Declaration

I hereby declare that this thesis comprises of my own work except where specifically stated to the contrary, and that it is not substantially the same as any thesis that has been submitted for any degree at any other university.

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Date: 16 Oct 12

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Certificate

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Dedicated to
My beloved Amma
& Nannagaru . . .

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1. β- amylase	Beta-amylase
2. ABA	Abscisic Acid
3. *Act 1*	Rice actin promoter
4. *Adh 1*	Alcohol dehydrogenase promoter;
5. BAP	6-Benzylaminopurine
6. *bar*	Bailophos resistance.
7. BI-BAC	Binary bacterial artificial chromosome
8. BSA	Bovine serum albumin
9. *Cah*	Cyanamide hydratase
10. *CaMV 35S*	Califlower mosaic virus 35S promoter.
11. *cat*	Chloramphenicol acetyl transferase
12. *CpTI-*	Cowpea trypsin inhibitor
13. CTAB	Cetyl-trimethyl ammonium bromide
14. dATP	Deoxy adenosine triphosphate
15. DMSO	Dimethyl sulfoxide
16. DNA	Deoxy ribonucleic acid
17. Dnase	Deoxy ribonuclease
18. EDTA	Ethylinediamine-tetraacetic acid
19. GA	Gibberilic acid
20. *Gfp*	Green fluorescent protein
21. GS	Glutamate synthase
22. GTC	Guanidine thiocyanate
23. GUS	β-glucuronidase (*uidA*)
24. *hpt*	Hygromycin phosphotransferase.
25. IAA	3-Indole acetic acid
26. IBA	Indole-3-propionic acid
27. Kn	Kinetin (6-Furfuryl aminopurine)
28. LS	Linsmayer and Skoog

29. luc	Luciferase gene
30. MARS	Matrix attachment regions
31. MBIM	Multiple bud induction medium
32. MMLV	Moloney murine leukemia virus
33. MOPS	3-[N-Morpholino] propanesulfonic acid
34. MS	Murashige and Skoog
35. NAA	1-Naphtylacetic acid;
36. NaoAc	Sodium acetate
37. NaOH	Sodium hydroxide
38. npt II	Neomycin phosphotransferase.
39. PAT	Phosphinothricin acetyl transferase.
40. PCR	Polymerase Chain Reaction
41. PIG	Particle-inflow gun.
42. PMI	Phosphomannose isomerase
43. PPT	Phosphinothricin.
44. RAPD	Random amplified polymorphic DNA.
45. Rnase	Ribonuclease
46. SDS	Sodium dodecyl sulphate
47. SDW	Sterile distilled water
48. SEGM	Somatic embryo germination medium
49. SEIM	Somatic embryo induction medium
50. SKT I	Soybean Kunitz trypsin inhibitor.
51. SPIs	Serine protease inhibitors
52. TAE	Tris acetate- EDTA buffer
53. TDZ	Thidiazuron; (1-phenyl-3-(1,2,3-thidiazol-5-yl) urea
54. TPIs	Thiol protease inhibitors
55. *Ubi*–	Maize ubiquitin promoter;
56. UV	Ultra Violet
57. VIP	Vegetative insecticidal protein

1 INTRODUCTION

Sorghum is cultivated on approximately 44 million hectares worldwide and is the fifth major cereal crop in the world after wheat, rice, maize and barley. It ranks fifth in acreage (3.5 %) and it accounts for 3.5 % of cereal production. It is cultivated in thirty-nine countries on six continents, with an estimated annual grain production of about 10,000 mt (FAO, 1999). All India figures for sorghum production indicate that 10.5 million tons are produced annually (FAO, 1996). It is the third most important cereal after wheat and rice in terms of total production. Its production area is 11.7 m ha out of a total of 186.4 m ha and this represents 6.67 % of the cropped area and sorghum represents 6% of national cereal consumption.

World's sorghum production at the beginning of the 1960's was about 40 million metric tonnes. Production increased and, by the mid-1970's, was around 65 million metric tonnes. It reached peak production of around 80 million metric tonnes in the mid 1970's and then declined to 66 million metric tonnes in early 1990's. Most of the global grain sorghum production occurs in arid or semi-arid regions, and is mostly a rainfed crop. It is grown in most of the countries of the world and in several climatic and edaphic zones. It is an important staple food crop in Africa, South Asia and Central America, especially of the poor. Sorghum is also an important source of animal feed and fodder. The term sorghum includes at least four groups of cultivated plants viz., grain sorghum; sweet sorghum as forage and for animal feed; Sudan grass for pasture, hay and silage, and lastly broom corn for making brooms. The cultivated sorghum had its origin in Africa somewhere in the region of present-day Sudan and Ethiopia. It includes five basic races, viz., *bicolor, guinea, caudatum, kafir* and *dura* (Harlan, 1972; Harlan and deWet 1972).

Traditional plant breeding has resulted in the development of sorghum cultivars with increased yield, enhanced grain quality, or improved resistance to abiotic and biotic stresses (Smith & Frederiksen, 2000).

The primary objective of most national and international sorghum improvement programs is to improve the yield and food quality, and to stabilize the sorghum production under dryland conditions with low or no purchased inputs. Although, the reported potential yield of this crop under optimum input condition is high (> 10 t ha^{-1}), grain yields in Asia and Africa are generally low (500-800 kg ha^{-1}). Despite its socioeconomic importance, sorghum received relatively little basic investigation. The limited resources applied to the sorghum have been concentrating on applications that could directly contribute to the continued success of in improvement of sorghum by conventional breeding. However, the shortage of useful genes available in the donor germplasm, and the difficulty of making wide crosses because of the sexual incompatibility, has marginalized the conventional breeding from making major impact on sorghum production rates. As a result, sorghum is still plagued with problems of diseases, insect pests and weeds. Nearly 150 insect species have been reported as pests on this plant. Sorghum is a host for more than 100 plant pathogens, including fungi, bacteria, virus and nematodes. Some of these pathogens cause more serious damage, and are more widespread diseases than others. Among the diseases of global importance, the following are noteworthy: grain mold, anthracnose, honeydew disease or ergot, root and stalk rots and sorghum downy mildew. Further, the parasitic weed - Striga, attacks the crop. Complete crop failures caused by Striga have been recorded in many regions of Africa

and India. All of these constraints to production have presented, and most continue to present, problems to growers and seed producers.

The advent of molecular technologies for the mapping and engineering of complex genomes now permit wholly new approaches to crop improvement. Where as on one hand, the conventional breeding has much to contribute to meet the growing demand, it is also essential to take full advantage of biotechnology, to enhance the efficiency of the crop improvement programmes.

1.1 Transgenic plants - vehicles for application of biotechnology

Biotechnology has contributed to the development of novel and exploitable methods to genetically control and alter plant development, performance, and products, in concert with traditional plant breeding. Genetic engineering is a key component of biotechnology, and it is defined as the production of targeted novel gene combinations by laboratory techniques. Genetic engineering allows the insertion of genes into plant cells, which can subsequently be regenerated into transgenic plants. The selected transgenic plants transmit the inserted genes to their progeny in a predictable manner and exhibit the phenotype conferred by the inserted gene(s). Genetic transformation is now widely accepted as method of choice for alien gene introduction for those traits, which are complex, and the natural variation in the gene pool of crops is inadequate. During the five-year period 1996 to 2000, the global area of transgenic crops increased by more than 25-fold, from 1.7 million hectares in 1996 to 44.2 million hectares in 2000 (Fig. 1). During this period the number of countries growing transgenic crops more than doubled,

increasing from 6 in 1996 to 9 in 1998, to 12 countries in 1999 and 13 in 2000 (James, 2000).

During the six-year period 1996 to 2001, herbicide tolerance has consistently been the dominant trait with insect resistance second. In the year 1999 alone, the economic advantage for the 2 million farmers who planted GM crops was estimated to be of the order of $700 million, with the additional benefits to consumers worldwide likely to bring the total benefits of GM crops in 1999 to $ 1 billion or more.

The proportion of transgenic crops grown in developing countries has increased consistently from 14 % in 1997, to 16 % in 1998, to 18 % in 1999 and 24 % in 2000. Thus, in 2000 approximately one quarter (Table 1) of the global transgenic crop area of 44.2 million hectares, equivalent to 10.7 million hectares, was grown in developing countries where growth continued to be strong between 1999 and 2000, in contrast to the expected. Further, the estimated global area of transgenic or GM crops for 2001, is 52.6 million hectares (hec.) or 130.0 million acres, grown by 5.5 million farmers in thirteen countries. 2001 is the first year when the global area of GM crops has exceeded the historical milestone of 50 million acres. In 2001, 13.5 million acres was grown in six developing countries (James, 2002).

In case of Sorghum, significant progress has been achieved at few places in the USA, Australia, Europe and India (Maqbool et al., 2001). Establishment of totipotent or morphogenic callus and cell cultures in cultivated and wild sorghums has been accomplished. However, successful genetic transfer of alien genes into sorghum has met with limited progress. So far, there is no single transgenic sorghum under commercial cultivation.

1.2 Interaction of technologies to produce transgenic plants

Transgenic plants result from the successful interaction of three technologies:

(i) Techniques to regenerate fertile plants from undifferentiated cells

(ii) Recombinant DNA techniques to isolate and characterize genes and to construct gene expression vectors for regulated expression of inserted genes.

(iii) Genetic transformation methodology.

The prerequisites for the application of *in vitro* techniques for the improvement of sorghum are efficient and reproducible methods of regeneration from callus, cell or protoplast cultures, long-term regeneration potential, genetic stability of callus cultures and genotype independent protocols. Following Strogonov et al. (1968), rapid advances in callus induction and regeneration of sorghum from *in vitro* cultured tissues has been achieved and subsequently improved. However, among all the important crops including cereals, sorghum is one of the last crops to be genetically transformed (Subudhi and Nguyen, 2000). To date, there are only few reports of successful recovery and analysis of transgenic sorghum plants (Maqbool et al., 2001). In the above attempts, frequency of plant recovery after transformation has been very low, making it difficult to facilitate genetic transformation on a routine basis.

Microprojectile bombardment is the most successful DNA delivery method to produce transgenic cereals (Bajaj, 2000). More so, Particle bombardment is a viable alternative for genetic transformation of cereals and in those plants, where agro-infection is difficult. It offers advantages like introduction of multiple genes and the simplicity of introducing genes. Since the development of the first particle delivery system by Klein et al. (1987), several modifications have come into being. To date, PDS-1000/He gene

delivery device of *Bio-rad* laboratories, Richmond, California has been the most successful device used for direct gene transfer. However, the device is expensive, has high operational costs, and the time interval between two bombardments is long. Further, there are complications related to intellectual property rights, as the device is only licensed and not sold. As an alternative, a particle inflow gun (PIG) was constructed indigenously with the help of scientists of University of Queensland in Australia, following the design of Finer et al. (1992).

In addition to the regeneration protocols and the system of DNA delivery, optimization of the factors for gene transfer, selection and post -transformation regeneration are important factors in order to increase the efficiency of transgenic production. The present study comprises the objectives to optimize the protocols towards successful DNA delivery into the sorghum cells using an indigenously made particle-inflow gun; efficient selection and post transformation recovery; analysis of the transgene integration and expression in the regenerants and their progeny.

1.3 Specific objectives of this thesis

1. Establishment of *in vitro* regeneration systems suitable for genetic transformation from various explants such as immature inflorescence immature embryos and shoot apices.
2. Establishment of assay systems for identification and evaluation of transgenic plants.
3. Optimization of parameters for particle bombardment of the above explants.
4. Analysis of transformants using histochemical, molecular and other tests and study of transgene inheritance in T_1 plants.

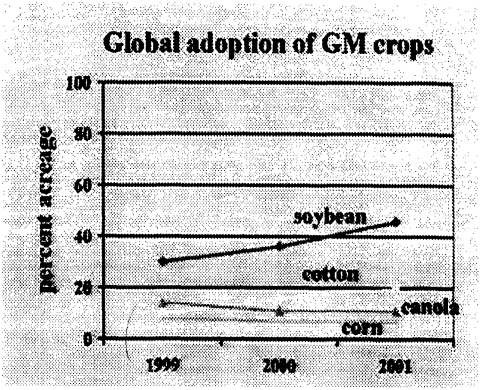


Fig.1. Global distribution of the transgenic crop area for the four major crops for the period 1996 to 2000.

Transgenic crop production area by country.		
Country	**Area planted in 2000 (millions of acres)**	**Crops grown**
USA	74.8	Soybean, corn, cotton, canola
Argentina	24.7	Soybean, corn, cotton
Canada	7.4	Soybean, corn, canola
China	1.2	Cotton
South Africa	0.5	Corn, cotton
Australia	0.4	Cotton
Mexico	Minor	Cotton
Bulgaria	Minor	Corn
Romania	Minor	Soybean, potato
Spain	Minor	Corn
Germany	Minor	Corn
France	Minor	Corn
Uruguay	Minor	Soybean

Table 1. **Global Transgenic crop production area by country for the year 2000.**

2 REVIEW OF LITERATURE

2.1 Tissue culture and regeneration studies in vitro

Explants derived form meristematic tissues at early stages of development are most amenable to tissue culture conditions (Puddephat et al., 1996). In cereals Immature embryos and immature inflorescences have been widely used as explants for successful plant regeneration (Bregiter et al., 1989; Bregiter et al., 1991). However, these are available only for a limited period of the year. Mature tissues such as seed embryos, and hypocotyls are readily accessible and year round sources for cereal explants (Cogner et al., 1982) so as do the shoots tips and shoot apices isolated from germinating seedlings. (Zhong et al., 1998).

2.1.1 Pathways of plant regeneration *in vitro*

In vitro plant regeneration can follow either of the following two different pathways: (i). Organogenesis involving the development of axillary buds following inhibition of apical dominance, or *de novo* organization of shoot meristems in callus cultures, and (ii). Somatic embryogenesis. In the latter case, regenerates arise from single cells either directly or follow the formation of a mass of proembryonic cells. Earlier reports in Gramineae described only shoot morphogenesis (Green, 1978). However, now extensive evidence is available of the regeneration of plants *via* somatic embryogenesis. Further it is suggested that, there exists a common pathway of regeneration in gramineae tissue cultures (Vasil, 1987).

2.1.2 Role of genotype on plant regeneration

The relationship between plant genotype and *in vitro* response is well known in cereals (Green, 1978). Further, there are many instances, both within the gramineae and in other angiosperms, where in plant regeneration was obtained in almost all the genotypes tested (Bajaj, 2000). These results strongly suggest that, the physiological state and the developmental stage of the explant are critical. The same is true for *in vitro* response in sorghum. In almost all the cases reported, employment of MS or LS basal medium supplemented with 2-4-D and /or kinetin resulted in successful morphogenic response (Table 2). Use of a variety of explants like immature embryos, immature inflorescences, and using shoot tips or apices (Table 2) have reported regeneration frequencies ranging from 0-100 % across the genotypes tested.

2.1.3 Genetic variability in plants regenerated *in vitro*

Larkin and Scowcroft (1981) termed the variation in tissue culture derived plants as 'somaclonal variation'. A variety of nuclear and cytoplasmic factors like point mutations, chromosomal rearrangements, recombination, DNA methylation and transposable elements, are responsible to its origin, and this is influenced by genotype, explant type, culture medium, and age of the donor plant (Jain, 2001). A majority of these variations are epigenetic in nature (Micke, 1999). In case of sorghum, somaclonal variation for leaf morphology and growth habit was reported, by Gamboorg et al. (1977). Similarly, Bhaskaran et al. (1983) obtained sodium chloride tolerant callus from mature seeds. Sorghum variety GAC, tolerant to aluminum in acid saturated soils was developed by Duncan et al. (1991), and Waskom et al. (1990) reported increased tolerance to acidic

soils and drought stress at field level. Maralappanavar et al. (2000) studied variation in both qualitative and quantitative characters like chlorophyll variation, altered phyllotaxy, branching phenotype, ear head weight and total grain weight in two sorghum cultivars, M35-1 and A-1. Out of a wide variety of molecular methods available for analysis of somaclonal variation in plants, RFLP and RAPD's are increasingly applied in the recent period (Jain, 2001; Henry, 1998 and Hashmi et al., 1997).

The positive aspect of such variation is its potential in crop improvement, if properly incorporated into the existing plant breeding programmes (Mythili et al., 1997). But from genetic transformation point of view, such a variation is unwanted. Therefore, a system which has no room for generation of somaclonal variation is the most suitable candidate (Birch, 1997).

2.1.4 Callus induction and regeneration from different explants of sorghum

Protocols were established for *in vitro* plant regeneration of sorghum from different types of explants like immature embryos, immature inflorescences and shoot tips or apices (Table 2) and also for mesophyll protoplasts of cultivated species (Sairam et al., 1999).

2.1.4.1 *Immature embryo*

One of the most popular and much widely adopted explant for tissue culture studies, as well as genetic transformation of sorghum is the immature embryo explant.

Protocols have been established for immature embryo based regeneration in sorghum (Table 2). Age of the immature embryo, measured as days after pollination (DAP) is found to be critical for *in vitro* response. In general, immature embryos were isolated

from spikelets harvested from plants 10-30 DAP for culture initiation. They reported callus induction and regeneration frequencies of 10-100 % and 10-90 % respectively, across the genotypes.

Apart from the age of the explant, influence of the size of immature embryo used for culturing was investigated in 20 genotypes by Ma et al. (1987). Kresovich et al. (1987) noticed no observable relationship between the size and induction potential, and they are of the opinion that, greenhouse microclimatology, and donor plant preconditioning, may confound the effects of age or the size of the embryo on *in vitro* response. For callus initiation, LS medium supplemented with 2,4-D and/or kinetin was used in majority of the reported cases, except Ma et al. (1987) who cultured immature embryos on MS medium supplemented with 2,4-D. For plant regeneration, embryogenic callus was transferred onto hormone free MS medium or medium supplemented with NAA and BAP. The frequency of callus induction and regeneration varied widely across the genotypes tested. Ma et al. (1987) from their study reported that, the ability of the plants to differentiate was heritable and is controlled by two gene pairs acting as a dominant trait.

Mature embryos were used as explants by El'konin et al. (1984) from genotypes Norghum 165 and Volzhskoe 2. Similarly, MacKinnon et al. (1986) used immature and mature embryos, of both grain and sweet sorghums, for *in vitro* culture. They observed no major differences in response with either grain or sweet sorghum cultivars except for difference in callus pigment colour. Similar observation was made by Kresovich et al. (1987). They are of the opinion that, this difference could be due to the phenotypic character of the donor plant.

2.1.4.2 *Immature inflorescence culture*

From the beginning, immature inflorescence have been identified as one of the source materials capable of producing embryogenic calli, with the capacity to regenerate into whole plants (Table 2). Length of the explant material has been shown to influence the ***in vitro*** response, and most of the reports mention the use of 10-40 mm length inflorescence for sorghum ***in vitro*** culture (Table 2). Kresowitch et al. (1987) reported a correlation between length and embryogenic response in some of the genotypes tested. However, this response was not consistent across all of them. This wide variation in the choice of lengths of the inflorescence can be attributed to difference in genotypes, and germplasm source. In most of the published reports, for callus initiation, LS medium supplemented with 2,4-D and/ or kinetin was used (Table 2). They have observed an average value of formation of embryogenic callus between 8-100 %. Callus initiation followed by transfer onto MS regeneration medium, with or without BAP, IAA, NAA and/or kinetin and culturing in light results in plant regeneration. Phenolics production resulting in death of the explants is one of the most common problem associated with immature inflorescence. Addition of 150 mg L^{-1} of asparagine to the medium, accompanied by frequent subculture has shown to mitigate this problem (Cai and Butler, 1990).

2.1.4.3 *Shoot tip or shoot apices as explant sources*

Shoot tips or shoot apices, when used as explants can overcome the seasonal limitation of the explant availability (Table 2). Shoot tips are isolated from seedlings germinated in dark under aseptic conditions.

For callus initiation, either LS medium supplemented with 2,4-D and kinetin (Seetharama et al., 2000), or for direct regeneration, MS medium supplemented with BAP, IAA and kinetin are used (Nahdi and De Wet, 1995). The entire process of regeneration was completed in 4-5 weeks, in direct regeneration as compared to somatic embryogenesis pathway, which took 5 weeks just for the embryos to germinate (Seetharama et al., 2000). In both the reported cases, the ability to regenerate varied among the genotypes tested. Using epicotyl and hypocotyls of sorghum, Gendy et al. (1996) attempted the TCL (thin layer system) of regeneration. They studied different factors that influence the regeneration potential in two sorghum commercial cultivars, Tamaran and Squirol.

As a further refinement to the above process, shoot apices isolated from the shoot tips are being cultured *in vitro*. In this technique, meristem tissue is reprogrammed to follow either organogenetic or somatic embryogenetic pathways of regeneration. This system of regeneration has been found to be rapid, comparatively genotype independent, and highly amenable for genetic transformation (Zhang et al., 1998).

Table 2. Some of the tissue culture work done in the past in cultivated sorghum.

S.No	Characteristics	Regeneration frequency	Reference
Immature embryo			
	Age in days after pollination (DAP)		
1	12-18	-	Gamborg et al (1977)
2	10-30		Thomas et al (1977)
3	10-14		Brar et al (1979)
4	14-17	90 %	Wang and Nguyen (1995)
5	10-14	26-69 %	Kresowhich et al (1987)
6	15-18	10-100 %	Saxena et al (2000)
7	21	-	MacKinnon et al (1986)
8	Milky stage	7 6-21 6 %	Sharma et al (1989)
9	9-12	0-40 %	Ma et al (1987)
Immature inflorescence			
	Length of the explant		
10	10-20 mm		Brettell et al (1980)
11	100-150 mm	47-95 %	Artha et al (1994)
10	1-10 mm	65-100 %	Murthy et al (1990)
11	16-105 mm	8 75%	Kresowitch et al (1987)
12	15 cms	20 -83 %	Muralappanavar et al (2000)
13	4-40 mm	8 70 %	Cai and Butler (1990)
Shoot tip/apices			
	Age after germination/length of the explant		
14	Shoot tips 24 h /0 2 - 0 5 mm	68%- 91 %	Nahdi and De Wet 1995
15	Shoot tip 3-4 days/ 3-4mm	18± 0 5 %	Seetharama et al 2000
16	Shoot apices -7-days	0 - 91 %	Zhong etal (1998)
Leaf bases			
	Age after germination/length of the explant	Regeneration frequency	Reference
17	1 5 mm		Wernicke and Brettel (1980)
18	5-7 mm/from week old seedlings	10-15 %	George and Eapen (1989)
Mature seeds			
	Explant size	Regeneration frequency	Reference
19	1-1 5 mm	11-48 5 %	Cai et al (1987)
20		6-80 %	Rao and Kishore (1989)
21		84 %	Lopez &Casas (1992)

2.1.4.4 *Protoplast culture*

Karunaratne and scott (1981) were the first to isolate protoplasts from one week old seedlings. Brar et al. (1980) and Chourey and Sharpe (1985) studied upto the level of protoplast divisions. Successful regeneration from cell suspension cultures was reported by Wei and Xu (1990) from two sorghum genotypes, and from mesophyll protoplasts by Sairam et al. (1999). The factors like growth conditions of the donor plant, genotype, and protoplast isolation methodology have been studied in detail by Murthy & Cocking (1988) and Sairam et al.(1999). However, among cereals sorghum protoplast culture is considered as one of the toughest *in vitro* techniques to establish (Mythli and Seetharama, 2000).

2.1.4.5 *Tissue culture studies with wild species of sorghum*

Wild species of sorghum are often the only source for high levels of insect resistance. Since they are not readily crossable with cultivated types, a technique like somatic hybridization needs to be developed. This is of critical importance when the resistance genes in wild sorghums are not identified, or resistance is distributed across whole genome.

A number of studies have been carried out on tissue culture of wild species, viz., *S. arundinaceaum* (Boyes and Vasil 1984; Guo and Liang, 1993), *S. almum* (George and Eapen, 1988), *S. versicolor* (Eapen and George, 1990), *S. plumosum*, *S. hewisonii* (Hegde and Kuruvinashetti, 1997). There are few reports on cell suspensions of cultivated and wild sorghums (Chourey and Sharpe, 1985; Wei and Xu, 1990), *S.dimidiatum* (Mythili etal., 1999).

However, due to the difficulty in establishing suspension and protoplast cultures, the strategy of somatic hybridization is being executed at a slow pace (Mythli and Seetharama, 2000)

2.2 Genetic transformation studies in plants with special reference to sorghum

Efficient plant transformation systems depend upon optimal levels of plant regeneration Besides an amenable tissue culture and regeneration system, following factors are also critical for successful transformation. (i) suitable explant, (ii) method of DNA delivery into cell, (iii) target gene in a suitable vector with reporter (to confirm alien gene introduction into cell or tissue) and selectable (to select only those cells into which foreign DNA has been incorporated) marker genes, and (iii) efficient testing method for confirmation of transformed phenotype It is equally important to ensure consistent inheritance of transgene in the progeny, lack of gene silencing or pleotropic effects, before efficient transgenics are developed

2.2.1 Genetic transformation of sorghum

Gene transfer to crop plants can be achieved using several methods such as direct DNA uptake, *Agrobacterium*-mediated DNA transfer (Christou 1995 and Zhao et al , 2000) and particle bombardment (Casas et al , 1993, Zhu et al , 1998) Owing to the difficulty in *Agrobacterium* mediated gene transfer, biolistic approach has been used extensively for the genetic transformation of the monocot species (Christou, 1995) Transformation systems have been described for all major cereals (Bajaj, 2000) including maize, oat, rice,

wheat and barley. In contrast, in the past one-decade, very little attention is given in developing transgenics of semi-arid crops like sorghum (*Sorghum bicolor* (L.) Moench) which can benefit millions of needy farmers and consumers in the developing world (Sharma et al., 2001 and Reddy & Seetharama, 2002). The protocols for producing transgenic sorghum are still being standardized using various methods of DNA transfer (Rathus et al., 2001). Therefore, genetic transformation of sorghum has not yet become routine and easy. Successful reports of sorghum transformation are few (Table 3) and there is an urgent need to standardize transformation protocols with popular varieties of sorghum.

Hagio et al (1991) reported transient expression of reporter genes in sorghum suspension cells by particle bombardment. Battraw and Hall (1991) used protoplasts for incorporating *npt-II* and *uidA* genes through electroporation. They studied transient expression of reporter genes using different factors such as linearizeation of the plasmid and effect of co-bom*bar*dment with two different gene constructs. However, their analyses of gene integration using PCR and Southern blots confined only putatively transformed calli, and no transgenic plants were regenerated. To date, there are only a few reports of successful recovery and analysis of transgenic sorghum plants. (Casas et al, 1993; 1997; Zhu et al., 1998; Zhao et al., 2000). Casas et al. (1993) reported transgenic plant production using microprojectile bom*bar*dment of immature zygotic embryos. Recently, Zhu et al. (1998) reported incorporation of rice *chitinase* gene into the immature embryo derived calli of *sorghum bicolor* and successful regeneration and molecular analyses of these transgenic plants. Apart from microprojectile mediated DNA

transfer, successful production of transgenic sorghum plants was reported by Zhao et al (2000) using *Agrobacterium* mediated DNA transfer.

2.2.2 Choice of the explant used for transformation

Pre-cultured immature embryos or isolated scutella with competent cells for somatic embryogenesis have been proven to be excellent targets for genetic transformation of cereals (Bommineni et al., 1997). However, earlier efforts to transform cereals involved the use of methods to facilitate direct DNA uptake into protoplasts. Since the regenerants are obtained from a single cell, the chances of obtained chimeras are almost nil. Battraw and Hall (1991) used cell suspension/ protoplasts of sorghum for electroporation. They obtained transformed calli. However, this callus turned out to be non-morphogenic. Cell suspensions were bombarded by Haigo et al. (1991), and they too ended up with non-morphogenic callus. Bombardment of cell suspension cultures directly eliminates the need for preparing protoplasts, and it reduces the formation of chimeras, which are often seen when embryos are bombarded. But, the disadvantage of the protoplast or cell suspensions is that the method is laborious, needs high skill, tends to be cultivar specific, and that too with very low regeneration frequency (Maheshwari et al., 1995). The first successful report of sorghum transformation came from Casas et al. (1993) using immature embryos. They were able to regenerate two plants, out of 600 bombarded embryos. To date, this has been the most tried explant in sorghum transformation (Casas

Table 3. Summary of attempts on genetic transformation of sorghum to date

S.No	Method of Transformation	Explant/ culture system	Objective, gene of interest.	Promoter used	Selection agent/ conc. used.	Observations and remarks	Reference
1	Electroporation	Cell suspension/ protoplasts	*npt II*	*CaMV 35S*	Kanamycin, 100 mgL-1	Stable transformation Non morphogenic callus regenerated	Battraw and Hall (1991)
2	PDS- 1000/He (Bio-Rad)	Cell suspension culture	*npt II / hpt*	*Adh 1/ CaMV 35S*	kanamycin/ hygromycin	Stable transformation Non morphogenic callus regenerated	Hagio et al (1991)
3	PDS- 1000/He (Bio-Rad)	Immature embryos	*bar*	*CaMV 35S*	Bialophos, 3 mgL-1	Plants regenerated at low frequency	Casas et al (1993)
4	PDS- 1000/He	Immature embryos/shoot tips	*npt II/dhdps-raec 1*	*Act 1/Adh 1/ CaMV 35S*	Kanamycin/ For more lysine content	Obtained > 10 plants Showed southern positive	Tadesse Y and Jacobs M (1999)
5	PDS- 1000/He	Immature inflorescence	*bar*	*CaMV 35S*	Bialophos	Plants regenerated at low frequency	Casas et al (1997)
6	PDS- 1000/He	Immature embryos/ inflorescence derived callus	*bar*		Bialophos	Plants regenerated at low frequency	Kononowicz et al (1995)
7	PDS- 1000/He	Immature embryos	*bar/chitinase*	*CaMV 35S*	Basta / 1-2 mgL-1	Showed southern positives	Zhu et al (1998)
8	PDS- 1000/He	Shoot meristems isolated from germinating seedlings	*bar/HVA 1*	*CaMV 35S*	Glufosinate/ 10 mgL-1	For drought tolerance Southern conformed. Further analysis on	Devi et al (1999)
9	PIG (Particle Inflow Gun)	Immature embryos/ inflorescence derived callus	*bar*	*CaMV 35S/Act 1*	bialophos/ 2 mgL-1	Single plant reported	Rathus et al (1996)
10	PIG	Immature embryo	*bar*	*CaMV 35S/Act 1*	Basta / 1-2 mgL-1	Casein hydrolysate was used for increasing regeneration frequency	Rathus & Godwin (2000)
11	PIG	Shoot meristems isolated from germinating seedlings	*bar/ Cry1A(b) & A(c)*	*CaMV 35S/Act 1*	Basta / 2 mgL-1	For insect resistance Southerns confirmed one plant	Gray et al (1999)
12	Agrobacterium						Godwin & Chikwamba (1993)
13	Agrobacterium (LBA4404)	Immature embryos derived callus	*gfp/ bar/ chitinases (G11, TLP & RC7)*		bialophos/ 3 mgL-1	Southerns not reported	Jeoung et al (1999)
14	Agrobacterium (LBA4404)	Immature embryos derived callus	*bar*	*Ubi*	PPT/5 mgL-1	2 1 % transformation frequency reported	Zhao et al (2000)

Abbreviations: npt II- neomycin phosphotransferase, *bar*- bialophos resistance, *gfp*- green fluorescence protein, hpt- hygromycin phosphotransferase, *Act* 1- rice actin promoter
Ubi - maize ubiquitin promoter, *Adh* 1- alcohol dehydrogenase promoter, *CaMV35S* - Cauliflower mosaic virus 35S promoter

et al., 1993; Zhu et al., 1998; Rathus et al., 1996; Jeoung et al., 2001; Zhao et al., 2000 and Rathus and Godwin, 2000). Apart from the above-mentioned explants, Tadesse and Jacobs (2001) have used shoot tips as explants. Of late, shoot meristems/ apices dissected out from germinating seedlings are being used as explants (Gray et al., 2001 and Devi et al., 2001).

2.2.3 Choice of promoters

Efficient transgene expression requires presence of a suitable promoter and a terminator. Heterologous promoters such as CaMV35S, rice actin, maize Ubiquitin promoters have been shown to regulate the expression of genes controlling them in cereals (Bajaj, 2000). In sorghum, all the above promoters have shown to be funtional (Table 3). However, Hagio et al. (1991) have observed that neither CaMV35S, nor maize *Adh1* are able to direct expression of introduced genes with high efficiency. For high levels of gene expression, either recombinant genes having two copies of promoter (Casas et al., 1993) or with added enhancers and introns have been used (Bajaj, 2000). Further, in *Agrobacterium* mediated transformation, use of intron helps distinguish actual gene transfer from bacterial contamination in transient GUS assays (Zhao et al. 2000).

2.2.4 Choice of reporter gene

Reporter genes help in analysis of the gene expression and its control, as well as protein trafficking. In general reporter genes codes for enzymes that can be used for anaysis of gene expression. The use of reporter genes has a long history and dates back to the early days of prokaryotic molecular genetics (Herrera -Estrella et al., 1983). So far, five

reporter genes have been successfully employed in cereal transformation. Reporter genes like β-glucuronidase (*uidA*) gene (Jefferson et al., 1987) that can be analysed by histochemical methods or fluorimetric methods and chloramphenicol acetyl transferase (*cat*) gene (Fromm et al., 1985) that can be analysed by radiochemical methods; or luciferase (*luc*) gene (Ow et al., 1986) and green fluorescent protein coding (*gfp*) gene using chemeluminescence methods. Fifth one is the R gene of maize, which is a transcriptional activator that regulates anthocyanin biosynthesis.

Transient expression studies based on these reporter genes help in establishment of systems for stable transformation. This is achieved by evaluating various gene transfer parameters 24-48 h after the event, based on the expression of these genes. Such studies, not only saves time and efforts, but also helps in rapid evaluation, and a chance to alter the transformation methodology. However, transient expression may not reflect the final integration of the transgene, and too much emphasis on these studies could be counter-productive Christou (1995). In sorghum genetic transformation, except, one report on transient expression of *cat* gene (Ou et al., 1986) and another on transient expression of R and C1 maize anthocyanin regulatory elements (Casas et al., 1993), β-glucuronidase (*uidA*) gene is the reporter gene that has been successfully used so far. This reporter gene has been used succesfully by Haigo et al. (1991); Battraw and Hall (1991); Zhu et al. (1998) and Zhao et al. (2000).

Assays based on R and C1 maize anthocyanin regulatory elements are non-destructive, compared to GUS assay, which is detrimental to the explant. However, Casas et al. (1993) reported that no clear-cut differentiation between transformed and non-transformed cells was observed using R and C1 maize anthocyanin regulatory elements

due to production of phenolic compounds. *uidA* gene having an intron that ensures genuine transient expression was used by Zhao et al. (2000).

2.2.5 Choice of selectable marker

In genetic transformation experiments, selectable markers allow identification of transformed cells based on selective growth of the transformants, when grown on medium containing the selection agents. The most common selection agents are antibiotics or herbicides Casas et al. (1995). The commonly used selection strategy for dicotyledons utilizes the antibiotic kanamycin, and the neomycin phosphotransferase II (*npt II*) gene isolated from *E. coli* (Bevan et al., 1983). Although this system was effective in tobacco and carrot (Hardegger and Sturm, 1998) transformation experiments, it has proven to be less effective for monocot transformation. The reason is that in monocots, growth is not significantly inhibited by these antibiotics (Dekeyser et al., 1989). Earlier attempts were made to genetically transform sorghum used neomycin phosphotransferase II (*npt II*) gene conferring resistance to the antibiotic kanamycin [Battraw and Hall, 1991; 100 mg L^{-1} and Hagio et al., 1991; 75-100 mg L^{-1} kanamycin]. Selection in these experiments was not satisfactory because of natural resistance to kanamycin shown by sorghum cell cultures. Therefore, attempts were made to look for alternate selection agents. Better results have been obtained with *E. coli* hygromycin phosphotransferase (*hph*) gene (Gritz and Davies, 1983). This has been succesfully used to select transformed maize (Walters et al., 1992) and rice (Li et al., 1993) tissues. Hagio et al. (1991) used 1-2 mg L^{-1} hygromycin for selection of transformed sorghum explants. Hygromycin, being highly photosensitive cannot be easily used as the selection agent

during plant regeneration and while selecting bombarded cultures like meristems, for which adequate exposure to light is important.

To date, the most successful and the most popular selection agent for sorghum has been the *bar* gene of *Streptomyces hygroscopicus*, which encodes the enzyme phosphinothricin acetyltransferase (PAT) conferring resistance to the herbicide phosphinothricin. This herbicide has been used as a selectable marker to obtain transgenic plants in most of the cereals and in sorghum (Casas et al., 1993; Rathus et al., 1996; Zhu et al., 1998; and Zhao et al., 2000). The concentration of selection agent used when using *bar* gene varies from 8-10 mg L^{-1} glufosinate, 2-3 mg L^{-1} bialophos, and 2-5 mg L^{-1} phosphinothricin (Table 3).

The obvious disadvantage with the use of these antibiotic and herbicide marker genes is the lack of wide public acceptance because of the environmental and health concerns like potential uncontrolled horizontal and vertical transfer to other organisms and its impact, the toxicity of the marker gene (protein) product and its breakdown product on both humans and other living forms, and the potential of the marker gene to cause environmental damage like the possible gene flow to wild species, which may result in the development of herbicide resistant weeds (Wilkinson, 1993).

(i) Devising methods to remove marker gene

It is indisputable that antibiotic or herbicide resistance genes account for the majority of selectable markers used (Yoder and Goldsbrough, 1994). Except for the existence of a clean biosafety certificate for *npt* II gene, no experimental data on other marker genes exists. Elimination of marker gene may result in easy public acceptance. To attain this,

several strategies have been devised (Yoder and Goldsbrough, 1994) These include transposon mediated repositioning of the marker gene (Goldsbrough et al , 1993), co-transformation of double T-DNA molecules (Komari et al , 1996) and site-specific recombination (Andrew et al , 1999, Dale and Ow, 1991) Yet another possibility is that, genetically modified plants containing selectable markers could be further modified with genes that encode antibodies against the marker gene protein product (Hiatt et al , 1989)

(ii) Employing alternate selection systems

As an alternate to this, selection systems, which overcome such obstacles can be adopted These include, the negative selection systems like amidohydrolase-auxin system which inhibits the formation of the root system (Béclin et al , 1993), and codA-5-fluorocytosine system, using selective agent 5-fluorocytosine that is converted to highly toxic 5-fluorouracil, which kills the modified plants (Stougaard, 1993)

The efficacy of phosphomannose isomerase (PMI) gene isolated from *E coli* was demonstrated by Joersbo et al (1998) They used Mannose as carbohydrate substrate, which is phosphorylated by a hexokinase to mannose-6-phosphate, whose accumulation in the non-transformed tissue results in severe growth inhibition

Recently, Weeks et al (2000) used a new selectable marker *Cah* for wheat transformation The gene *Cah* isolated from *Myrothecium verrucaria* encodes for the enzyme cyanamide hydratase, which converts cyanamide into urea For selection of the transformed calli, they used 37 5 mg L^{1} of cyanamide, in the culture medium The above mentioned markers may overcome the disadvantages posed by earlier markers and find increased number of applications in the coming days

2.2.6 Genes of agronomic interest

Gene transfer technology is aimed at introduction of genes of interest into plants, which is other wise not possible, or is a time consuming process, if carried out by conventional breeding It also enables to control the temporal and spatial expression of the transgene, there by allowing the micro level manipulation of the genome, aimed usually to confer a survival advantage to the plant. In this regard, herbicide resistance is the most common phenotype obtained by transformation in cereals, followed by biotic and abiotic stress resistance (Metz et al., 1998). In sorghum, more than half dozen groups have reported herbicide resistant transgenics (Table 3). Apart from this, transgenics conferring resistance to fungi by Zhu et al. (1998) and Jeoung et al. (2001); drought Devi et al (2001), Insect resistance Gray et al (2001) and nutritional quality improvement Tadesse and Jacobs (2001). Once the genetic transformation process becomes routine, more and more desired genes both of exogenous and endogenous origin (Table 4) can be transferred into sorghum, for is over all improvement

2.2.7 Microprojectile bombardment

Particle bombardment is an efficient method of genetic transformation of cereals, where in, biological molecules are driven at high velocity into the target tissue It offers advantages like introduction of multiple genes, the simplicity of introducing genes, and transformation in those plants, where agro-infection is difficult This process was initiated by J C Sanford and T M Kiein at Cornell University in 80's Their device included a barrel into which a gunpowder charge was fitted, which accelerated the coated tungsten powder placed at the tip of the macroprojectile (Sanford, 1988). The commercial

version of this device after a series of structural modifications was changed to a safer compressed helium system (Sanford, 1991).

The first set of successful applications of this process included bombardment of DNA and RNA into epidermal cells of onion (Sanford et al., 1987) and (Klein et al., 1987) few other reports also appeared in the same year (Klein et al., 1988a, 1988b; Christou *et al.*, 1988). These experiments mainly focused on transient expression, and once the method became routine, the utilization extended to genetic transformation of plants for which the existing methods of transformation like electroporation and agroinfection were considered difficult.

Currently, a number of instruments based on various accelerating mechanisms are in use. These include the original gun powder device (Sanford et al., 1987), an apparatus based on electric discharge (McCabe & Christou, 1993), a microtargetting apparatus (Sautter et al., 1991), a pneumatic instrument (Iida et al., 1990), an instrument based on flowing helium (Fenner et al., 1992; Takeuchi et al., 1992), and an improved version of both the original gun powder device utilizing compressed helium (Sanford., 1991). Hand held version of the original Biolistic R device and the *Accell* device are also in use. So far, biolistic transformation has allowed the recovery of transgenic fertile plants in several cereal crops such as rice (Chen et al., 1998), Maize (Brettschneider et al., 1997), wheat (Bommineni et al., 1997) oat and barley (Zhang et al., 1999) and sorghum (Casas et al., 1993 and Zhu et al., 1998).

2 2.7 1 *Parameters that affect DNA delivery*

Production of transgenic plants by particle bombardment can be divided into two process (i) That of introduction of DNA into cells with minimum tissue damage, and (ii) Regeneration from transformed cells Bombardment pressure, flight distance, amount of particles and DNA used per shot, and the number of shots per target Transformation is also affected by donor plant variables like, temperature, photoperiod and humidity, nature of explants (McCabe and Christou, 1993, Smith et al , 2001) Optimization of physical and biological parameters can increase the efficiency of these processes (Birich and Bower, 1994) Increased transient expression and stable transformation efficiencies result from treatment of the target tissues with osmoticum Vain et al (1993) Generally, gold or tungsten particles are used as micro-carriers in particle bombardment Size of the micro-carrier in the range of (0 5-1 0 μm) used for bombardment has an effect on transformation efficiency, as observed in wheat transformation (Altpeter et al , 1996) Of the two, tungsten particles are less expensive, but are more heterogeneous in size compared to gold But the disadvantage of using tungsten is that, it can catalytically degrade DNA over a period of time, and may be toxic to some cell types (Russel et al , 1992)

2.2.7.2 *Bombardment mediated co-transformation*

By means of microprojectile bombardment, a number of plasmids containing genes of interest and a selectable marker gene can be mixed and used for cotransformation (Chen et al , 1998) This has been employed successfully in a number of plant species including, maize (Gordon Kamm et al , 1990) oat (Pawlowski et al , 1998), rice (Kohli et al , 1998,

Chen et al , 1998 and Maqbool and Christou, 1999), and wheat (Barro et al , 1998) Chen et al (1998), and Maqbool and Christou (1999) reported cotransformation frequencies ranging from 20-80 % conformed by Southern blotting In addition, Gordon Kamm et al (1990) and Spencer et al (1992) reported cotransformation frequencies of 18 and 20 % determined on the basis of co-expression of two transgenes To the date, the largest number of plasmids transferred *via* particle bombardment is 12 in soyabean (Hadi et al , 1996) It has been observed that, there is no preferential integration of genes carried on different plasmids (Hadi et al , 1996, Maqbool and Christou, 1999) The copy number varied between 1-15 (Maqbool and Christou, 1999 and Kohli et al , 1998)

2.2.7.3 *Post-bombardment selection strategies*

The key to establishment of a successful transformation strategy lies in adoption of an effective and foolproof selection strategy It is an established rule that, post-bombardment selection should be just enough to allow the survival of transformed tissues, without hampering in any way with the regeneration process Again, this is case specific and determined by the type of marker gene used, and the type of explant transformed Different doses of the selection agent can be used to limit the number of non-transformed cells that survive due to cross-protection by the transformed cells This optimal concentration for selection, in turn depends on the species (Somers et al , 1992), which has to be evaluated experimentally, while taking into consideration, the effect of post-bombardment tissue damage on selection process (Taylor and Vasil 1991) Therefore, an important benchmark for using such selection strategy lies in the establishment of kill curves for the particular marker gene to be used, before hand For

sorghum, Battraw and Hall (1991) have reported that, 100 mg L^{-1} kanamycin, when used in the culture medium is sufficient to ensure selection of transformed protoplasts, while Haigo have used, as high as 500 mg L^{-1} kanamycin for effective selection for suspension cultures They have also bombarded *hpt* gene conferring resistance to antibiotic hygromycin, and used 50 mg L^{-1} in the selection medium Yet another marker gene, that has wide usage is the bar gene of *Streptomyces hygroscopicus*, which encodes the enzyme phosphinothricin acetyltransferase (PAT) conferring resistance to the herbicide Basta (or glufosinate, bialophos and phosphinothricin) (Casas et al , 1993, Zhu et al , 1998, and Zhao et al , 2000) The concentration of selection agent used against bar gene varies from 8-10 mg L^{-1} glufosinate, 2-3 mg L^{-1} bialophos, and 2-5 mg L^{-1} phosphinothricin Using this marker gene, Casas et al (1993) employed bialophos at 1-3 mg L^{-1} concentration Strategy followed here was the imposition of selection immediately after bombardment on 1 mg L^{-1}, or after 2 weeks of incubation on 3 mg L^{-1}, and maintenance of cultures further at this concentration during the rest of the culture period At times, it has been noted that, the above chemical selection proves too rigorous leading to loss of regenerative capacity of the transformed tissue as observed in barley (Stiff et al , 1995) and banana (Sagi et al , 1995)

2.2.8 *Agrobacterium*-mediated transformation

Genetic transformation of crop species using *Agrobacterium* is believed to be more practical, as the success rates of transformation are greater than with biolistics Further, unlike later, complex equipment is not involved However, for a long period of time monocotyledons have been considered outside the host range of *Agrobacterium* But,

advances in understanding the biology of the infection process and the availability of suitable gene promoters (Wilmink et al., 1995) as well as selectable markers improved transformation in monocotyledons (Smith and Hood, 1995). As a result, in the last decade, majority of the cereals like rice (Chan et al., 1993) wheat (Chen et al., 1997) maize (Zhao et al., 1998) and barley (Tingay et al., 1997) have been successfully transformed using this method of transformation. However, in sorghum only two reports have been published to date (Zhao et al., 2000 and Godwin and Chicwamba, 1993). Zhao et al.(2000) regenerated more than 100 transgenic events from transformed explants (Table 3). Thus, the feasibility of *Agrobacterium*-based method for sorghum transformation has been demonstrated. However, several variations and modifications need to be done, before this method of transformation can be handled on a routine basis.

2.2.9 Electroporation

The first attempt at electroporation mediated gene transfer into sorghum protoplasts was done by Ou-Lee et al. (1986). They reported transient expression of the reporter gene chloramphenicol acetyl transferase. Battraw and Hall (1991) electroporated protoplasts isolated from embryogenic suspension cultures to introduce *npt II* gene and the *hph* gene that were there on two different plasmids, along with *uidA* reporter gene. They obtained 77 resistant calli, but these calli failed to regenerate. Various factors effecting transformation frequencies like DNA concentration used, physical state of the plasmid (circular or linearized) were studied. However, this method of transformation is no more popular, for two reasons: (i) lack of reproducible genotype-independent cell suspension

or protoplast regeneration protocols, and (ii) development of superior and less cumbersome transformation methods like particle bombardment and *Agrobacterium*

2.2.10 Molecular mechanisms of transgene integration

The study of transgene structure and organization in transgenic cereal plants suggests a two-phase integration mechanism, resulting in a hierarchical organization At the most basic level, fragments of exogenous DNA may join together end-to-end, to form contiguous transgenes Such clusters integrate at breaks in the genome, which occur naturally in all cells Such breaks occur randomly, but integration takes place in easily accessible regions of the chromatin. The first integrated molecule attracts the integration of additional molecules to the same site leading to the formation of individual transgene clusters that are separated by short regions of genomic DNA (Kohli et al , 1998, 1999).

2.2.11 Molecular analysis of the transformants

Molecular methods like polymerase chain reaction (PCR) and Southern hybridization help in detection of the introduced gene Using the primers specifically designed to amplify the target sequence in the transgenic plant in PCR, rapid analysis of a large number of samples in relatively short period is possible (Bajaj, 2000) But, the disadvantages of screening the transformants using PCR is its inability to detect individual transformation events, which are crucial for estimating the transformation efficiency (Cacas et al , 1995) Apart from this, one may end up with spurious bands, which are often misleading when less stringent amplification conditions are employed These two practical problems can be overcome by performing Southern hybridization In

this, genomic DNA (digested and undigested) isolated from the transformant is blotted on to membrane, and hybridized with a radio labeled probe Usually, the probe is, a PCR amplified fragment or the restriction digestion released fragment of the introduced gene For digesting genomic DNA, the choice of restriction enzymes includes those that cuts the plasmid used for transformation once or twice (Zhao et al , 2000) The presence of the foreign gene can be shown by using an enzyme that cuts twice within the plasmid, followed by gel blot analysis Similarly, use of an enzyme that cuts only once within the inserted plasmid to indicate integration. In such an analysis, restriction fragments of varying sizes are expected, as a restriction site in the host DNA must also be cut in order to release a DNA fragment that can be detected by hybridization. Also in this analysis, the number of hybridizing bands reflects the transgene copy number

2.2.12 Functional analysis of the transformants

The next important step towards transgenic plant production is the expression analysis of the introduced transgene. A variety of strategies like the study of *uidA* gene expression in various plant parts, germination inhibition tests on medium with selection agent like hygromycin or Basta, *in vivo* application of herbicide, and immuno-detection of the protein using ELISA or western blotting (Bajaj, 2000) In case of genes of agronomic interest, like *Cry* or lectins, *in vitro* and *in vivo* bio-assays, where in resistance to the pests are evaluated basing on damage ratings For genes like chitinase, inoculum is added on to the plant at appropriate stage with the pathogen

2.2.13 Genetic variability in transformants

Somaclonal variation resulting in widespread genetic and phenotypic alterations in many species (Larkin and Scowcroft, 1981) is a common occurrence during *in vitro* tissue culture processes that involve a dedifferentiated callus phase Limitation in the delivery of transformable germplasm can be attributed mainly to somatic mutation and stable epigenetic changes that occur during transformation (Smith et al., 2001) Albinism, which occurs frequently in transformants, has been linked to changes in plastid DNA that occur during re-differentiation, suggesting that systems that limit the degree of differentiation and re-differentiation may enhance plastid genetic stability (Mouritzen and Holms, 1994). In rice and barley, evidence also exists that certain aspects of the transformation process exacerbate the mutagenic nature of the basic tissue culture process (Bregitzer et al., 1998) Instability of transgene expression, often attributable to transgene copy number, the genomic position of the transgene integration and to the degree of homology to endogenous genes (Matzke and Matzke, 1995), may also be related to the genomic instability (genetic and or epigenetic) induced during the *in vitro* culturing and transforming processes. Efficient commercial utilization of transformation technologies will require the ability to make stable genetic changes in many commercial varieties without disturbing their carefully selected agronomic and end-use characteristics.

2.2.14 Transgene expression and gene silencing

Stable integration and expression of introduced gene is essential to realize transgene advantage in the genetically modified crops Variation in transgene expression levels commonly observed in these plants reflect a number of influences such as position effect,

transgene structure, epigenetic silencing and co-suppression, and the presence of boundary elements, or MARS (matrix attachment regions) (Maqbool and Christou, 1999). Of more concern, is in terms of the practical application of transgenic plants in agriculture, are the plants showing apparent instability of transgene expression where by newly acquired traits can be lost, or expression levels may change (Finnegan and McElory, 1994). Loss of expression is attributed to gene silencing, rather than loss of the transgene. This occurs by various means, which parallel natural gene inactivation mechanisms. Methylation of the introduced DNA and homology-dependent ectopic pairing has been found to be the two major pathways that lead to transgene inactivation Iyer et al. (2000). During this process, integration intermediates become the targets for DNA methyltransferases that transfer a methyl group to 5' site of cytosine. Another occurrence, to which is often attributed gene inactivation and instability, is multiple tandemly arrayed copies of the transgenes, an event that occurs more frequently with direct DNA introduction methods (Flavell, 1994; Matzke and Matzke, 1995) than *Agrobacterium*-mediated methods. Also, evidence is rapidly accumulating that silencing of single copy foreign genes or multicopy transgenes integrated either at the same locus or at unlinked loci frequently cause silencing of themselves and of homologous host sequences. The frequency of silencing encountered in multicopy transformants has led to the speculation that enhanced DNA: DNA pairing of the repetitive elements in such complex inserts might act as a signal for detection, resulting in highly efficient silencing Iyer et al. (2000). To over come this, a number of approaches have been described for generation of single-copy transgenic lines. This includes the Agrolistic method that has been shown to generate reduced gene copy integration in tobacco and maize (Hansen and

Chilton, 1996; Hansen et al., 1997 and Srivastava & Ow, 2001), and the use of niacinamide to reduce recombination of extrachromosomal molecules (De Block et al., 1997). Therefore, suitable genetic transformation constructs need to be designed to avoid host surveillance processes and facilitate predictable single copy integration.

2.2.15 Inheritance studies in T_1 and T_2 progeny

Transgenes are generally expected to behave as dominant genes, due to their hemizygous state in the recipient genome, and thus segregate along typical 3:1 Mendelian ratio when selfed. This has been confirmed in several earlier reports as well (Barro et al., 1998 and Campbell et al., 2000).

Table 4. Genes isolated or cloned in sorghum

Gene family	Technique of isolation	Gene isolated	Reference
I *Genes involved in metabolic process*			
Protein kinases	Leaf cDNA clones (14 in number)	S6 kinases, SNF 1-like protein kinases & receptor like kinases	Annen et al. (1998)
Mesophyll-specific and bundle sheath specific gene sequences	DD-RT clones (25 in number)		Wyrich et al. (1998)
Phosphoenolpyruvate Carboxylase		CP21	Lepiniec et al. (1993)
II *Stress response (Biotic and Abiotic)*			
Glycine Betaine	cDNA clones	BADH 1 and BADH 15	Wood et al. (1996)
LRR proteins	cDNA clone	LRR	Hipskind (1996)
Sterols	PCR based cloning	Obtusifoliol 14α-demethylase	Bak et al. (1997)
Phytoalexins	PCR based cloning	PAL and CHS	Cui et al. (1996)
β-Glucosidase	cDNA clone	β-Glucosidase	Cicek and Esen (1998)
Cytochrome P450	PCR based cloning	A-type cytochrome P450	Bak et al. (1998)
Hydroxynitrile lyase	cDNA clone	HNL	Wajant et al. (1994)
III *Genes involved in growth and development*			
MADS box genes	cDNA clones	SbMADS1 and SbMADS2	Greco et al. (1997)
Opaque-2 gene		O2 gene	Pirovano et al. (1994)
Prolamines	Genomic clone	γ-kafirin	DeFreites et al. (1994)
Cysteine proteinase inhibitor	cDNA clone	cystatin	Li et al. (1996)
Vegetative protein	PCR based cloning	*Vp1*	Carrari et al. (2001)

Also, this has been crosschecked *via* backcrossing method using a non-transgenic parent to generate progeny segregation in 1:1 ratio (Fromm et al., 1990). In case of co-transformation experiments, it has been observed that, genes originating from different plasmids get forwarded together most of the times (Kohli et al., 1998; Chen et al., 1998; Pawlowski et al., 1998; Campbell et al., 2000). As an alternative to this, there are numerous reports describing aberrant transgene expression. In general, true integration of the foreign gene into the host plant genome can be proved by genetic analysis of the T_1 and T_2 populations.

2.3 Conclusions and future prospects

The establishments of callus and cell cultures from various explants, which are competent to express totipotency, have become routine. To enhance the tissue culture response further, identification of genes controlling the *in vitro* response and mapping these is highly desirable. Additional research on some of the factors like the production of phenolics (pigments) and appearance of abnormal root and leaf like structures from the callus which drastically decreases the regeneration frequency can help in the progress of *in vitro* response of sorghum. Parallel developments are needed in improvement of other transformation methods like the *Agrobacterium* method. Identification and isolation of genes controlling agronomically important traits like disease resistance and stress tolerance and designing better strategies for utilization of existing endogenous genes should be made an integral part of the development program (Table 4). Access to better gene promoters and self-excising selector (Zuo et al. 2001) regulatory systems, and

suitable breeding programs combining efficient *in vitro* manipulation techniques could soon lead to the production of sorghum cultivars with novel genetic traits.

Sorghum can be transformed with a number of candidate genes to increase resistance to biotic and abiotic stress factors, and the secondary gene pool of sorghum is a rich depository of resistance genes. Till the time these genes are isolated and fully characterized, its still desirable to consider cellular techniques like somatic hybridization to incorporate novel resistance genes into the pool of cultivated sorghum germplasm. The large-scale genome analysis of many organisms under way, in the long run we should be prepared to venture introducing novel sequences controlling plant growth and development to suit a variety of needs. Gradually it should be possible to pyramid multiple genes, *in vitro* within large vectors like binary bacterial artificial

Chromosomes (BI-BACs) that may be used for sorghum transformation. Such constructs will pave way for genetic engineering of metabolic pathways, and manipulation of such polygenic traits, there by enhancing the utility of transgenic approach.

3 MATERIALS AND METHODS

3.1 Plant material

The three sorghum genotypes M35-1 (land race cultivated during post-rainy season in India), 296B and BTx-623 (popular seed parents of hybrids) used in this study were maintained at ICRISAT.

3.2 Glassware

Culture flasks, beakers, measuring cylinders used for the present study were either *Borosil* or *Corning* made. Glassware was cleaned after prolong rinsing with diluted *teepol* (a detergent) followed by 4 - 5 rinses with tap water. The glassware was finally rinsed with distilled water and dried in hot air oven at 60 ^{0}C for 1h. before use. Apart from this glassware, disposable petri-plates of *Tarson* and *Granier* make were used extensively in this study.

3.3 Chemicals

All chemicals used in the preparation of culture media and also for other experiments were of analytical grade. Unless specified otherwise, all the chemicals were purchased from *Sigma* Chemical Co., USA.

3.4 Preparation of culture media

Basal medium formulations of Murashige and Skoog, (MS medium) (1962) and Linsmaier and Skoog, (LS) (1962) with phytohormones in various combinations were used in this study. The chemical composition of these media is given in Table 5. In order to reduce the time taken to weigh out each of the ingradient every time a medium is

Table 5. Formulations of MS and LS Culture Media.

	Concentration in culture medium (mg L^{-1})	
Constituent	**MS**	**LS**
KNO_3	1900	1900
NH_4NO_3	1650	1650
$MgSO_4 \cdot 7H_2O$	370	370
$CaCl_2 \cdot 2H_2O$	440	440
KH_2PO_4	170	170
$MnSO_4 \cdot 4H_2O$	22.3	22.3
KI	0.83	0.83
H_3BO_3	6.2	6.2
$ZnSO_4 \cdot 7H_2O$	8.6	8.6
$CuSO_4 \cdot 5H_2O$	0.025	0.025
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25
$CoCl_2 \cdot 6H_2O$	0.025	0.025
$FeSO_4 \cdot 7H_2O$	27.8	27.8
Na_2EDTA	37.3	37.3
Nicotinic acid	0.5	0.0
Pyridoxine-HCl	0.5	0.0
Thiamine-HCl	0.1	40
Myo-Inositol	100	100
Glycine	2.0	0.0
Sucrose	30000	30000

prepared, concentrated stock solutions were prepared and stored either at 4 ^{0}C (stocks of inorganic solvents) or at -20 ^{0}C (that of vitamins and hormones). Culture media was prepared by adding the appropriate amounts of stock solutions and growth regulators were added prior to the final volume adjustment. The pH of the medium was adjusted to 5.8 with 0.1 N NaOH or 0.1 N HCl, and the final volume made to 1 liter with distilled water. To gel the medium, 0.8 % (w/v) agar was dissolved by gently heating with constant stirring.

3.5 Sterilization

Autoclaving was done at 15-lbs/sq inch for 15 min. at 121^{0}C to sterilize media and glassware and other equipment. Thermo-liabile components used in the present study were filter sterilized through 0.22 μM filters and added under aseptic conditions to the culture medium after sterilization.

3.6 Aseptic conditions

All the operations related to tissue culture and transformation viz. Surface sterilization, inoculation, subculture, bombardment etc. were carried out in a sterile laminar flow hood cabinet (*Klenzoids*, Bombay). First, the bench and culture vessels were swabbed with 70 % ethyl alcohol. Next, culture vessels were placed on bench under UV light for 20 min. the devices used for inoculation and subculture were flame sterilized prior to use.

3.7 Statistical analysis

All the experiments were repeated and there were multiple replications for each treatment. Statistical analsis was performed using SIGMASTAT 3 software.

3.8 Establishment of in vitro regeneration systems for sorghum

A rapid and uniform regeneration system with high regeneration potential is a prerequisite for successful genetic transformation. The present study involves establishment and evaluation of three different regeneration systems based on shoot apices, immature embryos, and immature inflorescence, for their suitability to genetic transformation. All the experiments were repeated and there were multiple replications for each treatment.

3.8.1 Rapid regeneration from shoot apices

3.8.1.1 *Shoot apex isolation and in vitro culture*

Shoot tips were obtained from aseptically germinated seedlings of these genotypes. For surface sterilization, seeds were dipped in 100 ml distilled water with 2 drops of *Tween-20* and washed for 10 min. Following rinsing thrice with sterile distilled water, seeds were placed in 70 % ethanol for 1 min. and then they were collected in a sterile flask. Next, seeds were rinsed again with sterile water for 5 min. After decanting the water, seeds were placed in 0.1 % $HgCl_2$ for 7 min. under continuous stirring. Next, seeds were thoroughly rinsed again with sterile water twice for10 min. Finally, seeds were allowed to

germinate in dark on filter paper soaked with distilled H_2O till the shoot tip was isolated for *in vitro* culture

Shoot apices were excised from the hypocotyls of seedlings under dissection microscope. A cut was made at the base of the apex below the attachment of the largest unexpanded leaf. Membranous sheath was then separated from the primordial leaf. Parts of the unexpanded primordial leaves were left in place. Shoot apices were cultured using MS induction medium in 15-mm petri dishes by placing them horizontally on the MS medium. The petri dishes were sealed with *parafilm*, and were incubated in light (16h. per day) at 26 ^{0}C and 45 % relative humidity.

3.8.1.2 *Optimization of hormone combination for in vitro meristem culture*

Induction of organogenesis: Protocol for direct organogenesis of shoot meristem was standardized using different hormone combinations for their induction and proliferation. Initially, four hormone combinations, a control (without BAP) in basal MS formulation along with three levels of BAP (1.0, 2.0 and 4.0 mg L^{-1}) were tested. Next, the above combinations were fortified with different concentrations of either NAA (0, 0.1 and 0.2 mg L^{-1}) or 2,4-D (0, 0.1 and 0.2 mg L^{-1}) using one at a time and tested for induction of multiple buds. The induction response was recorded 14 days after initiation.

To study the effect of thidiazuron on multiple bud induction, 6 levels of TDZ (0., 0.0022, 0.011, 0.066, 0.022, 0.066, 0.11 and 0.154 mg L^{-1}) along with control (no TDZ) were added to MS medium with 4.0 mg L^{-1} BAP + 0.2 mg L^{-1} NAA. Proliferating buds attached to the enlarged meristems were transferred to MS basal medium supplemented with 3.0 mg L^{-1} BAP, 0.2 mg L^{-1} NAA and 0.066 mg L^{-1} TDZ. Three rounds of

subcultures were done at weekly intervals. During each subculture, the concentration of TDZ was reduced from 0.11 mg L^{-1} to nil by the time of the fourth passage (Table 6).

Induction of somatic embryogenesis: Incubation of shoot apices on multiple bud induction medium, followed by two rounds of subculture (passage1 and passage 2) similar to organogensis (Table 6) resulted in multiple bud formation.

Table 6: **Summary of Growth regulator combinations for shoot apex culture.**

A Common culture conditions for both pathways				
Weeks after culture initiation	*Passage details*	*Growth regulators used*		
		BAP (mg L^{-1})	TDZ (mg L^{-1})	NAA (mg L^{-1})
0	Induction medium	4 0	0 11	0 2
2	Passage 1	3 0	0 066	0 2
3	Passage 2	2 0	0 044	0 2
B Organogenesis pathway				
Weeks after culture initiation	*Passage details*	*Growth regulators used*		
		BAP (mg L^{-1})	TDZ (mg L^{-1})	NAA (mg L^{-1})
4	Passage 3	2 0	0 022	0 2
5	Passage 4	2 0	0	0 2
6	Passage 5	2 0	0	0 2
C Somatic embryogenesis pathway				
Weeks after culture initiation	*Passage details*	*Growth regulators used*		
		BAP (mg L^{-1})	TDZ (mg L^{-1})	NAA (mg L^{-1})
4	SE induction medium	4 0	0 0	0 5
7	Germination medium	4 0	0 0	0 2

For induction of somatic embryos, meristem cultures with 35 - 40 *de novo* shoot buds on them, were cut into 3-4 pieces and transferred onto somatic embryo (SE) induction medium (MS +4 0 mg L^{-1} BAP and 0 5 mg L^{-1} NAA)

3.8.1.3 *Rooting of the plantlets*

The elongated shoots derived from both organogenesis and somatic embryogenesis were rooted on half-strength MS medium with IBA (0 0, 0 5, 1 0 and 2 0 mg L^{-1}) and IAA (0 0, 0.1 and 0.2 mg L^{-1}). After 1 month of culture, rooted plantlets were removed from the magenta box, thoroughly rinsed with sterile water and transferred to jiffy cups containing autoclaved vermiculite mixture. Plants were hardened for ten days in the culture room maintained at 26 ^{0}C and 45 % relative humidity with a photoperiod of 16h light /8h dark. Next, plants were transferred to greenhouse and grow to maturity. Next, plants were transferred to greenhouse and grown to maturity

3.8.1.4 *Optimization of factors affecting the organogenic response of shoot meristem*

A factorial experiment was conducted with genotype BTx-623 to determine the effect of following three factors: seed size (large > 3 mm and small < 3 mm), method of germination (wet filter paper, and MS solid medium) and age of the explant (4 day to 10 day old seedlings) on the meristem proliferation. There were 28 treatments in all, with 20 explants per plate. The experiment was repeated once. Induction response was recorded after 2 weeks of culture. From day 4 to day 10, 20 seedlings from each treatment were used for meristem isolation.

3.8.1.5 *Histological studies of shoot meristem*

To study responses from enlarged shoot meristem such as callusing, direct organogenesis, direct somatic embryogenesis, explants at different stages of development were fixed in acetic acid: ethanol (1: 3) for 48h. Tissues were then dehydrated by passing through an ethanol series (first in 10 % ethanol, next in 20 %, like that till 100 % in 10 % increments). Explants were aspirated and infiltrated under vacuum for 5-8h. before embedding in paraffin. Finally, sections (5-8 µm thick) were stained with safranin and mounted on slides.

3.8.1.6 *RAPD analysis of in vitro regenerants*

DNA polymorphisms of shoot meristem derived plants were analyzed using the RAPD technique. Genomic DNA was extracted from R_0 plants, along with a control (field grown) plant as done in section (5.3.4.1).

DNA amplification: A total of 6 random decamer primers (OPA-01, OPA-03, OPD-01, OPE-01, OPL-03 and OPL-14) from *Operon Technologies*, were used for detecting polymorphism in genomic DNA. PCR amplifications were performed following the protocol of Williams et al. (1990). Amplification reactions were carried out in a thermocycler (*M. J. Research PTC 2000*) programmed for 38 cycles of amplification. Amplified products were analyzed on 1.8 % agarose gels.

3.8.2 Callus culture and regeneration from immature embryos

For surface sterilization, seeds were placed in 100 ml distilled water with 2 drops of *Tween-20* and washed for 10 min. Following rinsing thrice with sterile distilled water,

seeds were placed in 70 % ethanol for 1 min and then they were collected in a sterile flask. Next, seeds were rinsed again with sterile water for 5 min. After decanting the water, seeds were placed in 0.1 % $HgCl_2$ for 7 min under continuous stirring. Next, seeds were thoroughly rinsed again with sterile water twice for 10 minutes. Immature embryos (0.5-1.5 mm in size) were ascetically removed and placed on LS medium supplemented with 2-4-D (0.0, 1.5 and 2.5 mg L^{-1}) and Kinetin (0.0, 0.1 and 0.5 mg L^{-1}). After 18-21 days, primary callus measuring approximately 1mm in diameter was removed from the scutellum and used to establish embryonic callus. For the production of shoots, embryogenic calli were transferred to MS medium with BAP (0.0, 0.5 and 1.5 mg L^{-1}) and NAA (0.0, 0.1 and 0.2 mg L^{-1}). When shoots were 2-3 cm long, they were transferred on to half strength MS medium with 2 mg L^{-1} IBA and 0.2 IAA for rooting. Fully rooted plants after one month of culture were removed from the magenta box, thoroughly rinsed with sterile water and transferred to *jiffy* cups containing autoclaved vermiculite mixture. Plants were hardened for ten days in the culture room maintained at 26 °C and 45 % relative humidity with a photoperiod of 16h light /8h dark. Next, plants were transferred to greenhouse and grow to maturity. Next, plants were transferred to greenhouse and grown to maturity.

3.8.3 Callusing and regeneration from immature inflorescence

Young inflorescence ranging in length from 1.0 -1.5 cm was cut into 4 - 5 pieces and placed on LS medium supplemented with 2-4-D (0.0, 1.5 and 2.5 mg L^{-1}) and Kinetin (0.0, 0.1 and 0.5 mg L^{-1}) and incubated in dark at 26 °C. After three weeks, they were subcultured onto LS medium with 1.5 mg L^{-1} 2,4-D and 0.5 mg L^{-1} Kinetin and incubated

for 2 weeks. For formation of shoots, embryogenic callus was transferred on to MS medium with BAP (0.0, 0.5 and 1.5 mg L^{-1}) and NAA (0.0, 0.1 and 0.2 mg L^{-1}). Next, shoots were transferred on to half-strength MS rooting medium with 2 mg L^{-1} IBA and 0.2 mg L^{-1} IAA. Fully rooted plants after one month of culture were removed from the magenta box, thoroughly rinsed with sterile water and transferred to jiffy cups containing autoclaved vermiculite mixture. Plants were hardened for ten days in the culture room maintained at 26 ^{0}C and 45 % relative humidity with a photoperiod of 16h. light /8h. dark. Next, plants were transferred to greenhouse and grow to maturity.

3.9 *Establishment of in vitro and in vivo systems for identification and evaluation of transgenic plants*

Preparation of Basta solution

Basta is supplied in the liquid form as 13.5 % w/w solution (*Hoechst Schering AgrEvo* Ltd. Ankleshwar, India). Solution was diluted with distilled H_2O, sterilized by filtration (22 μM pore size) and used.

Preparation of phosphinothricin solution (10 mg/ml)

Phosphinothricin is supplied in powdered form. To 20.0 ml of distil. H_2O, 0.2 g phosphinothricin was added, and contents dissolved by stirring. Next, solution was sterilized by filtration (22 μM pore size) and stored at 4 ^{0}C in a dark bottle.

All the experiments were repeated and there were multiple replications for each treatment.

3.9.1 Kill curve with selection agents Basta and PPT

Kill curve experiments were performed to determine the dose response of callus and meristem explants to selection agents Basta and PPT. The objective was to ascertain concentration of selection agent used in the tissue culture media that allow the selective growth of transformed cells.

Lethal doses were determined for 40 day old calli, one day old shoot apices and tissue stratum loaded with somatic embryo's. Callus was transferred on to LS medium and shoot apices on to MS medium respectively, containing different concentrations of the selective agents Basta or PPT (0, 0.5, 1, 2, 3, 4, 8 and 12 mg $L^{-1}$and 2 mg L^{-1}). Selective agents were filter- sterilized and then added to the media. There were 10 explants per plate. Next, callus plates were incubated in dark and shoot apices in light for 1 week.

3.9.2 Determination of Basta concentrations that are lethal to the germination and growth of seedlings.

Seeds were surface sterilized as done in section (5.1.1.1) and were placed in petri dishes on sterile filter paper soaked with Basta solution, sealed with *parafilm*, and were incubated in light (16h. per day 7000 lux) at 26 ^{0}C and 45 % relative humidity.

Seven levels (100, 200, 300, 500,700, 1000, 2000 mg L^{-1}) of Basta solution dissolved in sterile distilled water were used along with control (sterile distilled H_2O). For each treatment, one plate containing 25 seeds, and 4 replications (a total of 100 seedlings per treatment) was used. Approximately, 5 ml of Basta solution was added to

each plate under aseptic condition. Percentage of germination was recorded at 24h intervals, over 4 days starting from third day of treatment.

3.9.3 Chlorophenol red assay

For chlorophenol red assay, we modified the protocol of Kramer et al., (1993). Young leaves of greenhouse grown sorghum plants at 5-leaf stage were used for the experiment. With the help of scissors, leaves were separated from the plant, surface-sterilized and cut into pieces of 3-cm size. Leaf pieces were next transferred to petri dishes at the rate of two pieces per plate, containing MS medium (Murashige and Skoog, 1962) and 4.0 M BAP and 0.5 M NAA, with different levels of Basta (0, 4.0, 8.0, 12.0, 16.0 and 32.0 mg L^{-1}). The solutions were distributed at the rate of 20.0 ml per plate, and for each treatment, there were two replications. For all the concentrations, initial pH was noted. At the end of incubation period, pH was measured again. Next, 25.0 µL of 0.5 % chiorophenol red (*Flow laboratories, Scotland*) was added to plates, and the gradation in colour intensity was noted visually.

3.9.4 Determination of dosage response of seedlings to Basta spraying

To ascertain Basta levels that are lethal to the growth of young sorghum plants, 7, 10, 14 and 30 days old glasshouse germinated plants of sorghum genotypes BTx-623, 296B and M35-1 were used. Five levels (50, 75, 100, 300 and 500 mg L^{-1}) of Basta solution dissolved in distilled water were used along with control. For each treatment, one pot containing 15-20 seedlings were sprayed. Solution of Basta was sprayed on the plants

using a sprayer. Approximately 25 ml of solution was applied from top to each pot. Observations were recorded from day 3 to day 7 from the start of the treatment.

3.9.5 **Determination of dosage response of plants to Basta application.**

The objective was to ascertain Basta levels that are lethal to the fully expanded 5 leafed Sorghum plant. For this, greenhouse germinated 30 day old sorghum plants of genotypes BTx-623, 296B and M35-1 at 5-leaf stage of development were used.

Eight levels of Basta solution (0.001, 0.025, 005, 0.01, 0.25, 0.5, and 1.0 %) along with control (0 %) dissolved in distilled water were used. For each treatment, one pot with 5 seedlings was used. Solution was applied by dipping upper 3 inches of the third and fifth leaf into a 25 ml beaker, three-fourth filled with Basta solution. Observations were recorded at 24-hour intervals over a period of 4 days from the start of the experiment. Area of the applied leaf that got scorched was accordingly recorded on a 0 to 9 scale (0-no scorching, 9-100 % scorching) against the treatment given.

3.9.6 Parameters affecting microprojectile bombardment

Several parameters of particle bombardment such as preparation of DNA samples, osmotic treatment of the explants, the flight distance of DNA coated tungsten particles, helium pressure - all affecting efficiency of transformation were studied using replicated samples.

3.10 Standardization of genetic transformation using particle inflow gun

Genetic transformation work was carried out using indigenously built particle inflow gun (PIG). This involves bombarding Tungsten particles coated with plant expression cassette into the explants. For protocol standardization we used a plasmid with *uidA* as reporter gene and PAT as marker gene under the control of 35S promoter, encoding enzyme, which confers resistance to herbicide phosphinothricin (PPT) and its analogues (Fig. 2). Explants bombarded so far are, callus derived from immature inflorescence, immature embryos and isolated shoot apices. All the experiments were repeated and there were multiple replications for each treatment.

Fig. 2. Schematic diagram of the PATGUS Gene construct used for genetic transformation of sorghum along with controlling elements. Construct was kindly gifted by Dr Ray - Wu of Cornell Univ. USA. PATGUS vector is 8.7 kb long, with *uidA* gene under the control of rice actin promoter and *bar* gene under the control of CAMV 35 promoter

Histochemical GUS assay of bombarded tissues was carried out for optimization of bombardment protocol. Selection of transformants was done on medium with herbicide Basta, but later PPT alone was used. For this, Basta and phosphinothricin kill curves were established.

3.10.1.1 *Preparation of Plasmid DNA for particle bombardment.*

Preparation of solutions

I. B Media per liter

Bacto-tryptone 10 g

Bacto-yeast extract 5.0 g

NaCl 5 g

pH of the medium was adjusted to 7.0 with 1 ml 1N NaOH. For solid medium 1.5 % agar was used. Antibiotics stock solutions were made at 50 mg/ml and stored at 20 ^{0}C.

$T_{10}E_1$ Buffer

Tris-Cl pH 8.0 - 10 mM

EDTA pH 8.0 - 1 mM

Ethidium bromide (10 mg/ml)

To 20.0 ml of distil. H_2O, 0.2 g ethidium bromide was added, and contents dissolved by stirring. Solution was store at 4 ^{0}C in a dark place.

0.5 M EDTA pH 8.0 (500 ml)

To 93.05 g of EDTA, 300 ml of distil. H_2O was added in a 1000 ml beaker, and the contents were dissolved while stirring. Sodium hydroxide was added to adjust the pH to 8.0, and the volume made to 500 ml with distil H_2O.

Protocol

3.10.1.2 *QIAGEN plasmid miniprep kit protocol*

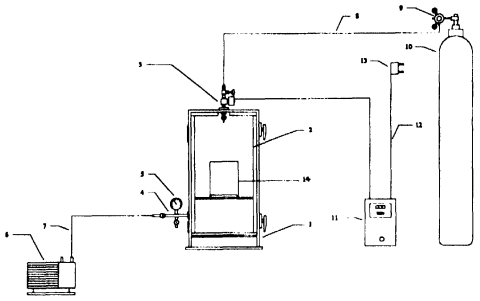
This protocol was designed for purification of plasmid DNA from 1-5 ml overnight cultures of *E. coli* in LB (Luria-Bertani) medium. Bacterial cells were grown overnight on a rotary platform at 270 rpm and 37 ^{0}C in microfuge tubes. Antibiotic ampicillin was added at 50 μg/ml concentration. Cells were harvested by centrifuging the tubes at 7500 rpm for 10 min. Pelleted bacterial cells were re-suspended in 300 μl of Buffer P1 (RNase A added) and transferred to a microfuge tube. To this, 300 μl of Buffer P2 was added and the tube was gently inverted 4-6 times and incubated at room temperature for 5 min. To this, 300 μl of ice cold Buffer P3 was added and the tube was mixed immediately 4-6 times and incubated on ice for 5 min. Next, the contents of the tube were centrifuged at 6000 rpm for 10 min. at 4 ^{0}C. During the centrifugation step, a *QIAGEN* tip 20 was equilibrated by applying 1.0 ml of QTB buffer. The supernatant from step 4 was now applied to the *QIAGEN*-tip 20 column by pipetting. Columns were then centrifuged for 30-60 sec. and the flow-through was discarded. Column was washed twice with Buffer QC adding 2 ml each time. Flow-through was discarded.

Finally, to elute the DNA from *QIAGEN* -tip 20 column, 800 μl of buffer QF was added to the column and let stand for 1 min. The flow through was collected in a sterile 1.5-ml microfuge tube. DNA was precipitated with 0.7 volumes of isopropanol. Next, microfuge tubes were centrifuged at 10,000 rpm for 30 min. Pellet was then washed with 70 % ethanol, air dried, and re-suspended in 20 μl of TE buffer pH 8.0.

3.10.1.3 *Working principle of the bombardment device*

Particle bombardment device is designed to pump tungsten or gold particles into target tissue under partial vacuum (using a timed burst of helium gas). The system consists of a thick-walled vacuum chamber having a door fitted with latches at two places (Fig. 3). The interior of the chamber has racked horizontal adjustable shelves (which are separated by 1.0 cm apart) to accommodate petridishes containing target tissue. An electronic control device, solenoid and metering valves were connected to the bombardment chamber to facilitate the conditions and acceleration of microprojectiles.

Fig. 3. Schematic diagram of the PIG used in the present study.



1. Vacuum Chamber 2. Target Shelf 3. Gun Assembly 4. Vacuum Control Assembly 5. Vacuum Gauge 6. Vacuum Pump 7. Vacuum Hose 8. Helium Hose 9. High Pressure Gas Regulator 10 Helium Cylinder 11. Control Station 12. Power Cord 13. Plug Pin. 14. Baffle.

Particle Inflow Gun

ENDEAVOUR ENTERPRISES

FIG-1 : Schematic Diagram

Prepared by : K. DURGA VARA PRASAD

NOTE : Restricted drawing - For limited Circulation

The helium gas cylinder with pressure gauge is connected to the needle adapter (fitted at the top of the chamber) *via* a solenoid valve. On the other side of the chamber, a vacuum pump is connected *via* a vacuum gauge with an opening to release the vacuum to outside.

For each bombardment, concentrated particle suspension is placed in the center of the pre-autoclaved screen of the syringe filter unit, which is then screwed into the needle adapter in the PIG. Next, petri-plate containing the target tissue is placed on the adjustable shelf at the desired distance from the tip of the filter unit. Baffles made of nylon screens (1.0 mm) are placed above the petri-plates. Helium solenoid opening time is set to 0.1 sec. Vacuum pump is turned on, and when vacuum is raised to the desired level (550-600 mm Hg) as indicated by the gauge, and the solenoid-activating switch is pressed. This results in a burst of helium gas, which accelerates the tungsten particles towards the target material. Following firing, the bombardment chamber is vented to atmospheric pressure, and DNA is loaded for another round of bombardment.

3.10.1.4 *Sample preparation*

Preparation of solutions

Preparation of tungsten particles

50 mg of tungsten particles were suspended in 1.0 ml of 96 % ethanol in a microfuge tube. Particle suspension was sonicated for 30-sec using a sterile tip to destroy particle aggregates. Next, microfuge tube was centrifuged for 1 min, and the supernatant removed. Particles were re-suspended in 1.0 ml of ethanol, and the centrifugation step was repeated. This was followed by three washes in 1.0 ml sterile distilled water as

described above. Finally, particles were suspended in 1.0 ml of SDW, made into 50.0 µl aliquots and stored at –20 ^{0}C.

Spermidine (100 mM)

Spermidine is supplied as a 0.92 g/ml density solution. 15.8 µl of spermidine was dissolved in 1 ml of deionized H_2O, filter sterilized and stored in aliquots of 25.0 µl at 20 ^{0}C.

Calcium chloride (2.5 M)

Eleven grams of $CaCl_2$ was initially dissolved in 10 ml of distil. H_2O, and the final volume made to 20 ml. Solution was next filter sterilized and stored at 20 ^{0}C

Protocol

DNA mixture for bombardment was prepared as follows: 50 µl of tungsten suspension was transferred into sterile microcentrifuge tube and vortexed; to this, 10 µl of plasmid DNA, 50 µl of 2.5 M $CaCl_2$ and 20 µl of 100 mM spermidine were added in that order. After addition of each component, tube was vortexed briefly. This mixture was then placed on ice for 5 min. When the tungsten along with the coated DNA settled down, 100 µl of the supernatant was discarded and the rest of the aliquot used for microprojectile bombardment.

3.10.1.5 *Osmotic treatment*

For microprojectile bombardment, the explants were placed on an osmotic containing 0.4 M mannitol and 0.4 M sorbitol) for 4, 8 or 12h. before bombardment with ten explants arranged over a sterile filter paper at the center of the petri-plate (3-cm diameter). After particle bombardment, explants were left on the osmoticum medium for - 0, 2, 4, 16, 24 or 48h. in the culture room, and later transferred to MS induction medium.

3.10.1.6 *Particle bombardment*

For each bombardment, 5 µl of the concentrated particle suspension was loaded into the syringe filter unit. Petridishes containing the explants were then placed on the adjustable shelf at 4, 7 or 15 cm distance from the tip of the filter unit. Explants were next bombarded under a helium gas pressure of 10, 15 or 18 kg/cm^2 under partial vacuum (600 mm Hg). Explants were analyzed for transient GUS expression according to the procedure of Jefferson et al. (1987).

3.10.2 Selection and Regeneration

Selection was imposed form 2 weeks after bombardment. Calli derived from immature inflorescence and immature embryos were selected on Basta, and shoot apices on medium with PPT. A three-step selection strategy was used for obtaining transformants. Explants were selected on 1.0 mg L^{-1} of selection agent for 10 days, and the surviving explants were sub cultured onto the same medium. Surviving explants were next selected on 2.0 mg L^{-1} of selection agent for another 2 x 10 days period (step 1 and step 2). Under step 3, after 6 weeks of selection, the surviving explants were transferred onto medium devoid of selection agent.

3.10.3 Confirmation of transgenics

3.10.3.1 *Histochemical test with X-Gluc*

Preparation of *X-Gluc* solution

For preparing 20 ml of *X-Gluc* reaction mix, the following solutions were added:

5.0 ml of phosphate buffer (50.0 mM) pH 7.0

1 ml of 0.1 M potassium ferrocyanide

1.0 ml of 0.1 M potassium ferricyanide

120 µl of Triton X- 100

12.3 ml of SDW.

Contents were filter sterilized and store at -20 ^{0}C.

Protocol

Explants were washed twice with 100 mM Na- Phosphate buffer, pH 7.0, and were then submerged in 200 µl of reaction mix in a sterile microfuge tube and vacuum infiltrated for 2 min. Next, these tubes were incubated at 37 ^{0}C for 24 - 48h. Destaining was done by transferring the explants from reaction mix to a second microfuge tube containing destaining solution (25 % glacial acetic acid and 75 % absolute ethanol) and incubated at 65 ^{0}C for 1h. GUS activity was estimated by counting the number of blue spots on the surface of bombarded explants.

3.10.4 **Molecular tests**

3.10.4.1 *Genomic DNA isolation and purification*

Preparation of solutions

CTAB extraction solution

2 % (w/v) CTAB

100 mM Tris. Cl, pH.8.0

20 mM EDTA pH.8.0

1.4 M NaCl

After adjusting the pH, buffer was stored at room temperature.

Rnase (2 mg/ml)

8.0 mg of RNase was dissolved in 4 ml of distil. H_2O, made into aliquots of 1 ml each and stored at –20 ^{0}C.

Proteinase K (2 mg/ml)

20 mg proteinase K, was dissolved in 10 ml of distl. H_2O, made into aliquots of 1 ml each and stored at –20 ^{0}C.

$T_{10}E_1$ Buffer

Tris-Cl pH 8.0 - 10 mM

EDTA pH 8.0 - 1 mM

Ethidium bromide (10 mg/ml)

To 20.0 ml of distil. H_2O, 0.2 g ethidium bromide was added, and contents dissolved by stirring. Solution was store at 4 ^{0}C in a dark place.

0.5 M EDTA pH 8.0 (500 ml)

To 93.05 g of EDTA, 300 ml of distil. H_2O was added in a 1000 ml beaker, and the contents were dissolved while stirring. Sodium hydroxide was added to adjust the pH to 8.0, and the volume made to 500 ml with distil. H_2O.

Sodium acetate (2.5M)

To 340 of sodium acetate, 400 ml of distl. H_2O was added, and the contents dissolved by stirring. pH was adjusted to 5.2 with 3M acetic acid and the volume made upto 1 liter with distil. H_2O prior to autoclaving.

Buffered phenol

The distilled phenol was removed from freezer, and allowed to thaw at 68 °C in a water bath. To this, 8-hydroxy quinoline was added to a final concentration of 0.1 %. The melted phenol was then extracted several times with an equal volume of 1M Tris (pH 8.0), until the pH of the aqueous phase was more than 7.6. Buffered phenol was stored at 4 °C.

Protocol

Genomic DNA was extracted from the young leaves of the *in vitro* regenerated (control and bombarded). The CTAB method of DNA extraction was followed. Young leaves (5.0 g) were harvested, midribs were removed, and the leaf blades were then cut into small pieces with scissors, lyophilized in liquid nitrogen and stored at -70 °C. The leaves were ground to fine powder in a mortar and pestle, and then transferred into a 50 ml centrifuge tube containing 15 ml of warm (65 °C) CTAB buffer. The contents were mixed well on a rotating shaker and incubated for 2h. at 65° C in a water bath with occasional mixing. The tubes were taken out, cooled to room temperature, and 10 ml chloroform-isoamyl alcohol (24:1) was added and mixed gently by inverting for 5-6 times and centrifuged at 6000 rpm for 20 min. The aqueous phase was transferred to a new 30 ml tube, to which 10 ml chloroform-isoamyl alcohol was added and mixed gently 5-6 times. Next, the extract was centrifuged at 6000 rpm for 20 min at 4 °C. The aqueous phase was then transferred to 30 ml centrifuge tube. To it 10 ml of isopropanol was added and mixed gently by vortexing for 5-6 times, and kept at -20 °C for 60 min. DNA was then spooled using a pasture pipette. Pellet was then washed with 100 % ethanol and the tubes were inverted on a paper-towel to allow ethanol to evaporate.

3.10.4.1.1 Purification of DNA with RNase

The above DNA was treated with 2 ml of TE buffer containing RNase (0.2 µg/ml) and incubated at 37 ^{0}C for 1h. After the RNase treatment, 200 µl of 5M NaCl was added followed by gentle shaking and incubation at 4 ^{0}C for 15-20 min. The tubes were then centrifuged at 6000 rpm for 20 min. at 4 ^{0}C. The aqueous phase was transferred to a 5 ml tubes. Next, 2 ml of phenol: chloroform (1:1) was added and tubes were centrifuged at 2500 rpm for 10 min. at 2 ^{0}C. Equal volume of chloroform: isoamyl alcohol (24:1) was added to the aqueous phase and centrifuged at 2500 rpm for 10 min. at 2 ^{0}C. For precipitation of DNA, 200 µl of 2.5 M sodium acetate pH 5.2 was added to the aqueous phase mixed well and 2 ml of absolute alcohol was added and again mixed well, incubated at -20 ^{0}C for 15-20 min. The precipitated DNA was spooled with a glass hook into a 1.5 ml microfuge tube and washed with 76 % and then 100 % alcohol. TE buffer was added to dissolve the pellet and stored at 4 ^{0}C.

Quantification of DNA

The quantity and purity of the DNA samples were determined spectro-photometrically by measuring the absorbance at 260 and 280 nm.

3.10.4.2 *Protocol of PCR for analysis of transformants*

The 1.8 kb DNA fragment of *uidA* gene was amplified by PCR using the forward primer 5'-GGTGGGAAAGCGCGTTACAAG 3' and reverse primer 5'-GTTTACGCGTTGCTTCCGCCA 3'. Polymerase chain reaction was carried out in a 25-µl reaction volume containing 25 ng of plasmid (used as positive control DNA) and 125 ng of plant DNA with 20 pM of each of the primer, 0.1 mM dNTP's, and 2.5 U of

Taq polymerase in 1X PCR buffer (PHARMESIA with 15 mM of $MgCl_2$), using MJ 200 thermocycler machine. Amplification was carried out at the following temperature: 94 ^{0}C for 1 min. (denaturation), 55 ^{0}C for 1 min. (annealing) and at 72 ^{0}C for 1.5 min. (extension) for 38 cycles.

3.10.4.3 *Genomic Southern hybridization analysis of transformants using radio-labeled probes*

Preparation of solutions

TBE buffer

Tris - Borate	0.089 M
Boric acid	0.089 M
EDTA	0.002 M

Loading buffer

10 % glycerol

0.02 % bromophenol blue

20X SSC

NaCl	175.3g
Sodium citrate	88.2 g
distl. H_2O	800 ml

pH was adjusted to 7.0 with 0.1 N NaOH, and the final volume made upto 1 litre prior to autoclaving.

Denatured salmon sperm DNA

The DNA was dissolved in distil. H_2O to a concentration of 10 mg/ml by stirring on a magnetic stirrer for 2 - 4h. DNA was sheared by passing it several times through an 18-gauge hypodermic needle, boiled for 10 min., made into aliquots and stored at -20 °C. Just before use, the DNA was heated for 5 min. in a boiling water bath and chilled quickly on ice.

50X Denhardts

Ficoll 5 g

PVP 5 g

BSA 5 g

Distil. H_2O to 500 ml.

Prehybridization solution

6X SSC

0.5 % SDS

100 µg/ml denatured salmon sperm DNA.

Protocol

For Southern analyses, genomic DNA (15 µg) of transgenics and control plants was digested with four restriction enzymes (single enzyme digestion with *Xba* I, and double digests with a combination of *Eco* RI and *Hind* III or *Eco* RI and *Bam* HI). Digestions were carried on according to the manufacturer's instructions (*Amersham*) with 2 units of enzyme per microgram of DNA over 15h. at 37°C. Aliquots of digested and purified

DNA were loaded onto a 0.8 % agarose gel in TBE buffer. After electrophoresis, the gel was denatured in 0.25 N HCl for 15 min. and subsequently in 0.4 N NaOH for 1h. DNA was next blotted onto a Hybond N$^+$ membrane (*Amersham*).

Restriction digested *uidA* fragment was used as probe at the rate of 75 ng of probe DNA per labeling reaction. PATGUS construct was digested with *Hind* III and *Bam* H1 to release the GUS coding region. Fragments were collected form 0.8 % LMP agarose gel, and purified using QIAGEN Gel Extraction Kit as per manufactures instructions.

Probes were labeled with the random primer DNA labeling kit (*NEBlot* Kit, *Biolabs*, New England) for 2h. with [^{32} dATP] to a specific activity of $1x10^9$ cpm per microgram. Prehybridization and hybridization were carried out in a buffer containing 6X HSB, 0.5 % SDS, 5X Denhart's and 25 % 5 μg ml^{-1} of sonicated and denatured salmon sperm DNA. The blots were pre-hybridized for 6h. at 65°C in 30 ml buffer. The prehybridization solution was then removed and replaced with 10 ml of buffer containing denatured radioactive probe at $1x10^6$ cpm per milliliter; hybridization was then performed overnight (16h.) at 65 °C. The blots were then washed at 65 °C for 30 min. each in 2X SSC, 0.5 % SDS and once in 2X SSC, 0.1 % SDS and finally once for 30 min. in 0.1X SSC, 0.1 % SDS. Blots were then wrapped in *Saran Wrap* and exposed for 4 days to *Hyperfilm* (*Kodak*) with intensifying screens at - 70 °C.

3.10.4.4 *Extraction of total RNA and DNase treatment*

Preparation of solutions

DEPC treatment of solutions

To every 100 ml of distil. H_2O, 0.2 ml DEPC solution was added, and the contents were vigorously shaken to get the DEPC into H_2O. Only this water was used for preparing the required solutions .All the required glassware was rinsing with this DEPC solution, and baked at 180 ^{0}C for 4h.

Sodium citrate (1M)

29.41g Sodium citrate was dissolved in 60.0 ml of DEPC treated H_2O and the contents dissolved by stirring. pH was adjusted to 7.0 and the volume final volume made upto 100 ml.

Lithium chloride (4M)

16.96 g of LiCl was dissolved in 60 ml of DEPC treated H_2O and the volume adjusted to 100 ml.

Sodium acetate (2M)

To 27.22 g of sodium acetate, 54 ml of DEPC treated H_2O was added, and the contents dissolved by stirring. PH was adjusted to 4.1 with glacial acetic acid and the volume made upto 100 ml with DEPC treated H_2O.

Water saturated phenol

The distilled phenol was removed from freezer, and allowed to thaw at 68 ^{0}C in a water bath. The melted phenol was then extracted several times with an equal volume DEPC treated H_2O, until the pH of the aqueous phase became acidic. solution was stored at 4 ^{0}C.

Denaturation solution

100.0 g GTC

117.2 ml H_2O.

5.5 ml of 1M sodium citrate buffer pH 7.0 (25 mM)

10.56 ml of 10 % sarcosyl

Final volume was made to 220.0 ml with DEPC treated water.

Working solution

To 50 ml GTC solution 0.36 ml of β- mercaptoetanol was added immediately before use.

Formaldehyde loading buffer

1 mM EDTA, pH 8.0

0.25 % (w/v) bromophenol blue

0.25 % (w/v) xylene cyanol

50 % (v/v) glycerol

10X MOPS running buffer

0.4 M MOPS, pH 7.0

0.1 M sodium acetate

0.01 M EDTA

Buffer was stored in a dark place.

Protocol

Total RNA was extracted from leaves of 5 T_0 transgenic plants along with control plant. The Guanidium–Thiocyante, Acid - Phenol method of RNA extraction was followed. Young leaves (1.0 g) were harvested, midribs removed, leaf blades were cut into small pieces of 1-2 cms and, immediately lyophilized in liquid nitrogen and stored at -80 ^{0}C until further use. Leaves were ground to fine powder in a mortar and pestle, and then

transferred into 50.0 ml centrifuge tubes containing 10.0 ml of extraction buffer (4.0 M guanidine thiocyanate, 25.0 mM sodium citrate (pH 7.0), 0.5 % sarcosyl, 0.1 M β-mercaptoethanol). Contents were mixed well, and 1.0 ml of 2M sodium acetate pH 4.0, followed by 10.0 ml of water-saturated phenol, and finally 2.0 ml of 49:1 chloroform/isoamyl alcohol were added. Tubes were vortexed briefly after adding each component, and finally incubated for 15 min. at 4 ^{0}C. At the end of incubation, samples were centrifuged at 4 ^{0}C for 20 min. Upper aqueous phase was transferred to a fresh tube, and equal volume of 100 % ice cold isopropanol was added, and tubes were incubated at -20 ^{0}C for 1h. RNA was pelleted by centrifugation at 10000 rpm, and the pellet was washed with 4M LiCl, followed by centrifugation at 10000 rpm. Pellet was re-dissolved by adding 3.0 ml of extraction buffer, and the homogenate was transferred to a fresh tube. RNA was now re-precipitated with equal volume of 100 % isopropanol, incubated at -20 ^{0}C for 1h. and centrifuged at 10000 rpm for 10 min. RNA pellet was washed with 75 % ethanol, vacuum dried for 10 - 15 min. and dissolved in 200.0 µl sterile DEPC treated water.

To 200.0 µl of total RNA, 10 Units of DNase I, Rnase- free (*Roche*) in 5.0 µl of 20X DNase buffer was added, and incubated at 37 ^{0}C for 2h. DNase was inactivated by adding 10.0 µl DNase inactivation reagent (*Ambion*) and incubated at room temperature for 2 min.

Quantification of RNA

The quantity and purity of the RNA samples were determined spectro-photometrically by measuring the absorbance at 260 and 280 nm. Integrity of the RNA was visually observed by electrophoresis on formaldehyde gel.

3.10.4.5 *Protocol of Reverse transcription*

First strand synthesis was performed in a total volume of 20.0 µl. Two independent cDNA-priming steps were tried. In the first method, cDNA synthesis was primed by oligo - dT M, and in the second method random primers (*Life Technologies*) were used to prime cDNA synthesis. Except this, all the other components and parameters were same. Reaction mixture (RT) consisted of 1.0 µl of 2.0 µM oligo dT primer (dT15 from Gene Hunter) or random hexamers (*Life Technologies*), 100 units of MMLV reverse transcriptase *(USB)*, 2.0 µl of 1mM dNTP 1.0 µl of 100 mM DTT, and 2 µl of 5X first strand buffer [125 mM Tris (pH 8.3), 7.5 mM $MgCl_2$ and 188 mM KCl].

Total RNA (0.5 µg) was heat denatured by incubating at 65 ^{0}C for 5 min., followed by addition of RT mix to the samples, and incubated at 37 ^{0}C for 120 min. cDNA - RNA hybrid was separated by heating the tubes at 75 ^{0}C for 5 min., and the products were stored at –20 ^{0}C until further use.

3.10.4.6 *PCR analysis of cDNA using uidA gene specific primers*

The 1.2 kb cDNA fragment of *uidA* gene was amplified by PCR using the forward primer 5'-GGTGGGAAAGCGCGTTACAAG 3' and reverse primer 5'-GTTTACGCGTTGCTTCCGCCA 3'. Polymerase chain reaction was carried out in a 25-µl reaction volume containing 25 pg of plasmid which served as positive control DNA) and 1.0 µl of cDNA with 20 pM of each of the primer, 0.1 mM dNTP's, and 2.5 U of Taq polymerase in 1X PCR buffer (*Pharmesia* with 15 mM of $MgCl_2$), using MJ 200 thermocycler machine. Amplification was carried out at the following temperature: 94 ^{0}C

for 1 min. (denaturation), 55 ^{0}C for 1 min. (annealing) and at 72 ^{0}C for 1.5 min. (extension) for 32 cycles.

To detect DNA contamination in the total RNA, the above PCR reaction was repeated after DNase treatment. In this, in the place of cDNA, total RNA (500 ng approx.) was added to the PCR mix.

PCR products were electrophoresed on 1.0 % agarose gel in 0.5 % TBE buffer, stained with Ethidium bromide (0.5 mg L^{-1}) and visualized under UV illumination.

3.11 Inheritance studies of transgenes

Progeny, from each of the four T_0 plants of the genotype 296B and from two T_0 plants of the genotype BTx-623, confirmed by Southern hybridization were used in inheritance studies. Plants were grown in the P_2 greenhouse at ICRISAT and subjected to GUS (histochemical), herbicide (dip, germination and chlorophenol red screening methods) and molecular (PCR and Southern) tests as described in earlier sections.

4 RESULTS

4.1 Establishment of in vitro regeneration systems for sorghum

In vitro response of three explants – isolated shoot apices, immature embryos and immature inflorescence were studied. For this, three sorghum genotypes M35-1, 296B and BTx-623 were used. In the present study, a rapid regeneration protocol from shoot meristem explant has been standardized by striking an optimal balance between NAA and Thidiazuron. The isolated shoot apices were manipulated to follow either organogenic or embryogenic pathway. Further, various factors influencing the efficiency of regeneration were studied. There was no intermediate callus formation in both cases. In the case of immature embryo and immature inflorescence, Linsmaier and Skoog (LS) medium supplemented with 2, 4-dichlorophenoxy acetic acid (2,4-D) and kinetin was used for induction of friable embryogenic calli. For regeneration, MS medium supplemented with BAP and NAA was employed. Regeneration pathway in the above two explants was *via* somatic embryogenesis.

4.1.1 Rapid regeneration from shoot meristem explants

4.1.1.1 *Optimization of hormone combination for in vitro meristem culture*

The morphogenic potential of isolated shoot apices was evaluated on MS basal medium supplemented with various concentrations of BAP, NAA, and TDZ. Among the three sorghum genotypes (BTx-623, M35-1 and 296B) tested for their induction (meristems producing multiple buds) and regeneration response (meristems that gave rise to plants),

induction percentage was maximum (80 ± 1.5 %) for genotype BTx - 623, while it was 72 ± 2 % for M35-1 and 54 ± 1 % for 296B. After completion of 6 weeks of culture covering 5 subcultures, 35-40 shoots per explant were obtained in the genotype BTx - 623.

Manipulation of BAP and NAA combinations after a common 4 weeks culture treatment resulted in direct somatic embryogenesis from the translucent outgrowths or tissue stratum of the multiple buds as described below. About 700 -1000 germinating somatic embryos could be seen on each explant. Among the three genotypes, BTx-623 showed the highest induction frequency of 80 ± 2 %, followed by M35-1 (65 ± 3 %) and 296B (61 ± 2 %). Ninety five percent of these somatic embryos developed into normal plantlets.

For induction of organogenesis on shoot apices, MS medium along with BAP and NAA were tried and the response was noted after 2 weeks of culture. Variation in BAP concentration (1.0 - 4.0 mg L^{-1}) had no significant effect on shoot development (Table 7). Few explants produced fleshy abnormal shoots and the remaining explants turned dark brown. On the other hand, use of BAP along with NAA caused the explants to remain green and this varied between 0.0 % (BAP 1.0 mg L^{-1} and NAA 0.1 mg L^{-1}) to 61 ± 8 % (BAP 4.0 and NAA 0.2 mg L^{-1}).

Table 7. **Optimization of phytohormone combinations for multiple bud formation on shoot meristem.**

Hormones used along with MS basal	**Induction response after 14 days. (Mean ± SE).**		
(Conc. in mg L^{-1}).	*M35-1*	*296B*	*BTx-623*
BAP (0.0)	0.0 *	0.0 *	0.0 *
BAP (0.0) + NAA (0.0)	0.0 *	0.0 *	0.0 *
BAP (1.0)	0.0 *	0.0 *	0.0 *
BAP (2.0)	0.0 *	0.0 *	0.0 *
BAP (4.0)	0.0 *	0.0 *	0.0 *
BAP (1.0) + NAA (0.1)	0.0 *	0.0 *	0.0 *
BAP (1.0) + NAA (0.2)	0.0 *	0.0 *	0.0 *
BAP (2.0) + NAA (0.1)	0.0 *	0.0 *	0.0 *
BAP (2.0) + NAA (0.2)	0.0 *	0.0 *	0.0 *
BAP (4.0) + NAA (0.1)	15 ± 1 % **	10 ± 1.5 % **	25 ± 3 % **
BAP (4.0) + NAA (0.2)	55 ± 8 % **	44 ± 8 % **	61 ± 8 % **
BAP (4.0) + 2,4- D (0.1)	30 ± 1 % ***	32 ± 1.5 % ***	38 ± 5 % ***
BAP (4.0) + 2.4-D (0.2)	59 ± 1%	55 ± 2 %	65 ± 3 %

All the explants turned brown; No meristem enlargement. ** Explants remain green; no meristem enlargement; no bud formation. *** Meristems enlarged; callus formation observed.

There was no enlargement of meristem and no callus formation on the above medium. BAP in combination with 2,4-D resulted in meristem enlargement, but was associated with some degree of callus formation. Upto 38 ± 5 % of the meristems enlarged when BAP (4.0 mg L^{-1}) and 2,4-D (0.1 mg L^{-1}) combination was used and 65 ± 3 % meristems enlarged when BAP (4.0 mg L^{-1}) and 2,4-D (0.2 mg L^{-1}) was added to MS basal medium. Among the three genotypes, BTx-623 was more responsive.

Based on the above results, the combination of BAP (4.0 mg L^{-1}) + NAA (0.2 mg L^{-1}) which could arrest browning of the meristem with no accompanying callus formation was selected. Next, this was fortified with different levels of TDZ (0, 0.002, 0.011, 0.022, 0.066, 0.11 and 0.154 mg L^{-1}) for induction (enlargement followed by formation of multiple leaf initials) and proliferation (formation of multiple bud initials) of the meristems. TDZ at concentrations of 0.002 to 0.022 mg L^{-1} initiated only 1-2 buds (Table 8). Beyond this concentration, the number of responding explants as well as the number of shoots formed per explant increased with increasing concentration of TDZ. Use of 0.066 mg L^{-1} TDZ resulted in 4-5 buds in M35-1 and BTx-623 genotypes, and 3-4 buds in 296B genotype.

Table 8: **Optimization of TDZ concentration for multiple bud formation.**

MS + BAP (4.0 mg L^{-1}) + NAA (0. 2 mg L^{-1})	Response after 14 days. (Mean ± SE).			No. of buds after 4 weeks		
With TDZ (mg L^{-1})	M35-1	296B	BTx-623	M35-1	296B	BTx-623
0.002	**	**	**	1 - 2	1 - 2	1 - 2
0.011	**	**	**	1 - 2	1 - 2	1 - 2
0.022	**	**	**	1 - 2	1 - 2	1 - 2
0.066	35 ± 2 % ***	32 ± 6 % ***	48 ± 6 % ***	4 - 5	3 - 4	4 - 5
0.11	72 ± 2 % ***	54 ± 1 % ***	80 ± 1.5 % ***	8 - 10	6 - 7	10 - 15
0.154	58 ± 2 % ***	51 ± 3 % ***	63 ± 2 % ***	6 - 8	4 - 5	8 - 10

' * Explants remained green. No meristem enlargement and no bud formation.

'** Explants remained green. Meristem enlargement and bud formation observed.

At a concentration of 0.11 mg L^{-1} M35-1formed 8-10 buds; 296B 6-7 and BTx - 623 formed 10-15 buds on bulged meristems. At a still higher concentration of TDZ (0.154 mg L^{-1}), the response declined in all the three genotypes (Table 8). Thus, 0.11 mg L^{-1} TDZ was an optimal concentration for multiple bud formation when added to MS basal medium with BAP (4.0 mg L^{-1}) + NAA (0.2 mg L^{-1}). Among the three genotypes, BTx- 623 was more responsive.

After induction, the concentrations of BAP and TDZ were brought down gradually at each passage as shown that in (Table 9). From Passage 4, TDZ was completely withdrawn from the culture medium, which resulted in about three-fold increase in the length and breadth of the bud initials within 1 week. Last round of subculture (Passage 5) using the same media composition helped further differentiation of the buds into individual plantlets of approximately 2 cm in size.

Table 9: **Growth regulator combinations used during each passage for plant regeneration through organogenic pathway.**

Passage details.	Weeks after culture initiation.	MS basal + Conc. of growth regulators used (in mg L^{-1})		
		BAP	TDZ	NAA
Ind. medium	0	4.0	0.11	0.2
Passage 1	2	3.0	0.066	0.2
Passage 2	3	2.0	0.044	0.2
Passage 3	4	2.0	0.022	0.2
Passage 4	5	2.0	Nil	0.2
Passage 5	6	2.0	Nil	0.2

4.1.1.2 *Rooting of the plantlets and transfer to greenhouse*

Out of the twenty four hormone combinations tried for rooting of shoots, successful root initiation upto 95 % of the 500 plants regenerating from organogenesis pathway was possible within 10 days of transfer on half-strength MS medium with IBA (2.0 mg L^{-1}) and IAA (0.2 mg L^{-1}) (Table 10). After 1 month of culture, rooted plantlets were removed from the magenta box, thoroughly rinsed with sterile water and transferred to jiffy cups (*Jiffy strips, Jiffy products (N.B) LTD. Shippagan. Canada.)* Containing autoclaved vermiculite mixture. After ten days of hardening in the culture room maintained at 26 ^{0}C and 45 % relative humidity with a photoperiod of 16h light /8h dark, plants were transferred to greenhouse. 85 - 90 % of the regenerants could be transferred to greenhouse successfully. At maturity, all the acclimatized plants (R_0) obtained through *in vitro* culture were fertile and uniform without any morphological variation and they set normal seed.

Table 10: **Percentage root formation in BTx - 623 genotype with different hormone combinations.**

	1/2 strength MS +					
IBA (mg L^{-1})	NAA (mg L^{-1})			IAA (mg L^{-1})		
	0.0	0.1	0.2	0.0	0.1	0.2
0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.5	0.0	0.0	0.0	0.0	0.0	0.0
1.0	0.0	12 ± 4	13 ± 2	0.0	53 ± 3	45 ± 1
2.0	0.0	22 ± 2	19 ± 1	0.0	65 ± 4	90 ± 2

*Values given are (Mean ± SE %).

4.1.1.3 *Morphogenesis of shoot meristem*

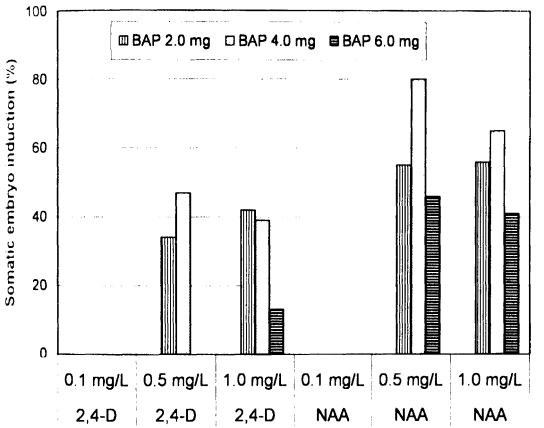
Regeneration of plants by organogenesis: 48 -72 hours after initiation of shoot apex cultures, the first leaf unfolded (Fig. 4A). At the end of first week of culture, 1-2 expanded leaf initials were seen. Unfolding of the primordial leaves followed by elongation of the leaf blade in the first week of culture was rapid. In addition, bulging of the lower meristem region was observed (Fig. 4B). This meristem bulging became prominent in the second week. Size of the meristem tissue increased 4-5 folds after 9-10 days after culture initiation, which also coincided with the appearance of additional leaf initials. These leaf initials were trimmed off when explants were 2-weeks old. This was followed by subculture onto fresh medium (Passage 1), which resulted in further proliferation of the meristem mass with production of additional leaf initials. Trimming of leaf initials for the second time was carried out at the end of 3 weeks of culture and such meristem masses were subcultured on to fresh medium (Passage 2). The production rate of leaf initials came down during this passage, with simultaneous appearance of dome-shaped buds all over the meristem mass (Fig. 4C). Four weeks after isolation, such responding meristematic masses containing multiple buds were dissected so as to produce 2 to 3 pieces and cultured (Fig. 4F) on to fresh medium (Passage 3).

Fig 4 Rapid and high efficiency regeneration system (organogenesis and somatic embryogenesis) from isolated shoot apices *in vitro*. (A) One day old isolated shoot apices with primordial leaves ***LP*** = *leaf primordia*; ***SM*** = *shoot meristem*. (B) One-week old explant showing bulged meristem portion and expanded primordial leaves. (C) Three- week old meristematic mass showing multiple buds and leaf initials. (D) Individual buds (shown in Fig.1C) producing 2-8 translucent tissue strata. (E) Each of the tissue stratum giving rise to many somatic embryos. (F) Meristematic clumps showing differentiating buds (G) Germinating somatic embryo's showing shoot apex (*SA*) surrounded by a pair of primary leaves ***(PL)***. (H) Differentiation of somatic embryos into platelets (I) Plantlets with well formed roots in magenta box. (J) Acclimatization of plantlets in the growth chamber. (K) Regenerated plants in greenhouse


LP
SAT
A
B
C
D
E
F
PL
G
H
I
J
K

Such enlarged meristems containing multiple shoots could root easily on rooting medium [half-strength MS medium with IBA (2.0 mg L^{-1}) and IAA (0.2 mg L^{-1})] (Fig. 4I). Fully rooted plantlets transferred into jiffy cups established well (Fig. 4J) and were kept in culture room maintained at 26 ^{0}C and 45 % relative humidity with a photoperiod of 16h. light /8h. dark for further acclimatization. After 1 week, hardened plantlets were transferred to greenhouse and grown to maturity (Fig. 4K).

Fig. 5 Comparison of effects of 2,4-D and NAA for somatic embryo induction frequency with different concentrations of BAP


BAP 2.0 mg
BAP 4.0 mg
BAP 6.0 mg
Somatic embryo induction (%)
100
80
60
40
20
0
0.1 mg/L 2,4-D
0.5 mg/L 2,4-D
1.0 mg/L 2,4-D
0.1 mg/L NAA
0.5 mg/L NAA
1.0 mg/L NAA

4.1.1.4 *Induction and differentiation of somatic embryos*

Direct somatic embryogenesis (without intermediary callus phase) from isolated shoot apices was tried. For this a two-step culture protocol was followed. Explants were first incubated on multiple bud induction medium (MBIM) for 2 weeks, followed by passage 1 (MS + 3.0 mg L^{-1} BAP, 0.066 mg L^{-1} TDZ and 0.5 mg L^{-1} NAA) and passage 2 (MS + 2.0 mg L^{-1} BAP, 0.044 mg L^{-1} TDZ and 0.2 mg L^{-1} NAA) (Table 11). After this, shoot apex cultures with 35-40 *de novo* shoot buds, were cut into 3-4 pieces and transferred on to somatic embryo induction medium (SEIM) (MS + 4.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA). After 4 weeks of culture on SEIM, The differentiating meristem with shoot buds on it enlarged further and developed translucent outgrowths all over its surface (Fig. 4D). On these outgrowths, numerous somatic embryos were seen (Figs. 4E). This combination was favored among all the 12 media tried (Fig. 5). Maximum induction efficiency of 80 % was observed on this phytohormone combination. Among the three genotypes, BTx-623 showed the highest induction frequency of 80 ± 2 %, followed by M35-1 (65 ± 3 %) and 296B (61 ± 2 %).

Table 11: **Growth regulator combinations used during each passage for plant regeneration through direct somatic embryogenesis pathway.**

Passage details.	Weeks after culture initiation.	Growth regulators used (in mg L^{-1})		
		BAP	TDZ	NAA
Ind. medium	0	4.0	0.11	0.2
Passage 1	2	3.0	0.066	0.2
Passage 2	3	2.0	0.044	0.2
SEIM	8	4.0	Nil	0.5
SEGM	9	2.0	Nil	0.5

These somatic embryos could be induced to germinate by transferring on to somatic embryo germination medium (SEGM) (MS medium + 4.0 mg L^{-1} BAP and 0.2 mg L^{-1} NAA (Fig. 4G). During the process of embryo development into plantlet 95 % of the embryos developed normally (Fig. 4H). While 5 % somatic embryos showed abnormal development like stunted growth and formation of curly leaves or both.

4.1.1.5 *Rooting of the plantlets and transfer to greenhouse*

Up to 95 % of the 400 plants regenerating from direct somatic embryogenesis pathway could be rooted successfully on half-strength MS medium with IBA (2.0 mg L^{-1}) and IAA (0.2 mg L^{-1}). After 1 month of culture, rooted plantlets were removed from the magenta box, thoroughly rinsed with sterile water and transferred to jiffy cups containing autoclaved vermiculite mixture. After ten days of hardening in the culture room maintained at 26 ^{0}C and 45 % relative humidity with a photoperiod of 16 h light /8 h dark, plants were transferred to greenhouse. 85 - 90 % of the regenerants could be transferred to greenhouse successfully. At maturity, all the acclimatized plants (R_0) obtained through *in vitro* culture were uniform without any morphological variation and they set normal seed.

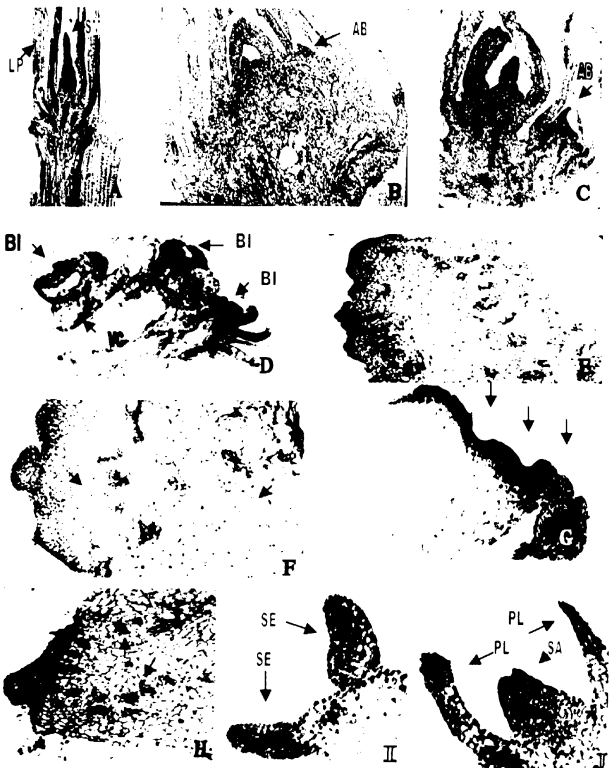
4.1.1.6 *Histological studies of in vitro cultured shoot apices*

Organogenesis: Anatomical studies were conducted to study the genesis of the multiple buds. Explant at the start of the culture showed shoot apex with two pairs of surrounding primordial leaves (Fig. 6A). Divisions on the periphery of the meristem resulted in formation of a protuberance after 7-8 days in the axial region (Fig. 6B). Along with this,

swelling of the lower part of the meristem was also noted. This protuberance differentiated into a bud initial with 2-3 pairs of overlapping leaf primordia by the end of second week (Fig. 6C). Buds became macroscopic and the vascular bundle connecting the adventitious shoots to the mother explant became clear as they advanced (Fig. 6D). As shown in the transverse section, there were 35-40 bud initials per shoot meristem (developed on the same lines) at different stages of differentiation at the end of fourth week (Fig. 6E).

Direct somatic embryogenesis: Histological examination of shoot apices cultured in SEIM showed the initiation of meristematic activity near the undulations of bulged meristems. Further developmental stages (Fig. 6G) revealed their direct origin from the epidermal and sub-epidermal layers. Section passing through translucent outgrowths of explants showed a clear demarcation of the meristematic zone as judged by the deeply stained nuclei and absence of vacuoles in the uniform cells (Fig. 6F).

Fig. 6 Anatomy (light microscopy) of sorghum organogenesis and somatic embryogenesis from *in vitro* cultured shoot apices. (A) LS of shoot apex at the start of the culture showing two pairs of primordial leaves (**LP**) surrounding shoot meristem (**SM**) (B) LS of one-week-old explant showing a differentiating bud initial, seen as a small protuberance in the axial region (***AB***). (C) LS of 2 weeks old meristematic mass showing a fully formed axillary bud (***AB***) surrounded by primordial leaf initials. (D) LS of meristematic mass showing multiple bud initials (***BI***). Vascular connection (***VC***) between buds and the mother tissue is clearly visible. (E) TS showing 35-40 buds at different stages of differentiation (F), Section passing through the translucent outgrowths showing highly meristematic cells on the upper side (*left side in the picture*) and non-meristematic, vacuolated region (*right side in the picture*) is in contact with the culture medium (G) Meristematic activity confined to epidermal and sub-epidermal layers of undulations or tissue stratum (H) TS showing numerous pro-embryos seen as deeply stained regions (I) TS of a germinated somatic embryo showing shoot apex (***SA***) and a pair of primary leaves (***PL***)

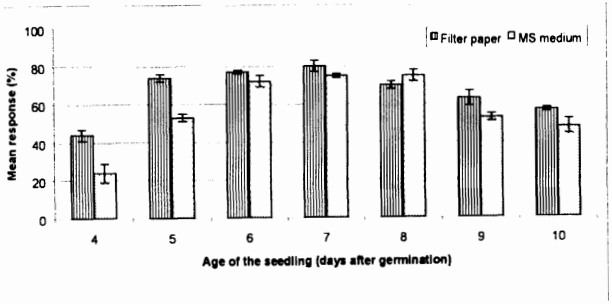

LP
A
AB
B
AB
C
BI
BI
BI
D
E
F
G
SE
SE
H
II
PL
PL
SA
J

This is in clear contrast to the non-meristematic zone showing amoeboid cells with large vacuoles. By the end of second week, many proembryos were seen in the section as isolated groups of darkly stained regions (Fig. 4H), differentiated from peripheral region of the translucent outgrowths. These proembryos further differentiated, and projected out from the translucent outgrowths (Fig. 6I). Such structures germinated, after 1 week of transfer onto somatic embryo germination medium, showing primordial leaves and shoot apex (Fig. 6J). Vascular tissue connection between embryo and mother tissue was not observed at any stage of embryo development.

4.1.1.7 *Factors effecting organogenesis*

Effect of three different factors on *in vitro* morphogenic potential of shoot apices was studied in genotype BTx-623. The difference in the induction efficiency between explants derived from seeds of small and large size was statistically not significant (data not shown). Seed germination was better on filter paper (90.0 %) than on MS medium (82.4 %). Induction response of the explants isolated from seeds germinated on filter paper was better in 6 out of the 7 developmental stages (4 to 10 days old seedlings) tested (Fig. 7). Developmental stage of the explants at the time of meristem isolation influenced the induction efficiency. Percentage of induction increased with the age of the explant from day 4 to day 7 (Fig. 7) and this response declined steadily when older explants were used for meristem isolation (tested up to tenth day following germination). Shoot tips showed gradual transformation from light yellowish on day 4 to pale green by day 7, which subsequently turned green by day 10. The size of the shoot tips varied from 2 to 5 mm during the course of experiment (day 4-10).

Fig 7 Effect of germination technique and age of the seedling on induction of multiple shoot buds form isolated shoot apices Induction response increased rapidly when 5-8 days old seedlings were used From day 9 onwards, there was decline in induction response Induction response from seedlings germinated on filter paper was marginally better than the ones germinated on MS medium

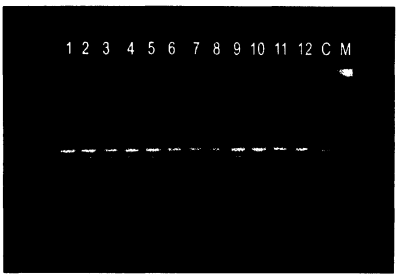

Filter paper
MS medium
Mean response (%)
100
80
60
40
20
0
4
5
6
7
8
9
10
Age of the seedling (days after germination)

As colour and size of the shoot tips were found to be reasonably good morphological indicators of differentiation stage (atleast of BTx-623 genotype studied), on each day tips of average length with uniform colour were chosen for meristem isolation. Over all, 5-8 days old explants isolated from seeds germinated on filter paper showed the highest induction response (70-80 %).

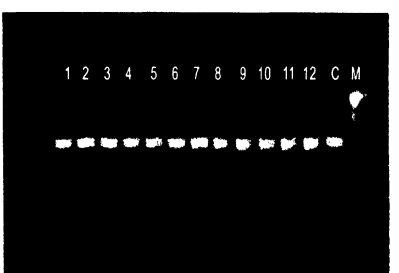
4.1.1.8 *RAPD analysis of in vitro regenerants*

RAPD profiles generated by using 6 random decamer primers (OPA-01, OPA-03, OPD-01, OPE-01, OPL-03 and OPL-14) from *Operon Technologies*, were monomorphic across all the plants (regenerants from shoot apices) tested. Four to six bands were amplified across the plants tesed. These profiles did not reveal any polymorphic bands among *in vitro* regenerated plants or between the *in vitro* regenerated plants and control plant (Fig. 8).

Fig. 8. RAPD analysis of regenerated plants from shoot apices of sorghum genotype BTx-623, using the random primer (A) OPA-3 and (B) OPL-14. Lanes 1-12 are the *in vitro* regenerated plants R_0. Lane 13 represents the seedling derived control plant "C", and lane "M" represents 1 kb DNA size marker


1 2 3 4 5 6 7 8 9 10 11 12 C M
2.6 kb
2.1 kb
1.7 kb
1.1 kb

(A)


1 2 3 4 5 6 7 8 9 10 11 12 C M
4.0 kb
2.8 kb
2.7 kb
1.6 kb

(B)

4.1.2 Regeneration system for immature embryos

Immature embryos of three sorghum genotypes M35-1, BTx-623 and 296B were used for this purpose. The percentage of embryogenic callus formation varied with the concentration of the 2,4-D and kinetin used in the culture medium (Table 12). Callus started appearing within a week of initiating cultures, and first subculture was done after 3 weeks on to the same medium (Fig. 9A). This resulted in the appearance of friable embryogenic callus within after 10-12 days of transfer (Fig. 9B). Highest frequency of embryogenic callus was produced on LS medium supplemented with 2.5 mg L^{-1} 2-4-D and 0.5 mg L^{-1} Kinetin (Table 12). The frequency of embryogenic callus varied from 90 ± 2 % in M35-1, 79 ± 3 % in 296B to 74 ± 1 % in BTx-623.

Table 12: **Optimization of phytohormone combinations for immature embryo culture.**

Hormone combinations with MS basal	**Frequency of embryogenic calli (Mean ± SE)**		
Conc. (in mg L^{-1})	M35-1	296B	BTx-623
2,4-D (0.0) + Kn (0.0)	16 ± 2	7 ± 1	4 ± 1.5
2,4-D (1.5) + Kn (0.1)	56 ± 2.5	53 ± 1	52 ± 1
2,4-D (2.5) + Kn (0.1)	85 ± 2	80 ± 1.5	69 ± 2.5
2,4-D (1.5) + Kn (0.5)	83 ± 3	72 ± 1	70 ± 1.25
2,4-D (2.5) + Kn (0.5)	90 ± 2	79 ± 3	74 ± 1
	Regeneration frequency (Mean ± SE)		
	M35-1	296B	BTx-623
BAP (0.0) + NAA (0.0)	12 ± 2	7 ± 1	4.5 ± 1
BAP (0.5) + NAA (0.1)	35 ± 2	27 ± 1	19 ± 4
BAP (0.5) + NAA (0.2)	42 ± 1	33 ± 4	28 ± 2
BAP (1.5) + NAA (0.1)	60 ± 2.5	52 ± 1	45 ± 1.5
BAP (1.5) + NAA (0.2)	81 ± 1	70 ± 3	62 ± 2

For the production of shoots, embryogenic calli were transferred onto MS medium with BAP and NAA. Within a week of transfer, callus turned green, and shoots started differentiating (Fig. 9C). Regeneration frequency varied with the concentration of the BAP and NAA used in the culture medium (Table 12). Of the five media that were tried, regeneration frequency was highest in MS with 1.5 mg L^{-1} BAP and 0.2 mg L^{-1} NAA (Table 12). The mean regeneration frequency was 81 ± 1 % for M35-1, 70 ± 3 % for 296B and 62 ± 2 % for genotype BTx- 623. After four weeks of culture, when the shoots were 2-3 cm long, they were transferred on to half-strength MS medium with 2.0 mg L^{-1} IBA and 0.2 mg L^{-1} IAA for rooting (Fig. 9D). Roots started appearing within a week of transfer onto this medium. After one month of culture, rooted plantlets were transferred into *jiffy* cups for hardening and after a week, to greenhouse to maturity (Fig. 9E-F).

Fig. 9 Plant regeneration from immature embryos in sorghum. (A) Immature embryo c
I.S medium showing callus initiation. (B) Three week old culture showing considerab
amount of callus. (C & D) Shoot differentiation on MS regeneration medium. (F) We
rooted plantlets in magenta box. (F) Acclimatized plants in greenhouse.

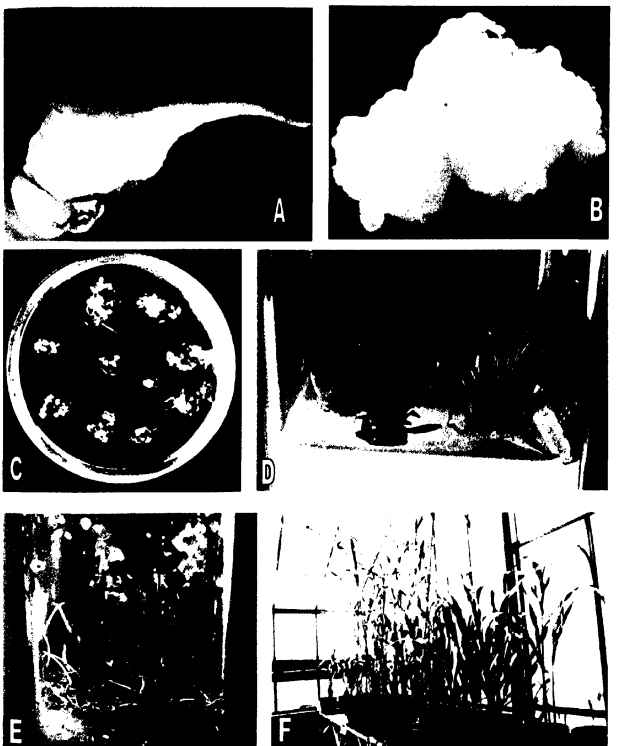

A
B
C
D
E
F

Fig. 10. Plant regeneration from immature inflorescence in sorghum. (A) Young inflorescence plated on LS callusing medium (B) Three week old culture showing considerable amount of callus (C) Differentiation of Embryogenic and nonembryogenic calli (5 days after culture initiation) (D-E) shoot differentiation on MS regeneration medium (F) Regenerated plantlets in culture bottles

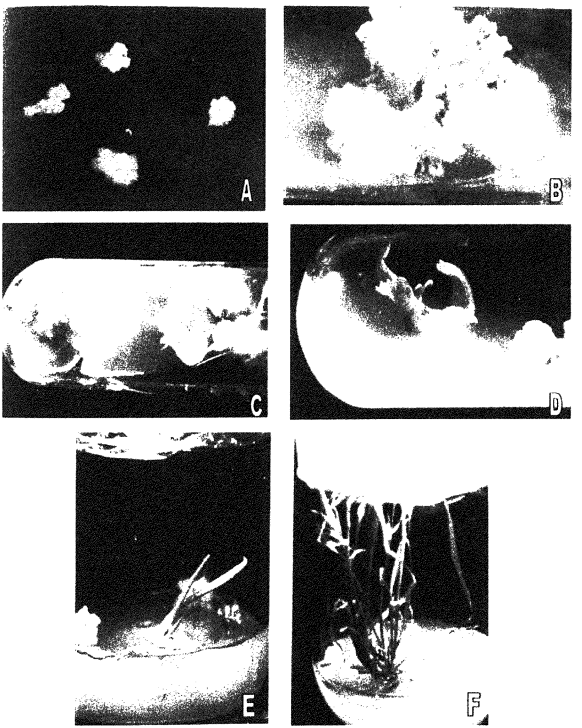

A
B
C
D
E
F

Table 13: **Optimization of phytohormone combinations for immature inflorescence culture.**

Hormone combinations with MS basal	**Frequency of embryogenic calli (Mean ± SE)**		
Conc. in mg L^{-1}	M35-1	296B	BTx-623
2,4-D (0.0) + Kn (0.0)	12 ± 2	5 ± 1	5 ± 1.5
2,4-D (1.5) + Kn (0.1)	69 ± 3	54 ± 1.5	50 ± 1
2,4-D (2.5) + Kn (0.1)	90 ± 3	85 ± 2	73 ± 2.5
2,4-D(1.5) + Kn (0.5)	85 ± 5	76 ± 3	69 ± 2
2,4-D (2.5) + Kn (0.5)	95 ± 2	90 ± 2	85 ± 2
	Regeneration frequency (Mean ± SE)		
	M35-1	296B	BTx-623
BAP (0.0) + NAA (0.0)	8 ± 2	4 ± 1	2.5 ± 0.5
BAP (0.5) + NAA (0.1)	26 ± 2	22 ± 1	20 ± 4
BAP (0.5) + NAA (0.2)	48 ± 1	39 ± 4	34 ± 2
BAP (1.5) + NAA (0.1)	60 ± 2.5	52 ± 1	45 ± 1.5
BAP (1.5) + NAA (0.2)	85.5 ± 1	75 ± 2	65 ± 2

At the end of 6 weeks of culture, embryogenic calli were transferred onto regeneration medium with BAP and NAA. Within a week of transfer, callus turned green, and shoots started differentiating (Fig.10D&E). Of the fifteen BAP and NAA combinations tried, the mean frequency of regeneration was highest on MS medium with 1.5 mg L^{-1} BAP and 0.2 mg L^{-1} NAA (Table 13). The mean regeneration frequency was 85.5 ± 1 % in M35-1, 75 ± 2 % in 296B and 65 ± 2 % in the BTx-623 genotype. After completion of 10 weeks of culture covering 2 subcultures, 5-7 shoots per explant were obtained.

Next, shoots were transferred onto half-strength MS rooting medium with 2 mg L^{-1} IBA and 0.2 mg L^{-1} IAA. Roots started appearing within a week of transfer onto this medium. After one month of culture, rooted plantlets were removed from the magenta box, thoroughly rinsed with sterile water and transferred to jiffy cups containing autoclaved vermiculite mixture. After ten days of hardening in the culture room maintained at 26 ^{0}C and 45 % relative humidity with a photoperiod of 16h. light /8h. dark, plants were transferred to greenhouse. 85 - 90 % of the regenerants could be transferred to greenhouse.

4.2 Establishment of transgenic evaluation systems

In vitro and *in vivo* screening systems were established against Basta and phosphinothricin (PPT) for three sorghum genotypes BTx-623, M35-1 and 296B. The minimum concentration of selection agent that can fully inhibit the growth of non-transformed cells or tissues was determined for each of the systems, and the lethal concentrations are tabulated (Table 14). For all these systems, the effect of genotype was statistically in significant (Table 15).

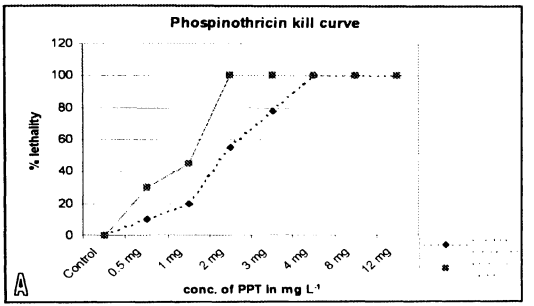
Table 14: **Basta lethal dose (LD, at 50 and 100 % kill) values in different assay systems.**

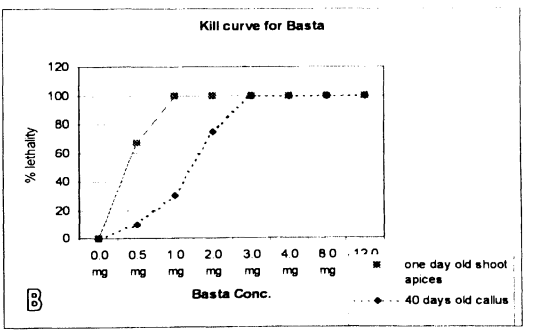
***In vitro* and *in vivo* assays**	**Basta conc. in mg L^{-1}**
Kill curve for embryogenic calli	
LD_{50}	1
LD_{100}	3
Seed germination inhibition test	
LD_{50}	300
LD_{100}	1000
Chlorophenol red assay	Color of the medium
Resistant tissues	Yellow
Susceptible tissues	Pink to red
Leaf spray test	
LD_{50}	50
LD_{100}	100
Basta *leaf dip test*	
LD_{50}	1000
LD_{100}	5000

4.2.1 Basta and phosphinothricin lethal dose determination for shoot apices

Presence of PPT and Basta affected the proliferation of explants. Fifty percent of the one day old shoot apices died (LD_{50}) on medium with 0.5 mg L^{-1} Basta or 4.0 mg L^{-1} of PPT and the explants showed 100 % mortality (LD_{100}) either at 1.0 mg L^{-1} Basta or 8.0 mg L^{-1} PPT respectively (Fig. 11). However, this inhibition varied with the developmental stage of the meristems. Germinating somatic embryos could not withstand to 2.0 mg L^{-1} PPT (LD_{100}) (Fig. 11A). Thus, germinating somatic embryos were more sensitive to selection agent than one day old shoot apices. Similarly, Basta and PPT retarded the growth and proliferation callus. Basta was more effective than PPT in retarding the growth of callus cultures also. A concentration of 3.0 mg L^{-1} Basta was sufficient to kill all the explants (LD_{100}) (Fig. 11B). In the case of PPT, 50 % of the explants died (LD_{50}) on the medium with 4.0 mg L^{-1} Basta, and 100 % explants died at 12.0 mg L^{-1} Basta.

Fig. 11. Kill curves with (A) phosphinothricin for shoot apice cultures, and (B) Basta, for callus. Explants were cultured on medium with selection agent. Presence of selection agent in the medium inhibited proliferation of explants. For one day old shoot apices LD_{50} was observed on medium with 2.0 mg L^{-1} and LD_{100} at 4.0 mg L^{-1} of PPT and for germinating somatic embryos, LD_{50} was on 1.0 mg L^{-1} and LD_{100} at 2.0 mg L^{-1} of PPT. For callus LD_{50} was observed on medium with 2.0 mg L^{-1} Basta and LD_{100} at 3.0 mg L^{-1} of Basta


Phospinothricin kill curve
% lethality
120
100
80
60
40
20
0
Control
0.5 mg
1 mg
2 mg
3 mg
4 mg
8 mg
12 mg
conc. of PPT in mg L-1
A


Kill curve for Basta
% lethality
120
100
80
60
40
20
0
0.0 mg
0.5 mg
1.0 mg
2.0 mg
3.0 mg
4.0 mg
8.0 mg
Basta Conc.
one day old shoot apices
40 days old callus
B

4.2.2 Determination of Basta concentrations that are lethal to seed germination and to growth of seedlings

Presence of Basta in the MS basal medium inhibited seed germination (Fig. 12A). The level of Basta in the medium had significant effect on germination percentage (Table 15). With increasing Basta concentration, germination percentage decreased. This effect reached a plateau after 4 days. Less than 50 % (47.2 % and 44.1 %) of the seeds germinated, on medium containing 100 and 200 mg L^{-1} of Basta and this further declined to less than 5 % (4.15-0.67 %) on medium with 300-700 mg L^{-1} Basta (Fig. 12B). There was total inhibition of germination on medium with 1000 mg L^{-1} of Basta or more. The genotypic effect was found to be insignificant (Table 15).

Fig. 1.2 Effect of Basta on seed germination. (A) Seeds germinated on medium with Basta and (B) graph showing the germination percentage. Inhibition of seed germination on a medium with different concentrations of the herbicide Basta. Note that the seeds germinated on medium without Basta (control) showed 100 % germination, while germination percentage decreased with increase in Basta concentration added to the medium and the effect reached a plateau after 4 days. Basta concentration is indicated as

Table 15: **Statistical analysis of genotype effect in screening systems.**

Source of Variation	296B	M35-1	BTx 623
(A) PPT kill curve			
Control	0	0	0
1 mg L^{-1}	24	30	20
2 mg L^{-1}	52	58	55
4 mg L^{-1}	98	100	100
8 mg L^{-1}	100	100	100
12 mg L^{-1}	100	100	100
(B) Seed germ			
0	100	100	100
100 mg L^{-1}	47.2	45.3	48.9
200 mg L^{-1}	44.1	42.6	43.2
300 mg L^{-1}	4.15	5.3	4.1
500 mg L^{-1}	1.32	2.8	1.14
700 mg L^{-1}	0.675	0.32	0.45
1000 mg L^{-1}	0	0	0
2000 mg L^{-1}	0	0	0
(C) C R assay			
0 mg L^{-1}	0.15	0.2	0.1
4 mg L^{-1}	0.32	0.4	0.3
8 mg L^{-1}	0.75	0.8	0.82
12 mg L^{-1}	1	1	1.1
16 mg L^{-1}	1.2	1.2	1.25
32 mg L^{-1}	1.45	1.4	1.2
(D) Basta spraying			
0 mg L^{-1}	0	0	0
50 mg L^{-1}	50	45	53
75 mg L^{-1}	85	82	75
100 mg L^{-1}	100	100	100
200 mg L^{-1}	100	100	100
(E) Leaf painting			
1%	9	9	9
0.50%	9	9	9
0.25%	9	9	8.9
0.10%	6.5	7.3	6.9
0.05%	2.7	3.4	3.2
0.025	2.5	2.8	2.5
0.01%	0.75	1.2	1.2
0 %	0	0	0

Data represents the mean value of three replications. The main effect, Basta was considered random.

Continued...

ANOVA results.

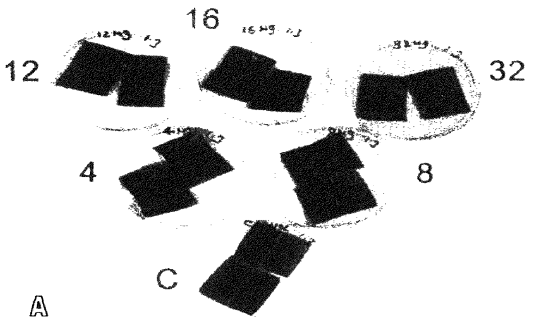
Source of Variation	SS	df	MS	F	P-value	F crit
(A) Phosphinothricin test						
Rows	28681.17	5	5736.233	1124.752	2.06E-13	3.325837
Columns	20.33333	2	10.16667	1.993464	0.186805	4.102816
Error	51	10	5.1			
Total	28752.5	17				
(B) Seed germination						
Rows	27552.93	7	3936.133	5394.734*	9.44E-21	2.764196
Columns	0.497565	2	0.248782	0.340971	0.716821	3.73889
Error	10.21475	14	0.729625			
Total	27563.65	23				
(C) C.R assay						
Rows	4.6104	5	0.92208	91.50645	5.11E-08	3.325837
Columns	0.002433	2	0.001217	0.120741	0.887536	4.102816
Error	0.100767	10	0.010077			
Total	4.7136	17				
(D) Spraying						
Rows	21482.67	4	5370.667	552.7273	8.42E-10	3.837854
Columns	7.6	2	3.8	0.391081	0.68858	4.458968
Error	77.73333	8	9.716667			
Total	21568	14				
(E) Leaf painting						
Rows	304.4544	7	43.49349	967.234**	2.87E-24	4.277865
Columns	0.316575	2	0.158287	3.975825	0.102331	6.514938
Error	0.445358	14	0.031811			
Total	305.2164	23				

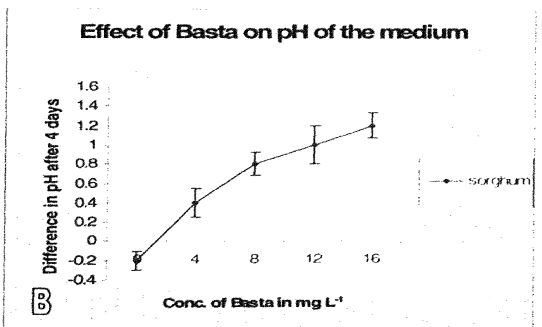
4.2.3 Chlorophenol red assay

At the end of 4 days of incubation, leaf pieces in the control plates remained green, while the pieces in Basta plates showed different levels of scorching.

In the control plate, pH decreased, while the plates with Basta showed a gradual increase in pH, which was directly proportional to the Basta concentration used (Fig. 13A). Presence of Basta in the culture medium, leads to an increase in the pH of the medium (alkalization) resulting from accumulation of ammonium ions to over 100-150 fold higher in control plants. This increase towards alkalization was studied by adding chlorophenol red. Culture plates showed a gradation of colour from yellow in control plates to pink, red and deep red with increasing concentrations of Basta in the medium (Fig. 13B). Similar response was observed in all the three genotypes (Table 15).

Fig. 15 Chlorophenol red assay. (**A**) Leaf pieces of 3-cm size incubated for 4 days in petri dishes containing MS salts + 4.0 M BAP and 0.5 M NAA, with different levels of Basta showing the senescing leaves pH lowered in the control plate (without Basta), while in the plates with Basta it showed a steady raise, and this raise was proportional to the Basta concentration added. This gradual increase was also evident, after addition of Chlorophenol red where in it transition from yellow color to red and deep red. (**B**) Values given are the means of pH difference after 3 days of incubation


16
12
32
4
8
C
A


Effect of Basta on pH of the medium
Difference in pH after 4 days
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
4
8
12
16
Conc. of Basta in mg L-1
sorghum
B

4.2.4 **Determination of dosage response of seedlings to spraying with Basta**

Spraying of Basta affected the viability of the seedlings. Viability of the plants decreased with increasing concentrations of Basta sprayed (Fig. 14A). On 7-day-old seedlings (2-leaf stage; second leaf collar visible), LD_{50} was observed at 50 mg L^{-1} of Basta. LD_{100} was observed when 100 mg L^{-1} and above concentrations were used. When 10-day old plants (third leaf emerging) were sprayed, LD_{50} was at 75 mg L^{-1} Basta. In case of two weeks old plant (3-leaf stage; third leaf collar visible), LD_{50} was 100 mg L^{-1}, and LD_{100} was 200 mg L^{-1} (Fig. 14B). The genotype effect was statistically insignificant (Table 15).

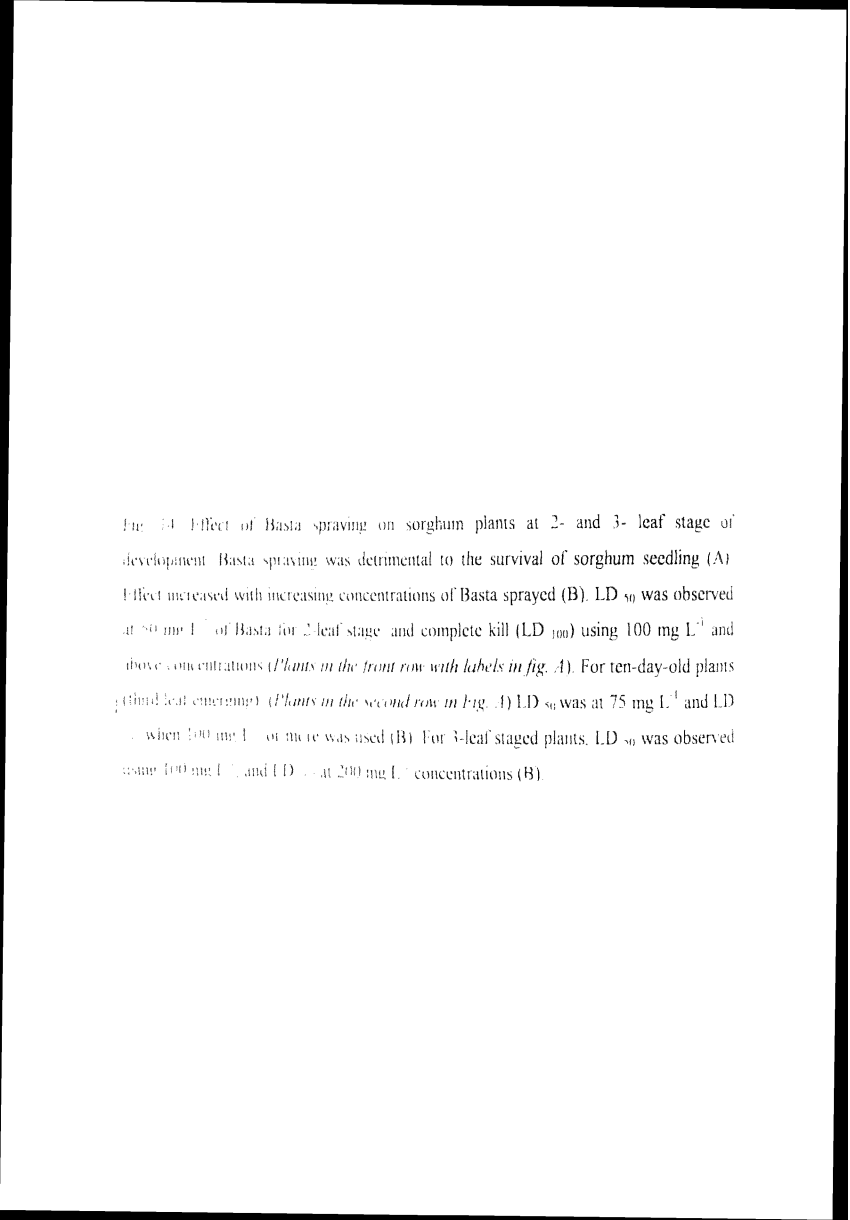
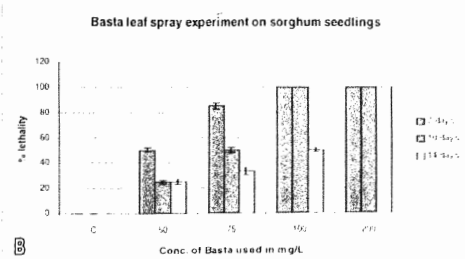


Fig. 34. Effect of Basta spraying on sorghum plants at 2- and 3- leaf stage of development. Basta spraying was detrimental to the survival of sorghum seedling (A). Effect increased with increasing concentrations of Basta sprayed (B). LD_{50} was observed at 50 mg L^{-1} of Basta for 2-leaf stage and complete kill (LD_{100}) using 100 mg L^{-1} and above concentrations (*Plants in the front row with labels in fig. A*). For ten-day-old plants (third leaf emerging) (*Plants in the second row in Fig. A*) LD_{50} was at 75 mg L^{-1} and LD ... when 100 mg L^{-1} or more was used (B). For 3-leaf staged plants, LD_{50} was observed using 100 mg L^{-1} and LD ... at 200 mg L^{-1} concentrations (B).


A
CONTROL
50
75
100
200

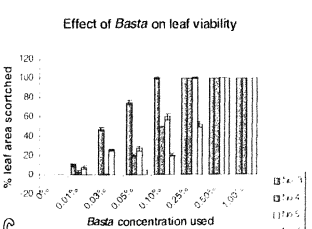

Basta leaf spray experiment on sorghum seedlings
% lethality
120
100
80
60
40
20
0
0
50
75
100
200
Conc. of Basta used in mg/L
B

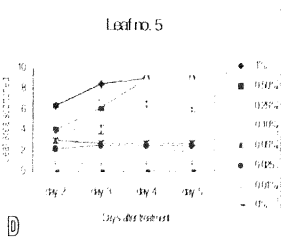
4.2.5 **Determination of dosage response of plants to Basta application**

Effect of Basta on the leaves was visible in the form of leaf scorching when painted on adaxial surface. Visual observations showed that more than 50 % of the third leaf area scorched when 0.1 % of Basta was applied (Fig. 15A). In case of fifth leaf, a concentration of 0.25 % was necessary to produce the same extent of scorching. However, when still higher concentrations (0.5 % and more) were painted, 100 % scorching was observed in treated (third and fifth leaves) as well as non-treated (fourth and sixth leaves) and plants died after 4 days of application (Fig. 15B-C). leaf scorching was observed from after 2 days of painting, and the effect reached a plateau after four days of application (15D). In all the genotypes, this effect was observed. And the genotypic effect was statistically insignificant (Table 15).

Fig. 3.8 Study of effect of Basta on leaf viability (A) Basta painted on leaves. Application of Basta reduced viability of leaf, and scorched area increased with increase in concentration of Basta. When higher concentrations were painted, whole plants died (A-C). Leaf scorching was observed from after 2 days of painting, and the effect reached a plateau after 4 days of application (D).


A
B
control
0.01 %
0.03 %
0.05 %
0.1 %
0.25 %
0.5 %
1.0 %


Effect of Basta on leaf viability
% leaf area scorched
120
100
80
60
40
20
0
Basta concentration used
C


Leaf no. 5
D

4.3 Optimization of genetic transformation using particle inflow gun

Genetic transformation was carried out using indigenously built particle-inflow gun. For protocol standardization, plasmid with *uidA* as reporter gene and PAT as marker gene under the control of 35S promoter was used. Explants bombarded included callus derived from immature embryos and isolated shoot apices. Histochemical GUS assay of bombarded tissues was carried out for optimization of bombardment protocol.

4.3.1 Parameters affecting microprojectile bombardment

Parameters affecting the bombardment efficiency like helium gas pressure, the flight distance of DNA-coated tungsten particles, osmotic treatment of explants and concentration of the plasmid DNA used have been optimized for bombarding immature inflorescence derived calli, immature embryos and shoot apices. The results are summarized in Table 16.

The pressure of helium gas used for acceleration of microprojectile significantly influenced the efficiency of transformation. However, there was no significant correlation between the explant (immature embryo and shoot meristem) and pressure factors (Table 16). A pressure of 15 kg cm^{-2} produced maximum number of blue spots with both the explant types than 10 kg cm^{-2} or 18 kg cm^{-2} helium pressure. The flight distance used between the target explant and the DNA holder had influenced the efficiency of transformation significantly (Table 16). Of the three distances tried, maximum frequency of transient expression was found at a flight distance of 7 cm for the above explants (Fig. 16).

Fig 16 Optimization of bombardment parameters for immature embryo, immature inflorescence derived callus and shoot apices explants.

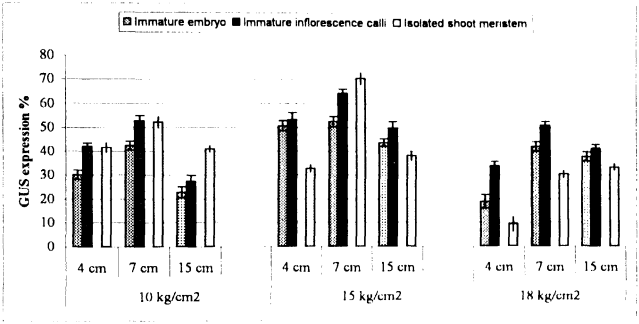
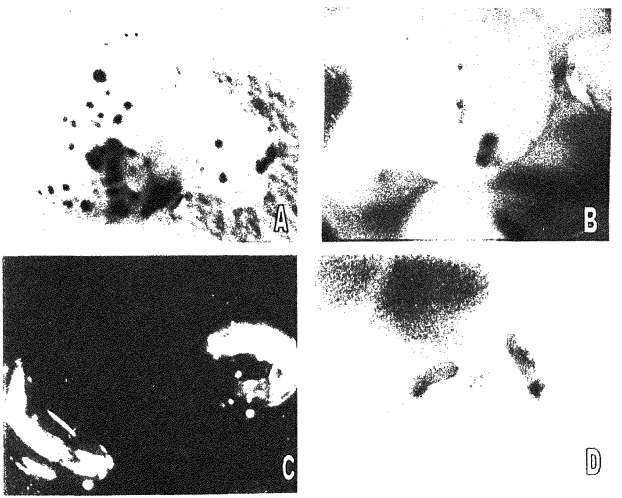

Immature embryo
Immature inflorescence calli
Isolated shoot meristem
GUS expression %
80
70
60
50
40
30
20
10
0
4 cm
7 cm
15 cm
10 kg/cm2
15 kg/cm2
18 kg/cm2

Table 16: **Conditions tested for the optimization of microprojectile DNA delivery in sorghum using PIG.**

S. No.	Variable	Conditions tested	Mean *uidA* spots	Optimum conditions	P value	Significance
1	Sample position from the stopping plate	4 cm 7 cm 15 cm	33.3 47.9 34.8	7 cm	0.232	Yes
2	Pressure	10 kg cm^{-2} 15 kg cm^{-2} 18 kg cm^{-2}	37.7 46.7 31.6	15 kg cm^{-2}	0.057	Yes
3	Type of explant	i. Immature embryo ii. Immature inflorescence iii. Isolated shoot apices	28.0 38.8 46.3		0.327	Yes

Out of the three pre-bombardment (incubation for 4, 8 or 12h) and six post bombardment (0, 2, 4, 16, 24 or 48h.) osmotic treatments, on MS medium with 0.4 M sorbitol and 0.4 M mannitol, maximum transient expression was observed when explants were left for 4h. before bombardment and 24h after bombardment (Fig. 16). Further, transformation efficiency varied with the type of the explant used for bombardment. The percentage of the explants showing transient expression and the effective area of transformation per single explant was more in isolated shoot apices and immature inflorescence derived callus (Fig. 17A-D) than in immature embryo explants.

Fig. 17 Transient GUS expression in bombarded (A) Immature inflorescence calli, (B) immature embryos and (C & D) shoot apices. Explants were transferred to *X-Gluc* solution after 24h of bombardment


A
B
C
D

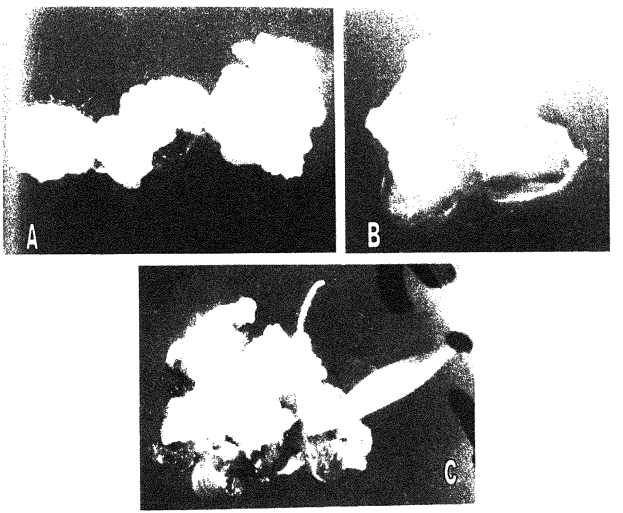
4.3.2 Selection and Regeneration of putative transgenic plants

Selection was imposed from 2 weeks after bombardment. This allowed the recovery and unhindered proliferation of the transformed cells, which are damaged during the process of delivering DNA. Further, immature embryos and callus derived from immature inflorescence was selected on Basta, and shoot apices on medium with PPT. A three-step selection strategy was used for obtaining transformants. Explants were selected on 1.0 mg L^{-1}, and next 2.0 mg L^{-1} of selection agent (calli on Basta and shoot apices on PPT) (step 1 and step 2). Subcultures were done at 10-day intervals, and there were two rounds of transfers on each concentration of selection agent. Under step 3, after 6 weeks of selection, the surviving explants were transferred onto medium devoid of selection agent

4.3.2.1 *Selection of bombarded calli on Basta*

During the first phase of selection on 1.0 mg L^{-1} Basta, growth rate slowed down, along with appearance of brown necrotic areas on the callus. After 3 weeks, healthy portions from these calli were transferred on to the medium with higher concentration of herbicide (2.0 mg L^{-1} Basta) (Fig. 18A-B). Only few surviving sectors were passed next on to MS regeneration medium with 2.0 mg L^{-1} Basta (Fig. 18C). Finally, the surviving plantlets, 3-4 cms in length were transferred onto rooting medium without Basta.

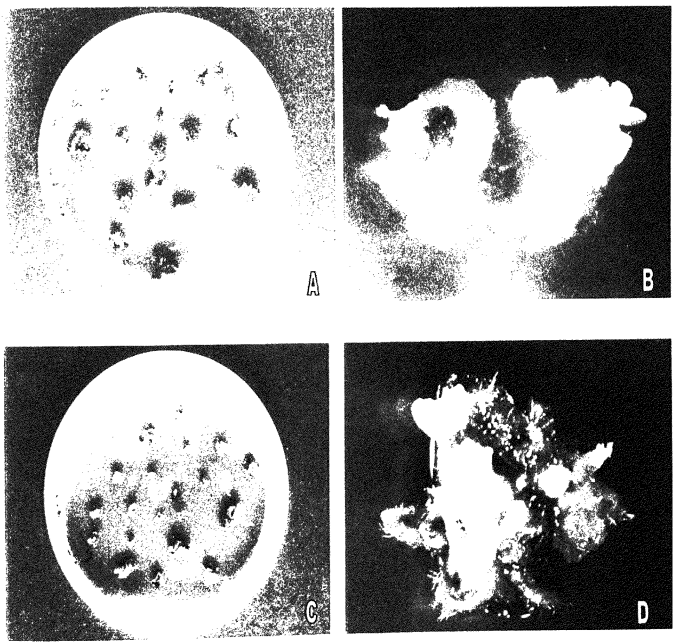
Fig. 18 Bombarded explants under selection on culture medium with Basta. (A) Showing callus from immature embryos on 1.0 mg L^{-1} Basta, (B) on 2.0 mg L^{-1} Basta and (C) showing regeneration from immature inflorescence callus on medium with 2.0 mg L^{-1} Basta


A
B
C

4.3.2.2 *Selection of bombarded shoot apices on phosphinothricin*

In case of shoot apices, during the initial phase of selection (15-45 days after bombardment), on MS medium with 1.0 mg L^{-1} PPT, there was a distinction between transformed and non-transformed sectors (tissue stratum) of meristems (Fig. 19A-B). Transformed sectors could be seen as lighter coloured growing tissue against a background of brown-coloured necrotic tissue. These small growing areas were then separated form the dead tissue and subcultured onto MS medium with 2.0 mg L^{-1} PPT. During this phase of selection, only the transformed sectors of tissue strata continued to grow and produce somatic embryos (Fig. 19C-D). Such structures with attached somatic embryos were then transferred onto SEGM with 2.0 mg L^{-1} PPT. Finally, the surviving plantlets (3-4 cm long) were transferred onto rooting medium free of PPT.

Fig. 19 Bombarded shoot apices regenerating on MS medium with phosphinothricin. (A & B) showing meristems on 1.0 mg L^{-1} PPT. (C & D) showing explants on 2.0 mg L^{-1} PPT


A
B
C
D

4.3.3 Acclimatization and transfer to greenhouse

After one month of culture, fully rooted plantlets were removed from the magenta box, thoroughly rinsed with sterile water and transferred to jiffy cups for hardening. Next, plants were transferred to P_2 greenhouse and grown to maturity. Transgenic plants were obtained from all the three genotypes used in genetic transformation studies: 296B plants were obtained from bombarded immature inflorescence derived calli, and plants from bombarded shoot apices of genotypes BTx-623 and M35-1. The selection efficiency for putative transgenics (calculated as the number of explants which regenerated into complete plants after selection/ total number of explants bombarded) for callus derived from immature inflorescence was 0.13 %, and for immature embryos and shoot apices it was 0.0 % and 2.7 % respectively. A total of 3846 calli pieces derived from immature inflorescence were bombarded in 38 individual experiments. Out of these, 1095 have passed 1.0 mg L^{-1} of Basta and 46 have passed 2.0 mg L^{-1} Basta concentration. A total of 5 plants of genotype 296B have been transferred to greenhouse, resulting in a selection efficiency of 0.13 %. Among the individual experiments, transformation efficiency varied between 0 - 0.2 %.

Out of the 450 immature embryos that were bombarded in 12 individual experiments, 87 explants passed through 1.0 mg L^{-1} of Basta. Further, nothing could be forwarded beyond callus phase as they failed to show shoots until the callus turned brown.

In the case of shoot apices, a total of 3919 explants were bombarded in 45 individual experiments. Out of these, 2358 has passed 1.0 mg L^{-1}selection, and 463

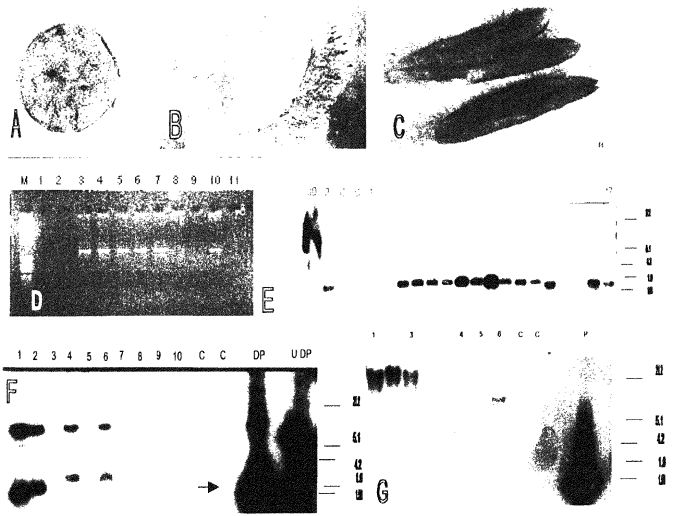
passed 2.0 mg L^{-1} PPT selection. A total of 108 (54 plants of genotype BTx-623 and 54 plants of genotype M35-1) plants have been transferred to greenhouse, resulting in a selection efficiency of 2.7 %. Among the individual experiments, selection efficiency varied between 0 - 15.2 % (SE ± 2.66).

4.4 Confirmation of transgenics

4.4.1 Histochemical assay of T_0 plants

Floral parts of transformed plants when dropped in *X-Gluc* solution developed blue colouration, after 24 - 48h of incubation. This histochemical localization of *GUS* activity in various floral parts like microspores (Fig. 20A), stigmas (Fig.20B), and anthers (Fig.20C), of these T_0 regenerants indicated the stable expression of the GUS coding sequences.

Fig. 20. GUS expression in floral parts and various stages of confirmation of putative transgenics. *uidA* gene expression in (A) pollen, (B) stigma and (C) anthers of transgenic plants (T_0). (D) PCR analysis of T_0 plants. Lane 1- control without DNA, lane 2- control without primer, lane # 3- 9 represents T_0 plants, lane 10- *PATGUS* plasmid DNA, lane 11- Untransformed (control) plant DNA. M - is the digested λ DNA marker. 1.2 kb size *uidA* fragment amplified in 5 out of the 7 plants; similar band was seen in plasmid (P) lane and there was no such band in control plant lane. Southern analysis of the putative transformants (E-F) λ DNA digested with *Eco*R1 and *Hind* III was used as molecular marker. (E) Genomic DNA was digested with *Eco* R1 and *Hind* III (double digest) and probed with *uidA* fragment. Corresponding bands were seen in transgenic plants and plasmid (P-in the figure) lanes, and absent in control plants (lane-C in the figure). Plant number 1 is an escape (no band) and UD (undigested genomic DNA from transgenic plant) lane shows thick band corresponding to high molecular weight DNA. (F) Single enzyme digest blot # 1-10 in the picture are T_0 plants; DP -digested plasmid and UDP -undigested plasmid DNA. Plasmid was digested with *Eco* R1 and *Hind* III and insert was added to the lane (indicated by arrow). Genomic DNA was restriction digested with the enzyme *Xba* I and hybridized with amplified *uidA* fragment. Bands between 1-5 were present in transgenic plant and plasmid (DP-in the figure) lanes, and absent in control plants (lane-C in the figure). (G) Single enzyme digest blot. Lane # 1-3 with undigested DNA and lane # 4 - 6 corresponding digested DNA of three T_0 plants; DP - digested plasmid. Plasmid was digested with *Eco* R1 and *Hind* III and insert was added to the lane (indicated by arrow). Genomic DNA was restriction digested with the enzyme *Xba* I and hybridized with amplified *uidA* fragment. Bands appeared in lane # 1-6 and in transgenic plant and plasmid (DP-in the figure), and absent in control plants (lane-C in the figure).


A
B
C
M 1 2 3 4 5 6 7 8 9 10 11
D
E
1 2 3 4 5 6 7 8 9 10 C C DP UDP
F
G

4.4.2 Molecular tests of T_0 plants

4.4.2.1 *PCR and Southern analysis of T_0 plants*

Polymerase Chain Reaction (PCR) demonstrated presence of the *uidA* gene in T_0 plants, and Southern hybridization of *uidA* probes with genomic DNA of putative transgenics, i.e. T_0 plants (Fig. 20 D-F). PCR using *uidA* specific primers resulted in amplification of the expected 1.2 kb *uidA* transcript in 101 plants derived from shoot apices (Fig. 20 D). All the above test material when subjected to Southern hybridization gave the expected 1.8 kb band when genomic DNA was digested with *Eco* R1 and *Hind* III (Fig. 20E). A thick band was seen in the lanes having high molecular weight undigested genomic DNA of the putative transformants in high molecular weight region (Fig. 20E). To detect the transgene copy number, genomic DNA was digested with *Xba* I enzyme, which cuts the PATGUS plasmid only once, thus linearzing it. The estimated copy number of the *uidA* gene varied between 1 to 5 in these plants (Fig. 20F-G). No bands were present in the non-transformed (control) plants. Further, all the plants showed different integration pattern indicating that, they belong to independent transformation events. However, there were differences in the band intensities among difference lanes. Bombardment of immature inflorescence derived calli resulted in generation of 5 putative transformants of genotype 296B. Molecular analysis using PCR and Southern hybridization have shown that four out of these five plants have stable integrated GUS transgene. Three independent transformation events were observed in these four transformants. Thus, the transformation efficiency (calculated as the number of transformation events/ total

number of explants bombarded Bombardment) for shoot meristem system was 2. 57 % while that for immature inflorescence was 0.07 %. All the confirmed transgenic plants were grown in the P_2 level greenhouse at ICRISAT to maturity.

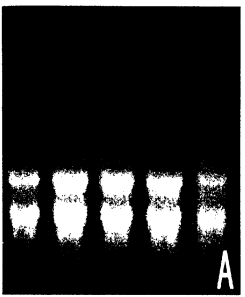
4.4.2.2 *Extraction of total RNA*

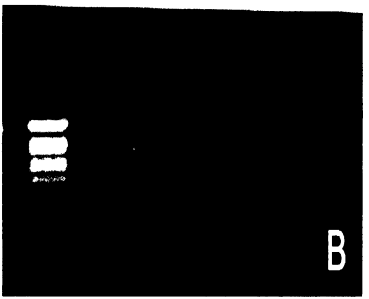
Total RNA extracted from sorghum leaf samples showed intact bands of 25S, and 17S RNA, when analyzed on 1.2 % formaldehyde agarose gel (Fig. 21A). The A260/280 of the total RNA was 1.9 to 2.0, in all the samples, indicating little contamination of the RNA by protein and polysaccharides. Yields in the range of 500 – 600 µg per 1.0 gram of leaf sample were obtained in all the samples extracted.

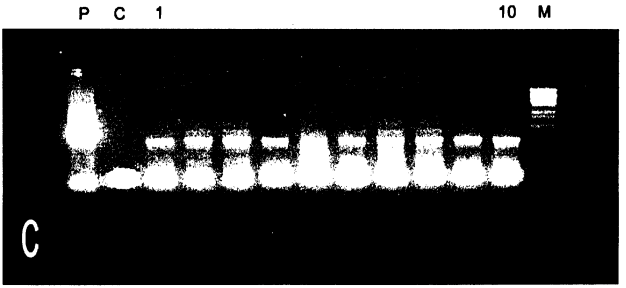
4.4.2.3 *RT-PCR analysis of cDNA using uidA gene specific primers*

PCR of first strand cDNA of 5 confirmed transgenics using *uidA* specific primers resulted in amplification of the expected 1.2 kb *uidA* transcript (Fig. 21C). PCR of extracted RNA was negative, confirming the absence of genomic DNA contamination in the samples after DNase treatment (Fig. 21C). Fragment amplification was similar from both (random hexamer and oligo-dT) priming methods, and there was no amplification in the control plant lane (Fig. 21B).

Fig 21 RT-PCR analysis of the transformants for *uidA* gene. (A) Formaldehyde denaturation gel electrophoresis analysis of total RNA. (B) PCR amplification of the extracted total RNA after DNase treatment. Gel showed no amplification, indicating that RNA is free of DNA contamination. M-is the *BstE* II digested λ DNA. (C) RT-PCR analysis of sorghum samples. GUS specific primers were used to amplify first strand cDNA products obtained from 5 T_0 transgenic plants. Two methods of first strand priming (random hexamers indicated as 1-5 and oligo-dT lanes indicated as 6-10) were done. In all the five plants, the expected 1.2 kb *uidA* fragment was present, as in the corresponding positive control (P). Priming by both the methods was identical, and effective in synthesizing transcripts longer than 1.0 kb as seen in the figure. There was no amplification in the control plant lane (C). M-is the *BstE* II digested λ DNA marker.


1 2 3 4 5
A


M 1
5
B


P C 1
10 M
C

4.5 Inheritance studies of transgenes

Histochemical and molecular analysis of T_1 plants of genotype 296B for *uidA* gene: T_1 progeny grown in P_2 greenhouse were examined for PPT resistance and GUS expression (Fig. 22). When histochemical GUS assay was performed using various floral parts of T_1 progenies, the segregation pattern of *uidA* gene expression agreed with the expected Mendelian ratios (Table. 17). Further, the results of Southern analyses perfectly agreed with those from GUS staining. T_1 progeny that showed blue colouration with *X-Gluc* substrate also showed the presence of *uidA* gene fragment when Southern hybridized with PCR amplified *uidA* gene fragment (Fig. 22G).

Fig. 22. Inheritance studies in T_1 progenies for PATGUS genes. (A) T_1 progeny in the greenhouse *uidA* gene expression in (B) anthers and (C) spikelets. (D), completely resistant plant is in the left and susceptible plant on the right. (E) Two resistant plant leaves along with a control (untreated) and susceptible plant leaves. (F) PCR analysis of the T_1 segregating progeny of genotype BTx-623. Southern hybridization pattern of T_1 transgenics (derived from T_0 plant # 3 genotype 296B). (A) T_1 progeny in the greenhouse *uidA* gene expression in (B) anthers and (C) spikelets. Herbicide resistance in (D), completely resistant plant is in the left and susceptible plant on the right. (E) Two resistant plant leaves along with a control (untreated) and susceptible plant leaves (F) PCR analysis of the T_1 segregating progeny of genotype BTx-623. Lane # 1-20 represents T_1 plants, and M- marker 1.2 kb size *uidA* fragment amplified in 17 out of the 20 plants, similar band was seen in plasmid (P) lane and there was no such band in control plant lane (C). (G) Southern hybridization pattern of T_1 transgenics (derived from T_0 plant # 3 genotype 296B) restriction digested with the enzyme *Xba* I and hybridized with *uidA* fragment. Lanes 1-20 are genomic DNAs from T_1 transgenics (Plant #s 1-20); Lane 21 control plant (296B), Lane 22 digested plasmid (PATGUS) DNA; Lane 23 undigested plasmid DNA; Lane 24 (M, λ *Hind* III digest).

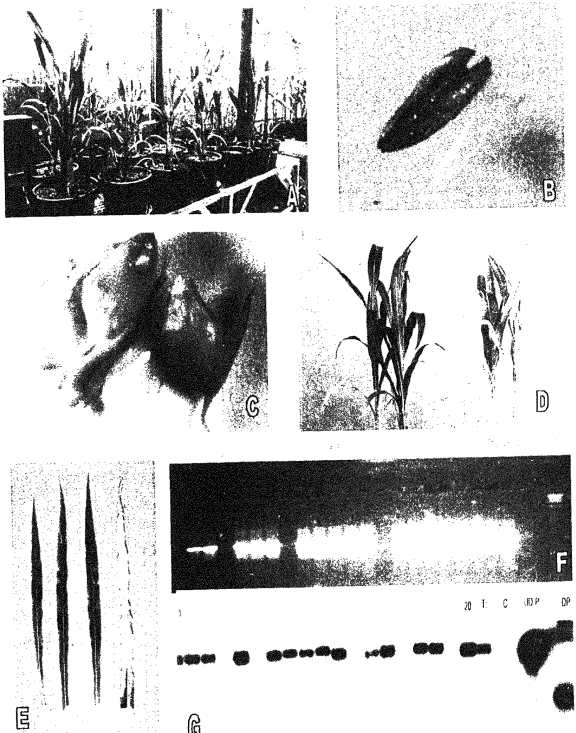

A
B
C
D
E
F
G
20
T
C
VRDP
DP
9.4
6.4
4.3
2.3

Table 17: **Segregation among 296B T_1 progeny for PATGUS transgenes.**

Plant NO (T_0).	**Total # of plants (T_1)**	**Herbicide resistance test**		**Observed ratio**	**Expected ratio**	**$\chi 2$**
		Resistant	**Susceptible**			
# 1	60	42	18	2.33: 1	3:1	0.8
# 2	56	44	12	3.66: 1	3:1	0.38
# 3	75	56	19	2.95: 1	3:1	0.005
# 5	68	53	15	3.53 :1	3:1	0.31
		Histochemical GUS staining reaction				
		GUS + ve	**GUS - ve**			
# 1	58	41	17	2.41:1	3:1	0.57
# 2	51	43	8	5.38:1	3:1	2.34
# 3	62	47	15	3.1: 1	3:1	0.02
# 5	63	46	17	2.7: 1	3:1	0.13

$\chi^2 > 3.84$, $P < 0.05$, 1 d.f.

Analysis of herbicide resistance in 60 T_1 296B plants: There was a clear demarcation between susceptible and resistant T_1 plants. From the control experiment, it is known that application of 0.5 % Basta results in > 90 % scorching of the treated leaf. In the present experiment, it was assumed therefore that, susceptible plants (that lack the transgene *PAT*) will show > 90 % leaf scorching, as do control plants, and the transgenic plants will withstand the herbicide effect. In T_1 progeny, apart from susceptible plants (Fig. 20D), two groups of resistant plants were observed. First group of plants showed 0 < 10% leaf scorching, and second group showed 20 - 30 % leaf scorching. Extent of scorching in susceptible plants was > 90 % as is the case with control plants. Based on statistical analyses, it was concluded that, 3 out of the 4 T_0 plant progeny tested showed segregation pattern on the expected 3:1 ratio of resistant: susceptibility (Table 17). Progeny of the T_0 Plant # 2 showed deviation from the expected lines (Table 17). However, this was less than the critical value χ^2 value at 5 % level.

Histochemical and molecular analysis of T_1 plants of genotype BTX-623 for *uidA* gene: 53 T_1 progeny of T_0 # 7 and 59 of T_0 # 8 grown in P_2 greenhouse was analyzed for the presence and expression of *uidA* gene (Fig. 22). When genomic DNA from these plants was amplified using *uidA* specific primers, 39 plants of T_0 # 7 and 44 plants of T_0 # 8 gave the expected 1.2 kb fragment (Fig. 22F). Further, all the positive plants showed blue colouration with *X-Gluc* substrate using various floral parts.

Analysis of herbicide resistance T_1 progeny of BTx-623 plants:

Transformants were examined for PPT resistance and GUS expression. Resistant and sensitive plants were distinguishable after 4 days of application of 0.5 % of Basta. In susceptible plants, the applied leaf showed > 90 % leaf area scorching, while the resistant plants were as healthy as non-treated plants (Fig. 22E). Further, GUS expression was seen in all the resistant plants. Thus, PPT resistance and GUS expression were tightly linked and showed a segregation ratio of 3:1 in both the plant progeny (Table 18).

Table 18: **Segregation among T_1 progeny of BTx-623 plants for PATGUS transgenes.**

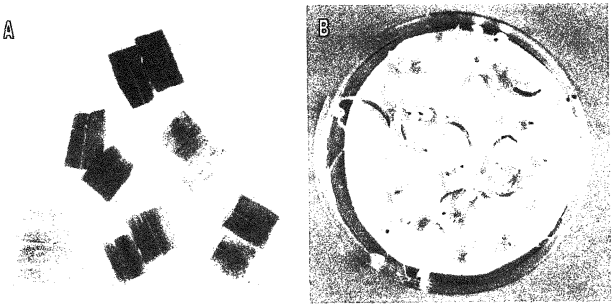
Plant NO (T_0).	Total # of plants (T_1)	Herbicide resistance test		Observed ratio	Expected ratio	$\chi 2$
		+ ve	- ve			
# 7	53	39	14	2.78	3:1	0.05
# 8	59	44	15	2.93	3:1	0.005
		Histochemical GUS staining reaction				
		+ ve	- ve			
# 7	53	39	14	2.78	3:1	0.05
# 8	59	44	15	2.93	3:1	0.005

$\chi^2 > 3.84$, $P < 0.05$, 1 d.f.

When leaf tips from the above T_1 progeny were incubated on medium with 32 mg L^{-1} Basta, browning was seen in some leaf tips, while some other remained largely green over the same period. Further, when chlorophenol red was added to the plates, culture plates with green leaf tips developed yellow color, while the plates with scorching leaf tips showed red to deep red coloration (Fig. 23A). Leaf tips obtained from progeny showing bar gene remained green, while the tips obtained from non-transgenic (without bar gene) progeny and control plants turned brown.

Germination percentage varied between 45 – 50 % when T_1 seed of the above T_0 plants was germinated on medium with 1000 mg L^{-1} Basta (Fig. 23B). On the other hand, seeds germinated on medium without Basta showed 100 % germination. Further, these plants with stood application of Basta solution indicating that, bar gene is being expressed in these plants.

Fig. 23 Screening of T_1 progeny resistant to Basta. Using (A) chlorophenol red assay. Leaf discs of resistant progeny showed no scorching as in plate no. 1-2, or little as in plate no 3 and upon addition of chlorophenol red remain yellow, while leaf discs of susceptible ones scorch (plates 4-6) and developed red color upon chlorophenol red addition (B) Seed germination inhibition test. Only the resistant seeds germinated while susceptible seeds did not


A
B

5 DISCUSSION

5.1 In vitro regeneration systems for sorghum

Genetic transformation work in cereals has been mostly based on biolistics, and slowly, *Agrobacterium* mediated transformation is acquiring prominence in monocots like rice (Bajaj, 2000). In contrast, in the past one-decade, very little attention is given to develop transgenics of tropical crops like sorghum (*Sorghum bicolor* (L.) Moench) that can benefit millions of needy farmers and consumers in the developing world (Sharma et al., 2001 and Reddy & Seetharama, 2002). There are many constraints for sorghum production, such as resistance to major insects and diseases, and drought that have eluded significant improvement by conventional means. Many useful wild relatives of the sorghum with desirable resistance genes exist, but there are considerable barriers for their hybridization with the cultivated sorghum accessions and efforts in the past decade in this area did not succeed. The large numbers of exotic genes those are available for genetic transformation can solve the specific crop-production problems of sorghum (Kononowicz et al., 1995; Tadesse and Jacob, 1999). Therefore, there is a strong and urgent need to develop technologies that will facilitate genetic transformation of sorghum.

The protocols for producing transgenic sorghum are still being standardized using various methods of DNA transfer (Rathus et al., 2000, Maqbool et al., 2001). To date, there are only a few reports of successful recovery and analysis of transgenic sorghum plants. (Casas et al., 1993; Zhu et al., 1998 and Zhao et al., 2000). Hagio et al. (1991) reported transient expression of reporter genes in sorghum suspension cells by particle bombardment. Battraw and Hall (1991) used protoplasts for incorporating *npt-II* and *uidA*

genes through electroporation. They studied transient expression of reporter genes using different factors such as linearization of the plasmid and effect of co-bom*bar*dment with two different gene constructs. However, their analyses of gene integration using PCR and Southern blots were confined to only putatively transformed calli, and no transgenic plants were regenerated. Casas et al. (1993) reported transgenic plant production using microprojectile bombardment of immature zygotic embryos. Recently, Zhu et al. (1998) reported incorporation of rice chitinase gene into the immature embryo derived calli of *Sorghum bicolor*, and successful recovery and molecular analyses of such transgenic plants. Apart from microprojectile mediated DNA transfer, successful production of transgenic sorghum plants was reported by Zhao et al. (2000) using *Agrobacterium*-mediated DNA transfer. Due to the low frequency of transformant recovery on selection agent leading to extremely low frequency of transformation genetic transformation of sorghum is not yet routine. Hence, very little has been achieved in genetic transformation of sorghum.

The present study was carried out to address some of the problems associated with sorghum transformation. This study has resulted in establishment of a novel and efficient regeneration protocol from shoot apices of germinated seedlings, apart from establishing callus based regeneration systems from immature embryos and immature inflorescence, using three popular sorghum varieties. Further, genetic transformation protocol using an indigenously built PIG has been standardized, which can be used to introduce genes of interest into sorghum plant.

In vitro response of three explants – isolated shoot apices, immature embryos and immature inflorescence were studied. Three popular sorghum genotypes, M35-1 (land

race cultivated during post-rainy season in India), 296B and BTx-623 (popular seed parents of hybrids) were used for this purpose.

In the present study, a rapid regeneration protocol from shoot apices has been standardized. The isolated shoot apices were manipulated to follow either organogenic or embryogenic pathway. Callus phase was avoided in both the cases.

Use of shoot apices from *in vitro* germinated seedlings facilitates the year round availability of explants (Zhong et al., 1998). Hence, this system could be efficiently used for genetic transformation due to increased frequencies of multiple buds and somatic embryo's capable of regeneration in a relatively short period.

By striking optimal balance between NAA (to avoid callus formation) and TDZ, the isolated shoot apices were manipulated to follow either organogenic or embryogenic pathway (Tables 9 & 11). Such morphogenic plasticity of the shoot meristem was reported by Zhong et al. (1998) by using shoot apices of sorghum seedlings obtained from eighteen commercial cultivars along with a portion of mesocotyl, leaf primordia, leaf sheath and one or two leaf pairs. However, in the present system, such heterogeneity of the explant was minimized by further isolation of shoot apices devoid of much of mother tissues from shoot apices, which also resulted in enhancing the rate of multiplication.

Plant growth regulators controlled the morphogenic competence, pathway and speed of regeneration from isolated shoot apices. The importance of auxin to cytokinin ratio in the control of regeneration is well known (Skoog and Miller, 1957). Such a process depends on cell or tissue competence (Christianson and Warinck, 1988). In earlier reports using shoot apex as an explant (Bhaskaran and Smith, 1988; Bhasakaran et

al., 1988; El'konin et al., 1984; Lusardi and Lupotto, 1990; Seetharama et al., 2000), embryogenic callus formation preceded the process of somatic embryogenesis. However, in order to minimize the generation of somaclonal variants commonly associated with callus-mediated regeneration, it is desirable to establish direct organogenesis or somatic embryogenesis with no callus formation. Such regeneration systems have been reported earlier in corn (Zhong et al., 1992), oat (Zhang et al., 1996), sorghum (Zhong et al., 1998), and pearl millet (Devi et al., 2000) and wheat (Ahmad et al., 2002). Further, a rapid and high frequency regeneration system will enhance the transformation potential as seen in genetic transformation of oats (*Avena sativa* L.), barley (*Hordeum vulgare* L.) Zhang et al. (1999) and Maize (*Zea mays* L.) (Zhong et al., 1996b). The system described here meets the entire above-mentioned criterion. All the three genotypes used in the present study showed uniform regeneration frequencies and thus the system is genotype independent with a marginal difference in induction responses. This is in agreement with the finding of Zhong et al. (1998).

During the first 2 weeks of culture, there was a significant expansion of shoot meristem region. At this stage, the bulged shoot meristem was trimmed and leaf primordia discarded. If the primordial leaves were not retained with the meristem during the first 2 weeks, meristems did not bulge, and there was no multiple bud formation. Earlier it has been demonstrated that if the apical dominance of existing buds is eliminated either physically or by using high cytokinin levels in the medium, a large number of shoots could arise *in vitro* (Polisetty et al., 1997). In our study, use of BAP and TDZ (which shows strong cytokinin properties) when combined with the technique of trimming leaf initials during *in vitro* culture, leading to such a response.

Thidiazuron is a substituted phenyl urea developed primarily as a cotton defoliant, which also exhibited strong cytokinin like activity in various cytokinin bioassays (Mok et al., 1982). TDZ also efficiently stimulated cytokinin-dependent shoot regeneration from a wide variety of plants (Malik and Saxena, 1992; Huetteman and Preece, 1993). The precise mechanism of action of TDZ is not known yet. However, two hypotheses have been proposed (Huetteman and Preece, 1993). First, TDZ could directly promote growth due to its own biological activity in a way similar to that of cytokinins. Second, it might effect accumulation of endogenous cytokinins (*via* a reduction in the rate of degradation) or increases the synthesis of endogenous cytokinins.

This study demonstrated that the addition of TDZ in the induction medium was effective for multiple bud formation from bulged meristems. The multiple bud formation was a branch-point from where it can lead to either organogenic or embryogenic pathway depending on the manipulation of plant growth regulators. Zhong et al. (1998) obtained direct somatic embryogenesis from shoot apices on medium supplemented with BAP and 2,4-D. In our experiments with isolated shoot apices, use of the above medium was always accompanied with a certain degree of callus formation. In the case of isolated shoot apices with high meristematic activity, it was observed that a weak auxin like NAA was more effective; in fact, it was more favored for signaling somatic embryogenesis, than a strong auxin like 2,4-D (Fig. 5). Moreover, replacement of NAA with 2,4-D induced somatic embryogenesis without any callus formation. Specific combinations of NAA and BAP were found to be highly effective for inducing somatic embryogenesis in previous studies as well by Tetu et al. (1990) in pea (*Pisum sativum*) and in tobacco (*Nicotiana tabacum* L); Gill and Saxena, (1993).

TDZ altered the shoot regeneration process from organogenesis to embryogenesis in leaf disc cultures of tobacco (*Nicotiana tabacum* L.); (Gill and Saxena, 1993). In the present study, though TDZ was not used in the somatic embryo induction medium (containing only BAP and NAA), it was used during the initial 4 weeks of culture, up to which a common route for both organogenic and embryogenic pathways was followed. This suggests that probably the carry-over effect of TDZ (present during initial 4 weeks) must have promoted the induction of direct somatic embryogenesis. Similar role of TDZ in somatic embryogenesis has also been reported in woody species like *Rubus* (Fiola et al., 1990) and *Vitis vinifera* (Matsuta and Hirabayashi, 1989).

A detailed anatomical study to establish the nature of regeneration is important, for its use for genetic transformation. Thus in the present study, extensive light microscopy studies were conducted on meristem cultures. These findings have clearly shown that shoot apices can be altered to follow either organogenic or somatic embryogenic pathways of regeneration. This is is in agreement with the conclusion drawn by Zhong et al. (1998) from their electron microscopic studies. Similar nature of regeneration has been reported in *Cicer arietinum L.* (Sagare et al., 1995). More so, these anatomical studies pave way for choosing the appropriate stage for transformation in order to minimize the recovery of chimeras following transformation.

The age of the seedling at the time of meristem isolation significantly influenced the induction potential of the isolated shoot apices (Fig. 7). When *in vitro* response from explants at seven stages (derived from 4-10 days old seedlings) were compared, the response showed a steep increase between day 4-5 and the response was maximum with 7-day-old explants, after which it declined (Fig. 7). Therefore, 5-7 day old explants are

optimum for isolated shoot meristem culture of sorghum. Age-dependent variation in *in vitro* responses was linked to difference in endogenous auxin levels (Cassels et al., 1982) or endogenous cytokinin levels (Josephina et al., 1990). It was reported that genetic and environmental factors alter the levels of endogenous hormones, which determine the *in vitro* responses (Narasimhulu and Chopra, 1988).

RAPD profiles of *in vitro* derived plants generated using 6 random primers did not show any polymorphism among them, and were similar to the control (plants in the field) plant profiles. This indicates that there is no somaclonal variation in regenerants from meristem cultures (Fig. 8). Similar results on absence of DNA variation of plants regenerated from callus, cell and protoplast cultures in poplar (Qiao et al., 1998), wheat (Chawdhury et al., 1994) and rice (Saleh et al., 1990) were reported earlier.

The uniformity of the explants, rapidity of regeneration and, minimized or no chances of formation of somaclonal variants, makes the system described here is useful for both developmental studies involving shoot meristem and genetic transformation.

Apart from the above explant, genetic transformation was carried out using Immature infloresence calli and immature embryos. For this, regeneration systems from the above two explants were also standardized. Efforts have been made in the past for optimization of *in vitro* culture media composition (Wernicke and Brettell, 1980; Cai and Butler, 1987; Hegde and Kuruvinashetti, 1995). The above studies indicate that, Murashige and Skoog (MS) media with 2,4-D is enough for callusing from different explants. However, in our earlier experiments using shoot tip (Seetharama et al., 2000) and scutellum (Sairam et al., 2000), Linsmaier and Skoog (LS) medium was found to be better than MS medium for callus induction. Therefore, in the present study, LS basal

media along with different hormone combinations was used for induction of friable embryogenic calli. LS medium supplemented with 2.5 mg L^{-1} 2, 4-dichlorophenoxy acetic acid (2,4-D) and 0.5 mg L^{-1} kinetin resulted in a callus induction frequency of 85 ± 2 to 95 ± 2 in immature inflorescence, and 74 ± 1 to 90 ± 2 from immature embryos (Tables 12 & 13).

For regeneration, MS medium supplemented with BAP and NAA was employed. MS medium supplemented with 2.5 mg L^{-1} BAP and 0.2 mg L^{-1} NAA was the optimal medium on which, a regeneration frequency of 62 ± 2 to 81 ± 1 was recorded from immature embryos derived callus and a regeneration frequency of 65 ± 2 to 85.5 ± 1 in immature inflorescence cultures (Tables 12 & 13). In both the systems, callus induction and regeneration frequency varied among the 3 genotypes, with M35-1 being the best responding genotype, followed by 296B and BTx-623 genotypes for both embryogenic callus production and regeneration. Seetharama et al. (2000) and Sairam et al. (2000) using sorghum shoot tips and scutellum as explants drew similar conclusions about the genotype response. Such genotypic variation is well known in sorghum (Wen et al., 1991 and Bhat and Kuruvinashetti, 1993). Regeneration pathway in the above two explants was *via* somatic embryogenesis.

5.2 Establishment of transgenic evaluation systems

Use of dominant selectable markers is an integral part of the transformation strategies. The sensitivity of plant cells to a selection marker depends on the genotype, the physiological condition, explant type, and tissue culture conditions. Therefore, the minimum concentration of selection agent that can fully inhibit the growth of non-transformed cells should be determined for each transformation and regeneration system. Similarly, identification of the functionality of the transgene in the mature transgenic plants, and tracking the inheritance of the transgenes in their progeny, usually involves time-consuming, laborious and expensive procedures such as PCR, Southern hybridization, enzyme assays and RT-PCR. As an alternative to this, direct in-planta assays for selectable marker gene activity, such as spraying whole plants or leaf painting with herbicide (Datta et al., 1992), germination of seed on selective media (Hiei et al., 1994) or *in vitro* leaf disc assays (Wang and Waterhouse et al., 1997) are in use. Working on similar lines, in the present study, three *in vitro* and two *in vivo* screening systems were established against phosphinothricin (Table 14). All the three sorghum genotypes BTx-623, M35-1 and 296B were used in establishing these systems. However, there was no significant genotypic effect on the levels of selection agent used in these systems.

5.3 Genetic transformation

Genetic transformation is now widely accepted as method of choice for alien gene transfer into selected cultivars for improving complex traits for which natural variation in the existing gene pool of crops is inadequate. Compared to all other major world crops, sorghum transformation is still in its infancy, and a lot more technical progress is yet to

be achieved. Ideally, genotype-independent protocols are preferred, but this is difficult to achieve. In sorghum, immature embryo is the most commonly used explant for genetic transformation (Casas et al., 1993; Zhao et al., 2000). Except for Battraw and Hall (1991) who for the first time reported transgene expression in electroporated protoplasts, and two reports on *Agrobacterium* mediated transformation, the most favored method of sorghum transformation is the particle bombardment (Table 3). Working on the same lines, in the present study shoot apices and callus derived from immature inflorescence were used extensively as targets for genetic transformation, apart from bombarding only a small number of immature embryos due to their limited availability.

5.3.1 Particle inflow gun *vs* Particle distribution system

Among a variety of gene guns that are currently available, the most popular instrument is the PDS-1000He from Bio-Rad. However, in the present study, an indigenously developed Particle in flow gun (PIG) was used for genetic transformation of sorghum.

PIG used in the present study is based on the principle of Finer et al. (1992). This model was borrowed from Queensland Univ., Australia. Particle in flow gun has been used in the past for successful transformation of crop species like cassava (Zhang & Puonti-Kaerlas, 2000) and rice (Abedinia et al., 2000). First and the foremost advantage of using this gun is the economy of the equipment. Cost of PIG is ten-fold less than PDS. Consumable materials used for bombardment in PIG are considerably less expensive than those used for PDS gun. Another advantage is the omission of membrane rupture disks in PIG, which helps reduce considerably the time interval between two consecutive bombardments.

5.3.2 Optimization of bombardment parameters

The efficiency of DNA delivery using the PIG bombardment device was studied and optimized by bombarding the immature inflorescence derived calli, immature embryos and shoot apices. Bombardment parameters were optimized based on transient GUS expression studies. For this, explants were stained in *X-Gluc* solution. β-glucuronidase is a hydrolase that catalyzes the cleavage of a whole range of β-glucuronidases. Many of these are commercially available as spectrophotomertic, fluorometric, and histochemical substrates. The gene for β-glucuronidase enzyme, which has been cloned (Jefferson et al., 1986), encodes a stable enzyme that has desirable properties for the construction and analysis of gene fusions.

The *E. coli* β-glucuronidase (*uidA*) gene has been developed as a reporter gene system for the transformation of crop plants. The *uidA* gene is commonly used as a gene fusion marker for analysis of gene expression in transformed plants (Jefferson et al, 1987). GUS assay is simple, economical, and has been successfully employed to detect gene activity in bombarded cells and tissues (Zhao et al., 2000), and regenerated plant parts like leaves (Miguel and Oliveirk, 1999) and floral parts (Curtis et al., 1999).

Several factors such as helium gas pressure used for accelerating the microprojectiles, flight distance of the projectiles (the distance microprojectile travels before buried into the target genome), and osmotic treatment of the explants were found to affect DNA delivery. Individual optimization experiments were carried out for each parameter to improve the protocol for particle bombardment. A maximum response of 10-15 GUS spots per explant were recorded for all the three explants when 15 kg cm^{-2}

pressure was used. Helium pressures beyond 15 kg cm^{-2} resulted in tissue damage, so was not favorable. On the other hand, at lower pressures, there was not enough tissue penetration. Therefore, a pressure of 15 kg cm^{-2} was found to be optimal for bombardment using PIG for the above explants (Fig. 16).

The flight distance (in physical terms, distance between the syringe filter unit and sample) was another factor that affected transient expression (Fig. 16). Transient expression was maximum when samples were placed at 7 cm from the filter unit. The receptivity of explant for transgene (measured as percentage of bombarded explants showing GUS spots) was more for isolated shoot apices followed by immature inflorescence and immature embryos (Fig. 17). This could be due to the highly uniform nature of shoot apices and immature embryos compared to inflorescence derived calli. It is known that, the efficiency of transformation using microprojectile bombardment is influenced by the stage of cell cycle (Iida et al., 1991 and Smith et al., 2001).

Osmotic treatment reduces the extent of cell damage following penetration of particles, there by improving the chance of cell recovery, compared to untreated explants. Pre- and post-osmotic treatment has been shown to increase the efficiency of both transient and stable transformation in number of other crop species like maize (Vain et al., 1993) and in asparagus (Carbrera-Ponce et al., 1997). Varying the duration of osmotic treatment of the tissues before and after bombardment has shown to affect transient expression. In this work, of all the osmotic treatments tried, a pre-bombardment osmotic treatment of 4 h on MS medium with 0.4 M sorbitol and 0.4 M mannitol followed by a post-bombardment osmotic treatment of 24h on the same medium resulted in maximum GUS expression.

5.4 Selection and regeneration of putative transgenics

Currently, the two genes *bar* and *pat* isolated from, *Streptomyces. hygroscopicus* and *S. viridochromogenes* are used as marker genes in plant transformation experiments. Both genes code for PAT proteins of 183 amino acids, and show 85 % DNA sequence homology. These PAT encoding genes under the control of suitable promoters are next introduced into the plants, and transformants are identified by selection against PPT or glufosinate or on any one of the commercial brands of these compounds like Basta, Finale or Radicale. Hoechst developed synthetically produced PPT, which bound to ammonium is an active ingredient of Basta. The chemical name of which is ammonium 4 – [hydroxy methyl phosphenoyl] – DL – homoalaninate. The Basta formulation includes 30 % wetting agent, which assists the herbicide to spread aids in its penetration. PPT is a structural analogue of glutamate, a substrate of glutamate synthase (GS). GS is the key enzyme in the nitrogen metabolism that assimilates ammonia produced by nitrate reduction, and recycles ammonia produced by process such as photorespiration and deamination. PPT inhibits GS irreversibly, resulting in inhibition of amino acid biosynthesis. PPT was used as selection agent at a concentration of 5.0 mg L^{-1} by Zhao et al. (2000). On the other hand, Rathus et al. (1996) used 2.0 mg L^{-1}; Casas et al. (1993) 3.0 mg L^{-1} and Jeoung et al. (1999) 3.0 mg L^{-1} of Bialophos. Zhu et al. (1998) & Gray et al. (1999) used 1-2 mg L^{-1} and 2.0 mg L^{-1} Basta in the culture medium for selection of transformants.

In the present study, Basta and PPT were used for selection of transformants. Basta (glufosinate ammonium) was found to be more effective than pure PPT. This could

be due to the activity of the wetting agent present in the commertial formulation. A three-step selection strategy was used for obtaining transformants. Explants were first selected on 1.0 mg L^{-1}, and next on 2.0 mg L^{-1} of selection agent. Subcultures were carried out at 10-day intervals, and there were two rounds of transfers on each concentration. Under step 3, after 6 weeks of selection, the surviving explants were transferred onto medium devoid of selection agent. Thus, the above used concentrations fall in line with the earlier published reports on sorghum genetic transformation (Table 3).

Selection of bombarded sectors on increasing concentrations of the selection agent were reported to allow better recovery of transformants (Casas et al., 1993; Zhang et al., 1998 and Zhou et al., 2000). Therefore, a three-step strategy was employed for transformants recovery. There was a non-selection period of two weeks immediately after bombardment. Similar non- selection strategy was followed by Casas et al. (1997) for bombarded immature inflorescence explants. This was followed by selection on 1.0 mg L^{-1} PPT for 3 weeks. Surviving tissues were selected next on 2.0 mg L^{-1} of PPT. Selection agent was withdrawn completely at the rooting stage. This further helped transgenic plant recovery. To minimize the chances of escapes Casas et al. (1993) and Zhang et al. (1998) used selection agent in the rooting medium. However, Casas et al. (1997) reduced the selection pressure at this stage in order to facilitate better plant recovery. In the present protocol, selection agent was completely with draw at rooting stage Even than, we observed good percentage (93.5 % from shoot apices and 80 % from immature inflorescence derived calli) of stable transformants among the recovered plants meaning that stage 1 and stage 2 selection procedures itself were effective. After a week

of acclimatization in the growth room, plants were transferred to soil and grown to maturity in greenhouse under optimal conditions.

5.5 Confirmation of transgenics

A total of 101 confirmed transgenic plants were regenerated form 3919-bombarded shoot apices and 4 plants from 3846 bombarded immature inflorescence derived calli.

Transformants were obtained from all the three genotypes [M35-1 a land race cultivated during post-rainy season in India; 296B and BTx-623 - popular seed parents of hybrids] used in this study. In the past, sorghum transformants have been obtained from plants belonging to - P898012 (a drought tolerant cultivar of *Pioneer* Co.) by Casas et al. (1993) and SRN 39 (a Striga resistant cultivar) by Casas et al. (1997). Zhu et al. (1998) were successful in generating transgenics from Tx 430 (Texas line) and Zhao et al. (2001) obtained plants from two sorghum lines P898012 (a public line) and PHI 391 (an elite line Pioneer Co.). Thus, availability of transformation protocols for the parent lines used in the present study will strengthen the ongoing efforts for sorghum crop improvement.

In this study, transformants were not regenerated from bombarded immature embryos, which so far has been the most successful explant for sorghum genetic transformation (Casas et al., 1993, Zhu et al., 1998 & Zhao et al., 2000). This could be due to the small number (450) of the immature embryos that were bombarded. But, comparable transient expression levels similar to the above reports were observed even in such a small number of bombarded immature embryos.

Distinctive bands were observed when *Xba*-I was used, demonstrating stable integration of the transgene in the plant genome. The number of transgene copies integrated into the genome of the transgenic plants varied from 1 to 5, as indicated by Southern hybridization results. This phenomenon of multiple copy integration is common in particle bombardment mediate genetic transformation. Copy numbers varying between 1-20 have been reported earlier in rice (Maqbool and Christou, 1999), soyabean (Haidi et al., 1996), oat and barley (Zhang et al., 1999).

There is a visible difference between the band intensities in the autoradiograph, which most likely could be due to difference in the amount of DNA loaded resulting from the presence of other A_{260} absorbing substances in these samples. Similar observations were made by Zhu et al. (1998). Along with the expected bands, few smaller bands were also visible in the Southern hybridization auto-radiographs of T_0 transformants. The presence of fragments of higher or lower molecular weights than expected from the original plasmids indicate two possibilities, one, that of multiple independent insertions occurring and the bands represent plasmid and chromosomal junction fragments; two, that the integrated fragments are long concatemeric plasmid chains in which rearrangements took place. Similar observations were made by Carbrera-Ponce et al. (1997) and further explained by Smith et al. (2001).

Plants regenerated were morphologically similar to control plants and set normal seed. All the transformants showed stable *uidA* gene expression in histochemical assays. Further expression of the *uidA* gene in these transformants was confirmed by RT-PCR analysis. Two methods of priming were done for first strand synthesis and both of them have given identical results (Fig. 21C). This was done to reduce the operational costs of

RT-PCR analysis so that instead of buying the commercial kit, individual components of the reaction mix can be bought and used effectively.

5.6 Inheritance studies

Earlier reports involving study of inheritance and expression of the transgenes have confirmed that transgenes behave in a hemizygous manner segregate in 3:1 dominant: recessive in selfed progeny (Kim et al., 1999; Maqbool and Christou, 1999; Zhang et al., 2000). Or 1:1 segregation in backcross experiments with non-transformed plants. There are also reports, which mention of aberrations in the inheritance ratios, within individual lines as well as the experiment as such (Sachs et al., 1996). Slight variations in the inheritance and the expression of transmitted transgene was observed among the multiple T_1 plants derived from a single transgenic line used in the present study. PATGUS genes segregated along simple Mendelian ratio that was confirmed by histochemical and Southern analysis (Fig. 22). When 0.5 % of Basta solution was applied on to the plants, 2 levels (high and low) of resistance were observed, which may be correlated to the levels of expression of the PAT gene in the plant. Transgene expression heterogeneity could reflect the influence of factors like position effects, gene rearrangement, gene silencing and co-suppression (Maqbool and Christou, 1999; Iyer et al., 2000; Zhu et al., 1998, Smith et al., 2001). Inheritance of the two transgenes was tightly linked, and the progeny that were resistant to Basta application also showed GUS expression. Due to the space constraint, T_1 progeny of only two BTx - 623 plants could be screened.

6 SUMMARY

Genetic transformation is now widely accepted as a method of choice for alien gene transfer into selected cultivars to improve complex traits for which natural variation in the crop gene pool is inadequate. To date, transformation systems have been described for cereals of major agricultural importance including maize, rice and wheat (Bajaj, 2000). Compared to all other major world crops, sorghum transformation is still in its infancy, and a lot more technical progress is yet to be achieved. Ideally, genotype-independent protocols are preferred, but this is difficult to achieve. In sorghum, immature embryos are the most commonly used explant for genetic transformation. To date, only four groups have been successful in transgenic sorghum plant recovery and analysis (Casas et al., 1993; Casas et al., 1997; Zhu et al., 1998 & Zhao et al., 2000). Casas et al. (1993) and, Zhu et al. (1998) obtained plant *via* microprojectile bombardment of immature zygotic embryos, and Casas et al., (1997) via bombardment of immature inflorescence. Zhao et al. (2000) recovered transformants after *Agrobacterium* infection of immature embryo derived callus. Except for the last mentioned report, recovery of transformants has been very low resulting in low transformation frequency (Maqbool et al., 2001). As a result of this, very little has been achieved in genetic transformation of sorghum. The present study was carried out to address some of the problems associated with sorghum genetic transformation.

In vitro response of three explants – immature inflorescence, immature embryos, and isolated shoot apices were studied. Three popular sorghum genotypes, M35-1 (land race cultivated during post-rainy season in India), 296B and BTx-623 (popular seed parents of hybrids) were used for this purpose.

In the present study, a rapid regeneration protocol from shoot apices has been standardized. The isolated shoot apices were induced to follow either organogenic or embryogenic pathway. Callus phase was avoided in both the cases. This could be achieved by striking an optimal balance between an auxin NAA and cytokinin thidiazuron.

Different factors influencing the induction as well as proliferation of meristems such as the method of germination and age of the explant were optimized. Induction response of the explants isolated from seeds germinated on filter paper was better in 6 out of the 7 developmental stages tested. Developmental stage of the explants at the time of meristem isolation influenced the induction efficiency. Percentage of induction increased with the age of the explant from day 4 to day 7 and this response declined steadily when older explants were used for meristem. Over all, 5-8 days old explants isolated from seeds germinated on filter paper showed the highest induction response of 70-80 %.

A detail light microscopy study revealed that, meristems could follow both organogenic and somatic embryogenic pathways of regeneration. RAPD profiles of the showed no polymorphism in the field grown R_0 plants. This indicated that the regeneration system developed during this study does not induce somaclonal variation.

Apart from the above explant, regeneration systems were established using immature embryos and immature inflorescence calli. Linsmaier and Skoog (LS) medium supplemented with 2, 4-dichlorophenoxy acetic acid (2,4-D) and kinetin was used for induction of friable embryogenic calli and somatic embryos. Callus induction and regeneration frequency varied among the 3 genotypes. For regeneration, MS medium supplemented with BAP and NAA was employed. Profuse regeneration *via* somatic

embryogenesis was achieved using both immature inflorescence and immature embryo explants with the former proving more totipotent that the latter.

Use of dominant selectable markers is an integral part of the transformation strategy. Sensitivity of plant cells to a selection marker depends on the genotype, physiological condition, explant type, and tissue culture conditions. In this study, three *in vitro* and two *in vivo* screening systems were developed and the minimum concentration of selection agent that can fully inhibit the growth of non-transformed cells was determined for each of these systems. Genotype effect was found to be non-significant for all the established systems.

In the present study, immature embryos shoot apices and callus derived from immature inflorescence were used extensively as targets for genetic transformation. An indigenously developed Particle inflow gun (PIG) was used for sorghum transformation.

The efficiency of DNA delivery using the PIG bombardment device was studied and optimized by bombarding calli derived from immature inflorescence, immature embryos and shoot apices with PATGUS plasmid. Individual optimization experiments were carried out for each of the bombardment parameter to improve the protocol for particle bombardment. The pressure of helium gas used for acceleration of microprojectiles significantly influenced (P= 0.00281) the efficiency of transformation. A maximum response of 10-15 GUS spots per explant was recorded for both immature embryo and shoot apices when 15 kg cm^{-2} pressure was used. Transient expression was maximum when flight distance was 7 cm from the filter unit. Further, a pre-bombardment osmotic treatment of 4h followed by post-bombardment osmotic treatment of 16h resulted in maximum GUS expression.

Transformation efficiency varied with the type of the explant used for bombardment. The receptivity of explant for transgene was more for isolated shoot apices followed by immature inflorescence and immature embryos.

In the present study, Basta and PPT were used for selection of transformants. Explants were first selected on 1.0 mg L^{-1}, and next on 2.0 mg L^{-1} of selection agent. A total of 101 confirmed transgenic plants were regenerated from 3919 bombarded shoot apices and 4 plants from bombarded immature inflorescence derived calli (3846 in number). However, in this study, transformants were not regenerated from bombarded immature embryos. This could have been due to the small number (450) of the immature embryos that were bombarded.

Histochemical (GUS expression in floral parts) and molecular analysis (PCR, RT-PCR and Southern) confirmed the transgenic nature of the regenerants. The number of transgene copies integrated into the genome of the transgenic plants varied from 1 to 5, as indicated by Southern hybridization results.

T_1 progeny grown in P_2 greenhouse were examined for PPT resistance and GUS expression. Resistant and sensitive plants were distinguishable after 4 days of application of 0.5 % Basta. In susceptible plants, the applied leaf showed > 90 % leaf area scorching, while the resistant plants were as healthy as non-treated plants. When histochemical GUS assay was performed using various floral parts of T_1 progenies, the segregation pattern of *uidA* gene expression agreed with the expected Mendelian ratios. Inheritance of the two transgenes was tightly linked, and the progeny that were resistant to Basta application also showed GUS expression. Further, T_1 progeny that showed blue coloration with *X*-

Gluc substrate also showed the presence of *uidA* gene fragment in PCR amplification and Southern hybridization.

Conclusions and future prospects

For practical use in breeding program, a large number of transformed lines are needed with high levels of transgene expression and its stable transmission to the subsequent generations. Efforts to reduce the time required for production of transgenic plants and the availability of an efficient genetic transformation system could lead to the production of a large number of transformants in a relatively short period, ultimately help in enhancing the quality and productivity of the sorghum plant. The regeneration system based on shoot apices described here has several other advantages like the ability to obtain target tissues from seeds, there by eliminating the necessity to grow donor plants under controlled conditions; and its relatively genotype independent application, apart from rapidity and high efficiency. The transformation method described here will help in recovery of transformants within 14-15 weeks after culture initiation. Transformants were obtained from all the three genotypes used in this study that and this method should facilitate the efforts to improve and enrich germplasm with desirable traits. Apart from these, the economical transgenic screening systems for phosphinothricin-based herbicides have been developed which will help in mass scale evaluation of putative transgenics, a pre-requisite for successful transgenic breeding programs. Thus, this protocol can be used effectively for over all improvement of sorghum.

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