Theor Appl Genet (1992) 83:679–683

THEORETICAL AND APPLIED GENETICS

Cucumber mosaic virus-tolerant transgenic tomato plants expressing a satellite RNA

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Received March 18, 1991; Accepted July 9, 1991
Communicated by K. Tsunewaki

Summary. A satellite RNA (T73-satRNA) gave reduced symptom severity on tomato plants when coinoculated with an ordinary strain of cucumber mosaic virus (CMV-O). cDNA for T73-satRNA was introduced into a binary vector (pTOK162) through a homologous recombination in an *Agrobacterium tumefaciens* cell, and then transferred to leaf disks of tomato. Stable integration and transcription of the cDNA in the regenerants were verified by Southern and northern blot hybridizations, respectively. Upon inoculation with CMV-O, the transformants exhibited very slight symptoms of CMV, grew normally, and finally set fruits, whereas untransformed wild-type tomato plants showed very severe symptoms, and their growth was retarded and formed few fruits. Agarose gel electrophoresis of total RNA from CMV-O-inoculated transformants detected RNA molecules corresponding to T73-satRNA.

Key words: *Lycopersicon esculentum* – Cucumber mosaic virus – Satellite RNA – Transgenic plants – *Agrobacterium tumefaciens*

Introduction

Cucumber mosaic virus (CMV) disease in tomato has been responsible for significant crop losses. Breeding of CMV-resistant tomato has been hampered by lack of suitable resistance sources in *Lycopersicon* species, although several investigators have tried to introduce the resistance from *Solanum lycopersicoides*, which has been reported to have the resistance (DeVerna et al. 1987).

Some plant viruses have a single-stranded small RNA molecule, so-called satellite RNA, which depends on the helper virus for its replication, shares no significant sequence homology with the virus genome, and is not necessary for the multiplication of the virus (Murant and Mayo 1982). Satellite RNAs of CMV are known to attenuate the symptoms induced by CMV in certain host plants (Waterworth et al. 1979; Takanami 1981; Gonsalves et al. 1982), and some isolates of the satellite RNA can cause severe stem necrosis on tomato plants (Kaper and Waterworth 1977). It has been demonstrated that transgenic tobacco plants expressing a CMV satellite RNA are tolerant to infection with CMV (Harrison et al. 1987).

We have also previously reported that transgenic tobacco plants that expressed a satellite RNA of CMV (Y-satRNA) showed symptom attenuation upon infection with CMV (Masuta et al. 1989). The use of transgenic plants expressing satellite RNA species that attenuate viral disease symptoms can be applicable to tomato, although Y-satRNA is not appropriate to tomato, because a CMV strain carrying this satellite causes severe necrotic symptoms on tomato (Takanami 1981; Masuta and Takanami 1989).

For this purpose, we have obtained other naturally occurring isolates of CMV satellite RNA, such as T73-satRNA or S19-satRNA, which can reduce severity of the symptoms in CMV-infected tomato (Masuta et al. 1990). Recently, Montasser et al. (1991) and Gallitelli et al. (1991) demonstrated that pre-inoculation or vaccination with a non-necrogenic satellite RNA, which can ameliorate symptoms of infection by CMV, resulted in effective protection of tomato plants from CMV disease.

In this paper, we report stable integration and expression of cDNA for T73-satRNA in transgenic tomato plants and a high level of tolerance to CMV in these transformants.

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Materials and methods

Plant materials

Seeds of an inbred cultivated tomato (*Lycopersicon esculentum* cv JTM-7) were obtained from Applied Plant Research Laboratory of Japan Tobacco Inc.

Satellite RNA

cDNA cloning and nucleotide sequence of T73-satRNA have been described elsewhere (Masuta et al. 1990).

Introduction of the cDNA for the satellite into plant expression and transformation vectors

A pUC119 containing full sequence of T73-sat cDNA (pUC119-73sat) was partially digested with *Eco*RI and *Bam*HI, and entire region of T73-sat cDNA was inserted into the *Eco*RI-*Bam*HI site of a plant expression vector, pLGV2382 (Herrera-Estrella et al. 1983), to give pLGV2382-73sat. The resulting pLGV2382-73sat was used for construction of two different plant transformation vectors described below. In the first one, pLGV2382-73sat was linearized with *Hin*dIII and inserted into the *Hin*dIII site of pGA643 (An et al. 1988) to give pTOK187. pTOK187 was introduced into LBA4404 by the freeze-thaw method (An et al. 1988). In the second one, a restriction fragment of pLGV2382-73sat digested with *Hin*dIII and *Pvu*II was blunt end-ligated with a 2.5-kb *Cla*I fragment carrying spectinomycin-resistance gene derived from Tn7 in a pBR322. The resulting plasmid containing the cDNA and spectinomycin resistance gene was referred to as pYS134. pYS134 was introduced into *Agrobacterium tumefaciens* (LBA4404), which carried pTOK162 (Komari 1990), by triparental matings (Ditta et al. 1980). By selecting for resistance to kanamycin and spectinomycin, LBA4404, which carried pYS134 cointegrated to pTOK162 by homologous recombination mediated by the regions of DNA derived from pBR322, was obtained. The cointegrated form of pYS134 and pTOK162 is referred to as pYS143 (Fig. 1).

Transformation

Procedures for leaf disk transformation were essentially the same as described by Masuta et al. (1989), except for the following modifications. Cotyledons of aseptically germinated seedlings were used for cocultivation with *Agrobacterium*. Medium for cocultivation with *Agrobacterium* consisted of basal salts of Linsmaier and Skoog (1965), vitamins of B5 (Gamborg et al. 1968), and 30 g/l glucose or sucrose. Medium for shoot induction from the tomato explants consisted of basal salts of Linsmaier and Skoog (1965), 0.3 mg/l indole acetic acid (IAA), 10 mg/l 2-isopentenyl adenine (2ip), 100 mg/l kanamycin, 250 mg/l cefotaxime, 30 g/l glucose or sucrose, and 9 g/l agar.

Southern hybridization

The procedures for isolation of DNA from plants and for Southern hybridization were those previously described (Komari et al. 1989). DNA was digested with *Bam*HI, separated by agarose gel electrophoresis, transferred onto Gene Screen Plus (Dupont), and hybridized with the randomly primed, 0.4-kb *Hin*dIII-*Eco*RI fragment from pUC119-73sat.

Northern hybridization

Total RNA was extracted from leaves, denatured with glyoxal and dimethyl sulfoxide as described by Thomas (1980), and separated by electrophoresis on a 1.5% agarose gel. The RNA was transferred to Gene Screen Plus and hybridized with the same 0.4-kb *Hin*dIII-*Eco*RI fragment as above.

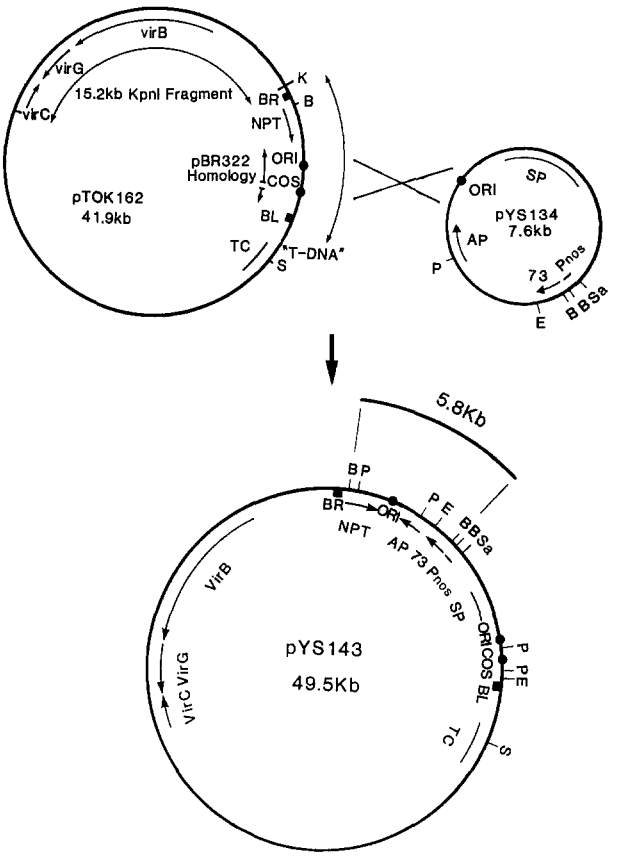

Fig. 1. Construction of pYS143. pYS134 carrying spectinomycin resistance gene derived from Tn 7 and cDNA of T73-satRNA was introduced into *Agrobacterium tumefaciens* (LBA4404) harboring pTOK162. pYS143 was generated by selecting for resistance to kanamycin of pTOK162 and spectinomycin of pYS134 through homologous recombination in the region of DNA derived from pBR322. Abbreviations: BR, right border; BL, left border; TC, tetracycline resistance gene; ORI, origin of replication of ColE1; COS, cos site of lambda phage; SP, spectinomycin resistance gene; AP, ampicillin resistance gene; P_{nos}, promoter of nopaline synthase gene; 73, cDNA of T73-satRNA; NPT, kanamycin resistance gene; VirB, VirC, VirG, virulence genes from plasmid pTiBo542; B, *Bam*HI; K, *Kpn*I; S, *Sal*I; Sa, *Sac*II; E, *Eco*RI; P, *Pst*I

Inoculation of CMV

Leaves were dusted with carborundum and inoculated with purified satellite-free CMV-O (10 μg/ml) followed by thorough rinse with tap water. Symptom development in the inoculated plants was observed until the fruiting stage. Total RNA from the infected leaf tissue was analyzed on a 1.5% agarose gel under non-denaturing conditions as described by Masuta et al. (1989).

Results

Culture conditions of leaf disks

Culture media varying in concentrations and combinations of phytohormones and in carbon source were ex-

Table 1. Effects of phytohormone and carbon source on the shoot forming efficiency from explants of cotyledons of tomato (JTM 7)

IAA (mg/l)	BA (mg/l)	2ip (mg/l)	Carbon Source (g/l)	Percent of explant forming shoots
0.3	5.0		Sucrose 30	53.3
1.0	20.0		Sucrose 30	6.7
0.3	20.0		Sucrose 30	0
0.3	5.0		Glucose 30	83.1
0.3	10.0		Glucose 30	42.6
0.3		10.0	Sucrose 30	16.7
0.3		30.0	Sucrose 30	5.0
0.3		10.0	Glucose 30	93.3
0.3		30.0	Glucose 30	71.7

For each combination, 60–75 explants were placed
Frequency of shoot formation was measured 3 weeks after culture
IAA, indole-3-acetic acid; BA, 6-benzylamino purine; 2ip, 2-isopentenyl adenine

Table 2. Superiority of plasmid, pYS143, harboring virulence genes from pTiBo542, and of cocultivation medium containing glucose

Plasmid	Medium for cocultivation	Leaf piece forming shoot	
		Number/total	%
pYS143	LS B5 sucrose	13/ 60	21.7
pYS143	LS B5 glucose	56/ 84	66.7
pTOK187	LS B5 sucrose	0/110	0
pTOK187	LS B5 glucose	0/120	0
pTOK162	LS B5 sucrose	21/ 72	29.2
pTOK162	LS B5 glucose	54/ 72	75.0

Number of shoots was counted 4 weeks after cocultivation with *Agrobacterium* carrying respective plasmid
pYS143 and pTOK162 carried virulence genes from pTiBo542, and pTOK187 was an ordinary binary vector
LS B5, inorganic salts of Linsmaier and Skoog (1965) and vitamin of B5 medium (Gamborg et al. 1968)

amined. As shown in Table 1, phytohormone combinations of 0.3 mg/l IAA and 10 mg/l 2ip or 0.3 mg/l IAA and 5 mg/l BA were suitable in terms of number of leaf disks producing shoots. The media containing glucose always resulted in a higher number of regenerants compared with those containing sucrose. Based on these findings, the culture medium containing 0.3 mg/l IAA, 10 mg/l 2ip, and 3% glucose was used in the subsequent transformation experiments. Usually from two to three shoots per leaf disk were obtained within 3 weeks on this medium.

Transformation of tomato leaf disks

Two different binary vectors which carried the cDNA for T73-satRNA were compared for the transformation frequency: pTOK187 is a derivative of a normal binary vector, and pYS143 is based on a special binary vector, pTOK162, carrying a DNA fragment derived from the virulence region of pTiBo542 (Komari 1990). LBA4404 (pTOK187) was not able to produce any kanamycin resistant shoots. In contrast, LBA4404 (pYS143) gave many shoots in the majority of leaf disks treated. Also, for the medium used in cultivation of *Agrobacterium* and leaf disks, two kinds of medium differing in their carbon source were compared. There was a significant difference between glucose and sucrose; the use of glucose in the place of sucrose enhanced the shoot regeneration by about threefold (Table 2).

Using the improved procedures for transformation and subsequent culture of tomato leaf disks described above, cDNA of T73-satRNA was introduced to tomato. Kanamycin-resistant shoots from the leaf disks treated with LBA4404 (pYS143) were transferred to the root-forming medium consisting of LS salts and 3% sucrose. Leaf segments of the putative transformants were placed on the medium containing kanamycin (300 mg/l) to confirm the resistance. The regenerated plants were grown in a greenhouse and subjected to further characterization.

Southern and northern hybridization

DNA and RNA were extracted from kanamycin-resistant regenerants. *Bam*HI fragments of the DNA were separated by electrophoresis, then transferred to nylon membrane. The 0.4-kb *Hin*dIII-*Eco*RI fragment from pUC119-73sat was used as the probe in both Southern and northern blots. The Southern blot hybridizations detected positive signals in the DNA from the two transformants, confirming that these transformants carried the cDNA for T73-satRNA (Fig. 2A). The size of the positive fragment (5.8 kb) was consistent with that expected from construction of pYS143 digested with *Bam*HI. One of the transformants, Sat 72, exhibited additional positive signals other than 5.8 kb. This indicates that rearrangement of the introduced gene has been taken place during the transformation process.

The northern blot hybridization revealed the presence of the transcripts from the cDNA for the satellite RNA in the two transgenic plants tested. Transcripts of 1.3 kb, which is of the expected size for the mRNA predicted from the original plasmid construction, were clearly identified (Fig. 2B).

CMV tolerance of transformants

Kanamycin-resistant regenerants, in which integration and expression of the cDNA for T73-satRNA were confirmed, were inoculated with an ordinary strain of CMV (CMV-O). Symptom development was surveyed up to

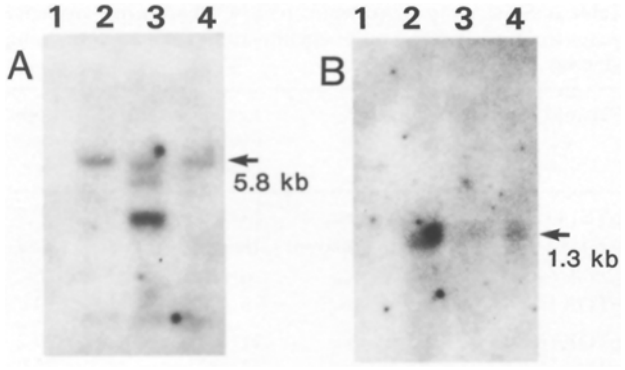

Fig. 2. **A** Southern hybridization. DNA was isolated from three transgenic and one wild-type plant, digested with *Bam*HI, subjected to electrophoresis on an agarose gel, transferred to nylon membrane, and probed with ^{32}P-labeled *Hin*dIII-*Eco*RI fragment from pUC119-73sat. *Lane 1*, a wild-type tomato (JMT 7); *lanes 2*, *3*, and *4*, transgenic plants, Sat 71, Sat 72, and Sat 73, respectively. **B** Northern hybridization. RNA was isolated from three transgenic and one wild-type plant, denatured with glyoxal and DMSO, separated by agarose gel electrophoresis, transferred to nylon membrane, and probed with ^{32}P-labeled *Hin*dIII-*Eco*RI fragment from pUC119-73sat. *Lane 1*, a wild type tomato (JTM 7); *lanes 2*, *3*, and *4*, transgenic plants, Sat 71, Sat 72, and Sat 73, respectively

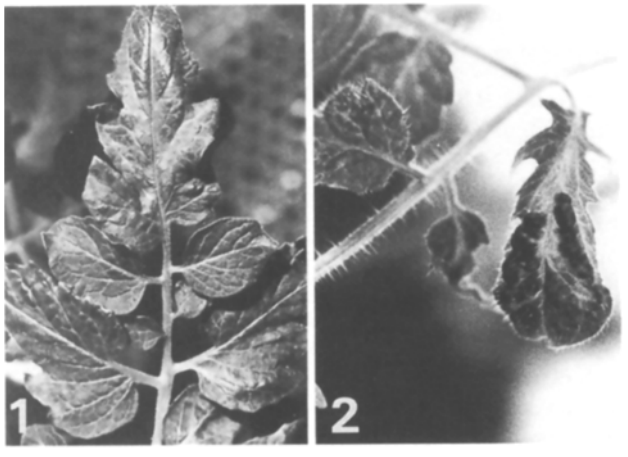

Fig. 3. Symptoms on the leaves of the transgenic plant inoculated with CMV-O. *1*, transgenic plant, Sat 71, inoculated with CMV-O; *2*, non-transgenic plant, JTM 7. Photograph was taken 2 weeks after inoculation

the fruiting stage. Although development of slight mosaic symptoms was observed in the inoculated transformants (Fig. 3), their growth was comparable to that of uninfected plants. Timing of fruit set and the number of fruits per plant in the infected transformants were similar to those of uninfected control plants. On the other hand, in the case of infected wild-type plants, the growth was severely retarded and no fruit set was observed (data not shown). These findings suggest that the level of tolerance of the transgenic tomato plants to CMV disease is almost enough.

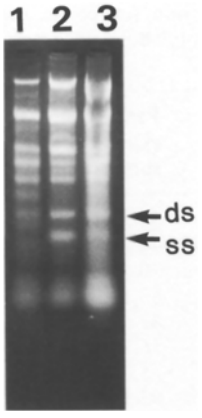

Fig. 4. Progeny-satellite RNAs produced in the transgenic plant after inoculation with CMV-O. *Lane 1*, transgenic plant, Sat 71, without inoculation; *lane 2*, transgenic plant, Sat 71, inoculated with CMV-O; *lane 3*, non-transgenic plant, JTM 7, inoculated with CMV-O plus T73-satRNA

Total RNA was extracted from the transgenic plants that were challenged with CMV-O and separated by agarose gel electrophoresis. The transgenic plants inoculated with CMV-O produced large amounts of single- and double-stranded RNA molecules corresponding to T73-satRNA (Fig. 4).

Discussion

Successful transformation of tomato by common Ti plasmid-based vectors has been described in the literature (McCormick et al. 1986; An et al. 1986; De Block et al. 1987). One of the reports (McCormick et al. 1986) described that there was a variation among genotypes of tomato in the ability to form shoots from transformed leaf pieces and in the length of time required in culture before shoots could be transplanted to soil. We have found that the tomato variety used in this study was not easily transformed with an ordinary vector. Komari (1990) reported that a binary vector carrying a DNA fragment from the virulence region of pTiBo542 had a wider host range and enhanced transformation ability, and suggested that this modified binary vector can be applied to plant species where it is difficult to introduce foreign genes by common binary vectors. The use of the modified vector resulted in higher efficiency of transformation of the particular variety of tomato, thus demonstrating the superiority of the vector.

Another point which leads to a high transformation rate was the use of glucose instead of sucrose in the media for the cocultivation and subsequent culture of tomato explants. The reason for this is not clear, but glucose may be more easily metabolized in tomato explants than sucrose.

Different isolates of satellite RNA of CMV induce quite different responses of host plants. For example, we have previously determined that Y-satRNA causes a

change of leaf color to bright yellow in tobacco and a severe necrosis in tomato when coinoculated with CMV-O (Takanami 1981; Masuta and Takanami 1989). Introduction and expression of various types of satellite RNA sequences in plants, such as tomato, provide unique materials for the studies on interaction between satellite RNA and host plants. Along this line, McGarvey et al. (1990) and Tousch et al. (1990) have successfully transferred the cDNA for a satellite RNA to tomato; the satellite was one of the strains that induces necrosis in tomato.

If production of CMV-tolerant tomato plants expressing satellite RNA is the objective, the choice of satellite RNA which strongly suppresses the symptom development is essential. We have screened many isolates of satellite RNA for the ability to attenuate symptom, and selected T73-satRNA as the best candidate (Masuta et al. 1990). Upon infection with CMV-O, a large quantity of unit-length T73-satRNA was produced in the transformants, and the symptoms of CMV disease were well suppressed. This is similar to the case of the transgenic tobacco plants expressing the cDNA for Y-satRNA (Masuta et al. 1989) and for T73-satRNA (T. Komari, Y. Saito, and C. Masuta, unpublished results). It is noteworthy that when a plant expressing the cDNA for a particular satellite RNA was inoculated with a satellite-free CMV strain, it followed the same pattern of symptom development as observed in a wild-type plant infected by the CMV containing the satellite RNA.

Although very close inspection of the infected transformants revealed very mild mosaic in the leaves, overall growth of the plants was not retarded at all, and there were no significant differences between the noninfected wild-type plants and the infected transformants in either growth rate or fruit production. On the other hand, CMV-infected wild-type plants were severely damaged and set few fruits. Therefore, the level of tolerance of the transgenic plants would be practically satisfactory.

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