TRANSGENIC PLANTS OF CC 84-75 RESISTANT TO THE VIRUS ASSOCIATED WITH THE SUGARCANE YELLOW LEAF DISEASE

By

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Abstract

The virus associated with the yellow leaf disease of sugarcane (ScYLV) was detected in Colombia in 1998. Since then it has been found in several commercial hybrids, especially in CC 84-75 which occupies second place in cultivated area in the Colombian sugar industry. Due to the susceptibility of most commercial hybrids and to the importance of CC 84-75, the possibility of producing transgenic plants with resistance to the virus was studied. Embryogenic callus of hybrid CC 84-75 was bombarded with plasmids pFM395 and pFM396 containing a ScYLV coat protein DNA fragment. After geneticin selection, 69 regenerates were obtained. Fifty-seven plants were positive for NPT II by PCR amplification. Forty-six plants out of 69 regenerates contained the coat protein coding fragment as confirmed by both PCR and Southern blot assays. Digestions with Bam HI, cutting only once within transgene, indicated the presence of six independent transformation events. Transformed plants were inoculated with ScYLV by Melanaphis sacchari and tested for infection at monthly intervals, using TBIA and RT-PCR, for 10 months after challenge. Thirty-seven plants out of 66 tested negative for ScYLV. A field test of resistant lines versus the original variety is underway.

Introduction

Yellow leaf disease (ScYLD) is a relatively new disease of sugarcane. ScYLD was first observed in 1968 and has subsequently been reported from a wide range of sugarcane growing countries throughout the world. Two etiological agents, sugarcane leaf yellows phytoplasma (ScLYP) and sugarcane yellow leaf virus (ScYLV), have been found to be associated with the disease. ScLYP predominates in Africa, while ScYLV appears to be the principal causal agent in the rest of the world. Losses as high as 50% have been estimated to occur in commercial fields as the result of virus induced YLD (Vega et al., 1997). This virus was first detected in Colombia in 1998. ScYLV has been found in several commercial hybrids grown in Colombia, especially CC 84-75, which is the second most widely grown in the country. In CC 84-75, losses as high as 0.21 tonnes of sugar per hectare have been calculated, when 1% of stalks are infected with the virus.

The use of viral coat protein transgenes for producing virus resistant plants is one of the most spectacular successes achieved in plant biotechnology. Since Powell-Abel et al., (1986) first reported CP mediated transgenic resistance against tobacco mosaic virus, numerous crops have been transformed to express viral CP and have been reported to show high levels of resistance in comparison with non-transformed plants (Dasgupta et al., 2003). Recently, ScYLV coat protein sequence-derived plasmid constructs have been reported (Moonan and Mirkov, 1999). Two of these constructs contain a ScYLV coat protein DNA fragment in sense (pFM395) and in antisense (pFM396) orientation under the control of the ubi-1 promoter. The goal of this work was to transform commercial hybrid CC 84-75 with the above constructs and to evaluate resistance to ScYLV in the transgenics obtained.

Materials and methods

Target tissues and culture conditions

Embryogenic calli were established from young leaf and meristematic tissue on MS medium (Murashige and Skoog, 1962) supplemented with 15% coconut water, 30 g/L sucrose, 3 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) (MSI) solidified with 2 g/L phytogel, after adjusting the pH to 5.7. Explants were
cultured in the dark at 28°C for 5 weeks. After this period, calli were subcultured and transferred to fresh medium under the same conditions. Embryogenic calli were selected and then sub-cultured for another 25 days. Initiation, selective subculture and proliferation of embryogenic callus before bombardment typically required 8 weeks. Segments of embryogenic calli of 4–8 mm diameter were placed on the same culture medium supplemented with 0.4 M mannitol and 0.4 M sorbitol as an osmotic treatment before bombardment (Vain et al., 1993).

**Plasmids**

Plasmids pFM395, pFM396 and Ubi-Km were provided by Dr E. Mirkov at Texas A&M Agricultural Experiment Station, Weslaco, TX, USA. Plasmid pFM395 contains an untranslatable ScYLV coat protein DNA fragment in the sense orientation and under the control of the maize ubiquitin (ubi-1) promotor, first exon and first intron followed by the nopaline synthase terminator (Figure 1).

Plasmid pFM396 contains the same untranslatable DNA fragment in the antisense orientation in relation to the ubiquitin promotor. Plasmid Ubi-km containing the NPT II gene (kanamycin resistance gene) was used to select calli resistant to the antibiotic.

![Restriction map of plasmid pFM395.](image)

**Bombardment**

Sense and antisense plasmids were bombarded together in order to get a higher frequency of resistant plants as suggested by Waterhouse et al., (1998). The plasmids were co-precipitated into gold particles. Gold particles in a suspension of 50% glycerol were mixed with 5 μL each plasmid DNA (1 μg/μL), 25 μL of CaCl₂ (2.5 M) and 10 μL of spermidine (0.1 M, free base form) in a microfuge tube at 4°C. The mixture was incubated for 15 min on ice and centrifuged briefly. After removing 50 μL of the supernatant, the gold pellet was resuspended in the remaining solution.

The plant expression vectors were introduced into calli of the sugarcane varieties using the biolistic particle delivery system (Bio-Rad). Particle bombardment was performed under vacuum in a BioRad PDS/1000 apparatus using a helium pressure of 1300 psi and approximately 2 μg of each plasmid DNA for each bombardment.

Embryogenic calli, arranged in a 2.5 cm-diameter circular area at the centre of a 9 cm diameter petri dish, were bombarded two times from a 6-cm distance. Optimisation of bombardment conditions was obtained in preliminary assays (data not shown) using the β-glucuronidase gene.
Selection conditions
After bombardment and incubation on medium containing osmotic solution, calli were transferred to MSI medium without antibiotic for 7–10 days for recovery. Calli were then put onto MSI plus 30 mg/L geneticin (Invitrogen) for four weeks. Calli that survived were transferred onto MSI plus 50 mg/L geneticin for five to ten weeks. Optimal antibiotic concentration for calli growth, and inhibition of plant regeneration from non-transformed material was determined previously (data not shown). Transformed plants were regenerated at 28°C by transferring calli to medium without 2,4-D under a 12 hours dark/light regime and were rooted under the same conditions. Selected plants were transferred to soil in plastic cups and moved to the greenhouse.

Polymerase chain reaction
Sixty-nine putatively transformed plants were subjected to PCR analysis. Sugarcane genomic DNA was isolated from leaf tissue according to Gilbertson et al., (1991). Integration of the transferred genes into the sugarcane genome was analysed by the PCR method using specific primers for the NPT II gene and specific primers for ScYLV coat protein DNA fragment included in plasmids pFM395 and pFM396.

Southern analysis
The 487-bp Pst I fragment from plasmid pFM395 was used to hybridise with genomic DNA extracted from leaf material of transformed and non-transformed control plants (Gilbertson et al., 1991). Total plant genomic DNA (20 μg per sample) was digested with Pst I and separated by electrophoresis on a 0.8% agarose gel and transferred to a nylon membrane Hybond N+ (Amersham International). Filters were hybridised with the Pst I fragment labelled with 32P-dATP using the random priming method described by Feinberg and Vogelstein (1983). Unincorporated nucleotides were removed with a Sephadex G-50 column. After hybridisation at 65°C overnight, the membranes were washed three times with different concentrations of saline sodium citrate (SSC) buffer (2XSSC, 1XSSC and 0.5XSSC) and exposed to Kodak X-ray film. To determine the number of copies of the gene inserted in the genome, genomic DNA was digested with a single cutter restriction enzyme (Bam HI).

Resistance through artificial inoculation of ScYLV
Transformed plants were multiplied vegetatively up to five plants per clone. A colony of Melanaphis sacchari aphids was established on non-transformed plants of variety CC 84-75 infected by ScYLV, as confirmed by RT-PCR, and the aphids were allowed to acquire the virus for 8 days. Then 20 aphids in their nymphal stage were taken to each of the five plants of the transformed clone. The aphids were left on the plants for 10 days, after which they were removed manually and the plants sprayed with an insecticide.

Detection of ScYLV in transformed and non-transformed plants
Plants were tested monthly after transmission for ten months by TBA and RT-PCR in order to detect the presence of virus. TBA was carried out as described (Schenck et al., 1997). ScYLV first strand cDNA was produced by RT of 4 μg of total plant RNA with Superscript (Invitrogen), in a 25-μL volume at 42°C with primer oFM361. PCRs were for 30 cycles of 95°C for 1 min, 58°C for 2 min and 72°C for 2 min using primer combination oFM 361 and oFM366 as described by Moonan and Mirkov (2002).

Results and discussion
Bombardment and plant regeneration
A total of 2489 embryogenic calli were bombarded in 13 experiments described in Table 1. Stepped selection on increasing concentrations of geneticin produced 756 and 60 resistant calli after selection at 43.3 μM and 72.2 μM respectively. From the resistant calli we were able to regenerate 69 geneticin resistant plants of which 66 were grown in a biosafety greenhouse (Table 1). To quickly screen for the presence of the NPT II transgene in regenerated sugarcane plants, PCR analysis was performed on leaf tissue. The efficiency of the geneticin selection was measured as the difference between the number of neo-positive plants by PCR (producing the expected band of 0.5 Kb – Table 1) (57) and the number of geneticin resistant plants (69). Twelve plants out of 69 (17%) were considered escapes from in vitro selection with geneticin. This percentage is considered high when compared to results published by Falco et al., (2000) who obtained four escapes out of 140 plants (2.85%), but low when compared to 39% obtained by Gallo-Meagher and Irvine (1996). Southern blot analysis revealed that the amplified band specifically hybridised to the NPT II coding region in the regenerated and not in non-transformed plants.
PCR analysis for the presence of the coat protein transgene was carried out in 57 plants containing amplifiable NPT II sequence. Forty-six out of 57 plants showed amplifiable coat protein sequence, producing the expected and specific band of 0.46 Kb as confirmed by Southern blot analysis.

**TABLE 1**—Summary of sugarcane transformation experiments in CC 84-75 commercial hybrid using plasmids pFM395, pFM396 and Ubi-km and verification by PCR amplification and Southern blot hybridisation.

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1: Number of bombarded calli
2: Number of calli growth in sublethal selection (30 mg/L)
3: Number of calli growth in sublethal selection (50 mg/L)
4: Number of plants regenerated
5: Number of plants transferred to greenhouse
6: Number of neo-positive plants by PCR
7: Number of plants PCR positive for transgene encoding viral coat protein
8: Number of plants Southern blot positive for coat protein transgene

**Southern analysis**

All PCR positive plants for the viral transgene were confirmed by Southern blot analysis (Table 1). Genomic DNA from each regenerated plant was digested with *Pst I*, which cuts out the coat protein coding region, contained a hybridising band of the expected size of 0.490 Kb. This result indicated that the inserted sequence had not been subjected to rearrangements (Figure 2, lanes d,e,k,l,m,v,x,y). However, some of these plants showed extra bands of a different size from the 0.490 band indicating DNA rearrangements (Figure 2, lanes b,c,f,j,n-u,w).

![Southern blot image](image-url)

**Fig. 2**—Southern hybridisation to detect ScYLV coat protein fragment in DNA digested with *Pst I*. Lane a: non-transformed control plant. Plants in lanes d,e,k,l,m,v,x,y contain inserted sequence of 0.490 Kb. Plants in lanes b,c,f-j,n-u,w showed extra bands indicating DNA rearrangements.
Digestion with Bam HI, which cuts only once within the transgene, revealed six transformation events. Two different events, event 1 in 15 plants and event 2 in 6 plants (Figure 3) showed 9.0 and 7.5 Kb fragments respectively, which are larger than the size of linearised pFM395 (5.3 Kb). Because the resulting hybridisation can contain fragments that could be larger than 20 Kb, other fragments may not be resolved using standard agarose electrophoresis conditions. However, these plants were finally considered to contain one copy but different insertions corresponding to two independent transformation events.

Sixteen plants revealed two bands, one of them smaller than 5.3 Kb, indicating rearrangements of the bombarded sequence in these lines. Bands larger than the linearised plasmid were 12 Kb and 10 Kb corresponding to two transformation events (Figure 4). Eight plants revealed more than two bands, some of them smaller than 5.3 Kb and a strong band of 8.2 Kb indicating a fifth independent transformation event (Figure 5). One plant showed a similar pattern to this displayed by these plants, but with some additional rearrangements included as indicated by the presence of small size bands (Figure 5, lane h). The number of integration sites in this work ranged from 1 to 5, low in number compared to data obtained by Bower et al. (1996) who observed from 1 to 14, but similar to the lines obtained by Falco (Falco et al. 2000) displaying from 1 to 3 copies.

Infection of transformed plants with ScYLV

Sixty-six regenerated plants in the greenhouse were challenged by inoculation with ScYLV. Thirty-seven out of 46 Southern positive plants were TBIA negative throughout all evaluations, whereas nine transformed plants were TBIA positive. These results were confirmed by RT-PCR using specific primers FM 361/366 and FM 359/323 (Moonan and Mirkov, 2002). The check plants (CC 84-75) showed disease symptoms as well as 4 out of 9 transformed TBIA positive plants. In five transformed TBIA
positive plants, disease symptoms were not present. The susceptibility of the nine transgenic plants could be due to several reasons such as gene silencing or DNA methylation of the transgene (Ingelbrecht et al., 1999). These reasons were not investigated in this study.

Fig. 4—Southern hybridisation of Bam HI digests indicating two different events displaying bands larger than linearised plasmid. Events 3 and 4 displayed 10 Kb and 12 Kb bands respectively.

Fig. 5—Southern hybridization of Bam HI digests revealing more than two bands in lanes a-g,i. Additional bands were observed in plant in lane h.
The main objective was to investigate the resistance and the breeding potential of transgenic clones obtained in order to be used in the near future as sugarcane parents in the breeding programs. A field test of resistant clones versus the original healthy and infected variety is underway.

Conclusions

1. Resistant plants to ScYLV were obtained by particle bombardment of CC 84-75 calli using plasmids constructed by E. Mirkov.
2. The method described in this paper is efficient to obtain resistant plants to the sugarcane yellow leaf virus. However, several rearrangements were evident, highlighting that it is important to develop Agrobacterium mediated transformation.
3. Number of escapes detected using geneticin selection system was moderate compared to others.

Acknowledgments

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REFERENCES


PLANTAS TRANGÉNICAS DE LA VARIÉTÉ CC 84-75 RÉSISTANTES AU VIRUS ASSOCIÉ À LA MALADIE DU YELLOW LEAF

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MOTS CLÉS: Canne à Sucre, Transgénique, la Maladie du Yellow Leaf, Résistance.

Résumé
Le virus associé à la maladie du yellow leaf de la canne à sucre (ScYLV) a été détecté en Colombie en 1998. Depuis lors on l’a trouvé dans plusieurs variétés hybrides industrielles, particulièrement dans la variété CC 84-75 qui occupe la deuxième place en superficie cultivée dans l’industrie sucrière colombienne. En raison de la sensibilité de la plupart des variétés hybrides industrielles et de l’importance de la variété CC 84-75, la possibilité de produire des cannes transgéniques résistantes au virus a été étudiée. Des cals embryogéniques de l’hybride CC 84-75 ont été bombardés avec les plasmides pFM395 et pFM396 contenant un fragment d’ADN de la protéine de capsid virale du ScYLV. Après une sélection sur génétique, 69 plantes ont été régénérées. Cinquante-sept étaient positif pour le NPT II après amplification par PCR. Quarante-six des 69 plantes régénérées avaient le fragment codant pour la protéine capsid, comme confirmé par les analyses PCR et l’hybridation Southern. Les digestions avec l’enzyme Bam HI, qui coupe une seule fois dans le transgène, indiquait six événements indépendants de transformation. Les plantes transformées ont été inoculées avec le ScYLV par Melanaphis sacchari et testées pour l’infection à intervalle mensuel pendant une période de dix mois après inoculation en utilisant les tests immuno-empreintes (IE) et la RT-PCR. Trente-sept plantes sur 66 se sont avérées négatives pour le ScYLV. Un essai au champ avec les lignées résistantes et la variété originale est en cours.

PLANTAS TRANSGENICAS DE LA VARIEDAD CC 84-75 RESISTENTES AL VIRUS ASOCIADO CON LA ENFERMEDAD DE LA HOJA AMARILLA DE CAÑA DE AZÚCAR

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PALABRAS CLAVE: Caña de Azúcar, Transgénicas, Enfermedad de la Hoja Amarilla, Resistencia.

Resumen
El virus asociado con la enfermedad de la hoja amarilla se detectó por primera vez en Colombia en el año 1998. A partir de allí se ha encontrado en varios híbridos comerciales especialmente en CC 84-75, el cual ocupa el segundo lugar en área de siembra en la industria colombiana. Debido a la susceptibilidad presente en varios híbridos comerciales y a la importancia de CC 84-75, se estudió la posibilidad de producir plantas transgénicas resistentes al virus. Callos embriogénicos de CC 84-75 se bombardearon con los plásmidos pFM395 y pFM396 los cuales contienen parte del gen que codifica para la cápsida del virus. Después de selección con genética, se obtuvieron 69 plantas regeneradas. Cincuenta y siete plantas fueron positivas para NPT II mediante PCR. Cuarenta y seis plantas de las 69 regeneradas tienen el fragmento que codifica para la cápsida viral como indicaron las pruebas de PCR e hibridación molecular. Digestiones con la enzima Bam HI la cual corta una sola vez dentro del transgen indicaron la presencia de seis eventos independientes de transformación. Las plantas se inocularon con el virus mediante el áfido Melanaphis sacchari y se evaluaron mensualmente usando TBIT y RT-PCR durante diez meses después de la infección. Treinta y siete plantas fueron negativas para el virus. Actualmente se están adelantando pruebas de campo de las plantas resistentes junto con plantas no transformadas.