Reports

Construction of effective inverted repeat silencing constructs using sodium bisulfite treatment coupled with strand-specific PCR

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RNA silencing has been exploited to produce transgenic plants with resistance to viral pathogens via posttranscriptional gene silencing (PTGS). In some cases, this technology is difficult to apply due to the instability of inverted repeat (IR) constructs during cloning and plant transformation. Although such constructs have been shown to be stabilized with introns and efficiently induce RNA silencing, we found that the *Pdk* intron did not stabilize *South African cassava mosaic virus* (SACMV) silencing constructs. Therefore, we developed a method for producing long SACMV IR constructs through bisulfite-induced base pair mismatches on the sense arm prior to IR assembly. Expression of SACMV BC1 mismatched IR constructs in the model test plant *Nicotiana benthamiana* resulted in a reduction in viral BC1 transcript levels, hence viral replication, upon SACMV infection. Mismatched SACMV AC1 IR constructs induced PTGS more efficiently in a *N. benthamiana* callus system than nonmismatched IR constructs. Our novel method for IR construct generation should be applicable to many sequences where the generation of these constructs has proven difficult in the past.

Currently, posttranscriptional gene silencing (PTGS) techniques are being applied for engineering resistance against viral plant pathogens. Many of these strategies involve the use of either sense or antisense constructs (1–7). Inverted repeat (IR) transgenes, whereby sense and antisense gene fragments are expressed from a single promoter in a head-to-head orientation, have been developed, and these IR constructs are reportedly more efficient inducers of PTGS than any of the previously used constructs (8). IR transgenes now form the basis of experimental RNA interference (RNAi) in plant systems, and the process has been termed IR-PTGS (9).

Despite potential applications of IR-PTGS, the inverted repeats generally result in genomic instability, particularly in prokaryotes and lower eukaryotes (10); with *Escherichia coli*, inverted repeats were shown to cause cell death in some cases, and deletions or rearrangements in others (10,11). DNA sequences in an IR conformation have the tendency to form a four-way

helical junction, known as a cruciform structure or Holliday junction (12). Several nucleases that specifically cleave Holliday junctions and other large secondary structures of DNA have been identified (13–15). In addition, these junctions are key intermediates for homologous recombination, and are therefore substrates of recombination enzymes (16,17). IR transgenes are therefore difficult to manipulate during cloning and plant transformation procedures, where the process depends on the replication and maintenance of the plasmid containing the construct.

To prevent formation of cruciform structures and stabilize DNA conformation during cloning, constructs containing a sufficiently large spacer sequence between the IR sense and antisense arms were tested. However, these spacer sequences significantly reduced PTGS induction efficiency (18). Subsequently, vectors were designed to stabilize the IR constructs by placing a spliceable intron from the *Pdk* gene of *Flaveria* between the sense

and antisense arms (19). However, our studies with *South African cassava mosaic virus* (SACMV) found the *Pdk* intron did not prevent SACMV IR construct instability during cloning and plant transformation (Taylor and Rey, unpublished). We believe that the difficulty in cloning the IR constructs may be due to the formation of Holliday junctions that can be created between two cruciform arms (12), altering the electrophoretic mobility of the DNA and rendering the DNA insensitive to restriction endonucleases (20). Instability of inverted repeats has been suggested as a likely cause of cloning failure (21,22). In addition, the large intron (~700 bp) increases the overall size of the T-DNA, and studies have shown that large-insert T-DNAs are prone to rearrangements and deletions in both *Agrobacterium* and plant cells (23).

Here we describe a method to produce long mismatched IR constructs, using SACMV BC1 and AC1 open reading frame (ORF)-derived sequences, wherein the sense

Figure 1. Method used to generate a SACMV BC1 mismatched inverted repeat expression cassette in the plant transformation vector pCambia 1303 (pC). SACMV, South African cassava mosaic virus; BC1, BC1 ORF on SACMV DNA-B component; CMV, cauliflower mosaic virus 35S promoter; GusA, β -glucuronidase marker gene.

arm is bisulfite-treated to randomly convert cytosine residues to thymine. When DNA is treated with sodium bisulfite, cytosines are converted into uracil. Subsequent amplification of the DNA fragment by PCR results in the uracils being replaced by thymine residues in the sense strand, resulting in a mismatched IR construct that should prevent cruciform formation of the DNA. The mismatched SACMV BC1 IR DNA could easily be cloned and sequenced and was expressed in the host plant *Nicotiana benthamiana*. When compared with a nonmismatched AC1 IR construct, the mismatched SACMV AC1 IR construct

showed improved efficiency in reducing SACMV replication.

Materials and methods

Isolation of gene fragments

SACMV is a geminivirus consisting of two circular ssDNA components [DNA-A and DNA-B, that have six ORFs (AC1, AC2, AC3, AC, AV1, and AV2) and two ORFs (BC1 and BV1), respectively] (24). A 222-bp fragment of the SACMV BC1 ORF (involved in movement of virus) was PCR-amplified using unmodified primers [BC1 (unmod) F (5'-AAACAT-

TCCACGGACATACG-3') and BC1 (unmod) R (5'-TGGTAG-CCCAATCT-GAGACCTT-3')] from 15 ng template DNA (full-length genomic SACMV DNA-B clone) (Figure 1) using 0.4 μ M each forward and reverse primer at an annealing temperature of 52°C. Amplification products were purified using a High Pure PCR Product Purification kit (Roche Applied Science, Mannheim, Germany).

Mutation of gene fragments and strand-specific amplification

Sodium bisulfite-mediated deamination reactions were performed on 120 ng BC1 PCR product using the EZ DNA Methylation-Gold kit (Zymo Research, Orange, CA, USA). Reactions were performed for 5, 10, or 15 min to determine deamination efficiency. Sodium bisulfite-treated SACMV BC1 fragments were PCR-amplified using modified primers [BC1 (mod+*XhoI*+*SpeI*) F (5'-GATCCTCGAGACTAGTAAAT-ATTCTACGGACATACG-3') and BC1 (mod+*BglII*) R (5'-GATCAGATCTTAG-TAGCCCAATCTAAGACCTTGT-3')] (Figure 1). These primers were designed to preferentially amplify the positive strand of the sodium bisulfite-treated DNA template. In addition, they result in restriction endonuclease sites that are added on to the 3'- and 5'-ends of the PCR products.

Assembly of the IR construct

Strand-specific PCR amplicons were each TA-cloned into the pTZ57R vector (InsT/A Clone PCR Product Cloning kit; Fermentas, Vilnius, Lithuania). Clones containing fragments that had been bisulfite-treated for 10 min [56% (39 of 70) C to T conversion] were screened for inserts in the sense orientation, and clones containing untreated fragments were screened for inserts in the antisense orientation by restriction endonuclease analysis. Selected clones were sequenced using the universal M13/pUC reverse primer. Clones containing the treated [pTZ57R/T-BC1* (-)] and untreated [pTZ57R/T-BC1 (+)] fragments were double-digested with *ScaI*/*BglII* and *ScaI*/*BamHI*, respectively, and resolved by agarose gel electrophoresis (Figure 1). Bands containing the appropriate restriction fragments were excised and extracted from the gel slice (MinElute Gel Extraction kit; Qiagen GmbH, Hilden, Germany). The inverted repeat was constructed by ligating (T4 DNA ligase; Fermentas) an equal ratio of the restriction fragments containing the untreated fragment to those containing the treated fragment (Figure 1). Four clones (pTZ57R/T-BC1) containing presumptive IR constructs were sequenced using both the

Figure 2. Properties of the SACMV BC1 mismatched inverted repeat construct. (A) A 222-nucleotide unstructured linear stretch (indicated in green) of SACMV BC1 mRNA folded according to a minimal secondary structure. (B) A six-nucleotide loop and terminal stem duplex with G:U wobbles are predicted by the secondary structure prediction algorithm mfold for the SACMV BC1 mismatched RNA hairpin. (C) The SACMV BC1 mismatched IR cloned into the pCambia 1303 plant transformation vector.

universal M13/pUC forward and reverse primers to confirm the presence of the mismatched IR sequence and to determine the number of C to T mutations. A single clone BC1hp7 (i) containing 56% C to T conversion (total of 17% mismatched base pairs) was subsequently used for cloning into pCambia 1303.

Generation of IR expression cassettes and transformation of *N. benthamiana*

The mismatched IR construct was released from BC1hp7 by restriction digestion with *Xho*I and *Xba*I and subsequently ligated (T4 DNA ligase; Fermentas) into *Xho*I/*Xba*I-restricted pART7 vector to generate an expression cassette. The IR expression cassette was PCR-amplified from pART7 [pART7 F (5'-TTAACGTTT-TACAATTTCCCATTCGC-3') and pART7 R (5'-GGAATTGTGAGCG-GATAAC-3')] and TA-cloned into pTZ57R (InsT/A Clone PCR product cloning kit; Fermentas). The IR expression cassette was released from the pTZ57R clone by digestion with *Eco*RI and *Hind*III and ligated to *Eco*RI and *Hind*III linearized pCambia1303 (Cambia, Brisbane, Australia) to generate pCambia 1303 SACMV BC1 (pC SACMV BC1) (Figure 1). *Agrobacterium tumefaciens* strain *Ag*I was transformed with a pCambia 1303

SACMV BC1 clone using the freeze-thaw method (25), and 40 *N. benthamiana* leaf discs were agro-transformed with *Ag*I-pCambia 1303 SACMV BC1 (26,27), 20 with pCambia1303 (vector alone control), and 10 with untransformed wild-type *Ag*I. Twenty-one pCambia 1303 SACMV BC1 transformants were regenerated in the presence of the selection antibiotic (hygromycin 50 mg/L) and an ethylene scrubber, potassium permanganate (28), and six vector-only and healthy control plant lines were regenerated.

Expression of mismatched BC1 IR cassette

T_1 progeny from T_0 transformed tobacco plant seeds were screened for expression of the transgene construct, which contains the hygromycin (*hptII*) marker gene, by real-time RT-PCR since transgene expression of hairpins is often difficult to detect as the hairpin is partially or fully cut by the RNAi machinery. Total RNA extractions were performed using Tri-Reagent method (Sigma-Aldrich, St. Louis, MO, USA), treated with DNase (1 U; Fermentas), resuspended in RNase-free water, and concentrations measured on a Nanodrop spectrophotometer (Nanodrop, Thermo Scientific, Wilmington, DE, USA). cDNA was reverse-transcribed

using a blend of oligo(dT) random hexamer primers (iScript Reverse Transcriptase; Bio-Rad Laboratories, Hercules, CA, USA) from total RNA isolated from leaf tissue and PCR-amplified [hptII F (5'-CTATTTCTTTGCCCTCGG-3') and hptII R (5'-TTCGATGATGCAGCTTGG-3')]. PCR controls included untransformed tobacco leaf tissue (negative) and pCambia 1303 vector (positive *hptII*). PCR products were run on a 2% agarose gel. Spatial expression of the transgene, in transgenic plants, was also monitored by histochemical staining of β -glucuronidase (GUS). Shoots were placed in staining buffer [1 M sodium phosphate, pH 7.0, 0.5 M EDTA, 10% Triton X-100, 50 mM $K_3Fe(CN)_6$, 0.1 M X-Gluc] at 37°C for 24 h, followed by several 50% ethanol washes.

Challenge of transformed plants and absolute quantification of BC1 transcription from replicating SACMV DNA-B

Of the 21 independent transgenic lines, four lines (C, D, L, M), showing strong hygromycin and GUS expression, were chosen for further study. Six plants of each line and six nontransgenic control plants were agro-infected using full-length SACMV DNA-A and DNA-B head-to-tail infectious dimers (24). A further six nontransgenic control plants were agro-infected with wild-type *Agrobacterium*. In order to quantify the levels of viral DNA-B molecules, expressed BC1 from replicating SACMV DNA-B was measured by absolute real-time quantitative PCR (qPCR). Twenty-three days postinfection (dpi) (and 28 dpi for wild-type control plants), total RNA was isolated from 6–8 leaf stage T_1 transformants using TRI Reagent (Sigma-Aldrich) and treated with RNAase-free DNase A (Fermentas). cDNA was synthesized from 30 μ g DNase-treated total RNA (quantified on a Nanodrop spectrophotometer; Nanodrop) (iScript cDNA synthesis kit; Bio-Rad Laboratories). Absolute quantitative PCR of the BC1 transcript was performed on 2 μ L (0.1 ng) cDNA using primers BC1 F (5'-TACGATAACCGACCCAGTTGCGTT-3') and BC1 R (5'-TGCGACTCAAAGGCCGATGTATGA-3') to amplify a 100-bp region situated upstream of the BC1 IR-target region (LightCycler FastStart DNA MasterPLUS SYBR Green I; Roche Applied Science). The number of BC1 transcript molecules present in each cDNA sample was calculated using the equation:

$$\text{Molecules of transcript} = \left[\frac{(\text{ng DNA})}{(8.58 \times 10^5 \text{ g/mol})} \right] \times (6.022 \times 10^{23} \text{ molecules/mol})$$

where x is the amount of BC1 cDNA present in grams, calculated from a standard curve. A standard curve was generated from dilutions (10 ng/ μ L to 1 pg/ μ L) of SACMV DNA-B in pBluescript spiked into healthy total nucleic acid (TNA) (29) from *N. benthamiana*. Real-time qPCR on extracted TNA (20 μ g/mL RNase-treated; Fermentas) and quantified on a Nanodrop 1000 spectrophotometer (Nanodrop) from challenged and unchallenged transgenic plants was also performed using AC1 primers [AC1 F (5'-GCACAAGCGTCGAT-3') and AC1 R (5'-CTGCCAGTATGCTT-AACGTCA-3')] to quantify the absolute amount of SACMV DNA-A molecules, using the same approach as described above, but no RT-PCR was required.

Particle bombardment of *N. benthamiana* callus tissue with SACMV AC1 mismatched and nonmismatched IR constructs

In order to compare the efficiency of SACMV knockdown between mismatched and nonmismatched IR constructs, a *N. benthamiana* callus-DNA bombardment system was performed, using a 193-nucleotide AC1 fragment derived from SACMV DNA-A (30). The mismatched AC1 IR construct was designed as described for BC1 above, and the nonmismatched AC1 IR construct was designed using the pHannibal system (19,30). Both the mismatched and nonmismatched IR inserts in pART7 vector were excised together with the CaMV 35S promoter and ocs 3'-terminator sequences to create minimal gene expression cassettes. The Helios Gene Gun System (Bio-Rad Laboratories) was used to co-bombard either of the minimal gene expression cassettes or pART7 only minimal cassette (CaMV 35S:MCS:ocs 3'; control; 0.25 μ g/shoot) together with challenging virus DNA [SACMV DNA-A and DNA-B infectious dimers in pBIN19 (24); 0.15 μ g DNA-A/shoot; 0.15 μ g DNA-B/shoot] into the callus tissue. All cartridges were prepared according to Helios Gene Gun System instruction manual. Four sets of eight calli each were bombarded once for each minimal gene expression cassette using 200 psi helium pressure under sterile conditions. After bombardment, calli were incubated for 16 h at 25°C in the dark, following which they were transferred to callus medium, pH 5.8 (MS medium plus 1% sucrose and 0.5% agar, supplemented with 50 μ M picloram; Sigma-Aldrich), and incubated at 28°C for a 12-h photoperiod in a growth chamber (Binder GmbH, Tuttlingen, Germany) for a further 4 days. DNA was isolated from bombarded callus tissue using a method adapted from Doyle and Doyle (1987) (29),

Figure 3. Inhibition of SACMV in *N. benthamiana* transformed with mismatched BC1 IR construct.

(A) i. and ii. Transformed shoots from a leaf disc of line D indicating positive GUS expression (blue color). iii. Negative control of untransformed wild-type indicates no GUS expression. (B) Quantification of viral BC1 mRNA by RT-qPCR in SACMV-challenged transgenic lines C, D, L, M, and transformed vector only control (pCambia) and wild-type control (WT 28 dpi) at 28 days dpi. Error bars represent standard deviation of four biological replicates. (C) Viral symptoms detected at 21 dpi. i. Infected wild-type untransformed plants (control) showing symptoms of stunting and leaf curling (arrow); and ii. infected transformed line M showing reduced symptoms.

quantified, and *DpnI*-treated to digest input viral DNA (30).

The absolute amount of replicating viral DNA present in bombarded callus tissue 4 days postbombardment was quantified in duplicate by absolute qPCR of a region of the AV1 ORF of SACMV DNA-A using a LightCycler 1.5 Instrument (Roche Applied Science) with SYBRGreen fluorescent dye (LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit; Roche Applied Science, Mannheim, Germany). Reactions were set up according to manufacturer's instructions, to which 6 μ L purified DNA and 2 μ M each PCR primer (AV1F: 5'-GCACAAGCGTCGAT-3'; AV1R: 5'-CTGCCAGTATGCTT-AACGTCA-3') were added. The following PCR conditions were used to amplify the 154-bp fragment: 5 min at 95°C, and 40 cycles of 5 s at 95°C, 10 s at 60°C, and 10 s at 72°C.

For quantification purposes, a standard curve was generated by amplification of SACMV DNA-A in pBluescript (24)

Figure 4. Absolute quantitative PCR of SACMV DNA-A copies in transformed *N. benthamiana* callus bombarded with mismatched or nonmismatched AC1 IR constructs. pART7 represents the plant transformation vector control. Data are the mean of four replicates, and error bars represent the standard error.

spiked into healthy tobacco total DNA, and the amount of replicating viral DNA per sample was calculated from the standard curve and expressed as molecules/ng DNA using the following equation, based on the assumption that one plasmid (DNA-A in pBS) has one copy or molecule of DNA-A, therefore if 100 ng plasmid DNA = 0.026 pmol plasmid, then using Avogadro's number, 1 pg plasmid DNA = 1.56×10^5 molecules:

$$\text{Molecules/ng DNA} = [\text{calculated amount of viral DNA (pg)} \times 1.56 \times 10^5 \text{ molecules/pg plasmid DNA}] / [\text{DNA added to PCR (ng)}]$$

Data was analyzed using Microsoft Excel and GraphPad InStat 3.01 software (GraphPad Software, San Diego, CA, USA). Standard errors of the means were calculated for four bombardment events (28). Control included bombardment with pART7 vector (no viral insert).

Results and discussion

In this study, mutagenesis was used to disrupt base pairing in the IR in transgene DNA constructs to prevent formation of cruciform structures. Bisulfite-induced mutagenesis resulted in G:U mismatches in the hairpin RNA sequence (Figure 2B), leaving the antisense strand unmodified. We hypothesized that IR or hpRNA secondary structure would not be affected and would be more stable than nonmismatched IR constructs. G:U base pairs are unusually

Table 1: Deamination efficiency (conversion rate of C to T) of sodium bisulfite on geminiviral sequences following a different treatment time-course.

Sequence	Time (min)	Conversion rate (% of total cytosine residues)
SACMV BC1	5	60
SACMV BC1	10	56
SACMV BC1	15	55
MSV AC1	150	82
SACMV AC1	150	100

stable in comparison to other non-Watson and Crick base pairings; in fact hairpins closed by G:U base pairs are approximately 0.7 Cal/mol more stable than loops closed by G:C or C:G base pairs (31). It has previously been shown that such G:U mismatches have no impact on RNAi knockdown efficacy in animal cells (32). Moreover, Krol et al. (33) describe Dicer processing of hairpins generated by complementary annealing of CNG repeats, suggesting a tolerance for duplex dsRNA substrates with wide-ranging stabilities.

Since geminivirus transcripts are known to have strong IR fold-back structures (34), we examined the secondary structure of the SACMV BC1 transcript and identified a region consisting of unstructured linear stretches of RNA (Figure 2A) that would be highly accessible to the RNA-induced silencing complex (RISC), thereby allowing efficient degradation of target transcripts. Hairpin constructs were designed to produce a population of an estimated 10–12 different small interfering RNAs (siRNAs) with affinity to the target transcript. A 222-bp fragment of the BC1 ORF was amplified by PCR and separated into two pools. One PCR pool was treated with sodium bisulfite while the second pool remained untreated (Figure 1). Bisulfite-catalyzed deamination only affects cytosine residues, therefore products amplified from the positive strand will contain cytosine to thymine mutations when read in the sense orientation, while negative strand products will contain guanine to adenine mutations when read in the sense orientation. Since we are interested in engineering C to T mutations, which lead to G:U mismatches in the RNA hairpin, primers were designed to allow for strand-specific PCR amplification of the positive strand of the mutated template DNA. Primer design ensured that the 3'-terminal nucleotide of the forward primer would be mismatched if bound to a mutated template strand, whereas the 3'-end of the reverse primer would be unaffected by mutations on the template strand, ensuring more efficient amplification of the positive strand. In this way, a single amplification product is produced with C to T mutations when read in the sense orientation. A single DNA strand of desired polarity was preferen-

tially amplified in 75% of the selected clones (data not shown). The antisense strand of the IR constructs was not mutated, as this may compromise the binding of subsequently formed siRNAs to target viral sequences.

In order to determine optimal exposure time to induce mutations in the sense arm, a range of methylation times was tested. Ten minutes exposure to bisulfite was found to catalyze an adequate mutation rate (C to T conversion of 56%). A final BC1 clone (BC1hp7) was selected with this C to T conversion rate (with a total of 17.5% mismatched base pairs). There was no notable difference in C to T conversion percentage introduced in the 222-nucleotide SACMV BC1 sequence between 5 (60%), 10 (56%), and 15 (55%) min (Table 1). A further study was conducted in our laboratory using the monopartite geminivirus *Maize streak virus* (MSV) and SACMV AC1 sequences, which highlighted the possibility of variation in conversion rate for different sequences. After 2.5 h, 80% of the cytosine residues in the MSV sequence (246 nucleotides) were converted to thymine, compared with all of the cytosines being mutated in the SACMV AC1 fragment (193 nucleotides) after the same exposure time (Table 1). It has previously been established that bisulfite reacts strongly with ssDNA and unpaired bases (35). Since the secondary structure of these viral sequences differ at 64°C (data not shown), we predict that the relative stabilities of secondary structures present at 64°C in a target DNA region will directly affect the degree of mutation by sodium bisulfite. While 2.5 h (manufacturer's recommendation) applies to screening for methylation in genomic DNA, our results indicate that for this application, shorter incubation times are adequate to introduce an acceptable number of mutations to stabilize the silencing construct during cloning; however, this still may have to be determined empirically, as well as the threshold number of mutations.

Inverted repeat constructs were successfully assembled and subcloned, first into the pART7 expression vector and then later into the pCambia 1303 binary plant transformation vector (Figure 2C). In addition, PCR amplification of the entire IR cassette was achieved without the need for DMSO or other agents that relax secondary structure.

Furthermore, automated sequencing, which is often difficult to achieve with tightly bound hairpins (36), could be performed using standard primers and conditions. In all cases, constructs were replicated and maintained in standard *E. coli* DH5 α without the need for IR-tolerant cells such as SURE Cells (Agilent Technologies, Palo Alto, CA, USA). In previous studies of IR constructs, the aforementioned molecular manipulations were not possible (37).

The effect of G:T mismatches on DNA thermodynamics has previously been determined, and the data was consistent with the "nearest-neighbor" parameters for Watson-Crick base pairs (38). In this controlled study, the effect of a single G:T mismatch was found to be context-dependent. For example, a single G:T mismatch may reduce helix stability when the mismatch is located between two AT base pairs. In addition, it was reported that G:T mismatches caused slight structural perturbations that were highly localized. If a single G:T base pair can reduce the stability of a helix, we believe that numerous G:T mismatches along the stem of an intrinsically unstable cruciform structure will significantly affect the thermodynamic favorability for formation of this structure. Our data are consistent with this theory, as our IR construct behaved like a linear molecule during molecular manipulations. In addition, our constructs were efficiently propagated by a strain of *E. coli* that does not support the replication of cruciform structures (14,16).

It was of utmost importance that mismatched IR constructs decrease the stability of DNA secondary structures without compromising the formation or folding of dsRNA, which is required to induce PTGS. Sequencing was done on numerous clones to confirm the presence of the SACMV BC1 mismatched IR sequence. Despite having 17.5% mismatched base pairs along the sense arm, a stable hairpin structure containing a long dsRNA stem and a six-nucleotide loop was predicted (Figure 2B). Further studies in our laboratory revealed that the RNA hairpin structure could be compromised around the loop region if a large number of G:U mismatches are localized there (39). G:U base pairs are unusually stable in comparison with other non-Watson and Crick base pairings and are processed as canonical base pairings by folding algorithms (40). It has also been shown that the orientation of the purine and pyrimidine bases in a base pair have a marked influence on the stability of a hairpin, especially at the closing base pair (41). For example, short hairpins closed by U:G base pairs, are 1.3 kcal/mol less stable than those

closed by G:U base pairs. Due to the unusual stability of G:U mismatches, especially when they are the closing base pair, they have been found to increase the relative stabilities of other non-Watson and Crick base pairings adjacent to them (42).

Four independent SACMVBC1-transgenic lines (C, D, L, and M) selected from a total of 21 lines all showed positive GUS expression. Temporal expression of GUS was consistently strong in emerging shoots from transformed leaf discs and rooted shoots from regenerated explants (line D is shown in Figure 3A, i and ii). Nontransgenic lines were GUS negative (Figure 3A, iii). Successful transformation was further indicated by the integration of the *hptII* reporter gene in all transgenic lines, as demonstrated by PCR (data not shown). RT-PCR was used to detect *hptII* mRNA in four or five replicates of each transgenic line; however only lines C and D had detectable expression levels (data not shown). In a study by Chellappan et al. (43) in cassava, four transgenic lines (A31, Y100, Y85, and Y44) were selected based on resistance to *African cassava mosaic virus* (ACMV). Resistance was more pronounced in lines Y44 and Y85, despite transcript levels being below detectable levels. Here we obtained similar results, where lines L and M showed pronounced reduction in symptoms and viral load (Figure 3B), despite undetectable transcript levels. It is possible that selective transgene silencing (methylation) of the *hptII* gene is induced by the presence of several homologous 35S promoter sequences when the transgene is integrated in high copy number. Spontaneous silencing due to methylation is a well-known phenomenon. The amount of viral BC1 transcript (indicating amount of DNA-B replication) was significantly reduced (87% to 100%) in lines C, D, L, and M compared with controls (Figure 3B), which corresponds with the lack of symptoms viewed on plants from these lines (Figure 3E, ii), compared with the infected control (Figure 3, D and E). In a similar study, significant SACMV knockdown was shown in *N. benthamiana* plants transformed with a SACMV AC1 mismatched IR construct. Ten independently produced SACMV challenged transgenic lines had significantly reduced levels (70% to 99%) of virus-derived AC1 compared with wild-type infected *N. benthamiana* (Taylor and Rey, unpublished).

Silencing is part of a recovery phenomenon, where plants intrinsically have the ability to induce virus-targeted PTGS, but that viruses are able to suppress PTGS. PTGS is not always expressed at certain times in infection and can be transient in some plant tissues, sometimes resulting in recovery (44). Efficacy of silencing may therefore be dependent on intrinsic features of the virus and its interaction with

the plant host. Off-target effects for both guide and sense strands have been reported in animals and plants (45,46). However there is no reason or evidence to suggest that a mutated versus a nonmutated sense strand would produce less or more nonviral off-target effects in targeted host DNA. This cannot be predicted. Should an off-target effect from any RNA silencing strategy cause any phenotypic changes in the plant, this would be of concern, but monitoring of postgenetic modification traits is always performed. In our study, we did not observe any morphological differences between wild-type and transgenic plants. One way to reduce the chances off-targets is to use shorter sequences, as we did in our studies.

In order to examine the efficiency of the mismatched IR construct to induce PTGS compared with the nonmismatched IR construct, we produced SACMV AC1 IR constructs in pHannibal (19) and performed a particle bombardment experiment. A minimal gene cassette (promoter:IR:terminator sequences) was introduced into *N. benthamiana* callus tissue together with SACMV DNA-A and DNA-B infectious dimers using the Helios Gene Gun System (Bio-Rad Laboratories). Minimal cassette technology is recognized as an important plant transformation tool for the production of marker-free transgenic plants (47). After bombarding, calluses were incubated for 4 days, following which DNA was isolated and the amount of replicating SACMV DNA was quantified by performing absolute qPCR. There was a significant reduction (three to four times reduction) in the amount of replicating SACMV DNA in callus tissues that were bombarded with the mismatched IR construct compared with the nonmismatched IR construct (Figure 4), thereby showing that the introduction of base pair mismatches in the sense arm of in IR sequence can improve the efficiency of the construct to induce PTGS.

Here we have demonstrated that introducing cytosine to thymine mutations on the sense arm by the mutagen sodium bisulfite

and subsequent strand-specific PCR can produce stable mismatched IR constructs. This chemical mutagen is ideal, as it specifically deaminates cytosine without affecting adenine, guanine, or thymine (48). To date, sodium bisulfite has commonly been used to screen genomic DNA for methylation or secondary structures, but we report the first application of this technology in the context of RNAi. We show for the first time the use of a stably expressed mismatched IR silencing construct to knockdown geminivirus replication in *N. benthamiana*. Furthermore, there was a notable improvement in the levels of PTGS, as demonstrated in lower viral loads with a mismatched AC1 IR compared with the corresponding nonmismatched IR construct. While addition of an intron in combination with a bisulfite approach could make the system more efficient, this was not tested. However, introns increase the size of *Agrobacterium* T-DNA in plant transformations, and large-insert T-DNAs can cause rearrangement and deletions (23), the stability and efficacy of the BC1 construct with a six-nucleotide loop demonstrated in this study may alleviate the need for introns. Applications in development of transgenic virus-resistant plants potentially expressing several siRNAs from a single long mismatched IR construct are promising and need to be further explored.

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Competing interests

The authors declare no competing interests.

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References

1. Day, A.G., E.R. Bejarano, K.W. Buck, M. Burrell, and C.P. Lichtenstein. 1991. Expression of an antisense viral gene in transgenic tobacco confers resistance to the DNA virus tomato golden mosaic virus. *Proc. Natl. Acad. Sci. USA* 88:6721-6725.
2. Bejarano, E.R. and P. Lichtenstein. 1994. Expression of TGMV antisense RNA in transgenic tobacco inhibits replication of BCTV but not ACMV geminiviruses. *Plant Mol. Biol.* 24:241-248.
3. Bendahmane, M. and B. Gronenborn. 1997. Engineering resistance against tomato yellow leaf curl virus (TYLCV) using antisense RNA. *Plant Mol. Biol.* 33:351-357.
4. Praveen, S., C.M. Kushwaha, A.K. Mishra, V. Singh, J. Varma, and A. Varma. 2005. Engineering tomato for resistance to tomato leaf curl disease using viral *rep* gene sequences. *Plant Cell Tissue Organ Cult.* 83:311-318.
5. Mubin, M., S. Mansoor, M. Hussain, and Y. Zafar. 2007. Silencing of the AV2 by antisense RNA protects transgenic plants against a bipartite begomovirus. *J. Virol.* 19:10.
6. Zhang, P., H. Vanderschuren, J. Fütterer, and W. Gruissem. 2005. Resistance to cassava mosaic disease in transgenic cassava expressing antisense RNAs targeting virus replication genes. *Plant Biotechnol. J.* 3:385-397.
7. Noris, E., G.P. Accotto, R. Tavazza, A. Brunetti, S. Crespi, and M. Tavazza. 1996. Resistance to tomoato yellow leaf curl geminivirus in *Nicotiana benthamiana* in plants transformed with a truncated viral C1 gene. *Virology* 224:130-138.
8. Chuang, C.-F. and E.M. Meyerowitz. 2000. Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* 97:4985-4990.
9. Brodersen, P. and O. Voinnet. 2006. The diversity of RNA silencing pathways in plants. *Trends Genet.* 22:268-280.
10. Gebow, D., N. Miselis, and H.L. Liber. 2000. Homologous and nonhomologous recombination resulting in deletion: effects of p53 status, microhomology, and repetitive DNA length and orientation. *Mol. Cell. Biol.* 20:4028-4035.
11. Brunier, D., B. Michel, and S.D. Ehrlich. 1988. Copy choice illegitimate DNA recombination. *Cell* 52:883-892.
12. Duckett, D.R., A.I.H. Murchie, S. Diekmann, E. von Kitzing, B. Kemper, and D.M.J. Lilley. 1988. The structure of the Holliday junction and its resolution. *Cell* 55:79-89.
13. Connelly, J.C., L.A. Kirkham, and D.R.F. Leach. 1998. The SbcCD nuclease of *Escherichia coli* is a structural maintenance of chromosomes (SMC) family protein that cleaves hairpin DNA. *Proc. Natl. Acad. Sci. USA* 95:7969-7974.
14. Leach, D.R. 1994. Long DNA palindromes, cruciform structures, genetic instability and secondary structure repair. *Bioessays* 16:893-900.
15. Sharples, G.J., S.N. Chan, A.A. Mahdi, M.C. Whitby, and R.G. Lloyd. 1994. Processing of intermediates in recombination and DNA repair: identification of a new endonuclease that specifically cleaves Holliday junctions. *EMBO J.* 13:6133-6142.
16. Ortiz-Lombardía, M., A. González, R. Eritja, J. Aymami, F. Azorin, and M. Coll. 1999. Crystal structure of a DNA Holliday junction. *Nat. Struct. Biol.* 6:913-917.
17. Lilley, D.M.J. and D.G. Norman. 1999. The Holliday junction is finally seen with crystal clarity. *Nat. Struct. Biol.* 6:897-900.
18. Smith, N.A., S.P. Singh, M.-B. Wang, P. Stoutjesdijk, A. Green, and P.M. Waterhouse. 2000. Total silencing by intron-spliced hairpin RNAs. *Nature* 407:319-320.
19. Helliwell, C. and P. Waterhouse. 2003. Constructs and methods for high-throughput gene silencing in plants. *Methods* 30:289-295.
20. Gillert, M., M.H. O'Dea, and K. Mizuuchi. 1983. Slow cruciform transitions in palindromic DNA. *Proc. Natl. Acad. Sci. USA* 80:5545-5549.
21. Lilley, D.M. 1981. In vivo consequences of plasmid topology. *Nature* 292:380-382.
22. Warren, G.J. and R.L. Green. 1985. Comparison of physical and genetic properties of palindromic DNA sequences. *J. Bacteriol.* 161:1103-1111.
23. Nakano, A., G. Suzuki, and K. Yamamoto. 2005. Rearrangements of large-insert T-DNAs in transgenic rice. *Mol. Genet. Genomics* 273:123-129.
24. Berrie, L.C., E.P. Rybicki, and M.E.C. Rey. 2001. Complete nucleotide sequence and host range of *South African cassava mosaic virus*: further evidence for recombination amongst begomoviruses. *J. Gen. Virol.* 82:53-58.
25. Holsters, M., D. de Waele, A. Depicker, E. Messens, M. van Montagu, and J. Schell. 1978. Transfection and transformation of *Agrobacterium tumefaciens*. *Mol. Gen. Genet.* 163:181-187.
26. Narváez-Vásquez, J., M.L. Orozco-Cárdenas, and C.A. Ryan. 1992. Differential expression of a chimeric CaMV-tomato proteinase inhibitor I gene in leaves of transformed nightshade, tobacco and alfalfa plants. *Plant Mol. Biol.* 20:1149-1157.
27. Koscianska, E., K. Kalantidis, K. Wypijewski, J. Sadowski, and M. Tabler. 2005. Analysis of RNA silencing in agroinfiltrated leaves of *Nicotiana benthamiana* and *Nicotiana tabacum*. *Plant Mol. Biol.* 59:647-661.
28. Park, S.W., J.H. Jeon, H.S. Kim, Y.M. Park, C. Aswath, and H. Joung. 2004. Effect of sealed and vented gaseous microenvironments on the hyperhydricity of potato shoots in vitro. *Sci. Hortic. (Amsterdam)* 99:199-205.
29. Doyle, J. J., and J.L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19:11.27-15.27.
30. Taylor, S.H. 2008. The development of SACMV-resistant cassava using pathogen-derived resistance strategies targeting the DNA-A component. PhD thesis. University of the Witwatersrand, Johannesburg, South Africa.
31. Giese, M.R., K. Betschart, T. Dale, C.K. Riley, C. Rowan, K.J. Sprouse, and M.J. Serra. 1998. Stability of RNA hairpins closed by wobble base pairs. *Biochemistry* 37:1094-1100.
32. Akashi, H., M. Miyagishi, T. Yokota, T. Watanabe, T. Hino, K. Nishina, M. Kohara, and K. Taira. 2005. Escape from the interferon response associated with RNA interference using vectors that encode long modified hairpin-RNA. *Mol. Biosyst.* 1:382-390.
33. Krol, J., A. Fiszler, A. Mykowska, K. Sobczak, M. de Mezer, and W. Krzyzosiak. 2007. Ribonuclease dicer cleaves triplet repeat hairpins into shorter repeats that silence specific targets. *Mol. Cell* 25:575-586.
34. Vanitharani, R., P. Chellappan, and C.M. Fauquet. 2005. Geminiviruses and RNA silencing. *Trends Plant Sci.* 10:144-151.
35. Gough, G.W., K.M. Sullivan, and D.M.J. Lilley. 1986. The structure of cruciforms in supercoiled DNA: probing the single-stranded character of nucleotide bases with bisulphite. *EMBO J.* 5:191-196.
36. Haqqi, T., X. Zhao, A. Panciu, and S.P. Yadav. 2002. Sequencing in the presence of betaine: improvement in sequencing of the localized repeat sequence regions. *J. Biomol. Tech.* 13:265-271.
37. Balliet, J.W., J.C. Min, M.S. Cabatingan, and P.A. Schaffer. 2005. Site-directed mutagenesis of large DNA palindromes: construction and in vitro characterization of herpes simplex virus type 1 mutants containing point mutations that eliminate the oriL or oriS initiation function. *J. Virol.* 79:12783-12797.
38. Allawi, H.T. and J. SantaLucia, Jr. 1997. Thermodynamics and NMR of Internal G/T Mismatches in DNA. *Biochemistry* 36:10581-10594.
39. Harmse, J. 2007. Engineering virus resistant transgenic cassava: the design of long hairpin RNA constructs against South African cassava mosaic virus. MSc. Dissertation, University of the Witwatersrand, South Africa.
40. Schroeder, S., J. Kim, and D.H. Turner. 1996. GA and UU mismatches can stabilize RNA internal loops of three nucleotides. *Biochemistry* 35:16105-16109.
41. Vecenie, C.J. and M.L. Serra. 2004. Stability of RNA hairpin loops closed by AU base pairs. *Biochemistry* 43:11813-11817.
42. Vecenie, C.J., C.V. Morrow, A. Zyra, and M.L. Serra. 2006. Sequence dependence of the stability of RNA hairpin molecules with six nucleotide loops. *Biochemistry* 45:1400-1407.
43. Chellappan, P., M.V. Masona, R. Vanitharani, N.J. Taylor, and C.M. Fauquet. 2004. Broad spectrum resistance to ssDNA viruses associated with transgene-induced gene silencing in cassava. *Plant Mol. Biol.* 56:601-611.
44. Chellappan, P., R. Vanitharani, J. Pita, and C.M. Fauquet. 2004. Short interfering RNA accumulation correlates with host recovery in DNA virus-infected hosts, and gene silencing targets specific viral sequences. *J. Virol.* 78:7465-7477.
45. Jackson, A.L., S.R. Bartz, J. Schelter, S.V. Kobayashi, J. Burchard, M. Mao, B. Li, G. Cavet, and P.S. Linsley. 2003. Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol.* 21:635-637.
46. Senthil-Kumar, M. and K.S. Mysore. 2011. Caveat of RNAi in plants: the off-target effect. *Methods Mol. Biol.* 744:13-25.
47. Kumar, S., L. Arul, D. Talwar, and S.K. Raina. 2006. PCR amplification of minimal gene expression cassettes: an alternative, low cost and easy approach to 'clean DNA' transformation. *Curr. Sci.* 91:930-934.
48. Mukai, F., I. Hawryluk, and R. Shapiro. 1970. The mutagenic specificity of sodium bisulfite. *Biochem. Biophys. Res. Commun.* 39:983-988.

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