NUTRITIONAL QUALITY IMPROVEMENT OF SORGHUM THROUGH GENETIC TRANSFORMATION

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Sorghum is one of the primary cereals in many parts of the world, and ranks fifth among the world's cereals, following wheat, maize, rice, and barley. It is one of the main staples for the world's poorest and most food-insecure people, supporting more than 300 million lives. It performs well in the hot and dry agro-ecologies of the semi-arid tropics. In these areas, sorghum is a dual purpose crop, where it is used for human consumption and as feed for animals. However, although widely used and consumed, this crop is known to have low nutritional quality, as a result of its characteristic low content in the essential amino acid lysine. This low content results from end-product feedback inhibition of two key enzymes, aspartate kinase (AK) and dihydrodipicolinate synthase (DHDPS), in the aspartate family pathway (Karchi *et al.*, 1994; Galili, 1995). In particular, since lysine inhibits the activity of the enzyme (DHDPS) specific for its own synthesis, it is a major drawback that has to be alleviated to improve plant nutritional value. In this case, incorporation of a lysine overproducing trait in sorghum through foreign gene transfer could have considerable nutritional advantage for the food supply in several developing countries. Of the different gene transfer methods available, the utility of particle bombardment techniques has proved to be important in extending recombinant DNA methodology to the improvement of the more recalcitrant *Gramineae* species. Using this method, regeneration of stably transformed transgenic cereals expressing different antibiotic or herbicide resistant genes has been confirmed. These achievements to obtain monocot plants expressing different antibiotic or herbicide resistant genes can also be extended to the production of plants expressing nutritionally important genes. Our aim is thus to introduce a mutated gene (*dhdps-r1*) (Ghislain *et al.*, 1995) coding for a feedback insensitive dihydropicolinate synthase, the first enzyme of the lysine-specific pathway, with the goal to produce transgenic sorghum plants with increased lysine content.

1. Genotype screening

Based on the possibility of DNA delivery into organized tissues by high velocity microprojectile bombardment, different sorghum genotypes were screened for better *in vitro* performance using shoot tip and immature embryo tissues. Two out of eleven genotypes with high frequency in embryogenesis and regeneration were selected for further manipulation.

2. Optimization of transformation conditions

To establish the conditions for sorghum transformation via particle bombardment, we have optimized various parameters, including the type and physiological condition of the explant, concentration of DNA and particles, particle accelerating pressure, target distance, and efficiency of selective agent. Different tissue types (immature embryos, mature embryos, shoot tips, proliferated pre-callus tissues, and organized calli) were bombarded at different velocities in relation to macrocarrier, microcarrier travel distance, gap width and chamber vacuum. The series of bombardment experiments were carried out using the gene construct pAHC25 (Christensen, H. Quail, 1996), with 0.83 µg DNA per bombardment as described by Sanford *et al.*, (1993). Parameters were evaluated in relation to transient GUS expression levels that were quantified by histochemical GUS staining 48 hours after bombardment. Compared to other tissue types in replicated experimental observations, immature embryos and shoot tips gave adequate and consistent results at 6 cm for target distance and helium pressure of 1300 and 1100 psi, respectively.

3. Promoter analysis transient expression studies

In situations where transformation of a recalcitrant species like sorghum is difficult, and where effective expression of an introduced gene is not proved, it is important to test the expression level in combination with different promoter sequences. In this regard, transient expression studies using five gene constructs (Act1 D-BAR, McElroy *et al.*, 1990; pBAR-GUS, Fromm *et al.*, 1990; pAHC25 Christensen, H. Quail, 1996; and p35S-GUS (the GUS-uid A gene under the transcriptional control of CaMV 35S promoter), encoding the GUS (*uidA*) gene under the control of Act1, Adh1, CaMV 35S and Ubi1 promoter sequences were carried out by bombarding shoot tips and immature embryos. As a result of blue staining tests at 24, 48 and 72 hrs after bombardment, Adh1 activity was found to be about 1.5 and 2 or 3 times less than those of Act1 and Ubi1 promoters respectively, while CaMV 35S showed an activity about 1.5 times lower than the Adh1 promoter. The number of transient expression blue spots increases between culture periods of 24 and 48 hrs. However, the trend of expression level difference over time among the different promoters seems similar. Following the GUS staining test, other tissues were bombarded in similar conditions and after 48 hr culture period, protein was extracted and GUS activity was measured by fluorescence assay. This comparative promoter activity analysis also supports the result we obtained with the histochemical staining test.

4. Stable transformation studies

Based on the conditions we optimized, stable transformation studies were carried out using three different gene constructs (Table 1).

After bombardment, target tissues (immature embryos and shoot tips) were cultured on callus induction medium in the presence of S-aminoethyl cysteine (AEC, analogue of lysine), AEC and geneticin, and geneticin only, as selective agents wherever applicable. After a series of stepwise selections, a total of 9 plantlets derived from AEC resistant calli, 5 plantlets derived from AEC plus geneticin resistant calli and 3 plantlets derived from geneticin resistant calli survived transfer to soil. At flowering stage, Act D-neo and pGV4042 transformants showed normal growth and inflorescence formation, while p35TPM transformants exhibited branched shoots and panicles with partially or totally sterile spikelets.

Table 1. Gene constructs used in stable transformation of sorghum

Construct	Promoter	Selectable marker gene	Selective agent
pAct D-neo	Act1	<i>nptII</i>	geneticin
p35TPM	CaMV 35S	<i>dhdps-r1</i>	AEC
pGV4042	CaMV 35S	<i>dhdps-r1</i>	AEC
	NOS		geneticin

5. Molecular analysis

Total DNA was isolated from the leaf tissues of the R0 plants, and the presence and integration of the foreign genes were examined by Southern hybridization. With the genomic DNA samples from all three groups of transformants, PCR was carried out using the primers for *dhdps* and *nptII* genes. A band of approximately 0.7 kb from the *dhdps* gene in 8 out of 9 p35TPM and 5 pGV4042 transformants, and of around 0.5kb from the *nptII* gene in 5 pGV4042 and 3 pAct1 D-neo transformants were obtained showing positive amplifications as expected. No amplification was obtained from DNA samples isolated from nontransformed wild type control plants. The amplified samples were subjected to Southern hybridization using *dhdps* and *nptII* radioactive probes, and positive signals were obtained with respect to the positive PCR amplifications.

In the case of the pGV4042 transformants whose progenies showed total sensitivity to geneticin (Table 2), the presence of the *nptII* gene was shown by positive PCR amplification, and was later confirmed by Southern hybridization. In addition, using the *nptII* primers, another PCR test was done on DNA samples extracted from AEC-sensitive seedlings and AEC-resistant plus geneticin-sensitive seedlings. As a result, no amplification was obtained from the former samples, whereas the latter ones gave positive amplification confirming the presence of the introduced *nptII* gene.

Moreover genomic DNA samples isolated from the nine p35TPM transformants and one nontransformed control were digested with *Bam* HI and *Hind*III. Digested samples, together with one positive control (probe DNA) were hybridized with a 1.7 kb *Bam*HI-*Hind*III fragment of the *dhdps* probe. Eight out of the nine lines gave positive signals, among which four showed one band of 1.7 kb, and other four extra bands of higher molecular weight in addition to the expected band. No band was observed in one line which was also negative to PCR amplification, and as well as in the wild type control.

6. Biochemical analysis

6.1 Enzyme activity

Crude extracts of protein from leaf samples of the p35TPM and pGV4042 transformants and wild type control were tested for DHDPS activity. 2.5 to 4.5 times more activity was obtained from the different lines compared to the wild type. In the line where we did not get a positive signal for the presence of the foreign gene, the activity was comparable with that of the wild type.

6.2 Inhibition test

Inhibition of the DHDPS enzyme activity by increasing lysine concentrations (0-100 μ M) was also tested in a few lines. Clear inhibition in the wild type control was observed, while the enzyme from the transgene did not show any inhibition.

7. Acknowledgments

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8. References

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