SUMMARY

Transgenic ‘San Marzano’ tomato lines transformed with a chimeric gene expressing a benign satellite RNA of Cucumber Mosaic Virus (CMV) were tested under conditions of natural infection, in southern Italy. Agronomic performance, virus susceptibility and spread of transgenic satellite RNA were monitored during the test. ELISAs and yield measurements showed that virus incidence was slightly lower in transgenic plants compared to controls. Satellite RNA was detected in melon, zucchini and pepper plants grown in the surroundings of the release site. Spread of the benign satellite RNA from transgenic tomatoes to surrounding host plants did not increase disease incidence or disease phenotype.

INTRODUCTION

Cucumber Mosaic Virus (CMV) has been the main agent of a severe disease causing significant crop losses in the Mediterranean region (Jordá et al., 1992). In southern Italy, the top quality tomato variety ‘San Marzano’ was particularly affected, with yield drop of about 300,000 tons and decrease of 12,500 cultivated ha (Gallitelli et al., 1991). Breeding for CMV resistance in tomato has not been successful till now, since natural sources of resistance are not readily available.

CMV is a single-stranded, positive-sense RNA virus, type member of the genus Cucumovirus in the family Bromoviridae, with a multipartite genome consisting of three genomic RNAs and a fourth subgenomic sequence (RNA 4) encoding the coat protein (Kaper and Waterworth, 1981; Palukaitis et al., 1992). Some strains of CMV carry an additional small single-stranded RNA, known as satellite RNA (satRNA) or CARNA 5 (for CMV-associated RNA 5), which depends on its helper virus for replication and spread. SatRNAs do not share any significant sequence homology with viral or plant RNAs and appear dispensable for any known viral function (Murant and Mayo, 1982). However, they can alter the symptoms induced by CMV, generally making them milder. In a few cases, however, specific satRNA variants, termed necrogenic, induce new symptoms, such as lethal necrosis in tomato, described by Kaper and Waterworth (1977).

The reported benign activity of many satRNAs has been exploited for biological control of CMV using different strategies. These include the use of CMV strains containing benign satRNAs in the field as vaccines (Gallitelli et al., 1991; Montasser et al., 1991) and the constitution of transgenic plants expressing satRNAs (Baulcombe et al., 1986; Jacquemond et al., 1988; McGarvey et al., 1990; Tien and Wu, 1991; Saito et al., 1992).
In a previous paper (Valanzuolo et al., 1994) we reported the constitution of transgenic ‘San Marzano’ lines expressing the satRNA Ra, and their behaviour in greenhouse tests when challenged with a satRNA-free strain of CMV. Sequencing of the original clone of satRNA Ra used for transformation revealed some differences (highlighted in Fig. 1) when compared to the published version (Devic et al., 1989). Due to these differences, the satRNA referred to in this paper was renamed CL 14.3.

Fig. 1. Nucleotide sequence comparison of the published Ra (Devic et al., 1989) and its revised form CL 14.3 satellite RNA. Differences between the two sequences are shaded. The box delineates the core of the necrogenic consensus (positions 309 to 334) used in analysis of the structural relationships between field-derived sequences. Y satellite sequence (Accession no. D00542), used as a reference for base numbering, is also shown.

Here we describe the results of a field test aimed at evaluating the agronomic performance of transgenic plants expressing CL 14.3 satRNA under natural infection pressure and the environmental risk associated with the satRNA technology. The strategy based on satellite-mediated biocontrol of plant viruses has been associated, in fact, with a number of potential environmental hazards, such as unpredictable effects induced by a benign satellite RNA on crops different from the target ones and its mutation from benign to pathogenic variants (Kaper and Tousignant, 1984; Palukaitis and Roossinck, 1996).

Most results relative to environmental impact have been obtained in laboratory conditions. Few reports coming from field experiments with transgenic plants are available (Yie and Tien, 1993; Stommel et al., 1998). It appeared interesting, therefore, to investigate the movement of a CMV satRNA from transgenic tomatoes to three different host species (pepper, melon and zucchini) growing in close proximity to the release site.

MATERIALS AND METHODS

Field test. The field trial was performed in Acerra (Campania region, southern Italy) in 1995. Seven hundred and twenty ‘San Marzano’ control tomato plants and the same number of genetically modified tomato plants (GMPs) checked to be homozygous for the CL 14.3 transgene, were planted on May in a 1440 m² field, split into 12 plots (1 plant m⁻²). The plants were of the following kinds: (i) controls: four hundred and eighty R₃ parental plants (third self-pollinated generation from TG02 regenerants) and 240 F₁ commercial hybrids (cv. ‘Ranco’, ‘San Marzano’ type). TG02 is a ‘San
Marzano' inbred line used as a target for genetic manipulation during our previous work; (ii) GMPs: four hundred and eighty R3 parental plants (third self-pollinated generation from TG02 transformants) and 240 F1 hybrids, obtained by crossing R3 transgenic plants (used as male parents) with a 'San Marzano' experimental line (TG210, used as female parent) were released as GMPs. R3 transgenic plants were distinguished in two groups (E and P), depending on the transgene pattern of integration in the host genome; (iii) trap plants: the plants were surrounded by six frames of trap plants (melon, zucchini, and pepper), all susceptible to CMV and used as indicators of potential spread of transgenic satRNA in the field (Fig. 2).

Fig. 2. Schematic representation of the Acerra field. Bar: 5 m.
A subset of 110 parental tomato plants (45 GMPs-E group, 45 GMPs-P group and 20 controls) were preinoculated at the two true leaf stage with a satellite-free CMV strain (CMV-FL) to promote early CL 14-3 satRNA replication. The plants were mechanically inoculated in the greenhouse with a virus suspension (40 ng μl⁻¹ in 30 mM Na₂HPO₄) six days before transplantation.

CMV resistance of released plants was evaluated on the basis of virus titre and crop yield.

**Sampling and ELISA test.** One hundred and fifty-six tomato plants, 13 per plot, in a geometrical ‘W’ configuration were chosen during transplantation and sampled on the occasion of each survey in order to include all the plant genotypes released. ELISA tests were performed during June and August on all sampled plants, using commercial kits (AGDIA, Elkhart, USA) to detect CMV, Alfalfa Mosaic Virus (AMV), Tomato Spotted Wilt Virus (TSWV), Potato Virus Y (PVY) and Tobacco Mosaic Virus (TMV). The same analyses were carried out on 43 pepper plants, while on 35 melon and 35 zucchini plants only the occurrence of CMV, Papaya Ringspot Virus (PRSV) and Zucchini Yellow Mosaic Virus (ZYMV) was checked by ELISA. Tomato production was evaluated, for the main chemical and physical traits and for yield, in each plot and for a limited sample of 39 controls and 39 GMPs - on single plants. Yield data were expressed as marketable fraction. Data for yield variables were analysed by ANOVA; after confirmation of the significance of the F-value, the data means were compared using the least significant difference (LSD) criterion. Percentage data underwent arc-sine transformation prior to analysis. Results are presented as untransformed data, but mean separations are based upon transformed data analysis.

One survey for the occurrence of CMV satRNAs was done two weeks before transplantation of GMPs: eighteen samples, representative of horticultural crops (tomato, potato) and several weed species, were harvested within a range of 50 m around the release site. Total RNA was extracted from leaf tissues and analysed by Northern blot hybridisation with a probe corresponding to the sequence of CL 14-3. This test was designed to detect the presence of any CMV satRNA sequence since CL 14-3 shares more than 80% sequence homology with other satRNAs, and mismatches are dispersed throughout the molecule.

The test field was sampled to evaluate the trend of viral infections, and to monitor the spread of satellite RNA from transgenic tomatoes to other host plants. About 10% of trap plants and 5% of tomato controls were sampled for this purpose. Two young leaves were put in a 2 ml Eppendorf tube and immediately dropped in liquid nitrogen before transportation to the lab.

**RNA extraction.** Frozen leaf samples, corresponding to about 100 mg of fresh tissue, were finely ground. Total RNA was isolated as described by Verwoerd et al. (1989) and blotted to a positively charged nylon membrane (Boehringer Mannheim).

Hybridisation was performed as previously described (Valanzuolo et al., 1994) using as probe a 359 bp DNA fragment including the CL 14-3 sequence labelled with digoxigenin-dUTP (Boehringer Mannheim). A ribosomal probe was also used as an internal control for checking RNA quality.

**cDNA synthesis, DNA amplification and sequencing.** RNA samples positive in Northern analysis were used as templates to obtain first-strand cDNA, and then to amplify the satRNA sequence. The primers were degenerate, annealing at the 5’ and 3’ conserved ends of satellite RNAs, and were 18 and 28 bases long respectively (upstream: 5’GTGGTGTTTGA(TT)(GG)GAGAATTGCCTGT(CT)(AG)GAG3’ downstream: 5’GGGTTCCTGT(CT)(ACG)GGAATG3’). The first strand cDNA was synthesized from 500 ng of total RNA primed with the downstream primer, using M-MuLV reverse transcriptase at 37°C for 1 hour.

Ten μl of the cDNA synthesis reaction were used as a template for the PCR reaction using 50 pmol upstream primer and 25 pmol downstream primer in 90 μl of total volume. The first two cycles were 1 min 94°C, 1 min 45°C, 2 min 72°C, followed by 35 cycles (1 min 94°C, 1 min 55°C, 2 min 72°C), and the final extension step (7 min 72°C).

PCR products were recovered after electrophoresis in 1.5 % Ultra Pure agarose gel stained with ethidium bromide. Fragments of 300-400 bp were cut and purified according to Koenen (1989). The purified fragments were directly sequenced with the Sequenase PCR products Sequencing kit (USB Corporation, Cleveland, USA).

**Sequences analyses.** Sequence analyses were carried out by directly comparing field-derived satRNA sequences to CL 14-3. All nucleotide positions refer to the homologous sites in the Y satRNA sequence (Devic et al., 1989). The specific domain in the 3’ region, including bases 309 to 334 (core of the necrogenic consensus of satRNA Y) was analysed for: (i) number of mismatches; (ii) presence/absence of G, U, and C respectively at positions 318, 323, and 325; (iii) presence/absence of the triplet UAA inserted between bases 322 and 323.
Positions 318, 323, 325 and the above mentioned triplet, all included within the necrogenic consensus, are very close to the definition of diagnostic, regarding the biological activity of a CMV-satellite RNA. With the notable exception of the satRNA R (benign even if different in only 1 base from the necrogenic consensus), the identity of the bases at these positions can be used to classify a sequence as necrogenic, benign A (structurally similar to necrogenic), or benign B (very distant from the necrogenic) (Devic et al., 1990). Sequences with UAA and bases different from G, U and C in the necrogenic domain were considered benign B, while sequences without UAA were considered not-B (necrogenic or benign A).

Pairwise alignments with the CL14-3 sequence were derived by application of the Wilbur and Lipman method (1983) using the Lasergene software (DNASTAR Inc). Multiple alignments were based on the Clustal algorithm (CLUSTALW, Higgins and Sharp, 1988). The phylogenetic tree was constructed by the neighbor joining (NJ) distance method (Saitou and Nei, 1987) and confidence limits to these inferences were placed by using the bootstrap procedure (Felsenstein, 1985). The programmes SEQBOOT, DNADIST, NEIGHBOR and CONSENSE, present in PHYLIP (Phylogeny Inference Package), version 3.5c (Felsenstein, 1989) were employed in this analysis, while TREEVIEW (Page, 1996) was utilised for displaying and printing trees.

RESULTS AND DISCUSSION

Field test. The CMV infection pattern in tomato plants is shown in Fig. 3. Differences between tomato genotypes were not statistically significant. The ELISA data indicated that AMV, more than CMV, was the major pest in tomato, with close to 80% infection, while CMV and ZYMV were the most frequent in zucchini and melon (Fig. 4). Fern leaf/shoestring but not systemic necrosis was observed on tomato, whereas mosaic and stunting were the most frequent symptoms of CMV in zucchini and melon. Pepper plants remained symptomless. In addition to viruses, fungal (Pyrenochaeta lycopersici) and red spider (Tetranychus urticae) attacks were noted.

This situation is consistent with descriptions of previous viral epidemics in Campania (Crescenzi et al., 1993). In the coastal area of Basilicata lethal necrosis, caused by necrogenic satRNA-bearing CMV strains, severely affected tomato fields. However, in Campania fern leaf/shoestring and fruit necrosis were prominent, sometimes combined in the same plant. Association of CMV with potyviruses (mostly PVY) was also frequently noticed (Gallitelli et al., 1988).
Yield data are reported in Fig. 5. Transgenic tomato plants yielded slightly more marketable fruit compared with controls, while no significant differences were observed in total production (data not shown). In particular, both lines of transgenic hybrids gave significantly higher yields, while, within the parental lines, only line E looked better than controls. The unequal performances of E and P are probably related to delayed ripening in P rather than differential virus susceptibility. The difference in ripening times, already apparent in greenhouse tests, was probably a consequence of different patterns of transgene integration since E and P have the same genomic background (Valanzuolo et al., 1994). Better results are shown by transgenic tomatoes preinoculated with the satellite-free strain FL in comparison with uninoculated parentals and hybrids. As previously shown (Valanzuolo et al., 1994) qualitative parameters (size, shape, pH, colour, optical residue, dry matter, etc.) of transgenic fruit were within the variability range of the control line.

The test was run in the typical ‘San Marzano’ tomato cropping area in order to evaluate performance in a realistic context. Unfortunately, field evaluation of a resistance trait directed against a specific pathogen is often hampered by the simultaneous presence of several other pests, as we experienced during our experiment. AMV especially (see Fig. 4) was serious. The satellite RNA approach, due to its specificity, can thus, in such a context, prove inadequate in protecting the crop.

In spite of this, all transgenic plants gave higher yields than controls in term of marketable production, as shown in Fig. 5, with best results coming from the preinoculated transgenic tomatoes. Preinoculation with the satellite-free strain FL, in fact, induces an amplification of satRNA in transgenic plants, probably as a consequence of viral RNA polymerase activity, using the transgenic satRNA transcript as a template. This hypothesis is consistent with the observation that, in satRNA transformed tomatoes, the transgenic transcript is hardly detectable in Northern blots, while a strong signal is obtained following preinoculation with the FL strain (Valanzuolo et al., 1994). Furthermore, CMV-FL can itself cross-protect against superinfecting strains of CMV, and thus improve resistance shown by all preinoculated transgenic plants (Fig. 3).

The reasons underlying the low steady-state level of the CL 14-3 transcript in transgenic tomatoes will be subject of further investigations.
Sequences analyses. Field-derived plant tissues were analysed in Northern blots before transplantation. No positive signals were detected, indicating the absence of satRNA-bearing CMV strains at that time (mid April).

Spread of satellite RNA from transgenic plants was monitored by analysing satRNA populations in neighbouring plants and control tomatoes.

SatRNAs from the field are a mixture of different sequences ranging in size from 337 to 344 bases. Quite surprisingly only two sequences were detected in control tomato plants. Results of the comparative analysis are summarised in Table 1. On the basis of specific sequence features in the region known as the necrogenic box (Devic et al., 1990) on the 3' side of the molecule, most sequences (51 out of 56) resembled members of the benign B group. These sequences displayed a degree of divergence from CL 14-3 ranging from 0 (identity) to 17.7% (clone P39), calculated as percentage of mismatches on the entire sequence. Average percentage of mismatches in the benign B group was estimated to be 4.0%. In the region 309-334 differences did not exceed 5. Eight sequences were found to be identical to CL 14-3. A group of 5 sequences, 4 isolated from zucchini and 1 from pepper, were characterised by a much higher degree of divergence from CL 14-3 (12.1% on average). At least 10 mismatches were present in the region 309-334 in which the triplet UAA was absent and positions 318, 323 and 325 were G,G,C. These features are shared with the satellite RNA OY2 (Hidaka et al., 1988) classified by Devic et al. (1990) as benign A.

Table 1. Classification of field-derived satRNAs.

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of sequences</th>
<th>Benign B</th>
<th>Not B</th>
<th>CL 14-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melon</td>
<td>20</td>
<td>17</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Pepper</td>
<td>9</td>
<td>7</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Zucchini</td>
<td>25</td>
<td>17</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Ctl Tomato</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>56</td>
<td>43</td>
<td>5</td>
<td>8</td>
</tr>
</tbody>
</table>

Fig. 5. Agronomic performance of different tomato genotypes expressed as marketable fraction. Ctl: control tomato plants; E, P: transgenic tomato lines; hyb: F1 hybrids; + FL: FL-preinoculated tomato plants. Bars with the same letters are not significantly different at the 5% level ($P = 0.05$). Comparisons are made within the same group (parentals, hybrids, preinoculated).
To highlight relationships between sequences found during the field test, a cladogram with four distinct branches, was drawn (Fig. 6). The sequences of B group constituted a robust lineage (with 82% bootstrap values), in which CL14-3 and other closely related sequences (different in no more than 2-5 bases) exhibited a large clade (63% bootstrap) indicated as $b_1$. These sequences showed an average mismatch of about 1.2%.

Fig. 6. Phylogenetic analysis of the satRNA field population. CMV sat Y (Accession no. D00542) was designated as the outgroup. The tree was derived with the neighbor-joining method based on the Kimura '2-parameter' model (Kimura, 1980). Numbers at forks represent the bootstrap values (%) based on 100 replications. T: tomato; P: pepper; M: melon; Z: zucchini.
Although resolved with a low bootstrap value (< 50%), a second clearly defined subgroup (b2) was formed by sequences differing in average of 6.2% from CL 14-3. A third minor branch, b3 (92% bootstrap), was formed by P39 and P17, both isolated from pepper and characterised by about 17.7% of divergence. Finally, the five above mentioned sequences from pepper and zucchini with GGC were clustered in the separate branch a, supported by a very high bootstrap value (100%). Branch a was composed of sequences that, on the basis of the above mentioned structural criteria (Devic et al., 1990), are not attributable to the group B. These sequences can probably be ascribed to group A of benign satellite RNAs, described by Devic et al., 1990 as structurally but not functionally related to necrogenic variants.

The complexity of this picture is probably due to two components, the infection by different satRNA-bearing CMV strains and the presence in the field of CL14-3 expressing and in some cases (FL preinoculated) overproducing transgenic tomatoes.

Important symptoms observed on plants harbouring satellite RNAs included mosaic, stunting and shoe-stringing (Table 2).

Modulation of CMV-incited symptoms by satRNA is well documented only in tomato, so we were unable to establish any positive correlation between the presence of particular satRNA sequences and the symptoms found in trap plants. The limited goal of our field test was to establish the capability of CL14-3 to spread from transgenic tomatoes to trap plants, including the possible appearance of symptoms not described in crops except tomato. Our data indicate that the complexity of the phytopathological situation in the field, rather than the presence of plants disseminating satRNA, can explain the observed symptomatologies.

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REFERENCES


Table 2. Symptoms detected on satRNA-harbouring plants.

<table>
<thead>
<tr>
<th></th>
<th>Tomato</th>
<th>Pepper</th>
<th>Melon</th>
<th>Zucchini</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign B</td>
<td>1 mo</td>
<td>2 mo</td>
<td>11 mo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 st</td>
<td>7 st</td>
<td>4 st</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 sy</td>
<td>2 dp</td>
<td>3 ifd</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 ln</td>
<td>10 ls</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 sy</td>
<td>1 sy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl 14-3</td>
<td>1 ls</td>
<td>3 st</td>
<td>3 mo</td>
<td>8 mo</td>
</tr>
<tr>
<td></td>
<td>1 sy</td>
<td>3 sy</td>
<td>5 st</td>
<td>2 st</td>
</tr>
<tr>
<td></td>
<td>1 sy</td>
<td>1 ifd</td>
<td>9 ls</td>
<td>2 lc</td>
</tr>
<tr>
<td>Not B</td>
<td>2 st</td>
<td>3 mo</td>
<td>1 st</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 ifd</td>
<td>2 ls</td>
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<td></td>
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</tbody>
</table>

Mo: mosaic; st: stunting; dp: delayed production; ln: leaf necrosis; ls: leaf shoestring; lc: leaf curling; ifd: internal fruit damage; sy: symptomless.

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