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RESEARCH ARTICLE

Genetic transformation of the euploid *Saccharum officinarum* via direct and indirect embryogenesis

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Abstract The genetic nature of foreign gene transfer and integration into commercial sugarcane hybrids with complex genetic backgrounds is unclear and unpredictable. A pure genetic background in genetic engineering approaches might lead to more predictable results within *Saccharum officinarum* than in a hybrid genome. *S. officinarum* plants (Badila and Black Cheribon genotypes) expressing a reporter gene was regenerated from microprojectile-bombarded leaf disks and callus. The concentration of auxin added to the basal media, both prior to bombardment and during regeneration, significantly affected callus induction and somatic embryogenesis. A ten-fold decrease in 2,4-D concentration resulted in somatic embryos forming directly from sugarcane leaf disks. Somatic embryos were produced via direct embryogenesis from 7 days old Black Cheribon leaf disks on 0.5 mg/l 2,4-D. A callus interface initiated on 3 mg/l 2,4-D from Badila leaf disks was preferred for indirect embryogenesis to produce the maximum number of transgenic Badila plants. These approaches lead to the successful establishment of genetic transformation systems for two *S. officinarum* genotypes that could be used in the future for gene function studies and breeding purposes.

Keywords *Saccharum officinarum*, somatic embryogenesis, plant transformation

Introduction

All commercial sugarcane cultivars, belonging to the genus *Saccharum* (Poaceae), grown today are complex aneuploid interspecific hybrids of mainly *Saccharum officinarum* and *S. spontaneum (*Simmonds 1976; Ming *et al*. 2006*)*. They account for about 75% of sugar production world-wide (FAO Sugar statistics). *S. officinarum* mainly contributes towards the sugar content and *S. spontaneum* towards disease resistance in modern cane hybrids (Britannica Encyclopedia 2009; Walker 1987). The sugarcane genome is characterized by a high level of polyploidy, with *S. officinarum* and *S. spontaneum* having basic chromosome numbers of x = 10 and x = 8, respectively (D'Hont *et al*. 1998; Irvine 1999). Cultivars are often aneuploids, with chromosome numbers between 100 and 130, of which 10 to 25% are contributed by the wild relative *S. spontaneum* (D'Hont *et al*. 1996). Clones with chromosome numbers outside of this range are rarely suited for commercial production (Ming *et al*. 2006). Interspecific chromosome exchanges also occur between the two species (D'Hont *et al*. 1996; Piperidis and D'Hont 2001; Cuadrado *et al*. 2004). Modern cultivars are essentially derivatives of no more than 15-20 genotypes of nobilized cultivars that can be traced back to the initial nobilized genetic base developed in Java and India (Roach 1989). Due to the genetic complexity of modern sugarcane varieties, traditional breeding methods are time consuming and it can take between 12 and 14 years to develop a new improved genotype (Liu *et al*. 1984; Ming *et al*. 2006). Most traits in sugarcane are also multigenic and/or multi-allelic and are quantitatively inherited making breeding of improved cultivars for increased resistance, yield and sugar content very difficult (Ming *et al*. 2006). Trait identification can also be difficult depending on the level of heterozygosity for the trait in a population. By using modern biotechnology tools it is possible to selectively incorporate / change a plant trait, e.g. insect resistance, without altering other traits. However, in a commercial sugarcane variety containing a mix of the *S.*

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officinarum and *S. spontaneum* genomes (ratio of ± 3:1), the nature of the interaction is unclear and unpredictable. The major part of this diversity are attributed to the chromosome complement inherited from *S. spontaneum* by random assortment of half its chromosomes, lack of preferential pairing and segregation of alleles with different dosage levels (Ming *et al*. 2006; D'Hort *et al*. 1996). Manipulating individual genes within this genetic environment carries the high risk that the gene expression will be modified or masked by the interaction between the genomes. Although this is realistic in commercial breeding trials, the question remains whether a "pure" genetic background in genetic engineering approaches might lead to more predictable results within *S. officinarum* than in a hybrid genome, or at least a more stable genetic background from which further breeding can be attempted.

Since the early nineties, much progress has been made in biotechnological aspects of sugarcane. Especially *in vitro* regeneration and genetic transformation protocols were established and are now routinely used around the world (Franks and Birch 1991; Bower and Birch 1992; Taylor *et al*. 1992; Arencibia *et al*. 1998; Arencibia and Carmona 2006). A wide range of commercial sugarcane cultivars have been reported to produce embryogenic callus *in vitro* (Chen *et al.* 1988; Taylor *et al*. 1992; Geijskes *et al*. 2003) but not all have a high transformation potential (Snyman *et al*. 1996). Many leading elite cultivars are recalcitrant to *in vitro* regeneration, and no transgenic versions of these cultivars have been produced to date (Ming *et al*. 2006). The aim of this study was therefore to explore the potential of genetically transforming two *S. officinarum* genotypes and two explant production routes for gene transfer namely i) direct or ii) indirect embryogenesis have been investigated. The noble genotype "Black Cheribon" originates from West Java and was planted as a commercial variety extensively during the later part of the nineteenth century (Mutsaers 2007). "Badila" is a chewing cane variety susceptible to a number of diseases and insect pests (Xu *et al*. 2008). Both genotypes are further known to have thick, barrel-shaped internodes, large juicy stalks and relative high sugar content. Here we report the successful *in vitro* regeneration and genetic transformation of the two *S. officinarum* genotypes "Badila" and "Black Cheribon".

Materials and Methods

Explant preparations and culturing conditions

Shoot tops of field grown *S. officinarum* genotypes, "Black Cheribon" and "Badila", were used as an explant source. Immature leaf rolls were isolated from tightly furled inner leaves after the aseptic removal of outer mature leaves. The basal part of the leaf roll, just above the apical meristem, was excised, cut into 2 mm thick transverse sections and placed on MS medium (Murashige and Skoog 1962) containing 2% sucrose, 2,4-D (0.5, 1, 2 or 3 mg/l; here after referred to as MS0.5, MS1, MS2, MS3, respectively), 0.5g/l casein and 0.22% gelrite, pH 6. Cultures were incubated in the dark at 24°C and placed on fresh medium every two weeks. For plant regeneration, tissue was placed on MS0 medium, lacking 2.4-D, in the light for 8 weeks or until roots has formed. Plantlets, 5 cm high with roots, were hardened off in soil containing pots in the glasshouse.

Plasmid construct

Plasmid pGFP-Ubi was 6 kB in size and contains a green fluorescent protein (GFP) reporter coding sequence under control of an ubiquitin promoter and a nos terminator sequence. The second 5.6 kB size plasmid, pEmuKN, contained the *npt*II coding sequence driven by the strong monocot Emu promoter sequence, allowing efficient selection on geneticin (Last *et al*. 1990).

Plant transformation

The particle inflow gun was constructed locally and was used for all DNA deliveries. Bombardment was done as described by Franks and Birch (1991) with modifications. Tungsten particles (M10; Biorad, CA) were sterilized with 100% ethanol, rinsed three times and re-suspended in sterile water. The tungsten suspension was mixed with 10 µl plasmid DNA (5 µg of each plasmid), 50 µl 2.5M $CaCl_2$ and 20 µl 0.1M spermidine. The mixture was incubated on ice and 100 µl of the aqueous layer removed prior to plant explant bombardment. For bombardment, 5 µl of the particle suspension was placed in the center of a 1 mm^2 metal grid above the explants. Target tissue was placed 16.5 cm below the particle source and covered with a metal grid. The chamber was set under a vacuum of 80 kPa and the particles were discharged when helium, 1000 kPa, was released by a timer relay, 0.05 s.

Plant tissue was placed on an osmoticum medium consisting of the basal MS medium with 0.2 M each of sorbitol and mannitol 4 h prior to and after bombardment (Vain and McMullen 1993). After bombardment, the plant tissue was maintained on MS medium for 3 days before selection started on 45 mg/l geneticin (Sigma, UK) for 8-10 weeks in the dark as outlined by Bower and Birch (1992). Explants at various stages of development were bombarded. This included leaf disks 7 days after initiation on MS0.5 (MS medium containing 0.5mg/l 2,4-D) and callus formed 6 weeks after initiation on MS3 (MS medium containing 3 mg/l 2,4-D). Ten plates of embryogenic callus portions, between 3-4 mm diameters, or seven plates containing leaf disks between 5-10 mm diameters, were arranged to cover a circle of 3 cm diameter tissue and bombarded for each treatment.

Results

Effect of 2,4-D on direct and indirect embryogenesis

Badila and Black Cheribon leaf disks were placed on four different concentrations of the auxin 2,4-D (0.5, 1, 2, or 3 mg/l) to initiate embryo formation with or without a callus interface. Black Cheribon leaf disks were highly embryogenic when placed on MS0.5 medium (Fig.1B). Light colored, smooth surfaced callus was observed when Badila leaf disks were placed on MS3 medium. Especially globular and pre-embryo-like structures were found for Badila leaf disks on medium MS3 after 5-7 weeks (Fig. 1C). On MS1 and MS2 medium both Black Cheribon and Badila leaf disks formed a combination of watery callus and limited shoots. These two mediums were not further used in the transformation experiments. Similar watery callus was also seen for Black Cheribon leaf disks placed on MS3 (Fig. 1A).

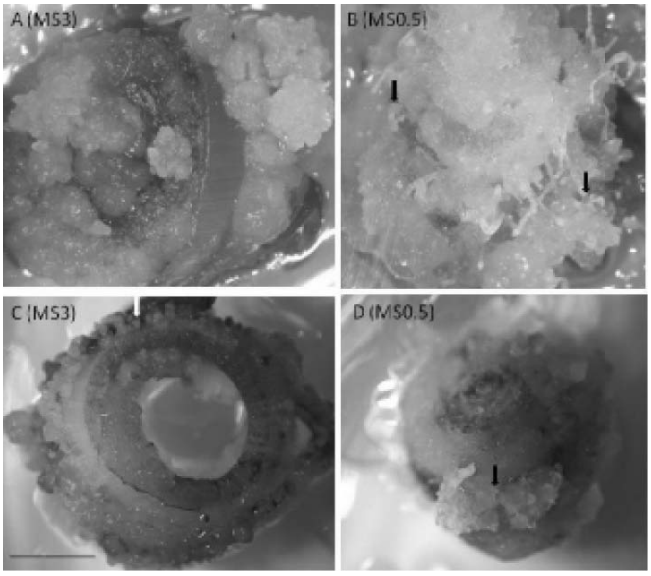

Fig. 1. Black Cheribon (top) and Badila (below) leaf disks showing direct embryogenesis (black arrows) and callus formation after 6 weeks on 0.5 and 3 mg/l 2,4-D, respectively. White arrow represents globular pre-embryo-like structures. Grey arrow represents watery callus. Scale = 5 mm.

Effect of direct or indirect embryogenesis on transformation efficiency

In experiment 1, seven plates of leaf disks from both Badila and Black Cheribon were bombarded 7 days after initiation on MS0.5 medium. In experiment 2, leaf disks of the two genotypes were allowed to form callus on medium MS3 for six weeks before the bombardment of ten plates of embryogenic callus occurred. Transient expression of the gfp coding sequence was found after two days for all four bombardment types in the two experiments (Badila on MS0.5 and MS3; Black Cheribon on MS0.5 and MS3) (Fig. 2). However, after 8 weeks on MS0.5 geneticin selection medium, Badila bombarded leaf disks showed no regeneration. This was in contrast to Black Cheribon bombarded leaf disks. For Black Cheribon a number of putative transgenic clones regenerated after 8 weeks on MS0.5 and 45 mg/l geneticin. This was followed by further regeneration in the light in the absence of 2,4-D (Fig. 3). For experiment 2, a number of transgenic clones, expressing the gfp coding sequence, developed from bombarded Badila callus after 8 weeks of selection (Fig. 4). Significantly less clones from bombarded Black Cheribon callus survived the selection period. Putative transformed regenerated Badila callus clones, which have been placed in the light on MS0 medium, developed plantlets within 6 weeks after removing auxin from the culture medium. All putative transformed clones were tested for integration of the transgene into the genome using PCR technique (Fig. 5).

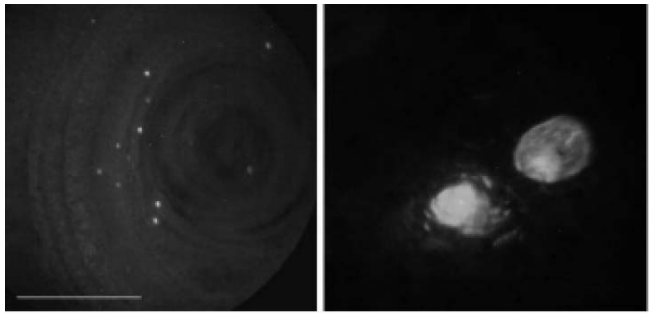

Fig. 2. Transient expression of the gfp coding sequence observed two days after bombardment of a seven day old Badila leaf disk. Scale = 5 mm.

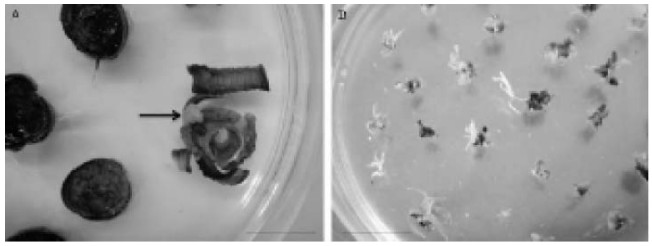

Fig. 3. (A) Limited callus formation found on Black Cheribon leaf disks co-bombarded with vectors pGFP-Ubi and pEmuKN, after 8 weeks on MS0.5 and 45 mg/l geneticin. (B) Direct Black Cheribon shoot formation from bombarded leaf disks placed in the light on 45 mg/l geneticin for selection in the absence of 2,4-D. Scale = 10 mm.

Discussion

A selected few sugarcane cultivars are routinely used as model systems, which are amenable to *in vitro* growth and genetic transformation. However, cultivars used for breeding purposes are not always as responsive as model genotypes and plant transformation can be problematic. In the past, sugarcane has been successfully transformed using either direct or indirect somatic embryogenesis routes (Bower and

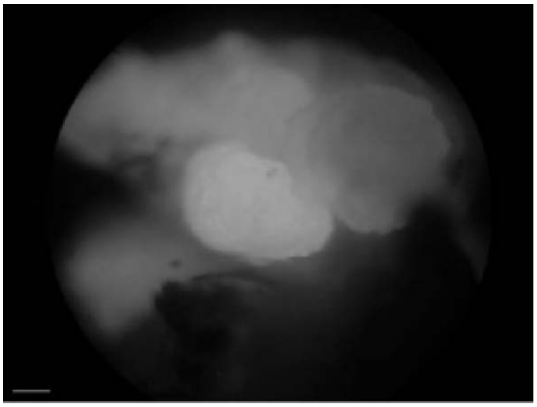

Fig. 4. Bombarded Badila callus showing stable gfp expression after 8 weeks on geneticin selection. Scale = 1 mm.

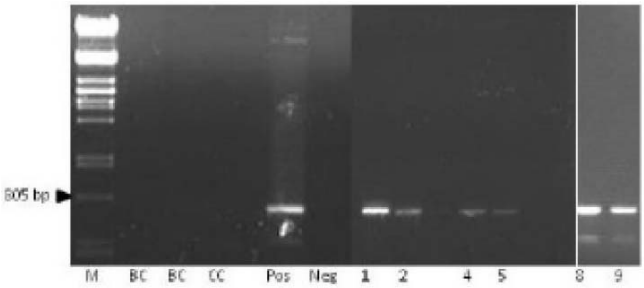

Fig. 5. PCR analysis of putative transformed Badila and Black Cheribon plants, using primers designed for amplification of pGFP-Ubi. Lanes 1 to 9 represent four gfp positive transformed Badila (1, 2, 4 and 5) and Black Cheribon (8 and 9) plants, respectively. M = Marker; BC= Badila Control; CC=Cheribon control; Pos = Positive plasmid control; Neg = Negative water control.

Birch 1992; Falco *et al*. 2000; Desai *et al*. 2004; Snyman *et al*. 2006). In this study, the most efficient route was investigated for direct DNA transfer into two *S. officinarum* genotypes, Badila and Black Cheribon. It was found that for Badila indirect embryogenesis was more efficient than direct regeneration. Out of ten plates of bombarded Badila embryogenic callus, six stable expressing transformed clones were developed. This indicates a transformation efficiency of 0.6 plants per bombardment. This result was comparable with a recent reported study on Badila transformation, which produced seven transgenic clones (Xu *et al*. 2008). The 2,4-D concentration for efficient callus production in Badila explants has been found to be 3 mg/l 2,4-D. This 2,4-D concentration differs slightly from the one previously described of 2 mg/l by Xu *et al*. (2008). Calli produced on 3 mg/l 2,4-D were typically globular and heart-shaped after 6 weeks of culturing and capable to regenerate plantlets. Transgenic Badila plants were ready to be hardened off in the glasshouse after a total of 20 weeks in culture. This is shorter than the time frame, 36 weeks, previously reported for the conventional system of sugarcane transformation (Bower *et al*. 1996; Birch 1997; Snyman *et al*. 2001).

For Black Cheribon, the most efficient transformation route was determined as direct somatic embryogenesis with no or very limited callus interface required. The auxin concentration was about ten-times lower in comparison to the concentration previously reported for sugarcane callus cultures (Franks and Birch 1991). However, auxin, especially 2,4-D, is known to cause somaclonal variation after prolonged exposure producing albino plantlets (Snyman *et al*. 1996). Therefore, lowering the amount of 2,4-D in the culture medium, as done in this study, has the advantage of limiting the possibility of producing somaclonal variants (off-type plantlets). Not only does this method of transformation have the potential to reduce somaclonal variation and phenotypic off-types, it greatly reduces the amount of time from the initial bombardment to the regeneration of plantlets. The decision to bombard both Badila and Black Cheribon leaf disks seven days after initiation was based on the report by Snyman *et al*. (2006) indicating high level of transformation efficiency at this embryo development stage. Globular, pre-embryoids only become visual on leaf disks after 14 days in culture therefore bombardment takes place prior to embryo development. Seven days after initiation, possible internal bacterial contamination could also be observed and eliminated if necessary. In general, direct embryogenesis decreases the time needed to produce transformed Black Cheribon sugarcane plants ready for hardening off to 15-18 weeks. This is comparable to the 18-20 weeks of sugarcane transformation using direct embryogenesis reported by Snyman *et al*. (2006).

Overall, we were able to develop a reproducible protocol for transformation and high frequency regeneration of *S. officinarum* through microprojectile bombardment via direct or indirect embryogenesis. The optimum conditions for embryonic culture induction and plantlet development were determined. The effect of different concentrations of 2,4-D on embryonic calli production using leaf base explants were determined and showed that the 2,4-D concentration had significant different effects on explants from responding genotypes. The procedure outlined here might be useful in future studies not only to unravel the complex carbon metabolite gene function studies in sugarcane but also, depending on the breeding value of the parent transgenic *S. officinarum* genotype, large numbers of transgenic progeny can be produced from crosses involving transgenic *S. officinarum* parents. For example, Butterfield *et al*. (2002) used transgenic sugarcane parents containing herbicide and virus resistance genes as males for crossing with non-transgenic sugarcane clones. Various genome analysis studies confirmed that *S. spontaneum* are distinctively more divers than *S. officinarum* (Sobral *et al*. 1994; Burnquist *et al*. 1992; Nair *et al*. 1999). Transgenic *S. officinarum* might therefore have the potential to act as a stable transgenic sugarcane parent, showing stable inheritance of transgenes, in effective breeding programs.

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