

## Review Article

# Genetic engineering of grain legumes: key transformation events

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### Abstract

A well defined, preferably simple, shoot regeneration protocol is a prerequisite for the production of transgenic plants. Grain legumes are one of the least amenable groups to transformation amongst dicotyledonous crops, although they are usually susceptible to *Agrobacterium* infection. Important parameters for successful transformation of grain legumes include the characteristics of the *Agrobacterium* strain used for inoculation of target plant tissues, the vectors which the bacterial strain carries, the co-cultivation period and a selection system combined with suitable explants that contain transformation competent cells. Particle bombardment is an alternative procedure for those legumes which fail to respond to *Agrobacterium*-mediated gene transfer.

### Introduction

Continued genetic improvement is a necessity for the development of crops with increased quality and yield. Methionine, for example, is the main limiting essential amino acid which influences the biological value of the protein in grain legumes. However, the correct balance in amino acid composition cannot be achieved by conventional breeding but requires the exploitation of genetic engineering techniques, since the latter offer the most promising strategy for increasing (by 5-10%) the concentration of methionine. This necessitates transfer into target legumes, by *Agrobacterium* or other means, of foreign genes encoding methionine-rich proteins, such as the Brazil nut 2S albumin or its homologue from sunflower. Indeed, such gene transfer experiments have demonstrated that the protein balance of grain legumes, such as lupins, can be corrected to FAO standards (Molvig *et al.*, 1997; Muntz *et al.*, 1998)

In crop plants, many of the shoot regeneration protocols have been developed in recent years specifically for exploitation in genetic manipulation experiments (Böhmer *et al.*, 1995). However, several requirements must be fulfilled in order to produce stably transformed plants. Initially, a suitable method is required to deliver foreign DNA to plant tissues, followed by the appropriate procedure for culturing tissues prior to the regeneration of shoots leading to the recovery of transgenic plants. Subsequently, the newly introduced gene(s) must be expressed in transgenic plants and, finally, the foreign DNA must be heritable and expressed reproducibly in succeeding seed generations.

### Systems for DNA delivery to crop plants

#### *Agrobacterium*-mediated transformation

The most widely used DNA delivery systems which have potential practical applications include those based on the natural gene transfer mechanism of the Gram negative soil bacterium *Agrobacterium*, with techniques such as particle bombardment and electroporation and/or chemical treatment of isolated protoplasts providing alternative approaches. Whilst these procedures differ in the way in which DNA is delivered into plant cells (De Block, 1993), they all require the use of cultured cells and tissues as recipients of foreign DNA. *Agrobacterium tumefaciens* and *A. rhizogenes* are the most frequently exploited gene transfer agents for generating transgenic plants in a wide variety of plant species (Hooykaas, 1989). These bacteria are well recognised plant pathogens which induce the agronomically-important diseases crown gall (incited by *A. tumefaciens*) and hairy root (incited by *A. rhizogenes*) in many dicotyledons (Kerstens and De Ley, 1984). Both diseases are caused by the transfer and stable integration of part (the transferred or T-DNA), of a large tumour- (Ti) or root-inducing (Ri) plasmid from the bacterium into the genome of recipient plant cells (Tinland, 1996). Of significance is the fact that foreign genes inserted between the T-DNA borders are also integrated, on the T-DNA, into recipient plant genomes.

In plasmids from wild-type strains of *Agrobacterium*, expression of oncogenicity genes on the T-DNA normally modifies the physiology of plant cells to undergo tumour formation. However, removal of such genes results in disarmed Ti or Ri plasmids, which can be used

to introduce foreign genes into plant cells without affecting their endogenous growth regulator balance. Subsequently, such cells may be induced to regenerate into phenotypically normal transgenic plants. Cointegrate vectors involve the insertion by homologous recombination of foreign gene(s) between the T-DNA borders of disarmed Ti plasmids and, to a lesser extent, Ri plasmids. A problem encountered with cointegrate vectors is their size, which makes their manipulation in the laboratory difficult. Consequently, the binary vector system is the one of choice, in which the disarmed T-DNA is placed on a small plasmid capable of being introduced and amplified in *Escherichia coli* and later transferred into *Agrobacterium* for plant transformation. The disarmed T-DNA usually has a multiple cloning site to facilitate foreign gene insertion. T-DNA transfer from the binary vector to plant cells is still controlled by virulence genes on the larger resident Ti plasmid, deleted of its T-DNA, within the *Agrobacterium* cell. Whilst the natural virulence of *agrobacteria* varies and hence their ability to infect plants, the virulence of some strains can be increased by the introduction of a supervirulent plasmid, such as pTOK47 carrying extra copies of some of the virulence genes, into the *Agrobacterium* cell, alongside the binary vector. Such supervirulent strains of *Agrobacterium* have been useful in transforming certain dicotyledons, such as lettuce (Curtis *et al.*, 1994). Super binary vectors, in which extra copies of virulence genes are on the binary vector itself, have also proven useful in the transformation of cereals, as in the case of rice (Hiei *et al.*, 1994, 1997). *Agrobacteria* carrying super binary vectors may also prove useful, in the future, in the genetic manipulation of "difficult-to-transform" dicotyledons, such as grain legumes. Certain disarmed strains of *A. tumefaciens* have been used extensively for several years to carry the binary and super binary vectors, an excellent example being LBA4404 (Hoekema *et al.*, 1983). The plant genotype is also a major factor which influences transformation.

#### Biolistics for gene delivery

Biolistics, particle bombardment or "gene gunning" is a technique which, unlike the use of *Agrobacterium*, is plant genotype-independent. The technique relies upon the acceleration of DNA coated particles (microprojectiles) into target cells. The microprojectiles usually consist of fragments of inert metal, usually gold, with diameters of 0.2-4.0  $\mu\text{m}$ . The most commonly used instruments for accelerating DNA coated particles are those powered by a burst of helium generated by a rupture-membrane mechanism (Kikkert, 1993), or by a shock wave generated by a high voltage discharge through a water droplet (McCabe and Christou, 1993). In both cases, a macrocarrier, upon which the DNA coated microprojectiles have been placed, is accelerated towards a perforated stopping screen. The macrocarrier is arrested by the stopping screen; the microprojectiles continue at high velocity, usually under vacuum, into the target tissue. Subsequently, DNA is released from the microprojectiles within the target cells and becomes integrated into plant genomic DNA, although the precise procedures involved in this process remain unclear.

#### Other approaches for gene transfer into plants

Electroporation and/or polyethylene glycol (PEG) treatment of DNA-protoplast mixtures has been exploited for transgenic plant production where a protoplast-to-plant regeneration system is available, but where the plant cells do not respond readily to *Agrobacterium* inoculation. In addition, isolated protoplasts are a useful experimental system for studying transient gene expression. For example, Giovanazzo *et al.* (1997) used protoplasts isolated from suspension cultured cells of *Phaseolus vulgaris* to investigate the expression and stability of a phaseolin gene sequence driven by a constitutive promoter. Accumulation of the correctly glycosylated and assembled protein was recorded in the protoplasts, the latter providing an excellent experimental system with which to study the

expression of wild-type as well as *in vitro* modified seed proteins. Other transformation approaches have been evaluated and their merits and limitations have been discussed (Southgate *et al.*, 1998), particularly in the case of monocotyledons. However, even in monocotyledons, the use of isolated protoplasts as recipients for foreign DNA insertion has been superseded recently, in several laboratories, by the availability of super virulent strains of *A. tumefaciens*, particularly those harbouring super binary vectors.

### Selection of transformed cells, tissues and regenerated plants

The inclusion of an antibiotic or a herbicide in the culture medium is normally used to select transformed cells and tissues from which transgenic plants are regenerated. The neomycin phosphotransferase (*nptII*) gene, conferring resistance to the aminoglycoside antibiotics kanamycin sulphate and geneticin (G418), has been exploited most extensively in plant transformation systems (Bevan *et al.*, 1983), although hygromycin resistance has been used for selection in the grain legume *Vicia narbonensis* (Pickardt *et al.*, 1991). In the case of herbicide-based selection, the *bar* gene for bialaphos resistance has given tight selection in several cases (Mohapatra *et al.*, 1999), but expression of this gene has not been assessed extensively in grain legumes. Expression of the  $\beta$ -glucuronidase (*gus*) gene (Jefferson *et al.*, 1987) remains a useful marker for rapid assessments of the success of gene delivery to plant cells, while expression of the green fluorescent protein (*gfp*) gene from the jellyfish *Aequorea victoria* (Molinier *et al.*, 2000) provides a very useful, non-destructive approach for monitoring gene transfer and expression in plant tissues. Recently, GFP has been used in the transient and stable transformation of embryogenic suspension cultures of soybean, following gene introduction by particle bombardment (Ponappa *et al.*, 1999).

### *Agrobacterium*-mediated transformation of grain legumes

As a group, grain legumes are less amenable to genetic manipulation *in vitro* compared with most other dicotyledonous crop species, particularly members of the Solanaceae (de Kathen and Jacobsen, 1995). Whilst several leguminous species are susceptible to *Agrobacterium* inoculation, relatively few grain legumes have been stably transformed using disarmed vectors carried by *A. tumefaciens*. A summary of the key transformation events relating to grain legumes is presented in Table 1. Overall, despite their economic importance, grain legumes have attracted less attention for genetic manipulation, compared with cereals, for example, using *in vitro*-based techniques. Possible exceptions are soybean (Jacobsen, 1992) and, to a lesser extent, pea (Bean *et al.*, 1997), with the generation of transgenic tissues and regenerated plants being well documented. Indeed, soybean was the first grain legume from which stable transgenic plants were obtained (Table 1). In the case of pea, the lateral cotyledonary meristems were used to develop a reproducible *A. tumefaciens*-mediated transformation system (Bean *et al.*, 1997). As these authors emphasised, the advantage of their system was that it utilised dry seed as starting material, while the highly regenerable cotyledonary meristems produced transgenic plants rapidly without an intermediate callus phase. Phenotypically normal, fertile plants contained functional transgenes which were inherited in a Mendelian fashion. Genetic engineering of the genera *Phaseolus* and *Vigna* has been reviewed by Nagl *et al.* (1997), while the integration into grain legumes of genes governing desirable traits, such as resistance to herbicides (Schroeder *et al.*, 1993; Russell *et al.*, 1993) and insects (Schroeder *et al.*, 1995; Chrispeels *et al.*, 1998) and increasing methionine to change the ratio of seed proteins (Saalbach *et al.*, 1994; Waddell *et al.*, 1994; Saalbach *et al.*, 1995; Muntz *et al.*, 1998), have also been reported (Table 1).

Table 1. A summary of the transformation of grain legumes.

| Plant species                               | Bacterial strain/procedure  | Explants   | Analysis/result   | Notes  | References                          |
|---|---|--|---|--|-------------------------------------|
| <i>Cicer arietinum</i> , various genotypes  | <i>A. tum.</i> strains A281, C58, A6; <i>A. rhiz.</i> strain R23.   | 7-d-old <i>ex vitro</i> seedlings; 10-d-old <i>in vitro</i> seedlings.                         | <i>In vitro</i> seedlings were more responsive than <i>in vivo</i> seedlings. Tumour and hairy root formation; Southern blot and opine analyses.                                    | Hypocotyl explants were more sensitive than other explants to <i>Agrobacterium</i> . Avirulent <i>Agrobacterium</i> strains failed to elicit any response. | Riazuddin and Husnain, 1993.        |
| <i>Cicer arietinum</i> , local ecotypes     | <i>A. tum.</i> strain LBA4404 carrying pBI121 with the <i>gus</i> and <i>nptII</i> genes. Co-cultivation of explants with <i>agrobacteria</i> for 20 min. | Embryonic axes devoid of apical domes.   | Acetosyringone treatment ineffective in stimulating transformation. Dot and Southern blot analyses; <i>gus</i> and <i>nptII</i> gene expression. Transgenic rooted plants obtained. | 4% transformation frequency. Origin of regenerants not discussed. First report of chickpea transformation.   | Fontana <i>et al.</i> , 1993.       |
| <i>Cicer arietinum</i> , various accessions | <i>A. tum.</i> strains C58C1/GV2260 carrying p35SGUSINT, and EHA101 harbouring pIBGUS.  | Embryo axes.   | GUS and Southern analyses. Kanamycin and phosphinothricin resistant transgenic plants. Rooting of transgenic shoots difficult.  | Grafting of transgenic shoots onto non-transformed stock plants resulted in recovery of phenotypically normal transgenic plants.                           | Krishnamurthy <i>et al.</i> , 2000. |
| <i>Glycine canescens</i>                    | <i>A. rhiz.</i> strain A4T and hypervirulent strain R1601.  | Hypocotyl sections of 12-d- and 35-42-d-old seedlings.   | Hairy root formation; NPTII and Southern analyses; gene integration and regeneration of transgenic plants from hairy roots.   | Young seedling explants are more responsive than older explants. <i>A. rhiz.</i> strain A4T failed to infect explants.                                     | Rech <i>et al.</i> , 1989.          |
| <i>Glycine argyrea</i> accession G1420      | <i>A. rhiz.</i> strains LBA9402, A4T and engineered strains R1601 and A4TIII.   | 9, 14 and 27 d-old seedling hypocotyl explants.  | Hairy root formation; regeneration of transgenic plants from hairy roots; opine and NPTII analyses.   | Explant age and bacterial concentrations important to maximise the transformation response.  | Kumar <i>et al.</i> , 1991.         |
| <i>Glycine max</i>                          | <i>A. tum.</i> with pTIT37-SE carrying <i>nptII</i> and <i>gus</i> genes; pTIT37-SE: pMON894 with <i>nptII</i> and glyphosate resistance genes.           | 4-10-d-old seedling cotyledons with their adaxial surfaces in contact with the culture medium. | GUS and Southern analyses. First stable, Kan resistant and glyphosate tolerant transgenic soybean plants by <i>Agrobacterium</i> -mediated transformation.                          | Plant genotype was important in response to <i>Agrobacterium</i> . Kan selection enriched transformed tissues. Mendelian gene inheritance of genes.        | Hinchee <i>et al.</i> , 1988.       |
| <i>Glycine max</i>                          | Delivery of <i>nptII</i> and <i>gus</i> genes by electric discharge particle acceleration device.   | Embryonic axes with exposed meristems devoid of leaf primordia.                                | GUS, NPTII and Southern analyses; gene expression and integration. First stably transformed soybean plant by particle bombardment.  | Transformation frequency 2-4%. Variable gene expression and some chimaeric plants.   | McCabe <i>et al.</i> , 1988.        |
| <i>Glycine max</i>                          | Delivery of <i>nptII</i> and <i>gus</i> genes by tungsten particles using PDS100 instrument.  | Shoot tips and embryonic cell suspensions.   | GUS and Southern analyses. Stable transformation of suspension cells leading to regeneration of somatic embryos and plants.   | Chimaeric gene expression in shoot tip explants. DNA coated particles penetrated into the second cell layer of bombarded shoot tips.                       | Sato <i>et al.</i> , 1993.          |
| <i>Lens culinaris</i>                       | <i>A. tum.</i> strain GV2260 carrying p35SGUSINT.   | Shoot apex consisting of apical dome, leaf primordia and part of the epicotyl.                 | GUS assay; transgenic shoots not regenerated. Pre-cultured explants exhibited less response than newly excised explants.  | Longer co-cultivation periods (>3 d) reduced the number of buds which developed and survived.  | Warkentin and McHugen, 1992.        |
| <i>Lens culinaris</i>                       | Electroporation of <i>in vivo</i> tissues.  | Intact plant tissues from nodal axillary buds; juvenile nodal meristems.                       | GUS and Southern analyses. Chimaeric shoots rooted to produce transgenic plants.  | Procedure also applicable to <i>Pisum sativum</i> , <i>Vigna unguiculata</i> and <i>Glycine max</i>  | Chowrira <i>et al.</i> , 1996.      |

Table 1. (Continued) A summary of the transformation of grain legumes.

| Plant species   | Bacterial strain/procedure  | Explants   | Analysis/result   | Notes  | References                     |
|---|---|--|---|--|--------------------------------|
| <i>Lupinus angustifolius</i> cvs. Unicorp and Merit.                    | <i>A. tum.</i> carrying the bar gene.   | Shoot apices producing multiple axillary shoots.   | Southern analysis. Herbicide resistant transgenic plants and seed progeny.  | Transformation frequency of 0.4-2.8%. Meristems required for successful transformation.  | Pigeaire <i>et al.</i> , 1997. |
| <i>Lupinus mutabilis</i> cv. Potosi                                     | <i>A. tum.</i> LBA4404 with a binary vector carrying gus and nptII genes, together with the supervirulent pTOK47. | Shoot apical meristems of 4-7-d old seedlings from which initial cell layers removed.      | GUS, NPTII and Southern analyses; integration and expression of gus and nptII genes. Production of Kan resistant plants.                    | Removal of apical cell layers promoted regeneration of buds. First transgenic sweet lupin plants.                                    | Babaoglu <i>et al.</i> , 2000. |
| <i>Lupinus mutabilis</i> cv. Potosi; <i>L. angustifolius</i> cv. Kubesa | Hypervirulent <i>A. rhiz.</i> R1601 carrying nptII gene.  | Intact hypocotyl and epicotyl explants from young seedlings.                               | NPTII and Southern analyses; gene integration and expression. Kan resistant transgenic hairy roots with rapid growth rates in both species. | First report of hairy root induction in both species. Hairy roots exhibit stable growth over 4 years, but fail to regenerate plants. | Babaoglu, 1996.                |
| <i>Phaseolus acutifolius</i> cv. Gray.                                  | <i>A. tum.</i> C58C1 carrying nptII, gus and orceLin-5a genes.  | Regeneration-competent callus derived from bud explants of <i>in vivo</i> cultured plants. | GUS and Southern analyses; transient expression of gus gene; integration and segregation of genes in progenies at a single locus.           | A new approach offering this species as a bridge for gene transfer to more important bean species.                                   | Dillen <i>et al.</i> , 1997.   |
| <i>Phaseolus vulgaris</i> cv. Goldstar                                  | Particle bombardment with pSAG-734 carrying the gus gene under the canalin gene.                                  | Seeds.   | GUS histochemical assay; production of transgenic plants.   | Organ and maturation stage specific gene expression.   | Kim and Minamikawa, 1997.      |
| <i>Phaseolus vulgaris</i> cv. Carioca                                   | Delivery of gus and 2S albumin genes using an electrical particle acceleration gun.                               | Embryonic axes excised from mature seeds.  | GUS, Western and ELISA assays; expression of gus and 2S genes.  | Transgenic plants not regenerated, but first demonstration of the expression of a nutritional protein in legumes.                    | Aragao <i>et al.</i> , 1992.   |
| <i>Phaseolus vulgaris</i>   | Electric discharge particle acceleration of pWRG2204 carrying gus and bar genes.                                  | Apical meristems derived from seeds incubated overnight in MS-based medium.                | GUS, Southern and Northern analyses; gus gene expression and stable transformation; herbicide resistant plants.                             | Germline transformed at low frequency (0.03%) to give transformed shoots and plants.   | Russell <i>et al.</i> , 1993.  |
| <i>Phaseolus vulgaris</i> L. cv. Dark Red Kidney                        | <i>A. tum.</i> EHA101 carrying pKYLX71GUS.  | Leaf disks and hypocotyl segments from 3-4- and 7-d-old seedlings.                         | NPTII ELISA and Southern analyses; Kan resistant callus from leaf disks with stable integration of DNA into cells.                          | Plants not regenerated from transformed tissues.   | Franklin <i>et al.</i> , 1993. |
| <i>Phaseolus vulgaris</i> , various cultivars and lines.                | <i>A. tum.</i> oncogenic and disarmed strains with gus reporter gene.   | Stab inoculation of nodal regions of germinating intact seedlings.                         | GUS and tumourigenesis assays; GUS activity in different tissues.   | Stab inoculation of apical meristems exposes the interior cell layers to agrobacteria.   | Levis and Bliss, 1994.         |
| <i>Phaseolus vulgaris</i> , various cultivars                           | <i>A. tum.</i> carrying gus and nptII genes.<br><i>A. rhiz.</i>   | Multiple buds from cotyledonary nodes.<br>Epicotyl explants.                               | GUS activity. Chimeric shoots produced<br>Transgenic hairy roots.   | Transgenic plants not regenerated.   | Barros <i>et al.</i> , 1997.   |
| <i>Pisum sativum</i> cv. Puget  | <i>A. tum.</i> wild-type strain C58; <i>A. rhiz.</i> wild-type strain 9402.                                       | Shoot apex, epicotyl and cotyledons.   | Histological analysis of tumours and hairy roots.   | Meristematic cells more responsive than other cell types to transformation.  | Hussey <i>et al.</i> , 1989.   |
| <i>Pisum sativum</i> . Various cultivars                                | <i>A. tum.</i> C58C1 carrying a binary vector.  | Thin cell layers from nodes.   | GUS assay; GUS activity in leaves of transformed shoots.  | Freshly excised tissues are more vulnerable to infection to <i>Agrobacterium</i> ; rooting of shoots is cultivar dependent.          | Nauerby <i>et al.</i> , 1991.  |

**Table 1.** (Continued) A summary of the transformation of grain legumes.

| Plant species   | Bacterial strain/procedure   | Explants  | Analysis/result  | Notes  | References                         |
|---|--|---|--|--|------------------------------------|
| <i>Pisum sativum</i> cvs. Greenfeast, Rondo                             | <i>A. tum.</i> strain AGL1 carrying <i>bar</i> and <i>nptII</i> genes.                                   | Immature embryonic axes lacking roots, co-cultivated for 4 d. | PAT assay and Northern blot; stable transformation (1.5-2.5%). First herbicide resistant pea plants.   | Mendelian inheritance of <i>bar</i> and <i>nptII</i> genes.  | Schroeder <i>et al.</i> , 1993.    |
| <i>Pisum sativum</i> cv. Puget  | <i>A. tum.</i> C58/3 carrying pSLJ1911 with <i>gus</i> and <i>nptII</i> genes.                           | Cotyledonary nodes.   | PCR, GUS and Southern analyses; multiple <i>gus</i> gene insertions; transformation frequency of 1.44%.  | Some plants escape Kan selection; high initial Kan levels reduce the efficiency of selection.  | Davies <i>et al.</i> , 1993.       |
| <i>Pisum sativum</i> cv. Greenfeast                                     | As in Schroeder <i>et al.</i> (1993) plus the <i>ai</i> gene.  | Procedure of Schroeder <i>et al.</i> (1993).                  | Northern Blot; insect infestation assay. First pea plant partially resistant to Pea Weevil.  | Gene expression restricted to cotyledons and embryonic axes.   | Schroeder <i>et al.</i> , 1995.    |
| <i>Pisum sativum</i> cvs. Bolero, Huka and Trounce.                     | <i>A. tum.</i> strain AGL1 with pLN27 carrying <i>bar</i> and <i>nptII</i> genes.                        | Immature cotyledons.  | PAT, Southern and cytological analyses. Transgenic herbicide resistant pea plants produced.  | Stable inheritance and expression of transgenes.   | Grant <i>et al.</i> , 1995.        |
| <i>Pisum sativum</i> cv. Puget  | <i>A. tum.</i> hypervirulent strain EHA105 carrying the binary vector pSLJ1561 with the <i>bar</i> gene. | Cotyledonary meristems  | PAT analysis and Southern analyses; herbicide painting. Transformants phenotypically normal following grafting to cv. Puget rootstock. Mendelian inheritance of the <i>bar</i> gene. | Rooting of putative transformants slow, erratic and unreliable, taking 6-12 weeks. Transformants phenotypically abnormal exhibiting unless grafting. | Bean <i>et al.</i> , 1997.         |
| <i>Vicia narbonensis</i> var. <i>narbonensis</i>                        | <i>A. tum.</i> strain C58C1 carrying pGV3850HPT.   | Small segment of epicotyl and shoot tips.                     | Southern blot analysis; hygromycin resistant transgenic shoots.  | All rooted shoots failed to undergo further development.   | Pickardt <i>et al.</i> , 1991.     |
| <i>Vicia narbonensis</i>  | <i>A. tum.</i> strain EHA101 with pGSGLOC1-2S carrying <i>gus</i> , <i>nptII</i> and 2S genes.           | Method of Pickardt <i>et al.</i> (1991).                      | GUS and Southern analyses; R2 progeny analysis; first stable transgenic plants which express <i>gus</i> , <i>nptII</i> and 2S genes.   | First expression of a nutritional protein in seed progeny.   | Saalbach <i>et al.</i> , 1994.     |
| <i>Vicia faba</i> , various cultivars                                   | <i>A. tum.</i> with Ri plasmid; <i>A. rhiz.</i> strains.   | Different sites on stem, stabbed to 2-3 mm depth.             | Tumour formation preceding hairy root formation after 10 d.  | Strain A4 (pRiA4) did not induce hairy roots, but strain A4T infected all cultivars.   | Siefkes-Boer <i>et al.</i> , 1995. |
| <i>Vigna aconitifolia</i> , <i>Vigna mungo</i> and <i>Vigna radiata</i> | Particle bombardment using pB1221 carrying <i>gus</i> and <i>nptII</i> genes.                            | Mature embryos with one cotyledon.                            | GUS assay; <i>gus</i> gene expression in cotyledonary meristematic region.   | Plants putatively transformed, but transformation not confirmed by Southern analysis.  | Bhargava and Smigochi, 1994.       |
| <i>Vigna mungo</i>  | <i>A. tum.</i> with binary vector pGA472.  | Leaf-derived calli  | NPTII and Southern analyses; transformed calli with gene integration.  | Transgenic plants not regenerated.   | Karthikeyan <i>et al.</i> , 1996.  |
| <i>Vigna unguiculata</i> cv. Blackeye                                   | Electroporation with plasmid carrying <i>gus</i> gene  | Embryonic axes from mature seeds.                             | GUS assay; microscopical analysis. GUS activity detected in various tissues.   | Various levels of transgene expression. Progeny tests not reported.  | Akella and Lurquin, 1993.          |
| <i>Vigna unguiculata</i> various accessions                             | <i>A. tum.</i> Strain C58 carrying pGV2260 and p35SGUSINT.   | Mature, ungerminated, excised embryos.                        | GUS and DNA dot blot analyses; chimaeric GUS-positive shoots regenerated.  | The majority of transgenic cells were located in the subepidermal layers of stems.   | Penza <i>et al.</i> , 1991.        |

(A. tum. = *Agrobacterium tumefaciens*; A. rhiz. = *Agrobacterium rhizogenes*; Kan = kanamycin sulphate).

Prior to the introduction of agronomically useful genes into a crop species by non-oncogenic strains of *Agrobacterium*, the plant response to bacterial inoculation is often evaluated by inoculating explants or intact plants with wild-type strains of *Agrobacterium*. These experiments provide insight into such parameters as the optimal co-cultivation period and the plant cells most competent for transformation within target tissues (de Kathen and Jacobsen, 1995). For example, Zhanyuan *et al.* (1997) investigated factors affecting transformation of common bean, using the *Agrobacterium* strains A2760 and EHA105 carrying the *gus* reporter gene to facilitate rapid screening of transformation. They confirmed an *Agrobacterium*-host plant interaction and obtained data relating to the influence of explant maturity, preculture and *Agrobacterium*-explant co-cultivation conditions, and the selection procedure on the transformation rate. In addition, it was suggested that preconditioning of explants on a medium containing 20  $\mu$ M benzyladenine prior to co-cultivation in darkness with concentrated suspensions of agrobacteria, was essential to optimise transformation rates. Phenolic compounds, such as acetosyringone, stimulate gene transfer from *Agrobacterium* to plant cells and, consequently, are often included in the bacterial culture medium and/or in the medium during co-cultivation of agrobacteria with plant cells.

The most successful transformation systems in grain legumes leading to the recovery of transgenic plants are those which have exploited embryonic axes (Schroeder *et al.*, 1993, 1995), stem nodal segments or cotyledon-hypocotyl sections (de Kathen and Jacobsen, 1990; Nauerby *et al.*, 1991; Davies *et al.*, 1993), and apical explants (Pickardt *et al.*, 1991; Russell *et al.*, 1993). Such explants all have terminal or axillary meristems and, hence, a high shoot regeneration capacity.

Whilst meristematic cells have been considered not to be competent or to have a low competence to *Agrobacterium*-based transformation in pea (de Kathen and Jacobsen, 1995), transgenic plants have been obtained, nevertheless, in this grain legume (Hussey *et al.*, 1989; Bean *et al.*, 1997) and also in chickpea (Fontana *et al.*, 1993; Krishnamurthy *et al.*, 2000), common bean (Levis and Bliss, 1994), sweet lupin (Babaoglu *et al.*, 2000) and blue lupin (Pigeaire *et al.*, 1997). The transformation procedure involves excision of the most apical meristematic layer(s) or stabbing of the apical meristems prior to inoculation with *Agrobacterium* (Table 1). For example, Pigeaire *et al.* (1997) transformed blue lupin with herbicide resistance by stabbing the apical dome and primordia of the third pair of leaves of shoot explants with *A. tumefaciens* carrying the *bar* gene. Interestingly, Babaoglu *et al.* (2000) could induce multiple shoots only after removal of the cell layer(s) of the apices of sweet lupin to a maximum depth of 300  $\mu$ m below the apical dome. Subsequently, such explants were inoculated with a super virulent strain (1065) of *A. tumefaciens* (Curtis *et al.*, 1994) to obtain transgenic shoots. The advantage of using such explants for *Agrobacterium* inoculation compared to other tissues is that the procedure is simple and relatively rapid, with the direct development of shoots from the inoculated explants without an intervening callus phase. The avoidance of a callus phase, followed by regeneration through shoots or somatic embryos, ensures a low incidence of somaclonal variation.

The inability of *Agrobacterium* to penetrate tissues and to adhere to the walls of cells capable of undergoing transformation and shoot regeneration, is probably one of the main factors limiting the transformation of many plants, including grain legumes. In some cases, a combination of procedures has increased the efficiency of transformation, as in subjecting plant tissues to short periods of ultrasound in the presence of bacteria. This treatment, called Sonication-Assisted *Agrobacterium*-mediated Transformation (SAAT), induces

the formation of channels in the target plant tissues, facilitating bacterial access to internal cells of the explants. Such a procedure has been implicated in being especially helpful in transforming meristems with high shoot regeneration potential, but which normally are physically impermeable to agrobacteria. The procedure is also applicable to embryogenic callus and suspension cells (Trick and Finer, 1997). Indeed, stable transformation of soybean embryogenic cell suspensions has been reported using this technique (Trick and Finer, 1998).

During the last decade, there has been an interest in developing *A. rhizogenes* as an alternative system to *A. tumefaciens* for introducing foreign DNA into plant cells. The ability to recover plants from transformed roots is an essential feature of this system. Porter (1991) reviewed the literature relating to the induction of transformed (hairy) roots in *Glycine* species, *Phaseolus vulgaris*, *Pisum sativum*, *Vicia faba*, *Vicia sativa*, *Vigna aconitifolia* and *Vigna unguiculata*, using different strains of *A. rhizogenes*. For example, the induction of transformed roots was reported in *Cicer arietinum* (Riazuddin and Husnain, 1993; Siefkes-Boer *et al.*, 1995), and in *Lupinus angustifolius* and *L. mutabilis* (Babaoglu, 1996). However, plants could only be regenerated from cultured, transformed roots of the wild soybeans *Glycine canescens* (Rech *et al.*, 1989) and *Glycine argyrea* (Kumar *et al.*, 1991), and from *Vigna aconitifolia* (Tepfer, 1990). Limited success has been reported from cultured hairy roots of pea (Saalbach *et al.*, 1994). Collectively, these results indicate that plant regeneration from transformed, cultured roots of grain legumes remains difficult and erratic.

### Microprojectile-mediated transformation of grain legumes

Although most effort has centered upon the use of *Agrobacterium* for introducing genes into grain legumes, there are also reports of the use of biolistics. Shoot apical meristems of mature seeds or whole embryos have been used extensively as target tissues for direct gene transfer by particle bombardment in *Glycine max* (McCabe *et al.*, 1988; Sato *et al.*, 1993), *Phaseolus vulgaris* (Russell *et al.*, 1993) and, with more limited success, in *Vigna* species (Bhargava and Smigocki, 1994) (Table 1). In the majority of cases, explants from near the shoot apex or the apex itself, have been the targets of choice (Christou, 1997) with the exception, recently, of soybean embryogenic cell suspensions which were transformed with the jellyfish *gfp* gene (Ponappa *et al.*, 1999). Apical meristems permit rapid multiple shoot production with minimum tissue culture compared with other types of tissues. More importantly, the genotype has less influence on plant regeneration. The transformation frequency in the case of biolistics is usually low compared to *Agrobacterium*-mediated gene transfer, while the selection of transformed cells and shoots following bombardment of apical explants may be more difficult than *Agrobacterium*-based procedures because of the complex organisation of the shoot apex (Yang, 1993). However, it has been reported that particle bombardment may be the preferred option for gene introduction into large-seeded grain legumes, circumventing the host specificity of many grain legumes to infection by *Agrobacterium* (Christou, 1994, 1995, 1997). Certainly, this technique may be useful for inserting foreign DNA into apical tissues of a species, in a variety-independent manner, when no other pathway of plant regeneration is available. The main limitation to this approach in some laboratories may be where there remains limited access to particle bombardment instruments (Christou, 1997).

An interesting concept is one which combines aspects of *Agrobacterium*-mediated transformation with biolistics. Thus, Hansen and Chilton (1996) described a novel "agrolistic" system in which viru-

lence genes from the Ti plasmid of *A. tumefaciens* were placed on one plasmid and the latter co-delivered by bombardment with a second plasmid carrying the T-DNA borders flanking the gene of interest. Virulence gene expression in *planta* induced T-DNA transfer similar to that occurring during normal *Agrobacterium*-mediated gene delivery. To date, only preliminary information has been obtained in soybean and the application of this approach to a range of legumes still requires further investigation.

### Concluding remarks

This review is intended to provide a brief overview and background information to the transformation of grain legumes since there is still a requirement, possibly through support from the European Union (EU), to develop simple, reproducible and efficient transformation procedures for those legumes cultivated in Europe. Certain parameters may emerge as being common to transformation protocols, such as the use of specific bacterial strains, the co-cultivation period, the bombardment conditions and the nature of the vectors, selectable markers and target explants. However, the rooting of transgenic shoots combined with poor shoot growth following rooting, have been identified as difficulties which remain common to the transformation of some grain legumes and which need to be resolved as soon as possible (Puonti-Kaerlas, 1993; de Kathen and Jacobsen, 1995; Ramsay, 1995).

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