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Generation of Sweet Orange Transgenic Lines and Evaluation of *Citrus psorosis virus*-derived Resistance against Psorosis A and Psorosis B

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Abstract

Citrus psorosis is a widespread serious disease of citrus caused by *Citrus psorosis virus* (CPsV). In Argentina and Uruguay, this disease is spread by an unknown vector and there is no natural resistance or tolerance to the disease. There are two types of psorosis, described according to the symptoms observed in citrus trees, psorosis A (PsA) and psorosis B (PsB). PsA protects against the severe effects of the more aggressive type PsB. We have applied pathogen-derived resistance to create a defence mechanism against this virus disease. Sweet orange transgenic lines were obtained containing three different genes of CPsV (*54k, 48k* and *24k* genes) taken from a PsA isolate (CPV-4). Fourteen lines were selected containing 1, 2 or 3 copies of the transgenes and evaluated for their acquired resistance against PsA (CPV 4 from USA) and PsB (CPsV 189-34 from Argentina) isolates. These lines were susceptible to both isolates when graft-infected, although one of the lines carrying the *cp* gene (CP-96 line) containing two copies of the transgene and expressing a low level of the coat protein showed a delay in symptom expression when inoculated with the PsB isolate.

Introduction

Citrus psorosis, first described by Swingle and Webber (1896), is a serious viral disease affecting citrus trees in many countries (Roistacher 1993). The distribution of the disease is well documented and occurs in North and South America, Africa and the Mediterranean Basin. In Argentina, it causes important losses (Danós 1990), and the budwood certification programme has been insufficient to control the disease, probably because it is spread by an unknown vector (Beñatena and Portillo 1984). In the field, characteristic symptoms in sweet orange (*Citrus sinensis* (L.) Osb.), mandarin (*C. reticulata* Blanco) and grapefruit (*C. paradisi* Macf.) are bark scaling on the trunk and chlorotic flecks and spots in young leaves (Roistacher 1991, 1993). Trunk-scaling symptoms appear only on trees that are 10–15 years of age, when they are at maximum of the fruit production.

The cause of the disease is *Citrus psorosis virus* (CPsV), the type member of genus *Ophiovirus* (Garcia et al. 1994). The virus is tripartite and its genome consists of three ssRNAs of negative polarity. CPsV RNA 1 contains two open reading frames (ORFs), one coding for a 24-kDa polypeptide of unknown function (24K) and, separated by an intergenic region, the putative RNA polymerase (280 kDa) (Naum-Ongania et al. 2003). RNA 2 contains one ORF coding for a polypeptide of 54.7 kDa of unknown function (54K) (Sanchez de la Torre et al. 2002), and the RNA 3 codes for the coat protein (CP) of 48.6 kDa (48K protein) (Sánchez de la Torre et al. 1998).

Fawcett and Klotz (1938) described two types of psorosis disease called psorosis A (PsA) and psorosis B (PsB), both being graft-transmitted and infecting trees systemically. PsA induces bark scaling restricted to the main trunk and limbs and chlorotic flecks and spots in young leaves. It can also cause dieback and reduced yield. PsB is more aggressive showing extensive bark scaling, affecting thin branches and limbs, blotching in old leaves, with brownish gum-impregnated eruptions on the leaf underside and pustules (Roistacher 1991). Chlorotic flecks and spots on young leaves and a shock reaction with shoot necrosis in the first flush occur with some isolates. These are used as diagnostic symptoms for biological indexing of psorosis in experimentally graft-inoculated sweet orange

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seedlings (Roistacher 1991). Roistacher (1991) described a specific diagnosis test for PsA using biological indexing that consists in a cross-protection test using PsB as a challenge. Sweet orange seedlings are graft-inoculated with tissue of the candidate tree and later grafted with a source of PsB. The PsA strain can prevent the multiplication of the PsB strain and consequently the appearance of the severe PsB symptoms. The mechanism of cross-protection between the two types of psorosis is unknown.

CPsV infects many different species of citrus and its relatives like *Poncirus trifoliata* (L. Raf.), which is a current rootstock used in Argentina and Uruguay. Moreover, no natural resistance or tolerance to the disease has been found. Pathogen-derived resistance has been applied to many plants to create a defence mechanism against viral diseases providing an alternative control (Prins et al. 2008). For this purpose, we have previously obtained citrus transgenic lines expressing the coat protein gene and challenged them with a PsA isolate described as mild isolate (CPV 4) (Zanek et al. 2008), but the plants were not tolerant to CPsV. In the current study, we have produced sweet orange plants expressing two other CPsV genes (*54k* and *24k* genes) and have evaluated the acquired resistance of these transgenic lines against PsA and PsB isolates.

Materials and Methods

Virus isolates, plasmid constructs and bacterial strain

The isolates used were CPV-4, a PsA isolate from Florida (USA) (Garnsey and Timmer 1980), but probably of Texas origin (Garnsey et al. 1976), and the CPsV 189-34, a PsB isolate from the collection from the Experimental Station Concordia – INTA, Argentina.

The full-length ORFs of the 24K, 48K and 54K from CPV-4 isolate were cloned into the pGEM T vector, digested with *BamH*I for the 24K and 54K and *Nhe*I and *EcoR*I for 48K (Zanek et al. 2008). The protruding end formed was filled and inserted between the *Cauliflower mosaic virus* (CaMV) 35 S promoter, with double enhancer and the *Alfalfa mosaic virus* (AMV) RNA 4 leader sequence, and nopaline synthase gene (*nos*) terminator sequence of plasmid pMOG180 (Mogen International). Correct cloning and insert orientation was confirmed by sequencing. The resulting expression cassette was then subcloned into the binary vector p35Sgusint (Vancanneyt et al. 1990) or pBin19sgfp (Chiu et al. 1996) at the *Hind*III (24K) or *EcoR*I (48K and 54K) site, generating p35Sgusint/48K (Zanek et al. 2008), pBIN19*sgfp*-24K and p35Sgusint/54K (see Fig. 1, Panels a and d). These plasmids were transferred to *Agrobacterium tumefaciens* strain EHA 105 by electroporation.

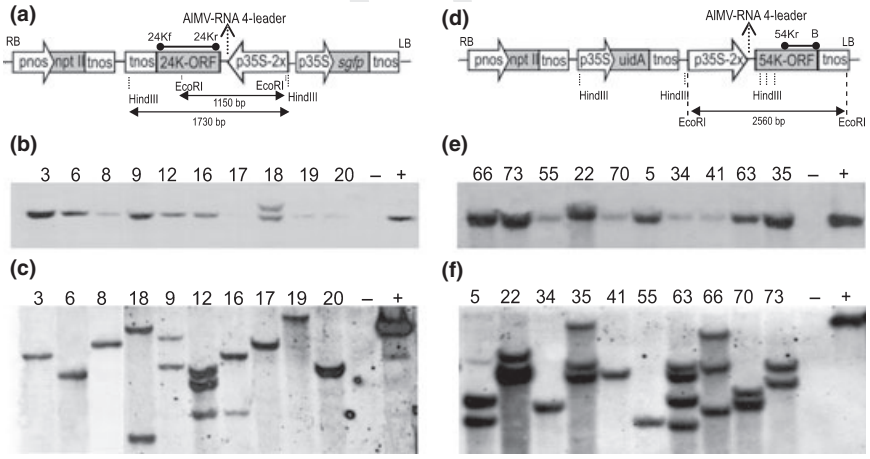


Fig. 1 (a) A schematic representation of the T-DNA from the binary plasmid pbin19*sgfp*/24K engineered to express the 24K gene from the CPsV strain CPV-4 in transgenic sweet orange plants. The gene is controlled by the CaMV 35S promoter (p35S) containing a duplicated enhancer region, the AMV RNA4 leader sequence (AlMV-RNA4-leader) and the nos terminator (tnos). The 24K coding region (ORF-24K) is flanked by the *nptII* gene between the nos promoter (pnos) and the tnos and by the *sgfp* gene between the p35S and the tnos. Transcription orientation of each gene is indicated by the arrows and EcoRI and HindIII restriction sites by vertical lines. The relative size of the 24K probe and the expected hybridization products is indicated. RB, right border; LB, left border. (b) Southern blot analysis of total DNA extracted from the transgenic lines 24K-3, 24K-6, 24K-8, 24K-9, 24K-12, 24K-16, 24K-17, 24K-18, 24K-19 and 24K-20 digested with HindIII, which releases the complete 24K gene. (c) Southern blot analysis of total DNA extracted from the same lines shown in Panel b digested with EcoRI, which shows the copy number of the transgene integrated. The band of 1150 bp is not shown. (d) A schematic representation of the T-DNA from the binary plasmid p35SGUSINT/54K engineered to express the 54K gene from the CPsV strain CPV-4 in transgenic sweet orange plants. The gene is controlled by the CaMV 35S promoter (35SP) containing a duplicated enhancer region, the AMV RNA4 leader sequence (AlMV-RNA4-leader) and the nos terminator (nosT). The 54K coding region (ORF-54K) is flanked by the nptII gene between the nos promoter (nosP) and the nosT and by the *uidA* gene (GUS) between the 35SP and the nosT. Transcription orientation of each gene is indicated by the arrows and the EcoRI and HindIII restriction sites by vertical lines. The relative size of the 54K probe and the expected hybridization products is indicated. RB, right border; LB, left border. (e) Southern blot analysis of total DNA extracted from the transgenic lines 54K-5, 54K-22, 54K-34, 54K-35, 54K-41, 54K-55, 54K-63, 54K-66, 54K-70 and 54K-73 digested with EcoRI, which releases the complete 54K gene. (f) Southern blot analysis of total DNA extracted from the same lines shown in Panel e digested with HindIII, which shows the copy number of the transgene integrated. The nylon membranes were probed with a digoxigenin-labelled fragment of the *24K or 54K* coding region, indicated as 24Kf-24Kr probe (24k dir/24k rev primers) and 54Kr/B probe (54krev and B primers)

Table 1
Primers used for PCR amplification to determine the presence of the transgenes

Gene	Primers	Sequence	Position
24K	24kdir	5′-CGGGATCCATGGCTGAATATATAG-3′	7529-7550
	24krev	5′-CGGGATCCAAGATTTTAATTTCAC-3′	8149-8128
CP	CPV1[a]	5′-GCTTCCTGGAAAAGCTGATG-3′	713-733
	CPV2[a]	5′-TCTGTTTTTGTCAACACACTC-3′	1312-1290
54K	A	5′-TAGATCCATGCTCAGTCACC-3′	950-968
	B	5′-GGTCACAGGTAGTAACAGTT-3′	1421-1401
	54krev	5-CCGGGATCCGARATCAYTCWATTTTG-3	
GUS	1GUS	5′-GGTGGGAAAGCGCGTTACAAG-3′	
	2GUS	5′-TGGATTCCGGCATAGTTAAA-3′	
GFP	1sGFP	5′-ATGGTGAGCAAGGGCGAGGA-3′	
	2sGFP	5′-GGACCATGTGATCGCGCTTC-3′	

[a]Barthe et al. 1998.

Transformation and regeneration

Transformation and regeneration was carried out as previously described (Peña et al. 1995, 2004; Zanek et al. 2008). Internodal stem segments from greenhouse-grown Pineapple sweet orange, 6–12 months old, were transformed by cocultivation with *A. tumefaciens* EHA 105 carrying the binary vectors pBIN19*sgfp*-24K, p35Sgusint/48K or p35Sgusint/54K. Selection of transformants was made on a culture medium containing kanamycin (100 μg/ml). The basal part of the shoots regenerated from the stem segments transformed with the vectors p35Sgusint/48K and p35Sgusint/54K was assayed for *uidA* gene expression (GUS) by overnight incubation in a 2-mM X-Gluc solution at 37°C (Jefferson et al. 1987). The shoots transformed with the vectors pBIN19*sgfp*-24K and pBin19sGFP/48K were examined under a stereomicroscope equipped with a Leica® fluorescence module. The positive shoot tips regenerated were grafted *in vitro* on decapitated and etiolated seedlings of Troyer citrange (*P. trifoliata* × *C. sinensis* (L. Osb.)). After 3–4 weeks, scions that had developed several leaves were screened for the presence of the 24K, 48K (CP) and 54K transgenes by PCR using primers designed from the viral genes and the *uidA* and *gfp* genes (see Table 1). A regenerated shoot without agro-infection was obtained and used as a regenerated non-transgenic control. The positive transgenic plantlets were then side-grafted onto vigorous 6-month-old seedlings of rough lemon (*Citrus jambhiri* Lush) and grown in a temperature-controlled greenhouse.

Polymerase chain reaction and Southern blot analyses

The presence of the transgenes was confirmed by PCR using specific primers (Table 1). For this purpose, total DNA was extracted from 20 mg of plantlet leaves according to McGarvey and Kaper (1991). PCR amplifications were carried out in 10 μl containing 1 μl of a 1 : 10 dilution of the DNA solution, 1 mM dNTPs, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl, pH 9.0, 0.1% Triton X-100, 10 μM of each primer and 2 units of Taq DNA polymerase (Promega). DNA was subjected to 35 cycles of 30 s at 94°C, 50 s at 48°C for CP gen, 50°C for 24 and 54K or 60°C for *gus* and *gfp* genes and 50 s at 72°C with a final extension step at 72°C for 3 min (Perkin-Elmer 2400 equipment). Amplified DNA fragments were electrophoresed on 2% agarose gels.

Southern blot assays were performed to analyse the integrity of the 24K and 54K genes and to estimate the number of copies that had been integrated in the plant genome. The 48K gene had been determined previously (Zanek et al. 2008). For that, DNA was extracted from 0.75 g of leaves according to Futterer et al. (1995), and aliquots of 20 μg were digested with *HindIII* or *EcoRI* to release the expression cassette and to determine the transgene copy number, respectively, for the 24K lines and with *EcoRI* or *HindIII*, respectively, for the 54K-lines. The digestion products were fractionated on 0.8% agarose gel and then blotted onto a positively charged nylon membrane (ROCHE) by capillarity using 20× SSC buffer. After fixation by baking at 120°C, membranes were hybridized with a digoxigenin-labelled fragment of the 24K gene (24dir-24rev) and 54K (B-54krev) using PCR according to the manufacturer's instructions (Boehringer-Mannheim). Hybridizations were performed in 5× SSC, 2× Denhardt, 0.1% SDS, 300 μg/ml denatured herring sperm DNA (Invitrogen) and 3 mg/ml denatured yeast tRNA (Sigma) overnight at 65°C. After three washing steps in 2× SSC, 0.2× SSC and 0.1× SSC plus 0.1% SDS at 65°C, bands were detected by a chemiluminescent substrate and exposed to X-ray film.

Analyses of the expression of the 54K and 24K transgenes

Due to the fact that mRNAs generated from the transgenes 54K and 24K were not detected by northern blot, we applied RT-PCR amplifying the complete ORFs from DNAse-treated RNA. Total RNA was extracted from 100 mg of fresh citrus tissue with TRIZOL (Life Technologies), following the manufacturer's instructions, and resuspended in 50 μl of RNase-free water. An aliquot of 8 μl of RNA suspension was incubated for 15 min at room temperature with 1 U of DNase I and 1 μl of buffer (200 mM Tris–HCl pH 8.4, 20 mM $MgCl_2$, 500 mM KCl). The reaction was stopped by adding 1 μl of 25 mM EDTA at 65°C for 10 min.

One-step RT-PCR was conducted in a 15-μl reaction mixture containing 1 mM dNTPs, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl pH 9.0, 0.1% Triton

X-100, 1μM of each primer, 16 units RNAsin® Ribonuclease Inhibitor (Promega), 12 units Super-
6 Script™III Reverse Transcriptase (Gibco BRL) and 1 unit of *Taq* DNA polymerase (Promega). The thermocycling conditions were as follows: 30 min at 48°C for RT, 4 min at 94°C for inactivation of the reverse transcriptase and initial denaturation, 36 cycles of 10 s at 94°C, 10 s at 50°C and 50 s at 72°C and a final elongation step of 4 min at 72°C in Perkin-Elmer 2400 equipment. PCR was conducted as negative control starting from the same samples of DNAse-treated RNA. PCR products were electrophoresed on 2% agarose gels and then blotted onto nylon membranes for Southern blot analysis as mentioned earlier.

Propagation and graft inoculation

Transgenic lines were propagated by bud grafting onto 6–8-month-old rough lemon seedlings as a vigorous rootstocks, ensuring fast growth of the transgenic scion. After 10 months, replicates of each line were graft-inoculated using pieces of infected bark tissue (bark pieces) carrying CPV-4 from which the transgenes were generated or the CPsV 189-34 isolate. Five to seven replicates of the transgenic lines were graft-inoculated with two bark pieces that remained in the plants throughout the challenge period. Two replicates of each line were not inoculated as negative controls. The plants were maintained in a light-controlled greenhouse at 22–24°C, 16-h photoperiod, and observed for the development of symptoms. After each observation, leaf tissues were collected and the presence of CPsV was determined. Controls for these experiments were a non-transformed and a regenerated non-transgenic Pineapple sweet orange (NT).

Virus detection

The progress of the infection in the challenged plants was evaluated by RT-PCR and Triple-Antibody Sandwich Immunoassay (TAS-ELISA) as described previously (Zanek et al. 2006). RT-PCR using the primers A and B, designed from CPsV RNA 2, was used to test the viral infection in the 48K (RNA 3) transgenic lines. For 24K and 54K lines, primers CPV1 and CPV2 (Barthe et al. 1998) from the RNA 3 were used to detect the virus. Total RNA was extracted from 100 mg of fresh tissue with TRIZOL, and one-step RT-PCR was conducted as described elsewhere. The thermocycling conditions were applied as conducted for transgene detection. PCR products were electrophoresed on 2% agarose gels. Both types of psorosis can be detected by the same techniques used for disease diagnosis, TAS-ELISA and RT-PCR (Alioto et al. 1999; Martin et al. 2006; Zanek et al. 2006).

Results

Sweet orange transformation and molecular analysis

Twenty-one transgenic plants carrying the viral genes were obtained by *A. tumefaciens*-mediated transformation with the plasmids p35Sgusint/48K or pBin19*sgfp*/48K, 15 lines with pBIN19*sgfp*-24K and 15 lines with the plasmid p35Sgusint/54K. Transformation efficiency (transgenic lines per inoculated explant) was variable, from 3.0 to 4.5%, as previously determined (Zanek et al. 2008). The phenotype of the lines was indistinguishable from the non-transformed Pineapple sweet orange plants. We analysed the integrity of the transgenes, the number of copies inserted in each of 10 transgenic 24K lines and 10 of the 54K lines and the mRNA expression. In Fig. 1 Panel b, a fragment of 1.73 kb confirmed the integrity of the *24k* transgene in all lines as determined by Southern blot. In line 18, the largest fragment observed is probably the result of rearrangements in the genome. Most of the transformed 24K lines, 24K-3, 24K-6, 24K-8, 24K-17, 24K-19 and 24K-20, had integrated one copy of the transgene, lines 24K-9, 24K-16 and 24K-18 two copies and line 24K-12 three copies (see Fig. 1, Panel c). The integrity of the transgene of the 54K lines is shown in Fig. 1, panel e. A fragment of 2.56 kb is present in all lines. The number of copies of the transgene was one in the lines 54K-34, 54K-41 and 54K-55, two in lines 54K-5, 54K-22, 54K-70 and 54K-73, three in lines 54K-35 and 54K-66 and four in the line 54K-63 (see Fig. 1, Panel f).

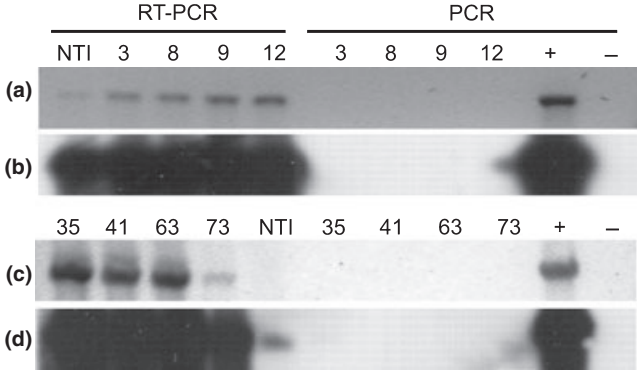
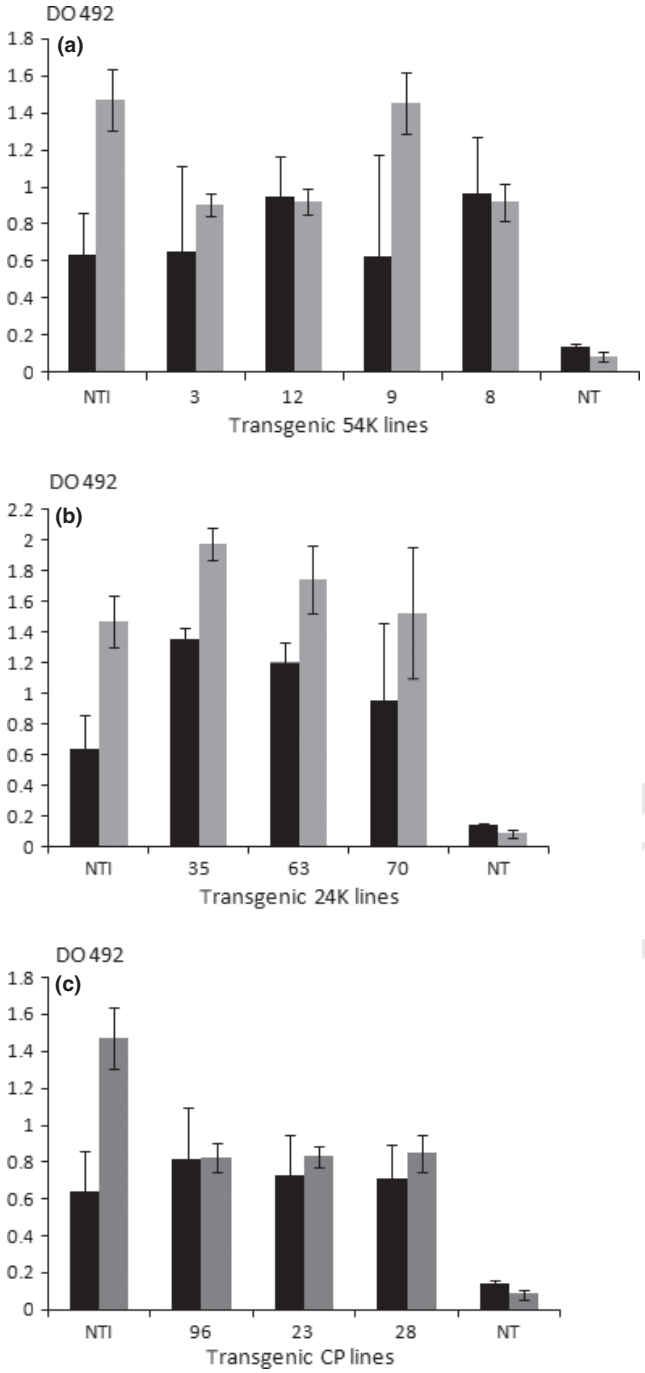


Fig. 2 Analysis of the RT-PCR and PCR products from total RNA extracted from lines 24K-3, 24K-8 24K-9 and 24K-12 (Panels a y b) and lines 54K-35, 54K-41, 54K-63 and 54K-70 (Panels c y d). Panel a and c show the results of electrophoresis and panels b and d show the Southern blot analysis from the same electrophoresis gel. Lines 2–5: RT-PCR amplification of DNAse-treated RNA extracted from transgenic plants. Lines 6–9: PCR amplification of the same DNAse-treated RNA samples. NTI: refers to RNA extracted from infected citrus. Positive (+) and negative (–) controls of the PCR amplification are indicated. The nylon membranes were probed with a digoxigenin-labelled fragment of the *24K or 54K* coding region (24K dir/24K rev probe and B/54Krev probe)

To determine the levels of the transgene expression, northern blots were performed for the CP lines (Zanek et al. 2008). Lines 24K and 54K were also analysed by northern blot, but the mRNAs were not detected. Thus, we conducted RT-PCR assays in total RNA samples previously treated with DNAse to determine the integrity of the mRNAs derived from the transgenes (Fig. 2 panels a and c), and the products were confirmed by Southern blot (Fig. 2 panels b and d). These results indicate that all tested 24K and 54K transgenic lines expressed the complete mRNA from the transgenes.



13 Fig. 3 Evaluation of transgenic lines challenged with psorosis A. The OD_{492} values refer to mean optical densities for six replicates infected with CPV-4 isolate for 24K-3, 24K-8, 24K-9 and 24K-12 (Panel a); 54K-35, 54K-63 and 54K-70 (Panel b); and CP-23, CP-28 and CP-96 (Panel c) transgenic lines in two successive flushes. *Black bars* first flush; *grey bars* second flush. NTI refers to the mean OD for the infected control. Non-transformed control refers to the mean OD for the non-infected control. Standard deviations are indicated

Challenge of the transgenic lines with a PsA isolate

To gain an insight into the response of the transgenic plants against viral infection, lines carrying the three selected genes of CPsV were challenged by graft inoculation with CPV-4, a PsA isolate homologous to the transgenes inserted in the plant genome. For the assay, four 24K lines 24K-3, 24K-8, 24K-9 and 24K-12 and three 54K lines 54K-35, 54K-63 and 54K-70 lines were inoculated. Three lines carrying the *cp* gene, CP-23, CP-28 and CP-96, had been evaluated previously (Zanek et al. 2008) and tested in parallel with lines 24K and 54K. The symptoms (rings, spots and flecking) were evaluated on three to four successive flushes for 4 months. After each observation, the presence of viral RNAs was determined by RT-PCR and TAS-ELISA confirming its accumulation in all assayed lines during 2 months of analysis (Fig. 3). All challenged lines had high OD values during the different determinations, similar to those of infected controls. Symptom expression was similar to the infected NT controls expected for CPV-4-infected seedlings. Table 2 summarizes the results of the RT-PCR, TAS-ELISA and symptom expression of all tested lines. Percentages of infection were from 80 to 100% similar to positive controls (89%). In addition, we did not observe any delay in the appearance of PsA symptoms in the transgenic lines.

Table 2
Evaluation of the resistance to the psorosis A (PsA) isolate (CPV-4) provided by the 24K, CP and 54K transgenic sweet orange lines

Transgene	Transgene copy number	Line	Plants infected with CPV4[a]	Plants infected with CPV4[a]
24K	1	3	100% (6)	100% (6)
	3	12	83% (6)	100% (6)
	2	9	100% (6)	100% (6)
	1	8	100% (6)	100% (6)
54K	3	35	80% (5)	100% (5)
	4	63	100% (6)	100% (6)
	2	70	100% (5)	100% (5)
CP	3	23	100% (6)	100% (6)
	1	28	100% (5)	100% (6)
NT	–	–	89% (9)	100% (9)

[a]Percentage of plants infected with CPV 4 analysed by Triple-Antibody Sandwich-ELISA, RT-PCR and expression of PsA symptoms; In brackets is indicated the total number of inoculated plants; NT, non-transformed control.

Challenge of the transgenic lines with a PsB isolate

Although transgenic plants did not protect against the PsA isolate, the ability of these lines to protect against PsB isolate CPsV 189-34 or at least to reduce symptom severity was assessed, considering that PsB is the most aggressive type of psorosis. In a preliminary experiment, two replicates of all lines previously challenged with CPV-4 (PsA isolate) were tested for resistance against the CPsV 189-34 isolate. The plants showed symptoms in the first two flushes (data not shown). A second experiment was performed with greater number of replicates (five to nine) of the lines CP-23, CP-34 and CP-96, 54K-41 and 54K-55, 24K-6, 24K-8 and 24K-9 using the same PsB isolate, CPsV 189-34.

The PsB symptoms evaluated were chlorotic mottling and gummy pustules on old leaves and branches. PsB symptoms usually take longer to be manifested than those of PsA isolates (leaf symptoms of shock, flecking and spots), and for that reason, the plants

Table 3
Evaluation of the resistance to the psorosis B (PsB) isolate (CPsV 189-34) provided by the 24K, CP and 54K transgenic sweet orange lines

Transgene	Line	Transgene copy number	Plants infected with PsB (1st flush[a])	Plants infected with PsB (2nd flush[a])
24K	6	1	67% (9)	89% (9)
	8	1	89% (9)	89% (9)
	9	2	71% (7)	71% (7)
54K	55	1	67% (9)	78% (9)
	41	1	60% (5)	60% (5)
	23	3	56% (9)	100% (9)
CP	96	2	33% (9)	78% (9)
	34	1	71% (8)	75% (8)
NT	–	–	80% (5)	80% (5)

[a]Percentage of plants infected with CPsV 189-34 analysed by Triple-Antibody Sandwich-ELISA, RT-PCR and expression of PsB symptoms in the first and second flushes; The total number of inoculated plants is indicated in brackets; NT, non-transformed control.

were observed for the severe symptoms for several months. Three months after inoculation, PsB symptoms were observed principally in young branches and, in some cases, pustules were evident on the underside of the mature leaves. Virus accumulation was tested in successive flushes, giving positive results in a high percentage of the plants transformed with the three constructs in the first flush (Table 3). Line CP-96 gave the lowest percentage of infection: only three of nine plants were infected.

In the second flush (tested 5 months after inoculation), the number of plants showing PsB symptoms and positive ELISA was very high. Many of the replicates died at 6 months, and in some others, the symptoms were observed for up to 1 year. Line CP-96 had also showed an increased number of infected replicates in this second flush.

Discussion

Not all genes from a pathogen are adequate when a pathogen-derived resistance strategy is applied (Valkonen et al. 2002), and therefore, we have generated transgenic citrus containing three genes of CPsV. We selected lines based on the number of copies and expression of the transgenes to assess the acquired resistance mechanism (PTGS or protein mediated). Line CP-23, which has three copies of the transgene, but did not express detectable levels of the CP protein, did not show resistance against the CPV 4 isolate, nor did the other CP lines containing one or two copies and expressing higher levels of the CP (Zanek et al. 2008). Lines carrying the genes 24K or 54K were selected to contain one, two and three copies of the transgenes, but they were also susceptible to both isolates. Therefore, no correlation was found between the selected viral genes and the number of copies inserted and the acquired resistance in the selected lines. Other examples in perennial species as plum and also citrus revealed variability in the resistance response (Scorza et al. 2001; Fagoaga et al. 2006; Febres et al. 2007) going from high levels of resistance to complete susceptibility, as the case of transgenic *C. macrophila* against *Citrus tristeza virus* (Batuman et al., 2006). We suspected that the level of expression of 24K and 54K proteins in the transgenic lines is very low, as well as the level of coat protein detected in the CP lines (Zanek et al. 2008). In addition, the PTGS mechanism has probably neither effectively expressed in the challenged lines. All these observations could explain the susceptibility of the lines against CPsV.

Cross-protection between PsA and PsB isolates was described much earlier (Fawcett and Klotz 1938), although neither the mechanism nor the viral genes involved are known. To assess this situation, we have tested transgenic lines carrying each viral gene taken from a PsA isolate with a PsB isolate. Sequences derived from the PsB isolate of this study (CPsV 189-34) exhibit around 80% sequence identity to regions of the PsA (CPV-4) genome. We were also concerned about the dependence of resistance induction on sequence identity between the transgene and the isolate used for challenge. Senthil-Kumar et al. (2007) reported that, in certain cases, a high level of resistance could be achieved by means of gene sequences having $<100\%$ identity between the inducer and the target sequence. Our previous observations (Martin et al. 2006) showed that CPsV sequences are highly conserved among isolates from different geographic regions of the world. All this information indicated that it would be likely to find resistance against other psorosis isolates. However, the fact that we found similar responses of the transgenic lines when infected with the homologous isolate (CPV-4) or with the non-homologous PsB isolate makes it unlikely that the limited resistance observed in these lines can be attributed to insufficient sequence identity between isolates.

Symptom attenuation in some lines could be expected, leading to PsA symptom expression when infected with PsB isolate, but all the lines showed at least some of the characteristic PsB symptoms. The dose of virus delivered to plants by graft inoculation was probably very high and could have overcome the potential protection of the lines assayed in the greenhouse.

The rate of virus multiplication of each isolate in the plant can be variable. PsB isolates take longer to manifest symptoms than PsA isolates, which could be the reason why the percentages of infected plants with CPsV 189-34 through different flushes were slightly lower than those of the infected with CPV-4. Line CP-96, harbouring two copies of the transgene and expressing a low level of CP, showed delayed symptom expression when inoculated with PsB isolate, but it was overcome by the virus multiplication.

We think that the viral inoculum dose used in all these experiments was high, because plants were graft-inoculated, which is unlike how CPsV is transmitted in the field. We think, therefore, that these transgenic lines should be assayed in the field to make conclusions about their resistance against psorosis.

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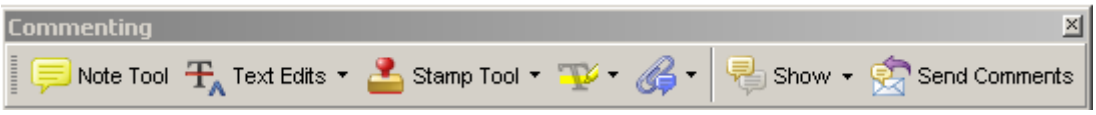

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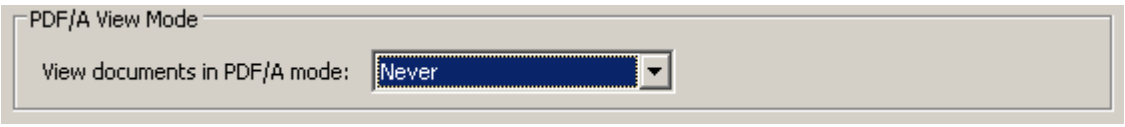

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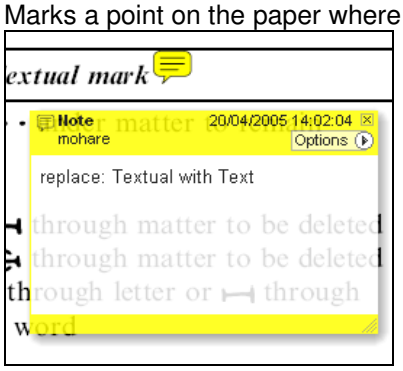

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1. Right click into area of either inserted text or relevance to note
2. Select Add Note and a yellow speech bubble symbol and text box will appear
3. Type comment into the text box
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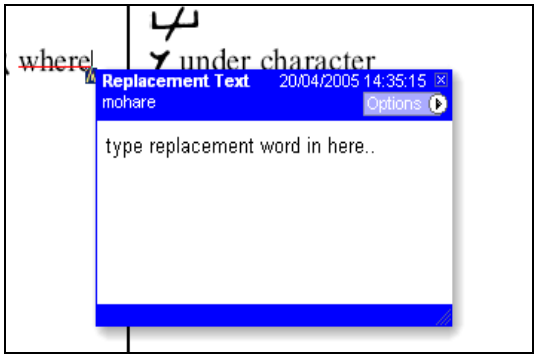

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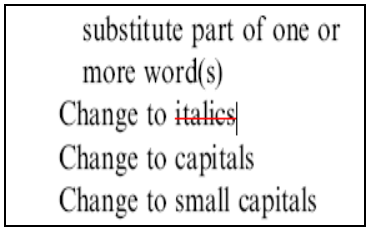

How to use it:

1. Select cursor from toolbar
2. Highlight word or sentence
3. Right click
4. Select Cross Out Text

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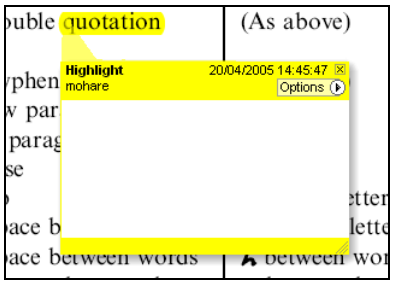

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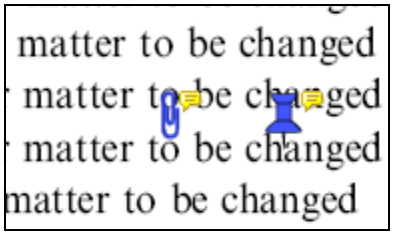

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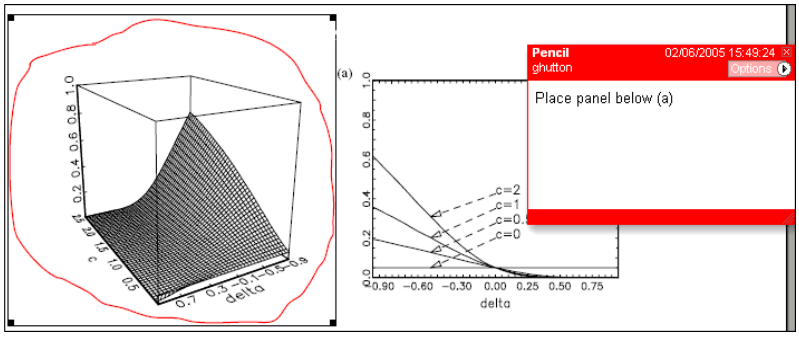

How to use it:

1. Select Tools > Drawing Markups > Pencil Tool
2. Draw with the cursor
3. Multiple pieces of pencil annotation can be grouped together
4. Once finished, move the cursor over the shape until an arrowhead appears and right click
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