

SHORT COMMUNICATION

# T-DNA transfer in meristematic cells of maize provided with intracellular *Agrobacterium*

Jesús Escudero<sup>1,\*†</sup>, Gunther Neuhaus<sup>2</sup>,  
Michael Schläppi<sup>1,‡</sup> and Barbara Hohn<sup>1</sup>

<sup>1</sup>Friedrich Miescher-Institut, Postfach 2543, CH-4002  
Basel, Switzerland, and

<sup>2</sup>Institute for Biology II, Department of Cell Biology,  
Schaenzlestrasse 1, D 79104 Freiburg, Germany

## Summary

***Agrobacterium* has been established as a tool for gene delivery to most dicotyledonous plant species. However, it is not generally efficient in monocotyledonous plant species, especially not in Graminae. In maize, *Agrobacterium*-mediated DNA transfer has been detected but early developmental stages in the plant proved incompetent as recipients. This research tests whether the lack of competence in young immature embryos of maize could be overcome by providing *Agrobacterium* in the interior of the plant cell. A microinjection technique was used to target single meristematic cells and prove competence to *Agrobacterium*. This response is dependent on the maize plant genotype.**

## Introduction

*Agrobacterium tumefaciens* is a gram-negative soil bacterium which was originally isolated from crown gall disease in dicotyledonous plants. Bacteria bind to plant cells and transfer to them a segment of DNA (the so-called T-DNA) from their large tumour-inducing plasmid (the so-called Ti plasmid; for review see Binns and Howitz, 1994; Hooykaas and Schilperoort, 1992; Winans, 1992; Zupan and Zambryski, 1995). This transkingdom gene transfer is initiated in the bacteria by the activity of Ti plasmid-encoded virulence (*vir*) genes, as a response to compounds released from plant cells (Cangelosi *et al.*, 1990; Stachel *et al.*, 1986). A T-complex, consisting of DNA and Vir proteins, is exported as single-stranded DNA to the plant cell (Tinland *et al.*, 1994), where it is most probably piloted to the nucleus by the VirD2 protein (Koukolíková-

Nicola *et al.*, 1993; Rossi *et al.*, 1993; Shurvinton *et al.*, 1992). Eventually, the T-DNA becomes inserted into the plant genome (Gheysen *et al.*, 1991; Mayerhofer *et al.*, 1991; see Tinland and Hohn, 1995, for a recent review). The natural T-DNA contains eukaryotic genes which are functionally inactive in the bacteria and which upon expression in the plant cell produce the characteristic cell proliferation of tumours.

Invasion by *Agrobacterium* produces neoplasia in most dicotyledonous but in only a few monocotyledonous species, most of which were therefore originally considered as non-hosts (De Cleene and De Ley, 1976). In the last 10 years, however, *Agrobacterium*-mediated transformation has been reported in several monocot plants including narcissus, asparagus, yam and recently in rice (Bytebier *et al.*, 1987; Chan *et al.*, 1993; Hiei *et al.*, 1994; Hooykaas-van Slogteren *et al.*, 1984; Schäfer *et al.*, 1987). In maize, the first strong evidence for T-DNA transfer was established using maize streak virus (MSV) as a reporter for successful infection, a technique baptized as agroinfection (Grimsley *et al.*, 1987). However, no routine system has been established yet for stable transformation of maize. The reason for this lack of success is presently unclear.

Studies on the competence of maize for T-DNA transfer using agroinfection showed that the frequency was highest when bacteria were injected close to the shoot meristem of 3-day-old seedlings (Grimsley *et al.*, 1988). Further analysis in immature embryos revealed a developmentally regulated susceptibility to agroinfection. The youngest embryo age analysed (about 10 days after pollination, DAP) was found non-competent (Schläppi and Hohn, 1992). The inoculated tissue present at this developmental stage is mainly composed of undifferentiated meristematic cells. Hence, the lack of competence in the early embryos could imply that *Agrobacterium* requires plant-derived factors which might be missing in meristematic cells. On the other hand, it could not be excluded in this case that availability of compounds activating bacterial virulence *in situ* was not sufficient for T-DNA transfer (Schläppi and Hohn, 1992).

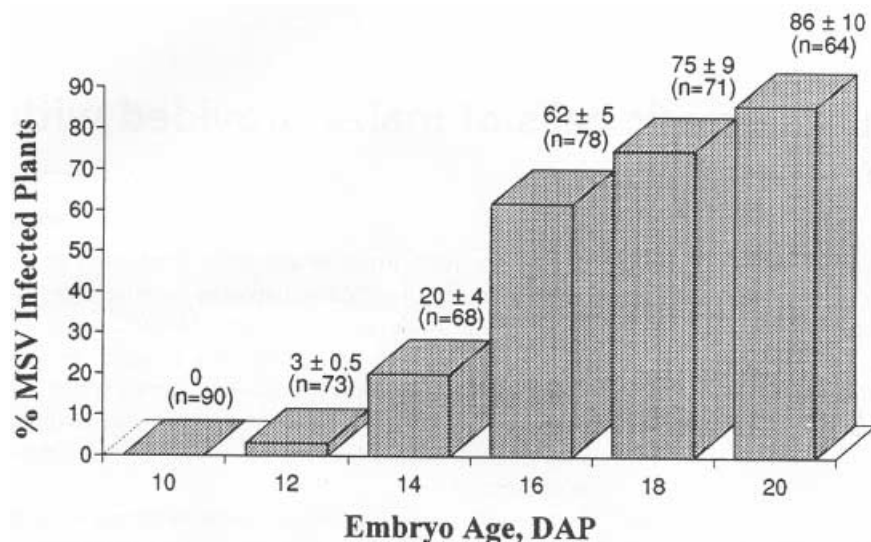
It has been shown recently that under conditions in which *vir*-genes are activated, T-DNA transfer from *Agrobacterium* can occur efficiently from within microinjected tobacco cells (Escudero *et al.*, 1995). Thus, transfer of T-DNA was demonstrated to happen intracellularly in the plant. In order to study the responsiveness of cells of young immature embryos to T-DNA transfer, we tested the competence of meristematic maize cells to intracellularly

Received 20 September 1995; revised 18 April 1996; accepted 13 May 1996.

\*For correspondence (fax +31 71 5 27 49 99).

†Present address: Institute of Molecular Plant Sciences, Clusius Laboratory, Leiden University, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands.

‡Present address: Marquette University, Department of Biology, Milwaukee WI 53233, USA.



**Figure 1.** Developmental response to T-DNA transfer of A188 immature embryos. Columns represent average values obtained when the embryos were vacuum infiltrated with a pre-induced C58C1 (pTiC58, pEAP37) bacterial suspension. Agroinfection frequencies are presented on the ordinate as the fraction of shoots showing viral symptoms 1 month after inoculation per total infiltrated embryos scored (% MSV infected plants). The age of immature embryos (Embryo age, DAP) is shown on the abscissa. Mean values, standard errors and number of plants assessed in three independent experiments are given above bars.

provided *Agrobacterium* cells. Microinjection of *Agrobacterium* was expected to circumvent the attachment step (Escudero *et al.*, 1995). If indeed in young embryos of maize a plant-cell receptor for bacterial attachment is missing, as has been suggested (Schläppi and Hohn, 1992), microinjection of agrobacteria may overcome this block and result in T-DNA transfer.

Our results suggest that single cells of the apical meristem of maize are susceptible to agroinfection by microinjected pre-induced bacteria. In addition we found an important role of the plant genotype in the competence for T-DNA transfer. Implications for *Agrobacterium*-mediated gene transfer to cereals and the use of the microinjection technique are discussed.

## Results

### *Susceptibility of meristematic maize cells to intracellular Agrobacterium*

Immature embryos of the A188 genotype, ranging in age from 10 to 20 days after pollination (DAP), were tested in several series for their competence for T-DNA transfer. Pre-induced bacteria (i.e. cultured in medium with the *vir*-gene inducer acetosyringone (AS)) were provided to the embryos using vacuum infiltration. Agroinfection was chosen as a sensitive assay for T-DNA transfer to the plant-cell nucleus; a duplicated (pEAP37) or partially duplicated (pLE1) MSV genome was used as T-DNA (Grimsley *et al.*, 1987; Schläppi and Hohn, 1992). Figure 1 summarizes the results: at 10 DAP, the earliest age of embryos tested, no agroinfection was found. At later ages, 12–20 DAP, increasing agroinfection rates were obtained. This pattern of response to T-DNA transfer depending on the age of the embryo shows that *vir*-gene induction of infectious

agrobacteria is not likely the limiting factor resulting in incompetence of the 10-DAP-old embryos.

Furthermore, the effect of different bacterial infection procedures on agroinfection was evaluated assaying the influence of wounding and AS treatment on the competence of 10 to 20-DAP-old immature embryos (Table 1). Although 10 DAP embryos remained incompetent under all five infection procedures (Table 1, columns a–e), either wounding or induction of virulence by AS were unequivocally required for agroinfection in older embryos (12–20 DAP). This indicated that albeit not sufficient, *vir*-gene activation is important for agroinfection independently of the nature of the inducer (either AS added in culture or natural inducers provided by the wounded maize tissue).

The results above suggested that agroinfection values represent a dependence of T-DNA transfer on plant development. To test the hypothesis that 10 DAP embryos are incompetent for agroinfection because of lack of bacterial attachment, we delivered whole bacteria within single cells of the L2 layer at the apical meristem of 10 DAP immature maize embryos. In several series of experiments in which bacterial strains carrying a nopaline wild-type Ti plasmid were microinjected into the maize line A188, we observed consistent expression of T-DNA in the plant, resulting in viral symptoms. Efficiency of infection varied with the bacterial strain used and correlated with conditions that are known to induce the virulence genes in agrobacteria, that is, culture in AS-containing medium at low pH (Table 2, lines 1 and 4), in agreement with studies done in tobacco (Escudero *et al.*, 1995). Conditions yielding agroinfected plants indicated that the mechanism responsible for this infection resembles typical T-DNA transfer, namely the need for: (i) *vir*-genes on the Ti plasmid (Table 2, line 3); (ii) bacterial preculture under inducing conditions (Table 2, line 2); (iii) a functional *virB* operon, known to be

**Table 1.** Percentage of MSV infected plants obtained from different infection procedures with *Agrobacterium* in immature embryos of the A188 maize line

Procedure	(a)	(b)	(c)	(d)	(e)
Apex wounding	-	+	-	+	+
Pre-induction of bacteria	-	-	+	+	-
Co-induction of bacteria	-	-	-	-	+
Embryo age (DAP)					
10	0	0	0	0	0
12	0	4 ± 0.6	2 ± 0.5	4 ± 0.6	5 ± 0.6
14	0	27 ± 4	18 ± 3	30 ± 4	38 ± 4
16	0	71 ± 8	67 ± 8	83 ± 10	86 ± 10
18	0	88 ± 10	83 ± 10	87 ± 10	90 ± 10
20	0	93 ± 10	89 ± 10	95 ± 10	95 ± 10
(Relative values)	(0)	(90)	(82)	(95)	(100)

The shoot apex in the embryo was wounded with a fine capillary. Acetosyringone was included in the 12 h bacterial culture (pre-induction of bacteria) or added to the bacterial suspension just before embryo infection (co-induction of bacteria). Mean agroinfection frequencies and standard errors are given from three independent tests where 50–60 plants were assessed for a particular infection procedure and embryo age. Relative values refer to frequencies obtained with infection procedure 'e'. Results correspond to C58C1 (pTiC58, pEAP37) bacterial strain but identical frequencies were obtained with C58C1 (pTiC58, pLE1).

**Table 2.** Agroinfection frequencies obtained from 10 DAP meristematic cells of immature embryos of the A188 maize line injected with *Agrobacterium*

Line	Injected material <sup>a</sup>	Number of injected plants <sup>b</sup>	Number of infected plants
1	Induced C58C1 (pTiC58, pEAP37)	117	28
2	Uninduced C58C1 (pTiC58, pEAP37)	95	0
3	Induced C58C1 (pEAP37)	89	0
4	Induced LBA4301 (pJK270, pEAP37)	102	5
5	Induced LBA4301 (pJK210, pEAP37)	98	0
6	pEAP37 DNA	80	0

<sup>a</sup>Bacteria were grown either under inducing or non-inducing conditions (see Experimental procedures) before injection into maize cells. C58C1 (pTiC58) (Watson *et al.*, 1975) and LBA 4301 (pJK 270) have a wild-type *vir* region whereas LBA 4301 (pJK210) (Rogowsky *et al.*, 1987) contains a mutated *virB* gene.

<sup>b</sup>Developing embryos were recovered efficiently after microinjection (approximately 98% survival) and plantlets were scored for viral infection 1 month after injection. Plasmid pEAP37 carrying an MSV dimer in the T-DNA was injected as pure DNA (approximately 10<sup>3</sup> molecules per maize cell). Results summarize four series of experiments.

required for T-DNA transfer (Table 2, compare lines 4 and 5). In contrast, injection of approximately 10<sup>3</sup> copies per cell of the binary plasmid as DNA did not result in agroinfection (Table 2, line 6).

These results demonstrate that T-DNA transfer to maize nuclei from microinjected agrobacterial cells had occurred. This represents evidence that undifferentiated cells in the

**Table 3.** T-DNA transfer frequencies obtained with different maize genotypes.

Maize line <sup>a</sup>	Agroinfection Bacteria microinjected into 10 DAP embryo meristems (%)	GUS activity Bacteria infiltrated to 16 DAP embryos (%)
A188	23 ± 3	60 ± 5
W23	0	3 ± 0.7
A188×W23	0	7 ± 0.6
88025-4A	0	8 ± 1.6

Wild-type C58C1 *Agrobacterium* strains cultured under inducing conditions, either harbouring MSV (pLE1) or GUS (pBG5) as T-DNA, were used. Agroinfection frequencies were calculated as the fraction of shoots showing MSV symptoms per total injected embryos, scored 1 month after inoculation. Frequencies of GUS activity were calculated as the fraction of shoots showing GUS expression (histochemical assay) per total infiltrated shoots.

<sup>a</sup>Comparable developmental stages of the immature embryos listed maize lines were isolated (length range 1–1.5 mm in microinjection assays; 3.5–4 mm, then germinated for 3 days, in infiltration assays). The results summarize three series of experiments with 20–25 embryos per genotype analysed in each case.

apical meristem of maize are sensitive to virulence-induced agrobacteria delivered within the plant cell.

#### *Genotype-specific response to intracellular Agrobacterium in maize*

The competence of 10 DAP embryos to injection-mediated agroinfection (see Table 2) seems to be a characteristic of certain maize genotypes such as line A188. The two other assayed maize genotypes W23 and 88025-4A did not result in detectable agroinfection levels (Table 3). Also in vacuum

infiltration experiments with agrobacteria carrying a *uid A* (GUS) gene as T-DNA, A188 proved to be the only genotype in which T-DNA transfer could be reproducibly observed (Table 3). Hence, it seems that the A188 line is especially competent for bacteria provided intra- and extracellularly.

In order to analyse the genetic component(s) of competence for T-DNA transfer we crossed the A188 and W23 maize lines and assayed immature embryos of the F<sub>1</sub> generation using MSV or GUS as transfer-marker genes. Both tests revealed a poor T-DNA transfer frequency (Table 3). Apparently, the 'competent genotype' of A188 behaves recessive to the 'non-competent genotype' of W23; this effect was independent of maternal inheritance since reciprocal crosses (i.e. ♀ A188 × ♂ W23, and ♀ W23 × ♂ A188) resulted in a similarly low T-DNA transfer efficiency (data not shown).

### Discussion

The results of our study indicate that the maize-*Agrobacterium* interaction is strongly influenced by two characteristics of the plant, an undefined genetic component and the particular stage of development. Plant-cell injection with pre-induced agrobacteria results in a positive response of otherwise silent 10 DAP embryos. Competence of this early developmental stage of embryos for *Agrobacterium* using the technique applied here can then be regarded as very efficient.

To explain the difference between results from microinjection and infiltration experiments, that is, intra- versus intercellular application of *Agrobacterium*, we propose that totipotent cells in the apical meristem might lack a key surface component (receptor) for recognition by agrobacteria. In the developing embryo of A188 maize this proposed receptor must become gradually accessible to the bacteria, possibly reaching its maximum number or activity at ages around 20 DAP (see Figure 1). This correlation between cell differentiation and competence for agrobacteria in the maize embryo suggests that problems currently envisaged in obtaining stable transformation (i.e. transgenic progeny) may in part be due to an irreversible arrest of susceptible plant cells at particular differentiated developmental stages. Thus, amplification of those transformation events (cell lineages) is prevented. Previous studies in which T-DNA-expressing cells could be visualized in various maize tissues (Shen *et al.*, 1993) showed no single pattern that could be attributed to genomic integrated T-DNA. This developmentally regulated competence could be one of the reasons why *Agrobacterium*-mediated stable and heritable transformation of maize is not a routine technique. Nevertheless, transfer and probably integration of T-DNA can take place in maize cells. Recently, efficient transformation of scutellum-derived calli of rice via *Agrobacterium* has been shown but apical

meristematic tissues of the same rice variovvars performed very poorly (Hiei *et al.*, 1994). On the other hand, direct comparison between plant species might be difficult since apparently the octopine bacterial strain LBA4404 could transform rice very efficiently but octopine-type *Agrobacterium* strains have been repeatedly reported avirulent in maize (Boulton *et al.*, 1989; Grimsley *et al.*, 1991; Escudero, unpublished data). However, a tissue-specific preference for particular bacterial strains can not be ruled out (Ritchie *et al.*, 1993).

The reason for the incompetence of immature embryos of maize lines other than A188 for T-DNA transfer from microinjected agrobacteria is not known at present. Probably a T-DNA complex is exported by the intracellular bacteria. Assuming that the translocation to the plant-cell nucleus of such a complex is a cellular process mediated by the VirD2 protein, incompetence to intracellular agrobacteria might be regarded as some cell-endogenous toxicity in maize affecting the viability of bacteria before intracellular T-DNA transfer can take place. Indeed, the effect of bacteriotoxic compounds inhibiting *Agrobacterium* in co-cultivation experiments with maize immature embryos has been previously reported (Schläppi and Hohn, 1992). The small inoculum size per single embryonic cell used in the microinjection experiments could account for this bacteriotoxic effect, in contrast to much higher inoculum titres ( $2-4 \times 10^6$  bacterial cells) used in former agroinfection experiments with germinated embryos. Even though agroinfection was detectable in the W23 as well as in the 88025-4A maize lines, the A188 genotype repeatedly proved to be more competent (Schläppi and Hohn, 1992).

The low competence of the A188 × W23 hybrid line for agroinfection suggests that inbreeding depression can not be responsible for the observed efficiency with which these maize lines respond to *Agrobacterium*. A similar effect has been observed for the hybrid between the maize lines W23 and K55. Using either MSV or GUS as T-DNA markers, K55 performed even worse in competence for T-DNA than W23, and the W23 × K55 hybrid was completely incompetent (Escudero, unpublished data).

From the study presented above we can conclude that microinjection of agrobacteria into the meristem of maize opens a new, yet genotype-specific, possibility for plant transformation. The agroinfection assay used, although extremely sensitive, does not allow selection of T-DNA integrated into the plant genome but it shows that the maize apical meristem is competent for *Agrobacterium*-mediated gene transfer if bacteria are provided intracellularly.

### Experimental procedures

#### *Bacterial strains, plasmids and manipulations*

*Agrobacterium tumefaciens* strains used are C58C1 (Watson *et al.*, 1975) and LBA4301 (Klapwijk *et al.*, 1979), both containing a

nopaline pTi plasmid. *Agrobacterium* were grown in YEB medium (Vervliet *et al.*, 1975) at 28°C with appropriate antibiotics (rifampicin, 20 mg l<sup>-1</sup>; kanamycin, 50 mg l<sup>-1</sup>; neomycin, 40 mg l<sup>-1</sup>; chloramphenicol, 50 mg l<sup>-1</sup>). *vir*-gene induction conditions have been described elsewhere (Escudero *et al.*, 1995). Briefly, bacteria grown overnight in YEB were washed and diluted in liquid M9 minimal medium (Sambrook *et al.*, 1989) to optical density at 600 nm (OD<sub>600</sub>) of 0.1. Induced cultures were grown for 12 h, at 28°C and 250 r.p.m., in liquid M9 pH 5.5 medium in the presence of 0.2 mM acetosyringone (AS), and uninduced cultures without AS at pH 7. *Agrobacterium* were then harvested, washed with 10 mM MgSO<sub>4</sub> and resuspended in 10 mM MgSO<sub>4</sub> or M9 pH 5.5 at OD<sub>600</sub>=1 (about 10<sup>6</sup> colony-forming units per µl) prior to use.

In agroinfection experiments, bacterial strains carrying binary plasmids pEAP37 (Grimsley *et al.*, 1987) or pLE1 (Schläppi and Hohn, 1992) were used. They contain an MSV dimer and partial dimer in the T-DNA, respectively. Plasmid pBG5-containing C58C1 (pTiC58) was used in experiments in which T-DNA transfer was monitored by GUS activity in maize cells. pBG5 contains a translational fusion to avoid β-glucuronidase expression in bacteria (Shen *et al.*, 1993). Plasmids were introduced into *Agrobacterium* strains using either tri-parental mating (Ditta *et al.*, 1980) or electroporation (Mattanovitch *et al.*, 1989). Standard cloning techniques were essentially as described (Sambrook *et al.*, 1989). All manipulations involving MSV were as described (Escudero and Hohn, 1994), and restricted to a BL3-containment laboratory.

#### Maize lines and cultivation

Inbred lines A188, W23, and 88025-4a have been described elsewhere (Schläppi and Hohn, 1992). Maize plants were propagated in a growth chamber under a 16 h light/8 h dark regime at 25–20°C, and 50–60% relative humidity. Manipulations concerning isolation and treatment of immature embryos were essentially done as described (Schläppi and Hohn, 1992).

#### Infection of immature embryos

Since immature maize embryos can differ in their developmental stage although coming from the same pollination, ears were harvested at a particular time (10–20 days after pollination (DAP)) and immature embryos were selected according to their developmental stage. This classification allowed us to make comparisons among the different maize lines. Freshly excised embryos were placed aseptically on to MS-agar medium (Murashige and Skoog, 1962) containing 3% sucrose and 1 mg l<sup>-1</sup> thiamine-HCl (named 3ST-MS medium) prior to infection with bacteria. Wounding of embryos to be infiltrated was done by puncturing the shoot apex with the tip of a glass capillary (approximately 2 µm in diameter). Infection was performed by dipping the embryos into bacterial suspensions at OD<sub>600</sub>=1 followed by application of vacuum (approximately 0.4 atm) for 5 min. Embryos were incubated on 3ST-MS plates in a chamber under growth conditions specified above. Besides wounding, bacterial *vir*-gene activation was assayed by the following treatments during the co-cultivation period: (i) infiltration with pre-induced bacteria, that is, induced culture; (ii) infiltration with co-induced bacteria, that is, uninduced culture resuspended in liquid M9 pH 5.5 medium supplemented with 0.2 mM AS. In treatments involving acetosyringone (AS), this inducer was also included in the agar medium at 0.2 mM.

In agroinfection experiments shoots were transferred 7 days after providing *Agrobacterium* to solidified 3ST-MS medium supplemented with 500 mg l<sup>-1</sup> cefotaxim and 500 mg l<sup>-1</sup> vancomycin,

in order to prevent bacterial growth. The frequency of plants showing MSV symptoms was determined 1 month after the embryos were infected. In experiments in which β-glucuronidase was used as the T-DNA marker gene, embryos were first germinated for 3 days in 3ST-MS agar-medium plates before infiltration with a bacterial suspension, as described (Shen *et al.*, 1993). After 3–4 days co-cultivation shoots were subjected to a histochemical GUS assay (Escudero *et al.*, 1995).

#### Microinjection

Isolated 10 DAP embryos were placed on petri dishes containing MS-agar medium and a single cell of the second meristematic layer (named L2) was injected in each embryo. The injection technique used is basically the same previously optimized and described elsewhere (for details see Lusardi *et al.*, 1994; Neuhaus *et al.*, 1994; Schnorf *et al.*, 1991). Briefly, after orientation of the embryo to expose microscopically the apical meristem, injection of L2 cells was optimal by targeting the injection capillary in between two cells of the first meristematic layer (named L1). Injection was performed with an Embryo Splitter system from Research Instruments (Basel, Switzerland) equipped with a high magnifying stereo microscope (SV8, Zeiss, Germany). The mechanical micromanipulator was provided with an injection glass capillary (tip diameter about 0.8 µm) connected to a microinjector (Eppendorf 5242 Microinjector) which was adjusted to deliver constant volumes of about 10 pl into the cells. In this way, inocula consisting of about 10 bacterial cells could be injected within single cells. Besides bacterial solutions, cells were injected with approximately 10<sup>3</sup> molecules of pEAP37 DNA dissolved in TE (10 mM Tris, 1 mM EDTA) as a control.

#### Acknowledgements

We thank the members of our groups for useful discussions and encouragement. We are indebted to Cal Kado for providing bacterial strains. Vicky Chandler and Ben Burr are kindly acknowledged for supplying maize lines. We also thank Georg Felix and Ferenc Nagy for critical comments on the manuscript. The technical assistance of Cynthia Ramos is very much appreciated. J.E. was partially supported by a fellowship from the Spanish Ministerio de Educación y Ciencia, while on leave of absence from Instituto Nacional de Investigaciones Agrarias, Madrid (Spain).

#### References

- Binns, A.N. and Howitz, V.R. (1994) The genetic and chemical basis of recognition in the *Agrobacterium*: plant interaction. In *Bacterial Pathogenesis of Plants and Animals* (Dangl, J.L., ed.). Berlin: Springer Verlag, pp. 119–138.
- Boulton, M.I., Buchholz, W.G., Marks, M.S., Markham, P.G. and Davies, J.W. (1989) Specificity of *Agrobacterium*-mediated delivery of maize streak virus DNA to members of the Gramineae. *Plant Mol. Biol.* **12**, 31–40.
- Boulton, M.I., Pallaghy, C.K., Chatani, M., MacFarlane, S. and Davies, J.W. (1993) Replication of maize streak virus mutants in maize protoplasts: Evidence for a movement protein. *Virology*, **192**, 85–93.
- Bytebier, B., Deboeck, F., DeGreve, H., Van Montagu, M. and Hernalsteens, J.P. (1987) T-DNA organization in tumor cultures and transgenic plants of the monocotyledon *Asparagus officinalis*. *Proc. Natl Acad. Sci. USA*, **84**, 5345–5349.
- Cangelosi, G.A., Ankenbauer, R.G. and Nester, E.W. (1990) Sugars

- induce the *Agrobacterium* virulence genes through a periplasmic binding protein and a transmembrane signal protein. *Proc. Natl Acad. Sci. USA*, **87**, 6708–6712.
- Chan, M.-T., Chang, H.-H., Ho, S.-L., Tong, W.-F. and Yu, S.-M.** (1993) *Agrobacterium*-mediated production of transgenic rice plants expressing a chimeric  $\alpha$ -amylase promoter/ $\beta$ -glucuronidase gene. *Plant Mol. Biol.* **22**, 491–506.
- De Cleene, M. and De Ley, J.** (1976) The host range of crown gall. *Bot. Rev.* **42**, 389–466.
- Ditta, G., Stanfield, S., Corbin, D. and Helinski, D.R.** (1980) Broad host-range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *Proc. Natl Acad. Sci. USA*, **77**, 7347–7351.
- Escudero, J. and Hohn, B.** (1994) Agroinfection. In *The Maize Handbook* (Freeling, M. and Walbot, V., eds). New York: Springer Verlag, pp. 599–607.
- Escudero, J., Neuhaus, G. and Hohn, B.** (1995) Intracellular *Agrobacterium* can transfer DNA to the cell nucleus of the host plant. *Proc. Natl Acad. Sci. USA*, **92**, 230–234.
- Gheysen, G., Villarroel, R. and Van Montagu, M.** (1991) Illegitimate recombination in plants: a model for T-DNA integration. *Genes Devel.* **5**, 287–297.
- Grimsley, N., Hohn, T., Davies, J.W. and Hohn, B.** (1987) *Agrobacterium*-mediated delivery of infectious maize streak virus into maize plants. *Nature*, **325**, 177–178.
- Grimsley, N., Ramos, C., Hein, T. and Hohn, B.** (1988) Meristematic tissue of maize plants are most susceptible to agroinfection with maize streak virus. *Bio/Technology*, **6**, 185–189.
- Grimsley, N., Jarchow, E., Oetiker, J., Schläppi, M. and Hohn, B.** (1991) Agroinfection as a tool for the investigation of plant-pathogen interactions. In *Plant Molecular Biology*, volume 2, NATO ASI series (Herrman, R., ed.). New York: Plenum Press, pp. 225–238.
- Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T.** (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J.* **6**, 271–282.
- Hooykaas, P.J.J. and Schilperoort, R.A.** (1992) *Agrobacterium* and plant genetic engineering. *Plant Mol. Biol.* **19**, 15–38.
- Hooykaas-van Slogteren, G.M.S., Hooykaas, P.J.J. and Schilperoort, R.A.** (1984) Expression of Ti plasmid genes in monocotyledonous plants infected with *Agrobacterium tumefaciens*. *Nature*, **311**, 763–764.
- Klapwijk, P.M., van Beelen, P. and Schilperoort, R.A.** (1979) Isolation of a recombination deficient *Agrobacterium tumefaciens* mutant. *Mol. Gen. Genet.* **173**, 171–175.
- Koukoliková-Nicola, Z., Raineri, D., Stephens, K., Ramos, C., Tinland, B., Nester, E.W. and Hohn, B.** (1993) Genetic analysis of the virD operon of *Agrobacterium tumefaciens*: a search for functions involved in the transport of T-DNA into the plant cell nucleus and in T-DNA integration. *J. Bacteriol.* **175**, 723–731.
- Lusardi, M.C., Neuhaus-Url, G., Potrykus, I. and Neuhaus, G.** (1994) An approach towards genetically engineered cell fate mapping in maize using the *Lc* gene as a visible marker. *Plant J.* **5**, 571–582.
- Mattanovitch, D., Ruker, F., da Câmara-Machado, A., Laimer, M., Regner, F., Steinkellner, H., Himmler, G. and Katinger, H.** (1989) Efficient transformation of *Agrobacterium* spp. by electroporation. *Nucl. Acids Res.* **17**, 6747.
- Mayerhofer, R., Koncz-Kalman, Z., Nawrath, C., Bakkeren, G., Cramer, A., Angelis, A., Redei, G.P., Schell, J., Hohn, B. and Koncz, C.** (1991) T-DNA integration: a mode of illegitimate recombination in plants. *EMBO J.* **10**, 697–704.
- Murashige, T. and Skoog, F.** (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–493.
- Neuhaus, G., Neuhaus-Url, G., Katagiri, F., Seipel, K. and Chua, N.-H.** (1994) Tissue-specific expression of as-1 in transgenic tobacco. *Plant Cell*, **6**, 827–834.
- Ritchie, S.W., Lui, C.-N., Sellmer, J.C., Kononowicz, H., Hodges, T.K. and Gelvin, S.B.** (1993) *Agrobacterium tumefaciens*-mediated expression of *gUSA*, in maize tissues. *Transgenic Res.* **2**, 252–265.
- Rogowsky, P., Close, T.J., Chimera, J.A., Shaw, J.J. and Kado, C.** (1987) Regulation of the vir genes of *Agrobacterium tumefaciens* plasmid pTiC58. *J. Bacteriol.* **169**, 5101–5112.
- Rossi, L., Hohn, B. and Tinland, B.** (1993) The VirD2 protein of *Agrobacterium tumefaciens* carries nuclear localization signals important for transfer of T-DNA to plants. *Mol. Gen. Genet.* **239**, 345–353.
- Sambrook, J., Fritsch, E.F. and Maniatis, T.** (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Schäfer, W., Görz, A. and Kahl, G.** (1987) T-DNA integration and expression in a monocot plant after induction of *Agrobacterium*. *Nature*, **327**, 529–531.
- Schläppi, M. and Hohn, B.** (1992) Competence of immature maize embryos for *Agrobacterium*-mediated gene transfer. *Plant Cell*, **4**, 7–16.
- Schnorf, M., Neuhaus-Url, G., Galli, A., Lida, S., Potrykus, I. and Neuhaus, G.** (1991) An improved approach for transformation of plant cells by microinjection: molecular and genetic analysis. *Transgenic Res.* **1**, 23–30.
- Shen, W.-H., Escudero, J., Schläppi, M., Ramos, C., Hohn, B. and Koukoliková-Nicola, Z.** (1993) T-DNA transfer to maize cells: Histochemical investigation of  $\beta$ -glucuronidase activity in maize tissues. *Proc. Natl Acad. Sci. USA*, **90**, 1488–1492.
- Shurvinton, C.E., Hodges, L. and Ream, W.** (1992) A nuclear localization signal and the C-terminal omega sequence in the *Agrobacterium tumefaciens* VirD2 endonuclease are important for tumor formation. *Proc. Natl Acad. Sci. USA*, **89**, 11 837–11 841.
- Stachel, S.E., Nester, E.W. and Zambryski, P.C.** (1986) A plant cell factor induces *Agrobacterium tumefaciens* vir gene expression. *Proc. Natl Acad. Sci. USA*, **83**, 379–383.
- Tinland, B. and Hohn, B.** (1995) Recombination between prokaryotic and eukaryotic DNA: integration of *Agrobacterium tumefaciens* T-DNA into the plant genome. In *Genetic Engineering*, volume 17 (Setlow, J.K., ed.). New York: Plenum Press, pp. 209–229.
- Tinland, B., Hohn, B. and Puchta, H.** (1994) *Agrobacterium tumefaciens* transfers single-stranded T-DNA into the plant cell nucleus. *Proc. Natl Acad. Sci. USA*, **91**, 8000–8004.
- Vervliet, G., Holsters, M., Teuchy, H., Van Montagu, M. and Schell, J.** (1975) Characterization of different plaque forming and defective temperate phages in *Agrobacterium* strains. *J. Gen. Virol.* **26**, 33–48.
- Watson, B., Currier, T.C., Gordon, M.P. and Nester, E.W.** (1975) Plasmid required for virulence of *Agrobacterium tumefaciens*. *J. Bacteriol.* **123**, 255–264.
- Winans, S.C.** (1992) Two-way chemical signaling in *Agrobacterium*-plant interactions. *Microbiol. Rev.* **56**, 12–31.
- Zupan, J.R. and Zambryski, P.** (1995) Transfer of T-DNA from *Agrobacterium* to the plant cell. *Plant Physiol.* **107**, 1041–1047.