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RNA2-encoded VP37 protein of *Broad bean wilt virus 1* is a determinant of pathogenicity, host susceptibility, and a suppressor of post-transcriptional gene silencing

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Abstract

Broad bean wilt virus 1 (BBWV-1, genus *Fabavirus*, family *Secoviridae*) is a bipartite, single-stranded positive-sense RNA virus infecting many horticultural and ornamental crops worldwide. RNA1 encodes proteins involved in viral replication whereas RNA2 encodes two coat proteins (the large and small coat proteins) and two putative movement proteins (MPs) of different sizes with overlapping C-terminal regions. In this work, we determined the role played by the small putative BBWV-1 MP (VP37) on virus pathogenicity, host specificity, and suppression of post-transcriptional gene silencing (PTGS). We engineered a BBWV-1 35S-driven full-length cDNA infectious clone corresponding to BBWV-1 RNA1 and RNA2 (pBBWV1-Wt) and generated a mutant knocking out VP37 (pBBWV1-G492C). Agroinfiltration assays showed that pBBWV1-Wt, as the original BBWV-1 isolate, infected broad bean, tomato, pepper, and *Nicotiana benthamiana*, whereas pBBWV1-G492C did not infect pepper and tomato systemically. Also, pBBWV1-G492C induced milder symptoms in broad bean and *N. benthamiana* than pBBWV1-Wt. Differential retrotranscription and amplification of the (+) and (−) strands showed that pBBWV1-G492C replicated in the agroinfiltrated leaves of pepper but not in tomato. All this suggests that VP37 is a determinant of pathogenicity and host specificity. Transient expression of VP37 through a potato virus X (PVX) vector enhanced PVX symptoms and induced systemic necrosis associated with programmed cell death in *N. benthamiana* plants. Finally, VP37 was identified as a viral suppressor of RNA silencing by transient expression in *N. benthamiana* 16c plants and movement complementation of a viral construct based on turnip crinkle virus (pTCV-GFP).

KEYWORDS

BBWV-1, determinant of pathogenicity, *Fabavirus*, infectious clone, *Secoviridae*, VSR

1 | INTRODUCTION

Broad bean wilt virus 1 (BBWV-1) is the member type of the genus *Fabavirus* in the family *Secoviridae*, which contains 81 virus species (Thompson *et al*., 2017). Members of the family *Secoviridae* have monopartite or bipartite single-stranded RNA genomes of positive polarity (ssRNA+) encoding polyproteins further processed into functional polypeptides (Sanfaçon, 2015). The genus *Fabavirus* contains seven viral species: *Broad bean wilt virus 1* (BBWV-1), *Broad bean wilt virus 2* (BBWV-2), *Cucurbit mild mosaic virus* (CMMV), *Gentian mosaic virus* (GeMV), *Lamium mild mosaic virus* (LMMV), *Grapevine fabavirus* (GFabV), and *Prunus virus F* (PrVF) (Kobayashi *et al*., 2003; Kobayashi *et al*., 2005; Dong *et al*., 2012; Rangel *et al*., 2013; Al Rwahnih *et al*., 2016; Villamor *et al*., 2017). Also, new putative fabaviruses have been reported such as *Cherry virus F* (CVF) and *Peach leaf pitting-associated virus* (PLPaV) (He *et al*., 2017; Koloniuk *et al*., 2018). BBWV-1 is distributed worldwide and infects important agronomic crops such as broad bean (*Vicia faba*), pepper (*Capsicum annuum*), tomato (*Solanum lycopersicum*), and spinach (*Spinacia oleracea*) as well as ornamental crops such as petunia (*Petunia* sp.) and narcissus (*Narcissus* sp.) (King *et al*., 2011; Ferriol *et al*., 2013). BBWV-1 has a bipartite genome (RNA1 and RNA2) that is encapsidated into virions with icosahedral morphology (Sanfaçon, 2015). RNA1 (c.5.8 kb) contains an open reading frame (ORF) encoding a polyprotein that 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 (c. 3.4 kb), like other viruses belonging to the genera *Fabavirus* and *Comovirus* in the family *Secoviridae*, has two in-frame successive translation initiation codons encoding alternative polyproteins differing only in their N-terminal extension length (Chen and Bruening, 1992; Lekkerkerker *et al*., 1996; Qi *et al*., 2002; Lin *et al*., 2014; Xie *et al*., 2016). The two RNA2-encoded polyproteins render by proteolytic cleavage the large and small coat proteins (LCPs and SCPs, respectively) and two putative movement proteins (MPs) with overlapping C-terminal regions and which only differ in size, c.47.2 (VP 47) and c.37 kDa (VP37). The synthesis in vivo of the two putative MPs of different size with overlapping C-terminal regions has not been demonstrated yet for BBWV-1 whereas it was reported for other members of the family *Secoviridae* such as the fabavirus BBWV-2, and the comoviruses *Bean pod mottle virus* (BPMV) and *Cowpea mosaic virus* (CPMV) (Chen and Bruening, 1992, Lekkerkerker *et al*., 1996; Qi *et al*., 2002; Lin *et al*., 2014). For these viruses, both MPs were involved in virus cell-to-cell movement (van Lent *et al*., 1990; Liu *et al*., 2009; Liu *et al*., 2011; Xie *et al*., 2016).

Interactions among BBWV-1 proteins and host factors have not been studied and there are no data about the involvement of viral products on the development of plant symptoms (determinants of pathogenicity). Different viral products have been associated with symptom development in some members of the family *Secoviridae* such as the two MPs of the fabavirus BBWV-2, the POL and 2A homing protein of the nepovirus *Grapevine fanleaf virus* (GFLV), the HEL of the comovirus BPMV, and the VP26 coat protein of the torradovirus *Tomato torrado virus* (ToTV) (Gu and Ghabrial, 2005; Vigne *et al*., 2013; Kong *et al*., 2014; Wieczorek and Obrępalska-Stęplowska, 2016a; Seo *et al*., 2017; Martin *et al*., 2018; Wieczorek *et al*., 2019).

Determinants of pathogenicity can also be suppressors of post-transcriptional gene silencing (PTGS) (Amin *et al*., 2011; Burgyán and Havelda, 2011). PTGS is an RNA sequence-specific degradation mechanism triggered by double-stranded RNA (dsRNA) or highly structured ssRNA molecules that are processed by the cellular machinery into short RNA fragments of 20–25 nucleotides (siRNAs) (Csorba *et al*., 2009). PTGS is a mechanism of gene regulation and defence against pathogens. Viral dsRNA replicative intermediates trigger PTGS, producing degradation of viral RNA and preventing a systemic infection. To counteract PTGS, viruses encode proteins that can suppress it (viral suppressors of RNA silencing, VSRs). Some examples of well-characterized VSRs are p19 of *Tomato bushy stunt virus* (TBSV), HC-Pro of several potyviruses, and p25 of *Potato virus X* (PVX) (Scholthof *et al*., 1995; Anandalakshmi *et al*., 1998; Lakatos *et al*., 2004; Chiu *et al*., 2010). In the family *Secoviridae*, different proteins depending on the virus species have been identified as VSRs (Ghoshal and Sanfaçon, 1995).

In the current work, we engineered a 35S promoter-driven cDNA infectious clone of BBWV-1 and a mutant construct knocking out VP37 to study its role on virus pathogenicity and host specificity. We evaluated the implication of BBWV-1 VP37 on plant symptom development by transient expression assays in *Nicotiana benthamiana* plants. Also, we assessed the VSR activity by transient expression in *N. benthamiana* 16c plants and by a movement complementation assay using a turnip crinkle virus construct (pTCV-GFP) (Powers *et al*., 2008).

2 | RESULTS

2.1 | BBWV-1 VP37 is a determinant of pathogenicity and host specificity

To study the role of BBWV-1 RNA2-encoded VP37, 35S-driven infectious cDNA clones of BBWV-1 isolate Ben were engineered and named pBBWV1-R1 and pBBWV1-R2 for RNA1 and RNA2 constructs, respectively (Figure 1a). An RNA2 mutant construct (pBBWV1-R2:G492C) knocking out VP37 was obtained by a single nucleotide substitution in its start codon at position 492 of pBBWV1-R2 (AUG > AUC, Met > Ile) (Figure 1b). Two infectious clones pBBWV1-Wt (pBBWV1-R1 + pBBWV1-R2) and pBBWV1-G492C (pBBWV1-R1 + pBBWV1-R2:G492C) were agroinfiltrated into *N. benthamiana*, broad bean, pepper, and tomato (30 plants per host). The systemic infection of BBWV-1 was assessed by reverse transcription (RT)-PCR from upper nonagroinfiltrated leaves. As control, the same number of plants for each species was mechanically inoculated with BBWV-1 isolate Ben or mock inoculated. Differences in host range and virus-induced symptoms were observed between pBBWV1-Wt and pBBWV1-G492C but not between pBBWV1-Wt and BBWV-1 isolate Ben (Figure 2a and Table 1). All *N. benthamiana*, broad bean, and tomato plants and 16 or 18 out of 30 pepper plants agroinfiltrated with either pBBWV1-Wt or BBWV-1 isolate Ben, respectively, were infected systemically, showing identical symptoms

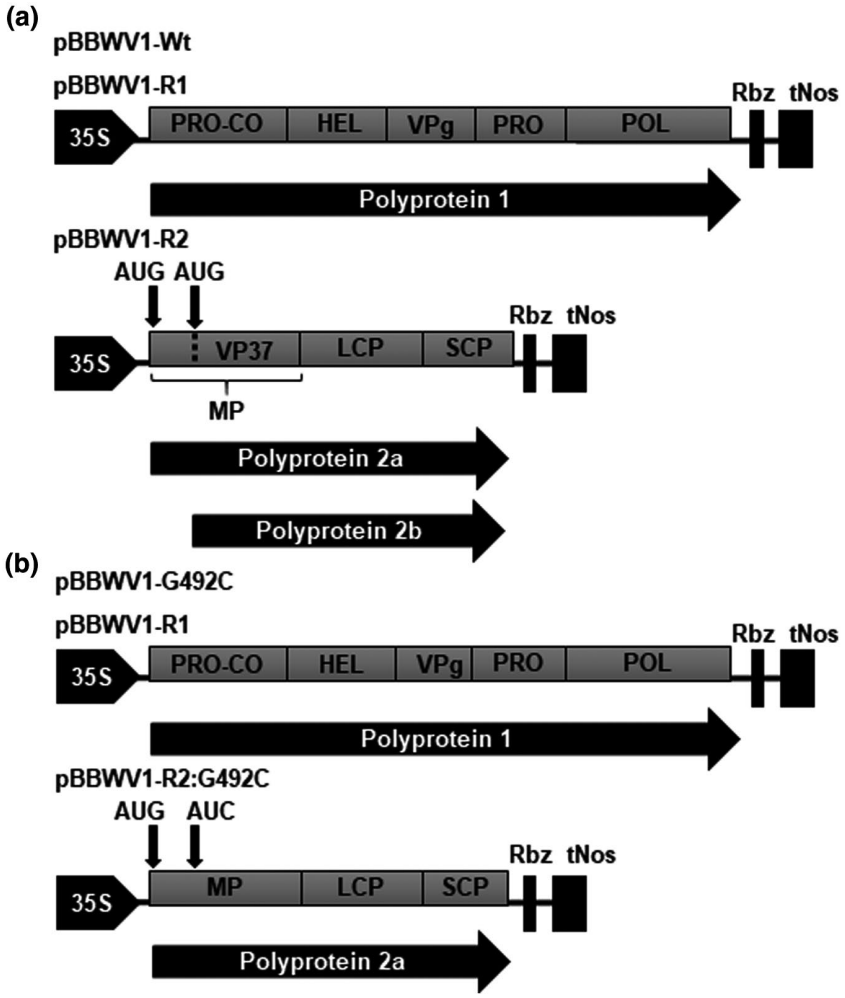

FIGURE 1 Schematic representation of cDNA constructs of broad bean wilt virus 1 (BBWV-1). (a) Combination of RNA1 (pBBWV1-R1) and RNA2 (pBBWV1-R2) cDNA constructs of BBWV-1 based on nucleotide sequence of isolate Ben used for the wild-type infectious clone (pBBWV1-Wt). The 35S promoter of cauliflower mosaic virus is upstream of the first nucleotide of each BBWV-1 cDNA. The hepatitis delta virus ribozyme (Rbz) and the nopaline synthase terminator (t-Nos) are engineered in tandem after a polyA tail. (b) Combination of RNA1 (pBBWV1-R1) and RNA2 (pBBWV1-R2:G492C) cDNA constructs with a single point substitution in the VP37 start codon at nucleotide position 492 (AUG > AUC, Met > Ile) used to obtain a BBWV-1 infectious clone not expressing VP37 (pBBWV1-G492C)

at 21 days postagroinfiltration (dpa). Symptoms consisted of stunting and severe mosaic in *N. benthamiana*, severe mosaic in broad bean, and slight mosaic in pepper plants. Tomato plants remained symptomless even though all plants were infected systemically by pBBWV1-Wt or BBWV-1 isolate Ben. All *N. benthamiana* and broad bean plants agroinfiltrated with pBBWV1-G492C were infected systemically, but showed milder symptoms (slight mosaic in *N. benthamiana* and a few chlorotic spots in broad bean). All mock-inoculated plants remained symptomless. None of the tomato and pepper plants agroinfiltrated with pBBWV1-G492C showed systemic infection (detected by RT-PCR) or symptoms.

Northern blot analysis of total RNAs obtained from upper non-infiltrated leaves at 12 dpa showed two RNAs of c.5.8 and 3.4 kb corresponding to genomic BBWV-1 RNA1 and RNA2, respectively, in the plants that were infected systemically by the virus (Figure 2b). These two RNAs were not observed in mock agroinfiltrated plants used as controls and the intensity of the hybridization signal was similar in northern blots of *N. benthamiana* and broad bean plants agroinfiltrated with either pBBWV1-Wt or pBBWV1-G492C. Also, quantitative real-time RT-PCR (RT-qPCR) showed no significant differences ($p > .05$) in the accumulation of RNA1 and RNA2 in *N. benthamiana* and broad bean plants infected with pBBWV1-Wt or pBBWV1-G492C (Figure S1).

Transmission electron microscopy (TEM) showed nonenveloped viral particles of 25–30 nm in diameter and icosahedral morphology, identical to those reported previously for BBWV-1 (Ikegami and Sharma, 2011), in plants agroinfiltrated with pBBWV1-Wt or pBBWV1-G492C (Figure 3a). Also, electrophoresis of RNA purified from virions revealed the presence of two RNAs (c.5.8 kb and c.3.4 kb) corresponding to BBWV-1 RNA1 and RNA2, respectively (Figure 3b). These results indicate that elimination of the start codon by the single mutation G492C in VP37, abolishing the synthesis of this protein, did not affect the virion formation. The presence of the mutation G492C in the viral progeny of all *N. benthamiana* or broad bean plants agroinfiltrated with pBBWV1-G492C was confirmed by sequencing an RT-PCR product of 388 bp (positions 220 to 607, GenBank KT988974) surrounding the mutation site.

To assess if BBWV1-G492C could replicate in these plants, pBBWV1-Wt and pBBWV1-G492C were agroinfiltrated into *N. benthamiana*, broad bean, pepper, and tomato (seven or eight plants per host). Both (+) and (−) RNA strands were quantified by RT-qPCR in the agroinfiltrated leaves at 6 dpa before the tissues became necrosed (Table 2). Both (+) and (−) RNA strands were detected in all plants of the four host species agroinfiltrated with pBBWV1-Wt whereas only in *N. benthamiana*, broad bean, and pepper plants agroinfiltrated with pBBWV1-G492C. In tomato plants agronfiltrated with pBBWV1-G492C,

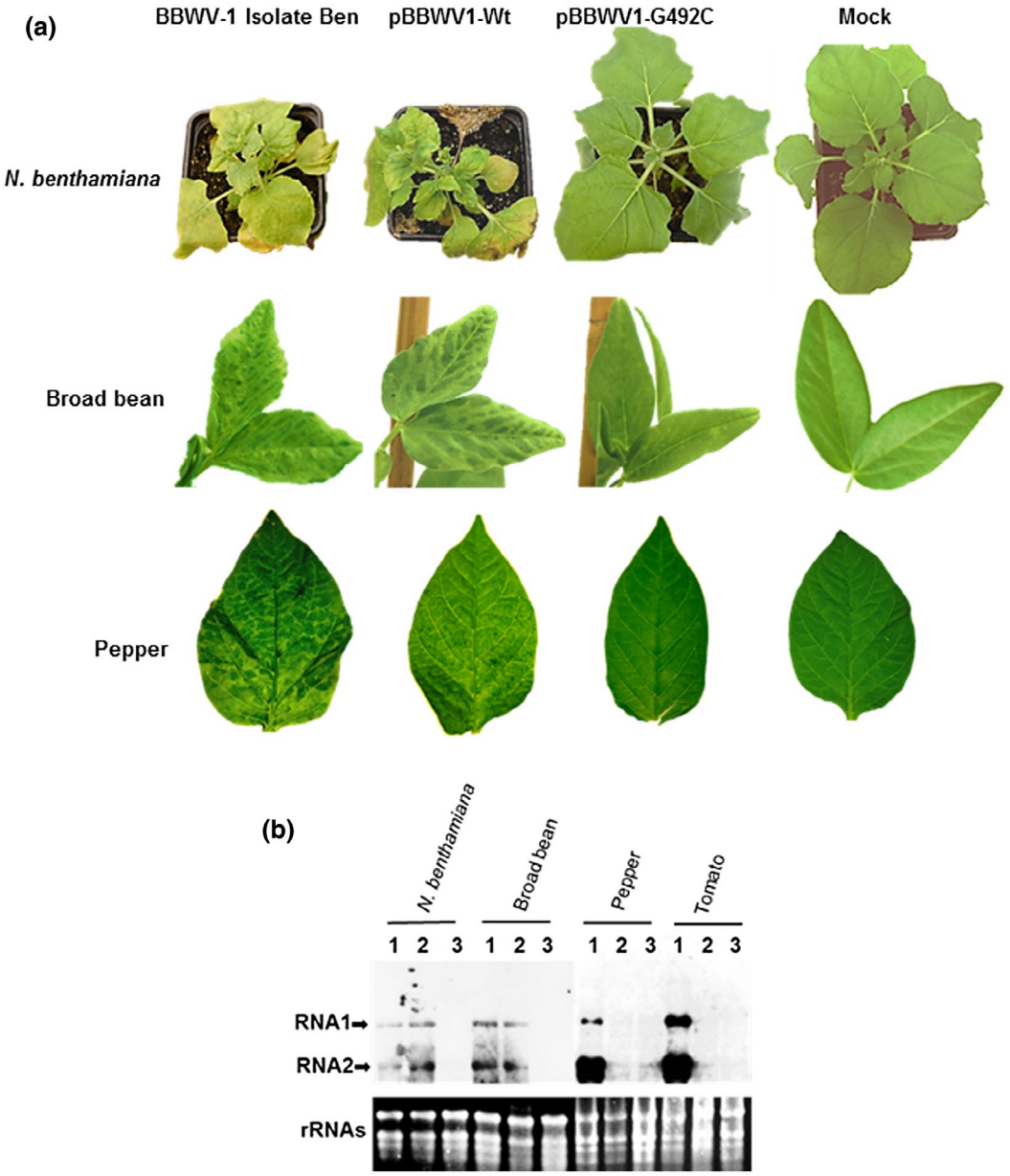

FIGURE 2 Biological characterization of pBBWV1-Wt and pBBWV1-G492C cDNA infectious clones in different herbaceous host species. (a) Symptoms induced by pBBWV1-Wt and pBBWV1-G492C in *Nicotiana benthamiana*, broad bean, and pepper plants in comparison with those induced by BBWV-1 isolate Ben at 21 days postagroinfiltration (dpa). (b) Northern blot analysis of plants agroinfiltrated with pBBWV1-Wt (lane 1), pBBWV1-G492C (lane 2), and mock-infiltrated used as negative control (lane 3) (at the top). Ribosomal RNAs (rRNAs) used as loading controls are shown at the bottom. Each lane corresponds to a pool of three total RNA samples

TABLE 1 Systemic infection of pBBWV1-Wt and pBBWV1-G492C cDNA infectious clones

	BBWV-1 isolate Ben	pBBWV-Wt	pBBWV1-G492C	Mock
Nicotiana benthamiana	30/30	30/30	30/30	0/30
Broad bean	30/30	30/30	30/30	0/30
Pepper	18/30	16/30	0/30	0/30
Tomato	30/30	30/30	0/30	0/30

Note: BBWV-1 isolate Ben and mock were used as infection controls. Values are numbers of infected plants/no. agroinfiltrated plants.

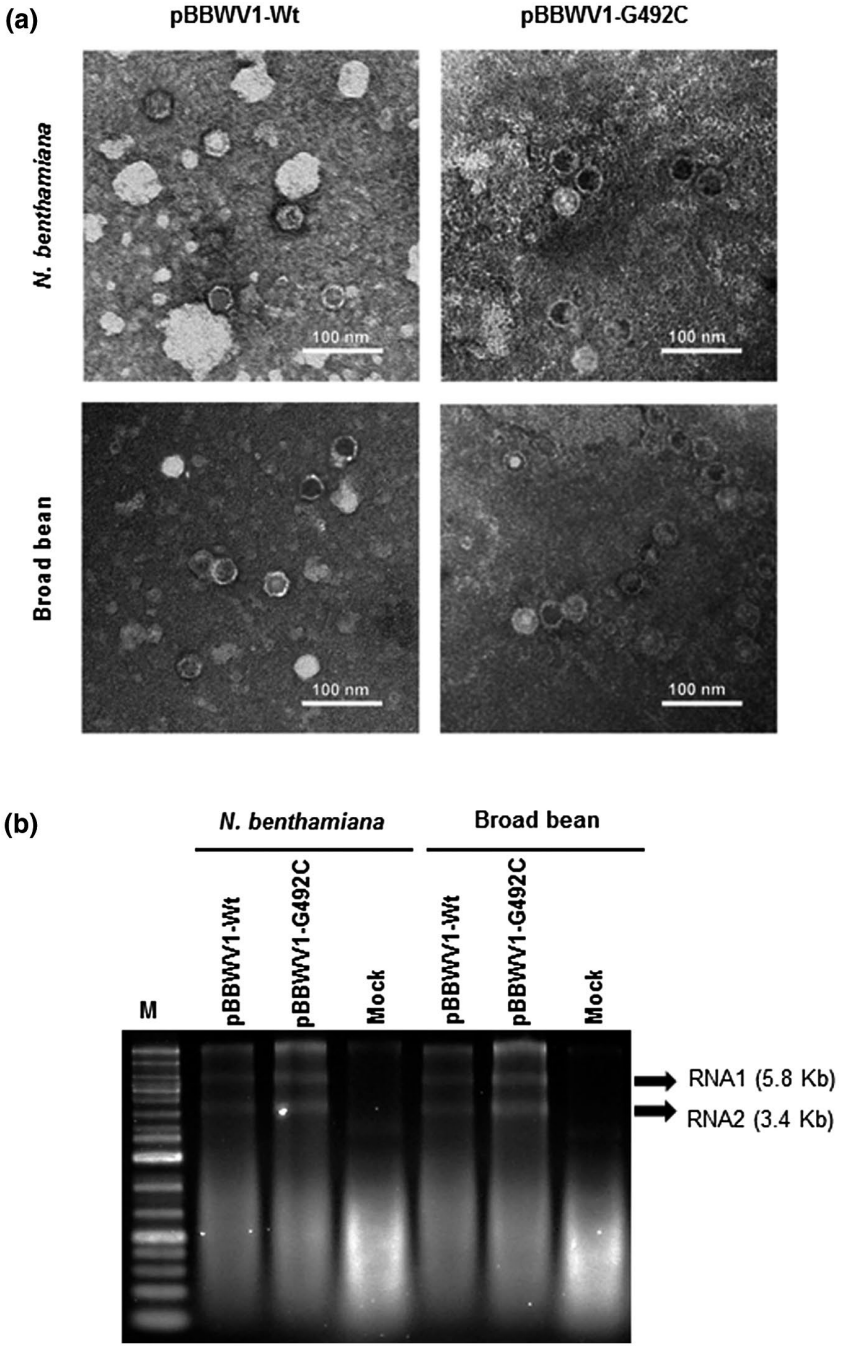

FIGURE 3 Purification of viral particles from *Nicotiana benthamiana* and broad bean plants agroinfiltrated with *Agrobacterium tumefaciens* cell suspensions containing pBBWV1-Wt and pBBWV1-G492C infectious cDNA clones. (a) Transmission electron micrographs showing nonenveloped viral particles of 25–30 nm with icosahedral morphology. The bar represents 100 nm. (b) Agarose gel analysis of genomic RNAs obtained from viral particles purified from *N. benthamiana* and broad bean plants agroinfiltrated with pBBWV1-Wt and pBBWV1-G492C. Mock-infiltrated plants were used as negative controls. M corresponds to 1 kb Plus DNA Ladder (Invitrogen). Each lane corresponds to a pool of three total RNA samples

(+) but not (−) RNA strands were detected. pBBWV1-G492C replicated 100 and 1,000 times less than pBBWV1-Wt in pepper and broad bean, whereas both cDNA infectious clones replicated similarly in *N. benthamiana*. In tomato, the replication of pBBWV1-Wt was also low.

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

The role of BBWV-1 VP37 as a determinant of pathogenicity was evaluated by transient expression of this protein in *N. benthamiana* plants using the pPVX202 viral vector (pPVX-VP37) (Sablowski *et al.*, 1995) and PVX empty viral vector (pPVX-Ø) as control of PVX infection (Figure 4a). Twenty *N. benthamiana* plants were inoculated mechanically with either pPVX-VP37 or pPVX-Ø, or mock-inoculated. All plants inoculated with pPVX-VP37 or pPVX-Ø became infected, but they showed different symptoms depending on the cDNA construct. *N. benthamiana* plants inoculated with pPVX-VP37 showed stunting, severe mosaic, and small necrotic lesions in upper noninoculated leaves at 7 days postinoculation (dpi) and seven plants died at 21 dpi. However, plants inoculated with pPVX-Ø showed only the typical PVX-induced symptoms consisting of mild mosaic, which disappeared at 21 dpi (Figure 4b). Mock-inoculated plants were symptomless. PVX genomic RNA with or without VP37 nucleotide

TABLE 2 Quantitative reverse transcription PCR amplification of both (+) BBWV-1 genomic and (–) replication-associated RNA1 strands of total RNA samples obtained from agroinfiltrated leaf areas of plants from different host species agroinfiltrated with pBBWV1-Wt or pBBWV1-G492C

Host	Type	RNA strand polarity	Copies[a]
Nicotiana benthamiana	pBBWV1-G492C	+	1.85×10^7
		–	6.59×10^5
	pBBWV1-Wt	+	1.59×10^7
		–	1.06×10^6
Broad bean	pBBWV1-G492C	+	2.19×10^8
		–	4.46×10^6
	pBBWV1-Wt	+	2.67×10^{11}
		–	4.15×10^7
Pepper	pBBWV1-G492C	+	8.52×10^5
		–	5.54×10^4
	pBBWV1-Wt	+	2.78×10^7
		–	5.00×10^5
Tomato	pBBWV1-G492C	+	8.41×10^4
		–	0
	pBBWV1-Wt	+	9.36×10^5
		–	1.43×10^4

[a]Number of BBWV-1 RNA1 copies/ng total RNA.

sequence was identified by differences of electrophoretic mobility in northern blot assays (Figure 4c). In addition, amplification products of expected size were obtained by RT-PCR with BBWV-1 VP37-specific primers from plants inoculated with pPVX-VP37 but not from those inoculated with pPVX-Ø (data not shown).

The presence of necrotic lesions in upper noninoculated leaves of *N. benthamiana* plants inoculated with pPVX-VP37 suggested the induction of systemic necrosis associated with programmed cell death (PCD). To confirm PCD, the accumulation of the superoxide ion (O_2^-), a reactive oxygen species (ROS) overproduced in PCD, was tested with the nitroblue tetrazolium (NBT) staining method (Grellet Bournonville and Díaz-Ricci, 2011) and the presence of dead cells by ROS toxicity was tested by trypan blue staining (Garcia-Marcos *et al*., 2013). Leaves with symptoms from *N. benthamiana* plants collected at 7 dpi and stained with NBT showed superoxide ion (O_2^-) dark-blue-coloured deposits and groups of dead cells in pPVX-VP37 inoculated plants but not in those inoculated with pPVX-Ø (Figure 5).

2.3 | BBWV-1 VP37 is an RNA silencing suppressor

The ability of BBWV-1 VP37 to suppress PTGS was studied by two different assays: (a) VP37 protein transient expression in transgenic *N. benthamiana* 16c plants, which constitutively express green fluorescent protein (GFP), and (b) movement complementation of pTCV-GFP construct (Roth *et al*., 2004; Powers *et al*., 2008).

Transient expression of foreign GFP in *N. benthamiana* 16c plants triggers PTGS, inducing the GFP mRNA degradation and dimming the green fluorescence. When foreign GFP is coexpressed with a PTGS suppressor, plant GFP expression and green fluorescence are recovered. BBWV-1 nucleotide sequence encoding for VP37 was amplified and cloned into a pCambia binary plasmid rendering p35S-VP37. *Agrobacterium tumefaciens* COR308 cells harbouring the cDNA construct or a plasmid expressing GFP named p35S-GFP were co-infiltrated into *N. benthamiana* 16c plants. The binary plasmid p35S-p19 expressing the p19 of TBSV and the empty binary vector (p35S-Ø) were used as positive and negative controls of PTGS suppression, respectively (Kong *et al*., 2014). Agroinfiltrated leaves were observed under a handheld UV lamp at 3 dpa (Figure 6a): all leaves co-agroinfiltrated with p35S-VP37/p35S-GFP showed green fluorescence in the agroinfiltrated areas 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 that was much more intense than in leaves agroinfiltrated with p35S-VP37/p35S-GFP. Moreover, the green fluorescence on the leaves co-agroinfiltrated with p35S-VP37/p35S-GFP decreased until disappearing at 6 dpa, whereas it remained constant on leaves co-agroinfiltrated with the positive control p35S-p19/p35S-GFP. Consistently, northern blots showed more GFP mRNA in leaves co-agroinfiltrated with p35S-VP37/p35S-GFP than in leaves co-agroinfiltrated with the negative control p35S-Ø/p35S-GFP but less than in leaves co-agroinfiltrated with positive control p35S-p19/p35S-GFP (Figure 6b). Also, siRNA derived from mRNA accumulated less in leaves co-agroinfiltrated with p35S-VP37/p35S-GFP than in leaves co-agroinfiltrated with the negative control p35S-Ø/p35S-GFP but more than in leaves co-agroinfiltrated with the positive control p35S-p19/p35S-GFP (Figure 6b).

To confirm VSR activity of VP37, *N. benthamiana* plants were agroinfiltrated with p35S-VP37, p35S-Ø, or p35S-p19. One day later, TCV-GFP RNA transcripts obtained from pTCV-GFP viral vector were mechanically inoculated on previously agroinfiltrated leaves. In the pTCV-GFP, the viral CP has been replaced by GFP, losing the

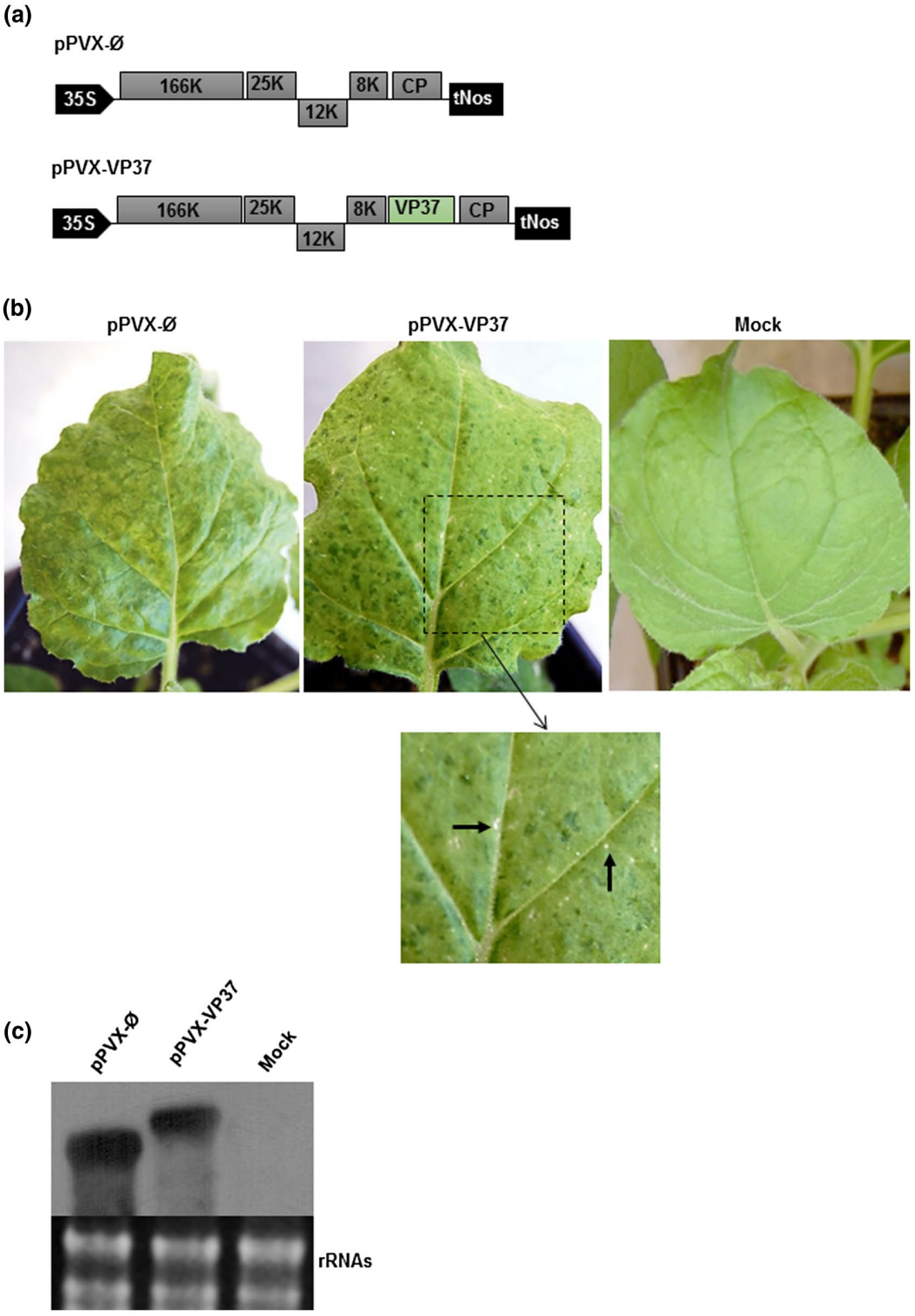

FIGURE 4 Transient expression of BBWV-1 VP37 in *Nicotiana benthamiana* plants using the pPVX202 viral vector. (a) Schematic representation of pPVX-derived cDNA constructs. (b) Symptoms induced by PVX-derived constructs in upper noninoculated leaves of *N. benthamiana* plants at 7 days postinoculation (dpi): mild mosaic in plants inoculated with pPVX-Ø and severe mosaic and small necrotic lesions (arrows) in those inoculated with pPVX-VP37. Mock-inoculated *N. benthamiana* plants were used as negative controls. (c) Northern blot analysis of upper noninoculated leaves of *N. benthamiana* plants inoculated with pPVX-Ø and pPVX-VP37 using a PVX-specific riboprobe. Ribosomal RNAs (rRNAs) used as loading controls are showed at the bottom. Each lane corresponds to a pool of three total RNA samples

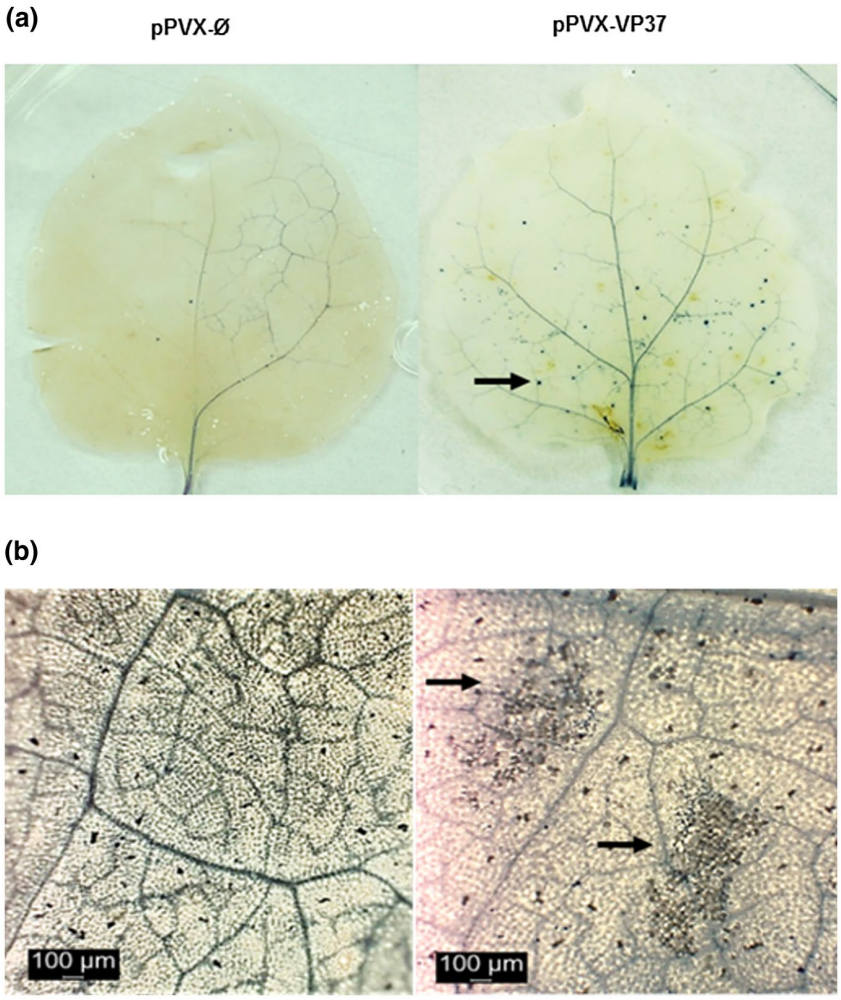

FIGURE 5 Systemic necrosis as consequence of programmed cell dead (PCD) induced by transient expression of BBWV-1 VP37 using the pPVX202 viral vector in upper noninoculated leaves of *Nicotiana benthamiana* plants. (a) Staining of superoxide ion (O_2^-) deposits with nitroblue tetrazolium (NBT) staining method (arrow). (b) Visualization of groups of dead cells using the trypan blue staining method (arrows)

ability to move to adjacent cells. However, TCV-GFP mobility can be recovered by complementation in trans with a PTGS suppressor of a different virus (Powers *et al*., 2008). Observation of inoculated leaves with a fluorescence stereomicroscope at 4 dpa revealed the presence of wide green fluorescence areas in leaves agroinfiltrated with p35S-VP37 or the positive control p35S-p19, which corresponded to TCV-GFP spread from the inoculated single cells. In contrast, leaves agroinfiltrated with the negative control p35S-Ø showed small green fluorescence spots corresponding to inoculated single cells (Figure 6c).

3 | DISCUSSION

Recently light has been shed on some crucial steps of the infectious cycle of several members of the family *Secoviridae* (Fuchs *et al*., 2017). In recent years, detection tools and studies of transmission by aphids, genetic variability, and evolution have been carried out for BBWV-1 (Ferrer *et al*., 2005; Ferriol *et al*., 2011, 2013, 2014, 2015; Panno *et al*., 2014). However, studies of molecular biology and virus–host interactions are lacking for BBWV-1 and other members of the genus *Fabavirus*. We developed 35S promoter-driven infectious cDNA clones of BBWV-1 RNA1 and RNA2 that improved the virus inoculation of our previous T7-driven infectious clones (Ferriol *et al*., 2016), facilitating the study of BBWV-1-encoded proteins in different host species. Here, we studied the role of BBWV-1 VP37 on important viral features such as plant symptom development, host specificity, virus replication, and formation of viral particles.

Agroinfiltration assays of BBWV-1 infectious clones with a mutated version of RNA2 knocking out VP37 (pBBWV1-G492C) showed that this protein is a determinant of pathogenicity, enhancing the severity of BBWV-1 induced symptoms in *N. benthamiana* and broad bean plants (Figure 2). 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, indicating that BBWV-1 VP37 was not required for virion formation (Figure 3). Sequence analysis of the viral progeny of *N. benthamiana* and broad bean plants systemically infected with pBBWV1-G492C indicated no reversion to the wild-type virus, supporting the relationship between plant symptoms and VP37 synthesis.

N. benthamiana and broad bean plants agroinfiltrated with pBBWV1-G492C were infected systemically, suggesting that BBWV-1 VP37 is not required for virus movement in these hosts. In contrast, it has been reported that the small MPs of fabavirus BBWV-2

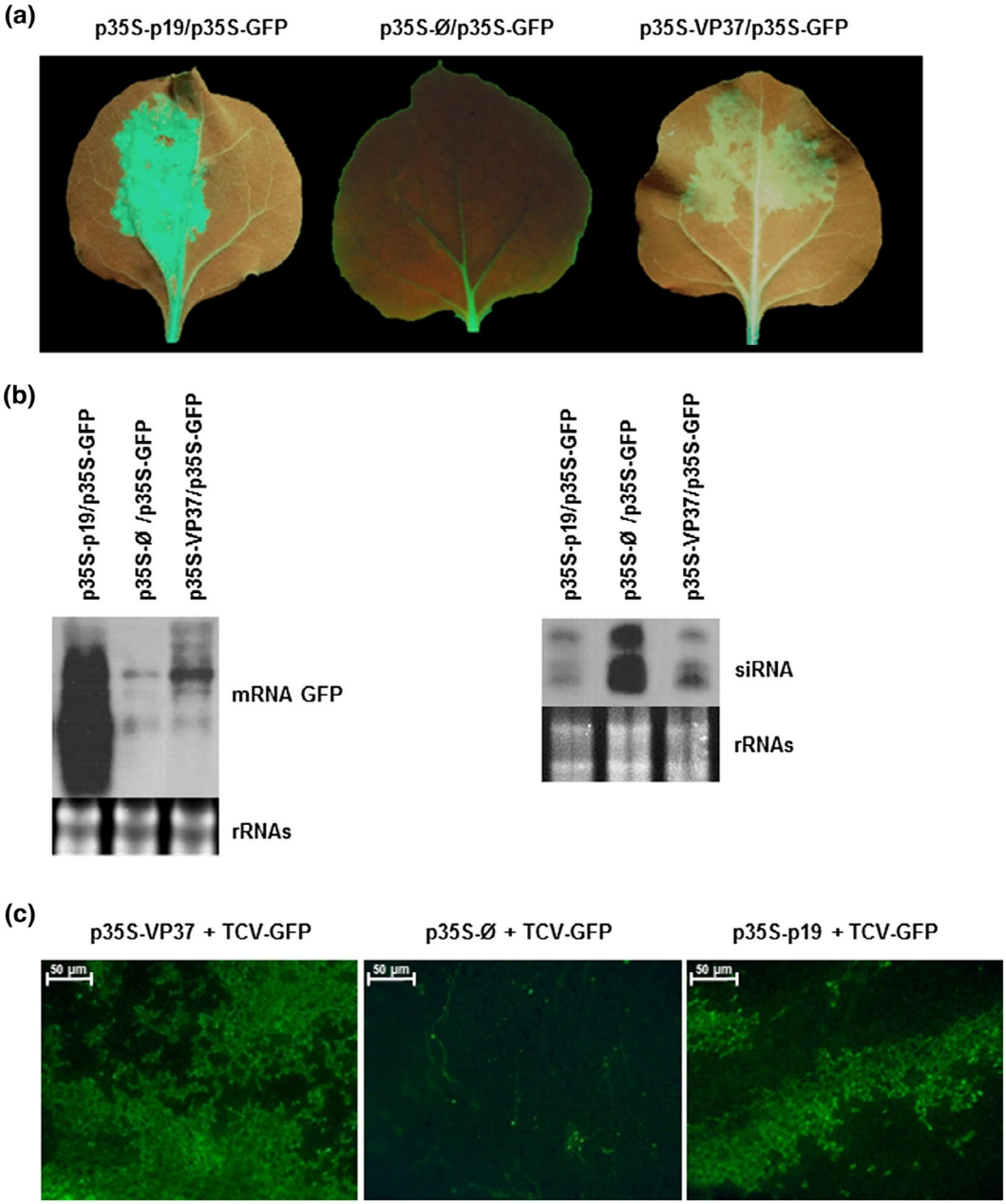

FIGURE 6 RNA silencing suppression activity of BBWV-1 VP37. (a) Leaves of *Nicotiana benthamiana* 16c plants lit with a handheld UV lamp at 3 days postagroinfiltration (dpa). Plants were agroinfiltrated with p35S-GFP in combination with p35S-VP37, p35S-p19, or p35S-Ø. (b) Northern blot analysis of green fluorescent protein (GFP) mRNAs and siRNAs extracted from agroinfiltrated tissue patches at 3 dpa. Ribosomal RNAs (rRNAs) used as loading controls are shown at the bottom. Each lane corresponds to a pool of three total RNA samples. (c) Visualization using a fluorescence stereomicroscope of *N. benthamiana* leaves agroinfiltrated with p35S-VP37, p35S-Ø, or p35S-p19 at 4 dpa. Agroinfiltrated leaves were mechanically inoculated with pTCV-GFP RNA transcripts 1 day later. Bars represent 50 µm

or comoviruses BPMV and CPMV (equivalent proteins to VP37 of BBWV-1) were involved in the cell-to-cell virus movement (van Lent *et al*., 1990; Liu *et al*., 2009; Liu *et al*., 2011; Xie *et al*., 2016). In contrast, tomato and pepper plants agroinfiltrated with pBBWV1-G492C were not infected systemically, which could be due to a lack of either virus replication or movement. RT-qPCR with specific primers for (+) and (−) BBWV-1 RNA1 strands showed no replication of pBBWV1-G492C in tomato because the (−) RNA strand was not detected in the inoculated leaves. BBWV-1 genomic ssRNA (+) strands act as mRNA, but for virus replication a dsRNA intermediate is formed and ssRNA (+) viral progeny is generated from its (−) replicative-associated RNA strand (Lisa and Boccardo, 1996). Replication of pBBWV1-G492C in pepper was very low so the virus concentration could have not reached a threshold to move efficiently to the upper leaves, in contrast to pBBWV1-Wt, which accumulated more efficiently in the inoculated leaf and moved systemically. In broad bean, pBBWV1-G492C replicated less than pBBWV1-Wt in the agroinfiltrated leaves (6 dpa) but the accumulation level of both

pBBWV1-G492C and pBBWV1-Wt was similar in upper leaves (12 dpa; Figure S1).

These results together with the lack of systemic infection in tomato and pepper plants agroinfiltrated with pBBWV1-G492C indicated the correlation between VP37 expression and host specificity, which might act at different levels depending on the host species. It was demonstrated that the ability of some bromoviruses to infect a particular host depends on the interaction between the viral MPs and plant plasmodesmata (Cilia and Jackson, 2004; Lucas, 2006; García and Pallás, 2015). However, little information is available in the family *Secoviridae*: it was demonstrated that the MP of torradovirus ToTV was crucial to infect tomato plants (Wieczorek and Obrępalska-Stęplowska, 2016b). The mutation G to C in the AUG start codon in pBBWV1-G492C might not completely abolish the expression of VP37 as reported in some plant systems showing expression at 2%–5% of efficiency (Gordon *et al*., 1992; Depeiges *et al*., 2006). Thus, the differences of symptoms and host specificity between pBBWV1-wt and pBBWV1-G492C might be due to low accumulation of VP37 rather than absence of expression.

The implication of BBWV-1 VP37 protein on plant symptom development was further studied by transient expression of this protein using a PVX viral vector (Sablowski *et al*., 1995). BBWV-1 VP37 enhanced the PVX-induced symptoms in *N. benthamiana* plants, causing stunting, severe leaf mosaic, and systemic necrosis mediated by activation of PCD (Figures 4 and 5). These results support the hypothesis that BBWV-1 VP37 protein is a pathogenicity determinant. 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 can spread in the plant despite the induction of cell death (Xu and Roossinck, 2000). In contrast, the hypersensitive response (HR) activated in plant–pathogen incompatible interactions impairs systemic infection by rapid death of infected cells (Goodman and Novacky, 1994). Although mechanisms underlying the systemic necrosis are poorly understood, it shares some features with HR as up-regulation of plant genes related to the production of toxic ROS species, such as hydrogen peroxide (H_2O_2), superoxide ion (O_2^-), and nitric oxide (NO), leads to death of plant cells (Van Breusegem and Dat, 2006; Komatsu, 2013). Transient expression of small MP of BBWV-2 using a PVX vector showed that it also enhanced the PVX-induced symptoms in *N. benthamiana* plants but did not induce systemic necrosis (Kong *et al*., 2014). There are therefore functional differences between the smallest MPs of BBWV-1 and BBWV-2 on plant symptom development despite both viruses belong to the genus *Fabavirus*. Systemic necrosis has been described for other proteins of different members of the family *Secoviridae* such as PRO-CO and HEL of BPMV and GFLV and the N-terminal region of the ToTV RNA1 polyprotein (Gu and Ghabrial, 2005; Wieczorek and Obrępalska-Stęplowska, 2016a). On the other hand, MPs of some viruses such *Banana bunchy top virus* (BBTV, genus *Babuvirus*) are determinants of pathogenicity and trigger systemic necrosis (Amin *et al*., 2011).

Determinants of pathogenicity such as the 2b protein of *Cucumber mosaic virus* (CMV, genus *Cucumovirus*) and the p23 protein of *Citrus tristeza virus* (CTV, genus *Closterovirus*) are often VSRs (Lewsey *et al*., 2010; Ruiz-Ruiz *et al*., 2013). How VSRs induce pathologic processes in infected plants is poorly understood, but some of them might act by modifying the PTGS-mediated plant gene expression, upsetting metabolic pathways linked to symptom manifestation (Wang *et al*., 2012). The VSR activity of BBWV-1 VP37 protein was demonstrated by two different approaches: transient expression of VP37 protein in *N. benthamiana* 16c plants and pTCV-GFP complementation assays (Roth *et al*., 2004; Powers *et al*., 2008). This latter approach has been successfully used to identify several plant and animal VSRs such as *Tomato bushy stunt virus* (TBSV) p19 protein, HC-PRO protein of *Tobacco etch virus* (TEV, genus *Potyvirus*), B2 protein of *Flock house virus* (FHV, genus *Alphanodavirus*), CMV 2b protein, E3L protein of *Human vaccinia virus* (VACV, genus *Orthopoxvirus*), and NS1 glycoprotein of *Human influenza virus* (FLUAV, genus *Influenzavirus*) (Powers *et al*., 2008). With regards to the family *Secoviridae*, several VSRs such as SCP of the comovirus CPMV, Vp20 CP of the cheravirus *Apple latent spherical virus* (ALSV), CP of the nepovirus *Tomato ringspot virus* (ToRSV), and p51 protein of the waikavirus *Maize chlorotic dwarf virus* (MCDV) (Cañizares *et al*., 2004; Yaegashi *et al*., 2007; Karran and Sanfacon, 2014; Stewart *et al*., 2017). In the genus *Fabavirus*, MP, small MP, and LCP of BBWV-2 have VSR activity and also were determinants of pathogenicity (Kong *et al*., 2014). In contrast, PRO-CO and HEL of BPMV, and POL of GFLV were pathogenesis determinants but not VSRs (Gu and Ghabrial, 2005; Vigne *et al*., 2013).

This work revealed that BBWV-1 has some differences with other members of the genus *Fabavirus* and the family *Secoviridae*. We have provided new insights into the VP37 interaction with its hosts, showing that it is a determinant of pathogenicity and host specificity. The VP37 protein seems to be a multifunctional protein, being involved in virus replication, movement, and PTGS suppression. Also, the 35S-driven infectious cDNA clones engineered in this work open a way to investigate virus–host–vector interactions and elucidate the functions of different BBWV-1-encoded proteins.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant and virus materials

BBWV-1 isolate Ben (Ferrer *et al*., 2005) was maintained in broad bean cv. Reina Mora (Fito) plants. *N. benthamiana*, broad bean cv. Reina Mora, tomato cv. Marmande, and pepper cv. Manolo plants were used for agroinfiltration or mechanical inoculation assays. All plants were kept in a growth chamber under conditions of 16 hr light at 24 °C and 8 hr darkness at 20 °C.

4.2 | Construction of full-length cDNA clones of BBWV-1

Full-length cDNA clones of RNA1 and RNA2 of BBWV-1 isolate Ben were constructed from BBWV-1 pBenR1 and pBenR2

T7-driven cDNA clones (GenBank accession numbers KT988973, KT988974) (Ferriol *et al*., 2016). The DNA fragments were obtained by PCR using CloneAmp HiFi PCR Premix (Takara Bio), gel purified with PCR clean-up gel extraction kit (Macherey-Nagel), and cloned into the binary plasmid pJL89 between the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase terminator (t-Nos) using In-Fusion HD Cloning Kit (Takara Bio). To obtain the full-length BBWV-1 RNA1, we divided the RNA1 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. The BBWV-1 RNA2 was cloned into plasmid pJL89 in one step. The resulting constructs were named pBBWV1-R1 and pBBWV1-R2 for the RNA1 and RNA2, respectively. To obtain a BBWV-1 RNA2 mutant construct that knocks out 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. The PCR product was ligated using T4 DNA Ligase (Promega) and the resulting construct was named pBBWV1-R2:G492C. All constructs were sequenced to confirm that no mutations were present. Equal volumes of *A. tumefaciens* COR308 cell suspensions containing pBBVW1-R1/pBBWV1-R2/pBIN35S-p19 (a pBIN binary plasmid expressing the TBSV p19 protein, which is a well-characterized VSR) or pBBWV1-R1/pBBWV1-R2:G492C/pBIN35S-p19 constructs were agroinfiltrated into *N. benthamiana*, broad bean, pepper, and tomato plants. Plant symptom development was monitored up to 21 dpi and photographed with a Nikon D40 Reflex Camera. PCR primers used for all of these procedures are shown in Table S1.

4.3 | Agroinfiltration procedure

Agroinfiltration experiments were carried out in *N. benthamiana* plants at the four- to six-leaf stage, and tomato, broad bean, and pepper plants at the two fully expanded cotyledons and two emerging true leaf stages. All plasmid constructs were transformed into *A. tumefaciens* COR308 cells and agroinfiltrated when OD_{600} = 1, following standard procedures (Martínez *et al*., 2014). Mock infiltration was done with agroinfiltration medium without transformed *A. tumefaciens* COR308 cells.

4.4 | 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 from transient expression assays in *N. benthamiana* 16c plants, total RNA was extracted from 0.25 g of agroinfiltrated leaves using TRI-reagent (Sigma). siRNAs were enriched from total RNAs by removing high molecular weight RNAs with a solution of 50% (wt/vol) PEG 8,000 (Sigma) and 0.5 M NaCl. Northern blot analysis of siRNAs was performed by electrophoresis of 3 μg enriched siRNAs in a 15% polyacrylamide gel containing 7 M urea, and electrotransferring it onto a positively charged nylon membrane (Roche) using 0.5 × TBE (80 mM Tris, 80 mM boric acid, 2 mM EDTA) at 25 V for 1 hr.

Northern blot of total RNAs was performed as previously described (Ferriol *et al*., 2015, 2016). Briefly, 4 μg of total RNAs were separated on a 0.9% formaldehyde agarose gel in 1 × MOPS buffer (5 mM sodium acetate, 1 mM Na_2EDTA, 20 mM MOPS, pH 7) and transferred by capillary action onto positively charged nylon membranes (Roche) in 20 × SSC buffer (3 M NaCl, 0.3 M sodium citrate). The membranes were UV cross-linked and hybridized with digoxigenin-labelled RNA probes specific for each virus as previously described (Ferriol *et al*., 2015). For BBWV-1 northern blot, an antisense 332-bp riboprobe, which hybridizes with the 5′-UTR regions of RNA1 and RNA2, was used (Ferrer *et al*., 2008). For PVX northern blot, a 432-bp antisense riboprobe complementary to PVX CP (positions 5,650 to 6,363, GenBank accession: EU021215) was synthesized by PCR with specific primers. For GFP northern blot, an antisense riboprobe complementary to the full-length GFP sequence was used (Renovell *et al*., 2012). The hybridization was developed with an antidigoxigenin alkaline phosphatase-conjugated antibody (Roche) and visualized with the CDP-Star Chemiluminescent Substrate (Roche). The PCR primers used to synthesize these probes are in Table S1.

4.5 | RT-qPCR

To quantify the viral titre of plants agroinfiltrated with pBBVW1-R1/pBBWV1-R2 (pBBWV1-Wt) and pBBWV1-R1/pBBWV1-R2:G492C (pBBWV1-G492C) two TaqMan (Sigma) probes specific for BBWV-1 RNA1 (positions 1,123 to 1,148) and RNA2 (positions 1,791 to 1,820) were designed using Primer Express (Applied Biosystems) software. Total RNAs were obtained from noninfiltrated 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. Previously, the concentration of total RNAs was adjusted to 10 ng/μl. Standard curves were obtained from 10-fold serial dilutions of the most concentrated viral RNA sample corresponding to a broad bean plant agroinfiltrated with pBBWV1-Wt (Figure S1). One-step RT-qPCR was performed in a LightCycler 480 (Roche) in a 25 μl reaction mix containing 50 ng total RNA, 15 U reverse transcriptase (Thermo Fisher Scientific), 2 U RNase Inhibitor (Thermo Fisher Scientific), 1 × LightCycler 480 Probes Master mix (Roche), 0.5 μM of each primer forward and reverse, and 0.1 μM of each BBWV-1 RNA1 or RNA2 TaqMan probe. Cycling conditions were RT at 48 °C for 30 min, cDNA denaturation at 95 °C for 10 min, and 40 cycles of DNA amplification at 95 °C for 10 s and 60 °C for 1 min. Virus titre was compared by multifactorial analysis of variance using Statgraphics software.

BBWV-1 replication was determined using a two-step RT-qPCR assay. *N. benthamiana*, broad bean, tomato, and pepper plants were agroinfiltrated with pBBWV1-Wt or pBBWV1-G492C. Total RNA

purified from agroinfiltrated areas at 6 dpa with the phenol:chloroform:isoamyl procedure was treated with TURBO DNase (Thermo Fisher Scientific) to remove the cDNA constructs. RT was performed with specific reverse or forward primer for the (+) or (−) strands of both BBWV-1 RNA1 and RNA2, 200 U SuperScript IV (Thermo Fisher Scientific), 2 U RNase Inhibitor (Thermo Fisher Scientific), and 0.25 μM of the respective specific strand primer. RT conditions were RNA denaturation at 65 °C for 10 min, RT at 55 °C for 30 min, and enzyme denaturation at 80 °C for 10 min. qPCR was performed in a 25 μl reaction mix containing 1 × LightCycler 480 Probes Master mix (Roche), 0.5 μM of each primer forward and reverse, 0.1 μM of each BBWV-1 RNA1 or RNA 2 TaqMan probes, and 2.5 μl of cDNA. Cycling conditions were cDNA denaturation at 95 °C for 10 min and 40 cycles of DNA amplification at 95 °C for 10 s and 60 °C for 1 min. RT and qPCR primers and TaqMan probes are shown in Table S1. As negative control, qPCRs were performed omitting the RT step.

4.6 | Virion purification, transmission electron microscopy, and RNA analysis

BBWV-1 virions were purified from 5 g of broad bean or *N. benthamiana* plants infected with pBBWV1-Wt or pBBWV1-G492C at 21 dpa, as previously described (Turina *et al*., 2007). Briefly, leaves were homogenized in extraction buffer (0.25 M potassium phosphate, 0.1% 2-mercaptoethanol) filtered through cheesecloth, kept on ice for 1 hr with 1% Triton X-100 and centrifuged at 8,000 × g for 10 min. The supernatant was stirred on ice for 2 hr with 8% PEG 8,000 and 1% NaCl, centrifuged at 8,000 × g for 10 min, the resulting pellets were resuspended in extraction buffer containing 15% sucrose and centrifuged at 235,000 × g for 3 hr. BBWV-1 virions were resuspended in extraction buffer and stained with 2% uranyl acetate. Negatively stained preparations were examined with a transmission electron microscope (JEOL JEM-1010). The presence of genomic RNAs (gRNAs) in the purified virion suspensions was confirmed by electrophoresis in 1% agarose gels and GelRed (Biotium) staining.

4.7 | Transient expression assays of BBWV-1 VP37 using a PVX-based vector

BBWV-1 nucleotide sequence encoding for the VP37 was amplified by RT-PCR with specific primers (containing restriction enzyme recognition sites) and the reverse primer also containing a UGA stop codon at the end of the sequence (Table S1). After enzymatic digestion, the amplified sequence was cloned into the pPVX202 expression vector (Sablowski *et al*., 1995) to obtain the pPVX-VP37 construct, which was transformed into *Escherichia coli* XL1-Blue Competent Cells (Agilent). The plasmid was purified using a Realplasmid Spin Miniprep kit (Real) and sequenced. Fourteen micrograms of each plasmid construct were mechanically inoculated in two leaves of each *N. benthamiana* plant using sodium phosphate buffer (10 mM Na_2HPO_4, 10 mM NaH_2PO_4, pH 7.2). PVX-empty vector (pPVX-Ø) was used as PVX infection control. As mock inoculation controls, *N. benthamiana* plants were mock-inoculated. Virus infection was assessed at 10 dpi by RT-PCR and northern blot analysis of total RNA samples obtained from upper noninoculated leaves (Table S1). Plant symptom development was monitored up to 21 dpi and photographed with a Nikon D40 Reflex Camera. The PCR primers used for all of these procedures are shown in Table S1.

4.8 | NBT and trypan blue staining

The presence of superoxide ion O_2^- accumulations and dead cells in upper noninoculated leaves of *N. benthamiana* plants inoculated with pPVX-Ø or pPVX-VP37 was determined at 7 dpi using the NBT and trypan blue staining methods, respectively (Grellet-Bournonville and Díaz-Ricci 2011; Garcia-Marcos *et al*., 2013). Briefly, apical noninoculated leaves were vacuum infiltrated for 1 min in NBT staining solution (10 mM sodium azide, 0.5 mM phosphate buffer pH 7.8, 2 mg/ml NBT) and then incubated in the same solution for 30 min. Samples were washed with 96% ethanol and photographed with a Nikon D40 Reflex Camera. For trypan blue staining, apical noninoculated leaves were boiled for 1 min in lactophenol–trypan blue staining solution (0.3 g/ml lactic acid, 25% glycerol, 0.25 g phenol, 0.5 mg/ml trypan blue) and decolourized with 2.5 g/ml chloral hydrate for 30 min. Stained leaves were photographed with a Leica stereoscopic microscope.

4.9 | Transient expression assay of BBWV-1 VP37 in *N. benthamiana* 16c plants

BBWV-1 nucleotide sequence encoding for VP37 was amplified with specific primers (reverse primers containing the UGA stop codon at the end). After enzymatic digestion, the amplified sequence was cloned into pCambia binary plasmid between the 2 × 35S CaMV promoter and the Nos-terminator, and the resulting construct was named p35S-VP37. The empty pCambia plasmid (p35S-Ø) was used as negative control. A pBIN binary plasmid containing the TBSV p19 protein (p35S-p19) was used as PTGS positive control. For GFP expression, a pBIN plasmid containing the GFP gene (p35S-GFP) was used. Plasmids were transformed into *A. tumefaciens* COR308 cells and equal volumes of *A. tumefaciens* COR308 cell suspensions containing p35S-p19/p35S-GFP (positive control), p35S-Ø/p35S-GFP (negative control), and p35S-VP37/p35S-GFP were agroinfiltrated into leaves of transgenic *N. benthamiana* 16c plants. Examination of fluorescence was performed after 3 dpa using a handheld UV lamp and photographed with a Nikon D40 Reflex Camera. The PCR primers used for all of these procedures are shown in Table S1.

4.10 | pTCV-GFP complementation assays

For TCV-GFP complementation assays, *N. benthamiana* plants were agroinfiltrated with constructs p35S-VP37, p35S-Ø (negative

control), and p35S-p19 (positive control). After 24hr, 5 μg per leaf of TCV-GFP transcripts generated using T7 RNA polymerase (Roche) from the pTCV-GFP construct were mechanically inoculated in leaves previously agroinfiltrated. Examination of fluorescence was performed at 4 dpa using a Leica fluorescent stereoscopic microscope MZ16 FA using a high-energy light source and a GFP filter.

4.11 | RT-PCR and sequencing

BBWV-1 systemic infection was determined by RT-PCR of total RNA samples obtained from upper nonagroinfiltrated leaves of plants agroinfiltrated with pBBWV1-Wt and pBBWV1-G492C at 12 dpa. BBWV-1 detection in agroinfiltrated leaves was performed by RT-PCR of total RNA obtained from agroinfiltrated areas of plants agroinfiltrated with pBBWV1-Wt and pBBWV1-G492C. Prior to RT-PCR, the obtained total RNA was treated with TURBO DNase (Invitrogen). RT-PCR primers were designed to amplify a BBWV-1 region surrounding the mutation site. 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 RNA extracts obtained from upper noninoculated leaves at 10 dpi of *N. benthamiana* plants inoculated with pPVX-VP37 and pPVX-Ø using a forward primer specific for the insert and a reverse primer designed in the virus CP region. In all cases, RT-PCR was performed in two steps using 20 ng of total RNA with SuperScript IV reverse transcriptase (Invitrogen) and *Taq* DNA polymerase (Invitrogen). Cycling conditions were RT at 50 °C for 30 min, denaturation at 95 °C for 2 min; 40 cycles at 95 °C for 20 s, 55 °C for 20 s, and 72 °C for 45 s; and a final extension of 72 °C for 5 min. 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 us to obtain the sequence of BBWV-1 full-length cDNAs. Sequencing of inserts in p35S-VP37 and pPVX-VP37 constructs was performed using PVX-specific primers contiguous to the cloning site. Sequences were obtained with an ABI 3130xl Genetic Analyzer (Thermo Fisher Scientific) and the PCR primers used for all of these procedures are shown in Table S1.

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CONFLICT OF INTEREST

The authors have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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