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Current Developments, Progress, Issues and Concerns in Producing Transgenic Peas (*Pisum sativum* L.)

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ABSTRACT

Pea (*Pisum sativum* L.) is an important grain legume crop and has a global economic value due to its protein content both for human and animal consumption. The productivity and value of peas has been successfully enhanced by the introduction of stably inherited traits such as pest, disease and herbicide resistance, and enhanced quality of pea proteins. In this review, we present an assessment of the current developments, progress, issues and concerns in developing transgenic pea lines, briefly highlighting the global pulse industry and legume genomics. This review discusses why *Agrobacterium* has been successful in pea, what other alternatives have been tested, the extent to which they have yielded transgenic pea lines, and their potential agronomic utility. The GM food aspect and research related to transgenic peas as a food and feed source have also been investigated.

Keywords: *Agrobacterium tumefaciens*, biolistics, chimaeric, transformant, transformation, transgene
Abbreviations: αAI, α-amylase inhibitors; **AMGT**, *Agrobacterium* (*tumefaciens*)-mediated gene transformation; **AMOVA**, Analysis of Molecular Variance; **AMV**, *Alfalfa mosaic virus*; **ANU**, Australian National University; **CaMV**, *Cauliflower mosaic virus*; **CSIRO**, Commonwealth Scientific and Industrial Research Organization (Australia); **CP**, Coat Protein; **FSANZ**, Food Standard Australia and New Zealand; **GM**, Genetically Modified; **JCSMR**, John Curtin School of Medical Research; **NMR**, Nuclear Magnetic Resonance; **PHA**, phytohemagglutinins; **PEMV**, *Pea enation mosaic virus*; **PSbMV**, *Pea seed-borne mosaic virus*; **PTGS**, post-transcriptional gene silencing

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INTRODUCTION

Pea (*Pisum sativum* L.) is a significantly important grain legume crop and has a global economic value because of its protein content both for human and animal food and nutrition source (Pniewski and Kapusta 2005; Hassan 2006). Global pea production exceeded 20 million tons in 2005 (Hassan 2006).

Pea has also been traditionally used as a classical model plant in fundamental studies related to genetics and plant breeding, biochemistry and molecular biology (Fehr 1993; Gaikwad *et al.* 1999; Wen *et al.* 1999; Malaysheva *et al.* 2001). One of the most important factors why pea is important as a legume crop is because of its high protein value (Pniewski and Kapusta 2005); also, the crop has very low concentrations of detectable anti-nutritive factors such as protease inhibitors, haemagglutinnins and alkaloids (Casey and Davies 1993; Jasinka and Kotecki 1993; Pniewski and Kapusta 2005). However, an important limitation of the crop, like other grain legume members, is that it is a poor dietary source of cysteine and methionine or, in other words, sulphur-containing amino acids (Schroeder *et al.* 1994; Pniewski and Kapusta 2005).

The prerequisites for the transfer of foreign genes into any plant species by genetic engineering are an efficient gene delivery system, an effective selectable marker for transformed tissue, and reliable regeneration of transformed tissue into a fertile plant (**Fig. 1**). Regeneration via embryo-

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Invited Review

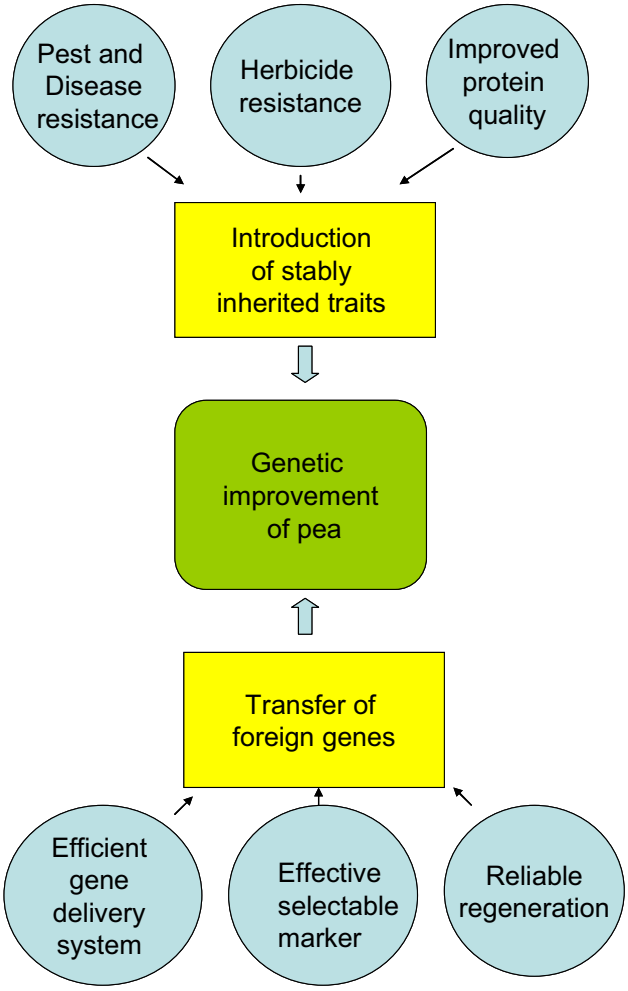

Fig 1 Understanding the introduction of stably inherited traits in peas by gene transfer as a means of quality improvement.

genesis or organogenesis has been described for a variety of pea explants, e.g. from immature leaflets (Mroginski and Kartha 1981; Rubluo *et al*. 1984), from cotyledonary nodes (Jackson and Hobbs 1990), from hypocotyls (Nielsen *et al*. 1991), from embryos (Kysely *et al*. 1987; Natali and Cavallini 1987; Tetu *et al.* 1990), from various organs of seedlings (Malmberg 1979; Hussey and Gunn 1984; Ezhova *et al*. 1985), and from protoplast cultures (Jacobsen and Kysely 1984; Puonti-Kaerlas *et al*. 1990; Lehminger-Mertens and Jacobsen 1991).

Grain legumes are a very difficult and challenging plant group to work with from the perspectives of higher regeneration and transformation rates (Grant *et al.* 1995). Significant achievements have been made in a number of legume members (as reviewed in Christou 1994). However, *Agrobacterium*-mediated gene transformation (AMGT) has been reported from other legumes for example in soybean (*Glycine max* (L.) Merr.) by Hinchee *et al.* (1988) and Chee *et al.* (1989); in chick pea (*Cicer arietinum* L.) by Fontana *et al.* (1993) and Ignacimuthu and Prakash (2006) and in lentil (*Lens culinaris* Medik.) by Khawar and Ozcan (2002). Both AMGT and biolistics approaches of gene transfer have now been well established in common bean, *Phaseolus vulgaris* L. (as reviewed in Aragão and Rech 2001).

Transgenic development is now a core research tool in plant biology and a practical tool for cultivar improvement. We are emerging from a period of plant transformation research dominated by the need to develop proven genetic transformation methods for the major experimental and economic plant species. The productivity and value of peas could be greatly increased by the introduction of stably inherited traits such as pest, disease and herbicide resistance, and improved protein quality (**Fig. 1**). There are verified

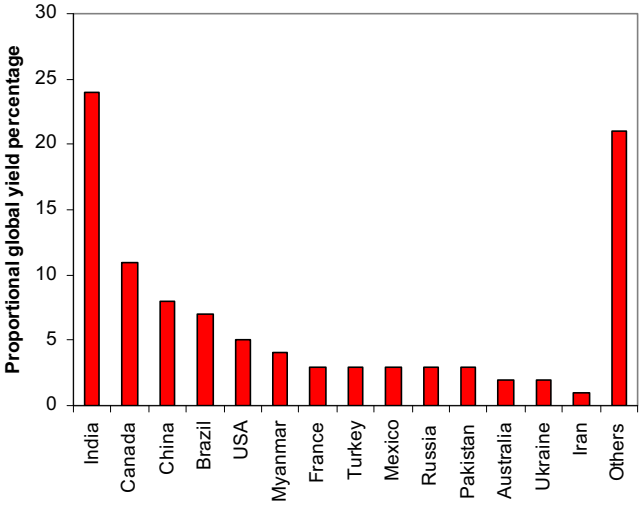

Fig. 2 Global profile of pulse production in different countries in 2005-2006. Figure redrawn with modifications from Pulse Canada (2007).

methods for stable introduction of novel genes into the nuclear genomes of different legume species. This review examines the criteria to verify pea transformation; the biological and practical requirements for transformation systems; the integration of tissue culture, gene transfer, selection, and regulatory environment and public perceptions.

GLOBAL PULSE INDUSTRY

Global pulse production has remained steady over the past 10 years at around 40 million tonnes per year (Pulse Canada 2007). At over 18 million tonnes in 2006, bean production accounted for the largest share of the world's pulse production followed by peas, chickpeas and lentil. India is the world's largest pulse producer, followed by Canada. The percentage proportion of global yield of four major legume/pulse crops in 2005-2006 were *Phaseolus vulgaris* (43%), *Pisum sativum* (26%), *Cicer arietinum* (21%) and *Lens culinaris* (10%) (Pulse Canada 2007). Brazil is one of the largest bean producers, but produces virtually no other pulses while countries like Canada, China, the U.S. and Mexico produce some of all the pulses. Turkey produces large amounts of lentils and chickpeas, but very few peas or beans while France and Russia produce peas (**Fig. 2**).

LEGUME TAXONOMY AND GENOME SIZE

The Fabaceae (= Leguminosae), or legumes, constitute the third largest family of flowering plants, comprising more than 650 genera and 18,000 species (Polhill and Raven 1981). The legumes are highly diverse and can be divided into three distinct subfamilies: Mimosoideae, Caesalpinioideae, and Papilionoideae. Although the pea genome is about five times larger than the *Medicago* genome, gene order seems well conserved and there is no evidence for large-scale segmental duplication as the underlying cause of the large genome size in pea (**Fig. 3**, **Table 1**).

Despite their close phylogenetic relationships, crop legumes differ greatly in their genome size, base chromosome number, ploidy level and self compatibility (**Fig. 3**). However, unlike many of the other major crop legumes, *Medicago truncatula* Gaertn. and *Lotus japonicus* (Regel) are of small genome size (**Table 1**), amenable to forward and reverse genetic analyses and well suited for studying biological issues important to the related crop legume species (**Fig. 3**, **Table 1**).

TRANSFORMATION STUDIES IN PEA

A number of pea cultivars are available all over the world that are adapted to specific bio-geographical regions and micro- and macroclimatic regimes. These cultivars carry a

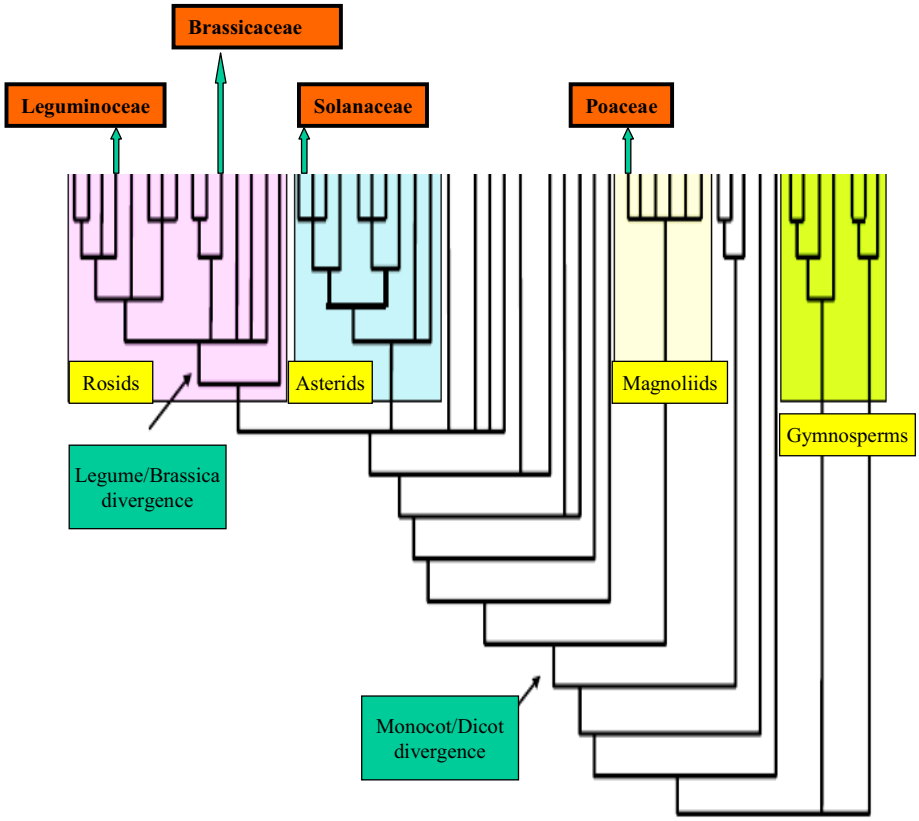

Fig. 3 Phylogenetic relationship of legumes with other angiosperms and gymnosperms. (Redrawn from Soltis *et al.* 1999 and Kellogg 1998)

Table 1 Chromosome number and genome size of major model and crop legumes. Table reproduced from Zhu *et al.* (2005) based on the plant DNA C-values database, available online at http://www.rbgkew.org.uk/cval/homepage.html.

Tribe	Genus	Species	Chromosome No.	Genome Size *Mb/1C*	Self-Compatibility
Trifolieae	*Medicago*	*M. truncatula*	2*n* = 2x = 16	466	Selfing
		M. sativa	2*n* = 4x = 32	1,715	Outcrossing
	Trifolium	*T. pretense*	2*n* = 2x = 14	637	Outcrossing
		T. repens	2*n* = 4x = 32	956	Outcrossing
	Melilotus	*M. officinalis*	2*n* = 2x = 16	1,103	Outcrossing
Viceae	***Pisum***	***P. sativum***	2*n* = 2x = 14	4,337	Selfing
	Vicia	*V. faba*	2*n* = 2x = 12	13,059	Selfing
	Lens	*L. culinaris*	2*n* = 2x = 14	4,116	Selfing
Cicereae	*Cicer*	*C. arietinum*	2*n* = 2x = 16	931	Selfing
Loteae	*Lotus*	*L. japonicus*	2*n* = 2x = 16	466	Selfing
Phaseoleae	*Phaseolus*	*P. vulgaris*	2*n* = 2x = 22	588	Selfing
	Vigna	*V. radiata*	2*n* = 2x = 22	515	Selfing
	Glycine	*G. max*	2*n* = 4x = 40	1103	Selfing
	Cajanus	*C. cajan*	2*n* = 2x = 22	858	Selfing

wide range of desirable morphologic and agronomic characters derived through conventional breeding. However, over time variability within different genotypes has been narrowed and desirable genetic characters have become difficult to establish through conventional breeding. Characters like disease resistance, higher yield, tolerance to specific biotic and abiotic stresses, production of specific, desirable, novel molecules like antibodies, nutraceuticals, medicinal compounds, biochemical enrichment of seeds, specific tolerance to particular disease, etc. are difficult and time consuming to achieve and/or address through conventional breeding programs (Puonti-Kaerlas *et al.* 1992; Bean *et al.* 1993; Davies *et al.* 1993; Schroeder *et al.* 1993; Grant *et al.* 1995; Bean *et al.* 1997; Nadoloska-Orczyk and Orczyk 2000; Pniewski and Kapusta 2005).

AMGT approach

It is at this crucial juncture that some sort of quick visible change or modification of the plant genome becomes essential to achieve desirable target(s) that quick and easy genetic transformation becomes essential to generate future cultivars. Modern realms of plant biotechnology such as genetic engineering and allied available technologies are therefore essential to address such issues. Genetic transformation through crown gall bacteria *A. tumefaciens* or the AMGT approach is thus a very traditional and handy, common approach to transfer desirable genes to target plant. However, it is important to note that there are a few important factors that are essential for successful and stable transformation via the AMGT approach. One of the important factors is an efficient regeneration system and the second is the availability of suitable virulent strain(s) of *A. tumefaciens* (Capell and Christou 2004; Pniewski and Kapusta 2005).

One of the serious limitations of the AMGT approach is the availability of a suitable, virulent strain of *A. tumefaciens*. Virulence of strains vary widely from one cultivar to another for the same crop in different agro-climatic regions; also, a particular strain infecting a particular crop in a particular region or laboratory may or may not equally virulent to the same crop or cultivar in another region or another laboratory. Growth conditions and other associated microbiological parameters may play an important role of widely divergent behaviour, virulence and integration profile of the same strain in different laboratories; in addition, there is the problem of genotypic and/or cultivar preference of the bac-

teria varying from crop to crop and from one cultivar to another within the same crop. However, virulent strains for other legumes have also been found to be effective in the stable transformation in peas: C58C1 (De Kathen and Jacobsen 1990), GV3101 (Puonti-Kaerlas *et al.* 1992) and EHA101 (Lulsdorf *et al.* 1991).

Additionally, AMGT is not a fool-proof genetic transformation method, meaning the integration patterns are not always desirable; genes may get inserted into transcriptionally inactive regions or may get truncated, may result in tandem repeats and in multi-copy insertions; often causing undesirable loss of important genetic traits, post-transcriptional gene silencing (PTGS) and undesirable mutations (de Kathen and Jacobsen 1990; Bean *et al.* 1993; Davies *et al.* 1993; Schroeder *et al.* 1993; Bean *et al.* 1997; Nadoloska-Orczyk and Orczyk 2000; Pniewski and Kapusta 2005).

OTHER TRANSFORMATION TECHNIQUES

Another gene transformation approach that has not yet been very well studied in pea transformation is the application of biolistics or particle bombardment. Although quite established in other grain legumes such as soybean (McCabe *et al.* 1988) and common bean (Russell *et al.* 1988; also reviewed in Aragão and Rech 2001), it has not yet been reported much in pea transformation. In our exhaustive review of the available primary literature, we have come across only a single research report on biolistic transformation work on peas and subsequent development of transgenic pea lines (Jones *et al*. 1998). Lowe *et al.* (1995) reported the importance of targeting multicellular tissues during particle bombardment based on their experience on developing maize (*Zea mays* L.) shoot meristems with successful transformation of every tested maize genotypes. Bean *et al*. (1997) indicated that such techniques could help in avoiding the genotype limitations due to *Agrobacterium* strain specificities of AMGT approach for transforming pea lines.

However, biolistic transformation of soybean stem meristems generated many chimaeras producing high number of non-resistant progenies causing non-Mendelian inheritance pattern of the introduced selectable marker gene (McCabe *et al.* 1988; Christou *et al.* 1989). Further analyses revealed that targeting multicellual tissues generate more chimaeras than true clonal transformants and that the transgene was not generated or transmitted through successive generations (Christou and McCabe 1992). Similar problems were also investigated in transgenic pea lines transformed via AMGT approach using mature seeds by Bean *et al.* (1997). Hence, it will be necessary to identify the appropriate pea tissue type for biolistic approach to have higher transformation efficiency and subsequent transmission of transgene to the following generation.

We could summarize by mentioning that the development of transgenic pea lines has been a daunting and challenging task for plant biotechnologists specializing in genetic engineering (Schroeder *et al*. 1994). The Swedish group of scientists from the University of Uppsala (Puonti-Kaerlas *et al.* 1990) was the first group to successfully develop transgenic pea lines through the AMGT approach. Several researchers from time to have reported different problems associated with pea transformation such as low regeneration rate through calli generation and poor transformation efficiencies, reduced fertility, abnormal and non-desirable morphological alterations and genetic variabilities like change in the level of chromosomal ploidy, chromosomal mosaicism, loss of transgene in subsequent generation or "transgene dilution" (De Kathen and Jacobsen 1990; Puonti-Kaerlas *et al.* 1990; Zubko *et al*. 1990; Puonti-Kaerlas *et al.* 1992; Bean *et al.* 1993; Davies *et al.* 1993; Schroeder *et al.* 1993; Grant *et al.* 1995; Bean *et al.* 1997; Nadoloska-Orczyk and Orczyk 2000; Pniewski and Kapusta 2005). A summary of the different approaches for generating transgenic pea lines is presented in **Table 2**.

PRACTICAL APPLICATION

Perrin *et al*. (2000) for the first time produced transgenic pea lines producing antibodies (scFvT84.66 at 9 µg/g fresh weight of seeds) in their seeds. The authors reported using cDNA coding for single-chain Fv fragment scFvT84.66 (derived from the monoclonal antibody T84.66, identifying the well-characterised tumour-associated carcinoembryonic antigen) expressed under the control of seed-specific legumin A promoter. The scFvT84.66 antibody was targeted into the pea endoplasmic reticulum and the authors reported stable integration of the transgene and higher accumulation of

Table 2 Summary table of different approached used by different research groups in generating transgenic pea lines.

Regeneration method	Targeted explant(s)	References
Organogenesis	Immature leaflets	Morginski and Kartha 1981
Organogenesis	Cotyledonary nodes	Jackson and Hobbs 1990
Organogenesis	Seedling organs	Ezhova *et al*. 1985
Organogenesis	Hypocotyls	Nielsen *et al.* 1991
Organogenesis	Cotyledon tissues adjacent to the axillary meristems of immature embryos	Pniewski *et al*. 2003
Embryogenesis	Embryos	Kysely *et al.* 1987; Natali and Cavallini 1987
Protoplast culture	Protoplasts	Jacobsen and Kysely 1984; Lehminger-Mertens and Jacobsen 1989
AMGT	Young plants	Hobbs *et al*. 1990
AMGT	Shoot culture and epicotyl	Pounti-Kaerlas *et al.* 1990
AMGT	Stem	Lulsdorf *et al.* 1991
AMGT	Nodes	De Kathen and Jacobsen 1990; Nauerby *et al*. 1991
AMGT	Root	Schaerer and Pilet 1991
AMGT	Protoplasts	Schaerer and Pilet 1991
AMGT	Embryonic axis and parts of epicotyl	Puonti-Kaerlas *et al*. 1989; Schroeder *et al*. 1993
AMGT	Cotyledonary node	Davies *et al*. 1993
AMGT	Immature cotyledons	Grant *et al*. 1993, 1995, 1998; Timmerman-Vaughan *et al*. 2001; Grant *et al*. 2003
AMGT	Dry, mature seed	Bean *et al*. 1995; Fei *et al*. 2003; Hettiarachchi *et al.* 2005; Khalafalla *et al*. 2005; Fei *et al*. 2006; Richter *et al.* 2006
AMGT	Thin slices from developing embryo axes	Polowick *et al.* 2000
AMGT	Meristems	Davies *et al.* 1993; Bean *et al.* 1997; Nadoloska-Orczyk and Orczyk 2000
AMGT	Slice of immature embryo, including embryonic axis and basal part of the cotyledon	Pniewski and Kapusta 2005
AMGT	Cotyledonary meristems	Perrin *et al*. 2000
Biolistics	Plants (no mention of exact plant part targeted)	Jones *et al.* 1998

Table 3 Summary table of transgenic pea plants generated in different studies.

Reference	*Agrobacterium* strain used	Plasmid and vector type used	Reporter/marker genes used	Selection media used with concentration	Explant used
Bean *et al*. 1997	EHA105	SLJ1561	*Bar*	Rifampicin (100 µg/mL) and tetracycline hydrochloride (10 µg/mL)	Lateral cotyledonary meristem
Schroeder *et al.* 1993	AGL1	SLJ1561	*Bar* + *npt*II	Phosphoinothricin (15 mg/mL)	Slices of immature cotyledons
Nadoloska-Orczyk and Orczyk 2000	LBA4404, C58C1, EHA105	35S-GUS-INT, GPTV-KAN, GPTV-HPT; GPTV-DHFR & GPTV-BAR	Reporter gene (*uid*A) and 4 selectable marker genes (*npt*II, *hpt*, *dhfr*, *bar*)	Hygromycin B (100 mg/L), methotrexate (1 mg/L), phosphoinothricin (2 mg/L) and kanamycin (50 mg/L)	Immature cotyledons
Grant *et al*. 1995	AGL1	LN27	*Bar*	Phosphoinothricin (10 mg/mL)	Immature cotyledons
Pniewski and Kopusta 2005	AGL0, AGL1, EHA105	P35SGIB	Reporter gene (*uid*A) and selectable marker gene *bar*	Kanamycin (50 mg/ L) and rifampicin (50 mg/ L)	Slice of an immature embryo, including embryonic axis and basal part of the cotyledon
Richter *et al*. 2006	EHA105	HKvst, SCP1	*Bar*	Phosphoinothricin (7.5 mg/mL)	Sliced embryonic axis

Table 3 (Cont.)

Reference	Pea cultivar used	Were all cultivars transformed	Transformation efficiency (%)	Survivability (%)	Transgenic events assessment
Bean *et al.* 1997	Puget	Yes	1.1 ± 0.43	NR	PCR + Southern blot
Schroeder *et al.* 1993	Greenfeast, Rondo	Yes	1.5-2.5	NR	PCR + Northern blot
Nadoloska-Orczyk and Orczyk 2000	Heiga, Laser	Yes	LBA 4404: 1 C58C1: 2.2 EHA105: 8.2	NR	PCR + Southern analysis
Grant *et al*. 1995	Bolero, Trounce, Huka, Bohatyr	Yes	Percentage transformation efficiency not clearly explained	Bolero-33.3, Trounce-18.2, Huka-100, Bohatyr-100	PCR + Southern analysis
Pniewski and Kopusta 2005	Agra, Kwestor, Pioneer, Cud, Kelwedonu, Delisa II, Konserwowy IHAR, Topaz, Grapis, Wiato	Variations in response among different cultivars	AGL0: 0.7-3.3 AGL1: 1.4-4.1% EHA105: 0.6-0.9	NR	PCR analysis
Richter *et al*. 2006	Baroness, Baccara	Variations in response among different cultivars	0.01-0.06	NR	PCR + Southern analysis; RT-PCR

NR = Not reported

scFvT84.66. Perrin *et al.* (2000) demonstrated the importance of grain legume seeds for synthesizing bioactive recombinant antibodies. This study for the first time established the possibility of using field peas in molecular farming of medicinally important bioactive, recombinant gene products for human food and animal feed in near future.

The AMGT approach used *A. tumefaciens* strain AgL1 (Schroeder *et al.* 1993; Grant *et al.* 1995), strain EHA105 (Nadoloska-Orczyk and Orczyk 2000) and three hypervirulent strains AgL0, AgL1 and EHA105 (Pniewski and Kapusta 2005) (see **Tables 3** and **4**). Again, Schaerer and Pilet (1991) reported high frequency of transgenic pea lines regardless of *Agrobacterium* species and strains used.

The success rate in the case of pea transformation is relatively low compared to other crops and there is still a need for an efficient transformation process that could give a better transformation percentage (Pniewski and Kapusta 2005). Polowick *et al.* (2000) also noted the importance of identifying suitable regeneration and transformation protocols for important pea cultivars and also for adaptation of those cultivars to specific agro-climatic zone(s). Initial pea transformation work, which were mostly restricted to antibiotic resistant neomycin phosphotransferase or *npt*II gene as a selectable marker (Puonti-Kaerlas *et al.* 1990; Davies *et al.* 1993; Grant *et al.* 1995), have been slowly and largely replaced or used in association with the more convenient herbicide resistant *bar* gene (Schroeder *et al.* 1993, 1994; Shade *et al.* 1994; Bean *et al.* 1995; Grant *et al.* 1995; Schroeder *et al.* 1995) or *gus* gene (Shade *et al.* 1994; Schroeder *et al.* 1995; Polowick *et al.* 2000) commonly driven by the CaMV 35S promoter. One of the important reasons for this is that pea tissue is strongly resistant to kanamycin, hence kanamycin media and *npt*II genes together do not constitute an effective selection media for screening putative transgenic pea lines to identify true and stable transgenic lines (Schroeder *et al*. 1993). Other related studies show that organ-specific promoters such as putative nodule-specific promoter (rolD) and putative root-specific promoters (LBC_3) has been successfully used in transformation of peas for soybean cytosolic glutamine synthetase gene (*GS1*) additionally fused to a constitutive promoter CaMV 35S (Fei *et al*. 2003, 2006).

Both Puonti-Kaerlas *et al.* (1990) and Schroeder *et al.* (1993) reported *npt*II to be ineffective in pea transformations while Davies *et al.* (1993) reported success using a hygromycin resiatance gene fused to the CaMV 35S promoter. The choice of the explants source, selective marker(s) and strains used for transfection along with presence of plant growth hormones in the cocultivation media are im-

Table 4 Summary table of the morphogenetic nature of transgenic pea plants generated in different studies.

References	Regeneration time	Morphological and agronomic changes observed	Flowering	Seed production	Yield trends	Transgene generation where transgene events measured	Any specific transgene silencing reported?
Bean *et al.* 1997	~ 9-12 weeks	NR	Yes	Harvested in 8 months	NR	T_0	No
Schroeder *et al.* 1993	~2.5-3.5 months	NR	Yes	Yes	NR	T_0 and T_1	No
Nadoloska-Orczyk and Orczyk 2000	NR	NR	Yes	Yes	NR	T_0 and T_1	No
Grant *et al*. 1995	~ 7 months	NR	Yes	Yes	NR	T_0 and T_1	No
Pniewski and Kopusta 2005	Not clearly explained	NR	Yes	NR	NR	T_0	No
Richter *et al*. 2006	Not clearly explained	No morphological alterations observed	Yes	Yes	NR	T_0, T_1 and T_7	No

NR = Not reported

Table 5 Comparison of three most common AMGT protocols used in generating transgenic lines in pea.

Protocol	Cultivar(s) used	Explant used	Selection agent	Duration (months)	Ploidy level in transformants	Transformation efficiency (%)	Reliability
Puonti-Kaerlas *et al.* 1990	Stivo, Puget	Shoot cultures and epicotyls	Hygromycin (antibiotic)	15 months	Tetraploids	15.0	Moderate
Schroeder *et al*. 1993	Greenfeast, Rondo	Slices of immature embryos	Phosphoinothricin (herbicide)	9 months	Not reported	1.5-2.5	High
Davies *et al.* 1993	Puget	Cotyledonary node	Kanamycin (antibiotic)	6-7 months	Not reported	1.4	Low

portant criteria for getting higher transformation efficiency rates to generate stable transgenic pea lines (Schroeder *et al.* 1993; also reviewed in Grant *et al.* 1995).

Bean *et al.* (1995) reported having consistent difficulties in generating healthy, transgenic pea plants with roots in their hormone induction media for rooting, since the process was erroneous, extremely slow, unreliable, with many putative transformants exhibiting dwarfness, early flowering with low seed set and premature senescence. However, they overcame the challenges by directly grafting the putative transformed shoots onto pea root stocks of pea cv. 'Puget' and got 95% success in transformation efficiency within a shorter time period and with the transformed plants showing normal phenotypic features. This was a significant achievement in AMGT mode of pea transformation and has now been rapidly adapted in different laboratories across the globe working on pea transformations (Bean *et al.* 1997; Nadoloska-Orczyk and Orczyk 2000; Timmerman-Vaughan *et al*. 2001; Fei *et al*. 2003; Grant *et al*. 2003; Pniewski and Kapusta 2005; Hettiarachchi *et al.* 2005; Khalafalla *et al*. 2005; Fei *et al*. 2006; Richter *et al.* 2006). Their approach made it possible to generate dry seed harvest from transgenic lines in eight months with transgenic shoot recovery in 12 weeks and was a substantial development over the methods reported earlier by Davies *et al*. (1993). A comparative account of the three most common AMGT protocols is presented in **Table 5**.

Pniewski and Kapusta (2005) reported that pea embryo axes are not quite suitable for transformation purposes because of their high mortality rate on selection media and low regeneration ability of cotyledon slices. However, the authors found a higher regeneration rate by using intact cotyledons similar to the protocol of Grant *et al.* (1995) and Nadoloska-Orczyk and Orczyk (2000). But their greatest success was from the use of mixed explants comprising both the pea embryo axis and the basal part of the cotyledon. However, the regeneration time of the plants developed through organogenesis in callus has been reported to be quite long extending over a few months and possibly developing *de novo* plants from an initial cell (Puonti-Kaerlas *et al.* 1982; Polowick *et al.* 2000; Pniewski and Kapusta 2005). Pniewski and Kapusta (2005) reported greatest success using *Agrobacterium* strain AgL0 with relatively high transformation rates of 0.7-3.3% followed by AgL1 with a transformation efficiency of 1.4-4.1% and EHA105 with only a transformation efficiency of 0.6-0.9% (**Tables 3** and **4**).

It is interesting to note the differences in the transformation efficiencies reported by other researchers using the same strains. A transformation efficiency of 0.7-2.5% using strain AgL1 was reported by Schroeder *et al.* (1993) and Grant *et al.* (1995). Nadoloska-Orczyk and Orczyk (2000) reported EHA105 to be a much more successful strain (3.16%) in their transformation study; similarly Polowick *et al*. (2000) reported a transformation efficiency of 0.1-2.4% with EHA105. The survey of the results from different research groups clearly indicates the wide range of fluctuations in transformation efficiencies within the same strain and the possible difficulty in establishing a uniform transformation and regeneration protocol for producing stable pea lines. On the other hand, it is also important to note that the fluctuation often reflects the difference in the way in which the transformation efficiency was calculated and not the influence of any biotic factors. Another important factor playing a role in explaining the wide fluctuations in transformation efficiencies may be because of the different selection media used by different research groups, such as the use of kanamycin at 3.4% (Grant *et al.* 1998), 5% (De Kathen Jacobsen 1990), or 8.2% (Nadoloska-Orczyk and Orczyk 2000); hygromicin at 4.9% (De Kathen Jacobsen 1990), or 15% (Puonti-Kaerlas *et al.* 1992); timentin at 3-4% (Pniewski and Kapusta 2005). In contrast, Nadoloska-Orczyk and Orczyk (2000) reported an inability to reduce the number of escapes in their study (only 30% plants were truly transgenic) by increasing the concentration of their selection agent since increasing concentrations seriously impaired plant regeneration and increased mortality (**Tables 3** and **4**).

Polowick *et al.* (2000), on the basis of their study in transforming eight Canadian genotypes using strain EHA105, concluded that transformation efficiency in the case of pea lines depended on the genotypes and vector used for the transformation process. Similar conclusions were also made by Pniewski and Kapusta (2005). The authors found that the transformation efficiency of specific pea genotypes studied not always necessarily correspond to their individual regeneration abilities. However, according to these authors such regenerative variation between different genotypes was not an important parameter for successful and stable integration and transformation in pea lines tested.

CLASSICAL MOLECULAR RESEARCH WORKS ON PEA

Plant physiology

Diamine oxidases (DAO) are copper-containing enzymes that have been specifically purified and characterized from legume members, where they occur at high concentrations (Medda *et al*. 1995). Based on their extensive *in situ* hybridization studies Wisniewski *et al.* (2000) found that the transcripts of DAO are abundant in all tissues of pea nodules infected with *Rhizobium leguminosarum*, detected both in the invasion zone and also in the host cells facilitating the bacteroids which in turn help in the process of nitrogen fixation of the pea plants, suggesting that DAO might have an important role in nodule formation. Further research work by Wisniewski and Brewin (2000) reported successful transformation in pea lines having a sequence coding for DAO (PSAO-1) in a sense orientation attached to tissue-specific promoter pENOD12A exhibiting strong co-suppression of DAO activity in the nodule and epicotyl extracts; the antisense constructs were reported to be relatively unaffected. Their work indicated that DAO may not have a special role in nodule formation as the number of nodules among transgenic lines and untreated control did not show any significant difference. Since the transformed lines were also found to be less sensitive to the inhibitory impacts of exogenous polyamines (PAs) like putrescine and exhibited low activity in the cross linking of matrix glycoprotein, the authors concluded that putrescine-derived products of DAO activity could possibly impair pea nodule formation. They further infected the co-suppressed transgenic lines with a *Rhizobium* strain B661, lipopolysaccharide defective mutant and observed further loss of nitrogen–fixation capacity and nodule impairment compared to non-modified control pea lines. Wisniewski and Brewin (2000) indicated, on the basis of their study, that the most plausible role of DAO in nodule development of legume members (like pea) may be due to their role in the regulation of the concentrations of diamines in host plant tissue. This is a good example of how transgenic pea lines have significantly contributed to the studies of fundamental physiology of nodule formation and the process of nitrogen-fixation.

Last and Gray (1990) successfully introduced pea plastocin gene into transgenic tobacco plants via AMGT. Molecular analyses revealed a single copy of the transgene in self-pollinating tobacco plants and equal amounts of pea and tobacco (*Nicotiana tabacum* L.) plastocyanin in homozygous were detected, indicating that the expression of tobacco plastocyanin was not hindered by the expression of the pea transgene. The transgene mRNA in tobacco could not be distinguished by northern blotting and S1 nuclease protection from the original pea mRNA. Pea plastocyanin devoid of any stress of tobacco plastocyanin obtained and then purified from transgenic tobacco lines were reported to be non-distinguishable from normal pea plastocyanin, both by the application of NMR spectroscopy and also N-terminal sequencing of protein.

Fungal disease resistance

Hassan (2006) demonstrated heterologous expression of a chitinase gene (*Chit30*) from *Streptomyces olivaceoviridis* ATCC 11238 for resistance to fungal infection in pea and tobacco plants using AMGT. The chimeric *N-Chit30* gene was cloned into binary vector pGreenII 0229 with selectable marker gene *bar*, *nos*-promoter and *nos*-terminator. Both the target and reporter genes were arranged divergently in the vector. The target gene was regulated by either constitutive 35S promoter from *Cauliflower Mosaic Virus* (CaMV) or *vst* promoter from grapes (*Vitis vinifera* L.) with a 35S terminator. The average transformation efficiency in pea was reported to be around 0.9%. The author reported detecting single copies of the target genes in most transgenic pea plants with two copies in some plants tested. The chitinase activity tested was found to be higher in transgenic peas (0.09-0.25 U/10 µg total protein) compared to transformed tobacco plants (0.07-0.14 U/10 µg total protein). Both crude extracts of transgenic pea and tobacco plants were successful in inhibiting the *in vitro* mycelial growth of test fungus *Trichderma harzianum* after 8 and 16 h, respectively, in comparison to their corresponding non-transformed controls. This is the first report of heterologous expression of chitinase gene from S. *olivaceoviridis* in stable transgenic pea and tobacco lines.

Viral disease resistance

A very successful case of inducing disease resistance against *Alfalfa mosaic virus* (AMV) in transgenic pea lines in New Zealand was reported by Timmerman-Vaugham *et al.* (2001). Successful transformations of AMV coat protein (CP) have been previously reported in tobacco (Tumer *et al.* 1987; Anderson *et al.* 1989; Xu *et al.* 1998), in tomato (*Lycopersicon esculentum* Mill.) by Tumer *et al.* (1987) and also in alfalfa (*Medicago sativa* L.) by Hill *et al.* (1991). Higher resistance against AMV were reported in case of tobacco (Tumer *et al.* 1987) and in the case of alfalfa (Hill *et al.* 1991). Hence transgenic pea produced by Timmerman-Vaugham *et al.* (2001) was the first reported case of successful AMV resistance in transformed peas. The researchers induced two chimeric CP gene constructs of AMV strain NZ1 (Lincoln) into pea via AMGT. Resistant plants showed detectable amounts of CP indicating that AMV resistance is CP-mediated; however, screening of the transgenic lines revealed that they were only partially resistant to the potent AMV strains 425 and NZ1 (Lincoln). The authors confirmed only partial resistance to AMV in transgenic lines compared to non-transformed controls based on their analyses of the disease severity of the germinated seedlings in the inoculated plots against two AMV strains NZ1 (Lincoln) and NZ4.

Another viral disease resistance study by Jones *et al.* (1998) revealed that 89% sequence similarity was necessary for the activation of the replicase gene, *Nib*, derived from *Pea seed-borne mosaic virus* (PSbMV) for expression in transgenic pea lines. The authors reported three pea lines in which an initial infection by homologous isolate (PSbMV-DPD1) was followed by higher resistance. The resistance was observed in the transgenic lines either in homo- or hemizygous condition with no significant yield loss. The authors also reported that resistance was linked to the loss of viral and transgene RNA, possibly associated with some post-transcriptional gene silencing (PTGS) mechanism. Jones *et al.* (1998) successfully bombarded plants (the authors did not clearly mention which exact plant part(s) was actually bombarded in their article; we assume that they possibly bombarded the leaves of the healthy field-grown plants) using plasmids carrying PSbMV CDNAs under the control of CaMV 35S promoter. The authors reported 100% success in infection rate via biolistics compared to 50-100% infection rate by manual inoculation. Furthermore, the biolistics generated infection could not be distinguished from infection generated through the standard sap inoculation approach. This article shows the possibility of using biolistic approach in production of transgenic pea lines and to our knowledge is the first paper to use biolistics for gene transfer in peas. It is quite unfortunate not to find any other major research work on pea transgenics using biolistics although it has a lot of future potentiality. In another study, Chowrira *et al.* (1998) reported developing transgenic pea lines using *in planta* ransformation via injection/electroporation of axillary meristems. The transgene construct inserted was a chimeric Pea Enation Mosaic Virus (PEMV) coat protein. The authors proved the presence of the transgene through standrad molecular biology techniques (PCR, Southern and Westen blotting). Subsequent T2-T4 generation plants exhibited slow or transient viral multiplications associated with reduced disease symptoms compared to their corresponding inoculated controls.

Insect disease resistance

Peas are used as an important intercrop in Australian agriculture and the annual pea crop approximately accounts for around AUS $120 million per annum (GMO Compass 2008). Pea weevil (*Bruchus pisorum*) is one of the major pea pests and accounts for about 30% annual loss of pea crops without the intervention of costly chemical pesticides thereby increasing the production cost per acre to substantial amounts (GMO Compass 2008).

Several plant proteins and molecules with insecticidal and entomopathogenic properties such as lectins like phytohemagglutinins (PHA), arcelin and α-amylase inhibitors (αAI), protease inhibitors, etc. are known to arrest the growth and development of insect pests on ingestion (Chrispeels and Raikhel 1991; Ignacimuthu and Prakash 2006). The genes for these proteins (PHA, arcelin and αAI) are located at a single locus in the common bean (*P. vulgaris*) genome and it is quite likely that these homologous genes may have evolved by duplication of the ancestral gene (Nodari *et al*. 1993). The bean α-AI protein has been reported to have the ability to inhibit some mammalian and insect amylases, but not plant enzymes; more importantly these inhibitors are host-specific, impacting only certain specific insect species (Bowman 1945; Chrispeels and Raikhel 1991; Nodari *et al*. 1993). The bean αAI has been reported to exist in two distinct isomeric forms, αAI-1 and AI-2. αAI-1 is found in common bean and the variant αAI-2 is found in some wild types of common bean; the major storage protein for αAI-1 is phaseolin while that of αAI-2 is arcelin (Moreno and Chrispeels 1989). αAI-1 has been reported to have the ability to successfully repressα-amylase activity in the midgut of the insect larvae (Suzuki *et al.* 1993; Ishimoto and Chrispeels 1996).

The development of pea weevil-resistant transgenic pea lines has been a success story of an international collaborative research project involving different research institutes and organizations from different countries. This project stood out as one of its kind as an international collaboration between Dr. Maarten J. Chrispeels at the University of California, San Diego (USA), Drs. Larry L. Murdock and Richard E. Shade at Purdue University West Lafayette (USA) and Drs. Hartmut H. Schroeder and Thomas J. Higgins of CSIRO at Canberra (Australia). The significant objective of this international project was to introduce three important novel traits in transgenic lines: resistance to the insect pea pest (pea weevil), herbicide resistance against Basta® and improvement of the nutritional level of the proteins in pea seed (Schroeder *et al*. 1994). Details are described below.

Legumes such as peas, chick peas, cow peas (*Vigna unguiculata* (L.) Walpers) and Azuki beans (*Vigna angularis* (Willd.)) are susceptible to invasive seed-attacking insects like the bruchid (Coleoptera: Bruchidae) members that thrive on the seed of such legumes and causing huge losses to their yield. But certain other legume members like common beans (*P. vulgaris*) are naturally resistant to bruchids due to the presence of special seed protein (α-amylase inhibitor or αAI-Pv) with very strong insecticidal properties, being toxic to the insect larvae (Schroeder *et al*. 1994). The principal mechanism of the action of αAI protein is that it inhibits the action of enzyme α-amylase in the midgut of the insect larvae, thereby interfering with the digestion of starch which in turn causes the larvae to starve as it can not process the food for its nourishment and further development (O'Neill 2005).

Previous studies indicated that slight alteration of diets by incorporating low amounts of kidney bean αAI-Pv inhibited certain bruchid species feeding on cowpea and azuki beans significantly (Ishimoto and Kitamura 1989; Huesing *et al*. 1991). Shade *et al.* (1994) for the first time tried to incorporate bean αAI-Pv into pea to check if that would confer resistance to the aggressive bruchid beetles. The researchers modified the common bean αAI-Pv promoter to a bean phytohematoglutinnin-L gene (*dlec2*)-driven promoter (strong seed-specific promoter) and introduced the modified gene into pea cv. 'Greenfeast' via AMGT. This particular chimeric construct has previously been reported to successfully express αAI-Pv proteins in transgenic tobacco seeds (Altabella and Chrispeels 1990). Shade *et al.* (1994) reported the successful development of transgenic pea lines with seeds containing 1.00-1.25% (w/w) αAI-Pv proteins showing resistance to brucid beetles like cowpea weevil (*Callasosobruchus maculatus* (Fabricius)) and Azuki bean beetle (*C. chinensis* (Linnaeus)).

The next target achieved by this collaborative research group was developing transgenic pea lines with bean αAI conferring resistance to the pea weevil (*Bruchus pisorum* (L.)) (Shade *et al.* 1994; Schroeder *et al.* 1995). They conducted bioassay experiments under greenhouse conditions to check whether the presence of αAI in transgenic pea seeds prevented insect infestations. Two bioassays were conducted, one with T_2 seeds of five transgenic lines and the other with T_5 seed of one transgenic line. In the first experiment, pea weevil eggs were simultaneously transferred into the pods of both transgenic lines and untransformed control. Infected seed were scored from the harvest of mature pods where no larval emergence was observed after40 days form the date of egg transfer. The amounts of αAI varied in the transgenic seed lines from undetectable to 3% of total soluble protein levels. These researchers suggested two possible reasons for such variations: seeds were tested form different transgenic lines and the αai transgene transferred segregated in the T_2 populations. In the second bioassay, the single transgenic line having the highest αAI content (3% of total soluble seeds) was used in the experiment to produce T_4 plants, being homozygous for the αai gene and produced a consistently higher concentration of the αAI proteins in all seeds. Immature pods were used for the experiment and no weevil larva emergence was noticed after 140 days; after 200 days of further incubation with zero emergence, weevil resistance in the transgenic pea line was confirmed (Shade *et al.* 1994; Schroeder *et al.* 1995).

In a later field investigation by the same group, Morton *et al.* (2000) reported that αAI-1 also sufficiently protected transgenic pea lines against pea weevil infestation under field conditions while pea lines expressing αAI-2 (sharing 78% amino acid sequence similarity to αAI-1) was capable of moderately suppressing pea weevil infestations. The authors further reported that αAI-1 inhibits 80% of pea weevil α-amylase over a pH range of 4.5-6.5; while αAI-2 could only inhibit 40% of pea weevil α-amylase, but its action was specifically restricted to a narrow pH range of 4.0-4.5. Morton *et al*. (2000) made a significant observation that while αAI-1 induces larval mortality during the 1st or 2nd instar stages, αAI-2 induces delay in the maturation of the pea weevil larvae.

Later, Sousa-Majer *et al.* (2004) studied the effects of water deficit and higher temperature on the production of αAI-1 in transgenic pea lines developed for pea weevil resistance compared to non-transformed controls under greenhouse conditions. The transgenic pea lines were deprived of water after first pod formation in the water deficit experiment compared to the controls which were watered throughout and produced 79% less seed. In the high temperature experiment, the transgenic lines exposed to a temperature regime of 32/27°C compared to 27/22°C regime of the controls (both groups had water deficit maintained at 1.3 kPa), generated 27% less seed than their corresponding control plants. Interestingly, they also reported that the αAI-1 concentration as a percentage of total protein was reduced on average by 36.3% in transgenic lines only under high temperature treatment. The researchers also investigated the percentage of weevil larvae emergence under temperature stress that reduced the concentration of αAI-1 in the transgenic lines. They reported 39% adult pea weevil emergence in transformed plants treated at a higher temperature then those treated at a lower temperature regime (1.2%), suggesting high temperature could certainly impair the resistance level of transgenic pea lines to pea weevil infestations.

CONCERNS REGARDING FOOD SAFETY OF TRANSGENIC PEA LINES

Elaborate studies were conducted to assess the food safety of the transgenic pea lines for its release for food and feed purpose. Genetically Modified (GM) food and feed are a global concern with respect to food and feed safety issues (GMO Compass 2008). According to the Online Free Dictionary, a GM organism is defined as "an organism whose genetic characteristics have been altered by the insertion of a modified gene or a gene from another organism using the techniques of genetic engineering" (http://medical-dictionary.thefreedictionary.com/Genetically+Modified+Crops). Pusztai *et al.* (1999) studied the effects of transgenic peas (expressing bean αAI-1) by fair-feeding rats with diets containing transgenic or parental peas at 300 and 650 g/kg respectively and at 150 g protein/kg diet fortified with essential amino acids. Another lactoalbumin diet was also tried with/without 0.9/0.2 mg bean αAI-1, the same as was present in transgenic pea lines. The weight gain and tissue weights of rats fed on either pea diets or that on lactoalbumin supplemented with 0.9 g αAI-1/kg were not significantly different form one another ($p < 0.05$) at the 300 g/kg level. The authors attributed the lower digestibilities of the proteins and the dry matter of transgenic pea diet compared to that of the standard lactoalbumin diet to the anti-nutritional factors present in pea seeds. But at a higher dosage (650 g/kg), the nutritional value of pea diet was lower that that of the lactoalbumin diet; but the difference between transgenic and parental pea lines was quite narrow. The authors suggested that neither the purified recombinant αAI-1 nor that occurring in transgenic lines inhibited *in vivo* starch digestion (although both equally inhibit starch digestion *in vitro*) in the rat intestine like the bean αAI-1. The authors therefore recommended transgenic peas as rat diets at lower inclusion (300 g/kg) without any principle harmful impact on the animal health.

Collins *et al.* (2006) conducted a comprehensive investigation on the impact of transgenic pea on a population of 18 individually housed pigs. The animals were provided a diet of 989.4 g/kg of basal wheat diet or a mix of 500 g/kg of basal wheat diet with 500 g/kg transgenic (expressing bean αAI-1) or non-transgenic pea for a period of 15 days. The ileal dry matter and starch digestibility of the experimental wheat, control pea and transgenic plants were reported as 78.3, 74.2 and 45.8% and 95.9, 95.2 and 42.2%, respectively. Although the apparent protein and amino acid digestibilities were similar between transgenic and non-transgenic pea lines, the ileal dry matter digestibility between non-transgenic (69.9%) and transgenic (12.7%) however, were significantly different ($p < 0.05$) suggesting that the transgene impacted carbohydrate metabolism in the animals (Collins *et al.* 2006). The transgene that inhibits carbohydrate digestion in the midgut of weevil larvae also proved to inhibit successful digestion of carbohydrates in the pig ileum. This could be considered as a serious blockade for the release of the transgenic pea lines as animal feed and also for human consumption. However, the authors reported higher fecal digestibility in the animals, possibly due to the fermentative properties of the hindgut microflora.

The authors suggested that transgenic peas could be an important protein source for the animals although with reduced energy availability. The authors advocated that the cost of transgenic pea lines would be less due to their strong insect resistance; however, heat processing of the feed was suggested if the animal owners would like to compensate for the low energy value of transgenic lines for the demand of high energy feed. The reduced energy value of transgenic pea may also bring down the economic value of the animals fed on it and runs the risk of transgenic pea lines not being very suitable as animal feed in the long run. Similar results were also reported by Lix *et al.* (2006) by feeding transgenic and non-transgenic peas to broiler chickens at 300 g/kg for 40 days with significantly ($p < 0.05$) reduced starch digestibilities in transformed lines (42.4%) in comparison to non-transformed lines (80.3%).

In spite of the fact that there has been great success with respect to the technology developed and protocols established by several leading research laboratories around the globe for developing transgenic peas with unique and agronomically important transgenes, the development has not been free of controversies. Transgenic pea lines developed for resistance against pea weevil has landed up in the latest controversy over the safety of GM foods and feeds, after Australia's Commonwealth Scientific and Industrial Research Organization (CSIRO) dropped the project in October, 2003 after immunologists at John Curtin School of Medical Research (JCSMR) of the Australian National University (ANU) reported that transgenic peas carrying *αAI* gene caused mild inflammation in the lungs of BALB/c mice by intragastric administration of 250 µL of seed-meal suspension at approximately 100 mg/mL (O'Neill 2005; Prescott *et al*. 2005). Prescott *et al*. (2005), through detailed structural analysis using MALDI-TOF-MS, reported that transgenic expression of the αAI proteins in transgenic peas were modified from original bean αAI proteins. The authors also showed that the pea αAI exhibited altered antigenic properties. Finally, the authors clearly demonstrated that exposure of the mice gastrointestinal tract to these altered αAI elicited immunogenicity and immunoreactivity, indicating its possible danger to be used as potenatial human food and animal feed (also reviewed in the editorial of *Nature Biotechnology* (2006) **24 (2)**. Available online: www.nature.com/nbt/v24/n1/full/nbt0106-2.html)

However, as previously discussed, long-term laboratory and field tests (transgenic peas were found to be 99.5% resistant in field studies) indicated that transgenic pea lines are safe for both human consumption and animal feed and was detrimental only to the pest pea weevil (Pusztai *et al.* 1999; Smith 2005; GMO Compass 2008). The αA1 protein is reported to be easily inactivated during cooking and hence transgene integration in the transformed lines was considered as a safe, eco-friendly and sustainable strategy against aggressive insect pest damages and subsequent yield loss (Ignacimuthu and Prakash 2006).

However, researchers suggested that the bean α-amylase protein was slightly different than that ofα-amylase produced by the transgenic pea lines due to a different glycosylation process (O'Neill 2005). A detailed investigation initiated after the scientists at CSIRO and JCSMR detected minor differences in the surface structure of transgenic pea α-amylase protein. An elaborate feeding test was conducted where one group of laboratory mice was fed transgenic pea while the lungs of the other group was exposed to aerosols from transgenic peas with adequate controls for both treatments (GMO Compass 2008). Researchers at JCSMR reported that they detected elevated blood antibody levels in the mice fed on transgenic pea and above average levels of inflammation in the lungs of animals exposed to the transgenic pea aerosol compared to their corresponding controls (O'Neill 2005; Smith 2005).

The researchers speculated that the minor alteration in the structure of the pea αA1 protein due to a different glycosylation process may be the possible reason behind the mild inflammation observed in the laboratory animals (GMO Compass 2008). Based on these findings, CSIRO decided to shelve the transgenic pea project, anticipating possible future risks and health hazards such as allergy and inflammation in humans although Food Standard Australia and New Zealand (FSANZ), the organization responsible for monitoring food and feed safety standards of GM crops under study, indicated that a positive animal test under laboratory conditions does not necessarily mean immediate threats to human health (O'Neill 2005; Smith 2005; GMO Compass 2008).

This is quite a blow to the tremendous technological progress made in the past few decades on the development of transgenic pea lines. Similar cases have been reported before when US researchers withdrew transgenic soybean lines expressing Brazil nut genes with the possibility of alleviating allergic responses in humans and when potato

varieties were retracted from markets in US and Sweden since the tubers had higher concentrations of toxicogenic glycoalkaloids harmful for human consumption (O'Neill 2005; GMO Compass 2008). However, it is important to note that the team leader of the CSIRO transgenic pea project Dr. Higgins suggested that shelving of the project is not a backwards step since the technology developed could be and is being used for developing transgenic lines in other related legume crops (O'Neill 2005; GMO Compass 2008).

However, in spite of the unfortunate termination of the transgenic pea project in Australia there is some welcome news from other parts of the world on biotech progress in transgenics. In Gatersleben, Germany a small biotech firm Novoplant has developed four transgenic pea lines each producing specific antibodies for a particular infectious disease and are expected to be ready for release in 2010 (GMO Safety 2007). Since antibiotics have been banned in the EU from mixing in animal feed, the animal feed industry has been looking for viable alternatives for developing improved feeds that could protect the animals from infectious diseases.

The transgenic pea lines developed by Novoplant could certainly provide a sophisticated mode of developing strong immunity in the bodies of the animals fed on such crops by improving their antibody levels against certain infectious microbial agents. According to Novoplant, this 'passive inoculation' approach is a step towards enhancing the animal's 'own immune system' (GMO Safety 2007). In 2006, *αA1* gene from bean was successfully introduced into another very important legume crop, chickpea (*Cicer arietinum*) in India (Ignacimuthu and Prakash 2006), indicating the harvesting of the 'ripe fruit of technology' developed for transgenic peas is now being extended to other related legume crops in a short time span. Since legumes constitute a very important and cheap plant protein source in underdeveloped and developing countries, it is important to look for opportunities to promote innovative technologies for developing newer disease resistant cultivars with better agronomic traits for future sustainability. Legumes not only provide cheap food and feed sources for poor economies, they also allow local and regional farmers to increase their profit margins since they improves the soil nitrogen profile through nitrogen fixation and being disease resistant can cut down substantially on the cost of application of expensive fertilizers and pesticides.

OTHER FOOD-RELATED ISSUES

In a very interesting study, Chalton *et al.* (2004) applied nuclear magnetic resonance (NMR) spectroscopy to transgenic pea lines to identify whether 'unintended' biochemical changes could be detected, that may have result from the incorporation of a transgene in the plant genome. Multivariate analysis of the NMR spectral data clearly indicated a significant ($p < 0.05\%$) difference between the T_3 transgenic and non-transformed control (wild type) plants.

However, it was not clear whether the difference was because of the transformation process (due to the transfer of the transgene) or due to some 'undefined' factor or criteria or parameter. The author did a further extensive investigation between null segregant (devoid of any insertion) and transformed lines using T_4 plants (3rd selfed generation from the original transformant T_1 population). Nested Analysis of Molecular Variance (AMOVA) showed no significant difference ($p > 0.05\%$) between the transgenic plants (T_4 plants) and the non-transgenic plants (wild types and null segregants pulled together) suggesting that the presence of the transgene does not indicate any significant difference in the constitution of the pea leaf extracts (transgenic *vs.* non-transgenic) profiled using NMR. Differences were more pronounced in T_3 than T_4 plants. The authors finally concluded on the basis of detailed statistical analyses that the principle reason for the difference was due to the fact that "the transformation process selects for a subset of individuals able to undergo transformation and selection procedures, and that their descendants *have a restricted variation in metabolite profile*, rather that the *presence of the transgene itself* generates these differences." In other words, both AMOVA and multivariate analyses conducted on the NMR data suggests that the difference is primarily due to the subsequent selection and reduction in variation of the process of transformation and culture techniques used in generating the transgenic plants and not the transgene itself.

Additionally, since the null segregant group also differed significantly ($p < 0.05\%$) from the wild type group, it was concluded that factors other then the transgene actually had a primary impact on the differences observed. However, in the future, further improvements in techniques may help us gain a better estimate of how the transgene impacts the metabolomic profiles of the transformed plants and to what extent and thereby strengthen the neglected view of metabolomics. It may also help us in future to take bold and more scientific decisions regarding food safety issues associated with Genetically Modified (GM) food and feeds and GM crops overall. More research is necessary in this arena to strengthen our understanding of the molecular aspects of food and feed safety.

Due to recent advances in biotechnology and genetic engineering, the development and commercialization of (GM) crops has broadly become an important concern with regards to food safety and human and animal health. Strict regulations and monitoring have been put in place to look into the safety of incorporating GM crops in human and animal food chains by Australia and New Zealand, EU member countries, USA, Canada, Mexico, Brazil and Japan; and fairly recently by India (Halford 2003; Prescott and Hogan 2006; GMO Compass 2008).

Pea is a predominantly self-pollinating crop plant (Fehr 1993), having cleistogamous flowers that open around 24 h post-pollination (Cooper 1938), although the rate of cross-pollination in peas has been estimated to be only around 1% (Gritton 1980). However, with the development of transgenic crops and their rapid commercialization there are constant concerns regarding the possibility of transgene migration into wild relative(s) and/or other commonly available commercially-grown lines all over the globe (Polowick *et al.* 2002).

Hence, to assess the possible frequency of transgene migration, Polowick *et al.* (2002) studied the frequency of outcrossing in the field from a transgenic pea variety (PLP1) into three non-transgenic lines ('Carneval', 'Monatana' and 'Tipu') in 1997 and 1999. Normal leaf form and overexpressd *gusA* gene were used as markers for pollen migration. However, due to heterogeneity of the commercial seed stocks, leaf morphology on its own was not a reliable indicator of pollen migration from transgenic lines into non-transgenic lines. The authors reported that only 0.06% of plants sampled out of a substantial population (~9000 plants) scored positive for both markers with an outcrossing rate of 0.07% and all the positive plants were detected in trap plots close to the plots growing transgenic lines. No outcrossing was detected in plots 5 m away from the plots reported having outcrossing in spite of the fact of prevailing wind and the presence of potential insect pollinators. Low outcrossing in peas could be due to the fact that the stigma is responsive to the pollen until 1 day after the wilting of all the pea petals (Warnock and Hegerdon 1954; Polowick *et al.* 2000). Due to low rate of outcrossing detected in this study, developing a meaningful distribution model of distances covered by the pollen grains was difficult (Polowick *et al.* 2000). Similar studies on chickpea by Yayyar *et al.* (1995) reported 0.14% outcrossing; however, 20-30% outcrossing has been reported in case of canola (*Brassica napus* L.) by Rakow and Woods (1987) indicating a crop-specific pattern in outcrossing percentages.

Although only very long term studies (~10 years) could certainly and specifically indicate or establish any specific genetic, agronomic or treatment effect scientifically beyond any doubt at the same time it is important to keep in mind that such studies are usually expensive and labour intensive to conduct and difficult to operate over a long period of

time. However, reasonablly based on the Polowick *et al.* (2000) study it could be mentioned that the possibility of transgene migration from transgenic pea lines to related crops and non-transgenic pea varieties is relatively low, although it warrants more of such multi-location trials to enhance safety standards to a reasonable level. In another study, Dostalova *et al*. (2005) using non-GM pea cvs. 'Zekon' (used as trap variety) and 'Arvika' (used as pollen donor), convincingly established that probability of outcrossing in commercial pea varieties are extremely low. These researchers detected practically no outcrossing in their experiment in Czech Republic even after screening 40,000 F1 plants per annum for two years (2002 and 2003).

CONCLUSIONS AND FUTURE DIRECTIONS

We conclude that the AMGT approach has been quite extensively investigated and applied in developing transgenic pea lines across the globe in different institutes and laboratories more than other available genetic transformation methods. Although well established in several other legumes, the application of biolistics or particle bombardment has not been exploited thoroughly in pea transgenics. AMGT has its own merits and demerits and its own success stories as discussed above with specific problems of virulence of *Agrobacterium* and its genotype specificity. The possibilities of pea improvement through gene silencing via RNAi technology or gene transfer by isolated microspore culture that has been successful in a number of other related crop species and in some cereals to a limited extent, needs to be investigated in pea too; while looking for better alternative approaches or methods of efficient and stable pea transformation that can compete with efficiency of existing AMGT protocols.

A key regulator of starch synthesis in legume seeds is the ratio of hexose to sucrose in the developing embryo environment (Rolletschek and Borisjuk 2005). During recent years we have advanced towards a better understanding of the mechanisms of assimilate uptake and the regulation of seed development in peas. Recent molecular approaches provide initial information on genes involved in seed-specific assimilate transport and the regulation of their expression. This information, based on the knowledge of biochemical pathways of storage product synthesis as well as seed physiology, will allow us a more integrated understanding of seed-specific transport in relation to pea seed development and metabolism (Borisjuk and Wobus 2005). We have recently transferred a cell wall invertase gene under seed coat specific promoter for the modification of sucrose and starch ratio in mature pea seeds, and we are currently testing its effectiveness in conferring production of increased total starch and modified starch granules and amylase and amylopectin ratio. Changing metabolic pathways by transgenics may have a significant potential for manipulating seed growth and development and thus for agricultural yield of pea in the near future.

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