

Gene Transfer in Legumes

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1 Introduction

In the last few decades, plant breeders, cell biologists and genetic engineers have joined forces and used a number of different techniques to unravel the secrets buried within the plant genome aiming at plant improvement. Breeders have traditionally relied on mass selection breeding following sexual crosses between

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the individuals possessing the best desirable characteristics and examining the best possible combinations within the resulting progenies over several years for the creation of new varieties. Cell biologists strive to develop tools for producing genetic novelties through the regeneration of plants (from protoplasts, cells, tissues and organs) following diverse in vitro selection treatments and then evaluate their conformity compared to the mother plants (wild type) as well as their specific biochemical, genetic and physiological status. Genetic engineers develop methods and techniques for the isolation and successful insertion of genes controlling desirable traits into plants by transformation.

Plant transformation may be defined as the sequence of delivery, integration and expression of foreign genes into the plant cells which will ultimately regenerate into a whole plant. This ability to introduce and express or inactivate specific genes in the plant genomes provides a new and powerful experimental tool for validating gene function, particularly in relation with various plant physiology mechanisms and processes that have not been resolved so far using other biochemical approaches. Another non-negligible application of this approach is that of obtaining and transferring genes that are not available to a given species due to sexual incompatibility from other plants, from microorganisms or even animals.

The process involves choosing a trait, identifying and isolating the gene(s) encoding it. To be functional, such gene(s) must include the regulatory regions ensuring their correct expression in the plant. Then, a reliable protocol must be devised and followed for the transformation of genes into plants, and the DNA sequences introduced must subsequently be integrated, expressed and maintained in the genome throughout subsequent cell divisions and progenies. Finally, transformed cells must be competent for regeneration into whole plants.

Gene delivery systems used to date can be divided into direct gene transfer (mediated by physical or chemical forces for delivery of the gene into plant protoplasts, cells and even tissues) and *Agrobacterium*-mediated gene transfer, where either *A. tumefaciens* or *A. rhizogenes* is used as vectors for introducing the foreign gene into the plant genome.

In this context, recombinant DNA technology has revolutionised biotechnology in such a way that plant transgenesis is now a relatively mature approach, and plant biotechnology provides today not only novel genotypes which carry agronomically useful genes for biotic and abiotic stresses, but also others that improve plant nutrition or increase yield components.

2 *Agrobacterium*-Mediated Transformation

In *Agrobacterium*-mediated transformation, the natural infecting capacity of the bacterium is used to transfect genes into plant cells (Chilton 2001; Somers et al. 2003; Sangwan et al. 2010), and this approach has become a tool used in plant breeding for crop improvement.

One significant limitation of the *Agrobacterium* gene transfer system is the fact that large groups of commercially important plants and some legumes among them are not hosts for *Agrobacterium* whereby this gene transfer system is not efficient for them.

A critical step for successful transformation with *Agrobacterium tumefaciens* is the co-cultivation of plant cells and bacteria to allow gene transfer, generally done by mixing plant cells with bacteria in vitro for a few days, after which bacteria are removed and cells or organs are regenerated to plants using adequate culture media. Alternatively, whole plants are dipped in *Agrobacterium* and subsequently allowed to grow under natural conditions, as in the floral dip transformation method of *Arabidopsis thaliana* (Clough and Bent 1998). This, unfortunately, has only been applicable to a few plant species to date including the legume *Medicago truncatula* (Trieu et al. 2000).

Due to the quite high recalcitrance of legumes to *Agrobacterium*-mediated transformation, numerous studies were focused on the optimisation of co-cultivation conditions (light regime, temperature, co-cultivation period, application of physical treatments such as sonication and vacuum, electroporation, mechanical pre-wounding or treatment by macerating enzymes). The co-cultivation step is a crucial and complex process where two different biological elements (plant explants and *Agrobacterium*) share the same space and conditions; thus many parameters should be tested to satisfy both partners and guarantee a successful outcome (Hansen and Wright 1999). A range of chemical substances which work for instance through the reduction of the resistance of the recipient cell (thiol compounds, anti-oxidants) or by facilitating their penetration through the cell wall (macerating enzymes) (Svabova and Griga 2008, and references therein) have been studied. A “classical” co-cultivation substance is acetosyringone (AS), a compound which is essential for induction of virulence genes. AS was used in several studies with legumes, e.g. De Kathen and Jacobsen (1990) found that AS did not increase the transformation ratio, Grant et al. (1995) used AS but did not compare its effect with the control, while Nadolska-Orczyk and Orczyk (2000) recorded a negative effect of AS but a positive influence of 5-azacytidine. A series of experiments with co-cultivation substances used previously for other leguminous (soybean, common bean, peanut) as well as non-leguminous crops (tobacco, sunflower, grapevine) were carried out by Svabova and Griga (2008). Subsequently, the effect of application of acetosyringone, L-cysteine, dithiothreitol, glutathione, cellulase and pectinase in various concentrations and combinations was examined. The only combination significantly improving responses was the addition of 100 µM AS and 50 mg L-cysteine to the co-cultivation medium.

In legumes, all methods for gene transfer are based on specific in vitro techniques used to foster the genetically modified cells to regenerate into plants, depending on the organ and the cells used for transformation. Regeneration either proceeds directly from meristems existing in the original explant or adventitiously, through organogenesis or somatic embryogenesis, which can both be direct or include an intervening callus phase, with such callus generating embryos or shoots following subculture on an appropriate medium.

Only a small fraction of the target cells are actually transformed. Thus, genetic modification requires a selection mechanism ensuring that the genetically modified cells are favoured to grow and divide over wild-type cells. Usually, this is achieved by coupling the gene(s) of interest to genes conferring resistance to antibiotics or herbicides, whereby adding antibiotics or herbicides precludes the division and growth of non-transformed cells. For most crops, such selection treatment is applied during a callus phase after which plants are regenerated via adventitious shoot formation or somatic embryogenesis, depending on the species. However, selection mechanisms are not always fully efficient with this procedure and hence regenerated plants may contain transgenic cells but also either a mixture of transgenic and wild-type cells (chimeras) or only wild-type cells (escapes). A proper selective concentration of selective substances should decrease the number of escape plants to a minimum. Several conclusions were obtained in experiments focused on the utilisation of various genes for resistance to antibiotics as selective genes, particularly kanamycin. Puonti-Kaerlas (1992), De Kathen and Jacobsen (1990) as well as Schroeder et al. (1993) found kanamycin less effective for selection than hygromycin and phosphinotricin. On the other hand, Grant et al. (1998) deemed kanamycin in higher concentrations and in combination with successive selection steps as effective. Similar results were published by Morton et al. (2000).

Generally, in large seeded legumes regeneration systems omitting callus culture are more effective than those where embryos or calli are initiated. The conversion of undifferentiated cell growth (calli) to the process of differentiation and induction of embryo- or organogenesis is a normally challenging task. A method of direct use of germinating seeds was first described on soybean by Chee et al. (1989). Such “Non in vitro-tissue regeneration” system, i.e. favouring the growth of pre-existing meristems in the explants, seems to be very suitable for application in transformation protocols of pea where, in spite of the chimaeric nature of the regenerated plants, the transgenes were transmitted to the progeny (Bean et al. 1997; Svabova et al. 2005, 2008).

3 Direct Gene Transfer Methods

Direct DNA transfer through physical or chemical methods provides an alternative to *Agrobacterium*, and it is the only way to introduce genes into the chloroplast genome (Clarke et al. 2011).

Examples of direct gene transfer methods are particle bombardment and electroporation, but also polyethylene glycol (PEG) treatment. In particle bombardment, small metal particles are coated with the sequences of interest and are shot into plant cells. In electroporation, plant cells and DNA are together in a solution and an electric stimulus is used to transfer DNA into the plant cells, whereas in PEG-mediated treatment protoplasts (and more rarely cells) are co-cultured with plasmids in the presence of PEG.

3.1 Electroporation

Electroporation is a simple and rapid method that involves applying electrical pulses to a suspension of protoplasts and DNA, placed between electrodes in a suitable cuvette. When a cell is exposed to an electric field, pores are formed through an enhancement of its transmembrane potential (Cole 1968; Neumann and Rosenheck 1973) which depends on the cell radius, the electric field strength delivered and the angle between the normal vector of the membrane and the direction of the electric field applied (Chang 1992). The transient pores in the plasmalemma allow the DNA to enter the cell and nucleus. The method has been used to introduce genes into protoplasts isolated from a range of different species and seems to be a universal method of gene transfer into prokaryotic and eukaryotic cells.

Developing procedures by which plants could be efficiently and successfully regenerated from single cells (protoplast) and organised tissues is the prerequisite for practical genetic engineering through electroporation-mediated protoplast transformation and for crop improvement. Protoplast regeneration requires techniques for efficient and reproducible protoplast isolation, induction of sustained protoplast proliferation in culture and shoot morphogenesis in resulting calli (Ochatt and Power 1992).

In pea, the major limitation of recovering stable transformants by protoplast electroporation was the requirement for an efficient protoplast-to-plant regeneration scheme. Puonti-Kaerlas et al. (1992) stably transformed protoplasts from two different pea cultivars (Belman and Filby) by direct gene transfer using electroporation; they recovered transgenic calli when hygromycin resistance was used as the selective trait, but no transformants were obtained when kanamycin resistance was used as selective marker. *Gus* gene was used to assess transformation efficiency using histochemical staining, and the transgenic nature of the calli selected for resistance against antibiotics was confirmed by DNA analysis. The effect of the field strength on survival and division rates of the protoplasts was also studied. Unfortunately, plants could not be regenerated from the transformed calli.

Lehminger-Mertens and Jacobsen (1993) and Böhmer et al. (1995) described the successful regeneration of plants from leaf and lateral shoot bud protoplasts of pea (*Pisum sativum*). Thus, protoplast-derived calli regenerated shoots but were unable to produce roots and could only be induced to develop fertile plants following grafting onto recipient pea seedlings as rootstocks. Later on, Ochatt et al. (2000) isolated and cultured viable protoplasts from five pea genotypes in an attempt to predict their expressable totipotency. The protoplast-derived tissues exhibited great differences in proliferation and their competence to regenerate shoots, both within and between genotypes. Flow cytometric analysis of DNA content of calluses at different developmental stages showed a correlation between endoreduplication and the ability to regenerate shoots, since fertile plants were regenerated only from calluses with a normal DNA level. This technique can serve as a tool for the early prediction of plant regeneration competence from protoplasts.

Electroporation-mediated gene transfer is a simple and rapid method involving application of electrical pulses to a suspension of protoplasts and DNA placed between electrodes in a suitable cuvette. However, it depends upon certain physical and chemical forces to introduce foreign genetic material into the host genome, including capacitance and field strength, duration and shape of electrical pulses, buffer composition and temperature, type of chimaeric gene constructs and the concentration and form of DNA.

During electroporation, weak electric fields are generally insufficient to create pores in the membrane and thus to promote incorporation of the plasmid and expression of the reporter gene in electroporated protoplasts. On the other hand, too strong an electric field will provoke irreparable plasma membrane damage, due to irreversible membrane breakdown, and thus causing extensive cellular death and virtually no transformation. Maximum transient gene expression has been reported under electric field strengths causing more than 50 % reduction in protoplast viability (Fromm et al. 1985; Hauptmann et al. 1987; Oard et al. 1990; Quecini et al. 2002).

Despite protoplasts being wall-less cells, which renders them sensitive to stress (particularly mechanic and osmotic ones), electroporation has been reported as a means of increasing a certain number of cellular processes in protoplasts and tissues. Stimulation of direct embryogenesis was reported in protoplast-derived *Medicago* tissues (Dijak et al. 1986). Likewise, electroporated protoplasts of a range of species, including legumes, exhibited an increased rate of cell division and faster and sustained microcallus growth compared to non-electropulsed protoplasts (Rech et al. 1987). Ochatt et al. (1988) reported a higher frequency of plant regeneration and root and shoot proliferation from electroporated protoplasts of the cherry rootstock Colt, and a similar response was observed with the medicinal species *Solanum dulcamara* (Chand et al. 1988). More recently, electroporation was shown to improve the production of embryos from isolated microspores of pea and other legumes (Ochatt et al. 2009) and also from intact anthers (Ribalta et al. 2012). Rech et al. (1988) observed a significant increment of DNA synthesis in electroporated protoplasts, while specific transgene amplification in shoots regenerated from electroporated protoplasts has also been reported in *Stylosanthes guianensis* (Quecini et al. 2002).

Transient gene expression following protoplast electroporation was reported only seldom in pea (Hashimoto et al. 1992; Puonti-Kaerlas et al. 1992; Ochatt et al. 2005), and the determination of the optimum electrical parameters permitting entry of the foreign information into protoplasts whilst permitting their subsequent viability and proliferation competence was a prerequisite for success in this domain. Conversely, there are quite a few examples of electroporation applied for transient and (less frequently) stable gene transfer in other legume species, as will be discussed crop-wise below.

3.2 Biolistic

Transgene delivery into pea plant cells by the biolistic approach was reported by several authors (Molnár et al. 1999; Warkentin et al. 1992). Recently, a series of

experiments were carried out with Biolistic® PDS-1000/He Particle Delivery System (Biorad laboratories, Hercules, USA.) in our laboratory to check the mean frequency of transformation. Alternatively, integrated transformation by using particle bombardment in combination with *Agrobacterium*-mediated approach (Droste et al. 2000) was tested. Transient *gus* gene expression after 7 days was shown by 6 % of explants after biolistic and by 33 % after composite approach. The limiting factor in this system was the necessity to use relatively little objects to put into the biolistic device. We had to use culture of apical meristems where the mere regeneration capacity is limited to 5 %, and a further decrease of explant viability was caused by application of the transformation protocol which led us to reject use of the biolistic method for mass production of transformants.

The use of the biolistic approach for successful stable transformation was first demonstrated with soybean (McCabe et al. 1988; Christou et al. 1989), and this approach has since been exploited on several occasions in this crop, as recently reviewed by Dickins et al. (2003). Likewise, particle bombardment has yielded transgenic plants of bean (Russell et al. 1993; Aragao et al. 1996; Aragao and Rech 1997), alfalfa (Filipe Pereira and Erickson 1995), peanut (Wang et al. 1998), faba bean (Ismail et al. 2001; Metry et al. 2007), chickpea (Kar et al. 1997) and *Vigna* species (Bhargava and Smigocki 1994). All these examples will be discussed individually in the sections devoted to each of these species below.

In the coming paragraphs we shall discuss the state of the art on *Agrobacterium*-mediated gene transfer of grain legumes in recent years and focus also on direct gene transfer in this group of species, aimed at both stable and transient genetic transformation.

4 A Brief Retrospective of Genetic Transformation of Legumes

Production of transgenic plants has been reported in a broad range of legume species (reviewed by Somers et al. 2003), including pea, and despite being generally regarded as recalcitrant to transformation (Ochatt et al. 2000, 2005; Svabova and Griga 2008), some appeared easier to transform than others (Somers et al. 2003).

Even when many of the available adventitious regeneration systems have been used for transformation of legumes (Somers et al. 2003; Ding et al. 2003), the most successful regeneration methods to date have been based on non-adventitious regeneration. This is probably so because, in legumes, adventitious methods have a very low efficiency (Ochatt et al. 2000; Somers et al. 2003), are only applicable to a few genotypes within a species (Trinh et al. 1998; Ochatt et al. 2000; Chabaud et al. 2007) or risk yielding plants of low quality due to somaclonal variation (Ochatt et al. 2001; Ochatt 2008).

Below, we give a brief overview of the methods and results of gene transfer in both forage and grain legumes.

4.1 Forage Legume Crops

4.1.1 Alfalfa

Alfalfa (*Medicago sativa* L.) is the most important forage pasture legume cultivated in the world and has been the object of a number of transgenesis studies (Table 1).

Atanasov and Brown (1984) reported a system for successful regeneration of plants from protoplasts from either leaf mesophyll or cotyledon-derived cell suspension cultures, which divided and formed colonies on Kao medium, followed by somatic embryo formation on a high auxin/low cytokinin medium. Monteiro et al. (2003) also reported plant regeneration from leaf-derived protoplast cultures of several alfalfa cultivars via somatic embryogenesis. Because of its high efficiency, this procedure can be used for electroporation-mediated protoplast transformation of alfalfa.

A preliminary study of transient gene expression in alfalfa protoplasts for functional analysis of *cis*-elements affecting expression of an elicitor-inducible bean chalcone synthase gene (*CHS*) promoter was carried out by Harrison et al. (1991). After electroporation of cell suspension protoplasts, they were able to identify the regulatory sequences for protein binding in CHS promoter using chloramphenicol acetyltransferase (CAT) assay.

Du et al. (1994) demonstrated *A. tumefaciens*-mediated transformation in highly embryogenic clones of alfalfa. Petiole and stem explants were transformed with different *A. tumefaciens* strains harbouring various vectors with *nptII* as selectable marker gene. Transgenic plants, from kanamycin-resistant callus, were verified for transgene integration by PCR and Southern hybridisation but no information on seed formation and progeny was provided. Petiole explants gave the best results and A281 was the most effective *A. tumefaciens* strain.

The first report on production of transgenic alfalfa expressing insecticidal protein was presented by Thomas et al. (1994). Petioles and leaf segments were inoculated with *A. tumefaciens* strain LBA 4404 with pAN70 plasmid, containing cDNA encoding the anti-elastase proteinase inhibitor (PI) from *Manduca sexta*. Transgenic plants developing from kanamycin-resistant callus were proven for their transgenic nature by Southern blot analysis. Western blot analysis confirmed the expression of anti-elastase protein, which was also evident by reduced thrip damage on transgenic plants. Mendelian segregation of *nptII* and *PI* gene was observed in progeny while transformation efficiency was 10 %.

Desagnés et al. (1995) described a strategy for genetic transformation of commercial breeding lines of alfalfa. Leaf discs were co-cultivated with *A. tumefaciens* strains C58, A281, LBA4404 harbouring plasmid with *nptII* gene as selectable marker. Kanamycin-resistant plants, developed from callus via somatic embryogenesis were screened for transgene integration by PCR, Southern hybridisation and recalling assays. When they crossed kanamycin tolerant T₀ parents, transgene inheritance to the progeny followed a segregation ratio near 1:1, consistent with a single pseudo-dominant gene being responsible for the kanamycin

Table 1 Alfalfa (*Medicago sativa* L.) transformation

<i>Agrobacterium</i> strain ^a	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
LBA4404	Regen-SY	Leaves	<i>GUS</i>	–	Samac et al. (2004)
<i>A. rhizogenes</i> NCPPB 1855, pUC19	Europe and Adriana	Roots	<i>Agropine/T-DNA</i>	–	Spano et al. (1987)
C58-R1000	<i>Not given</i>	Protoplasts	<i>CAT</i>	–	Harrison et al. (1991)
	Rangelander, Regen-S/C2-4	Petiole and stem	<i>T-DNA, NPT-II</i>	20	Du et al. (1994)
LBA4404	Moapa 69 and Cuf-IO1	Leaf and petiole	<i>nptII</i>	10	Thomas et al. (1994)
LBA4404	RYS1 and RSY27	Ovaries and leaves	<i>nptIII/GUS</i>	–	Micallef et al. (1995)
LBA4404	Regen-SY-27	Leaves	<i>nptII</i>	20–50	Austin et al. (1995)
C58, A281, LBA4404	1.5, 8.8 and 11.9	Leaves	<i>nptII</i>	60	Desgagnés et al. (1995)
A281 and LBA 4404	Zajcarska 83	Somatic embryos	<i>nptII</i> and/or <i>GUS</i>	30	Ninkovic et al. (1995)
pKANGUS and pFFI9K	C2-4	Petioles and stems	<i>nptIII/GUS</i>	–	Pereira and Erickson (1995)
LBA 4404, ABI and AGL1	Rangelander, <i>M. falcata</i> Ladak,	Leaves	<i>nptIII/GUS</i>	60	Samac (1995)
<i>A. rhizogenes</i> 9402	Baoding, Rangelander	Hairy roots	<i>NPT II</i>	43–60	Lü et al. (2000)
LBA4404 and EHA101	<i>M. falcata</i> 47/1-5 and 47-150	Leaves	<i>nptIII/GUS</i>	5–10	Shao et al. (2000)
LBA4404/pBI11	Zajcarska 83	Immature embryos	<i>hpt gene/GUS</i>	1–14	Ninkovic et al. (2004)
LBA4404/pTOK233					
LBA4404	Regen-SY	Leaves	<i>nptIII/GUS</i>	–	Samac et al. (2004)
vector pLITAB357	Regen-SY	Leaf disks	<i>nptII</i>	–	Tesfaye et al. (2005)
vector PSG529	RegenSY-27	Leaves	<i>NptII</i>	–	Calderini et al. (2007)
pPZP-hemL-nptII LBA4404	RSY1	Leaves	<i>NptII</i>	54	Rosellini et al. (2007)
LBA 4404	WL357HQ	Young seedlings	<i>GUS</i>	17.5	Weeks et al. (2008)
LBA 4404 vector p2GS-P5CS	Xinjiang Daye	Cotyledons, hypocotyls	<i>PPT</i>	6	Zhang et al. (2010)
LBA4404 and AGL1	RSY1	Leaves	<i>nptIII/GUS</i>	10.4	Ferradini et al. (2011a)
AGL1	RSY1	Leaves	<i>MsGSAgr</i>	42–43	Ferradini et al. (2011b)

^aAll strains of *A. tumefaciens* unless indicated otherwise

tolerance. This study demonstrated that when genotypes are carefully screened for regenerability and when the best strain/genotype combinations are used, the success rates of alfalfa transformation could be maximised.

In order to observe the strain specificity of *A. tumefaciens* for alfalfa transformation, Samac (1995) inoculated greenhouse plants with several *A. tumefaciens* wild-type strains. The genotypes showed varying degree of susceptibility and resistance to these strains calculated by observation of stem tumour induced in inoculated plants. She then carried out in vitro transformation of leaf disc explants from different genotypes to verify the transformation capacity of disarmed *A. tumefaciens* strains i.e. LBA 4404, ABI and AGL1. Based on GUS screening, a significant genotype–strain interaction was observed, with more transformants when genotypes were transformed by strain LBA4404 than with either of the other strains.

In order to produce high levels of industrially important enzymes, Austin et al. (1995) developed genetically engineered alfalfa expressing bacterial and fungal proteins by inoculating leaf discs with *A. tumefaciens* strain LBA4404 containing plasmids with genes coding manganese-dependent lignin peroxidase (Mn-P) from the fungus *Phanerochaete chrysosporium* and alpha-amylase mature protein from bacterium *Bacillus licheniformis*. Transgenic plants (produced in 10–12 weeks with transformation efficiency ranging from 20 to 50 %) were screened for kanamycin resistance. Expression of *B. licheniformis* alpha-amylase was confirmed by alpha-amylase assay and that of *P. chrysosporium* Mn-P by western blot analysis. Field performance of the transformants was assessed. Mn-P was shown to segregate in sexual progeny derived after crossing transgenic plants with wild-type ones.

Micallef et al. (1995) compared the previously described alfalfa transformation method using leaf discs (Austin et al. 1995) with a new one using excised ovaries as explants. They co-cultured the explants with *A. tumefaciens* strain LBA4404 harbouring vector pDM4715 containing *nptII* under the control of the CaMV35S promoter and *gus* driven by the MAC promoter. Transgenic, kanamycin resistant, plants were obtained from callus induced from explants. Transformation of ovaries was less efficient and more time consuming in terms of callus production compared with transformation of leaf tissue. Backcrossing of transformants with a cultivated variety was carried out to improve transgenic alfalfa. Heritability studies of transgene by using *gus* as a dominant genetic marker suggested that three backcrosses were optimal as the progeny contained 94 % cultivar germplasm and could be used as parents of a new cultivar.

Use of biolistic approach for successful stable transformation of alfalfa was reported for the first time by Pereira and Erickson (1995), who bombarded calli derived from petiole and stem sections with vectors harbouring *nptII* and *gus* as selectable marker. Transformants developed from kanamycin-resistant calli were assayed for *GUS* gene expression by histochemical and fluorometric assays. PCR and Southern hybridisation confirmed transgene insertion and integration in the genome. The transgene was segregated in Mendelian fashion but regeneration and transformation frequency was considerably low.

First evidence of alfalfa hairy root transformation was reported by Spano et al. (1987) when plant stems from different alfalfa genotypes were inoculated with

A. rhizogenes strain NCPPB 1855, harbouring the agropine type Ri plasmid pRi 185. Adventitious hairy roots developed regardless of genotype and gave rise to calli. Auxin (2,4-D or NAA) was shown to inhibit hairy root growth while promoting callus formation directly from inoculated explants. Plant regeneration, via somatic embryogenesis, was only achieved with a highly regenerable genotype. When compared with non-transformants, T-DNA from Ri plasmid completely changed the root structure in transgenic plants. The same phenotypic alteration of transgenic plants from wild types was observed by Lü et al. (2000) when transformation of alfalfa was carried out with the aim of improving sulphur-amino acid content. Cotyledon explants were infected with *A. rhizogenes* strain 9402 with plasmid pBF649 containing a gene encoding protein of high sulphur-amino acid content (HNP) under CaMV35S promoter and *nptII* gene as selectable marker under control of nos promoter. Kanamycin-resistant plants derived from somatic embryos were proven for transgene insertion and integration by Northern hybridisation and recallusing assay. Assay of *HNP* gene showed that transgenic plants had significantly higher contents of sulphur amino acids compared with the control. They also demonstrated a negative correlation between age of hairy roots and embryogenesis frequency in alfalfa plants. Segregation of transgene in the progeny was Mendelian.

The susceptibility of alfalfa somatic embryos to *A. tumefaciens* infection was reported by Ninkovic et al. (1995). They selected somatic embryos as explants source for transformation of alfalfa via *A. tumefaciens* strains A281 and LBA 4404. Well-developed embryos were separated and cloned. Under constant kanamycin selection, clones were selected for proliferation by repetitive somatic embryogenesis. Transgenic nature of kanamycin-resistant clones was confirmed by *nptII* and/or GUS assay and Southern hybridisation. Unfortunately no seeds resulted from these transformants as all plants died due to adverse weather conditions when transferred to soil. By modifying and improving this protocol, Ninkovic et al. (2004) were able to regenerate high frequency of transformed alfalfa plants when somatic embryos were transformed with *A. tumefaciens* LBA4404 carrying the novel superbinary vector pTOK233. Based on hygromycin selection, up to 14 % transformation efficiency was achieved for *M. sativa* L. cv. Zajecarska 83. Such high transformation efficiency was attributed to the presence of an extra set of *vir* genes in superbinary vector pTOK233.

Shao et al. (2000) developed and compared two efficient regeneration protocols after transformation of tetraploid lines of alfalfa (*M. falcata* L.). Leaf explants transformed with LBA4404 and EHA101 harbouring different plasmids, all containing *nptII* as selectable, regenerated kanamycin-resistant plants either via direct somatic embryogenesis or via indirect production of somatic embryos from embryogenic callus. The former regeneration path proved to be more efficient but both systems led to production of transgenic plants as confirmed by recallusing assay on kanamycin, PCR and Southern blot analysis.

Samac et al. (2004) compared the activity of five constitutive promoters by *A. tumefaciens*-mediated stable transformation in the highly regenerable alfalfa clone Regen-SY. Transformation was carried out as described by Austin et al.

(1995). Two marker genes, i.e. *gus* and endochitinase gene (*ech42*) from biocontrol fungus *Trichoderma atroviride* conferring resistance against fungal pathogens were used to monitor the expression driven by promoters in different plant tissues. Based on GUS expression (measured by histochemical staining and quantified by fluorometric assay) and endochitinase activity, cassava vein mosaic virus (CsVMV) promoter was suggested to be useful for high level transgene expression in alfalfa. Although heterologous protein activity in tissues of transformed plants was several fold greater than in controls, none of the transgenic plants showed a consistent increase in disease resistance compared to controls when inoculated with *Phoma medicaginis* var. *medicaginis*, the causal agent of spring black stem and leaf spot in alfalfa.

Using the same protocol as Austin et al. (1995), the same endochitinase gene (*ech42*) fused in frame of a white lupin signal peptide acid phosphatase (APase) under CsVMV promoter was heterologously expressed in alfalfa by Tesfaye et al. (2005). Signal peptide caused the expression of endochitinase in plant rhizospheres. Relative RT-PCR was employed for determination of endochitinase mRNA transcript level in leaf and root tissue of transgenic alfalfa plants.

In order to exploit the potential to improve the quantity and quality of alfalfa forage, onset of leaf senescence was efficiently delayed in alfalfa when leaf disc explants were inoculated with *Agrobacterium* culture containing *ipt* gene translationally fused with the senescence-specific promoter SAG12 (Calderini et al. 2007). Transgene integration into transformants was determined by Southern analysis while expression of transgene was measured by RT-PCR. Transformed plants stayed green for a longer time hence providing forage for longer.

Rosellini et al. (2007) reported the development of an herbicide/antibiotic marker free genetic transformation system in alfalfa. A novel selectable marker gene (SMG) *hemL* encoding a mutant form of the enzyme glutamate 1-semialdehyde aminotransferase (GSA-AT) was transferred via *A. tumefaciens* [with the protocol by Austin et al. (1995)] and its efficiency was then compared with *nptII* selection system. Gabaculine (the selective substance for *hemL* gene) and kanamycin (the selective substance for *nptII* gene) were used to screen the transformants. Gabaculine-based system was more efficient than the conventional, kanamycin-based system. Inheritance of *hemL* gene in the progeny was Mendelian.

Since last 2 decades, an extensive research rendered the genetic transformation of Alfalfa possible, but it remains strongly genotype dependent and is restricted to a few highly regenerable genotypes. A simple, efficient, genotype independent and marker free *in planta* transformation protocol for successful alfalfa transformation was developed by Weeks et al. (2008). Young decapitated seedlings were immersed and vacuum infiltrated or vortexed vigorously in *A. tumefaciens* LBA 4404 suspension supplemented with sand. Rigorous vortexing significantly enhanced transformation frequencies over vacuum infiltration. Developing plants were assayed for GUS expression by histochemical staining and fluorometric analysis. This novel method has several advantages over conventional methods as it limits the time, materials and resources needed and is applicable to commercial varieties.

The capacity of regeneration of various local alfalfa cultivars was evaluated using different explant sources on different media by Zhang et al. (2010). Xinjiang Daye cultivar showed better frequency of callus formation while using hypocotyl explants. Frequency of callusing and shoot differentiation was further increased on medium with glutamine, shortening the period of regeneration. They then used this system to transform alfalfa via *A. tumefaciens* strain LBA 4404 containing vector p2GS-P5CS carrying two glutamine synthetase (GS) genes. Basta-resistant transgenic plants were proved for transgene integration by PCR analysis and Southern hybridisations. Effective expression of GS in transformants suggested it as a selectable marker in plant genetic transformation for screening of transformants hence avoiding the use of herbicides or antibiotic resistance genes.

Ferradini et al. (2011a) compared two different marker free *Agrobacterium*-mediated transformation methods in alfalfa, i.e. marker-less transformation and co-transformation. In the former, leaf explants were transformed with a vector carrying *nptII* and *gus* markers. Somatic embryos were regenerated without selection followed by kanamycin selection for the second cycle of regeneration from these embryos. The percentage of transgenic embryos was determined by GUS staining and PCR screening of T₁ progenies. In the latter technique, explants were co-transformed with two vectors carried separately in *Agrobacterium* cultures. After crossing the primary transformants with a non-transgenic pollinator, marker-free segregants were obtained in T₁ progenies, confirmed by Southern hybridisation. Although the transformation efficiency using both marker-free approaches was low, these methods can be used as a tool for production of marker-free plants.

Instead of using bacterial-derived SMGs during genetic transformation, Ferradini et al. (2011b) used an alfalfa-derived mutant of glutamate 1-semialdehyde aminotransferase (*MsGSAgr*) gene and compared its efficacy with mutated *Synechococcus elongates* GSA (Rosellini et al. 2007). Inoculated explants produced green embryos in the presence of 30- μ M gabaculine while all controls died on this media. Stable integration and expression of *MsGSAgr* were assessed by tailed PCR and gabaculine resistance. This novel plant-based marker system is promising for future studies.

4.1.2 Barrel Medic

Successful genetic transformation of *Medicago truncatula* was reported for the first time just under 20 years ago (Thomas et al. 1992; Table 2). *M. truncatula* was proposed as a model system for legume genomics (Cook 1999), taking advantage of the superior in vitro regeneration (via somatic embryogenesis) and transformation characteristics of a few genotypes, among which are Jemalong 2HA, M9-10a and R108-1. Indeed, the limiting step in the entire process is generally the in vitro regeneration of transformed cells into transgenic plants. Transformation protocols can be classified according to the nature of the regeneration process either via embryogenesis or organogenesis. The most efficient protocols currently available use embryogenesis. However, the efficiency of somatic embryogenesis is highly

Table 2 Barrel medic (*Medicago truncatula* Gaertn.) transformation

<i>Agrobacterium</i> strain ^a	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
EHA101	Jemalong	Leaves	<i>npII</i>	1 plant recovered	Thomas et al. (1992)
LBA4404	Jemalong A17	Cotyledons	<i>Bar</i> , <i>npII</i>	3.11	Trieu and Harrison (1996)
LBA4404	Jemalong A17, 2HA1, 2HA3-9, 10-3	Leaflets	<i>npIII</i> , <i>GUS</i>	2–29	Chabaud et al. (1996)
A281	R 108-1, Ghor, 131-1, 139-2, E4258	Hypocotyls, roots, petioles, leaflets	<i>GUS</i>	4.5–5	Hoffman et al. (1997)
EHA105	R108-1(c3)	Young leaves	<i>GUS</i>	2–3	Trinh et al. (1998)
EHA105	Jemalong A17	Young seedlings, flowering plants	<i>npIII</i> , <i>bar</i>	4.7–76 % (flower infiltration) and 2.9–27.6 (seedling infiltration)	Trieu et al. (2000)
<i>A. rhizogenes</i> A4T pLP100 pM1ENOD11–gusA binary vector	Jemalong J5	Hypocotyls, epicotyls and seedling radicles	<i>npIII</i> , <i>GUS</i>	33–63 (hairy roots)	Boisson-Dernier et al. (2001)
EHA105	R108-1 (c3)	Young leaves	<i>GUS</i>	Not given	Scholte et al. (2002)
EHA105	R-108-1, Jemalong J5	Floral organs	<i>npIII</i> , <i>GUS</i> , <i>GFP</i>	High but not precised	Kamaté et al. (2000)
LBA 4404, C58pMP90, C58pGV2260, AGL1	Jemalong 2HA	Leaflets	<i>npIII</i> , <i>GUS</i>	26	Chabaud et al. (2003)
EHA105 or AGL1 with pCB302-phas-GUS	A17	Cotyledons	<i>PPT</i> , <i>GUS</i> , <i>GFP</i>	3–15	Zhou et al. (2004)
EHA105	Jemalong M9-10a	Leaflets	<i>npIII</i> , <i>GUS</i>	25–45	Araujo et al. (2004)
LBA4404	R-108-1	Embryo clusters from cotyledons and petiole base	<i>npIII</i> , <i>GUS</i>	9	Iantcheva et al. (2005)
AGL1, C58Cl, EHA105, LBA4404	R-108	Root segments	<i>bar</i> , <i>GUS</i>	10.6–41.3	Crane et al. (2006)
EHA105	Jemalong M9-10a	Leaves	<i>GFP</i> , <i>GUS</i>	15.7	Duque et al. (2007)
EHA105	R-108	Leaves	<i>npIII</i> , <i>GUS</i>		Scaramelli et al. (2009)
EHA105	Jemalong M9-10a	Leaves	<i>npIII</i> , <i>ipt</i>	7.8–11.7	Confalonieri et al. (2010)

^aAll strains of *A. tumefaciens* unless indicated otherwise

genotype dependent. Thomas et al. (1992) and Rose et al. (1999) selected and used genotype 2HA, which belongs to *M. truncatula* ssp. *truncatula*, the same as genotype M9-10a used by several authors (Santos and Fevereiro 2002; Araujo et al. 2004; Confalonieri et al. 2010), while Trinh et al. (1998) worked with genotype R108, sometimes assigned to *M. truncatula* ssp. *tricycla* (Chabaud et al. 2007). Being the genotype used for genome sequencing and for mutagenesis and TILLING programs, Jemalong is also the genotype of choice when requiring genetic crossing of transgenic plants with characterised mutants.

In addition to the use of such highly embryogenic genotypes, using hypervirulent strains of *A. tumefaciens*, such as AGL1 or EHA105 (see above), can also significantly increase transformation efficiency. Several agents have been reported for the selection of transformed cells including kanamycin, hygromycin or phosphinothricin (Chabaud et al. 2007).

Hashimoto et al. (1992), Trinh et al. (1998), Scholte et al. (2002), among others, obtained barrel medic transformants using leaves as explants and subsequently regenerating the produced callus into plants through somatic embryogenesis. An efficient transformation procedure for barrel medic has also been developed by Kamaté et al. (2000), where flower parts were used as explants followed by regeneration through embryogenesis, while Trieu and Harrison (1996), aimed at reducing the tissue culture work involved, developed a new method based on cotyledonary node explants, followed by regeneration of multiple shoots from the pre-existing meristems. Some time later, Trieu et al. (2000) reported two *in planta* procedures for transformation based on infiltration of flowers and seedlings but were later unable to reproduce them, nor were they corroborated by other laboratories.

4.1.3 *Lotus japonicus*

Lotus japonicus is a wild legume that has become a model plant for genome studies in legumes, particularly in reference to rhizobial and arbuscular mycorrhizal symbiosis. As such, several teams have undertaken its genetic transformation over the last 20 years (Table 3).

Handberg and Stougaard (1992) optimised the regeneration protocol of *L. japonicus* and then assayed tissue susceptibility to *A. rhizogenes* and *A. tumefaciens*. Hypocotyls challenged with *A. rhizogenes* gave hairy roots capable of regeneration. When transformed with various disarmed strains of *A. tumefaciens*, 90 % of hypocotyl explants gave to transgenic callus based on kanamycin/hygromycin resistance. Hygromycin-based selection was more efficient and rapid. When the wild-type plant was pollinated with pollen from transformed plants, 25 % of plants in the progeny were hygromycin resistant.

In order to study the behaviour of maize transposable elements in *L. japonicus*, Thykjaer et al. (1995) carried out *A. tumefaciens*-mediated transformation with transposable element *Ac* following the protocol reported by Handberg and Stougaard (1992). Kanamycin-resistant calli showed variegated sectors after GUS

Table 3 *Lotus japonicus* L. transformation

<i>Agrobacterium</i> strain ^a /method	Genotype/Cultivar	Explants	Selection agent/marker genes	Transformation efficiency (%)	References
LBA4404, C58, or GV2260	Gifu B-129, B-177	Hypocotyls, leaves	Hygromycin, kanamycin	Not given	Handberg and Stougaard (1992)
SLJ1931 T-DNA	Gifu B-129	Cotyledons	<i>npII</i> , <i>GUS</i>	42	Thykyjaer et al. (1995)
C58CI	Gifu	Plantlet stems at cotyledon	<i>npII</i>	Not given	Oger et al. (1996)
LBA4404	Gifu B-129-56	Hypocotyls	<i>GUS</i>	70	Stiller et al. (1997)
MSU440	Gifu	Roots, hypocotyls	<i>GUS p35S-hpt-tml</i>	23.5, 13.3	Martirani et al. (1999)
AGL1	Gifu B-129	Hypocotyls	<i>Bar</i>	70	Lohar et al. (2001)
EHA101	Gifu B-129	Hypocotyls	<i>GUS/HPT</i>	11–20	Aoki et al. (2002)
<i>A. rhizogenes</i> LBA1334	Gifu B129	Hairy roots	<i>GUS</i>	60	Kumagai and Kouchi (2003)
AGLO and AGL1	GIFU B-120	Roots	<i>Hpt/GUS</i>	58	Lombari et al. (2003)
LBA 4404/GPTV	Gifu	Hypocotyls	Geneticin	90	Edwards et al. (2004)
LBA 1334	Gifu	Hypocotyls	<i>GUS</i>	62	Diaz et al. (2005)
LBA4404	MG-20	Hypocotyls	<i>GUS</i>	Not given	Kato et al. (2005)
<i>A. rhizogenes</i>	Gifu ecotype F9	Roots	<i>Hygromycin/GUS</i>	Not given	Lombari et al. (2005)
<i>A. tumefaciens</i>	Not given	Hypocotyls	<i>Hygromycin/GUS</i>	Not given	Tirichine et al. (2005)
<i>A. rhizogenes</i> , <i>A. tumefaciens</i>	Not given	Hypocotyls	<i>npII</i> , <i>hptII</i> , <i>bar</i>	Not given	Udvardi et al. (2005)
EHA105, AGL1, LBA4404	Gifu B-129	Hypocotyls, cotyledons	<i>Hygromycin</i>	0.9–12.8	Wang et al. (2010)

^aAll strains of *A. tumefaciens* unless indicated otherwise

staining, hence showing somatic activity of *Ac* in *L. japonicus* calli. Regenerated primary transformants were selfed and GUS expression in the cotyledons of the progeny suggested that maize transposable elements were mobile in *L. japonicus* and that the transposition mechanism operating in *L. japonicus* was similar to that in maize. Mobility and reinsertion of *Ac* element were also proved by Southern hybridisation.

Oger et al. (1996) developed a novel method of genetic transformation based on a hormone independent regeneration system. They inoculated decapitated cotyledons with a disarmed *A. tumefaciens* strain harbouring plasmid carrying nopaline synthase (*nos*) gene and *nptII* gene conferring kanamycin resistance. Nopaline presence was investigated by high voltage paper electrophoresis analysis. The transgenic nature of the nopaline positive kanamycin-resistant plants was confirmed by PCR and Southern hybridisation but no information about progeny was provided.

The susceptibility of *L. japonicus* to a large number of wild-type strains of *A. rhizogenes* for production of hairy roots was evaluated by Stiller et al. (1997), and strains 9402 and AR10 proved to be most virulent. To assess the applicability of the hairy root system for molecular analysis of plant genes involved in nodulation, hypocotyl explants were inoculated with these strains carrying binary vectors with *uidA* and *luc* as selectable marker genes driven under CaMV35S promoter. About 80 % of hairy root lines regenerated fertile transgenic plants. Expression of *gusA* and *luc* was investigated through histochemical staining and luciferase assay. Seventy percent of *A. rhizogenes*-induced hairy roots were capable of normal nodulation. Martirani et al. (1999) used the high frequency hairy root induction ability of *A. rhizogenes* for T-DNA tagging of nodulation and root-related genes in *L. japonicus*. Wounded root explants were inoculated with *A. rhizogenes* with vector p35S-*gusA*-int. The study of wound sites indicated that transgenic roots developed via direct organogenesis from the primary root near the wound site. Plant lines expressing intense GUS activity in the root meristem and the vascular tissues in nodulation-specific manner were developed from hairy roots induced from inoculated explants. Kumagai and Kouchi (2003) used a hairy root-based transformation system for studying the post-transcriptional gene silencing of genes expressed in roots and nodules of *L. japonicus*. After inoculation with *Mesorhizobium loti*, transgenic plants showed GUS expression in root nodules driven either by CaMV35S promoter or *lj27* promoter (specific for nodule-infected cells). Expression of *gus* transgene in nodules and other root parts was silenced by transforming them with *A. rhizogenes* strain LBA1334 harbouring pHKN30 or pHKN31 plasmid expressing hairpin RNAs with sequences complementary to the *gus* coding region. *Gus* transgene was either completely silenced or its activity was significantly decreased in transformed tissues when its expression was observed in root nodules of hairy roots as compared to control plants. From these results, Lohar and Bird (2003) suggested *L. japonicus* as a powerful model legume to study functional genomics of nodule formation and plant–nematode interactions. The above protocols work well but it took long time for rooting of transformants, hence production of T₀ seeds. To overcome this, Díaz et al. (2005) developed a modified protocol for rapid production of transgenic plants, thus facilitating the study of root

nodule regulating genes. After transformation with *A. rhizogenes*, hairy roots were induced that could be inoculated with *M. loti* after 2 weeks. The nodules thus emerged were comparable to those of wild type, and expression studies of genes involved in nodulation could be completed within 2 months.

Lohar et al. (2001) reported a simple *A. tumefaciens*-based protocol for genetic transformation of *L. japonicus* using herbicide resistance marker. Hypocotyl explants were transformed with *A. tumefaciens* strain AGL1 equipped with binary vector pTAB10 with *bar* as selectable marker gene driven by CaMV35S promoter. PPT-resistant plants checked for transgene insertion by PCR were morphologically normal. The transgene was segregated in the progeny as demonstrated by chlorophenol red assay and phosphinotricin spray.

An optimised *A. tumefaciens*-mediated transformation system of *L. japonicus* based on antibiotic selection and efficient regeneration was developed by Aoki et al. (2002) using strain EHA 101 harbouring binary vector carrying *GUS* reporter gene and two selectable marker genes, i.e. *nptII* and *hpt* conferring resistance to kanamycin and hygromycin, respectively. Hygromycin-resistant shoots were regenerated from calli. This optimised protocol ensured survival of only the transformed plants on selection medium, as *GUS* activity was observed in all of the transformed shoots while all non-transformants were dead.

Lombardi et al. (2003) obtained transgenic *L. japonicus* plants when regeneration-competent dedifferentiated root explants were inoculated with AGLO and AGL1 strains of *A. tumefaciens* containing *hpt* as selectable marker and *gus* as reporter gene. Transformation efficiency as demonstrated by *GUS* expression was several fold than that with the transformation procedure using hypocotyl explants. It took 4 months to get transformed plants. After selfing the primary transformants *hpt* segregated in Mendelian fashion.

Edwards et al. (2004) used the transformation protocol developed by Thykjaer et al. (1995, 1998) for structural manipulation of a plant cell wall polysaccharide by downregulating the Galacto-mannan glycosyltransferases (GMGT) responsible for its polymerisation. *L. japonicus* GMGT cDNA cloned in various orientations driven under dual 35 S promoter was expressed in seed endosperm of transformed plants. Geneticin-based antibiotic selection was employed to obtain transgenic plants. Transgenic lines exhibited galactomannans with higher mannose/galactose values in their seeds, consistent with post-transcriptional gene silencing.

Kato et al. (2005) modified the *A. tumefaciens*-based transformation method for *L. japonicus* genotype MG-20 by optimising hormone concentration (auxin and cytokinin), and used this method for heterologous expression of an allergin protein used in immunotherapy, in mature plants. Hypocotyl explants were transformed with *A. tumefaciens* strain LBA4404 with pDerf1 plasmid containing *Derf1* gene from American house dust mite *Dermatophagoides farina*. Based on Southern hybridisation results, expression of *Derf1* protein in transformants was confirmed by RT-PCR. The size of plant synthesised *Derf1* protein was exactly comparable to that of native *Derf1* purified from *D. farina* proved by Western blotting.

An *A. tumefaciens*-based protocol with improved regeneration was reported by Tirichine et al. (2005). After co-culturing explants with *Agrobacteria*, hypocotyl

callus was selected for geneticin resistance. Shoots were induced from transgenic calli on media with cytokinin (BAP) and were rooted on auxin (NAA) containing media.

Lombardi et al. (2005) compared several methods for genetic transformation of *L. japonicus* using various explants and regenerating putative transformants on different media. When using dedifferentiated roots as explants for *A. tumefaciens*-based transformation, the advantage laid in the availability of explants that can be stored at 4 °C for long time and are hence readily available when needed. When using *A. rhizogenes*-based hairy root transformation method, the study of root or nodulation-related genes is facilitated.

In some previous protocols, transformation frequency remained low due to quite a high rate of false positives. Wang et al. (2010) employed an efficient screening system based on hygromycin followed by regeneration on medium supplemented with auxin (IAA) to efficiently select and regenerate the transformants. They then tested three strains, i.e. LBA4404, AGL1 and EHA105 for their susceptibility to transform *L. japonicus*. Among them, EHA105 which had never been tested previously for *L. japonicus* gave a tenfold higher transformation efficiency than the other two strains. This improved transformation, selection and regeneration system was then used for silencing of different *CYCLOIDEA* genes in *L. japonicus* through RNAi technology.

4.2 Miscellaneous Forage Legumes

Trifolium repens, the white clover, a forage legume native to Europe, North Africa and West Asia is among the first legume species where *A. tumefaciens*-mediated transformation was successfully carried out (White and Greenwood 1987). Stolon internode segments were transformed with two non-oncogenic *A. tumefaciens* strains, LBA4404 and GV3850. Kanamycin-resistant calli were assayed for in situ neomycin phosphotransferase II activity. Transgenic nature of cells and shoots was further assessed by the presence of nopaline from *nopaline synthase* gene as well as *nptII* specific probe hybridisation to DNA fragments. In order to improve the nutritional quality of legumes, Ealing et al. (1992) followed the same protocol to overexpress a chimaeric gene encoding the pea albumin 1 (PAI) protein rich in sulphur amino acids under 35 S CaMV promoter into the white clover mediated via *A. tumefaciens*. In transgenic plants, the abundance and stability of the PAI protein was assessed by immunoselection of in vivo [³⁵S] Na₂SO₄-labelled plant proteins.

Medicago varia, a forage legume also known as bastard alfalfa, is also among the first forage legume species whose successful *A. tumefaciens*-mediated transformation was reported (Deak et al. 1986). Kanamycin-resistant transformed plants were regenerated through somatic embryogenesis from callus induced from stem cuttings. The transgenic nature of developing plants was confirmed by neomycin phosphotransferase activity and southern hybridisation.

Astragalus sinicus, a model forage legume generally known as Chinese Milkvetch, is very widely grown as a green-manure-cum-forage legume. Tryptophan (Trp) is an essential amino acid since it is not synthesised by animals and must be obtained in the diet of non-ruminants such as pigs, poultry and humans. In order to increase the Trp contents in legumes, the *A. rhizogenes*-mediated hairy root transformation of *A. sinicus* was carried out by Cho et al. (2000), overexpressing Trp feedback-insensitive ASA2 gene. Northern-blot hybridisation demonstrated constitutive expression of p35S-ASA2 gene in the transgenics. Expression of the transgene resulted in a 1.3- to 5.5-fold increase in free Trp. Overexpression of this gene in *A. sinicus* can be used as a new tool for studying the regulation of Trp biosynthesis in legumes.

Stylosanthes guianensis, the Brazilian lucerne, is a forage legume cultivated in tropical and subtropical regions of the world. In order to enhance foliar blight resistance, Kelemu et al. (2005) carried out *Agrobacterium*-mediated transformation of rice chitinase gene under the control of CaMV35S promoter in leaf segment explants. Transformed calli were selected on medium containing kanamycin, and regeneration was via somatic embryogenesis. Ten-week-old plants were artificially inoculated with sclerotia and were then evaluated for their reactions to *Rhizoctonia solani*, starting 6 days after inoculation. Thus, transgenic plants had a higher level of resistance to *R. solani* than did control plants. DIG-labelled chitinase probe during Dot blot analysis confirmed the stable inheritance of rice-chitinase gene to the progenies along with resistance to *Rhizoctonia* foliar blight disease.

Lotus corniculatus, the Bird's-foot trefoil, is a perennial, fine-stemmed, leafy legume native to grassland temperate Eurasia and North Africa with and increasing importance in agriculture as pasture and hay crops in recent years. Tanaka et al. (2008) obtained transgenic *L. corniculatus* from Superroot-derived leaves via *A. tumefaciens*-mediated transformation. Transgenic nature of kanamycin-resistant calli was verified by GUS staining, but transformation efficiency was low and the process from gene transfer to PCR identification took 6 months. Jian et al. (2009) developed an improved and highly efficient *A. rhizogenes*-mediated transformation of Superroot-derived *L. corniculatus* by introducing the efficient Superroot regeneration system. Transgenic nature of plants was verified by GUS staining as well as GFP observation, with a frequency up to 92 % based on detection of GUS activity. Transgene integrity and stability were examined by Southern (DIG method) and Western blot analysis.

4.3 Grain Legume Crops

4.3.1 Soybean

Soybean was the first grain legume for which transgenic plants were produced via both *Agrobacterium*-mediated transformation (Hinchee et al. 1988; Parrott et al. 1989; Di et al. 1996) and particle bombardment (McCabe et al. 1988; Christou et al.

Table 4 Soybean (*Glycine max* [L.] Merr.) transformation

<i>Agrobacterium</i> strain/method	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
pTiT37-SE	Century, Cobb, Douglas, Harosoy, Heilongjiang 10, Heilongjiang 26, J103, Lee, Manchui, Manitoba Brown, McCall, Peking, PI 283332, Williams 82	Cotyledons	<i>nptII</i>	6	Hinchee et al. (1988)
LBA4404, EHA101		Immature embryos	<i>nptII</i>	0.03	Parrott et al. (1989)
Plasmid pZA300	Clark 63	Immature cotyledon	<i>Hpt</i> , <i>GUS</i>	0.29–0.68	Dhir et al. (1991)
electroporation		protoplasts			
Biolistic—pMON 10026 or pMON 13671 plasmids	Williams 82, Fayette	Shoot tips from immature green seeds, embryogenic cell suspensions	<i>nptII</i> , <i>GUS</i>	0.4	Sato et al. (1993)
Biolistic—vector pIC35/A with <i>BtCryIAC</i> gene	Jack	Somatic embryos	<i>Hph</i> , <i>BtCryIAC</i>	Many plants recovered	Stewart et al. (1996)
				no precise figure given	
Biolistic—12 plasmids	Fayette	Embryogenic suspension	<i>HPH</i> , <i>GUS</i>	Not given	Hadi et al. (1996)
Z707		Cotyledonary nodes	<i>nptII</i>	1.25	Di et al. (1996)
Biolistics—plasmids 35 S-SGFP-TYG-nos (pUC18) and HBT-SGFP-TYG-nos (pUC18)	Chapman	Embryogen suspension	<i>Hygromycin B</i> , <i>GFP</i>	Not given, no fertile plants recovered	Ponappa et al. (1999)
AGL1	Bert	Axillary buds, cotyledonary nodes	<i>bar</i>	0.7	Olhofs and Somers (2001)
EHA101	Bert	Cotyledonary nodes	<i>hph</i>	16.4	Olhofs et al. (2003)
EHA101		Cotyledonary nodes	<i>bar</i>	2–6	Paz et al. (2004)

(continued)

Table 4 (continued)

<i>Agrobacterium</i> strain/method	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
EHA105	Bert, Harosoy, Jack, Peking, Thorne, Williams, Williams 79, Williams82, Clark, Delsoy 5710, Essex, Ogden Nannong88-1, Nannong 18-6, Yu23, Nannong 87C-38	Cotyledonary nodes	<i>mpII</i> , <i>GFP</i>	1.39–2.20	Xinping and Deyue (2006)
EHA105, LBA4404, KYRT1	Hefeng 25, Hefeng 35, Hefeng 39, Heinong 37, Heinong 43, Dongnong 42, Lefeng 39	Embryonic tips	<i>Bar</i> , <i>GUS</i>	4.29–18	Dang and Wei (2007)
EHA105	Kariyutaka	Cotyledonary nodes	<i>Bar</i> , <i>GFP</i>	1	Sato et al. (2007)
EHA105	Kariyutaka	Cotyledonary nodes	<i>Bar</i> , <i>GUS</i>	4.4	Yamada et al. (2010)

1989), and the plant recovery approaches used ranged from somatic embryogenesis from immature seeds to non-adventitious organogenesis from cotyledonary nodes of seedlings or germinating seeds (Table 4). Nevertheless, genetic transformation of soybean remains non-routine, as most methods show a reduced efficiency of 0.2–2 % and, very rarely, up to 5–6 %. The low efficiency of T-DNA transfer itself and the regeneration systems used to recover transformed plants are the two main limiting factors. The first was improved through the use of more virulent *Agrobacterium* strains (Torisky et al. 1997) and addition of e.g. L-cysteine, dithiothreitol (DTT) and sodium thiosulfate during co-cultivation of explants with the bacteria (Olhoft and Somers 2001; Olhoft et al. 2001, 2003). Conversely, progress made in improving regeneration (Somers et al. 2003) is scanty, as somatic embryogenesis is highly genotype specific and associated with extensive somaclonal variation among regenerated plants (Parrott et al. 1989). Based on proliferation of meristems pre-existing in the cotyledonary node, organogenesis is less genotype dependent and was thus adopted by several groups (Dan and Reighceri 1998). However, meristems are complex structures where meristematic cells represent only a very low proportion of the total cells in the explant, and the recovery of transgenic plants capable of transmitting the target genes to subsequent generations is very low with this method (Christou et al. 1990).

Dang and Zhi-Ming (2007) developed an optimised method for *Agrobacterium*-mediated transformation for expression of binary insect resistance genes, where they examined the effects of several factors by measuring transient expression levels of β -glucuronidase and the number of resistant explants with phosphinothricin selection. The hypervirulent *A. tumefaciens* strain KYRT1 proved to be better than EHA105 and LBA4404. Improved transformation efficiencies were obtained when embryonic tips were incubated with an *Agrobacterium* suspension for 20 h, in an acidic medium, and then co-cultivated at 22 °C in the dark for 5 days. By combining the best treatments, transgenic plants of seven soybean cultivars were obtained, most of which were fertile, with a transformation frequency ranging from 4.3 to 18 %. Analysis of T1 plants showed inheritance and stable integration of transgenes, coupled with a high resistance to cotton bollworm. More recently, Yamada et al. (2010) used cotyledonary nodes wounded with a micro-brush whereby they increased the frequency of transformation.

Direct transformation by particle bombardment has been reported on soybean (Dickins et al. 2003). The first transgenic soybean plant developed by direct gene delivery was created in 1988 (McCabe et al. 1988). The first commercially available transgenic Roundup Ready soybean was also developed by particle bombardment (Padgett et al. 1995). Due to the importance of soybean as a crop, several other transformation methods were examined, including whole plant transformation, pollen tube and pollen transformation, *in planta* electroporation or protoplast-based transformation (Dickins et al. 2003, and references therein).

Paz et al. (2004) studied the conditions required for an efficient *Agrobacterium*-mediated transformation of cotyledonary node explants. They were able to increase the transformation efficiency up to threefold by using cysteine and DTT during co-cultivation. Similar results of increase in transformation efficiency were obtained

by Xinping and Deyue (2006) who transformed multiple soybean cultivars by infecting cotyledonary nodes with *A. tumefaciens* while adding thiol compounds during co-cultivation. Zeng et al. (2004) refined the use of antioxidants during co-cultivation and glufosinate selection in *Agrobacterium*-mediated transformation of soybean genotypes which are otherwise difficult to transform.

Sonication of explants has also been used before co-cultivation to improve the transformation efficiency. Santarem et al. (1998) reported an optimisation of transient gene expression following the sonication of immature cotyledons co-cultured with *Agrobacterium* strains.

Townsend and Thomas (1996) patented a method for the *Agrobacterium*-mediated transformation of cultured soybean cells. Among several factors identified, they discovered that temperature during co-cultivation of explants with agrobacteria is an important factor for efficient transformation. They showed that usual temperatures (26–28 °C.) for the culture of soybean cells are inappropriate for efficient transformation. Conversely, a lower temperature resulted in highly effective transformation.

4.3.2 Common Bean

Phaseolus vulgaris usually known as common/dry bean is amongst important food legume serving as an important source of protein and calories in the developing world. Veltcheva et al. (2005) reviewed the problems and progress for in vitro regeneration and genetic transformation of common bean (Table 5).

In 1991, McClean et al. demonstrated the susceptibility of a wide range of dry bean genotypes to infection by *A. tumefaciens* strains A 208, A 281 and LBA 4001. They also transformed cotyledonary nodes of *P. vulgaris* cv Othello with *A. tumefaciens* strain C58Z707 but were unable to regenerate plants from kanamycin-resistant calli. They also established kanamycin-resistant root cultures from hypocotyl tissues infected with the *A. rhizogenes* strain A4RS. Transgenic nature of calli and roots was confirmed by Southern hybridisation.

Russell et al. (1993) used electric-discharge-mediated particle acceleration to produce transgenic navy beans. Apical meristems were bombarded with DNA-coated gold particles under partial vacuum and high voltage discharge. Transgenic plants were recovered through de novo shoot formation. Since the DNA was delivered to organised tissue, many of the problems associated with recovering plants from protoplasts or callus were avoided. Multiple shoots were screened for GUS enzyme activity through histochemical staining followed by PAT activity by herbicide spraying to recover transgenic plants at a rate of 0.03 % germline transformed plants/shoot. DNA presence was confirmed by PCR and Southern blot analysis.

Particle bombardment for production of transgenic dry bean was conducted by Aragao et al. (1996). They co-transformed different genes linked (in the same plasmid) or unlinked (in different plasmids) coated on tungsten particles into embryonic axes using a locally built high pressure helium-driven particle

Table 5 Common bean (*Phaseolus vulgaris* L.) transformation

<i>Agrobacterium</i> strain/method	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
A208, A281, LBA4001, C58Z707, A4RS	Pinto, black, white, kidney and Cranberry types	Cotyledonary nodes, hypocotyls	<i>nptII</i>	Not given	McClean et al. (1991)
Biolistic	Seafarer	Apical meristems	<i>bar</i> , <i>GUS</i> , <i>PAT</i>	0.03 germline transformed plants/shoot	Russell et al. (1993)
Biolistic	Olathe	Eembryonic axes	<i>GUS</i>	0.9	Aragao et al. (1996)
C58CIR	Admires, Great Northern Tara, Xan-159	Cotyledonary leaves	<i>nptII</i> , <i>aadA</i> , <i>GUS</i>	Not given	Kapila et al. (1997)
Biolistic	–	Embryonic axes	<i>bar</i>	0.3–0.8	Vianna et al. (2004)

acceleration device. Transformed plants were continuously selected on kanamycin and GUS activity. The average frequency of transformation was 0.9 %. The progeny of the self-fertilised transgenic plants was screened by PCR analysis and southern blotting. Seventy-seven percent of these transformed plants segregated in a Mendelian fashion.

Biotic stress plays an important role in reducing bean yields, and Aragao and Rech (1997) transformed apical meristems by the biolistic method with a construct containing viral antisense RNAs and regenerated them via organogenesis. The transgenic plants showed delayed and faded symptoms of Bean golden mosaic geminivirus (BGMV).

Development of an efficient transient gene expression system is indispensable in order to study the expression of different gene promoters and proteins. Kapila et al. (1997) optimised vacuum infiltration-based *A. tumefaciens*-mediated transient expression in bean leaves. Higher final *Agrobacterium* cell density ($OD_{600} = 2.4$) proved to be an important factor for the success of transient expression in bean leaves.

Optimisation of factors influencing *A. tumefaciens*-mediated transformation using several genotypes of common beans was studied by Zhang et al. (1997). When explants derived from mature seeds of susceptible genotypes were injured, precultured and then transformed with *A. tumefaciens* strain A2760, a transformation efficiency of 4 % was achieved as proven by GUS staining. Genotype, explant (age, type and source), pre-cultivation conditions, *Agrobacterium* strains, co-cultivation conditions, selection system and conditions for regeneration are important factors influencing *A. tumefaciens*-mediated transformation (Nagl et al. 1997).

Development of vector free genetic transformation system of legumes and hence avoiding the presence of antibiotic resistance genes is very desirable. Vianna et al. (2004) developed transgenic bean plants by introducing a 1.5-kb linear DNA fragment carrying the *bar* gene using the biolistic method of Aragao et al. (1996).

Transformation frequency was comparable with that using entire circular plasmid. Southern blot analyses revealed a similar pattern complexity in transgenic plants obtained with either the entire plasmid or a DNA fragment. Stable transmission of transgene to the progeny followed the Mendelian ratio (3:1). This method presented a novel approach to get transgenic legumes containing only the gene responsible for a desirable trait.

4.3.3 Peanut

Direct DNA delivery via microprojectile bombardment has become an established approach for gene transfer into peanut (*Arachis hypogaea* L.) (Table 6). Wang et al. (1998) bombarded embryogenic cultures from three peanut cultivars with two plasmid constructs containing a uidA gene controlled by either a soybean vegetative storage protein gene promoter or a CaMV35S promoter. GUS transient expression proved useful to predict stable transformation and confirmed that image analysis provides an efficient method for semi-quantitation of transient expression. They regenerated over 200 transgenic plants from 38 cell lines of which about half were fertile. GUS expression driven by the vspB promoter was modulated by chemical and positional information.

Sharma and Anjaiah (2000) developed a procedure for regeneration via adventitious shoot bud formation from cotyledons excised from mature seeds, and they combined it with transformation to produce one or more independently transformed shoots with an efficiency of up to 55 % of the explants. In parallel, Rohini and Rao (2000) co-cultivated wounded embryo axes with one cotyledon cut off, they were allowed to develop without selection pressure and, ultimately, primary transformants were transferred to the glasshouse with GUS used as reporter gene for identification of transgenic plants; they later used the same approach to generate transformants with variable responses towards leaf spot disease (Rohini and Rao 2001). This procedure subsequently inspired various teams, and Anuradha et al. (2006) used it for transformation with a *gus::nptII* fusion gene-based vector. Likewise, this methodology was also adopted by other teams working with several grain legume species other than peanut, including pea (Svabova and Griga 2008).

Yang et al. (2003) tested the bacterial mercuric ion reductase gene, *merA*, as an alternative selectable marker system. *MerA* reduces toxic Hg(II) to the volatile and less toxic metallic mercury molecule, Hg(0), and renders its source Gram-negative bacterium mercury resistant. A codon-modified version of the gene, *MerApe9*, was cloned into a plant expression cassette containing the ACT2 promoter from *Arabidopsis thaliana* and the NOS terminator. The expression cassette also was inserted into a second vector containing the hygromycin resistance gene driven by the UBI3 promoter from potato. Stable transgenic plants were recovered from somatic embryo tissues bombarded with the plasmid containing both genes. Expression of *merA* as mRNA was detected by Northern blot in leaf tissues of transgenic plants, but not in somatic embryos. Western blot showed production of the mercuric ion reductase protein in leaf tissues.

Table 6 Peanut (*Arachis hypogaea* L.) transformation

<i>Agrobacterium</i> strain/method	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
Biolistic	Georgia Runner, Florunner, MARC-1	Embryogenic cultures	<i>Hph</i> , <i>GUS</i>	36.5	Wang et al. (1998)
C 58	J-11, ICGS-11, Robut-33-1, ICGS-76, ICGS-44	Cotyledon explants	<i>npII</i> , <i>GUS</i>	55	Sharma and Anjiah (2000)
LBA 4404	TMV-2	Embryo axes	<i>npII</i> , <i>GUS</i>	3.3	Rohini and Rao (2000)
LBA 4404	TMV-2	Embryo axes	<i>npII</i>	5.33	Rohini and Rao (2001)
C 58	JL-24	Cotyledon explants	<i>npII</i> , <i>DREBI</i>	Not given, 71 confirmed transgenic plants analysed	Bhatnagar-Mathur et al. (2007)
EHA105	JL-24	Embryo axes	<i>npII</i>	7 plants	Anuradha et al. (2008)
EHA101	JL-24	Cotyledon explants	<i>HptII</i>	17	Tiwari et al. (2008)
C 58	JL-24	Cotyledon explants	<i>Marker free</i>	24-34	Bhatnagar et al. (2010)
LBA 4404	Golden	Embryonic nodes	<i>Bar</i>	38	Iqbal et al. (2011)
LBA 4404	Golden, BARI-2000	Cotyledonary nodes	<i>Hpt</i>	42	Iqbal et al. (2012)
EHA101 LBA 4404	JL-24	Cotyledon explants	<i>Hpt</i> , <i>npII</i> , <i>GUS</i>	81	Tiwari and Tuli (2012)

Qiusheng et al. (2005) studied the effects of antioxidants on the plant regeneration and GUS expression frequency in peanut explants co-cultured with *A. tumefaciens*. The explants cultured on medium containing different antioxidants were able to produce more shoots and showed no browning of callus and an increased rate of shoot formation as compared to controls. Moreover, antioxidants were able to promote buds and shoots to grow into plantlets. The transformation efficiency as observed through GUS histochemical staining was also significantly increased by using antioxidants.

Most recently, a Rice chitinase-3 under enhance version of CaMV35S was introduced into peanut through *Agrobacterium*-mediation transformation using strain LB4404 harbouring the binary vector (pB1333-EN4-RCG3) containing the chitinase (chit) and hygromycin resistance (hpt) gene as selectable marker (Iqbal et al. 2011, 2012). Putative transgenic shoots regenerated were examined for the presence of the integrated rice chitinase gene and hygromycin resistance and the integration pattern of transgene in the nuclear genome was confirmed through Southern hybridisation. Viable and fertile T0 transgenic plants were produced with over 42 % transformation frequency. T1 plants were tested for resistance against *Cercospora arachidicola* by infection with the microspores. Transgenic strains exhibited a higher resistance than the non-transgenic control, and chitinase gene expression in highly resistant transgenic strains was correlated with fungal pathogen resistance. Also very recently, Tiwari and Tuli (2012) optimised conditions for peanut transformation to reach a transformation efficiency of 81 %.

4.3.4 Faba Bean

As with the other protein legume species, improvement of faba bean using genetic engineering has been limited due to the difficulties in developing an efficient and reproducible regeneration system (Table 7). Nevertheless, in the last 2 decades several regeneration protocols were developed for *Vicia faba* L. (Di Antonio et al. 1988, Fakhrai et al. 1989; Khalafalla and Hattori 2000; Hanafy et al. 2005). Consecutively, faba bean transformation procedures were also developed, particularly those using *Agrobacterium* inoculation of meristematic tissues (Böttinger et al. 2001; Hanafy et al. 2005). *Agrobacterium*-mediated gene transfer with faba bean and related species counts a number of examples, and the subject was recently the object of a comprehensive review (Hanafy et al. 2008).

The first gene transfer studies with *Vicia faba* L. were focused on induction of tumours by *Agrobacterium* virulent strains carrying the Ti plasmid. During further culture of this faba explants, intensive root proliferation was observed (Di Antonio et al. 1988; Siefkes-Boer et al. 1995). Jelenic et al. (2000) inoculated the stems of seedlings of three broad bean cultivars with *A. tumefaciens* wild-type strains (A281 and B6S3), transconjugant strains (C58C1(pArA4abc) and C58C1(pArA4b)), the B6S3 root and shoot mutants (GV3101(pGV2255), GV3101(pGV2215) and GV3101(pGV2235)) and *A. rhizogenes* wild-type strains (8196 and 15834). Cultivars differed in susceptibility to strains and a plant genotype::

Table 7 Faba Bean (*Vicia faba* L.) transformation

<i>Agrobacterium</i> strain ^a /method	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
<i>A. tumefaciens</i> strains A281, B6S3, GV3101, C58C1, <i>A. rhizogenes</i> strains 8196, 15834	Lobab Lippoi, Topolo, Oslje	excised cotyledons, leaves, intermodal stem segments	–	20–90 % based on induced tumour	Jelenic et al. (2000)
EHA101	G 461, G 674	Cotyledons, Shoot apex	<i>npII</i> , <i>GUS</i>	0–50 % based on Kanamycin resistance	Ismail et al. (2001)
EHA101, EHA105	Mythos	internodal segments	<i>npII</i> , <i>GUS</i>	0.025	Böttinger et al. (2001)
EHA101, EHA105	Mythos, Albatross, Giza2, Giza 429, Giza blanka, Giza 716	embryo axes, internode segments, leaf explants	<i>npII</i> , <i>bar</i>	0.15–2.0	Hanafy et al. (2005)
Biolistic	Giza2, G3, G40, G429, G461, G716, G834, Misr1, Sakhal1, Nubarial	Embryo axes, Shoot apex	<i>Bar</i> , <i>GUS</i>	2	Metry et al. (2007)
Biolistic	Orafuku	Leaves	<i>GFP</i>	Not given	Melhorn et al. (2008)

^aAll strains of *A. tumefaciens* unless indicated otherwise

strain interaction was detected but only unorganised tumour tissue was obtained. In vitro transformation with Ri plasmid-containing bacterium strains was similarly unsuccessful.

Böttinger et al. (2001) reported the first reliable transformation method for faba bean (*Vicia faba* L.), using stem segments from aseptically germinated seedlings as explants undergoing callusing and with transgenic shoots regenerated from such stem calluses at a frequency of 10–30 % of the initial explants used.

Using microprojectile bombardment, cotyledon and callus explants of *Vicia faba* L. were transformed (Ismail et al. 2001) and transformants were evaluated for NPTII presence by PCR Southern blot hybridisation and GUS assay. Transformation of *Vicia faba* L. using microprojectile bombardment with the plasmid pCG1258 containing *bar* and *gus*-intron genes was developed also using mature embryos (Metry et al. 2007). Faba bean cultivars were utilised to produce transgenic faba bean (2 %). Expression of the transgenes was evaluated by PCR, Southern blotting and GUS assay.

Hanafy et al. (2005) developed a system based upon direct shoot organogenesis after transformation of meristematic cells from embryo axes with *A. tumefaciens* strain EHA105/pGlsfa harbouring a binary vector with a gene encoding a sulphur-rich sunflower albumin (*SFA8*) linked to the *bar* gene, and strain EHA 101/pAN109 carrying the binary plasmid containing the coding sequence of a mutant aspartate kinase gene (*lysC*) from *E. coli* in combination with *nptII* gene. The coding sequences of *SFA8* and *LysC* genes were fused, respectively, to the seed-specific promoters *V. faba* legumin B4 (LeB4) and phaseolin. Seven phosphinothricin (PPT) resistant clones were recovered, with integration, inheritance and expression of transgenes confirmed by Southern blot, PCR, enzyme activity assay and Western blot.

Youssef et al. (2007) transformed embryogenic axis explants with *A. tumefaciens* strain LBA-4404 harbouring AFP plasmid which contains defensin gene under the control of CaMV 35S promoter, NOS terminator and *gus* intron as a reporter gene. The integration of the defensin transgene into the genome of faba bean plants was confirmed by PCR assay, and *gus* gene expression indicated that some of the putative shoots were indeed transformed.

In 2008, Melhorn et al. examined the competence of guard cells to synthesise ABA, using two Arabidopsis enzymes of ABA biosynthesis, p35S::AtNCED3–GFP and AAO3–GFP, that were introduced into guard cells of broad bean leaves. AtNCED3–GFP expression was detected at the chloroplasts, while GFP and AAO3–GFP were expressed in the cytosol. The stomatal aperture was decreased in AtNCED3–GFP and AAO3–GFP-transformed guard cells. This indicated that ABA biosynthesis is stimulated by heterologous expression of AtNCED3 and Arabidopsis aldehyde oxidase 3 (AAO3) proteins, which both seem to be regulatory enzymes for ABA biosynthesis in these cells. Stomatal closure by the expression of AtNCED3 and AAO3 also suggested that the substrates of these enzymes are present and native ABA-biosynthesis enzymes are active in the guard cells.

4.3.5 Chickpea

Chickpea regeneration is possible with varying degrees of success but, to date, there have been few successful reports of production of transgenic chickpea plants using *Agrobacterium*-mediated transformation (Fontana et al. 1993; Krishnamurthy et al. 2000; Polowick et al. 2004; Sarmah et al. 2004; Senthil et al. 2004; Tewari-Singh et al. 2004) and only one on biolistic transformation (Kar et al. 1997), as detailed in Table 8. Most of these articles showed variations in transformation efficiency, number of transgenic plants recovered, rooting efficiency and subsequent establishment of the plants in the greenhouse and inheritance of transgenes in the progeny. Thus, Fontana et al. (1993) claimed a 4 % success but obtained only 3 GUS positive transgenic chickpea plants, whereas several teams (Kar et al. 1997, Krishnamurthy et al. 2000; Tewari-Singh et al. 2004) reported a 0.4–4 % frequency of *Agrobacterium*-mediated transformation based on multiple shoot formation from embryo axis explants and coupled with recovery of transgenic plants. Of these, though, only Krishnamurthy et al. (2000) and Tewari-Singh et al. (2004) showed transgene inheritance in T1 generation of transformed plants.

Recently, Polowick et al. (2004) also showed successful *Agrobacterium*-mediated transformation of chickpea, at a 1.3–3.1 % frequency of recovery of rooted transgenic plants but these were obtained after a prolonged tissue culture period, which could lead to somaclonal variation. Senthil et al. (2004) showed 2.0–13.3 % transformation efficiency, but performance of in vitro rooted plants in the glasshouse was inconsistent, requiring grafting of shoots, which was very laborious. Finally, Bhattacharjee et al. (2010) transformed four different chickpea genotypes with three different strains of *Agrobacterium* (EHA105, AGL1 and LBA4404) harbouring the binary vector pCambia1301 with reporter genes (*gus*, *hpt*) driven by CaMV35S promoter and efficiently regenerated rooted plants whose T2 progeny expressed both reporter genes in the expected 3:1 inheritance.

4.3.6 Lentil

Lentil (*Lens culinaris* Medik.) is a grain legume produced in Asia, the Middle East and parts of North and South America as a source of protein in human diets. This crop has also been studied within the context of gene transfer by a number of groups (Table 9).

In order to assess the susceptibility of lentil to crown gall transformation, Warkentin and McHughen (1991) used four strains of *A. tumefaciens*, i.e. C58, Achh5, GV3111 and A281. All these strains were capable of inducing tumours at a high frequency on shoot apex explants when infected in vivo and on excised shoot apices in vitro, which were capable of growth on hormone free medium, a characteristic of tissue transformed with oncogenic *Agrobacterium* strains (Braun 1958). Their results suggested that disarmed versions of any one of these strains could be suitable for the recovery of transgenic lentil plants from shoot apex explants. As

Table 8 Chickpea (*Cicer arietinum* L.) transformation

<i>Agrobacterium</i> strain/ transformation method	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
A281, R1601	Pusa-256	Leaf, stem	<i>nptII</i>	Not given	Srinivasan and Sharma (1991)
LBA4404	Local ecotype (Italy)	Embryo axis lacking apical meristem	<i>nptII</i> , <i>GUS</i>	4	Fontana et al. (1993)
LBA4404	ICC-4918	Immature cotyledon	<i>GUS</i>	Not given	Ramana et al. (1996)
LBA4404	Red chickpea Canitez 87 MB-10	Shoot primordial of mature embryo	<i>nptII</i> , <i>GUS</i>	Not given	Alinkut et al. (1997)
C58C1, EHA101	PG1, PG12, Chafa	Embryo axes	<i>nptII</i> , <i>bar</i> , <i>GUS</i>	0.4	Krishnamurthy et al. (2000)
LBG66	CDC Yuma; Kabuli-type	Sliced embryo axis	<i>nptII</i> , <i>GUS</i>	3.1	Polowick et al. (2004)
EHA101	P-362, P-1042, P-1043	Embryo with 1/2 cotyledon	<i>bar</i> , <i>nptII</i> , <i>GUS</i>	0.7–1.2	Tewari-Singh et al. (2004)
C58, LBA4404, AGL1	Kabuli (ICCV5), Desi (H208, ICCL87322, K850 and Annigeri)	Embryo axis slices	<i>bar</i> , <i>GUS</i>	5.1	Senthil et al. (2004)
GV 2260, GV 3850, LBA 4404, EHA 105	C 235, BG 256, Pusa 362, Pusa 372	Cotyledonary nodes	<i>nptII</i> , <i>GUS</i>	1.12	Sanyal and Amla (2008)
LBA4404	K850	Embryogenic axis	<i>nptII</i> , <i>GUS</i>	0.3	Ignacimuthu and Prakash (2006)
Biolistic	Chaffa PG12 (MPKV, Rahuri), ICCC37 and ICC32	Epicotyl	<i>nptII</i> , <i>GUS</i>	18	Indurker et al. (2007)
LBA4404	ICC 10943, ICC 10386	Decapitated embryo axis	<i>hptII</i>	9–26	Pathak and Hamazah (2008)
GV3101	C-235	Embryo with 1/2 cotyledon	<i>GUS</i>	3	Patil et al. (2009)
AGL-1	ICCV 89314	Embryo with 1/2 cotyledon	<i>nptII</i>	0.066	Chakraborti et al. (2009)
C58	C 235 (desi type)	Axillary meristem explants	<i>nptII</i> , <i>GUS</i>	65	Bhatnagar-Mathur et al. (2009)
EHA105, AGL1, LBA4404	Pusa-256, KWR-108, Pusa-1003	Cotyledons, cotyledonary-nodes	<i>Hpt</i> , <i>GUS</i>	7.56–25.56	Bhattacharjee et al. (2010)
LBA4404	Desi (P-362)	Cotyledonary nodes	<i>nptII</i>	1.69–2.77	Mehrotra et al. (2011a)
LBA4404	Desi (P-362)	Mature embryonic axes	<i>nptII</i> , <i>GUS</i>	3.6	Mehrotra et al. (2011b)

Table 9 Lentil (*Lens culinaris* Medik.) transformation

<i>Agrobacterium</i> strain/ method	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
C58, Ach5, GV3111, A281	Laird	Shoot apices	–	81–100 based on tumour induction	Warkentin and McHughen (1991)
Biolistic A281	Laird, CDC 599-23 Emre 20, Malazgirt, Akm 565, Akm 302, Akm 362 and so on (total 21 genotypes)	Cotyledonary node Stem, leaf	<i>bla</i> , <i>ALS</i> <i>GUS</i>	3.1 0–82 based on tumour induction	Gulati et al. (2002) Khawar and Ozcan (2002)
GV2260	Sultan-1	Cotyledonary node	<i>npII</i> , <i>GUS</i>	95 based on transient expression	Mahmoudian et al. (2002)
LBA4404	Barimasur-2, Barimasur-4	Cotyledonary node, decapitated embryo, immature embryo, epicotyl	<i>npIII</i> , <i>GUS</i>	Not given	Sarker et al. (2003)
A281, 15834	Sultan, Erzurum 89, Akm 565, 93CI003	Cotyledon node, epicotyl, shoot meristem	<i>GUS</i>	0–90 based on tumour induction	Dogan et al. (2005)
EHA105, C58CI, KYRT1	Sultan-1	Cotyledonary node	<i>npIII</i> , <i>GUS</i>	2.3	Akcaay et al. (2009)
EHA105	L-4076	Whole seed	<i>npIII</i> , <i>GUS</i>	0.9	Chopra et al. (2011)

strain A281 induced the heaviest tumours, Khawar and Ozcan (2002) used this hypervirulent strain for inoculation of several explants from 21 genotypes of lentil. Based on their responses in terms of different parameters including % of tumour formation, tumour diameter and tumour weight, three were best when leaf explants were used, four with stem explants and 14 formed tumours on both leaf and stem explants. Low but reproducible levels of GUS expression could be obtained for confirmation of such induced tumours.

Warkentin and McHugen (1993) studied the regeneration of cotyledonary nodes and reported very low potential of these explants for transformation by *A. tumefaciens*. In order to overcome this difficulty, Gulati et al. (2002) developed a reproducible system for lentil transformation using highly regenerable cotyledonary node meristems by biolistic method. Rooting of shoots was achieved through grafting. They bombarded herbicide resistance gene acetolactate synthase (*ALS*) into lentil cotyledonary nodes. Putative primary transformants and selfed progeny plants were screened by leaflet painting using metsulfuron herbicide while transgene insertion was confirmed through PCR and Southern hybridisation.

As vacuum infiltration has been reported to enhance transformation frequency in *Agrobacterium*-mediated gene transfer in some legumes (Trieu et al. 2000), Mahmoudian et al. (2002) used it for transfer of *Agrobacterium* cells into lentil cotyledonary nodes without affecting the regeneration potential of shoots. Transient expression of *gus* transgene on agro-infected explants was demonstrated through histochemical staining 3 d after co-cultivation.

Sarker et al. (2003) tested a number of explants such as cotyledonary nodes, decapitated embryos, immature embryos and epicotyls for their regeneration ability following *A. tumefaciens*-mediated transformation. Histochemical staining showed that epicotyl explants exhibited highest transgene expression followed by decapitated embryos, which were found to be more effective in formation of multiple shoots and were thus suggested as suitable explants for lentil transformation. Unfortunately, as various other authors, they were also unable to root the transformed shoots.

Bayrac (2004) investigated the regeneration of several tissues on various media via indirect organogenesis. He then carried out *A. tumefaciens*-mediated transformation using peeled cotyledonary nodes, cotyledonary petioles, shoot tips and roots as explant. Shoot tips showed the highest percentage of GUS expression. Root formation was only achieved in media with NAA/IAA.

Dogan et al. (2005) compared tumour and root formation ability of several tissues from different lentil cultivars after inoculation with *A. rhizogenes* and *A. tumefaciens* strains. The frequency of tumour formation from cotyledon node explants was higher compared than from shoot meristems. Rooting was only observed in cultivar Erzurum 89 while *A. rhizogenes*-mediated hairy roots were induced only in dark.

Production of disease-resistant lentil would help to increase its production as it is susceptible to many biotic stresses. Fungus-resistant lentil was developed by Hashem (2007) by transforming decapitated embryos with one cotyledon with *Ri-gip* gene coding polygalacturonase inhibitory protein, conferring resistance

against fungal pathogens followed by an optimised regeneration system, with a very high transformation efficiency of about 35 %. Micro-grafting was used for rooting transformants. She was among the first to develop a marker free transformation system in legumes, by removing *bar* gene and PGIP gene was kept in T-DNA cassette before carrying out transformation. Fungus-resistant marker free plants were demonstrated via semi-quantitative polygalacturonase-inhibition assay.

For optimisation of lentil transformation, Akcay et al. 2009 used a combination of several treatments with three *A. tumefaciens* strains, i.e. EHA105, C58C1 and KYRT1 to deliver T-DNA into cotyledonary node tissues. As compared to EHA105 and C58C1, KYRT1 was found about threefold more efficient for producing transient GUS expression on cotyledonary petioles. Among several treatments used, wounding of explants, use of an optimised transformation protocol with the application of acetosyringone and vacuum infiltration and gradual selection resulted in a high percentage of stably transformed shoots. Fertile transgenic plants were obtained through grafting transgenic shoots on rootstocks. The transgene insertion and expression were confirmed through PCR, RT-PCR and Southern hybridisation, and the transgenes were segregated in Mendelian fashion.

During production of transgenic lentil, micrografting has been extensively used to recover transformed plants, and there is a need to develop an efficient and reproducible regeneration protocol that can lead to root induction from developing shoots without passing through the laborious work of micrografting. Chopra et al. (2011) developed a simple and genotype independent in vitro regeneration system of lentil capable of root induction. They then used it for transformation using sonication-assisted *A. tumefaciens* (SAAT) transformation (first report of this method in lentil). A supervirulent *A. tumefaciens* strain EHA105 was employed for transferring the T-DNA containing *nptII* and *uidA* genes into whole seeds using sonication and vacuum infiltration, and 40 % of the kanamycin-resistant transfected shoots produced through direct shoot organogenesis were able to root on a medium with IBA and kanamycin. Transgene insertion and activity in leaves and roots were detected by PCR and GUS histochemical assay, respectively.

Most recently, in order to enhance drought and salinity tolerance, Khatib et al. (2011) introduced *DREB1A* gene driven by the rd29A promoter into lentil decapitated embryo explants followed by shoot regeneration from the apical meristems and cotyledonary buds via direct organogenesis. Subsequently, basta-resistant putative transgenic explants were micro-grafted onto non-transformed rootstocks to establish transgenic plants. Transgene insertion and inheritance to the progeny were evaluated through PCR and Southern blot analysis. Expression of *DREB1A* gene in transgenic plants was induced by salt stress and was confirmed through RT-PCR.

4.3.7 Pea

Studies of the interactions between *Agrobacterium tumefaciens* and *A. rhizogenes* and pea started in the late 1980s and early 1990s (Hobbs et al. 1989; Hussey et al.

1989; Schaerer and Pilet 1991). Simultaneously, the first complete transgenic pea plants were regenerated from transformed protoplast cultures by Puonti-Kaerlas et al. (1989) soon followed by De Kathen and Jacobsen (1990, 1995) who regenerated transgenic pea plants from epicotyls and cotyledonary nodes.

Thenceforth, a number of protocols have been reported for *Agrobacterium*-mediated gene transfer in pea (Bean et al. 1997; Grant et al. 1998; Nadolska-Orczyk and Orczyk 2000; Pniewski and Kapusta 2005; Polowick et al. 2000; Schroeder et al. 1994; Svabova et al. 2008), as listed in Table 10. In the procedure described by Polowick et al. (2000), segments of the embryogenic axis are used. Bean et al. (1997) and Nadolska-Orczyk and Orczyk (2000) used cotyledonary nodes, whereas Grant et al. (1995, 2003) used immature cotyledons as starting material. In all procedures, multiple shoots are formed after infection with *Agrobacterium tumefaciens*, some of which are genetically modified. In these systems, about 2–5 % of the initial seeds used for transformation formed transgenic plants. Limitations of the currently available transformation protocols are associated with the regeneration systems used, which either result in polyploidy and sterility, or in the production of high numbers of escapes and chimeric plants, or have a low repeatability (Puonti-Kaerlas et al. 1990; De Kathen and Jacobsen (1990); Davies et al. 1993; Schroeder et al. 1993; Bean et al. 1997; Nadolska-Orczyk and Orczyk 2000; Polowick et al. 2000; Grant et al. 2003).

Despite the fact that successful pea transformation was reported 20 years ago (Puonti-Kaerlas et al. 1990), the efficiency of transformation protocols is still relatively low (in the range 0.1–6.5 %—Davies et al. 1993; Schroeder et al. 1993; Bean et al. 1997; Jones et al. 1998; Polowick et al. 2000; Grant et al. 2003; exceptionally over 10 %—Nadolska-Orczyk and Orczyk 2000). Particularly, co-cultivation additives were not studied systematically (only sporadic data on acetosyringone use are available—De Kathen and Jacobsen 1990; Lulsdorf et al. 1991; Grant et al. 1995; Nadolska-Orczyk and Orczyk 2000; Svabova et al. 2005, 2008).

Krejci et al. (2007) also reported *Agrobacterium*-mediated gene transfer in pea. They compared three different methods for pea transformation by using stem segments, axillary buds and embryonic segments as explants, respectively. When using stem segments as explants, no shoot or plantlet regenerated from callus. Conversely, a large number of regenerated shoots were obtained from the other two methods. They were able to get some transient expression of *uidA* gene but they could not achieve the stable incorporation of transgene in the plants.

The importance of producing transgenic peas (as for other grain legumes with a high content of seed protein) increased with the demand to develop plant-made vaccines. Expressing recombinant proteins in transgenic plants has been actively sought for the past 20 years, resulting in a fast and flexible production system (Penney et al. 2011). Plant-derived pharmaceuticals can be used against various human diseases, including cancer (Pujol et al. 2007), hepatitis B and C, measles, cholera (Mishra et al. 2008) or AIDS (Floss et al. 2008), as well as for veterinary purposes. In peas, there are several reports about pea-derived vaccines against rabbit haemorrhagic disease virus (Mikschofsky et al. 2009), intestinal infections

Table 10 Pea (*Pisum sativum* L.) transformation

<i>Agrobacterium</i> strain/ method	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
GV3101	Filby, Petra, Puget, Stivo, Vreia	Axenic shoot culture and epicotyl nodes	<i>npII</i>	28 plants	Puonti-Kaerlas et al. (1990)
C58C1, A281, A8683	Madria	Epicotyl segments and nodes	<i>GUS</i> , <i>npII</i>	5	De Kathen and Jacobsen (1990)
C58/3	Puget	Germinating seeds	<i>npII</i> , <i>GUS</i>	1.44	Davies et al. (1993)
AGL1	Greenfeast, Rondo	Immature seeds	<i>npII</i> , <i>bar</i>	1.5–2.5	Schroeder et al. (1993)
AGL1	Bolero, Trounce, Bohatyr, Huka	Immature cotyledons	<i>npII</i> , <i>bar</i>	1.47	Grant et al. (1995)
AGL1	94-A26, Bolero, Hadlee, Crown, Courier, 89T46, UK	Immature cotyledons	<i>npII</i>	0.8–3.4	Grant et al. (1998)
EHA105	Puget	Cotyledonary nodes	<i>bar</i>	1.1 ± 0.43	Bean et al. (1997)
AGL1, KYRT1	Greenfeast, Laura	Immature Seeds	<i>αAI-1</i> , <i>αAI-2</i>		Morton et al. (2000)
LBA4404, C58C1, EHA105	Laser, Heiga	Cotyledonary nodes	<i>uidA</i> , <i>npII</i> , <i>hpt</i> , <i>dhfr</i> , <i>bar</i>	4.2–3.6	Nadolska-Orczyk and Orczyk (2000)
EHA105	Greenfeast, CDC Vienna, S2-90-25E, 93- 4-18G, MP1338, MP1382, AWPNZ66, AWP1512	Embryogenic axis segments	<i>uidA</i> , <i>npII</i> , <i>pat</i>	0.6	Polowick et al. (2000)
EHA105	Puget, BC1/17	Cotyledonary meristems	<i>bar</i>	0.13–0.8	Welham and Domoney (2000)
AGL1, KYRT1	Bolero, Lincol, 97-B19	Immature cotyledons	<i>npII</i> , <i>GUS</i>	0.2–13	Grant et al. (2003)
AGL0, AGL1, EHA105	Several edible and fodder Polish cultivars	Immature embryos	<i>bar</i>	4.1	Pniewski and Kapusta (2005)
EHA105	Adept, Kommet, Lantra, Olivín, Oskar, Tyrcys	Cotyledonary nodes, seeds	<i>npII</i> , <i>uidA</i>	18.1 % for cotyledonary node, 31.6 % for seed GUS expression	Svabova et al. (2005)
EHA105	Vladan, Havel, Citrad, Zazrak, Cezar, Puget	Stem segments, Axillary buds, embryonic segments	<i>Bar</i> , <i>npII</i> , <i>GUS</i>	0.5	Krejci et al. (2007)
EHA105 with plasmids pGT89 and pBIN19	Adept, Kommet, Menhir	Cotyledonary nodes	<i>Bar</i> , <i>npII</i> , <i>GUS</i>	0.1–1.0	Svabova and Griga (2008)
Electroporation	Belma, Filby	protoplast	<i>Hph</i> , <i>GUS</i>	1–2.2	Puonti-Kaerlas et al. (1992)

in pigs (Novoplant 2007; <http://www.gmo-safety.eu>) and coccidiosis in chickens (Zimmerman et al. 2009).

For efficient and large-scale production of recombinant proteins in plants, transient expression by agroinfection has a number of advantages over stable transformation, as simple manipulation, rapid analysis and high expression efficiency are possible. In pea, Fan et al. (2011) using the pea early browning virus converted a virus-induced gene silencing system into an efficient agroinfection system by converting the two RNA genomes of the virus into binary expression vectors for *Agrobacterium* transformation. They vacuum infiltrated germinating pea seeds with 2–3 cm roots with *Agrobacteria* carrying the binary vectors, and expression of the gene for GFP as marker and the gene for the human acidic fibroblast growth factor (aFGF) was obtained in 80 % of infiltrated developing seedlings. Maximal production of recombinant proteins was achieved 12–15 days after infiltration, i.e. half the time for the production cycle of plants for harvesting the recombinant protein. Thus, compared to the leaf injection method, vacuum infiltration of germinated seeds is highly efficient and allows large-scale production of plants transiently expressing recombinant proteins. The synthesised aFGF was purified by heparin-affinity chromatography and its mitogenic activity on NIH 3T3 cells was shown to be similar to a commercial product.

4.3.8 Lupin

In one of the first examples of gene transfer with lupin, Molvig et al. (1997) succeeded in recovering plants of narrow-leaved lupin *Lupinus angustifolius* L. that expressed a sunflower albumin gene and thus had an enhanced nutritional value with an increase in the methionine levels (Table 11).

Working with the same lupin species, Pigeaire et al. (1997) produced transgenic plants from co-cultivated shoot apices, with an average transformation frequency of 0.4–2.8 %, depending on genotype. Similarly, Li et al. (2000) generated transgenic yellow lupin (*Lupinus luteus* L.) shoots, developed from existing meristems in the embryo axes explants, but these failed to root in vitro and had to be grafted onto rootstocks from seedlings of non-transgenic *L. angustifolius* L. Transformation efficiency was low (0.05–0.75 %).

Babaoglu et al. (2000) found that transformation competent cells from which buds developed were located at the periphery of the apical meristems and, based on this, they used a procedure for regenerating transgenic plants of *Lupinus mutabilis* following *A. tumefaciens*-mediated gene delivery. Kanamycin-resistant plants obtained expressed β -glucuronidase activity, and integration of the *nptII* and β -glucuronidase genes into the genome was confirmed by non-radioactive DNA–DNA hybridisation.

Pniewski et al. (2006) developed an improved transformation protocol with seedlings and hypocotyls of yellow lupin, reaching 44 % efficiency rate, which they used to produce calli and tumours that produced a small surface antigen of

Table 11 Lupin (*Lupinus* sp.) transformation

<i>Agrobacterium</i> strain ^a /method	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
<i>A. tumefaciens</i>	Warrah	Cotyledon with sliced embryonic axis	<i>Bar</i> , <i>GUS</i>	0.01	Molvig et al. (1997)
AGL0, LBA4404, K61, EHA101	Unicrop, Illyarie, Yandee, Wandoo, Danja, Gungurru, Yorrel, Warrah, Merrit	Embryonic axis	<i>Bar</i> , <i>GUS</i>	2.8 % for Unicorp, 0.4 % for Merrit	Pigeaire et al. (1997)
Strain 1065 based on strain LBA 4404	Potosi	Part of hypocotyl and epicotyl with apical meristem, extreme tip of the apical dome	<i>npII</i> , <i>GUS</i>	0.04	Babaoglu et al. (2000)
AGL0	Teo, Teo101, Wodjil, Popiel, Motiv 369, Juno, WDT 6174, WDT 6179	Lower hypocotyl and radicle wa	<i>Bar</i>	0.05–0.75	Li et al. (2000)
Derivatives of <i>A.</i> <i>rhizogenes</i> strain A4T	Ultra	Radicle sections	<i>GFP</i> , <i>GUS</i>	27–32	Uhde-Stone et al. (2005)
C58, A281 77, Ach5, GV3101, EHA105 and LBA4404	Ventus	Truncated seedlings	<i>npII</i> , <i>GUS</i>	44	Pniewski et al. (2006)
<i>A. rhizogenes</i> strain A4TC24	Ultra	Hypocotyl region	<i>GUS</i>	Not given	Zinn et al. (2009)

^aAll strains of *A. tumefaciens* unless indicated otherwise

Hepatitis B Virus, the final goal being the production of an oral vaccine that could be administered as a portion of plant tissue.

Finally, Zinn et al. (2009) produced transgenic white lupin and *Arabidopsis* and studied deletion and mutation constructs linked to the β -glucuronidase (*gus*) reporter gene, in order to understand the structure of the LaSAP1 promoter and explore the role of the P1BS motif. In this context, Uhde-Stone et al. (2005) had shown that white lupin transformation provides a homologous system to directly study gene expression in proteoid roots, since transgenic white lupin roots have a similar morphology to normal roots, as both develop proteoid rootlets and express P-deficiency responsive genes.

4.3.9 Cowpea

Cowpea (*Vigna unguiculata* L.) is a major food legume for the poor in Asia and especially in Africa, where a number of experiments on gene transfer have also been performed (Table 12).

The first report on the susceptibility of cowpea to *Agrobacterium* was by Garcia et al. (1986, 1987), who demonstrated that *A. tumefaciens* was capable to transform cowpea cells derived from leaf callus that became kanamycin resistant, but they were unable to generate transgenic plants from them. Transgenic cowpea callus cells were also obtained by Penza et al. (1991) following co-culture of mature embryos with hypervirulent *A. tumefaciens* A281. Based on GUS expression, they showed that transformed cells were mostly located in the subepidermal regions of the plant stems.

Muthukumar et al. (1996) also tried to produce transformed cowpea by indirect DNA transfer using *A. tumefaciens* strain LBA4301; 15–19 % of the co-cultivated explants produced shoots on selection medium supplemented with hygromycin. Although transgene insertion and integration in primary transformants were confirmed by Southern hybridisation, there was no germination of their seeds and transgene inheritance.

Several years later, a biolistic approach was used to transform embryonic axes by Ikea et al. (2003). Putative transgenic plants were obtained after bombardment with a construct containing *nptII* and *gus* driven by CaMV35S promoter. Plants developing under constant kanamycin screening were assayed for GUS expression. Southern hybridisation confirmed the T-DNA insertion in primary transgenics. Though the transformation efficiency was low, this was the first report to demonstrate transgene inheritance to the progeny, but it did not follow Mendelian rule nor was transgene expression very stable.

Different conditions that significantly affect genetic transformation in cowpea were optimised by Popelka et al. (2006) using different plant tissues as explant. They then used this knowledge to transform cotyledonary nodes via *A. tumefaciens*. Nodes were grown on auxin-free medium in early stages and were transferred for shoot initiation to medium with low cytokinin level. A strict phosphinotricin-based selection procedure was followed to ensure that there were no escapes from the selection regime. Transgene insertion and functioning were verified using Southern

Table 12 Cowpea (*Vigna unguiculata* L.) transformation

<i>Agrobacterium</i> strain/ method	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
C58CI	Not mentioned	Leaf discs	<i>nptII</i>	–	Garcia et al. (1987)
LBA4301	C-152	De-embryonated cotyledons	<i>hpt</i>	15–19 % based on Hygromycin resistance	Muthukumar et al. (1996)
Biolistic	IT83D-442	Embryonic axes	<i>bar</i> , <i>GUS</i>	0.57	Ikea et al. (2003)
AGL1	Sasaque, Holstein, Ebony	Mature and immature embryos, shootapex, embryonic axes with cotyledons	<i>bar</i> , <i>nptII</i> , <i>hpt</i> , <i>GUS</i>	0.05–0.15	Popelka et al. (2006)
EHA105	IC-202786, IC-257438, IC- 259159, IC-243501, V-240, V-130, V-585	Cotyledonary nodes	<i>nptII</i> , <i>GUS</i>	0.76	Chaudhury et al. (2007)
Biolistic	Paraguacu, Pitiuba, Rouxinol, CE-11	Embryonic axes	<i>GUS</i> , <i>ahas</i>	0.90	Ivo et al. (2008)
LBA4404	Pusa Komal	Cotyledonary nodes	<i>nptII</i> , <i>GUS</i>	1.67	Solleti et al. (2008a)
LBA4404, GV2260, EHA105, AGL1	Pusa Komal, Pusa Safed, Pusa Sam-pada, Rambha, V-16, V-240, V-130, V-585	Cotyledonary nodes	<i>nptII</i> , <i>GUS</i>	1.64	Solleti et al. (2008b)
pGV2260, pGV3850	IT86D-1010	Embryos	<i>bar</i> , <i>GUS</i>	2.5–3.9	Adesoye et al. (2010)

hybridisation and PAT assay with a transformation efficiency of 0.05–0.15 %. This protocol was the first where transgene inheritance to the offspring followed the Mendelian fashion. Co-culture, a critical step during transformation, was further enhanced by Chaudhury et al. (2007) by wounding of cotyledonary node explants prior to inoculation with *A. tumefaciens* EHA105 harbouring a binary vector pCambia2301, which contains *gus* and *nptII* genes both driven by the CaMV35S promoter, whereby they reported an improved transformation efficiency of 0.76% of explants and coupled with transgene inheritance to the progeny.

Ivo et al. (2008) used a novel selection system based on imazapyr, a herbicidal molecule that provides tolerance to *ahas* gene (a mutated gene from *Arabidopsis thaliana* used as a marker), and is capable of systemically translocating and concentrating in the apical meristem of the plant. They mixed this novel selection system with *gus* staining to screen transformants when embryonic axes were bombarded through the biolistic method. Transgenic plants expressing *gus* and *ahas* genes were obtained with ameliorated transformation efficiency of 0.9 % and transmitting traits to the progenies following Mendelian segregation.

Solleti et al. (2008a) transferred alpha-amylase gene to cowpea, with an inhibitory effect for certain pest attacks, in what is probably the first report on heterologous expression of a gene that confers a desirable agronomical trait in cowpea. The transformation method was improved by introduction of additional virulence genes in *A. tumefaciens* strain LBA4404 for efficient T-DNA delivery to regenerating cells. An improved regeneration system with optimised hormonal balance and geneticin selection was employed, which reduces the escapes and promotes regeneration of proliferating transformed cells. All these factors when combined boosted transformation efficiency to 1.67 %. Solleti et al. (2008b) then compared different *A. tumefaciens* strains for their ability to transform various cowpea genotypes employing this same approach.

Adesoye et al. (2010) used vacuum infiltration when embryo explants were inoculated in *Agrobacterium* liquid culture to enhance transformation, as vacuum infiltration had been reported to improve the transformation efficiency in pea (Svabova and Griga 2008). Co-cultivation of explants was carried out on selection-free medium. Double selection using hygromycin and phosphinotricin resulted in development of GUS positive plants with a much higher transformation efficiency, i.e. 2.5–3.9 %, than previously reported.

4.3.10 Mungbean

Mungbean (*Vigna radiata* L. Wilczek) is an important protein-rich grain legume, principally grown in tropical and sub-tropical countries. The very first assay for transformation of mungbean was reported by Pal et al. (1991), who regenerated shoots from cotyledon explants of *Agrobacterium* susceptible genotypes via organogenesis (Table 13). Based on GUS assay, 4–5 % of the kanamycin-resistant shoots were transgenic, but rooted and fertile transgenic plants were not recovered.

Table 13 Mungbean (*Vigna radiata* L., Wilczek) transformation

<i>Agrobacterium</i> strain/method	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
Not given	Not given	Cotyledons	<i>npII</i> , <i>GUS</i>	4–5	Pal et al. (1991)
Biolistic	Marechal	Cotyledons, mature embryos	<i>npII</i> , <i>GUS</i>	Not given	Bhargava and Smigocki (1994)
LBA4404, EHA105, C58C1	K-851, Pusa-105, ML-5, ML-323, ML-337, PDM-11, PS-7	Hypocotyls, primary leaves, cotyledonary nodes	<i>npII</i> , <i>GUS</i>	0.9	Jaiwal et al. (2001)
K599, EHA105	Kamphaeng Saen 1 (KPS 1), Suranaree 1 (SUT 1)	Cotyledons, cotyledonary nodes, cotyledonary roots	<i>npII</i> , <i>hpt</i> , <i>GUS</i>	Only one shoot regenerated	Suraninpong (2002)
K599, EHA105	Kamphaeng Saen 1 (KPS 1), Suranaree 1 (SUT 1)	Cotyledons	<i>hpt</i> , <i>GUS</i>	31.25 based on gus staining	Suraninpong et al. (2004)
C58C1	NM92, NCM 209, NM98	Hypocotyls, primary leaves, roots, cotyledonary nodes	<i>npII</i> , <i>GUS</i>	40–80 based on GUS	Tazeen and Mirza (2004)
C-58	K-851, LGG-407	Primary leaves	<i>hpt</i> , <i>GUS</i>	2	Mahalakshmi et al. (2006)
EHA105	PUSA-105	Cotyledonary nodes	<i>npII</i> , <i>GUS</i>	1.51	Sonia et al. (2007)
LBA4404	BINA mug-5	Cotyledonary leaves, cotyledon attached with embryonal axis (CAEA)	<i>npII</i> , <i>GUS</i>	Not given	Islam and islam (2010)

Gulati and Jaiwal (1994) reported rapid multiple shoot formation at high frequency from cotyledonary nodes using varying combination of growth regulators but regenerated plants were generally stunted, unbranched and flowered precociously. Bhargava and Smigocki (1994) used biolistic to introduce *gus* and *nptII* genes into ungerminated mature embryos of different *Vigna* species. GUS expression was observed in cotyledonary meristematic regions 18 h after bombardment. They were able to root some kanamycin-resistant transformed shoots but no information on seeding of these plants was provided. Nagl et al. (1997) reviewed the progress made with the regeneration and genetic transformation of *V. radiata*.

Establishment of optimised conditions necessary for regeneration and efficient *A. tumefaciens*-based transformation is a prerequisite for production of transgenic plants of *V. radiata* as reported by Jaiwal et al. (2001) for the first time. They co-cultivated hypocotyl and primary leaves with *A. tumefaciens* strains LBA4404 (pTOK233), EHA105 (pBin9GusInt) and C58C1 (pIG121Hm), all containing *nptII* and *gusA* marker genes under CaMV35S promoter. Stable fertile transformants were identified within 4–6 weeks from kanamycin-resistant GUS positive calli and node explants. Molecular analysis of putative transformed plants by PCR and Southern blot revealed the integration and expression of transgenes in primary transformants and their progeny. Choice of the explants, transformation vectors and use of selective agent were the most important factors. Cotyledonary nodes were the best explant source, as shoot regeneration occurs through direct shoot organogenesis avoiding somaclonal abnormalities.

Mungbean production is commonly affected by different insect pests. In this context, Suraninpong (2002) made an attempt to transform various mungbean tissue sources with *A. rhizogenes* K599 and *A. tumefaciens* EHA 105 harbouring plasmid pCambia 1301 containing cholesterol oxidase gene (*choA*), coding a potent insecticidal protein active against boll weevil larvae. GUS expressing transformed hairy roots developed from cotyledonary root explants could not be regenerated into plants. In 2004, Suraninpong et al. improved the same method to get a transformation efficiency of 31.25 % in hairy roots based on GUS staining; only one, sterile, GUS-positive shoot could be regenerated from such roots.

Tazeen and Mirza (2004) studied different factors to standardise the *A. tumefaciens*-mediated transformation protocol for *V. radiata* using *A. tumefaciens* strain C58C1 harbouring a binary vector p35SGUSINT containing *NPTII* gene as selectable marker and GUS as a reporter gene. Kanamycin-resistant explants and regenerated calli were screened by transient and stable GUS expression, respectively, achieving up to 70 % transient GUS expression at pH 5.8 after 3 days of co-culturing in 2-day-old explants. Higher transformation efficiency (80 %) was achieved using primary leaves than hypocotyl (60 %) or root (40 %) explants but they were also unable to regenerate shoots from calli.

The lack of an efficient and reliable regeneration system for successful acclimatisation and growth of mungbean plants to maturity has slowed its improvement via tissue culture selection and transformation. Mahalakshmi et al. (2006) obtained the first stably transformed mungbean plants after developing a genotype independent, high frequency plant regeneration protocol from primary leaf

explants. Hygromycin-resistant transformed plants were morphologically similar to seed-germinated plants. Stable integration of the transgene in the primary transgenics and stable inheritance to progeny was confirmed through PCR and Southern hybridisation.

Successful production of insect resistant phenotypically normal and fertile mungbean expressing insect resistant gene was reported for the first time by Saini et al. (2007). They transformed cotyledonary node explants with *A. tumefaciens* EHA105 harbouring α -amylase inhibitor gene of *Phaseolus vulgaris* with insecticidal nature, and *bar* as a selectable marker, and obtained plants via direct shoot organogenesis. Stable integration and expression of the *bar* gene in T₀ plants was shown by PCR-Southern analysis and PPT leaf paint assay, respectively. Presence of the α -amylase inhibitor gene was also confirmed by Southern blot analysis while inheritance of both transgenes to the progeny was evidenced by PCR. Among different conditions, preculture and wounding of the explants, use of acetosyringone during co-cultivation and PPT-based selection of transformants played vital role for achieving an enhanced transformation frequency of 7.81 %.

Islam and Islam (2010) compared cotyledonary leaf and cotyledon attached with embryonic axis (CAEA) as explants for transformation of local mungbean varieties using *A. tumefaciens* strain LBA4404. Based on kanamycin selection and GUS assay, CAEA showed better response towards transformation than the cotyledonary leaf, obtained after 45 min of infection with Agrobacterial suspension having an OD₆₀₀ of 1.3 and 3 days of co-cultivation.

4.3.11 Blackgram

Vigna mungo, blackgram, is a large-seeded grain legume grown in developing countries of southeast Asia, notorious for its recalcitrance to in vitro regeneration and, not unexpectedly, this has adversely affected attempts at its genetic transformation (Table 14).

Transformed blackgram calli obtained by Karthikeyan et al. (1996) after co-culture of segments of primary leaves with two strains of *A. tumefaciens*, i.e. LBA4404 and EHA105 exhibited neomycin phosphotransferase activity but were unable to regenerate plants.

Saini et al. (2003) established an efficient plant regeneration method through direct multiple shoot organogenesis from cotyledonary-node explants without cotyledons, which they used for *A. tumefaciens*-based transformation of *V. mungo*. An optimal selection system enabled them to produce kanamycin-resistant and GUS-expressing transgenic plants; however, the transformation frequency remained low, i.e. 1 %. They proposed that this low yield of transformants using cotyledonary node explants was due to a limited number of meristematic cells whose capability for regeneration was short lived. In this context, the current trend in genetic transformation of recalcitrant grain legumes has been to target meristems as a source of totipotent cells (Somers et al. 2003). Saini and Jaiwal (2005) first reported *A. tumefaciens*-mediated transformation of the shoot apical meristem in

Table 14 Blackgram (*Vigna mungo* L.) transformation

<i>Agrobacterium</i> strain/method References	Genotype	Explants	Selection marker and	reporter genes	Transformation efficiency (%)
LBA4404, EHA105	Co5	Primary leaves	<i>nptII</i>	10–23 % Kanamycin- resistant callus	Karthikeyan et al. (1996)
EHA105	Pusa-1	cotyledonary nodes with and without cotyledons	<i>nptII</i> , <i>GUS</i>	1	Saini et al. (2003)
EHA105	PS-1, Pusa-2, Vamban-1, Co-5, T-9	shoot apices,	<i>nptII</i> , <i>GUS</i>	1–6.5	Saini and Jaiwal (2005)
LBA4404	Vamban 3	Immature cotyledonary nodes, shoot-tips	<i>nptII</i> , <i>bar</i> , <i>GUS</i>	2.6–7.6	Muruganantham et al. (2007)
EHA105	PS-1	cotyledonary nodes	<i>nptII</i> , <i>GUS</i>	4.31	Saini and Jaiwal (2007)

legumes by establishing an efficient regeneration system from mature seed-derived embryo shoot apices. Optimisation of co-culture conditions and wounding of explants led to an efficiency of 6.5 %, and they later (Saini and Jaiwal 2007) optimised the conditions affecting *A. tumefaciens*-mediated transformation of blackgram. They concluded that inoculation of pre-cultured and mechanically injured cotyledonary node explants for 30 min with *A. tumefaciens* at a density of 10^8 cells/cm³ followed by co-culture on SR medium for 3 days was most beneficial.

Muruganantham et al. (2005) introduced a different efficient plant regeneration system from immature cotyledonary nodes from 18 DAP seeds, later used (Muruganantham et al. 2007) to get transformed blackgram plants from both cotyledonary-node and shoot-tip explants from immature seeds. After selection on phosphinothricin, transformation efficiency was 7.6 %.

For transformation via either *A. tumefaciens* or biolistic method, embryogenic cultures can serve as good target tissues because such cultures provide a high level of cell exposure. Muruganantham et al. (2010) used primary leaf explants of *V. mungo* for the induction of embryogenic callus. Liquid shaken culture of embryogenic calluses led to plant regeneration via somatic embryogenesis. This regeneration method can be applied for genetic improvement of this crop through transformation.

4.3.12 Pigeonpea

Pigeonpea (*Cajanus cajan* L.), the main food legume of the semi-arid tropics because of its richness in protein, has also been genetically transformed (Table 15). Geetha et al. (1999) first reported its *A. tumefaciens*-mediated transformation using shoot apices and cotyledonary nodes as explants followed by direct regeneration,

Table 15 Pigeonpea (*Cajanus cajan* L.) transformation

<i>Agrobacterium</i> strain/method	Genotype	Explants	Selection marker and reporter genes	Transformation efficiency (%)	References
LBA4404	HyderabadC	Shoot apices, cotyledonary nodes	<i>nptII, GUS</i>	45–62 % Kanamycin- resistant shoots	Geetha et al. (1999)
GV2260	Pusa 855	Embryonic axes	<i>nptII</i>	1	Lawrence and Koundal (2001)
Biolistic	ICPL 87, ICPL 88039, ICPL 87119, ICPL 85063, ICPL 88009, ICPL 87091, ICPL 2376, ICPL 87051, ICPL 91011, ICPL 332, and ICPL 84031	Leaves	<i>nptII, GUS</i>	50 % of selected plants	Dayal et al. (2003)
LBA4404	T-15-15	Decapitated mature embryo axes	<i>nptII, GUS, GFP</i>	1.7–6.7 % Kanamycin- resistant shoots	Mohan and Krishnamurthy (2003)
LBA4404	ICPL 87	Seed-derived calli	<i>nptII, GUS</i>	20–25 % based on randomly selected plants	Thu et al. (2003)
C-58	ICPL 87	Axillary meristem	<i>nptII, GUS</i>	54–70 % based on regenerated plants	Sharma et al. (2006)
LBA4404	LRG 30, ICPL 87, ICPL 85063	Plumules, nodes, cotyledons	<i>nptII, GUS</i>	36–80 % explants based on GUS staining	Surekha et al. (2007)

albeit at very low frequency, of transformed plants and coupled with integration of T-DNA into the genome of transgenic plants confirmed by Southern hybridisation.

Transgenic pigeon pea plants resistant to chewing insects, expressing the cow-pea protease inhibitor gene, were successfully obtained by Lawrence and Koundal (2001) through indirect regeneration from callus derived from embryonic axes. Expression of transgene was verified through Northern hybridisation. The frequency of transformed shoots (T0) was less than 1 %.

A significant improvement was achieved through an efficient plant regeneration method (>90%) from leaf explants of pigeonpea by Dayal et al. (2003), who also showed genetic transformation of leaf explants using biolistics; 90% of the bombarded explants exhibited transient expression of the uidA gene and 50% of the selected plants that were transferred to the glasshouse showed positive gene integration.

An efficient regeneration protocol for pigeonpea based on callus induction and differentiation on high concentration of cytokinin from seed explants was developed by Thu et al. (2003). They transformed pigeonpea using both *A. tumefaciens*-mediated gene transfer and biolistics. Stable transmission and expression of transgene in the progeny was confirmed through GUS assays, PCR and Southern hybridisation.

Mohan and Krishnamurthy (2003) reported efficient *A. tumefaciens*-mediated transformation of pigeonpea based on a reliable regeneration method by direct organogenesis from mature embryo-derived explants using *nptII* as selectable marker and GUS and GFP as reporter genes. Southern hybridisation of plants confirmed the stable integration of *GFP* gene in transgenics. In parallel, calli were also induced from those Agro-infected explants. Transformed callus showed GFP and GUS expression but were unable to develop plants.

The first record on the successful production of pest-resistant pigeonpea was reported by Sharma et al. (2006) who transformed axillary meristem explants with the *Bt cryIAb* gene driven by double-enhanced CaMV35S promoter, also containing fused *uidA* and *nptII* genes driven by CaMV35S promoter as selectable marker genes. Transformed organogenic tissues differentiated into shoot buds. GUS staining and southern blot analysis proved the transgenic nature of the progeny. Expression of the transgene in progeny was proved using RT-PCR. Stable expression of Cry1Ab protein was observed even in the T2 generation using ELISA. Here, 60 % of the independently transformed plants showed positive gene integration and expression and 65 % of the transformants showed single copy inserts of the introduced gene.

Surekha et al. (2007) showed effects of numerous pigeonpea genotypes, using various explant sources and different *A. tumefaciens* strains on pigeonpea transformation frequencies. Transformation frequency was evaluated using GUS staining and it depends upon genotype, explant and *A. tumefaciens* strain. They were also the first to prove endogenous glucuronidase activity in pigeonpea that is restricted to the root meristems region.

A very informative review on the progress of tissue culture and genetic transformation research in pigeon pea [*Cajanus cajan* (L.) Millsp.] was recently published by Krishna et al. 2010.

4.4 Miscellaneous Grain and Medicinal Legume Species

Lathyrus sativus L., grasspea, is an important grain legume cultivated extensively in tropical and sub-tropical parts of the world and is well adapted to adverse agricultural conditions (Vaz Patto et al. 2006). Despite all these features, only limited efforts have been made to exploit the potential of this grain legume because of the presence of a neurotoxic amino acid that causes neurolathyrism in humans with prolonged consumption (Ochatt et al. 2007). Barik et al. (2005) developed an efficient and reproducible procedure for *Agrobacterium*-mediated genetic transformation of grasspea using epicotyl segments as explants. Two different strains of *Agrobacteria*, i.e. EHA 105 and LBA 4404 were used, and up to 36% transient expression was achieved based on the GUS histochemical assay. Southern hybridisation of genomic DNA of the kanamycin-resistant GUS-expressive shoots proved the integration of the transgene. In T1, the plants segregated in a typical 3:1 Mendelian ratio.

Parkia timoriana (DC.) Merr., popularly known as “tree bean”, is a very large legume tree distributed from northeast India to Irian Jaya and is the most widely distributed species of the Indo-Pacific region. Its pods are consumed as vegetable and salad while branches and wood are used as firewood or as timber. The first report of successful in vitro regeneration and establishment in the field for transgenic *P. timoriana* came from Thangjam and Sahoo (2012). They used cotyledonary node explants for transformation experiments with *A. tumefaciens* strain EHA105 harbouring a binary vector pCambia2301 which contains β -glucuronidase (GUS) with an intron in the coding region and neomycin phosphotransferase (nptII) gene, both driven by CaMV35S promoter. Explants were grown on constant kanamycin selection. Transient GUS expression in cotyledonary explants was scored after 3-day co-cultivation using histochemical GUS assay. Transformants were verified by PCR and southern hybridisation.

Mucuna pruriens, known as velvet bean or cowitch, is a tropical legume used in agriculture and horticulture that has a range of medicinal properties. Sathyanarayana et al. (2012) reported a simple and reliable method for the genetic transformation of *M. pruriens* mediated via *A. tumefaciens*. Leaf discs were used as explants and regeneration from callus was achieved through somatic embryogenesis. Transformed plants were confirmed through histochemical GUS staining and PCR.

Sesbania drummondii, known as Rattlebush, Rattlebox and Poison bean, is a medium-sized perennial shrub native to coastal areas of the United States. It is an important source for phytopharmaceuticals. Padmanabhan and Sahi (2009) developed an efficient method for its *A. tumefaciens* genetic transformation followed by

successful regeneration of shoots through organogenesis from cotyledonary node explants. GUS staining followed by PCR and Southern blotting was done to evaluate the integrity of transgene.

5 Concluding Remarks and the Way Forward

From the recent advances in genetics and genomics it is clear that gene transfer is emerging as a major player in the understanding of gene function and its validation, and also that it may play an important role for the generation of genetic novelties that, pending appropriate legislation and stringent control of the performance of transgenic plants and inheritance of introduced traits once in the field, should find their way into the breeding strategies for a number of crops. Progress achieved to date in the use of transgenic legumes worldwide is rather limited and this contrasts markedly with the expectations of both farmers and breeders (Dita et al. 2006; Eapen 2008). Legumes will still require a large research input worldwide until efficient protocols permitting their routine transformation are available.

To date, transgenic plants of grain legumes have mostly been produced up to the T0 generation levels only and more rarely up to later progeny levels (T1, T2, etc.). This would, however, be a major prerequisite for the successful commercialisation of any transgenic pulse crop, as the stability and inheritance of transgenes introduced must be ascertained under field conditions. The fact that most pulse crops are mainly cleistogamous reduces the risk of transgenes being horizontally transferred to related species but does not entirely preclude it, and all proof in this context will have to be provided if pulse crops are to find their way into commercial exploitation as both food and feed.

With the novel post-genomic tools, new opportunities exist now for a better understanding of many metabolic pathways and for the identification of genes involved therein. This will undoubtedly contribute to the development of grain legumes with a higher nutritive value, but also to an improved yield and tolerance to various biotic and abiotic stresses. In this respect, the study of transcription factors involved in the development of the embryo and in seed filling will play a crucial role and contribute to improving the nutritional value of grain legumes, i.e. by suppressing anti-nutritional compounds and toxins. However, transgenic legume research must still gain pace and the commercialisation of genetically modified legume crops will still require several years before it becomes a reality.

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