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Transgenic Approach to Improve the Resistance of Sugarcane to *Sugarcane Yellow Leaf Virus* (SCYLV)

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ABSTRACT

Genetic transformation of sugarcane using various methods has been reported. In this review, an overview of the *sugarcane yellow leaf virus* (SCYLV) will be presented. The technical approaches used to create transgenic sugarcane through transformation, selection and regeneration will be reviewed. The genetic engineering methods for sugarcane include *Agrobacterium*-mediated transformation, electroporation and particle bombardment. In particular, work in Hawaii and Florida with constructs conferring resistance to sugarcane yellow leaf virus will be highlighted. Finally, thoughts about the future of sugarcane transformation will be discussed.

Keywords: *sugarcane yellow leaf virus*, genetic transformation
Abbreviations: G418, geneticin; **PEG**, polyethyleneglycol; **PMI**, phosphomannose isomerase; **SCYLV**, *sugarcane yellow leaf virus*; **TBIA**, tissue blot immunoassay; **YLS**, yellow leaf syndrome

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SUGARCANE YELLOW LEAF VIRUS

Sugar cane yellow leaf virus (SCYLV) is a *Polerovirus* (Moonan *et al*. 2000) and is a member of the *Luteoviridae* family that also contains barley yellow dwarf virus, soybean dwarf virus, potato leaf roll virus, and pea enation mosaic virus-1. SCYLV has a single-stranded positive sense RNA genome and is phloem-limited (Schenck *et al.* 1997; Vega *et al.* 1997; Lehrer *et al.* 2007). Analysis of viral RNA-sequences revealed that SCYLV belongs to a distinct subgroup that probably originated from the recombination of two other *luteovirus* species (Moonan *et al.* 2000). Five different genotypes of SCYLV have been reported (Moonan and Mirkov 2002) and also there has been shown to be variation in pathogenicity among some of these genotypes (Abu Ahmad *et al.* 2007).

The virus is transmitted from plant to plant by aphids in a persistent, circulative and non replicative manner (Moonan *et al*. 2000; Daugrois *et al.* 2011). Research has been conducted to examine the conditions which favour the spread of the virus. One example is the study by Daugrois *et al*. (2011) in Guadeloupe who investigated environmental conditions and aphid vector dynamics in relation to disease spread. Aphid species *Melanaphis sacchari*, *Rhopalosiphum maidis*, *R. rufiabdominalis* are known to be vectors of SCYLV (Schenck and Lehrer 2000). However, in Hawaii, for example, only *Melanaphis sacchari* is considered important for widespread dispersal of the virus (Lehrer *et al.* 2007).

In the late 1980s, severe leaf yellowing symptoms were seen in fields of sugarcane in Hawaii (Schenck and Lehrer 2000). Although the cause was originally unknown, it was thought to be a disease of the roots or vascular system as it would begin as bright yellowing of the abaxial surface of the midrib on the fourth or fifth youngest leaf and would spread downwards till most of the leaves were affected. Initial reports were that a clostero-like virus was associated with the leaf yellowing. However, viral particles found in the phloem of symptom showing leaves were characterized as those of a *luteovirus* (Vega *et al.* 1997; Scagliusi and Lockhart 2000) and it was named *sugarcane yellow leaf virus* (SCYLV).

Subsequently, similar leaf yellowing symptoms were reported in other sugarcane growing areas. Examples include Florida and Louisiana (Comstock *et al.* 1994), Australia and Brazil (Borth *et al.* 1994; Vega *et al.* 1997) and Africa (Cronje *et al.* 1998). It should be noted, however, that the symptoms attributed to SCYLV are not specific and can in fact be caused by various biotic and abiotic stresses (Lockhart and Cronje 2000). For example, the leaf yellowing documented in samples from eight African countries by Cronje *et al*. (1998) was not caused by SCYLV but instead by a phytoplasma. Presently, SCYLV has been reported to infect sugarcane worldwide (Vega *et al.* 1997; Moutia and Saumtally 1999).

A number of different terms have been used to name the symptoms caused by SCYLV, including yellow wilt, yellow leaf syndrome (YLS) and more recently yellow leaf (YL). The most characteristic symptom is a yellowing of the leaf midrib but the midrib can also turn pink (Daugrois *et al.* 2011). Discoloration of the leaves often occurs while the lamina is still green and symptoms can also include shor-

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tening of terminal internodes and necrosis of older leaves (Ahmad *et al.* 2007). Ultimately there is a reduction in biomass and reduced plant vigor (Rassaby *et al.* 2003). In a study by Lehrer *et al.* (2009), it was found that germination and early shoot growth of virus-infected plants was retarded and even without visible yellowing symptoms there was a reduction in total biomass. This, Lehrer *et al.* (2009) suggested that it could be due to the shortening of internodes as seen by Rassaby (2003). Plants of cultivars with SCYLV symptoms were reported to show an increase in the sucrose content of their leaves (Lehrer and Komor 2008), which was interpreted as the result of inhibition of assimilate translocation due to blocking of phloem vessels.

Antibodies specific for purified SCYLV were produced to assay for the presence of the virus in sugarcane tissues (Scagliusi and Lockhart 2000). Using this antibody in subsequent tissue blot immunoassays, both symptomatic and asymptomatic plants were found to contain the virus (Schenck *et al.* 1997; Schenck and Lehrer 2000). In Hawaii, SCYLV is widespread in all susceptible cultivars and has been shown to be spread by certain common aphid species (Lehrer *et al.* 2007; Zhu *et al.* 2010a). Often SCYLV is spread by planting seed pieces from infected plants. Virus-free seed cane can be produced from meristem tip culture, but the maintenance of virus-free fields for producing seedcane may be problematic as plants can quickly become infected if viruliferous aphids are present because of adjoining infected fields. In one instance, nearly half of SCYLV-free H87-4094 plants growing near a SCYLV diseased sugarcane field which was infested with aphids became infected and symptomatic within 18 weeks, while SCYLV-free plants in plots located far from any other sugarcane remained uninfected 12 months after planting (Lehrer *et al.* 2007).

GENETIC TRANSFORMATION OF SUGARCANE

Traditional plant breeding, coupled with biotechnological approaches, has been extensively used to increase crop yields to produce improved varieties which are resistant to diseases and pathogens and more productive. Important traits such as resistance to certain noxious pests and diseases appear to be absent from the available sugarcane cultivars (Arencibia *et al.* 1997). The use of plant transformation to introduce resistance genes into plant genomes may provide solution to this problem. However, the lack of a reproducible system for stable transformation and regeneration of sugarcane was initially an obstacle to efficient genetic engineering. Fortunately, it was discovered that sugarcane is capable of producing regenerable calli and protocols were developed for somatic embryogenesis and organogenesis using callus derived from a number of different tissues. Recent work demonstrates the generation of callus from immature inflorescences (Gallo-Meagher *et al*. 2000), young leaves (Hoy *et al*. 2003) and seed (Chengalrayan *et al.* 2005). Improved methods which were later developed using rice, e.g. (Hiei *et al.* 1994) also helped in sugarcane transformation.

Early work in sugarcane focused on the use of protoplasts and polyethylene glycol (PEG) treatment. Chen *et al.* (1987) used polyethylene glycol-treated protoplasts to produce kanamycin-resistant cell clusters. Poor reproducibility and low efficiency, one per 10^6 treated protoplasts (Chen *et al.* 1987), were a deterrent to using this method. Transgenic plants were not recovered using this system.

Three separate methods have now been used to generate transgenic sugarcane plants; particle bombardment, electroporation, and an *Agrobacterium*-mediated system. The first successful recovery of transgenic plants was by Bower and Birch (1992) who used particle bombardment method to transform cell suspensions and embryogenic callus. Also in 1992, Arencibia *et al.* stably transformed sugarcane using electroporation of meristem tissue. This group later went on to publish a method to produce transgenic sugarcane plants by electroporation of a cell suspension derived from embryogenic calli (Arencibia *et al.* 1995). Transformation efficiency for electroporation was higher than the PEG-mediated system with one transformant per 10^2 to 10^4 protoplasts being reported by Rathus and Birch (1992). The first successful use of an *Agrobacterium*-mediated method was reported in 1998 by Arencibia *et al*. Elliott *et al.* (1998) extended this work and incorporated use of the visual selection marker, GFP. In 2004, Manickavasagam *et al.* published a system for using *Agrobacterium* to transform axillary buds of sugarcane for herbicide resistance. With almost 50% of buds being transformed this was the highest transformation efficiency ever reported for sugarcane. More recently, the *Agrobacterium*-mediated method was used by Zhang *et al.* to introduce a trehalose synthase gene to improve drought resistance (2006) and by Zhangsun *et al.* (2007) to introduce GNA for resistance to woolly aphid. However, particle bombardment is the method which has been most widely used for sugarcane transformation. This can be seen in the paper by Lakshmanan *et al*. (2005) who reviewed sugarcane biotechnology and listed markers and traits engineered into sugarcane and the technologies used for transformation. Relative ease of use and availability of the equipment were a driving factor. Also, the fact that it could be used with a wide range of target tissues and different genotypes added to its popularity. More recent accounts of the use of particle bombardment include Bower *et al.* (1996) who introduced several marker genes including *luc*, *aphA* and *bar* genes and also Gallo-Meagher and Irvine (1996) who introduced the *bar* gene for herbicide resistance. Later, Jain *et al.* (2007) published the first report of the use of a *phosphomannose isomerase* (PMI)/mannose system for selection of transgenic sugarcane plants.

TRANSFORMATION OF SUGARCANE FOR RESISTANCE TO SCYLV

Although all three methods mentioned above have proven successful for sugarcane transformation, researchers have reported varying levels of success with each transformation method. Bower *et al.* (1996) claim that particle bombardment is very efficient, producing tens of independent transgenic lines per bombardment. Elliot *et al* (1998) suggest that sugarcane transformation using *Agrobacterium* methods is lower than that described for microprojectile bombardment. Hotta *et al.* (2010) suggest that more research should go into *Agrobacterium*-mediated systems as they are simple, inexpensive, and can transfer large DNA fragments with low copy number and little rearrangement. Basnayake *et al.* (2011) investigated the amenability to tissue culture of several Australian sugarcane cultivars and favours particle bombardment, saying it has the potential to cross from an experimental phase to a routine and practical application for a diverse set of cultivars.

Particle bombardment has certainly been the method of choice to generate sugarcane with improved resistance to SCYLV. Lakshmanan *et al.* (2005) list microprojectile as the transformation system used by Rangel *at al.* (2003) with SCYLV coat protein. Two groups have recently published accounts of sugarcane transformation for improved virus resistance. One group is based in Florida (Gilbert *et al.* 2009) and another in Hawaii (Zhu *et al.* 2010b).

Gilbert *et al.* (2009) reported co-transformation of two transformation vectors using particle bombardment. One construct harboured a fragment of the SCYLV coat protein in an antisense orientation driven by a maize ubiquitin promoter; the other construct contained a modified antibacterial Cecropin B gene, also under the maize ubiquitin promoter. Transformed cell lines containing the *npt*II selectable marker gene were selected using geneticin (G418, 50 mg/L) in the tissue culture medium and kanamycin spray on plants in the field (3 g/L). In a field trial, two transgenic lines with a transformation control (*NPT*II only), the non-transformed parent and a tissue culture control, were compared for various measurements. They investigated yield over two ratoon periods and also measured disease incidence. In

these trials, yields of the two transformed lines were lower than that of the non-transformed plants (6.5 to 8.7 tons less sucrose/ha/yr). Using TBIA to detect SCYLV, they reported 98% infection in the non-transformed plants, only 5% infection in one transformed line and the other line was virus free. One should note that since TBIA is a relatively insensitive assay, it is reasonable to assume that the reported virus free line may have contained a low virus titer so that both transformed lines had a low virus titer. Researchers concluded that although their results showed the potential of genetic transformation to engineer desirable traits into sugarcane, they also indicated the need for thorough field evaluation of yield traits in transgenic plants.

Zhu *et al.* (2010b) used particle bombardment to co-transform a single SCYLV susceptible cultivar, H62-4671, with two transformation constructs. One construct contained an untranslatable gene of the SCYLV coat protein in a sense orientation and driven by a maize ubiquitin promoter while the other construct contained the *npt*II selectable marker under a sugarcane ubiquitin promoter. Transformed plants were selected in tissue culture using geneticin (G418, 100 mg/L). Reaction to SCYLV of transformed plants was tested by determining the level of SCYLV in the plant leaves following plant inoculation with viruliferous aphids. Six out of nine transgenic lines had at least 10^3-fold lower virus titer than the non-transformed, susceptible parent line. This resistance level, measured by virus titer and symptom development, was similar to that of the resistant cultivar, H78-4153.

Although both groups used TBIA to identify the virus in sugarcane leaves, Gilbert *et al.* (2009) detected lower virus titer in all transformed lines, including the transformation control plants, i.e. the ones containing only the *npt*II selectable marker. In contrast, Zhu *et al.* (2010b) found no difference between plants containing *NPT* II and the non-transformed H62-4671.

Field yield tests of SCYLV-free lines obtained by meristem culture showed that the absence of SCYLV in a commercial cultivar had higher yield than the line that had not been freed of virus infection, at least in a one year crop cycle (Lehrer *et al*. 2009). Similarly, a field trial comparing yields of plots of cv. H65-7052 carrying low and high SCYLV-titer showed that plots with plants of higher virus titer developed YLS symptoms and yielded 54% as much cane fresh weight and 60% as much calculated sugar weight compared to plots with plants of low virus titer (Zhu *et al*. 2010a). These results support the concept that genetic transformation is a valid approach to produce high yielding sugarcane lines with resistance to SCYLV.

PARTICLE BOMBARDMENT PROTOCOL FOR TRANSFORMATION OF SUGARCANE FOR RESISTANCE TO SCYLV

The two groups in Florida and Hawaii ostensibly followed the same transformation protocol. Embryogenic calli were the sugarcane explants used for transformation. Calli were established from leaf whorls containing the shoot apical meristem using media termed MS3, 1x Murashige and Skoog with macro and micronutrients (Murashige and Skoog 1962) (Caisson Labs) with 3 mg/L 2,4-D. For maintenance, a medium termed MS1 was used which contains 1 mg/L 2,4-D.

The protocol used by the Hawaii group is outlined below. Four to seven days prior to bombardment, calli were cut into 3–4 mm pieces and transferred to fresh MS1 medium. They were transferred to MS1 supplemented with sorbitol and mannitol (MS1 with 0.2 M sorbitol and 0.2 M mannitol) 4 h prior to bombardment. Each plate was bombarded twice, at a distance of 6.5cm with a disk rupture pressure of 1500 psi (PDS-1000He, Bio-Rad). The Florida group used tungsten instead of gold as a DNA carrier. One day after bombardment, calli were transferred to MS1 medium for recovery. After 5 days, the calli were placed on selection medium, MS1 with an antibiotic. The cultures were maintained under a controlled temperature and light regime (approximately 22.8°C with 14 h light). Calli were maintained on selection medium for 12–16 weeks, and plantlets were regenerated from selected calli on MS0 (MS medium, no hormones) with antibiotic selection.

The work carried out by the Hawaiian group was based on an earlier paper by Ma *et al*. (2000), while the Florida group based their work on that of Franks and Birch (1991). Further information tracing the development and optimization of microprojectile systems for plant transformation can be found in an account by Birch and Franks (1991).

FINAL NOTE

SCYLV disease remains a problem in areas where sugarcane is grown. Preventing virus spread by controlling aphids in the field is not practical. Certainly in Hawaii, insecticides are not used on sugarcane plantations (Schenck and Lehrer 2000). SCYLV disease symptoms are not a reliable measure for the presence of the virus, because some cultivars can exhibit high virus titer with only marginal symptom expression (Lehrer and Komor 2008; Zhu *et al.* 2010a). Improved detection methods with higher sensitivity than the tissue blot immunoassay (TBIA) method have detected the virus in cultivars previously judged on the basis of TBIA, to be immune to virus infection (Zhu *et al.* 2010a).

Production of SCYLV-resistant cultivars through conventional breeding has been hindered by the fact that the currently grown highest yielding cultivars, which would otherwise be ideal as parental lines, are all susceptible to SCYLV. Genetic engineering has the ability to correct this one negative trait to allow current cultivars to serve as parents. In addition genetic transformation can be designed to alter several additional traits simultaneously, such as water and fertilizer requirements, sucrose content, and resistance to other pests and diseases. Genetic engineering may be the most promising way to reduce the incidence of SCYLV in the field.

Although the main approach to sugarcane improvement has been through classical breeding, genetic engineering can alter characteristics not necessarily possible through breeding. An example would be the expression of enzymes, a trait which may become more important as sugarcane is considered as an energy crop or biofactory. It therefore seems reasonable to assume that genetic engineering will continue to be an important tool for sugarcane improvement. However, the process is not without problems and limitations and further development of techniques is still required. Key areas which currently constrain high-throughput sugarcane transformation include the low transformation efficiency, transgene silencing and inactivation, somaclonal variation and the long time taken for regeneration and commercial release (Hotta *et al.* 2010). Commercial release of transgenic sugarcane requires legislative and public perception issues to be addressed. Technology which avoids antibiotic selection, such as the PMI/mannose system, improved vector systems which do not incorporate any non-transgene DNA and targeted and controlled expression of transgenes will all help address these issues. Currently different commercial sugarcane varieties are grown in different parts of the world. This is demonstrated in the work in Florida and Hawaii to improve resistance to SCYLV. The two groups had the same final aim but used different sugarcane varieties. Each commercial sugarcane variety for a given area would have to be transformed and this could be problematic as they have different characteristics and transformation and regeneration capacities.

The outlook for sugarcane improvement through genetic engineering remains bright. Hotta *et al.* (2010) were optimistic, suggesting that over the next few years the first commercial transgenic sugarcane with herbicide and insect resistance will become available. The release of transgenic sugarcane with improved resistance to SCYLV will hopefully follow on from this.

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