Specificity of resistance to pea seed-borne mosaic potyvirus in transgenic peas expressing the viral replicase (NIb) gene

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Transgenic pea lines carrying the replicase (NIb) gene of pea seed-borne mosaic potyvirus (PSbMV) were generated and used in experiments to determine the effectiveness of induced resistance upon heterologous isolates. Three pea lines showed inducible resistance in which an initial infection by the homologous isolate (PSbMV-DPD1) was followed by a highly resistant state. Resistance was observed in plants in either the homozygous or hemizygous condition and resulted in no overall yield loss despite the initial infection. Resistance was associated with a loss of both viral and transgene RNA, which is indicative of a mechanism based upon post-transcriptional gene silencing. There was no correlation between the steady-state levels of transgene RNA and ability of the plants to show resistance. To test the specificity of the resistance, plants were also inoculated with the most distantly related sequenced PSbMV isolate, NY. PSbMV-NY varied between experiments in its ability to induce resistance, suggesting that the sequence identity in the NIb gene is borderline for the specificity required for triggering gene silencing. Upon challenge inoculation of virus-free recovered leaves, the specificity of the induced resistance varied between the two isolates and indicated that the virus and transgene additively determined the resistant state. These results suggest that the sequence requirements for triggering gene silencing may differ from those involved in the degradation process.

Introduction

The strategy of transforming plants with sequences derived from viral RNA has proved to be successful in generating resistance to many viruses and in several plant species (reviewed in Lomonossoff, 1995; Baulcombe, 1996; Baulcombe & English, 1996; Palukaitis & Zaitlin, 1997; van den Boogaart et al., 1998). Different viral sequences have been employed in these strategies and there appear to be no clear rules to indicate which sequences will, and which will not, give resistance. The two most common sequences employed to date for the generation of virus-resistant plants are viral coat protein and replicase sequences. However, it is likely that all viral sequences have the potential to induce resistance through mechanisms of post-transcriptional gene silencing. For neither coat protein nor replicase transgenics is the mechanism of resistance fully understood. Of importance in the practical application of the technology is the breadth of resistance conferred. Coat protein sequences tend to produce a broader spectrum of resistance (i.e. active against other isolates and other viruses of the same group) than do replicase sequences, which often give resistance that is highly sequence specific (discussed in Lomonossoff, 1995).

Pea seed-borne mosaic virus (PSbMV) is a member of the genus Potyviridae. It infects legumes and can cause major yield loss especially when infection is via the seed. Natural recessive resistances to PSbMV exist (Alconero et al., 1986; Provvidenti & Alconero, 1988a) but have yet to be introgressed into commercial pea lines. The strategy of engineered resistance against potyviruses has been used successfully using viral coat protein, sense (Lindbo & Dougherty, 1992) and antisense (Hammond & Kamo, 1995), Nla protease (Maiti et al., 1993; Vardi et al., 1993), NIb replicase (Audy et al., 1994) and VPg sequences (Swaney et al., 1995). However, the majority of the work has been performed in Nicotiana tabacum and N. benthamiana and it remains to be determined how effective these strategies will be in legumes. To date, there is only one example of pathogen-derived resistance generated in legumes; this used the coat protein precursor gene of a comovirus, bean-pod mottle virus, for the production of transgenic soybean (Di et al., 1996). We aimed to determine whether the strategy of...
engineering resistance via introduction of viral sequences could be employed against PSbMV in pea and to show the effective breadth of resistance.

We report here the generation of transgenic pea lines carrying PSbMV replicase (Nlb) sequences that exhibit resistance to PSbMV. The induced resistance was associated with the absence of viral RNA and a reduction in transgene RNA levels in upper leaves and is typical of virus-induced gene silencing events described previously (Guo & Garcia, 1997; Lindbo et al., 1993). Although the plants showed induced resistance following inoculation with different isolates, chal- lenge inoculation of resistant tissue suggested that different rules determined the effectiveness of the targeted degradation of the viral RNA in the cytoplasm.

### Methods

#### Viruses and plants. The PSbMV isolates used in this study were DPD1 (pathotype 1), NY (pathotype 4) (Johansen et al., 1996) and L-1 (pathotype 2; Provvidenti & Alconero, 1988). Isolates DPD1 and NY were maintained as cDNA clones that could be used to initiate an infection after inoculation onto susceptible pea (Pisum sativum cv. Vedette) plants. Isolate L-1 was maintained as infectious virus by passage on the same plants. Pea plants (cv. Vedette and the transgenic cv. Puget) were grown in a glasshouse at 18 ± 5 °C with a 14 h supplemented photoperiod. Transgenic plants were grown under contained conditions.

#### Virus inoculations. Virus inoculations for isolates DPD1 and NY were performed using plasmids carrying full-length PSbMV cDNAs under the control of the CaMV 35S promoter (Johansen, 1996). Introduction of the DNA into the nucleus of test plants was achieved by microprojectile bombardment using a portable hand-held gun (Gal-On et al., 1997). Gold particles (0.95 μm) were coated with DNA as described previously (Christou et al., 1991). The suspension was sonicated briefly and then loaded into a length of Teflon tubing; after drying the gold uniformly onto the inside surface of the tubing, short lengths were used as ‘bullets’ in the hand-held gun. The gold was discharged using helium gas at a pressure of 300 p.s.i. The use of this method of inoculation ensured an infection rate of 100% as opposed to 50–100% for manual inoculation of cv. Puget. Such a particle delivery inoculation method resulted in an infection that was indistinguishable in all respects from an infection initiated from a standard sap inoculation.

Manual virus inoculations were performed by lightly rubbing extracts of infected pea leaves in 50 mM sodium phosphate buffer pH 7.0 onto the surface of expanded leaves dusted with carbonbium. Virus infections were identified by DAS-ELISA (Ding et al., 1992) using a rabbit polyclonal antiserum raised to virus particles, and by northern analysis for PSbMV RNA (see below).

#### DNA constructs for transformation. All the cloning steps were based upon standard molecular biology protocols (Sambrook et al., 1989). The following clones provided the starting material for making four different transformation constructs (Fig. 1).

1. **PSbMV DPD1 cDNA clones pPS13 and pPS34** (Johansen et al., 1991) were the sources of the Nlb gene (nt 7328–8888), the 5′ untranslated region (nt 1–143) and the 3′ UTR (nt 9761–9924) of PSbMV RNA.

2. **Vector pJIT60** (Guerrineau et al., 1992) provided the expression cassette with the 2 × CaMV 35S promoter and the CaMV poly(A) terminator.

3. **Vector pSLJ501** (J. Jones, unpublished data) is a modified expression vector based upon published materials (Jones et al., 1992). The key component of this vector is a nos (nopaline synthase) promoter–bar gene–ocs3′ terminator cassette with appropriate restriction enzyme sites to allow the bar gene to be replaced with PSbMV sequences.

4. **Binary vector Eños:bar** (a gift from P. Mullineaux) was the recipient for the expression cassettes. Eños:bar consists of an 815 bp T-DNA based on the lacZ′ gene and a multiple cloning site polylinker from pBlueScript (Stratagene) flanked by synthetic right and left T-DNA border repeats. The vector carries the nos promoter–bar gene–ocs3′ terminator cassette for selection. The bar gene encodes a modifying enzyme, phosphinothricin acetyltransferase (PAT), which confers resistance to the herbicide BIALOPHOS and the related compound phosphinothricin (PPT) (De Block et al., 1987).

**Cloning of 5′ UTR-Nlb with engineered translational start and stop casons.** PSbMV cDNA nt 1–143 (5′ UTR) were amplified from pPS34 by PCR using adapted primers with restriction sites 5′ EcoRI–Dral and 5′ BsmII–Ncol adjacent to nt 1 and 143, respectively. The amplified fragment was cloned as an EcoRI–BsmII fragment into pGEM-3 (Promega) to give p5′ UTR. PSbMV cDNA nt 6912–7773 (including the N-terminal half of Nlb) from pPS13 was cloned as a BgIII–SacI fragment into similarly digested pBlueScript KS– (Stratagene) and subjected to site-specific mutagenesis with the SCULPTOR kit (Amersham) to introduce a BamHI site immediately before the start codon of the Nlb coding sequence at nt 7328. The clone was then digested with BamHI and SacI and the nt 7328–7773 fragment recloned into pBlueScript KS (–), to give pBamN-Nlb. To combine the 5′ UTR and N-term-Nlb, p5′ UTR was digested with Ncol, blunt-ended with Klenow (which generated the required ATG start codon) and the fragment released with EcoRI. After digestion of pBamN-Nlb with BamHI, incubation with mung bean nuclease to give a blunt end and digestion with EcoRI, it was ligated with the 5′ UTR fragment to give p5′ UTR-Nlb.

The C-terminal half of Nlb with a translational stop codon was obtained from a parallel series of cloning steps that resulted in the 3′ UTR (nt 9761–9924) being placed after the Nlb coding region. This gave the sequence …GTT CGA TTG CAA ggc TAAATCCGTAIGTATTT-TTATGACGTCTAAT… where the last Nlb codon was followed by an additional ‘gcc’ codon before the translational stop (bold). The sequence following included a SalI site (underlined) from the 3′ UTR that was used in subsequent cloning steps; the remainder of the 3′ UTR was not used. The construct originated from PSbMV cDNA nt 6812–9106 from pPS13) cloned as a BgIII fragment in pBlueScript KS (–), the latter modified with BglI linker inserted into the unique EcoRV site. This clone was then modified by site-directed mutagenesis using the SCULPTOR kit to introduce a Ndel site (GGCCGC) at the junction between the Nlb and coat protein coding regions (nt 8891), to give p–NlbNaeN-CP. The Ndel site was used to make clone p–NlbStop3′UTR by ligating in a primer-adapted PCR fragment of the 3′ UTR that donated a TAA stop codon between Nlb and the 3′ UTR.

To combine the 5′ UTR and the N- and C-terminal halves of Nlb, the SacI (nt 7773–SacI (polylinker) fragment from p–NlbStop3′UTR was ligated into SacI-digested (nt 7773) p5′ UTR-Nlb, to give p5′ UTR-NlbStop3′UTR.

**Cloning of Nlb for expression in transformed plants.** To construct 35S-Nlb (Fig. 1), the HindIII (nt 8647)–SacI fragment from p5′ UTR-NlbStop3′UTR was ligated into HindIII/SmaI-cut pJJIt60. The resulting clone was digested with HindII and ligated with the HindIII (5′ polylinker–nt 8647) fragment also from p5′ UTR-NlbStop3′UTR. The complete expression cassette was excised by digestion the flanking polylinkers with ApaI and SpeI. After producing blunt ends by incubation with T4 DNA polymerase, the cassette was inserted into the Eños:bar binary vector which had been digested previously with Xhol and T4 DNA polymerase to similarly give blunt ends.
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**Fig. 1.** Four constructs (a) were used for the generation of PSbMV NiB-transgenic peas. The intermediate clones used in the construction of the expression cassette are illustrated in (b). Boxes (not to scale) show the components of the PSbMV genome where hatched box = NIb and the filled box = 3’UTR; the direction of the coding sequence for NIb is shown (arrow). Restriction sites used in the cloning steps are shown on the respective clones (A, Asp718; Ba, BamHI; Bc, BclI; Bg, BglII; D, DraI; E, EcoRI; EV, EcoRV; H, HindIII; N, NcoI; Nc, Ncol; Sc, ScaI; Sp, SphI; X, XhoI). Nt coordinates refer to the sequence of PSbMV DPD1 (Johansen et al., 1991). Engineered restriction sites used to produce translational start (ATG) and stop (TAA) codons are shown in bold.

35S:NIbIb and 35S:NIbbI (Fig. 1) were produced by ligating the XhoI (nt 8075)–ScaI digested and blunt-ended fragment from p5′UTRNbstop3’UTR into Smal-digested plasmid vector pIT60. The two possible inserts gave the Ib and bI orientations. After digestion of these clones at the SalI site in the polylinker and treatment to give blunt ends, they were ligated with the EcoRV (5′polylinker)–ScaI fragment of p5′UTRNbstop3’UTR. The complete expression cassettes were cloned into the E6nos:bar binary vector as for 35S:NIb.

To create Nos:NIb (Fig. 1), the bar gene was removed from pSLJ501 by XhoI/Smal digestion and replaced with the XhoI (nt 8075)–SalI fragment from p5′UTRNbstop3’UTR. After digestion with XhoI (nt 8075), the resulting clone was ligated with the XhoI (5′polylinker)–XhoI (nt 8075) fragment also from p5′UTRNbstop3’UTR. The complete expression cassette was excised using the flanking enzyme sites for BclI and PstI, blunt-ended and inserted into XbaI-cut and blunt-ended E6, as before.

All of the binary vectors were inserted into *Agrobacterium tumefaciens* AGL1 (Lazo et al., 1991) by electroporation. The fidelity of the cloning steps was checked by sequencing the regions containing the inserted translational start and stop codons and by restriction analysis of the final constructs.

### Production of transgenic peas

Pea cv. Puget was transformed as described by Bean et al. (1997). Briefly, lateral cotyledonary meristems of germinating seeds were inoculated with *A. tumefaciens* strain AGL1 carrying the transformation constructs. Shoots developing from the lateral meristems were subjected to selection with 5 mg/l PPT. Shoots surviving the selection were grafted onto non-transgenic seedlings used as root stocks. Primary transformants were analysed by painting leaves with the herbicide HERBIACE (Meija Seika Ltd, Japan) (3 mg/l) and by small scale DNA preparation (Ellis, 1994) followed by PCR using NiB specific primers complementary to nucleotides 7332–7354 (A: 5′
CGGGATGACGCATGGCTAGAG 3') and 8885–8861 (B: 5' CAAT-
CGAACCTTGATTGATCCATC 3').

- **DNA blot analysis.** Genomic DNA was extracted from leaf material and analysed as described by Ellis (1994). Ten µg of DNA was digested overnight with HindIII followed by separation by gel electrophoresis, transfer onto Hybond-NX membrane (Amersham) and Southern hybridization. DNA fragments for use as probes were generated by PCR and labelled with [32P]dCTP by the method of Feinberg & Vogelstein (1983).

- **RNA blot analysis.** Total RNA was isolated from pea leaves using the RNA ISOLATOR kit (Genosys) and 10 µg was used for Northern blot analysis. Denaturing RNA gels were run as described by Gründermann & Koepf (1994) followed by transfer to Hybond-NX membrane and hybridization with radioactive probes as described above.

**Results**

**Transformation constructs and transgenic pea lines**

Four transformation constructs were made for this study (Fig. 1). All four constructs carried the viral 5'UTR and the entire replicase (Nb) gene from PSbMV isolate DPD1. Two constructs differed only in the promoter that was used for expression of the Nb gene; 35S:Nb had double CaMV 35S promoter sequences whereas nos:Nb had the nopaline synthase (nos) promoter. The other two constructs carried not only the 5'UTR and entire Nb gene driven by the double 35S promoter but also had the 3' 816 bp of the Nb gene present as either a direct (35S:Nbb) or inverted (35S:Nbbl) repeat to give transcriptional, but not translational, fusions. Constructs 35S:Nbbl and 35S:Nbb were made based on observations that transgene repeats are a common feature in lines that have resistance, or show co-suppression of the transgene (data not shown). Southern analysis of genomic DNA (data not shown) was used to determine the number of transgene loci, and to confirm that the primary transformants represented independent transformation events. The analysis showed that for the 35 primary transformants, one, two and three transgene loci were present in twenty-six, five and four plants, respectively.

**Screening for virus resistance**

Seeds were collected from self-pollinated primary transformants and the F1 progeny for each line was tested for virus resistance. Prior to virus inoculation using biolistics, DNA from each plant was analysed by PCR for the Nb transgene to assess the segregation pattern (data not shown). The nature of the pea transformation procedure is such that chimeric primary transformants can be produced which fail to pass the transgene to the next generation, or result in a non-Mendelian pattern of inheritance (Bean et al., 1997). An approximate 3:1 segregation ratio for the Nb transgene was observed in all but three lines. These three lines gave a lower than 3:1 ratio (indicative of a chimeric primary transformant), so a greater number of plants were included to ensure that 15 or 16 Nb-positive plants per line were tested. Non-transgenic segregants were also included in the resistance testing and used as internal controls for infection.

Plants were analysed for PSbMV-DFP1 infection by DAS-ELISA, 10 and 30 days post-inoculation (Table 1). At 10 days post-inoculation all plants were ELISA positive and had visible symptoms of PSbMV infection (i.e. vein clearing and slight mosaic). At 30 days post-inoculation most lines continued to show infection of new leaves. However, a proportion of the plants of three lines were now completely virus-free in their upper leaves (Table 1). These upper leaves gave ELISA readings similar to an uninfected control plant and were devoid of virus symptoms. The recovery from virus infection was observed for 1/17 35S:Nb lines (#2) and 2/8 35S:Nbb lines (#31 and #44). The lines generated with the other two constructs (nos:Nb and 35S:Nbb) showed no resistance. The plants of the three lines that showed recovery from initial infection (16/20 plants for line #2, and 15/20 plants for lines #31 and #44) corresponded to all those plants that were PCR positive for the Nb transgene, indicating that the recovery is related to presence of the transgene. The F1 plants that had lost the transgene by segregation remained infected to the same extent as the other transgenic lines and the control non-transgenic plants. Plants of lines #2, #31 and #44 also were inoculated using standard manual inoculation and again virus-free leaves emerged following an initial systemic infection. There was no difference in the timing of recovery or appearance of recovered

<table>
<thead>
<tr>
<th>Table 1. Resistance of transgenic pea lines to PSbMV isolate DPD1</th>
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<tr>
<td>Transgene</td>
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<tr>
<td>-----------</td>
</tr>
<tr>
<td>35S:Nb</td>
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<tr>
<td>nos:Nb</td>
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<td>35S:Nbb</td>
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<td>35S:Nbbl</td>
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* Virus accumulation in the uppermost pair of fully expanded leaves was assessed by DAS-ELISA. Lines with plants having an ELISA reading equivalent to that of a control non-infected plant were considered to be resistant.
† One line had 16 out of 20 F1 plants with background ELISA readings for the uppermost leaf pair.
‡ The two lines each had 15 out of 20 F1 plants showing background ELISA readings for the uppermost leaf pair.
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Table 2. Specificity of PSbMV resistance induction and targeting

<table>
<thead>
<tr>
<th>Pea line</th>
<th>Resistance to PSbMV-NY at +30 days*</th>
<th>Resistance of DPD1 recovered plants to infection with:†</th>
<th>Resistance of NY recovered plants to infection with:‡</th>
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<tbody>
<tr>
<td>#2</td>
<td>0/6, 7/7, 9/9‡</td>
<td>6/6</td>
<td>3/3, 3/3, 3/3</td>
</tr>
<tr>
<td>#31</td>
<td>0/6</td>
<td>0/6</td>
<td>NT</td>
</tr>
<tr>
<td>#44</td>
<td>0/6</td>
<td>0/6</td>
<td>NT</td>
</tr>
<tr>
<td>Puget</td>
<td>0/6§</td>
<td>0/6§</td>
<td>0/6§</td>
</tr>
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* Virus accumulation in the uppermost pair of expanded leaves was assessed by DAS-ELISA. Plants having an ELISA reading equivalent to a control non-infected plant were considered to be resistant.
† Results of three independent experiments.
‡ Recovered portions of plants previously infected with either the DPD1 or NY isolates of PSbMV were reinoculated with DPD1 or NY. The uppermost expanded leaves were assessed by DAS-ELISA 10 days post-inoculation.
§ Resistance for control inoculations on non-transgenic not previously inoculated cv. Puget.
NT, Not tested.

Evidence for an induced state of virus resistance

Recovered portions of #2, #31 and #44 plants were reinoculated with PSbMV-DPD1 and analysed by ELISA 10 days later. Control non-transgenic and uninoculated #2, #31 and #44 transgenic plants of the same age were challenged in parallel. No virus was detected by ELISA in the re-inoculated leaf or younger leaves of recovered plants. Virus was detected by ELISA in the inoculated control non-transgenic or transgenic plants, although all the latter (infected older #2, #31 and #44 plants) eventually recovered. The appearance of recovery in these plants, when inoculated for the first time at a much later stage of growth, indicates that the potential for the induction of resistance is independent of the age of the challenged plant.

Specificity of the recovery phenotype

In order to test whether the inducible resistance was active against other PSbMV isolates, homozygous F2 plants of line #2 were inoculated with different PSbMV isolates. Of the sequenced PSbMV isolates (Johansen et al., 1996), isolate NY shows the least (89%) identity with isolate DPD1 in the Nib gene. Isolate NY also represents a different pathotype (pathotype 4) from DPD1 (pathotype 1), as defined by their reaction to natural resistance genes in pea (Alconero et al., 1986). Isolate L-1 (pathotype 2; Provvidenti & Alconero, 1988a) has been sequenced in the Nib gene region (I.E. Johansen, unpublished data) and shows 92% identity with isolate DPD1. As seen for DPD1, isolate L-1 induced resistance on line #2 plants (data not shown). For isolate NY, in one experiment, recovery was not observed and there was no difference between infections of line #2 and non-transgenic control plants. However, in two other experiments PSbMV-NY infection was followed by recovery in all the plants as indicated by the emergence of virus-free leaves (Table 2).

The specificity of the induced resistance was tested by challenging recovered leaves with the most divergent isolates. Hence, line #2 plants previously infected with isolate DPD1 or isolate NY, and displaying recovery, were challenged with the reciprocal isolates; non-transgenic control plants of an equivalent age were similarly challenged. All control plants could be infected with either isolate. In two experiments, leaves showing recovery following initial infection with PSbMV-DPD1 all remained resistant to the same isolate but were susceptible to PSbMV-NY. In contrast, virus-free leaves obtained following initial infection with PSbMV-NY were all resistant to subsequent challenge with either isolate (Table 2).

Transgene expression prior to and following recovery

Northern blot analysis performed on total RNA preparations from all of the transgenic lines indicated that in many lines there was no detectable Nib transgene RNA accumulation (data not shown). Nib RNA was only detectable in 6/35 lines (Fig. 2a); the reason for the lack of expression from the majority of lines was not investigated. Nib transcript was always detected in the lines in which recovery occurred...
Fig. 2. RNA Northern analysis of transgenic lines. Total RNA (10 µg) was separated by gel electrophoresis, blotted and subjected to hybridization with a 32P-labelled probe specific for NiB sequences (a, c) and Rubisco small subunit (rbc) sequences (b). Lanes in panels (a) and (b) are labelled according to transgene line number or P for non-transgenic cv. Puget. R and S indicate the resistant (recovery) or susceptible phenotype seen after infection with PSbMV DPD1. Approximately equal loadings of RNA (b) contrast with the wide variation of NiB RNA accumulation in the transgenic plants (a); no NiB RNA was detected in lines #4 and #33, or other transgenic lines (not shown). Panel (c) shows the effect of infection on the steady-state levels of transgene NiB RNA in the three transgenic recovery lines (#2, #31 and #44); — indicates tissue from uninfected plants and + indicates fully recovered tissues from PSbMV DPD1-infected plants. In panels (a) and (c), NiB RNA is larger from lines #31, #35 and #44 owing to the repeat sequence at the 3’ end of the transgene; NiB probes frequently identified a range of smaller bands that were interpreted as specific breakdown products of the NiB RNA.

Fig. 3. Appearance of a transgenic line #2 F1 plant at 10 weeks after inoculation with PSbMV DPD1 (right) in comparison with an un inoculated line #2 F1 plant (left) and an infected non-transgenic cv. Puget plant (centre). At maturity, the symptoms of infection on the resistant line #2 plants are no longer apparent and the growth is equivalent to the uninfected plant.

Performance of recovered resistant plants

Plants which had undergone recovery attained a final height equivalent to that of a non-infected transgenic, or non-transgenic plant (Fig. 3) and set equivalent amounts of seed.
Seed was collected from the PSbMV-DPD1 recovered plants and progeny tested for resistance to PSbMV-DPD1. These progeny of the recovered plants behaved in an identical way to the parental plants, such that an initial infection was followed by recovery in the upper leaves. F2 and F3 progeny of lines #2 and #31 which had never been infected was also tested. In all cases, inoculation of PCR-positive plants resulted in recovery, indicating that the transgene-mediated resistance was stable over several generations although the need to establish a recovery state was reset at each generation.

Discussion

Thirty-five transgenic peas carrying the PSbMV-DPD1 replicase (NIb) gene were produced. Of these, three lines gave rise to what has been described previously for other plant–virus combinations as a ‘recovery phenotype’, in which plants are infected initially but later produce new leaves that are all symptom- and virus-free (Lindbo et al., 1993). The recovery phenotype was observed in all transgene-positive progeny of the three lines, indicating that the transgene is active in both the homozygous and heterozygous states. This, and the fact that all three lines contain only a single copy of the transgene, suggest that plants such as these could be used directly for breeding resistance into commercial pea lines.

Experiments by others with different potyviruses and plant species also have demonstrated similar induced resistance. In most experiments, some plants with extreme resistance also were observed (Cassidy & Nelson, 1995; Swaney et al., 1995), although it has been suggested that these represented stronger induced resistance (Tanzer et al., 1997). Goodwin et al. (1996) demonstrated a relationship between copy number of transgenes and resistance. Using the coat protein of tobacco etch virus (TEV), they showed that lines carrying three or more insertions were highly resistant to TEV whereas lines with one or two insertions showed the ability for inducible resistance. The fact that the majority of lines generated in our study carried only single T-DNA insertions may explain why extreme resistance was not observed, although it is also possible that too few lines were analysed.

While the three ‘recovery’ lines had single T-DNA integrations, two of them carried the transgene with a direct repeat of the 3’ half. Sijen et al. (1996) demonstrated an increase from 20 to 60% in the frequency of cowpea mosaic virus (CPMV)-resistant N. benthamiana when transformation was performed with a CPMV transgene carrying a direct repeat, as compared with a construct lacking the repeat. Although the frequency of pea lines with the recovery ability was higher with the construct carrying a direct repeat (2/8 for 35S::NIbblb) than with the simple construct (1/17 for 35S::NIb), we feel that the numbers are too low to conclude that this difference is significant. Clearly the repeats did not reduce the frequency of resistance and such complex T-DNA arrangements may be of use in increasing the possibility of obtaining resistance.

The recovery phenotype we have observed is characterized by the absence of viral RNA and a dramatic reduction in transgene RNA in the upper portion of the plant. This is typical of virus-induced gene silencing events based upon a post-transcriptional degradation of RNA in the cytoplasm (Lindbo et al., 1993; Guo & Garcia, 1997). Of the six lines in which NIb transcript was detectable by Northern blot analysis, three showed the ability for induced resistance and three did not. There was no correlation between the steady state level of NIb transgene RNA accumulation and ability to recover. Hence, our results do not fit with a model for resistance induction based upon a threshold of transgene RNA accumulation (Lindbo et al., 1993; Dougherty et al., 1994). We propose that presence of the virus triggers the post-transcriptional silencing and that additional factors must account for the differences between the various NIb expressing lines. Such factors may include genome position effects or transgene structure.

To determine the breadth of resistance, we inoculated line #2 plants with different PSbMV isolates. Since few PSbMV isolates have been sequenced in the NIb region we selected two heterologous isolates (L-1 and NY; pathotypes 2 and 4, respectively) which differed from DPD1 (pathotype 1) in their response to natural resistance genes. Strain NY was also the most sequence divergent strain. Recovery from PSbMV infection in line #2 was observed following inoculation with isolate DPD1, L-1 and also isolate NY, although recovery from isolate NY infection was not observed in all experiments. The NIb genes of DPD1 and NY share 89% nucleotide identity (Johansen et al., 1996). It appears that the triggering of the recovery mechanism has sequence specificity in that it is most active against the isolate (i.e. DPD1) from which the transgene was derived, or very closely related isolates. Mueller et al. (1995) showed RNA-mediated resistance to be effective against viruses with sequence identity of 88% or greater. At 89%, the level of identity may be such that other factors (e.g. virus accumulation or environmental conditions) play a role in determining whether recovery is initiated or not.

Two further and subtly different pieces of information were provided by experiments to test the specificity of the resistance. First, they showed that the specificity of RNA degradation after the onset of recovery was determined additively by both transgene source and the inducing virus strain. Hence, induction of resistance by the transgene source isolate (DPD1) provided protection against itself but not against PSbMV-NY, while induction by PSbMV-NY provided protection against both isolates. This was surprising, especially as the inducing virus could not be detected by ELISA or RT–PCR in the recovered tissue. A popular view of the mechanism of post-transcriptional gene silencing is that the process is triggered, RNA degradation takes place in the cytoplasm, and the silencing can be transmitted systemically via a sequence-specific signal (Palauqui et al., 1997; Voinnet & Baulcombe, 1997). We have also demonstrated the existence of a remote signal for gene silencing in line #2 plants (Jones et al.,
1998), although the precise nature of the signal has not been resolved. The data described in this paper would suggest that both the transgene and the inducing virus serve as sources for the signal. The second piece of information relates to differential sequence requirements for a virus to trigger recovery and to provide a target for degradation after recovery. Hence, whereas PsbMV-NY could trigger recovery with a PsbMV-DPD1 specific transgene, it was not targeted for degradation when recovery was triggered by the homologous PsbMV-DPD1 virus. We can conclude, therefore, that the sequence identity required to trigger the process can be less than that required for degradation. This information will be important when considering the effectiveness of gene-silencing based resistance in the field.

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