GENETIC ENGINEERING FOR GRAIN LEGUME IMPROVEMENT

J.E. Grant, G.M. Timmerman, A.J. Conner

DSIR Crops
Private Bag
Christchurch, New Zealand

ABSTRACT

Progress toward the genetic engineering of grain legumes such as peas is well advanced. Using tissue culture technology, whole plants can be efficiently regenerated from individual cells of immature cotyledons from pea cultivars important in New Zealand. These cultivars are also excellent hosts for Agrobacterium-mediated transformation. Using this approach we have selected transformed hairy root cultures of pea that express two foreign genes: kanamycin resistance and - glucuronidase. We are currently attempting to combine our regeneration system with the selection of transformed cells to produce transgenic plants. Once developed, we intend to exploit this technology for the development of pest and disease resistance in peas. Our immediate target is pea seed-borne moasic virus using the now well established approach of "coat protein mediated virus resistance". To date we have cloned, sequenced and manipulated the coat protein gene of this virus to permit expression upon transformation into plants, and are currently attempting gene transfer to peas.

Additional key words: *tissue culture, regeneration, transformation,* Agrobacterium tumefaciens, Agrobacterium rhizogenes, *tumours, hairy roots, insect resistance, virus resistance, pea seed-borne mosaic virus.*

INTRODUCTION

Plant genetic engineering is a multidisciplinary area of scientific research. It requires co-operation and involvement of scientists with a wide range of skills, including molecular biology, biochemistry, microbiology, tissue culture and genetics. Once genetically modified plants are obtained there is a great deal of testing required not only to determine if the plants are only altered in the particular desired trait, but also what effects may possibly occur when the plant is released into the environment. In this phase of research the skills of plant breeding, agronomy, food technology and ecology all become important.

In this paper we present an overview of genetic engineering technology as it applies to grain legume improvement, with the main emphasis on peas (*Pisum sativum*). We focus on the current status and direction of research, rather than discuss the various technologies available. (For reviews see Conner *et al.*, 1990; Gasser & Fraley, 1989). To achieve successful genetic engineering of plants several lines of research must intersect. These include the ability to regenerate plants from individual cells, the ability to transform genes into plant cells, the cloning of target genes to be transferred and the linking of their coding regions to appropriate regulatory elements.

REGENERATION OF PEAS IN TISSUE CULTURE

Organogenesis: There are a number of reports of successful regeneration via organogenesis in peas (Mroginski & Kartha, 1981; Rubluo *et al.*, 1984; Hussey & Gunn, 1984; Natali & Cavallini, 1987). All these reports used immature tissue as explant material. This included embryos, leaflets, plumules and shoot apices. In general the reports indicate a low efficiency of regeneration with up to 37 % of explants responding, but generally the response reported was significantly lower. The appearance of adventitious buds or shoots occurred after a relatively long period in culture (from six weeks onwards; Natali & Cavallini, 1987; Mroginski & Kartha, 1987). Of the plants produced

from tissue culture and examined cytologically, there was a high frequency of tetraploidy and aneuploidy among the expected diploids.

At Crop Research Division we have developed a medium (modified from Hinchee *et al.*, 1988) that successfully allows regeneration for New Zealand conditions and cultivars. Shoot regeneration is rapid and although there are marked genotype differences, all cultivars tested have regenerated plants. For cultivars 'Bohatyr' and 'Pania' grown under greenhouse conditions, 70 % of explants produced shoots (Table 1). Our method uses the distal two-thirds of immature cotyledons with embryos (including the cotyledonary nodes) removed. The most responsive stage of development of the seed is at the "green pea" stage, i.e. when the cotyledons fill the seed and at the optimal time for picking fresh eating peas. The cotyledons are placed with their flat surface in contact with the culture medium. After 10-14 days some callus develops and shoot primordia are clearly visible. Cotyledons continue to respond for up to two months. Shoots can be excised, rooted and successfully transferred to soil. Upon subsequent subculture of remaining cotyledonary material further shoots develop.

Somatic embryogenesis: Kysely *et al.* (1987) reported whole plant regeneration via somatic embryogenesis in peas using immature embryos or shoot apex segments as explants. Induction of the somatic embryos required 2,4-D or picloram, and genotypic differences were evident. The frequency of embryo production was low with only an average of two somatic embryos for every zygotic embryo cultured. Of the nine plants recovered, six were diploid and three were tetraploid.

Protoplast isolation and regeneration: Recent work on protoplast isolation and regeneration to shoots and plants has been successful. Puonti-Kaerlas & Eriksson (1988) used a bead culture system and found the cultivar 'Filby' gave an 80 % response (i.e. development of mini-cell colonies). Shoot regeneration was obtained in cultivars 'Petra' and 'Stivo', but these shoots were not able to be rooted.

Lehminger-Mertens & Jacobsen (1989) produced protoplasts from embryo axes of mature seeds. Somatic embryos were produced on the protoplast derived calli by using strong auxins (2,4-D and picloram) in association with increased osmolarity in the medium. Cultivars 'Belmen' and 'Brite' gave relatively high rates of embryo induction (20 - 30 %). The authors were able to induce the somatic embryos to mature and germinate. The plants so far produced have a normal phenotypic appearance.

AGROBACTERIUM-MEDIATED TRANSFORMATION

Agrobacterium-mediated transformation has been the most successful method of gene transfer into plant cells. This system takes advantage of the natural genetic engineering ability of the bacterium. Two main species of *Agrobacterium* are used for gene transfer: *A. tumefaciens* and *A. rhizogenes*. Each work on similar principles, but the outcomes vary.

Agrobacterium tumefaciens: In its natural state this bacterium infects wound sites and causes the formation of crown gall tumours on many dicotyledonous plant species. During tumourigenesis a specific segment of bacterial tumour-inducing (Ti) plasmid, the T-DNA, integrates into the nuclear DNA of the plant cells. These plant cells express the T-DNA genes which encode enzymes responsible for phytohormone biosynthesis (causing the tumorous growth) and for opine production. The phytohormone biosynthesis genes on the T- DNA can be deleted to produce "disarmed' strains. In their place coding regions of other genes under the control of appropriate plant regulatory sequences can be inserted. Such "disarmed" strains of bacteria are still capable of gene transfer, and since they do not induce tumours, complete plants can be regenerated from the transformed cells using tissue culture technology.

In the disarmed strains the genes inserted usually include a gene for antibiotic or herbicide resistance, which allows transformed cells to be conveniently selected by growing the plant material on an antibiotic or herbicide supplemented medium. For peas we have established that 100 mg/l of kanamycin is an appropriate concentration for selecting transformed cells. This level inhibits the growth in culture of wild-type cells and causes existing shoot material to become chlorotic.

We have also established that peas (and other legumes) are a good host for *Agrobacterium* (Table 2). We have not yet selected kanamycin-resistant cell cultures of pea using disarmed strains of *Agrobacterium*. Puonti-Kaerlas *et al.* (1989) reported the selection of kanamycin-resistant cells following cocultivation of pea shoot cultures with a modified Agrobacterium tumefaciens strain, but they were unable to regenerate shoots. Recently the regeneration of transgenic pea plants with kanamycin resistance and -

Table 1: **Analysis of pea regeneration from immature cotyledons after three weeks in tissue culture (Grant & Frew, unpublished results).**

	Pea genotype	A Standard*	B +3 mmol glutamine	C B + 56.8 umol asparagine	D A + 100 mgl^{-1} inositol
1	Bohatyr	31 %	70 %	42 %	33 %
2	Almota	15 %	-	-	-
3	OSU 442-15	40 %	10 %	33 %	19 %
4	017	32 %	0 %	-	20 %
5	985-990	38 %	52 %	-	-
6	Pania	44 %	47 %	41 %	70 %
7	FR80-1724	9 %	3 %	4 %	4 %
8	Whero	25 %	-	-	-

* Standard medium: B5 macro and micro salts, vitamins; MS iron; 1.15 mgl^{-1} BAP; 2.0 % sucrose; 0.8 % Difco agar; pH 5.8.

Table 2: **Host range of *Agrobacterium tumefaciens* strains on pea genotypes grown in the glasshouse: - no response; + tumours formed (Grant & Frew, unpublished results).**

Pea genotype	*Agrobacterium tumefaciens* strain				
	Nt*	Ach5	A281	C58	LBA4404**
Rover	-	+	+	+	-
Pania	-	+	+	+	-
Whero	-	+	+	+	-
985-990	-	+	+	+	-
599-600	-	+	+	+	-

* Control inoculations without *A. tumefaciens*. ** A disarmed strain of *A. tumefaciens* ACH5

glucuronidase activity has been claimed following cocultivation of epicotyl and nodal explants from etiolated pea seedlings with modified *A. tumefaciens* (de Kathen & Jacobsen, 1990). Another grain legume, *Glycine max* (soya bean) has also been successfully transformed by co-cultivation of *A. tumefaciens* with cotyledon explants (Hinchee *et al.*, 1988).

***Agrobacterium rhizogenes*:** *Agrobacterium rhizogenes* is a promising alternative to *A. tumefaciens* for obtaining transformed plants. Essentially this bacterium acts in the similar manner to *A. tumefaciens*, but instead of tumour induction via the Ti plasmid, *A. rhizogenes* generally produces roots via the Ri (root-inducing) plasmid. The roots produced are known as "hairy roots" because phenotypically the roots have a fine, highly branched appearance.

We have examined the host range of several *A. rhizogenes* strains on a range of pea genotypes (Table 3). Only one strain of *A. rhizogenes*, A4T, gave the typical hairy root response. The other six strains gave tumours or no response.

Table 3: **Host range of *Agrobacterium rhizogenes* strains on pea genotypes: - no response; t tumours; hr hairy roots (Conner & Williams, unpublished results).**

Pea genotype	*Agrobacterium rhizogenes* strains						
	TR7	TR101	TR107	8196	15834	A4	A4T
Proton	t	t	-	-	t	-	hr
11/2	t	t	-	-	t	-	hr
Summit	t	t	-	-	t	-	hr
Canterbury 39	t	t	-	-	t	-	hr
Whero	t	t	-	-	t	-	hr
Puke x Whero	t	t	-	t	t	-	hr
Maro	t	t	-	-	t	-	hr
142a	t	t	-	-	t	-	hr
Bohatyr	t	t	-	-	t	-	hr
Birte	t	t	-	-	t	-	hr

We have used the A4T strain of *A. rhizogenes* containing the binary vector pKIWI 110 (Janssen & Gardner, 1990) to produce transformed hairy root cultures of the cultivar Pania. The modified T-DNA of pKIWI 110 contains genes for kanamycin resistance and -glucuronidase. We therefore selected the transformed hairy roots on medium with kanamycin and further confirmed their transformed nature by detecting expression of - glucuronidase using a simple histochemical test.

An advantage of using *A. rhizogenes* transformation is that hairy roots from many species can be readily regenerated into plants without having to create disarmed strains. *Glycine canescens*, a wild relative of soybean, has been regenerated from hairy roots (Rech *et al.*, 1989). Other legumes in which this is possible include Lotus (Jensen *et al.*, 1986), Medicago (Sukhapinda *et al.*, 1987) and Stylosanthes (Manners & Way, 1989). We are currently attempting to regenerate pea plants from our transformed hairy roots.

Even if we are unable to regenerate plants from these hairy roots, transformation of pea with *A. rhizogenes* offers a very easy and convenient system in which we can study the expression of foreign genes in the grain legumes. As molecular biologists construct gene vectors for grain legume transformation, we will be able to assess rapidly which of the various versions constructed show the highest expression in peas.

OTHER APPROACHES TO TRANSFORMATION

While our efforts to genetically engineer peas have so far concentrated on using Agrobacterium to transfer the genes, we will in the near future be looking at two further options for transformation. One approach involves direct DNA uptake into protoplasts as there are now reports of regeneration of whole plants from pea protoplasts (Lehminger-Mertens & Jacobsen, 1989). A second approach involves the gene gun, where DNA coated particles (tungsten or gold) are accelerated into plant tissue. Both of these approaches have been successfully used to transform other grain legumes (Kohler *et al.*, 1987; McCabe *et al.*, 1988).

GENES TARGETED FOR TRANSFER TO PEAS

Once a transformation system is established for peas, the transfer of agriculturally useful genes can be achieved by constructing vectors with the appropriate gene adjacent to a kanamycin-resistant selectable marker gene. The primary focus of our genetic engineering programme is the transfer of genes for pest and disease resistance into arable and vegetable crop plants, including peas (Conner *et al.* 1990). In the

longer term we anticipate applications for improvements in grain quality, especially nutritional composition.

Insect resistance: The major insect pests of pea for which we are targeting resistance are Heliothis, Bruchus and Etiella. Two approaches which have been successful against similar pests in other crops offer considerable potential. These include the use of genes encoding insecticidal BT proteins from the bacterium *Bacillus thuringiensis* and proteinase inhibitor genes from other plants. The activities of specific proteins are currently being screened against the targeted pests by other DSIR divisions, after which the appropriate genes will be cloned for subsequent transfer to peas.

Virus resistance: It is now well established that genetic engineering technology can be used to genetically manipulate plants for resistance to viral diseases. This involves the integration and expression in plant genomes of DNA sequences corresponding to specific genetic components from plant viruses, particularly the viral coat protein gene(s). Although the mechanisms of this viral protection are not completely understood, a number of crops have been protected against infection by tobacco mosaic virus, alfalfa mosaic virus, cucumber mosaic virus, soybean mosaic virus and potato virus Y. For grain legumes our initial interest is in developing resistance to pea seed-borne mosaic virus. This virus causes important disease in the world trade of pea and lentil seed, affecting yield, quality, and appearance. Because of its seed-borne nature, it has been transmitted internationally.

To produce virus-resistant peas, we are making use of the "coat protein mediated virus resistance" phenomenon first reported by Powell-Abel *et al.* (1986) for tobacco mosaic virus. We have cloned a portion of the viral genome containing the pea seed- borne mosaic virus coat protein gene and determined its nucleotide sequence (Timmerman *et al.*, 1990). The pea seed-borne mosaic virus coat protein is encoded at the carboxy-terminal end of a long polypeptide, as is the case with other potyviruses. So that this gene can be expressed in pea plants without other viral gene products, it was necessary to modify the gene extensively. An ATG codon in an optimal translation initiation context was added to the start of the coat protein gene. This was done using the polymerase chain reaction (Saiki *et al.*, 1988) and specially designed oligonucleotide primers. The modified coding region was then inserted between a cauliflower mosaic virus 35S promoter sequence and a 3' poly (A) addition sequence in a plasmid expression vector which also carried a gene for expression of kanamycin resistance in plant tissues. These two linked genes have been inserted into disarmed T-DNA, and mated into the two species of Agrobacterium discussed above. Experiments are currently underway to transform two crops, peas and potatoes, with this coat protein gene. Expression of the modified coat protein gene will first be characterised in transgenic potato plants using immunodot or Western blotting techniques (Towbin *et al.*, 1979).

Transgenic pea plants expressing the gene will be tested for their susceptibility to infection by this virus and other related viruses under controlled glasshouse and field trial conditions. The development of virus-resistant germplasm using this technology will result in the production of clean pea seed for international trade.

REFERENCES

Conner, A.J., Grant, J.E., Williams, M.K., Abernethy, D.J., Tynan, J.L., Dommisse, E.M., Pither, M.D., Timmerman, G.M., Cooper, P.A., Frew, T.J., Terada, M. & Christey, M.C. 1990. Genetic engineering of vegetables. *Horticulture in New Zealand 1*, 3-7.

Gapen, S., Köhler, F., Gerdemann, M. & Schieder, O. 1987. Cultivar dependence of transformation rates in moth bean after co-cultivation of protoplasts with *Agrobacterium tumefaciens*. *Theoretical Applied Genetics 75*, 207-210.

Gasser, C.S. & Fraley, R.T. 1989. Genetically engineering plants for crop improvement. *Science 244*, 1293-1299.

Hinchee, M.A.W., Connor-Ward, D.V., Newell, C.A., McDonnell, R.E., Sato, S.J., Gasser, C.S., Fischoff, D.A., Re, D.B., Fraley, R.T. & Horsch, R.B. 1988. Production of transgenic soybean plants using Agrobacterium-mediated DNA transfer. *Bio/Technology 6*, 915-922.

Hussey, G. & Gunn, H.V. 1984. Plant production in pea (*Pisum sativum* L. cvs. Puget and Upton) from long-term callus with superficial meristems. *Plant Science Letters 37*, 143-148.

Janssen, B-J. & Gardner, R.C. 1990. Localised transient expression of GUS in leaf discs following cocultivation with Agrobacterium. *Plant Molecular Biology 14*, 61-72.

Jensen, J.S., Marcker. K.A., Otter, L. & Schell, J. 1986. Nodule specific expression of a chimeric soybean leghaemoglobin gene in transgenic *Lotus corniculatus*. *Nature, London 321*, 669-674.

Kathen, A. de & Jacobsen, H-J. 1990. Transformation of pea using binary and coinegrate vectors of *Agrobacterium tumefaciens* strains. *Journal of Cellular Biochemistry 14E*, 282.

Kohler, F., Golz, C., Eapen, S., Kohn, H. & Schieder, O. 1987. Stable transformation of moth bean *Vigna aconitifolia* via direct gene transfer. *Plant Cell Reports 6,* 313-317.

Kysely, W., Myers, J.R., Lazzeri, P.A., Collins, G.B. & Jacobsen, H-J. 1987. Plant regeneration via somatic embryogenesis in pea (*Pisum sativum* L.). *Plant Cell Reports 6,* 305-308.

Lehminger-Mertens, R. & Jacobsen, H-J. 1989. Plant regeneration from pea protoplasts via somatic embryogenesis. *Plant Cell Reports 8,* 379-382.

Manners, J.M. & Way, H. 1989. Efficient transformation with regeneration of the tropical pasture legume *Stylosanthes humilis* using *Agrobacterium rhizogenes* and a Ti plasmid binary vector system. *Plant Cell Reports 8,* 341-345.

McCabe, D.E., Swain, W.F., Martinell, B.J. & Christou, P. 1988. Stable transformation of soybean (*Glycine max*) by particle acceleration. *Bio/Technology 6,* 923-926.

Mroginski, L.A. & Kartha, K.K. 1981. Regeneration of pea (*Pisum sativum* L. cv. Century) plants by in vitro culture of immature leaflets. *Plant Cell Reports 1,* 64-66.

Mroginski, L.A. & Kartha, K.K. 1984. Tissue culture of legumes for crop improvement. *Plant Breeding Review 2,* 215-264.

Natali, L. & Cavallini, A. 1987. Regeneration of pea (*Pisum sativum* L.) plantlets by in vitro culture of immature embryos. *Plant Breeding 99,* 172-176.

Powell-Abel, P., Nelson, R.S., De, B., Hoffman, N., Rogers, S.G., Fraley, R.T. & Beachy, R.N. 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science 232,* 738-743.

Puonti-Kaerlas, J. & Erikkson, T. 1988. Improved protoplast culture and regeneration of shoots in pea (*Pisum sativum* L.). *Plant Cell Reports 7,* 242-245.

Puonti-Kaerlas, J., Stabel, P. & Eriksson, T. 1989. Transformation of pea (*Pisum sativum* L.) by *Agrobacterium tumefaciens. Plant Cell Reports 8,* 321-324.

Rech, E.L., Golds, T.J., Husnain, T., Vainstein, M.H., Jones, B., Hammatt, N., Mulligan, B.J. & Davey, M.R. 1989. Expression of a chimaeric kanamycin resistance gene introduced into the wild soybean *Glycine canescens* using a cointegrate Ri plasmid vector. *Plant Cell Reports 8,* 33-36.

Rubluo, A., Kartha, K.K., Mroginski, L.A. & Dyck, J. 1984. Plant regeneration from pea leaflets cultured in vitro and genetic stability of regenerants. *Journal of Plant Physiology 117,* 119-130.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, Gl.T., Mullis, K.B. & Erlich, H.A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science 239,* 487-491.

Sukhapinda, K., Spivey, R., Simpson, R.B. & Shahin, E.A. 1987. Ri plasmid as a helper for introducing vector T-DNA into alfalfa plants. *Plant Molecular Biology 8,* 209-216.

Timmerman, G.M., Calder, V.L. & Bolger, L.E.A. 1990. Nucleotide sequence of the coat protein gene of pea seed-borne mosaic potyvirus. *Journal of General Virology.* (In press).

Towbin, H., Staehelin, T. & Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Sciences, USA 76,* 4350-4354.