III.5 Chickpea

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1 Introduction

1.1 Importance

Amongst the grain crops, grain legumes (also known as pulses or food legumes) rank third behind cereals and oilseeds in world production. With a worldwide production of 55×10^6 t, pulses represent an important dietary constituent for humans and animals and play an often under-estimated role as break crops that fix nitrogen. Legumes associate with nitrogen fixing bacteria and play a central role in low input agricultural production systems, particularly on small-scale farms (Graham and Vance 2003). Grain legumes are cultivated mainly in developing countries where they accounted for 61.3×10^6 ha in 2002, compared to 8.5×10^6 ha in developed countries. Chickpea (*Cicer arietinum* L.) ranks fourth among the pulses on a global scale, behind soya, peanut and common bean. However, chickpea, also known as garbanzo bean, can be the most important crop at a regional level, especially in semi-arid areas of the world.

This ancient crop probably originated over 7000 years ago in Turkey and spread from there to the Middle East, South Asia and North Africa, where it became an important crop. The small-seeded desi-type chickpea now accounts for about 85% of world production (7.8×10^6 t) and is the principal type grown in India, Pakistan, Iran, Afghanistan and Ethiopia. The less common, large-seeded Kabuli type is grown in the Middle East, India, Mexico as well as in North America, Australia and Spain. Chickpeas are mostly consumed as a mature pulse (cooked whole, dehulled or as flour), but are also served as a vegetable (immature shoots and seeds). Seeds average about 20% protein, 55% carbohydrate and 5% fat and represent a basic food crop in many developing countries. In India, especially, they have a high economic value. Similar to other legumes, proteins of chickpeas are high in lysine, but low in methionine and cysteine. However, combined with cereals they result in a well balanced diet of energy and protein.

Chickpea is a classic low-input crop that often completes its lifecycle in drought and heat stress with no more input than seeds and labour.

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1.2 Crop Constraints

The average yield of chickpea is about 0.8 t ha^{-1} (Sharma and Lavanya 2002), but its estimated yield potential is 5 t ha^{-1}. Drought stress, poor management practices and diseases are the main yield limiting factors in chickpeas. Fungal diseases, such as *Ascochyta* blight, *Rhizoctonia* root rot, *Pythium* rot, *Fusarium* wilt and white mold, as well as bacterial blight and certain viruses can cause considerable damage to the crop. The exudation of malic and oxalic acids from granular hairs covering leaves, stems and pods, make chickpea less susceptible to direct damage from aphids and other insects. However, damage due to the pod borer *Helicoverpa armigera* is a major threat. Stored chickpeas are highly susceptible to bruchid beetle attack (*Callosobruchus maculatus*, *C. chinensis*). Germplasm with some degree of resistance to bruchids has been identified, but it appears to be correlated with undesirable physical characteristics of the seeds, such as dark colour, roughness, altered chemical composition and thickness of the seed coat (Schalk 1973; Ahmed et al. 1991, 1993; Pacheco et al. 1994).

The goals of current breeding programmes are to increase productivity by upgrading the genetic potential of germplasm and by reducing the impact of abiotic and biotic factors, such as diseases, insects, drought and cold. Singh (1997) produced a detailed review of breeding achievements and specific breeding goals in chickpea. Classic breeding techniques may be limited by the availability of desired traits in chickpea germplasm or the linkage of desired traits with undesired characteristics. For instance, dark colour, roughness, altered chemical composition and thickness of the seed coat may make chickpeas resistant to bruchids, but also less desirable for human consumption. Biotechnology, particularly genetic transformation, offers the advantage of introducing unlinked resistance genes into elite germplasm and has the potential to complement existing breeding programmes.

Two popular strategies for gene transfer to plants (Potrykus 1990; De Block 1993; Songstrad et al. 1995; Barcelo and Lazzeri 1998) are the *Agrobacterium* method (Zupan and Zambryski 1997; Gheysen et al. 1998) and direct DNA introduction by micro-particle bombardment (Sanford et al. 1987). The efficient production of transgenic plants requires stringent selection procedures supported by a selectable marker gene that confers resistance to agents such as antibiotics or herbicides. The pre-requisites for successful application of gene technology in plants have been outlined earlier (Popelka et al. 2004).

2 Advances in Chickpea Tissue Culture

An efficient tissue culture protocol is the basis for successful genetic transformation. It is characterised by easy and uncomplicated ways to obtain tissue

Table 1. In vitro culture systems for chickpea and their predicted suitability for genetic transformation. *EA* Embryonic axes, *gS* germinated seeds, *hyp* hypocotyl, *Imm cot* immature cotyledon, *CLS* cotelydon-like structures. *ABA* Abscisic acid, *B5* Gamborg et al. (1968), *BA* N6-benzyladenine, *BAP* benzylaminopurine, *2,4-D* 2,4-dichlorophenoxyacetic acid, *GA3* gibberellic acid A3, *IAA* indole-3-acetic acid, *IBA* indole-3-butyric acid, *K* kinetin, *MS* Murashige and Skoog (1962), *NAA* α-naphthalene acetic acid, *2,4,5-T* 2,4,5-trichlorophenoxyacetic acid, *TDZ* thidiazuron, *Z* zeatin, *2-iP* 2-isopentenyladenine

Genotype	Explant	Important media components	Result	Reference	Predicted suitability
Nabin	Hyp	Pre-soaking seeds with B5 and BA	Shoots	Islam et al. (1999)	Moderate
ICC 640	Imm cot	B5; 2,4-D; 2,4,5-T; NAA; IAA; BA; K; Z; ABA	CLS	Shri and Davis (1992)	Moderate
BG 362, 329, 267, 256 and C 235	EA	MS; B5; BA	Shoots	Polisetty et al. (1997)	Moderate
Nabin	Internode	MS; B5; 2,4-D; BAP; NAA; K; IAA	Callus, shoots	Huda et al. (2000)	Low
C 235	EA of gS	MS; 2-iP; TDZ; K; GA3; IBA; NAA	Shoots	Jayanand et al. (2003)	High

explants with the capacity to produce a large number of independent, healthy and fertile plants. In chickpeas, two distinct regeneration strategies have been described. The first is via somatic embryogenesis, e. g. by induction of embryogenic callus growth with auxin-type growth regulators. The second is *via* organogenesis, e. g. by induction of multiple shoot formation with cytokinin-type growth regulators. In chickpea, different genotypes and explants including hypocotyls, cotyledons and embryonic axes have been cultured. Furthermore, a wide range of tissue culture media have been tested (Table 1). In several early attempts, regeneration of shoots was achieved, but the number of shoots per explant was low and this limited the likelihood of recovering transgenic shoots. Jayanand et al. (2003) described an efficient protocol for the regeneration of whole chickpea plants using embryonic axes after removal of the shoot and root tips as well as the axillary bud. Culture on medium containing low concentrations of thidiazuron (TDZ), 2-isopentenyladenine and kinetin yielded an average of 40 shoots per responding explant. The formation of vigorous roots on in vitro-generated shoots can be a further hurdle. Rooting in vitro has been developed by Fratini and Ruiz (2003) and Jayanand et al. (2003) and, for many situations, may be a suitable alternative to the grafting technique (Murfet 1971) used in several transformation protocols (Krishnamurthy et al. 2000; Sarmah et al. 2004; Senthil et al. 2004; Polowick et al. 2004; Sanyal et al. 2005).

3 Advances in Chickpea Transformation

Early transformation experiments which relied on callus cultures failed due to poor shoot regeneration but demonstrated the potential of *A. tumefaciens* as a transformation vector for chickpea (Islam et al. 1994). The first report of successful chickpea transformation (Fontana et al. 1993) after co-cultivation of embryonic axes with *A. tumefaciens* included molecular evidence for the transgenic nature of at least two independent plants. Genes encoding β-D-glucuronidase (GUS) and neomycin phosphotransferase II (*npt*II) were expressed and the transgenes were transmitted at least to the T_2 generation. Subsequently, using similar experimental protocols (Table 2), the formation of multiple shoots from different genotypes and the production of primary transgenic plants was reported (Kar et al. 1996; Krishnamurthy et al. 2000). Multiple shoot formation was achieved on MS medium supplemented with 6-benzylaminopurine (BAP; Krishnamurthy et al. 2000), BAP and α-naphthalene acetic acid (NAA; Kar et al. 1997) or BAP, NAA and kinetin (Fontana et al. 1993). Transgenic plants were selected via multiple cycles in vitro on media containing kanamycin (Fontana et al. 1993; Kar et al. 1996) or phosphinothricin (Krishnamurthy et al. 2000). Transformation frequencies and reproducibility in these early breakthroughs were low and limited their practical applicability. However, both transformation frequency and reproducibility have been improved recently in four separate studies (Polowick et al. 2004; Sarmah et al. 2004; Senthil et al. 2004; Sanyal et al. 2005), enabling the routine application of transformation technology to chickpea.

The four protocols are compared in Table 3 and it can be seen that they are very similar. Senthil et al. (2004) and Sanyal et al. (2005) pre-germinated seeds for 2 days or 20 days on medium containing TDZ or BAP, respectively, while the other authors imbibed the seeds in water overnight before preparation of explants (Polowick et al. 2004; Sarmah et al. 2004). All four protocols rely on embryonic axes as explant source, either halved and still attached to the cotyledon (Fig. 1A; Sarmah et al. 2004), sliced longitudinally (Fig. 1B; Polowick et al. 2004; Senthil et al. 2004) as described earlier for *Pisum sativum* L (Schroeder et al. 1993) or as an excision with exposed cells of the L2 layer (Sanyal et al. 2005). In addition, Sanyal et al. (2005) performed a pre-conditioning of explants for 24 h prior to cocultivation (Table 3; Sanyal et al. 2005). Multiple shoots were induced by the growth regulator BAP (Fig. 1C) and Senthil et al. (2004) further supplemented all culture media with TDZ in decreasing concentrations with time (Table 3). Selection was commenced early during the shoot induction phase (Polowick et al. 2004; Sarmah et al. 2004; Sanyal et al. 2005) with constant or increasing kanamycin concentrations during the tissue culture process [200 mg l^{-1} (Sarmah et al. 2004), 50–150 mg l^{-1} (Polowick et al. 2004), 100–200 mg l^{-1} (Sanyal et al. 2005)]. Further evidence for the transformation of embryonic axes was obtained by testing for the transient expression of the screenable marker gene encoding GUS (Fig. 1D). Using phosphinothricin (PPT) as selection agent, Senthil et al. (2004) started the selection process at

Table 2. Approaches to genetic transformation of chickpea. All genotypes are desi types unless otherwise indicated. *At Agrobacterium tumefaciens*, *CNs* cotyledonary nodes, *EA* embryonic axes, *MPB* micro-particle bombardment, *NPE* not possible to estimate, *PGIP* polygalacturonase-inhibiting protein gene. *gus* or *uid*A: Gene encoding β-glucuronidase, *bar*: gene encoding phosphinothricin acetyl transferase, *cry1*Ac: gene for crystal protein toxin from *B. thuringiensis*, *npt*II: gene encoding neomycin phosphotransferase II, *αAI*1: gene for α-amylase inhibitor 1 from *P. vulgaris*

Genotype	Explant	Method of gene transfer	Expressed genes	Evidence provided	Reproducibility and robustness	References
Local ecotype	EA	*At* (LBA 4404)	*npt*II, *uid*A	Gus expression in T_0; gene integration and expression in T_0 (Southern and Western)	Low	Fontana et al. (1993)
ICCV 1, ICCV 6 Desi	EA	*At* (LBA 4404)	*npt*II, *uid*A	Gus expression in T_0; gene integration and expression in T_0 (Southern and Western)	Low	Kar et al. (1996)
ICCV 1, ICCV 6	EA	MPB	*npt*II, *cry1*Ac	*Cry1*A(c) expression in T_0; transmission to T_1 (PCR)	Low	Kar et al. (1997)
Turkey, Chafa	EA	*At* (EHA 101 and C58C1)	*npt*II, *pat, gus*	Gus expression in T_0; transmission to T_1 (PCR)	Low	Krishnamurthy et al. (2000)
P 362, P 1042, P 1043	EA	*At* (EHA 101)	*npt*II, *uid*A, *bar*	Gus expression in T_0; transmission to T_1 (PCR)	Low	Tewari-Singh et al. (2004)
Semsen	EA	*At* (AGL1)	*npt*II, *αAI*1	Transmission and expression in T_1 (Southern and western blot); functionality of *αAI*1	Good	Sarmah et al. (2004)
ICCV5 (Kabuli), H208, ICCL87322, K850	Sliced EA	*At* (AGL1)	*bar*, PGIP, *uid*A	Transmission and expression of GUS gene in T_1–T_3	Good	Senthil et al. (2004)
CDC Yuma (Kabuli)	Sliced EA	*At* (EHA105)	*uid*A, *npt*II	Transmission and expression of GUS gene in T_1–T_3	Good	Polowick et al. (2004)
C 235, BG 256, Pusa 362, Pusa 372	L2 layers of CNs	*At* (LBA4404)	*uid*A, *npt*II, *cry1Ac*	Transmission and expression of *npt*II and *cry1Ac* in T_1; functionality of *cry1Ac*	Good	Sanyal et al. (2005)

Table 3. Comparison of four robust transformation protocols for chickpea. *AS* Acetosyringone, *B5* Gamborg et al. (1968), *BAP* 6-benzylaminopurine, *bm* basal medium, *cef* cefotaxime, *IBA* indole-3-butyric acid, *kan* kanamycin, *kin* kinetin, *MES* 2-(*N*-morpholino)ethanesulfonic acid, *MS* Murashige and Skoog (1962), *NAA* α-naphthalene acetic acid, *PPT* phosphinothricin, *TDZ* thidiazuron, *tim* timentin, *vit* vitamins

	Sarmah et al. (2004)	Senthil et al. (2004)	Polowick et al. (2004)	Sanyal et al. (2005)
Explant pre-culture	Over-night imbibition	Germination for 2 days	Over-night imbibition	20-day-old seedlings
Germination medium	Water	MS-bm with B5 vit, pH 5.7, 10 µM TDZ	Water	MS-bm, 1 mg l^{-1} BAP
Explant preparation	Halved embryonic axes	Longitudinal slices of embryonic axes (4–5 per embryo)	Longitudinal slices of embryonic axes (5–6 per embryo)	Exposed and pre-conditioned[a] tissue after transverse excision of main root, shots and shoot buds, exposing L2 layer
Infection	In liquid *Agrobacterium* suspension for 1 h	In liquid *Agrobacterium* suspension for 35 min and 150 rpm	In liquid *Agrobacterium* suspension for 1–2 h	In liquid *Agrobacterium* suspension combined with 60 s sonication and 20 min vacuum treatment
Co-culture	3 days at 24 °C, 16 h light	4 days at 22 °C, dark	4 days at 25 °C, 16 h light	48 h at 24 °C, 16 h light
Medium	B5-bm, 10 mM MES, 1 mg l^{-1} NAA, 1 mg l^{-1} BAP, 100 µM coniferyl alcohol, pH 5.8	MS-bm, B5-vit, 10 µM TDZ, pH 5.7	B5-bm, 100 µM AS, pH 5.7	MS-bm, 100 µM AS, 1 mg l^{-1} BAP, 200 mg l^{-1} L-cysteine, 100 mg l^{-1} DTT
Wash transfer	4× with water, blotted	None	Minimum 1 h in 300 mg l^{-1} tim solution	In MS-bm, 1 mg l^{-1} BAP, 500 mg l^{-1} cef or carbenicillin
Shoot initiation	2 weeks	3 weeks	2 weeks, then many cycles of 3 weeks	15 days
Medium	MS-bm, 0.5 mg l^{-1} BAP, 0.5 mg l^{-1} kinetin, 0.05 mg $l1^{-1}$ NAA, 10 mM MES, 200 mg l^{-1} kan, 150 mg l^{-1} tim	MS-bm, B5-vit, pH 5.7, 5 µM TDZ, 500 mg l^{-1} cef	B5-bm, 150 mg l^{-1} tim, 3 mg l^{-1} BAP, 50 mg l^{-1} kan	MS-bm, 1 mg l^{-1} BAP, 100 mg l^{-1} kan, 500 mg l^{-1} cef, 0.02 g l^{-1} silver nitrate

Table 3. (continued)

	Sarmah et al. (2004)	Senthil et al. (2004)	Polowick et al. (2004)	Sanyal et al. (2005)
Shoot elongation or proliferation	10–14 days	3× to 4× 4-week subcultures	3 cycles of 3 weeks each	2× 15 days
Medium	MS-bm, 0.5 mg l^{-1} BAP, 0.5 mg l^{-1} kin, 10 mM MES, 200 mg l^{-1} kan, 150 mg l^{-1} tim	MS-bm, B5-vit, 2.5 µM TDZ, 500 mg l^{-1} cef, 8.9 µM BAP, 0.1 µM NAA, 2.5 mg l^{-1} PPT	MS-bm, B5-vit, 1 mg l^{-1} BAP, 50–75 mg l^{-1} kan	MS-bm, 1 mg l^{-1} BAP, 100 mg l^{-1} kan, 0.02 g l^{-1} silver nitrate
Shoot elongation and multiplication	Minimum of six cycles of 10–14 days	3–4 weeks	None	2× 15 days
Medium	MS-bm, 0.1 mg l^{-1} BAP, 0.1 mg l^{-1} kin, 10 mM MES, 200 mg l^{-1} kan, 150 mg l^{-1} tim	MS-bm, B5-vit, 8.9 µM BAP, 0.1 µM NAA, 2.5 µM TDZ, 500 mg l^{-1} cef, 2.5 mg l^{-1} PPT	None	MS-bm, 1 mg l^{-1} BAP, 150–200 mg l^{-1} kan, 0.02 g l^{-1} silver nitrate
Rooting	In vitro shoots rooted for 2 weeks or grafted	Shoots of 1.5–2.0-cm for 3–4 weeks or grafted	Until roots form (1–3 weeks)	In vitro shoots rooted or grafted
Medium	MS-bm, 1 mg l^{-1} IBA, 10 mM MES	MS-bm, B5-vit, 2.5 µM NAA, 2.5 µM TDZ, 500 mg l^{-1} cef	B5-bm, 0.18 mg l^{-1} NAA, 150 mg l^{-1} kan	None
Transfer to greenhouse	Rooted shoots or grafted shoots on rootstocks with a silicon ring	Rooted shoots or grafted shoots on rootstocks	Rooted shoots	Rooted shoots or grafted shoots on rootstocks
Transgenic plants per seed	0.72%	2.0–13.3% (4–5 explants per seed)	3.1% (5–6 explants per seed)	5.18–13.2% (not clear)
Time required	6–9 months	5–6 months	4.5–13 months	Not specified

[a] Explants were 24 h pre-cultured on solidified MS-bm, 1 mg l^{-1} BAP, 4 µM L-glutamine and L-arginine, 200 mg l^{-1} L-cysteine.

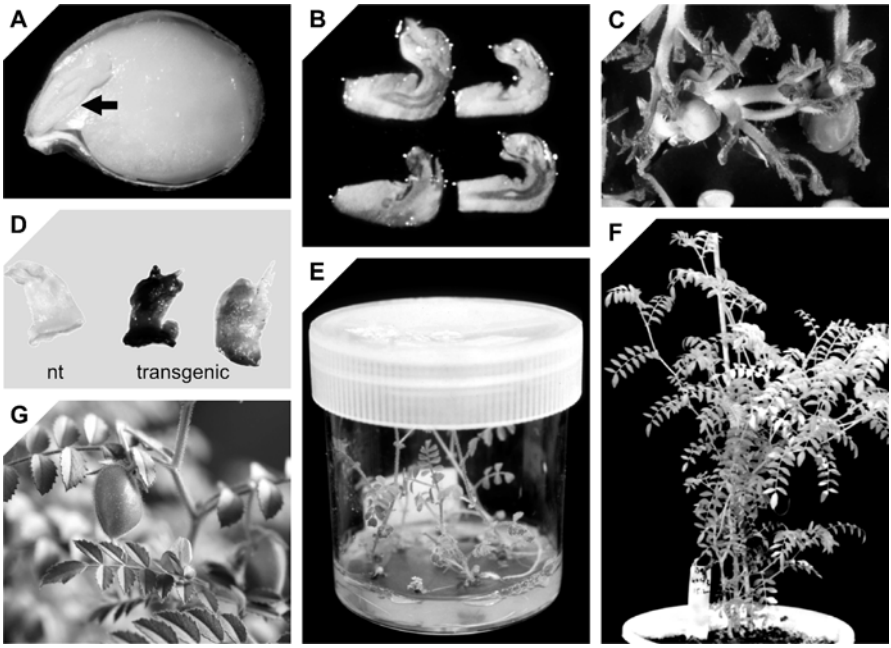

Fig. 1. Transformation of chickpeas. **A** Halved chickpea cotyledon with attached embryonic axis (*arrow*). Sarmah et al. (2004) used this explant after removal of the ends of the shoot and root. **B** Sliced embryonic axes (Schroeder et al. 1993) were used by Polowick et al. (2004) and Senthil et al. (2004). **C** Multiple shoots induced on halved embryonic axes. **D** Transient GUS expression in embryonic axes of chickpeas (*nt* non-transformed control explant). **E** Shoots surviving the selection process induce roots on rooting medium. **F–G** Transgenic chickpea plants established in the glasshouse are normal in phenotype and fertile

a later stage, with 2.5 mg l^{-1} PPT. Surviving shoots were either transferred to rooting medium (Table 3; Fig. 1E; Polowick et al. 2004; Sarmah et al. 2004; Senthil et al. 2004) or grafted onto seedlings (Sarmah et al. 2004; Senthil et al. 2004; Sanyal et al. 2005) and finally transferred to soil in the glasshouse (Fig. 1F,G). The four systems all appear equally useful and have the following important elements in common, namely: (1) mature seeds (imbibed or pre-germinated) are the preferred explant source, (2) embryonic axes contain the target tissue, (3) submersion of explants in liquid *Agrobacterium* suspension followed by several days of co-culture on semi-solid medium, (4) frequent sub-cultures on selective medium for shoot initiation, elongation and rooting and (5) transfer of rooted shoots to soil in the glasshouse. In summary, many different genotypes can now be transformed, including both desi and Kabuli types; and the slight differences in growth regulator type and concentration between the three reports may be only a reflection of the different starting cutivars or lines.

4 Application of Transformation Technology to Chickpea and Its Potential

It is expected, with these reliable transformation protocols available, that a number of potentially useful genes will be introduced into chickpeas in the near future, providing excellent opportunities for plant improvement against insect pests and other constraints, such as quality related traits (White et al. 2000; Wang et al. 2003). Besides the large family of *Bacillus thuringiensis*-derived genes (Krattiger 1997), there are a significant number of useful genes available from other organisms including higher plants (Schuler et al. 1998). The latter includes genes for lectins (Murdock et al. 1990), diverse proteases (Ryan 1990), protease inhibitors (Hilder et al. 1987) and α-amylase inhibitors (Shade et al. 1994).

So far, there have been three reports of trangenic chickpeas expressing genes for protection against insect pests. Transgenic chickpea plants produced with the biolistic transformation approach expressed the bacterial *cry*1Ac gene from *B. thuringiensis* (Kar et al. 1997). Insect feeding trials with one primary transgenic plant demonstrated an inhibitory effect on growth of larvae of the chickpea pod-borer *H. armigera* (Kar et al. 1997). Transmission to T_1 progeny was demonstrated, although further analysis has not been reported. The *Agrobacterium* method was used to introduce a seed-specific α-amylase inhibitor (α *AI*1) gene from *Phaseolus vulgaris* L (Sarmah et al. 2004). Stable transmission and expression of the transgene in subsequent generations was demonstrated (Table 2). The high level of expression of the α*AI*1 gene protected chickpea seeds from insect damage by severely inhibiting the development of cowpea weevils (*C. maculatus*) and adzuki bean weevils (*C. chinensis*; Sarmah et al. 2004). Finally, Sanyal et al. (2005) tested the toxicity of T_0 and T_1 plants expressing the *cry1Ac* gene. In bioassays, larvae of *H. armigera* ceased feeding on transgenic chickpea leaves after 2 days and showed high mortality after weight decreases of 40–90%. In T_1, many lines showed complete protection against the insects (Sanyal et al. 2005).

While progress in genomics research will continue delivering interesting and useful genes, intellectual property rights, regulatory approval as well as biosafety concerns will determine the future. It will be important to ensure that biosafety regulations and regulatory compliance systems are in place in each of the countries using the technology before regulatory agencies are faced with requests for release of the transgenic crop. Risk assessments for non-target species associated with the crop (Romeis et al. 2004) will also need to be concluded. Regulatory packages are in place for pesticides and will similarly be applied to transgenic plants expressing insecticidal genes (Hill and Sendashonga 2003). Further, issues relating to food safety, labelling, traceability, trans-border movement and trade must be addressed concurrently, as technological advances described here mean that transgenic chickpeas with useful genes are now in the pipeline for small-scale field trials and potentially for broad-acre release.

5 Conclusion and Outlook

The prospects are now excellent for the genetic improvement of chickpeas using gene technology. There are at least four closely related transformation protocols from which to choose and all the indications are that the systems are robust and reproducible. It is possible to predict that genes for protection against major insect pests, such as pod borer, will soon be installed in chickpea germplasm for use in varieties adapted to local conditions around the world. These will be followed by other genes including those for other biotic stresses as well as genes that will overcome abiotic stresses such as drought, soil acidity and frost. Similarly, transformation protocols are available for other important legumes, such as soybean (Olhoft and Somers 2004), groundnut (Yang et al. 1998; Li et al. 2000), lupin (Tabe and Molvig 2006), cowpea (Popelka et al. 2006) and garden pea (McPhee et al. 2004).

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