TOWARDS ANTIBIOTIC RESISTANCE-FREE TRANSGENIC SUGARCANE

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Abstract

The sugarcane transformation programme at SASEX depends on the use of a negative selection gene, which confers resistance to the antibiotic geneticin. Expression of this antibiotic resistance gene results in the selective development of transgenic cells on a medium containing the antibiotic, which is lethal to the untransformed cells (negative selection). The presence of the resistance gene is superfluous and undesirable once the transgenic plant has been produced, and its elimination is a priority for the future release of commercially acceptable genetically modified sugarcane plants. Alternatively, a positive selection gene such as the *manA* gene that encodes the enzyme phosphomannose isomerase (PMI) can be employed. When expressed, the *manA* gene confers a metabolic advantage on the transgenic cells, enabling them to grow on a mannose-containing medium. Initial results indicated that a mixture of mannose and sucrose is needed to support callus growth. MS medium supplemented with 5 g/L mannose and 10 g/L sucrose was shown to prevent growth of untransformed sugarcane cells and was subsequently tested for the selection of sugarcane calli transformed with the *manA* gene. Putative transformants have been regenerated from the mannose selection regime tested, namely 5 g/L mannose in combination with either 5 or 10 g/L sucrose.

Keywords: sugarcane, sugarcane transformation, selection gene, antibiotic resistance gene, positive selection, phosphomannose isomerase

Introduction

Following any transformation procedure, a few transformed cells exist among a bulk of untransformed tissue. The ability to differentiate between transformed and untransformed cells is important for the efficient recovery of transgenic cells. Selection genes are co-delivered to the plant genome along with the gene(s) of interest to selectively promote the growth of transgenic cells. Negative selection systems that rely on the use of antibiotic or herbicide resistance genes have traditionally been chosen for the transformation process.

The sequence *aph (3′) II* that encodes the enzyme neomycin phosphotransferase II (NPT II) and confers resistance to aminoglycoside-type antibiotics (e.g. kanamycin, neomycin and geneticin) is to date the most widely used selection gene in plant transformation and the gene currently used at SASEX. One disadvantage associated with antibiotic resistance as the basis of selection is that a great number of transformed cells die because of toxic substances excreted from the necrotic untransformed cells, resulting in only a fraction of transformed cells being recovered (Haldrup *et al*., 2001). Moreover, the presence of antibiotic resistance genes is both undesirable and unnecessary in the transgenic plant after transfer of desirable trait(s). The removal of antibiotic resistance genes would eliminate potential regulatory

difficulties in the future release of commercially acceptable genetically modified sugarcane plants. However, this is a technically difficult procedure.

Alternatively, positive selection systems can be used to avoid the use of antibiotic resistance genes. These systems are based on conferring to plant cells the capacity to convert a compound not previously easily metabolised into one that will induce a positive response, through the insertion and expression of a selection gene. This approach is termed 'positive selection' because the transgenic cells are given a metabolic advantage over the untransformed cells that eventually die (Penna *et al*., 2002). The phosphomannose isomerase (PMI) selection system is based on positive selection of plant cells expressing the *manA* gene (encodes PMI enzyme), where mannose is used as a selective agent. The PMI system has been tested with success in several plant species (reviewed by Reed *et al*., 2001). Mannose selection differs from antibiotic resistance selection systems in that (i) the toxicity of mannose is not mediated by the selective compound itself, but is considered to be a result of its phosphorylation to mannose-6-phosphate by endogenous hexokinase, by which the untransformed cells are thought to be starved for phosphate and ATP (Pego *et al*., 1999), and (ii) transgenic cells convert mannose to an easily metabolised compound, fructose-6-phosphate (Privalle *et al*., 2000). The purpose of this study was to develop and optimise tissue culture conditions using the PMI selection system for sugarcane transformation.

Results and discussion

To assess the effect of mannose on the growth of untransformed embryogenic calli, mannose dose response experiments were conducted. Sugarcane leaf roll discs were cultured on MS basal medium (Murashige and Skoog, 1962) containing 2,4-Dichlorophenoxyacetic acid (2,4-D, 3 mg/L) for eight weeks to generate embryogenic calli. These calli were exposed to a range of mannose/sucrose ratios for eight weeks. To monitor callus growth, callus mass was recorded initially and at every fortnightly subculture. It was demonstrated that mannose inhibits the growth of untransformed callus, and at 5 g/L mannose callus growth was severely repressed (Figure 1). The inability of sugarcane callus to grow on mannose is likely due to a deficiency in endogenous PMI activity, the decreasing energy supply through phosphorylation of mannose by hexokinase (Pego *et al*., 1999), or the presence of a mannose-induced endonuclease that results in DNA laddering and eventual death of plant cells (Stein and Hansen, 1999). The inhibitory effect of mannose was alleviated by the presence of sucrose, indicating that an external sucrose source is necessary for the growth of untransformed callus (Figure 1). When sucrose was supplied at a level of 20 g/L excellent callus growth was achieved. It became evident that a mixture of mannose and sucrose in the media allowed for better callus growth than mannose as the sole carbon source.

Once the levels of mannose/sucrose preventing growth of untransformed callus were established, two selection plasmids were co-delivered to embryogenic calli by microprojectile bombardment (Snyman *et al*., 1996). The pEmuKN selection plasmid (Chamberlain *et al*., 1994), bearing the *aph (3′) II* selection gene allowed selection on medium containing geneticin and the pNOV2820 selection construct (Syngenta), carrying the *manA* gene enabled selection on a mannose-containing medium. Selection of bombarded calli was performed on media containing different selective agents: 45 mg/L genticin, 5 g/L mannose in combination with either 5 or 10 g/L sucrose and sucrose only (10 and 20 g/L) controls for eight weeks, with sub-culturing onto fresh medium every two weeks. Plantlets were regenerated on medium (MS without 2,4-D) containing the same selective agents. Necrotic activity of untransformed cells associated with the toxic effect of the antibiotic was observed in the geneticin selection regime, whereas no necrosis was detected in the mannose selection

regimes. Plant regeneration efficiency was determined as the number of plants regenerated per number of bombardments and expressed as a percentage. Although regeneration efficiencies obtained on mannose selection are lower than those obtained for the conventional geneticin selection (Table 1), the mannose selection system can be improved to recover more plants, avoid the use of the antibiotic resistance gene and hence eliminate its presence in transgenic sugarcane plants.

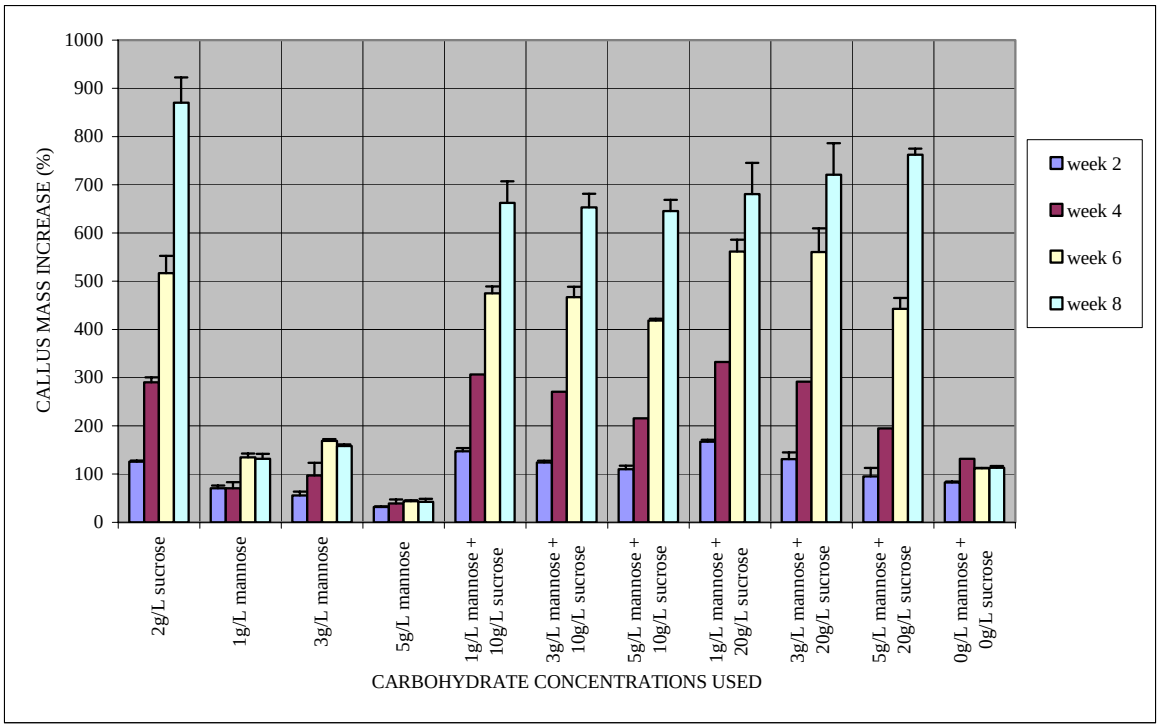

Figure 1. The effect of different mannose/ sucrose ratios on the growth of untransformed embryogenic callus. Eight-week old callus was placed onto MS medium supplemented with a range of mannose/sucrose ratios for eight weeks. Callus was massed at every two-week subculture. Each data point represents the average of three replicates and the error bars represent the 95% confidence level.

Table 1. Comparison of regeneration efficiencies obtained from the different selection regimes. Callus was bombarded with pEmuKN and pNOV2820 selection plasmids. Selective agents were present throughout the selection and regeneration periods. Plant regeneration efficiency was determined as the number of plants regenerated per number of bombardments and expressed as a percentage.

Selection regime	Regeneration efficiency (%)
10 g/L sucrose	60
20 g/L sucrose	340
45 mg/L geneticin	60
5 g/L mannose + 5 g/L sucrose	3.3
5 g/L mannose + 10 g/L sucrose	6.7

Conclusion and future work

A suitable mannose selection regime of 5 g/L mannose combined with 10 g/L sucrose has been established from which putative transgenic sugarcane plants have been regenerated, suggesting that the PMI selection system might prove a useful alternative to the antibiotic

resistance based selection system currently used at SASEX. Molecular analysis of regenerated plants will be carried out to confirm transgene presence and thus the efficacy of selection.

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