TRANSGENIC RESISTANCE TO TOBACCO RINGSPOT VIRUS

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Summary. – The coat protein (CP) gene including the 3'-untranslated region (UTR) of RNA2 of a cherry isolate of Tobacco ringspot virus (TRSV) was utilized in a CP-mediated resistance (CP-MR) strategy. To facilitate construction of plant expression vectors the sequence context of the CP gene translation initiation codon was modified at the 5'-end of the coding sequence by including an initiation codon. The gene was ligated to a version of the Cauliflower mosaic virus (CaMV) 35S promoter with a duplicated enhancer. The cloned CP gene was used to transform *Nicotiana tabacum* cv. Xanthi, as a systemic and local lesion host. The transgenic plants showed different level of resistance ranging from complete resistance to reduction in symptom severity post inoculation with the cherry isolate of TRSV. A CP gene transcript was detected in different tissue of transgenic lines, but translation product was undetectable by Western blot analysis or enzyme-linked immunosorbent assay (ELISA). Interestingly, 100% of seed transmission was blocked in a resistant line, which offers important prospects for engineering TRSV into economically important crops as soybean with 100% seed transmission.

Key words: coat protein gene; coat protein-mediated resistance; Tobacco ringspot virus; post transcriptional gene silencing

Introduction

TRSV is the type species of the genus *Nepovirus* (the family *Comoviridae*). The virus can cause economically important diseases in such diverse crops as blueberry, cucurbits, grapes and soybean (Murant *et al.,* 1996). Of the many diseases caused by TRSV the bud blight of soybean is the most severe and causes the greatest losses. Yields may be reduced by 25–100%, depending on the time of infection (Stace-Smith, 1985). The screening of more than 700 soybean germplasm lines using grape and soybean bud blight isolates of TRSV has failed to identify resistance or tolerance to the virus (Lee *et al.,* 1996). In addition to cultivated plants, the virus infects many biennial and perennial weed hosts, often symptomlessly (Stace-Smith, 1985), these potentially acting as reservoirs of infection and as hosts for vectors. TRSV is transmitted by the nematode *Xiphinema americanum* and also by insects (several species of arthropods) and spider mites (non-specifically), along with seed and pollen (Stace-Smith, 1985).

The TRSV genome consists of two positive-sense linear single-stranded RNA (ssRNA) that are encapsidated separately into polyhedral particles of 28 nm in diameter: the larger RNA (RNA1, approximately 7500 nt) (Rezaian and Francki, 1974) and the smaller RNA of known sequence (RNA2, 3900 nt) (Buckley *et al*., 1993). The main goal of this research was to use gene transfer techniques to investigate possible resistance strategy using CP-MR approach. The feasibility of the CP-MR against nepoviruses has been demonstrated for Arabis mosaic virus (Bertioli *et al.,* 1991), Grapevine chrome mosaic virus (Brault *et al.,* 1993), Grapevine fan leaf virus (Bardonnet *et al.,* 1994),

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Abbreviations: CaMV = Cauliflower mosaic virus; CP = coat protein; CP-MR = coat protein-mediated resistance; ELISA = enzyme-linked immunosorbent assay; p.i. = post inoculation; PTGS = post transcriptional gene silencing; ssRNA = single-stranded RNA; TEV = Tobacco etch virus; TomRSV = Tomato ringspot virus; TRSV = Tobacco ringspot virus; wt = wild type; UTR = untranslated region

and TRSV (Yepes *et al.,* 1996). It was hoped that this strategy would reduce the spread of TRSV in economically important crops by limiting the availability of virus inoculum in a plant tissue.

Materials and Methods

Virus. A cherry isolate of TRSV obtained from Birmingham University was used in this study.

cDNA synthesis. Total RNA isolation, cDNA synthesis, cloning and sequence of this isolate have been described previously (Hamdollah Zadeh and Foster, 2001). In designing primers the TRSV CP gene was modified at the 5'-end of the coding sequence (Acc No. L09205, GI. 535773) to simultaneously introduce an *Nco*I (CC**ATG**G) site with methionine initiation codon.

Cloning of a CP gene construct into plant expression vector. A 2016 nt cDNA clone containing the TRSV CP gene and 3'-UTR of RNA 2 was excised by digestion with *Xho*I and *Bam*HI and then directionally cloned into the plant expression vector pRLT2-GUS (Carrington and Freed, 1990). Digestion with these enzymes removed the *uidA* and Tobacco etch virus (TEV) 5'-UTR vector sequences. pRTL2GUS is a derivative of the pRT series of pUC19-based plant expression vectors (Topfer *et al.,* 1987), which contains the CaMV 35S promoter with a duplicated enhancer region, TEV 5'-UTR, a multiple cloning site and CaMV 35S derived terminator sequences. The constructed plasmid designated p35 S -cp was excised with *Hin*dIII which cleaves immediately upstream of the CaMV 35S promoter and immediately downstream from the CaMV 35S poly(A) signal. The obtained fragment was subcloned into an uique *Hin*dIII site of pBin19. Colonies were screened using restriction digests and PCR amplification of the entire construct between the forward primer M13 (-27) (5'-CAGTCAC GACGTTGTAAAAC GACGGCC-3') ad the reverse primer RSPL-M13 (5'-CACACAG GAAACAGCTATGACC-3'). Recombinant binary plasmid is illustrated in Fig. 1.

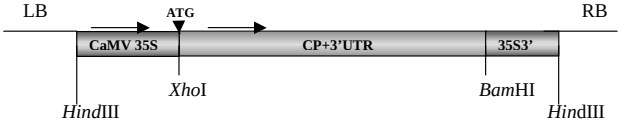

Fig. 1

Diagrammatic representation of T-DNA of the binary construct generated for plant transformation

The left border (LB) and right border (RB) demonstrate the position of this construct within the T-DNA region of pBin19. Transfer of T-DNA originates at RB and proceeds towards LB. The cloning and diagnostic restriction sites are indicated.

The procedure based on the freeze-thaw method of Hofgen and Willmitzer (1988) was used for transformation of *Agrobacterium tumefaciens* strain LBA4404. To confirm the presence of the insert in transformed *Agrobacterium* cells, PCR analysis was carried out on a number of colonies using a combination of primers from the promoter and CP regions of the constructs, in addition to Southern blot analysis. The 35S forward primer for the promoter region was 5'-CCCGGGAAGCTTGGTGCGTGCAG-3' (nt 100–123) and the reverse primer P5 from the CP region was 5'-CCA ACTAACCA ACCATG-3' (complementary to nt 1653–1636 downstream from the end of the CP ORF). The PCR profile used was 30 cycles of 94°C/1 min, 52°C/1 min, and 72°C/3 mins.

Southern blot analysis. Genomic DNA was extracted and used for restriction digestion with appropriate enzyme to confirm the presence of inserts in constructs and transgenic plants. For RNA analysis, a sap extract of transgenic and wild type (wt) plants was spotted on a nylone membrane.

Northern blot analysis. RNA was denatured and blotted onto a nylon membrane.

Hybridization probe for Southern, sap dot blot and Northern blot analyses was full-length cDNA of CP gene that was prepared using the Amersham Readyprime kit according to the manufacturer's protocol.

Transformation of Nicotiana tabacum. N. tabacum L.cv. Xanthi was employed for tobacco leaf disc transformation using the procedure described by Gallois and Marinho (1995). A total of 60 independent lines and 4 plants per line by subculturing were generated. Some plants transformed with binary vectors not harbouring the CP gene were used as control for resistance analysis.

Characterization of transgenic R_0 plants. Analysis of plants for the presence of the trasgene was carried out by PCR after isolation of plant genomic DNA. PCR primers (kanamycin forward: 5'-TCAAGTAAGTCCCGTGGCCT-3' and kanamycin reverse: 5'-TACGGACGAACGGCTTATAG-3' were designed for the kanamycin resistance gene neomycin phosphotransferase II (nptII)). For the screening of transgenic lines for the CP gene, PCR was carried out using the primer P1 (5'-GG*CTCAGC*CATGGGTGCTGTGA CAGTTGTTC-3') designed for the start of the CP gene and the primer P5 (above). The non-complementary bold sequence in the P1 primer represents an *Xho*I site, which was included to facilitate cleavage of this gene and its insertion into transformation vectors. The following *Nco*I site contained the first initiation codon (ATG) of the CP gene. The same PCR conditions were used (see above).

To correlate the reaction of a transgenic line to the presence of transgene, samples of leaves, anther and pollen of selected lines were examined using Southern, Northern/RT-PCR/, sap dot blot, and Western blot analyses and ELISA to determine the transgene integration to the plant genome, transgene expression and a translated product, respectively. In RT-PCR analysis to prevent genomic contamination of RNA, extracted total RNA was treated with RNase-free DNase and recovered with ethanol precipitation. Alternatively, all RNA samples were also subjected to PCR (with the same condition as with cDNA) to check for the possible amplification of genomic DNA. For the pollen RT-PCR the primers P3 (5'-GCGGAAGTGTCAAGAGTG-3') and P5 (5'-CCAACTAAC CAACCATG-3') were used. The PCR conditions were as follows: one cycle of 94°C/2 mins, 52°C/1 min, and 72°C/2 mins followed by 29 cycles of 94°C/1 min, 52°C/1 min and 72°C/1 min. A product of approximately 0.9 kb was obtained.

For anther reaction the primers used were P1 (5'-GCTCGAGC CATGGGTGCTGTGACAGTTGTTC-3') and oligo(dT)*Not*I (5'-CAATTCGCGGCCGC$(T)_{15}$-3'). The reaction proceeded in 30 cycles of 94°C/1 min, 55°C/1 min and 72°C/3 mins. A product of approximately 2 kb was obtained.

Challenge inoculation of transgenic plants. All transgenic lines were examined for resistance to TRSV. Ten or eleven-week-old plants were mechanically inoculated with the cherry isolate at high concentration of inoculum (plant sap diluted 1:5 in a phosphate buffer). This concentration was chosen to guarantee a strong infection in transgenic lines. For each transgenic line, 2 plants (fully expanded leaves) were inoculated and 1 plant was mock-inoculated. In addition, a wild type control and vector-transferred plants were inoculated at the same time in the same manner. Also seedlings of *Chenopodium quinoa,* a local lesion host, were inoculated to monitor inoculum strength. The inoculated leaves were analyzed at day 10 post inoculation (p.i.) and the systemic leaves were analyzed at days 20 and 30 p.i. Final symptom recording at day 50 p.i. was carried out to determine the overall level of resistance and phenotype of each line. To determine presence of the virus within infected plants (at day 10 p.i.) and the virus systemic spread (at days 20 and 30 p.i.), sap dot blot hybridization was carried out.

The choice of this detection system was dictated by the fact that the hybridization assays allow independent quantification of accumulation of each of the viral RNAs. For each transgenic line a sample containing one leaf disc from each inoculated leaf at day 10 p.i. and a systemic sample containing one leaf disc from the youngest systemic leaf at days 20 and 30 p.i. were analyzed. Leaf samples were also taken from mock-inoculated plants of each line at the same time points and treated in the same manner. These corresponded to the numbers 1, 2 (inoculated plants) and 3 (mock-inoculated plant) for each line tested shown in Fig. 6. The leaf extracts were spotted onto nylon membrane and the viral RNAs were analyzed by hybridization with a probe corresponding to the TRSV CP gene. CP expression in transgenic line was analyzed in samples from infected and mock-inoculated plants for each line by ELISA using antibodies provided by DZMS (Germany) and AGDIA (USA). The samples were prepared at days 10, 20 and 30 p.i. to compare the protein level over time in mock-inoculated plants and the extent of virus spread in infected transgenic lines. The samples were prepared using a 1.5 ml tube lid to cut two 1.5 cm circles of tissue. The samples were homogenized and centrifuged to extract the plant sap. The sap of mock-inoculated and TRSV-infected wild type plants (setting positive-negative thresholds) were included in each experiment. The wells were loaded with each plant sample induplicate and A_{405} was read.

Segregation analysis and seed transmissibility of TRSV in transgenic lines. Once flowering commenced flower heads were bagged with paper bags to prevent cross-pollination and carried on to R_1 generation. To estimate the number of transgene(s) loci within each transformant, a genetic approach was used to determine the segregation of the introduced kanamycin resistance marker gene (*npt*II) in the progeny of selfed mock-inoculated plants of transgenic lines described by Wilkinson *et al.* (1996). The population ratio of kanamycin-resistant segregants was counted and used to estimate the number of T-DNA loci in the plant genome. Surface sterilized seeds were sown on half strength MS medium supplemented with sucrose and 200 µg/ml kanamycin or without kanamycin as a control for comparison of seed germination. The population ratio of kanamycin-resistant/kanamycin-sensitive was counted and used to estimate the number of T-DNA loci in the plant genome. To determine seed transmission of TRSV, 100–200 surface sterilized seeds from transgenic lines (two resistant lines, 35S-cpH and 35S-cpF, one delayed line, 35S-cpC, and one enhanced susceptible line, 35S-cpI) and TRSV-infected plants were germinated on media with (transgenic selection) or without (wild type) kanamycin before establishing the seedlings in soil. Three to four week-old selected kanamycin-resistant seedlings were transplanted into pots in such a way to ensure that no mechanical contact occurred between them. To determine the time of virus activation and detection in seedlings, extracts of germinated seedling from infected wild type at one week, and thereafter weekly until 6 weeks, were indexed on *C. quinoa,* as a local lesion host for TRSV. Hundred seedlings from each transgenic line and infected wild type were analyzed for viral infection. For each seedling, duplicate samples were tested by ELISA. Interpretation of ELISA values was established with the commonly used standards in plant ELISA (Gillet *et al.,* 1986). Absorbance values were considered positive if the A_{405} values greater than twofold values obtained for healthy control seedlings were considered positive.

Statistical analysis was carried out using the Genstat statistics package (Payne *et al.,* 1987). Differences between transgenic groups were evaluated by the least significant difference test (LSD) after analysis of variance. $P \leq 0.05$ was considered significant.

Results

Characterization of transgenic lines

Plants were regenerated from separate calli to ensure recovery of independent transformation events. Initially, a PCR method utilizing *npt*II and CP gene specific primers was used to confirm transgenic nature of transformant. A total of sixty lines were generated and transgenic status was confirmed by genomic DNA extraction and subsequent PCR for the *npt*II gene. Nine lines (35-cpA, 35S-cpB, 35S-cpC, 35S-cpE, 35-cpF, 35S-cpH, 35S-cpI, 35S-cpO, 35S-cpP) were selected for further analysis.

Similarly Southern blot analysis confirmed integration of transgene into plant genome in selected lines. To determine the transgene expression, total RNA were prepared from leaf, anther and pollen. Anthers were examined at anthesis, and one or two days prior to anthesis. A single transcript of the expected size (~2.3 kb for the CP gene, 3'-UTR and its boarding sequence) was detected in leaf samples of all lines (Fig. 2). In majority of lines there was no real difference in the strength of signal (except 35S-cpA and 35S-cpF which may related to loading unequal amount of RNA). This confirmed the results of sap dot blot analysis (described below) and was in agreement with the constitutive expression pattern of the CaMV 35S promoter. Interestingly, transgene RNA was detected in anthers and pollen from only one line (35S-cpH) of four lines (35S-cpC, 35S-cpF, 35S-cpI) tested by Northern blot analysis and RT-PCR (Fig. 3.). Detection of transgene RNA from anther and pollen in this line confirmed the variable activity of expression pattern of the CaMV 35S promoter in independent lines, which has been reported previously (Wilkinson *et al.,* 1997).

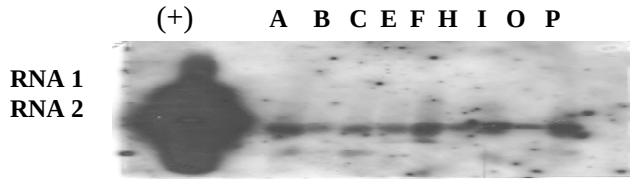

Fig. 2

Northern blot analysis to detect the CP transcript in transgenic lines

Letters correspond to independent transgenic lines and lane (+) is a wt TRSV-infected control plant. The samples were prepared from leaf tissues of mock-inoculated transgenic plants. The probe contained the CP gene and 468 nt corresponding to the 3'-UTR of RNA2 of TRSV (common for both RNAs of TRSV), so it hybridized to both viral RNAs in control lane.

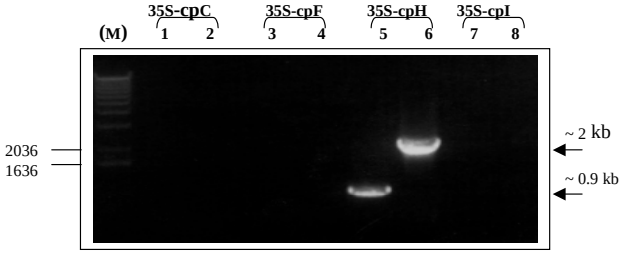

Fig. 3

RT-PCR of CP transcript from anther and pollen RNA in CaMV 35S RNA transgenic lines

PCR carried out on oligo(dT)Not_{15}I primed first-strand cDNA of anther and pollen. cDNA from pollen (lanes 1, 3, 5 and 7) and anther (lanes 2, 4, 6 and 8).

In protein analysis by ELISA, all mock-inoculated lines demonstrated low readings as did mock-inoculated wild type plants. The data indicated that the CP expression level in transgenic lines was not remarkable (≤ 0.003) and there was no significant increase in CP expression over time in transgenic lines. There were no significant differences between transgenic lines with the wild type plant (P = 5%) in the absorbance level (LSD = 0.0132).

Western blot analysis of infected wild type and transgenic plants using two anti-CP antibodies proved unsuccessful. In dot immunobinding assay both these antisera (titer 1:1000) reacted with intact TRSV from wild type infected plants but did not react with denatured CP. These antibodies were probably raised to native target protein, and consequently were not suitable for use as probes in Western blot analysis, where the target protein has been denatured.

Evaluation of transgenic lines for resistance

All mock-inoculated transgenic plants were indistinguishable from mock-inoculated wt plants, in terms of external morphological characters such as height (Table 1.) and color. The inoculated lines exhibited a broad range of symptoms. Disease severity was evaluated using visual assessment and a rating scale of 0 to 4 in which 0 was no local or systemic symptoms through to 4 which was a large number (>15) of ringspots on systemic leaves (Fig. 4). Chlorotic lesions appeared in control plants (wt and pBin19-transformed) at day 10 p.i. and became necrotic with typical rings. Systemic symptoms with typical rings developed in upper leaves at days 15–20 p.i. (rating of 3) and the plants recovered at days 45–50 p.i. In transgenic

Table 1. Response of transgenic lines to TRSV

Transgenic line	Inoculated leaf (day 10 p.i.)	Systemic leaf (day 20 p.i.)	Systemic leaf (day 30 p.i.)	Phenotype (day 50 p.i.)	Sap dot blot analysis		Height (mm) at flowering stage	
					Virus infection/ spread	Transgene transcript	Mock-inoculated	TRSV-inoculated
wt (1)	CLL+NLL	1	3	Susceptible	++++	–	416	362
V-transformed (1)	CLL+NLL	1	3	wt, susceptible	++++	–	408	381
35S-cpA	CLL	0	1	Delay in symptom expression	+	+	410	390
35S-cpB	NLL	3	4	Enhanced wt, susceptible	+++	?	394	305
35S-cpC	CLL	0	1	Delay in symptom expression	++	+	388	372
35S-cpE	CLL	0	0	Resistant	+	++++	418	403
35S-cpF	CLL	0	0	Resistant	+	+	412	418
35S-cpH	CLL	0	0	Resistant	++	+	422	416
35S-cpI	NLL	3	4	Enhanced wt, susceptible	++++	+	388	296
35S-cpO	CLL	1	3	wt, susceptible	++++	-	434	380
35S-cpP	CLL	1	3	wt, susceptible	+++	+	428	368

The response determined at days 10, 20 and 30 p.i. The phenotype and height of lines are presented at day 50 p.i.
The height of infected transgenic lines is an average of 2 plants. Disease severity was rated 0-4. CLL = chlorotic local lesion; NLL = necrotic local lesion.

lines, comparing to wt/vector-transformed plants four distinct phenotypes were observed:

(i) Resistant, rating of 0 on systemic leaves, indistinguishable from the mock-inoculated plant of the same line and the wt plant.

(ii) Delay (7–10 days) in symptom expression and slight stunting and rating of 2.

(iii) wt level, susceptible, stunting and rating of 3 on systemic leaves.

(iv) Enhanced wt, very susceptible, sever stunting and rating of 4.

The incidence of the resistant phenotype was 33% (3/9) and that of the delay phenotype, the wt susceptible phenotype and the enhanced wt was 22% (2/9) each. The plant response and phenotype of transgenic lines are summarized in Table 1.

Results of sap dot blot hybridization at day 10 p.i showed that all transgenic lines became infected upon inoculation with TRSV. At day 30 p.i. (Fig. 5), the hybridization signals from all lines were weaker than those from wt and vector-transferred plants except the line 35S-cpO, which displayed a high signal of virus RNAs relatively similar to wt-infected plants (visual assessment of hybridization signal was rated as 1+ to 4+). This apparently low level of viral RNAs in majority of transgenic lines could be due to low rate of virus replication/spread. Three lines with resistant phenotype (35S-cpE, 35S-cpF and 35S-cpH) displayed initially chlorotic local lesion on inoculated leaves but showed considerable reduction in viral accumulation in systemic leaves. The durability of the reduction in viral replication was monitored at day 30 p.i. At recovery stage (day 50 p.i.), as common symptom of nepovirus infection, these plants were symptomless as compared with recovered wt-infected plants. At this stage these lines were tested for presence of virus by the infectivity assay on *C. quinoa.* The inoculum was prepared from the youngest symptomless leaf of each plant. Chlorotic lesions were produced on leaves of indicator plant from inocula of wt-infected plants, where the infectivity assays from 35S-cpE, 35S-cpF and 35S-cpF on *C. quinoa* were negative.

In mock-inoculated plants, trace signal of the transgene RNA appeared in all lines except 35S-cpO (though in Northern blot analysis the transcript of transgene was detected in this line). The high signal in 35S-cpE in mock-inoculated plant and combined low transgene transcript and viral RNA (considering high homology between the transgene and viral RNAs) in infected plants of this line (plants 1 and 2) may be explained by an RNA threshold model based upon a homology-dependent post transcriptional gene silencing (PTGS) degradation mechanism (Guo and Gacia, 1997).

In ELISA data from infected lines at day 10 p.i. all lines showed a reading comparable to wt-infected plants (Fig. 6). 35S-cpI showed the highest reading approximately equal to

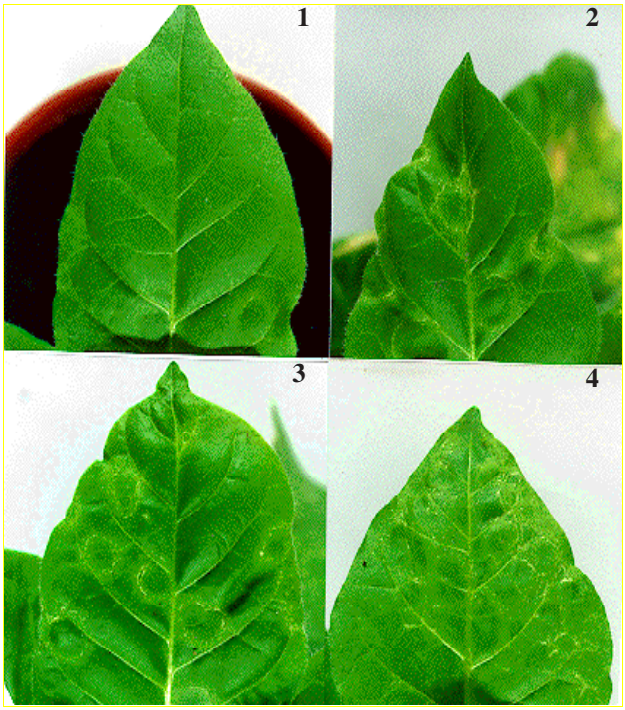

Fig. 4

Rating scale applied to transgenic lines

Rating 0–4 based on the number of ringspots produced on systemic leaves from a similar position of *N. tabacum* transgenic plants at days 20 and 30 p.i. in which 0 = no symptoms (not shown), 1 = below 5 ringspots; 2 = 5–10 ringspots; 3 = 11–15 ringspots; and 4 = over 15 ringspots.

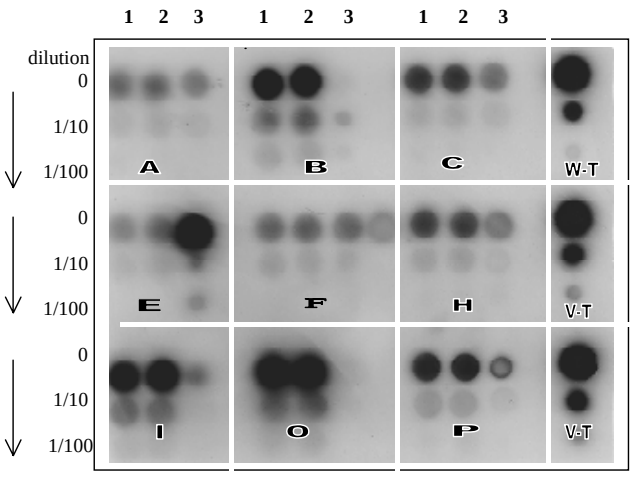

Fig. 5

Sap dot blot hybridization of transgenic lines to determine infection progress on systemic leaves at day 30 p.i.

Tenfold serial dilutions of sap samples were prepared. Extracts of leaf tissues from mock-inoculated plants of related lines were also assayed for the transgene transcript. Plants 1 and 2 represent two infected plants, while plant 3 was mock-inoculated (the same line). V-T stands for vector-transformed plants.

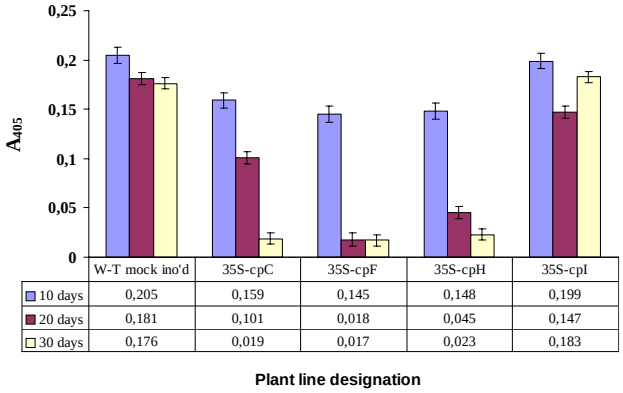

Fig. 6

ELISA analysis of transgenic lines

Average ELISA values of duplicate samples at days 10, 20 and 30 p.i. were corrected by subtracting the wt and transgene readings as background to show ELISA readings related to viral CP.

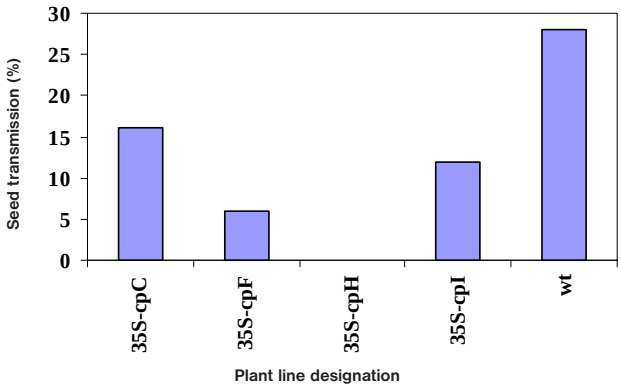

Fig. 7

TRSV transmission (%) in transgenic and wt plants

the reading generated in a wt-infected plant. At day 20 p.i. all lines demonstrated a considerable decrease in their ELISA reading. 35S-cpF and 35S-cpH showed the lowest ($P < 0.05$) and 35S-cpI showed the highest level of ELISA reading approximately equal to that of the wt-infected plant. Whilst 35S-cpC showed a relatively high ELISA reading, there were little or no symptoms on systemic leaves of this line. At day 30 p.i. 35S-cpC, 35S-cpH and 35S-cpF demonstrated very low readings (<0.03) and the reading of 35S-cpI was higher than that of wt-infected plants (Fig. 6).

Segregation analysis and seed transmissibility

Segregation analysis of 35S-cpC (delayed phenotype), 35S-cpF and 35S-cpH (resistant phenotype) and 35S-cpI (enhanced susceptible) showed that 35S-cpI possessed two transgene loci. The three other lines contained one transgene locus.

Progeny seedlings (50 seedlings per line) derived from R_0 self-fertilized TRSV-infected plants were tested by ELISA. The incidence of TRSV seed transmission in wt-infected plants was 28% and the rate of seed transmission in all tested transgenic lines was less than that in the wt-infected plant (≤16%) (Fig. 7). The line 35S-cpF exhibited a resistance response in R_0 and also showed a low rate of seed transmission (6%). This is probably due to low level of virus replication/spread in this line and consequently to a less successful virus entry into reproductive tissue. 35S-cpH showed a resistance response in R_0 and it also showed 0% of seed transmission. This could be due to the presence of transgene or its product in the leaf and stem, thereby preventing either virus replication and/or virus movement within the plant. This in turn could reduce systemic infection and prevent infection of the floral organs and subsequent establishment in gametes. Alternatively, a 100% prevention of TRSV seed transmission in 35S-cpH could be due to the expression of transgene in reproductive organs (anther and pollen) (Fig. 2), resulting in the blockage of seed transmission.

The incidence of virus transmission in 35S-cpC, which exhibited the delay phenotype, was higher (16%) than the rate of seed transmission in the enhanced susceptible line, 35S-cpI (12%). Both were lower than the rate of seed transmission in wt plants. The low rate of seed transmission in these two lines in comparison with wt-infected plant could be due to constitutive expression of the transgene in plant. In 35S-cpC, delay and mild symptom expression did not restrict virus movement to reproductive tissue. It is suggested that a milder interaction results in more successful seed transmission (Johansen *et al.*, 1994). In 35S-cpI that the low rate of seed transmission could be due to the inability of the infected pollen to fertilize the egg cells.

Discussion

A TRSV CP gene construct was linked to the CaMV 35S constitutive promoter. The construct contained a 2016 nt RT-PCR product representing the 3'-end of TRSV RNA2 and CaMV 35S 3'-terminator signal (Hamdollah Zadeh and Foster, 2001). CP gene was ligated to a version of the CaMV 35S promoter with a duplicated enhancer, which has been shown to lead to a higher level of transcription (Kay *et al.*, 1987). This promoter was used in this project for two main reasons: (i) As a strong and commonly used promoter to generate transgenic lines for the CP-MR approach. (ii) The

CaMV 35S promoter (or derivatives of it) has been involved in generating transgenic lines against many plant viruses, some of which were pollen and seed transmitted. However, the promoter has not been used to evaluate the prevention of seed transmissibility in progeny of transgenic lines.

The generated lines (R_0) were challenged and their response was evaluated. The expectation was that the plants containing this construct would display reasonable resistance against TRSV infection depending on expression level of the transgene. The strong and constitutive expression of the transgene should reduce systemic infection and therefore decrease the chance of the virus spreading and reaching reproductive tissue. Hence, due to the low level of virus replication, healthy and viable pollen and consequently a low rate of seed transmission was also anticipated in resistant lines.

Of nine selected transgenic lines three (35S-cpE, 35S-cpF and 35S-cpH) were found to be resistant to TRSV infection. The other six lines (35S-cpA, 35S-cpB, 35S-cpC, 35S-cpI, 35S-cpO and 35S-cpP) exhibited variable levels of responses, which varied from moderately resistant that showed a delay in symptom expression to enhanced susceptibility with remarkable stunting.

The transgene transcript was detected in all nine transgenic lines and there was no correlation between the steady-state levels of transgene RNA and the ability of the plants to show resistance.

TRSV was transferred through seed in transgenic plants with variable efficiency ranging from 0% to 16%. This was consistent with the phenotype of primary transformants in resistance lines. It is likely that factors involved in the resistance of transgenic lines influenced the ability of the plants to block virus transmission.

It was interesting that the transgene transcript was detected in anther and pollen from the 35S-cpH line which exhibited 100% prevention of seed transmission. Therefore, this line could potentially offer the highest resistance to TRSV interaction. It is possible that the site of transgene integration in this line may influence the expression pattern in floral organs. For example, floral transcriptional enhancer may cause the CaMV 35S promoter to become activated (Wilkinson *et al.,* 1998). It is also possible that the variation between transgenic lines in the CaMV 35S promoter expression in anther and pollen of transgenic lines could be influenced by the structure of CaMV 35S promoter used in this study. This version of CaMV 35S promoter has been reported to confer developmentally regulated expressions (Wilkinson *et al.,* 1998). The CP gene translation product was not detectable in any of the transgenic lines by ELISA. It is possible that CP could be present in very low concentration, i.e. under the ELISA detection limit, or even could be absent e.g. due to gene silencing. However, this cannot discount the role of C in mediating resistance. It is possible that the polyclonal antibody raised against intact particles of TRSV was unable to react with individual CP molecules. Alternatively, the CP molecules may occur in a configuration that was not recognized by the polyclonal antibody to TRSV.

Previous reports of engineering resistance to nepoviruses (Bertioli *et al.,* 1991; Brault *et al.,* 1993; Bardonnet *et al.,* 1994) have demonstrated that resistance correlated with the expression of *CP* gene in *Nicotiana* spp. transgenic lines. However, lines with no or low levels of CP were not tested. In another study using the *CP* gene and 3'-UTR of RNA 2 of Tomato ring spot virus (TomRSV), and analysing over 170 independent lines, there was a high level of resistance without detectable *CP* gene expression (Yepes *et al.*, 1996). The authors did not correlate the mechanism of resistance to the expected translated protein. Similarities between the 3'-end of RNA2 (for example lack of the CP motif in nepoviruses) in some regions between TRSV isolates and TomRSV (Buckley *et al.,* 1993; Hamdollah Zadeh and Foster, 2002) may account for the same mechanism of resistance in the resistant lines investigated in this study.

Many models have proposed that PTGS is initiated by the accumulation of more than threshold amounts of a specific RNA or by events that may lead to the production of aberrant RNA as the silencing signal (Lucy *et al.,* 2000). Clearly, if any PTGS mechanism is involved in TRSV resistant lines, current data does not allow us to distinguish between these alternatives. Further nuclear run-on analysis to determine the rate of transcription level and also Northern blot analysis to determine steady state level of a transcript in progeny from resistant lines would be valuable to understand the underlying mechanism(s).

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