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Biolistic Mediated Sorghum (*Sorghum bicolor* L. Moench) Transformation via Mannose and Bialaphos Based Selection Systems

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Abstract: The efficiency of the bialophos (*bar*) and phosphomannose isomerase (*pmi*) selectable markers in microprojectile mediated transformation of P898012, a sorghum inbred line generally considered amenable to *in vitro* manipulation was investigated. Two plasmids containing *bar* gene, encoding bialaphos resistance and *manA* gene, for phosphomannose isomerase, both under the control of maize ubiquitin promoter and nopaline synthase terminator were used to transform Immature Zygotic Embryos (IZE) of sorghum via particle bombardment using a particle inflow gun. Transgene integration in putatively transgenic plants was confirmed by PCR and Southern blot analysis. The expression of the bar gene in transgenic plants was demonstrated by the BASTA leaf painting assay. Present results indicated a transformation efficiency of 0.11 and 75% escapes in the apparent bialaphos resistant plants. A transformation efficiency of 0.77% was observed on *manA* selection and all plants recovered contained the *man*A gene. Besides an improved transformation efficiency, mannose selection offers a more environmentally sound system as sugar metabolism is considered ecologically neutral should it inadvertently be transferred to wild sorghum species.

Key words: Mannose selection, particle bombardment, phosphomannose isomerase (*pmi*), sorghum

INTRODUCTION

Sorghum bicolor L. Moench is the fifth most important cereal crop globally and the second most important cereal in Africa, after maize. In developed countries, sorghum is mainly used as a livestock feed, but in the developing countries of Africa, Asia and the Middle East, it is a principal source of energy, protein, vitamins and minerals (Chakauya *et al*., 2009). Sorghum is also an important component in traditional farming systems, providing food security to 400 million people in these countries (Andrews and Bramel-Cox, 1993). Sorghum is drought tolerant crop and is thus well adapted to the semi-arid tropics where droughts cause frequent failures of other crops (O'Kennedy *et al*., 2006; Chakauya *et al*., 2006). To date, most of the varietal improvement on this crop has mostly been done through conventional breeding. Precision breeding facilitated by genetic transformation offers a much faster approach to achieving genetic gains for a broad range of traits.

Sorghum has been considered as a recalcitrant cereal with regards to tissue culture regeneration and transformation (Zhu *et al*., 1998). Because of its relatively lower importance compared to maize, rice, wheat and barley, sorghum has not received as much attention in terms of efforts of improvement using advanced biotechnology tools as other cereals. Consequently, fewer laboratories have reported sorghum transformation and tissue culture based manipulations (Kaeppler and Pederson, 1997; Zhu *et al*., 1998; Zhao *et al*., 2000, 2003; Able *et al*., 2001; Emani *et al*., 2002; Tadesse *et al*., 2003; Gao *et al*., 2005; Howe *et al*., 2006). A key requisite in the development of a genetic transformation protocol for any species is a reliable method of distinguishing transformed from non-transformed tissue. This is done through co-transformation of the genes of interest with selectable marker gene which confers unique metabolic properties to transgenic tissues, allowing them to grow on media where non-transgenic tissue cannot thrive. When generating sorghum transgenic plants using biolistic mediated transformation, the selection step is crucial since it necessitates careful *in vitro* culturing of mechanically damaged explants to recover and regain tissue totipotency (Grootboom *et al*., 2008).

Until recently, only two selectable marker systems based on the *bar* or *pmi* genes have been employed to produce transgenic sorghum. The *bar* gene was isolated from the bacterium *Streptomyces hygroscopicus*, encodes

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the enzyme Phosphinothricin Acetyl Transferase (PAT) and confers resistance to the herbicides BASTA and bialaphos. This selection gene had been used to achieve stable transgene integration in sorghum (Casas *et al*., 1993, 1997; Zhu *et al*., 1998; Zhao *et al*., 2000; Able *et al*., 2001; Emani *et al*., 2002). Despite the advantage of allowing stringent selection of calli in culture to generate bialaphos-resistant plants, *bar* selection seems to be a leaky selection system resulting in escapes (Gao *et al*., 2005). Moreover, there is a challenge of public acceptance of herbicide resistance because of the possibility of gene transfer to wild sorghum relatives such as the Johnsongrass and shattercane via pollen. This is particularly a problem in Africa where wild sorghum relatives are endemic.

Positive selectable marker genes not only eliminate the risk of herbicide and antibiotic resistance genes spreading to wild relatives, but also enhance transformation efficiencies of several crops. *E. coli* phosphomannose isomerase (*pmi*) gene converts mannose-6-phosphate, a carbon source that most plants cannot metabolize, into easily metabolizable fructose-6-phosphate (Miles and Guest, 1984). Therefore, transgenic cells can utilize the fructose-6-phosphate, while untransformed cells starve because of lack of a carbon source. This selectable marker gene was first used in sugarbeet transformation (Joersbo *et al*., 1998) and has subsequently become popular with cereal transformation such as, maize (Negrotto *et al*., 2000; Wright *et al*., 2001), rice (Lucca *et al*., 2001), wheat (Wright *et al*., 2001) pearl millet (O'Kennedy *et al*., 2004) and sorghum (Gao *et al*., 2005). Also *pmi*-based selection was reported to improve the transformation efficiency (Wright *et al*., 2001; Negrotto *et al*., 2000; O'Kennedy *et al*., 2004; Gao *et al*., 2005).

The study presented here provides data on the use of both the *bar* and *manA*-based selection systems in sorghum transformation using particle bombardment. To our knowledge, this is a first report on transgenic sorghum plant generated using particle inflow gun, while comparing *bar* and mannose selection.

MATERIALS AND METHODS

The research was conducted at the Council of Scientific and Industrial Research (CSIR), Pretoria, South Africa between April and December in 2007.

Plasmids: Two plasmids were used for transforming the sorghum, pAHC25 (Christenson and Quail, 1996) is a dual expression vector which contains the *uidA* reporter gene that encodes the β-glucuronidase (GUS) enzyme and for selection, the *bar* gene (Wehrmann *et al*., 1996), that encodes the enzyme Phosphinothricin Acetyl Transferase (PAT), which confers herbicide resistance. PAT inactivates the herbicidal compound phosphinothricin (PPT) found in several herbicide formulation, such as BASTA® (Hoechst AG, Germany) The plasmid pNOV3604-ubi, obtained from Syngenta carries the *manA* gene which confers resistance to mannose (Rebecca and Brian, 2001). Both genes are driven by the maize ubiquitin promoter with nopaline synthase terminator (NosTer). All plasmid DNA preparations were carried out using the Qiagen Maxiprep Kit (Southern Cross Biotechnologies, South Africa) according to the manufacturer's recommendation.

Explant preparation and transformation: Sorghum public line P898012 was grown until flowering in a greenhouse in soil mix of red soil, rough sand and compost in equal proportion. Panicles were harvested 12-14 days after pollination and surface-sterilized in 70% (v/v) ethanol for 3 and 15 min in 2.5% sodium hypochlorite solution containing 0.1% (v/v) of the surfactant Tween 20 before a thorough rinse with sterile distilled water. Immature zygotic embryos (IZE), 0.8-1.2 mm long were aseptically excised from these seeds and used to initiate type-I embryogenic callus cultures. Briefly, the IZEs were placed on Callus Induction Medium (CIM) with the scutellum side facing up and the embryogenic axis in contact with the medium for 6-8 days prior to transformation. The CIM used prior to selection contains L3 based salts and vitamins, 20 mM L-proline, 2.5 mg L^{-1} of the auxin 2,4-dichlorophenoxyacetic acid, the carbon source maltose and 4 g L^{-1} Gelrite as the gelling agent.

For transformation the Particle Inflow Gun (PIG) was used on IZEs that were pre-cultured for 6-8 days on CIM (0-1 cm diameter) and then placed in the middle of a 9 cm petri dish containing CIM supplemented with 0.2 M D-sorbitol and 0.2 M D-mannitol for 3-4 h as described by Vain *et al*. (1993). Bombardment mixtures were prepared by the precipitation of plasmid DNA on 1 μm tungsten particles with 2.5 mM $CaCl_2$ and 0.1 M spermidine-free base as described by O'Kennedy *et al*. (1998). Sixteen hours post bombardment, all calli were transferred to CIM without osmoticum. For plasmid DNA delivery the following parameters were used: helium pressure of 900 kPa, 0.16 μg $shot^{-1}$ plasmid DNA, 500 μm nylon mesh screen placed 8 cm above the target tissue and a vacuum of approximately -87 kPa was applied with a time duration of 50 m sec.

Selection and regeneration of transformants: Bialaphos selection was initiated seven days after bombardment by

placing the formed embryogenic calli on bialaphos (2 mg L^{-1}) containing CIM for four weeks. Thereafter, cultured embryos that produced somatic embryos were transferred to callus maturation and subsequently plant regeneration and root formation media with two-week subculturing intervals.

Similarly, mannose selection was initiated at seven days after bombardment by transferring calli to CIM containing 9 g L^{-1} mannose and 12 g L^{-1} maltose for four weeks. For callus maturation, they were subcultured onto RRM for two weeks with the same mannose concentration but double the maltose concentration (24 g L^{-1}), followed by a two week period with the initial selection regime (9 g L^{-1} mannose and 12 g L^{-1} maltose). For both selection systems the growth chamber temperature and light conditions for both callus induction and regeneration were 25°C and 1.8 μE m^{-2} sec^{-1}, respectively. However, regenerating shoots (>1 cm long) were placed under full light conditions 18 μE m^{-2} sec^{-1}. Rooted plantlets (4-6 cm long) were hardened off in the greenhouse and analyzed by PCR.

DNA extraction: Genomic DNA was extracted from putative transgenic sorghum leaf material using the mini extraction procedure of Dellaporta *et al.* (1983).

PCR and southern analysis: For PCR analysis of putative bialaphos resistant plants the primers: BAR forward: 5′-CATCGAGACAAGCACGGTCAACTTC-3′ and BAR reverse: 5′-CTCTTGAAGCCCTGTGCCTCCAG-3′ were used to amplify the *bar* gene while the primers: PMI forward: 5′-CGTTGACTGAACTTTATGGTATGG-3′ and PMI reverse: 5′-CACTCTGCTGGCTAATGGTG-3′ were used to amplify the *manA* gene.

For Southern analysis, five micrograms of sorghum genomic DNA from a representative 8 transgenic plants, was digested with *Hind*III and separated on agarose gel and analyzed as described by O'Kennedy *et al.* (2004). A PCR digoxigenin (DIG)-labeled probe (*bar* or *manA* gene cassette) was prepared using the PCR DIG probe synthesis kit as described by the supplier (Roche Diagnostics, South Africa).

BASTA leaf painting assay: Putative bialaphos resistant lines were confirmed to be transgenic and expressing the *bar* gene using a BASTA leaf painting assay. Seedlings were assayed when 4-5 leaves had fully emerged from the whorl. A solution of 1% (w/v) BASTA, 0.1% (v/v) Tween 20 was applied to the upper and lower surfaces of the first fully emerged leaf. The plants were scored three days after painting.

RESULTS

In this experiment we used a public genotype (P898012) to generate transgenic plants using particle bombardment and two selection systems: bialaphos or mannose selection. Out of a total of 2609 IZEs (Table 1) that were bombarded for *bar* selection, 12 plants survived the bialaphos selection but only three were PCR positive, (Fig. 1a, b). Results from the bialaphos leaf painting assay showed that the leaves from the three PCR positive plants were green and healthy after being exposed to BASTA, while leaves from the other nine plants were burnt and yellowish. This result confirmed that expression level of the *bar* gene was high enough to confer resistance to the herbicide BASTA even at the plant level. The data suggests a transformation efficiency of 0.12% and 75% escapes. Interestingly, the embryos cultured on *bar* selection produced more phenolic compounds compared to embryos without selection or on mannose selection which necessitated more frequent subculturing.

In comparison to the bialaphos selection system, 27 putative transgenic plants (Table 1) recovered with

Table 1: Efficiency of biolistic transformation of Sorghum using either bialaphos or mannose selection

Parameters	Bialaphos	Mannose
No. of IZE	2609	3499
No. of resistant plants	12	61
No. of transgenic plants	3	27
No. of escape plants	9	0
Transformation efficiency (%)	0.11	0.77

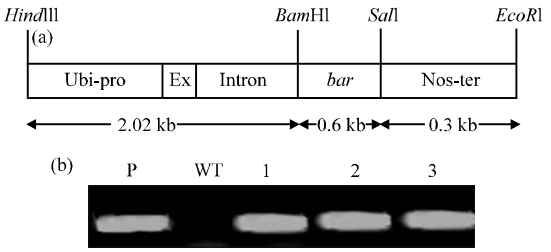

Fig. 1: Molecular analysis of independent bialaphos resistant T_0 plants. (a) Schematic diagram of pAHC25 construct used for sorghum transformation. The pAHC25 (9706 bp) plasmid contains the *bar* gene, encoding bialaphos resistance under the control of the maize Ubi1 promoter (Ubi-pro), first exon (*Ex*) and the first intron and the nopaline synthase terminator (Nos-ter). (b) PCR analysis of T_0 plants. Lanes P- positive control (pAHC25 plasmid DNA), WT: Wild type sorghum plant DNA and lanes 1-3: Three independent transformants

mannose selection were also PCR positive (0.77% transformation efficiency). Southern blot analysis with a *manA* gene probe showed two different gene integration patterns in the 8 plants analyzed. The presence of distinctive bands (Fig. 2a-c) demonstrates integration of the transgene in the plant genome. Figure 2b and c show PCR and Southern analysis results of a representative eight of the *pmi* expressing transgenic plants. For the purpose of this study multiple positive plants regenerated from a single embryogenic cell were considered the same transgenic event. Mannose selection therefore resulted in a 6.4-fold improvement in transformation efficiency compared to bar. Moreover, the mannose selection pressure gave a 100% inhibition on non-transformed callus cultures at 6 weeks after selection. Further, the transgenic plants generated using the two systems did not show any phenotypic differences attributable to the transgenes. In summary, our results show that more transgenic plants were recovered with mannose selection compared to that obtained with bar selection using particle bombardment experiment in sorghum IZEs.

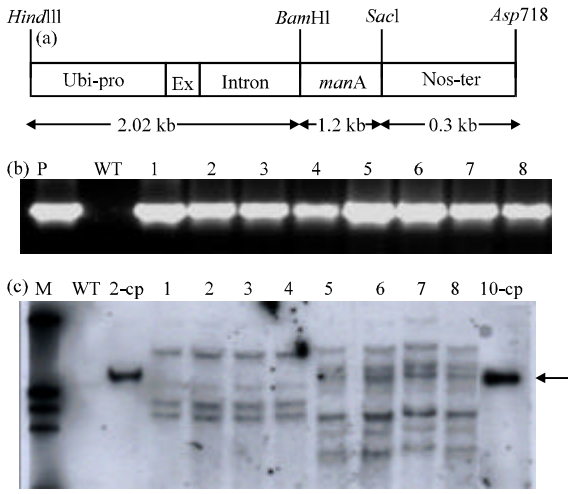

Fig. 2: Molecular analysis of independent mannose resistant T_0 plants. (a) Schematic diagram of pNOV3604ubi construct used for sorghum transformation. The pNOV3604ubi (6210 bp) plasmid contains the *man*A selectable marker gene under the control of the maize Ubi1 promoter (Ubi-pro), first exon (*Ex*) and the first intron and the nopaline synthase terminator (Nos-ter). (b) PCR analysis of T_0 plants. (c) Southern blot analysis of T_0 plants. The blot was hybridized to DIG-labeled PCR *man*A probe. Plasmid DNA representing two copies (2-cp) or ten (10-cp) of the introduced transgene was mixed with *Bam*H1 digested genomic DNA from WT plant (WT), BamHI digested transgenic T_0 plant DNA (5 μg L^{-1}ane) from eight mannose resistant plants (1-8). Lanes P: Positive control (pNOV3604) and M: Molecular weight marker

DISCUSSION

In this study we compared the *bar* and *manA* selection systems for generating transgenic sorghum via particle bombardment. The transformation efficiency of 0.11% we achieved with the *bar* gene is within the range previously reported in the literature for sorghum using biolistic techniques (Casas *et al*., 1993; Able *et al*., 2001). However, we observed a high number of escapes about 75% of the putative transgenics even under the optimum concentration of bialaphos (2 mg L^{-1}). This is higher than reported by Casas *et al.* (1993) using bialaphos concentration of 1 to 3 mg L^{-1} in maintenance media. It is possible that the poor efficiency of selection in our study could be a combination of genotype of sorghum that we used for transformation and growth conditions. Nevertheless, the *bar* gene has now been reported as a leaky selection system under certain conditions (Gao *et al*., 2005).

This study also investigated a positive selection system based on the *ManA* gene and 0.77% transformation efficiency was achieved, which is a 6.4-fold increase when compared with the bialaphos selection system. In addition, no escapees were found when the mannose selection was applied. The importance of reducing escapees in the transformation systems should be overemphasized. Reduction in number of escapees has a concomitant effect on saving time and resources necessary for the analysis of transformed plants (O'Kennedy *et al*., 2004). Furthermore, we observed that the embryos cultured on bar selection produced more phenolic compounds compared to the embryos without selection or mannose selection. It could be that the cells were subjected to stress through the conversion of the selective agent *bar* to a detoxified derivative and might have in turn, started to release phenolic compounds; unlike in mannose selection were cells convert the sugar and is readily available giving the cells a metabolic advantage.

However, the transformation efficiencies achieved in this study are still below the transformation efficiencies already reported for *pmi* in sorghum. Using *Agrobacterium*-mediated gene transfer, Gao *et al.* (2005) reported an average transformation efficiency of 2.91, with a range of up to 2.28 and 3.3% using genotypes Pioneer 8505 and C401, respectively. However, there's very limited

information on sorghum transformation, either *Agrobacterium*-based or biolistics and therefore efforts to rectify are commendable.

Our findings have biosafety implications in South Africa where there is little information available on transgenic systems of indigenous crop plants. The potential risk of the transfer of antibiotic and herbicide resistance genes from genetically modified crops into the environment or gut of microbes (Gao *et al*., 2005) has necessitated the shift towards positive selection systems especially for sorghum, a crop with several wild relatives in Africa. Moreover, there is a general concern that the *bar* gene can be transmitted via pollen to wild relatives of sorghum producing herbicide resistant weeds such as Johnson grass and shattercane. Consequently, *bar* resistant commercial sorghum might be difficult to pass the regulatory huddle especially in South Africa. Furthermore, the *pmi* gene as a selectable marker has biosafety advantages. It has been reported that it has no allergenic or any sequence homology to known oral allergens and no endogenous PMI activity has been detected in grasses, making it the best candidate for generating transgenic food crops.

In conclusion, we generated transgenic sorghum plants using bar and *pmi* genes and demonstrated that the latter produces better transformation efficiencies and has a more appealing biosafety profile. To our knowledge this is a first report comparing the bar and *pmi* based selection systems using biolistic transformation of a public sorghum line, under the same experimental conditions.

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