Half-Embryo Cocultivation Technique for Estimating the Susceptibility of Pea (*Pisum sativum* L.) and Lentil (*Lens culinaris* Medik.) Cultivars to *Agrobacterium tumefaciens*

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Abstract

Longitudinally sliced embryonic axes from pea and lentil mature seeds cocultivated with *A. tumefaciens* carrying a *gus* reporter gene in its T-DNA provided a convenient means to evaluate the efficiency of gene transfer to tissues in different cultivars and cocultivation conditions. Use of this technique demonstrated wide variation in susceptibility to *Agrobacterium* among several pea and lentil commercial genotypes.

Index Entries: *Agrobacterium tumefaciens*; gene transfer; lentils; peas; susceptibility.

1. Introduction

Pea and lentil are among the most important crops used for human and animal nutrition in many areas of the world. Thus, it is not surprising that there is considerable interest in the genetic improvement of these legumes, both by conventional breeding and by genetic engineering. *Agrobacterium*-mediated genetic transformation of pea has been reported in a few articles, with varying degrees of success *(1–6)*. On the contrary, there are no reports describing the stable transformation of lentil, although *Agrobacterium*-mediated gene transfer into tissues has been shown *(7)*.

Thus, the genetic manipulation via recombinant DNA technology cannot be considered routine in these two leguminous plants. Further, it has been shown that different pea genotypes radically differ in their susceptibility to *Agrobacterium* *(8,9)*. Therefore, it is important to determine gene transfer efficiency before starting the lengthy *Agrobacterium*-mediated transformation process with hitherto untested pea and lentil cultivars. The studies by Hobbs et al. *(8)* and Lulsdorf et al. *(9)* on susceptibility to *Agrobacterium tumefaciens* were either based on crown-gall induction by fully virulent strains or in vitro selection experiments with callus tissue involving several time-consuming steps. In this article, we describe the use of half-embryonic axes cocultivated with *Agrobacterium* carrying a *gus* reporter gene to evaluate quickly the response of seven pea and four lentil cultivars to tissue transformation attempts.

2. Materials and Methods

Tested pea cultivars included Paloma, Umatilla, Puget, Almota, Code 90, Columbia and Alaska 81, whereas lentil cultivars were Eston, Palouse, Brewer, and Chilean. *A. tumefaciens* strains used were C58 and EHA105, both known to transfer their T-DNA to pea tissues *(8,9)*. Both strains carried the chimeric *gus* gene p35SGUSINT between the left and right T-DNA borders of their binary component, as described in *(10)*. The supplier of VIR functions was pGV2260 in the case of C58 and disarmed pEHA105 in the case of EHA105. Bacteria were grown overnight in liquid LB medium at pH 7.2 or 5.6, with or without 20 μ*M* acetosyringone.

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Pea, lentil, and cowpea seeds (the latter used as positive control) were surface-sterilized by three sequential treatments: immersion and stirring for 10 min in 0.525% sodium hypochlorite with a few drops of Tween-20, 1-min immersion and stirring in 75% ethanol, and three rinses in sterile double-deionized water at room temperature. Cotyledons were excised from imbibed seeds and isolated embryonic axes sliced longitudinally with a sterile scalpel as previously described in **ref. *11***. Embryo halves were then placed in drops of overnight *A. tumefaciens* cultures and soaked for 2 min. Treated embryos were transferred to plates containing hormone-free MS medium at pH 5.8 and cocultivated for 48 h at 26°C. Embryos were washed with liquid Murashige-Skoog (MS) medium supplemented with 500 mg/L Claforan™ and plated on solid MS medium containing the same dose of the antibiotic, with or without 200 μ*M* acetosyringone. Plants were incubated at 23°C under fluorescent light at 80 $\mu E/m^2$ s with a 16-h period.

After 1–4 wk of growth, whole plants were stained for GUS activity with the chromogenic substrate X-glu, as in ***(12)***. Negative controls consisted of identically treated half embryos except that incubation and cocultivation with *Agrobacterium* was omitted. No staining for GUS was ever detected in these controls. Experiments were done in duplicate or triplicate with batches of 6 or 12 embryos. Plants showing blue sectors were scored as positive and the average number of sectors per positive plant were recorded. Positive controls consisted of cocultivated cowpea (*Vigna unguiculata* Walp) embryos previously shown to produce plants consistently displaying transgenic sectors in their stems and leaves at an average frequency higher than 50% (***11***, and our own unpublished data).

3. Results

Figure 1 demonstrates the various steps involved in our technique applied here to pea. An imbibed seed with one cotyledon removed and embryonic axis ready to be severed from the remaining cotyledon is shown in **Fig. 1A**. Shown next are longitudinally sliced embryos at the stage of early development as used for cocultivation (**Fig. 1B**). These half-embryos can be grown in vitro after cocultivation with *A. tumefaciens* (**Fig. 1C**) and stained for GUS activity (**Fig. 1D**). Lentil half-embryos responded very similarly. Cells staining for GUS were observed in both stems and leaflets, whereas no transgenic sectors were found in roots.

Table 1 shows the proportion of plants staining positive for GUS in six different pea genotypes. It can be seen that genotypes vary remarkably in their response to *Agrobacterium*. Umatilla produced on the average over 50% plants displaying transgenic sectors, whereas Columbia and Alaska 81 produced none. Four lentil cultivars also showed large differences in their response to *A. tumefaciens* (**Table 1**). In the latter case, however, no variety was found to be completely insensitive.

Factors known to induce the *vir* regulon of *Agrobacterium*, such as acid pH of the growth medium and acetosyringone, have been shown to increase transformation efficiency, mostly in Solanaceae. **Figure 2A** shows that cocultivating pea half-embryos of the Puget variety in the presence of acetosyringone tripled the number of GUS-positive plantlets. Also, growing *Agrobacterium* cells at pH 5.6 with or without acetosyringone prior to the cocultivation step did significantly enhance this cultivar's susceptibility. This is incontrast with observations made with Eston lentil (**Fig. 2B**) where all treatments, and in particular cocultivation in the presence of acetosyringone, had a deleterious effect on susceptibility to *Agrobacterium* as compared to controls simply cocultivated on MS medium with bacteria grown at pH 7.2. A negative effect of acetosyringone on transformation was also observed by De Kathen and Jacobsen ***(2)*** with Madria pea.

Further, our results also show that plants originating from cocultivated half-embryos should be examined for GUS activity after at least 2 wk of growth following the cocultivation step. Indeed, **Table 2** indicates that in pea and lentil, the number of GUS-positive sectors detected 1 wk after cocultivation is significantly higher than after 2 or 4 wk of growth. In all likelihood, many sectors

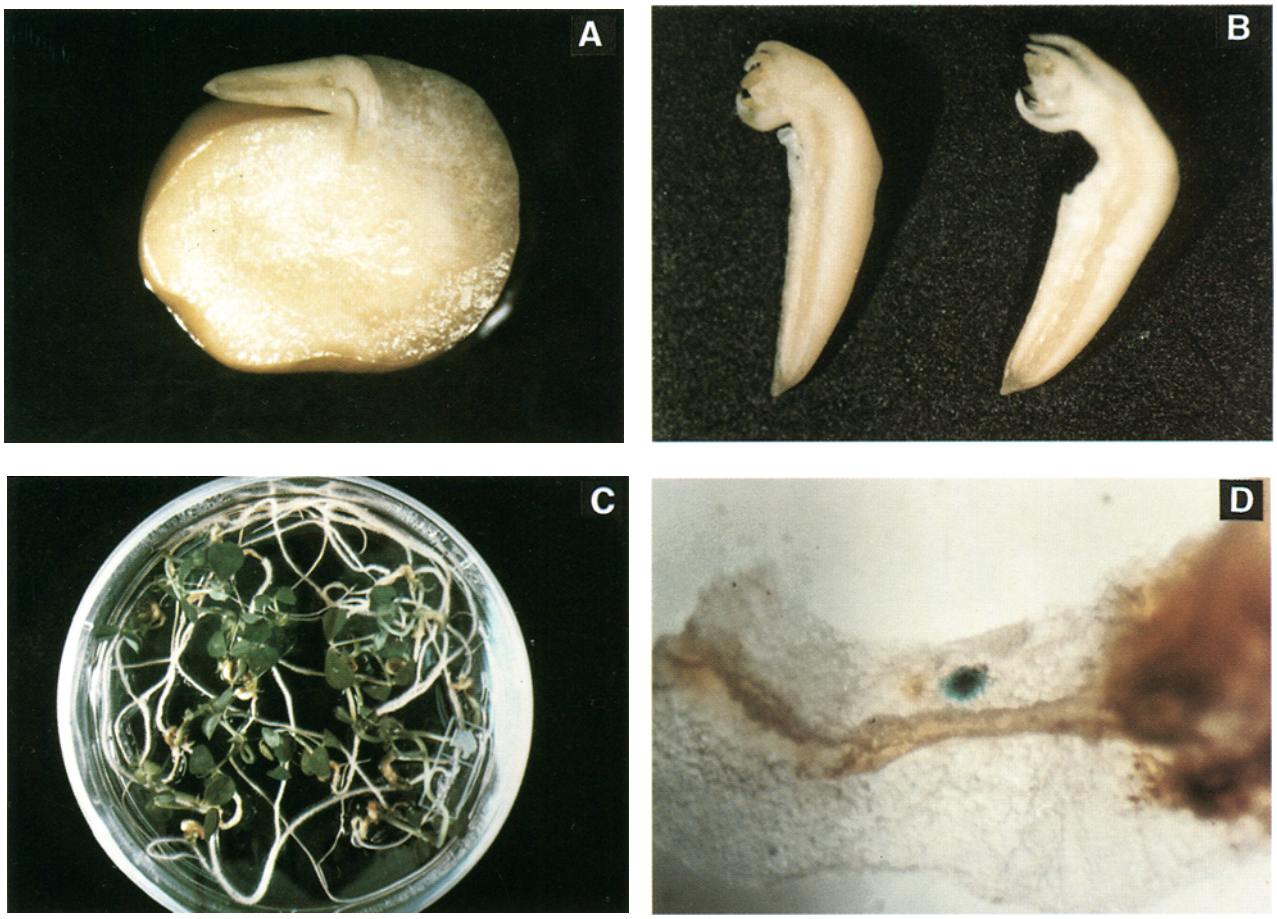

Fig. 1. Representation of four of the steps involved in our cocultivation technique. **(A)** Sliced imbibed pea seed showing a half-embryonic axis still attached to one of the cotyledons; **(B)** Developing half-embryos ready for the *Agrobacterium* cocultivation step; **(C)** Plantlets growing on MS medium after cocultivation; **(D)** GUS-positive sector in plantlet stem.

Table 1
Response of Pea and Lentil Cultivars to Cocultivation with *A. tumefaciens* C58 (pGV2260/p35SGUSINT)[a]

Pea cultivar		Lentil cultivar	
Umatilla	54.9 ± 11.4 (3.3)	Eston	41.2 ± 7.8 (1.8)
Paloma	43.3 ± 11.3 (6.4)	Palouse	28.4 ± 5.0 (2.3)
Almota	21.3 ± 9.4 (2.0)	Chilean	24.6 ± 12.0 (5.6)
Code 90	5.7 ± 2.1 (1.0)	Brewer	16.3 ± 10.6 (0.9)
Columbia	0		
Alaska 81	0		

[a]Figures represent the percent GUS-positive plants with standard deviations. Numbers in parentheses represent the average number of transgenic sectors per positive plant. Data were collected 4 wk after cocultivation and are the result of three independent experiments.

seen after the first week were transiently transformed and disappeared later on. Similar observations were made with *Arabidopsis* leaf disks cocultivated with *Agrobacterium* ***(10)***.

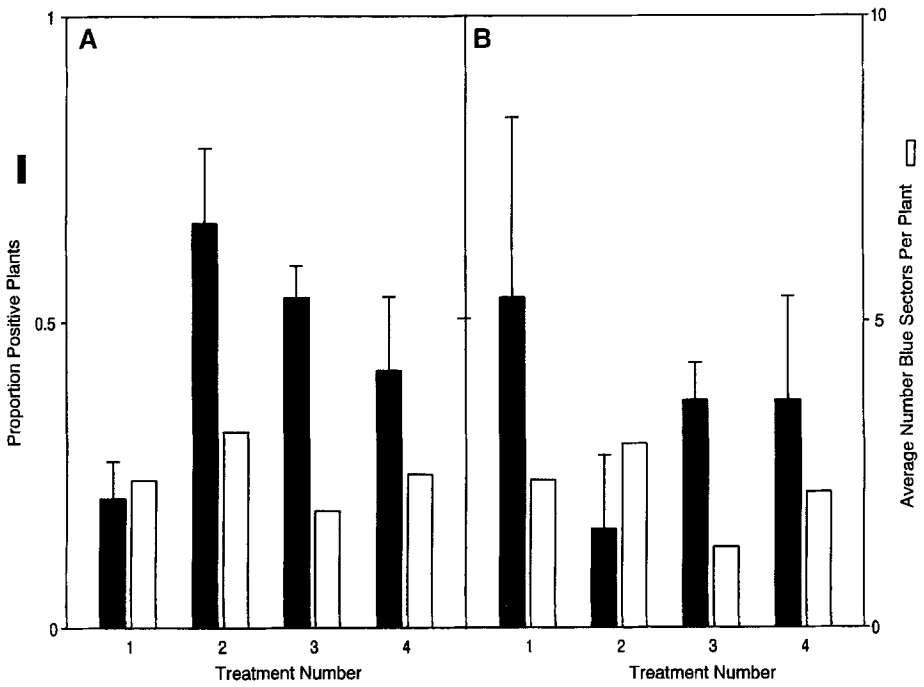

Fig. 2. Effects of different parameters on the susceptibility to *A. tumefaciens* EHA105 (p35SGUSINT) of **(A)** Puget pea and **(B)** Eston lentil. Data were scored after 2 wk and are the result of two independent experiments. Closed bars represent the proportion of GUS-positive plants with standard deviations and open bars represent the average number of transgenic sectors per positive plant. 1, Bacteria grown in LB medium at pH 7.2 and cocultivation in standard MS salts; 2, Bacteria grown in LB medium at pH 7.2 and cocultivation in MS medium with 200 μ*M* acetosyringone; 3, Bacteria grown in LB medium at pH 5.6 and cocultivation in standard MS medium; 4, Bacteria grown in LB medium supplemented with 20 μ*M* acetosyringone at pH 5.6 and cocultivation in standard MS medium.

Table 2
Variation of the Average Number of Transgenic Sectors per GUS-positive Plant as a Function of Time after Cocultivation[a]

Genotype	Week 1	Week 2	Week 4
Paloma (pea)	14.2	3.7	5.0
Eston (lentil)	3.4	1.3	1.5

[a]Batches of 6 (pea) and 12 (lentil) half-embryos were cocultivated with *A. tumefaciens* EHA105 (p35SGUSINT), allowed to grow for 1, 2, and 4 wk and stained for GUS activity.

Finally, we compared response to *A. tumefaciens* in pea and lentil after cocultivation with strains C58 and EHA105, the latter being known as "hypervirulent," at least in Solanaceae. Eston and Palouse lentil essentially showed no increased susceptibility when cocultivated with EHA105 (not shown). In contrast, Paloma pea cultivar displayed enhanced response when cocultivated with EHA105: in an experiment done in triplicate with batches of six half-embryos, 50% of the seedlings stained positive for GUS, as opposed to 25% when cocultivated with C58.

4. Conclusions

In conclusion, the technique described here allowed us to evaluate easily and reasonably

quickly the performance of pea and lentil genotypes in cocultivation experiments with *Agrobacterium*. Given the wide variety of responses observed with different genotypes, cocultivation conditions and bacterial strains, randomly selected parameters would be unlikely to lead to successful stable transformation of any pea or lentil cultivar. Data obtained with our technique can help choose the most responsive cultivars, *A. tumefaciens* strains, and cocultivation conditions for genetic-engineering purposes within a reasonable amount of time. We also believe that our protocol is applicable to other plant species where viable embryos can be isolated from mature seeds and sliced to provide access for the bacterial cells.

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References

1. Davies, D. R., Hamilton, J., and Mullineaux, P. (1993) Transformation of peas. *Plant Cell Rep.* **12,** 180–183.
2. De Kathen, A. and Jacobsen, H.-J. (1990) *Agrobacterium tumefaciens*-mediated transformation of *Pisum sativum* L. using binary and cointegrate vectors. *Plant Cell Rep.* **9,** 276–279.
3. Puonti-Karleas, J., Eriksson, T., and Engstrom, P. (1990) Production of transgenic pea (*Pisum sativum* L.) plants by *Agrobacterium tumefaciens*-mediated gene transfer. *Theor. Appl. Genet.* **80,** 246–252.
4. Schroeder, H. E., Schotz, A. H., Wardley-Richardson, T., Spencer, D., and Higgins T. J. V. (1993) Transformation and regeneration of two cultivars of pea (*Pisum sativum* L.) *Plant Physiol.* **101,** 751–757.
5. Grant, J. E., Cooper, P. A. McAra, A. E. and Frew, T. J. (1995) Transformation of peas (*Pisum sativum* L.) using immature cotyledons. *Plant Cell Rep.* **15,** 254–258.
6. Schroeder, H. E., Gollasch, S., Moore, A., Tabe, L.M., Graig, S., Hardie, D. C., Chrispeels, M. J., Spencer, D. and Higgins, T. J. V. (1995) Bean alpha-amylase inhibitor confers resistance to the pea weevil (*Bruchus pisorum*) in transgenic peas (*Pisum sativum* L.). *Plant Physiol.* **107,** 1233–1239
7. Warkentin, T. D. and McHughen, A. (1992) *Agrobacterium tumefaciens*-mediated beta-glucuronidase (GUS) gene expression in lentil (*Lens culinaris* Medik.) tissues. *Plant Cell Rep.* **11,** 274–278
8. Hobbs, S. L. A., Jackson, J. A., and Mahon, J. D. (1989) Specificity of strain and genotype in the susceptibility of pea to *Agrobacterium tumefaciens*. *Plant Cell Rep.* **8,** 274–277
9. Lulsdorf, M. M., Rempel, H., Jackson, J. A., Baliski, D. S., and Hobbs, S. L. A. (1991) Optimizing the production of transformed pea (*Pisum sativum* L.) callus using disarmed *Agrobacterium tumefaciens* strains. *Plant Cell Rep.* **9,** 479–483
10. Vancanneyt, G., Schmidt, R., O'Connor-Sanchez, A., Willmitzer, R., and Rocha-Soza, M. (1990) Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. *Mol. Gen. Genet.* **220,** 245–250
11. Penza, R., Lurquin, P. F., and Filippone, E. (1991) Gene transfer by cocultivation of mature embryos with *Agrobacterium tumefaciens:* Application to cowpea (*Vigna unguiculata* Walp). *J. Plant Physiol.* **138,** 39–43
12. Jefferson, R. A. (1987) Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol. Biol. Rep.* **5,** 385–387