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Hairpin-based virus resistance depends on the sequence similarity between challenge virus and discrete, highly accumulating siRNA species

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Abstract Virus resistance can be effectively generated in transgenic plants by using the plant's silencing machinery. To study the specificity of gene-silencing-based resistance, homozygous tobacco (*Nicotiana tabacum* L.) plants containing a 597-nt hairpin RNA construct of the *Potato Virus Y* (PVY) replicase sequence were challenged with a variety of PVY strains. The transgene-carrying tobacco line was immune to five potato PVY strains with high sequence similarity (88.3–99.5%) to the transgene. Infection with more distant tomato and pepper PVY field strains (86–86.8% sequence similarity) caused delayed symptom appearance in the transgenic tobacco. Transgene production of small interfering (si) RNA was detected by northern blot and measured using a custom-designed microarray for the detection of small RNAs. siRNA accumulation peaks were observed throughout the inverted-repeat transgene. In the resistance-breaking tomato and pepper strains there were nucleotide differences in the sequences correlated to siRNA transgene accumulation, indicating the role of siRNA specificity in resistance breaking. The log of transgene siRNA signal intensity increased with probe GC content, indicating that the accumulating siRNA molecules were GC-rich. Sequence similarity of highly accumulating siRNAs with the target virus strain appears to be important for both resistance and resistance-breaking characteristics.

Keywords *Potato virus Y* · PVY · Transgenic resistance · Tobacco · Hairpin RNA construct · Viral strain specificity

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Introduction

Viral resistance in transgenic plants can be protein- or RNA-based (Prins et al. 2008). In general, resistance based on transgenic expression of a protein confers relatively broad resistance to related viral strains (Fitchen and Beachy 1993). In contrast, resistance based on the expression of RNA is more efficient, but sequence-dependent and related to mechanisms of

post-transcriptional gene silencing (PTGS) (Goldbach et al. 2003; Prins et al. 2008).

RNA silencing is the plant's basal defence against RNA viruses based on the recognition of viral dsRNA, processing by DICER and the RNA-induced silencing complex (RISC), and amplification by RNA-dependent RNA polymerase (Brodersen and Voinnet 2006; Ding and Voinnet 2007). Accumulation of viral dsRNA may be due to RNA secondary structure, replicative form in which the viral (+) and (-) strands are associated (Mlotshwa et al. 2008), or amplification by an endogenous RNA-dependent RNA polymerase (Garcia-Ruiz et al. 2010). Based on the natural phenomenon of PTGS, transgenic plants have been engineered to produce dsRNA, via the introduction of an inverted-repeat construct encoding a hairpin RNA (hpRNA) (Wesley et al. 2001). The dsRNA activates the plant's silencing machinery, which then processes it with Dicer-like (DCL) enzymes. It is likely that a mechanism similar to that used for the recognition, targeting and processing of viral RNA is used for the processing of hpRNA, producing a population of predominately 21–24nt small interfering RNAs (siRNAs). The 21- and 22-nt siRNAs are produced by the action of DCL4 and DCL2, respectively (Eamens et al. 2008; Fusaro et al. 2006; Henderson et al. 2006). The 24-nt siRNAs produced by DCL3 action are required for direct methylation (Daxinger et al. 2009). In transgenic plants, 21-nt viral siRNA (vsiRNA) were dominant following infection with an RNA virus (Szittya et al. 2003). The selected strand of the resulting siRNA, the guide strand, is loaded into RISC, while the passenger strand is degraded. The guide-strand-activated RISC specifically recognizes and digests the target viral RNA (Ding and Voinnet 2007; Voinnet 2005). Since hpRNA is produced in the nucleus, in contrast to viral RNA which is cytoplasm-processed; therefore, hpRNA is expected to be cleaved also by DCL3, which is less important in the silencing of viruses (Garcia-Ruiz et al. 2010).

A hpRNA construct with a virus fragment inserted in opposing orientations confers a high level of resistance (in 90% of transgenic plants) to a homologous virus (Fusaro et al. 2006; Wesley et al. 2001). In many cases, the level of RNA-based resistance is positively correlated to accumulation of the transgene siRNA (Bucher et al. 2006; Chen et al. 2004; Kalantidis et al. 2002). PTGS-based viral resistance has been demonstrated for several plant virus families, including potyviruses (Chen et al. 2004; Kreuze et al. 2008; Missiou et al. 2004; Vanderschuren et al. 2009).

Potato virus Y (PVY) is the type member of the genus *Potyvirus*, with a single (+) strand RNA genome. It infects many solanaceous species (Singh et al. 2008) and replicates via RNA intermediates. PVY exhibits much sequence diversity (Glais et al. 2002; Singh et al. 2008), which may depend on the host plant. Some of the PVY strains that infect potato are unable to infect pepper or tomato, and vice versa (Romero et al. 2001; Singh et al. 2008); however, most PVY strains are able to infect tobacco (Singh et al. 2008). Such diversity could complicate the production of transgenic plants with PTGS-based resistance, and therefore conserved regions (e.g. coat protein (Missiou et al. 2004)) are selected for the construction of hpRNAs. A high level of viral resistance was demonstrated to three different PVY subgroups by transgenic potatoes bearing a coat protein-hpRNA construct (Missiou et al. 2004). Different parts of the potyvirus genome can be used to produce virus-resistant plants by hpRNA constructs: coat protein (Missiou et al. 2004), NIa protease (Smith et al. 2000) or P1 (Di Nicola-Negri et al. 2005) sequences.

The new deep-sequencing technologies and the use of microarrays for the detection of specific small RNAs facilitate the investigation of the roles of small RNAs in virus resistance (Kreuze et al. 2009). Moreover, this information is expected to enable more effective engineering of virus resistance using specific sequences of known effectiveness for gene silencing. In the current study, we found a correlation between resistance-breaking in transgenic plants expressing a hpRNA PVY construct and specific variation in the resistance-breaking viral strains which match definite siRNA populations expressed in the transgenic plants.

Materials and methods

Plant inoculation and maintenance of virus strains

Tobacco (*Nicotiana tabacum* L. cv. Samsun NN) plants were maintained in a temperature-controlled greenhouse at 25°C with supplementary lighting

before and after inoculation. Tobacco seedlings were used as source plants to maintain cultures of PVY strains. Dry leaves from tobacco plants inoculated with PVY strains confirmed by sequencing were maintained at -80°C. Inocula were prepared by grinding young leaves of source plants 7 to 10 days post-inoculation (dpi) in distilled water. Young plants (with three leaves) were mechanically inoculated following dusting with carborundum and kept in the greenhouse as above. Viruses were monitored by visual symptoms (mosaic, stunting), ELISA, RT-PCR and back-inoculation of tobacco plants.

Virus strains

A range of PVY strains was used, including five potato strains (WP, N, H, O, 52): PVY^{H} and PVY^{52} are tuber-necrotic strains; PVY^{O} gives mosaic symptoms on tobacco leaves, while PVY^{N} produces necrotic symptoms on tobacco leaves. The replicase sequence (597 nt) of strain PVY^{WP} is 99% similar to that of PVY^{N} (Genebank accession number D00441). Strain PVY^{H} is most closely related (97%) to a Hungarian strain (accession number M95491). PVY^{N} is most closely related (98%) to a strain with accession number AY166867. PVY^{O} is most closely related (99%) to accession number AJ585195 from Scotland. PVY^{52} is most closely related (97%) to accession number EF026074 from the northeastern US. Three additional PVY field strains were used: Israeli tomato and pepper strains most homologous to a Canary Islands PVY strain (accession number AJ439545) (94 and 95% sequence similarity, respectively, in the replicase region, and sharing 95% sequence similarity), and another Egyptian tomato field strain which is 95% similar to an Iranian strain (accession number EF455803) that has been reported not to infect potato. The 5′-non-translated regions of the five PVY potato strains were characterized (Rosner and Maslenin 2006), and leaf symptoms of these strains are shown in Supplementary Figure 1 (Additional material on line).

Construction of the binary vector pRepIR-PVY for gene silencing

A 597-nt fragment of the replicase sequence of PVY N strain (accession number D00441) containing the GDD motif was isolated by PCR amplification. Each primer was designed with two additional restriction sites to permit cloning in both orientations. Forward primer: 5′-AAAGGTACCGGATCCTGTGATGCT GATGGCTCACAGTT-3′ (sense, with added *Kpn*I *and BamH*I sites). Reverse primer: 5′-AAACTG CAGGCGCCCCAGGACTCTATCATAGCTG-3′ (antisense, with added *Pst*I and *Nar*I). The amplified replicase sequence was initially cloned in a pGEM-TEasy plasmid vector (Promega, Madison, WI). We generated a pBlueScript KS vector template for cloning a target sequence in inverted-repeat orientation harbouring the 190 nt of the catalase intron (accession number AF234293) flanked by *BamH*I and *Pst*I on one side and *Nar*I and *Kpn*I on the other. The PCR product was cloned in two orientations into the modified KS vector as follows. The cloned replicase sequence was excised with *Pst*I/*BamH*I and inserted in a forward (sense) orientation beside the intron sequence in pBlueScript KS plasmid cleaved with the same enzymes. In step II, the PVY replicase clone was excised with *Kpn*I/*Nar*I and inserted in a reverse (antisense) orientation into the construct from step I cleaved with the same enzymes. The whole PVY unit (inverted repeat and intron) was excised from the KS-based plasmid by *BamH*I and *Kpn*I and inserted into the *BamH*I and *Kpn*I sites of pCambia 2301 binary vector containing β-D-glucuronidase and NPTII genes (accession number AF234316) (http://www.cambia.org/), and the SVBV promoter (Wang et al. 2000) instead of the 35 S promoter (see Fig. 1b). The binary vector pRepIR-PVY was verified by sequencing and digestion by the appropriate restriction enzymes.

Agrobacterium-mediated transformation of tobacco

The preparation of pRepIR-PVY-transformed tobacco plants was similar to the *Agrobacterium tumefaciens*-mediated transformation process (Maldonado-Mendoza et al. 1996), using greenhouse-grown leaves of tobacco cv. Samsun NN.

T_1 seedling segregation assay and homozygote selection

Seeds of the T_1 generation were sown in the greenhouse and GUS enzyme activity was measured in the leaves of young seedlings (Jefferson 1987). Seedlings expressing GUS and fully resistant to PVY

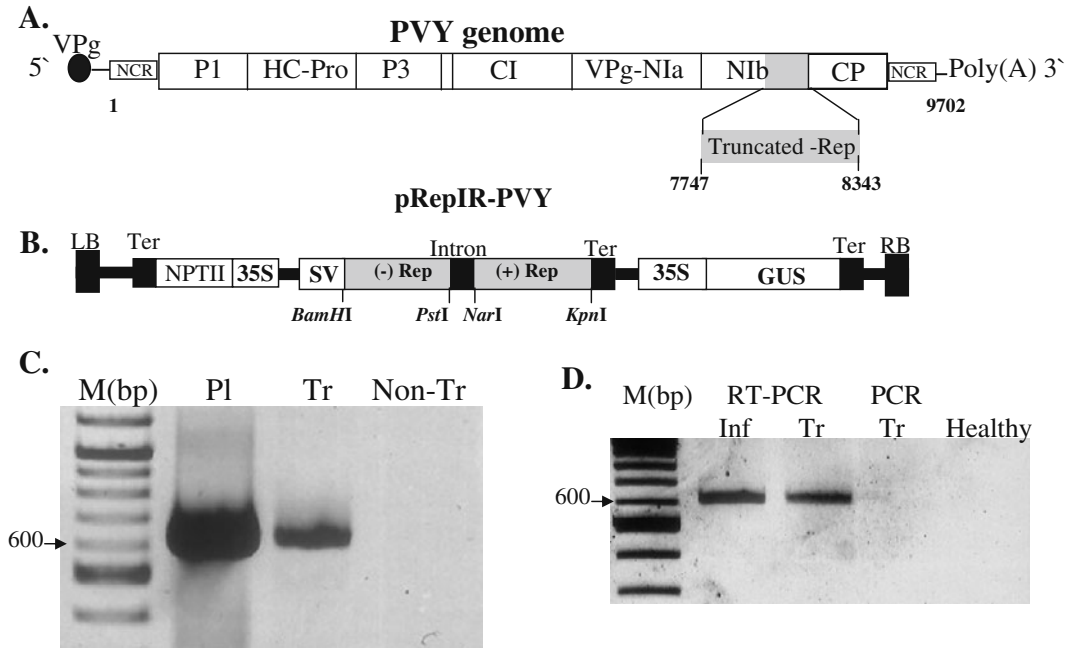

Fig. 1 Construct used for plant transformation. **a** Schematic outline of the PVY genome, showing the segment of the NIb replicase sequence cloned for the construction of the vector. **b** Schematic diagram of the T-DNA of the binary vector derived from pCambia 2301 (pRepIR-PVY). On the left border (LB) is the NPTII gene, under control of the *Cauliflower mosaic virus* 35 S promoter (35 S) and the NOS terminator (Ter), then the virus resistance gene consisting of the promoter from the *Strawberry vein banding virus* (SV), the replicase fragment (Rep) in two orientations [(+) and (-)] separated by the maize catalase intron, followed by the NOS terminator. Adjacent to the right border (RB) of the T-DNA is a β-D-glucuronidase (GUS) gene controlled by a 35 S promoter and NOS terminator. **c** PCR confirmation of transformation of the tobacco line. Left to right: Molecular weight markers (bp) (M), pCddRep-PVY plasmid control (Pl), extract from transgenic plant (Tr), extract from non-transgenic plant (Non-Tr). **d** Transgene-derived RNA expression was detected in transgenic plants by RT-PCR from total plant RNA. Left to right: Molecular weight markers (bp) (M), RT-PCR of infected (Inf) and transgenic (Tr) plant extracts, PCR of transgenic plant after DNase treatment of extract (PCR Tr), and healthy plant extract. Arrow defines expected product size in base-pairs

inoculation were selected. Seeds of these lines were used for the next generation (T_2).

Evaluation of virus resistance

Homozygous T_2 seedlings were screened for virus resistance. Three biological experiments were performed. For each experiment, 10 seedlings were inoculated. Inoculated seedlings were kept for several weeks under greenhouse conditions. Responses of the tobacco transgenic line to inoculation were determined by visual observation of symptoms, and by testing for the presence of PVY by ELISA (BioReba Reinach, Switzerland) and RT-PCR with the primers from the coat protein sequence 5′-GTGCAGCAAT GATAGAATCCTG-3′ forward position 8340; reverse 8900 5′-GCACCAAACCATAAGCCCATTC-3′. Further resistance analysis was performed by mechanical back-inoculation to control *N. tabacum* cv. Samsun NN, 21 dpi.

Small-RNA-specific microarray

Microarrays were printed by LC-Sciences (Houston, TX) with 25-nt probes of the PVY replicase transgene in both its sense and antisense orientations. Sense and antisense probe pairs were numbered according to their 5′ sense coordinates. A microarray was printed with 25-nt probes, representing the whole transgenic region: SVBV promoter, (+) and (-) PVY replicase sequences, and the intron. Each probe was shifted 1 nt from the previous probe, thereby tiling the transgene. We printed 1,437 probes in each orientation (2,874 in total). Printed negative controls included sequences from the PVY coat protein. Small RNA array hybridization was performed by LC-Sciences with

small RNA fractionated with the mirVana miRNA Isolation Kit (Ambion, Austin, Texas) from 20 μg total RNA from transgenic or non-transgenic tobacco leaves. RNA was extracted from pools of five plants at the 4-leaf stage (5 g per sample), ground in liquid nitrogen, and extracted in TRI-reagent (MRC, Cincinnati, OH). RNA was spiked with several internal control RNAs for in-chip normalization. RNAs from transgenic and non-transgenic tobacco leaves were labelled with different chromophores. Only data from transgenic tobacco leaves are shown, as there was no signal observed from the control leaf RNA microarray hybridization. Further description of the microarray technology is provided in Additional material on-line.

Detection of siRNA by northern blot hybridization analysis

Total leaf RNA was extracted using TRI-reagent according to the manufacturer's instructions from tobacco leaves 10 dpi, non-infected transgenic plants, and *N. benthamiana* leaves subjected to agro-infiltration (Shiboleth et al. 2007) with the same construct 4 dpi. Total RNA (10 μg) in 50% formamide was loaded per lane of a 15% acrylamide-8 M urea gel buffered in 20 mM 3-[N-morpholino]propanesulfonic acid (MOPS) pH 7, electrophoresed in MOPS buffer and electrotransferred in water (Bio-Rad Semi-dry) to a Hybond NX (GE) nylon membrane. RNA was crosslinked to the membranes with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Pall et al. 2007). For detection of PVY^{H}-siRNA, blots were hybridized with radiolabeled transcripts using T7 RNA polymerase (Fermentas, Lithuania) and α-^{32}P-UTP, transcribed from the pBluescript vector (linearized with *Bam*HI) containing 597 nts of the truncated NIb (as above). Hybridization was performed overnight in 2X SSC, 1% SDS and 100 μg ml^{-1} herring sperm DNA (ICN, Costa Mesa, California) at 45°C, and the membrane was then washed in 2X SSC, 0.1% SDS at 40°C twice for 15 min each.

Results

Characterization of transgene expression

Transgenic tobacco plants transformed with T-DNA from a binary construct based on pCAMBIA 2301 were regenerated. This construct contained a 597-nt inverted-repeat hpRNA derived from PVY N strain's replicase sequence (position 7747–8343) (accession number D00441) (Fig. 1a and b). The replicase fragment was cloned in two orientations as described in Materials and Methods (Fig. 1b). The kanamycin resistance (*nptII*) and β-D-glucuronidase (*gus*) reporter genes served to select for transgenic shoots and to screen for homozygotes in T_2 transgenic plants. In this study, we used a single transgenic line (TR-repPVY), in which the segregation of GUS activity in T_1 plants was close to 3:1, and segregation of virus resistance was 1:3, indicating a single genomic insertion. Selfing of the resistant line generated progeny 100% bearing the transgene, indicating homozygosity. This generation (T2) was fully virus-resistant, and only this was used in the work reported here. Presence of the transgene in the transformed plants was demonstrated by PCR amplification of the plant DNA using replicase-specific primers (Fig. 1c, Tr). The size of the amplified product was consistent with that of the original insert from the binary plasmid construct. No PCR products were observed from the non-transgenic control plants (Fig. 1c, Non-Tr). The replicase gene-silencing construct in the plant's genome was expected to yield an RNA transcript initiated by the SV promoter. RNA expression was detected in the transgenic plants by RT-PCR from total plant RNA (Fig. 1d, RT-PCR/Tr), which yielded a product similar to that from virus-infected plants (Fig. 1d, RT-PCR/Inf) and which was absent from non-transformed healthy plants (Non-Tr) (Fig. 1d). To eliminate the possibility that this product originated directly from transgene DNA (and not RNA), the RNA preparations were subjected to direct amplification by PCR, and yielded no product (Fig. 1d).

To demonstrate that the hpRNA construct is functional and processed by the Dicer machinery, *N. benthamiana* leaves were infiltrated with *Agrobacterium* harboring the pRepIR-PVY construct (Fig. 2), and accumulation of siRNA of the PVY insert was revealed by northern blot, while siRNA was not detected from the binary vector without the PVY insert. Northern blot analysis of total RNA extracted from the transgenic plant line showed a positive reaction with the PVY probe for siRNA. A vsiRNA extract from PVY-infected tobacco was used as a positive control, and low molecular weight RNA extracted from non-transgenic uninfected tobacco

plants served as a negative control (Fig. 2). The transgene siRNA migrated similarly to the PVY vsiRNA (Fig. 2) and to 21 nts DNA primers (not shown) indicating that these products are of similar mass (21–24 nts). However, further analysis would be required to determine the exact size of the transgene-derived siRNA.

Resistance to different PVY strains: pepper and tomato PVY strains break the transgenic resistance

Resistance of the selected transgenic tobacco T_2 homozygous line was tested against eight PVY strains: five potato strains (WP, N, H, O, 52) and three PVY field strains recovered from tomato and pepper. The PVY potato strains shared high sequence similarity with the transgene's replicase region: from 99.5% (strain PVY^{WP}) to 88.1% (PVY^{52})(Fig. 3; Table 1). Lower similarities were found with the tomato (86.8 and 86%) and pepper (86.3%) strains (Fig. 3; Table 1). All of the strains were 100% infective in control (non-transgenic) tobacco, producing symptoms of plant stunting and leaf mosaic (Fig. 4, Supplementary Figure 1). PVY^{52} and PVY^{H} produced more severe symptoms, including leaf necrosis. The transgenic tobacco was challenged with all eight strains by sap inoculation (Table 1). The transgenic plants were symptomless when challenged with the potato PVY strains (Fig. 4): virus accumulation could not be detected by ELISA, RT-PCR, or back-inoculation to non-transgenic tobacco (Table 1). In contrast, the tomato and pepper strains were detected in the transgenic plants by ELISA, RT-PCR and back-inoculation, and symptom development was observed following a 10-day delay (relative to non-transgenic controls) (Table 1). Transgenic plants inoculated with any potato PVY strain were symptomless 21 dpi. Inoculation with a tomato or pepper PVY strain resulted in leaf mosaic symptoms 16 dpi. Control non-transgenic tobacco displayed symptoms to all PVY strains 6 dpi. There is therefore a moderate level of resistance to PVY strains more distantly related to the transgene. The resistance response to the PVY strains was similar in three experimental repeats. We did not observe any variation in the levels of resistance of individual plants inoculated with PVY.

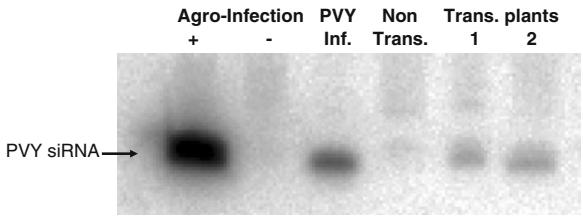

Fig. 2 Northern blot detection of transgene production of PVY siRNA. Left to right: PVY siRNA production is observed following *Agrobacterium*-mediated expression of the transgene after infiltration of a *N. bethamiana* leaf (+) [but not in the control (-)], in PVY-infected tobacco (PVY Inf) but not in a non-inoculated non-transgenic plant (Non Trans). PVY-siRNA production is observed in transgenic plants (Trans plants 1 and 2). The blot was probed with ^{32}P-labeled sense RNA transgene transcript

Genetic variation in PVY strains

To better understand resistance breaking, we examined the distribution of sequence variation among the PVY strains, compared to the relative accumulation of siRNA produced by the transgene. The distribution of nucleotide differences between the transgene and the various PVY strains varied (Fig. 3). For example, the sequence of the PVY^{O} strain, which is comparatively different from the transgene (88% sequence similarity), showed only three modifications in the first 100 nts (positions 34, 46, 47). However, between 100 and 200 nts, there were nine changes, and between 200 and 300 nts there were 15 nts alterations in the PVY^{O} strain compared to the transgene. In contrast, in the pepper strain, in the first 100 nts there were 11 sequence changes compared to the transgene, in the region from 100 to 200 nts there were 10 variations, and there were 15 changes between 200 and 300 nts (Fig. 3).

Accumulation of transgene siRNA and its sequence

We analyzed the population of transgene-homologous siRNAs which accumulated in the non-inoculated transgenic tobacco using a custom-designed (micro-array) chip for the detection of small RNAs, with 25-nt probes covering the PVY sense and antisense sequences. The probes on the chip were temperature-optimized using a Locked Nucleic Acid design so that the binding affinity would be sequence-independent. Each probe was moved 1 nt along the transgene from the previous probe, thereby mapping siRNA production by the whole transgene. Total small RNA from transgenic and control tobacco was labeled with

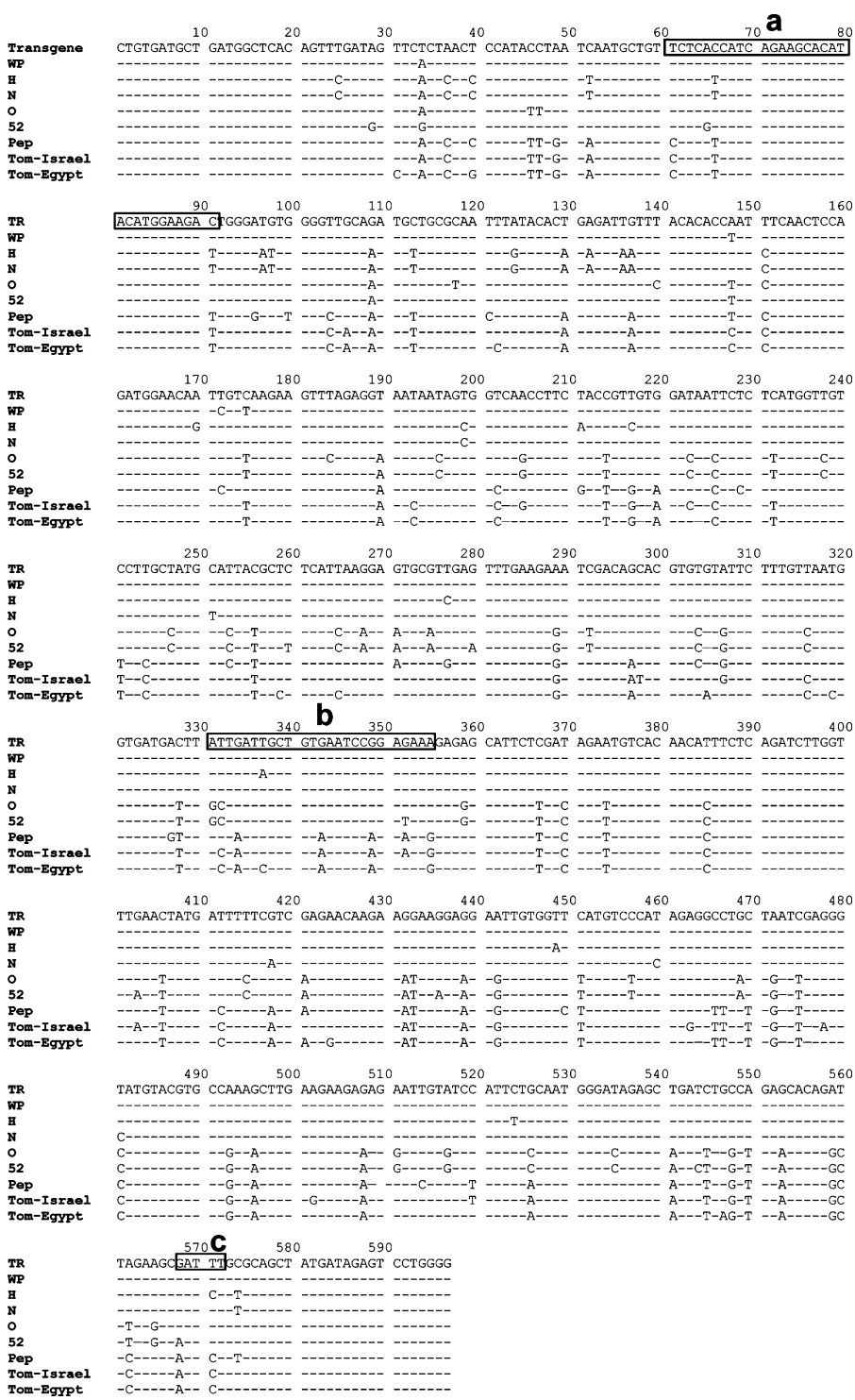

Fig. 3 Sequence alignment of 8 PVY strains compared to the transgene. Israeli "tomato" strain variations which might be responsible for resistance breaking opposite major siRNA peaks are marked by bars a, b and c

different fluorescent probes. Relative fluorescent signals were measured from each set, and the data recorded for each sequence. A small-RNA signal related to the hpRNA could be detected in the transgenic plants (Fig. 5), but not in the non-transgenic controls (not shown). Signal peaks and troughs were observed throughout the PVY transgene. We observed 13 discrete peaks of siRNA accumula-

Table 1 PVY strains used in this work, their nucleotide similarity (%) with the transgene, and the results of ELISA, RT-PCR, symptom expression, and back inoculation tests

PVY strains	Similarity to the transgene (%)	Evaluation of transgenic resistance			
		ELISA		RT-PCR	Back-inoculation to control
		Tr	Non-Tr	Tr	
WP	99.5	–	+	–	–
N	96.3	–	+	–	–
H	94.6	–	+	–	–
O	88.6	–	+	–	–
52	88.1	–	+	–	–
tomato strain Is	86.8	+	+	+	+
tomato strain Eg	86.0	+	+	+	+
pepper strain	86.3	+	+	+	+

Tr transgenic plant, *Non-Tr* non-transgenic plant, *Is* Israel, *Eg* Egypt

tion (Fig. 5, peaks A-M, ≥1,000. Peak details are listed in Supplementary Table 1, Additional material on-line).

Interestingly, although the hpRNA construct is expected to produce a single fully matching dsRNA with a highly negative ΔG value, peak probe responses were generally not similar between the (+) and (-) strands (e.g. peaks F and G, Fig. 5). Production of siRNA against the sense strand was, overall, about twice that against the antisense strand (Fig. 5). siRNA peaks against the sense strand were sometimes accompanied by lesser siRNA peaks to against the antisense strand, and vice versa (e.g. Fig. 5, peaks A, H, L). Peaks were not phased by 21 to 24 nt, or any multiple of these values. In some regions, a very weak siRNA response was found (e.g. Fig. 5, nts 350–400). Major siRNA peaks were generally observed in zones of high GC content (≥40% GC, peaks A-M), while high GC content alone did not predict the siRNA peak (i.e. Fig. 5, nt 190–200). The probes covering the promoter and intron regions generally did not respond, indicating processing of the dsRNA as expected.

Among the non-resistance-breaking strains, PVY^{52} exhibited the most changes compared to the transgene, while among the resistance-breaking stains, the Israeli tomato strain showed the least variation from the transgene (Fig. 3 areas marked a, b and c). Only a few mutations (nts 61, 66, 91 [against peak A]; 334, 343, 349, 355 [against peak F]; 568, 571 [against peak M]) were observed in the Israeli tomato strain that were not observed in PVY^{52}. These may be

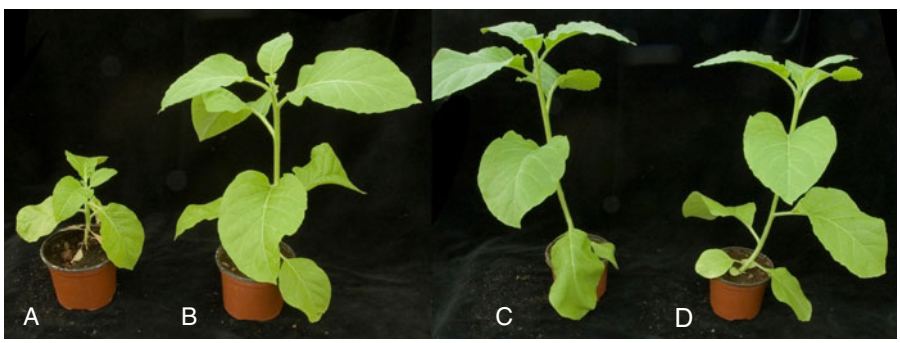

Fig. 4 Resistance of transgenic tobacco to inoculation by PVY^{N}. **a** Control tobacco cv. Samsun NN inoculated with PVY^{N}. **b** Non-inoculated control tobacco. **c** Inoculated transgenic tobacco. **d** Non-inoculated transgenic tobacco. Photographs were taken 3 weeks after inoculation

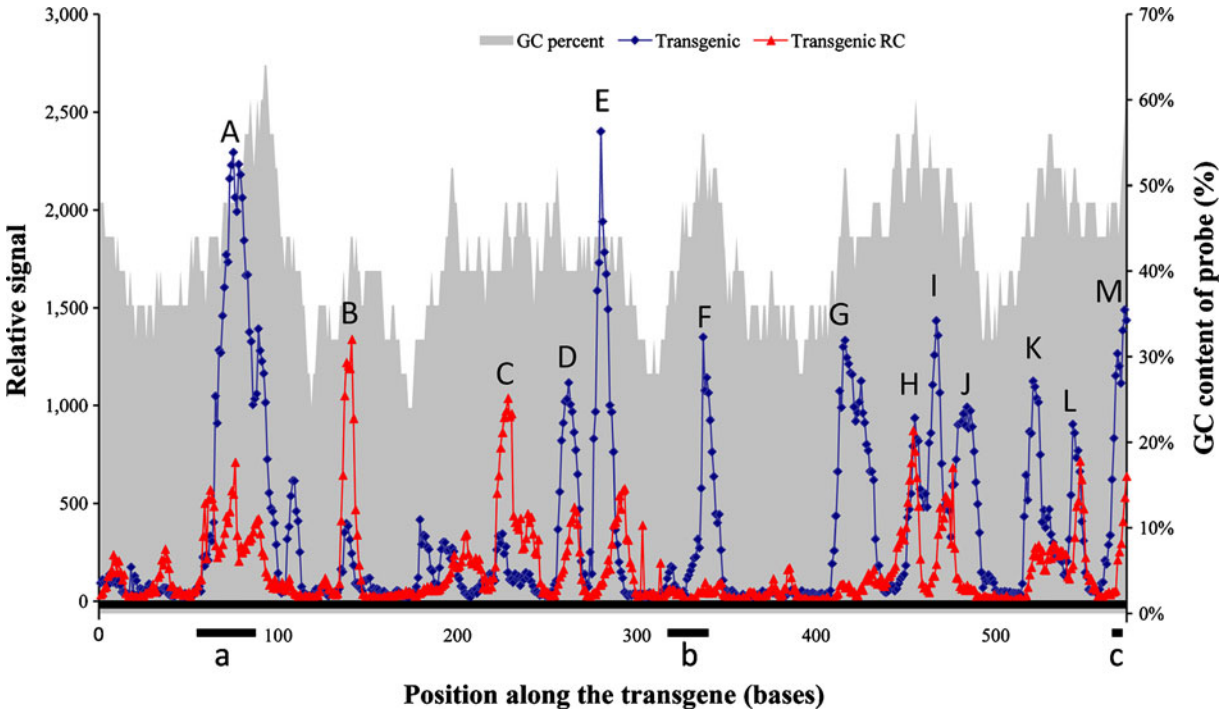

Fig. 5 Accumulation of siRNA across the transgene, GC-content and viral strain divergence. Relative siRNA accumulation across the transgene was plotted for both sense (Transgenic, red) and antisense (Transgenic RC, blue) strands. The signal for the 25-nt probe starting with that nucleotide (i.e. 5' terminus) is plotted, starting with nucleotide 1 at the left, and finishing with nt 573, which is the last point for which the 25-base probe consists only of PVY replicase sequence. Each point was corrected by subtraction of the relevant background. On the right-hand axis, the GC-content for each 25-nt probe is plotted (gray background on graph). Israeli "tomato" strain variations which might be responsible for resistance breaking (compared to the "O" strain) opposite major siRNA peaks are marked by bars a, b and c. Peaks of siRNA accumulation are labeled A-M. Only data from transgenic tobacco leaves are shown, as there was no signal observed from the control leaf RNA microarray hybridization

involved in resistance breaking, as they were located at peaks of siRNA accumulation (Figs. 3 and 5, areas a, b, c).

To examine the relationship between GC content and probe response, probe GC content was plotted against the mean log signal for both sense and antisense probes (Fig. 6), and a sigmoid correlation was observed for both. Therefore, in the log phase increased siRNA GC content is directly linked to greater siRNA accumulation (Fig. 6).

Discussion

RNA-mediated resistance in transgenic plants has been shown to confer a high level of resistance against plant RNA viruses (Eamens et al. 2008; Prins et al. 2008). The specificity of RNA-based resistance allows it to be effective against related viral strains, but this aspect has not been thoroughly investigated. Most RNA-mediated resistance is based on PTGS, where the active molecule is a small RNA, generated from a dsRNA molecule originating from the transgene (Ding and Voinnet 2007). Such dsRNA can be produced from an aberrant RNA, or transcribed from a hpRNA inverted-repeat construct (Ruiz-Ferrer and Voinnet 2009). Additionally, it has been shown that a single small RNA (an artificial miRNA of 21 nts viral RNA) accessible to RISC is sufficient for the generation of viral resistance (Duan et al. 2008).

We looked for a correlation between PVY strain sequence similarity and accumulating siRNA populations originating from the transgene which provide resistance. Gene silencing is a highly sequence-specific mechanism and it would therefore be expected to differentially affect viral strains whose nucleotide sequences vary within the transgene region (Chen et al. 2004). Here we demonstrate that a PVY

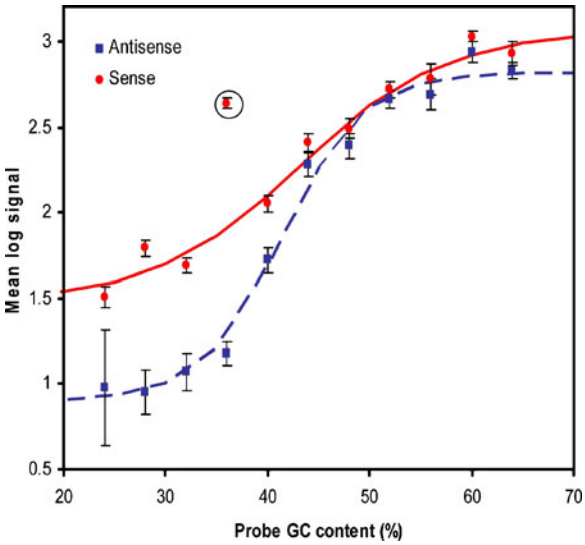

Fig. 6 Probe GC content correlates with log signal intensity. Probe GC content (%) was plotted against the log signal for each point on the transgene (using the data from Fig. 5). For each level of GC content, the log signal means and standard error were calculated and are shown for sense probes (*circles*, *solid line*), and antisense probes (*squares*, *broken line*). The lines are sigmoid curves fitted by the Sigmaplot program to the function $Y = Y_0 + a/1 + e^{-((X-X_0)/b)}$, where for sense and antisense, respectively: a=1.572, 1.917; b=7.039, 3.956; X_0= 43.094, 41.482; Y_0=1.485, 0.904; r^2=0.9782, 0.9888; for both curves P<0.0001. The encircled point was treated as an outlier and not included in the curve fitting

hpRNA transgene from the relatively conserved replicase sequence confers immunity to five PVY potato strains sharing 88 to 99% sequence similarity in that region. Tomato and pepper PVY strains, which exhibited a lower degree of sequence similarity (86%), were able to partially overcome the viral resistance, with delayed symptom appearance.

We demonstrated accumulation of transgene siRNA by two separate methods: northern blot and microarray. The northern blot demonstrated the presence of transgene-derived siRNA that migrated similarly to the siRNA produced during a PVY infection. Using the microarray, we were able to examine the siRNA population in much greater detail, as previously shown (Shiboleth et al. 2007). We examined the population of transgene (+) and (-) siRNA in this homozygotic line by tiling probes at 1-nt intervals over the length of the transgene. vsiRNA does not accumulate uniformly in dicotyledonous plants but in "hotspots", with strand preferences [(+) higher than (-)] (Duan et al. 2008; Ho et al. 2007; Molnar et al. 2005; Qi et al. 2009; Shiboleth et al. 2007) which may be due to DICER preference or RISC stability (Duan et al. 2008; Ho et al. 2007), or RNA regions lacking secondary structure (Qi et al. 2009). However, in virus-infected monocotyledons, an equal quantity of vsiRNA is found against each strand (Ho et al. 2008; Mlotshwa et al. 2008). Similar to the vsiRNA, we found that there are "hotspots" (Fig. 5) and strand specificity in transgene siRNA accumulation. If DICER preferences and RISC stability are related to sequence, then we can assume that transgenic vsiRNA will generally accumulate similarly to vsiRNA, except if there is a specific secondary structure interfering with DICER processing, as shown with the 3′ non-coding region of CMV (Duan et al. 2008; Ho et al. 2007).

We mapped differences between resistance-breaking and non-resistance-breaking viral strains to pinpoint sequence differences in the "hotspots" (Fig. 3). The most diverse of the PVY potato strains compared to the transgene was PVY^{52}, which nevertheless did not break the transgenic resistance. Much of the PVY^{52} strain divergence was in zones of transgene siRNA accumulation (peaks B-E, G-L). Nevertheless, at peaks A, F and M (Figs. 3 and 5) there were stretches of full sequence similarity between the transgene and PVY^{52}. We therefore assume that the transgene siRNA accumulating in these three peaks is responsible for the viral immunity. A unique siRNA designed as a miRNA is sufficient to generate RNA-virus-resistant plants (Duan et al. 2008; Zhang et al. 2006), which supports our speculation that a single peak, e.g. peak A, could be sufficient to confer resistance to PVY^{52}. Interestingly, the other potato-infecting PVY strains (e.g. PVY^{H} and PVY^{N}) showed variations that were similar to that of the tomato strain opposite peak A, but were fully homologous with transgene peaks D-F, and almost completely homologous with transgene peaks G-L. In the tomato and pepper strains, we found unique mutations (Fig. 3, areas a, b, c) against peaks A, F and M relative to PVY^{52}, and we therefore assume that those mutations, together with the HC-Pro suppressor, can overcome transgenic resistance. To prove the notion of resistance specificity would require an analysis of mutations at these points in an infective PVY clone. We assume that resistance breaking may not be due to a single or a few changes

(i.e. only in Peak A), but rather in many (accumulative) alterations. Each change should be checked individually, and then together, to permit a full analysis.

Notably, the plant treats the transgene like viral RNA (preferably accumulating siRNA against the positive strand) because this is a viral sequence: therefore there are features that the plant can recognize, including GC content, which probably contribute to greater stability in RISC of the siRNA against the (+) strand vs. the siRNA against the (-) strand (Ho et al. 2007).

In conclusion, the degree of sequence similarity of siRNA with the target virus is related to the resistance or resistance-breaking phenomena. In addition, we do not observe a preference for 5′-U of the transgene siRNA as was found for microRNA, and in general do not observe a bias to a specific 5'-nucleotide (data not shown). Therefore it is not clear which AGOs are involved in processing duplex RNA from a transgene. Better knowledge of these features would enable the design of improved gene-silencing constructs and plants that are resistant to multiple viruses.

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