GENETIC ENHANCEMENT OF NUTRITIONAL QUALITY OF GRAIN SORGHUM

A Grootboom and M M O'Kennedy

CSIR Bio/Chemtek, P O Box 395, Pretoria 0001, South Africa,
E-mail: mokennedy@csir.co.za

Sorghum (*Sorghum bicolor* L. Moench) ranks fifth, worldwide in production among cereals and it is unique in that it is adapted to semi-arid environments. Sorghum grain with enhanced protein quality could contribute significantly to improving the nutritional value of the diets of people and livestock dependent on sorghum as a major protein source. The current study aims at genetically enhancing the nutritional quality of grain sorghum by the introduction of genes encoding the methionine-rich maize beta-zein and the lysine-rich barley chymotrypsin inhibitor CI-2 proteins, with the goal to produce transgenic sorghum plants with elevated lysine and methionine contents. A biolistic and *Agrobacterium*-mediated transformation protocol for selected grain sorghum lines was established. This would form the technological basis for nutritional quality improvement of grain sorghum.

Abbreviations
2,4-D = 2,4-dichlorophenoxyacetic acid, IAA = indolacetic acid , IBA = Indole-3-butyric acid, NAA = α-Naphthalene acetic acid

INTRODUCTION

Sorghum (*Sorghum bicolor* L. Moench) is one of the main staples of the world's poorest an most food-insecure people, supporting more than 300 million lives in Africa and Asia. Grain sorghum is becoming even more of an important staple food in Africa in the face of growing food scarcity and several prolonged droughts. However, in the past, the results of global sorghum research have not substantially benefited African farmers mainly because of the inferior quality and poor commercial value of the released lines. Although sorghum is widely used and consumed, this crop is known to have low nutritional quality, because of its characteristic low lysine content.

Most agricultural crops that have been genetically improved are temperate crops. The production of transgenic sorghum plants via particle bombardment of immature zygotic embryos has been reported only recently[1, 2, 3]. In addition, transgenic sorghum plants were produced via *Agrobacterium*-mediated transformation using immature zygotic embryos as explant[4]. Furthermore, transgenic sorghum plants were obtained by particle bombardment of immature inflorescences[5] and shoot tips[2] introducing mainly reporter and selectable marker genes. Only recently, a functional gene which codes for a feedback insensitive dihydropicolinate synthase, the first enzyme of the lysine-specific pathway, was introduced into the genome of sorghum with the goal of producing transgenic sorghum plants with increased lysine content[2].

An efficient transformation protocol for sorghum would form the technological basis for nutritional quality improvement of grain sorghum. As a first step towards achieving this goal, two transformable sorghum genotypes were selected in this study, and five African sorghum genotypes were assessed on various tissue culture media compositions to identify additional highly regenerable and transformable African genotypes. Subsequently, parameters were optimised for the stable introduction of the herbicide selectable marker gene, *bar*, using the particle inflow gun. Furthermore, plant expression vectors containing the reporter gene *uid*A (GUS), the selectable marker genes bar or *hpt* II, as well as the lysine-rich CI-2 gene under control of the gamma-zein promoter were introduced into the sorghum genome via *Agrobacterium*-mediated transformation of selected sorghum genotypes.

EXPERIMENTAL

Explant source

Immature zygotic embryos (IZE) were used as explants. Ten to twelve days after pollination, sorghum spikelets were sterilized with 70% ethanol for 5 minutes followed by the addition of 2.5% sodium hypoclorite solution (70% commercial bleach) containing 0.1% of the surfactant Tween 20, and shaking on a rotary orbital shaker for 15 minutes. After the immature spikelets were rinsed aseptically 5 times with sterile distilled water, the 0.8-1.2 mm long immature zygotic embryos were removed from the rachis and plated (90mm diameter Petri plates) on the surface of the media with their axis side in contact with the medium i.e. scutellum side facing up.

Tissue culture

All tissue culture was performed under aseptic conditions. All cultures were transferred to fresh medium every 2 weeks except when changes were found necessary due to repeated formation of phenolics or medium contamination. Until the

onset of embryogenesis, tissues remained on callus initiation medium. When embryogenic tissue formed, the calli were transferred to a callus maintenance and/or maturation medium. Subsequently, the somatic embryos produced were transferred to regeneration media.

Three media regimes were used to culture sorghum and designated G2+L-Proline, CAPD and that of Tadesse[2]. Each is described in detail below. G2+L-Proline callus induction medium is described by Gless and co-workers[6] and contains L3 salts and vitamins as described by Jahne[7], 2.5 mg l^{-1} 2,4-D, maltose as a carbon source, 4 g l^{-1} gelrite as solidifier and supplemented with 20 mM L-Proline (Pro). Cultures initiated on this medium were matured on maturation medium for a period of two weeks. The maturation medium was composed of G2+L-Proline medium without 2,4-D and L-Proline, containing 6% maltose instead of 3% maltose. The matured cultures were then regenerated and rooted on G2+L-Proline induction medium without 2,4-D and L-Proline. The total tissue culture period was 9-10 weeks.

The CAPD medium regime was based on the media described by Casas *et al.*[1] with various modification developed by CSIR: CAPD2 and CAPD1 consist of MS[8] basal medium supplemented with modified B5 vitamins[1], 30 g l^{-1} sucrose, 2 (CAPD2) or 1 mg l^{-1} (CAPD1) 2,4-D, 3.3 g l^{-1} Ammonium nitrate (NH_4NO_3), 2 g l^{-1} L-Proline, 1 g l^{-1} Asparagine, 100 ml l^{-1} coconut water and solidified with 8 g l^{-1} agar. CCM medium contained only 1 mg l^{-1} 2,4-D with the addition of 0.5 mg l^{-1} kinetin but did not contain NH_4NO_3, L-Proline, Asparagine and coconut water. CMR was identical to CCM but with the addition of 1 mg l^{-1} IAA and 10 mg l^{-1} silver nitrate. The CSE medium was identical to CMR with the omission of silver nitrate. CRE+H medium consisted of half strength MS basal salts supplemented with modified B5 vitamins, 0.5 mg l^{-1} IBA and 0.5 mg l^{-1} NAA with 20 g l^{-1} sucrose.

Callus production was induced by culturing the immature zygotic embryos on CAPD2 (10-14 days) and subsequently CAPD1 (4-7 days). Calli were maintained on CCM (4-7 days) before a 2-3 weeks culturing on CMR. The 1-2 cm shoots were then transferred to CSE for shoot elongation until the shoots were about 5cm in height. CRE+H was then used to culture the 5cm shoots until root formation and subsequent hardening-off. The total tissue culture period was approximately 15 weeks.

Tadesse's medium is described in his PhD thesis[2]. The total tissue culture period of Tadesse was approximately 25-30 weeks.

The incubation conditions for all cultures were at 24-25˚C under low-light conditions (1.8 $\mu Em^{-2}s^{-1}$), except regenerated shoots ($\geq$ 5cm), which were incubated under higher light intensity conditions (9 $\mu Em^{-2}s^{-1}$). The plantlets forming roots with shoot height ranging from 1-10cm were hardened-off to the greenhouse.

Microprojectile bombardment

Cultured immature zygotic sorghum embryos (pre-cultured for 6-8 days) were used as explant source for transformation experiments. Pre-cultured immature zygotic embryos were placed in the middle (0 - 1 cm diameter) of a 9 cm petri dish, containing induction media supplemented with approximately 25% increased solidifier, plus 0.2 M D-Sorbitol and 0.2 M D-Mannitol described by Vain and co-workers[9]. A bombardment mixture was prepared by precipitating plasmid DNA on

tungsten particles as previously described[10]. All experiments were conducted with the particle inflow gun (PIG). Sixteen hours after bombardment, bombarded tissue was transferred to the respective media omitting D-Sorbitol and D-Mannitol. DNA delivery parameters were as follows: 900-1200 kPa, 0.16 or 0.5 µg DNA per shot, 17 cm from the target tissue and a 500 µm nylon mesh screen placed 8 cm above the target. A vacuum of approximately -87 kPa was applied and the bombardment mix particles on the filter syringe were discharged when the helium was released following activation of the solenoid. The timer duration was 50 milli seconds. The bombardment mix introduced into calli used in each treatment contained 0.1mg tungsten per shot.

Plasmid DNA

Bombardment experiments were conducted with pAHC25, a dual expression vector containing the *uidA* reporter gene encoding β-glucuronidase (GUS), and the selectable *bar* gene encoding phosphinothricin acetyltransferase (PAT). PAT inactivates the herbicide Herbiace® by acetylation of phosphinothricin (PPT), the active component of these herbicides that inhibits nitrogen metabolism in plants. Both the *uidA* and *bar* genes are individually under the control of the maize ubiquitin promoter[11].

Selection and regeneration of transformants

Selection for phosphinothricin (PPT)-resistant sorghum tissue was initiated 7 days after bombardment by placing the cultured immature embryos on callus induction medium containing 2 mg/l of bialaphos (phosphinothricin based selective agent). After 4-6 weeks on selection medium, cultured embryos that produced white compact calli, presumably somatic embryos, were transferred to regeneration selection medium containing 2 mg l^{-1} bialaphos. Regenerating putative transgenic plants were subcultured at 2-3 weeks intervals until they reached 8-10 cm in height. These were then hardened-off as described by O'Kennedy *et al.*[12].

DNA extraction and southern blot analysis

Genomic DNA was extracted from putative transgenic sorghum leaf material using the mini extraction procedure of Dellaporta[13]. Five micrograms of sorghum genomic DNA either undigested or digested with restriction enzymes were separated on an agarose gel and analysed by Southern blotting as described by O'Kennedy *et al.* [12]. The *bar* genes of pAHC25 were labelled with digoxigenin (DIG) by the PCR DIG probe synthesis kit as described by the supplier (Roche).

Herbicide application

A 2% Basta® (200g/l of the active ingredient, glufocinate ammonium), 0.01% Tween 20 solution was applied to both surfaces of selected leaves of transgenic sorghum plants as described by O'Kennedy and co-workers[12]. Glufosinate and its commercial formulation, Basta® and the tripeptide, bialaphos, or its commercial formulation Herbiace® are both phosphinothricin (PPT)-based selective agents.

***Agrobacterium*-mediated transformation and regeneration procedure**

The transformation protocol was based on that of Zhao *et. al.*[4]. *Agrobacterium* strain LBA4404 was used in all experiments. Freshly isolated immature embryos of genotype 214856 were immersed in *Agrobacterium* suspension (OD 0.1 – 0.3) for 15 minutes, dried on sterilised filter paper, transferred onto solid callus induction medium (SCIM) containing 100µM Acetosyringone (AS), and cocultured in the dark for 3-8 days. The immature embryos were then rested on SCIM containing 100 mgL^{-1}

carbenicilin for a week. Selection was conducted afterwards on SCI medium supplemented with 100 mgL^{-1} carbenicilin, 0 to 50.0 mgL^{-1} hygromycin, 10 to 100 mgL^{-1} geneticin (G418) and 0 to 10.0 mgL^{-1} PPT or bialaphos for a period of 3 weeks. Surviving calli were transferred onto regeneration medium (SRM) with selection agents for regeneration. Gus transient expression was examined after 3 days of coculture.

Several binary vectors were used for *Agrobacterium* mediated transformation. A lysine-rich chymotrypsin inhibitor (CI-2) gene or modified versions of it, driven by γ-zein promoter, were cloned into the plant expression vector pCAMBIA 1303 and 3201 (CAMBIA; Australia). The vectors contain the *hpt*II or *bar* gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter as selection markers and g*usA:GFP* or *gus*A driven by the 35S promoter as reporter genes. In addition, vectors containing genes encoding a 15kD β-zein (Z15) or a 15kD β-zein fused with a 10kD δ-zein (Z15+10), under the control the high molecular weight glutenin or zein promoters were used. The latter vectors contain the *nptII* gene and the *bar* gene, respectively, as selectable marker genes.

RESULTS AND DISCUSSION

Tissue culture ameanability

Two sorghum genotypes identified in previous studies to be transformable, were selected for this study: the Ethiopian genotype 214856 and genotype Sensako 85/1191 that belongs to Monsanto South Africa.. In addition, four selected African sorghum genotypes were screened i.e. Kadaga, Kapaala (both Ghanaian), SA 2861 and SA 4322 (both South African) on three different tissue culture media as described in materials and methods. The regeneration potential is indicated as the regenerants per plate (15 explant) value (Table 1). Sensako 85/1191 on G2 + L-Proline was found to be the most regenerable genotype (90.22), followed by genotype 214856 on G2 + L-Proline (36.22). Genotype 85/1191 was far less responsive on the medium described by Tadesse (19.5). In contrast, genotype SA 4322 cultured on the G2 + L-Proline medium regime produced no regenerants, but produced 11.56 ± 11.44 and 53.44 ± 23.18 regenerants on Tadesse and CAPD media regimes, respectively. All of the F_0 sorghum lines that were hardened-off displayed normal vegetative growth and fertility i.e. formed viable seeds. The medium G2 + L-proline produced regenerants in a shorter period (75-90 days) compared to the media regimes of Tadesse (90-120 days) or CAPD (140-175 days). From these regenerability results, the following sorghum lines for biolistic transformation experiments were chosen: Sensako 85/1191 and 214856 on G2 + L-proline media regime and SA 4322 and SA2861 on CAPD tissue culture media regime.

Table 1

Biolistic-mediated transformation

Stable integration of the selectable marker gene, bar, was confirmed in T_0 and T_1 plants by southern blot analysis of one transgenic sorghum transformation event of genotype Sensako 85/1191. Table 2 shows the results of the micro-projectile bombardment transformation of sorghum lines with pAHC25. From the 1462 IZE bombarded, two (both Sensako 85/1191) transgenic sorghum plants have been obtained to date. The Southern blot hybridization results of the four putative

transgenic Sensako plants resulted in two transgenic plants (plant no. 3 and no. 4). All four putative transgenic plants tested were both CPR and PCR positive for the bar gene (results not shown). Figure 1 shows the Southern blot hybridization results of one of these two plants

Table 1: Results of the micro-projectile bombardment transformation with pAHC25 and selection on media supplemented with 2mg/l bialaphos. Parameters that resulted in transgenic plants are typed in bold.

Genotype	medium	# embryos bombarded	Helium pressure	Preculture period	ng DNA per shot	# of regenerants	Basta resistant	Southern blot positive
85/1191	G2+L-Pro	512	**900**-1200	**6**,7 or 8	**0.16** or 0.5	9	2	2
214856	G2+L-Pro	255	900-1200	7	0.5		0	0
SA 2861	CAPD2	250	900-1200	7	0.5		0	0
SA 4322	CAPD2	445	900-1200	7	0.5		0	0

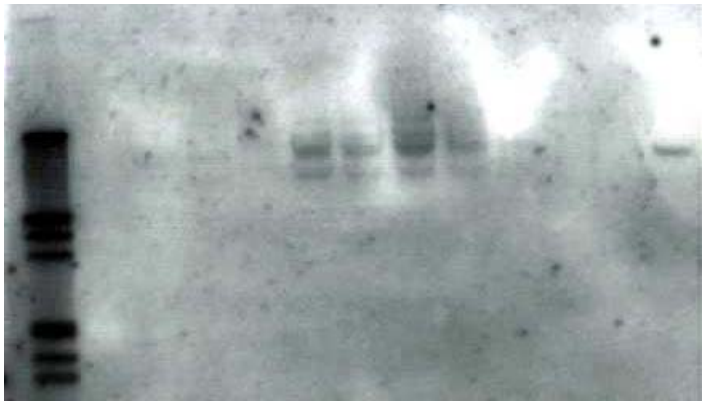

Figure 1 Southern blot hybridisation results of a transgenic plant

(transgenic plant no.3) with its eight T_1 progeny. The DNA banding pattern resembles the banding pattern of transgenic plant no. 4, suggesting that the two plants arose from a single transformation event and were therefore clones. These two plants were from a single plate bombarded at a helium pressure of 900 kPa and 0.16µg DNA introduced into 6 day pre-cultured IZE, thus giving a transformation efficiency of not even 0.1%. Figure 1 confirmed the unique integration pattern of the bar gene into the T_1 progeny of transformation event no. 3. Figure 2 shows the results of selected leaves of the T_0 transformation events no. 3 and 4., painted with 2% of the herbicide Basta®. A typical Mendelian 3:1 segregation was obtained for the T_1 plants (6 resistant and 2 susceptible plants). The Basta® paint results corresponded to the Southern blot analysis i.e. the 6 basta resistant plants hybridized to a bar gene probe (lanes 5-10 on Figure 1). Although two of these samples showed very faint bands on the Southern blot (lanes 5 and 10), hybridization signals were obtained. Two of the four putative transgenic plants were confirmed by Southern blot analysis to be stably transformed. The Basta® susceptible plants showed no hybridization with the bar probe (lanes 11&12). Therefore, a reliable transformation system for sorghum genotype 85/1191 was established.

Figure 2:T_0 transgenic plants displaying resistance to a 2% Basta® painting in contrast to the control (C) plant. Susceptibility or resistance was determined 5 days after painting with the herbicide Basta®. T_1 progeny of no. 3 plants were tested in a similar way and the herbicide resistance phenotype displayed a 3:1 Mendelian segregation

Agrobacterium-mediated transformation and tissue culture regime

Agrobacterium transformation conditions were optimised by assessing transient GUS expression. The infection of immature embryos with *Agrobacterium* at a concentration of 0.2-0.3 OD_{600} and a cocultivation period of 4 or 5 days yielded the highest transient GUS expression. Longer cocultivation periods gave similar transient GUS expression, but resulted in overgrowth of *Agrobacterium* which caused problems for further growth and selection of calli.

The resistance of calli of the sorghum genotypes 214856, Kapaala and Sensako 85/1191 to phosphinothricin (PPT), bialaphos, geneticin (G418) and hygromycin were evaluated in order to obtain the best selective agent for the selection procedure. Low concentrations of PPT (1-2 mg l^{-1}) had little effect on callus growth. More than 50% of the treated calli survived in medium supplemented with 10 mg l^{-1} PPT. The reason may be the presence of casein hydrolysate in the culture medium[14]. Although the callus looked healthy after 4 weeks, the development of callus in the medium supplemented with 6, 8 and 10 mg l^{-1} of PPT was delayed. The size of callus was two times less than the control. In the medium with 2 mg l^{-1} bialophos, more than 50% of the calli survived after 25 days of culture, but further development into regenerable callus was observed in SRM with the same bialaphos concentration. Therefore, 2 mgL^{-1} bialaphos could be used for selection.

Callus quality was less affected by geneticin and the continuous development of embryogenic callus was observed even at relatively high concentrations of 75 and 100 mg l^{-1}. However, geneticin 100 mg l^{-1} fully suppressed shoot and root formation, if applied during the regeneration process.

Calli treated with hygromycin rapidly showed the appearance of browning, especially in the part of callus directly contacting the medium, even at the lowest concentration of 12.5 mg l^{-1}. After 25 days, 70% of callus pieces were necrotic on the medium supplied with 25 mg l^{-1}.

The above-described conditions were used for *Agrobacterium*-mediated transformation of Sensako, 214856 and Kapaala. So far, ten independent Sensako plants were recovered after transformation with the *Agrobacterium* strain containing the binary vector Z15+10 and selection for 8 weeks. PCR using primers specific for the Z15+10 construct were positive for eight out of the ten putative transgenic plants (Figure 3). Southern and progeny analysis of those plants are being performed.

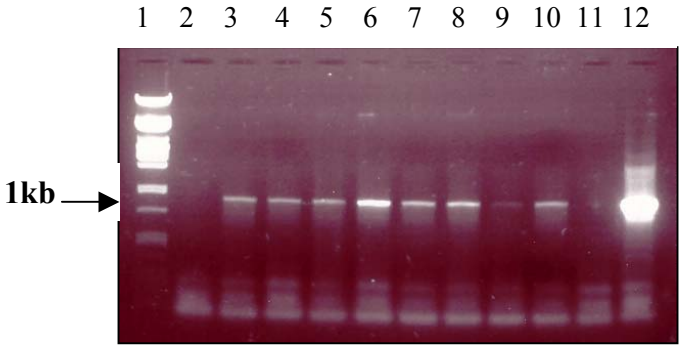

Figure3:
PCR analysis of independently regenerated Sensako shoots after transformation with Agrobacterium containing the Z15+10 binary vector.
1: Marker;
2-10: independent lines;
11:Negative control (DNA from wild type plant as template); 12: positive control (plasmid DNA as template).

Conclusion

In this study, stable transformation with both *Agrobacterium*- and biolistic mediated transformation form the technology basis for nutritional quality improvement of grain sorghum. However, the ultimate goal of this project is the production of transgenic grain sorghum with improved nutritional value due to elevated lysine and methionine contents. Four constructs containing genes encoding either methione-rich or lysine rich proteins, driven by strong seed specific promoters, were prepared for particle bombardment-mediated transformation of grain sorghum. One construct containing the wild type CI-2 gene driven by the maize gamma-zein (γ-zein) promoter was prepared with signal peptides and a C-terminal KDEL ER-retention signal to increase protein stability. Three constructs were prepared containing the genetically engineered CI-2 gene, with additional lysine substitutions in a reactive loop or hairpin region, driven by the maize gamma-zein (γ-zein) promoter and KDEL ER-retention signal (Dr Jane Forsyth and Prof PR Shewry, IACR-Rothmsted, UK). Two constructs were prepared containing the methionine-rich beta-zein gene or fusion protein gene driven by the HMW or gamma-zein promoter, respectively (Prof G Galili, Weizmann Institute of Science, Israel and Dr R Amir, Migal Galilee Technology Center, Israel). In order to detect the recombinant protein in the future transgenic sorghum, an epitope tag was fused in frame to the methionine-rich protein genes.

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