Replicase-Derived Resistance Against *Pea early browning virus* in *Nicotiana benthamiana* Is an Unstable Resistance Based upon Posttranscriptional Gene Silencing

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Virus resistance in *Nicotiana benthamiana* plants containing a translatable *Pea early browning virus* (PEBV) 54K sequence from the 201K replicase gene has been reported previously. Resistant plants contain multiple transgene copies divided between two loci. Analysis of a genetic series containing the two loci in separate homozygous or heterozygous condition suggest that only one of the loci is necessary to induce the resistance. The resistance observed in R2 and R3 generations of lines containing both transgene loci in homozygous condition became less consistent in R4 and R5 generations. This inversely correlated with steady-state transgene transcript levels of the segregating populations. The use of recombinant *Potato virus X* vectors carrying PEBV 54K sequences showed that the resistance is based upon posttranscriptional gene silencing, is non-strand specific, and recognizes 3' located sequences within the PEBV 54K sequence.

Pathogen-derived resistance, as formulated by Sanford and Johnston (1985), predicts that the expression of pathogen-derived products in a host can disrupt essential pathogenic processes and result in resistance to the pathogen (Baulcombe 1996; Fitchen and Beachy 1993; Lomonosoff 1995). Generally, two types of resistance can be distinguished (Baulcombe 1996; Van den Boogaart et al. 1998), one that requires the presence of transgene-derived protein (Brederode et al. 1995; Lapidot et al. 1993; Powell-Abel et al. 1986) and the other requiring the presence of transgene-specific RNA (De Haan et al. 1992; Lindbo and Dougherty 1992; Van der Vlugt et al. 1992). This second type of resistance, called "RNA-mediated resistance," is similar to a more general mechanism, "posttranscriptional gene silencing" (PTGS) (English et al. 1996; Mueller et al. 1995).

In 1992, pathogen-derived resistance was obtained for the *Pea early browning virus* (PEBV) isolate SP5 (MacFarlane and Davies 1992). PEBV (Boulton 1996) belongs to the *To-*

braviridae spp., an important family of plant pathogens typified by *Tobacco rattle virus* (TRV). The genome of tobaviruses is divided in two single-stranded, positive-sense RNA molecules, each encapsidated separately by the coat protein. RNA-1 of PEBV encodes two replicase proteins, the larger (201K) being expressed by readthrough of the opal (UGA) termination codon at the end of the smaller protein (141K) (MacFarlane et al. 1989). In an attempt to generate resistance against PEBV isolate SP5, MacFarlane and Davies (1992) transformed *Nicotiana benthamiana* plants with the readthrough portion of the replicase genes, or the "54K sequence." Southern blot analysis revealed a complex integration pattern with multiple copies of the 54K sequence present. Susceptible plants within a segregating R2 population had a higher 54K transcript level compared with resistant plants and were missing a particular integrated T-DNA. Resistant plants showed a high resistance phenotype, or immunity. The resistance was effective against an uncharacterized isolate of PEBV and *Broad bean yellow band virus* (a serotype of PEBV) (Robinson and Harrison 1985) but not against TRV or *Pepper ringspot virus* (the third member of the tobaviruses). Although the sequence similarities between these isolates and SP5 are unknown, the former characteristics are often associated with RNA-mediated resistance (Baulcombe 1996; Van den Boogaart et al. 1998). A modified form of the 54K sequence, resulting in a prematurely terminated 54K protein, was unable to induce resistance, leading to the suggestion that the protein rather than the RNA could be responsible for the resistance (MacFarlane and Davies 1992).

To analyze the mechanism(s) underlying the resistance to PEBV in plants transgenic for the PEBV 54K sequence, the resistance was followed over several generations and combined with DNA and RNA analysis. Such approaches have been used to characterize RNA-mediated resistance or PTGS (Dougherty et al. 1994; Goodwin et al. 1996; Smith et al. 1994). A series of progeny lines was generated by self-fertilizing the original transformant or by crossing with non-transformed plants. Genetic analysis revealed that resistance segregated with a complex locus recognizes a minimum sequence of the 3' end of the 54K sequence for the incoming virus and operates by a PTGS mechanism. Furthermore, detailed analysis of the resistance over four generations revealed that the resistance occasionally breaks down.

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RESULTS

T-DNA analysis of transgenic *N. benthamiana* containing a translatable PEBV 54K sequence.

Transgenic *N. benthamiana* line pRok54.3.2, as described by MacFarlane and Davies (1992), served as the starting material for subsequent studies. This line was transformed with the readthrough portion of the replicase genes from PEBV, the 54K sequence, inserted between a *Cauliflower mosaic virus* (CaMV) 35S promoter and a NOS terminator. After inoculation of segregating R2 plants (R0 being the primary transformant), the resistance against PEBV appeared to segregate with a particular integrated copy of the transgene (MacFarlane and Davies 1992).

To investigate these correlations and the underlying mechanism(s), R2-segregating plants were analyzed for their T-DNA organization and tested for their resistance to PEBV by following symptom development and using an enzyme-linked immunosorbent assay (ELISA) to detect the viral coat protein. This enabled the selection of individual susceptible and resistant plants, which were used to generate homozygous lines for further characterization of the resistance.

Genomic DNA was isolated from individual R2 plants, digested with *Hind*III (there is one site within the transferred DNA), and probed for the complete PEBV 54K sequence (Fig. 1). Two patterns of hybridization could be distinguished. DNA extracted from plants 1, 18, 19, and 20 gave only a single band of approximately 4.9 kb, whereas DNA from plants 4, 5, 7, 8, 9, 10, 11, 12, 13, and 17 gave three bands, one of which was apparently the same size as the single band found in plants 1, 18, 19, and 20. The segregation pattern of the transgenes indicates that they are dispersed between two loci (presumably located on two different chromosomes): locus A, which gives rise to the 4.9-kb band seen in all plants, and locus B, which produces two additional bands (3.7 and 5.5 kb) found in plants 4, 5, 7, 8, 9, 10, 11, 12, 13, and 17. Cosegregation of the 3.7- and 5.5-kb bands has been observed consistently in R3 and R4 generations (results not shown), supporting the notion that these two bands come from the same locus. The observed pattern suggests that the R1 parental plant, from which the analyzed R2 plants are derived, was homozygous for locus A and heterozygous for locus B.

All ten plants giving rise to three bands were immune to infection by PEBV, whereas three out of the four plants containing only the single band were susceptible. This suggests that resistance generally correlates with the presence of three bands. The exception to this is plant 1, which gave only a single hybridizing band but appeared to be resistant. There are two possible explanations for this apparent discrepancy: plant 1 is genuinely different from the other single-band plants (18, 19, 20) or somehow escaped infection during the inoculation process and was actually susceptible. The latter explanation is plausible because a small percentage of such escapes is observed consistently, even with nontransformed *N. benthamiana*.

To determine the influence of the integration pattern on resistance and to investigate whether plant 1 is genuinely different from the other plants that only contain locus A, plants 1 (pRok54.3.2.1), 10 (pRok54.3.2.10), and 19 (pRok54.3.2.19) were investigated. These three plants constitute the representative genotypes-phenotype combinations. Southern blot

analysis and segregation of kanamycin resistance (the 54K sequence is adjacent to the NPTII gene, resulting in cosegregation) of progeny plants from backcrossings with nontransgenic *N. benthamiana*, showed that plants 1 and 19 were homozygous for locus A, whereas plant 10 was homozygous for locus A and locus B (results not shown).

Pooled genomic DNA from R3 progeny, derived from plant 1, 10, and 19 (obtained by selfing), was digested with restriction endonuclease *Hind*III, *Eco*RI (both cleave only once within the T-DNA), or a combination, and probed for the complete PEBV 54K sequence (Fig. 2). The progeny of plants 1 and 19 have the same integration pattern, confirming the obtained hybridization pattern for the R2 generation (Fig. 1B). The single band obtained for *Hind*III and *Eco*RI digests suggests a single 54K transgene at locus A. Analysis of locus B in the progeny of plant 10 suggests three possibilities: i) three transgene copies are arranged as head-to-head-tail-to-tail repeats (Fig. 2C); ii) a tail-to-tail repeat with 3.5 kb of intervening DNA (plant DNA or vector DNA not containing the 54K sequence) containing a *Hind*III site after 750 nucleotides (nt); iii) a head-to-head repeat with 5.2 kb of intervening DNA containing an *Eco*RI site after 250 nt. With the use of a different enzyme (*Kpn*I), two bands of 3 and 5 kb were obtained (results not shown), strongly supporting the first option of transgene integration. No large deletions for the promoter-transgene-terminator region were observed (as illustrated by the *Hind*III + *Eco*RI digest), suggesting that the promoter-transgene-terminator cassette is integrated correctly into the plant genome, although small aberrations cannot be excluded.

Fig. 1. T-DNA analysis and the correlation with resistance against *Pea early browning virus* isolate SP5. **A**, Origin of the different plants tested (described here and in MacFarlane and Davies 1992). **B**, Genomic DNA isolated from transgenic R2 plants or nontransformed *Nicotiana benthamiana* (N), digested with *Hind*III, and probed for the 54K sequence. Numbers represent individual R2 kanamycin-resistant plants. Presence (+) or absence (-) of PEBV SP5, as determined by enzyme-linked immunosorbent assay and symptom scoring, is indicated.

PEBV 54K-mediated resistance is unstable over multiple generations.

To gain insights into the relation among transgene dosage, transcript levels, and resistance, homozygous and heterozygous progeny from plants 1, 10, and 19 were analyzed over a number of generations. As observed in the R2 generation, plants containing only locus A (plants 1 and 19, in homozygous or heterozygous condition) were generally susceptible, although there were a small number of exceptions observed in all generations (the inoculation efficiency was 97 to 100%) (Fig. 3A). The similarity in resistance and the identical pattern in Southern blots for plants 1 and 19 suggests that the presence of the transgene in plant 1 does not confirm resistance. This supports the earlier suggestion that the resistance observed for plant 1 in the R2 generation is most likely an es-

cape from inoculation (although it should be noted that PTGS in plant 1 cannot be excluded, particularly because results presented later in this paper suggest that factors other than transgene integration influence PTGS).

R3 progeny from plant 10 showed a high degree of resistance to PEBV infection in a heterozygous (20% susceptibility) and a homozygous state, with only one plant infected out of 50 inoculated (2% susceptibility). Southern blot analysis demonstrated that this infected plant had the same T-DNA organization as the parental plant. Virus isolated from this

Fig. 2. T-DNA organization in pRok54.3.2. **A**, Physical map of the T-DNA construct used to generate transgenic *Nicotiana benthamiana*. RB = right border; LB = left border; CaMV35S = CaMV35S promoter; NOS = NOS 3' polyadenylation region; Hi = *HindIII*; K = *KpnI*; P = *PvuII*; Hp = *HpaII*; E = restriction endonuclease designation (*EcoRI*). **B**, Southern blot analysis of pRok54.3.2.1, pRok54.3.2.10, and pRok54.3.2.19 (N is nontransgenic *N. benthamiana*). Genomic DNA was isolated from R3 homozygous plants; digested with *HindIII*, *EcoRI*, or both; and probed with a complete PEBV 54K DNA probe for the 54K sequence. **C**, Deduced organization of the T-DNA loci in pRok54.3.2.10. Potential products for *HindIII* and *EcoRI* digest are indicated. Arrow shows the orientation of the transgene; squiggly line indicates intervening DNA (plant or vector). Note that **A** and **C** are not drawn to scale.

Fig. 3. Inheritance of 54K-mediated resistance in R2, R3, and R4 progeny of 54K transgenic plants. **A**, pRok54.3.2.1, pRok54.3.2.10, and pRok54.3.2.19 were used for self-fertilization and crossings with nontransgenic *Nicotiana benthamiana*. The number of plants that became infected is shown as percentages (actual number is given below the percentage number). Arrow indicates the parental plant. Below "s" indicates a susceptible plant was used to create progeny; below "r," a resistant plant was used to create progeny. **B**, Methylation analysis of pRok54.3.2.10 segregating R4 and R5 populations. Genomic DNA was digested with *HpaII* and *PvuII* and probed for the 54K sequence. **C**, Transcript analysis of segregating plants of pRok54.3.2.10 R4 (from a susceptible parent) and R5 generations. Total RNA was isolated, followed by production of cDNA of polyadenylated transcripts. RACE polymerase chain reaction was performed followed by Southern blot analysis of amplified 54K cDNAs. sus. par. = susceptible parent; res. par. = resistant parent; s or S = susceptible; se = selfed; r or R = resistant; N = nontransgenic *N. benthamiana*; *N = crossed with nontransgenic *N. benthamiana*.

plant was unable to break through the resistance of other homozygous plant 10 plants, ruling out the possibility that the infected plant contained a resistance-breaking virus variant (results not shown). Progeny plants from this single, susceptible plant showed an enhanced susceptibility (60%) in the R4 generation, whereas progeny from a resistant R3 plant remained completely resistant. The segregation resistance-susceptibility was transmitted to the R5 generation by susceptible (progeny 45% susceptible) and resistant (progeny 25% susceptible) R4 parents.

To determine whether the segregation observed in the R5 and R4 (from a susceptible parent) in plant 10 correlated with the methylation pattern, genomic DNA was digested with *Pvu*II, located in the 5' part of the PEBV 54K sequence (Fig. 2A) and *Hpa*II, located in the 3' part of the PEBV 54K sequence (Fig. 2A), and probed for the 54K sequence. *Pvu*II and *Hpa*II are sensitive to symmetrical cytosine methylation. A typical Southern blot is shown in Figure 3B. Analysis of genomic DNA digested with *Hpa*II and *Pvu*II followed by probing for the 54K sequence showed differences when resistant plants were compared with susceptible plants within the different populations. When plants were compared over several generations by parental origins, however, no consistent differences could be correlated with the resistance.

Leaf tissue of the same developmental age as that of the inoculated tissue was used for transcript analysis. Attempts to visualize 54K transcripts by Northern blot analysis of total RNA or poly(A)+ mRNA were unsuccessful, although transcripts could be detected after cDNA of polyadenylated transcripts was subjected to polymerase chain reaction (PCR) followed by Southern blot analysis (RACE PCR). Transcripts were detected in all plants except resistant R5 plants, and an inverse correlation was observed between 54K transcript levels and resistance (Fig. 3C). This was confirmed by a TaqMan assay (PE Biosystems, Norwalk, CT, U.S.A.) (results not shown). The observation of a higher transgene transcript level in susceptible plants is consistent with resistance operating through PTGS.

PEBV 54K-mediated resistance does not require locus A.

To determine whether locus A is required to establish the resistance, plants containing only locus B were generated by backcrossing (selected by Southern blot analysis) (Fig. 4) and subsequent selfing of heterozygous plant 10. Lines that were either homozygous or heterozygous for locus B, termed 1W5 homozygous or 1W5 heterozygous, respectively, were selected on the basis of kanamycin segregation data. When inoculated with PEBV, homozygous plants showed 6% susceptibility (1 out of 16 plants became infected), whereas heterozygous plants showed 5% susceptibility (1 out of 19 plants became infected). Thus plants homozygous for locus B showed resistance equivalent to homozygous plants containing locus A and B (Fig. 3).

Previous analysis with RACE PCR (MacFarlane and Davies 1992) suggested an inverse correlation between resistance and transgene transcript levels, which is typical of RNA-mediated resistance. To confirm and investigate this inverse relationship, RACE PCR was performed on total RNA from plants 1, 10, 19, and 1W5 in homozygous condition. Transgene transcripts could be detected in plants 1 and 19 (Fig. 5), which confirmed the inverse relation between trans-

gene transcript levels and resistance. The two bands observed for plants 1 and 19 (Fig. 5) correspond to single- and double-stranded DNA. Lines 10 and 1W5 were resistant and showed undetectable levels of transcripts, although transcripts were detected in plant 10 with RACE PCR (Fig. 3C) and TaqMan (results not shown).

The T-DNA organization of plant 10 is the sum of plants 1 (or 19) and 1W5. If transgene silencing was not occurring, one would expect the transgene transcript levels to be the sum of the two lines. Clearly, transcript levels in plant 10 are much lower.

Resistance in 54K transgenic plants and PTGS.

Genetic analysis of lines transgenic for the PEBV 54K sequence indicate that resistance to PEBV operates through a silencing mechanism. To rule out the possibility that protein may be involved, two types of constructs were made and inserted into a PVX vector (Baulcombe et al. 1995). The first type contained the full-length PEBV 54K sequence, either in sense or antisense orientation. To determine whether the resistance recognizes specific sequences or merely a specific length of homology, a second range of constructs was produced. These constructs contained sequences from the 5' or 3' ends of the 54K sequence, with identical sizes. If the resistance develops on the basis of interference with protein products of PEBV, the mechanism would be unable to interfere with the infection of a heterologous virus, even if it carried the sequence used to create the resistance.

Infectious transcripts were inoculated onto the three homozygous representative lines of plant 1 (locus A), 1W5 (locus

Fig. 4. T-DNA organization of backcross progeny of heterozygous pRok54.3.2.10 with nontransgenic *Nicotiana benthamiana*. Genomic DNA was digested with *Hind*III and probed for the 54K sequence. Eight lines are shown, of which pRok54.3.1W5 (lane 5) was used in subsequent experiments. Arrows indicate the bands associated with locus A and B.

Fig. 5. Analysis of 54K transcripts from representative 54K transgenic lines. Total RNA was isolated from representative lines 30 days post-germination. RACE polymerase chain reaction was performed on cDNA of polyadenylated transcripts, followed by Southern analysis of amplified 54K cDNAs. 1, 10, 19, 1W5 = pRok54.3.2.1, pRok54.3.2.10, pRok54.3.2.19, and pRok54.3.1W5, respectively; N = nontransgenic *Nicotiana benthamiana*. Two bands observed for pRok54.3.2.1 and pRok54.3.2.19 are single- and double-stranded DNA molecules (MacFarlane and Davies 1992).

PVX containing the 54K sequence derived from the 5' part was able to replicate on homozygous plants 10 and 1W5, suggesting that these 54K sequences were neither recognized nor targeted by the resistance mechanism. PVX containing 3'-located sequences of the 54K sequence was unable to replicate in homozygous plants 10 and 1W5, demonstrating that these 54K sequences were targeted by the resistance mechanism. The shortest 54K sequence shown to confer resistance contained the 3' terminal 370 nt, suggesting that the recognized sequence is located within this region. It should be noted, however, that this sequence was not very efficiently recognized in homozygous plant 1W5. This finding is similar to that obtained with other systems (English et al. 1996; Sijen et al. 1996). Because some of the constructs represented deletions of similar length on the 5' or 3' end, it is unlikely that targeting develops on the basis of recognition of the minimal length of the PEBV 54K sequence.

DISCUSSION

Previously it was shown that the resistance in pRok54.3.2 was highly effective and correlated with multiple T-DNA insertions and low levels of transgene-derived RNA (MacFarlane and Davies 1992). Detailed analysis in this study showed that the transgenes were divided between two loci, which was supported by Southern blot analysis over multiple generations. Locus A contained a single transgene copy, whereas locus B most likely contained three transgenes arranged in a head-to-head-tail-to-tail configuration.

The resistance observed in 54K-transgenic plants is induced only by locus B, which displays a number of characteristics generally associated with PTGS. The first is that locus B can transinactivate locus A. Transgene transcript levels of locus A are much higher when locus B is absent compared with transgene transcript levels when locus B is present. Furthermore, the transgene integration structure of locus B is characteristic for PTGS. Locus B displays two transgene integration characteristics generally associated with PTGS. The first is a transgene copy number. There seems to be a general correlation between a transgene copy number (or transgene transcript levels) and silencing. In a study similar to the one described in this paper, a genetic series of plants containing transgenes

divided among different loci showed that resistance correlated with copy number (Goodwin et al. 1996).

The second characteristic of locus B is the presence of inverted repeats. PTGS is often correlated with complex T-DNA integrations that frequently contain repeats (Cluster et al. 1996; Hamilton et al. 1998; Sijen et al. 1996; Stam et al. 1997; Stam et al. 1998). Indeed, Sijen et al. (1996) showed that transformation with a direct repeat of the cowpea mosaic virus movement protein increased the percentage of resistant plants

Fig. 6. Analysis of recombinant PVX accumulation. **A**, Northern blot analysis of the accumulation of pP2C2S-PET3 on nontransgenic *Nicotiana benthamiana* and pRok54.3.2.10. Although the accumulation of this construct is shown, all the constructs inoculated on all the lines and controls were analyzed in this manner. Plasmid pP2C2S is the wild-type PVX containing a duplicated coat protein promoter (Baulcombe et al. 1995). RNA was isolated from systemic leaves of inoculated plants and probed with a DNA probe for the 54K sequence. **B**, Deletion analysis of the PEBV 54K sequence with a PVX vector. RNA was isolated from systemic leaves of nontransformed and transgenic *N. benthamiana* pRok54.3.2.1 and pRok54.3.2.10 (homozygous R3 generation plants). Arrow shows whether the insert is in the sense or antisense orientation. For the complete PEBV 54K sequence, the number of the first and last nucleotide is shown; for other constructs, either the last (PEF1) or first nucleotide (PET1) is shown. Values indicate the number of infected plants (as determined by Northern blot analysis) per total number of plants inoculated.

to 60% compared to 20% for single-copy insertions. Hamilton et al. (1998) showed that 96% of plants transformed with an ACC oxidase gene containing two additional upstream inverted copies of its 5' untranslated region showed gene silencing compared with 15% of plants without the repeat. Unfortunately, no conclusions can be drawn as to which of the two characteristics mentioned is responsible for the induction of resistance.

The characteristics of the resistance, immunity, strain specificity, and a low accumulation of transgene-derived RNA are characteristic of RNA-mediated resistance (Baulcombe 1996; Lomonosoff 1995; Van den Boogaart et al. 1998). In their initial report, MacFarlane and Davies (1992) suggested that the 54K protein might mediate the resistance on the basis of the observation that modified forms of the 54K sequence, resulting in a prematurely terminated 54K protein, were unable to induce resistance. Only a low number of lines were tested, however, and plants transformed with an untranslatable version of the 54K sequence were subsequently shown to be resistant (C. Wallis, G. P. Lomonosoff, and J. W. Davies, *unpublished results*).

The suggestion of an RNA-based mechanism also is supported by the finding that the resistance can act against PVX carrying PEBV 54K sequences. As was observed in other systems (English et al. 1996; Sijen et al. 1996), the target sequence is located in the 3' part of the 54K sequence and the recognition is not strand specific. Although the positive and negative strand of the 54K sequence is produced during the replication process of PVX, the negative strand is generally regarded to be located in the replication complex and is thought to be inaccessible. The location of the target in the 3' end is not a general rule, however, because multiple targets in the coding region have been observed for other systems (Jacobs et al. 1999; Marano and Baulcombe 1998). The resistance mechanism seems less effective against PVX containing the complete 54K sequence or derivatives (ranging from zero out of five plants infected to two out of five plants infected) compared with resistance against PEBV SP5 (98% resistance in the R3 generation). The reason for this is unclear, but it may reflect differences in the levels of virus accumulation or the site of replication between PEBV and PVX carrying PEBV sequences.

The resistance as observed in plants homozygous for both loci appears to be unstable. Although initially all plants showed the resistance (R3 and R4 plants from a resistant parent) in subsequent generations, the resistance started to segregate. Transgene analysis showed no obvious changes in transgene integration structure between generations. PTGS is reset during meiosis (Balandin and Castresana 1997; Dehio and Schell 1994) and should therefore display a stable phenotype over multiple generations. The reason behind the instability reported here is unclear, but not unprecedented. Instability was observed for plants containing a fragment of the *Plum pox virus* genome (Guo et al. 1998). Unfortunately, these transgenic plants were not examined by Southern blot analysis, so it is unclear how the T-DNA is integrated and whether fragments of the T-DNA are present at different loci. This could lead to complications because the zygosity was determined with kanamycin resistance data that does not necessarily correspond to the zygosity of the transgene. Additionally, the instability only concerned the R1 and R2 generation. R3

transgenic plants were consistent in their phenotype, which depended on the phenotype of the R2 parent (i.e., if the parent was resistant, the progeny would be resistant, and vice versa), in contrast with the instability in R5 54K-transgenic plants. The instability was related to levels of transgene transcripts as observed here, and resistant plants had a lower level of transgene mRNA as compared with susceptible plants. The low levels of RNA, however, are most likely a consequence of PTGS, so the underlying mechanism responsible for the segregation is unknown.

Instability of silencing also was detected in rice containing a *bar* transgene that encodes bialaphos herbicide resistance (Kumpatcha and Hall 1998). Here the instability was correlated with extensive methylation in the promoter region. Smith et al. (1994) also showed that genetically identical plants can show differences in their phenotype. A double-haploid family of transgenic plants containing an untranslatable potato virus Y (PVY) coat protein showed differences in its response to PVY inoculation. This correlated to differences in steady-state mRNA levels and the methylation state of the transgene. PTGS is quite sensitive to changes in the rate of transcription (English and Baulcombe 1997) and, although differences in transgene steady-state transcript levels have been observed here, it is not clear whether they are a cause or a consequence of the segregation of the silencing. No correlation of methylation and resistance was found here, however, and only a low number of sites have been analyzed. Further instances of PTGS instability have since been reported (Tenllado and Díaz-Ruiz 1999), stressing the need for multigeneration testing as has been stated by others (Kumpatcha and Hall 1998).

MATERIALS AND METHODS

Plant material and inoculation with PEBV.

The transgenic *N. benthamiana* plant line pRok54.3.2 contains the readthrough portion or 54K sequence of the replicase genes of PEBV (MacFarlane and Davies 1992). Nontransgenic *N. benthamiana* plants were used as controls. Seed was sown and kept for approximately 4 weeks in the containment greenhouse under natural-light conditions before inoculations. Transgenic seed was germinated on MS medium (Murashige and Skoog 1962) containing 500 µg of kanamycin per ml. After 2 weeks, the seedlings were transferred to soil and kept in the greenhouse for approximately 2 weeks before inoculations.

To generate heterozygous progeny from a homozygous parent, pollen from a transgenic homozygous parent was transferred to the stigma of nontransgenic *N. benthamiana*. Heterozygous seeds were then distinguished from nontransgenic seeds (resulting from self-pollination) by germination on MS medium containing 500 µg of kanamycin per ml, as described above.

PEBV isolate SP5 was propagated in and purified from the systemic host *N. benthamiana*, according to MacFarlane et al. (1989). The virus was resuspended in 30 mM sodium phosphate buffer, pH 7.0. To challenge a plant, a leaf was mechanically inoculated with carborundum as an abrasive and PEBV at a concentration of approximately 1 mg of inoculation buffer (20 mM phosphate, pH 7.2; 1 mM EDTA) per ml. After inoculation with 20 µl of virus suspension, the leaflet was rinsed with water. The inoculation efficiency with this method

was always 97 to 100%. Inocula also were applied to *Chenopodium amaranticolor* (a local lesion host for PEBV) to quantitate the infectivity of each inoculum. A concentration of 1 mg of purified PEBV per ml corresponded to approximately 500 to 1,000 lesions per 20 µl of inoculum per leaf.

Detection of PEBV in infected tissue.

Systemic leaves (younger than the inoculated leaf) were homogenized with a mechanical grinder in coating buffer (10 mM Na₂CO₃, 35 mM NaHCO₃). The insoluble material was discarded following centrifugation (5 min at full speed in a microfuge). The sample was applied to a Nunc-Immuno Plate MaxiSorp plate (Nalge Nunc International, Rochester, NY, U.S.A.), followed by a direct ELISA assay. Detection of viral coat protein was achieved by reaction with rabbit anti-PEBV serum followed by a goat anti-rabbit antibody conjugated with alkaline phosphatase with p-nitrophenylphosphate in diethanolamine buffer (pH 9.8) as a substrate. Plates were washed with phosphate buffered saline (0.02 M phosphate; 0.15 M NaCl; 0.003 M KCl, pH 7.4) containing 0.05% Tween-20, and absorbance values were recorded at 405 nm.

Generation of recombinant PVX vectors and infection on plants.

Constructs pP2C2S-PEF4, pP2C2S-PEF3, pP2C2S-PEF2, and pP2C2S-PEF1 (Fig. 6) were created by PCR with primers V1177 (sense nucleotides 211 to 231 in the 35S promoter of pMon530) (Rogers et al. 1987) and V4097 (antisense nucleotides 388 to 366 in NOS terminator of pMon530) (Rogers et al. 1987) from template DNA pMon54.4367, pMon54.4455, pMon54.4566, and pMon54.4708, respectively (C. Wallis, G. P. Lomonosoff, and J. W. Davies, *unpublished data*).

Constructs pP2C2S-PET1 to PET4 (Fig. 6) were made by PCR with primers V4172 (sense nucleotides 4,714 to 4,746 of 54K sequence), V4173 (sense nucleotides 4,856 to 4,888 of 54K sequence), V4174 (sense nucleotides 4,967 to 4,999 of 54K sequence) and V4175 (sense nucleotides 5,055 to 5,085 of 54K sequence), respectively, in combination with V4171 (antisense nucleotide 5,425 to 5,401 of 54K sequence) from template pMon54.3 (a pMon530 transformation vector containing the complete PEBV 54K sequence) (C. Wallis, G. P. Lomonosoff, and J. W. Davies, *unpublished data*). Plasmid pP2C2S-PE+ was constructed with primers V4170 (sense nucleotides 3,993 to 4,025 of 54K sequence) and V4171 from template pMon54.3. Plasmids pP2C2S-PET5 and pP2C2S-PET7 were constructed with PCR primers 991103 (antisense nucleotides 5,238 to 5,212 of 54K sequence) and 991105 (antisense nucleotides 5,146 to 5,123 of 54K sequence), respectively, in combination with primer V4175. Plasmids pP2C2S-PET6 and pP2C2S-PET9 were made by PCR primers V4171 and 991107, respectively (antisense nucleotides 5,332 to 5,312 of 54K sequence), in combination with 991104 (sense nucleotides 5,239 to 5,260 of 54K sequence). pP2C2S-PET8 was constructed with PCR primers 991106 (sense nucleotides 5,147 to 5,167 of 54K sequence) and 991103. pP2C2S-PET10 was made with PCR primers 991108 (sense nucleotides 5,333 to 5,352 of 54K sequence) and V4171. Plasmids pP2C2S-PET5 to PET10 were generated from template pMon54.3.

PCR reactions contained 5 ng of template DNA; 10 mM Tris-HCl, pH 8.5; 50 mM KCl; 1.5 mM MgCl₂; five units of *Taq* polymerase; 250 µM dATP, dCTP, dTTP, and dGTP; 2.5

mM primer (each); and volume was created with sterile water to 50 µl. Cycling conditions were 5 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 65°C, and 2 min at 72°C. The last cycle ended with a final extension of 10 min at 72°C. The PCR program was run on an Omnigene thermal cycler (Hybaid Ltd., Ashford, Middlesex, U.K.).

For cloning, the PCR products were digested with *Cla*I (present in the 5' primers) and *Eco*RV (present in the 3' primers) and ligated in a *Cla*I- and *Eco*RV-treated pP2C2S-vector (Baulcombe et al. 1995) with 1 U of T4 DNA ligase in a ligation buffer + ATP for 14 h at 15°C. The ligation mixture was used to transform *Escherichia coli* GM119, after which colonies were checked by PCR with primers V3793 (antisense nucleotide 5,917 pP2C2S) and V3794 (sense nucleotides 5,450 to 5,472 pP2C2S) and restriction analysis.

In order to produce infectious transcripts, 25 µg of template DNA was linearized with *Spe*I. In vitro transcription was performed as described by Chapman et al. (1992). One-fifth of the transcription reaction per plant was used for inoculation, as described earlier.

Extraction of genomic DNA and Southern blot analysis.

The isolation of genomic DNA was performed essentially as described by Dellaport et al. (1985). PCR-generated PEBV 54K DNA fragments were labeled with ³²P-dCTP and the Random Primers DNA Labeling System (Gibco BRL, Paisley, U.K.). Labeled DNA was separated from unincorporated nucleotides with a Nick column (Pharmacia, Milton Keynes, U.K.), according to the supplier's instructions.

For Southern blots, approximately 10 µg of genomic DNA was digested overnight with 50 U of the appropriate restriction enzyme, followed by transfer and hybridization as described in Sambrook et al. (1989).

Extraction of RNA and Northern analysis.

Total RNA was extracted with the RNeasy plant kit (Qiagen, Hilden, Germany), according to the instructions of the manufacturer. Poly(A)+ mRNA was isolated from total RNA with the Oligotex mRNA mini kit (Qiagen). For viral RNA detection, approximately 1 µg of total RNA was loaded. RNA was run in a 1% formaldehyde agarose gel and transferred by capillary blotting to a Hybond NX nitrocellulose membrane (Boehringer Mannheim, Indianapolis, IN, U.S.A.), according to standard methods (Sambrook et al. 1989). Hybridization with a DNA probe, specific for the complete PEBV 54K sequence, was performed as described in Sambrook et al. (1989).

Transgene-derived transcripts of the PEBV 54K sequence were analyzed by 3' RACE PCR. Total RNA was isolated as described above and hybridized to the 3' oligonucleotide V3469 (GACTCGAGTCGACATCGA(T₁₇)). cDNA was produced with Superscript reverse transcriptase (Gibco BRL), according to the manufacturer's instructions. Transcript cDNAs were detected with primers V4175 (CCATCGATAGCATTCTGCGGGAAGTTTTTG) and V3468 (GACTCGAGTCGACATCGA), followed by PCR as described before with an annealing temperature of 55°C. PCR products were visualized by Southern blot analysis as described in Sambrook et al. (1989). Controls were included to ensure that no residual genomic DNA or viral RNA was amplified.

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