Evidence that resistance in squash mosaic comovirus coat protein-transgenic plants is affected by plant developmental stage and enhanced by combination of transgenes from different lines

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Three transgenic lines of squash hemizygous for the coat protein genes of squash mosaic virus (SqMV) were shown previously to have resistant (SqMV-127), susceptible (SqMV-22) or recovery (SqMV-3) phenotypes. Post-transcriptional gene silencing (PTGS) was the underlying mechanism for resistance of SqMV-127. Here, experiments conducted to determine the mechanism of the recovery phenotype and whether enhanced resistance could be obtained by combining transgenes from susceptible and recovery plants are reported. Upper leaves of SqMV-3 plants were sampled for Northern analysis at 17, 31 and 45 days after germination (DAG) and a proportion of plants were inoculated with SqMV. SqMV-3 plants inoculated at a young stage (17 DAG) showed susceptible or recovery phenotypes. However, a number of plants inoculated at later developmental stages (31 or 45 DAG) were resistant to infection. Resistance of recovery plants was due to PTGS that was activated at a later developmental stage, independent of virus infection. Similar results were observed with plants grown under field conditions. To investigate the interactions of transgenes, progeny of crosses between SqMV-127, -3 and -22 were inoculated with SqMV. Progeny with the transgene of line 127 were resistant. However, a number of plants with transgenes from the recovery and susceptible lines or the self-pollinated recovery line were resistant even when inoculated at a young stage. Northern analysis suggested that resistance was due to PTGS. The results reveal that the timing of PTGS and consequent resistance of the transgenic plants were affected by their developmental stage and the interaction of transgene inserts.

Introduction

RNA-mediated virus resistance has been shown to be the result of post-transcriptional gene silencing (PTGS) in transgenic plants (Dougherty & Parks, 1995; Baulcombe, 1996; Baulcombe & English, 1996; Dawson, 1996; Prins & Goldbach, 1996; Beachy, 1997; van den Boogaart et al., 1998). This type of virus resistance is also known as homology-dependent resistance (Mueller et al., 1995). Short complementary RNAs synthesized from the transgene mRNA by a plant-encoded RNA-directed RNA polymerase are thought to be the element that accounts for the features of sequence specificity of RNA-mediated virus resistance (Dougherty & Parks, 1995; Smith et al., 1994; Schiebel et al., 1998). Recently, Hamilton & Baulcombe (1999) have detected specific antisense RNAs of 25 nucleotides in length in plants showing PTGS. Several models have been proposed to account for PTGS and the resulting virus resistance. These include the threshold model (Dougherty & Parks, 1995; Smith et al., 1994), the aberrant RNA and ectopic pairing model (English et al., 1996; Baulcombe & English, 1996) and the double-stranded RNA-mediated model (Metzlaff et al., 1997; Montgomery & Fire, 1998; Waterhouse et al., 1998; Bass, 2000).

Squash mosaic virus (SqMV) is a seed-borne, beetle-transmitted comovirus with isometric virus particles about 30 nm in diameter (Campbell, 1971). The bipartite viral genome of comoviruses consists of two single-stranded, positive-sense RNA molecules, designated as bottom-component RNA (B-RNA) and middle-component RNA (M-
RNA, of about 6000 and 4200 nucleotides, respectively (Goldbach & Wellink, 1996). Each RNA contains a genome-linked protein (VPG) at the 5’ end and has a polyadenylated tract at the 3’ end (Goldbach & Wellink, 1996). Both RNAs are translated into polypeptides from which the functional proteins are derived by proteolytic cleavages. The M-RNA encodes the coat proteins (CP) (42 and 22 kDa) and cell-to-cell movement proteins (Franssen et al., 1982; Wellink & van Kammen, 1989) while the B-RNA encodes the VPG, replicase and protease proteins (Lomonossoff & Shanks, 1983). The sequence for the SqMV CP genes has been determined and is located in the 3' region of the M-RNA (Hu et al., 1993; Haudenshield & Palukaitis, 1998).

Recently, we reported that transgenic squash lines expressing both CP genes of the melon strain of SqMV displayed varying reactions to SqMV (Pang et al., 2000). The susceptible line SqMV-22 had a high steady-state transcript level but low transcription rate compared with the resistant line SqMV-127, which showed PTGS. Line SqMV-3, designated as a recovery line, initially showed systemic infection but newly developing leaves were free of symptoms 20–40 days after inoculation and these leaves were resistant to SqMV infection (Pang et al., 2000).

This communication reports the further characterization of the recovery phenotype of SqMV-3 and the phenotypes of plants that contain combinations of transgene inserts from lines SqMV-127, -3 and -22. The recovery phenotype of SqMV-3 was observed under greenhouse and field conditions and is due to PTGS being activated at later developmental stages rather than being induced by virus infection. We also show that resistant progeny can be obtained by combining transgenes from the susceptible SqMV-22 and recovery SqMV-3 lines.

Methods

Plant material. Transgenic squash lines with both the 42 and 22 kDa CP genes of the melon strain of SqMV (SqMV m-88) were produced previously (Pang et al., 2000). R₃ transgenic squash were crossed with a non-transformed inbred line to generate R₄ plants that were hemizygous for the transgenes. Three lines with distinct phenotypes were identified (Pang et al., 2000). Plants of the resistant line (SqMV-127) were resistant to SqMV infection. Plants of the recovery phenotype line (SqMV-3) initially developed systemic symptoms when inoculated at the cotyledon stage but leaves that developed 20–40 days after inoculation did not show symptoms. The susceptible line (SqMV-22) developed symptoms that persisted and spread throughout the plant. These R₃ transgenic squash (SqMV-127, -3 and -22), progeny from their self-pollination (SqMV-127 × 127, -3 × 3 and -22 × 22) and crosses SqMV-3 × 127, SqMV-22 × 127 and SqMV-3 × 22 were used in this study. Seeds were germinated in artificial potting mixture and the seedlings were assayed by nptII ELISA to identify the non-transgenic segregants.

Inoculation of transgenic plants. The virus isolate, SqMV m-88, was obtained originally from infected melon seeds (Hu et al., 1993) and typed as the melon strain by biological comparisons with the type strain (Nelson & Knuhtsen, 1973). Inocula were prepared by propagating the virus in zucchini squash (Cucurbita pepo L.) and grinding infected leaves in 0.01 M phosphate buffer (pH 7.0). Fifteenfold-diluted leaf extracts were immediately rubbed onto the three upper carborundum-dusted leaves of squash plants and the inoculated leaves were subsequently rinsed with water. Plants were observed for symptoms every other day for at least 60 days. The reaction of transgenic squash to SqMV was confirmed by a double antibody sandwich (DAS)-ELISA (Clark & Adams, 1977) with a polyclonal antibody against the virion of SqMV (Hu et al., 1993).

ELISA, Northern and Southern blot analysis of transgenic plants. Antibodies to SqMV (Hu et al., 1993) were used in DAS-ELISA (Clark & Adams, 1977) to detect virus infection and an nptII ELISA kit (5 Prime to 3 Prime) was used to detect the nptII enzyme in transgenic plants. Total DNA isolated according to the method of Doyle & Doyle (1990) was used in Southern blots. The number of transgene inserts was estimated by the number and the size of expected fragments generated by digestion with BglII and EcoRI or BglII and Xhol. A BglII site is located in the 42 kDa CP gene just after its initiation codon and another is located in the polylinker of the pGA482G vector (Pang et al., 2000). Thus, digestion with BglII generates a fragment with the 22 kDa CP transgene plus plant genomic DNA. The Xhol and EcoRI enzymes were used to reduce the size of the 22 kDa CP transgene plus genomic DNA fragment by cutting within the plant genomic DNA. Restriction enzyme-digested DNA was separated on agarose gels and then blotted onto GeneScreen Plus membrane (Dupont). For Northern blots, total RNA was isolated by using the procedure described by Napoli et al. (1990). Ten μg total RNA was applied in each well and separated by electrophoresis on a formaldehyde-containing agarose gel (Sambrook et al., 1989). The agarose gels were stained with ethidium bromide to compare the relative amounts of total plant RNA in each well. The Northern and Southern blots were probed with only the 22 kDa CP because Pang et al. (2000) had shown previously that the 22 kDa CP and the 42 kDa CP transgenes were coordinately expressed in the transgenic lines under investigation. Hybridization conditions for Southern and Northern blots were chosen according to the protocol of the GeneScreen Plus membrane (Dupont) and random-primer methods (Feinberg & Vogelstein, 1983) were used to generate probes specific to the 22 kDa CP. Images of some autoradiograms were photographed with a COHU CCD camera, model 4915-2000. Signals were quantified by using the US National Institutes of Health Image program version 1.59.

Isolation of nuclei and nuclear run-on transcription assays. Isolation of nuclei and nuclear run-on transcription assays were performed essentially as described by Dehio & Schell (1994). The same amount of labelled RNA was used for hybridization to replicated Southern blot membranes that contained 0.2 μg restriction enzyme-digested, electrophoretically separated fragments of the 22 kDa CP, 42 kDa CP, actin and nptII genes prepared similarly, as described previously (Pang et al., 1997).

Field evaluation. The R₄ seeds of three transgenic squash lines as well as those of non-transformed controls were germinated in a plastic house on July 19, 1995 and seedlings were screened by nptII ELISA to identify the non-transgenic segregants from the R₄ transgenic populations. Plants (48 per line) from each of the three transgenic squash lines and the non-transformed control were planted in the field on July 28, 3 feet (~ 1 m) apart within the row and 6 feet (~ 2 m) between rows, by using a randomized block design. Twelve to fourteen plants for each transgenic lines and non-transformed plants were inoculated at 1, 3 or 5 weeks post-transplantation. SqMV m-88-infected leaf extracts (1 g infected tissue in 15 ml buffer) were applied to the upper leaves of squash plants. Observations were made twice a week until the fruits became mature. The reaction of transgenic squash to SqMV was confirmed by DAS-ELISA as described above.
Results

Recovery phenotype is due to activation of PTGS at later developmental stages rather than to virus infection

We observed previously that some transgenic SqMV-3 plants displayed a recovery phenotype when the virus was inoculated on the cotyledons of small (10-day-old) plants but showed resistance when inoculated at a later developmental stage (Pang et al., 2000). These results may have been due to virus infection, as reported by Lindbo et al. (1993), or due to PTGS at later developmental stages. In order to test these alternatives, transgenic R₁ plants of SqMV-127, -3 and -22 were germinated in a greenhouse for 10 days and subsequently transplanted to a plastic house. Plants were inoculated at 1, 3 and 5 weeks post-transplantation, which was 17, 31 and 45 days after germination (DAG).

As expected, all inoculated SqMV-22 progeny developed systemic symptoms, while nearly all (25/27) SqMV-127 progeny displayed resistant phenotypes regardless of when the plants were inoculated (Table 1). However, the reactions of SqMV-3 progeny ranged from susceptible (4/9) to recovery (5/9) phenotype when small plants (17 DAG) were inoculated, while some SqMV-3 plants were resistant (3/15) when large plants (31 and 45 DAG) were inoculated (Table 1).

In order to investigate whether the resistance or recovery phenotype of SqMV-3 was due to PTGS that was induced at later developmental stages, Northern assays were performed with RNAs isolated from upper leaves of plants before and after inoculation with SqMV. Plants were selected from those shown in Table 1. Plants showing susceptible phenotypes had high levels of CP22 transcript at all developmental stages tested (Fig. 1a) and, as expected, high levels of SqMV genomic RNAs were detected after inoculation. In contrast, the plants showing the recovery phenotype contained high levels of CP22 transgene RNA at the early stages (17 and 31 DAG) but low levels at later stages (45 DAG; Fig. 1b). These plants had high levels of SqMV genomic RNAs in leaves at 31 DAG but none in newly developed leaves at 45 DAG (Fig. 1b). Moreover, this low level of CP22 transcripts in the leaves that developed later was also observed in plants (two out of six tested) that were not inoculated (Fig. 1d). The leaves that displayed PTGS (Fig. 1b, d; Table 1) could not be infected by subsequent SqMV inoculation. Taken together, our data suggested that the recovery phenotype of SqMV-3 was due to PTGS induced at later stages of plant development independent of virus infection.

Table 1. Reactions of transgenic squash inoculated with SqMV

<table>
<thead>
<tr>
<th>Line</th>
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<tr>
<td></td>
<td>n</td>
<td>S</td>
<td>rec</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>8</td>
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<tr>
<td>SqMV-22</td>
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<td>5</td>
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<tr>
<td>SqMV-127</td>
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<td>2</td>
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Fig. 1. Northern blot analysis of recovery transgenic line SqMV-3. Total RNA was isolated from the second or third leaf from the top of R₁ progeny of SqMV-3 at 17, 31 and 45 DAG (10 µg per lane) and analysed by Northern blots which were probed with the CP 22 kDa gene. (a) Inoculated plant that was susceptible to SqMV. (b) Inoculated plant that showed recovery phenotype 20 days after inoculation. (c) Non-inoculated plant that did not show development-induced silencing. (d) Non-inoculated plant that showed development-induced silencing. In (a) and (b), SqMV m-88-infected leaf extracts (1:15) were applied to the three upper leaves of squash plants immediately after a leaf was removed for RNA isolation. M-RNA of SqMV (g RNA; ca. 3400 bp) from virus infection and the product of the CP 22 kDa transgene (CP; ca. 900 bp) are indicated.
Combination of transgene inserts from different lines results in resistant phenotype

The recovery line SqMV-3 was of particular interest because it appeared as a resistant phenotype except that the resistance was activated at a later developmental stage. Thus, we were interested to determine whether resistance could be obtained at an earlier stage, similar to that of line SqMV-127, by combining transgene inserts from SqMV-3 and the susceptible line SqMV-22. In other words, could enhanced resistance be obtained by combining transgenes from lines with less resistance? Southern blots showed that lines SqMV-3, -22 and -127 have two, one and two transgene inserts, respectively. Phenotypes are indicated as R (resistance), r (recovery) or S (susceptible). The DNA bands (from top to bottom) in (a) are 7-0, 4-8 and 4-3 kb and in (b) are 6-8, 5-8 and 5-4 kb.

Table 2. Transgene patterns in progeny from crosses between lines SqMV-127, -3 and -22

The transgene loci were determined by Southern blot as shown in Fig. 2. n, Number of plants tested.

<table>
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<tr>
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<td>3</td>
</tr>
<tr>
<td>127</td>
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<td>22SP</td>
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<td>22</td>
<td>13</td>
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Table 3. Reactions to SqMV m-88 of progeny from crosses between lines SqMV-127, -22 and -3

Transgenes were identified by Southern blot. SqMV m-88-infected leaf extracts (1:15) were applied to the three upper leaves of squash plants at 10–17 DAG. Plants were observed for at least 60 days. See Table 1 for description of susceptible (S), recovery (rec) and resistant (R) phenotypes. SP, Self-pollinated; n, number of plants tested.

<table>
<thead>
<tr>
<th>Cross</th>
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<th>S</th>
<th>rec</th>
<th>R</th>
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</tr>
<tr>
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Enhanced homology-dependent virus resistance

Fig. 3. Northern analysis of progeny from self-pollination and crosses. Leaves were harvested for RNA isolation 10–17 days after germination. Ten µg per lane total RNA were used for Northern blots, which were probed with the 22 kDa CP gene of SqMV. Lanes: 1–3, progeny from SqMV-3 x 127; 4–6, progeny from SqMV-22 x 127; 7–9, progeny from SqMV-3 x 22. Lanes 10–11, 12–13 and 14–15 are the progeny from self-pollinated (SP) plants of SqMV-127, -3 and -22, respectively. Lane 16, a non-transformed squash. Their reactions to SqMV are shown below the Northern bands as resistant (R), susceptible (S) or recovery (r). The transgene patterns of the crossed progeny were determined by Southern analysis and are shown at the top of the lanes. The signal intensities were quantified by NIH-Image program and normalized with the non-transformed controls. The low (L) expressers had density reading between 0 and 30 while the high (H) expressers had density readings between 67 and 146. The size of the 22 kDa CP gene transcript is ca. 900 bp.

(127 x 127, 127 x 3, 127 x 22) with the transgene from line 127 showed resistant (28/35) or recovery (5/35) phenotypes, with the exception of two plants. Northern assays of leaf RNA isolated from some of these progeny before the plants were inoculated showed that the progeny of 3 x 127, 22 x 127 and 127 x 127 that were resistant accumulated low levels of transgene RNA (Fig. 3, lanes 2–4 and 6). In contrast, progeny that contained only the transgene from line 3 or line 22 were susceptible. These susceptible plants accumulated high levels of transgene RNA (Fig. 3, lanes 1, 5 and 7).

Interestingly, resistant phenotypes were observed in progeny from the cross between recovery SqMV-3 and susceptible SqMV-22 as well as from the self-pollinated recovery SqMV-3 (3 x 3) (Table 3). However, only plants with a combination of transgenes from line 3 and 22 or plants of self-pollinated line 3 showed the resistant phenotype (Table 3). Northern analysis of some of these progeny showed that resistant plants had low steady-state levels of the 22 kDa CP transgene transcript (Fig. 3, lanes 8, 9 and 13). In order to determine whether the reduced steady-state CP transgene mRNA levels in the resistant plants were due to post-transcriptional down-regulation of the transgenes, nuclear run-on transcription analyses were performed before inoculation, with the endogenous actin gene as a control. The 22 and 42 kDa CP genes were found to be much more efficiently transcribed in resistant SqMV-3 x 22 (Fig. 4a) and self-pollinated SqMV-3 (Fig. 4d) plants than in susceptible SqMV-22 (Fig. 4a) and -3 (data not shown) plants. Also, resistant SqMV-127 (Fig. 4c) and recovery SqMV-3 (Fig. 4b) plants showed high levels of transcription of the CP genes. These results clearly showed that the resistant phenotypes observed were due to the PTGS mechanism.

Fig. 4. Nuclear run-on transcription analysis on transgenic squash progeny. Labelled nuclear RNAs were hybridized to restriction enzyme-digested nptII (lane 1), actin gene (lane 2), 42 kDa CP (lane 3) and 22 kDa CP (lane 4) fragments separated on an agarose gel and blotted onto a membrane. The nuclei used in the assays were isolated from individual plants of (a) susceptible (S) SqMV-22, (b) recovery (rec) SqMV-3, (c) resistant SqMV-127, (d) resistant self-pollinated SqMV-3 (SP), (e) resistant SqMV-3 x 22 and (f) control non-transformed Pavo squash. Samples for nuclear run-on analysis were taken from leaves at the third or fourth position from the top of small squash plants at the 4–7 leaf stage (10–17 DAG), except for the recovery SqMV-3 plant. In this latter case, a sample was taken from an upper symptomless leaf of a SqMV-3 plant that showed recovery following inoculation with SqMV.

Resistant and recovery phenotypes are stable under field conditions

A field trial was conducted to evaluate the stability of the SqMV resistant and recovery phenotypes. Twelve to fourteen plants for each transgenic line and non-transformants were inoculated at 1, 3 or 5 weeks after transplanting. As shown in
showed that the recovery phenotype of SqMV-3 was due to
These results were consistent with greenhouse data, which
showed recovery phenotype. Furthermore, 17–21% of SqMV-
progeny inoculated at 1 week were resistant, but 21% of them
exhibited the resistant phenotype. None of the SqMV-3
susceptible to SqMV infection at any developmental stage
under field conditions

### Discussion

<table>
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<tr>
<th>Genotype</th>
<th>Plants showing resistance (%) after inoculation at:</th>
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<tr>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
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<td>0 (7)</td>
</tr>
<tr>
<td>SqMV-3</td>
<td>0 (21)</td>
</tr>
<tr>
<td>SqMV-127</td>
<td>79 (7)</td>
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Table 4, controls and almost all SqMV-22 R<sub>1</sub> plants were
susceptible to SqMV infection at any developmental stage (Table 4). In contrast, 79–93% of the SqMV-127 progeny
exhibited the resistant phenotype. None of the SqMV-3 progeny inoculated at 1 week were resistant, but 21% of them
showed recovery phenotype. Furthermore, 17–21% of SqMV-
3 progeny were resistant when inoculated at 3 or 5 weeks.
These results were consistent with greenhouse data, which
showed that the recovery phenotype of SqMV-3 was due to
PTGS induction at a later developmental stage.

Our results with transgenic squash expressing CP genes of
SqMV have shown that (i) SqMV CP transgene inserts from a
resistant line can silence the homologous transgenes from
recovery and susceptible lines; (ii) resistant progeny can be
obtained from crosses of susceptible and recovery lines (3 × 22)
and a self-pollinated recovery line (3 × 3); (iii) the recovery
phenotype of SqMV-3 is due to the activation of PTGS at later
developmental stages rather than by infection of SqMV; and
(iv) virus resistance in transgenic plants was similarly stable
under greenhouse and field conditions. Although other
laboratories, as well as ours, have observed the influence of one
or more of these factors in transgenic plants, this is the first
report where all of these factors have been studied or observed
in plants transformed with the same viral transgene construct.

Our results demonstrate that the recovery phenotype of
transgenic squash expressing the CP genes of SqMV is due to
the activation of PTGS at later plant developmental stages
(Table 1; Fig. 1) rather than by virus infection, as reported by
Lindbo et al. (1993) and Guo & García (1997). Thus, at least
two types of recovery phenotypes can be observed in
transgenic plants: virus-induced (tobacco etch virus, Lindbo
et al., 1993; plum pox virus, Guo & García, 1997) and
development-induced recovery (SqMV, this research). The
relationship of PTGS and consequent virus resistance to the
age of the leaves was observed in transgenic lettuce expressing
the nucleocapsid gene of tomato spotted wilt tospovirus (Pang
et al., 1996). In that study, transgenic hemizygotes and, in some
cases, homozygotes consisted of unsilenced lower leaves and
silenced upper leaves. However, no recovery phenotypes were
observed with plants that were inoculated at the earliest stage.
In addition, developmental PTGS has already been observed in
transgenic plants (Vaucheret et al., 1995; Kunz et al., 1996;
Balandin & Castresana, 1997). For example, Balandin
& Castresana (1997) showed that gene silencing occurs a few
weeks after seed germination and is maintained throughout
vegetative growth and floral development in all leaves of the
plant.

The recovery line SqMV-3 contained two inserts of CP
genes and young plants hemizygous for the CP transgene
displayed susceptible or recovery phenotypes. However,
increasing the gene dosage by self-pollination or by crossing
with the susceptible phenotype (SqMV-22) resulted in plants
that displayed the resistant phenotype. This resistant pheno-
type was due to activation of PTGS at an early stage, as
observed by nuclear run-on experiments (Fig. 4; Pang et al.,
2000). These results confirm the transgene-dosage effects on
PTGS and virus resistance that have been reported in the
literature (Smith et al., 1994; Goodwin et al., 1996; Mueller
et al., 1995; Pang et al., 1996). Thus, our data suggest that
resistant plants can be obtained by combining transgenes from
susceptible and recovery phenotypes. This approach could
have practical applications.

We have shown that the presence of the transgene insert of
the resistant line SqMV-127 can silence homologous trans-
genes from recovery and susceptible transgenic lines, as
evidenced by lowered SqMV-3/-22 transgene transcript
accumulation in progeny from crosses of SqMV-3 × 127 and
SqMV-22 × 127 (Table 3; Fig. 3). A similar observation was
reported by Mueller et al. (1995), who showed that virus
resistance in transgenic tobacco expressing the RNA poly-
merase gene of potato virus X is associated with the ability of
the transgene to silence homologous transgenes from sus-
ceptible plants.

It has been reported the PTGS can be affected by
environmental factors (Pang et al., 1996; Vaucheret et al.,
1995; Kunz et al., 1996). Consideration of these factors could
be important, as engineered resistance in crops needs to be
effective under field conditions. Our field experiments showed
that SqMV resistance is apparently stable under field con-
ditions. These observations suggest that transgenic SqMV
resistance under field conditions should have practical appli-
cations (this work; Pang et al., 2000). This development is
especially significant since natural resistance to SqMV in
squash and melons has not been identified (Provvidenti,
1993; Campbell, 1971).
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References


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