REVIEW
of
LITERATURE

2. REVIEW OF LITERATURE

2.1. *AGROBACTERIUM*-MEDIATED TRANSFORMATION IN CEREALS

Genetic transformation of crop species using *Agrobacterium* is believed to be more practical, as the success rates of transformation are greater than with biolistics. *Agrobacterium tumefaciens* naturally infects only dicotyledonous plants. Monocotyledonous plants remained inaccessible to genetic manipulation for many years. 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, wheat, maize and barley have been successfully transformed using this method of transformation. However, in sorghum Zhao *et al*., (2000) regenerated more than 100 transgenic events from transformed explants. Thus, the feasibility of *Agrobacterium*-based method for sorghum transformation has been demonstrated.

A. tumefaciens is a Gram-negative, non-sporing, motile, rod-shaped bacterium, belongs to the genus *Agrobacterium* of the family *Rhizobiaceae*, which includes both saprophytic and pathogenic species. *Agrobacterium tumefaciens* is a pathogenic bacterium that has a naturally evolved mechanism to transfer genes into the chromosomes of host plant cells. Crown gall is a disease naturally occurring among perennial plants caused by *Agrobacterium* specific segment of the Ti-plasmid, T-DNA, might be engineered by initial disarming (removal of the bacterial tumorigenic genes contained in the T-DNA) and insertion of a selectable marker and genes of interest. Other important factors required for successful *Agrobacterium*-mediated transformation are the bacterial virulence (*vir*) genes, regulating mechanism of host inoculation, and phenolic inducers of virulence, such as

acetosyringone, that are released by wounded plant cells, triggering expression of the *vir* genes and initiating transformation process. *Agrobacterium*-mediated transformation is advantageous in terms of the relative simplicity of the protocol and minimal equipment cost. The yields of transgenics obtained are usually 10-30%, the transferred genes are often present in single or low copy, incorporated in a stable manner, and inherited in the plant's progeny (Gould, 1997). It was thought that *Agrobacterium* was incapable of transferring large (>10 kb) tracts of DNA, until it was demonstrated that the BAC fragments of 120-150 kb could be transferred (Hamilton *et al*., 1996).

Until recently it was believed that *Agrobacterium*-mediated plant transformation could only be used successfully with dicotyledonous plants, being natural hosts of *A. tumefaciens*; monocotyledonous plants including cereals were excluded from the range of *A. tumefaciens* hosts partly due to demonstrated inability to produce signaling compounds for activation of *vir* genes (Usami *et al*., 1987) and lack of a typical wound response. But the idea of principal possibility of DNA transfer to monocot plants via, *A.tumefaciens* has found its enthusiastic investigators.

Maize was the first of the cereal species shown to be susceptible to *Agrobacterium* infection (Graves and Goldman, 1986 and Grimsley *et al*., 1987). Ishida *et al*. (1996) took the initiative and reported stable transformation of maize cultivar A188 and its hybrids after co-cultivation of freshly isolated immature embryos with *Agrobacterium* harboring a similar super-binary vector to that developed by Hiei *et al.* (1994). Reproducible protocols for *Agrobacterium* mediated maize transformation have used super-binary vectors (Ishida *et al*., 1996; Negrotto *et al*., 2000 and Zhao *et al*., 2001). Frame *et al*. (2002) demonstrated that maize could also be transformed using *Agrobacterium tumefaciens* carrying a standard binary vector after producing fertile transgenic maize plants at a frequency of 5.5%. The developed system resulted in transgenic maize plants with

transformation frequencies ranging from 5 to 30%. Vega *et al*. (2008) reported the enhanced *Agrobacterium*-mediated transformation of immature zygotic embryos of maize Hi- II using standard binary vectors. This improved transformation process employs low-salt media in combined use with antioxidant L-cysteine alone or in combination with dithiothreitol (DTT) during the *Agrobacterium* infection stage. Three levels of N6 medium salts, 10, 50 and 100%, were tested. Average of 18% transformation frequencies were achieved from a large number of experiments using immature embryos grown in various seasons.

Chan *et al.* (1993) obtained a few transgenic rice plants by inoculating immature embryos with a strain of *Agrobacterium tumefaciens*. They demonstrated the inheritance of the transferred DNA to progeny plants by Southern hybridization, but analyzed the progeny of only one transformed plant. In a landmark report, Hiei *et al*. (1994) resolved the controversy and provided unequivocal evidence for the stable transformation of Japonica rice with *Agrobacterium* after molecular and genetic analysis of large numbers of R0, R1 and R2 progeny. It is now understood that a number of factors are of critical importance in the *Agrobacterium* mediated transformation of rice. These technical requirements explain why it was initially so difficult to apply this technique to rice. A super-binary vector containing an extra copy of each *vir* B, *vir* C and *vir* G from Ti plasmid pTiBo 542 in *Agrobacterium* strain LBA4404 was demonstrated to be most effective for the transformation of rice. The majority of these reports used immature embryos as an explant for *Agrobacterium* mediated transformation of rice. Other explants, such as inflorescences (Dong *et al*., 2001), have also been used for *Agrobacterium* mediated transformation of rice. Terada *et al*. (2004) established an efficient and large-scale *Agrobacterium* mediated rice transformation protocol that generated around 103 stable transformants routinely from 150 seeds. Their transformation procedure clearly demonstrates that it is possible to

perform efficient gene targeting in rice. In recent years, *Agrobacterium* mediated transformation of rice has also emerged as a reliable and highly reproducible method for transferring genes of interest into the rice genome (Giri and Laxmi, 2000; Ignacimuthu *et al*., 2000; Tyagi and Mohanty, 2000 and Bajaj and Mohanty, 2005). Rui-feng *et al* (2006) demonstrated that additional copies of *vir* G and *vir* E genes in *A. tumefaciens* obviously enhance the transformation efficiency of the large DNA fragment using the BIBAC vector in rice though different *A. tumefaciens* strains also affected the transformation.

Cheng *et al*. (1997) produced stable transgenic wheat plants within 3 months by co-cultivating freshly isolated immature embryos, pre-cultured immature embryos and embryogenic calli with *Agrobacterium* strain C58 harboring a binary vector containing the *nptII* gene for selection and *uidA* as a reporter gene, both under the control of an enhanced *cauliflower mosaic virus* (CaMV) 35S promoter. In addition to acetosyringone, the presence of a surfactant during inoculation of the tissue with *Agrobacterium* was found to be an important factor for the efficient delivery of T-DNA into wheat. Although the transformation efficiency of 1.12% was 10-fold lower than that reported by the same laboratory using particle bombardment, molecular analysis of transgenic plants revealed that approximately 35% of the transgenic plants received a single copy of the T-DNA without rearrangement. Later, the same method as developed by Cheng *et al*. (1997) was also employed in other laboratories for the production of transgenic wheat (Guang and Zhong 1999; Weir *et al*., 2001) (Table 2.1). Khanna and Daggard (2002) also demonstrated the superiority of a super-binary vector in *Agrobacterium* mediated wheat transformation. They produced transgenic wheat plants with transformation frequencies ranging from 0.3 to 3.3%. Cheng *et al*. (2003) further exploited the fact that an explant such as an immature embryo with active cell division can enhance T-DNA delivery (Cheng *et al*., 1997; Hu *et al*., 2003 and Wu *et al*., 2003) in order to increase the recovery of stable

transgenic plants in wheat. Following desiccation of plant tissues post-*Agrobacterium* infection and the use of paromomycin and glyphosate selection, they produced stable transgenic wheat plants with frequencies ranging from 4.8 to 19%.

Agrobacterium-mediated transformation of barely was reported by Tingay *et al*. (1997), who used a non-super-virulent strain of *Agrobacterium* carrying a binary vector and produced transgenic plants with 4.2% transformation frequency. Wu *et al*. (1998) demonstrated that cultured microspores of barley, or cell suspension cultures derived from immature embryos, could also be used as an alternative to immature embryos for genetic transformation. Following the method of Tingay *et al*. (1997), which is based on the infection of immature embryos with *Agrobacterium*, a number of laboratories reported the successful production of transgenic barley plants with transformation frequencies ranging from 0.5 to 6.3% (Patel *et al*., 2000; Trifonova *et al*., 2001; Wang *et al*., 2001 and Fang *et al*., 2002).

Table 2.1: Genetic transformation of major cereals using *Agrobacterium tumefaciens*

Species	Target tissue	Efficiency (%)	Promoter/ reporter gene	Promoter/ selectable marker gene	Reference
Maize	IE	5-30	*CaMV35S:gus*	*CaMV35S Ubi1:pmi:bar*	Ishida *et al*. (1996)
Maize	IE	30	-		Negrotto *et al*. (2000)
Maize	IE	0.8-7.1	*Ubi1:gus*	*CaMV35S:bar*	Zhao *et al*. (2001)
Maize	IE	7	*CaMV35S:gus*	*CaMV35S:bar*	Frame *et al*. (2002)
Maize	IE	-	*2xCaMV35S:gus/ Nos:gfp/Ubi1:moGFP*	*LIR:RepA/CaMV35S:bar/Ubi1:moGFP:pinII/CaMV35S:bar+Ubi1:FLP:PinII*	Gordon *et al*. (2002)
Maize	IE	-	*Ubi1:gus*	*CaMV35S:bar*	Miller *et al*. (2002)
Maize	IE	13.6	–	*Ubi1:PPO (Y426M + S305L) Ubi1:pmi//ubi:PPO*	Li *et al*. (2003)
Maize	IE	1-18.9	*Gfp*	*e35S/HSP70:nptII/Act1:cre//e35S:nptII/35S/HSP70:npt11*	Zhang *et al*. (2003)
Maize	IE	3.3	–	*CaMV35S:bar CaMV35S:NPK1*	Shou *et al*. (2004)
Maize	IE	3-13.4	–	*Act1:epsps-cp4*	Huang *et al*. (2004)
Maize	EC	12	-	*CaMV35S:als*	Yang *et al*. (2006)
Maize	IE	6.4 B104) 2 (B114) 8 (Ky21)	*Gus*	*CaMV35S:bar*	Frame *et al*. (2006)
Maize	IE	-	*Gus*	*CaMV35S:bar*	Vega *et al*. (2008)
Maize	IE	0.5-24	*Gus*	*CaMV35S ,Pat*	Hensel *et al*. (2009)
Maize	IE	6.45	CaMV35S ,P35S-*gus*	CaMV35S ,P35S-bar	Takavar *et al*. (2010)
Rice	IE,EC	12.8-28.6	*CaMV35S:gus*	*Nos-P:nptII CaMV35S:hpt*	Hiei *et al*. (1994)
Rice	IE	1-5	*CaMV35S:gus*	*CaMV35S:nptII CaMV35S:hpt*	Aldemita and Hodges (1996)
Rice	EC	-	*CaMV35S:gus*	*CaMV35S:hp*	Dong *et al*. (1996)
Rice	EC	-	*CaMV35S:gus*	*Nos-P:nptII/CaMV35S:hpt*	Komari *et al*. (1996)

Species	Target tissue	Efficiency (%)	Promoter/ reporter gene	Promoter/ selectable marker gene	Reference
Rice	ISM	2.8	-	*CaMV35S:bar/Act1:bar Nos-P:nptII*	Park *et al*. (1996)
Rice	SC	4.8-22	*CaMV35S:gus*	*CaMV35S:hpt*	Rashid *et al*. (1996)
Rice	SC	-	-	*Nos-P:hpt,Ubi1:bar*	Toki *et al*. (1997)
Rice	EC	-	*Osg6B:gus*	*Osg6B:hpt*	Yokoi *et al*. (1997)
Rice	SC	-	*CaMV35S:gus*	*CaMV35S:hpt*	Khanna and Raina (1999)
Rice	EC	-	*CaMV35S:gus*	*CaMV35S:hpt*	Mohanty *et al*. (1999)
Rice	EC	-	*Ubi1:gus*	*CaMV35S:hpt*	Upadhyaya *et al*. (2000)
Rice	SC	-	-	*CaMV35S:hpt*	Datta *et al*. (2000)
Rice	EC	-	-	*CaMV35S:hpt*	Jeon *et al*. (2000)
Rice	EC	7	*Ubi1:gus*	*CaMV35S:hpt/Nos-P:nptII*	Dai *et al*. (2001)
Rice	EC	41	-	*CaMV35S:pmi*	Lucca *et al*. (2001)
Rice	EC	9-19	*CaMV35S:gus*	*CaMV35S:hptNos-P:nptII*	Kumaria *et al*. (2001)
Rice	EC	16-19	*CaMV35S:gus*	*CaMV35S:hpt/Nos-P:nptII*	Kumaria and Rajam (2002)
Rice	EC	6	-	*CMPS:pmi*)	He *et al*. (2004)
Rice	EC	-	*GOS2:gfp*	*CaMV35S:hpt/Ubi1:bar*	Breitler *et al*. (2004b)
Rice	EC	-	*CaMV35S:gus*	*`Act1:hpt*)	Terada *et al*. (2004)
Rice	EC	-	-	*CaMV35S:nptII*	Kim *et al*. (2005)
Rice	EC	4-7.2	-	*hpt, nptII*	Rui-Feng *et al*. (2006)
Rice	EC	30-50	-	-	Nishimura *et al*. (2007)
Rice	EC	22.2	*CaMV35S:gus*	*Hpt*	Tyagi *et al*. (2007)
Rice	EC	4	*CaMV35S:gus*	*nptII*	Nazim-Ud-Dowla *et al*. (2008)
Rice	EC	5-10	glutenin HMW, gus	*hpt*	Wagiran *et al*. (2010)
Rice	IE	1.00-5.83	Ubi	*Mannose*	Thi *et al*. (2011)
Wheat	PCIE, EC	1.4-4.3	*CaMV35S:gus*	*CaMV35S:nptII*	Cheng *et al*. (1997)
Wheat	IE	3.7-5.9	–	*Ubi1:nptII*	Guang-Min and Zhong-Ti (1999)

Species	Target tissue	Efficiency (%)	Promoter/ reporter gene	Promoter/ selectable marker gene	Reference
Wheat	Sn-cell	1.8	*CaMV35S:gfp*	*CaMV35S:bar*	Weir *et al*. (2001)
Wheat	IE	1.2-3.9	*Ubi1:gus*	*Ubi1:bar*	Khanna and Daggard (2002)
Wheat	PCIE	4.4	–	*Act1:aroA:CP4/CaMV35S:aroA: CP4*	Hu *et al*. (2003)
Wheat	PCIE ,EC	4.8-19	*CaMV35S:gus*	*CaMV35S: nptII/CaMV35S:CP4*	Cheng *et al*. (2003)
Wheat	IE	0.3-3.3	*Ubi1:uidA*	*Ubi1:bar*	Wu *et al*. (2003)
Wheat	IE	0.4-0.13	*CaMV35S:gus/Act1:gus*	*CaMV35S:hpt/NOS:nptII Ubi1:bar*	Miti1 *et al*. (2004)
Wheat	IE	1-12.6	*CaMV35S:gus/ Act1:gus*	*NOS:nptII/CV35S:hpt/Ubi1:bar*	Przetakiewicz *et al*. (2004)
Wheat	IE	2-10	*sgfp* (S65T), *Ubi-1*	*CaMV35S,Hpt*	Hensel *et al*. (2009)
Wheat	IE	6.3-12.3	*Ubi-1,GusA*	*Ubi 1,bar*	He *et al*. (2010)
Barley	IE	4.2	*Act1:gus*	*Ubi1:bar*	Tingay *et al*. (1997)
Barley	Sn-cells	-	*CaMV35S:gus CaMV35S:C1/LC*	*CaMV35S:hph CaMV35S:bar*	Wu *et al*. (1998)
Barley	IE	6.0	*GluB1:gfp/Hor2-4:gfp/Act1:gfp*	*Ubi1:bar*	Patel *et al*. (2000)
Barley	IE	0.5-2.9	*CaMV35S-gfp CaMV35S:hpt*	*Ubi1:bar*	Wang *et al*. (2001)
Barley	IE	1.7-6.3	*CaMV35S:gus*	*Ubi1:bar*	Trifonova *et al*. (2001)
Barley	IE	3.4	*Ubi1:gfp*	*CaMV35S:hpt*	Fang *et al*. (2002)
Barley	IE	-	–	*Ubi1:bar*	Stahl *et al*. (2002)
Barley	IE	4.4-9.2	*Ubi1:gfp/Ubi1:gus*	*CaMV35S:hpt*	Murray *et al*. (2004)
Barley	IE	-	*Act1:gus/Ubi1:luc*	*Ubi1:bar*	Travella *et al*. (2005)
Barley	APC	-	*Ubi1:gus/Ubi1:gfp*	*CaMV35S:bar/CaMV35S:hpt*	Kumlehn *et al*. (2006)
Barley	IE	25	*Luc*	*hpt* gene	Joanne *et al*. (2008)
Barley	IE	20-86	*sgfp* (S65T), *Ubi-1*	*CaMV35S,Hpt*	Hensel *et al*. (2009)
Sugarcane	SpS (ms)	10-35	*CaMV35S:gus*	*Ubi1:bar*	Enríquez-Obregón *et al*. (1998)

Species	Target tissue	Efficiency (%)	Promoter/ reporter gene	Promoter/ selectable marker gene	Reference
Sugarcane	EC	-	*AMY3:gus*	*CaMV35S:hpt*	Arencibia *et al*. (1998)
Sugarcane	EC	-	*CaMV35S:gfp*	*CaMV35S:bar*	Elliott *et al*. (1998)
Sugarcane	EC	-	*CaMV35S:mgfp-ER*	*Nop:aphA*	Elliott *et al*. (1999)
Sugarcane	AB	50	*CaMV35S:gus CaMV35S:bar*	*CaMV35S:nptII*	Manickavasagam *et al*.(2004)
Sugarcane	MT, EC	-	*CaMV35S:gus/AMY3:gus*	*Ubi1: bar/CaMV35S:hpt*	Carmona *et al*. (2005)
Sugarcane	EC	-	*RSs-1-GNA*	*CaMV35S: hpt ,bar, nptII*	Dongting *et al*. (2007)
Sugarcane	Leaf disc	28	CaMV35S	*npt*II	Raviraj *et al*. (2009)
Sugarcane	EC	0.8-4.8	*Ubi1:gus*	*Ubi1:nptII*	Joyce *et al*. (2010)
Rye	IE	1.0-4.0	–	*Ubi1:nptII*	Popelka *et al*. 2003)
Rye	IE	2-4	*sgfp* (S65T), *Ubi-1*	*CaMV35S,Hpt*	Hensel *et al*. (2009)

Abbreviations:AB: axillary buds, ***Act*1:** rice actin promoter, ***Adh*1:** maize alcohol dehydrogenase gene promoter, ***als*:** chlorsulphuron resistance gene, ***AMY*3:** α-amylase Promoter, **APC:** androgenetic pollen cultures, ***aph*A:** geneticin resistance gene, ***CP*4:** 5-enolpyruvylshikimate-3-phosphate synthase gene from *Agrobacterium* strain CP4, ***bar* or *pat*:** phosphinothricin acetyl transferase, ***cre*:** cyclization recombination, which encodes a site-specific DNA recombinase, ***CaMV35S*:** cauliflower mosaic virus promoter, ***CMPS*:** pmi gene promoter, ***CP*4** and ***GOX*:** glyphosate oxidoreductase genes, ***C1/Lc*:** anthocyanin biosynthesis regulatory genes, ***e35S*** or **2x *CaMV35S*:** enhanced or double-enhanced *CaMV35S* promoter, **EC:** embryogenic calli; ***epsps-cp4*:** EPSPS synthase gene from *Agrobacterium* sp. strain CP4, ***gfp*:** green fluorescent protein, ***GluB*-1:** rice glutelin B-1 promoter, **GNA:** galanthus nivalis agglutinin *GOS2*, rice *GOS2* promoter; ***GST*-27:** maize glutathione *S*-transferase gene, ***gus*** or ***uidA*:** β-glucuronidase gene, ***H2B*:** maize histone gene promoter, ***Hor2-4*:** barley B1 hordein promoter, ***hpt*:** hygromycin phosphotransferase gene, ***HSP17.5E*:** promoter from soybean, **IE:** immature embryos, **ISM:** isolated shoot meristem, ***LIR*:** native promoter of *Rep*A, ***luc*:** luciferase gene, ***mgfp-ER*:** modified *gfp* gene version, ***mogfp*:** maize codon-optimized version of *gfp*, **MSC:** mature seeds-derived calli, **MT:** meristematic tissue, ***Nop*** or ***NospP*:** nopaline synthase promoter, ***NPK1***: *Nicotiana* protein kinase gene, ***nptII*:** neomycin phosphotransferase II, ***Osg6B*:** promoter of an anther tapetum-specific gene, **PCIE:** precultured immature embryos, ***pinII*:** proteinase inhibitor, ***pmi*** or ***man*A:** phosphomannose isomerase, ***PPO*:** *Arabidopsis* mutant proto porphyrinogen oxidase gene (Y426M + S305L), ***R*:** anthocyanin synthase gene, ***RepA*:** replication-associated protein, ***RSs-1*:** rice sucrose synthase-1, **SC:** scutellum-derived calli; **Sn-cells:** suspension cells, **SpS (ms):** spindle sections (meristematic sections), ***Ubi1*:** maize ubiquitin promoter.

2.2. TISSUE CULTURE AND *IN VITRO* REGENERATION STUDIES

The development of an efficient regeneration system is a pre-requisite for genetic transformation of plants. 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 and 1991). However, these are available only for a limited period of the year. Mature tissues such as seed embryo and hypocotyls are readily accessible year round sources for cereal explants (Cogner *et al*., 1982) and are the shoots tips and shoot apices isolated from germinating seedlings (Zhong *et al*., 1998).

2.2.1. Role of genotype on plant regeneration

The relationship between plant genotype and differential *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. Use of a variety of explants like immature embryos, immature inflorescences, and using shoot tips or apices have reported regeneration frequencies ranging from 0-100% across the genotypes tested.

2.2.2. Role of growth regulators in *in vitro* plant regeneration

Plant growth regulators (PGR) controlled the morphogenic competency, pathway and speed of regeneration from isolated shoot meristems (Harshavardhan *et al*., 2002 and Zhong *et al*., 1998). The ratio of auxin to cytokinin in the control of regeneration was well

documented by Skoog and Miller (1957). Polisetty *et al*., (1997) reported that eliminating the apical dominance of existing buds physically or by using high cytokinin levels in the culture medium, a large number of shoots could raise *in vitro*. Two type of cytokinin-like plant growth regulators are available; one is phenyl urea derivative including N- phenyl-N' (-1, 2, 3-thidiazol-5-yl) urea (Thidiazuron, TDZ) and the other is a naturally occurring purine-based derivative including N6-benzylaminopurine (BAP). TDZ efficiently stimulated cytokinin-dependent shoot regeneration. Two hypotheses regarding the mechanism of action of TDZ are that (i) TDZ could directly promote growth due to its own biological activity in a way similar to that of cytokinins, (ii) it might affect the accumulation of endogenous cytokinins (by reducing the rate of degradation) or increase the synthesis of endogenous cytokinins (Huetteman and Preece, 1993).

Shan *et al*., (2000) demonstrated that TDZ is capable of promoting callus regeneration and it has potential for enhancing the regeneration of cereal and grass species. Gupta and Conger (1998) observed *in vitro* differentiation of multiple shoot clumps from intact seedlings in switch grass when TDZ was used together with 2,4-D. The addition of TDZ in the induction medium was effective for multiple bud formation from bulged meristems and promoted the induction of direct somatic embryos on shoot apices of sorghum (Harshavardhan *et al*., 2002). It was reported that TDZ altered the shoot regeneration process from organogenesis to embryogenesis in leaf disc cultures of tobacco (Gill and Saxena, 1993). Role of TDZ in somatic embryogenesis induction was also reported in woody species (Fiola *et al*., 1990). Higher levels of 2,4-D (1-2.5 mg l^{-1}) with low levels of BAP (0.05-0.5 mg l^{-1}) or kinetin were required to promote the formation and proliferation of embryogenic callus from shoot apices of sorghum in the studies of Bhaskaran *et al*. (1988); Bhaskaran and Smith (1990); Lusardi and Lupotto (1990) and Nahdi and deWet (1995). Sudhakar *et al*. (2009) reported that the combination of 1.5 mg l^{-1} of BAP, TDZ with 1.0 mg l^{-1} IAA enhanced multiple shoot production in sorghum.

Zhong *et al*. (1998) reported that low levels of BAP induced the formation of axillary buds while high levels (2-4 mg l^{-1}) of BAP stimulated the differentiation of adventitious buds from shoot apices of sorghum. The addition of a low level (0.5 mg l^{-1}) of 2, 4-D in the BAP containing media, not only triggered the higher frequency of adventitious shoot formation, but also resulted in efficient embryogenesis directly from the shoot apical domes of cultured sorghum shoot apices.

The use of MS medium supplemented with (2-4 mg l^{-1}) BAP and (0.5 mg l^{-1}) 2,4-D was always accompanied with certain degree of callus formation (Harshavardhan *et al*. 2002). However, replacement of 2, 4-D with NAA resulted in the effective induction of somatic embryos without any callus formation. Induction of callus formation in rice and sorghum was reported earlier with higher concentrations of 2,4-D i.e., 3 mg l^{-1} (Abe and Futsuhara, 1985) and 2 mg l^{-1} (George and Eapen, 1988). Seetharama *et al*. (2000) reported the induction of friable embryogenic calli and somatic embryos from shoot tips cultures by culturing on Linsmaier and Skoog (LS) medium supplemented with 2,4-D (2 mg l^{-1}) and kinetin (0.1 mg l^{-1}) indicating the role of 2,4-D in induction of indirect somatic embryogenesis.

The embryogenic calli from scutella of immature embryos was induced in 10-15 days after the embryos were placed on M S medium with 0.25 mg l^{-1} zeatin and 0.5 mg l^{-1} 2,4-D and that the induction was poor in medium with 2,4-D alone (Sairam *et al*. (2000). It was also seen that age-dependent variation *in vitro* responses was linked to differences in endogenous auxin levels (Cassels *et al*., 1982) or endogenous cytokinin levels (Josphina *et al*., 1990). Harshavardhan *et al*. (2002) reported that isolated meristems from seven-day-old germinating seedlings were optimum for sorghum *in vitro* plant regeneration.

2.2.3. 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 Gamborg *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, M 35-1 and A1. 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 (Hashmi *et al*., 1997; Henry, 1998 and Jain, 2001).

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).

2.3. Successful transformation strategies

Besides an amenable tissue culture and regeneration systems, the following factors are also critical for development of transgenic plants:

- Suitable target gene in a convenient vector with reporter (to conform alien gene introduction into target cell or tissue) and selectable (to eliminate those cells into which foreign DNA has not been incorporated) marker genes;
- Method of DNA delivery into the cell

- Efficient testing method to confirm transformation events (stable integration)
- Adopting strategies to address transgene silencing.

It is equally important to ensure consistent inheritance and expression of the transgene in the progeny. Lack of pleotropic effects and consideration of Biosafety issues are important before useful transgenic can be commercialized.

2.3.1. Choice of the explants used for transformation and plant regeneration

Protoplast, suspension cell cultures, immature embryos, immature inflorescences and shoots tips from germinating seedlings are used as explant material to introduce various transgenes into sorghum genome. The first report of direct DNA uptake into protoplasts of sorghum by electroporation was carried out by Ou-Lee *et al.,* (1986) and latter by Battraw and Hall (1991). Hagio *et al.,* (1991) reported stable transformation from suspension cell cultures of *Sorghum vulgare* through micro projectile bombardment. Though these above investigators reported expression of the integrated foreign gene in detectable amounts, attempts to regenerate them in to whole plants were not attempted. Bombardment of cell suspension cultures directly has advantage as it eliminates the need for preparing protoplasts and reduces the formation of chimeras which are often seen when embryos are bombarded. The disadvantage of the protoplast or cell suspensions is that the method is laborious, needs special skills, tends to be cultivar specific and have very low regeneration frequency.

Casas *et al.* (1993) were first to report successful transformation of sorghum immature embryos via biolistic method of gene transfer. They reported that among the surviving calli, only a few exhibited shoot development indicating regeneration of plants at low frequency. Since then, immature embryos or immature embryo derived calli has been the more popular explants for sorghum transformation (Zhu *et al.,* 1998; Rathus and Godwin, 2000; Zhao *et al.,* 2000; HillAmbroz and Weeks, 2001 and Jeoung *et al.,* 2004). The frequency of plant regeneration reported so far, does not seem to be sufficient for genetic

transformation on a routine basis (Harshavardhan *et al.,* 2002). Shoot tips from germinating seedlings are also widely used explants in sorghum transformation (Tadesse and Jacobs, 2004; Tadesse *et al.,* 2003; Gray *et al.,* 2004 and Devi *et al.,* 2004). Explants derived from meristematic tissues at the early stages of development are most amenable to tissue culture conditions. Tissues such as seed embryos and shoot apices isolated from germinated seedlings are readily accessible and form year long source for cereal explants. Highly uniform meristematic tissues are desirable for genetic transformation to minimize chimeras and somaclonal variants. So, meristematic shoot tip along with a pair of primordial leaves are reprogrammed *in vitro* to produce multiple shoots or large number of somatic embryos.

Eventually, high regeneration frequency and efficient protocols for *in vitro* regeneration of sorghum have been developed which formed the basis for genetic transformation studies (Harshavardhan *et al.,* 2002 and Girijashankar *et al.,* 2005). Bombarding of immature inflorescence derived callus is followed but the regeneration frequency being low made it least preferred explant (Kononowicz *et al.,* 1995; Rathus *et al.,* 1996 and Casas *et al.,* 1997). Of late, the simple and easy way of generating transgenic sorghum with the use of transformed pollen is developed (Wang *et al.,* 2007). In this method, pollen from sorghum was transformed by a novel genetic transformation approach using mild ultra sonication. The treated pollen is then used to pollinate stigmas of desired genotypes. Taking into consideration the different explants used, cultured immature embryos and shoot tips of sorghum are the two explants of choice which have been predominantly used for sorghum genetic transformation.

2.3.2. Choice of promoters

Transgene expression efficiency is dependent on the promoter regulating it, which also depends on the plant species that is being examined (Able *et al.,* 2001). Promoter heterologous for sorghum such as *CaMV35S* promoter, rice *Actin* promoter and maize

Ubiquitin promoter have been used in cereals (Bajaj, 2000). *Actin1* gene promoter from rice and *ubiquitin1* gene promoter from maize are the two-monocot promoters showing naturally high constitutive activity (McElroy and Brettell, 1994). In general, the *CaMV35S* is a strong promoter in dicot species but its expression rate is very much less in monocots, in which several folds relative increase of gene expression could be achieved by the insertion of introns in the untranslated region behind the 35S promoter (Gallie and Young, 1994 and Vain *et al*., 1996).

The *ubiquitin1* and *actin1* promoter constructs have one native intron incorporated in the transcription unit, which has been implicated in elevating the mRNA abundance and enhancing the gene expression in transformed cereal cells (Callis *et al*., 1987 and Luehrsen and Walbot, 1991). The potato proteinase inhibitor II promoter region has been used with enhanced expression needed by the *Act1* intron- to drive its own coding and 3' terminator regions in transgenic rice plants, allowing damage reduction and increased protection against the pink stem borer (*Sesamia inferens*) (Duan *et al*., 1996).

Depending upon the plant species being examined, the promoter will also be an important factor in evaluating optimal transgene expression (Able *et al*., 2001). *CaMV35S* promoter was approximately seventy times less effective than the Emu promoter, which significantly increased transient *GUS* expression in intact sugarcane cells (Franks and Birch, 1991). Last *et al*., (1991) found that the recombinant Emu promoter (based on the truncated maize *Adh1* sequence) significantly increased transient transgene expression when compared to eleven other promoters in several types of cereal cells (including maize and wheat). Similar studies have been conducted evaluating the *Ubiquitin, Actin1, Adh1* and *CaMV35S* promoters in other monocotyledonous species, including rice (Cornejo *et al*., 1993). They found that the Ubiquitin promoter enhanced levels of transgene expression between 7.0 and 10.3 times more than *Adh1, Actin* and *CaMV35S* promoters. It was hypothesized that poor promoters may be one of the potential reasons for the lack of

effective sorghum transformation. Optimal promoter sequences should be identified to increase the transformation frequency in sorghum.

Transgenic rice plants with enhanced resistance to yellow stem borer were produced using synthetic *cry1Ab* gene under the control of *CaMV35S* promoter (Alam *et al*., 1998), synthetic *cry1Ac* gene under the control of *ubiquitin1* promoter (Nayak *et al*., 1997), synthetic *cry1Ab* (Datta *et al*., 1998) and fused *cry1Ac/Ab* genes under the control of rice *actin1* promoter (Tu *et al*., 1998).

The expression of a wild type truncated *cry1Ab* gene under the control of the mannopine synthetase *Mas* 2 promoter has been achieved in an early report where transgenic tobacco plants were obtained (Vaeck *et al*., 1987). Though significant tolerance against the lepidopteran pest, *Manduca sexta* was attained, expression in these plants reached very low levels (0.004%) presumably due to the use of a native *Bt* gene, and stimulation by wounding was not demonstrated. The isolation and characterization of cDNA and genomic clones encoding a serine proteinase inhibitor protein, the maize (*Zea mays* L.) proteinase inhibitor (*mpi*), has been reported. The *mpi* transcripts were undetectable in unwounded vegetative tissues (leaves, roots) or in flower tissue in maize plant (Cordero *et al*., 1994).

HillAmbroz and Weeks (2001) found that a strong promoter can increase the number of transformation events recoverable among sorghum cells. However, Hagio *et al*. (1991) have observed that neither CaMV35S, nor maize *Adh1* are able to direct expression of introduced genes with high efficiency. Similarly, the dual *35S cauliflower mosaic virus*, rice *Actin-1*, maize *A*lcohol dehydrogenase-1 (*Adh1*) and maize *Ubiquitin-1* promoters driving the GUS gene expression was compared by HillAmbroz and Weeks (2001). They reported that the levels of quantified GUS expression was much higher in wheat embryos than in sorghum embryos and concluded that none of the promoters could generate sufficient expression to allow sorghum transformation to occur at a practical frequency.

Ubiquitin1, *Actin1* and *CaMV35S* promoters regulate the transient GUS expression (Able et *al*., 2001). They reported that a significant high number of GUS foci were obtained with Ubiquitin construct, compared to Actin and CaMV35S constructs. Jeoung *et al*., (2002) in their attempt to optimize the parameters for use of *gfp* and *uidA* as visual markers in sorghum transformation evaluated different promoters controlling the expression of these two genes. The order of promoter strength as measured by green fluorescent protein (GFP) expression in calli was highest in *ubi1* followed by *CaMV35S* and HBT (HBT-a chimeric promoter with the 35S enhancer fragment fused to the basal promoter that includes the *TATA* box, transcription initiation site and 5' untranslated region) and the order of promoter strength for GUS expression was highest in *ubi1*followed by *CaMV35S, act1and adh1*.

Tadesse *et al*., (2003) examined the activity of four heterologous promoters was determined both by *GUS* histochemical staining and enzymatic activity assay in immature embryos and shoot tips. The strength of these promoters was highest in *ubi1* followed by *act1D, adh1* and *CaMV35S*.

The use of wound-inducible promoters to direct the expression of genes encoding insecticidal proteins in transgenic crops has been proposed in order to save energy of the plant and delay the occurrence of resistance among the target insect population (de Maagd *et al*., 1999). In maize, the *mpi* gene proved to be inducible by mechanical and fungal wounding as well as by methyl jasmonate or abcisic acid treatments. Induction occurs both locally and systemically, proving the first evidence of such a type of regulation for a monocot wound-inducible gene. Like other wound-inducible proteinase inhibitors, whose expression is also developmentally regulated in storage and/or reproductive organs, Mpi protein accumulates in maize embryos. Such features make it an attractive candidate to direct the expression of insecticidal genes in transgenic plants (Breitler *et al*., 2001). They illustrated for the first time that wound-inducible expression of a *cry1B* under the control

of maize proteinase inhibitor gene –689/+197 (C1) fragment afforded full protection to transgenic rice plants and showed 100 % mortality of stripped stem borer second instar larvae.

Girijashankar *et al.,* (2005) used wound induced promoter maize protease inhibitor gene (*mpiC1*) for genetically transformed sorghum (genotype Btx 623) using particle bombardment method. Insect bioassays (leaf disc assay) of T_1 generation plants carrying *mpi cry1Ac* showed reduction in leaf damage (60%), larvae weight (25%) and increased larval mortality (40%) when compared to the control plants. In response to mechanical wounding, the Cry1Ac toxin is expressed at low levels in leaves (1-8 ng/g of leaf tissue). On the other hand, *cry* genes under the control of the constitutive promoter from maize i.e., maize *polyubiquitin1* promoter could drive the expression at low levels compared to the wound-inducible promoter *mpiC1*. From the preliminary studies, the wound inducible maize protease inhibitor promoter (*mpiC1*) was found to be 14.55 fold stronger in expressing the transgene *cry1Ac* than the *ubiquitin1* promoter (Girijashankar *et al.,* 2005).

2.3.3. 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 analysis of gene expression. The use of reporter genes has a long history and dates back to the early days of prokaryotic molecular genetics (Herrera *et al*., 1983). So far, six reporter genes have been successfully employed in cereal transformation (Table 2.2). Reporter genes like β-glucuronidase (*uidA*) gene (Jefferson *et al*., 1987) that can be analyzed by histochemical methods or fluorimetric methods, chloromphenicol acetyl transferase (*cat*) gene (Fromm *et al*., 1990) that can be analyzed by radiochemical methods, luciferase (*lux*) gene and green fluorescent protein coding (*gfp*) gene using chemiluminescence methods(Ou-Lee *et al*., 1986).

β-*glucuronidase* (GUS) is the most widely used reporter system in sorghum transformation starting from initial transformation attempts (Hagio *etal*., 1991) till the latest reports of Wang *et al.,* 2007. The main disadvantage of using GUS as a reporter system is that it requires a destructive assay that precludes further proliferation and regeneration of identified transformed tissues.

The second most important reporter system is GFP which acquired importance because of its nondestructive visualization systems that can facilitate the recovery of identified transformed tissues (Bhat *et al.*,2010; Gurel *et al.*,2009; Gao *et al.*, 2005a, b; Jeoung *et al.,* 2004 and Able *et al.,* 2001). Green fluorescent protein, comprises of 238 amino acids (26.9 kDa), originally isolated from the jellyfish *Aequorea victoria*. The use of *gfp* as reporter system in transformation studies along with agronomically important genes proved to be a failure in sorghum as no somatic embryos were formed on the sectors selected on bialophos co-bombarded with *gfp* and *bar* genes. Further investigations are needed to ascertain the toxicity of GFP to sorghum cells (Able *et al.,* 2001 and Jeoung *et al.,* 2002). The other disadvantage in using GFP is the need for costly equipment like fluorescent stereomicroscope for detection of GFP.

However, both the reports suggested that GFP was superior over GUS and can be used for early and reliable detection of transgenic events in efficient transformation protocols. In contrast, the main advantage of using *uid*A gene as reporter system is the non-involvement of expensive equipment and ease of detection that involves visualization of the GUS expression (*uid*A) after treatment with the substrate, X-Gluc. However, use of R and C1 maize anthocyanin regulatory elements (Casas *et al.,* 1993) and *luc*, a fire fly luciferase (Kononowicz *et al.,* 1995) as reporter genes in sorghum transformation is also reported.

Table 2.2: Reporter genes used in genetic transformation system

Gene	Enzyme /protein encoded	Reference
Cat	Chloramphenicol acetyl transferase	Herrera *et al*., (1983)
LacZ	β-Galactosidase	Helmer *et al*., (1984)
*npt*II	Neomycin phosphotransferase	Reiss *et al*., (1984)
Lux	Luciferase	Ow *et al*., (1986)
*Uid*A	β-Glucuronidase	Jefferson *et al*., (1987b)
Gfp	Green florescent protein	Chalfie *et al*., (1994)

2.3.4 Choice of selectable marker for transformation.

The development of reliable transformation system for the production of transgenic sorghum plants depends on the efficient expression of the introduced selectable marker genes (Tadesse *et al.,* 2003). The key to establish a successful transformation strategy lies in the adoption of an effective and foolproof selection strategy. The usefulness of a selectable marker gene to optimize sorghum transformation system that will eventually allow the introduction of agronomically important traits to sorghum by genetic transformation was demonstrated (Casas *et al.,* 1993and Harshavardhan *et al.,* 2003).

Transgenic sorghum tissues growing in vitro are screened against three broad categories of selection markers such as antibiotics, herbicide and nutrient assimilation. Five different selection markers were utilized in sorghum transformation. They include *cat, npt II, hpt, bar* and *man*A (Table 2.3) Neomycin phosphotransferase II (*npt II*) gene isolated from *E.coli* conferring resistance to the antibiotic Kanamycin is one of the commonly used selection strategy for sorghum (Howe *et al.,* 2006, Tadesse and Jacobs, 2004, Battraw and Hall, 1991).

Hagio *et al.,* (1991) reported the use of Hygromycin B phosphotransferase (*hpt*) as well as Neomycin phosphotransferase II (*nptII*) genes to confer Hygromycin and

Kanamycin resistance, respectively. Till date, the most successful and popular selection marker is the *bar* gene, derived from *Streptomyces hygroscopicus* which encodes the enzyme phosphinothricin acetyltransferase (PAT) conferring resistance to the herbicide phosphinothricin (PPT) or its analogues *Basta* (with its active ingredient glufosinate ammonia) or bialophos (Harshavardhan *et al.,* 2003). The selectable marker *bar* gene is isolated from *Streptomyces hygroscopicus* codes for phosphinothricin acetyl transferase (PAT) proteins of 183 amino acids and shows 85% DNA sequence homology with another marker gene *pat* isolated from *S. viridochromogenes*.

Phosphinothricin inhibits *glutamine synthetase* (GS) irreversibly, resulting in inhibition of amino acid biosynthesis. Almost all the transformation studies conducted earlier in sorghum used *bar* gene as selectable marker gene except for Battraw and Hall (1991), who used *nptII* and Hagio *et al.* (1991), where *nptII* and *hph* genes were used. The herbicide phosphinothricin is used as selection agent at a concentration of 5.0 mg l^{-1} (Zhao *et al.,* 2000) while others used low concentrations of the same i.e. 2-3 mg l^{-1} (Rathus *et al.,* 1996 and Casas *et al.,* 1993). Bialophos (a tripeptide) and Basta (shows nonsystemic and localized effect) are the derivatives of PPT. Upto 3.0 mg l^{-1} of Bialophos (Casas *et al.,* 1993, Jeoung *et al.,* 2004 and Groot boom *et al.,* 2010) and 2.0 mg l^{-1} Basta (Zhu *et al.,* 1998 and Gray *et al.,* 2004) was used in the culture medium for selection of putative transformants. Cassas *et al.* (1997) reported that in the absence of bialaphos in the selection medium the morphogenesis of sorghum immature inflorescences was primarily *via*, embryogenesis, while on the other hand; organogenesis was more predominant in callus maintained on herbicide selection. So, the selection agent used can influence the transgenic plant regeneration pathway in sorghum.

Recently, *man*A gene from *Escherichia coli* coding for phosphomannose isomerase enzyme, is used as the selectable marker gene while the disaccharide mannose is the selection agent (Gurel *et al.,* 2009 and Gao *et al.,* 2005 b). The conversion of mannose to a

metabolizable fructose carbon source is beneficial to plants. This is an efficient and non-destructive method of screening the transformed sorghum plants under *in vitro* conditions and is gaining popularity.

Table 2.3: List of selectable markers used in genetic transformation systems

Gene	Enzyme encoded	Selective agent(s)	Reference
Hpt	Hygromycin phosphotransferase	Hygromycin B	Van den Elzen *et al*., (1985)
*npt*II	Neomycin phosphotransferase	Genticin (G418) Kanamycin	Bevan *et al*., (1983) Herrera *et al*., (1983)
PPT	Phosphinothricin acetyltransferase	Phosphinothricin (*Bialophos*)	De Block *et al.,* (1987)
Als	Acetolactate synthase	Chlorosulphuron Imidazolinones	Haughn *et al*., (1988)
Coda	coda-5-fluorocytosine	5-fluorocytosine	Stougaard *et al*., (1993)
Pmi	Phosphomannose isomerase	Mannose	Miles and Guest, (1984)
Cah	Cyanamide hydratase	Cyanamide	Weeks *et al*., (2000)

2.3.3. Selection of transgenics

The efficiency of the transformation is not even high in the most successful transfer systems where only a fraction of the cells exposed to *Agrobacterium* integrate the foreign DNA into their genomes. As known by the basic studies of *Agrobacterium* mediated transformation, the process is purely random and single cell process, a successful gene transfer does not guarantee stable integration and expression of the foreign gene, even by using signals for the regulation of the transgene expression. Therefore, systems to figure out the transformed cells, tissues or organisms from the untransformed ones are indispensable to regenerate the truly genetically transformed organisms.

The resistance genes for antibiotics allow the transformed cells expressing them to be selected from the populations of non-transformed cells. As a part of this system, a selective toxic agent that interferes with the cellular metabolism is applied to a population of putatively transformed cells (Angenon *et al*., 1994). The population of the cells that has been transformed and expresses a resistance gene is able to neutralize the toxic effect of the selective agent, either by detoxification of the antibiotic through enzymatic

modification (Joersbo *et al*., 1998, Wang *et al*., 2001 and Jaiwal *et al*., 2002) or by evasion of the antibiotic through alteration of the target (Jaiwal *et al*., 2002).

2.4. ANALYSIS OF TRANSGENIC PLANTS

2.4.1. Detection of transgene in the transformants

Molecular methods like polymerase chain reaction (PCR) and Southern hybridization help in detection of the introduced gene. Using the specific primers 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). The disadvantages of screening the transformants using PCR are its inability to detect individual transformation events, which are crucial for estimating the frequency of transformation (Casas *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 Southern hybridization, 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 cut the plasmid used for transformation once or twice (Zhao *et al*., 2000). The presence of the foreign gene in the target cell's genome can be identified 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 will 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 (Bhat and Srinivasan, 2002).

2.4.2. Transgene inheritance studies

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). 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 and 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 population.

2.4.3. Mechanisms of transgene integration into a host genome

The analysis of molecular biological and cell biological characteristics of transgenic loci will provide invaluable information not only on the mechanism of the integration of transgenes in to the host genome, but also for better understanding of the molecular evolution of the genome. This information is also indispensable for improving genetic transformation technology for recalcitrant plant species (Morikawa *et al*., 2002). Whole-genome duplication is an important evolutionary mechanism (Ohno, 1973) and recombination is a pervasive force at all levels of plant evolution (Clegg *et al*., 1997).

Recent comprehensive analyses by Blanc *et al*., (2000) and Vision *et al*., (2000) suggest that a large proportion (>60%) of the Arabidopsis genome results from duplication. These studies suggest endogenous recombination-enhancing DNA elements are important to shaping plant genomes. Morikawa *et al*., (2002) studied the mechanism of transgene integration into a host genome by particle bombardment and reported that it is conceivable that MARs (nuclear matrix attachment regions) such as TJ1, which is located in a

transgenic locus and increases transformation frequency, contribute to DNA rearrangement and the revolution of plant genomes. 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 and Kohli *et al*., 1999).

2.4.4. Genetic instability in transformants

Somaclonal variation resulting in widespread genetic and phenotypic alterations in many species is a common occurrence during *in vitro* tissue culture processes that involve a de-differentiated callus phase (Larkin and Scowcroft, 1981). 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). 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, may also be related to the genomic instability (genetic and or epigenetic) induced during the in vitro culturing and transforming processes (Matzke and Matzke, 1995).

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).

2.4.5. Transgene silencing

Genetic engineering relies on stable integration, desired level of expression and predictable inheritance of the introduced transgene while transgene silencing phenomenon appears to be a major obstacle in the path of transformation efforts in sorghum (Emani *et al.*, 2002). Stable integration and expression of introduced gene is essential to realize transgene advantage in the genetically modified crops. Variation in the candidate gene expression levels are commonly observed in transgenic plants. Once the transgene gets integrated into the genome of the host plant the expression is influenced by the structure, position, epigenetics, silencing, co-suppression and the presence of boundary elements or MARs (Matrix Attachment Regions).

Transgene silencing has been observed in dicotyledons and monocotyledons. Loss of expression is attributed to gene silencing, rather than loss of the transgene in sorghum (Krishnaveni *et al.,* 2004). This occurs through various means which parallels 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 methyl transferases that transfer a methyl group to 5' site of cytosine.

Another reason for gene inactivation and instability is the occurrence of multiple tandemly arrayed copies of the transgenes. This kind of gene inactivation occurs more frequently with direct DNA transformation methods (Flavell, 1994; Matzke and Matzke, 1995) than with *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 genes 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 by Iyer *et al*. (2000). To overcome this, a number of approaches have been described for generation of single-copy transgenic lines. This includes the *Agrolistic* method (bombardment of explants followed by *Agrobacterium* infection) that has been shown to generate reduced gene copy integration in tobacco and maize (Hansen and Chilton, 1996; Hansen *et al*., 1997 and Srivastava and Ow, 2001), and the use of niacinamide to reduce recombination of extra-chromosomal molecules (De Block *et al*., 1997).

A reporter gene silencing event in *Nicotiana tobacum* that has following distinctive combination of following features (a) silencing occurs by a post-transcriptional process, (b) silencing correlates with DNA methylation, and (c) the *de novo* methylation is not restricted to cytosines located in the symmetrical motifs CG and CXG (Ingelbrecht *et al*. (1994).

It was hypothesized that, where the loss of gene expression is post- transcriptional, antisense RNA could be formed on accumulated, inefficiently processed RNAs by an RNA-dependent RNA polymerase or from a chromosomal promoter. This could cause the observed loss of homologous mRNAs and possibly the modification of homologous genes. It has been suggested that mechanisms evolved to help silence the many copies of transposable elements in plants. In the same way, multiple copy genes that are part of the normal gene catalog of a plant species must have evolved to avoid these silencing mechanisms or their consequences (Flavell, 1994).

RNA-directed DNA methylation was presumed to be responsible for the methylation observed in protein coding regions of post-transcriptionally silenced genes (Aufsatz *et al*., 2002). It was also seen that high molecular weight RNAs and small interfering RNAs (siRNA) induce systemic post-transcriptional gene silencing (PTGS) in plants. The silent state in transgenic plants can spread systemically, implying that mobile silencing signals

exist. Neither the chemical nature of these signals nor their exact source in the PTGS pathway is known (Klahre *et al*., 2002).

It is generally recognized that a silencing-inducing locus can efficiently reduce the expression of genes that give rise to transcripts partially homologous to those produced by the silencing-inducing locus (primary targets). Interestingly, the expression of genes that produce transcripts without homology to the silencing-inducing locus (secondary targets) can also be decreased dramatically *via*, transitive RNA silencing (Rockville, 2003).

2.5. *BACILLUS THURINGIENESIS*-A GAIN TO GENETIC TRANSFORMATION

Bacillus thuringiensis (*Bt*) is a rod shaped, aerobic, motile, gram-positive, endospore-forming bacillus initially isolated in Japan by Shigetane Ishiwatari from diseased *Bombyx mori* larvae and named the bacterium *Bacillus sotto* in 1901. A decade later Ernst Berliner (1915) isolated a similar organism from diseased granary populations of *Ephestia kuehniella* Zell (Mediterranean flour moth) larvae obtained from Thueringen, Germany and named the bacterium *Bacillus thuringiensis* (*Bt*) (Lenin *et al*., 2001).

Application of *Bt* was started in 1928 when a *Bt* strain isolated from *Ephestia sp*. was tested on European corn borer, *Ostrinia nubilalis* (Kumar *et al*., 1996). Although *Bt* powder was quite affective at eliminating insect pests, the problems associated with topical applications of this powder include the risk of dilution by rain; degradation by the sun; and the fact that the crops have to be constantly monitored to determine when insecticides were needed (Gatehouse, 1999). The advancement in genetic engineering and molecular biology in the early eighties led to the cloning of *Bt* crystal protein (*cry*) gene from *Bt* subsp. *Kurstaki* in to *E. coli* by Schnepf and Whiteley (1981). Since then, more than 100 *cry* genes have been successfully cloned and added to the growing list (Wasano *et al*., 2001). Initially several *cry* genes were expressed in plant colonizing microorganisms to target the stem and root dwelling insect pests. Today, the most efficient way to deliver *cry* genes seems to be the development of transgenic plants expressing those (De Cosa *et al*., 2001).

2.5.1. Classification of *cry* genes

In general, the term *cry* was followed to designate the genes encoding crystal proteins and Cry for toxins (first given by Heid *et al*., 1982). The *cryI* genes code for lepidopteran specific bipyramidal crystal proteins having molecular range of 130-140 kDa. The *cryII* class of genes encodes about 65-71 kDa proteins, which form cuboidal inclusions and are toxic to lepidopteran and dipteran larvae. The *cryIII* class encodes 73 kDa coleopteran specific proteins. The *cryIV* class of genes is composed of a mixed group of dipteran-specific crystal protein genes encoding polypeptides with predicted molecular mass of 135, 128, 74 and 72 kDa respectively. The *cryV* class of genes encodes a protein having molecular weight of about 80 kDa. They showed toxicity towards coleopteran and lepidopteran larvae. The *cryVI* class on the other hand is reported to exhibit activity against nematodes (Feitelson *et al*., 1992) (Table 2.4).

(a) Some of the important features of revised nomenclature (Fig. 2.1) (Crickmore *et al*., 1998).

(b) The *cry* genes whose products share <45% amino acid sequence homology are characterized by different Arabic numbers, designated as primary ranks (eg. *cry1*, *cry2* etc.).

(c) The *cry* genes of same primary rank whose products show <78 % amino acid sequence homology are differentiated by secondary ranks by using upper case letters (eg. *cry1A, cry1B* etc.).

(d) The *cry* genes of same primary and secondary ranks whose products share <95% amino acid sequence homology receive separate tertiary rank, designated by lower case letters (eg. c*ry1Aa, cry1Ab* etc.)

(e) The *cry* genes whose products are different in amino acid sequence, but are more than 95% identical to each other are given separate quaternary ranks by another Arabic number (eg. *cry1Aa1, cry1Aa2* etc.).

Lepidopteran larvae are susceptible to various insecticidal crystal proteins (ICPs), or δ-endotoxins, produced by a number of strains of the spore–forming soil bacterium *Bacillus thuringienesis* (*Bt*) (Frutos *et al*., 1999). Insecticidal crystal proteins are formed during sporulation stage of the bacterium's life cycle. Crystal formation occurs during the last part of stage II. Electrons microscopic studies revealed the presence of nascent inclusions even at stage I (Abdel-Hameed *et al*., 1990). The sequences among a number of toxins were investigated (Hofte and Whiteley, 1989); and they found five well conserved regions designated as blocks from one to five.

The fact that the crystal toxin consists of three domains was confirmed by X-ray crystallographic studies by Grochulski *et al*. (1995). The structures of the three Domains are all significant in the bacterium's lethality to insects. Domain 1 is composed of 7 α-helices, which can bore holes in the insect's gut; Domain 2 has three anti-parallel beta-pleated sheets, which help the antigens bind to the gut; and Domain 3 is tightly packed moiety, which prevents binding of the gut protease (Sharma *et al*., 2004).

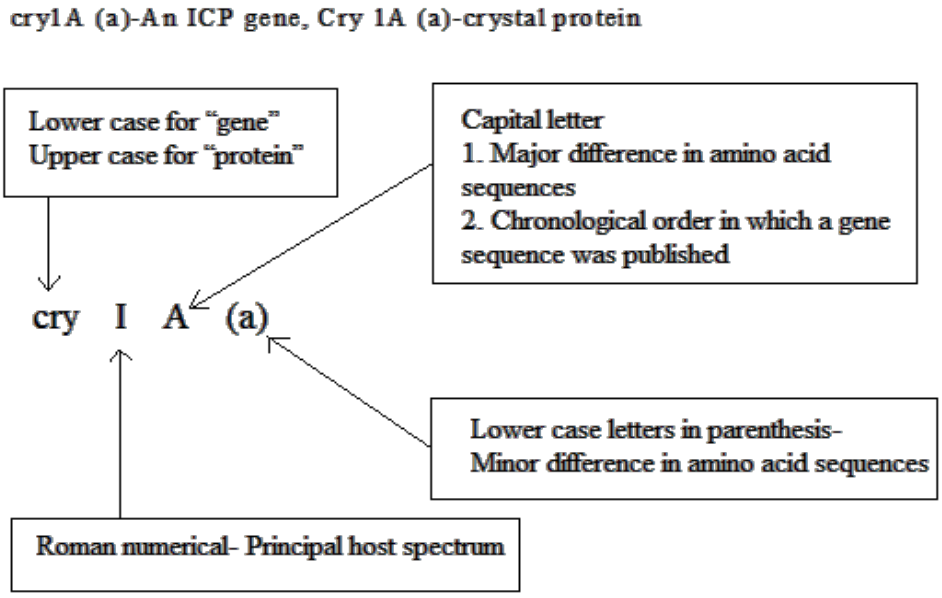

Figure 2.1: ICP gene and protein nomenclature

Table 2.4: Nomenclature for crystal protein gene of *Bacillus thuringiensis* and activity spectrum against insects

Gene	Protein	Subspecies (strain)	Activity Spectrum
cryI	CryI	CryI kurstaki (HD-1), aizawai, sotto	Lepidoptera
cryII	CryII	CryII kurstaki (HD-1), kurstaki (HD-263)	Lepidoptera and Diptera (mosquitoes)
cryIIIA	CryIIIA	Tenebrionis	Coleoptera (chrysomelids)
cryIIIB	CryIIIB	Japonensis	Coleoptera (scarabaeids)
cryIV	CryIV	Israelensis	Diptera (mosquito and black flies)
cryV	CryV	-	Lepidoptera and Coleoptera
cryVI	CryVI	-	Nematodes

The involvement of Domain II in receptor binding was supported by site directed mutagenesis and segment swapping experiments (Jurat-Fuentes and Adang, 2001). The β-sandwich structure of Domain III plays a number of roles; the main function consists of maintaining the structural integrity of the toxin molecule by shielding the molecule from proteases during proteolysis within the gut of the target pest (Li *et al*., 1991).

Gill *et al*. (1992) reported that the larger protoxin of about 130 to 140 kDa undergoes proteolysis yielding a toxic fragment of 60 to 70 kDa derived from N-terminal half of the protoxin. Structurally, the activated toxin can be divided into three structural regions: (1) N-terminal region, which is the toxic domain (amino acid sequence 1-279) consisting of several conserved hydrophobic regions; (2) a conserved C-terminal region (amino acid sequence 461-695) and (3) a variable region between these two regions that contains most of the amino acid differences. Because the bacterium is composed of several crystal proteins, the toxins produced have a unique “targeting affect”. Due to the fact that currently only one of the crystal protein toxins can be affectively isolated from the bacterium at once, the natural insecticide is lethal to only a few species, depending on which protein is isolated (Tabashnik, 1997).

2.5.2. Mode of action of *Bt* protein on ingested larva

Bioactivity of *Bt* is dominated by the ICPs and can be summarized in the following stages (Fig. 2.2).

- Ingestion of spores and ICP by a susceptible insect larva
- ICP dissolve in the alkaline midgut
- Activation of the ICP by proteases
- Irreversible binding of the C-terminal domain of activated ICP to specific receptors in the midgut cell membrane
- Insertion of the N-terminal domain of activated toxin in the cell membrane and formation of pores and channels in the gut membrane, followed by destruction of epithelial cells and paralysis of the digestive system
- Spore germination and septicemia; Larval death (within an hour to a few days) from starvation or septicemia

2.5.2.1. pH of the midgut

The midgut pH must be strongly alkaline (pH>9.5) for dissolution of the crystals, for most of the Cry toxins (Bradley *et al*., 1995), while some of the coleopteran specific toxins function at a much lower pH (Koller *et al*., 1992 and Bauer, 1995). Rate and extent of crystal solubilization influence the toxicity levels in different hosts, and pH may influence the effectiveness and specificity of some toxins (Bradley *et al*., 1995).

The lepidopteran and dipterans midgut are highly alkaline, whereas the coleopterans midgut are neutral to acidic. It has been postulated that expression of a truncated (pre-solubilized) form of *Bt* gene in transgenic plants removes the need for the initial gut barriers of solubilization and therefore may imply a higher risk of toxicity in both target and non-target organisms (Hilbeck, 2002 and Stotzky, 2002).

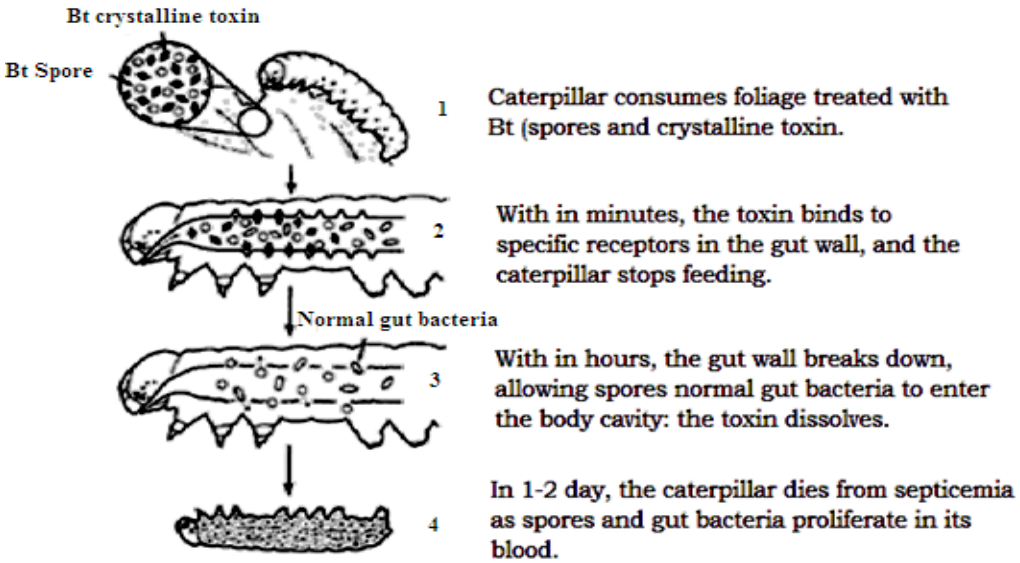

Figure 2.2: Mode of action of *Bt* crystalline protein on ingested larval intestine

2.5.2.2. Mid-gut enzymes

Mid gut enzymes are essential for processing of Cry protoxins to produce the active toxin core. In relation to ultimate toxicity, proteolysis is both enzyme and time critical. If the toxin processing is not completed optimally, which may take place if there is rapid movement of food through the insect gut, or if the toxin is exposed to enzymes for an extended period, ultimate activity may be composed and mid-gut lumen of lepidopteron insect larvae have been shown to contain a variety of alkaline proteases (Lightwood *et al*., 2000). Cry1 crystals are solubilized to release protoxins (130-140 kDa), which are subsequently processed to 55-66 kDa proteins by gut enzymes (Jurat-Fuentes and Adang, 2001). The main digestive proteases of lepidopteron and dipterans are serine proteases, and those of coleopteran are mainly cysteine and aspartic proteases (De Maagd *et al*., 2001).

2.5.2.3. Mid-gut structure

Cry toxins pass through the peritrophic membrane and bind reversibly to receptors on the brush border membrane of the midgut cells. Finally, irreversible binding is linked to insertion of part of the toxin (Domain I) into the midgut membrane (Jurat-Fuentes and Adang, 2001). There is a positive correlation between the toxin activity and ability to bind

brush border membrane vesicles (BBMV) (Gill *et al*., 1992) and the toxicity is correlated with the receptor number rather than receptor affinity (Van Rie *et al*., 1989) a large portion of the molecule (Domain I) inserts into the membrane, forming low selective ion channels (Knowles and Dow, 1993; Luo *et al*., 1999 and Miranda *et al*., 2001). The formation of toxin-induced pores in the columnar cells of the membrane allows rapid fluxes of ions. The pores are K+ selective (Hendrickx *et al*., 1991).

Carroll and Ellar, (1993) observed that midgut permeability in the presence of *cry1Ac* was altered for cations, anions and neutral solutes and water. Knowles and Dow, (1993) suggested that *Bt* toxins lead to cessation of K^+ pump that results in the swelling of columnar cells and osmotic lysis. The disruption of gut integrity leads to death of the insect through starvation or septicemia. These pores possess both selective (only K^+ passes through) and nonselective (Na^+ and anions pass through) properties depending on the pH (Schwartz *et al*., 1993). The lepidopteron insect midgut is alkaline and the pores probably permit K^+ leakage. Formation of this cation selective channel destroys the membrane potentials (English and Slatin, 1992), thus resulting in midgut necrosis, degeneration of peritrophic membrane and epithelium and ultimately bacterial septicemia, which occurs after larval death due to toxins (Sneh and Schuster, 1981 and Salama and Sharaby, 1985). Channels lead to osmatic swelling, cell lysis, damage to the mid-gut haemocoel barrier and leading ultimately to the death of the host (Federici and Bauer, 1998).

2.5.3. Genes from *Bacillus thuringiensis*

Bacillus thuringiensis (*Bt*) is a gram positive, aerobic and an endospore forming bacterium recognized by its parasporal body (known as crystal) that is proteinaceous in nature and possesses insecticidal properties. These insecticidal proteins, synthesized during sporulation are tightly packed by hydrophobic bonds and disulphide bridges. The most common shape is a bi-pyramidal structure. *B. thuringiensis* was discovered from diseased silkworm (*Bombyx mori*) larvae by Ishiwata, (1901). It was re-isolated, in a diseased

Mediterranean flour Moth population (Berliner, 1915) and designated as *BT*. Further research by Steinhaus, (1951) on *Bt* led to renewed interest in biopesticides, as a results of which, more potent products such as Sporeine, Thuricide and Dipel were introduce. The use of conventional *Bt* insecticides was found to have limitations like narrow specificity, short shelf- life, low potency, lack of systemic activity and the presence of viable spores (Lambert and Pferoen, 1992).

Bt strains can be characterized by a number of techniques including serotyping, crystal serology, crystal morphology, protein profiles, peptide mapping, DNA probes and insecticidal activity. The most useful scheme of classification of *Bt* toxins is based primarily on morphology of toxin gene sequence and the spectrum of insecticidal activity. Hofte and Whiteley (1989) has classified 42 *Bt* genes into 14 distinct types and grouped them into four major classes. The classes are *cryI* (lepidopteron specific) *cryII* (lepidopteron and Diptera specific), *cryIII* (Coleopteron specific) and *cryIV* (Dipteron specific). Feitelson, (1992) added two new major classes; *cryV* and *cryVI*. This nomenclature failed to accommodate genes that were highly homologous to known genes but with a different insecticidal spectrum, e.g., *cryIIA* and *cryIIB* were included in diptera-specific class though it is known that *cryIIB* is active against diptera. Crickmore *et al*., (1998) have introduced a systematic nomenclature for classifying the *cry* genes and their protein products.

Most *cry* genes retain the name as signed by Hofte and Whiteley, (1989) with a substitution of Arabic for Roman numerals (eg, *cry1Aa*) to accommodate the newly discovered genes. So far more than 150 cry toxins have been cloned and tested for their toxicity on various insect species. *Bt* based genes are usually plasmid borne (Gonzalez *et al*., 1981 and Gonzalez and Carlton, 1984), but also chromosomally located (Carlson and Kolsto, 1993). The genes encoding the δ-endotoxins of *Bacillus thuringiensis* are located on plasmids ranging in size from 11.3 kb to 19.44 kb (Ward and Ellar, 1983; Gonzalez and

Carlton, 1984; Hoflack *et al*., 1997 and Rolle *et al*., 2005). *Bt* toxin protein has three domains (Chona and Kalpan, 1990 and Convents *et al*., 1990), the domain I is required for toxicity (Chen *et al*., 1995) and domain II is important for specificity (Dean *et al*., 1996 and Smedley and Ellar, 1996) and domain III near the carboxyl end was not defined, is speculated that it may have a role in the processing of protoxin and channel forming function (Wolfersberger *et al*., 1996 and Schwartz *et al*., 1997). However, experiments involving reciprocal exchange of domain segments between toxins have produced evidence suggesting that in a number of cases, Domain III may also be a determinant of insect specificity/receptor binding (De Maagd *et al*., 1996).

In addition to Cry proteins, several strains of *Bt* are known to produce insecticidal proteins during vegetative phase of growth. These proteins are called vegetative insecticidal proteins (Vip). The first *vip* genes described were *vip3Aa1* and *vip3Aa2* isolated from *Bt* strain AB88 and AB424 (Koziel *et al*., 1996). Vips possess toxicity of the same magnitude as that of δ-endotoxins against the susceptible insects. They induce gut paralysis, followed by complete lysis of the gut epithelium cells, resulting in larval mortality (Yu *et al*., 1997). They represent second generation of insecticidal to *Bt* δ-endotoxins (Estruch *et al*., 1997). The Vips have shown a broad insecticidal spectrum, including activity towards a wide variety of lepidopteran and also coleopteran pests (Koziel *et al*., 1996; Estruch *et al*., 1996; Warren, 1997; Selvapandiyan *et al*., 2001 and Bhalla *et al*., 2005).

2.5.4. *Bt* Genes for resistance against stem borers

Over 150 insect species are known to attack sorghum and reduce the yield (Jotwani *et al*., 1980). Insects attacking sorghum can be grouped into soil pests, foliage feeders, stem feeders and storage pests (Leuschner, 1985). Among the various species of insects known to cause damages to sorghum crop, the shoot fly (*Atherigona soccata* Rondani); stem borers (*Chilo partellus* Swinhoe and *Chilo zonellus*); and the sorghum midge (*Contarinia*

sorghicola Coquillett) cause extensive damage to the seedlings, fully grown plants and earheads respectively (Raina, 1985). Grain yields, particularly of the semi-arid tropics, generally quoted low (500-700 kg/ha) due to pest attack (Davies, 1982).

Grover and Pental, (2003) in their survey in India, identified that out of the major important problems in cultivation of sorghum, shoot fly accounts to 24%, stem borers 17% and sorghum midge 7% of sorghum crop damage. Several species of stem borer attack sorghum in different sorghum growing regions (Nwanze, 1997), of which the spotted stem borer, *Chilo partellus* (Lepidoptera: Pyralidae) is the most predominant in Indian sub-continent (Ingram, 1958; Jotwani and Young, 1972 and Singh and Rana, 1989). The spotted stem borer causes US $334 million annual loss to sorghum alone in the semi-arid tropics (ICRISAT 1992).

2.5.5. Transgenic plants with synthetic *Bt* genes

Genes from *Bacillus thuringiensis* are best known for their low expression in plants. Initial efforts to express *Bt* genes in plants using standard approaches yielded transgenic plants that produced little or no *Bt*- toxin mRNA or protein. The problem is that the wild-type *Bt*-toxin genes have an A/T content of approximately 65% similar to that of many A/T rich plant RNA-processing signals, in particular those for polyadenylation, mRNA decay and splicing.

The mRNA and protein levels were eventually increased by resynthesizing the genes to be more ‘plant like”. In most cases, this included changing the codon usage to a plant-preferred codon bias, which also has the effect of raising the G/C content of the wild-type gene (Diehn *et al*., 1998). To achieve high-level expression of the *cry* genes in crop plants, it is necessary to modify the coding sequence, such as the removal of sequences responsible for gene silencing and polyadenylation signals followed by optimization of codon usage. The truncated version of such synthetic *Bt* genes further increase the expression levels, which has been reported in maize (Koziel *et al*., 1993; Armstrong *et al*.,

1995; Williams *et al*., 1997 and Bohorova *et al*., 1999) and rice (Fujimoto *et al*., 1993; Cheng *et al*., 1995; Wunn *et al*., 1996; Nayak *et al*., 1997 and Datta *et al*., 1998) cotton (Perlak *et al*., 1991).

Since the first report on the introduction of *Bt*-derived *cry* genes into tobacco (Barton *et al*., 1987) and tomato (Fischhoff *et al*., 1987 and Vaeck *et al*., 1987), there has been a rapid increases in the transformation of other crop plants to achieve resistance against insect pests, at least ten different *Bt* genes, viz., *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1Ba*, *cry1Ca*, *cry1H*, *cry2Aa*, *cry3A*, *cry6A* and *cry9C* have been engineered into different crop plants (Schuler *et al*., 1998). Cotton plants with *cry1Aa* (Benedict *et al*., 1996), *cry1Ac* (Adamczyk *et al*., 2001b) against *H.armigera* and *H. viriscens*, corn transgenics with *cry1Aa* against *H.zea* (Lynch *et al*., 1999) and *O.nubilalis* (Burkness *et al*., 2001).

Rice plants with *cry1Ab* and *cry1Ac* for resistance to yellow stem borer and stripped stem borer (Cheng *et al*., 1995), hybrid rice plants with *cry1Ac* and *cry1Ab* together for leaf folder and yellow stem borer (Tu *et al*., 2000), rice plants with *cry1Ac* for stem borer (Nayak *et al*., 1997). Tobacco plants with *cry1Ab* and *cry1Ac* against *S.exgua*, *M.sexata* and *H.viriscens* (Van der salm *et al*., 1994 and Stewart *et al*., 1996) and chickpea with *cry1Ac* (Kar *et al*., 1997 and Sanyal *et al*., 2005) for resistance to *H.armigera* were produced. In sorghum, Girijashankar *et al*., (2005) with *cry1Ac* for resistance to sorghum spotted stem borer (*Chilo partellus*).

2.6. GENETIC TRANSFORMATION OF SORGHUM

Research on sorghum transformation began with the first reports of DNA introduction into protoplasts (Battraw and Hall, 1991) and particle bombardment of a non-regenerable cell suspension (Hagio *et al.*, 1991). 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. Parameters influencing the stable transformation of sorghum protoplasts with a chimeric *neomycin phosphotransferase* II

(*nptII*) and β-*glucuronidase* genes by electroporation were investigated (Battraw and Hall, 1991).

Sorghum vulgare represents the first stable transformation using biolistic method as route for delivery of DNA Hagio *et al.*, (1991). They have demonstrated that expression of the *hph* and *nptII* genes that conferred resistance to selectable levels of hygromycin and kanamycin, respectively, for the isolation of transformed sorghum cells. Their results extend the utility of the biolistic method as a useful DNA delivery system for the transformation of monocots. The first transgenic sorghum plants were obtained by microprojectile bombardment of immature embryos (Casas *et al.*, 1993) and immature inflorescence derived calli (Casas *et al.*, 1997) with a relatively low transformation frequency of 0.2%, a protracted time in culture of 7 months, and used a sorghum genotype, PI898012, which has poor agronomic traits.

Casas *et al.*, (1993) used the R and C1 maize genes encoding anthocyanin transcription factors to optimize DNA delivery parameters in sorghum immature embryos. Frequency of transient *gus*A expression was less than 20 blue foci per embryo. Their findings indicated that transient gene expression level in sorghum was lower than in maize due to genotype effect or interactions between genotype and acc eleration pressure, or other inherent characteristics of sorghum scutellar tissue. Kononowicz *et al.*, (1995) utilized immature embryos and immature inflorescences were utilized as explants for particle bombardment and plant regeneration. Genotype specific differences in the response of primary explants to regeneration protocols have been found among sorghum genotypes utilized in their studies. Selected genotypes are PS98012 (Immature embryos) SRN 39 (Immature inflorescence).

The first successful report in sorghum transformation using *Agrobacterium*- mediated was reported by Zhao *et al.*, (2000). They have tested a number of parameters involved in *Agrobacterium*-mediated transformation of sorghum using the public line P898012.

Sorghum line P898012 is reported by many because is capable of producing good/better embryogenic, quality callus from immature embryos and was very responsive to coconut water (Casas *et al.,* 1993, Kaeppler and Pedersen, 1997 and Carvalho *et al.,* 2004).

Zhao *et al.,* (2000) studied several parameters those are effect of media on callus formation and maintenance, effect of antibiotics on sorghum cultures, effect of *Agrobacterium* concentration and co-cultivation time on delivery of T-DNA into embryos and effect of agro infection on callus response. Their results enabled them to determine the optimal conditions and provided them a base line conditions in subsequent experiments to achieve stable transformation of sorghum using *Agrobacterium*. They observed N6 medium not only decreased callus response from embryos (76% with MS and 20% with N6) and also increased the production of phenolic pigments. Overall transformation frequency was 2.1%. They reported optimization of media and other conditions for transformation of sorghum using *Agrobacterium*. However, they didn't optimize with respect to the Acetosyringone concentration and used a single concentration of 100µM of the phenolic compound to induce *vir* operon.

The results of Jeoung *et al*. (2002) while comparing the transformation of reporter genes, *gfp* and *gus,* for *Agrobacterium* and biolistic transformation, recorded that *gfp* gene was superior to *gus* gene for transgene expression in transiently transformed materials in both methods of transformation. Using GFP as the screenable marker, they optimized the sorghum transformation with respect to the conditions for transformation, type of explants, promoters, and inbreds. Similarly, Able *et al*., (2001) utilized the *gfp* gene to optimize sorghum transformation and regeneration *via* particle bombardment. They also optimized the conditions for biolistic transformation, such as the distance between the rupture disk and the target tissue, helium inlet aperture and pressure of helium gas used for accelerating the micro-projectiles, and the age of tungsten and spermidine solution used in these experiments.

Emani *et al.,* (2002) have made use of the cytidine analog, 5-azacytidine (azaC), in reversing the methylation-mediated transgene silencing in sorghum. Their results represented the existing evidence of methylation-based transgene silencing in sorghum. Cases of methylation-mediated transgene silencing have been reported in dicots (Matzke *et al.,* 1995) and in cereal crops like rice (Kumpatla *et al.,* 1997, Kohli *et al.,* 1999 and Fu *et al.,* 2000) and wheat (Demeke *et al.,* 1999). It was possible to activate *gus*A 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). Emani *et al.* (2002) suggested that methylation-based silencing is frequent in sorghum and probably responsible for several cases of transgene inactivation reported earlier in sorghum.

Tadesse *et al*. (2003) optimized transformation parameters in sorghum *via* biolistics. For optimization, they used four types of explants of sorghum based on transient GUS expression. They tested physical parameters including acceleration pressure, target distance, gap width and microprojectile travel distance. The sorghum explants studied were immature and mature embryos, shoot tips and embryogenic calli. In addition, the activity of four heterologous promoters was determined both by histochemical staining and determining enzymatic activity to assay GUS expression in immature embryos and shoot tips. The highest GUS expression was attributed to the promoter *Ubi1*, followed by *Act1D, Adh1* and *CaMV35S* promoters, in the decreasing order. The optimized bombardment conditions were applied for selecting phosphinothricin or genticin resistant *in vitro* cultures in order to generate transgenic plants.

Visarada *et al*. (2003) reported that with immature embryos, calli- derived from immature inflorescence, and shoot apices were ideal target tissues for obtaining high *gus* expression with *Agrobacterium*-mediated transformation in sorghum. Highest transient *gus* expression was reported in immature embryos (100%) followed by freshly isolated shoot apices (93.3%) and immature inflorescence calli (60%).

Carvalho *et al.* (2004) identified four factors that mostly influence sorghum transformation: (i) the sensitivity of immature sorghum embryos to *Agrobacterium* infection (ii) the growth conditions of donor plant (iii) type of explant and (iv) co-cultivation medium. The authors reported the necrotic response which developed in explants after co-cultivation. Immature embryos of sorghum proved to be very sensitive to *Agrobacterium* infection and they found that the level of embryo death after co cultivation was the limiting step in improving transformation efficiency. They suggested particular attention should be given to the number of *Agrobacterium* in the inoculum, the selection of sorghum genotypes and explants less sensitive to *Agrobacterium* infection. Increased percentages 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 coconut water is a very responsive to P898012, its use in other genotypes may require different composition, because many genotypes were reported without response to coconut water (Kaeppler and Pederson, 1996).

Gao *et al.* (2005a) transformed grain sorghum with a visual reporter gene (*gfp*) a green fluorescent protein and a target gene *tlp*, encoding thaumatin like protein. They described 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 earlier report 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.,* 2005 b). Both the genes were under the control of maize *ubi*1 promoter. The *E.coli* phosphomannose isomerase gene (*pmi*) was used as the selectable marker gene and mannose was used as the selection agent. In their study, using mannose selection necrotic calli were rarely observed. Mannose

selection had less negative effects on plant regeneration. Gao *et al.,* (2005 a and b) did not observe gene silencing for either the *gfp* gene or the *tlp* gene in T_0 and T_1 generations.

Girijashankar *et al.,* (2005) report the successful production of transgenic sorghum through microprojectile bombardment method and expression of the *cry1Ac* gene from *Bacillus thuringiensis* (*Bt*) under the control a wound-inducible promoter from the maize protease inhibitor gene (*mpiC1*). This promoter is functional in sorghum and drives expression of the *cry1Ac* gene at low levels which in turn can confer partial protection against the neonate larvae of the spotted stem borer (*Chilo partellus*).

Howe *et al.* (2006) carried out stable transformation experiments in sorghum immature embryos of Tx430 and C2-97 genotypes using a novel C58 strain of *A.tumefaciens* implementing *npt*II as a selectable marker. They reported the transformation frequencies of 0.3 to 4.5% for the successful experiments, with an average transformation frequency of approximately 1% for both genotypes.

Nguyen *et al.* (2007) developed an improved regeneration protocol suitable for sorghum transformation. The improvements focused on limiting the production of phenolic compounds and the use of suitable culture vessels for each developmental stage in plant regeneration from immature embryo derived calli. The addition of activated charcoal in the callus induction medium reduced the production of black pigment; however it also inhibited the callus formation from immature embryo explants. A one-day 4°C treatment of immature seeds significantly improved the callus formation from immature embryos and reduced the need for frequent subculture. *Agrobacterium*-mediated transformation using the improved regeneration protocol and the hygromycin phosphotransferase gene as selectable marker resulted in the recovery of 15 transgenic plants from 300 initial immature embryos with transformation efficiency of 5%.

Zhang *et al*. (2009) carried out stable transformation experiments in sorghum using immature inflorescences of 115, ICS21B and 5-27 with *cry1Ab* gene, *hygromycin*

resistance gene and *gus* gene *via Agrobacterium* mediated approach. After selecting the plants with increasing concentration of hygromycin, a total of 21 independent transgenic plant lines, 52 transgenic plants were regenerated. The average stable transformation efficiency was 1.9%. The integration and transcription of *cry1Ab* gene in transgenic sorghum was confirmed by PCR analysis, Southern blotting and RT-PCR analysis.

Gurel *et al* (2009) reported that a number of parameters related to *Agrobacterium*-mediated infection were tested to optimize transformation frequencies of sorghum. They tried the following factor, to improve *Agrobacterium* transformation efficiency.

(a) Using different temperatures and centrifugation conditions to pre-treat immature embryos prior to *Agrobacterium* infection,

(b) Altering cooling temperatures following heat treatment of immature embryos

(c) Varying temperatures during and after centrifugation

(d) Pre treating spikes in the cold prior to immature embryo isolation.

The effects of the different treatments on frequencies of transient and stable transformation were determined by monitoring GFP expression during callus formation and mannose selection and by conducting PCR, DNA hybridization and western analyses of regenerated shoots.

According to Nguyen *et al*. (2007) a 1-day, 4°C pre-treatment of immature seeds significantly improved callus formation from immature embryo of an African red sorghum cultivar and reduced the need for frequent sub culturing due to reduction of phenolics. But according to Gurel *et al*. (2009), 1-day pre-treatments at 4°C of two US sorghum lines, P898012 (Type II) and Tx430 (Type I), did not significantly increase frequency of immature embryo survival or callus induction; in fact with Tx430 the frequency decreased significantly after 1-day of pre-treatment. Cold pre treatment, however, did reduce phenolic production, most likely due to effects of low temperature on reducing key enzyme activities (polyphenol oxidases and peroxidases) that are involved in phenolic compound

synthesis (Dicko *et al*., 2006). Using different heating times at 43°C prior to infection showed 3 min was optimal. Centrifuging immature embryos with no heat or heating at various temperatures decreased frequencies of all tissue responses; however, both heat and centrifugation increased de-differentiation of tissue. The most optimal treatment, 43°C for 3 min, cooling at 25°C and no centrifugation, yielded 49.1% GFP-expressing calli and 8.3% stable transformation frequency. Transformation frequencies greater than 7% were routinely observed using similar treatments over 5 months of testing.

Indra *et al*. (2010) carried out stable *Agrobacterium*-mediated transformation experiments in sorghum immature embryos of CO25, TNS586. *Agrobacterium* strains used were LBA4404 harboring pCAMBIA-*ubi-chi11* (chi gene from genomic clone), EHA105 harboring pCAMBIA-ubi RC7 (chi gene from cDNA clone) with *bar* gene and *EHA105* harboring pMKU-RF2 (*chitinase* and gus reporter gene) for producing fungal resistance in sorghum plants. They tested two selection agents (hygromycin and bialaphos), among these two bialaphos was found as a suitable selection agent. By comparing the two genotypes, CO25 shows better transformation frequency (1.0-2.0%)

Shridhar *et al*. (2010) carried out *Agrobacterium*-mediated transformation with pKU352NA vector containing *SgfpS65T*, an improved *gfp* reporter gene and i*Ac* encoding transposase and the transformation efficiency was 4.28%.

Kumar *et al*. (2011) investigated and describe the parameters related to co-cultivation, culture and regeneration that have allowed them to obtain transgenic sorghum plants in as little as 2.5 months.

(a) The transformation efficiency was increased 2.9-fold when L-cysteine was included in the medium during the co-cultivation step.

(b) The transformation efficiency was 2.8-fold higher when they used the modified *Agrobacterium*-induction medium, including lower phosphate and acidic pH of the AB minimal medium.

(c) Incorporation of an additional binary vector, harboring extra copies of *vir* G and *vir* C genes, in the *Agrobacterium* did not confer any improvements in the transformation of sorghum.

(d) Characterization of transgene activity provided some interesting results suggesting that *CaMV35S* promoter activity in T_0 generation is very low during the early stages of development of a transgenic sorghum plant, and is not indicative of the expression level during the later stages of development or in the next generation.

Guoquan liu *et al*. (2012) developed a highly efficient microprojectile transformation system for sorghum by using immature embryos (IEs) of inbred line Tx430. Co-bombardment was performed with the neomycin phosphotransferase II (*npt*II) gene and the green fluorescent protein (*gfp*) gene, both under the control of the maize Ubiquitin1 (*ubi*1) promoter. They report the highest recorded sorghum transformation efficiency to date of 20.7%. This result is more than 70 times higher than the first successful transformation rate of 0.286% reported via microprojectile transformation, and more than 25 times higher than the biolistic sorghum transformation efficiencies cited by Grootboom *et al*. (2010) (0.11–0.77%) and Raghuwanshi and Birch, (2010) (0.09%).

Both the systems of transformation, *i.e.*, *Agrobacterium* and particle bombardment though successful in sorghum, both systems have their own merits and limitations. Effective method till date remains to be the *Agrobacterium* based transformation, with higher transformation efficiency which ranged from 2.1-7.0% from reports of Zhao *et al.* (2000), Gao *et al.* (2005), Howe *et al.* (2006), Nguyen *et al.* (2007), Gurel *et al.* (2009), Shridhar *et al*. (2010) and Kumar *et al*. (2011). The summarized information on genetic transformation of sorghum is presented in Table 2.5.

Table 2.5: Summary of attempts on genetic transformation of sorghum

Method	Explant/ culture system	Gene of interest	Promoter	Selection agent/ conc. Used	Efficiency %	Reference
Ep	Protoplasts	*Cat*	CaMV 35S/Copia	Chloramphenicol		Ou-Lee *et al.* (1986)
Ep	CS/ protoplasts	*nptII*	CaMV35S	Kanamycin 100 mg L^{-1}		Battraw and Hall (1991)
PB	CS	*nptII/hpt/uidA*	Adh1/ CaMV35S	Kanamycin/ hygromycin		Hagio *et al*. (1991)
PB	IE	*bar/ uidA*	CaMV 35S	Bialophos 3mg L^{-1}	0.08	Casas *et al*. (1993)
PB	IE /IC	*bar/uidA/iuc*		Bialophos	-	Kononowicz *et al*. (1995)
PB	IE /IC	*bar*	CaMV35S/Act1	Bialophos 2 mg L^{-1}	-	Rathus *et al*. (1996)
PB	IC	*bar/uidA*	CaMV 35S	Bialophos	0.33	Casas *et al*.(1997)
PB	IE	*bar/chitinase1*	CaMV35 S	Basta 1-2 mg L^{-1}	0.09	Zhu *et al*.(1998)
PIG	IE	*bar*	CaMV 35S/Act 1	Basta 1-2 mg L^{-1}	-	Rathus and Godwin (2000)
AM	IE	*bar*	Ubi1	PPT 5 mg L^{-1}	2.1	Zhao *et al*. (2000)
PB	IE	*uidA/bar/gfp*	Act1/ Ubi/ CaMV35S	Bialophos 2 mg L^{-1}	1.0	Able *et al*. (2001)
PB	IE	*uid*	Ubi1/ Act1/Adh1/ CaMV 35S	-	-	Hill -Ambroz et.al., (2001)
PB	IE	*uidA/gfp*	Ubi/ Act1/Adh1/CaMV35S	Observing GFP expression	-	Jeoung *et al*. (2002)
PB	IE	*Uid/ bar*	Act1/ Ubi1	PPT 5 mg L^{-1}	-	Emani *et al* (2002)
PB	IE /ST	*nptII/dhdps-raec1*	Act1/Adh1/ CaMV35S,Ubi1	Kanamycin/ lysine	1.3	Tadesse and Jacobs (2003).
PB	SM	*bar/HVA I*	CaMV35S	Glufosinate 10 mg L^{-1}	-	Devi *et al*. (2004)
PIG	SM	*bar/cryIAb/cryIB*	CaMV35S/Act1	Basta 2 mg L^{-1}	-	Gray *et al*.(2004)
AM	IE	*bar/ T1p, rice chitinase G11*	Ubi1	Bialophos 3 mg L^{-1}	-	Jeoung *et al*. (2004)
AM	IE	*gfp/bar/tlp/ rice chitinase G11*	CaMV35S	Hygromycin	-	Carvalho *et al*. (2004)

Method	Explant/ culture system	Gene of interest	Promoter	Selection agent/ conc. Used	Efficiency %	Reference
PB	ST	*uid/bar/cryIAc*	MpiC1	Basta 2 mg L^{-1}	1.5	Girijashanker *et al*. (2005)
AM	IE	*gfp/tlp*	Ubi1	-	2.5	Gao *et al*.(2005a)
AM	IE	*gfp/manA*	Ubi1	Mannose	2.88-3.3	Gao *et al*.(2005b)
AM	IE	*npt II/uidA*	-	-	0.3 to 4.5	Howe *et al*.(2006)
AM	IE	*Hpt*	-	Gentamycin/Paromycin	5	Nguyen *et al*. (2007)
sonication	Pollen	*npt II/uid A*		Hygromycin	-	Wang *et al*. (2007)
AM	IE	*gfp, manA*	Ubi1	Mannose	7.0	Gurel *et al*.(2009)
AM	IE	*Bar*	CaMV35S/ ZeinCZ19BI	PPT 2.5 mg L^{-1}	0.4 - 0.7	Lu *et al*. (2009)
AM	IF	*gus/cry1Ab/ hpt*	-	Hygromycin	1.9	Zhang *et al*.(2009)
PB	IZE	*bar, manA*	Ubi1	Bialophos 2 mg L^{-1}	0.77	Grootboom *et al*. (2010)
PB	IE	*hpt, luc*	Ubi1	Hygromycin/Geneticin	0.09	Anshu *et al*. (2010)
AM	IE	*Rice chitinase/ bar/hpt*	Ubi1	Bialophos 2 mg L^{-1} & Hygromycin	1.0-2.0	Indra *et al*. (2010)
AM	IF	*SgfpS65T*	Ubi1	Hygromycin	4.28	Shridhar *et al*. (2010)
AM	IE	*uidA*	Ubi1/CaMV 35S	Hygromycin	4.0	Kumar *et al*.(2011)
PB	IE	*npt II/gfp*	Ubi1	Geneticin	20.7	Guoquan *et al*.(2012)

Abbreviations: *cat*: *c*hloramphenicol acetyl transferase, ***nptII*:** neomycin phosphotransferase, ***bar*:** bialophos resistance, ***gfp*:** green fluorescence protein; ***hpt*:** hygromycin phosphotransferase, ***act*1:** rice *actin* promoter, ***ubi:*** maize *ubiquitin1* promoter, ***adh*1:** alcohol dehydrogenase promoter, **CaMV35S:** Cauliflower mosaic virus 35S promoter, **PPT:** phosphinothricin, ***luc:*** Luciferase, ***manA:*** phosphomannose isomerase, **CS:** Cell suspension, **IE:** immature embryo, **IC:** inflorescence callus, **IF:** immature inflorescence, **ST:** shoot tips, **SM:** shoot meristems, **IZE:** immature zygotic embryo, **PB:** particle bombardment, **AM:** *Agrobacterium* mediated, **Ep:** Electroporation, **SEC:***:*Scutellum-derived embryogenic calli