Genetic Transformation of Sour Orange and Alemow with Non-translatable CP, p27-CP and 3'-end Sequences from *Citrus tristeza virus* Found in Veracruz, Mexico

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ABSTRACT. *Citrus tristeza virus* (CTV) is the most important virus affecting citrus worldwide. CTV isolates from Veracruz State, Mexico, were chosen to clone sequences for transformation. Five constructs of non-translatable versions of the p25 gene, the p27 and the p25 genes together (p27-CP) and the 3'-end in antisense were used in genetic transformation of sour orange and Alemow to obtain rootstocks resistant or tolerant to CTV. Internodal stem segments of sour orange and Alemow seedlings were transformed with *Agrobacterium tumefaciens* strains EHA105 and Agl1 harboring pCAMBIA 1301, pCAMBIA 2201 and pCAMBIA 2202 plasmids with CTV non-translatable-CP, p27-CP and 3'-end sequences. Six transgenic shoots obtained were positive for CTV sequences using PCR assays.

Citrus tristeza virus (CTV) is the most economically important virus affecting citrus production worldwide (5). Different isolates can cause diverse disease syndromes that vary from no visible symptoms, to stem pitting and fruit size reduction, as well as to the quick decline and death of scions grafted on sour orange (1, 10). The single-stranded, positive-sense genomic RNA of CTV is encapsidated by a major capsid protein of 25 kDa (CP) and a minor capsid protein of 27 kDa (p27) (3). The CTV RNA has ten other genes (7, 13). In Mexico, CTV was first detected in 1983 in commercial citrus in Tamaulipas and then was found at nurseries in Veracruz in 1986-1987. In both cases infected trees were eradicated by incineration as part of the official eradication program (14).

One strategy of management for this virus is the use of important citrus rootstocks that are genetically tolerant or resistant to this virus. Research has shown that expression of viral sequences in sense or antisense orientation, individually or simultaneously, can induce gene silencing, producing plants that are resistant to the viral source of the transgenes as well as to closely related strains of the virus (16).

In Spain transgenic Mexican lime plants expressing the CTV CP gene showed some resistance to CTV (2). With this in mind our strategy was to transform sour orange and alemow individually with three different CTV sequences to increase the chances of producing tolerant plants. The sequences for transformation were amplified from a CTV isolate from Veracruz and included a non-translatable version (NTCP) of the major (p25) coat protein gene, both the minor and the major coat protein genes (p27-CP) together and the 3' end of the CTV genome. All sequences were introduced in anti-sense orientation and are non-translatable (12). Plasmids for transformation and the resulting constructs are shown in Table 1.

Sequencing of the cloned CTV genes showed that the sequences for

TABLE 1
PLASMIDS AND BACTERIAL STRAINS USED FOR TRANSFORMATION OF SOUR ORANGE AND ALEMOW WITH THE INDICATED CTV SEQUENCES

Agrobacterium strain	Vector	Construct[a]	Plant selectable marker[b]	Bacteria selectable marker[c]
Agl 1	pCAMBIA 2201	FMV_3'END-AS	Kan	Cap
Agl 1	pCAMBIA 2201	FMV_NTCP-AS	Kan	Cap
Agl 1	pCAMBIA 2202	FMV_p27-CP-AS	Kan	Cap
Agl 1	pCAMBIA 2201	FMV_p27-CP-AS	Kan	Cap
EHA 105	pCAMBIA 1301	FMV_p27-CP-AS	Hyg	Kan

[a]FMV = FMV 34S promoter; NTCP = nontranslatable major CP in antisense orientation; p27-CP = minor and major capsid proteins in antisense orientation; 3'END = 3'-terminal region in antisense orientation.
[b]Kan, Kanamycin; Hyg, Hygromycin.
[c]Cap, chloramphenicol; Kan, Kanamycin.

NTCP and p27-CP were identical to the Florida mild isolate T30 (Genbank Accession AF260651) and the sequences for the 3'-end were similar to T30 and to T385 (Genbank Accession Y18420), a mild isolate from Spain (12).

Agrobacterium-mediated transformation was done as described (6, 8), with the following modifications: i) Inoculation by immersion involved shaking for 2 h at 110 rpm, ii) Coculture period was for 72 h, and iii) Selection media was MS supplemented with BA, cefotaxime and kanamycin (pCAMBIA 2201 and 2202) or hygromycin (pCAMBIA 1301). Putative transgenic shoots transformed with pCAMBIA 2201 were further analyzed by β-glucuronidase (GUS) assay as described (11), or green fluorescent protein (GFP, present in pCAMBIA 2202) expression. GUS-positive or GFP-positive shoots were transferred to rooting medium or were *in vitro* grafted onto sour orange or rough lemon seedlings.

Extraction of plant DNA from transformants for polymerase chain reaction (PCR) assays was performed as described (9, 15). The quantity, purity, and integrity of the genomic DNA were determined by electrophoresis with appropriate standards. PCR assays to amplify CTV sequences were performed on GUS positive and negative transformants and on non-transformed controls. The nontranslatable CP (NTCP) gene was amplified using forward primer V16 (5'-TTA TTA TGC GGC CGC ATG GAC TAA TAA ACA AAG AAA TTG AAG-3') and reverse primer VF12 (4). The p27-CP sequence was amplified using forward primer VF53 (5'-GTC ATA TGA GCA GAG ACG TGG C-3') and reverse primer VF54 (5'-TGA AAC TCC ACC ATC CCG ATA-3'). The 3'-end was amplified using forward primer VF36 (5'-TAT ATA CTC GAG ATG AGG TAC ATG AGT TCT TAG TCA CAC C-3') and reverse primer VF37 (5'-ATA TAT GGG CCC TGG ACC TAT GTT GGC CCC CCA ATA GG-3').

We were able to transform sour orange and alemow with CTV sequences using *A. tumefaciens* strains Agl 1 and EHA 105 (Table 2). Explants in media in vertical positions regenerated more shoots than explants in horizontal position, although more escapes were regenerated. Strain EHA 105 on the hygromycin selection media produced 5-33% GUS-positive shoots and 0-25% GUS-positive shoots were obtained with the Agl 1 strain and the kanamycin selection. Some GUS-positive sour orange transformants also were PCR-positive for the indicated sequence (Table 2). However, a few of the plants that were negative in the GUS assay

TABLE 2
TRANSGENIC SHOOT PRODUCTION FROM STEM SEGMENT EXPLANTS WITH DIFFERENT CTV SEQUENCES

Citrus species	Construct	*A. tumefaciens* strains	pCAMBIA plasmid	Explant position	GUS (+) shoots	PCR (+) shoots*
Sour orange	FMV_p27-CP	EHA 105	1301	Vertical	5	0
	FMV_p27-CP	EHA 105	1301	Horizontal	36	0
	FMV_p27-CP	Agl 1	2201	Vertical	13	0
	FMV_p27-CP	Agl 1	2201	Horizontal	11	1
	FMV_p27-CP	Agl 1	2202	Vertical	20	0
	FMV_NTCP	Agl 1	2201	Horizontal	20	1
	FMV_3'END	Agl 1	2201	Horizontal	31	4
Alemow	FMV_p27-CP	EHA 105	1301	Horizontal	2	0
	FMV_3'END	Agl 1	2201	Horizontal	2	0

*Initial results.

were positive in PCR for NTCP or 3'END (data not shown). This indicates it may be necessary to analyze all putative tranformants by PCR, even if they were negative in the GUS assay. Some GUS positive and GUS-negative sour orange shoots are currently on regeneration media, planted in soil or grafted onto sour orange, rough lemon or Volkamer lemon in the greenhouse. GUS positive Alemow shoots were not grafted because they easily regenerated roots.

This is the first report of production of sour orange plants stably transformed with sequences from a Mexican CTV isolate. Although the NTCP cloned from a Mexican isolate is identical to this sequence of the Florida isolate T30, sour orange has not been transformed previously with the NTCP of T30 in antisense orientation.

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