

Transient Expression of Homologous Hairpin RNA Interferes with Broad bean wilt virus 2 Infection in *Nicotiana benthamiana*

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Broad bean wilt virus 2 (BBWV2), genus *Fabavirus*, subfamily *Comovirinae*, family *Secoviridae*, causes damage in many economically important horticultural and ornamental crops. Sequence alignments showed several conserved sequences in 5' non-coding regions (5' NCRs) of RNA 1 and RNA 2 in all BBWV2 strains characterized so far. Based on this observation, we generated a hpRNA construct (pIR-BBWV2) harboring an inverted repeat containing a 210 bp cDNA fragment homologous to 5' NCR portion of BBWV2 RNA 1 to investigate the silencing potential for its ability to interfere with a rapidly replicating BBWV2. *Agrobacterium*-mediated transient expression of the IR-BBWV2 had a detrimental effect on BBWV2 infection, showing no distinct symptoms in non-inoculated leaves of the agroinfiltrated *Nicotiana benthamiana* plants. BBWV2 genomic RNAs were not detected by RT-PCR from tissues of both the inoculated leaves and upper leaves of the agroinfiltrated plants. Accumulation of virus-derived small interfering RNAs was detected in the inoculated leaf tissues of *N. benthamiana* plants elicited by transient expression of IR-BBWV2 indicating that RNA silencing is responsible for the resistance to BBWV2.

Keywords : Agroinfiltration, Broad bean wilt virus 2, Resistance, RT-PCR, Small-interfering RNA, Transient expression

Introduction

Pathogen-derived resistance (PDR) is a specific resistance of plants to pathogens by introducing a pathogen into the plant genome. It is widely shown that the PDR to virus infection are relevant to RNA silencing known well for homology-dependent selectively degradation of RNA (Baulcombe, 2005; Hull, 2002). The RNA silencing machinery recognizes several features of viral infections involving the formation of double-stranded (ds) RNA and initiates a response that degrades viral RNA and eventually enables the plant to recover from virus infection. The dsRNA

triggers degradation of homologous RNAs and is diced into small interfering (si) RNAs of 21–25 nts in length. The siRNAs then act as guide sequences to recognize complementary RNAs for their degradation (Voinnet, 2005; Waterhouse *et al.*, 2001). RNA silencing is also activated by transgenes expressing inverted-repeat (IR) structures that produce dsRNA (Chuang and Meyeriwitz, 2000; Waterhouse *et al.*, 2001) or aberrant transcripts that could be templates for a cellular RNA-dependent RNA polymerase activity (Lipardi *et al.*, 2001). In addition, RNA silencing can be induced by expression of hairpin (hp) RNA in plants, and a variant of this construction which also encodes a spliceosomal intron inserted between the hpRNA arms (so called intron-hpRNA) induced RNA silencing with almost 100% efficiency when directed against RNA virus or endogenous plant genes (Pandolfini *et al.*, 2003; Smith *et al.*, 2000). Transient expression triggered by infiltration of *Agrobacterium tumefaciens*

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cultures (so called agroinfiltration) into leaf tissue has been widely used to induce RNA silencing (Johansen and Carrington, 2001; Llave *et al.*, 2000; Voinnet *et al.*, 1999).

Broad bean wilt virus 2 (BBWV2), genus *Fabavirus*, subfamily *Comovirinae*, family *Secoviridae*, causes damage in many economically important horticultural and ornamental crops (Koh *et al.*, 2001; Lisa and Boccardo, 1996; Qi *et al.*, 2000; Wang *et al.*, 2008; Xu *et al.*, 1988). Since some BBWV2 isolates identified cause severe damages in pepper production, BBWV2 is one of harmful viruses for pepper production in Korea (Lee *et al.*, 2000; Cho *et al.*, 2007; Choi *et al.*, 2001; Choi *et al.*, 2005). BBWV2 has a wide host range and is transmitted by aphids in a non-persistent manner. BBWV2 virion is icosahedral particles, composed of two proteins (the large and small coat proteins; LCP and SCP) and a genome composed of two single-stranded positive-sense RNA molecules of about 6 and 4 kb (Lisa and Boccardo, 1996). Both RNAs are translated into single polyproteins from which functional proteins are divided by proteolytic cleavage. RNA 1 encodes proteins involved in genome replication and expression, and RNA2 encodes the movement protein and the two CPs (Lisa and Boccardo, 1996). RNA silencing has been efficiently used to generate resistance

against plant viruses in many ornamental plants (Bucher *et al.*, 2006; Hammond *et al.*, 2006; Tenllado *et al.*, 2004) and in different host systems to obtain resistance against several other viruses (Abhary *et al.*, 2006; Di Nicola-Negri *et al.*, 2005; Lennefors *et al.*, 2006; Pooggin *et al.*, 2003; Tenllado *et al.*, 2003; Vanitharani *et al.*, 2003). Particularly, transgenic expression of pathogen-derived sequences encoding hpRNAs that undergo to an efficient RNA silencing is a new and agricultural sustainable strategy to obtain virus-resistant plants (Smith *et al.*, 2000). However, it is not known if transient expression of a hpRNA could block multiplication and spread of a widely replicating BBWV2 in non-transgenic plants.

In this study, we show that transient expression of a hpRNA construct using agroinfiltration allows high resistance to BBWV2 in *Nicotiana benthamiana*. RNA silencing is responsible for this resistance to BBWV2 and this approach makes it possible to construct transgenic crops conferring resistance against BBWV2.

Materials and Methods

Plasmid construction

A cDNA fragment homologous to 210 bases of 5'

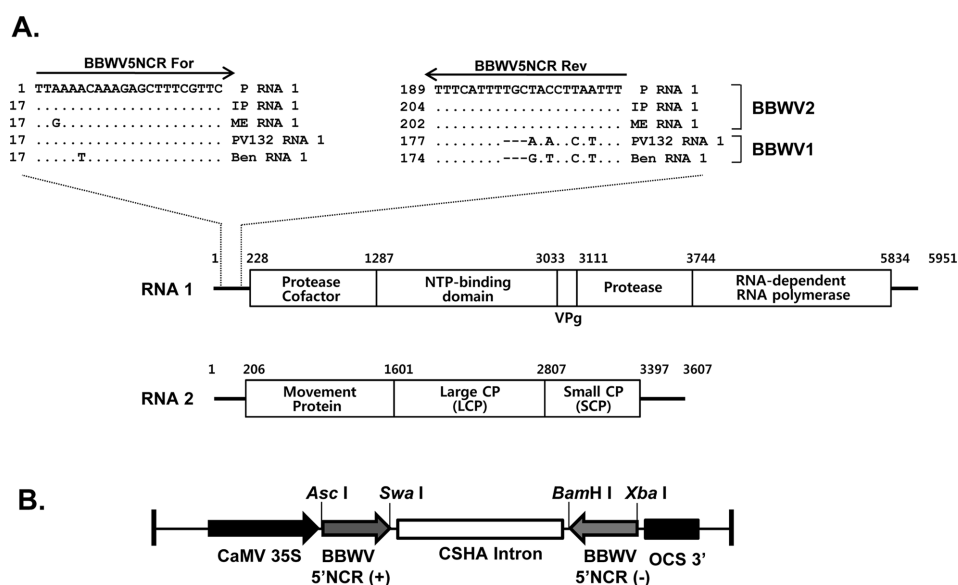


Fig. 1. Schematic representations of BBWV2 genome and constructs used in *A. tumefaciens*-mediated transient expression. **(A)** Primers used in this study were designed by multiple alignments of 5' NCRs between BBWV2 strains and BBWV1 strains. Nucleotide (nt) positions in 5' NCRs are shown above BBWV2 genome. Name and RNA source of each strain are shown on the right. Nucleotide positions were indicated on the left. The nt identical to the consensus are indicated by dots within the alignment and the nt different from the consensus are indicated by dashes within the alignment. Accession numbers deposited to GenBank are as follows: BBWV2-P RNA 1 (in this study), BBWV2-IP RNA 1 (AB023484), BBWV2-ME RNA 1 (NC 003003), BBWV1-PV132 RNA 1 (AB084450), and BBWV1-Ben RNA 1 (AY781171). **(B)** A construct (pIR-BBWV2) containing hpRNA sequences contained the CaMV 35S promoter (a black arrow) and the 3'-termination sequences of octopine synthase (black square). The sense (+) and antisense (-) cDNA fragments homologous to the 5' NCR sequences of BBWV2 RNA 1 are represented by blue arrows. The cDNA fragments encoding sense or antisense 5' NCR sequences are separated by a chalcone synthase (CHSA) intron derived from *P. hybrida*. Restriction enzyme sites used for cloning are shown above the construct.

noncoding region (5' NCR) portion of RNA 1 of a Korean BBWV2 strain pepper (BBWV2-P) was synthesized by RT-PCR using the primers showing several conserved sequences sites in 5' NCRs of RNA 1 in all BBWV2 strains and BBWV1 characterized so far, as described previously (Fig. 1A; Yoon *et al.*, 2002). BBWV2-P that induces mosaic symptoms in *Nicotiana* species was obtained from PVGB (<http://www.virusbank.org>). Briefly, cDNA synthesis using BBWV2 genomic RNAs (approx. 100 ng) purified from virions was performed in a 20 μ l volume of 1x Superscript III reaction buffer (Invitrogen, USA), containing 0.5 mM dNTP mix, 5 mM DTT, 40 U RNaseOut, and 200 U of SuperScript III Reverse Transcriptase (Invitrogen, USA) 20 pmole gene-specific reverse primer (BBWV5NCRRev; 5'-*TCTAGA*ATTTAAATAAATTAAGGTAGCAAAAATGAAA) complementary to positions 189-210 (bold) in the RNA1 of BBWV2 strain P. An *Xba* I (italics) and *Swa* I (underline) restriction sites were created in the primer BBWV5NCRRev to facilitate further cDNA cloning. The tenth volume of the RT reaction was used for PCR amplification with the primer BBWV5NCRRev and a forward primer (BBWV5NCRFor; 5'-*GGATCCGAATTCTT*AAAACAAAGAGCTTTCGITC) corresponding to positions 1-22 (bold) in the RNA 1 of BBWV2-P. *Bam*H I (italics) and *Acs* I (underline) restriction sites were created in the primer BBWV5NCRRev to facilitate further cDNA cloning. PCR amplification was performed in 50 μ l of 1x Platinum[®] *Taq* DNA polymerase reaction buffer containing 1 mM MgCl₂, 0.2 mM dNTP, 10 pmole of each primer and 1 U of Platinum[®] *Taq* DNA polymerase according to manufacturer's instructions (Invitrogen, USA). The thermal cycles were as follows: 2 min at 94°C (1 cycle); 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C (35 cycles).

In the first cloning step, amplified RT-PCR products were digested with *Acs* I-*Swa* I restriction enzymes and ligated into *Acs* I-*Swa* I sites in pFGC5941 plasmid (http://www.chromdb.org/tnai/vector_info.html), producing pFGC-half-BBWV2. For the second cloning step, RT-PCR fragments amplified were digested with *Bam*HI-*Xba*I restriction enzymes and cloned into the pFGC-half-BBWV2 digested with the same enzymes, generating pIR-BBWV2 (Fig. 1B). This second ligation inserts the PCR product in inverted orientation with respect to first cloned fragment, yielding an IR separated by the *ChsA* intron (Genbank no. AY310901). Structure of the cloned construct was verified by a combination of restriction enzyme mapping and sequencing analysis. The pIR-BBWV2 construct was transferred to *A. tumefaciens* strain C58C1 by freeze-thaw transformation method (An *et al.* 1985). pFGC5941 containing no cDNA insert was also introduced into *A. tumefaciens* strain C58C1 to use a negative control.

Agoinfiltration and virus inoculation

Leaves of *N. benthamiana* were infiltrated with *A. tumefaciens* as described previously (Canto *et al.*, 2002). Initially, 4 transformants per the construct were tested for agroinfiltration. A single colony of *A. tumefaciens* strain C58C1 containing the binary construct was inoculated to LB media supplemented with 10 mM MES (pH 5.6), 20 μ M acetosyringone (Fluka, USA) and antibiotics (rifampicin 10 μ g/ml, ampicillin 50 μ g/ml and kanamycin 50 μ g/ml). The cells were grown at 28°C overnight. The cells of the overnight culture were collected by centrifugation, resuspended in infiltration buffer (10 mM MgCl₂, 10 mM MES (pH 5.6), and 150 μ M acetosyringone) for a final OD₆₀₀ of 0.5. *A. tumefaciens* culture was incubated at room temperature for at least 2 hour, and then infiltrated into leaves of *N. benthamiana* using a 1 ml disposable syringe without a needle.

BBWV2-P was maintained in *N. benthamiana* by mechanical inoculation. Three days after agroinfiltration, the infiltrated leaves of *N. benthamiana* plants were inoculated with a 1:3 dilution of plant sap from BBWV2-infected *N. benthamiana* plants. Inoculation of plants was done on two fully expanded leaves by gently rubbing the leaf surface with the inoculum using Carborundum[®] as an abrasive. For back-inoculation experiment, *Chenopodium quinoa* plants were mechanically inoculated with sap inoculum prepared from upper leaves of sample plants and control plants at 14 dpi. The inoculated plants were kept in a growth room with a 16 h light and 8 h dark cycle at 25°C, and the development of viral infection symptoms was monitored until 28 days post-inoculation (dpi).

Analysis of viral RNA in plants

Total RNA was extracted from inoculated leaves at 3 days after BBWV2 inoculation and from upper leaves at 7 dpi, with Trizol reagent and phenol/chloroform (25:24, v/v), as described previously (Choi *et al.*, 2011). Further precipitation, purification and DNase I treatment were performed as standard protocols (Sambrook *et al.*, 1989). RNA concentrations were measured photometrically with a NanoDrop (Thermo Scientific, USA) and RNA quality was analyzed by 1.2% agarose-formaldehyde gels electrophoresis. To detect RNA 2 of BBWV2-P from the infiltrated and the upper leaves of *N. benthamiana*, RT-PCR analysis was carried out using a forward primer corresponding to nt 1237 to 1260 and a reverse primer complementary to nt 1867 to 1890, as described previously (Choi *et al.*, 2005). Primers specific for the *N. benthamiana* gene encoding elongation factor 1 α (Nb-EF1 α) were used for PCR amplification of a housekeeping gene (Choi *et al.*, 2011). The amplified RT-PCR product was separated on 1.5% agarose gel and stained in edithium bromide

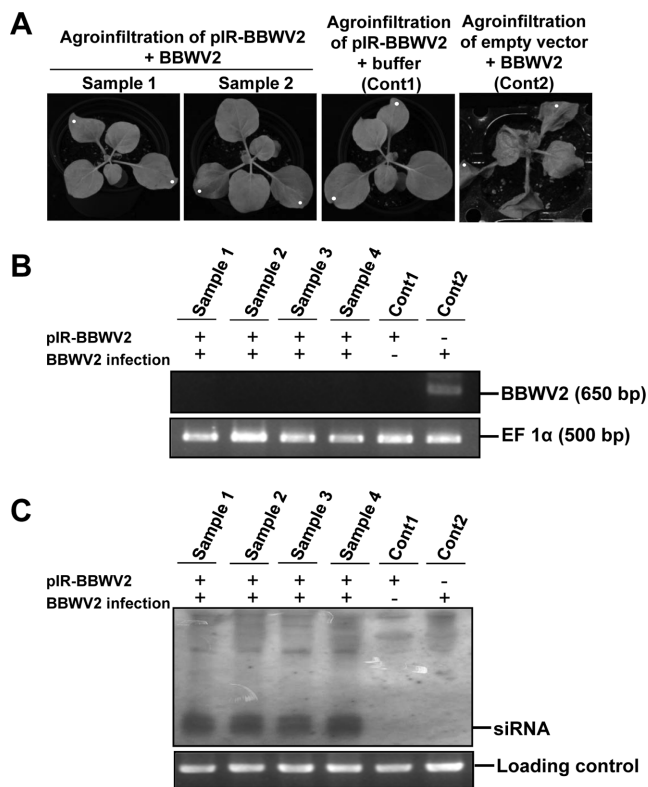


Fig. 2. Specific interference with BBWV2 infection by transiently expressed hairpin RNA. (A) Symptoms of plants agroinfiltrated with IR-BBWV2, Cont1-plants and Cont2-plants 7 days post-inoculation of BBWV2. Cont2-plants showed wilt symptoms in the inoculated leaves and mosaic symptoms in the non-inoculated upper leaves. The inoculated leaves that had been agroinfiltrated with pIR-BBWV2 or the empty vector are indicated by circline dots. (B) RT-PCR analysis of BBWV2 RNA 2. Total RNAs were used for BBWV2 detection in the non-inoculated leaves of each sample and controls. The absence (–) or the presence (+) of agroinfiltration and BBWV2 inoculation is indicated on each lane. (C) Northern blot analysis of low molecular weight RNAs extracted from *N. benthamiana* plants infiltrated with *A. tumefaciens* containing pIR-BBWV2 or the empty vector and challenge-inoculated with BBWV2 after 3 days. Samples were taken from the inoculated leaves at 3 days after the challenge-inoculation. Similar amounts (2 µg) of the low-molecular-weight RNA samples were fractionated by 15% PAGE-7M-urea gel, and the blot was hybridized with a DIG-labeled RNA probe specific for BBWV2 RNA 2. Signal of the blot was detected using DIG-detection kit and equivalent loading of samples was confirmed by staining the gel with ethidium bromide before transfer.

mechanically inoculated BBWV2, two leaves of *N. benthamiana* plants were infiltrated with *A. tumefaciens* culture carrying pIR-BBWV2 or the empty vector. At 3 dpi, both plants infiltrated with *A. tumefaciens* cultures carrying IR-BBWV2 and plants infiltrated with *A. tumefaciens* cultures carrying the empty vector (hereafter Cont2-plants) were inoculated with sap inoculum of BBWV2-P that was directly inoculated on the entire infiltrated

Table 2. BBWV2-infectivity assay on leaves of *N. benthamiana* agroinfiltrated with pIR-BBWV2 construct or empty vector

Assay No.	pIR-BBWV2/BBWV2		Cont1 ^c		Cont2 ^d	
	IT ^a	R (%) ^b	IT	R (%)	IT	R (%)
Expt. 1	1/15	93.3	0/4	100	6/6	0
Expt. 2	0/6	100	0/2	100	5/5	0

^aThe number of BBWV2-inoculated leaf/tested plant. Infection of BBWV2 was confirmed by RT-PCR analysis.

^bNumber of resistant plants as a percentage of the total inoculated plants.

^cTransient expression of pIR-BBWV2+buffer.

^dTransient expression of empty vector+BBWV2.

leaves dusted with Carborundum®. As a negative control, plants infiltrated with *A. tumefaciens* cultures carrying pIR-BBWV2 were mechanically inoculated with buffer alone (hereafter Cont1-plants). In several independent experiments, Cont2-plants that had been infiltrated with *A. tumefaciens* harboring the empty vector showed wilt symptoms on the infiltrated leaves and mosaic symptoms in upper leaves at 7 dpi (Fig. 2A). In contrast, all inoculated plants that had been infiltrated with pIR-BBWV2, except a plant remained symptomless throughout the entire testing period (4 weeks), similar to Cont2-plants inoculated with buffer alone (Fig. 2A and Table 2).

RT-PCR analysis confirmed this observation in upper leaves at 7 dpi. BBWV2 RNA 2 was not detectable in plants agroinfiltrated with IR-BBWV2 construct and in Cont1-plants, whereas cDNA fragment corresponding to sequences of BBWV2 RNA 2 was synthesized from the Cont2-plants (Fig. 2B). The amplified cDNA fragments of the Nb-EF1α were synthesized from all samples tested, Cont1-plants, and Cont2-plants (Fig. 2B). These results indicate BBWV2-genomic RNAs were specifically degraded by transient expression of IR-BBWV2 in plants, suggesting that interference with BBWV2 infection conferred by transient expression of IR-BBWV2 is likely to be due to the activation of RNA silencing.

Moreover, biologically active BBWV2 was absent in homogenates of the upper leaves of plants agroinfiltrated with IR-BBWV2 or in those of Cont1-plants, as confirmed upon back-inoculation of another systemic host *C. quinoa*. In contrast, *C. quinoa* plants inoculated with a sap inoculum from Cont2-plants expressed typical BBWV2 symptoms at 14 dpi (data not shown). These results suggest that the resistance response of plants elicited by hpRNA sequences in pIR-BBWV2 have detrimental effect on the replication process of BBWV2. It worthwhile to mention that the interference with BBWV2 infection triggered by the IR-BBWV2 construct was sequence-specific, since inoculations of *Cucumber mosaic virus* (CMV strain Fny) and *Tobacco*

mosaic virus (TMV strain U1), unrelated to the genus *Fabavirus*, on plants that had been agroinfiltrated with the pIR-BBWV2 had no effects on timing and severity of symptoms in upper leaves at 7 dpi, respectively. CMV RNAs or TMV RNA in upper leaves of the plants agroinfiltrated with the IR-BBWV2 was detected, similar to those of plants agroinfiltrated with the empty vector or wild-type *N. benthamiana* plants (data not shown). Again, this result supports that none of amplified cDNA fragment of BBWV RNA 2 in plants expressed transiently IR-BBWV2 was due to specific degradation of BBWV2-genomic RNAs by the activation of RNA silencing (Fig. 2B).

To verify whether transient expression of IR-BBWV2 through agroinfiltration could inhibit BBWV2 infection once the virus had been inoculated on plants, *N. benthamiana* plants were first inoculated with BBWV2 and, 24 h later, *A. tumefaciens* harboring pIR-BBWV2 or the empty vector was infiltrated into the inoculated leaves or the upper, non-inoculated leaves. In both cases, all plants displayed typical disease symptoms at 7 dpi, and BBWV2 RNA2 was detected from upper-most leaves of both sample plants and control plants (data not shown). Therefore, transient expression of pIR-BBWV2 prior to virus inoculation is required to interfere with BBWV2 infection.

Since homology-dependent resistance to virus infection is a characteristic feature of RNA silencing, we further tested whether interference with BBWV2 infection conferred by transient expression of IR-BBWV2 could be attributable to RNA silencing. The LMW RNA fraction was extracted from BBWV2-inoculated leaves that had been agroinfiltrated with IR-BBWV2 and BBWV2-inoculated leaves of Cont2-plants. The buffer-inoculated leaves of Cont1-plants were used as negative controls. DIG-labeled RNA probe specific for the BBWV2 RNA2 sequences cloned in pGEM-half-BBWV2 was produced by T7 RNA transcription. It is worthwhile to mention that this RNA probe is not able to hybridize the sequences of the inserted cDNA of BBWV2 RNA1 in pIR-BBWV2, but not the sequences of BBWV2 RNA 2. Thus, the probe enables us to exclude siRNA species generated from expression of IR-BBWV transgene transferred by *A. tumefaciens*. As shown in Fig. 2C, 21-23 nts siRNA species that are a hallmark of RNA silencing were detected in plants that had been infiltrated with IR-BBWV2 but were absent in Cont1-plants and Cont2-plants. This result clearly shows that the activation of RNA silencing plays a crucial role in the interference with BBWV infection in leaves expressed transiently IR-BBWV.

Discussion

We have shown that *A. tumefaciens*-mediated transient expression of a homologous hpRNA results in resistance to BBWV2 infection in *N. benthamiana*. Numerous studies have indicated that IR constructs of transgenes can effectively induce RNA silencing to trigger knockout of specific gene expressions (Ali *et al.*, 2010; Allen *et al.*, 2004; Gavilano *et al.*, 2006; Johansen and Carrington, 2001; Kusaba *et al.*, 2003; Liu *et al.*, 2002; Meli *et al.*, 2010; Pandolfini *et al.*, 2003; Segal *et al.*, 2003; Smith *et al.*, 2000; Xiong *et al.*, 2005). In addition, a number of studies showed that IR constructs of transgenes containing virus-derived sequences transformed to plants can effectively protect plants from challenging viruses (Abhary *et al.* 2006; Bucher *et al.* 2006; Chen *et al.*, 2004; Di Nicola-Negri *et al.* 2005; Hammond *et al.* 2006; Lennefors *et al.* 2006; Pooggin *et al.* 2003; Simón-Mateo and García, 2011; Tenllado *et al.* 2003; Tenllado *et al.*, 2004; Vanitharani *et al.*, 2003; Wang *et al.*, 2000). In particular, transient expression of a hpRNA homologous to sequences of *Pepper mild mottle virus* (PMMoV) caused inhibition of PMMoV accumulation (Tenllado *et al.*, 2003). *A. tumefaciens*-mediated transient expression of a hpRNA derived from the triple gene block (TGB) p1 of *Potato virus X* (PVX) induced RNA silencing of the TGB p1 gene and resulted in the interference of PVX infection (Takahashi *et al.*, 2006). Transient expression of a CP hairpin RNA also induced interference of PVX. The TGBp1 hpRNA showed more efficient interference of PVX infection than the CP hpRNA, but the interference was induced in the infiltrated leaves but not in the upper non-infiltrated leaves. *A. tumefaciens*-mediated transiently expression of a hpRNA homologous to the CP gene of *Potato virus Y* (PVY) showed a complete and specific interference with aphid transmission of PVY in leaf tissues of *N. benthamiana* (Vargas *et al.*, 2008).

Although a viral silencing suppressor of BBWV2 has not been directly identified so far, it is worthwhile to mention that BBWV2 SCP may act as a suppressor of RNA silencing suppressor, as demonstrated by *Cowpea mosaic virus* (CPMV) SCP (Cañizares *et al.*, 2004). It suggested that the CPMV SCP is involved in binding to siRNAs in a manner analogous to p19 of tombusviruses, since the silencing suppressor activity of CPMV SCP requires the C-terminal residues that interact with RNA (Cañizares *et al.*, 2004). In this view, our siRNA detection resulted from Cont2-plants suggests that BBWV2 SCP can strongly bind viral siRNAs generated in the process of infection (Fig. 2C). Interestingly, our result suggests that BBWV2 SCP does not appear to inhibit signaling

of RNA silencing induced by the IR-BBWV2 construct. In the pIR-BBWV2, the region homologous to BBWV2 RNA 1 includes the 5' NCR portion before the AUG initiation codon of the first BBWV2 polyprotein coding region (Fig. 1A). In general, since 5' NCR in positive-sense single strand RNA viruses contains promoter sequences that are crucial for the synthesis of negative-sense strands, it is plausible that transient expression of IR-BBWV2 efficiently interferes with replication process of BBWV2 genomic RNAs. The inhibition of replication process of BBWV2 RNA 1 significantly caused poor translation of the BBWV2 polyprotein encoding RNA-dependent RNA polymerase. Considering that hpRNAs less than 100 nts in length are usually efficient in eliciting RNA silencing (Wesley *et al.*, 2001) and that viral RNA genomes are usually several kb long, it would be feasible to design several different hpRNAs from the sequences of BBWV2 genomic RNAs. RNA silencing is an adaptive mechanism of defense and the viral genome is both a target for RNA degradation and a template for amplification of RNA silencing. Thus, resistance results from the interplay of host mechanisms and virus inoculum and replication. Damage and economic losses in pepper production, Korea, are due to systemic BBWV2 infection and disease (Lee *et al.*, 2000; Cho *et al.*, 2007; Choi *et al.*, 2001; Choi *et al.*, 2005). To our knowledge, conventional breeding has limited to produce commercial varieties of BBWV2-resistant pepper. We expect that the IR-BBWV2 construct can be used to obtain transgenic crops that are highly resistant to BBWV2. These transgenic pepper plants will contribute to reducing damage from the virus.

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