Constitutive Expression of Untranslatable Versions of the p25 Coat Protein Gene of *Citrus tristeza virus* (CTV) in Transgenic Mexican Lime Plants Does Not Confer Resistance to the Virus

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ABSTRACT. To develop RNA-mediated resistance (RMR) against *Citrus tristeza virus* (CTV), Mexican lime plants were transformed with two modified versions of CTV p25 gene: i) the complete p25 ORF derived from the severe strain T305, mutated to make its mRNA untranslatable, and ii) an untranslatable fragment of the p25 gene fused to the green fluorescent protein (*gfp*) gene. Forty-eight independent transgenic lines were obtained in total, and Southern blot analyses revealed that all incorporated the corresponding p25 sequence. Some of them showed post-transcriptional gene silencing (PTGS) of the p25 transgene, which has been associated to RMR in other transgenic plant-virus interactions. After graft-inoculation with the homologous CTV strain, transgenic plants developed symptoms and virus accumulated at similar rates than in non-transgenic control plants. Factors potentially involved in this protection failure are discussed.

In citrus areas where severe isolates of *Citrus tristeza virus* (CTV) are common, cross-protection with mild CTV isolates (4) is the unique system to reduce yield losses in sensitive varieties, but this approach has been successful only in some areas, and protection afforded is sometimes temporary. Pathogen derived resistance (PDR) was proposed by Sanford and Johnston in 1985 (11) as a tool to get protection against pathogens using pathogen-derived genes and/or their products. Since the initial report of coat protein (CP)-mediated resistance against *Tobacco mosaic virus* (10), this approach has proved to be applicable to engineer resistance in many virus-plant systems. RNA-mediated resistance (RMR) is one of the most effective PDR approaches to control plant virus diseases through genetic engineering. It often confers high degree of protection or even immunity against the challenging virus. To develop RMR against CTV, we generated transgenic Mexican lime plants carrying modified versions of CTV p25 major CP gene. Transgenic plants were graft-inoculated with the homologous CTV isolate and their response to virus infection was studied.

The full-length p25 ORF from the severe strain T305 of CTV (8) was RT-PCR amplified, and the corresponding cDNA was cloned into the expression vector pMOG 180 (Mogen International) between the 2× 35S promoter plus the *Alfalfa mosaic virus* RNA 4 leader sequence and the nopaline synthase gene (*nos*) terminator sequence. The p25 sequence was modified by introducing two consecutive stop codons three nucleotides downstream the start codon to make its transcripts untranslatable. The p25 expression cassette was subcloned into the plant transformation vector pBI 121 (Clontech), between the 35S/*uidA*/*nos* and *nos*/*nptII*/*nos* marker cassettes. This plasmid was incorporated into *Agrobacterium tumefaciens* and the bacterial vector was used to generate transgenic lines termed as B-1 to B-35 (Fig. 1a). A second transformation vector was constructed by RT-PCR amplification of a p25 fragment comprising nucleotides +349 to +683, which was also mutated by introducing a stop codon at nucleotide +354, and subsequent cloning of this

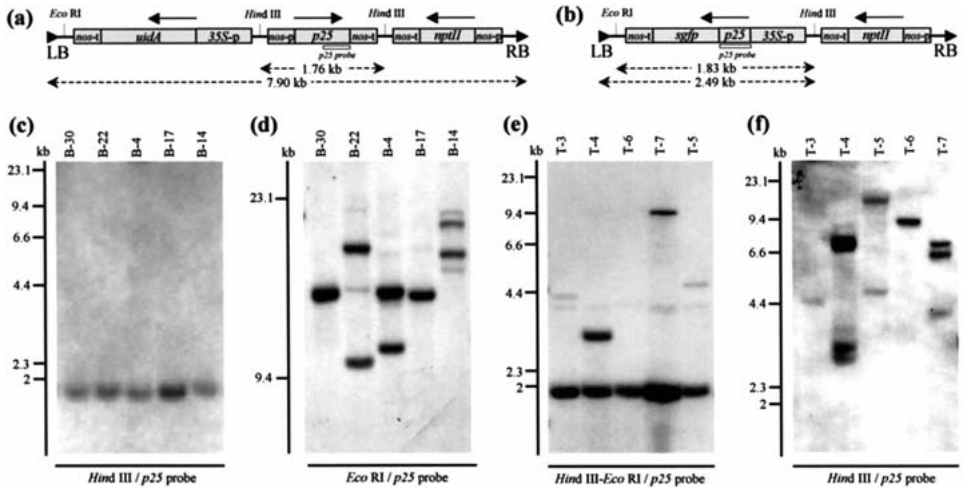

Fig. 1. Schematic representation of T-DNAs from transformation vectors used to generate transgenic plants and Southern blot analysis of representative lines. (a) Plant transformation vector pBI 121 carrying the untranslatable version of the complete p25 gene used to generate B lines. (b)) Plant transformation vector pBin 19-*sgfp* carrying the untranslatable version of a fragment of the p25 gene used to generale T lines. Southern blot analysis of represen tative lines B-4, B-17, B6-22 and B-30 (c,d) and T-3, T-4, T-5, T-6, and T-7 (e-f). DNA was digested with *Hin*d III (c,f), *Eco* RI (d) and *Hin*d III-*Eco* RI (e) and hybridized with a p25 specific probe. Size of DNA fragments is expressed in kilobases. Arrows indicate the transcription sense.

sequence into pBin 19-*sgfp*, fused to the *sgfp* marker gene and under the control of the 35S promoter and *nos* terminator. Once in *Agrobacterium*, this plasmid was used to produce transgenic lines termed as T-1 to T-13 (Fig. 1b).

Transgenic Mexican lime plants were obtained as described in Domínguez et al. (7), and transformants, were selected based on PCR amplification of a fragment of the p25 sequence. Forty-eight independent transgenic lines were generated, 35 harboring the full-length untranslatable p25 gene sequence and 13 carrying the truncated version. To confirm their transgenic nature, Southern blot analysis was performed. DNA was extracted from leaves according to Dellaporta et al. (6), and aliquots (20 µg) of *Hin*d III, *Eco* RI or *Hin*d III/*Eco* RI-digested samples were separated by electrophoresis in 1% agarose gels, blotted to nylon membranes, and fixed by UV irradiation. The blots were hybridized with a DIG-labeled fragment of the p25 gene (+349 to +683) prepared by PCR following the supplier instructions (Boehringer-Mannheim). Southern blot analysis revealed that all transgenic lines incorporated at least one intact copy of the corresponding p25 expression cassette (Fig. 1c, e, and data not shown). This analysis also showed that multiple integration was the most frequent integration pattern (Fig. 1d, f, and data not shown). Northern blot, methylation and nuclear run-on transcription studies revealed that some B lines had the p25 transgene silenced at post-transcriptional level (data not shown), which has been widely associated to RMR (1, 2). This mechanism involves post-transcriptional degradation of the transgene transcripts and of highly homologous viral RNAs incoming in the cytoplasm of the transgenic cells, which often confers high degree of protection or even immunity against the virus.

Transgenic and non-transgenic control Mexican limes were graft-

propagated onto Carrizo citrange rootstocks. Eight to ten homogeneous plants from each transgenic line and the same number of non-transformed lime controls were graft-inoculated with CTV T305 and evaluated for resistance. CTV T305 causes severe symptoms in Mexican lime, as vein clearing, leaf cupping, stem pitting, vein corking and marked stunting (8). All plants were periodically tested for the presence of CTV in the scion by PC-RT-nested-PCR (9) and, upon virus detection, the bark chip grafted was removed to limit the inoculum dose. CTV symptoms and virus accumulation were monitored in new leaves of three consecutive flushes, which spanned over a 1-yr period. Symptom development and intensity was visually scored, and virus accumulation was estimated by a semiquantitative DAS-ELISA (3). Symptom onset started in the first flush after inoculation, and the transgenic plants developed typical CTV T305 symptoms basically in the same way as the non-transformed controls. Symptom intensity and virus accumulation in both type of plants were comparable throughout the three flushes investigated (Table 1 and data not shown). Several factors could contribute to the lack of protection observed even in lines showing PTGS before virus inoculation: i) the high dose of virus delivered to plants by graft-inoculation would overcome the potential protection afforded by constitutive expression of viral sequences in transgenic cells, ii) CTV replication is restricted to phloem and phloem-associated cells and PTGS may not occur in these cells, as proposed by De Haan (5), iii) genetic divergence of some RNA variants within the same CTV strain could exceed 10%, and thus escape the RMR mechanism, iv) the variable level of transgene expression during growth and development of transgenic plants could affect the RMR mechanism, v) CTV genome

TABLE 1
SYMPTOMATOLOGY IN TRANSGENIC MEXICAN LIME PLANTS CARRYING UNTRANSLATABLE VERSIONS OF THE CTV p25 CP GENE AFTER GRAFT-INOCULATION WITH CTV T305

	1st flush		2nd flush		3rd flush	
Transgenic line	Plants with symptoms/ total[a]	Symptoms intensity[b]	Plants with symptoms/ total[a]	Symptoms intensity[b]	Plants with symptoms/ total[a]	Symptoms intensity[b]
B-1	5/10	1.8	6/10	1.5	10/10	1.4
B-2	3/10	2.0	6/10	1.5	10/10	1.3
B-3	7/10	2.4	9/10	2.0	10/10	2.0
B-4	5/10	2.4	8/10	2.1	10/10	1.7
B-5	5/10	2.4	6/10	2.3	10/10	1.4
B-14	5/9	1.2	7/9	1.3	9/9	2.0
B-17	9/10	2.1	10/10	1.8	10/10	2.1
B-22	6/8	1.7	6/8	2.6	8/8	2.1
B-30	6/8	2.2	7/8	2.0	8/8	1.9
T-3	5/8	1.3	6/8	1.6	8/8	2.0
T-4	4/8	1.3	8/8	1.6	8/8	2.3
T-5	6/8	1.5	7/8	1.3	8/8	2.1
T-6	5/8	1.2	6/8	1.7	8/8	2.3
T-7	7/8	1.8	7/8	2.0	8/8	2.5
Control[c]	8/10	1.9	9/10	2.0	10/10	2.2

[a]Number of plants that show symptoms / total inoculated plants.
[b]Average intensity of symptoms in symptomatic plants. 0: asymptomatic; 1: mild; 2: medium; 3: severe.
[c]Non transgenic plants of Mexican lime inoculated with CTV T305.

could encode protein/s breaking PTGS and consequently RMR, or vi) untranslatable mRNAs could be recognized as non-sense RNAs by the plant cell translational machinery and degraded once in the cytoplasm.

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