Regeneration and genetic transformation of grain legumes: An overview

Atika Chandra and Deepak Pental*

Department of Genetics, University of Delhi South Campus, Benito Juarez Road, New Delhi 110 021, India

Grain legume crops like pigeonpea, chickpea, mungbean, groundnut and soybean are extensively grown in the rainfed and dryland areas of India. These legume crops are a source of dietary protein, especially for the largely vegetarian population of sub-tropics. Despite large acreage under these crops, total productivity remains low and has been stagnating for the last few decades. A number of biotic and abiotic stresses are severely affecting full realization of the yield potential of these crops. There is need to increase productivity and enhance the nutritional value of these pulse crops. Cultivars resistant to biotic and abiotic stresses and which have better protein quality and quantity are needed. Grain legumes have a narrow genetic base since they are essentially self-pollinated (although cross-pollination does take place, it is at very low frequency). Thus, there is need to widen the genetic base and incorporate desirable characters. There is an urgent need to use transgenic technologies for improvement of leguminous crops. Worldwide, soybean is the only transgenic grain legume being cultivated in nearly 63% of the total area under transgenics[1]. Routine transformation protocols are limited in most grain legumes. The low success has been attributed to poor regeneration ability (especially via callus) and lack of compatible gene delivery methods, although some success has been achieved in soybean. This review is an attempt to summarize the studies on regeneration and genetic transformation in soybean, pigeonpea, chickpea, pea, groundnut, and *Vigna* spp. and to identify the hurdles being faced in the efficient recovery of transgenic plants. The review presents a comparative account of explants used, mode of regeneration (organogenesis v/s embryogenesis), gene delivery techniques and recovery of transgenics in crops considered here.

Plant tissues regenerate *in vitro* through two pathways, namely 'organogenesis' wherein shoot buds are organized by concerted meristematic activity of a number of cells and 'embryogenesis', where usually single cell or a small cluster of cells undergo differentiation to produce somatic embryos similar to zygotic embryos. The regeneration of complete plants via tissue culture has made it possible to introduce foreign genes into plant cells and recover transgenic plants. Morphogenesis could occur directly from the explant or indirectly via the formation of a dedifferentiated callus (Figure 1). However the different pathways of regeneration, viz. organogenesis from callus (pathway I), embryogenesis from callus (pathway IV), organogenesis directly from explants (pathway II) and embryogenesis from explants in a direct mode (pathway III) vary in their amenability to different gene delivery techniques.

Although many different techniques (electroporation of intact tissues, silicone carbide whiskers, etc.) have been tested for gene delivery to plant cells, two major methods, namely *Agrobacterium*-mediated and particle bombardment, have been extensively employed for genetic transformation of crop plants. Regeneration via the callus lends itself easily (compared to explants regenerating directly) to *Agrobacterium*-mediated transformations, while direct regeneration is more amenable for particle bombardment. Organogenesis from an unorganized callus (pathway I) has only been reported in soybean[2] and pea[3], where shoots were recovered from callus tissues at a low frequency. Thus, this pathway, although amenable to *Agro-*

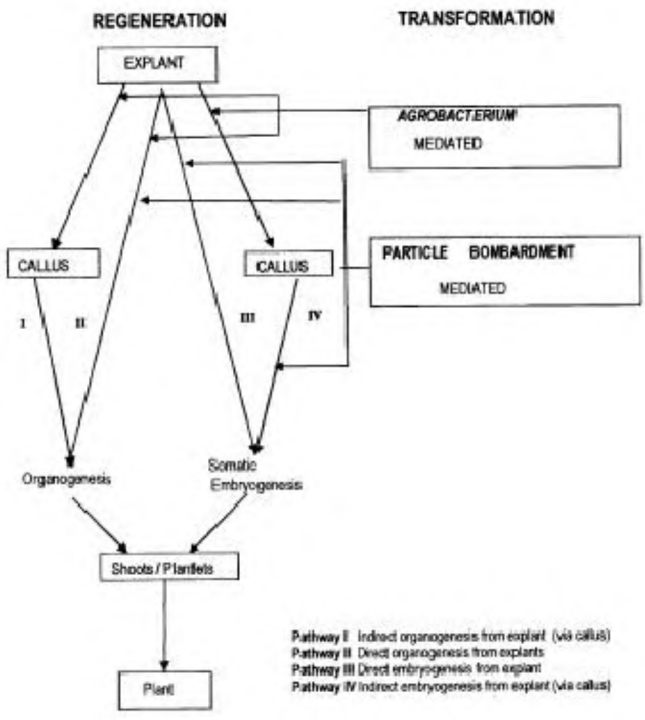

Figure 1. Flow chart showing different pathways of *in vitro* regeneration and their amenability to the two major methods of gene delivery.

*For correspondence. (e-mail: dpental@hotmail.com)

bacterium transformation, could not be used extensively for the recovery of transgenic plants in the two crops.

Regeneration and transformation using direct organogenesis pathway

In the legume crops considered here, direct organogenesis of shoots from cotyledonary nodes, shoot apices, leaflets and embryo axes is the most common regeneration pathway.

Cotyledonary nodes from mature seeds have been most responsive for the induction of multiple shoots via organogenesis in soybean[4–6], pigeonpea[7,8], chickpea[9,10], pea[11], and *Vigna* spp.[12–15]. However the number of shoots recovered from these explants is low with the exception of pigeonpea. In soybean, pea, chickpea and *Vigna* between 5 and 10 shoots have been recovered per explant while in pigeonpea up to 45 shoots were recovered from each cotyledonary node explant[7]. Most of the explants are responsive to cytokinins, especially BAP and thidiazuron (TDZ). Morphogenesis of shoots via organogenesis is sometimes preceded by formation of intermediate structures, i.e. the 'cotyledon-like structure (CLS)' in chickpea[16], 'caulogenic buds'[17] in groundnut and 'morphs' in pigeonpea (unpublished).

The axillary meristems at the junction of the cotyledon and the embryo axes contain cells that are competent for regeneration and hence could be useful targets for gene delivery. *Agrobacterium*-mediated transformation of cotyledonary node explants has been reported in soybean[18–20], pigeonpea[21,22], chickpea[23–25], pea[26–28], groundnut[29,30] and *Vigna* spp.[31,32]. Since the infectivity of *Agrobacterium* needs wound response, explants and media regimes that allow regeneration after wounding have been preferred for transformation. Injury is created by incising through the meristematic region[26] or by pricking the node with needles[33] prior to infection. A strong host genotype and strain interaction exists and very few genotypes of the crop are infected by a given *Agrobacterium* strain. In soybean, extensive studies on susceptibilities of different cultivars to different strains have been conducted[34–36]. The octopine strain, C58 and derivatives of the supervirulent succinamopine strain Bo542 have been found to be most effective. Differential susceptibility of genotypes to *Agrobacterium* infection has also been reported for pigeonpea[37], chickpea[38], pea[39] and groundnut[40].

Early studies on transformation of soybean reported very low frequencies (0.1–1.2%)[41,42] of recovering transgenics. However, with improvement in *Agrobacterium* strains and the use of sonication-assisted-*Agrobacterium* transformation, higher frequency of transformation has been reported[18,43,44] (2% of the infected cotyledonary node explants gave transformed shoots with the new *Agrobacterium* strain KYRT1[18]). The use of glufosinate as a selective agent has also been reported to enhance the recovery of transgenics[19] in soybean. A novel method of genotype independent *Agrobacterium*-mediated transformation of cotyledonary nodes and subsequent regeneration of shoots has been patented[45]. In this method, the induction of virulence genes and the use of sequential inoculations have been shown to enhance the frequency of transformation and achieve infections in non-susceptible cultivars.

In pigeonpea, 62% of cotyledonary node explants and 45% of shoot apices were reported to be transformed[21] using LBA4404 strain. However, this is a preliminary report and no confirmed transgenics are available. Recently, strain GV2260 has been used to recover transgenic plants carrying the cowpea protease inhibitor[22]. Transgenic nature of regenerants was characterized by Northern blotting to confirm the presence of mRNA. Transformation frequency reported in this study was ~ 1%.

In early attempts at recovering transgenics of chickpea with *Agrobacterium* transformation of cotyledonary nodes, frequencies of 2–4% were reported[23,24], but in a recent report[25], transformation efficiency of only 0.4% was reported.

A low frequency of transformation has been reported in pea. However, in a recent study using a new binary vector, pGreen[46] and cotyledonary node explants, transformation frequency of 3.5% was achieved. Enhanced recovery of transgenic plants by including 5-azacytidine in the selective medium has also been reported[28]. In this study, abnormal transgenic shoots were recovered on media with kanamycin as selection agent, while selection on phosphinothricin containing medium proved more useful for regenerating normal plants.

In groundnut, a non-tissue culture approach has been employed for recovering transgenics[33]. In this study, embryo axes lacking one cotyledon were wounded by pricking with needles and infected with *Agrobacterium* strain LBA4404 (pKIWI105). Interestingly, the infectivity of *Agrobacterium* could be enhanced by use of tobacco leaf extract instead of acetosyringone. Transgenic groundnut plants have also been recovered[29,33] from leaf explants infected with EHA101 (pBI121), but frequency was very low (0.02–0.03%). Transmission of the transgene (*gus*) to the progeny was shown[33].

For transformation of *Vigna sesquipedalis*, cotyledonary node explants were infected with strain EHA105 and transformation frequency of up to 2% was reported[31]. Transformation frequencies of 23% with LBA4404 and 10% with EHA105 for leaf discs of *V. mungo* have been reported[47]. However, transformed calli did not give rise to any shoots. In *V. unguiculata*, transformed callus[48] was recovered by leaf disc transformation with strains C58C1 (pGV3850) and C58C1 (pGV3850 :: 1103neo). Transformation of mature embryos with strain C58 (pGV2260/p35S*gus*int)[49] gave rise to chimeric plants. Cotyledon explants infected with strain LBA4301 (pUCD2340) yielded six transformants[50]. However, using shoot apices from mature dry seed, transgenic plants[51] were recovered

at a frequency of ~ 2%. In *V. radiata*, nearly 80–100% transformation frequency of cotyledon and hypocotyl explants has been observed with *A. rhizogenes* strain LBA9402, but transformed tissue gave rise to roots only[52]. In a recent report[32], transgenic calli and shoots were recovered from primary leaves and cotyledonary nodes respectively. Transformation frequency of up to 50% was reported using strain EHA105 (pBin*gus*int) and hypocotyl explants, however the callus did not regenerate shoots. Cotyledonary nodes infected with strain LBA4404 (pTOK233) gave rise to transgenic plants at an overall efficiency of 0.9%. Integration of foreign genes was confirmed by Southern analysis.

The particle bombardment method has been used for genetic transformation of many grain legume species. Shoot apical meristems have been shown to form multiple shoots in soybean[53], groundnut[54] and *V. radiata*[55]. In soybean, bombarded apices were allowed to grow into plants that flowered and set seed. The seeds were tested for GUS expression to confirm the transgenic nature of the parent plant. No *in vitro* selections were done and thus a large number of plants had to be screened. Although transformations were at low frequencies[56,57] ~ 0.4–4%, transgenic plants could be recovered. However, a large proportion of these were chimeras. Southern analysis of T_0 plants and their progeny revealed that mostly two to hundred copies of the gene were integrated in the genome. However these copies were linked and co-segregated as a single locus in the progeny. At present all the transgenic plants of soybean under commercial cultivation have been generated via particle bombardment.

Particle bombardment of shoot apices of mature embryonic axes in two cultivars of groundnut followed by induction of multiple shoots yielded an average of five shoots per explant[58]. Mostly, chimeric plants were produced at frequencies of 6–8% but of these, only 0.6–2.3% of shoots expressed GUS in a uniform manner.

No reports on biolistic transformation of pigeonpea and chickpea are available while only one report[59] exists on the bombardment of embryo axes of pea. In the work on pea, the authors have basically evaluated the effect of different promoter sequences on transient expression of reporter gene.

Transient expression of *gus* gene has also been studied in meristematic zones and complete seedlings of bombarded embryo axes of mature seeds of *V. mungo*, *V. aconitifolia and V. radiata*[60]. Recovery of stably transformed plantlets on medium supplemented with kanamycin was reported, but no detailed molecular analyses have been presented.

Regeneration and transformation via embryogenesis

The embryogenic mode of regeneration has been reported in soybean[61–69], groundnut[70–75], pea[76] and chickpea[9]. While in soybean[61,62] and groundnut[70], embryogenic callus cultures have been established, in pea[76], pigeonpea[68,69,70], groundnut[73] and chickpea[9], direct somatic embryos have been observed. Induction of somatic embryos via suspension cultures has been observed in pigeonpea[80] and *Vigna* spp.[81].

In early work on the induction of somatic embryos on explants of soybean, abnormal structures, which lacked well-developed meristems, were recovered. These were categorized as 'neomorphs'[61]. However, while further optimization of the culture media has resulted in an efficient protocol for induction of somatic embryos[62], eventually fertility of the regenerants has been found to be low[63] using this protocol. Embryogenic suspension cultures of soybean have also been found amenable for *Agrobacterium*-mediated transformation using sonication (SAAT)[44]. However, all regenerants were found to be sterile.

Bombardment of zygotic embryos and embryogenic cultures has also been reported in groundnut[75]. The phenomenon of repetitive somatic embryogenesis or somatic-embryo cycling has been reported in soybean[64,65] and groundnut[71], where the primary somatic embryos have been used to initiate fresh embryogenic cultures. Supra-optimal concentrations of auxins (especially 2,4-D and NAA) were reported to be more effective for induction of embryogenic calli and repetitive somatic embryogenesis in soybean and groundnut. In contrast, sub-optimal concentrations of IAA, NAA supported callus formation. Although varying degree of genotypic influence on regeneration has been reported in different legume species (e.g. soybean[68,] groundnut[74]) optimizing culture regimes for a particular genotype is also possible, as has been shown for soybean[69].

TDZ, has been shown to induce somatic embryos in pigeonpea[78], pea[76], groundnut[73] and shoots in pigeonpea[79], soybean[6] and groundnut[82]. However these have not been used for developing reproducible transformation protocols.

Conclusions

As has been described above, in the legume crops reviewed here, the most prevalent mode of regeneration is via direct organogenesis from cotyledonary node explants. In general, the callus-mediated regeneration systems are highly amenable to *Agrobacterium*-mediated transformation while the direct regeneration systems are more or less recalcitrant to *Agrobacterium*-mediated transformation. However, by creating microinjuries some success has been achieved in transformation of cotyledonary node explants[43,45]. A ready-reckoner of the available regeneration systems and transformation protocols for these six crops is collated in Table 1. Only those regeneration systems that are highly amenable to the process of

genetic transformation can provide protocols for recovery of transgenic plants at a high frequency. Some of the important reports have been tabulated to present the nature of transgenic plants recovered (Table 2).

In soybean, gene delivery via particle bombardment of apical meristems has been preferred[67]. As this protocol relies on growing large number of shoots (recovered after bombardment of apical meristems) in the greenhouse and screening the progeny for presence of the reporter gene, it is very labour-intensive. This method does not utilize *in vitro* selections but depends on transmission of transgenes to the progeny through transformation of the cells in the meristem (the L2 layer) that contribute to the development of gametic tissues. The segregation of the transgene in the progeny has been usually detected by GUS expression. Details of soybean transgenics that have been commercialized and are being grown is presented in Table 3. Transgenics raised through the biolistic method in soybean are known to carry multiple copies that are often tandemly arranged and as a consequence co-segregate. High transient expression in bombarded embryo axes of *Vigna* spp was reported[57], but transmission of transgene to the progeny needs to be evaluated.

Although the biolistic gene delivery has been successful in producing transgenic lines, *Agrobacterium*-mediated transformation is the method of choice as the integrations of the transgene are more well-defined compared to those in biolistic transformations. Also, the copy number of transgenes is low and tandem integrations of multiple copies are less frequent (multiple copy insertions at a single locus are highly prone to methylation-based gene silencing in plants). *Agrobacterium*-mediated transformation of the explant or the callus initiated from the explant has been successful in many crop plants, but in the grain legumes such protocols exist for a few species only, as in groundnut and pea. The efficient recovery of transformed plants depends not only on the mode of regeneration and choice of transformation procedure but also on selectable markers. Although kanamycin has been the most favoured selectable agent, it has not proved an efficient selectable marker for grain legumes. The development of efficient uptake of selective agents by the regenerating tissues has increased recovery of transformed shoots, as has been shown by efficient selection in soybean on glufosinate containing medium[19].

Future research: breaking the impasse

As evident from the above survey, the major grain legume crops especially for the tropics face an impasse in transgenic production. Transgenics are needed for genetic enhancement as part of plant breeding programmes. The need is for single copy transgenics expressing the transgene stably and capable of transmitting it to the progeny. The focus of transformation protocols should be on the recovery of 'useful transgenics'[83].

Poor regeneration (compared to model systems like tobacco, mustard) and especially poor regeneration via callus contributes to this impasse. It is concluded from the review of literature, that all the four modes of regeneration are not available in legume crops and consequently there are constraints on the choice of gene transfer methods that could be used. Overall, as pathway II is the most common regeneration pathway in grain legumes, *Agrobacterium*-mediated transformation which has been successful in other dicot crops has not been as efficient in grain legumes. Only limited success in soybean has been achieved via pathway II. In systems where embryogenic callus can be raised efficiently, recovery of plantlets is high like in soybean. Embryogenic suspension cultures have been reported in pigeonpea and *V. unguiculata*. However, eventual success in developing transgenic plants will depend on the conversion of embryos to plants. This

Table 1. Assessment of available regeneration* and transformation systems in the six grain legumes

Explant	Crop plant						Transformation method
	Soybean	Peanut	Pea	Chickpea	Pigeonpea	Vigna	
Cotyledons	†(I, II)	×	×	†(III)	×	†(I)	*Agrobacterium*
	×	×	×	×	×	×	Particle bombardment
Cotyledonary nodes	†(II)	×	†(II)	†(II)	†(II)	†(II)	*Agrobacterium*
	†(II)	×	×	×	×	×	Particle bombardment
Apical meristem	×	×	×	×	×	†(II)	*Agrobacterium*
	†(II)	†(II)	×	×	×	×	Particle bombardment
Embryogenic callus/cell suspensions	†(IV)	×	×	×	×	×	*Agrobacterium*
	†(IV)	†	×	×	×	×	Particle bombardment
Embryo axes	×	×	×	×	×	†(II)	*Agrobacterium*
	†(II)	×	†	×	×	†	Particle bombardment

†Available/tested by transient expression.
×Not available.
*Regeneration pathway (I–IV as described in Figure 1) in case transgenics were raised.

Table 2. Particle bombardment and *Agrobacterium*-mediated transformation of soybean, pigeonpea, chickpea, pea, peanut and *Vigna* spp. Some important reports

Plant	Explant and mode of regeneration	Strain/plasmid	Frequency of transformation	Transgenic plants and progeny analyses	Reference
Soybean	Somatic embryogenesis from zygotic embryo cotyledons	EHA105 (pHIG)	0.03%	Three fertile plants were recovered. Southern hybridization revealed these to be from the same transformation event and carried three copies. Segregation of GUS in 48 plants of selfed progeny revealed single locus inheritance.	Yan *et al.*[89]
Soybean	Shoot organogenesis from cotyledonary node	EHA105, EHA101 Plasmids pPTN101, pPTN105, pPTN140	3%	Total of 7 T_0 transformants were recovered. Southern analysis on progeny was performed.	Zhang *et al.*[19]
Soybean	Direct shoot recovery from apical meristems	Particle bombardment	Not reported	Progeny from a single line was tested further and selections were made from these.	Padgette *et al.*[67]
Pigeonpea	Shoot organogenesis from cotyledonary node	LBA4404(pBI21)	62%	Southern analyses of three primary transformants revealed these to be the same transformation event. Progeny was not analysed.	Geetha *et al.*[21]
Pigeonpea	Shoot organogenesis from callus	GV2260 harbouring binary plasmid pBin19 with cowpea protease inhibitor gene and *nptII* gene under 35 S promoter	1.25%	Northern analyses have been presented for 4 transformed plants. Further work on transmission of genes to progeny is underway.	Lawrence and Koundal[22]
Chickpea	Shoot organogenesis from cotyledonary node	LBA4404(pBI121)	1%–1.5%	Southern analyses of six primary transformants have been reported. No study on transmission of transgene to progeny has been done.	Kar *et al.*[24]
Chickpea	Shoot organogenesis from cotyledonary nodes of mature embryo axes	GV2260(p35S GUSINT), EHA101 (pIBGUS)	0.4%	Southern analyses of T_0 plants and PCR for T_1 have been presented.	Krishnamurthy *et al.*[25]
Pea	Shoot organogenesis from immature cotyledons	LBA4404(pBI121)	8%	Seventeen primary transformants were analysed by PCR and Southern hybridizations. Progeny of nine plants was studied for segregation of transgenes.	Orczyk and Orczyk[28]
Pea	Shoot organogenesis from lateral cotyledonary meristems	LBA4404 (pGreen0229)	3.5%	Seven transgenic plants were recovered	Hellens *et al.*[91]
Peanut	Shoot organogenesis from embryo axes	LBA4404 (pKIWI105)	3.3%	Five transgenics from three independent events and their T_1 and T_2 progeny were analysed with PCR, Southern hybridization, assay for *nptII* and *uidA* genes.	Rohini and Rao[33]
V. radiata	Shoot regeneration from cotyledonary nodes	LBA4404(pTOK233), C58C1 (pIG121Hm), EHA105 (pBin9GusInt)	0.9%	A total of ten shoots were recovered, which flowered and set seed. Southern analysis was performed on T_0 plants and contained the gene of interest.	Jaiwal *et al.*[32]
V. sesquipedalis	Shoot organogenesis from cotyledonary node	EHA101 (pIBGUS-INT)	2%	Southern analyses of primary transformants were performed and integration of transgenes was observed. Progeny screened for GUS expression revealed 3:1 segregation. Number of transgenics not mentioned.	Ignacimuthu[31]
V. unguiculata	Multiple shoots from de-embryonated cotyledons	LBA4301 (pUCD2340)	15–17%	Six primary transformants were recovered. Southern hybridization was done to confirm presence of transgene. Four transgenics set seeds but none of the seeds germinated.	Muthukumar *et al.*[50]

Table 3. Transgenic soybean under cultivation (Source, Agbios Inc.; www/agbios.com)

Event	Origin of transgenic	Gene of interest	Company	Description	Year and country of cultivation*
GTS 40-3-2 (RoundUp Ready)	Particle bombardment	EPSPS gene from *Agrobacterium tumefaciens*	Monsanto	Glyphosate herbicide tolerant	1996, USA
A2704-12,-21 (Liberty resistant/Ignite	Particle bombardment	*Pat* gene from *Streptomyces viridochromogenes*	Aventis CropScience	Phosphinothricin herbicides (active ingredient is glufosinate ammonium)	1996, USA
A5547–127	Particle bombardment	*Pat* gene from *Streptomyces viridochromogenes*	Aventis CropScience	Phosphinothricin herbicides (active ingredient is glufosinate ammonium)	1998, USA
GU262	Particle bombardment	*Pat* gene from *Streptomyces viridochromogenes*	Aventis CropScience	Phosphinothricin herbicides (active ingredient is glufosinate ammonium)	1998, USA
W62, W98	Particle bombardment	*Bar* gene from *Streptomyces hygroscopicus*	Aventis CropScience	Phosphinothricin herbicides (active ingredient is glufosinate ammonium)	1998, USA
G94-1, G94-19 and G168	Particle bombardment	*GmFad2-1* gene, *gus* gene	Du Pont Canada Agricultural Products	High oleic acid in oil	1997, USA

*Year and country where clearance was granted as food or feed crop.

conversion is extremely poor for pigeonpea and *Vigna* species. Thus developing protocols to enhance regeneration still remains an important goal in grain legume crops.

Alternatively, protocols could be developed where poor regeneration systems are complemented by increasing the efficiency of gene delivery and gene expression. The continued search for infective *Agrobacterium* strains can lead to development of new disarmed strains which can provide higher transformation efficiencies, as has been observed in case of the KYRT1 strain. Particle-gun mediated transformation remains a viable alternative and has been used in some of the legume crops to generate transgenics although at low frequencies. The development of new vectors[32,84], like pTOK233 could enhance transformation frequencies.

Another major hurdle is of selection of transgenics *in vitro*. In most of the crops discussed here, kanamycin has been the selective agent of choice although phosphinothricin has been used successfully in pea. Besides testing other herbicides like glufosinate and 'Positive selection' strategies employing cytokinins (*ipt* gene[85]), xylose isomerase gene (*xyl* gene[86]) and phosphomannose isomerase gene (*pmi* gene[87]) need to be tested on grain legume crops.

Thus, concerted efforts are needed on improving *in vitro* regeneration or developing non-tissue culture approaches to genetic transformation. A recent report shows that model forage legume species *Medicago truncatula* can be transformed by floral dip method[88]. Although grain legumes produce fewer seeds compared to forage legumes, it may be worthwhile to test this method on mungbean and other grain legumes. Development of new disarmed *Agrobacterium* strains that are highly infective on specific grain legume crops could be another useful alternative. Development of transgenics in major grain legume crops of India, i.e. groundnut, pigeonpea, chickpea and mungbean still remains dogged by poor regeneration and low frequency transformation protocols and this situation needs to be rectified if transgenics of these crops are to be developed.

1. James, C., International Service for the Acquisition of Agri-biotech Applications (ISAAA), 2001, Brief No. 24.
2. Barwale, U. B., Kerns, H. R. and Widholm, J. M., *Planta*, 1986, **167**, 473–481.
3. Bohmer, P., Meyer, B. and Jacobsen, H.-J., *Plant Cell Rep.*, 1995, **15**, 26–29.
4. Cheng, T.-Y., Saka, H. and Voqui-Dinh, T. H., *Plant Sci. Lett.*, 1980, **19**, 91–99.
5. Barwale, U. B., Meyer Jr. M. M. and Widholm, J. M., *Theor. Appl. Genet.*, 1986, **72**, 423–428.
6. Kaneda, Y., Tabei, Y., Nishimura, S., Harada, K., Akihama, T. and Kitamura, K., *Plant Cell Rep.*, 1997, **17**, 8–12.
7. Shiva Prakash, N., Pental, D. and Bhalla-Sarin, N., *Plant Cell Rep.*, 1994, **13**, 623–627.
8. Franklin, G., Jeyachandran, R., Melchias, G. and Ignacimuthu, S., *Curr Sci.*, 1998, **74**, 936–937.
9. Murthy, B. N. S., Victor, J., Singh, R. P., Fletcher, R. A. and Saxena, P. K., *Plant Growth Regul.*, 1996, **19**, 233–240.
10. Subhadra, Vashishat, R. K., Chowdhury, J. B., Singh, M. and Sareen, P. K., *Indian J. Expt. Biol.*, 1998, **36**, 1276–1279.
11. Jackson, J. A. and Hobbs, S. L. A., *In vitro Cell. Dev. Biol.*, 1990, **26**, 835–838.
12. Mathews, H., *Plant Cell Tissue Org. Cult.*, 1987, **11**, 233–240.
13. Gulati, A. and Jaiwal, P. K., *Plant Cell Rep.*, 1994, **13**, 523–527.
14. Ignacimuthu, S., Franklin, G. and Melchias, G., *Curr. Sci.*, 1997, **73**, 733–735.
15. Das, D. K., Shiva Prakash, N. and Bhalla-Sarin, N., *Plant Sci.*, 1998, **134**, 199–206.

16. Shri, P. V. and Davis, T. M., *Plant Cell Tissue Org. Cult.*, 1992, **28**, 45–51.
17. Chengalrayan, K., Mhaske, V. B. and Hazra, S., *Plant Sci.*, 1995, **110**, 259–268.
18. Meurer, C. A., Dinkins, R. D. and Collins, G. B., *Plant Cell Rep.*, 1998, **18**, 180–186.
19. Zhang, Z., Xing, A., Staswick, P. and Clemente, T. E., *Plant Cell Tissue Org. Cult.*, 1999, **56**, 37–46.
20. Donaldson, P. A. and Simmonds, D. H., *Plant Cell Rep.*, 2000, **19**, 478–484.
21. Geetha, N., Venkatachalam, P. and Lakshmi Sita, G., *Plant Biotechnol.*, 1999, **16**, 213–218.
22. Lawrence, P. K. and Koundal, K. R., *Curr. Sci.*, 2001, **80**, 1428–1432.
23. Fontana, G. S., Santini, L., Caretto, S., Frugis, G. and Mariotti, D., *Plant Cell Rep.*, 1993, **12**, 194–198.
24. Kar, S., Johnson, T. M., Nayak, P. and Sen, S. K., *Plant Cell Rep.*, 1996, **16**, 32–37.
25. Krishnamurthy, K. V., Suhasini, K., Sagare, A. P., Meixner, M., de Kathen, A., Pickardt, T. and Schieder, O., *Plant Cell Rep.*, 2000, **19**, 235–240.
26. Jordan, M. C. and Hobbs, S. L. A., *In vitro Cell. Devl. Biol.*, 1993, **29**, 77–82.
27. Bean, S. J., Gooding, P. S., Mullineaux, P. M. and Davies, D. R., *Plant Cell Rep.*, 1997, **16**, 513–519.
28. Nadolska-Orczyk, A. and Orczyk, W., *Molecular Breeding*, 2000, **6**, 185–194.
29. Cheng, M., Jarret, R. L., Li, Z., Xing, A. and Demski, J. W., *Plant Cell Rep.*, 1996, **15**, 653–657.
30. Cheng, M., Jarret, R. L., Li, Z. and Demski, J. W., *Plant Cell Rep.*, 1997, **16**, 541–544.
31. Ignacimuthu, S., *Indian J. Exptl. Biol.*, 2000, **38**, 493–498.
32. Jaiwal, P. K., Kumari, R., Ignacimuthu, S., Potrykus, I. and Sautter C., *Plant Sci.*, 2001, **161**, 239–247.
33. Rohini, V. K. and Sankara Rao, K., *Plant Sci.*, 2000, **150**, 41–49.
34. Byrne, M. C., McDonnell, R. E., Wright, M. S. and Carnes, M. G., *Plant Cell Tissue Org. Cult.*, 1987, **8**, 3–15.
35. Owens, L. D. and Cress, D. E., *Plant Physiol.*, 1985, **77**, 87–94.
36. Delzer, B. W., Somers, D. A and Orf, J. H., *Crop Sci.*, 1990, **30**, 320–322.
37. Rathore, R. S. and Chand, L., *Int. Chickpea Pigeonpea Newsl.*, 1997, **4**, 38–39.
38. Islam, R., Malik, T., Husnain, T. and Riazuddin, S., *Plant Cell Rep.*, 1994, **13**, 561–563.
39. Hobbs, S. L. A., Jackson, J. A. and Mahon, J. D., *Plant Cell Rep.*, 1989, **8**, 274–277.
40. Lacorte, C., Mansur, E., Timmerman, B. and Cordiero, A. R., *Plant Cell Rep.*, 1991, **10**, 354–357.
41. Hinchee, M. A. W. *et al.*, *Biotechnology*, 1988, **6**, 915–922.
42. Di, R., Purcell, V., Collins, G. B. and Ghabrail, S. A., *Plant Cell Rep.*, 1996, **15**, 746–750.
43. Trick, H. N. and Finer, J. J., *Transgenic Res.*, 1997, **6**, 329–336.
44. Trick, H. N. and Finer, J. J., *Plant Cell Rep.*, 1998, **17**, 482–488.
45. Thomas, L. A. and Townsend, J. A., Patent No. WO9402620, 1994.
46. Hellens, R. P., Edwards, E. A., Leyland, N. R., Bean, S. and Mullineaux, P. M., *Plant Mol. Biol.*, 2000, **42**, 819–832.
47. Karthikeyan, A. S., Sarma, K. S. and Veluthambi, K., *Plant Cell Rep.*, 1996, **15**, 328–331.
48. Garcia, J. A., Hille, J. and Goldbach, R., *Plant Sci.*, 1986, **44**, 37–46.
49. Penza, R., Lurquin, P. F. and Filippone, E., *J. Plant Physiol.*, 1991, **138**, 39–43.
50. Muthukumar, B., Mariamma, M., Veluthambi, K. and Gnanam, A., *Plant Cell Rep.*, 1996, **15**, 980–985.
51. Sahoo, L., Sushma, Sugla, T., Singh, N. D. and Jaiwal, P. K., *Plant Cell Biotech. Mol. Biol.*, 2001, **1**, 47–54.
52. Jaiwal, P. K., Sautter, C. and Potrykus, I., *Curr Sci.*, 1998, **75**, 41–45.
53. Ponsamuel, J., Huhman, D. V., Cassidy, B. G. and Post-Beittenmiller, D., *Plant Cell Rep.*, 1998, **17**, 373–378.
54. Yang, Y-S., Wada, K. and Futsuhara, Y., *Plant Sci.*, 1990, **72**, 101–108.
55. Gulati, A. and Jaiwal, P. K., *Plant Cell Tissue Org. Cult.*, 1992, **29**, 199–205.
56. Christou, P., Swain, W. F., Yang, N.-S. and McCabe, D. E., *Proc. Natl. Acad. Sci. USA*, 1989, **86**, 7500–7504.
57. Sato, S., Newell, C., Kolacz, K., Tredo, L., Finer, J. and Hinchee, M., *Plant Cell Rep.*, 1993, **12**, 408–413.
58. Brar, G. S., Cohen, B. A., Vick, C. L. and Johnson, G. W., *Plant J.*, 1994, **5**, 749–753.
59. Warkentin, T. D., Jordan, M. J. and Hobbs, S. L. A., *Plant Sci.*, 1992, **87**, 171–177.
60. Bhargava, S. C. and Smigocki, A. C., *Curr. Sci.*, 1994, **66**, 439–442.
61. Christianson, M. L., Warnick, D. A. and Carlson, P. S., *Science*, 1983, **222**, 632–634.
62. Finer, J. J. and Nagasawa, A., *Plant Cell Tissue Org. Cult.*, 1988, **15**, 125–136.
63. Hadi, M. Z., McMullen, M. D. and Finer, J. J., *Plant Cell Rep.*, 1996, **15**, 500–505.
64. Liu, W., Moore, P. J. and Collins, G. B., *In vitro Cell. Dev. Biol.*, 1992, **28P**, 153–160.
65. Liu, W., Torisky, R. S., McAllister, K. P., Avdiushko, S., Hildebrand, D. and Collins, G. B., *Plant Cell Tissue Org. Cult.*, 1996, **47**, 33–42.
66. Cho, M.-J., Vodkin, L. O. and Widholm, J. M., *Plant Biotechnol.*, 1997, **14**, 11–16.
67. Padgette, S. R. *et al.*, *Crop Sci.*, 1995, **35**, 1451–1461.
68. Bailey, M. A., Boerma, H. R. and Parrott, W. A., *In Vitro Cell. Dev. Biol.*, 1993, **29P**, 102–108.
69. Bailey, M. A., Boerma, H. R. and Parrott, W. A., *Plant Sci.*, 1993, **93**, 117–120.
70. Baker, C. M., Durham, R. E., Burns, J. A., Parrott, W. A. and Wetzstein, H. Y., *Plant Cell Rep.*, 1995, **15**, 38–42.
71. Durham, R. E. and Parrot, W. A., *ibid*, 1992, **11**, 122–125.
72. Murthy, B. N. S. and Saxena, P. K., *ibid*, 1994, **14**, 145–150.
73. Saxena, P. K., Malik, K. A. and Gill, R., *Planta*, 1992, **187**, 421–424.
74. Chengalrayan, K., Mhaske, V. B. and Hazra, S., *Plant Cell Rep.*, 1998, **17**, 522–525.
75. Ozias-Akins, P., Schnall, J. A. and Anderson, W. F., *Plant Sci.*, 1993, **93**, 185–194.
76. Griga, M., *Biol. Plant.*, 1998, **41**, 481–495.
77. Patel, D. B., Barve, D. M., Nagar, N. and Mehta, A. R., *Indian J. Exp. Biol.*, 1994, **32**, 740–744.
78. Sreenivasu, K., Malik, S. K., Ananda Kumar, P. and Sharma, R. P., *Plant Cell Rep.*, 1998, **17**, 294–297.
79. Eapen, S., Tivarekar, S. and George, I., *Plant Cell Tissue Org. Cult.*, 1998, **53**, 217–220.
80. Anbazhagan, V. R. and Ganapathi, A., *ibid*, 1999, **56**, 179–184.
81. Prem Ananda, R., Ganapathi, A., Ramesh Anbazhagan, V., Vengadesan, G. and Selvaraj, N., *In vitro Cell. Dev. Biol.*, 2000, 1–7.
82. Victor, J. M. R., Murch, S. J., KrishnaRaj, S. and Saxena, P. K., *Plant Growth Regul.*, 1999, **28**, 9–15.
83. Birch, R. G., *Annu. Rev. Plant. Physiol. Mol. Biol.*, 1997, **48**, 297–326.
84. Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T., *Plant J.*, 1994, **6**, 271–282.
85. Ebinuma, H., Sugita, K., Matsunaga, E., Yamakado, M. and Komamine, A., *Plant Biotechnol.*, 1997, **14**, 133–139.
86. Haldrup, A., Petersen, S. G. and Okkels, F. T., *Plant Cell Rep.*, 1998, **18**, 76–81.
87. Joersbo, M., Peterson, S. G. and Okkels, F. T., *Physiol. Plant.*, 1999, **105**, 109–115.
88. Trieu, T. A. *et al.*, *Plant J.*, 2000, **22**, 531–541.
89. Yan, B., Srinivasa Reddy, M. S., Collins, G. B. and Dinkins, R. D., *Plant Cell Rep.*, 2000, **19**, 1090–1097.

ACKNOWLEDGEMENT. Research work on grain legumes in authors' laboratory was supported in part by the Department of Biotechnology, Government of India, under the project BT/RD/7/43/93.