Chapter 3

Transformation of sorghum for suppression of selected seed proteins

3.1 Abstract

Sorghum [*Sorghum bicolor* (**L.**) Moench] is recalcitrant to gene manipulation, thus, few laboratories have reported reliable and reproducible transformation methods. Because of biosafety concerns for using the herbicide and antibiotic resistance genes, the selection of a selectable marker for selection of transgenic tissue and plants is very important. Two tissue culture selection systems that employ the *bar* gene, encoding bialaphos resistance, and the *manA* gene, for phosphomannose isomerase, both under the control of maize ubiquitin promoters and nopaline synthase terminator sequences were compared to improve the transformation efficiency of sorghum via particle bombardment using a particle inflow gun. A total of 3 and 27 transgenic plants were recovered for bialaphos and mannose selection, respectively. This translates to transformation efficiencies of 0.11% and 0.77% for the *bar* and *manA* genes, respectively. The expression of the *bar* gene in the three transgenic plants was demonstrated by the BASTA leaf painting assay. Results further indicated that 75% of putative bialaphos resistant plants were escapees, while none was recorded for *manA* gene. Using the mannose selection system, the RNAi approach was selected as a strategy to suppress a subset of seed endosperm proteins that are implicated in poor lysine content and protein digestibility. The target genes, all carried on the same expression cassette, were co-expressed as an RNAi construct into the sorghum genome by particle bombardment using the Bio-Rad gun (Biolistic PDS-1000/He). A total of 11 independent transgenic events were generated at an average transformation efficiency of 3.36%. The PCR and Southern blot hybridization analyses of seven selected transgenic events revealed a 100% stable co-integration of the selectable marker gene with the target expression construct. Transgenic lines 2 and 4 displayed multiple transgene insertions, i.e. more than five copies, while events 1, 3 and 5 displayed simpler transgene integrations, i.e less than five copies. Two transgenic events,

namely 6 and 7 show transgene rearrangement or truncation at the 5' promoter end of the transgene cassette.

3.2 Introduction

A suggestion was aired by Birch and Bower (1994) that one of the most important aspects for successful particle bombardment in generating transgenic plants was an efficient selection of transformants. In sorghum, for a long time, the selectable marker gene *bar*, an herbicide resistance gene was used in most sorghum transformation reports. The *hpt* (hygromycin phosphotransferase) gene was only used once by Hagio (1991). The use of the *pmi* (phosphomannose isomerase) gene was only recently reported by Gao *et al*. (2005). In optimizing the biolistics technique, there are a number of parameters that have to be considered in order to maximize the transformation efficiency output. These include the bombardment distance, the preculture period, the explant type, the DNA concentration and helium pressure. As already reviewed in Chapter 1, the *bar* gene was isolated from *Streptomyces hygroscopicus*. It encodes the enzyme PAT that confers resistance to the herbicides BASTA and bialaphos. This gene has been used to select for stable transgenic sorghum plants (Casas *et al*., 1993, 1997; Zhu *et al*., 1998; Zhao *et al*., 2000; Able *et al*., 2001; Emani *et al*., 2002).

The *E. coli pmi* gene enables plant cells to metabolize mannose (Miles and Guest, 1984). The mannose selection system is considered superior to the bialaphos selection system due to the selection process mode of action. The *pmi* selectable marker gene has been successfully used in plant transformation (Joerbo *et al*., 1998; Negrotto *et al*., 2000; Wright *et al*., 2001; Lucca *et al*., 2001; Wright *et al*., 2001; O'Kennedy *et al*., 2004; Gao *et al*., 2005) and there is strong

indication that this selection system results in higher transformation efficiency in comparison to the bialaphos selection system. The use of the biolistics method employing the Bio-Rad and the mannose selection system will yield a transformation efficiency that exceeds 2.5%.

To date, 9 of the eleven reports for stable sorghum transformation included a reporter gene and/or visual marker gene expression. This includes expression of the *gus* and *gfp* genes (Hagio *et al*., 1991; Casas *et al*., 1993 and 1997; Able *et al*., 2001; Emani *et al*., 2002; Godwin and Chikwamba, 1994; Zhao *et al*., 2000; Gao *et al*., 2005; Howe *et al*., 2006). Only two reports involve genes for agronomically important traits, viz. *chitinase*, which impacts pathogen resistance (Zhu *et al*., 1998), and *Cry1Ac*, which confers insect resistance (Girijashankar *et al*., 2005). Both reports formed a strong basis for improving sorghum cultivation for agricultural purposes. In addition, only one report involves the use of the positive selectable marker gene *ManA* (Gao *et al*., 2005) via *Agrobacterium*-mediated transformation and resulting in the highest reported average transformation efficiency of 2.91%, ranging up to 3.3, to date has been reported using this method. This chapter is divided into two parts. The objective for the first part was a comparison between the *bar* and *manA*-based selection systems to optimize the sorghum transformation using particle bombardment. The objective for the second part was to transform sorghum with a dual trait expressing DNA construct (RNAi vector) for the improvement of seed nutritional value through the elevation of the lysine content as well as enhancement of protein digestibility via down-regulation of a subset of kafirins and the lysine ketoglutarate reductase (LKR) genes. The RNAi hairpin construct used targets the co-suppression of the enzyme LKR and selected kafirins (δ-kaf-2, γ-kaf-1 and -2) to improve lysine content and seed protein digestibility.

3.3 Materials and Methods

3.3.1 Explant preparation and transformation

Sorghum public line P898012 was grown in the greenhouse in a soil mix of red soil, rough sand and compost (1:1:1) until flowering. Seeds were harvested 12-14 days after pollination and surface-sterilized in 70% (v/v) ethanol for 3 min 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 in length, were excised from these seeds and used to initiate type-I embryogenic callus cultures as described by O'Kennedy *et al*. (2004). For this, the IZEs were placed on CIM with the scutellum side facing upwards 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 of the auxin 2,4-dichlorophenoxyacetic acid (2,4-D), the carbon source maltose, and 4 g/l gelrite as the gelling agent (see Chapter 2 and annexure).

3.3.2 Plasmids

Two plasmids were used for transforming sorghum. Plasmid pAHC25 (Christenson and Quail, 1996) contains the bialaphos resistance gene *bar* (Figure 3.2 A). The plasmid pNOV3604-ubi, obtained from Syngenta, USA, carries the *man*A gene (O'Kennedy *et al*., 2004) which confers resistance to mannose selection (Figure 3.1 A). Both genes are driven by the maize ubiquitin promoter and the nopaline synthase terminator (Nos-ter). All plasmid DNA preparations were carried out using the Qiagen Maxiprep Kit (Southern Cross Biotechnologies, South Africa) according to the manufacturer's recommendation.

The plasmid pABS encoding the RNAi co-suppression cassette is shown in Figure 3.1 A, B and C. This was generously supplied by Drs Rudolf Jung and Kimberly Glassman from Pioneer Du Pont, Iowa, USA (members of the ABS consortium). The seed endosperm specific promoter from maize 19GZ was used to drive the co-suppression of the LKR, δ-kaf-2, γ-kaf-1 and -2 genes. The rice ADH1 intron was used as a hairpin part of this double stranded DNA transgene. The target genes were isolated from a cDNA library of a developing seed. cDNA clones from a developing seed were analyzed and sequenced to generate expressed sequence tags (EST). These ESTs were classified on the basis of sequence homology to known protein sequences. Domains for silencing targeted genes were selected from the EST sequences (Jung, 2007) and the 5'-3' sense strand sequences are displayed in Figure 3.1 C. Selected domains of the LKR and kafirin genes were cloned in tandem and designed into a hairpin construct in which the tandemly cloned domains and the inverted versions are separated by the ADH-1 intron to form a loop and cloned into a binary vector through Gateway cloning (Invitrogen, USA).

The γ-kaf-1 gene sequence translates into a 186 amino acid long protein that contains only one lysine residue and a total of 12 sulphur containing cysteine residues (see NCBI protein sequence database, Accession number AAS73290. The γ-kaf-2 protein is a 211 amino acid long preprotein that comprises one lysine and 13 cysteine residues (Accession number CAA44347). The δ-kaf-2 protein is 187 amino acid long containing 2 lysine and 14 cysteine residues. This δ-kaf-2 protein sequence has not been identified and reported into any database to date. These three target proteins are implicated as major contributors to the seed lysine content deficiency problem and

the low seed protein digestibility due to their high content of cysteine, thus, increasing the di-sulphide bond formation potential with other proteins.

To create plasmid pABS (Figure 3.1 B) minimal transgene cassettes (MTCs), the full plasmid was prepared by endonuclease digestion with E*co*RI to remove the backbone carrying the kanamycin resistant gene. For the *pmi* MTC, a double digest of A*sp* 718 and H*ind* III released the backbone carrying the ampicillin resistance gene. These kafirins were identified and chosen on the basis of their inherently poor lysine content and contribution to poor protein digestibility as a result of cross-linkages with other polymers in grain endosperm.

3.3.3 Transformation

3.3.3.1 *Transformation with bar and pmi*

The particle inflow gun (PIG) was used for bombarding 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 plate containing CIM supplemented with 0.2 M D-sorbitol and 0.2 M D-mannitol for 3-4 hrs 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 hrs post bombardment, all calli were transferred to CIM without D-sorbitol and D-mannitol. For plasmid DNA delivery the following parameters were used: helium pressure of 900 kPa, 0.16 µg/shot 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 timer duration of 50 millisec.

3.3.3.2 *Transformation with RNAi construct*

The Bio-Rad gun (Biolistic PDS-1000/He) was used for transformation of IZEs. Bombardment experiments were carried out with both the selectable marker and target constructs introduced into the same bombardment mixture. Each co-bombardment mixture contained the target and selectable marker genes (RNAi and the *pmi*) of 22 ng and 18 ng, respectively. A total of 222 and 105 IZEs were co-bombarded with MTCs and closed circular plasmid constructs, respectively. Bombardment mixtures were prepared by the precipitation of plasmid DNA on 0.7 µm gold particles with 2.5 mM $CaCl_2$ and 0.1 M spermidine-free base. All IZEs were placed on osmoticum medium (0.2 M D-sorbitol and 0.2 M D-mannitol) for 3-4 hrs before bombardment. Sixteen hrs post bombardment, all calli were transferred to CIM without osmoticum. For plasmid DNA delivery, a Helium pressure of 900 kPa and a total plasmid DNA of 0.09 µg/shot were used, i.e. 0.05 µg target genes + 0.04 µg selectable marker gene. Bombardments were carried out by following the manufacturer's (Bio-Rad) recommended method with a chosen distance of 7.5 cm between the macro-carrier and the target tissue. Proliferating IZEs subjected to bombardment treatment were placed back on CIM for seven days before the selection stage.

3.3.4 Selection and regeneration of transformants

3.3.4.1 *Bialaphos*

Bialaphos selection was initiated seven days after bombardment by placing the formed embryogenic calli on bialaphos (2 mg/l) containing CIM for four weeks. Thereafter, cultured embryos that produced somatic embryos were transferred to medium J callus maturation, cultured for two weeks (2 mg/l) and subsequently on medium J RRM (2 mg/l) with a two-week sub-culturing intervals until rooted plantlets formed.

3.3.4.2 *Mannose selection*

Similarly, mannose selection was initiated at seven days after bombardment by transferring calli to CIM containing 9 g/l mannose and 12 g/l maltose for four weeks. For callus maturation, they were subcultured onto medium J maturation for two weeks with the same mannose concentration but with double the maltose concentration (24 g/l), followed by a two-week period with the initial selection regime (9 g/l mannose and 12 g/l maltose) on RRM. 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^2s^1, respectively. However, regenerating shoots (≥ 1 cm long) were placed under light (18 µEm/$^2s^1$). Rooted plantlets (4-6 cm long) were hardened off in the greenhouse and analyzed by PCR. Due to the phenolic compounds produced by some sorghum cultures, sub-culturing was performed every seven days as required.

3.3.5 DNA extraction

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

3.3.6 PCR analysis

The PCR reaction mix consisted of: 50 ng genomic DNA or 1ng plasmid (pABS) DNA, 1/10 of the final reaction volume of a 10x PCR buffer (Bioline, Inqaba Biotech, SA), 1.5 mM MgCl, 160 µM dNTPs, 400 nM forward primer, 400 nM reverse primer, 4 units *Taq*-DNA-polymerase (Bioline, Inqaba Biotech, SA), and H_2O to a final volume of 25µl.

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. The primer annealing temperatures for the *bar* and *pmi* genes were 64°C and 60°C, respectively.

For PCR analysis of the pABS transgene, a 503 bp fragment was amplified using the following primers: forward 5'-GTTACGTGACCCGGACCGAA-3' and reverse 5'-ACGCCGAAGATCGCCTGGTA-3'. This sequence region is the 3' end of the transgene, hereby called the confirmation region (c-r) region (PCR 2 region in Figure 3.1 B).

A thermo-block from PE Applied Biosystems (GeneAmp PCR System 9700) was used to perform PCR reactions using the following parameters: Denaturation of DNA at 94°C for 5 min, followed by 10 cycles of DNA denaturation at 94°C for 30 sec, primer annealing at 65°C for 45 sec and DNA extension at 72°C for 59 sec, followed by 25 cycles of DNA denaturation at 94°C for 30 sec, primer annealing at 55°C for 45 sec and DNA extension at 72°C for 59 sec and a final DNA extension at 72°C for 7 min, after which the samples were held at 4°C until analyses by gel electrophoresis.

3.3.7 Southern blot analysis

The Southern blot analysis of putative plants for the *bar* and *pmi* genes was carried out as follows: 5 µg of sorghum genomic DNA were digested with the restriction enzyme *Hind*III and

the digests were separated on 0.8% agarose gel and blotted as described by O'Kennedy *et al*. (2004). A PCR digoxigenin (DIG)-labelled probe (*bar* or *manA* gene cassette) was prepared using the PCR DIG probe synthesis kit using the PCR primers and conditions already stated in section 3.3.6 as described by the supplier (Roche Diagnostics, SA). Following separation by 0.8% agarose gel electrophoresis at 60V for 3-4 hrs, DNA was transferred and fixed onto positively charged nylon membranes from Roche (Sambrook *et al*., 1989), pre-hybridization for 3-4 hrs (5 X SSC, 50% formamide, 0.1% sodium-lauroylsarcosine, 0.02% SDS, 2% Bio-Rad blocking reagent). Probe hybridization was performed for 16 hrs at 42°C. Following membrane washes at 65°C (0.5 X SSC, 0.1% SDS), membrane blocking was done for 45 min with blocking buffer (1% blocking reagent, 0.1M maleic acid, 0.15M NaCl pH 7.5). The unbound probe was thoroughly washed-off with wash buffer (0.1M maleic acid, 0.15M NaCl pH 7.5, 0.3% Tween-20). Probe-target DNA fragment binding was detected by using the Bio-Rad CDP-Star secondary antibody (Bio-Rad, South Africa). The membranes were exposed on X-ray films (Kodak, USA) and developed at room temperature for visualization.

For the RNAi construct southern blot analysis, two different probes were prepared. The z-k target sequence mentioned above and the c-r targeting the 3' end (see the PCR 2 region in Figure 3.1). The c-r probe was prepared using the primers stated in section 3.3.6. The DNA probe for the z-k region was PCR DIG-labelled using the following primers: forward 5′-ACAATGATGAGCCTCCTATG-3′; and reverse 5′-CCTTTATGTTGGCATCAAAA-3′. The PCR reaction mixture was the same as mentioned in section 3.3.6 but the cycle parameters used were as follows: Denaturation of DNA at 94°C for 5 min, followed by ten cycles of DNA denaturation at 94°C for 30 sec, primer annealing at 60°C (pABS) for 45 sec and DNA extension

at 72°C for 59 sec, followed by 25 cycles of DNA denaturation at 94°C for 30 sec, primer annealing at 50°C for 45 sec and DNA extension at 72°C for 59 sec and a final extension at 72°C for 7 min, after which the samples were held at 4°C until analyses by gel electrophoresis.

After the autoradiography step, the z-k probe was stripped off from the membranes using a stripping buffer (0.1 X SSC, 0.1% SDS) at 90-95°C for 10 min. The membranes were re-probed with the c-r specific probe to investigate the extent of transgene integration, i.e. to determine if any DNA re-arrangements took place on the transgene in the process of integration. This is believed to be especially prevalent when using MTCs instead of full circular plasmid constructs (Fu *et al*., 2000; Kohli *et al*., 1998; Pawlowski and Somers, 1998). After stripping off the c-r probe, the membranes were re-probed for the *pmi* gene.

3.3.8 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 plant. 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. Negative plants show yellowing and necrosis on the leaves, while the leaves from the resistant lines remain green and healthy. This indicates not only that the *bar* gene in plasmid pMON19477 was expressed in these plants, but also that the expression levels were high enough to confer resistance to the herbicide BASTA® at the plant level.

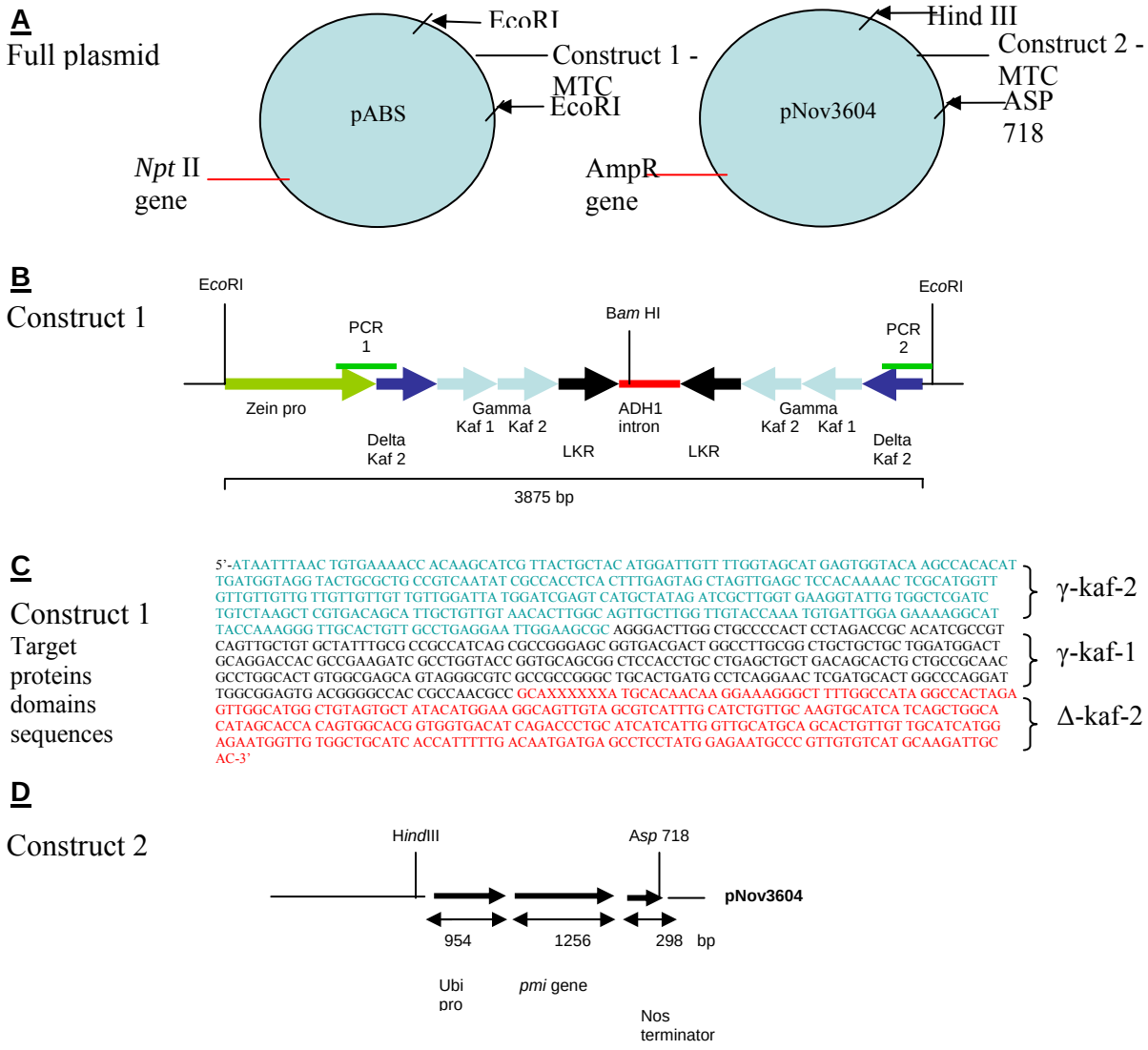

Figure 3.1 Maps of constructs used for transformation of sorghum P898012 IZEs. (A) Whole, circular, closed plasmid versions with backbone DNA sequences present. (B) The MTC construct for the co-suppression of 3 kafirins (1 δ- and 2 γ-kafirins) and LKR proteins, (C) The selected target gene domains sequences to generate siRNAs for the 3 kafirin genes, and (D) selectable marker gene, *pmi*.

3.4 Results

3.4.1 Transformation with *bar* and *pmi* genes

In this part of the study, the public line P898012 was used to generate transgenic plants using particle bombardment and two selection systems: bialaphos or mannose selection. Out of a total of 2609 IZEs (Table 3.1) that were bombarded for bialaphos selection, 12 plants survived the bialaphos selection but only three plants were PCR positive showing insertion of the *bar* gene (Figure 3.2 B). Results from the BASTA® 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 (Figure 3.3). This result confirmed that the expression level of the *bar* gene was high enough to confer resistance to the herbicide BASTA®. This data display a transformation efficiency of 0.12% and 75% escapes. Also, the embryos cultured on bialaphos selection produced more phenolic compounds compared to embryos cultured on medium without selection or on mannose selection. This necessitated more frequent subculturing of every 7 days.

In comparison to the bialaphos selection system, the 27 putative transgenic events (Table 3.1) recovered with the mannose selection were all PCR positive (0.77% transformation efficiency). No false positive plants escaped the selection system. These plants displayed different gene integration patterns when analyzed by Southern blotting, while some showed identical integration patterns (Figure 3.4).

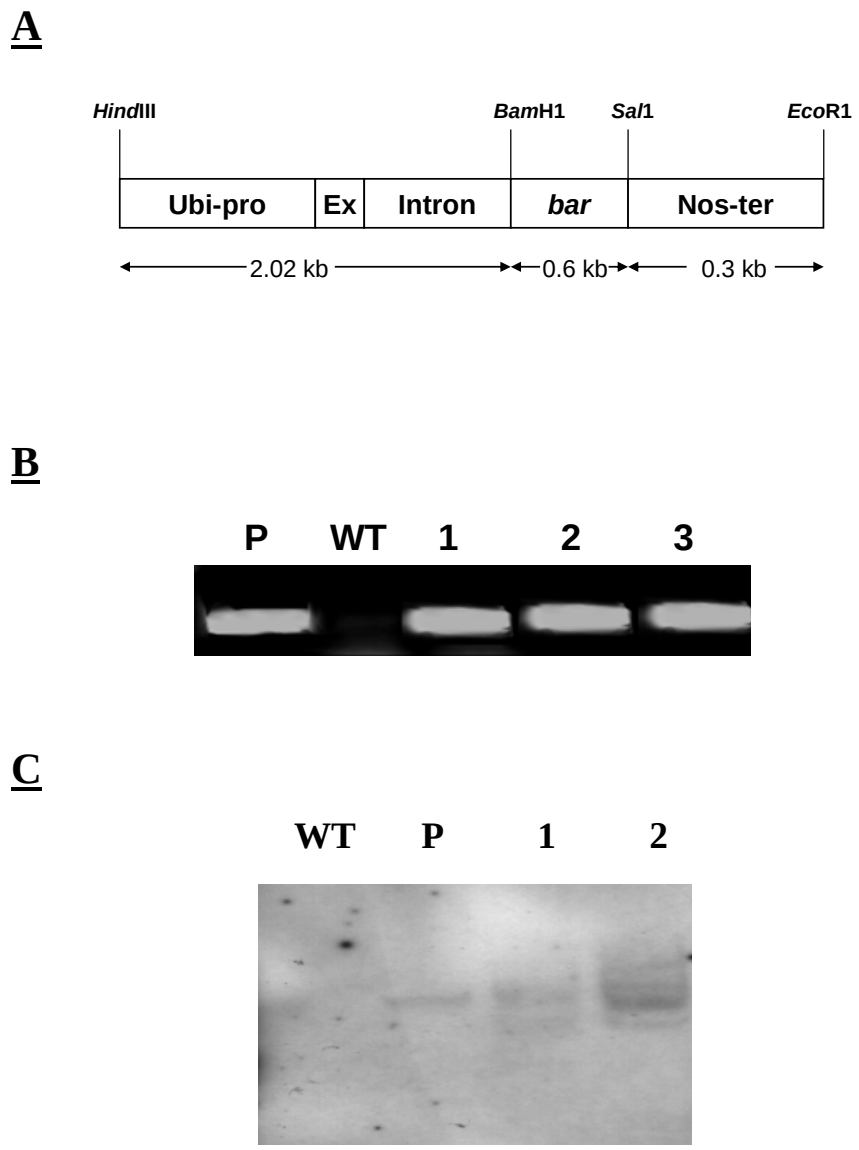

Figure 3.2 Molecular analysis of independent mannose resistant T_0 plants. (A) Schematic diagram of plasmid pAHC25 construct used for sorghum transformation. The pAHC25 (9706 bp) plasmid contains the *bar* gene, encoding BASTA® resistance under the control of the maize Ubi1 promoter (Ubi-pro), first exon (*Ex*), 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 represents wild-type sorghum plant DNA, and 1 to 3 – putative transgenic plants. (C) Southern blot analysis of T_0 plants. The blot was hybridized with DIG-labeled PCR *bar* DIG-labelled probe. Plasmid DNA representing two copies of the introduced transgene was mixed with *SacI* digested genomic DNA from WT plant (WT), *Sac*I digested transgenic T_0 plant DNA (5 µg/lane) from 2 of the 3 transgenic plants. Lane P represents positive control (pAHC25).

C T_0 T_0

Figure 3.3 T_0 transgenic plant leaves displaying resistance to a 2% BASTA® painting in comparison to a control plant leaf (C). Susceptibility or resistance was determined five days after painting with the herbicide BASTA®.

Table 3.1 Efficiency of biolistic transformation of sorghum using either bialaphos or mannose selection.

Parameters	**Bialaphos**	**Mannose**
Number of IZE	2609	3499
Number of resistant plants	12	61
Number of transgenic events	3	27
Number of escape plants	9	0
Transformation efficiency %	0.11	0.77

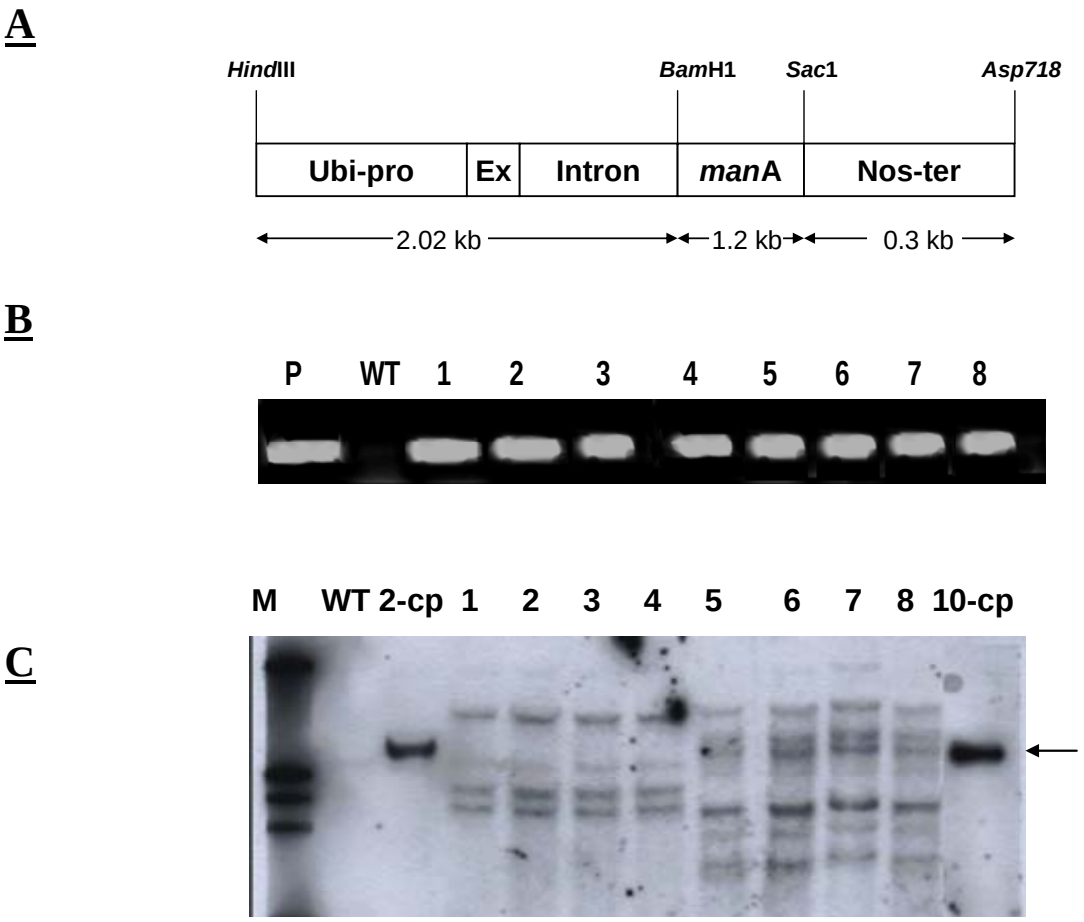

Figure 3.4 Molecular analysis of independent mannose resistant T_0 plants. (A) Schematic diagram of pNOV3604ubi construct used for sorghum transformation. The pNOV3604ubi (6210 bp) plasmid contains the *man*A selectable marker gene under the control of the maize Ubi1 promoter (Ubi-pro), first exon (*Ex*) and the first intron and the nopaline synthase terminator (Nos-ter). (B) PCR analysis of T_0 plants. (C) Southern blot analysis of eight T_0 plants, representing two transgenic events. The blot was hybridized with DIG-labelled PCR *man*A DIG-labelled probe. Plasmid DNA representing two (2-cp) and ten copies (10-cp; arrow) of the introduced transgene was mixed with *Hind*III digested genomic DNA from wild-type plant (WT), *Hind*III digested transgenic T_0 plant DNA (5 µg/lane) from eight *pmi* expressing plants (1-8). Lane P represents positive control (pNOV3604) and lane M represents DNA size marker. Approximately four to eight *ManA* gene copy integrations were observed.

Figures 3.2 and 3.4 (B and C) show PCR and Southern blot analysis results of a representative two *bar* and eight *pmi* expressing transgenic plants, respectively. Mannose selection resulted in a 6.4-fold improvement in transformation efficiency compared to bialaphos selection. Moreover, the mannose selection pressure gave a 100% inhibition on non-transformed callus cultures six weeks after selection. Further, the transgenic plants generated using the two systems did not show any visible phenotypic differences.

3.4.2 Transformation with RNAi construct

3.4.2.1 *Recovery of transgenic plants*

Two different transformation experiments were conducted. In the first experiment, MTCs, both target and selectable marker genes, were used in co-bombardment experiments. The second experiment involved closed, whole circular plasmid constructs (see Table 3.2 and Figure 3.1) in a mixture with the selectable marker gene. Fertile T_0 plants were produced through successful type I callus formation that resulted in rooted plantlets within a period of 150 days (Figure 3.5). The definition of transformation efficiency is based on the percentage of the number of independent events recovered per explant number bombarded. Using MTCs, 7 events resulting in 19 plants at a transformation efficiency of 3.2% were recovered while 4 transgenic events, resulting in 42 plants, were produced at 3.8% transformation efficiency for full plasmids (Table 3.2).

3.4.2.2 *PCR and Southern blot hybridization*

Based on plant and seed availability, seven transgenic events were chosen for further analysis. The PCR and Southern blot hybridization results of these events are shown in Figures 3.6 and

3.7, respectively. Until the Southern blot hybridization results were obtained that displayed different integration patterns for these plants, they were assumed identical (result from same callus cell) and were assigned with the same event number. Figure 3.6 shows PCR analyses results of 7 transgenic events, with some having at least 2 plants from the same event analyzed subject to leaf material availability. Transgene presence in the genome was detected by PCR in all chosen samples. For quality control purposes, the negative controls (lanes N and W) displayed no visible bands as expected. The positive controls (lanes P and PS) showed the expected reference DNA product of 503 bp as expected from the primer pairs used.

The Southern blot hybridization results confirmed transgene integration into the genome. The probes used were DIG-labelled by PCR and targeted three transgene regions. One of these targets for probe hybridization was the *pmi* internal fragment sized 965 bp (Figure 3.7, C and F). This was to confirm the selectable marker gene integration. The two target DNA sequences for pABS construct were named the z-k (Figure 3.7, A and D) and c-r (Figure 3.7, B and E). The former forms part of the 5'- end flanking the promoter and the first target kafirin, the δ-kafirin-2. The c-r forms part of the 3'- end of the gene cassette, i.e. the antisense oriented δ-kafirin-2. This approach was adopted to investigate any possible re-arrangements that could have taken place in order to understand the molecular process of gene integration and expression. Transgene re-arrangement at the promoter-end was observed in transgenic events 6 and 7 and these two transgenic events did not show any cross-reaction between genomic DNA fragments and the z-k DNA probe, i.e. no bands in Southern blot analysis. This demonstrated that transgene re-arrangement occurred at the 5'- end of the transcription cassettes.

Table 3.2 Transformation results displaying successful production of transgenic sorghum plants via particle bombardment. Transformation by using MTCs was supplemented by using closed, circular and longer version of the transcription cassettes. Eleven transgenic events were produced at an average 3.36 % transformation efficiency.

Experiment	**Number of explants**	**Number of transgenic events**	**Number of plants**	**Trans-formation efficiency**
Minimal transgene cassettes bombardment	222	7	19	3.2%
Closed, circular plasmids bombardment	105	4	42	3.8%

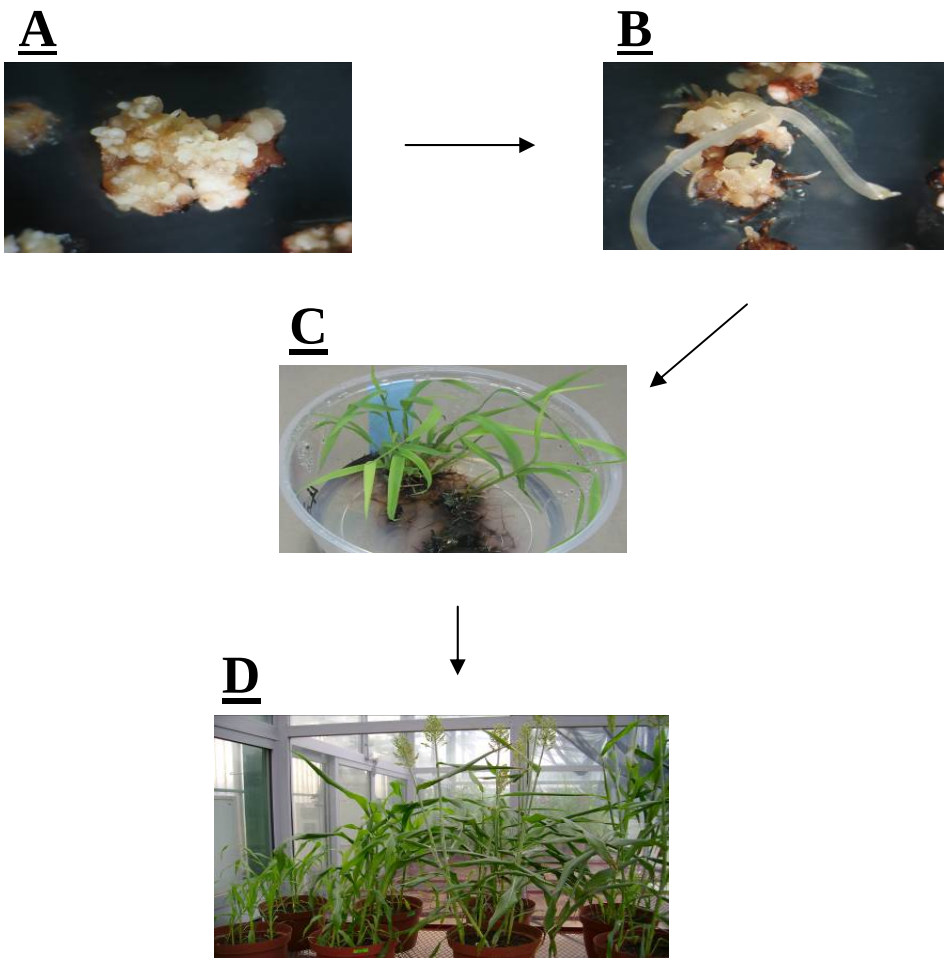

Figure 3.5 Tissue culture and regeneration of putative transgenic plants selected on mannose. (A) Selection of somatic embryos produced by type I callus 28 days after bombardment. (B) Shoot and root formation of mature transgenic callus on regeneration medium 54 days after bombardment. (C) Transgenic plantlets with defined roots and shoots 71 days after bombardment. (D) Fertile T_0 transgenic plants resembling a wild-type plant in the greenhouse 141 days after bombardment.

The effectiveness of the probe stripping process was confirmed by comparison of the binding profiles of the pABS probes (top and middle panels) with the pNov3604 probe (bottom panel, C and F). The profiles were clearly different for most transgenic events. This was confirmed by different integration of the selectable marker gene to the target genes. Therefore, successful probe stripping and re-probing were achieved.

Regarding the copy number determination, the results should be considered as qualitative because only estimated values could be determined. Because the c-r region is duplicated due to the sense and antisense nature of the RNAi construct, it is assumed that the c-r probe will bind both the sense and antisense target sequences, i.e. two times. An estimation of 3 to 12 copies of transgene integration was observed. The copy number estimation takes into account, the two times probe binding for a single transgene insertion. Accurate copy number determination can be performed via a real-time PCR technique.

Contrary to expectations when using MTCs (Fu *et al*., 2000; Kohli *et al*., 1998), events 2 and 4 displayed multiple integrations, while events 1 and 3 show slightly simpler banding patterns. The simplest integration pattern was displayed by one of the two closed, circular plasmid construct, i.e. event 5 (blue lanes). Event numbers 6 and 7 displayed no probe binding for the 5'- end z-k region (D) indicating possible re-arrangement of the transgene cassette's promoter region. However, the 3'- end confirmation region was present in the two events' samples (E). The *pmi*-specific probe also positively confirmed transgene presence.

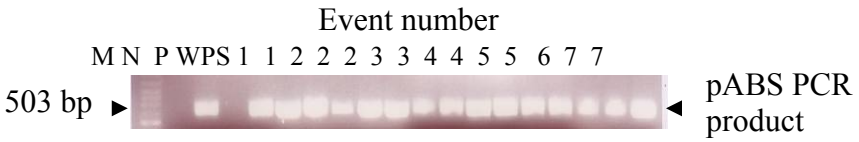

Figure 3.6 The PCR results for 14 of the 61 putative transgenic plants produced by co-bombardment of IZEs with pNov3604 and pABS MTCs. A 1.2% agarose gel electrophoresis was loaded and labelled as follows: (M) 100bp ladder molecular size marker; (N) negative control without template DNA added in a reaction mix; (P) corresponds to 1ng of pABS target construct added as the only template DNA used as a positive control; (W) 50ng wild-type P898012 genomic DNA added as template used as a second negative control; (PS) wild-type P898012 genomic DNA spiked with 1ng of target construct used as a spiked positive control. Fourteen putative transgenic events resulting in 61 plants were produced. Transgene presence was observed in all samples that were analyzed.

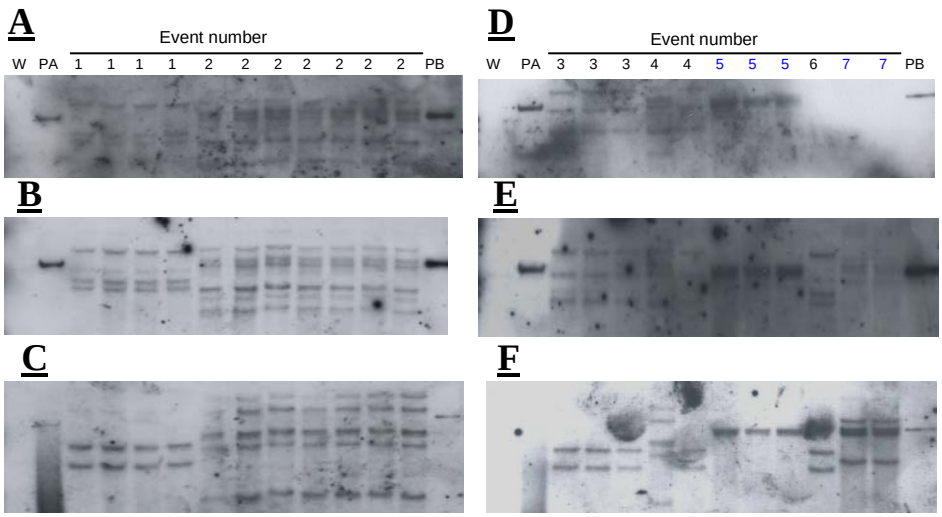

Figure 3.7 The Southern blot hybridization analysis of 22 of the 61 T_0 P898012 plants produced after co-bombardment with pNOV3604 and pABS constructs. (A) The first 11 putative transgenic samples (left panel i.e. A, B and C) were probed for the 5' promoter region, and (B) re-probed for a 3' transgene confirmation region. (C) The last probe used was specific for *pmi*. The last 11 samples (C, D and E – right panel) were analyzed on a second membrane to confirm the complete transgene integration. The same order of probe binding was employed, i.e. (D) for 5' promoter region, (E) for the confirmation region and (F) for the *pmi* gene. A total of 61 plants which resulted in 10 transgenic events were produced. Sample labelling was as follows: (W) wild-type P898012 genomic DNA sample representing the negative control; (PA) wild-type genomic DNA spiked with 2 copies of the pNov3604 and pABS constructs and digested with B*am*HI used as a positive control; (PB) wild-type genomic DNA spiked with 10 copies of the pNov3604 and pABS constructs and digested with B*am*HI used as a second positive control. Events 5 and 7 (lane numbers labelled in blue) were bombarded with closed, circular plasmid.

3.5 Discussion

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. This is especially important for sorghum, a crop with several wild relatives in Africa. Positive selection systems are callus tissue friendly as their mode of action is not poisonous but acts on starving non-transgenic tissue. In this study, a comparison was made between two selection systems for generating transgenic sorghum via particle bombardment. Although the *bar* gene has been so far the commonly used selection marker for sorghum transformation via biolistics, using this gene has resulted in variable transformation efficiencies ranging from 0.08% (Casas *et al*., 1993) to 1% (Able *et al.,* 2001). The result in this study of 0.12% transformation efficiency from the experiment is therefore within the range already described in the literature. However, the *bar* gene creates a leaky selection which results in a high number of escapes (Gao *et al*., 2005). This was also confirmed in this study because 75% of putative transgenic plants were false positives. Moreover, there is a general concern that the *bar* gene can be transmitted via pollen to wild relatives of sorghum, thus, producing herbicide resistant weeds such as Johnsongrass and shattercane. Consequently, using the *bar* gene in commercial sorghum might not pass the regulatory hurdle in South Africa.

The study further explored the use of a positive selection system based on the *ManA* gene. A 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 cannot 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).

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 and *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; this reported transformation efficiency is superior when compared to 0.77% found in this study. However, using the Bio-Rad gun, an improvement of this value to 3.36 was found. This was produced via transformation with an RNAi construct plasmid targeting the introduction of two traits, namely, enhanced seed lysine content through suppression of LKR and seed protein digestibility improvements through down-regulation of δ-kafirin-2, γ-kafirins-1 and -2.

Transgene re-arrangement for transgenic events 6 and 7 was observed. These two transgenic events were produced with MTC and closed circular plasmid bombardment, respectively. However, the extent of this re-arrangement was not established in this study and further sequence probe analysis would be required to elucidate the effects this might have on transgene expression. Transgenic events 6 and 7 were carefully observed in the subsequent transgene expression analyses. This is because the seed-specific promoter used here is expected to direct successful transcription of the double-stranded RNA molecule. Truncation in these transgenic lines is likely to lead to unsuccessful transcription due to the loss of the RNA polymerase II identification site and subsequent binding to kick-start transcription.

MTCs were used in one experiment because they reportedly result in higher transformation efficiencies and simpler transgene integration patterns (Fu *et al*., 2000; Kohli *et al*., 1998). However, closed circular plasmid constructs were also used. Another reason for using full plasmids is the possibility of DNA cleavage or damage at the MTCs DNA ends. Although particle bombardment is extensively used to introduce genes into crop plants, the mechanism of transgene integration is still not well understood. However, a few reports have shed some light on the mechanism of transgene integration (Pawlowski and Somers, 1996; Riggs and Bates, 1986; Bates *et al*., 1990; Finer and McMullen, 1990; Kartzke *et al*., 1990; Kohli *et al*., 1998; Pawlowski and Somers, 1998).

In general, direct DNA delivery frequently results in multiple, intact, and rearranged transgene copies at one genomic locus. For example, in oats, Pawlowski and Somers (1998) reported on the interspersion of transgenic DNA with host genomic DNA at a number of loci and attributed this transgene clustering in one locus to a number of DNA replication forks at that particular locus. The consequence of this is, likely, the lack of successful segregation between the individual transgenes by recombination. This multiple transgene copy integration is therefore the main reason that particle bombardment is considered inferior to *Agrobacterium* method.

During the biolistic transformation procedure, two stages are believed to provide the opportunity for transgene re-arrangement. The first stage is just before the integration into the nucleic genomic DNA and the second stage is the mechanical shearing or damage during particle preparation and bombardment processes. In the former stage, the host cell's nuclease activities are believed to be responsible for these re-arrangements. Due to DNase degradation of

transgenes from the DNA ends, it is expected that whole plasmids would be better protected from mechanical shearing and bombardment process. The endonucleases would also have to act a little longer on whole plasmids than on MTCs with free ends due to longer plasmid DNA sequences, thus, lessening the damage on the transgenes in full plasmids.

For the purposes of this study, the number of integrated transgene copies is not important to pursue. This is due to the endogenous gene silencing approach employed here, instead of a transgene expression approach. The multiple transgene copy integration in the latter approach would likely result in minimal expression due to unintended multiple transgene silencing, while no negative expression effects would be observed in the former approach. In general, information available on sorghum transformation, *Agrobacterium*-based or biolistic, is very limited and efforts to rectify the situation are commendable. This study 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 the best of my knowledge this is a first report of the use of *pmi* in combination with biolistic transformation of a public sorghum line P898012.

3.6 References

Able J.A, Rathus C. and Godwin I.D. 2001. The investigation of optimal bombardment parameters for transient and stable transgene expression in sorghum. *In vitro Cellular and Developmental Biology – Plant* **37**:341-348.

Casas A.M., Kononowicz A.K., Zehr U.B., Tomes D.T., Axtell J.D., Butler L.G., Bressan R.A. and Hasegawa P.M. 1993. Transgenic sorghum plants via microprojectile bombardment. *Proceedings of the National and Academy of Sci*ence USA. **90**: 11212-11216.

Casas A.M., Kononowicz A.K., Haan T.G., Zhang L., Tomes D.T., Bressan R.A. and Hasegawa P.M. 1997. Transgenic sorghum plants obtained after microprojectile bombardment of immature inflorescences. *In vitro Cellular and Developmental Biology* **33**: 92-100.

Christenson A.H. and Quail P.H. 1996. Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Research* **5**: 213-218.

Dellaporta S.L., Wood J. and Hicks J.B. 1983. A plant DNA minipreparation: version II. *Plant Molecular Biology Reports* **1**:19-21

D'Halluin K., De Block M., Denecke J., Janssens J., Leemans J., Reynaerts A. and Botterman J. 1992. The *bar* gene as selectable and screenable marker in plant engineering. *Methods in Enzymology* **216**: 415–426.

Emani C., Sunilkumar G. and Rathore K.S. 2002. Transgene silencing and reactivation in sorghum. *Plant Science* **162**: 181-192.

Gao Z., Jayaraj J., Muthukrishnan S., Claflin L. and Liang G.H. 2005. Efficient genetic transformation of sorghum using a visual screening marker. *Genome* **48**: 321-333.

Gao Z. S., Xie X. J., Ling Y., Muthukrishnan S. and Liang G. H. 2005. *Agrobacterium tumefaciens*-mediated sorghum transformation using a mannose selection system. *Plant Biotechnology Journal* **3**: 591-599.

Hagio T., Blowers A.D., and Earl E.D. 1991. Stable transformation of sorghum cell cultures after bombardment with DNA-coated microprojectiles. *Plant Cell Reports* **10**: 260-264.

Howe A., Sato S., Dweikat I., Fromm M. and Clemente T. 2006. Rapid and reproducible *Agrobacterium*-mediated transformation of sorghum. *Plant Cell Reports* **25**: 784-791.

Joersbo M., Donaldson I., Kreiberg J., Pedersen S.G., Brunstedt J. and Okkels F.T. 1998. Analysis of mannose selection used for transformation of sugar beet. *Molecular Breeding* **4**: 111-117.

Jung R. 2007. Grain quality through altered expression of seed proteins. United States Patent application number 20080134361.

Kresovich S., McGee R.E., Panella L., Reilley A.A. and Miller F.R. 1987. Application of cell and tissue culture techniques for the genetic improvement of sorghum, Sorghum bicolor (L.) Moench: progress and potential. *Advances in Agron*omy **41**: 147-170.

Lucca P., Hurrell P. and Potrykus I. 2001. Genetic engineering approaches to improve the bioavailability and the level of iron in the rice grain. *Theoretical and Applied Genetics* **102**: 392-397.

Ma H. and Liang G.H. 1987. Plant regeneration from cultured immature embryos of *Sorghum bicolor* (L.) Moench. *Theoretical and Applied Genetics* **73**: 389-394.

Miles J.S. and Guest J.R. 1984**.** Nucleotide sequence and transcriptional start point of the phosphomannose isomerase gene (*manA*) of Escherichia coli. *Gene* **32**: 41-48.

Negrotto D., Jolley M., Beer S., Wenck A.R. and Hansen G. 2000. The use of phosphomannose-isomerase as a selectable marker to recover transgenic maize plants (*Zea mays* L) via *Agrobacterium* transformation. *Plant Cell Reports* **19**: 798-803.

O'Kennedy M.M., Burger J.T. and Botha F.C. 2004b. Pearl millet transformation system using the positive selectable marker gene phosphomannose isomerase. *Plant Cell Reports* **22**: 684-690.

O'Kennedy M.M., Burger J.T., Watson T.G. 1998 Stable maize transformation using Particle Inflow Gun. *South African Journal of Science* **94**:188-192.

Vain P., McMullen M.D. and Finer J. 1993b. Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. *Plant Cell Reports* **12**: 84-88.

Wright M., Dawson J., Dunder E., Suttie J., Reed J., Kramer C., Chang Y., Novitzky R., Wang H. and Artin-Moore L. 2001. Efficient biolistic transformation of maize (*Zea Mays* L.) and wheat

(*Triticum aestivum* L.) using the phosphomannose isomerase gene, *pmi*, as the selectable marker. *Plant Cell Reports* **20**: 429-436.

Zhao Z-U, Cai T., Tagliani L., Miller M., Wang N., Pang H., Rudert M., Schroeder S., Hondred D., Seltzer J. and Pierce D. 2000. *Agrobacterium*-mediated sorghum transformation. *Plant Molecular Biology* **44**: 789-798.

Zhu H., Muthukrishnan S., Krishnaveni S., Wilde G., Jeoung J.M. and Liang G.H. 1998. Bioilistic transformation of sorghum using a rice chitinase gene. *Journal of Genetics and Breeding* **52**: 243-252.