ISSN 0975-6299 Vol.1/Issue-4/Oct-Dec.2010

International Journal of Pharma and Bio Sciences

AGROBACTERIUM MEDIATED TRANSFORMATION OF SORGHUM BICOLOR FOR DISEASE RESISTANCE

INDRA ARULSELVI. P[1,2] *, P.MICHAEL[1], S. UMAMAHESWARI[1] AND S. KRISHNAVENI[2]

[1] Department of Biotechnology, Periyar University, Salem, India.
[2] Department of Plant Molecular Biology and Biotechnology, CPMB, TNAU, Coimbatore, India.

**** Corresponding author*** iarulselvi@gmail.com

ABSTRACT

Sorghum serves as staple food for millions of people in Asia. It ranks as the sixth most planted crop and it is vulnerable to fungal diseases resulting in decreased grain quality and yield loss. *Agrobacterium* strains used were LBA4404 harbouring pcambia-ubi-chi11 (rice chitinase), EHA105 harbouring pcambia-ubi RC7 (rice chitinase) with *bar* gene and EHA105 harbouring pMKURF2 (rice chitinase gene) having *hph* gene for producing fungal resistance in sorghum plants. pCRC7 harbouring chitinase gene driven by ubiquitin promoter was found to be more efficient than pCG11 and pMKURF2.Out of the concentrations of acetosyringone tested, 200µM showed maximum transformation efficiency. Out of the two selection agents tested, bialaphos was found as a suitable selection agent. The transgenic nature of the sorghum calli and plants were shown by transient *gus* expression, their ability to survive in the selection medium and by western blot. The transformation frequency was found to be very low.

KEYWORDS

Sorghum bicolor, chitinase gene, *Agrobacterium* mediated, acetosyringone, transformation frequency

INTRODUCTION

Sorghum was first domesticated in East Africa several thousand years ago and it ranks as the sixth most planted crop in the world next to wheat, rice, maize, soybean and barley[1]. Food grain production of 270 million tonnes will be required by the year 2010-11. For some of the crops there is a need to increase the yield by 30% - 50 %. Comparative analyses of sorghum yield data, obtained from plants in field trials and in production agriculture, indicate that the genetic potential for biomass production is not being realized properly[2]. This yield limitation is, in large part, due to constraints imposed by biotic and abiotic stresses.

One of the solutions, scientists agree globally, is biotechnological intervention, which can play a vital role in not only increasing the crop yield but also improving the quality. This will be possible through development of varieties, which are resistant to diseases and pests. Recent demonstrations of *in planta* resistance to insects, viruses and phytopathogens through genetic transformation[3] portend the future for biotechnology in sorghum crop improvement

programs that will augment traditional breeding efforts and improve cultural management practices. The near term impact of biotechnology on agricultural production can be envisaged because of advances in tissue culture and transformation technologies that have resulted in the production of transgenic plants of all cereals[4], including sorghum[5].

Sorghum plant is affected by several diseases and pests. Its impact on crops is potentially devastating with total crop losses. Sorghum is plagued by diseases, especially in higher–yielding environments. Yield losses of sorghum from fungal diseases such as stalk rot, sooty stripe and charcoal rot cause severe economic hardship in the US and many developing countries[6]. There is a need to develop sorghum plants that are resistant to fungal diseases. By introducing PR-protein genes such as chitinase and glucanase through gene transfer techniques transgenic sorghum plants having enhanced disease-resistance could be generated.

Due to the simplicity of the transformation system and precise integration of transgenes, *Agrobacterium* Ti-plasmid-based vectors continue to offer the best system for plant transformation. Binary vectors have been improved by the incorporation of supervirulent *vir* genes, matrix attachment regions (MAR) and the insertion of introns in marker genes and reporter genes. With these improvements and with the use of acetosyringone, transformation of monocotyledonous plants using *Agrobacterium* has almost become a routine process[7]. But only few laboratories have attempted transformation of sorghum using *Agrobacterium*. In the present study, *Agrobacterium* mediated transformation was carried out in sorghum for disease resistance.

MATERIALS AND METHODS

(i) Genotypes, vectors and strains:

Sorghum genotypes (CO25, TNS586) were obtained from Department of Millets, Tamil Nadu Agricultural University (TNAU), Coimbatore. EHA105-pMKU-RF2 harbouring chitinase and gus reporter gene was gifted by Dr. Veluthambi, Madurai Kamaraj University, Madurai. PCam-ubi-RC 7 in *E. coli* (Chi gene from cDNA clone), pCam-ubi-chi 11 in *E. coli* (Chi gene from genomic clone) and pRK 2013 in *E. coli* were gifted from Dr. Muthukrishnan, Kansas State University, USA.

(ii) Kill curve experiments for selection of suitable selectable markers:

The vectors in *Agrobacterium* strains (LBA4404, EHA105) used in this experiment are having either bialaphos or hygromycin as selectable marker. The low concentrations of the selectable marker may lead to false-positive results. To avoid the false-positive results, kill curve experiments were carried out for selecting the suitable concentration of the selectable markers. For this, different concentrations of bialaphos / hygromycin were introduced in the callus induction medium and shoot regeneration medium and explants were placed in the medium.

(iii) Optimization of acetosyringone concentration to increase the transformation efficiency:

To determine the transformation efficiency in sorghum acetosyringone was added to I_6 medium in different concentrations viz., 100 µM, 200 µM and 300 µM and 48 h old *Agrobacterium* culture was inoculated. Scutellum-derived embryogenic calli were placed in the above solution for 30 min to 1 h for cocultivation. After soaking, the calli were dried in filter paper and plated in I_6 medium for 3-4 days in darkness.

(vi) Agro infection, cocultivation, resting, selection and plant regeneration:

Agrobacterium strain harbouring the plasmid (with antifungal genes and selectable marker) were grown with kanamycin (50µg/ml) for 16-20 h at 27°C till the O.D reached 0.5 at 600 nm. The grown cultures were centrifuged at 4500 rpm for 10-15 min and the pellets were taken. Enough quantity I_6 medium (liquid) was added, washed and spun twice to get a pellet.

Particular volume of I_6 broth was added and 200µM concentration acetosyringone (1M) was added. The calli were then soaked in the above solution for 30 min to 1 h. After soaking, the calli were dried in filter papers; plated in I_6 medium and incubated in the same for 3-4 days. After incubation, the calli were removed and washed in sterile distilled water with cefotaxime (400µg ml^{-1}) and after washing plated in I_6 medium containing cefotaxime (400-µg ml^{-1}) for a week. The calli were placed in selection medium (bialaphos 1 mgl^{-1} and hygromycin 10 mgl^{-1}). Bialaphos resistant callus lines were maintained by subculture on callus induction medium with bialaphos (2 mgl^{-1}) and transferred to shooting medium for regeneration. Selection of the calli on hygromycin medium produced intensive rooting even at 25 mgl^{-1} concentration hence limited further use of hygromycin. Plants were regenerated from bialophos – resistant callus lines by transferring the embryogenic callus to shoot regeneration medium with 2 mgl^{-1} bialaphos at 26°C under fluorescent lights. Surviving calli and shoots that resisted the selection pressure of 2mgl^{-1} bialaphos were transferred to rooting medium containing bialaphos (2mgl^{-1}) and after one subculture to normal rooting medium without bialaphos.

(v) Transient gene expression:

Histochemical GUS assay was carried out by a modified method [8]. For the GUS assay, the material was immersed in the GUS substrate mixture immediately followed by vaccum treatment for 10 min and then incubated at 37°C for 12h in the dark. The numbers of blue GUS foci were counted under stereomicroscope.

(vi) Western blotting:

Putative transgenic calli and leaves (~100-200 mg) were quickly frozen in liquid nitrogen, powdered and ground into a paste using 0.1 M potassium phosphate buffer, pH 6.5. Crude extracts were obtained by centrifugation at 15,000 g at 4°C for 10 min. During extraction, phenyl methyl sulfonyl fluoride was added to a level of 1mM as a protease inhibitor. Crude extracts were subjected to western blotting after determining the protein content.

RESULTS

(i) Kill curve experiments to select a suitable marker for sorghum transformation:

For cereals mostly hygromycin has been used as a selection agent in the medium. But no reports were so far available about suitable marker for selection of transgenic sorghum tissue. Experiments conducted with hygromycin and bialaphos on calli induction and plant regeneration of non transformed sorghum tissue revealed non transformed, control tissue can tolerate upto the level of 1mg l^{-1} of bialaphos. To avoid false-positive results kill curve experiments were carried out for selecting the suitable concentration of the selectable marker with immature embryos and calli as explants and the results are presented in Tables 1 and 2. The *Agrobacterium* cocultivated explants were selected at a bialaphos concentration of 2 mg l^{-1} and higher in our studies. But hygromycin plates showed extensive root system even at a concentration of 25mg l^{-1}, thus avoiding its further use as selection agent in sorghum transformation.

Table 1

Effect of hygromycin on callus proliferation and shoot regeneration of sorghum immature embryos

a. Effect on callus proliferation

I_6+hygromycin (mg/l)	Number of embryos	Proliferating calli (%)	Regeneration medium without hygromycin	Number of calli	Calli showing shoots (%)
I_6	40	87	I_6 +2kt+0.5 NAA	38	63.8
I_6 +25	42	11	I_6 +2kt+0.5 NAA	22	0**
I_6 +50	50	10.0	I_6 +2kt+0.5 NAA	10	0**
I_6 +75	40	10.0	I_6 +2kt+0.5 NAA	20	0**
I_6 +100	44	8.0	I_6 +2kt+0.5 NAA	22	0**

* Immature embryos precultured 1 week in I_6 medium
** All calli showed intensive roots and turned black.

b. Effect of hygromycin on shoot regeneration of non-transformed calli

Shoot regeneration medium (SRM) + Hygromycin (mg/L)	Number of calli	Number of calli showing green pigm/shoots	% of shoot regeneration	Length of the shoots	
				After a month	After 2 months
SRM	38	34	89.50	2 mm	4 mm
SRM +25	50	0*	0*	-	-
SRM + 50	40	5	Extensive roots	-	-
SRM+75	40	10**	Extensive roots	-	-
SRM + 100	35	0	Extensive roots turned black	-	-

* 80% of the calli showed roots and turned black
** Surviving ; no pigmentation

Table 2

Callus induction and plant regeneration in 'bialaphos' selection medium

a. Effect of bialaphos on callus proliferation of immature embryos precultured on I_6 medium

I_6+bialaphos (mg l^{-1})	Number of embryos	Proliferating calli (%)	Regeneration medium (without bialaphos)	Number of calli	Calli with shoots (%)
0	48	96	I_6 +1kt+0.5 NAA	38	63.8
2	46	6	I_6 +1kt+0.5 NAA	2	10.00
3	34	4	I_6 +1kt+0.5 NAA	1	0
5	24	0	I_6 +1kt+0.5 NAA	0	0

* Immature embryos precultured 1 week in I_6 medium.

b. Effect of bialaphos on shoot regeneration of non-transformed calli

Shoot regeneration medium (SRM) + bialaphos (mg/L)	Number of calli	Number of calli showing green pigm/shoots	% of shoot regeneration	Length of the shoots	
				After a month	After 2 months
SRM (I_6 +1Kt+0.5NAA)	48	48	100	1-2mm	4 mm
SRM +2mg	56	36 (only green pigmentation)	64	no shoots	-
SRM + 3 mg	48	No green colouration*	0	0	-
SRM+5 mg	52	0	0	Turned black	-

* Calli survived for 2 months without proliferation or shoot induction; Kt : Kinetin, NAA : Naphthalene acetic acid

(*ii*) *Optimization of acetosyringone concentrations for Agrobacterium-mediated transformation in sorghum*

In our attempts to optimize the concentration, 3 different concentrations of acetosyringone (100-200µM) were tried and the optimal concentration of acetosyringone was selected based on more number of calli survived on selection medium and *gus* transient expression (Table 3).Our results indicated that 200µM concentration of acetosyringone was suitable for sorghum transformation. After agroinfection, the calli were kept in the dark at 26°C for 3-4 days for cocultivation.

Table 3
Optimization of acetosyringone concentration for sorghum transformation

Inbred line	*Agrobacterium* strain	AS (µM)	Number of blue foci / callus	Percentage Calli survived in selection medium
CO25	EHA 105 harboring pMKURF2	100	20	18
		200	30	22
		300	22	15

(iii) Transformation using pMKURF2 in EHA105:

PMKURF2 harbours rice chitinase; *gus* as reporter gene and *hph* as selectable marker gene. The number of blue foci per callus was found to be 25-30 (Fig.1). The cocultivated calli were transferred to selection medium containing hygromycin at a concentration of 25 mgl^{-1}.

Fig. 1
Transient gus gene expression in calli of Co 25

(iv) Transformation using pCambia G11 in LBA4404:

The results of the transformation frequency of *Agrobacterium* - mediated transformation in sorghum using Co 25 was given in Table 4. By tissue culture techniques, CO25 and TNS586 sorghum genotypes were selected for the transformation study. Very few cocultivated calli were transferred to selection medium showed active proliferation. During the second round of selection, some pieces of calli grew vigorously; the growth of some were inhibited and some turned black and eventually died within a month. The resistance was due to the expression of the *bar* gene.

Table 4
Transformation frequency of Agrobacterium–mediated transformation in sorghum using CO25

***Agrobacterium* strains**	**No. of calli used for co-cultivation**	**No. of proliferation on selection medium**	**No. of calli showing shoots**	**No. of putative transgenics**	**Transformation frequency percentage**
LBA4404 G11	120	18	12	2	1.66
EHA 105 RC7	110	22	14	3	2.70
EHA 105 pMKU RF2	105	19	11 (intensive roots)	-	-

The bialaphos-resistant calli were transferred to shoot regeneration medium containing the same level of bialaphos and grown under light. Out of the calli transferred to shooting medium, only a few plants regenerated (Fig.2). Comparing the two genotypes, CO 25 showed better transformation frequency (1.0-2.0%). With respect to TNS 586 though very few putative transgenic plants were obtained, they were found to be chitinase-negative by western blot (Fig. 3).

(v) Transformation using pCambia RC 7 in EHA105:

Selection of the transformants after cocultivation of the embryogenic calli derived from CO 25 with pCambia RC 7 was done as specified above. The number of bialaphos-resistant calli or shoots obtained with this construct is comparatively more than with pCambia G11 which in turn may be due to the nature of *Agrobacterium* strain used (Fig. 2).

Fig.2
Shoot regeneration from embryogenic calli in selection medium

Fig. 3
Western Blot analysis of primary transgenic material of sorghum

1	:Extract from TNS 586
2&3	:Crude extract from bialaphos-resistant transgenic sorghum (pC Chill)
4	:Negative control
5	:Extract from transgenic material harbouring rice chitinase (positive control)
6&7	:Extracts from bialaphos-resistant transgenic sorghum (pC RC7)
8	:Marker proteins

DISCUSSION

In our study, we were not able to include many varieties of sorghum with different strains of *Agrobacterium* due to difficulties associated with regeneration system of the varieties tried. Comparing the different strains of *Agrobacterium* (LBA 4404, EHA 101 and EHA 105), EHA 105 appeared to result in highest frequency of sorghum transformation. Our results are in agreement with the results reported by Jeoung *et al.*[9]. The optimum concentration of acetosyringone may also vary with genotype to genotype and strain to strain.

We have used the *Agrobacterium* concentration of 1 x 10^9cfu ml^{-1} in our experiment as it was optimised to show higher gus expression[1]. We have used a cocultivation period of 72 h (3 days). This cocultivation period has been used in many cereals for *Agrobacterium*-mediated gene transfer. So far no optimised cocultivation period with embryogenic calli has been reported for sorghum transformation. Zhao *et al.*[1] conducted experiments with 3, 5 and 7 days cocultivation and reported 5 and 7 days were resulting in higher percentage of '*gus*' expression but they have used immature embryos as explants in their study. Jeoung *et al.*[9] adopted a cocultivation period of 3-4 days in optimization of sorghum transformation parameters. Progress in sorghum transformation in this area is needed to enhance the frequency of transformation.

The survival of calli in selection medium after 2 weeks culture in non-selection medium also indicated the toxicity of bialaphos at concentrations higher than 1 mg ml^{-1}. These results are in agreement with the results obtained by Casas *et al*.[10] whereas the embryogenic calli of other cereals such as barley, rye and rice [11,12,13] were found to resist 2-5 µg ml^{-1} of bialaphos. Wheat calli were found to tolerate 40 µg ml^{-1} of bialaphos concentration as reported by Chen *et al.*[4]. Krishnaveni *et al.* [15]; Vasil *et al*. [16] and Zhu *et al.* [17] in their study indicated the bialaphos level for selection of the transgenic material from cereals.

Though the number of calli survived on selection medium were more in our experiment only a very few calli showed shoot initiation; all eventually died. The low frequencies of shoot induction as experienced by us were also noticed in barley[11], Wheat[18] and rice [19]. This low frequency may be attributable to the acceleration of the development of phytotoxic activity of the herbicide on exposure to light.

Western blot analyses showed the presence of the chitinase proteins in bialaphos-resistant transgenic sorghum material and found to be absent in the control, nonbialaphos-resistant material. Not all bialaphos-resistant calli showed positive reaction with antisera raised against chitinases, which also suggests the inactivation of expression of transferred gene by DNA methylation in sorghum cells[20]. This in turn indicates that the loci containing active forms of these genes are not necessarily linked in the transgenic material. Presumably, different integration events can result in the inactivation of one gene without affecting the other. The low frequency of transformation may be attributed to many factors such as explant, agroinfection, cocultivation conditions, selection pressure, medium effects etc.

Our experiments provide the cumulative evidence for transformation of sorghum cells with *bar* gene and chitinase proteins - (i) by the growth of selected lines on bialaphos containing media and (ii) by chitinase protein expression in the transgenic material of sorghum by Western blot analysis. Further study will, however, be needed to evaluate these primary transgenic sorghum materials for their resistance to pest and pathogen in green house and field trials.

REFERENCES

1. Zhao ZY, Cai T, Tagliani L, Miller M, Wang N, Pang H, Rudert R, Schroder S, Hondred D, Seltzer J, and Pierce D, *Agrobacterium*-mediated sorghum transformation. Plant Mol Biol, 44: 789-798, (2000).
2. D. Krieg, and R.J Lascano. Agronomy Monograph no. 30. In: Madison and Wisconsin (eds.), Agronomy Monograph no. 30, American Society of Agronomy CSSA-SSSA, 1990, pp. 719-739.
3. Perlak FJ, Stone TB, Muskoff YM, Peterson LJ, Parker GB, McPherson SA, Wyman J, Love S, Reed G, Biever D, Fischhoff DA, Genetically improved potatoes: protection from damage by Colorado potato beetles. Plant Mol. Biol, 22: 313-32, (1993).
4. I.K. Vasil. Cellular and molecular genetic improvement of cereals. In. Terzi, T. (ed.), Current issues in plant molecular biology, Kluwer Academic Publishers, Neterlands,1995, pp.5-18.
5. Kononowicz AK, Casas AM, Tomes DT, Bressan RA, Hasegawa PM, New vistas are opened for sorghum improvement by genetic transformation. African Crop Sci. J, 3: 171-180, (1995).
6. Krishnaveni S, Jeoung JM, Muthukrishnan S, Liang GH, Transgenic sorghum plants constitutively expressing a rice chitinase gene show improved resistance to stalk rot. J. Genet. And Breed, 55: 151-158, (2001).
7. Veluthambi K, Aditya KG, Arun S, The current status of plant transformation technology. Current Science, 84: 368-380, (2003).
8. Jefferson RA, Kavanagh TA, Bevan MW, *gus* fusions (β glucuronidase) as a sensitive and versatile-gene fusion marker in plants. EMBO J, 6 : 3901-3907, (1987).
9. Jeoung JM, Krishnaveni S, Muthukrishnan S, Trick HW, Liang GH, Optimization of sorghum transformation parameters using genes for green fluorescent protein and β-glucuronides as visual markers. 136 (2002).
10. Casas AM, Kononowicz AK, Zehr UB, Tomes DT, Axtell JD, Butler LG, Bressan RA, Hasegawa PM, Transgenic sorghum plants via microprojectile bombardment. Proc. Natl. Acad. Sci. USA, 90: 11212-11216, (1993).
11. Wan Y, Lemaux PG, Generation of large numbers of independently transformed fertile barley plants. Plant Physiol, 104 : 37-48, (1994).
12. Castillo AM, Vasil V, Vasil IK, Rapid production of fertile transgenic plants of rye (*Secale cereale* L.). Bio Technology,12: 1366-1371, (1994).
13. Rathore KS, Chowdhury VK, Hodges TK, Use of *bar* as a selectable marker gene and for the production of herbicide-resistant rice plants from protoplasts. Plant Mol. Biol,21:871-884,(1993).
14. Chen WP, Xu X, Liang GH, Muthukrishnan S, Chen PD, Liu DJ, Gill BS, Introduction and constitutive expression of a rice chitinase gene in bread wheat using biolistic bombardment and the bar gene as a selectable marker. Theor. Appl. Genet, 97: 1296-1306, (1999).
15. Krishnaveni S, Jeoung JM, Muthukrishnan S, Liang GH, Transgenic sorghum plants constitutively expressing a rice chitinase gene show improved resistance to stalk rot. J. Genet. and Breed, 55: 151-158, (2001).
16. Vasil V, Srivastava V, Castillo AM, Fromm MC, Vasil IK, Rapid production of transgenic wheat plant by direct bombardment of cultured immature embryos. Bio/Technology, 11 : 1553-1555.
17. Zhu H, Muthukrishnan S, Krishnaveni S, Wilde G, Jeoung JM, Liang GH, Biolistic transformation of sorghum using a rice chitinase gene. J. Genet. and Breed, 52: 243-252, (1998).
18. Weeks TJ, Anderson OD, Blechl AE, Rapid production of multiple independent lines of fertile transgenic wheat (*Triticum aestivum*). Plant Physiol, 102: 1077-1084 (1993).
19. Rathore KS, Chowdhury VK, Hodges TK, Use of *bar* as a selectable marker gene and

for the production of herbicide-resistant rice plants from protoplasts. Plant Mol. Biol, 21:871-884, (1993).

20. Bochardt A, Hodal L, Palmgren G, Mattsson O, Okkels FT, DNA methylation is involved in maintenance of an unusual expression pattern of an introduced gene. Plant Physiol 99 : 409-414, (1992).