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Agronomic performance and genetic characterization of sugarcane transformed for resistance to sugarcane yellow leaf virus

R.A. Gilbert [a,*], N.C. Glynn [b], J.C. Comstock [b], M.J. Davis [c]

[a] University of Florida, EREC, 3200 E. Palm Beach Rd., Belle Glade, FL 33430, United States
[b] USDA-ARS, Sugarcane Field Station, 12990 US Hwy. 441, Canal Point, FL 33438, United States
[c] University of Florida, CREC, 700 Experiment Station Rd., Lake Alfred, FL 33850, United States

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ABSTRACT

Sugarcane yellow leaf virus (SCYLV, a *Poleovirus* of the *Luteoviridae* family) is already widespread in Florida, and resistance in the Canal Point (CP) sugarcane population is limited. Genetic transformation of sugarcane for disease resistance holds promise but tissue culture and transformation processes produce undesirable agronomic characteristics necessitating thorough field evaluation. A 3-year sugarcane (a complex hybrid of *Saccharum* species) field study was conducted in Belle Glade, FL with the following objectives: (1) thoroughly evaluate the agronomic performance of two transgenic lines transformed for SCYLV resistance (6-1, 6-2) compared with parental cultivar control CP 92-1666, (2) determine level of SCYLV resistance in the transgenic lines, and (3) characterize genetic differences in the transgenic lines compared with CP 92-1666 using simple sequence repeat (SSR) genotyping. Sugarcane yields of CP 92-1666 were superior to both transgenic lines, as well as tissue culture (C-1) and nptII marker gene (20-1) controls, in the plant cane, first ratoon and second ratoon crops. CP 92-1666 recorded an average of 6.5–8.7 tons sucrose ha^{-1} yr^{-1} more than genotypes subjected to tissue culture and biolistic transformation. However, SCYLV infection rates in transgenic lines were only 0–5%, compared with 98% in CP 92-1666. Kanamycin field assays indicated that selectable marker gene nptII was stably expressed in all co-transformed lines. SSR genotyping showed 35 additional fragments to be present and 25 existing fragments absent among 6-1, 6-2, C-1 and 20-1 compared with CP 92-1666. Although all clones had unique genotypes, the four regenerated clones showed a greater genetic distance from the donor clone CP 92-1666 (mean GD 0.4) than to one another (mean GD 0.03). This study reports the first successful gene transfer of SCYLV resistance in sugarcane and the first report of variations in microsatellite repeat number associated with regeneration from embryogenic callus. Our results highlight the potential of genetic transformation methods to incorporate desirable traits into sugarcane, combined with the necessity of thorough agronomic evaluation of transgenic genotypes. Transgenic lines 6-1 and 6-2 are being used as parents in crosses designed to combine SCYLV resistance from these genotypes with agronomic characteristics of high-yielding materials.

1. Introduction

Sugarcane yellow leaf virus symptoms in sugarcane are characterized by a yellowing of the abaxial surface in the upper leaves. In severe cases growth is stunted leading to a fan-like appearance in the plant. These symptoms are caused by a luteovirus (Scaglusi and Lockhart, 2000; Moonan et al., 2000; Smith et al., 2000), which has been associated with a phytoplasma in some regions (Aljanabi et al., 2001). There are several virus strains which are associated with SCYLV and these may differ in infection capacity and virulence (Ahmad et al., 2006, 2007). The virus is transmitted by aphid vectors (Schenck and Lehrer, 2000; Lehrer et al., 2007), and may also be spread through infected vegetative planting material.

Sugarcane yellow leaf was first described less than 20 years ago in Hawaii (Schenck, 1990), but despite its relatively recent discovery has been reported in more than 30 countries (Lockhart and Cronje, 2000). Sugarcane yellow leaf virus symptoms mimic abiotic stresses which made field identification difficult; however, recently published disease surveys indicate that SCYLV has the potential to spread quite rapidly. Incidence of SCYLV infection has been reported in 89% of grower fields in Florida (Comstock et al.,

* Corresponding author. Tel.: +1 561 993 1535; fax: +1 561 993 1582.
E-mail address: ragilber@ufl.edu (R.A. Gilbert).

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1999), in 90% of cultivars surveyed in Ecuador, Guatemala and Honduras (Comstock et al., 2002), in 98% of stalks surveyed in a susceptible cultivar in Reunion (Rassaby et al., 2004), in 73% of cultivars surveyed in Colombia (Victoria et al., 2005), and in 62% of stalks surveyed in the central valley of Costa Rica (Moreira et al., 2006).

There are few published studies on SCYLV effects on sugarcane growth and yield. Physiological changes in SCYLV infected plants include a reduction in leaf area and a decrease in leaf chlorophyll and N content (Izaguirre-Mayoral et al., 2002). Rassaby et al. (2003) reported a 19–37% yield reduction in the first ratoon crop in two susceptible cultivars in Reunion, but yields of cultivar R570 were not affected by SCYLV.

The Canal Point (CP) sugarcane clones occupy more than 90% of total sugarcane area in Florida and generate more than $2 billion in total economic activity. Yield trial data indicate a 4–7% yield loss in cultivars infected with SCYLV (Flynn et al., 2005), thus the spread of SCYLV is a major concern. Traditional breeding techniques to incorporate SCYLV resistance are likely to be lengthy since the degree of SCYLV resistance in the CP sugarcane population appears to be limited. A survey of SCYLV incidence in the CP program indicated that 67% of Stage IV clones (the final field testing stage) and 98% of grower collection clones were infected with the virus (Comstock et al., 1999).

Meristem tip culture of virus-free plants (Fitch et al., 2001) and meristem tissue culture of infected varieties (Parmessur et al., 2002) have reduced SCYLV and phytoplasma pathogens in regenerated plants. However the benefits of meristem tissue culture techniques appear to be transitory in the field under Florida conditions (Comstock and Miller, 2005).

Molecular breeding techniques using microprojectile or *Agrobacterium* mediated gene transfer have been used to incorporate herbicide (Gallo-Meagher and Irvine, 1996; Falco et al., 2000; Leibbrandt and Snyman, 2003), disease (McQualter et al., 2004; Gilbert et al., 2005), and pest (Arencibia et al., 1999; Li-Xing et al., 2006) resistance into sugarcane. Rangel et al. (2005) recently reported successful incorporation of SCYLV resistance into Colombian cultivar CC84-75 through microprojectile gene transfer. While these techniques have the potential to improve the efficiency of sugarcane crop improvement (Lakshmanan et al., 2005), variability in agronomic traits of transformed clones due to somaclonal variation caused by tissue culture and transformation procedures (Arencibia et al., 1999; Carmona et al., 2005; Gilbert et al., 2005; Vickers et al., 2005) necessitate thorough field evaluation. To our knowledge there are no published reports of agronomic evaluation or field disease resistance of sugarcane genetically modified for resistance to SCYLV.

Embryogenic regeneration is known to cause genetic variability in plants, and most reports of variability have involved the analysis of Random Amplified Polymorphic DNA (RAPD) markers. In sugarcane, RAPD markers were used successfully to identify genetic variations following regeneration of embryogenic callus (Taylor et al., 1995) or indeed meristem cultures (Zucchi et al., 2002). Whereas, no genetic variability resulting from the regeneration of sugarcane embryogenic callus was detected by Chowdhury and Vasil (1993) using the analysis of restriction fragment length polymorphisms (RFLPs). The results of RAPD analyses are well known to be difficult to reproduce (MacPherson et al., 1993; Mcunier and Grimont, 1993) making their application in genome analysis limited. A more robust marker system would appear to be the analysis of simple sequence repeats (SSRs) which involves the detection of variations in repeat number at specific loci within the genome. The analysis of SSRs has been applied widely in genome analysis because SSRs are highly polymorphic, easy to analyze, and reproducible (Li et al., 2002; Ellegren, 2004).

The objectives of this study were to (1) evaluate the agronomic characteristics of two transgenic sugarcane genotypes bioengineered for resistance to SCYLV compared with the non-transformed parent clone CP 92-1666, (2) determine field expression of co-transformed SCYLV resistance and selectable marker genes, and (3) identify any genetic differences between transformed clones and parent controls using SSR genotyping.

2. Materials and methods

2.1. Plasmids

Plasmids Ubi-Km and pFM396 were provided by E. Mirkov at the Texas A&M Agricultural Experiment Station, Weslaco, TX. Plasmid pMBP39-22 (Huang et al., 1997) was provided by L. Owens at the Beltsville Agricultural Research Center, USDA, Beltsville, MD. Plasmid pFM396 contains an untranslatable SCYLV coat protein DNA fragment in the antisense orientation and under control of the maize ubiquitin promoter, first exon and first intron, followed by the nopaline synthase terminator. The MB39 gene in pMBP39-22 was amplified by polymerase chain reaction (PCR) using primers CCF2 (5′-TTGAAGATCTCGAGCCATGGGGAAGAAGAGCCACA-3′) AND CCR2 (5′-TTGACTCGAGATCTTATCCTAGCGCTTTGGCTTGC-3′) introducing Xho I restriction sites at both ends of the fragment. The MB39 gene encodes for modified Cecropin B which is an antimicrobial peptide. The MB39 gene and the ScYLV coat protein genes should act independently. The following program was run: initial denaturation for 3 min at 95 °C, followed by 40 cycles of 45 s at 95 °C, 1 min at 52 °C, and 1 min at 72 °C. The final extension step was 8 min at 72 °C. All reactions had a final volume of 20 μL and contained Tris buffer (1.5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris, pH 8.3), 0.2 mM of each DNTP, 10 pM of each primer, 1 unit of *Taq* polymerase and 5 ng of sample. The MB39 fragment was then restricted with Xho I and ligated into the Sal I site of pFM396 yielding pZY-CSA. Plasmid Ubi-Km contained the nptII gene under control of the maize ubiquitan promoter and was used to select calli for resistance to kanamycin.

2.2. Bombardment and regeneration of transgenic plants

Embryogenic callus was established from young leaf spindle tissues and maintained on MS medium (Murashige and Skoog, 1962) with 0.3% Gellrite and 3 mg/L 2,4-D (MS3 medium). Callus was co-bombarded with pZY-CSA and Ubi-Km-coated tungsten microprojectiles by procedures described by Franks and Birch (1991), except a particle inflow gun constructed and operated as described by Gray et al. (1994) was used. Following bombardment, the calli were put onto MS3 medium for 1 week, and then transferred to MS medium with 1 mg/L 2,4-D and 50 mg/L geneticin for 2 weeks. Plantlets were regenerated on MS medium without 2,4-D containing 65 mg/L geneticin at 28 C under alternating 12 h light and 12 h dark and then transferred to soil. The presence of the MB39 and anti-sense SCYLV coat protein genes in putatively transformed plants was determined by PCR amplification of a 571 bp fragment of the MB39 and anti-sense SCYLV coat protein genes using the CCF1 (5′-TCCGGCCATCAGCC-GAAATGGAAAGT-3′) and FM385F (5′-TGCTAACCGTCGTTGACT-GACTC-3′) primer pair.

2.3. Experimental design

Following transformation and selection, transgenic plants and controls were initially planted in the field in unreplicated plots at