Expression of Different Genes to Confer Resistance to Pathogens in Transgenic Citrus Plants

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ABSTRACT. Novel approaches to control diseases and pests in citrus involving the use of transgenic plants with pathogenic-related (PR) genes and viral sequences are explored. Genetic transformation of sour orange, Mexican lime and Volkamer lemon with oryzacystatin I gene, chitinase/glucanase genes and viral sequences (untranslatable p27 and coat protein genes from Mexican isolates of *Citrus tristeza virus*), has been achieved, using *Agrobacterium tumefaciens* strains EHA 105 and LBA4404 and *A. rhizogenes* A4. Plasmids pCAMBIA 2301 and 1301 were used as transformation vectors. Regenerated shoots and roots representing independent transgenic events were assayed histochemically for GUS activity. The presence of transgenes was confirmed by PCR assays. Apical portions of GUS-positive regenerated shoots were shoot-tip grafted in *vitro* onto sour orange. Under greenhouse controlled conditions, shoots of 0.5-1.0 cm in length were grafted onto virus-free, 1-yr-old Volkamer lemon rootstocks. Nontransformed scions were also grafted as controls. Challenges with mild and severe CTV isolates will be done in plants containing oryzacystatin I gene and viral sequences. Plants expressing chitinase/glucanase genes will be challenged with severe strains of *Fusarium* spp. Recovery of plants showing different degrees of tolerance is expected and will be selected for further analysis.

Citrus trees are highly susceptible to the ravages of insects, pests and plant diseases. Three novel approaches involving the use of citrus transgenic plants to control citrus diseases are described in this article: a) usage of cysteine proteinase inhibitors such as cystatins which are strong, natural inhibitors of cysteine proteinase activity (4). Closteroviruses encode a polyprotein that is processed by proteolysis into individual polypeptides (2, 7), three proteinases have been shown to be involved in this processing mechanism, one of them is thought to be of cysteine type. One of the best characterized plant cystatins, identified from rice (*Oriza sativa* L.) seeds, is oryzacystatin I (4, 15). b) The second approach involves gene silencing mechanism. It has been reported that transgenes could become post-transcriptionally inactivated; resulting in co-suppression of homologous endogenous genes. RNA-mediated virus resistance is reminiscent of co-suppression; the viral RNA becomes a target for degradation (16). c) The third approach includes the expression of pathogenesis-related (PR) proteins, which are induced by plants in response to pathogen infection. For instance, ß-1,3-glucanase and chitinase proteins are part of a mechanism of defence against fungi and viruses (8). Expressing them alone or in combination can increase the resistance of the plant against fungal pathogens (6). Glucanase and chitinase can hydrolyze ß-1,3-glucans and chitin, respectively, which are major components of the cell walls of many pathogenic and potentially pathogenic fungi (3).

In this study, we report the genetic transformation of different C*itrus* varieties with oryzacystatin I, chitinase/glucanase and untranslatable *Citrus tristeza virus* (CTV) coat protein and p27 genes through *Agrobacterium*-mediated gene transfer.

MATERIALS AND METHODS

Seeds of sour orange, Mexican lime and Volkamer lemon were cultured as previously described (5, 9). Disarmed *Agrobacterium tumefaciens* strain EHA 105, LBA4404 and *A. rhizogenes* A4 were used in transformation protocols. Two plasmid vectors were used:

a) pCAMBIA 2301, containing a chimeric gene for neomycin phosphotransferase II (NPTII) for selection on medium containing kanamycin and the scorable marker *uidA* (the gene for β-glucoronidase, GUS) which allows simple histochemical visualization of transformation by the presence of a blue color after staining with the appropriate substrate. This plasmid was cloned with: 1) A cassette containing oryzacystatin I gene, CaMV 35S promoter and the rubisco terminator (rbcs3'). This plasmid (pCAMBIA2301-ORYZACYSTATIN I) was inserted into *A. tumefaciens* EHA 105 (Fig. 1). 2) A cassette containing oryzacystatin I gene, a phloem limited promoter (Rol-C) and rbcs3' terminator. This plasmid (pCAMBIA2301-ORYZACYSTATIN I–RolC) was inserted into *A. rhizogenes* A4 (Fig. 2). 3) A cassette containing chitinase and glucanase genes, CaMV 35S promoter and the rbcs3' terminator. This plasmid (pCAMBIA2301-CHITINASE/GLUCANASE) was inserted into *A. tumefaciens* LBA4404 (Fig. 3).

b) pCAMBIA 1301, containing a chimeric gene for hygromycin for selection on medium containing hygromycin B and the scorable marker *uidA*. This plasmid was (pCAMBIA1301-FMV-p27_CP) used to clone a cassette with the CTV major coat protein (CP) and p27 genes from a Mexican isolate from Veracruz, both in antisense orientation. The cassette for the viral genes contained FMV promoter and CaMV 35S terminator. This plasmid was inserted into *A. tumefaciens* EHA 105 (Fig. 4).

The transformation methodology has been previously described (5, 9, 12, 13). Shoots and roots that regenerated on selection media were assayed histochemically for GUS activity (9). The extent of the section that stained blue was recorded

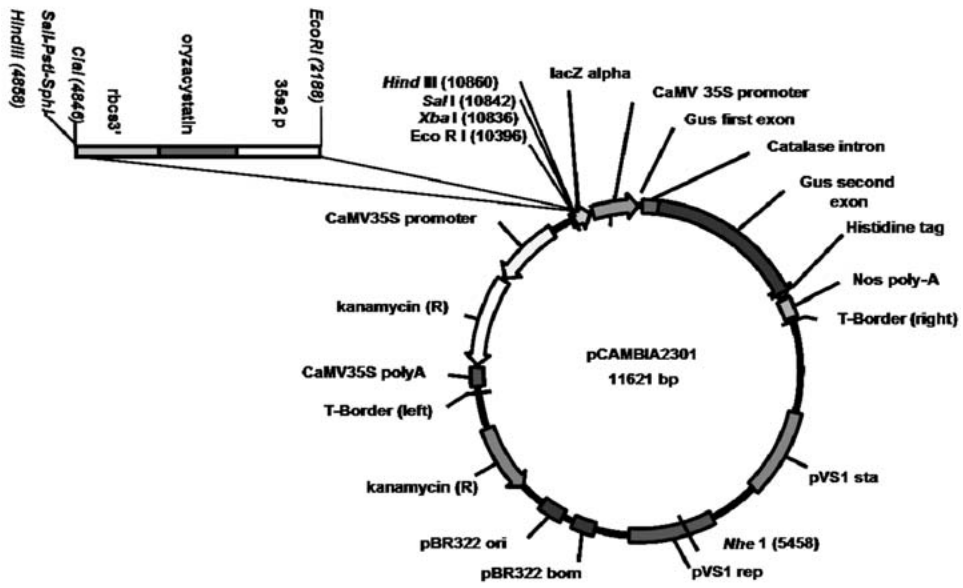

Fig. 1. Genetic map of pCAMBIA 2301-ORYZACYSTATIN I. Plasmid used in transformation experiments containing genes that codify for oryzacystatin I, neomycin phosphotransferase II and *uidA* proteins.

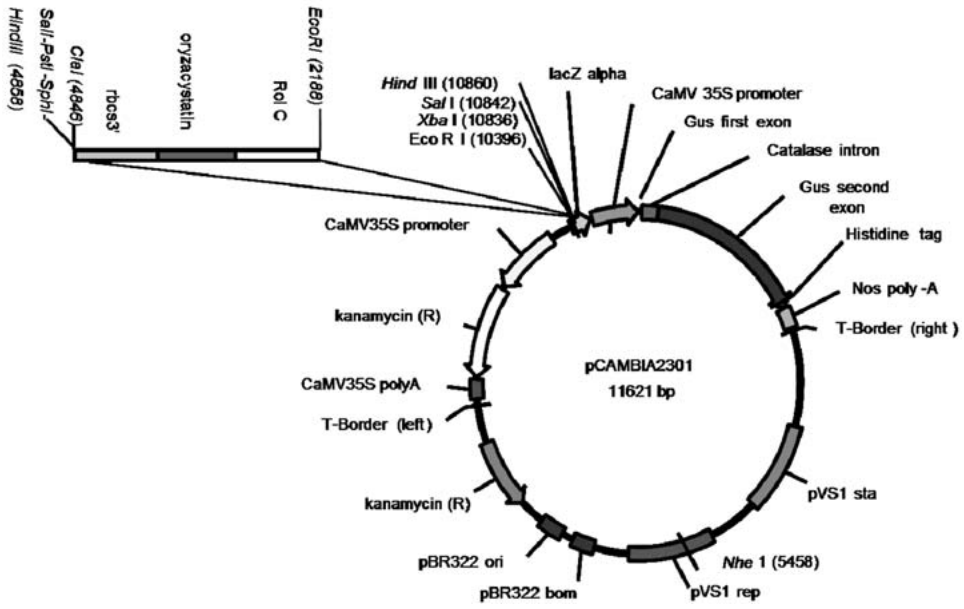

Fig. 2. Genetic map of pCAMBIA 2301-ORYZACYSTATIN I-RolC. Plasmid used in transformation experiments containing genes that codify for oryzacystatin I with a phloem limited promoter, neomycin phosphotransferase II and *uidA* proteins.

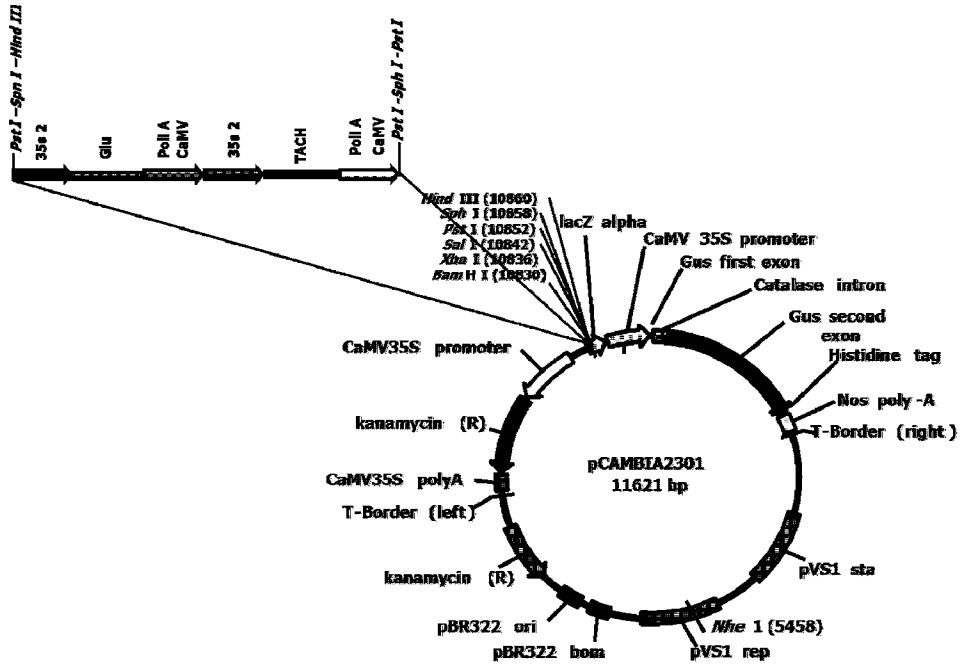

Fig. 3. Genetic map of pCAMBIA 2301-CHITINASE/GLUCANASE. Plasmid used in transformation experiments containing genes that codify for chitinase, glucanase, neomycin phosphotransferase II and *uidA* proteins.

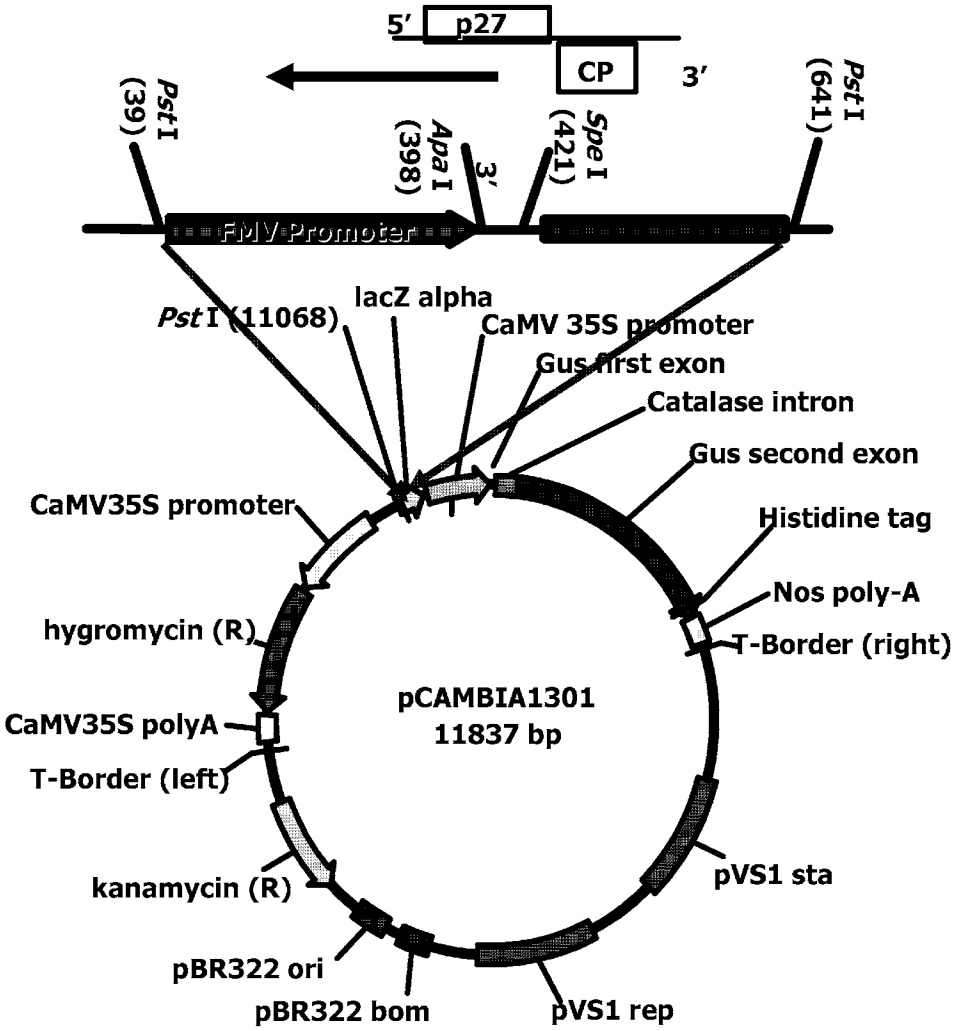

Fig. 4. Genetic map of pCAMBIA 1301-FMV-p27_CP. Plasmid used in transformation experiments containing genes that codify hygromycin B and the scorable marker *uidA* genes. This plasmid was cloned with a cassette with the CTV major coat protein (CP) and p27 genes in antisense orientation.

either as sector (for partial staining) or completely stained.

For PCR analysis, DNA was isolated from leaves using a CTAB extraction method (14). Approximately 5 ng of DNA were added to a 45 µl reaction using specific primers for each gene: primers for amplification of a 346 bp oryzacystatin I gene fragment were 5'-ATGTCGGAGCGACGGAG-3' and 5'-ACACATAGGATCGAGATGG-3', for amplification of a 1.6 Kb FMV-p27_CP gene fragment primers were 5'-GTCATATGAGCAGAGACGTGGC-3' and 5'-TGAAACTCCACCATCCCGATA-3'. Specific primers for amplification of an 800 bp glucanase gene were used (10, 11). Following each PCR, 20 µl of each reaction were loaded onto a 0.8% agarose gel and visualized with UV-ethidium bromide.

To recover transgenic plants, the GUS positive shoots and roots obtained were rooted or induced to produce adventitious shoots, respec-

tively. Apical portions of the transgenic shoots were tip grafted *in vitro* onto 4 weeks old *C. aurantium* seedlings. Shoots of 0.5-1.0 cm in length were grafted onto virus-free, one-year old *C. volkameriana* rootstocks and kept under controlled greenhouse conditions. Inhibition assays of a cysteine type enzyme (papain) will be performed in order to verify the activity of the oryzacystatin I protein. Western blot assays will be performed to plantlets that amplified the oryzacystatin I and chitinase/glucanase genes. Challenge with CTV will be done in the plants containing oryzacystatin I gene, chitinase/glucanase genes and the CTV viral sequences. Plants expressing the chitinase/glucanase will be challenge with *Fusarium* spp.

RESULTS AND DISCUSSION

We have achieved transformation of sour orange, Mexican lime and Volkamer mediated by *A. tumefaciens* and *A. rhizogenes* with the different sets of sequences.

Results of some experiments are shown in Table 1 and Figs. 5-7. Results (No. of shoots, time of appearance and phenotypic characteristics) are similar to those reported by (5, 9) for *A. tumefaciens* and (13) for *A. rhizogenes*.

The majority of the GUS + shoots showed chimeric staining ranging from a few blue dots to almost completely blue. Thirty-two plantlets (Fig. 5A) and 7 roots (Fig. 5B) amplified the expected 346 bp fragment from oryzacystatin I. One plantlet amplified the expected 800 bp fragment from chitinase/glucanase (Fig. 6). One plantlet amplified the expected 1600 bp fragment from P27-CP (Fig. 7). Although some of the initially regenerated shoots derived from chimeric tissue we found solid transformed portions of them, this allowed us to generate new plants that were completely transgenic. Micrografting (12) was a very efficient method for establishing the transgenic plants (data not shown). Transformation efficiencies were similar to those reported elsewhere (5, 9, 13). We expect that at least some of the recovered plants will be disease tolerant.

These PCR results partially demonstrate that we have successfully used *A. tumefaciens* and *A. rhizogenes* to obtain transgenic *Citrus* plants with the four different sets of sequences. To our knowledge production of transgenic plants of Volkamer lemon has not been previously reported.

TABLE 1
GUS POSITIVE SHOOTS OBTAINED WITH THE DIFFERENT TRANSFORMATION VECTORS

Cultivar	*Agrobacterium* strain	Vector	GUS positive shoots	
			Sector	Complete
C. *aurantium*	*A. tumefaciens* LBA4404	pCAMBIA2301-CHITINASE/GLUCANASE	12	0
C. *volkameriana*	*A. tumefaciens* EHA 105	pCAMBIA1301-FMV-p27_CP	15	0
C. *volkameriana*	*A. rhizogenes* A4	pCAMBIA2301-ORYZACYSTATIN I-RolC	0	7
C. *aurantifolia*	*A. tumefaciens* EHA 105	pCAMBIA2301-ORYZACYSTATIN I	7	0
C. *volkameriana*	*A. tumefaciens* EHA 105	pCAMBIA2301-ORYZACYSTATIN I	10	5
C. *aurantium*	*A. tumefaciens* EHA 105	pCAMBIA2301-ORYZACYSTATIN I	14	1

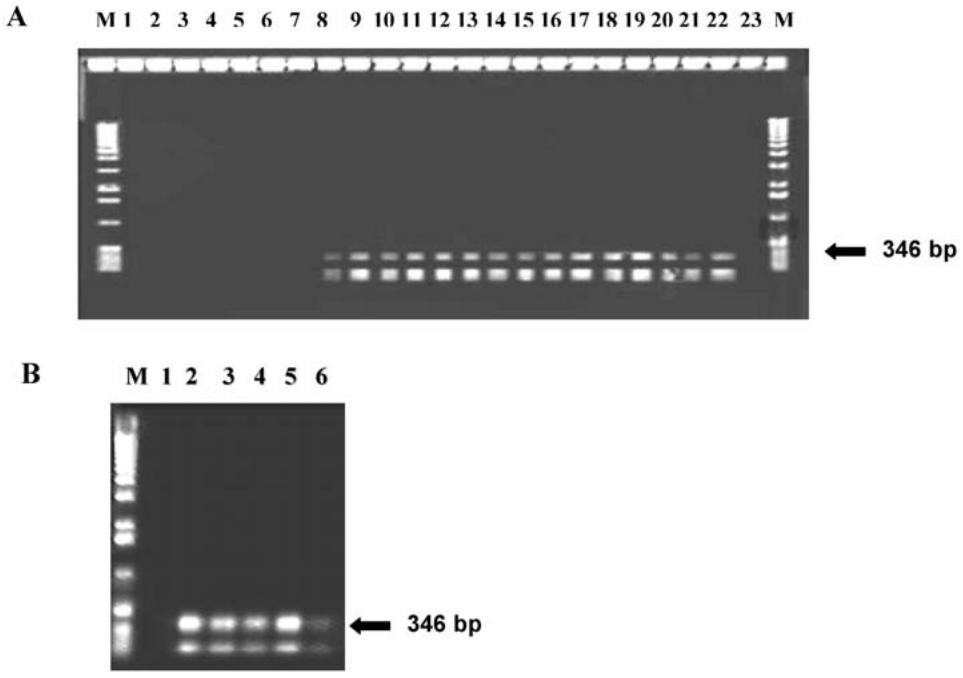

Fig. 5. PCR analysis of DNA from transgenic plantlets transformed with the oryzacystatin I gene. (A) Lanes: M, 1Kb marker DNA; 1, negative control; 2-7 DNA from non-transformed plantlets; 8-21, DNA from transgenic plantlets transformed with *A. tumefaciens*; 22, positive control DNA and 23, empty lane. Arrow to the right indicates 346 bp Oryzacystatin I amplicon. (B) Lane: M 1Kb marker; 1, negative control; 2-5, DNA from plantlets transformed with *A. rhizogenes*; 6, positive control DNA. Arrow to the right indicates 346 bp Oryzacystatin I amplicon.

The introduction of infection-specific factors into citrus plants offers a promising approach for the control of a wide range of infections. Cysteine

Fig. 6. PCR analysis of DNA from transgenic plantlets transformed with the chitinase/glucanase genes. Lanes: 1, negative control; 2-5 DNA from non-transformed plantlets; 6, DNA from *A. tumefaciens* transformed plantlet; 7, DNA from positive control and M, 1Kb marker. Arrow to the right indicates 800 bp chitinase/glucanase amplicon.

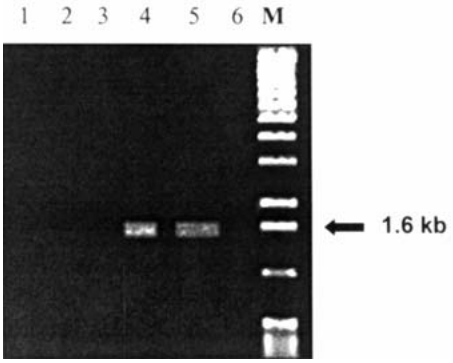

Fig. 7. PCR analysis of DNA from transgenic plantlets transformed with the CP and p27 genes from CTV. Lanes: 1, negative control; 2 and 3 non-transformed plantlets DNA; 4, DNA from transgenic plantlet; 5, DNA from positive control and 6, empty lane. Lane M is 1Kb marker DNA. The arrow to the right indicates the 1.6 kb p27-CP amplicon.

proteinase inhibitors represent an attractive option for a safe defense strategy against specific infection because they don't compromise the usage of pathogen-derived resistance. This is the first report of citrus (sour orange, Mexican lime and Volkamer lemon) transformed with the genes that code for protection against viral and fungal diseases.

ACKNOWLEDGMENTS

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