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Use of silencing reporter and agroinfiltration transient assays to evaluate the potential of hpRNA construct to induce multiple tospovirus resistance

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Abstract

Tospoviruses are devastating plant viruses causing severe economic losses in a diverse range of crops worldwide. Here, we describe the development and evaluation of an RNA interference (RNAi) broad-spectrum virus resistance strategy based on a unique and short hairpin-RNA-generating construct (pNhpRNA). This construct was designed from a region of the nucleocapsid gene (*N*) of *Tomato spotted wilt virus* (TSWV) that showed a high sequence identity to the corresponding region in the related species *Groundnut ringspot virus* (GRSV) and *Tomato chlorotic spot virus* (TCSV). To test the effectiveness of the pNhpRNA construct, we developed a silencing reporter assay based on three fusion proteins in which the complete viral *N* gene sequence from each of the three tospoviruses was fused in frame to the green fluorescent protein (GFP) sequence. Co-agroinoculation of these constructs with pNhpRNA into leaves of *Nicotiana benthamiana* resulted in a strong silencing phenotype determined by GFP decay and suppression of the three *N* genes at the RNA and protein levels. To test the potential of the pNhpRNA construct to generate virus-resistant plants, we infiltrated the whole shoots of *N. benthamiana* with pNhpRNA. When these infiltrated plants were mechanically inoculated with the mentioned viruses 100, 70, and 60 % resistance phenotypes to TSWV, GRSV, and TCSV, respectively, were observed. The induction of a broad tospovirus resistance with a simple construct and a minimized off-target effect are the main contributions of pNhpRNA.

Additional key words: broad-spectrum resistance, *Nicotiana benthamiana,* tobacco, transgene evaluation.

Introduction

In plants, RNA interference (RNAi), which is based on sequence-specific degradation of RNA molecules, is an important mechanism of defense against viruses (Waterhouse *et al.* 2001, Ritzenthaler 2005). This mechanism can be exploited to produce transgenic virus-resistant plants by introducing an inverted repeated viral sequence in a chimeric construct. The transcripts of the transgene form double-stranded RNA (dsRNA), the so-called "hairpin" RNA (hpRNA), which is recognized by the plant RNA silencing machinery. In this pathway, dsRNA is processed by specific nucleases, mainly Dicer-Like-2, into small interfering RNAs (siRNAs) 21 - 24 nt long. These siRNAs are recruited to the RNA-induced silencing complex (RISC) which guides the cleavage of homologous RNA molecules (Eamens *et al.* 2008). This process can inhibit viral infection resulting in a resistance equivalent to immunity (Dietzgen and Mitter 2006). The mechanism of RNAi has been well studied, and the RNAi strategy has been used to generate resistance to a broad range of viruses in many host plants (reviewed in Auer and Frederic 2009, Simon-Mateo and García 2011, Duan *et al.* 2012).

Tospoviruses are among the ten most devastating viruses worldwide and cause substantial economic losses in vegetable and ornamental crops (Prins and Goldbach 1998). Members of the virus genera *Tospovirus, Cytorhabdovirus, Nucleorhabdovirus, Dichorhabdovirus, Ophiovirus, Tenuivirus, Varicosavirus*, and *Emaravirus* are all negative sense single stranded (ss) RNA viruses that infect plants (Kondo *et al.* 2006, Kormelink *et al.*

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Abbreviations: GFP - green fluorescent protein; GRSV - *Groundnut ringspot virus*; hpRNA - hairpin RNA; RISC - RNA-induced silencing complex; RNAi - RNA interference; TCSV - *Tomato chlorotic spot virus*; TSWV - *Tomato spotted wilt virus.*
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2011, Mielke-Ehret and Mühlbach 2012). Negative sense ssRNA viruses have an anti-sense genome that is transcribed by their own RNA-dependent RNA polymerase into positive sense RNA. This complicated transcription process means that it is difficult to use purified viral RNA as infectious material and to generate full-length infectious clones. Consequently, there are significant methodological restrictions in studying these viruses. The genus *Tospovirus* is the only plant-infecting genus in the family *Bunyaviridae*. Species in this genus have a single-stranded RNA genome consisting of three RNA segments; small (S), medium (M), and long (L) RNAs. The S RNA encodes a nucleocapsid protein (N), which has also been involved in intracellular movement (Feng *et al*. 2013), and a non-structural protein (NSs) that functions as RNA-silencing suppressor (Takeda *et al*. 2002) and determinant for thrips transmission (Margaria *et al*. 2014). The M RNA encodes precursors of glycoproteins (Gn and Gc) (Sin *et al*. 2005) and a non-structural protein (NSm) involved in cell-to-cell movement and hypersensitive response to tomato resistance (R) gene-encoded protein Sw-5 (Hallwas *et al*. 2014). The L RNA segment encodes an RNA-dependent RNA polymerase (Kormelink *et al.* 2011).

Because of serious losses caused by this broad group of viruses, the development of virus-resistant plants is a promising strategy for disease control. This is the case for tomato and pepper since R-gene based resistance to *Tomato spotted wilt virus* (TSWV) has been successfully applied in commercial cultivars. The R-genes utilized in these crops are the NBS-LRR class *Sw5b* and *Tsw* genes, respectively (Black *et al.* 1991, Stevens *et al.* 1991, Boiteux 1995). However, this conventional breeding strategy is restricted to tomato and pepper. Several research groups have produced tospovirus-resistant plants based on pathogen-derived resistance, mainly specific to the type member of the genus, TSWV. For example, tospovirus-resistant plants are produced by expressing the N or NSm proteins in transgenic tobacco, tomato, and peanut (Prins *et al.* 1995, 1997, Ultzen *et al.* 1995, Li *et al.* 1997). The expression of short peptides (Rudolph *et al.* 2003), and truncated or antisense RNAs corresponding to the *N* gene in tobacco and chrysanthemum yield virus-resistant plants (Prins *et al.* 1996, Sherman *et al.* 1998). Short *N* RNA regions, chimeric transcripts of *N* gene regions of related species, and chimeric "L" sub-genome derived hairpins have also been used to produce multiple tospovirus-resistant plants (Pang *et al.* 1997, Jan *et al.* 2000, Bucher *et al.* 2006, Lin *et al.* 2011, Peng *et al*. 2014).

Because of the sequence-specific nature of RNAi, the engineered construct must contain sequences of each virus target to confer resistance. This is a major limitation in the applications of pathogen-derived resistance. This constraint is particularly challenging when a short single chimeric transgene construct is preferred, but several virus species are present in the field. This is the case in Argentina, where TSWV, *Groundnut ringspot virus* (GRSV), and *Tomato chlorotic spot virus* (TCSV) which have not been reported to cause mixed infections, are present in the same geographical area and share many horticultural and ornamental crops as hosts (Gracia *et al.* 1999, Williams *et al.* 2001).

Agroinfiltration (Schöb *et al.* 1997) has been employed to test the ability of a hairpin construct to generate resistance to a tobamovirus and a potyvirus in a local assay, involving the infiltration of an *Agrobacterium tumefaciens* culture harboring a hairpin transgene construct, followed by virus inoculation and analysis of local and systemic symptoms (Tenllado and Díaz-Ruíz 2001, Vargas *et al.* 2008). Here, we describe the development of a dual transient platform combining a silencing reporter system and a whole-plant agro-infiltration method to induce and evaluate virus resistance to TSWV, GRSV, and TCSV in *N. benthamiana*. We designed an hpRNA construct (pNhpRNA) targeting a region of the *N* gene sequence conserved among the three tospovirus species (79.8 - 80.3 % sequence identity). In the silencing reporter assay, the targeted viral gene (*N* gene) fused in frame to a fluorescent reporter *GFP* gene was co-agroinoculated with the pNhpRNA construct resulting in a strong silencing phenotype determined by GFP decay and suppression of the three *N* genes at the RNA and protein levels. To test the ability of the pNhpRNA construct to generate transgenic virus-resistant plants, we adapted the "Magnifection" system (Marillonet *et al.* 2005) by agroinfiltrating shoots with an *A. tumefaciens* culture harboring the pNhpRNA construct.

Materials and methods

Cloning a binary vector containing a TSWV *N* gene hairpin construct: Full-length protein N coding sequences of TSWV, GRSV, and TCSV were aligned to identify conserved regions (Fig. 1 Suppl., bases 418 - 590). We selected a 173-nt region from the *N* gene of TSWV that showed 79.8 and 80.3 % identity to the corresponding region of the TCSV *N* gene and the GRSV *N* gene, respectively. This region showed a higher sequence similarity than the complete *N* gene sequence among the three viruses (the complete *N* gene sequence of TSWV showed 75.6 % identity to that of TCSV, and 76.9 % to that of GRSV). The 173-nt *N* region was amplified by PCR using a pair of primers that inserted restriction sites to allow cloning the fragment in both orientations. The forward primer sequence was 5´-AGC ACAGTGCAAACTTTCCCT-3´ (*Kpn*I or *Sac*I sites added to the 5' end) and the reverse primer sequence was 5´-GTGAGGCTTGCCATAATGCTG-3´ (*Xho*I or *Bam*HI added to the 5' end). The amplified *N* fragment was cloned in sense and antisense orientations into a

pBlueScript KS vector flanking both sides of a 190-nt sequence of the castor bean catalase modified intron (accession No. AF234293). The complete *Kpn*I-*Sac*I unit containing the *N* gene region in the sense orientation, the *Cat1* intron, and the antisense *N* region were excised and inserted into the binary vector pCambia2301. This vector, containing β-D-*glucuronidase* and *NPTII* genes (acc. No. AF234316; http://www.cambia.org/), was modified to incorporate a *35S* promoter and the *NOS* terminator, between the H*ind*III and E*co*RI in the multi-cloning site. The *Kpn*I-*Sac*I unit was incorporated between the 35S promoter and the NOS terminator. The make-up of the binary vector was checked by digestion with restriction enzymes and sequencing. The resulting plasmid was denoted pNhpRNA and contained the inverted-repeat conserved 173-nt region of the TSWV *N* gene.

Silencing reporter assay: We developed three different silencing reporter constructs containing the N protein genes of TSWV, GRSV, or TCSV fused in frame to the *GFP* gene. The complete *N* sequence of the three tospoviruses was amplified and modified by PCR using primers (forward: 5′-CCATGGCTAAGGTTAAGCTC ACTAAG-3′ and reverse: 5′-AGATCTGCAAGTTCT GCAAGTTTTGCC-3′ for TSWV; forward: 5′-CCA TGGTCAAGCTCACAAAAGAAAAC-3′ and reverse: 5′-AGATCTGCAACACCAGCAATCTTGGC-3′ for GRSV; and forward: 5′-CCATGGCTAAGGTCAAGC TCACCAGA-3′ and reverse: 5′-GGATCCGCAACA CCTGAAATTTTGGCTT-3′ for TCSV). All three resulting PCR products had the first two coding triplets of the *N* gene replaced by the *Nco*I restriction site and the translation stop codon was eliminated. After cloning these fragments into pTOPO2.1, their sequences were verified. Then, the inserts were excised with *Nco*I and *Bgl*II (TSWV and GRSV) or *Nco*I and *Bam*HI (TCSV) and incorporated into the binary vector pCambia1302 containing the *GFP* gene and the *NPTII* gene (acc. No. AF234298.1) fused to the open reading frame of *GFP*. The binary vectors pTSWV-N:GFP, pTCSV-N:GFP, and pGRSV-N:GFP were verified by digestion with appropriate restriction enzymes and sequencing. *Agrobacterium tumefaciens* L. (strain GV2260) cells were transformed by electroporation with pNhpRNA, pTSWV-N:GFP, pGRSV-N:GFP, pTCSV-N:GFP, pCambia1302, and pCambia2301.

For leaf infiltration, cultures of transformed *A. tumefaciens* were inoculated into 5 cm^3 of a Luria-Bertani (LB) medium containing 50 mg dm^{-3} kanamycin and grown overnight at 28 °C with shaking at 200 rpm. The bacterial cultures were harvested by centrifugation at 4 800 *g* and 24 °C for 15 min. The pellets were re-suspended in an infiltration medium (10 mM $MgCl_2$ + 10 mM MES, pH 5.5) and the concentration was adjusted to absorbance A_{600} = 1.00. The re-suspended *A. tumefaciens* cells were kept at room temperature for 2 h, then mixed at a 1:1 ratio [pTSWV-N:GFP + pNhpRNA; pTSWV-N:GFP + empty vector (pCambia2301); pTCSV-N:GFP + pNhpRNA; pTCSV-N:GFP + pCambia2301; pGRSV-N:GFP + pNhpRNA; pGRSV-N + pCambia2301; pNhpRNA + non-fused GFP construct (pCambia1302)] and infiltrated into tissues from the abaxial side of leaves of *N. benthamiana* at the five- to six-leaf stage using a syringe. The infiltrated leaves were harvested at 0, 2, 4, and 6 d post inoculation (dpi) and kept at -80 °C until further analysis by confocal microscopy, DAS-ELISA, and real-time quantitative PCR. A total of 15 biological replicates were evaluated for N transcript and N protein expression for each tospovirus treatment at respective evaluation time points. The comparisons were made between fusion constructs + empty vector *versus* fusion construct + pNhpRNA at each evaluation time by *ANOVA* using the *InfoStat* statistics software (Di Rienzo *et al.* 2011). Differences in treatments at specific evaluating times were considered significant for $P < 0.0001$; results from individual experiments are presented as means ± SE.

Whole-plant agroinfiltration: *N. benthamiana* plants were grown in a greenhouse at a temperature of 24 °C, a 16-h photoperiod, an irradiance of 300 μmol m^{-2} s^{-1}, and an air humidity of 70 % until they reached the five to six leaf stage. Cultures of *A. tumefaciens* strain GV2260 transformed with pNhpRNA were inoculated into 100 cm^3 of a LB medium with 50 mg dm^{-3} kanamycin and grown overnight at 28 °C with shaking at 200 rpm. The bacterial cultures were harvested by centrifugation (4 800 *g* and 24 °C for 15 min) and the pellet was re-suspended in 1 dm^3 of an infiltration medium (10 mM $MgCl_2$ + 10 mM MES, pH 5.5) to obtain a 10^{-1} dilution. A beaker containing the infiltration medium was placed in a vacuum chamber (30 cm diameter) with shoots immersed in the solution. Vacuum of 80 kPa was applied for 2 min and then the pressure was gradually returned to atmospheric pressure. The infiltrated plants were returned to the greenhouse and grown under standard conditions. Two days after infiltration, 2 leaves per plant of 120 infiltrated plants were mechanically inoculated with TSWV. The inocula were prepared from infected *Nicotiana tabacum* plants ground in a 10 mM phosphate buffer 1:5 (m/v) with 0.1 % (m/v) sodium sulphite. The same procedure was followed with GRSV and TCSV assays. Two types of controls were performed; 3 sets of 30 whole agroinfiltrated plants with empty vectors challenged with TSWV, GRSV, and TCSW, respectively, and whole agroinfiltrated plants mock-inoculated. The assayed plants were monitored for virus-induced symptoms at 15 dpi and analyzed by DAS-ELISA and real-time quantitative RT-PCR and followed for symptom development for 30 d.

Confocal microscopy: Monitoring and imaging GFP fluorescence were conducted using a *Nikon eclipse* model *Ti* of confocal microscope (*Nikon*, Tokyo, Japan) and images were analyzed using the *Nis-Elements* software (*Nikon*). The excitation wavelength was 488 nm and emission was captured at 497 - 520 nm.

Viral N protein immunodetection: Virus-inoculated leaves from agroinfiltrated *N. benthamiana* were analyzed by DAS-ELISA with a polyclonal antiserum against the N proteins of TSWV, GRSV, and TCSV as described by Williams *et al.* (2001). Samples were analyzed at 0, 2, 4, and 6 dpi for the silencing reporter assay and at 15 dpi for the whole-plant agroinfiltration assay.

Real-time RT-qPCR: Total RNA was extracted from plant tissues using a *TRIzol* reagent (*Invitrogen*, Grand Island, NY, USA), according to the manufacturer's instructions. The real-time PCR experiments were performed in a *Rotor-Gene 6000* thermocycler (*Corbett Life Science*, Sydney, Australia). The amplification protocol was as follows: 60 min at 55 °C, 15 min at 95 °C, 40 cycles of 20 s at 94 °C, 20 s at the corresponding annealing temperature, 30 s at 72 °C followed by a plate read, then a melting curve of 50 to 95 °C with 0.2 °C steps, hold 2 s followed by a final extension step at 72 °C for 10 min.

To amplify viral *N* genes, we designed the following primers: TS-forward: 5′-GACTTTCAGAAGGCT TGATAG-3′ and TS-reverse: 5′-CCATAGGCTTGA ATCAAAGGA-3′ (TSWV-N), TC-forward: 5′-GAC TTTTAGAAGGCTTGATAG-3′ and TC-reverse: 5′-CCATAAGCTTGAACAAGAGGA-3′ (TCSV-N), GR-forward: 5′-GACTTTCAGAAGGCTTGATAG-3′ and GR-reverse: 5′-CGTAAGCTTGGACCAAAGGG-3′ (GRSV-N). Elongation factor 1 (*EF1*) was used as reference gene and it was amplified using a primer set and conditions validated previously (Yang *et al.* 2004). To confirm the presence or absence of the virus in inoculated plants by real-time PCR, RNA samples were amplified with the corresponding pair of primers (TS-F/TS-R; TC-F/TC-R; or GR-F/GR-R), and the cycle cut-off corresponding to the risk of a false positive result of 1 % (F(x) = 0.01) at 37.45 cycles was determined according to Chandelier *et al.* (2010). A Ct value lower than 37.45 indicated the virus presence, and a Ct value higher than 37.45 indicated the virus absence.

Results

We developed a construct using a sequence of the *N* gene conserved among TSWV, GRSV, and TCSV with the objective of generating tospovirus-resistant transgenic plants. The alignment of the *N* sequences of the three tospoviruses revealed a region of 173 nt with a relatively high sequence identity. The selected sequence was isolated from TSWV by PCR amplification. We generated an hpRNA construct (Fig. 1*A*) containing the conserved sequence amplified from the TSWV *N* gene cloned in the sense and antisense orientations, flanking both sides of the castor bean catalase intron sequence. This sequence was also selected due to its low predicted off-target potential, as determined by *in silico* software simulations. In our hands, the siRNA scan tool (Xu *et al.* 2006), using the *N. benthamiana* Gene Index of the Computational Biology and Functional Genomics Laboratory database at the Dana-Farber Cancer Institute (DFCI Gene Index) as target, determined 20 and 12 putative off-targets for the complete TSWV *N* gene and the three fused 150 bp fragments of TSWV, GRSV and TCSV *N* gene constructs, respectively (Prins *et al.* 1995, Bucher *et al.* 2006). However, when the siRNA scan analyzed the 173 bp conserved region of the pNhpRNA construct, the number of putative off-targets was strongly reduced to only one (Fig. 2 Suppl.).

To evaluate the effectiveness of the pNhpRNA construct in a transient expression system, we designed two different and complementary assays based on agroinfiltration. Firstly, we developed a GFP-silencing reporter assay to evaluate the effectiveness of pNhpRNA to silence the *N* gene of the three different tospoviruses. For this purpose, three constructs (pTSWV-N:GFP, pGRSV-N:GFP, and pTCSV-N:GFP) were constructed by fusing each of the target *N* genes in frame to the *GFP* gene (Fig. 1*A*). Each one of these constructs was co-agroinfiltrated with pNhpRNA into *N. benthamiana* leaves, and GFP fluorescence decay was evaluated as indicator of silencing the *N* gene of TSWV, GRSV, and TCSV (Figs. 1*B* and 2). An efficient and consistent GFP expression inhibition was observed by confocal microscopy at 4 dpi (Fig. 2) in the half of the *N. benthamiana* leaves co-agroinfiltrated with pNhpRNA and the fusion reporter constructs. The expression of GFP was inhibited by pNhpRNA not only when co-infiltrated with pTSWV-N:GFP which had a 100 % sequence similarity to the hpRNA region, but also with the related viral fusions pTCSV-N:GFP and pGRSV-N:GFP which had only 79.8 and 80.3 % sequence identities to the hpRNA region, respectively. The sequence-specific nature of the inhibition induction phenomenon was verified by the persistence of GFP expression when pNhpRNA was co-agroinfiltrated with the non-fused GFP construct (pCambia1302).

These leaves were further analyzed by DAS-ELISA with specific antibodies against the N proteins of TSWV, GRSV, and TCSV and by RT-qPCR specific to the respective *N*-gene sequences at 0, 2, 4 and 6 dpi. At 4 dpi, the N protein was not detected in any of the leaves co-agroinfiltrated with fusion reporters and pNhpRNA, whereas the N protein was detected from 2 dpi in the leaves co-agroinfiltrated with the fusion reporters together with the empty vector (Fig. 3*A*,*C*,*E*). Starting at 2 dpi, the amount of viral transcripts markedly decreased but were not completely absent in the leaves co-agroinfiltrated with the viral fusion constructs and pNhpRNA (Fig. 3*B*,*D*,*F*). Strong and uniform expression of the pNhpRNA construct was confirmed by GUS staining the infiltrated leaves (Fig. 3 Suppl.).

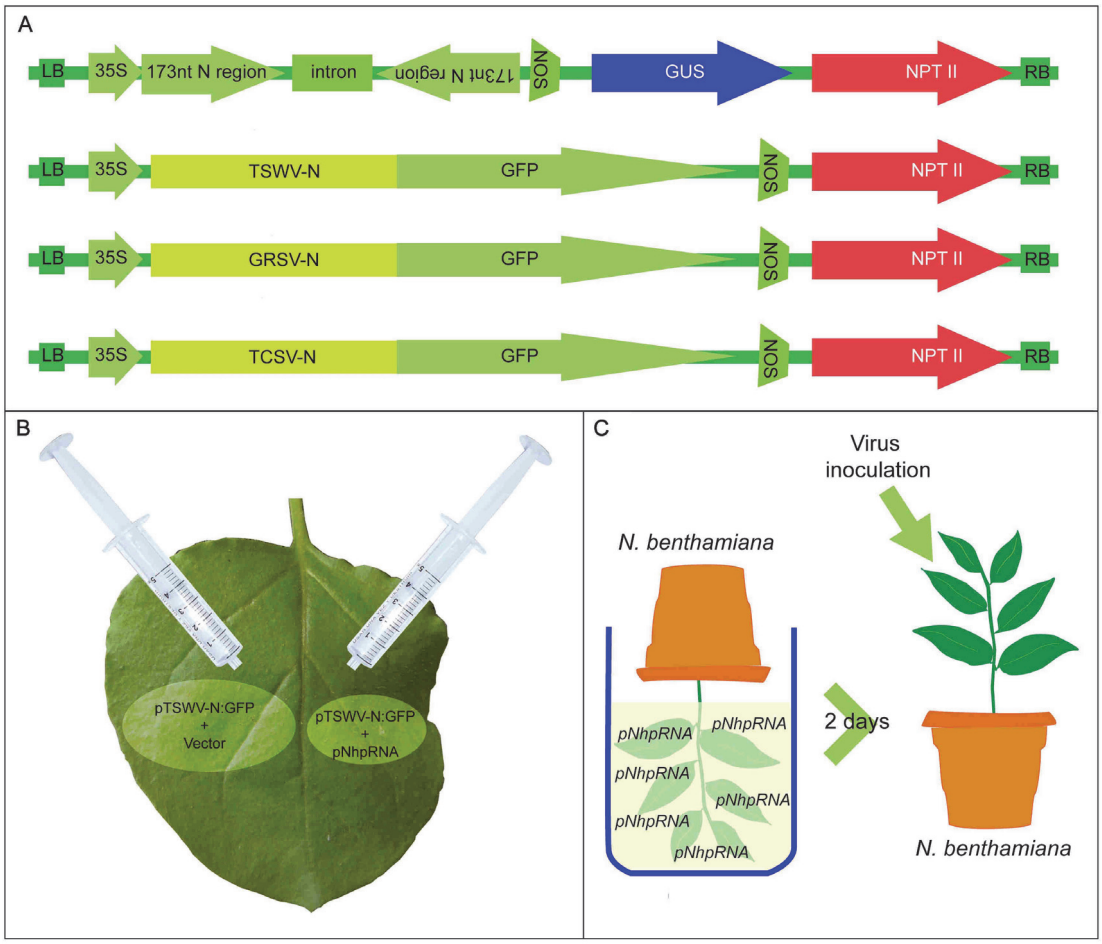

Fig. 1. *A* - Gene constructs to induce and evaluate multiple tospovirus resistance: pNhpRNA corresponds to a 173-bp conserved sequence of the *N* gene from TSWV cloned in sense and antisense orientations either side of the castor bean *Catalase* intron; TSWV-N:GFP, TCSV-N:GFP, and GRSV-N:GFP are the target *N* genes of each tospovirus fused to *GFP*; LB - left border, 35S - CaMV 35s promoter, NOS - nopaline synthase terminator, *GUS* - Gus reporter gene cassette, *NPTII* - neomycin phosphotransferase II gene, RB - right border. *B* - A schematic picture of a silencing reporter assay procedure in which one half of a leaf was co-agroinfiltrated with a silencing construct (pNhpRNA) and reporter fusion constructs (pTSWV-N:GFP, pTCSV-N:GFP, or pGRSV-N:GFP), and the other half of the leaf was co-agroinfiltrated with fusion constructs and the vector (pCambia2301). *C* - A schematic picture of a whole-plant agroinfiltration method. Shoots were submerged in *A. tumefaciens* cultures harboring pNhpRNA or a vector (pCambia2301) and subsequently mechanically inoculated with tospoviruses.

The second transient assay employed to assess the effectiveness of the pNhpRNA construct was the whole-plant agroinfiltration and subsequent tospovirus challenge. We developed a new rapid *in planta* assay in which shoots were infiltrated with *A. tumefaciens* harboring pNhpRNA or with an empty vector (in this case pCambia2301). Two days after whole-plant agro-infiltration, the plants were independently inoculated with the challenging tospoviruses. At 15 dpi, the inoculated plants were evaluated to confirm the presence or absence of viral infection by observation of symptoms, DAS-ELISA, and RT-qPCR analyses of the systemically virus-infected leaves (Figs. 1*C* and 4*A*). The strong and uniform expression of the pNhpRNA construct throughout the plant was confirmed by GUS staining assay of the multiple infiltrated leaf samples (Fig. 4 Suppl.). A high proportion of plants agroinfiltrated with pNhpRNA showed virus resistance. Among the agroinfiltrated plants, 100 % showed resistance to TSWV, 70 % were resistant to GRSV, and 60 % were resistant to TCSV. In contrast, all of the plants infiltrated with the empty vector showed typical tospovirus symptoms when inoculated with the respective viruses (Fig. 4*B-C* and Table 1). The absence of tospoviruses in plants with a healthy phenotype was confirmed by DAS-ELISA and RT-qPCR analysis (Table 1). Together, these results confirm the effectiveness of the pNhpRNA construct to induce multiple resistance to TSWV, GRSV, and TCSV in a transient platform.

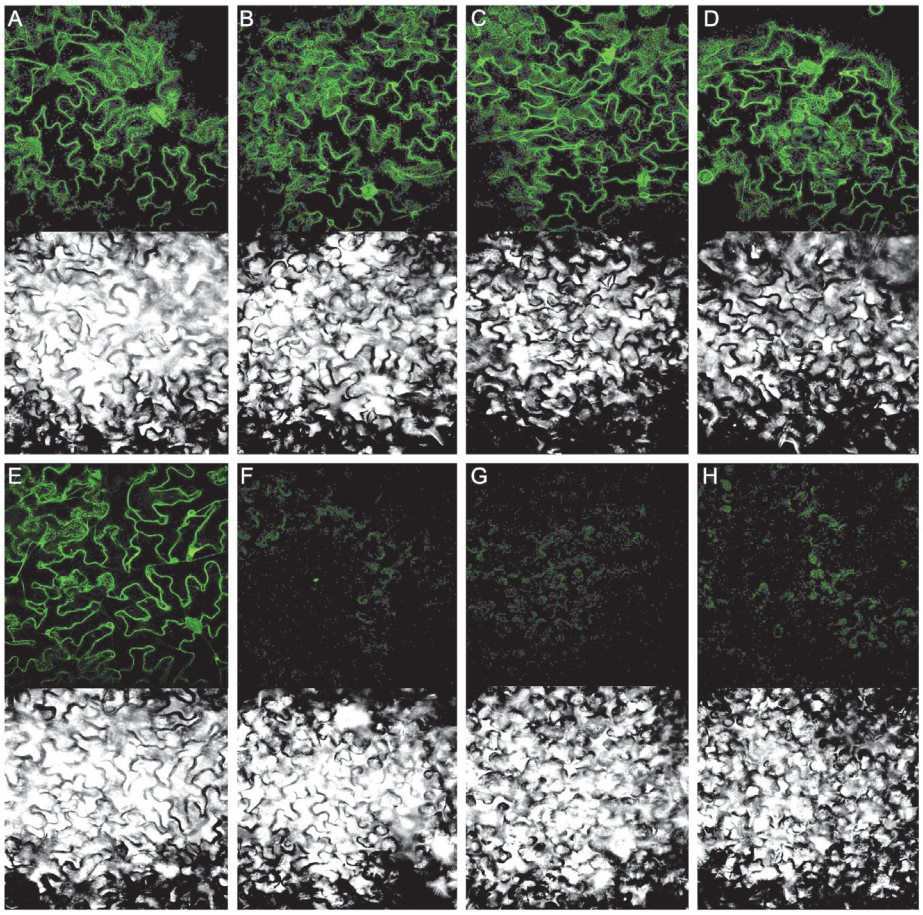

Fig. 2. Confocal microscopy of sections of co-agroinfiltrated plant leaves evaluated by the silencing reporter assay. The expression of GFP was observed in the following treatments: pGFP + Vec (pCambia1302 + pCambia2301) (*A*), pTSWV-N:GFP + Vec (*B*), pTCSV-N:GFP + Vec (*C*), pGRSV-N:GFP + Vec (*D*), and pGFP + pNhpRNA (*E*). Suppression of GFP expression indicating silencing the target *N* gene induced by pNhpRNA was observed in the following treatments: pTSWV-N:GFP + pNhpRNA (*F*), pTCSV-N:GFP + pNhpRNA (*G*), and pGRSV-N:GFP + pNhpRNA (*H*).

Discussion

Hairpin-RNA constructs have been widely and successfully used to generate virus resistance in numerous plant species (Waterhouse *et al.* 1998, Wesley *et al.* 2001, for review see Dietzgen *et al.* 2006, Xie *et al.* 2014). There are several reports of the use of the *N* gene to induce gene silencing and tospovirus resistance (Gielen, *et al.* 1991, Mackenzie and Ellis 1992, Herrero *et al.* 2000, Magbanua *et al.* 2000). In this study, we focused on multiple tospovirus resistance, since in Argentina, several tospoviruses occur in the same geographic area. The use of transgenic plants targeting only one tospovirus species would not be particularly useful when several viruses detrimentally affect crop. Accordingly, we designed an hpRNA construct from a sequence of the *N* gene conserved among three tospoviruses and tested the capacity of pNhpRNA to induce gene silencing and confer resistance to multiple tospoviruses. We reasoned that the length of the sequence of hpRNA to induce resistance to multiple viruses should be longer than 59 nt (the minimum length of the *N* gene of TSWV reported in the literature that is able to generate virus resistance), but not as long as the complete *N* gene sequence which may undergo a high off-target effect (Jan *et al.* 2000, Xu *et al.* 2006). The off-target effect of RNAi technology may have two possible outcomes; intended broadening the spectrum of a resistance phenotype to related viral species, and undesired silencing host genes with similar sequences, a phenomenon that has been described for sequences with an identity of over 70 % (Miki *et al.* 2005).

Our results show that pNhpRNA was effective in generating *N. benthamiana* plants resistant to TSWV, GRSV, and TCSV in transient assays and potentially should be equally capable of generating transgenic plants resistant to these three tospoviruses. Gaba *et al.* (2010) have reported that a broad-spectrum resistance to *Potato virus Y* is limited when identity between a target sequence and a hairpin sequence is less than 88 %, and Peng *et al.*

(2014) have reported that a heterologous hairpin-mediated resistance can be achieved to tospovirus species with sequence identity ranging from 66 - 69 %. We achieved gene silencing and virus resistance with target sequences showing less than an 81 % identity. It is worth noting that all of the whole plants agroinfiltrated with pNhpRNA, which shared a 100 % identity with the TSWV *N* gene, were resistant to TSWV. In contrast, only 60 and 70 % of the plants agroinfiltrated with pNhpRNA were resistant to TCSV and GRSV, the two virus species with a lower sequence identity in the *N* gene region. This apparent but not straightforward correlation between the level of resistance and the percentage identity between the hpRNA and the virus target sequence suggests that there was a more dynamic interaction between these homologous sequences and reinstated the importance of empirical testing gene silencing predicted targets. In a previous study, RNA-mediated virus resistance using the *NSs* and *NSm* gene sequences of TSWV was less effective than that using the *N* gene sequence against

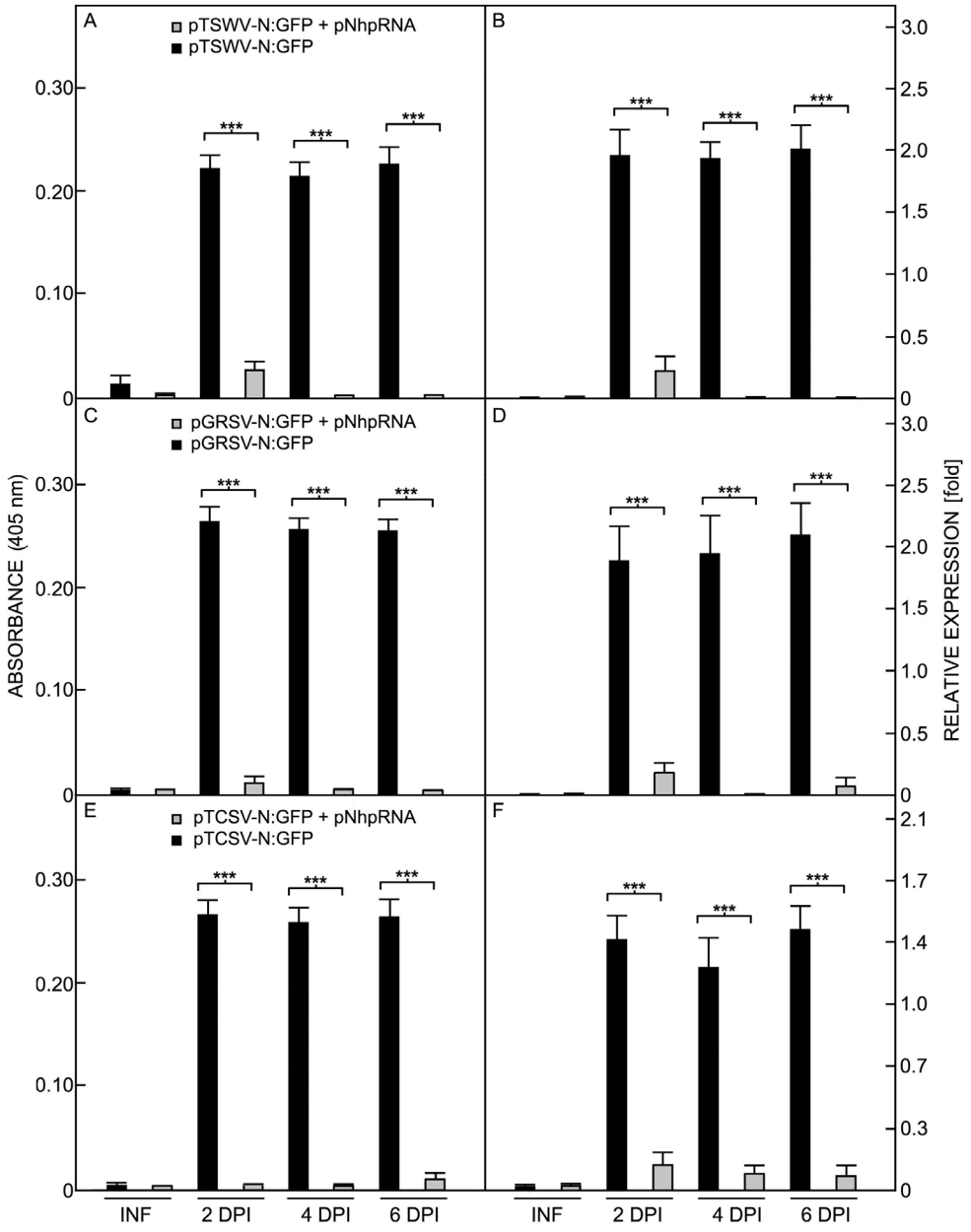

Fig. 3. A silencing reporter assay. The immunodetection by DAS-ELISA of the TSWV (*A*), GRSV (*C*) and TCSV (*E*) N protein in co-agroinfiltrated leaves at 0 (INF), 2, 4, and 6 dpi. The relative accumulation of TSWV (*B*), GRSV (*D*) and TCSV (*F*) *N* transcripts as determined by RT-qPCR at the same time points. The transcripts of the *N* gene were normalized to that of *EF1*. The comparison was made between the fusion construct + empty vector *versus* the fusion construct + pNhpRNA at each evaluation time point. Means ± SE of 15 biological replicates per treatment at each evaluation time point, *** - significant differences at $P < 0.0001$ determined by *ANOVA*. *Black bars* - fusion reporter construct + empty vector (pCambia2301); *grey bars* - fusion reporter construct + pNhpRNA.

TSWV (Sonoda *et al.* 2003). These results suggest that RNA-mediated virus resistance is not only defined by the degree of sequence homology between a gene-silencing-inducing construct and a virus target, but may be also affected by other factors such as structures of the target genome that determines RISC accessibility (Duan *et al.* 2008).

There are several studies on the persistence of plant virus resistance mediated by artificial micro RNAs (amiRNAs) (Simon-Mateo and García 2006, Lafforque *et al.* 2011, 2013), but few studies on the longevity of hpRNA-mediated resistance. The spontaneous mutation rate in the amiRNA target of a plant virus has been estimated to be approximately 6×10^{-5} per replication event (De la Iglesia *et al.* 2012). In this system, viral evolution in fully susceptible plants results from an equilibrium between mutation and genetic drift, whereas evolution in partially resistant plants originates from more complex dynamics involving mutation, selection, and drift (Martinez *et al.* 2012). We suggest that the use of hpRNA constructs with conserved sequences is a better strategy to develop virus-resistant transgenic plants. Since the putative target sequence is longer in hpRNAs than in short amiRNAs, the hpRNA strategy reduces the potential escape of emerging viral mutants.

It is becoming more evident that gene target selection and sequence identity constraints should be determined empirically in order to develop transgenic viral resistant plants (Mubin *et al.* 2011). Here, we describe two improved complementary and versatile techniques to evaluate the ability of a candidate construct to induce virus resistance, in this case, to multiple tospoviruses in

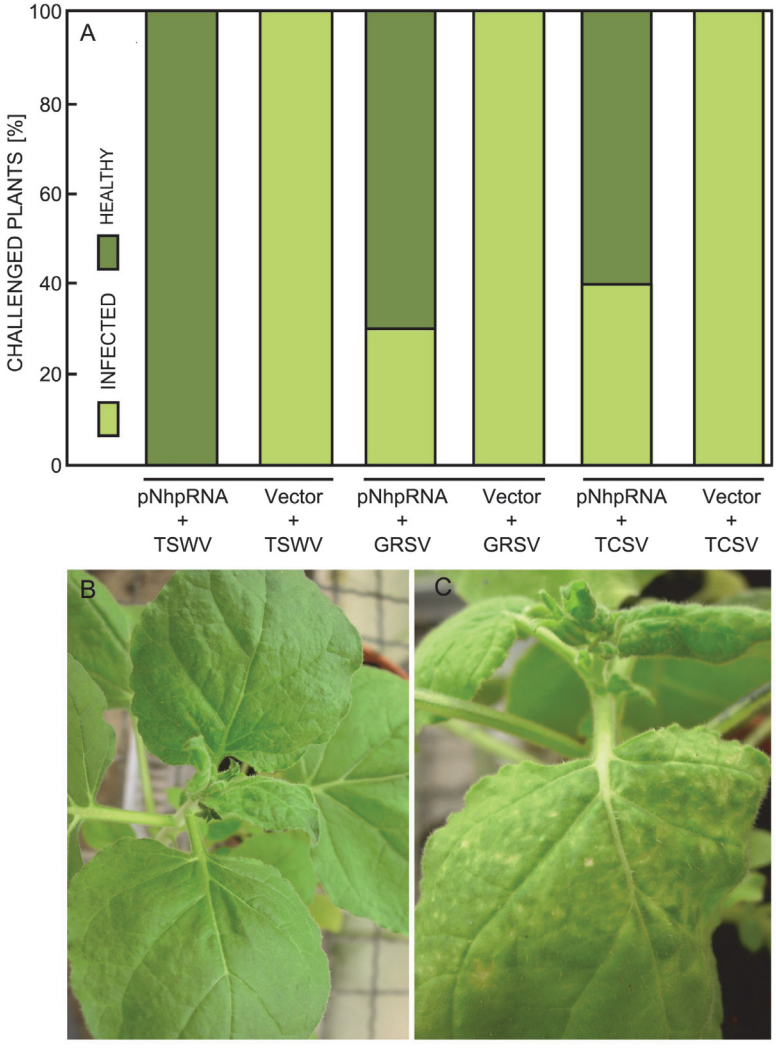

Fig. 4. *A* - The generation of virus-resistant plants by whole-plant agroinfiltration: *light colour bars* - percentage of tospovirus-infected plants at 15 dpi; *dark colour bars* - percentage of uninfected plants after agroinfiltration of aerial parts with pNhpRNA or an empty vector (pCambia2301) and tospovirus inoculation at 15 dpi. The virus infection was determined by evaluation of viral symptoms, DAS-ELISA, and RT-qPCR of systemic leaves. Representative plants of *N. benthamiana* showing no symptoms (pNhpRNA + TSWV inoculated) (*B*) and a symptomatic plant (vector + TSWV inoculated) (*C*).

Table 1. Tospovirus challenge experiments in the whole-plant agroinfiltration assay. * - Gene construct agroinfiltrated and virus species inoculated; † - the number of symptomatic plants/total plants assayed (percentage of plants showing tospovirus symptoms); ‡ - the number of plants tested positive by DAS-ELISA/total plants assayed (percentage of plants tested as positive by DAS-ELISA assay); § - the number of plants in which *N* gene transcripts were detected by qRT-PCR/total number of plants evaluated (percentage of positive plants in RT-qPCR analysis).

Construct + virus*	Symptoms†	DAS-ELISA‡	qRT-PCR§
Vector + TSWV	30/30 (100 %)	30/30 (100 %)	30/30 (100 %)
Vector + GRSV	30/30 (100 %)	30/30 (100 %)	30/30 (100 %)
Vector + TCSV	30/30 (100 %)	30/30 (100 %)	30/30 (100 %)
pNhpRNA + TSWV	0/120 (0 %)	0/120 (0 %)	0/120 (0 %)
pNhpRNA + GRSV	35/120 (30 %)	35/120 (30 %)	35/120 (30 %)
pNhpRNA + TCSV	47/120 (40 %)	47/120 (40 %)	47/120 (40 %)

N. benthamiana. The silencing reporter assay can be used to confirm the ability of a construct to inhibit gene target expression, whereas the whole-plant agro-infiltration method can be used to test the potential of a construct to induce resistance against one or more viruses. The first approach to evaluate the silencing-inducing ability of a pNhpRNA construct using GFP-target fusion constructs is rapid and reliable. We performed a preliminary experiment to evaluate our pNhpRNA construct by local agroinfiltration, and then challenged the plants with TSWV, GRSV, and TCSV according to the protocol described by Tenllado and Diaz-Ruiz (2001). In this experiment, 100 % of the plants were infected with the tospoviruses (data not shown) so we assumed that the 4-d lag between the infiltration and virus inoculation was exceedingly long. Then, we speculated that the silencing machinery of the plant could be boosted to interfere with viral transcripts by infiltrating the whole aerial parts of the plant simultaneously. This strategy resulted in a high-synchronized expression of pNhpRNA. We anticipated that multiple transformation foci throughout the plant would enhance the silencing response as result of multiple short-distance signalling events through cell-to-cell movement of the silencing signal (Himber *et al.* 2003). We suggest that the whole-plant agroinfiltration method has a higher resemblance to a stable transgenic state than a localized agroinfiltration, and that it allows for a rapid evaluation of several constructs in *N. benthamiana*.

The generation of tospovirus-resistant cultivars using a biotechnological technique is an important development from several viewpoints. Firstly, from an economic perspective, since these viruses cause substantial crop losses, and several resistance-breaking isolates have already been reported in different countries (Thompson and Van Zijl 1995, Latham and Jones 1998, Canady *et al*. 2001, Aramburu and Marti, 2003, Ciuffo *et al*. 2005, Gordillo *et al*. 2008, Zaccardelli *et al*. 2008). Secondly, from an environmental perspective, because tospoviruses are currently controlled by targeting their thrips vectors with insecticides. Thirdly, from a biological perspective, because this strategy can be useful to analyse the intriguing biochemical and biological uniqueness of this virus genus (Scholthof *et al.* 2011). Both techniques presented here provide a transient platform for rapid and efficient testing the effectiveness of a genetic construct to generate plant virus resistance before committing to the arduous and challenging work of producing stable transgenic plants.

The selection of the most appropriate strategy in order to generate virus resistant transgenic plants its extremely challenging due to the diversity of approaches and a prolonged time required for efficacy, durability, and safety assessment. In this direction, the dual transient platform presented here allows rapid testing a candidate transgene construct for virus resistance before committing to the arduous and challenging task of producing stable transgenic plants.

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