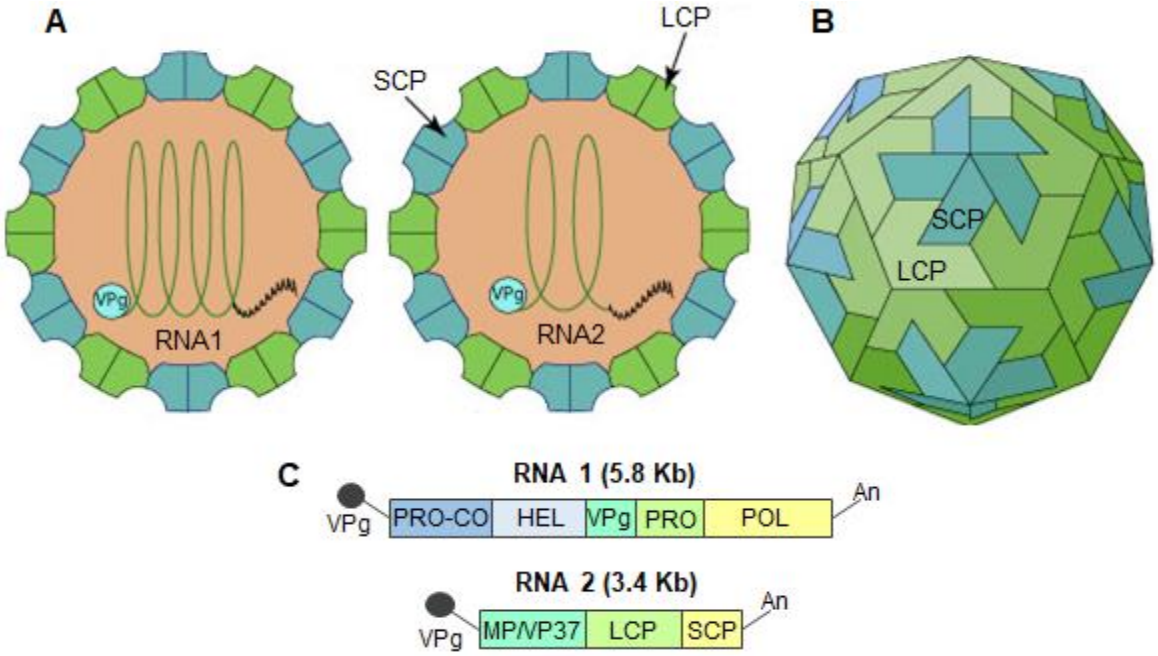

Figure 1.4 Virion and genome organization of *Broad bean wilt virus 1*. **(A)** Virions of BBWV-1 containing RNA1 and RNA2 with a 5´-VPg protein and a 3'-polyA tail. **(B)** Virion structure showing the two large and small capsid proteins. **(C)** RNA1 and RNA2 genomic organization. Images were readapted from http://viralzone.expasy.org.

Chapter 2

Interest and objectives

Among plant diseases, viral infections represent one of the major threats to worldwide agriculture, mainly those infecting horticultural crops. Plant viruses cause important economic losses and their control is based on the prevention and the containment of the virus spread. Virus infections rely on complex interactions with plant host and vectors. As obligate parasites, the relationships between the viruses and the host plants are essential to complete the viral life cycle and for the successful transmission to other plants. For this reason, understanding the mechanisms involved in virus-host interactions and pathogenesis is essential to develop efficient control strategies.

This doctoral thesis was based on the study of the biological properties of different viral isolates of *Broad bean wilt virus 1* (BBWV-1), type member of the *Fabavirus* genus, belonging to the *Secoviridae* family. In addition, the implication of RNA2-encoded proteins on plant defence mechanisms and symptom development were studied. BBWV-1 is worldwide distributed and infects several plant species, including important horticultural crops, such as pepper (*Capsicum annuum*), tomato (*Solanum lycopersicum*) and broad bean (*Vicia faba*). BBWV-1 is transmitted by more than 20 species of aphids in a non-persistent manner. BBWV-1 infection can be relevant in pepper and broad bean crops of the Mediterranean basin. Currently available research studied the mechanisms of the viral infectious cycle and the interactions with host and insect vectors of different viruses belonging to *Secoviridae* family (Fuchs *et al.*, 2017). Regarding BBWV-1, only few transmission and molecular variability studies were performed (Ferrer *et al.*, 2005; Ferriol *et al.*, 2013; Ferriol *et al.*, 2014) and recent reverse genetics systems are available (Ferriol *et al.*, 2016). However, BBWV-1 viral variants have not been characterized biologically, and the role played by the viral proteins in the pathological processes, that leads to plant disease, have not been determined.

The first part of this doctoral thesis was addressed to study the biological characteristics of BBWV-1 viral isolates. For years, the taxonomic classification of fabaviruses has undergone numerous changes and information such as host range, induced plant symptoms or transmission vectors has been scarce and confusing. Thus, in order to obtain more information about BBWV-1, four viral isolates belonging to different phylogenetic clusters and originating from different countries were analysed. The ability of these BBWV-1 isolates to infect different plant species was tested and the induced symptomatology for each viral isolate was recorded. Moreover, it was studied if viral accumulation over time was correlated to induced-plant symptoms.

The second part of this thesis was addressed to the study of BBWV-1-plant interactions through the use of cDNA infectious clones. Infectious clones are important biotechnological tools that can be used for the evaluation of the virus life cycle and the study of host-virus interactions. Hence, 35S-driven infectious cDNA clones for BBWV-1 RNA1 and RNA2 were engineered, to evaluate the role of BBWV-1-encoded VP37 protein in symptom determination, viral accumulation and virion formation. In addition, the obtained results were complemented by other approaches, such as transient expression in *Nicotiana benthamiana* plants through a viral vector based on *Potato Virus X* (PVX) sequence. Moreover, VP37 role in suppression of post-transcriptional gene silencing (PTGS) was estimated through a transient expression assay in *N. benthamiana* 16c and a complementation assay based on the use of a viral vector based on the *Turnip crinkle virus* (TCV) sequence. The construction of a BBWV-1 cDNA infectious clone opens the possibility to engineer a viral vector to perform genetic reverse studies on important horticultural crops such as tomato, pepper and broad bean for further studies.

Finally, the third part of this thesis was based on the study of the role of the other BBWV-1 RNA2-encoded MP, LCP and SCP proteins in virus-plant interactions. Several studies, performed on viruses belonging to *Secoviridae* family, identified pathogenicity determinants in RNA2 (Canizares *et al.*, 2004; Kong *et al.*, 2014; Kwak *et al.*, 2016; Seo *et al.*, 2017). In order to identify pathogenicity determinants, transient expression assay through a PVX viral vector was performed in *N. benthamiana*. Moreover, RNA2-encoded proteins were investigated to identify suppressors of PTGS trough transient expression assay in *N. benthamiana* 16c and a complementation assay using the TCV viral vector.

The manuscript of this doctoral thesis was organised in different sections according to the main goals of this work:

1. Biological characterization and accumulation of BBWV-1 isolates

2. Evaluation of BBWV-1 RNA2-encoded VP37 as host and pathogenicity determinant

3. Evaluation of BBWV-1 RNA2-encoded MP, SCP and LCP proteins in suppression of post-transcriptional gene silencing and induction of plant symptoms

Chapter 3

Biological characterisation and viral accumulation of BBWV-1 isolates

The research work presented in this chapter was included in the paper:

Biological characterization and viral accumulation of *Broad bean wilt virus 1 (*BBWV-1) isolates, C. Carpino C, L. Elvira-González, I, L. Rubio L, E. Peri, S. Davino, L. Galipienso L (*Annals of Applied Biology*, under revision).

3.1 Introduction

The *Secoviridae* family was introduced in the ninth report of the International Committee on Taxonomy of Viruses (ICTV) in 2009 (https://talk.ictvonline.org/). The *Secoviridae* family groups all plant-infecting picorna-like viruses from the *Comovirinae* sub-family (comprising the genera *Comovirus*, *Nepovirus* and *Fabavirus*), with the genera *Cheravirus*, *Sadwavirus*, *Sequivirus*, *Torradovirus* and *Waikavirus* (Sanfaçon, 2015). The *Fabavirus* genus comprises five virus species: *Broad bean wilt virus 1* (BBWV-1), *Broad bean wilt virus 2* (BBWV-2), *Lamium mosaic virus* (LMMV), *Gentian mosaic virus* (GeMV) and *Cucurbit mild mosaic virus* (CuMMV). The taxonomic classification of the *Fabavirus* genus has been confusing for years since viral isolates of the same virus species were called with different names depending on the host species or the country where they were detected. Indeed, *Broad bean wilt virus* (BBWV) was first described in infected broad bean (*Vicia faba*) plants in Australia (Stubbs, 1947) and later on it was identified and classified as a different species depending on the host species and the geographic area where it was reported. Viral species such as *Parsley virus 3* (PVS3) (Frowd & Tomlinson, 1970) *Nasturtium ringspot virus* (NRSV) (Boccardo & Conti, 1973) and *Petunia ringspot virus* (PeRSV) (Rubio-Huertos, 1968) were demonstrated to be a unique viral species named BBWV (Frowd & Tomlinson, 1972; Sahambi *et al.*, 1973; Doel, 1975). Further serological and nucleotide sequence analysis showed that BBWV isolates could be separated in two different species, BBWV-1 and BBWV-2 (Uyemoto & Provvidenti, 1974; Kobayashi *et al.*, 1999; Kobayashi *et al.*, 2003; Murphy *et al.*, 2012).

BBWV-1 infects important horticultural and ornamental crops worldwide. However, information about the biological properties of BBWV-1 isolates, such as host range or induced plant symptoms, is very scarce and unclear. BBWV-1 shows higher genetic diversity than most plant viruses with frequent events of recombination and reassortment (Ferriol *et al.*, 2014). However, the relationship between genetic variation and differences in biological behaviour among BBWV-1 isolates has not been studied yet. For this purpose, four BBWV-1 isolates (Ben, PV0548, B41/99 and NSRV) clustering in different phylogenetic groups (Ferriol et al., 2014) were evaluated for symptoms, viral infectivity and accumulation in broad bean and pepper plants.

3.2 Materials and methods

3.2.1 BBWV-1 isolates and mechanical inoculation in herbaceous hosts

BBWV-1 isolates Ben, PV0548, B41/99 and NSRV were multiplied and maintained in broad bean plants. These BBWV-1 viral isolates were collected from different hosts and countries and grouped into different phylogenetic clades (Ferriol et al., 2014) (Table 3.1). To exclude possible mixed infections with other viruses, BBWV-1-infected broad bean plants were analysed for the most common viruses infecting broad bean: the absence of *Bean common mosaic virus* (BCMV), *Bean common necrotic virus* (BCMNV), *Bean yellow mosaic virus* (BYMV) and *Broad bean V virus* (BBVV) infection was tested by RT-PCR with degenerated generic primers of potyviruses (Hu et al., 2010). The absence of *Alfalfa mosaic virus* (AMV), *Cucumber mosaic virus* (CMV) and *Tomato spotted wilt virus* (TSWV) was assessed by DAS-ELISA using specific antibodies (Loewe). Absence of BBWV-2 was evaluated by molecular hybridization with a specific digoxigenin labelled riboprobe (Ferriol et al., 2015).

In order to determine the host range, infectivity rates, induced symptomatology and virus accumulation, different BBWV-1 isolates were mechanically inoculated in 10 plants of the following varieties: tomato var. Marmande (Fito) (*Solanum lycopersicum*), broad bean var. Reina Mora (Fito) (*Vicia Faba*), pepper var. Manolo (Fito) (*Caspicum annuum*), common bean var. Helda (Mascarell) (*Phaseolus vulgaris*), cucumber var. Granada (Fito) (*Cucumis sativus*), melon var. Pinonet (Mascarell) (*Cucumis melo*) and *Nicotiana benthamiana.* All plants were inoculated at two true leaf stage and the experiment was repeated twice. Mechanical inoculation was performed by homogenization of 1 g of broad bean plant tissues infected with each BBWV-1 isolate in the inoculation buffer (Na_2HPO_4 0.01 M, NaH_2PO_4 0.01 M, pH 7.2). Homogenized extracts were rub-inoculated using carborundum. Prior to inoculation, viral concentration in the inoculum source corresponding to BBWV-1 infected broad bean plants was estimated by quantitative RT-PCR (RT-qPCR) and the viral concentration of each BBWV-1 isolate was adjusted to 3×10^7 BBWV-1 RNA1 copies per ng of total RNA in the inoculation buffer. As a mock inoculation control, 10 plants of each plant species were inoculated only with inoculation buffer. Plants were maintained in a growth chamber under conditions of 16 h of light at 24°C and 8 h of darkness at 20°C. BBWV-1 infection was assessed at 16 days post inoculation (dpi) by molecular hybridization with a virus-specific riboprobe as previously described (Ferrer *et al.*, 2008). Symptomatology of BBWV-1 infected plants was recorded at 16, 32 and 40 dpi.

Table 3.1 BBWV-1 isolates used in this study. Geographical origin, collection host and date and GenBank accession numbers of the published nucleotide sequences are listed for each virus isolate.

BBWV-1 isolate	Geographical origin	Collection host	Collection date	Accession Number
PV0548 (SV-3-88)	Syria	*Vicia faba*	1998	JF440100, JF440076 JF440124, JF440053
Ben	Spain	*Capsicum annuum*	2001	AY781172 AY781171
B41/99	Bulgaria	*Capsicum annuum*	2000	JF44009, JF440068 JF440115, JF440036
NSRV	Italy	*Unknown*	1996	JF440097, JF440074 JF440121, JF440049

3.2.2 RNA extraction

For RT-qPCR and molecular hybridization assays, 0.1 g of apical non-inoculated leaves were grinded in a power homogenizer TissueLyser (Qiagen, Hilden, Germany) with liquid nitrogen. Total RNA was extracted using a phenol:chloroform:isoamyl alcohol standard protocol followed by ethanol precipitation as previously described (Ferriol *et al.*, 2011) and resuspended in 40 µl of RNase free water. For RT-qPCR analysis, the concentration of total RNAs was measured with UV–vis Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific) and adjusted to 10 ng/µl.

3.2.3 Virus quantitation by real-time RT-PCR

BBWV-1 virus accumulation was estimated by RT-qPCR from total RNA extracts obtained from apical leaves of five broad bean and pepper plants infected with Ben, PV0548, B41/99 and NSRV at 4, 8, 16, 24 and 32 dpi. Two replicates for each measurement were done. For absolute quantification, RT-qPCR was performed using a primer set and a TaqMan minor groove binding (MGB) probe which was designed in a conserved region of BBWV-1 RNA1, as described by Ferriol *et al.*, 2011. RT-qPCR was performed in a LightCycler480 (Roche) and each reaction contained 50 ng of total RNA, 1xTaqMan One-Step Master Mix (Roche), 15 U Multiscribe Reverse Transcriptase (Invitrogen), 2 U RNase Inhibitor (Invitrogen), 0.5 µM of each forward and reverse primer and 0.25 µM of the TaqMan minor groove binding (MGB) probe. RT-qPCR cycling conditions comprised reverse transcription

at 48°C for 30 min, incubation at 95°C for 10 min, and 45 cycles of 95°C for 2 sec, 58°C for 8 sec and 60°C for 10 sec. Data were analysed by multifactorial ANOVA test (P-value > 0.05) with Stargraphics software (Statgraphics Technologies, Inc.). For each assay, a mock inoculation, a healthy plant and a blank sample were included as negative controls. The calibration curve was obtained as described Ferriol et al., 2011. Briefly, an amplicon equivalent to RT-qPCR product was obtained by conventional RT-PCR using a forward primer which contained the T7-promoter sequence. Positive-sense RNA transcripts were synthetized with Megascript T7 Kit (Ambion) and were treated with Turbo RNase-free DNase (Ambion) following the manufacturer's instructions. Transcripts were purified by ethanol precipitation and the number of ssRNA copies was evaluated. 10-fold serial dilutions containing 10^{10} to 10^{1} RNA copies of ssRNA copies were prepared and used to obtain a calibration curve with an amplification efficiency of 99% in a range equivalent to 10^{3} to 10^{10}.

3.3 Results

3.3.1 Infectivity of different BBWV-1 isolates

Infectivity (proportion of infected plants) of BBWV-1 isolates Ben, B41/99, NSRV and PV0548 were almost identical (Table 3.2). All four BBWV-1 isolates infected all inoculated broad bean and *N. benthamiana* plants. Infections rates in tomato were 50 % for isolate Ben and isolate B41/99, 60 % for isolate NSRV and 40 % for isolate PV0548. In pepper, infectivity rates were higher than in tomato, being 95, 85, 80 and 70 % for isolates Ben, B41/99, NSRV and PV0548, respectively. In contrast, none of the BBWV-1 isolates were mechanically transmitted to cucumber, common bean and melon.

Table 3.2 Infectivity of BBWV-1 isolates on different host. Percentages of infected plants were calculated from two independent assays of 10 plants for each specie.

BBWV-1 isolate	Tomato	*N. benthamiana*	Broad bean	Pepper	Common bean	Cucumber	Melon
PV0548	40 %	100 %	100 %	75 %	0 %	0 %	0 %
Ben	50 %	100 %	100 %	95 %	0 %	0 %	0 %
B41/99	50 %	100 %	100 %	85 %	0 %	0 %	0 %
NSRV	60 %	100 %	100 %	80 %	0 %	0 %	0 %

3.3.2 Symptomatology of different BBWV-1 isolates

Plants symptoms induced by the BBWV-1 isolates showed differences depending on the host plant, the viral isolate and the infection time.

In broad bean, PV0548-infected plants showed at 16 dpi a middle leaf mosaic and a nervial chlorosis that disappeared at 40 dpi, remaining only the middle mosaic (Fig. 3.1). At this infection time, new leaves showed a slight distortion. Ben-infected plants showed intense nervial chlorosis that evolved to middle mosaic and slight leaf distortion in old and new leaves at 40 dpi. B41/99 and NSRV-infected plants showed similar symptoms which consisted in intense nervial chlorosis and a leaf distortion that increased in severity until 40 dpi. New leaves of B41/99 and NSRV-infected plants showed strong distortion and leaf curling. In pepper, BBWV-1 isolates induced symptoms that usually started at 10 dpi and remained constant up to 40 dpi (Fig. 3.2), which consisted in mosaic and leaf distortion whose severity was dependent on the viral isolate. Ben-infected plants showed a severe mosaic, PV0548 and NRSV-infected plants showed a mild mosaic whereas B41/99 infected plants showed only a slight mosaic that was almost imperceptible.

In *N. benthamiana* plants, the four BBWV-1 isolates induced the same symptoms consisting in stunting, chlorosis, severe mosaic and finally plant death (data not shown). Tomato plants infected with BBWV-1 isolates remained asymptomatic like the mock-inoculated controls.

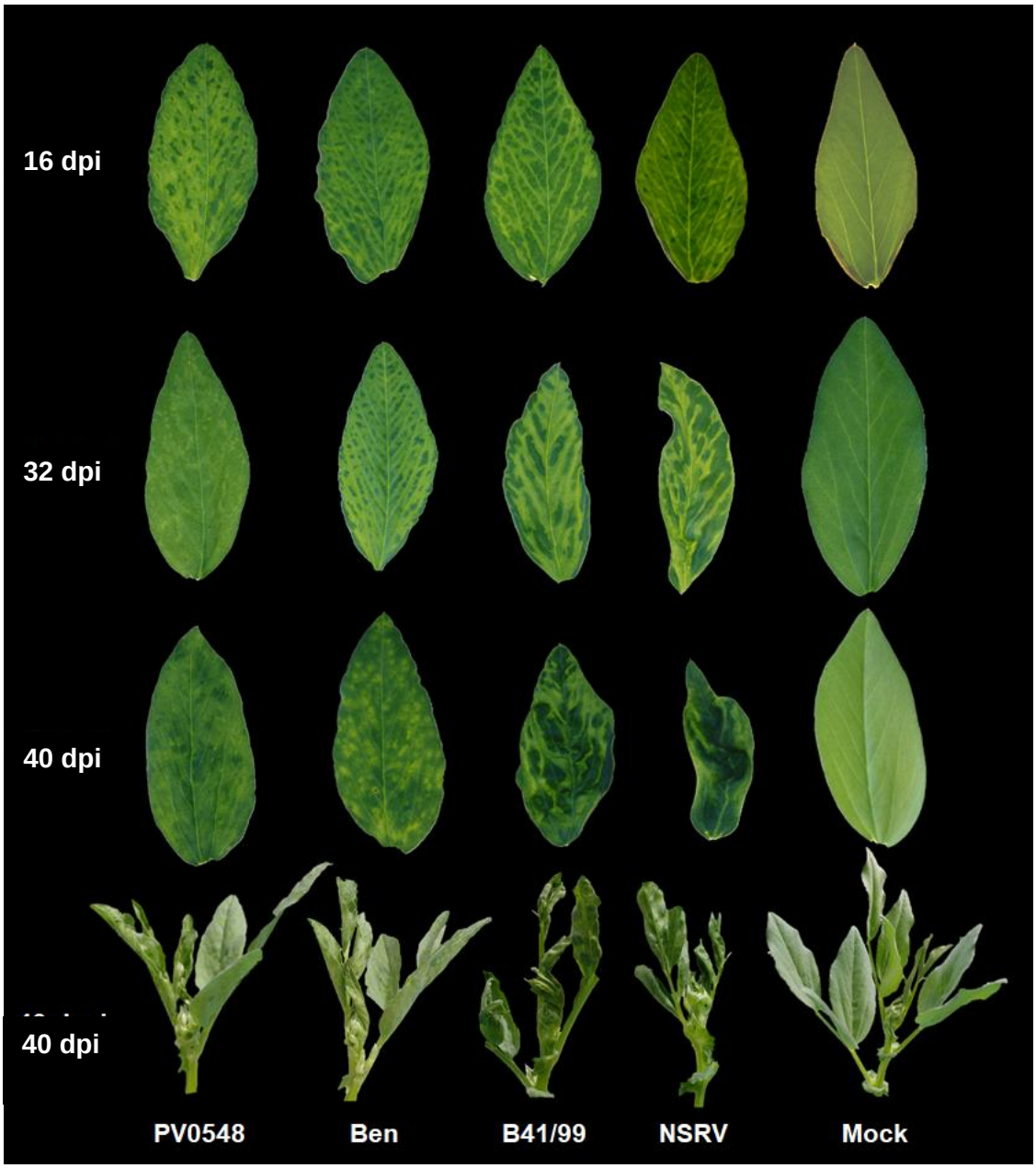

Figure 3.1 Nervial chlorosis, mosaic and leaf distortion symptoms observed at 16, 32 and 40 dpi in broad bean leaves infected with BBWV-1 isolates Ben, B41/99, NSRV and PV0548. At the bottom, symptoms of the new leaves at 40 dpi.

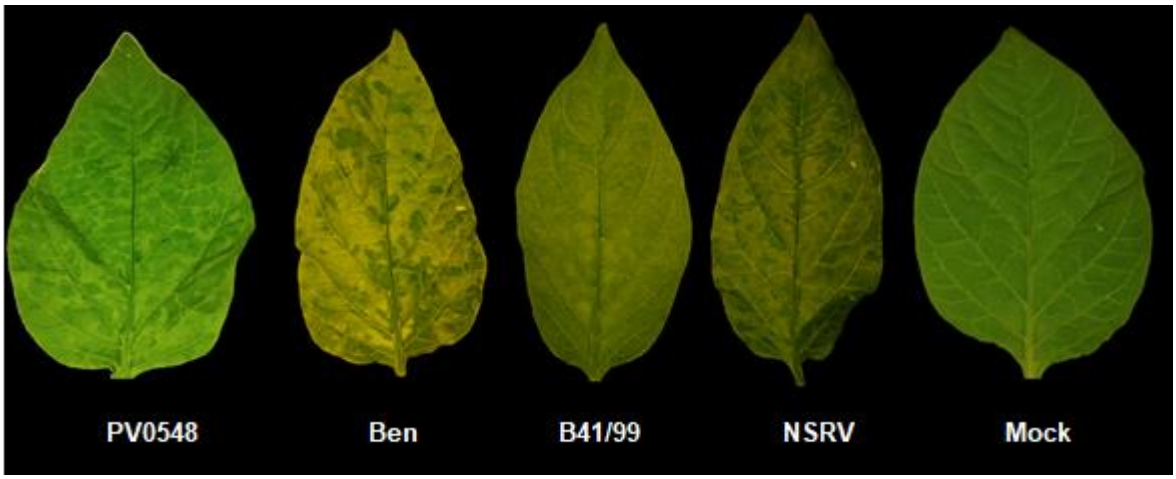

Figure 3.2 Mosaic and distortion leaf symptoms observed in pepper plants infected with BBWV-1 isolates (Ben, B41/99, NSRV and PV0548) at 20 dpi.

3.3.3 Accumulation of different BBWV-1 viral isolates

To study if the differences of plant symptoms severity, induced by PV0548, Ben, B41/99 and NSRV, were correlated to viral replication, the virus accumulation over time of each BBWV-1 isolate was estimated in the inoculated brad bean and pepper plants (Fig. 3.3). The mean values of viral concentration at 16, 24 and 32 dpi, obtained through ANOVA statistical analysis revealed that accumulation of Ben, PV0548 and B41/99 was higher in broad bean than in pepper plants (10 times higher for the two first isolates and 100 times higher for the last one) whereas accumulation of isolate NSRV was the same in both plant species. NSRV was also the isolate that showed lower accumulation values in the two host species.

BBWV-1 isolates showed different virus accumulation depending on host species. This result is in concordance with a previous study which assessed that Ben showed different virus accumulation over time in broad bean and *Chenopodium quinoa* plants (Ferriol *et al.*, 2011).

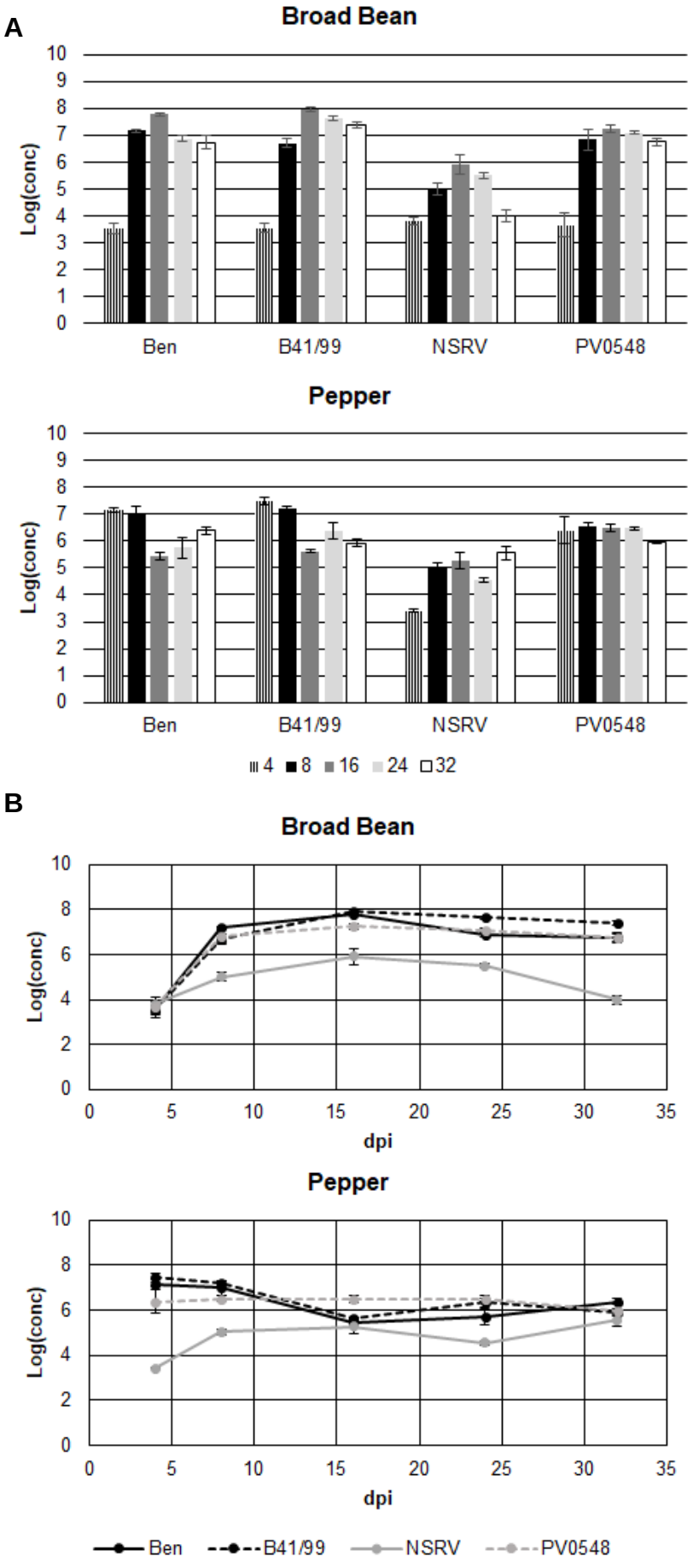
A
Broad Bean
Log(conc)
Ben
B41/99
NSRV
PV0548
Pepper
4
8
16
24
32
B
Broad Bean
dpi
Pepper
Ben
B41/99
NSRV
PV0548

Figure 3.3 (A) Column and **(B)** lineal graphic representations of viral accumulation over time in pepper and broad bean plants inoculated with BBWV-1 isolates PV0548, Ben, B41/99 and NSRV. Viral accumulation is represented as logarithm of BBWV-1 RNA1 copies per ng of total RNA. Vertical bars represent standard errors.

In broad bean, virus concentration was similar between the four viral isolates at 4 dpi (3.43×10^3, 3.47×10^3, 6.34×10^3 and 4.57×10^3 RNA1 copies/ng of total RNA for Ben, B41/99, NSRV and PV0548, respectively). At 8 dpi, virus concentration strongly increased for isolate Ben, B41/99 and isolate PV0548 (1.48×10^7, 5.04×10^6 and 6.68×10^6 copies/ng of total RNA, respectively) whereas NSRV concentration increased only to $1{,}00 \times 10^5$ copies/ng of total RNA. The maximum accumulation peak was recorded for all four isolates at 16 dpi reaching concentrations of 5.94×10^7, 8.88×10^7, 1.73×10^7 and 7.96×10^5 copies/ng of total RNA for Ben, B41/99, PV0548 and NSRV, respectively. Virus concentration decreased slightly at 24 and 32 dpi to values of 7.48×10^6, 2.44×10^7, 5.62×10^6 and 5.62×10^6 copies/ng of total RNA for Ben, B41/99, PV0548 and NSRV, respectively.

In pepper, viral accumulation of BBWV-1 isolates was different to broad bean, with strong differences between some isolates. At 4 dpi, viral accumulations of isolates Ben, B41/99 and PV0548 were similar (1.44×10^7, 2.51×10^7 and 2.49×10^6 copies/ng of total RNA, respectively) but higher than of isolate NSRV (2.27×10^3 copies/ng of total RNA). Virus concentrations of Ben and B41/99 decreased to reach concentrations at 16 dpi of 2.49×10^5 and 4.28×10^5 copies/ng of total RNA, respectively. In contrast, NSRV viral concentration increased to 1.71×10^5 copies/ng of total RNA up to 16 dpi. From 16 dpi up to 32 dpi, isolate Ben concentration increased to 2.39×10^6 copies/ng of total RNA whereas concentration of B41/99 increased to 2.34×10^6 copies/ng of total RNA at 24 dpi and then decreased to 8.43×10^5 copies/ng of total RNA at 32 dpi. NSRV concentration decreased from 16 dpi to 24 dpi reaching values of 3.50×10^4 copies/ng of total RNA and increased to reach the maximum value of 3.56×10^5 copies/ng of total RNA. PV0548 did not show noticeable changes of virus accumulation values during all quantitation assay.

3.4 Discussion

Information about the biological properties of viruses belonging to *Fabavirus* genus is scarce and unclear, and no systematic comparison of different viral isolates has been performed. To delve on the study of the biological behaviour of BBWV-1, the induced symptoms, infectivity and accumulation of four BBWV-1 isolates (PV0548, Ben, B41/99 and NSRV) were evaluated. The BBWV-1-isolates used in this study were collected in different countries and clustered in different phylogenetic groups. PV0548, Ben, B41/99 and NSRV were able to infect broad bean, pepper, tomato and *N. benthamiana* plants. However, none of BBWV-1 isolates was able to infect common bean, cucumber and melon plants in spite of these species have been reported as natural hosts of other isolates of BBWV-1 or BBWV-2 in Germany, Italy, USA and China (Provvidenti, 1983; Lisa *et al.*, 1986; Edwardson & Christie, 1991; Dong *et al.*, 2012). With respect to tomato, it was reported that BBWV-1 or BBWV-2-infected plants showed symptoms of mosaic, chlorosis, reduced growth and deformation but viral isolates were not identified (Blancard, 2012). In the current work, BBWV-1 isolates PV0548, Ben, B41/99 and NSRV infected tomato plants but induced no symptoms.

The severity of the symptoms induced by BBWV-1 depended on the host species and on the virus isolate. The differences of symptom severity between BBWV-1 isolates were observed in broad bean and pepper plants but not in *N. benthamiana* plants. No correlation between accumulation of BBWV-1 isolates PV0548, Ben, B41/99 or NSRV and induced symptoms on broad bean or pepper plants was observed. In broad bean plants, Ben, B41/99 and PV0548 showed similar viral accumulation up to 32 dpi, despite of inducing symptoms of different severity. NSRV, accumulated significantly less than Ben or PV0549 that induced symptoms similar to B41/99 and more severe than those of Ben or PV0549. In pepper, Ben and B41/99 induced the most and the less severe mosaic symptoms, respectively. Both BBWV-1 isolates showed similar virus accumulation up to 32 dpi. In contrast, mosaic symptoms induced by NSRV were more severe than those induced by B41/99, also if NSRV accumulation was less than B41/99 at 4, 8 and 24 dpi.

One model which correlates virulence and pathogen replication is the trade-off hypothesis. This model is based on the concept that virulence is directly proportional to viral accumulation in host tissues. Consequently, the pathogen evolves in order to achieve the optimal replication and transmission rates for a specific host (Anderson & May, 1982). For vector-borne plant viruses, virulence can be affected by parasite accumulation both in the

host and in the vector. Consequently, virus accumulation increases virulence and virus transmission to another host (Froissart *et al.*, 2010). This controversial model is under debate (Alizon *et al.*, 2009) and several studies failed to prove a direct correspondence between virulence and virus replication, whereas others reported a positive correlation between both parameters. Studies performed with different CMV strains infecting *Arabidopsis thaliana* plants showed that virulence, measured as plant growth and seed production, was uncoupled to virus replication (Pagán *et al.*, 2007). Supporting these results, studies performed with *Prunus necrotic ringspot virus* (PNRS) (Moury *et al.*, 2001) suggested that virus accumulation and symptom severity were unrelated. In addition, an analysis of different strains of *Cucumber vein yellowing virus* (CVYV, *Ipomovirus* genus) demonstrated that symptom severity was not correlated to viral titre (Galipienso et al., 2013). In contrast, studies performed with *Papaya ringspot virus* (PRSV, *Potyvirus* genus) strains in zucchini squash, squash and watermelon plants (Pacheco *et al.*, 2003) correlated viral multiplication and symptom severity. The results obtained with BBWV-1 isolates supported the hypothesis that virulence, measured as symptom severity, does not correlate with virus replication. Further studies are needed to identify the BBWV-1 products that are implicated on virulence and plant symptom development.

Chapter 4

Role of BBWV-1 RNA2 encoded VP37 protein in the induction of plants symptoms and suppression of post-transcriptional gene silencing

The research work presented in this chapter was included in the paper:

Role of *Broad bean wilt virus 1* RNA2-encoded VP37 protein in the induction of plant symptoms and suppression of post-transcriptional gene silencing. C. Carpino, I. Ferriol, L. Elvira-González, V. Medina, L. Rubio, E. Peri, S. Davino, L. Galipienso (*Phytopathology*, under revision)

4.1 Introduction

Broad bean wilt virus 1 (BBWV-1) RNA2 (~ 3.4 kb) has two "in frame" successive AUG initiation codons encoding for two alternative polyproteins, named 2a and 2b. RNA2-encoded polyproteins render by proteolytic cleavage identical large and small coat proteins (LCPs and SCPs, respectively), and putative MPs of different sizes that share the same C-terminal amino acid sequence: the longest, named MP, is encoded by polyprotein 2a and has a molecular weight of 47 kDa whereas the smallest, named VP37, is encoded by polyprotein 2b, has a molecular weight of 37 kDa and lacks the first 90 amino acids. There are not experimental evidences of *in vivo* accumulation of VP37 for BBWV-1, nevertheless there are studies regarding some members of *Secoviridae* family that demonstrated the presence of this additional protein. Accumulation of MPs of different sizes has been assessed for *Bean pod mottle virus* (BPMV, *Comovirus* genus), *Cowpea mosaic virus* (CPMV, *Comovirus* genus) and *Broad bean wilt virus* 2 (BBWV-2, *Fabavirus* genus) (Lekkerkerker *et al.*, 1996; Hoffmann *et al.*, 2001; Qi *et al.*, 2002; Lin *et al.*, 2014). In all cases, the viral product originated from the second polyprotein encoded by RNA2 was associated with the formation of tubular structures and cell to cell movement (Van Lent *et al.*, 1990; Liu *et al.*, 2009; Liu *et al.*, 2011; Xie *et al.*, 2016).

Until now there are no available data about the role played by different viral products of BBWV-1 on the induction of plant symptoms. Studies with reassortant full-length cDNA infectious clones, containing the genomic RNA1 and RNA2 of two BBWV-2 isolates, that induce symptoms of different severity in *Nicotiana benthamiana* and pepper plants, showed that BBWV-2 RNA2 contained the factors responsible for enhancing BBWV-2-induced symptoms in both plants species (Kwak *et al.*, 2016). Further studies assessed that one single amino acid substitution in the C-terminus of BBWV-2 RNA2-encoded MP protein was sufficient for altering the virus symptom severity (Seo *et al.*, 2017). However, experiments performed with different strains of BPMV (*Comovirus* genus), and *Grapevine fanleaf virus* (GFLV, *Nepovirus* genus), identified the RNA1-encoded PRO-CO, HEL and POL proteins as determinants of symptom severity (Gu & Ghabrial, 2005; Vigne *et al.*, 2013). Transient expression of BPMV RNA1-encoded proteins PRO-CO and HEL, through a *Potato virus* X (PVX) vector, induced systemic necrosis in infected *N. benthamiana* plants reinforcing the hypothesis that these viral proteins could be involved on plant symptom induction (Gu & Ghabrial, 2005). Finally, transient expression of the RNA1-encoded N-terminal fragment of *Tomato torrado virus* (ToTV, *Torradovirus* genus), using a PVX vector, induced necrotic

lesions on agroinfiltrated leaves and systemic necrosis in *N. benthamiana* plants (Wieczorek & Obrępalska-Stęplowska, 2016b).

Several viral proteins involved in plant symptom induction have also been identified as suppressors of post-transcriptional gene silencing (PTGS) (Amin *et al.*, 2011; Burgyán & Havelda, 2011). PTGS is a RNA-specific degradation mechanism, used in plants for regulation of endogenous gene expression and for defence against pathogens. During viral infection, double-stranded RNA (dsRNA) structures of viral genomes activate PTGS, causing the degradation of viral RNA and preventing the spread of the virus within the plant. To counteract PTGS, viruses have evolved encoding proteins which have the ability to suppress this defence mechanism (viral suppressors of RNA silencing, VSRs). Within the *Secoviridae* family, both PRO-CO and HEL proteins of BPMV, as well as POL of GFLV, were associated with plant symptom induction but did not act as VSRs (Gu & Ghabrial, 2005; Vigne *et al.*, 2013). Similar experiments performed with SCP protein of CPMV or MP, VP37 and LCP proteins of BBWV-2 showed that these proteins had PTGS suppressor activity (Canizares *et al.*, 2004; Kong *et al.*, 2014).

In the current work, a full-length BBWV-1 cDNA infectious clone and a BBWV-1 cDNA mutant construction that lacks VP37 protein start codon were developed to study the role played by this protein in plant symptom induction and host specificity. In addition, transient expression assays of BBWV-1 VP37 protein using a PVX vector enhanced the PVX-induced symptoms in *N. benthamiana* plants and activated the systemic necrosis associated with programmed cell death (PCD). Finally, the ability of BBWV-1 VP37 protein to suppress PTGS was determined by transient expression assay in *N. benthamiana* 16c plants and by a movement complementation assay using a *Turnip crinkle virus* construct (pTCV-GFP) (Powers *et al.*, 2008).

4.2 Materials and methods

4.2.1 Plant and virus materials

Commercial plants of broad bean var. Reina Mora (Fito), pepper var. Manolo (Fito), tomato var. Marmande (Fito) were used in all the assays. BBWV-1 isolate Ben (Ferrer *et al.*, 2005; Ferriol *et al.*, 2014) was maintained in broad bean and *N. benthamiana* plants. Plants

were grown in a growth chamber under conditions of 16 h of light at 24°C and 8 h of darkness at 20 °C.

4.2.2 Construction of full-length genome cDNA clones of BBWV-1

To obtain BBWV-1 cDNA infectious clones suitable for agroinfiltration in different host plants, RNA1 and RNA2 full-length cDNAs of BBWV-1 were cloned into the binary plasmid pJL89 between the *Cauliflower mosaic virus* (CaMV) 35S promoter and the *Hepatitis delta virus* (HDV) ribozyme followed by the nopaline synthase terminator (t-Nos). Briefly, BBWV-1 RNA1 and RNA2 were amplified from BBWV-1 pBenR1 and pBenR2 T7-driven cDNA clones (GenBank accession numbers KT988973, KT988974) (Ferriol *et al.*, 2016) using CloneAmp HiFi PCR Premix (Takara Bio) with forward and reverse primers containing sequences overlapping the pJL89 binary vector. To obtain full-length BBWV-1 RNA1, RNA1 was divided into two segments which were separately cloned into pJL89 plasmid, and then reassembled with a further ligation reaction to obtain the full-length genomic RNA1 whereas RNA2 was cloned into plasmid pJL89 in one step. All the primers used for amplification are listed in Table 4.1. pJL89 vector was opened by inverse PCR using CloneAmp HiFi PCR Premix (Takara Bio) using specific pJL89 forward primer that anneals to the HDV ribozyme region and reverse primer that anneals to the promoter region. All the resulting PCR products were gel purified with PCR clean-up Gel extraction kit (Macherey-Nagel) and ligation reaction was performed using In-Fusion HD Cloning Kit (Takara Bio) following manufacturer's instructions. The resulting plasmids were named pBBWV1-R1 and pBBWV1-R2 for the RNA1 and RNA2, respectively. To obtain a mutant construct of BBWV-1 RNA2 with a truncated expression of VP37 protein, a single point modification at nucleotide position 492 of pBBWV1-R2 (AUG>AUC, Met>Ile) was introduced by inverse PCR using specific primers. PCR product was ligated using T4 DNA Ligase (Promega) and the resulting plasmid was named pBBWV1-R2:G492C. All plasmids were sequenced to confirm that no mutations were present, and transformed into *Agrobacterium tumefaciens* COR308 cells via electroporation.

Table 4.1 Primers used for the development of full-length cDNA clones of BBWV-1. Underlined letters indicate single point modifications.

Name	Sequence (5'-3')	Function
PJL89-R	CCTCTCCAAATGAAATGAACTTCCTTATATAGAGGA	Inverse PCR to open PJL89
PJL89-F	GGGTCGGCATGGCATCTC	
RNA1-AF	GTTCATTTCATTTGGAGAGGTGATTTAAAAAATTTTTAA ATCAAACAGCTTTCGTTCGGAA	PCR to amplify RNA1 (segment A) of BBWV-1
RNA1-AR	TGGAGATGCCATGCCGACCCCGACGTGTAGAGAGCTAT TTCAGAA	
RNA1-BF	AAATAGCTCTCTACACGTCGGCTAATTTGCCCAAAGCCC C	PCR to amplify RNA1 (segment B) of BBWV-1
RNA1-BR	TGGAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTTT TTTTCCTCTATTAAGTAAACTATTTTCCT	
RNA1-A1R	TGGGGCTTTGGGCAAATTAGCCGACGTGTAGAGAGCTAT TTCAGAATTCT	Inverse PCR to open pBBWV1-R1A
RNA2_34_F	GTTCATTTCATTTGGAGAGGTGATTTAAAAAATTTTTAAA TCAAACAGCTTTCGTTCCGAAA	PCR to amplify RNA2 of BBWV-1
RNA2_34_R	TGGAGATGCCATGCCGACCCTTTTTTTTTTTTTTTTTTTT TTTCCTTTATTTAGCGAAATATTTCC	
SPM-R	Phosphate-CTCGCTCTTCCTCGATTTTGATAAG	Inverse PCR to obtain pBBWV1-R2:G492C
SPM-F	Phosphate-TCAATTTGACAAAAACTGAGACT	

4.2.3 Agroinfiltration of cDNA BBWV-1 constructions

Equal volumes of *A. tumefaciens* COR308 cell suspensions containing pBBVW1-R1 + pBBWV1-R2 + p35S-p19, a pBIN binary plasmid expressing the p19 VSR of *Tomato bushy stunt virus* (TBSV), or pBBWV1-R1 + pBBWV1-R2:G492C + p35S-p19 constructs were agroinfiltrated into *N. benthamiana* with four to six leaf stage and tomato, broad bean and pepper plants with two fully expanded cotyledons and two emerging true leaf stage following standard procedures (Martínez *et al.*, 2014). Briefly, bacterial cultures obtained from individual colonies were grown in agitation (180 rpm) overnight at 28°C in induction medium (LB, MES 10 mM, acetosyringone 20 μM and the appropriate antibiotic). Cell cultures were centrifuged at 3500 rpm for 15 minutes and resuspended with concentration adjusted to OD600=1 in infiltration medium (sterile water, $MgCl_2$ 10 mM, MES 10 mM, acetosyringone 150 μM), incubated in agitation (20 rpm) for 3 h at room temperature and infiltrated with a needless syringe. Mock infiltration was done infiltrating the infiltration medium without cell suspensions. BBWV-1 infection was assessed at 10 days post agroinfiltration (dpa) by RT-PCR and northern blot analysis of total RNA extracts obtained from upper non inoculated leaves. Plant symptom development was monitored up to 21 dpa and photographed with a Nikon D40 Reflex Camera.

4.2.4 Real-time quantitative RT-PCR

Relative RT-qPCR was performed to quantify the viral titre of pBBWV1-Wt (pBBVW1-R1/pBBWV1-R2) and pBBWV1-G492C (pBBWV1-R1/pBBWV1-R2:G492C) infected-plants. Two specific TaqMan (Sigma) probes for BBWV-1 RNA1 (positions 1.123 to 1.148) and RNA2 (positions 1.791 to 1.820) were designed using Primer ExpressTM (Applied Biosystems) software (Table 4.2). Total RNAs were obtained from non-infiltrated upper leaves of broad bean and *N. benthamiana* plants agroinfiltrated with pBBWV1-Wt (pBBVW1-R1/pBBWV1-R2) and pBBWV1-G492C (pBBWV1-R1/pBBWV1-R2:G492C) at 12 dpa. Total RNAs were normalized at the same concentration. Standard curves were derived from 10-fold serial dilutions of the most concentrated total RNA sample of pBBWV1-Wt broad bean infected-plant. Relative concentrations were expressed in arbitrary units. RT-qPCR from total RNA extracts was performed in a LightCycler480 (Roche) using LightCycler480 TaqMan One-Step Master Mix (Roche) following manufacturer's instructions. Cycling conditions were: RT at 48°C for 30 min, denaturation at 95°C for 10 min and 40 cycles at 95°C for 10 sec, 59°C for 10 sec and 72°C for 15 sec. Multifactorial ANOVA statistical analysis was performed using Statgraphics software.

Table 4.2 Primers used for relative RT-qPCR of RNA1 and RNA2 and the corresponding TaqMan probes.

Name	Sequence (5'-3')	Function
R1_Ta	[6FAM]CCAAAGGATCAGAGGAATCCC AGGGA[TAM]	Taqman probe for relative RT-qPCR of RNA1
R2_Ta	[6FAM]AGGCAGGCTTCTTGGAATACAA CATGTCAA[TAM]	Taqman probe for relative RT-qPCR of RNA2
R1_Ta_F	TTGGGTAATCTCTTTGGCAAATC	Relative RT-qPCR RNA1
R1_Ta_R	GCATAGTGGTTGGGCCAAAT	
R2_Ta_F	GCAGCACTTGGCTCCGATT	Relative RT-qPCR RNA2
R2_Ta_R	GGTTCAATTGCTGGGTTCCA	

4.2.5 Virion purification and analysis

Virions were purified from 5 g of broad bean or *N. benthamiana* plants infected with pBBWV1-Wt or pBBWV1-G492C infectious clones at 21 dpa, as previously described (Turina *et al.*, 2007). Briefly, leaves were homogenized in extraction buffer (potassium phosphate 0.25 M, 2-ME 0.1 %) filtered through cheesecloth, let on ice 1 h with 1% Triton X-100 and centrifuged at 8000 x g for 10 min. Supernatant was stirred on ice 2 h with 8%

PEG8000 an 1% NaCl, centrifuged at 8000 x g for 10 min, resuspended in extraction buffer containing 15% sucrose and centrifuged at 235000 x g for 3 h. Pellet was resuspended in extraction buffer. Purified virion suspensions were stained with 2% uranyl acetate and examined with a transmission electron microscope (JEOL JEM-1010). The presence of gRNAs in the purified virion suspensions was confirmed in a 1% agarose gel and stained with GelRed (Biotium).

4.2.6 PVX-based vector expression assay

For transient expression of BBWV-1 encoded VP37 protein trough a PVX viral vector, the BBWV-1 corresponding gene was amplified with iProof High-Fidelity DNA Polymerase (Biorad) using specific primers that contained specific enzyme recognition sequences and a start and stop codons to allow the correct protein expression (Table 4.3). The resulting PCR product was digested and cloned into the PVX plasmid pVX202 (Sablowski *et al.,* 1995) rendering pPVX-VP37 construct that was transformed into *Escherichia coli* X blue (Agilent Technologies) and sequenced to assess the presence of BBWV-1 VP37 gene. As pVX202 lacks the T-DNA sequence, this construct is not suited for agroinfiltration. Consequently PVX constructions were purified with Realplasmid Spin Miniprep kit (Real). Fourteen μg of each plasmid construct were mechanically inoculated in two leaves of each *N. benthamiana* plants using sodium phosphate buffer using inoculation buffer (Na_2HPO_4 0.01 M, NaH_2PO_4 0.01 M, pH 7.2). Ten *N. benthamiana* plants were mechanically inoculated with pPVX-VP37 and PVX-empty vector (pPVX-Ø) which was used as PVX infection control. As negative control of inoculation, ten *N. benthamiana* plants were mechanically mock-inoculated with only inoculation buffer. Each experiment was repeated twice. Virus infection was assessed at 10 days post inoculation (dpi) by RT-PCR and northern blot analysis of total RNA extracts obtained from upper non inoculated leaves. Plant symptom development was monitored up to 21 dpi and photographed with a Nikon D40 Reflex Camera.

4.1.7 NBT and Trypan blue staining

Presence of superoxide ion O^{2-} accumulations and dead cells in upper non-inoculated leaves of pPVX-Ø and pPVX-VP37 inoculated *N. benthamiana* plants was determined at 7

dpi using nitro blue tetrazolium (NBT) and trypan blue (TB) staining method, respectively (Grellet Bournonville & Díaz-Ricci, 2011; Garcia-Marcos *et al.*, 2013). Briefly, for NBT staining, apical non-inoculated leaves were vacuum infiltrated for about 1 min in staining solution (sodium azide 10 mM, phosphate buffer 0.5 mM, pH=7.8, NBT 2mg/ml) and then incubated in the same solution for 30 min in slow agitation. After removing staining solution, samples were washed with 96% ethanol until complete removal of chlorophyll. For TB staining, leaves were boiled 1 min in lactophenol-trypan blue staining solution (lactic acid 0.25 %, glycerol 0.25 %, phenol 0.25 %, trypan blue0.5 mg/ml) and decolorized with 2.5 g/ml of chloral hydrate for at least 30 min. Photos were taken with a stereoscopic microscope Leica.

4.2.8 Transient expression assay in *N. benthamiana 16c*

For transient expression assay in *N. benthamiana* 16c, a binary plasmid containing the VP37 full-length gene was engineered. Gene encoding for VP37 protein was amplified with specific primers that contained specific restriction enzyme recognition sequences and which were used to introduce a stop codon at the end of VP37 gene (Table 4.3). VP37 was engineered between the duplicated 2x35S CaMV promoter and the Nos-terminator of the pCambia binary plasmid and the resulting construct was named p35S-VP37. The empty pCambia binary plasmid (p35S-Ø), was used as negative control. A pBIN binary plasmid containing the TBSV p19 protein, (p35S-p19) was used as a positive control. For green fluorescent protein (GFP) expression, a pBIN plasmid containing the GFP gene (p35S-GFP) was used (kindly provided by Dr. José Guerri). Plasmids were transformed into *A. tumefaciens* COR308 cells and equal volumes of cells suspensions containing p35S-p19/p35S-GFP (positive control), p35S-Ø/p35S-GFP (negative control), and p35S-VP37/p35S-GFP were infiltrated into leaves of transgenic *N. benthamiana* 16c plants (three true leaves stage) as described in the section 4.2.4. Examination of fluorescence was performed after 3 dpa using a handheld UV lamp (Black Ray model B100AP; UV Products, Upland, CA, U.S.A.) and images were taken with a CANON EOS 300D digital camera using a yellow filter (Jos. Schneider Optische Werke, B+W Filter, Bad Kreuznach, Germany). Agroinfiltrated areas of plant leaves were collected for RNA extraction.

Table 4.3 Primers used for the development of binary plasmids and PVX constructions expressing BBWV-1 VP37. Lower case letters indicate restriction enzyme sequences. Underlined letters indicate insertions.

Name	Sequence (5'-3')	Function
MP-2F	AT*ggatcc*ATGGAGGAAGAGCGAGT	PCR to amplify VP37 of BBWV-1 to obtain p35S-VP37
MP-R	AT*ggatcc*CTATTGCCCATATTTGTAA	
MP2_EagI_F	AC*cggccg*ATGGAGGAAGAGCGAGT	PCR to amplify VP37 of BBWV-1 to obtain pPVX-VP37
MP1_SalI_R	AC*gtcgac*TCATTGCCCATATTTGTAATCTATTCC	

4.2.9 pTCV-GFP complementation assay

For TCV complementation assay, *N. benthamiana* leaves (4 true leaves stage) were individually agroinfiltrated with constructs p35S-VP37, p35S-Ø (negative control) and p35S-p19 (positive control). One day later, TCV-GFP transcript was generated using T7 RNA Polymerase (Roche) from pTCV-GFP construct previously linearized with XbaI restriction enzyme (Fermentas) following manufacturer's instructions. The obtained transcript was mechanically inoculated on previously agroinfiltrated leaves (5 µg of transcript per leaf) using inoculation buffer (Na_2HPO_4 0.01 M, NaH_2PO_4 0.01 M, pH 7.2). Leaves were observed at 4 dpa with a Leica MZ16 FA fluorescence stereomicroscope (Leica Microsystems, Heerbrugg, Switzerland) using a high-energy light source and a GFP filter.

4.2.10 RNA extraction and northern blot hybridization

Total RNA was extracted from 0.1 g of leaf tissue using a phenol:chloroform:isoamyl alcohol standard procedure followed by ethanol precipitation (Ferriol *et al.*, 2011). To obtain siRNAs extracts for transient expression assays in *N. benthamiana 16c* plants, total RNA was extracted from 0.25 g of agroinfiltrated leaves using Tri-Reagent (Sigma) following manufacturer's instructions. SiRNAs were enriched from total RNAs by removing high molecular weight RNAs with a solution of 50% (w/v) PEG 8000 (Sigma) and 0.5 M NaCl.

Northern blot analysis of BBWV-1 genomic RNA, PVX genomic RNA and of GFP mRNA was performed as previously described (Ferriol *et al.*, 2015; Ferriol *et al.*, 2016). Briefly, denaturing electrophoresis of total RNAs (4 µg) was performed on a 0.9 % formaldehyde agarose gels in 1 X MOPS buffer (sodium acetate 5 mM, Na_2EDTA 1 mM, MOPS 20 mM, pH 7) and transferred by capillarity onto a positively charged nylon

membrane (Roche) in 20 X SSC buffer (NaCl 3 M, sodium citrate 0.3 M). Northern blot analysis of siRNAs was performed by denaturing electrophoresis separating enriched siRNA extracts (3 μg) in a 15% polyacrylamide gels containing 7 M urea. siRNAs were transferred onto a positively charged nylon membrane using 0.5 X TBE electrotransfer buffer (Tris 89 mM, boric acid 89 mM, EDTA 2 mM, Invitrogen) applying 25 V for 1 h. Membranes were UV cross-linked and hybridized with digoxigenin-labelled RNA probes as previously described (Ferriol *et al.*, 2015). For BBWV-1 northern blot, an antisense 332-bp riboprobe which hybridizes with the 5'-UTR region of RNA1 and RNA2 was used (Ferrer *et al.*, 2008). For PVX northern blot, a 432-bp antisense riboprobe complementary to PVX CP (positions 5650 to 6363, GenBank accession: EU021215) was synthetized using specific primers (Table 4.4). For GFP northern blot an antisense riboprobe complementary to full-length GFP sequence was used (Renovell *et al.*, 2012). Hybridized membranes were developed using Anti-Digoxigenin Alkaline Phosphatase conjugated antibody (Roche) and hybridization reaction was visualized with CDP-Star Chemiluminescent Substrate (Roche).

4.2.11 RT-PCR and sequencing

BBWV-1 detection was performed by RT-PCR of total RNAs extracts obtained from upper non agroinfiltrated leaves at 12 dpa of *N. benthamiana*, broad bean, tomato and pepper plants agroinfiltrated with pBBWV1-Wt and pBBWV1-G492C. Primers are listed in Table 4.4. The PCR products obtained from pBBWV1-G492C agroinfiltrated plants were also sequenced to determine the presence of mutation G492C in the viral progeny. PVX detection was performed by RT-PCR of total RNAs extracts obtained from upper non inoculated leaves at 10 dpi of *N. benthamiana* plants inoculated with pPVX-VP37 and pPVX-Ø using the specific primers for the CP of PVX (Table 4.4). To detect the presence of BBWV-1 VP37 gene in upper non-inoculated leaves in plants inoculated with pPVX-VP37, the same primers used to obtain PVX constructions were employed (Table 4.3). In all cases, two-step RT-PCR was performed using 20 ng of total RNA with SuperScript IV Reverse Transcriptase (Invitrogen) and Taq DNA Polymerase (Invitrogen) following manufacturer's instructions.

Complete sequencing of pBBWV1-R1, pBBWV1-R2 and pBBWV1-R2:G492C constructs was performed with primers specific for PJL89 or gRNA1 and gRNA2 of BBWV-1 that covered overlapping regions and allowed to obtain the sequence of BBWV-1 full-length cDNAs. Sequencing of inserts in p35S-VP37 and pPVX-VP37 constructs was

performed using specific primers contiguous to the cloning sites. All sequences were obtained with an ABI 3130xl Genetic Analyzer (Thermo Fisher Scientific).

Table 4.4 Primers used for detection of PVX and BBWV-1 systemic infection by RT-PCR. Moreover, primers for the development of PVX riboprobe are listed.

Name	Sequence (5'-3')	Function
PVX_3R_T7	TTAATACGACTCACTATAGTGGACTGT TGTTAG TTAAC	This primer contains T7 promoter sequence and was used in combination with PVX-3F to obtain a PCR product used for production of PVX specific riboprobe
MP1_F	ATGCCGTTGATTCTTCT	RT-PCR to detect systemic infection of BBWV-1 infectious constructions
MP_3R	CTTGTGGCAGCAAAGATC	
PVX_3F	ATGTCAGCACCAGCTAGCAC	RT-PCR to detect systemic infection of pPVX-Ø.
PVX-3R	GTG GAC TGT TGT TAG TTA AC	

4.3 Results

4.3.1 BBWV-1 VP37 protein is involved in plant symptom induction and host specificity

To determine the role of RNA2-encoded VP37 protein in the viral cycle of BBWV-1, a full-length cDNA clone of wild type BBWV-1 and a mutant construction that lacks VP37 expression were engineered. RNA1 and RNA2 genomic RNAs (gRNAs) of BBWV-1 were cloned in the PJL89 binary vector between the CaMV 35S promoter and the HDV ribozyme (Rbz) followed by the Nos-terminator. BBWV-1 gRNAs were obtained from a T7-driven cDNA clone of BBWV-1 isolate Ben (Ferriol *et al.*, 2016). The final constructions were named pBBWV1-R1 (containing gRNA1) and pBBWV1-R2 (containing gRNA2). A mutant construct that did not express the putative RNA2-encoded VP37 protein, was obtained introducing a single point modification in VP37 start codon at nucleotide position 492 of the pBBWV1-R2 (AUG>AUC, Met>Ile) and the resulting construct was named pBBWV1-R2:G492C.

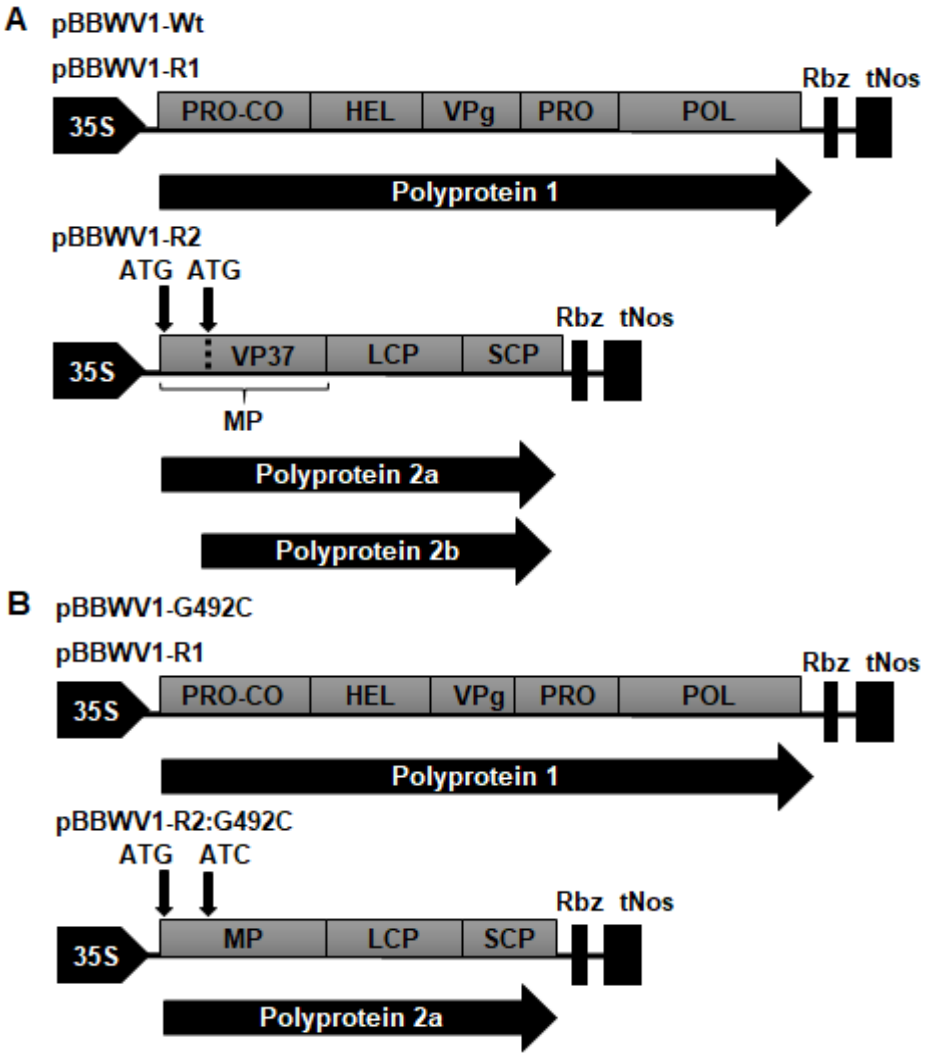

Figure 4.1 Schematic representation of the BBWV-1 infectious constructions. **(A)** cDNA constructs of BBWV-1 containing gRNA1 (pBBWV1-R1) and gRNA2 (pBBWV1-R2) used to elaborate the wild-type infectious clone (pBBWV1-Wt). **(B)** cDNA constructs of BBWV-1 containing gRNA1 (pBBWV1-R1) and mutant gRNA2 (pBBWV1-R2:G492C) with a single point substitution in the VP37 start codon at nucleotide position 492 (AUG>AUC, Met>Ile) used to obtain BBWV-1 infectious clone that did not express the putative VP37 protein (pBBWV1-G492C).

Both BBWV-1 infectious clones were composed by a combination of pBBWV1-R1/pBBWV1-R2 and pBBWV1-R1/pBBWV1-R2:G492C constructs and were named pBBWV1-Wt and pBBWV1-G492C, respectively (Fig. 4.1) and were agroinfiltrated into thirty *N. benthamiana*, broad bean, pepper, and tomato plants, together with a binary construction expressing the VSR p19 of TBSV. As controls, also 30 *N. benthamiana*, broad bean, pepper, and tomato plants were mechanically inoculated with BBWV-1 isolate Ben and mock agroinfiltrated. Detection of BBWV-1 was performed by RT-PCR of total RNA

extracted from apical leaves at 12 dpa. Development of plant symptomatology was monitored until 21 dpa revealing differences in host range and induced symptomatology (Fig. 4.2, Table 4.5). pBBWV1-Wt infectious clone infected all *N. benthamiana*, broad bean and tomato plants (100%) and 16 pepper plants (53%) inducing the same symptomatology than BBWV-1 isolate Ben. Symptomatology consisted in stunting and severe mosaic in *N. benthamiana* plants, severe mosaic in broad bean plants and slight mosaic in pepper plants, whereas tomato plants remained totally asymptomatic, in spite of being systemically infected with pBBWV1-Wt. pBBWV1-G492C mutant construct infected all *N. benthamiana* and broad bean plants (100%), but none of tomato and pepper plants (0%). Moreover, *N. benthamiana* and broad bean plants infected with pBBWV1-G492C were characterized by milder symptoms than those induced by pBBWV1-Wt at 21 dpa and consisted in slight mosaic (sometimes imperceptible) in *N. benthamiana* and few chlorotic spots in broad bean plants. RT-PCR of total RNA extracted from agroinfiltrated tomato and pepper leaves was performed to evaluate if the absence of pBBWV1-G492C systemic infection in these plant hosts was caused by lack of movement or replication. Results indicated that pBBWV1-G492C was not able to replicate in tomato and pepper plants. Northern blot of total RNA extracted from apical non-agroinfiltrated leaves was performed and revealed the presence of two bands (~ 5.8 and 3.4 Kb) corresponding to gRNA1 and gRNA2 (Fig. 4.2 B), in all the plant species infected with both infectious constructions. These two bands were not observed in plants which were not infected and in the mock-agroinfiltrated plants used as controls. In addition, signal intensity of the hybridization signal of northern blot of *N. benthamiana* and broad bean plants, infected with either pBBWV1-Wt or pBBWV1-G492C, was similar suggesting that the differences of symptomatology were not correlated with differences in viral accumulation. To assess that differences of intensity of induced symptomatology between pBBWV1-Wt and pBBWV1-G492C was not influenced by virus titre, BBWV-1 viral accumulation was estimated in *N. benthamiana* and broad bean plants infected with both infectious clones by quantitative RT-PCR (RT-qPCR).

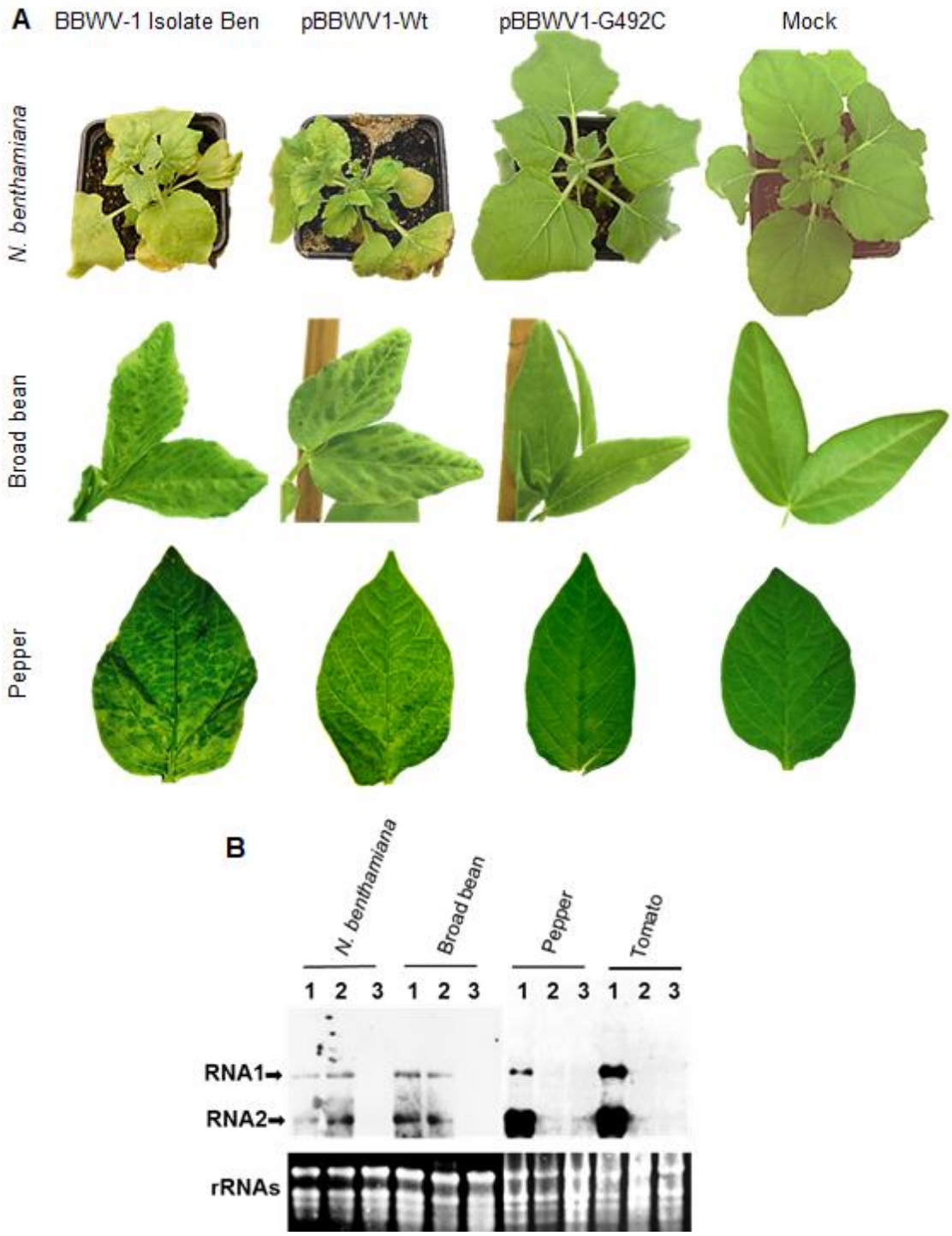

Figure 4.2 Biological characterization of pBBWV1-Wt and pBBWV1-G492C cDNA infectious clones in different herbaceous host species. **(A)** Symptoms induced by BBWV-1 Ben viral isolate and pBBWV1-Wt and pBBWV1-G492C infectious clones in *N. benthamiana*, broad bean and pepper plants at 21 dpa. **(B)** Northern blot analysis of upper non-inoculated leaves of *N. benthamiana*, broad bean, tomato and pepper plants agroinfiltrated with pBBWV1-Wt (lane 1), pBBWV1-G492C (lane 2) and healthy plant negative control (lane 3). Ribosomal RNAs loading controls (rRNAs) are indicated below.

Table 4.5 Symptomatology and infectivity results of different plant host species agroinfiltrated with pBBWV1-Wt and pBBWV1-G492C infectious clones. Percentages were calculated from one assays comprising 30 plants for each plant species.

	pBBWV1-Wt		pBBWV1-G492C	
Plant species	**Infectivity**	**Symptomatology**	**Infectivity**	**Symptomatology**
N. benthamiana	100 %	Stunting, sever mosaic and leaf necrosis	100 %	Slight stunting and chlorosis
Broad bean	100 %	Severe mosaic	100 %	Few chlorotic spots
Pepper	55%	Slight mosaic	0 %	-
Tomato	100 %	Asymptomatic	0 %	-

Relative quantification was performed using specific Taqman probes for each BBWV-1 gRNA1 and gRNA2 with appropriate primers, using two replicates of total RNA extracted at 12 dpa from upper non-agroinfiltrated leaves of five *N. benthamiana* and five broad bean plants infected with pBBWV1-Wt and pBBWV1-G492C. To evaluate relative viral accumulation standard curves were derived from 10-fold serial dilutions of the most concentrated total RNA sample of broad bean agroinfiltrated with pBBWV1-Wt (Fig. 4.3 A). The obtained data were analysed by multifactorial ANOVA test (P-value > 0.05). Results revealed that differences in viral accumulation between pBBWV1-Wt and pBBWV1-G492C agroinfiltrated *N. benthamiana* and broad bean plants were not statistically relevant (Fig. 4.3 B) indicating that variation in the intensity of the induced symptomatology was not related to viral accumulation.

Virions from *N. benthamiana* and broad bean plants agroinfiltrated with pBBWV1-Wt and pBBWV1-G492C were purified and observed by transmission electron microscopy (TEM) to assess if the single point mutation in VP37 protein start codon (G492C) altered BBWV-1 virion formation. Both *N. benthamiana* and broad bean plants agroinfiltrated with either pBBWV1-Wt or pBBWV1-G492C were characterized by the presence of non-enveloped viral particles of 25-30 nm in diameter and icosahedral morphology and were identical to those reported for BBWV-1 viral isolate (Ikegami & Sharma, 2011), suggesting that VP37 was not involved in virion formation (Fig. 4.4 A). RNAse free 1% agarose gel of total RNA extracted from purified virions revealed the presence of two bands (~5.8 kb and 3.4 kb) corresponding to BBWV-1 gRNA1 and gRNA2, respectively (Fig. 4.4 B).

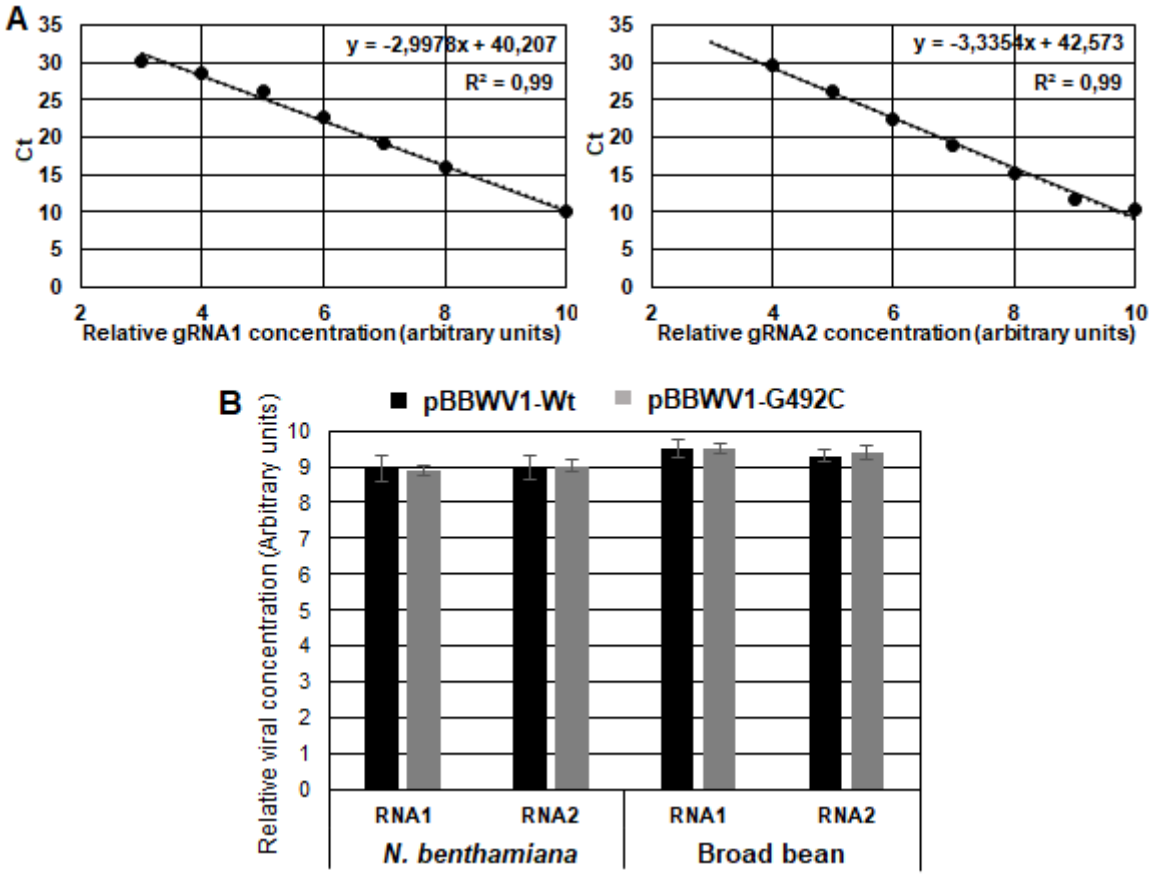

Figure 4.3 Graphical representations of relative quantitation of pBBWV1-Wt and pBBWV1-G492C. **(A)** Relative standards curves of BBWV-1 gRNA1 (left) and gRNA2 (right). Equations and correlation coefficient (R^2) are indicated. Vertical axes represent the threshold cycle (Ct) and horizontal axes represent 10-fold serial dilutions of total RNA from pBBWV1-Wt infected broad bean plant**. (B)** Relative quantitation of BBWV-1 gRNA1 and gRNA2 from nucleic acid extracts obtained from *N. benthamiana* and broad bean plants agroinfiltrated with either pBBWV1-Wt or pBBWV1-G492C. Bars represent the standard error of the mean.

The presence of mutation G492C was confirmed in the viral progeny of all pBBWV1-G492C agroinfiltrated plants. RT-PCR of total RNA extracted from apical non-agroinfiltrated leaves was performed and the resulting PCR product, (positions 220 to 607, GenBank KT988974) obtained with primers surrounding the mutation site, was sequenced. Results indicated that all *N. benthamiana* and broad bean plants agroinfiltrated with pBBWV1-G492C contained the G492C mutation and no reversion to the wild-type sequence occurred.

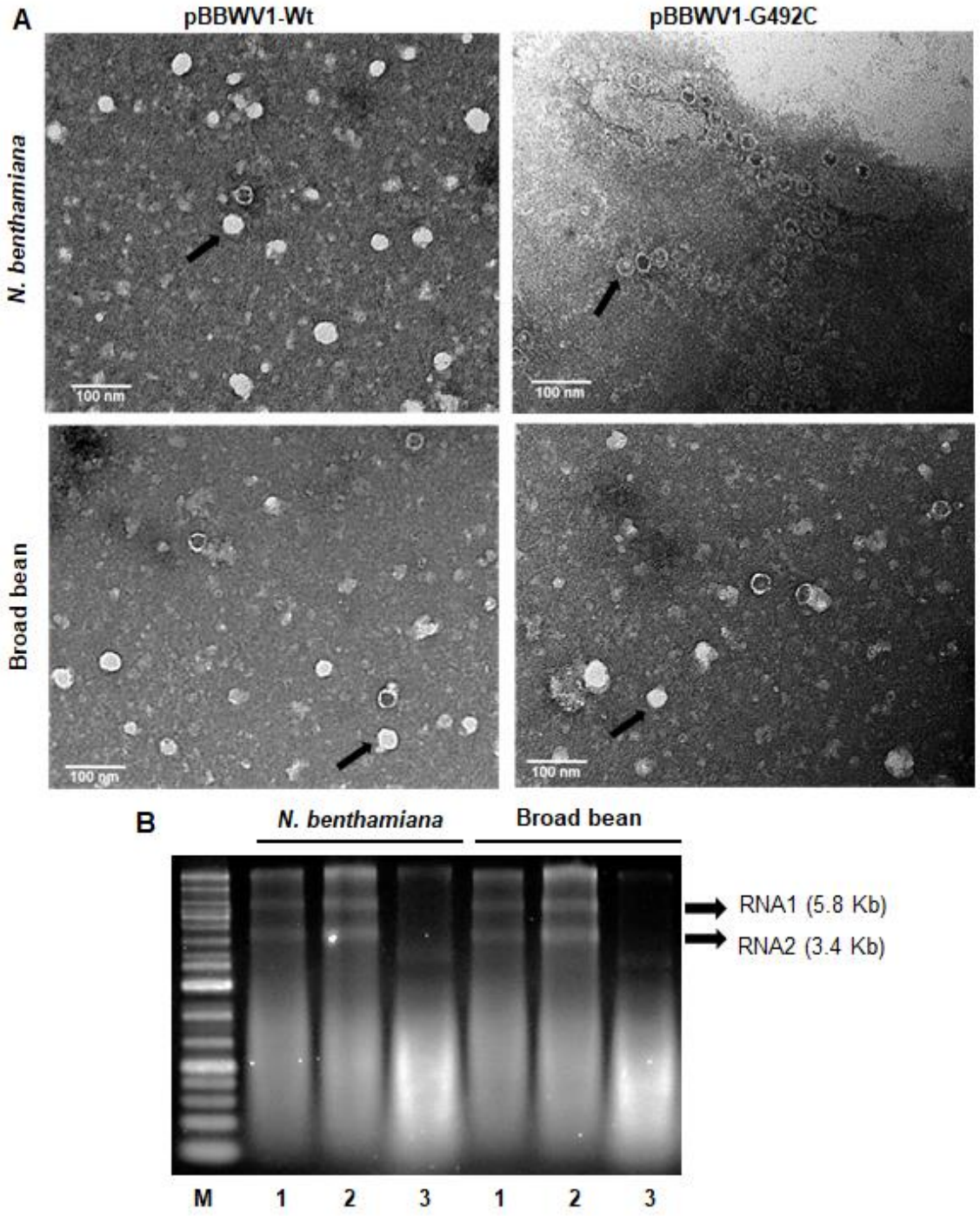

Figure 4.4 (A) Transmission electron micrographs showing purified viral particles from *N. benthamiana* and broad bean plants agroinfiltrated with pBBWV1-Wt and pBBWV1-G492C infectious clones. Arrows indicate viral particles of 25-30 nm with icosahedral morphology. Bars represent 100 nm. **(B)** 1% agarose non-denaturing gel of gRNAs obtained from purified particles of *N. benthamiana* and broad bean plants infected with pBBWV1-Wt (line 1) pBBWV1-G492C (line 2) and healthy plants negative control (line 3). M corresponds to 100 bp molecular weight marker (Invitrogen).

4.3.2 BBWV-1 VP37 protein enhances the PVX-induced symptoms and activates the systemic necrosis associated with programed cell death in *N. benthamiana* plants

BBWV-1 RNA2-encoded VP37 implication on plant symptom induction was evaluated by transient expression in *N. benthamiana* plants through the viral vector based on PVX sequence (Sablowski *et al.*, 1995). VP37 was cloned into pPVX202 viral vector rendering pPVX-VP37 and PVX empty viral vector (pPVX-Ø) was used as a control of PVX infection (Fig. 4.5 A). pPVX-VP37 or pPVX-Ø constructs were mechanically inoculated in twenty *N. benthamiana* plants. As inoculation negative control, twenty *N. benthamiana* plants were mechanically inoculated with only the inoculation buffer. PVX systemic infection was determined by RT-PCR of total extracted RNA from apical non-inoculated leaves at 10 dpi whereas plant symptom development was monitored up to 21 dpi. pPVX-VP37 and pPVX-Ø infected all inoculated plants but induced different symptomatology (Fig. 4.5 B). At seven dpi, plants inoculated with pPVX-VP37 showed stunting, severe mosaic and small necrotic lesions in apical non-inoculated leaves, and some plants died at 21 dpi. pPVX-Ø inoculated plants showed only typical symptomatology that consisted of mild mosaic symptoms which became imperceptible at 21 dpi. Mock-inoculated plants were asymptomatic. Northern blot analysis of PVX gRNA obtained from total RNA extracted from apical non-inoculated leaves at 10 dpi was performed and revealed differences of electrophoretic mobility depending on the presence of VP37 gene (Fig. 4.5 C). RT-PCR of total RNA extracted from apical non-inoculated leaves performed with VP37 specific primers revealed the presence of VP37 in pPVX-VP37 inoculated plants but not in those inoculated with pPVX-Ø.

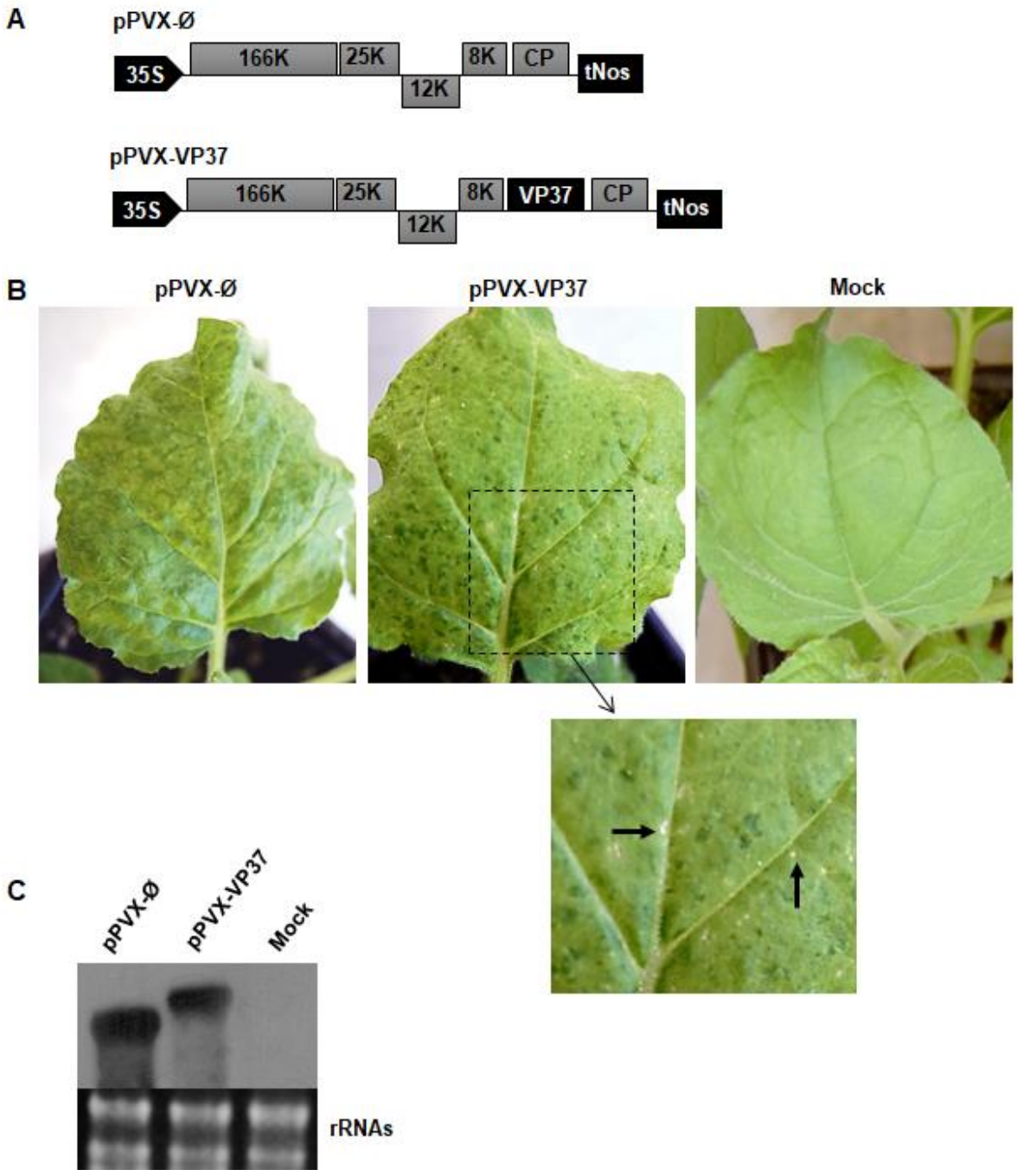

Figure 4.5 Transient expression of BBWV-1 VP37 protein in *N. benthamiana* plants using the PVX viral vector. **(A)** Schematic representation of pPVX-derived constructs. **(B)** Symptoms induced by PVX-derived constructs in *N. benthamiana* plants at 7 dpi: mild mosaic observed in upper non-inoculated leaves of *N. benthamiana* plants inoculated with pPVX-Ø and severe mosaic and small necrotic lesions (indicated with arrows) in upper non-inoculated leaves of *N. benthamiana* plants inoculated with pPVX-VP37. Mock-inoculated plats were used as negative controls. **(C)** Northern blot from upper non-inoculated leaves of *N. benthamiana* plants inoculated with pPVX-Ø and pPVX-VP37 using a PVX-specific riboprobe. Ribosomal RNAs (rRNAs) loading controls are showed.

The necrotic lesions that appeared in apical non-inoculated leaves of *N. benthamiana* plants inoculated with pPVX-VP37 suggested the activation of programmed cell death (PCD) related to systemic necrosis. To assess the activation of defence-associated responses histochemical and biochemical analysis were performed. The presence of the superoxide ion (O^{2}_{-}), a reactive oxygen species (ROS) associated to PCD activation, was tested by NBT staining method. NBT reacts with superoxide ion (O_2^-) and forms insoluble precipitates visualized as dark-blue-coloured deposits (Grellet Bournonville & Díaz-Ricci, 2011). Additionally, TB staining method was used to stain dead cells (Garcia-Marcos *et al.*, 2013). TB is a colour dye able to penetrate selectively through the damaged cell wall of death cells. At seven dpi apical non-inoculated symptomatic leaves of *N. benthamiana* plants inoculated with pPVX-VP37 and pPVX-Ø were collected and stained. Staining with NBT revealed the presence of superoxide O_2^- dark-blue-coloured deposits in pPVX-VP37 inoculated plants but not in those inoculated with pPVX-Ø (Fig. 4.6 A). TB staining showed that in pPVX-VP37 inoculated plants dark-blue-coloured groups of dead cells were present, but not in those inoculated with pPVX-Ø (Fig. 4.6 B).

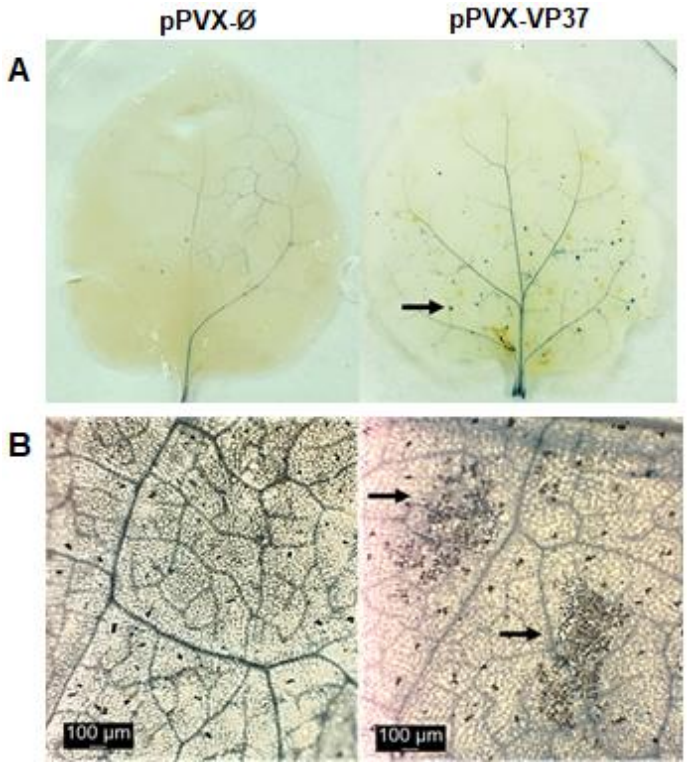

Figure 4.6 Systemic necrosis produced by transient expression of BBWV-1 VP37 protein using PVX viral vector in upper non-inoculated leaves of *N. benthamiana* plants at 7 dpi. **(A)** Staining of superoxide ion (O_2^-) deposits with NBT (arrow). **(B)** Visualization of groups of dead cells using the TB staining method (arrows). Bars represent 100 µm.

4.3.3 BBWV-1 VP37 protein is an RNA silencing suppressor

The ability of VP37 protein to supress PTGS was studied by VP37 transient expression assay in *N. benthamiana* 16c plants which constitutively express GFP and by an assay of movement complementation of a viral vector based on TCV, namely pTCV-GFP construct (Powers *et al.*, 2008).

Transient expression of foreign GFP in *N. benthamiana* 16c plants induces degradation of mRNA transcribed from GFP transgene via PTGS activation and elimination of the plant green fluorescence. When foreign GFP is co-expressed with a protein which has the ability to suppress PTGS, expression of GFP is restored and the plant emits green fluorescent light. BBWV-1 VP37 gene was cloned into a binary plasmid between the duplicated CaMV 35S promoter and the Nos-terminator rendering the p35S-VP37 construct that was co-agroinfiltrated with a plasmid expressing GFP named p35S-GFP into *N. benthamiana* 16c plants. The binary plasmid p35S-p19 expressing the TBSV p19 protein, a well-characterized VSR and the empty binary vector (p35S-Ø) were used as positive and negative controls of PTGS suppression, respectively (Zamore, 2004). At 3 dpa, agroinfiltrated leaves were observed under a handheld UV lamp (Fig. 4.7 A): all leaves co-agroinfiltrated with p35S-VP37/p35S-GFP showed a green fluorescence in the agroinfiltration area whereas it was not observed on leaves co-agroinfiltrated with the negative control p35S-Ø/p35S-GFP. Leaves co-agroinfiltrated with the positive control p35S-p19/p35S-GFP showed green fluorescence in the agroinfiltrated areas much more intense than those showed in leaves agroinfiltrated with p35S-VP37/p35S-GFP. The emission of green fluorescence on the leaves co-agroinfiltrated with p35S-VP37/p35S-GFP decreased until disappearing at six dpa whereas it remained on leaves co-agroinfiltrated with the PTGS suppression control p35S-p19/p35S-GFP. Consistent with these observations, northern blot analysis showed that GFP mRNA level was higher in leaves co-agroinfiltrated with p35S-VP37/p35S-GFP than in leaves co-agroinfiltrated with the negative control p35S-Ø/p35S-GFP but lower than those co-agroinfiltrated with positive control p35S-p19/p35S-GFP (Fig. 4.7 B). Conversely, siRNA derived from mRNA accumulated a lower level in leaves co-agroinfiltrated with p35S-VP37/p35S-GFP than in leaves co-agroinfiltrated with negative control p35S-Ø/p35S-GFP but in this case higher than those co-agroinfiltrated with the positive control p35S-p19/p35S-GFP (Fig. 4.7 C).

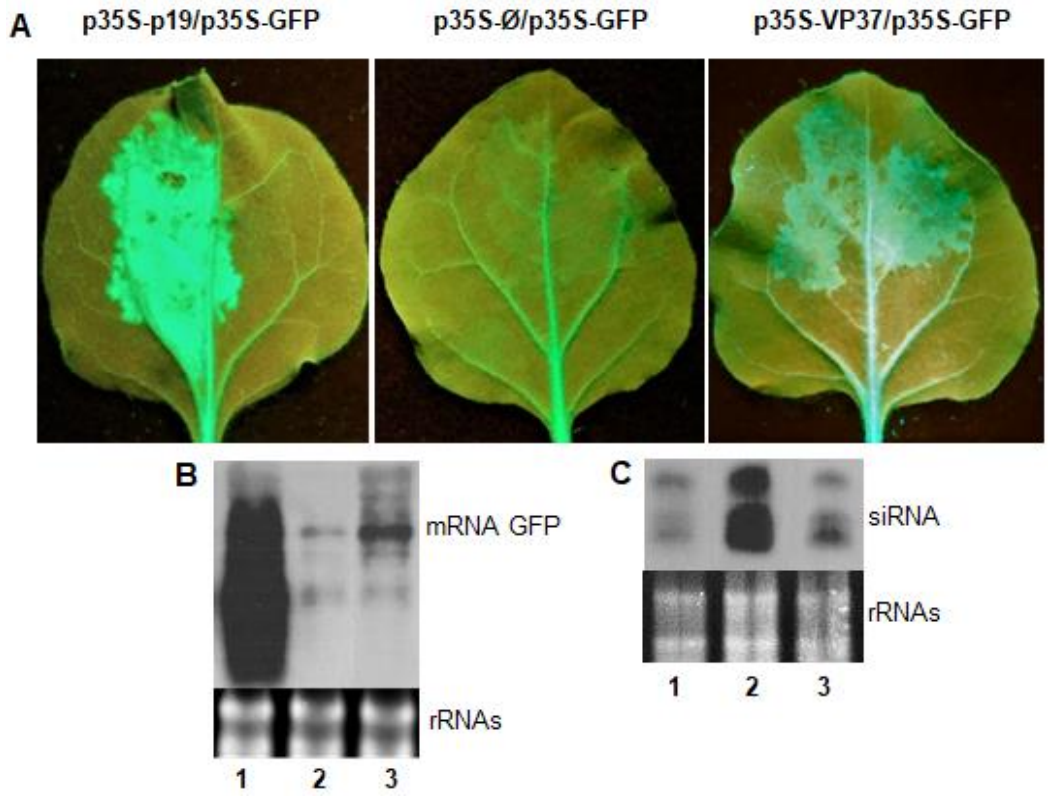

Figure 4.7 RNA silencing suppression activity of BBWV-1 VP37. **(A)** Leaves of *N. benthamiana* 16c plants observed under UV light at 3 dpa. The corresponding co-agroinfiltrated constructions are indicated above each picture. (**B)** Northern blot analysis of GFP mRNAs and **(C)** siRNAs extracted from agroinfiltrated tissue patches at 3 dpa co-agroinfiltrated with p35S-p19/p35S-GFP (line 1), p35S-Ø/p35S-GFP (line 2) and p35S-VP37/p35S-GFP (line 3). Ribosomal RNAs (rRNAs) loading controls are indicated below each panel.

To confirm the VSR activity of VP37, *N. benthamiana* plants were agroinfiltrated with p35S-VP37, p35S-Ø or p35S-p19 and one day later TCV-GFP RNA transcripts obtained from pTCV-GFP viral vector were mechanically inoculated on previously agroinfiltrated leaves. In the pTCV-GFP construct, the viral CP has been replaced by GFP, losing the ability to move to adjacent cells that can be restored by complementation in "trans" with a PTGS suppressor. Observation of inoculated leaves with a fluorescence stereomicroscope revealed at four dpa the presence of wide green fluorescence areas in leaves agroinfiltrated with p35S-VP37 or p35S-p19 corresponding to TCV-GFP spread from the inoculated single cells whereas leaves agroinfiltrated with p35S-Ø showed GFP green small fluorescence spots corresponding to single cell foci (Fig. 4.8).

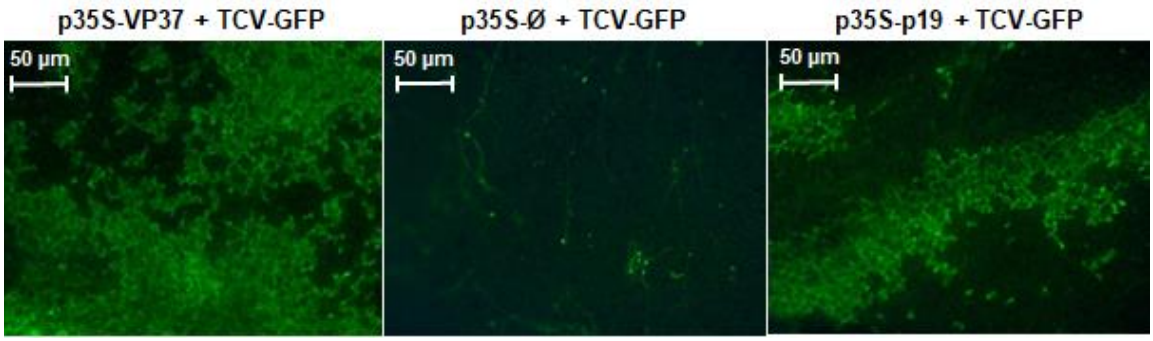

Figure 4.8 Visualization under a fluorescence stereomicroscope of *N. benthamiana* leaves agroinfiltrated with p35S-VP37, p35S-Ø or p35S-p19 constructs and mechanically inoculated one day later with TCV-GFP RNA transcripts. Plants agroinfiltrated with either p35S-VP37 or p35S-p19 showed groups of cells expressing green fluorescent protein. Bars represent 50 µm. Observation was performed three days after mechanical inoculation of TCV-GFP transcripts.

4.4 Discussion

Recent research on members of *Secoviridae* family has shed light in many crucial steps of the infectious cycle of these viruses and its interactions with its plant host and insect vectors (Fuchs *et al.*, 2017). However, little attention has been paid to the molecular biology of members of *Fabavirus* genus, mainly because reverse genetics systems for BBWV-1 and BBWV-2 were not available until recent (Ferriol *et al.*, 2016; Tasaki *et al.*, 2016). Some studies of BBWV-1 virus transmission and molecular variability have been performed (Ferrer *et al.*, 2005; Ferriol *et al.*, 2013; Ferriol *et al.*, 2014) and detection methods have been developed (Ferrer *et al.*, 2008; Ferriol *et al.*, 2011; Panno *et al.*, 2014; Ferriol *et al.*, 2015). However, studies regarding virus-virus, virus-host and virus-vector interactions have not been done. To determine the role of BBWV-1-encoded proteins in the infectious cycle of BBWV-1, 35S-driven infectious cDNA clones were engineered for BBWV-1 gRNA1 and gRNA2. Co-infiltration of both cDNA clones (pBBWV1-Wt) in *N. benthamiana*, broad bean, tomato and pepper plants showed the biological properties of the wild-type virus BBWV-1 isolate Ben. Thus, the function(s) of the putative BBWV-1 RNA2-encoded VP37 protein were studied, creating a mutant construct with a truncated expression of the VP37 protein, inserting a nucleotide change (G-C) at the nucleotide position 492 that changed the start codon AUG (Met) to AUC (Ile) (pBBWV1-G492C). The results obtained in this work showed that BBWV-1 VP37 protein enhanced the severity of induced symptoms and also

determined the virus host specificity. pBBWV1-G492C was only able to infected systemically *N. benthamiana* and broad plants, suggesting that VP37 protein was not involved in long-distance movement of BBWV-1 in these herbaceous hosts. Sequence analyses of the viral progeny of *N. benthamiana* and broad bean plants systemically infected with pBBWV1-G492C indicated that no reversion to the wild-type virus sequence occurred and indicated a relationship between plant symptoms and VP37 synthesis. However, lack of systemic infection of BBWV-1 in pBBWV1-G492C agroinfiltrated pepper and tomato plants could be caused by the suppression of long-distance and/or cell-to-cell virus movement or the impairment in the virus replication. Studies with the BBWV-2 VP37 and homologous proteins of viruses belonging to the *Comovirus* genus, suggested that these proteins were associated with cell-to-cell virus movement (Van Lent *et al.*, 1990; Liu *et al.*, 2009; Liu *et al.*, 2011; Xie *et al.*, 2016). However, the role(s) played by BBWV-1 VP37 protein in virus movement remains to be determined. Results of RT-qPCR showed no differences in virus titre among *N. benthamiana* and broad bean plants infected with either pBBWV1-Wt or pBBWV1-G492C suggesting that BBWV-1 VP37 protein was not involved in virus replication in *N. benthamiana* and broad bean plants. In addition, identical viral particles to those reported for BBWV-1 were purified from *N. benthamiana* and broad bean plants agroinfiltrated with BBWV1-Wt or pBBWV1-G492C suggesting that BBWV-1 VP37 protein was not required for virion formation.

Implication of BBWV-1 VP37 protein on plant symptom development was assessed by its transient expression using a PVX viral vector (Sablowski *et al.*, 1995). BBWV-1 VP37 protein enhanced the PVX-induced symptoms in *N. benthamiana* plants even inducing systemic necrosis mediated by activation of PCD. These results were in agreement with those obtained using the BBWV-1 full-length cDNA infectious clones and suggested that BBWV-1 VP37 protein was involved on plant symptom induction. Systemic necrosis is one of the most severe symptoms in plant-pathogen compatible interactions that eventually results in plant death. In this interaction, the pathogen is able to spread into the plant in spite of the induction of cell death (Xu & Roossinck, 2000). In contrast, hypersensitive response (HR) is activated in plant-pathogen non compatible interactions and systemic infection is impaired by a rapid death of infected cells being the pathogen confined in small plant areas (Goodman & Novacky, 1994). Although mechanisms underlying systemic necrosis are poorly understood, several studies showed that it exhibits several features of HR such as the activation of PCD with up regulation of plant genes related with production of ROS species whose toxicity leads to death of plant cells (Van Breusegem & Dat, 2006; Komatsu, 2013).

Result of transient expression of BBWV-2 VP37 using a PVX vector showed that this protein was able to enhance PVX-induced symptoms, causing severe mosaic and leaf crinkling of *N. benthamiana* plants but on the contrary to BBWV-1, it did not induced systemic necrosis in this host (Kong *et al.*, 2014). For other members of *Secoviridae* family, similar experiments of transient expression of VP37 homologous proteins have not been performed. However, transient expression of RNA1-encoded proteins such as the PRO-CO and HEL of BPMV, HEL of *Grapevine fanleaf virus* (GFLV, *Nepovirus* genus) and the N-terminal region of the RNA1 of *Tomato torrado virus* (ToTV, *Torradovirus* genus) enhanced the PVX-induced symptoms and necrotic lesions on agroinfiltrated leaves as well as systemic necrosis in *N. benthamiana* plants (Gu & Ghabrial, 2005; Wieczorek & Obrępalska-Stęplowska, 2016a). In the case of other plant viruses, transient expression using PVX vectors of the MP protein of *Banana bunchy top virus* (BBTV, *Babuvirus* genus) or the βC1 protein of *Chili leaf curl betasatellite* (ChLCB) determined that both viral proteins enhanced the PVX-induced symptoms but BBTV MP activated also the systemic necrosis and PCD in *N. benthamiana* plants (Amin *et al.*, 2011; Tahir & Mansoor, 2011).

Viral proteins involved on induction of plant symptoms have often the ability to suppress PTGS; some examples are the *Cucumber mosaic virus* (CMV, *Cucumovirus* genus) 2b protein and the *Citrus tristeza virus* (CTV, *Closterovirus* genus) p23 protein (Lewsey *et al.*, 2010; Ruiz-Ruiz *et al.*, 2013). How viral suppressors of RNA silencing (VSRs) induce pathologic process in the infected plants is still under study but some of them might act modifying the plant gene expression mediated by PTGS upsetting metabolic pathways linked to symptom manifestation (Wang *et al.*, 2012). Transient expression of BBWV-1 VP37 protein in *N. benthamiana* 16c plants showed that it had VSR activity. These results were confirmed by an additional pTCV-GFP complementation assay which has been used to confirm and identify several plant and animal VSRs such as TBSV p19 protein, HC-PRO protein of *Tobacco each virus* (TEV, *Potyvirus* genus), B2 protein of *Flock house virus* (FHV, *Alphanodavirus* genus), CMV 2b protein, E3L protein of *Human Vaccinia virus* (VACV, *Orthopoxvirus* genus) and NS1 glycoprotein of *Human Influenza virus* (FLUAV, *Influenzavirus* genus) (Powers *et al.*, 2008). With regard to the *Fabavirus* genus, transient expression of BBWV-2 small MP on *N. benthamiana* plants showed that it was also a VSR. In addition, these assays revealed that MP and LCP had also VSR activity. In contrast, transient expression assays in *N. benthamiana* 16c of different CPMV RNA2 full-length cDNA infectious clones with deletions at the C terminus of SCP showed that the last 24

amino acids of this protein is essential for the VSR activity (Canizares *et al.*, 2004; Liu *et al.*, 2004).

Viruses belonging to *Fabavirus* genus have many commonalities with members of the *Secoviridae* family, but also unique features that make them excellent tools to understand some of the major steps of the plant virus infectious cycle. Engineering a reverse genetic system for BBWV-1, provided new insights on the VP37 protein function(s), showing that it is involved in symptom development and host specificity. This improved reverse genetic system opens a way to perform studies of other BBWV-1 RNA1 and RNA2-encoded proteins which implications on virus movement, replication, encapsidation or development of pathological process remains still unknown.

Chapter 5

Role of BBWV-1 RNA2-encoded MP, SCP and LCP proteins in the suppression of PTGS and induction of plant symptoms

Part of the research work presented in this chapter was included in the paper:

Role of *Broad bean wilt virus 1* RNA2-encoded MP, SCP and LCP proteins in the suppression of post-transcriptional gene silencing. C. Carpino C, L. Elvira-González, I, L. Rubio L, E. Peri, S. Davino, L. Galipienso (*European Journal of Plant Pathology*, under revision)

5.1 Introduction

Plant viral infection triggers the anti-viral plant defence mechanisms such as induction of hypersensitive response (HR) and systemic necrosis, causing the deregulation of plant gene expression, of hormones related to cell cycle and post-transcriptional gene silencing (PTGS) (Csorba *et al.*, 2009). HR and systemic necrosis are associated with the accumulation of reactive oxygen species (ROS) which react with cellular components causing cellular damage and inducing programmed cell death (PCD) (Van Breusegem & Dat, 2006). PTGS is a sequence-specific degradation mechanism used for regulation of plant gene expression and for defence against different pathogenic organisms including plant viruses. However, viruses have evolved to counteract this mechanism and most plant viruses express proteins which have the ability to supress PTGS. These viral proteins are called viral suppressors of gene silencing (VSRs) and can interfere with different steps of gene silencing pathway either interfering with effector proteins of PTGS or by sequestrating siRNAs (Silhavy & Burgyán, 2004). Some VSRs can also be involved in the development of plant symptoms and can act as pathogenicity determinants. One way is enabling the virus spread and accumulation, increasing the virus symptomatology, or alternatively, affecting the host siRNA-induced gene silence pathway and in consequence upsetting the expression of host genes involved in essential metabolic or developmental processes (Senda *et al.*, 2004; Ding & Voinnet, 2007; Wang *et al.*, 2012).

In the fourth chapter of this doctoral thesis, it was demonstrated that *Broad bean wilt virus 1* (BBWV-1) RNA2-encoded VP37 protein was a VSR and acted as a pathogenicity determinant. In the current work, the other BBWV-1 RNA2-encoded proteins were studied: The ability of MP, SCP and LCP proteins to suppress PTGS was assessed by transient expression assay in transgenic *Nicotiana benthamiana* 16c plants and by a complementation assay using a viral vector based on *Turnip crinkle virus* expressing the GFP protein (pTCV-GFP). Moreover, the role played by BBWV-1 SCP protein on the enhancement of PVX induced symptoms and activation of systemic necrosis associated with PCD was assessed in *N. benthamiana* plants using a viral vector based on the sequence of *Potato virus* X (PVX).

5.2 Materials and methods

5.2.1 Plasmid construction

BBWV-1 RNA2 MP, SCP and LCP genes were amplified from a previously obtained BBWV-1 infectious cDNA clone (GenBank accession numbers KT988973, KT988974) (Ferriol *et al.*, 2016) using iProof High-Fidelity DNA Polymerase (Biorad) with specific primers that contained BamHI enzyme recognition sequences and start and a stop codons to allow correct protein expression (Table 5.1). The resulting PCR products were digested with BamHI enzymes and cloned between the duplicated 35S promoter (2x35S) of *Cauliflower Mosaic Virus* (CaMV) and the nopaline synthase terminator (t-Nos) within the binary plasmid pCambia using T4 ligase (Promega). The resulting constructs p35S-MP, p35S-SCP and p35S-LCP were transformed into *Escherichia coli* X blue (Agilent Technologies) and sequenced. As positive controls, p35S-VP37 and p35S-p19, for transient expression of BBWV-1 VP37 and *Tomato bushy stunt virus* (TBSV) p19 VSRs were used. The plasmid pCambia without insert (p35S-Ø) was used as negative control and p35S-GFP, a binary pBIN plasmid containing GFP sequence was used for full-length GFP expression.

For transient expression of BBWV-1 encoded MP, LCP and SCP proteins through a PVX viral vector, the BBWV-1 corresponding genes were amplified with iProof High-Fidelity DNA Polymerase (Biorad) using specific primers that contained specific enzyme recognition sequences and a start and stop codons to allow the correct protein expression (Table 5.1). The resulting PCR products were digested and cloned into the PVX plasmid pVX202 (Sablowski *et al.*, 1995) rendering pPVX-MP, pPVX-LCP and pPVX-SCP constructs. Empty plasmid pVX202 (pPVX-Ø) was used as negative control whereas pPVX-VP37 was used as positive control. All constructs were transformed into *E. coli* X blue (Agilent Technologies) and sequenced to assess the presence of BBWV-1 genes and mechanically inoculated into 10 plants of *N. benthamiana* as described in section 4.2.6. As mock inoculation control, *N. benthamiana* plants were mechanically inoculated using only the inoculation buffer. Symptomatology was monitored up to 21 days post inoculation (dpi) and non-inoculated upper leaves were collected at 10 dpi for total RNA extraction. All the obtained constructions were sequenced with an ABI 3130xl Genetic Analyzer (Thermo Fisher Scientific). Specific primers for vector constructions or internal primers of the inserts were used for this purpose.

Table 5.1 Primers used for the development of binary plasmids and PVX constructions expressing BBWV-1 coding sequences. Lower case indicates restriction enzyme sequences. Underlined letters indicate insertions.

Name	Sequence (5'-3')	Function
MP1-F	AT*ggatcc*ATGCCGTTGATTCTTCT	PCR to amplify MP of BBWV-1 to obtain p35S-MP
MP-R	AT*ggatcc*CTATTGCCCATATTTGTAA	
LCP-F	AT*ggatcc*ATGGCTCTCATGGAAGAG	PCR to amplify LCP of BBWV-1 to obtain p35S-LCP
LCP-R	AT*ggatcc*CTATTGTCCCTTTGCAAGGC	
SCP-F	AT*ggatcc*ATGGCTCATATGAACCTAGCAG	PCR to amplify SCP of BBWV-1 to obtain p35S-SCP
SCP-R	AT*ggatcc*CTAATATTTAAACTCCTTAAAGAC	
MP1_EagI_F	AC*cggccg*ATGCCGTTGATTCTTCTTTG	PCR to amplify MP of BBWV-1 to obtain pPVX-MP
MP1_SalI_R	AC*gtcgac*TCATTGCCCATATTTGTAATCTATTCC	
LCP_EagI_F	AC*cggccg*ATGGCTCTCATGGAAGAGGAC	Amplification of LCP for cloning into pPVX.
LCP_SalI_R	AC*gtcgac*TCATTGTCCCTTTGCAAGGC	
SCP_EagI_F	AC*cggccg*ATGGCTCATATGAACCTAGCAGAC	Amplification of SCP for cloning into pPVX.
SCP_SalI_R	AC*gtcgac*CTAATATTTAAACTCCTTAAAGACAC	

5.2.2 *N. benthamiana* 16c transient expression assay

For transient essays in *N. benthamiana* 16c plants, p35S-MP, p35S-SCP, p35S-LCP, p35S-VP37, p35S-p19, p35S-Ø and p35S-GFP constructs were transformed into *Agrobacterium tumefaciens* COR308 and agroinfiltrated as described in section 4.2.8. Cells suspensions containing equal volumes of p35S-MP/p35S-GFP, p35S-LCP/p35S-GFP, p35S-SCP/p35S-GFP and p35S-VP37/p35S-GFP were co-infiltrated into leaves of transgenic *N. benthamiana* 16c (3 true leaves stage). Combination of p35S-VP37/p35S-GFP and p35S-p19/p35S-GFP constructs was used as positive controls and p35S-Ø/p35S-GFP as negative control. Green fluorescence was observed at 3 days post agroinfiltration (dpa) with a under a long-wavelength UV lamp (Black Ray model B100AP; UV Products, Upland, CA, U.S.A.) and images were taken with a CANON EOS 300D digital camera using a yellow filter (Jos. Schneider Optische Werke, B+W Filter, Bad Kreuznach, Germany). Agroinfiltrated areas of plant leaves were collected for RNA extraction.

5.2.3 RNA extraction, northern blot and RT-PCR

For northern blot and RT-PCR assays, plant total RNAs and siRNAs were obtained and northern blot analysis of PVX genomic RNA (gRNA) and of GFP mRNA and siRNAs was performed as described in section 4.2.10. For the detection of PVX constructions, RT-

PCR of total RNA extracted from apical non-inoculated leaves of plants inoculated with the different PVX construction was performed with the same primers used to obtain PVX constructions (Table 5.1).

5.2.4 NBT and TB staining

Non inoculated upper leaves of *N. benthamiana* plants inoculated with the different PVX constructs were collected at 7 dpi and analysed for production of superoxide O^{2-} using Nitro Blue Tetrazolium (NBT) staining method (Grellet Bournonville & Díaz-Ricci, 2011) and dead cells of upper non-inoculated leaves of plants inoculated with PVX constructs were stained at 7 dpi using trypan blue (TB) staining method (Garcia-Marcos *et al.*, 2013) as described in section 4.2.7.

5.2.5 pTCV-GFP complementation assay

For TCV complementation assay, *N. benthamiana* leaves (4 true leaves stage) were individually agroinfiltrated with p35S-MP, p35S-SCP and p35S-LCP. p35S-VP37 and p35S-p19 constructs were used as positive controls and p35S-Ø as negative control. One day later, TCV-GFP transcripts were generated and mechanically inoculated on previously agroinfiltrated leaves as described in section 4.2.9. Leaves were observed at 4 dpa with a Leica MZ16 FA fluorescence stereomicroscope using a GFP filter. All plants used in the experiments were maintained in a growth chamber with 16 h light at 24 °C and 8 h of darkness at 20 °C. Plant symptoms were visualized up to 21 dpi.

5.3 Results

5.3.1 BBWV-1 encoded MP and SCP proteins are suppressors of post-transcriptional gene silencing in *N. benthamiana* plants

The ability of BBWV-1 RNA2 encoded MP, LCP and SCP proteins to supress PTGS was studied by transient expression assay in *N. benthamiana* 16c plants which contain the GFP transgene and express constitutively this protein. Agroinfiltration of these plants with

a binary vector expressing GFP activates PTGS inducing GFP mRNA degradation and accumulation of GFP siRNAs. GFP mRNA degradation results in a reduction of GFP green fluorescence and an increase of chlorophyll red fluorescence under UV light. Co-expression of a VSR inhibits the GFP mRNA degradation by suppression of PTGS, reducing the levels of siRNA and restoring the GFP green fluorescence under the UV light (Johansen & Carrington, 2001). BBWV-1 MP, LCP and SCP genes were individually cloned into a binary plasmid between the duplicated CaMV 35S promoter and the Nos-terminator to obtain p35S-MP, p35S-LCP and p35S-SCP constructs (Fig. 5.1). Each one of these constructs was agroinfiltrated into leaves of *N. benthamiana* 16c plants in combination with a binary plasmid expressing GFP named p35S-GFP. Binary plasmids expressing VP37 protein of BBWV-1 and p19 protein of TBSV (p35S-VP37 and p35S-p19 constructs, respectively), well-characterized VSRs showing different levels of PTGS suppression activity (Carpino *et al.*, unpublished, Zamore, 2004) were used as positive controls whereas empty binary plasmid (p35S-Ø) was used as negative control. Agroinfiltrated leaves were observed under hand-held UV lamp and photographed at 3 dpa. (Fig. 5.2). Leaves co-agroinfiltrated with p35S-SCP/p35S-GFP or p35S-MP/p35S-GFP showed mild green fluorescence in the agroinfiltrated areas that remained imperceptible at 5-6 dpa. In contrast, p35S-LCP/p35S-GFP leaves did not show green fluorescence in agroinfiltrated areas as in leaves of p35S-Ø/p35S-GFP co-agroinfiltrated negative controls. In all cases, the intensity of green fluorescence was lower than that observed in leaves co-infiltrated with both p35S-VP37/p35S-GFP and p35S-p19/p35S-GFP positive controls.

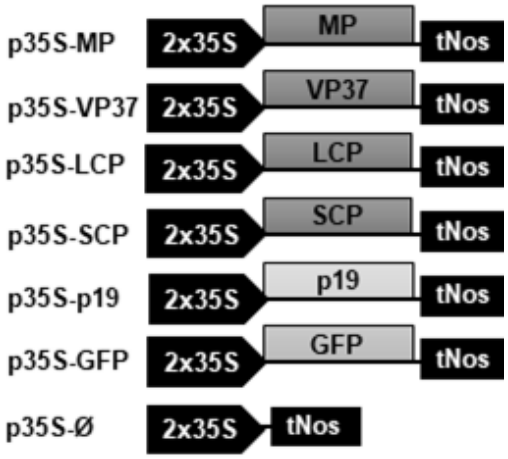

Figure 5.1 Schematic representation of binary constructs used in the transient expression assays in *N. benthamiana* 16c.

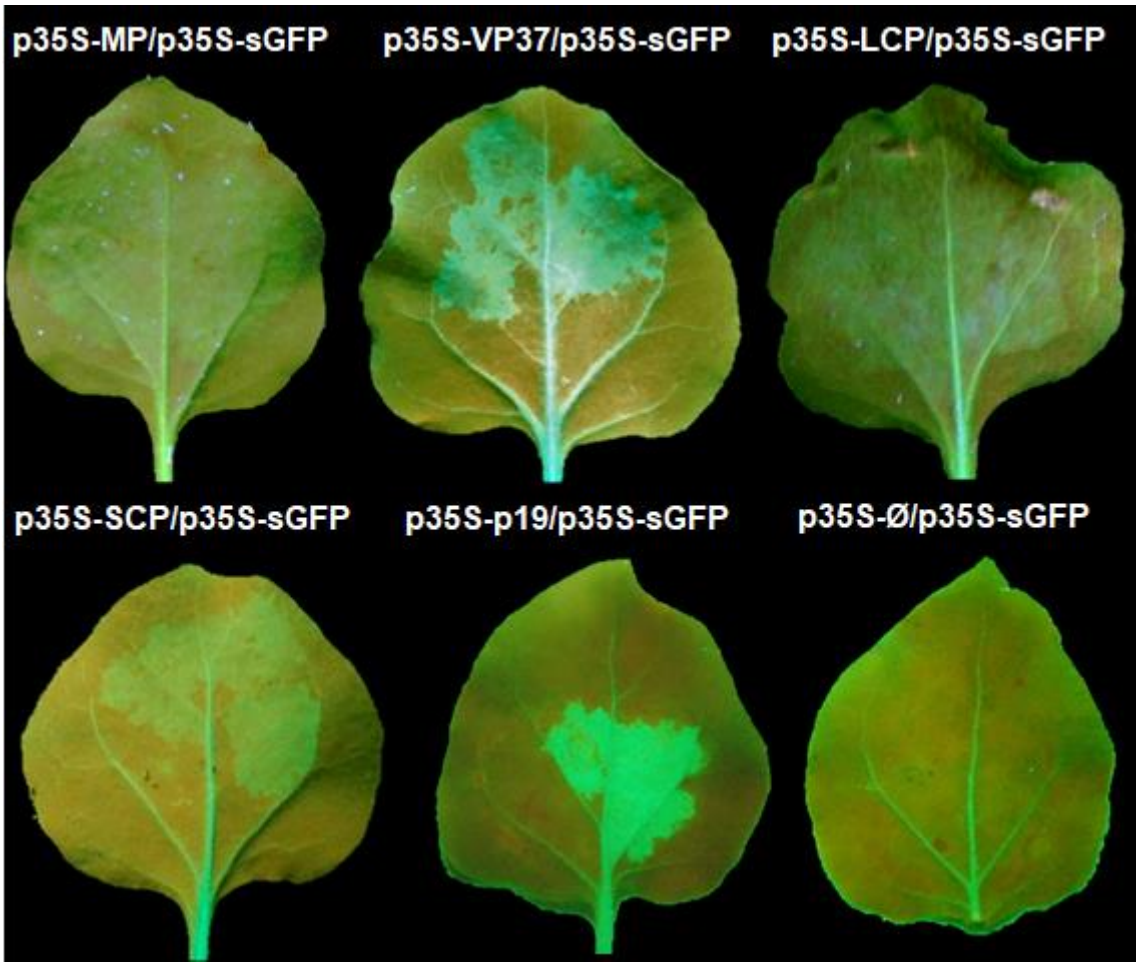

Figure 5.2 RNA silencing suppression activity of BBWV-1 RNA2-encoded proteins. GFP green fluorescence of *N. benthamiana* 16c leaves co-agroinfiltrated with p35S-GFP in combination with the different binary constructs (indicated above each picture) and observed under UV light at 3 dpa.

GFP mRNA and siRNAs accumulation was determined by northern blot analysis of total RNAs extracted at 3 dpa from agroinfiltrated leaf areas using a GFP complementary riboprobe (Fig. 5.3). Levels of GFP mRNA were similar in leaves co-agroinfiltrated with p35S-MP/ p35S-GFP, p35S-SCP/p35S-GFP and p35S-VP37/p35S-GFP (positive control) and higher than leaves co-agroinfiltrated with p35S-LCP/p35S-GFP or p35S-Ø/p35S-GFP (negative control). Leaves co-agroinfiltrated with p35S-p19/p35S-GFP (positive control) showed very high levels of GFP mRNA whereas it was low in leaves co-agroinfiltrated p35S-LCP/p35S-GFP or p35S-Ø/p35S-GFP (negative control). Consistent with these results, GFP siRNAs accumulation was similar in leaves co-agroinfiltrated with p35S-MP/ p35S-GFP and p35S-SCP/p35S-GFP and p35S-VP37/ p35S-GFP (positive control) and lower than in leaves co-agroinfiltrated with p35S-LCP/p35S-GFP or p35S-Ø/p35S-GFP (negative control). Very low GFP siRNAs accumulation was detected in leaves co-

agroinfiltrated p35S-p19/p35S-GFP (positive control) whereas leaves co-agroinfiltrated with p35S-Ø/p35S-GFP (negative control) or p35S-LCP/p35S-GFP showed high GFP siRNAs accumuation. Taken together, the results of these experiments assessed that both BBWV-1 SCP and MP proteins but not BBWV-1 LCP protein had the ability to supress the PTGS. In addition, the level of PTGS suppression of MP and SCP seemed similar to VP37 and lower than TBSV p19 protein.

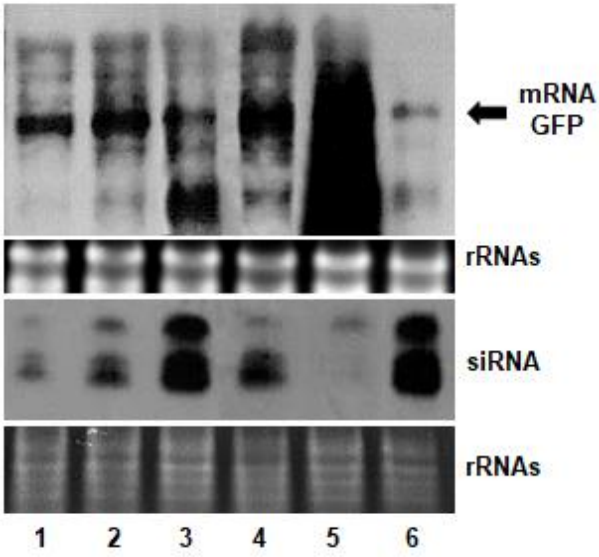

Figure 5.3 Northern blot analysis of GFP mRNA (arrow) and siRNAs extracted from leaf areas of *N. benthamiana* 16c agroinfiltrated with whit p35S-MP/p35S-GFP (lane 1), p35S-VP37/p35S-GFP (lane 2), p35S-LCP/p35S-GFP (lane 3), p35S-SCP/p35S-GFP (lane 4), p35S-p19/p35S-GFP (lane 5) and p35S-Ø /p35S-GFP (lane 6). Ribosomal RNA loading controls are showed at the bottom of the corresponding assays.

To confirm the results obtained by transitory expression assay in *N. benthamiana* 16c plants, a complementation assay was performed using a viral vector based on TCV sequence (pTCV-GFP) in which CP, a well-known VSR, was substituted by GFP gene. Inoculation of TCV-GFP transcripts generates infection foci restricted to single cells, since TCV construct is unable to move to adjacent cells. However, it was demonstrated that transient expression of a VSR restored the TCV-GFP movement by complementation 'in trans', increasing the foci area (Powers *et al.*, 2008). *N. benthamiana* plants were agroinfiltrated with p35S-MP, p35S-LCP and p35S-SCP. As positive control, *N. benthamiana* plants were agroinfiltrated with p35S-VP37 and p35S-p19 whereas for negative controls, plants were agroinfiltrated with p35S-Ø. One dpa, TCV-GFP RNA transcripts were mechanically inoculated on previously agroinfiltrated leaves and at 4 dpa plants were observed with a fluorescence stereomicroscope (Fig. 5.4).

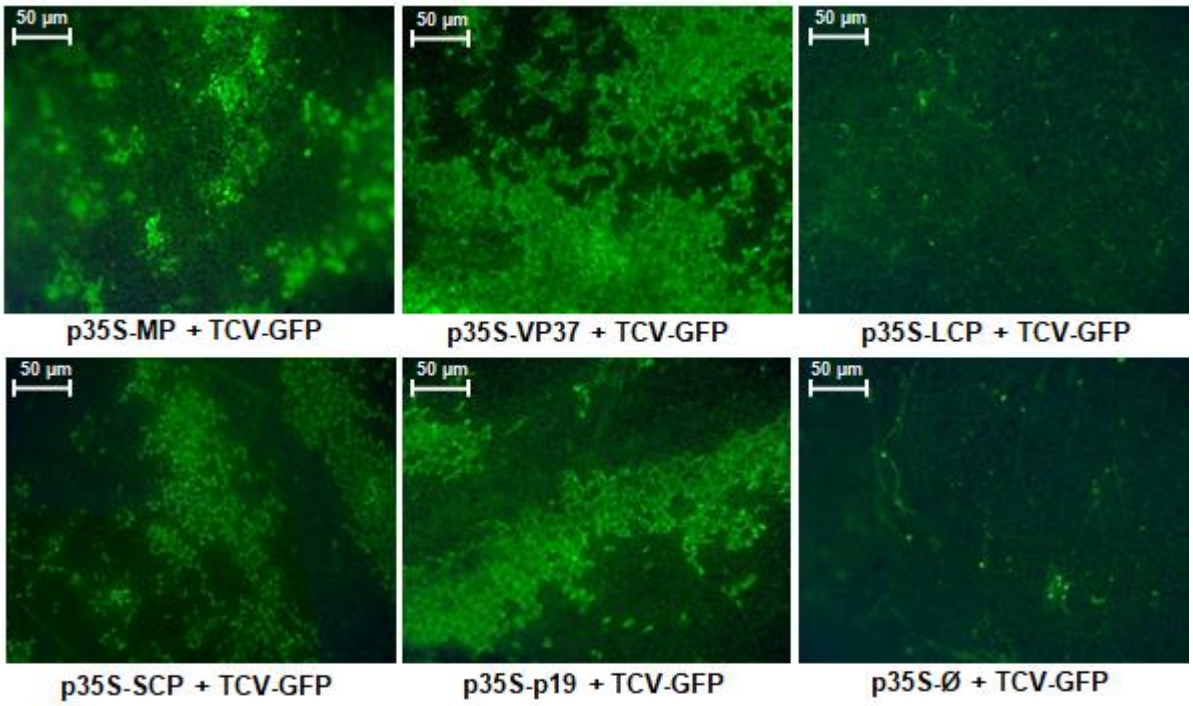

Figure 5.4 GFP green fluorescence under UV fluorescence stereomicroscope of TCV-GFP infection areas of *N. benthamiana* leaves agroinfiltrated with the different binary constructs.

Leaves agroinfiltrated with p35S-MP and p35S-SCP showed extended GFP green fluorescence areas comparable to positive controls agroinfiltrated with p35S-VP37 and p35S-p19, indicating that BBWV-1 MP and SCP proteins restored the TCV-GFP movement and suggesting that these proteins had VSR activity. In contrast, leaves agroinfiltrated with p35S-LCP showed GFP green fluorescence restricted to single cell foci as the negative controls agroinfiltrated with p35S-Ø pointing out that TCV-GFP movement was not restored and suggesting that BBWV-1 LCP had not VSR function. Therefore, results of TCV-GFP movement complementation assay were in concordance with those obtained by transient expression assay in *N. benthamiana* 16c plants, reinforcing the hypothesis that BBWV-1 SCP and MP proteins had the ability to supress PTGS whereas BBWV-1 LCP protein did not.

5.3.2 BBWV-1 SCP protein enhances the PVX-induced symptoms and activates the systemic necrosis associated with programed cell death in *N. benthamiana* plants

In order to identify whether BBWV-1 MP, LCP and SCP proteins were involved in plant symptoms development and in consequence could act as determinants of pathogenesis,

a transient expression assay of these proteins was performed using the PVX viral vector (Sablowski *et al.*, 1995). This system has been widely used to identify determinants of pathogenesis of different plant viruses, including the BBWV-1 VP37 protein as showed in the fourth chapter of this doctoral thesis. BBWV-1 MP, LCP and SCP were cloned into pPVX202 vector obtaining pPVX-MP, pPVX-LCP and pPVX-SCP constructs. pPVX-Ø empty and pPVX-VP37 were used as negative and positive controls, respectively (Fig. 5.5 A). Each construct was mechanically inoculated in twenty *N. benthamiana* plants and twenty *N. benthamiana* plants were mechanically inoculated with the inoculation buffer as inoculation negative control. PVX systemic infection was determined at 10 dpi by RT-PCR of total RNA extracted from upper non inoculated leaves using PVX-specific primers, whereas plant symptom development was monitored up to 21 dpi. All pPVX-SCP, pPVX-VP37 and pPVX-Ø inoculated plants were infected showing different symptoms depending on the inoculated construct (Fig 5.5 B) but none of pPVX-MP and pPVX-LCP inoculated plants resulted systemically infected. Presence and integrity of BBWV-1 SCP and VP37 sequences was determined by RT-PCR and sequencing of total RNA extracted from symptomatic upper non inoculated leaves.

RT-PCR of total RNA extracts of upper leaves revealed that both PVX-MP and PVX-LCP had lost the ability to replicate, probably due to recombination of these constructs in the infected plant cells. At 10 dpi, pPVX-Ø inoculated plants used as negative controls showed typical symptoms of PVX infection such as mild leaf mosaic that almost disappear at 21 dpi whereas pPVX-VP37 and pPVX-SCP inoculated plants showed symptoms of stunting, sever mosaic and small necrotic lesions in non-inoculated upper leaves up to 21 dpi. On the other hand, pPVX-MP, pPVX-LCP and mock-inoculated plants which were no systemically infected remained asymptomatic during all assay.

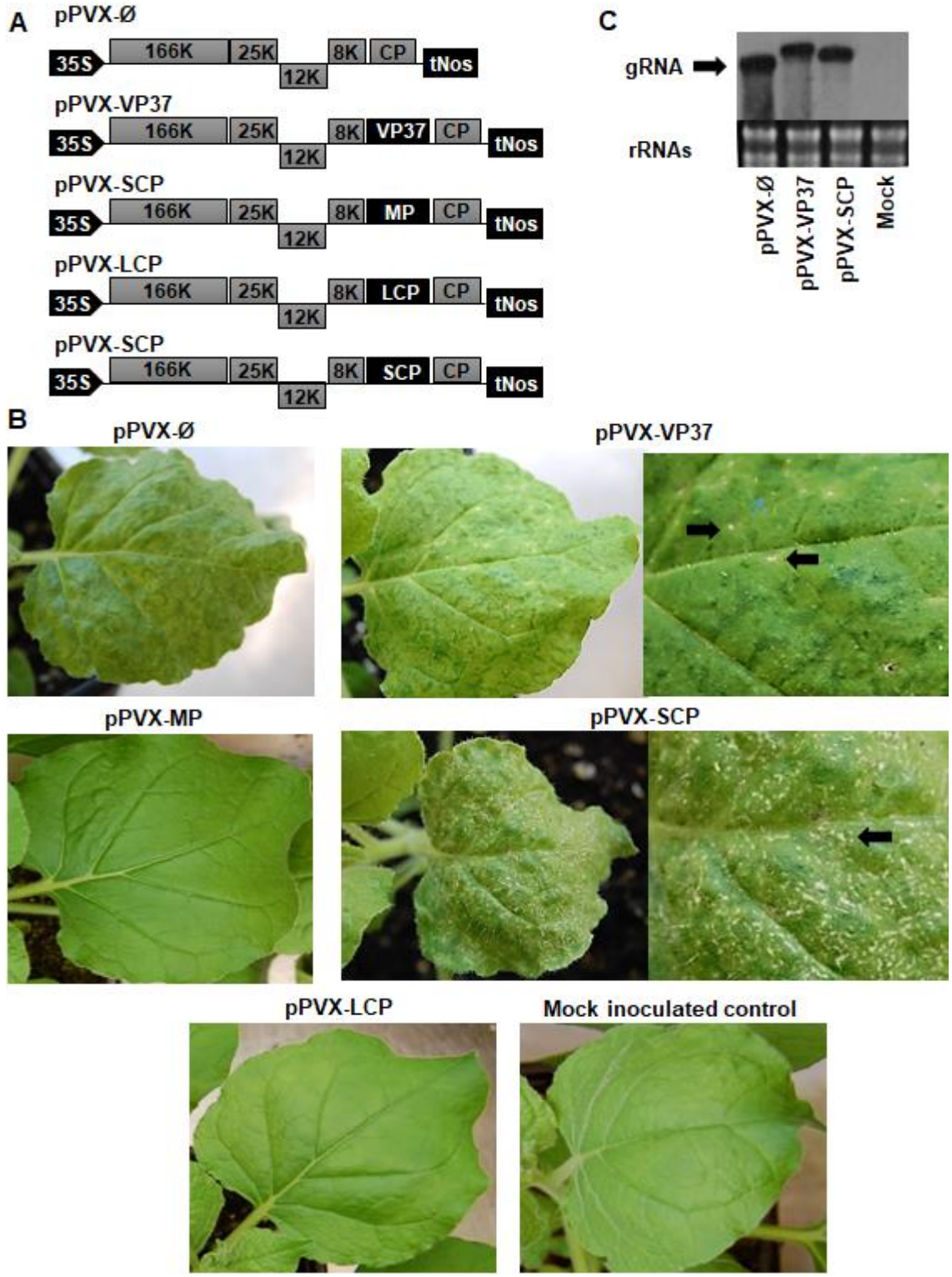

Figure 5.5 Transient expression assay of BBWV-1 RNA-2 encoded proteins using PVX viral vector. **(A)** Schematic representation of PVX constructs. **(B)** Upper non-inoculated leaves of *N. benthamiana* plants inoculated with PVX constructions at 7 dpi. Necrotic lesions induced by pPVX-VP37 and pPVX-SCP are showed with more detail at the right (arrows). **(C)** Northern blots of total RNA obtained from *N. benthamiana* plants inoculated with different PVX constructs. Ribosomal RNA loading controls are showed at the bottom.

Northern blots assays using a PVX specific riboprobe of total RNA extracts obtained from non-inoculated upper leaves of *N. benthamiana* plants agroinfiltrated with pPVX-SCP, pPVX-VP37 and pPVX-Ø revealed the presence of PVX genomic RNA (gRNA) showing different electrophoretic mobility in concordance with sizes of BBWV-1 SCP and VP37 cloned nucleotide sequences (Fig. 5.5 C). Thus, results presented here assessed that BBWV-1 SCP was a determinant of pathogenicity enhancing the PVX-induced symptomatology even inducing necrotic lesions.

Upon pathogen entry, to limit disease spread, plants develop a systemic defence mechanism triggered by viral products that leads to upset of the plant hormone levels (salicylic acid, jasmonic acid and nitric oxide), to ROS accumulation and to final induction of PCD (Iakimova *et al.*, 2005; Komatsu *et al.*, 2010; Mandadi & Scholthof, 2013). In order to analyse the necrotic lesions of upper non inoculated leaves of plants inoculated with pPVX-SCP, accumulation of superoxide ion (O^{2}_{-}), a reactive oxygen species (ROS) associated to PCD activation, was determined by NBT staining method (Grellet Bournonville & Díaz-Ricci, 2011). Moreover, TB was used to stain death cells (Garcia-Marcos *et al.*, 2013). Symptomatic leaves of *N. benthamiana* plants collected at 7 dpi and stained with NBT showed the presence of superoxide O^{2}_{-} dark-blue-coloured deposits in pPVX-SCP and pPVX-VP37 inoculated plants but not in pPVX-Ø inoculate plants used as negative controls (Fig. 5.6 A). Related to superoxide O^{2}_{-} dark-blue-coloured deposits, dead cell groups were visualized by TB (Fig. 5.6 B). Taken together, results showed that BBWV-1 SCP protein was a determinant of pathogenicity enhancing PVX symptomatology and inducing systemic necrosis in upper non-inoculated leaves of *N. benthamania* plants as consequence of activation of defence response mechanism and PCD such as BBWV-1 VP37 protein which was used in this assay as positive control.

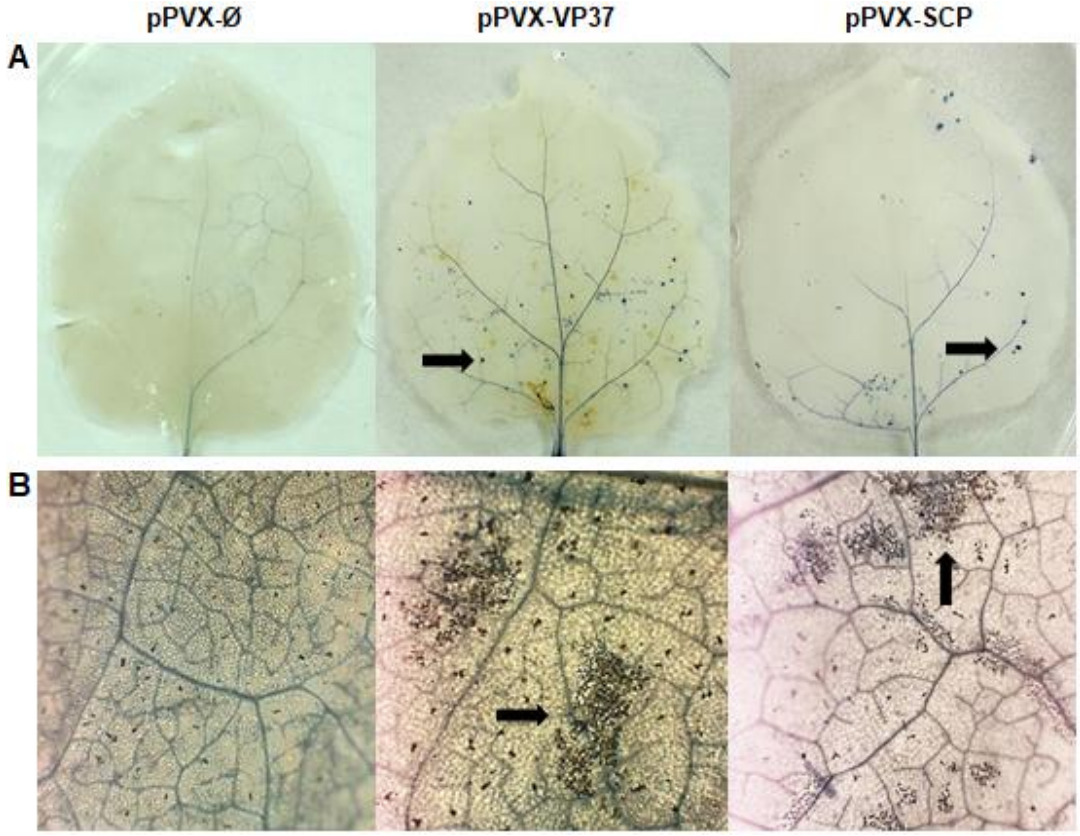

Figure 5.6 Analysis of systemic necrotic lesions of upper non-inoculated *N. benthamiana* plants produced by transient expression of BBWV-1 VP37 (positive control) and SCP proteins using the PVX viral vector at 7 dpi. As negative control, pPVX-Ø construct was used. **(A)** NBT staining of superoxide O^{2-} deposits. **(B)** TB staining of groups of dead cells.

5.4 Discussion

Viral infections rely on complex interaction between viral factors and host components, causing physiological alterations that lead to disease manifestation in the plant. One of the most important plant-virus interaction, consists in the counteraction of plant defence mechanism by the invasive viruses. The other one attends to physiologic changes produced by the pathogenic viruses on infected plants leading to manifestation of plants disease. BBWV-1 infects important horticultural and ornamental crops and causes significant economic losses. The interactions between BBWV-1 and host plants have been poorly studied. The role played by BBWV-1 RNA2-encoded VP37 protein in PTGS suppression and plant symptom development was studied in chapter four of this thesis. In the current work, the VSR' activity of the other BBWV1 RNA2 encoded proteins and their involvement in plant disease manifestation was investigated. Transitory expression assays

in *N. benthiamiana* 16c revealed that both SCP and MP, but not LCP proteins had the ability to supress PTGS. These results were confirmed by a complementation assay using the pTCV-GFP viral construct. Among *Secoviridae* family, several studies were performed to identify VSRs and it was seen that either capsid or movement proteins of viral species belonging to different genera could act as VSRs. The Vp20 protein, one of the three capsid proteins of *Apple latent spherical virus* (ALSV, *Cheravirus* genus), the SCP protein of *Cowpea mosaic virus* (CPMV, *Comovirus* genus) and also the CP protein of *Tomato ringspot virus* (ToRS, *Nepovirus* genus) were identified as VSRs by transient expression of these proteins in *N. benthamiana* 16c (Canizares *et al.*, 2004; Liu *et al.*, 2004; Yaegashi *et al.*, 2007; Karran & Sanfaçon, 2014). On the other hand, the movement protein p51 of *Maize chlorotic dwarf virus* (MCDV, *Waikavirus* genus) has been recently identified as VSR (Stewart *et al.*, 2017). With regard to *Fabavirus* genus, information about VSRs is scarce and both MPs and LCP proteins of BBWV-2 were identified as VSRs (Kong *et al.*, 2014). The fourth chapter of this thesis showed that BBWV-1 RNA2-encoded VP37 had PTGS suppressor activity revealing that BBWV-1, such as BBWV-2, encodes multiple VSRs to suppress RNA silencing during plant infection in a complex way to counteract host defence response. However, both viral species seem to follow different strategies in spite of having close phylogenetic relationships. BBWV-1 MP and VP37 proteins had the ability to suppress PTGS like those of BBWV-2 whereas opposite results were obtained for LCP and SCP proteins of both viruses. Indeed, BBWV-1 SCP had VSR activity but that of BBWV-2 did not.

To determine if BBWV-1 RNA 2 encoded MP, LCP and SCP acted as pathogenicity determinants, a transient expression assay using the pPVX202 viral vector was performed. PVX-based vectors represent a widely used system to study the proteins of plant viruses. It has been used to identify determinants of pathogenesis of different plant viruses such as the CTV p23 protein, the TBSV p19 protein, the *Plum pox virus* (PPV, *Potyvirus* genus) HC-Pro protein, the *Tobacco mosaic virus* (TMV, *Tobamovirus* genus) ORF6, and T*omato leaf curl New Delhi virus* (ToLCNDV, *Begomovirus* genus) nuclear shuttle protein (Scholthof *et al.*, 1995; Canto *et al.*, 2004; González-Jara *et al.*, 2005; Hussain *et al.*, 2005; Amin & Falk, 2009). Results obtained in the current work revealed that SCP protein, like BBWV-1 VP37 protein, was a determinant of pathogenicity inducing necrotic lesion in upper non-inoculated leaves of *N. benthamania* plants as consequence of activation of PCD. Unfortunately, transitory expression of BBWV-1 MP and LCP using pPVX202 was not possible and further experiments to clarify the role played by these viral proteins on plant symptom development

must be performed using other PVX viral vectors. Studies performed on viruses belonging to *Secoviridae* family but from different genera than *Fabavirus* localized the pathogenicity determinants among RNA1 encoded proteins. For *Bean pod mottle virus* (BPMV, *Comovirus* genus) experiments with chimeric infectious clones indicated that the RNA1 encoded protease co-factor (PRO-CO) and the C-terminal fragment of putative helicase (HEL) were determinants of symptom severity (Gu & Ghabrial, 2005). Moreover, the RNA1 encoded N-terminal 11k protein of *Tomato torrado virus* (ToTV, *Torradovirus* genus) enhanced PVX-induced symptomatology inducing strong local and systemic necrosis lesions although not further experiments to determinate the activation of HR and systemic necrosis performed (Wieczorek & Obrępalska-Stęplowska, 2016a). Finally, RNA1 encoded RNA polymerase was identified as symptom determinants using reassortants of *Grapevine fanleaf virus* (GFV, *Nepovirus* genus) inducing symptoms of different severity (Vigne *et al.*, 2013). Regarding on *Fabavirus* genus, the information about determinants of pathogenesis is still very limited. Assays using reassortant full-length cDNA infectious clones containing genomic RNA2 molecules of two BBWV-2 isolates inducing different plant symptoms showed that genomic RNA2 contained the determinants of pathogenesis (Kwak *et al.*, 2016). Further studies demonstrated that BBWV-2 MP, VP37 and LCP proteins enhanced PVX-induced symptomatology in *N. benthamiana* whereas BBWV-2 SCP protein did not (Kong *et al.*, 2014). In addition, at difference to SCP and VP37 of BBWV-1, none them induced necrotic lesions on leaves.

In conclusion, BBWV-1 and BBWV-2, both belonging to the *Fabavirus* genus, use different strategies to counteract the host defence response and to induce plant symptoms. However, for both viruses, the viral proteins that have the ability to supress PTGS are also determinants of pathogenesis enhancing PVX symptoms in *N. benthamiana* plants. It was reported that some VSRs can affect the micro RNA (miRNA) induced gene silencing pathway, upsetting the expression of important plant genes related to developmental processes, biotic and abiotic stress responses, and nutrient homeostasis (Dunoyer & Voinnet, 2005; Tatineni *et al.*, 2011; Wang *et al.*, 2012). Consequently, BBWV-1 and BBWV-2 VSRs could also induce physiological abnormalities related with viral infection such as plant stunting, chlorosis, leaf deformation and systemic necrosis. Further studies in order to determine the interaction of BBWV-1 SCP protein and VP37 proteins with endogenous miRNAs or with proteins involved in gene silencing pathway must be performed.

Chapter 6

General conclusions

1. BBWV-1 isolates Ben, B41/99, NSRV and PV0548, originated from different countries and clustered in different phylogenetic groups (Ferriol et al., 2014) induced different symptoms. On broad bean (*Vicia faba*), plants infected with PV0548 showed a middle leaf mosaic and a nerval chlorosis in the initial stage of the infection which disappeared at 40 dpi and the new leaves showed a slight distortion. Ben-infected plants showed intense nerval chlorosis that evolved in middle mosaic and slight leaf distortion in old and new leaves. B41/99 and NSRV infected plants showed similar symptoms consisting of intense nerval chlorosis and leaf distortion that increased in severity until 40 dpi. New leaves of plants infected with these BBWV-1 isolates showed also strong distortion symptoms and leaf curling. On pepper (*Capsicum annum*), Ben-infected plants showed intense leaf mosaic; PV0548- and NRSV-infected plants showed mil mosaic whereas B41/99-infected plants only showed an almost imperceptible mosaic. On *Nicotiana benthamiana* plants, the four BBWV-isolates induced the same symptoms consisting in stunting, chlorosis, severe mosaic and plant death. Finally, tomato (*Solanum lycopersicum*) plants infected with the four BBWV-1 isolates remained asymptomatic.

2. No differences in infectivity between the four assayed BBWV-1 isolates were found on broad bean, pepper, tomato or *N. benthamiana* plants. None of these viral isolates infected common bean (*Phaseolus vulgaris*) cucumber (*Cucumis sativus*) and melon (*Cucumis melo*), which were previously reported as hosts for other BBWV-1 or BBWV-2 isolates.

3. RNA accumulation of BBWV-1 isolates Ben, B41/99, NSRV and PV0548 over time on broad bean and pepper was uncorrelated with symptom severity.

4. A BBWV-1 full-length cDNA infectious clone, based on the sequence of BWWV-1 isolate Ben (pBBWV1-Wt) was obtained. A mutant construct (pBBWV1-G492C) lacking the putative VP37 RNA2-encoded protein was engineered introducing one point mutation in RNA2 alternative start codon (G492C). Agroinfiltration assays in different plant host species showed that pBBWV1-Wt had the same biological properties than the original BBWV-1 isolate Ben in broad bean, tomato, pepper and *N. benthamiana* plants. However, pBBWV1-G492C was unable to infect pepper and tomato plants and induced milder symptoms than pBBWV1-Wt on broad bean and *N.*

benthamiana plants. These results suggest that the BBWV-1 VP37 protein is involved on host specificity and symptom development.

5. Viral quantitation of *N. benthamiana* and broad bean plants inoculated with pBBWV1-Wt and pBBWV1-G492C revealed that BBWV-1 VP37 protein was not involved in virus replication.

6. Non-enveloped viral particles of 25-30 nm in diameter and icosahedral morphology, identical to those reported for BBWV-1, were purified from *N. benthamiana* and broad bean plants agroinfiltrated with BBWV1-Wt or pBBWV1-G492C suggesting that BBWV-1 VP37 protein has no role in virion formation.

7. Transient expression of BBWV-1 RNA2-encoded proteins using the *Potato virus X* (PVX) vector pPVX202, showed that VP37 and small capsid protein (SCP) enhanced PVX-related symptoms and induced systemic necrosis associated with programed cell death (PCD) in *N. benthamiana* plants. These results confirm those obtained with the BBWV-1 full-length cDNA infectious clones and reinforce the hypothesis that VP37 and SCP are pathogenicity determinants.

8. Transient expression in transgenic *N. benthamiana* 16c plants and assays of movement complementation of *Turnip crinkle virus*-GFP viral construct (pTCV-GFP) showed that BBWV-1 VP37, MP and SCP proteins are post-transcriptional gene silencing (PTGS) suppressors.

References

Acosta-Leal R, Duffy S, Xiong Z, Hammond R, Elena SF, 2011. Advances in Plant Virus Evolution: Translating Evolutionary Insights into Better Disease Management. *Phytopathology* 101, 1136-48.

Alizon S, Hurford A, Mideo N, Van Baalen M, 2009. Virulence evolution and the trade-off hypothesis: history, current state of affairs and the future. *Journal of Evolutionary Biology* 22, 245-59.

Amin HA, Falk B, 2009. *Citrus tristeza virus*–p23 gene correlated with the pathogenicity in non citrus hosts. *Egypt.J.Phytopathol* 37, 83-98.

Amin I, Ilyas M, Qazi J, Bashir R, Yadav JS, Mansoor S, Fauquet CM, Briddon RW, 2011. Identification of a major pathogenicity determinant and suppressors of RNA silencing encoded by a South Pacific isolate of *Banana bunchy top virus* originating from Pakistan. *Virus genes* 42, 272-81.

Anderson PK, Cunningham AA, Patel NG, Morales FJ, Epstein PR, Daszak P, 2004. Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. *Trends in Ecology & Evolution* 19, 535-44.

Anderson RM, May R, 1982. Coevolution of hosts and parasites. *Parasitology* 85, 411-26.

Baulcombe D, 2004. RNA silencing in plants. *Nature* 431, 356-63.

Blancard D, 2012. Tomato diseases: identification, biology and control: A Colour Handbook. *CRC Press.*

Boccardo G, Conti M, 1973. Purification and properties of *Nasturtium ringspot virus*. *Journal of Phytopathology* 78, 14-24.

Boualem A, Dogimont C, Bendahmane A, 2016. The battle for survival between viruses and their host plants. *Current opinion in virology* 17, 32-8.

Boyer J, Haenni A, 1994. Infectious transcripts and cDNA Clones of RNA Viruses. *Virology* 198, 415-26.

Burgyán J, Havelda Z, 2011. Viral suppressors of RNA silencing. *Trends in plant science* 16, 265-72.

Canizares MC, Taylor KM, Lomonossoff GP, 2004. Surface-exposed C-terminal amino acids of the small coat protein of *Cowpea mosaic virus* are required for suppression of silencing. *Journal of general virology* 85, 3431-5.

Canto T, MacFarlane SA, Palukaitis P, 2004. ORF6 of *Tobacco mosaic virus* is a determinant of viral pathogenicity in *Nicotiana benthamiana*. *Journal of general virology* 85, 3123-33.

Casteel CL, Falk BW, 2016. Plant virus-vector interactions: more than just for virus transmission. *Current Research Topics in Plant Virology.* Springer, 217-40.

Castrovilli S, Savino V, Castellano M, Engelbrecht D, 1985. Characterization of a grapevine isolate of broad bean wilt virus. *Phytopathologia Mediterranea*, 35-40.

Chapman EJ, Prokhnevsky AI, Gopinath K, Dolja VV, Carrington JC, 2004. Viral RNA silencing suppressors inhibit the microRNA pathway at an intermediate step. *Genes & development* 18, 1179-86.

Chen X, Bruening G, 1992. Nucleotide sequence and genetic map of *Cowpea severe mosaic virus* RNA 2 and comparisons with RNA 2 of other comoviruses. *Virology* 187, 682-92.

Chiu M, Chen I, Baulcombe DC, TSAI C, 2010. The silencing suppressor P25 of *Potato virus X* interacts with Argonaute1 and mediates its degradation through the proteasome pathway. *Molecular plant pathology* 11, 641-9.

Conti M, Masenga V, 1977. Identification and prevalence of pepper viruses in Northwest Italy. *Journal of Phytopathology* 90, 212-22.

Csorba T, Pantaleo V, Burgyán J, 2009. RNA silencing: an antiviral mechanism. *Advances in Virus Research* 75, 35-230.

Csorba T, Bovi A, Dalmay T, Burgyan J, 2007. The p122 subunit of *Tobacco Mosaic Virus* replicase is a potent silencing suppressor and compromises both small interfering RNA- and microRNA-mediated pathways. *Journal of virology* 81, 11768-80.

Culver JN, 2002. *Tobacco mosaic virus* assembly and disassembly: determinants in pathogenicity and resistance. *Annual Review of Phytopathology* 40, 287-308.

Davino M, Areddia R, Polizzi G, Grimaldi V, 1989. Observations on pitting in pepper fruit in Sicily. *Difesa delle Piante* 12, 65-73.

Deleris A, Gallego-Bartolome J, Bao J, Kasschau KD, Carrington JC, Voinnet O, 2006. Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense. *Science (New York, N.Y.)* 313, 68-71.

Ding S, Voinnet O, 2007. Antiviral immunity directed by small RNAs. *Cell* 130, 413-26.

Doel T, 1975. Comparative properties of type, nasturtium ringspot and petunia ringspot strains of B*road bean wilt virus*. *Journal of General Virology* 26, 95-108.

Domingo E, 1994. Mutation rates and rapid evolution of RNA viruses. *The Evolutionary Biology of Viruses*, 161-84.

Dong S, Xiang H, Shang Q, Li D, Yu J, Han C, 2012. Complete genomic sequence analysis reveals a novel fabavirus infecting cucurbits in China. *Archives of Virology* 157, 597-600.

Duffy S, Shackelton LA, Holmes EC, 2008. Rates of evolutionary change in viruses: patterns and determinants. *Nature Reviews Genetics* 9.

Dunoyer P, Voinnet O, 2005. The complex interplay between plant viruses and host RNA-silencing pathways. *Current opinion in plant biology* 8, 415-23.

Edwardson JR, Christie RG, 1991. CRC handbook of viruses infecting legumes. *CRC Press.*

Elena SF, Bedhomme S, Carrasco P, Cuevas JM, de la Iglesia F, Lafforgue G, Lalic J, Prosper A, Tromas N, Zwart MP, 2011. The evolutionary genetics of emerging plant RNA viruses. *Molecular plant-microbe interactions : MPMI* 24, 287-93.

Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA, 2005. Virus taxonomy: VIIIth report of the International Committee on Taxonomy of Viruses. *Academic Press.*

Ferrer RM, Luis-Arteaga M, Guerri J, Moreno P, Rubio L, 2007. Detection and identification of species of the genus *Fabavirus* by RT-PCR with a single pair of primers. *Journal of virological methods* 144, 156-60.

Ferrer R, Escriu F, Luis-Arteaga M, Guerri J, Moreno P, Rubio L, 2008. New molecular methods for identification of *Broad bean wilt virus 1*. *Molecular and cellular probes* 22, 223-7.

Ferrer R, Guerri J, Luis-Arteaga M, Moreno P, Rubio L, 2005. The complete sequence of a Spanish isolate of *Broad bean wilt virus 1* (BBWV-1) reveals a high variability and conserved motifs in the genus Fabavirus. *Archives of Virology* 150, 2109-16.

Ferriol I, Rangel E, Panno S, Davino S, Han C, Olmos A, Rubio L, 2015. Rapid detection and discrimination of fabaviruses by flow-through hybridisation with genus-and species-specific riboprobes. *Annals of Applied Biology* 167, 26-35.

Ferriol I, Rubio L, Pérez-Panadés J, Carbonell E, Davino S, Belliure B, 2013. Transmissibility of *Broad bean wilt virus 1* by aphids: influence of virus accumulation in plants, virus genotype and aphid species. *Annals of Applied Biology* 162, 71-9.

Ferriol I, Ruiz-Ruiz S, Rubio L, 2011. Detection and absolute quantitation of *Broad bean wilt virus 1* (BBWV-1) and BBWV-2 by real-time RT-PCR. *Journal of virological methods* 177, 202-5.

Ferriol I, Ambrós S, da Silva DM, Falk BW, Rubio L, 2016. Molecular and biological characterization of highly infectious transcripts from full-length cDNA clones of broad bean wilt virus 1. *Virus research* 217, 71-5.

Ferriol I, Ferrer RM, Luis-Arteaga M, Guerri J, Moreno P, Rubio L, 2014. Genetic variability and evolution of *Broad bean wilt virus 1*: role of recombination, selection and gene flow. *Archives of Virology* 159, 779.

Froissart R, Doumayrou J, Vuillaume F, Alizon S, Michalakis Y, 2010. The virulence–transmission trade-off in vector-borne plant viruses: a review of (non-) existing studies. *Philosophical Transactions of the Royal Society B: Biological Sciences* 365, 1907.

Frowd J, Tomlinson J, 1970. *Nasturtium ringspot virus* in Parsley crops in Britain. *Plant Disease Reporter* 54, 734-5.

Frowd J, Tomlinson J, 1972. Relationships between a parsley virus, *Nasturtium ringspot virus* and *Broad bean wilt virus*. *Annals of Applied Biology* 72, 189-95.

Fu ZQ, Dong X, 2013. Systemic acquired resistance: turning local infection into global defense. *Annual review of plant biology* 64, 839-63.

Fuchs M, Schmitt-Keichinger C, Sanfaçon H, 2017. Chapter Two-A Renaissance in Nepovirus Research Provides New Insights Into Their Molecular Interface With Hosts and Vectors. *Advances in Virus Research* 97, 61-105.

Fujita Y, Mise K, Okuno T, Ahlquist P, Furusawa I, 1996. A single codon change in a conserved motif of a bromovirus movement protein gene confers compatibility with a new host. *Virology* 223, 283-91.

Galipienso L, Janssen D, Rubio L, Aramburu J, Velasco L, 2013. Cucumber vein yellowing virus isolate-specific expression of symptoms and viral RNA accumulation in susceptible and resistant cucumber cultivars. Crop protection 43, 141-5.

García JA, Pallás V, 2015. Viral factors involved in plant pathogenesis. *Current opinion in virology* 11, 21-30.

Garcia-Marcos A, Pacheco R, Manzano A, Aguilar E, Tenllado F, 2013. Oxylipin biosynthesis genes positively regulate programmed cell death during compatible infections with the synergistic pair potato virus X, potato virus Y and Tomato spotted wilt virus. *Journal of virology* 87, 5769-83.

Gomez P, Sempere RN, Elena SF, Aranda MA, 2009. Mixed infections of Pepino mosaic virus strains modulate the evolutionary dynamics of this emergent virus. *Journal of virology* 83, 12378-87.

González-Jara P, Atencio FA, Martínez-García B, Barajas D, Tenllado F, Díaz-Ruíz JR, 2005. A single amino acid mutation in the *Plum pox virus* helper component-

proteinase gene abolishes both synergistic and RNA silencing suppression activities. *Phytopathology* 95, 894-901.

Goodman RN, Novacky AJ, 1994. The hypersensitive reaction in plants to pathogens: a resistance phenomenon. *American Phytopathological Society* (APS).

Gracia O, Gutierrez LS, 1982. *Broad bean wilt virus* in pepper crops in Argentina. *Phytopath.Medit* 21, 107-9.

Grellet Bournonville CF, Díaz-Ricci JC, 2011. Quantitative determination of superoxide in plant leaves using a modified NBT staining method. *Phytochemical Analysis* 22, 268-71.

Gu H, Ghabrial SA, 2005. The *Bean pod mottle virus* proteinase cofactor and putative helicase are symptom severity determinants. *Virology* 333, 271-83.

Haas G, Azevedo J, Moissiard G, Geldreich A, Himber C, Bureau M, Fukuhara T, Keller M, Voinnet O, 2008. Nuclear import of CaMV P6 is required for infection and suppression of the RNA silencing factor DRB4. *The EMBO journal* 27, 2102-12.

Heath MC, 2000. Hypersensitive response-related death. *Programmed cell death in higher plants.* : Springer, 77-90.

Hoffmann K, Qiu WP, Moyer JW, 2001. Overcoming host- and pathogen-mediated resistance in tomato and tobacco maps to the M RNA of Tomato spotted wilt virus. *Molecular plant-microbe interactions : MPMI* 14, 242-9.

Hu W, Huang C, Lee S, Wu C, Chang Y, 2010. Detection of four calla potyviruses by multiplex RT-PCR using nad5 mRNA as an internal control. *European Journal of Plant Pathology* 126, 43.

Hull R, Davies J, 1983. Genetic engineering with plant viruses, and their potential as vectors. *Advances in Virus Research* 28, 1-33.

Hull R, 2009. Comparative plant virology. *Academic press.*

Hull R, 2013. Plant virology. *Academic press*.

Hussain M, Mansoor S, Iram S, Fatima AN, Zafar Y, 2005. The nuclear shuttle protein of *Tomato leaf curl New Delhi virus* is a pathogenicity determinant. *Journal of virology* 79, 4434-9.

Iakimova ET, Michalczuk L, Woltering EJ, 2005. Hypersensitive cell death in plants-its mechanisms and role in plant defence against pathogens. *Journal of fruit and ornamental plant research* 13, 135.

Ikegami M, Sharma P, 2011. *Fabavirus. The Springer Index of Viruses, Springer,* 355-9.

Islam W, Zaynab M, Qasim M, Wu Z, 2017a. Plant-virus interactions: Disease resistance in focus. *Hosts Viruses* 4, 5-20.

Islam W, Zhang J, Adnan M, Noman A, Zainab M, Jian W, 2017b. Plant virus ecology: a glimpse of recent accomplishments. *Appl.Econ.Environ.Res* 15, 691-705.

Johansen LK, Carrington JC, 2001. Silencing on the spot. Induction and suppression of RNA silencing in the Agrobacterium-mediated transient expression system. *Plant Physiology* 126, 930-8.

Kang BC, Yeam I, Jahn MM, 2005. Genetics of plant virus resistance. *Annu.Rev.Phytopathol.* 43, 581-621.

Karran RA, Sanfaçon H, 2014. *Tomato ringspot virus* coat protein binds to ARGONAUTE 1 and suppresses the translation repression of a reporter gene. *Molecular Plant-Microbe Interactions* 27, 933-43.

Kobayashi YO, Kobayashi A, Nakano M, Hagiwara K, Honda Y, Omura T, 2003. Analysis of genetic relations between *Broad bean wilt virus 1* and *Broad bean wilt virus 2*. *Journal of general plant pathology* 69, 320-6.

Kobayashi Y, Nakano M, Kashiwazaki S, Naito T, Mikoshiba Y, Shiota A, Kameya-Iwaki M, Honda Y, 1999. Sequence analysis of RNA-2 of different isolates of *Broad bean wilt virus* confirms the existence of two distinct species. *Archives of Virology* 144, 1429-38.

Komatsu K, 2013. Studies on molecular mechanisms underlying virus-induced systemic necrosis associated with host defense responses. *Journal of General Plant Pathology* 79, 455-7.

Komatsu K, Hashimoto M, Ozeki J, Yamaji Y, Maejima K, Senshu H, Himeno M, Okano Y, Kagiwada S, Namba S, 2010. Viral-induced systemic necrosis in plants involves both programmed cell death and the inhibition of viral multiplication, which are regulated by independent pathways. *Molecular Plant-Microbe Interactions* 23, 283-93.

Kong L, Wang Y, Yang X, Sunter G, Zhou X, 2014. *Broad bean wilt virus 2* encoded vp53, vp37 and large capsid protein orchestrate suppression of rna silencing in plant. *Virus research* 192, 62-73.

Kwak H, Lee Y, Kim J, Kim M, Kim J, Choi H, Seo J, 2016. A determinant of disease symptom severity is located in RNA2 of *Broad bean wilt virus 2*. *Virus research* 211, 25-8.

Lakatos L, Csorba T, Pantaleo V, Chapman EJ, Carrington JC, Liu YP, Dolja VV, Calvino LF, Lopez-Moya JJ, Burgyan J, 2006. Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. *The EMBO journal* 25, 2768-80.

Lecoq H, 1998. Control of plant virus diseases by cross protection. *Plant virus disease control*.

Lekkerkerker A, Wellink J, Yuan P, Van Lent J, Goldbach R, van Kammen AB, 1996. Distinct functional domains in the *Cowpea mosaic virus* movement protein. *Journal of virology* 70, 5658-61.

Lewsey MG, González I, Kalinina NO, Palukaitis P, Canto T, Carr JP, 2010. Symptom induction and RNA silencing suppression by the *Cucumber mosaic* virus 2b protein. *Plant signaling & behavior* 5, 705-8.

Lewsey M, Surette M, Robertson FC, Ziebell H, Choi SH, Ryu KH, Canto T, Palukaitis P, Payne T, Walsh JA, 2009. The role of the *Cucumber mosaic virus* 2b protein in viral movement and symptom induction. *Molecular Plant-Microbe Interactions* 22, 642-54.

Lin J, Guo J, Finer J, Dorrance AE, Redinbaugh MG, Qu F, 2014. The *Bean pod mottle virus* RNA2-encoded 58-kilodalton protein P58 is required in cis for RNA2 accumulation. *Journal of virology* 88, 3213-22.

Lisa V, Boccardo G, 1996. Fabaviruses: broad bean wilt and allied viruses. *The plant viruses*. Springer, 229-50.

Lisa V, Dellavalle G, Peressini S, 1986. *Broad bean wilt virus* in fagiolo in Friuli-Venezia Giulia. *Informatore Fitopatologico.* 4, 31-2.

Liu C, Ye L, Lang G, Zhang C, Hong J, Zhou X, 2011. The VP37 protein of *Broad bean wilt virus 2* induces tubule-like structures in both plant and insect cells. *Virus research* 155, 42-7.

Liu C, Meng C, Xie L, Hong J, Zhou X, 2009. Cell-to-cell trafficking, subcellular distribution, and binding to coat protein of Broad bean wilt virus 2 VP37 protein. *Virus research* 143, 86-93.

Liu L, Grainger J, Cañizares MC, Angell SM, Lomonossoff GP, 2004. *Cowpea mosaic virus* RNA-1 acts as an amplicon whose effects can be counteracted by a RNA-2-encoded suppressor of silencing. *Virology* 323, 37-48.

Liu L, Chen X, 2016. RNA quality control as a key to suppressing RNA silencing of endogenous genes in plants. *Molecular plant* 9, 826-36.

Lockhart B, Betzold J, 1982. *Broad bean wilt virus* in begonia in Minnesota. *Plant Diseases* 66.

Lucas WJ, 2006. Plant viral movement proteins: agents for cell-to-cell trafficking of viral genomes. *Virology* 344, 169-84.

Makkouk KM, Kumari SG, Bos L, 1990. *Broad bean wilt virus:* host range, purification, serology, transmission characteristics, and occurrence in faba bean in West Asia and North Africa. *European Journal of Plant Pathology* 96, 291-300.

Makkouk K, El-Muadhidi M, Kumari S, 2001. First record of *Beet western yellows virus*, *Chickpea chlorotic dwarf virus* and *Faba bean necrotic yellows virus* affecting faba bean (*Vicia faba*) crops in Iraq. *Plant Pathology* 50, 793-.

Mali V, Vyanjane N, Ekbote A, 1977. *Petunia ringspot virus* disease-a new record for India. *Current science* 46, 567-8.

Mandadi KK, Scholthof KB, 2013. Plant immune responses against viruses: how does a virus cause disease? *The Plant Cell* 25, 1489-505.

Marrou J, Duteil M, Leclant F, Cadilhac B, Esvan C, 1976. Mise en évidence du virus du flétrissement de la fève Broad bean wilt (BBWV), dans des laitues présentant des symptômes de marbrure. *Ann.Phytopathol* 8, 461.

Martínez C, Coll-Bonfill N, Aramburu J, Pallás V, Aparicio F, Galipienso L, 2014. Two basic (hydrophilic) regions in the movement protein of *Parietaria mottle virus* have RNA binding activity and are required for cell-to-cell transport. *Virus research* 184, 54-61.

Mehle N, Tušek Žnidarič M, Tornos T, Ravnikar M, 2008. First report of *Broad bean wilt virus 1* in Slovenia. *Plant Pathology* 57, 395-.

Melcher U, 2000. The '30K'superfamily of viral movement proteins. *Journal of General Virology* 81, 257-66.

Merai Z, Kerenyi Z, Kertesz S, Magna M, Lakatos L, Silhavy D, 2006. Double-stranded RNA binding may be a general plant RNA viral strategy to suppress RNA silencing. *Journal of virology* 80, 5747-56.

Molnar A, Melnyk CW, Bassett A, Hardcastle TJ, Dunn R, Baulcombe DC, 2010. Small silencing RNAs in plants are mobile and direct epigenetic modification in recipient cells. *Science (New York, N.Y.)* 328, 872-5.

Moury B, Cardin L, Onesto J, Candresse T, Poupet A, 2001. Survey of *Prunus necrotic ringspot virus* in Rose and Its Variability in Rose and Prunus spp. *Phytopathology* 91, 84-91.

Moya A, Holmes EC, González-Candelas F, 2004. The population genetics and evolutionary epidemiology of RNA viruses. *Nature Reviews Microbiology* 2, 279-88.

Murphy FA, Fauquet CM, Bishop DH, Ghabrial SA, Jarvis A, Martelli GP, Mayo MA, Summers MD, 2012. *Virus taxonomy: classification and nomenclature of viruses. Springer Science & Business Media.*

Nagyová A, Subr Z, 2007. Infectious full-length clones of plant viruses and their use for construction of viral vectors. *Acta virologica* 51, 223.

Nault LR, 1997. Arthropod transmission of plant viruses: a new synthesis. *Annals of the Entomological Society of America* 90, 521-41.

Ng Jck, Perry Kl, 2004. Transmission of plant viruses by aphid vectors. *Molecular Plant Pathology* 5, 505-11.

Ng JC, Falk BW, 2006. Virus-vector interactions mediating nonpersistent and semipersistent transmission of plant viruses. *Annu. Rev. Phytopathol.* 44, 183-212.

Pacheco DA, Rezende JAM, Piedade, Sônia Maria de Stefano, 2003. Biomass, virus concentration, and symptomatology of cucurbits infected by mild and severe strains of Papaya ringspot virus. *Scientia Agricola* 60, 691-8.

Pagán I, Alonso-Blanco C, García-Arenal F, 2007. The relationship of within-host multiplication and virulence in a plant-virus system. *PLoS One* 2, e786.

Panno S, Ferriol I, Rangel EA, Olmos A, Han C, Martinelli F, Rubio L, Davino S, 2014. Detection and identification of Fabavirus species by one-step RT-PCR and multiplex RT-PCR. *Journal of virological methods* 197, 77-82.

Peyret H, Lomonossoff GP, 2015. When plant virology met Agrobacterium: the rise of the deconstructed clones. *Plant biotechnology journal* 13, 1121-35.

Popp J, Hantos K, 2011. The impact of crop protection on agricultural production. *Studies in Agricultural Economics* 113.

Power AG, 2000. Insect transmission of plant viruses: a constraint on virus variability. *Current opinion in plant biology* 3, 336-40.

Powers JG, Sit TL, Qu F, Morris TJ, Kim K, Lommel SA, 2008. A versatile assay for the identification of RNA silencing suppressors based on complementation of viral movement. *Molecular Plant-Microbe Interactions* 21, 879-90.

Prins M, Laimer M, Noris E, SCHUBERT J, Wassenegger M, Tepfer M, 2008. Strategies for antiviral resistance in transgenic plants. *Molecular plant pathology* 9, 73-83.

Provvidenti R, 1983. Reaction of bean cultivars to *Broad bean wilt virus*. *Annual Report of the Bean Improvement Cooperative*.

Provvidenti R, Robinson R, Shail J, 1984. Incidence of *Broad bean wilt virus* in lettuce in New York State and sources of resistance *HortScience* 19.

Qi Y, Zhou X, Huang X, Li G, 2002. In vivo accumulation of *Broad bean wilt virus 2* VP37 protein and its ability to bind single-stranded nucleic acid. *Archives of Virology* 147, 917-28.

Renovell Á, Vives MC, Ruiz-Ruiz S, Navarro L, Moreno P, Guerri J, 2012. The *Citrus leaf blotch virus* movement protein acts as silencing suppressor. *Virus genes* 44, 131-40.

Rist D, Lorbeer J, 1989. Occurrence and overwintering of *Cucumber mosaic virus* and *Broad bean wilt virus* in weeds growing near commercial lettuce fields in New York. *Phytopathology* 79, 65-9.

Roossinck MJ, 1997. Mechanisms of plant virus evolution. *Annual Review of Phytopathology* 35, 191-209.

Rosciglione B, Cannizzaro G, 1977. Reperimento del virus dell'avvizzimento della Fava nell'isola di Linosa/Occurrence of *Broad bean wilt virus* in Linosa island. *Phytopathologia Mediterranea*, 140-2.

Rubio L, Luis-Arteaga M, Cambra M, Serra J, Moreno P, Guerri J, 2002. First Report of *Broad bean wilt virus 1* in Spain. *Plant Disease* 86, 698-.

Rubio-Huertos M, 1968. Further studies on ultrastructure of plants infected with *Petunia ringspot virus*. *Protoplasma* 65, 465-76.

Ruiz-Ruiz S, Soler N, Sánchez-Navarro J, Fagoaga C, López C, Navarro L, Moreno P, Peña L, Flores R, 2013. *Citrus tristeza virus* p23: determinants for nucleolar localization and their influence on suppression of RNA silencing and pathogenesis. *Molecular Plant-Microbe Interactions* 26, 306-18.

Sablowski RW, Baulcombe DC, Bevan M, 1995. Expression of a flower-specific Myb protein in leaf cells using a viral vector causes ectopic activation of a target promoter. *Proceedings of the National Academy of Sciences of the United States of America* 92, 6901-5.

Sahambi H, Milne R, Cook SM, Gibbs A, Woods R, 1973. Broad bean wilt and nasturtium ringspot viruses are related. *Journal of Phytopathology* 76, 158-65.

Sanfaçon H, 2015. Secoviridae: A Family of Plant Picorna-Like Viruses with Monopartite or Bipartite Genomes. *eLS*.

Scholthof HB, Scholthof KB, Jackson AO, 1995. Identification of *Tomato bushy stunt virus* host-specific symptom determinants by expression of individual genes from a potato virus X vector. *The Plant Cell* 7, 1157-72.

Schroeder W, Provvidenti R, 1970. A destructive blight of Spinacea olerácea incited by a strain of the *Broad Bean wilt virus*. *Phytopathology* 60, 1405-6.

Scott S, Barnett O, 1984. Some properties of an isolate of *Broad bean wilt virus* from dogwood (*Cornus florida*). *Plant Disease* 68, 983-5.

Senda M, Masuta C, Ohnishi S, Goto K, Kasai A, Sano T, Hong JS, MacFarlane S, 2004. Patterning of virus-infected Glycine max seed coat is associated with suppression of endogenous silencing of chalcone synthase genes. *The Plant Cell* 16, 807-18.

Seo J, Kwak H, Choi B, Han S, Kim M, Choi H, 2017. Movement protein of B*road bean wilt virus 2* serves as a determinant of symptom severity in pepper. *Virus research*.

Shukla DD, Teakle DS, Gough KH, 1980. Periwinkle, a latent host for broad bean wilt and cucumber mosaic viruses in Australia. *Plant Disease* 64, 802-3.

Silhavy D, Burgyán J, 2004. Effects and side-effects of viral RNA silencing suppressors on short RNAs. *Trends in plant science* 9, 76-83.

Silhavy D, Molnar A, Lucioli A, Szittya G, Hornyik C, Tavazza M, Burgyan J, 2002. A viral protein suppresses RNA silencing and binds silencing-generated, 21- to 25-nucleotide double-stranded RNAs. *The EMBO journal* 21, 3070-80.

Simón-Mateo C, García JA, 2011. Antiviral strategies in plants based on RNA silencing. *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms*.

Stewart LR, Jarugula S, Zhao Y, Qu F, Marty D, 2017. Identification of a maize chlorotic dwarf virus silencing suppressor protein. *Virology* 504, 88-95.

Stubbs LL, 1947. A destructive vascular wilt virus disease of broad bean (Vicia faba L.) in Victoria. *Journal of the Department of Agriculture Victoria* 46, 323-32.

Tahir MN, Mansoor S, 2011. βC1 of chili leaf curl betasatellite is a pathogenicity determinant. *Virology journal* 8, 509.

Tasaki K, Atsumi G, Nishihara M, Sekine K, 2016. Development of a *Broad bean wilt virus 2*-based expression vector for gentian. *Scientia Horticulturae* 201, 279-86.

Tatineni S, Robertson CJ, Garnsey SM, Dawson WO, 2011. A plant virus evolved by acquiring multiple nonconserved genes to extend its host range. *Proceedings of the National Academy of Sciences* 108, 17366-71.

Turina M, Ricker M, Lenzi R, Masenga V, 2007. A severe disease of tomato in the Culiacan area (Sinaloa, Mexico) is caused by a new picorna-like viral species. *Plant Disease* 91, 932-41.

Uyemoto J, Provvidenti R, 1974. Isolation and identification of two serotypes of B*road bean wilt virus*. *Phytopathology* 64, 1547-8.

Van Breusegem F, Dat JF, 2006. Reactive oxygen species in plant cell death. *Plant Physiology* 141, 384-90.

Van den Bosch F, Jeger MJ, Gilligan CA, 2007. Disease control and its selection for damaging plant virus strains in vegetatively propagated staple food crops; a theoretical assessment. *Proceedings.Biological sciences* 274, 11-8.

Van Lent J, Wellink J, Goldbach R, 1990. Evidence for the involvement of the 58K and 48K proteins in the intercellular movement of *Cowpea mosaic virus*. *Journal of General Virology* 71, 219-23.

Vigne E, Gottula J, Schmitt-Keichinger C, Komar V, Ackerer L, Belval L, Rakotomalala L, Lemaire O, Ritzenthaler C, Fuchs M, 2013. A strain-specific segment of the RNA-dependent RNA polymerase of *Grapevine fanleaf virus* determines symptoms in Nicotiana species. *Journal of General Virology* 94, 2803-13.

Wang M, Masuta C, Smith NA, Shimura H, 2012. RNA silencing and plant viral diseases. *Molecular Plant-Microbe Interactions* 25, 1275-85.

Weidemann H, Lesemann D, Paul H, Koenig R, 1975. Das *Broad Bean Wilt-Virus* als Ursache für eine neue Vergilbungskrankheit des Spinats in Deutschland. *Journal of Phytopathology* 84, 215-21.

Whitfield AE, Falk BW, Rotenberg D, 2015. Insect vector-mediated transmission of plant viruses. *Virology* 479, 278-89.

Wieczorek P, Obrępalska-Stęplowska A, 2016a. The N-terminal fragment of the *Tomato torrado virus* RNA1-encoded polyprotein induces a hypersensitive response (HR)-like reaction in *Nicotiana benthamiana*. *Archives of Virology* 161, 1849-58.

Wieczorek P, Obrępalska-Stęplowska A, 2016b. A single amino acid substitution in movement protein of *Tomato torrado virus* influences ToTV infectivity in *Solanum lycopersicum*. *Virus research* 213, 32-6.

Xie L, Shang W, Liu C, Zhang Q, Sunter G, Hong J, Zhou X, 2016. Mutual association of *Broad bean wilt virus 2* VP37-derived tubules and plasmodesmata obtained from cytological observation. *Scientific reports* 6, 21552.

Xu P, Roossinck MJ, 2000. *Cucumber mosaic virus D* satellite RNA-induced programmed cell death in tomato. *The Plant Cell* 12, 1079-92.

Yaegashi H, Yamatsuta T, Takahashi T, Li C, Isogai M, Kobori T, Ohki S, Yoshikawa N, 2007. Characterization of virus-induced gene silencing in tobacco plants infected with *Apple latent spherical virus*. *Archives of Virology* 152, 1839-49.

Yang G, Qiu BS, Liu XG, Li Y, Wang XF, 2002. Nonsense mutations of replicase and movement protein genes contribute to the attenuation of an avirulent *Tomato mosaic virus*. *Virus research* 87, 119-28.

Yankulova M, Kaitazova P, 1979. *Broad bean wilt virus*-a new virus on pepper in Bulgaria. *Gradinarska i Lozarska Nauka* 16, 48-57.

Zamore PD, 2004. Plant RNAi: How aViral Silencing Suppressor Inactivates siRNA. *Current Biology* 14, R198-200.

Zhang X, Yuan YR, Pei Y, Lin SS, Tuschl T, Patel DJ, Chua NH, 2006. *Cucumber mosaic virus*-encoded 2b suppressor inhibits *Arabidopsis* Argonaute1 cleavage activity to counter plant defense. *Genes & development* 20, 3255-68.

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