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Short Communication

Multiple virus resistance at a high frequency using a single transgene construct

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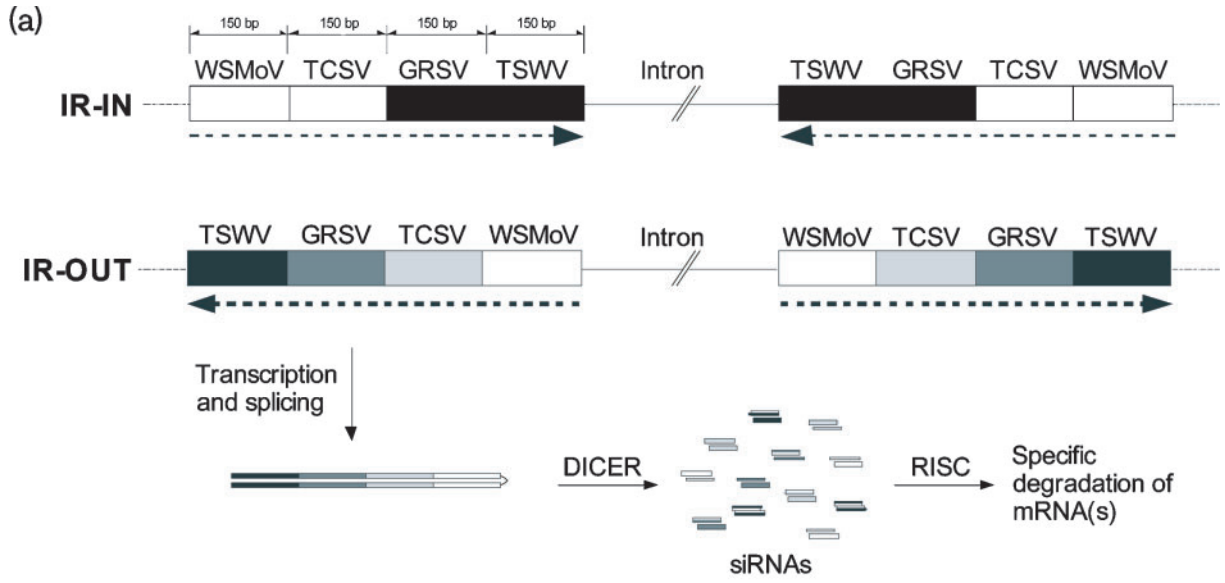
RNA silencing is a natural antiviral defence in plants, which can be exploited in transgenic plants for preprogramming virus recognition and ensuring enhanced resistance. By arranging viral transgenes as inverted repeats it is thus possible to obtain strong repression of incoming viruses. Due to the high sequence specificity of RNA silencing, this technology has hitherto been limited to the targeting of single viruses. Here it is shown that efficient simultaneous targeting of four different tospoviruses can be achieved by using a single small transgene based on the production of minimal sized chimaeric cassettes. Due to simultaneous RNA silencing, as demonstrated by specific siRNA accumulation, the transgenic expression of these cassettes rendered up to 82 % of the transformed plant lines heritably resistant against all four viruses. Thus RNA silencing can be further improved for high frequency multiple virus resistance by combining small RNA fragments from a series of target viruses.

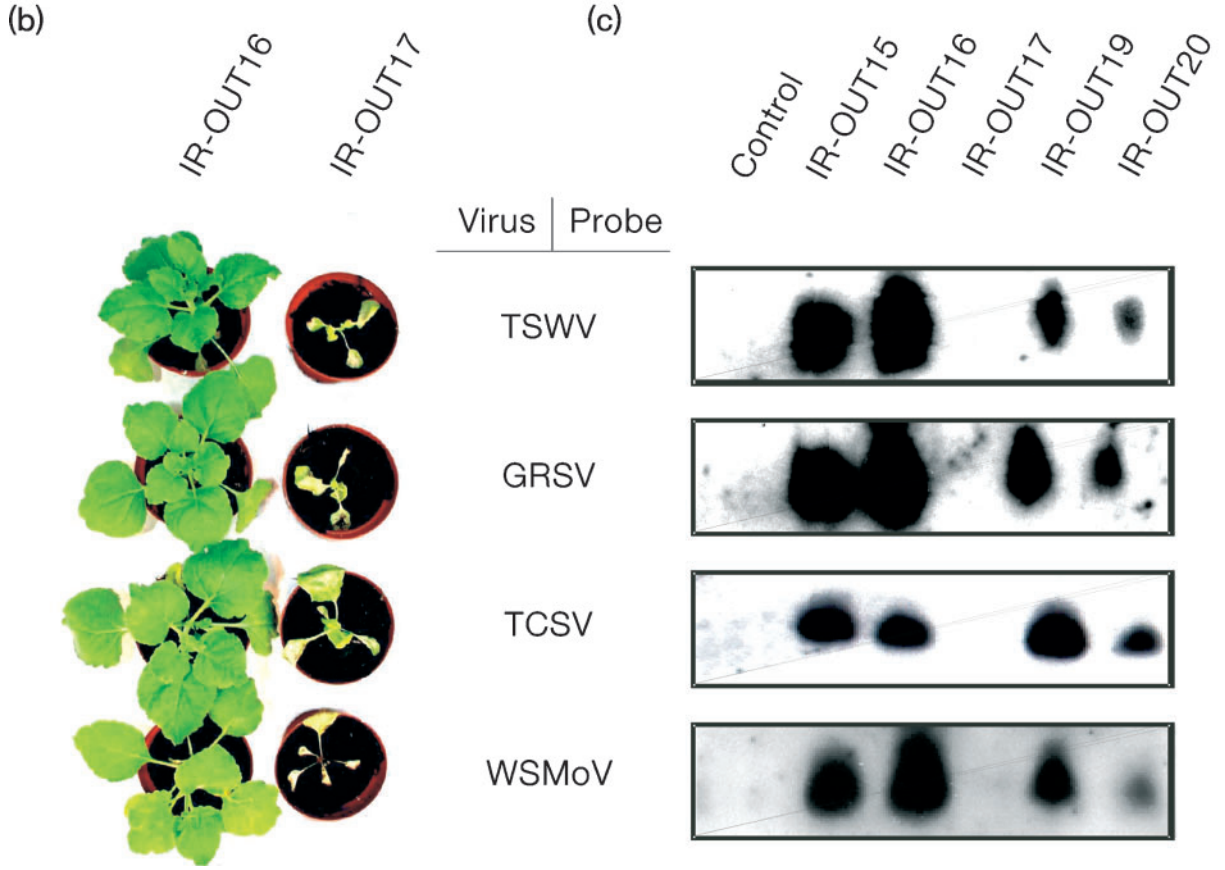
In plants, RNA silencing has been an important tool to render plants resistant to a large range of viruses (reviewed by Tenllado *et al.*, 2004). Originally, sense- or antisense-mediated RNA silencing resulted in a maximum resistance frequency of 20 %, but often far lower frequencies were obtained (Smith *et al.*, 2000). Moreover, not all viral genes used in transgenic constructs rendered plants resistant. A significant improvement in RNA silencing could be obtained by introducing inverted repeat transgenes, resulting in double-stranded RNA transcripts (dsRNAs). Compared to the originally used (single) sense transgenes, these dsRNAs render the system very efficient in that a much higher frequency of transformed plant lines expressing such dsRNA constructs will display efficient gene knockdown or virus resistance (Smith *et al.*, 2000; Waterhouse & Helliwell, 2003). It is perceived that this is due to the dsRNAs being fed directly into the silencing pathway at the level of the RNaseIII-like enzyme Dicer and therefore there is no reliance on the action of plant-encoded RNA-dependent RNA polymerase proteins, such as RDR6, required for the production of dsRNAs in (single) sense transgene-induced RNA silencing (Beclin *et al.*, 2002). Using inverted repeats, it has proved possible to use transgenes that had previously seemed unsuitable for sense RNA-mediated silencing (Chen *et al.*, 2004).

Tospoviruses rank among the most detrimental plant viruses worldwide, causing significant economic losses in the cultivation of vegetables and ornamental crops (Prins & Goldbach, 1998). Previous work in transgenic plants has shown that from all genes of *Tomato spotted wilt virus* (TSWV) only the N and NS_M gene constructs resulted in resistance, albeit at low frequencies (Prins *et al.*, 1996). Subsequent work (Pang *et al.*, 1997; Jan *et al.*, 2000) showed that sequences as short as 110 nt from the TSWV N gene were sufficient to efficiently induce RNA silencing, and therefore virus resistance, but only when fused to a carrier mRNA such as green fluorescent protein (GFP).

To obtain broad (tospo)virus resistance at a high frequency, a new approach was taken, which is described here. We aspired to use N gene sequence fragments of the four major tomato-infecting tospoviruses, TSWV, *Groundnut ringspot virus* (GRSV), *Tomato chlorotic spot virus* (TCSV) and *Watermelon silver mottle virus* (WSMoV), in a single small chimaeric hairpin (hp) RNA construct. As the overall homologies of the N genes of the tospoviruses used in this study range between 40 and 80 % (Prins & Goldbach, 1998), effective RNA silencing against one virus is insufficient to protect against the other viruses (De Haan *et al.*, 1992). Primers were designed to amplify sequential 150 bp N gene segments of TSWV, GRSV, TCSV and WSMoV and to overlap where the segments of the cassette should be fused (see Table S1, available as supplementary material in JGV Online). After a first round of PCR, the products were used in another round of PCR, resulting in the fusion of the two segments. Using this technique, the four segments were fused, resulting in a chimaeric N gene cassette of 600 bp. Successive parts of the tospoviral N genes were chosen to

A table of primer sequences used in fusion-PCR is available as supplementary material in JGV Online.


(a)
150 bp
150 bp
150 bp
150 bp
WSMoV
TCSV
GRSV
TSWV
Intron
TSWV
GRSV
TCSV
WSMoV
IR-IN
IR-OUT
TSWV
GRSV
TCSV
WSMoV
Intron
WSMoV
TCSV
GRSV
TSWV
Transcription and splicing
DICER
RISC
Specific degradation of mRNA(s)
siRNAs


(b)
IR-OUT16
IR-OUT17
(c)
Control
IR-OUT15
IR-OUT16
IR-OUT17
IR-OUT19
IR-OUT20
Virus
Probe
TSWV
GRSV
TCSV
WSMoV

reduce the risk of intramolecular homologous recombination either in bacteria or plants. To prevent possible complications due to translatability of the transgene, the start codon sequence was omitted in the amplified sequence, while subsequent potential start codons were out-of-frame. Primers MH17–MH20 (Table S1, available as supplementary material in JGV Online) were used to amplify the 500 bp intron sequence of the *Arabidopsis thaliana* actin2 gene, and the quadruple chimaeric tospovirus cassettes, flanked by *Bam*HI and *Not*I sites, were cloned into inverted repeat (IR) arrays around the intron as described previously (Chen *et al.*, 2004), resulting in the sense-antisense IR-IN and the antisense-sense IR-OUT (Fig. 1a). The intron was inserted to stabilize the construct during cloning and to enhance the effectiveness of the transcript in inducing hpRNA-mediated silencing (Smith *et al.*, 2000). The IR constructs were cloned into a modified pBIN19 binary vector containing the restriction sites *Asc*I and *Pac*I between the right and left border. Finally the binary vector was transformed into *Agrobacterium tumefaciens* LBA 4404. Plasmid DNA was rescued from *A. tumefaciens* and sequenced. *A. tumefaciens* clones were finally used to transform small leaf explants of *Nicotiana benthamiana*, as described by Horsch *et al.* (1985). Each resulting transgenic plant line originated from a single independent callus.

Sixteen independent IR-OUT and 16 IR-IN lines of transgenic *N. benthamiana* were generated and their self-fertilized seeds (S1 progeny) were harvested. Following selective germination on kanamycin (100 mg l^{-1}), progeny plants were transferred to the greenhouse and challenged with the tospoviruses TSWV, GRSV, TCSV and WSMoV either separately or in combination. Isolates of TSWV, GRSV, TCSV and WSMoV as well as antisera have been described by de Ávila *et al.* (1992) and Heinze *et al.* (1995), respectively. To prevent the occurrence of symptom-attenuating defective viruses (Inoue-Nagata *et al.*, 1997), tospovirus isolates were regularly transmitted by thrips and maintained on *N. benthamiana* plants for no more than five sequential mechanical passages. Inoculum for infection of the transgenic plants was obtained by grinding systemically infected *N. benthamiana* leaves in 0·5 % Na_2SO_3 and was applied to the two top leaves of four-leaf-stage plants with carborundum powder. In mixed inoculation experiments equal amounts of diluted sap of systemically infected plants were combined. Non-transformed control plants were inoculated following the inoculation of the transgenic plants. To avoid escapes, inoculations of young emerging leaves were repeated after a week. The plants were then monitored for 30 days for symptom development, and

Table 1. Resistance analysis of the IR-OUT and IR-IN lines

At least six S1 plants of all the lines were inoculated with each virus or with a mix of all viruses. Resistance was assessed phenotypically and by ELISA 3 weeks post-infection.

Line	Inoculated virus(es) (% resistance)				
	TSWV	GRSV	TCSV	WSMoV	Mix
IR-OUT4	100	100	100	100	83
IR-OUT5	0	20	11	10	0
IR-OUT6	100	100	100	100	100
IR-OUT7	94	100	100	100	100
IR-OUT8	100	100	100	100	100
IR-OUT9	100	100	88	70	67
IR-OUT10	94	90	89	80	67
IR-OUT11	100	100	100	100	100
IR-OUT12	100	100	100	100	83
IR-OUT13	7	0	44	10	33
IR-OUT14	100	100	100	100	100
IR-OUT15	100	93	100	100	100
IR-OUT16	100	100	100	100	100
IR-OUT17	0	0	0	11	0
IR-OUT19	100	100	100	100	33
IR-OUT20	100	100	100	83	83
IR-IN5	100	100	100	70	100
IR-IN6	92	92	100	100	100
IR-IN7	8	0	10	8	0
IR-IN8	100	100	100	100	100
IR-IN9	100	100	89	100	100
IR-IN10	100	100	100	92	100
IR-IN11	0	0	17	25	0
IR-IN12	8	0	0	8	0
IR-IN13	9	0	0	0	0
IR-IN14	83	92	100	100	100
IR-IN15	100	100	100	100	100
IR-IN16	0	0	0	10	0
IR-IN18	100	67	100	100	100
IR-IN19	100	100	100	100	80
IR-IN20	100	100	100	100	80
IR-IN21	17	25	20	75	33

Fig. 1. (a) Schematic representation of hairpin RNA constructs IR-IN and IR-OUT used to obtain transgenic resistance against four tospoviruses. Four 150 bp consecutive fragments of the N gene of four different tospoviruses were fused to form a chimaeric gene and arranged as inverted repeats. The inward-facing construct is named IR-IN and the outward construct IR-OUT. Upon transcription and splicing the RNA is expected to form a hairpin structure which is diced into siRNAs. This results in the production of a mixed population of siRNAs originating from the four different N genes. (b) Transgenic plants showing multiple virus resistance. The photograph shows two examples of transgenic plant lines inoculated with four different tospoviruses. IR-OUT16 is a line which is resistant to all viruses, as verified by visual observation and ELISA 30 days post-inoculation, and shows no symptoms. IR-OUT17 is an example of a fully susceptible line. (c) siRNA analysis of several transgenic plant lines. The siRNA blots shown on the right were extracted from transgenic non-inoculated IR-OUT lines. Enriched siRNAs were analysed with four different probes each covering a 150 bp segment of the N gene of each tospovirus. The control was total siRNA extracted from a non-infected, non-transgenic *N. benthamiana* plant.

analysed by ELISA (de Ávila *et al.*, 1991) to exclude symptomless infections, using four times the background value as a cut-off point. While all non-transgenic plants readily developed virus symptoms, 81 % of all IR-OUT lines showed high resistance (Fig. 1b and Table 1). In nearly all cases where an IR-OUT line was resistant to a single tested virus, it was also resistant to the other three viruses. Moreover, most plants showed resistance to infection using a mixture of the four different tospoviruses. Interestingly, the resistance incidence for the IR-IN lines, though still high, was lower than for the IR-OUT lines, as 63 % of the lines were found to be resistant to all four viruses. This phenomenon of lower silencing induction by inwardly oriented IR constructs has also been observed by others (Chen *et al.*, 2004; K. Kalantidis, personal communication). It is possible that ribosome scanning or shunting of the sense RNA prevents proper folding of the RNA into perfect dsRNA and thereby suppresses effective Dicer function. For both IR-IN and IR-OUT lines, progeny generations of resistant plants (S2 and S3) all remained 100 % virus-free, as determined by ELISA, despite a repeated inoculation using a mixed inoculum of all four viruses.

To confirm that RNA silencing was indeed the underlying mechanism of the observed virus resistance, the presence of small interfering (si) RNAs derived from the four segments of the transgene cassette were examined in resistant and susceptible plant lines. To this end, total nucleic acids of transgenic plants and control plants were extracted prior to virus inoculation and enriched for small RNAs using PEG precipitation (Hamilton & Baulcombe, 1999; Bucher *et al.*, 2004). Twenty micrograms of total small RNA per sample was separated on a 15 % PAGE gel and analysed by Northern blotting using specific digoxigenin (Roche)-labelled double-stranded DNA probes derived from each of the four 150 bp segments of the transgene cassette. As these fragments represent non-homologous subsequent parts of the N gene, no cross-hybridization to segments of the other viruses occurred (results not shown). siRNAs originating from the different parts of the transgene could readily be detected, but exclusively in virus-resistant transgenic lines (Fig. 1c). Similar to the non-transgenic controls, none of the susceptible plants accumulated detectable amounts of siRNAs from any part of the transgene, even though the presence of the genomic DNA copy of the transgene was demonstrated by PCR (data not shown). This might indicate transcriptional gene silencing of the transgene.

The feasibility of obtaining high-frequency resistance based on RNA silencing against multiple viruses simultaneously has been demonstrated. The data presented show that by using RNA silencing, virus resistance frequencies of over 80 % of all transformed plant lines can be achieved. It was shown that the plants became resistant to four different tospoviruses at once, when producing abundant siRNAs originating from each segment of the cassette. It has been shown before that the detection of specific siRNAs in transgenic plant lines expressing an hpRNA construct correlated with virus resistance (Kalantidis *et al.*, 2002; Chen *et al.*, 2004). Since our constructs are entirely hpRNA-based, they do not rely on the inclusion of random carrier sequences such as GFP RNA, as used by Jan *et al.* (2000). Using the constructs presented here, the size limitation problem (Pang *et al.*, 1997) can be overcome by linking fragments of as many viral sequences as required. Transgenic mRNAs did not accumulate in plant lines expressing hpRNA constructs (results not shown) as these were apparently efficiently diced to siRNAs, thereby minimizing the risk of recombination or complementation events in the field.

Overall, the work presented here demonstrates a simple procedure to obtain broad virus resistance at a high frequency by RNA silencing, using a single transgene construct of limited size. By extending the transgene construct with additional viral sequences, the broadness of the resistance can be extended further. Due to the high frequency of this multiple virus resistance, the approach can be applied first and foremost to tomato in which the four tospoviruses of this study present a major problem. In addition, other susceptible plant species can thus be protected. As many of the tospoviruses have a broad host range, this presents a major advantage.

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