9 Advances in Genetic Transformation

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Abstract

Transgenic sorghum has trailed behind other cereals in progress due to tissue culture limitations, lack of model genotypes, low regeneration, and lack of sustainability of regeneration through sub-cultures. Particle bombardment and *Agrobacterium*-mediated methods are frequently preferred methods for production of transgenic sorghum. Immature embryos and shoot apical meristems are the most suited as target material for genetic transformation. Transformation efficiency is improved through tailored in vitro protocols in desirable genotypes. Many agronomically important traits were introduced in sorghum genotypes to improve quality of grain and forage and to increase resistance to biotic and abiotic stresses. Despite several improvements in transgenic technology and its application for sorghum crop improvement, so far there are no reports on the release and cultivation of transgenic sorghum. Deployment of innovative genetic modification technologies that can keep away from GMO classification and biosafety concerns in sorghum can benefit the producers and consumers of sorghum.

Keywords

Sorghum • Transgenics • Tissue culture • Regeneration • Marker genes

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9.1 Introduction

Success in developing improved cultivars by genetic engineering requires an efficient gene transfer, stable integration, and predictable expression of the transgene. With the advent of genetic transformation techniques based on recombinant DNA technology, it is now possible to insert genes that confer resistance to a number of biotic stresses and to several abiotic factors into the plant genome efficiently which in turn improve the yield. Agronomically useful genes available across genera can be incorporated into the sorghum genome through gene transfer techniques along with reproducible tissue culture protocols to produce transgenic sorghum plants with enhanced yield and nutritional quality.

Though significant progress has been achieved in genetic transformation in cultivable crops, there have been very few reports on sorghum. Sorghum has trailed behind other cereals in the progress toward genetic transformation due to limitations in tissue culture, such as low regeneration frequency and accumulation of phenolic pigments. Being the most recalcitrant crop for tissue culture, regeneration, and genetic transformation, sorghum has lagged behind in the application of transgenic approach for genetic improvement as compared to other cereal crops. To date, there is no transgenic sorghum under commercial cultivation. Attempts were made in sorghum to transform with marker, selectable, and agronomically useful genes. The summary of reports on genetic transformation of sorghum is presented in Table 9.1. Despite several advancements in tissue culture techniques of sorghum, the genetic transformation is by no means either routine or easy.

9.2 Genetic Transformation

Three different methods of genetic transformation have been reported in sorghum, *viz*., protoplast-mediated transformation, particle bombardment, and *Agrobacterium*-mediated transformation.

9.2.1 Protoplast-Based Transformation

The first report of genetic transformation of sorghum described the introduction of DNA into protoplasts by electroporation and selection of transformed cells, without achieving plant regeneration (Battraw and Hall 1991). Parameters influencing the stable transformation of sorghum protoplasts with a chimeric neomycin phosphotransferase II (*nptII*) and *β-glucuronidase* genes by electroporation were investigated. Sorghum cell suspensions can be established initially, but they do not sustain regeneration for longer periods. The cells tend to elongate and lose their regeneration potential making isolation and regeneration of protoplasts difficult. Limitations of protoplast method are that it is laborious, needs special skills, is genotype specific, and has low regeneration ability. To overcome these difficulties, leaf mesophyll tissues were used for isolation of protoplasts in dicots. Sairam et al. (1999) isolated protoplasts and regenerated plants from protoplasts isolated from leaf tissues of sorghum seedlings. However, further work on mesophyll protoplasts and genetic transformation was not followed up.

9.2.2 Particle Bombardment

The biolistic approach has been used extensively for the gene transfer in most of the monocot crops, including sorghum owing to its advantages over *Agrobacterium*-mediated approach. Hagio et al. (1991) demonstrated the first stable expression of the *hph* and *nptII* genes that conferred resistance to selectable levels of hygromycin and kanamycin, respectively. Their results extended the utility of the biolistic method as a useful DNA delivery system for the transformation of sorghum. The first transgenic sorghum plants were obtained by Casas et al. (1993, 1997) through microprojectile bombardment of immature embryos and immature inflorescence-derived calli, but with a relatively low transformation frequency

Table 9.1 Genetic transformation studies in sorghum

Target gene (s)	Transformation method	Explant	Genotype	Transformation efficiency (%)	Reference
gus, *nptII*	E	P	NK300	–	Battraw and Hall (1991)
gus, *hph*, *nptII*	PB	IE[a]	–	–	Hagio et al. (1991)
bar, *gus*	PB	IE	P898012	0.08	Casas et al. (1993)
bar, *gus*	PB	IF	P898012	0.33	Casas et al. (1997)
bar, *chiII*	PB	IE	Tx430, SRN 39	0.09	Zhu et al. (1998)
bar, *gus*	AT	IE	P898012, PHI391	2.1	Zhao et al. (2000)
bar, *gfp*	PB	IE	P898012	1.0	Able et al. (2001)
bar, *chiII*	PB	IE	Tx430, SRN39	–	Krishnaveni et al. (2001)
bar, *gus*	PB	IE	Tx430	0.18	Emani et al. (2002)
bar, *gus*	PB	IE	214856, 213108	1.3	Tadesse et al. (2003)
gfp, *G11*, *tlp*	AT	IE	Tx430, C401, Wetland	–	Jeoung et al. (2002)
gfp, *tlp*	AT	IE	Tx430, C401	2.5	Gao et al. (2005a)
*man*A	AT	IE	P8505, C401	2.88, 3.3	Gao et al. (2005b)
Bt cry1Ac	PB	SA	BTx623	1.5	Girijashankar et al. (2005)
nptII, gusplus	AT	IE	Tx430, C2-97	0.3–4.5	Howe et al. (2006)
gus, *hpt*	AT	IE	Sensako 85/1191	5.0	Nguyen et al. (2007)
Sgfp, *man*A	AT	IE	P898012	7.7	Gurel et al. (2009)
bar, *gus*	AT	IE	P898012	0.4, 0.7	Lu et al. (2009)
mtlD	PB	SM	SPV462	4.0–7.0	Maheswari et al. (2010)
hpt	PB	IE	Ramda	0.09	Raghuwanshi and Birch (2010)
bar, *pmi*	PB	IE	P898012	0.77	Grootboom et al. (2010)
gus, *hpt*	AT	IE	P898012, RTx430	1.1–7.2	Kumar et al. (2011)
nptII, *gfp*	PB	IE	Tx430	20.7	Liu and Godwin (2012)
Bt cry1Aa, *cry1B*	PB and AT	IE	CS3541, 296B, SSV84, RSSV9	0.1–0.3	Visarada et al. (2014)
moPAT, pmi, *dsRED*	AT	IE	Tx430	8–13.4	Wu et al. (2014)
gus	AT	IE	P898012	–	Urriola and Rathore (2014)

[a]Reported from a suspension culture of *Sorghum vulgare*

bar phosphinothricin acetyltransferase, *chiII* rice chitinase, *gfp* green fluorescence protein, *gus* β-glucuronidase, *hph* hygromycin phosphotransferase, *mtlD* mannitol-1-phosphate dehydrogenase, *manA/pmi* phosphomannose isomerase, *moPAT* codon-modified phosphinothricin acetyltransferase gene, *nptII* neomycin phosphotransferase II, *tlp* thaumatin-like protein

E electroporation, PB particle bombardment, AT *Agrobacterium*-mediated transformation, P protoplast, IE immature embryos, IF inflorescences, SA shoot apices, SM shoot meristem

(0.2 %), a protracted time in culture (7 months), in the genotype, PI898012, which has poor agronomic traits.

Maize genes encoding anthocyanin transcription factors (R and C1) were used by Casas et al. (1993) to optimize DNA delivery parameters in sorghum immature embryos. Frequency of transient expression of *gus* was less than 20 blue foci/embryo, which indicated that transient gene expression level in sorghum was lower than in maize due to genotype effect or interactions between genotype and acceleration pressure or other inherent characteristics of sorghum scutellar tissue. Immature embryos and immature inflorescences were used as explants by Kononowicz et al. (1995) for particle bombardment and plant regeneration. Genotype-specific differences in the response of primary explants to regeneration protocols have been found among sorghum genotypes studied and the genotypes PS98012 (immature embryos) and SRN39 (immature inflorescence) were found to be promising.

Optimization of transformation conditions, parameters for microprojectile bombardment, and strength of promoters were reported by Tadesse et al. (2003) in the genotype 214856, which is capable of producing a good-quality callus and less susceptible to phenolic pigments during selection on geneticin. Transgenic sorghum was produced by the combined use of optimized bombardment conditions, strong monocot gene promoters, and stepwise antibiotic selection. Physical and biological parameters that give the highest transient expression of the introduced *gus* reporter gene without compromising the frequency of somatic embryogenesis and regeneration capacity were studied. Variation in transient gene expression level was observed with different types of explants, and with different pressures. It was concluded that immature embryos and shoot tips were the best explants to target potential progenitor cells that are competent for embryogenesis. Evaluation of transient expression of the *gus* reporter gene under the control of a number of parameters using four different promoters revealed that the activity of all three monocot promoters (*ubi*1, *act*1, and *adh*1) was higher in sorghum than that of the CaMV 35S promoter (Tadesse et al. 2003). However, maize *ubi*1 and rice *act*1 gene promoters were preferred for stable expression of foreign genes in sorghum immature embryo and shoot tip explants. The transformation frequency ranged between 0.5 and 1.3 % for shoot tips and immature embryos, respectively. Very slow or no root formation was observed on shoots regenerated under selection on phosphinothricin or geneticin.

A low incidence of transgenic sorghum has been obtained using particle bombardment of callus explants of the genotypes, P898012 (Casas et al. 1993; Rathus et al. 2004), SRN 39 (Casas et al. 1997) and Tx430 (Zhu et al. 1998). This indicates that an efficient genotypes-independent plant regeneration system is vital for the development of transformation protocols for sorghum. Sorghum line, P898012, is reported by many because it is capable of producing a good/better embryogenic, quality callus from immature embryos and was very responsive to coconut water (Casas et al. 1993; Kaeppler and Pederson 1997; Carvalho et al. 2004). Frequently reported sorghum genotypes for successful transformation are P898012, Tx430, and C401 (Table 9.1). Immature embryos are the most preferred target tissues in sorghum. However, they are not available round the year due to the photoperiodic sensitivity of many agronomically important genotypes. Improved methods for rapid isolation and culture of immature embryos can aid in large-scale transformation experiments (Raju et al. 2007). Multiple shoot buds induced from shoot apical meristems are promising target tissues for genetic transformation, which mitigate the issues of regeneration and availability round the year. Callus cultures derived from immature inflorescences in sorghum, though have high regeneration at 30–50 days, decrease rapidly further making the recovery of transgenic plants a challenge. A highly efficient microprojectile transformation system for sorghum has been developed by Liu and Godwin (2012) using immature embryos of inbred line Tx430. After optimization of tissue culture media and parameters of microprojectile transformation, 25 independent transgenic events were obtained from 121 bombarded immature embryos with an

average transformation frequency of 20.7 % in three independent experiments, the highest frequency reported so far.

Use of the cytidine analog, 5-azacytidine (azaC), in reversing the methylation-mediated transgene silencing in sorghum was demonstrated by Emani et al. (2002). It was possible to activate *gus* gene expression in T_1 seedlings and in calli derived from immature T_1 and T_2 embryos by the treatment of 5-azacytidine (azaC). The investigators suggested that methylation-based silencing is frequent in sorghum and probably responsible for several cases of transgene inactivation reported earlier in sorghum.

9.2.3 *Agrobacterium*-Mediated Transformation

Agrobacterium-mediated transformation is the most preferred method among the researchers due to its advantages in the production of transgenic plants with single or low-copy inserts. The first successful report in sorghum transformation using this method was reported by Zhao et al. (2000) using the public line P898012, in which four factors that influence sorghum transformation the most were identified, *viz*., (1) sensitivity of immature sorghum embryos to *Agrobacterium* infection, (2) growth conditions of donor plant, (3) type of explant, and (4) cocultivation medium. Necrotic response in explants after cocultivation is a critical factor to improve the transformation efficiency. Immature embryos of sorghum proved to be very sensitive to *Agrobacterium* infection, and it was found that the level of embryo death after cocultivation was the limiting step in improving transformation efficiency. Hence, attention should be given to the number of *Agrobacterium* cells in the inoculum, selection of sorghum genotypes, and explants less sensitive to *Agrobacterium* infection. Increased percentage of embryos that formed callus (recovery of callus), reduced pigment production, and improved callus growth were observed by the addition of coconut water to the medium (Carvalho et al. 2004). Though the genotype P898012 is responsive to coconut water, its use in other genotypes may require different composition, since many genotypes did not respond to coconut water (Kaeppler and Pederson 1996). Various explants were used by Visarada et al. (2003) to explore genetic transformation of sorghum using *Agrobacterium*-mediated transformation resulting in transient *gus* expressions. However, immature embryos and calli derived from immature inflorescences were found to be ideal target tissues for obtaining high *GUS* expression. In a recent study by Wu et al. (2014), high transformation frequencies (10–33 %) were obtained through elevated copper sulfate and 6-benzylaminopurine in the resting and selection media by *Agrobacterium* infection of immature embryos using the genotype Tx430.

Optimal conditions were determined and baseline conditions in subsequent experiments were provided by Zhao et al. (2000) to achieve stable transformation of sorghum using *Agrobacterium*. It was observed that N_6 medium not only decreased the callus response from embryos (76 % with MS and 20 % with N_6) but also increased the production of phenolic pigments. The overall transformation frequency was 2.1 %. The optimization of media and other conditions for the transformation of sorghum using *Agrobacterium* was reported and a transformation frequency of 2.1 % was achieved. However, acetosyringone concentration was not optimized since a single concentration (100 μM) was used to induce *vir* operon.

Addition of amino acid L-cysteine in the plant tissue culture medium aided the recovery of putative transformants and improved the transient *gus* expression and stability of transformed explants (Sai Kishore et al. 2004). Production of transgenic sorghum plants through tailored in vitro protocols is possible in choice genotypes. However, decontamination of *Agrobacterium* by employing subtle treatments aided the recovery of transgenic plants in recalcitrant genotypes. In sorghum the resistant calli obtained after selection did not regenerate probably due to the toxic effects of decontaminating agents and selection pressure (Sai Kishore et al. 2011). Moreover,

sweet sorghums secrete polyphenols into the culture medium leading to decrease in regeneration, which was overcome by frequent sub-culturing in the initial stages of post-transformation.

In planta method of *Agrobacterium*-mediated transformation helps in overcoming the hurdle of regeneration after selection. This method was demonstrated in sorghum by Elkonin et al. (2009) through the generation of transgenic plants and inheritance of the transgene to T_1 generation. Our research group developed a simplified method of *in planta* genetic transformation by a floral-dip method in sorghum, which does not involve vacuum infiltration and uses *Agrobacterium* suspension derived from solid cultures. Two independent transgenic sorghum lines were developed in sweet sorghum genotypes and the gene integration and expression were studied till T_5 generation. However, the expression of *Bt* protein in *in planta* derivatives was low. *In planta* method is simple, easy, and economic without the problem of regeneration after selection. Supplementation of agro-infiltration liquid with special components like L-cysteine and Tween-20 had a promotive effect on transformation (unpublished results).

Parameters were optimized for transformation by *Agrobacterium*-mediated and particle bombardment by Prasad Sant (2011) using the sorghum cultivars, *viz*., SA281, 296B, and Tx430. *Agrobacterium* strain LBA4404 at an inoculum density of 0.5 OD_{600nm}, heat treatment at 43 °C for 3 min., inoculation media (pH 5.2) with the surfactant Pluronic F-68 (0.02 % w/v), and a 3-day cocultivation period in dark at 22 °C was found to be optimum resulting in high frequencies of transient GFP expression in immature embryos and callus derived from immature inflorescences. The optimum conditions for particle bombardment, *viz*., use of 3–7 day-old immature embryos and 4 week-old callus from immature inflorescences, pre- and post-bombardment osmoticum treatment of 4 h and 0.6 μm gold microparticles, 1,500 kPa helium pressure, and a target distance of 15 cm, resulted in transient GFP expression for up to 14, 30, and 50 days for SA281, 296B, and Tx430, respectively. Though particle bombardment resulted in less tissue necrosis compared to *Agrobacterium*-mediated transformation, no stable transformed plants were regenerated.

Both the systems of transformation, i.e., *Agrobacterium* and particle bombardment, though successful in sorghum, have their own merits and limitations. With *Agrobacterium-based* transformation, the higher transformation efficiency achieved so far ranged from 2.1 to 4.5 % (Howe et al. 2006; Gao et al. 2005b; Zhao et al. 2000). Effective method till date remains to be the microprojectile bombardment with higher transformation efficiency of 20.7 % (Liu and Godwin 2012).

In addition to the above mentioned methods of genetic transformation, pollen-mediated method was reported in sorghum by Wang et al. (2007), in which the plasmid DNA and pollen were submerged in a 0.3 mol/l sucrose solution, subjected to ultrasonication and then used for pollination of stigmas from the male sterile line A2V4A. Confirmation of gene integration was supported by the evidence of Southern blotting.

9.3 Tissue Culture and Regeneration

A wide range of sorghum explants, either for somatic embryogenesis or organogenesis, have been tested to establish a dependable tissue culture system for regeneration, which include mature embryos, immature embryos, immature inflorescences, seedlings, leaf fragments, and anthers. However, genetic transformation achieved in sorghum so far has been established with embryogenic cultures initiated from immature embryos or immature inflorescences. Maintenance of consistent quality of the callus on medium for an extended time (2–3 months or longer) and regeneration of fertile plants are essential for successful plant transformation.

MS medium supplemented with different combinations and concentrations of plant growth hormones were used by Pola et al. (2008) to culture immature embryos. Embryogenic callus was initiated on MS medium supplemented with 2 mg l^{-1} 2,4-D and 0.5 mg l^{-1} kinetin; the addition of kinetin significantly enhanced embryogenesis. Proficiency

in the development of embryogenic callus, induction of somatic embryo, and shoot regeneration was observed with 2 mg l^{-1} BAP. The regenerated shoots readily rooted on half-strength MS medium containing 1 mg l^{-1} NAA and were successfully transferred to the soil, which subsequently produced the seeds. Since the regeneration frequency of the bombarded calli derived from immature inflorescence is low, it is the least preferred explant (Kononowicz et al. 1995; Casas et al. 1997; Jogeswar et al. 2007). High-quality callus from immature inflorescence of sorghum genotypes is high, but the efficiency is still very low. It was improved to 1.01 to 3.33 % by modification of bombardment parameters (Brandao et al. 2012).

An attempt was made by Prasad Sant (2011) to establish an efficient and reproducible tissue culture and transformation system using five different sorghum cultivars, *viz*., SA281, 296B, SC49, Wray, and Rio. Regenerable embryogenic cell lines could be established only from SA281 and 296B. In addition, embryogenic cell lines were established using florets of immature inflorescence as explants from SA281, 296B, as well as Tx430 and regenerated using sorghum callus induction media (SCIM) and Vam's wonderful regeneration media (VWRM) with the inflorescences from plants at the FL-2 stage (where the last fully opened leaf was two leaves away from the flag leaf) giving the best in vitro response. Moreover, the responses of immature inflorescences were robust in tissue culture and independent of season and growth condition. The optimum combination of plant growth regulator standardized for the micro-propagation of in vitro regenerated plantlets was 1.0 mg l^{-1} BAP and 0.5 mg l^{-1} NAA.

Apart from immature embryos and inflorescences, shoot tips from germinating seedlings are also widely used as explants in sorghum transformation (Girijashankar et al. 2005; Tadesse et al. 2003). Recently, an efficient protocol for regeneration in sorghum through somatic embryogenesis from shoot tip explants was developed by Amali et al. (2014) with the highest frequency of embryogenic callus formation (99 %), when explants were cultured on MS medium supplemented with 2.5 mg l^{-1} 2,4-D, 0.25 mg l^{-1} kinetin and 500 mg l^{-1} of casein hydrolysate. Sub-culturing of embryogenic callus on MS medium supplemented with 2.5 mg l^{-1} 2,4-D, 0.25 mg l^{-1} kinetin, 500 mg l^{-1} of casein hydrolysate, and 500 mg l^{-1} of L-proline resulted in the highest mean number of somatic embryos (33.3). The highest regeneration of plantlets (21.4 per embryogenic callus) was obtained in MS medium supplemented with 4 mg l^{-1} benzylaminopurine. A maximum number of roots (12.4) and root length (5.7 cm) were observed in half-strength MS medium supplemented with 1.0 mg l^{-1} indole-3-butyric acid and 0.8 g l^{-1} activated charcoal. The survival rates of in vitro grown plantlets transferred to greenhouse were up to 70 %, which were morphologically similar to in vivo plants.

Explants derived from meristematic tissues at the early stages of development are most amenable to tissue culture conditions (Sai Kishore et al. 2006). Highly uniform meristematic tissues are desirable for genetic transformation to minimize chimeras and somaclonal variants. Cultured immature embryos and shoot tips are the two explants of choice that have been predominantly used for sorghum genetic transformation. Two parental lines (CS3541 and 296B) that are less responsive to tissue culture were transformed by Visarada et al. (2014) by identifying the critical factors like 3d pre-cultured immature embryos as target tissues, small-size explants, treatment with L-cysteine during cocultivation, recovery of transformed embryos after antibiotic washes, and regeneration *via* multiple shoot bud induction.

The supply of immature inflorescences and embryos is not available throughout the season as sorghum flowering occurs for a few days only, thereby providing a small window for the collection of explants. To overcome this problem, Pola (2011) used leaf discs that will be available at any season for optimizing callus induction and regeneration. Efficient callus induction was obtained on MS media supplemented with 2 mg l^{-1} 2,4,5-T and 1 mg l^{-1} NAA and 0.5 mg l^{-1} zeatin, while better shoot regeneration (62.2 ± 4.6 shoots per explants) was achieved in MS medium supplemented with 2.5 mg l^{-1} thidiazuron + 1.0 mg l^{-1} BAP and 0.5 mg l^{-1} IAA. Similarly, better root induction was observed with 1.0 mg l^{-1} NAA followed by their

transfer to half-strength MS medium. Various reports on tissue culture techniques and different explants with regeneration ability in sorghum are presented in Table 9.2.

Table 9.2 Summary of tissue culture studies in sorghum

Explant	Experiment	Conclusion	Reference
Immature embryos	Callus induction medium – MS medium supplemented with L-proline, L-asparagine, potassium dihydrogen phosphate, $CuSO_4$, and 2,4-D Regeneration medium – MS medium supplemented with BAP, IAA, and $CuSO_4$ Rooting medium – MS medium supplemented with NAA, IAA, IBA, and $CuSO_4$	Optimized callus induction, regeneration, and rooting medium to reduce phenolic production.	Liu and Godwin (2012)
Germinated seeds	Shoot induction on MS medium supplemented with different concentrations of IAA, BAP, and kinetin	Developed an efficient regeneration system using two cultivars Yuantian No. 1 and M81E	Zhao et al. (2010)
Immature embryos	Regenerated callus using MS medium containing 2 mg l^{-1} 2, 4-D, 0.5 mg l^{-1} kinetin, 10 mg l^{-1} $AgNO_3$, 400 mg l^{-1} casein hydrolysate, and 200 mg l^{-1} each of L-asparagine and L-proline	Developed a protocol for long-term maintenance of callus cultures and succeeded in maintaining the embryogenic callus cultures up to 57 weeks	Pola et al. (2009)
Immature embryos	Heat treatment of immature embryos (IEs) at various temperatures for 3 min prior to *Agrobacterium* infection	Optimized a 3-min heat treatment at 43 °C prior to infection. Both heat and centrifugation increased dedifferentiation of tissue	Gurel et al. (2009)
Immature zygotic embryos	Callus initiation and regeneration potential of five sorghum genotypes on specific nutrient media	Identified the best genotype, nutrient medium combination for satisfactory regeneration	Grootboom et al. (2008)
Leaf	Shoot regeneration using different growth hormonal combinations	The highest number of somatic embryos was produced from leaf segments on MS medium supplemented with 2.0 mg l^{-1} 2,4,5-trichloro acetic acid and 1.0 mg l^{-1} zeatin	Pola et al. (2007)
Immature inflorescence	Effect of various growth regulators on somatic embryogenesis in three genotypes (SPV462, SPV839, and M35-1)	High frequency of somatic embryogenesis was obtained on MS medium supplemented with 2 mg l^{-1} 2,4-D and 0.5 mg l^{-1} kinetin	Jogeswar et al. (2007)
Immature embryos	Cold pretreatment of the immature seeds from which embryo explants were excised and by the use of activated charcoal	Developed an improved regeneration protocol suitable for transformation by limiting the production of phenolic compounds and the use of suitable culture vessels for each developmental stage in plant regeneration	Nguyen et al. (2007)

(continued)

Table 9.2 (continued)

Explant	Experiment	Conclusion	Reference
Shoot tip	Evaluated 24 sorghum genotypes and 3-day- and 5-day-old shoot tips for tissue culture response	Age of the seedlings in relation to the number of actively dividing cells that contain greater potential for in vitro response was reported for the first time using electron microscopy	Saikishore et al. (2006)
Immature embryos Immature inflorescences	A factorial experiment was conducted with a combination of 2,4-D and kinetin in 8 genotypes of *S. sudanese* and *S. bicolor*	Genotypic limitation of plant regeneration can be overcome by the addition of kinetin in callus induction media. Immature inflorescences regenerated at a higher frequency (2.71 shoot $callus^{-1}$) than immature embryos (1.26 shoot $callus^{-1}$)	Gupta et al. (2006)
Shoot tip	Somatic embryogenesis and multiple shoot clumps in Indian sorghum genotypes	Somatic embryogenesis was reported by combination of 2,4-D and 6-benzyl adenine. Multiple shoot clumps were produced using BAP alone in 1-week-old shoot tips	Syamala and Devi (2003)
Shoot meristem	Multiple shoot bud induction	Multiple shoot bud induction on enlarged meristems was achieved using higher concentrations of TDZ, BAP, and NAA	Harshavardhan et al. (2002)
Immature embryo	Eleven genotypes of sorghum were experimented for their response in tissue culture	Adventitious shoot regeneration from immature embryos was achieved	Hagio (2002)
Immature embryo	Influence of phytohormones, $AgNO_3$, and maltose/sucrose as carbon source on callus induction	Callus induction with respect to regeneration was observed only by the modification of phytohormones alone. Fertile plants at high efficiency were obtained	Oldach et al. (2001)
Shoot tip	LS medium with 2,4-D and kinetin was used for induction of friable callus MS media with BAP and IAA was used for germination of somatic embryos	Somatic embryogenesis was observed in shoot tip-derived callus by the incubation of shoot tip for 6–7 weeks	Seetharama et al. (2000)
Young panicle Immature embryo	Explants were cultured on modified MS and N_6 media supplemented with Asn and Pro with different conc. of NO_3^-, NH_4^+, and PO_4^{3-}	Level of NO_3^- and NH_4+ ratio was the critical factor for the formation of embryogenic calli. Increase of the PO_4^{3-} level increased the regeneration ability	Elkonin and Pakhomova (2000)
Shoot meristem-derived callus	Regeneration of wild sorghum from suspension cultures with shoot meristem-derived callus	High frequency of somatic embryogenesis (80 %) was obtained from small clusters (300–400 wm) maintained in liquid medium with 0.25 mg l^{-1} 2,4-D	Mythili et al. (1999)

(continued)

Table 9.2 (continued)

Explant	Experiment	Conclusion	Reference
Shoot tip	Multiple shoot induction	Multiple shoot induction using BAP and 2,4-D was achieved in 18 genotypes	Zhong et al. (1998)
Immature inflorescence	For identification of high-quality callus producing genotypes, 41 diverse inbred sorghum lines were tested	Seven elite inbred genotypes and five non-elite genotypes were selected for high-quality callus based on mean rating	Kaeppler and Pederson (1997)
Seedlings	Importance of kinetin in induction of embryogenic calli from seedlings through transverse thin cell layers	Callus induction, somatic embryogenesis, and plant regeneration were reported in two cultivars from epicotyls of 15-day-old seedlings. Callus response is dependent on genotype	Gendy et al. (1996)
Immature embryo	Cefotaxime, asparagine, and praline effects on production of embryogenic callus and to enable the frequency of plant regeneration	Even though these compounds did not promote callus induction or growth of callus, they influenced the plant regeneration in sweet sorghum genotypes with high anthocyanin	Rao et al. (1995)
Immature inflorescence	Eight genotypes tested by modification of MS medium for induction and regeneration of cultures	Genotypic differences were observed in pigment production, embryogenic callus formation, shoot differentiation, and maintenance of regeneration capacity. Embryogenic calli were formed at a frequency of 8–70 % depending on the genotype	Cai and Butler (1990)
Immature inflorescence and shoot tips	Callus induction and plant regeneration	Immature inflorescence followed by shoot tips favor good callus induction and plant regeneration	Murty et al. (1990a, b)
Seedlings	Plant regeneration from 4–5-month-old callus cultures	Somaclonal variation was examined in the SC2 and SC3 generation of eight different clones and one non-tissue-cultured parental line	Bhaskaran et al. (1987)
Mature embryos	Callus and plant regeneration were induced from shoot portions of mature embryos of 5 high-tannin cultivars	Plants were regenerated on MS medium supplemented with asparagine and kinetin with a regeneration frequency of 11–48 %	Cai et al. (1987)
Anthers	Microspore-derived callus production at different temperatures	Callus was produced only from anthers incubated at 33 °C	Rose et al. (1986)
Seedlings	Sorghum plant regeneration from aluminum selection medium	Callus cultures of germinated seed	Smith et al. (1983)

9.4 Marker Genes

The most widely used selectable markers in cereal transformation are the genes encoding hygromycin phosphotransferase (*hpt*), phosphinothricin acetyltransferase (*pat* or *bar*), and neomycin phosphotransferase II (*nptII*). Use of these marker genes under the control of constitutive promoters such as CaMV 35S promoter or the *ubi*1 promoter from maize works as efficiently for selection of *Agrobacterium-transformed* cells as for biolistic-mediated transformation. The positive selectable marker *pmi* (*phosphomannose isomerase*) has been shown to be effective in the transformation of many monocots including sorghum (Gao et al. 2005a). Expression of green fluorescence protein (*gfp*) on sorghum regeneration has been reported by Able et al. (2001) using particle bombardment, in which the distance between the rupture disk and target tissue, helium inlet aperture and pressure of helium gas, and age of tungsten and spermidine were studied. The sorghum genotype Tx430 yielded the most foci/callus in transient expression, while C401 was the least amenable for stable transformation. The use of *gfp* as a reporter for optimizing the transient expression during *Agrobacterium*-mediated transformation of sorghum was demonstrated by Jeoung et al. (2002). Using two different reporter genes, the suitability of different inbred lines (Tx430, C401, CO25) was determined using different promoters, type of explants, and inbreds during the early transformation process involving both biolistic and *Agrobacterium*-mediated transformation. The results indicated that *gfp* can be used effectively as a reporter for optimizing the conditions for successful transient expression during transformation with *Agrobacterium*.

Grain sorghum was transformed by Gao et al. (2005a) with a visual reporter gene (*gfp*) and a target gene (*tlp*), encoding thaumatin-like protein, and they reported the successful use of GFP screening for efficient production of stably transformed sorghum plants without using antibiotics or herbicides as selection agents. Transformation efficiency of 2.5 % was observed, which was greater than that reported earlier by Zhao et al. (2000). A dual-marker plasmid containing the selectable marker gene, *man*A, and the reporter gene, *sgfp*, was used to transform immature sorghum embryos by employing *Agrobacterium-mediated* transformation system (Gao et al. 2005b). Both the genes were under the control of a maize *ubi*1 promoter. The phosphomannose isomerase gene *pmi* was isolated from *E. coli* and used as the selectable marker gene and mannose was used as the selection agent. Necrotic calli were rarely observed in mannose selection and it had less negative effects on plant regeneration. Gene silencing of either the *gfp* gene or the *tlp* gene in T_0 and T_1 generations was not observed by Gao et al. (2005a, b). Stable transformation experiments in sorghum immature embryos of Tx430 and C2-97 genotypes were carried out by Howe et al. (2006) using a novel strain of *A. tumefaciens* (C58) implementing *nptII* as a selectable marker, resulting in transformation frequencies in the range of 0.3–4.5 % with an average transformation frequency of ~1 %.

In most reports on genetic transformation of sorghum, it was observed that the expression of introduced *gus* gene was either very poor or totally absent. GUS enzyme activities were very low in sorghum cells compared to other *gus* gene-transformed monocot cells (Hagio et al. 1991). The majority of the *gus* transformed cells did not stain blue upon incubation with histochemical substrate X-Gluc (Battraw and Hall 1991). Moreover, GUS activity that was high in transient assays could not be detected later than 3 weeks after bombardment (Casas et al. 1993, 1997) suggesting that methylation of transgene might have occurred in sorghum cells, leading to the inhibition of the expression of the reporter gene. Jeoung et al. (2002) studied the optimization of parameters for use of *gfp* and *uid*A as visual markers and evaluated different promoters. It revealed the order of promoter strength for GUS expression as *ubi*1 > CaMV 35S > *act*1 > *adh*1. Another reporter gene, *luc*, a firefly luciferase, was used in sorghum transformation studies by Kononowicz et al. (1995).

Five different selection markers, which include *cat*, *npt II*, *hpt*, *bar*, and *man*A, representing three broad categories of selection markers (antibiotic resistance, herbicide resistance, and nutrient assimilation), were utilized in sorghum transformation. The conversion of mannose to a metabolizable six-carbon source is beneficial to plants and is an efficient and non-destructive method of screening the transformed sorghum plants under in vitro conditions. Co-bombardment was performed by Liu and Godwin (2012) with *nptII* and *gfp* genes, both under the control of the maize *ubi*1 promoter using immature embryos of sorghum resulting in high transformation efficiency.

9.5 Economically Important Genes

Transgenic technology in sorghum is extended to combat biotic and abiotic stresses and to improve the quality. Agronomically important gene, *chi ll*, encoding rice chitinase under the constitutive CaMV 35S promoter was transferred to sorghum to impart resistance to stalk rot (*Fusarium thapsinum*) by Zhu et al. (1998) and Krishnaveni et al. (2001). Transgenics for resistance to anthracnose were reported by Kosambo-Ayoo et al. (2011). Transgenic sorghum plant expressing *cry1Ac* gene under the control of the wound-inducible promoter for the protection against spotted stem borer was reported in the cultivar BTx623 through microprojectile bombardment of shoot apices after selection and regeneration through embryogenic pathway (Girijashankar et al. 2005). The study showed that the *mpiC1* promoter from maize is functional in sorghum and drives the expression of *cry1Ac* gene, but at low levels to confer partial protection against the neonate larvae of the spotted stem borer. In another study, the T_0 transgenic plants generated using *ubi1–cry1Ab* and *ubi1–cry1Ac* were found to be chimeric in nature, which may be due to the lack of the transgene *cry* in the reproductive parts (Girijashankar and Swathisree 2009). Promising *Bt* transgenic possessing *cry1Aa* gene conferring resistance to stem borer in the grain sorghum genotype M 35-1 was reported by Ratnala (2013). Transgenic sweet sorghum derivatives through backcross breeding using a promising transgenic event in the genotype M 35-1 were developed recently by Indukuri (2014), which showed promising levels of resistance in whole-plant bioassays for *Chilo partellus*. Transgenic sweet sorghum plants in the genetic background of SSV84 and RSSV9 were generated through particle bombardment and *in planta* method of *Agrobacterium*, which exhibited moderate to low levels of resistance in insect feed assays in the laboratory (Visarada et al. 2013). Transgenic sorghum expressing two different classes of *Bt* toxin proteins, Cry1Aa and Cry1B, in two elite and recalcitrant genotypes (CS3541 and 296B) was developed, which showed high levels of expression (35–500 ng/g protein) with commensurate resistance to stem borer (Visarada et al. 2014). Transgenic sorghum plants possessing *cry1C* gene showed 10–13 % leaf damage and 97–100 % larval mortality in the insect bioassays with *Chilo partellus* neonate larvae (Ignacimuthu and Premkumar 2014). Promising transgenic lines were identified in the field trials for resistance to stem borer by Balakrishna et al (2013).

High-lysine sorghum lines were generated by Tadesse et al. (2003) in the genetic background of the Ethiopian genotype 214856 through the over-expression of *dhdps-raec*1 mutated gene, which encodes the insensitive form of the *dihydrodipicolinate synthase*, the key regulatory enzyme of the lysine pathway. Marker-free transgenic sorghum plants harboring *lysyl tRNA synthetase* gene for enhanced lysine content in sorghum seed was developed by Lu et al. (2009). Contrary to the expectation, introduction of glycine-rich RNA-binding protein gene atRZ-1a from *Arabidopsis*, Bcl-2 mRNA sequence 725–1,428 representing the 3′ non-coding region of the gene from humans and rice Ca-dependent protein kinase 7 (OsCDPK7) could not improve the cold tolerance in sorghum (Mall 2010). Transgenic sorghum events expressing HMW-GS showed improvement in protein digestibility of the uncooked ground grain, and the downregulation of alpha kafirin showed the presence of distorted protein bodies in the transgenic seed (Mall 2010).

The lysine content of sorghum grain was increased by Tadesse and Jacobs (2004) through the introduction of a mutated *dhdps-rl* gene encoding a feedback-insensitive *dihydrodipicolinate synthetase* enzyme by microprojectile bombardment of immature embryos and shoot tips leading to accumulation of more amount of lysine. The *dhdps-raec1* mutated gene encodes an insensitive form of the *dihydrodipicolinate synthase*, the key regulatory enzyme of the lysine pathway. The over-expression of this gene could lead to the elevated lysine content in sorghum and improvement of nutritional quality of this crop. Lu et al. (2009) reported transgenic sorghum plants harboring a modified tRNAlys, and sorghum lys1 tRNA synthase elements for improving the lysine content in sorghum seeds. However, there was no mention on the expression of the lysine gene or amino acid content.

To impart resistance to abiotic stresses like drought, *HVA1* gene from barley was inserted into the sorghum genome through biolistic transformation by Devi et al. (2004), which accumulated barley 3 LEA protein under induced stress. Sorghum line SPV462 was transformed with the *mtlD* gene encoding for *mannitol-1-phosphate dehydrogenase* from *E. coli* to enhance tolerance to water deficit and NaCl stress (Maheswari et al. 2010). Transgenic sorghum plants maintained a 1.7–2.8-fold higher shoot and root growth, respectively, at 200 mM NaCl stress compared to untransformed control plants and demonstrated that the engineering mannitol biosynthetic pathway into sorghum can impart enhanced tolerance to water deficit and salinity. Transgenic sweet sorghum with altered lignin content was obtained by manipulating the expression of *caffeoyl-CoA-O-methyltransferase* (*CCoAOMT*) and *caffeic acid-O-methyltransferase* (*COMT*) toward production of easily degradable plant material for biofuel production (Basu et al. 2007). A young forage crop of sorghum produces HCN in the leaf tissues proving toxic to animals on grazing. Low HCN transgenic forage lines containing safe levels (<200 ppm) of HCN were developed by Pandey et al. (2010) through downregulation of dhurrin gene.

9.6 Transformation Efficiency

High-efficient transformation systems are essential for GM product development as well as gene expression studies. Much effort was applied to extend the host range of *Agrobacterium* to monocotyledonous species. *Agrobacterium* infection of seedlings/immature embryos of cereal species was employed to evaluate the competency of these explants under various conditions. Following this *Agrobacterium*-mediated transformation, protocols were developed for many important monocotyledonous crops. Transformation frequency was improved when the parameters were further optimized, by modification of medium components and optimization of coculture and resting timing periods and by the addition of *Agrobacterium* growth-inhibiting agent or bactericide such as silver nitrate. Inclusion of silver nitrate in coculture medium enhanced stable transformation in maize (Zhao et al. 2001). Silver nitrate significantly suppressed the *Agrobacterium* growth during coculture without compromising T-DNA delivery and subsequent T-DNA integration. The suppressed *Agrobacterium* growth on the target explants could facilitate plant cell recovery and resulted in increased efficiency of transformation (Cheng et al. 2003).

To date, the published reports of successful monocot transformation *via Agrobacterium* used only three different strains, i.e., LBA 4404, disarmed C58, and EHA 101 and its derivatives (EHA105, AGL0, and AGL1). Anti-necrotic compounds such as ascorbic acid, cysteine, and silver nitrate for preincubation of the explants were emphasized to be useful for efficient transformation. Super-virulent strains or superbinary vectors and acetosyringone in inoculation and coculture media were suggested to be important for efficient transformation in cereal species. Chemicals such as acetosyringone for *vir* induction are recommended in most of the cereal transformation protocols. Antibiotics such as cefotaxime, carbenicillin, and timentin have been used regularly in the *Agrobacterium*-mediated transformation of cereal crops following coculture

to suppress or eliminate *Agrobacterium*. Cefotaxime worked well in *Agrobacterium-mediated* transformation of rice and maize initially, and later it was found that cefotaxime at a concentration of 250 mg l^{-1} had a detrimental effect to maize callus (Ishida et al. 1996). Zhao et al. (2000, 2001) used carbenicillin (100 mg l^{-1}) in their experiments on sorghum and maize. Hill-Ambroz and Weeks (2001) studied the expression levels of various constitutive promoters in sorghum.

Removal of myoinositol from the callus culture media in combination with a cold-shock pretreatment and the addition of L-Gln prior to and during *Agrobacterium* infection resulted in about 84 % of the treated calli being stably transformed in *Lolium* (Zhang et al. 2013). Omission of myoinositol from the callus culture media was associated with the failure of certain pathogenesis-related genes to be induced after *Agrobacterium* infection. The addition of a cold-shock and supplemental Gln appeared to have synergistic effects on infection and transformation efficiencies. Nearly 60 % of the stably transformed calli regenerated into green plantlets. It is now possible to transform even difficult monocots using tailor-made gene constructs and promoters, suitable *A. tumefaciens* strains, and a proper understanding of the entire process of efficient regeneration (Sood et al. 2011). Still, there are many challenges to reach high-efficient transformation systems in sorghum that require genotype-independent regeneration and transformation methods.

9.7 Future Prospects

Despite the advances in technology, the transgenic sorghum is not released for cultivation. Innovative genetic modifications are developed to keep away from the classification of GMOs and biosafety concerns. These modifications such as zinc finger nuclease technology, cisgenesis, RdDM, and other technologies are dependent on transgenic technology for the introduction of DNA segments. Genotype-independent and high-throughput transformation systems are the key challenges to attain highly efficient genetic transformation of sorghum. Site-directed integration of transgenes in sorghum can be a reality with the complete sequence of sorghum genome available. Apart from this, selection of effective events plays an equally important role for transgenic sorghum to see the farmer fields. Science and the arts of tissue culture, genetic transformation, and, more importantly, plant breeding have to be integrated to reach effective transgenics of sorghum. There is growing interest in including sorghum as health food to alleviate the lifestyle disorders. With the research partnerships involving public and private organizations, we can anticipate improvement of sorghum nutritional quality with enhanced levels of vitamins, minerals, and protein and also tolerance to biotic and abiotic stresses leading to the genetic improvement of sorghum in the coming years.

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