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T-DNA transfer to maize cells: Histochemical investigation of β-glucuronidase activity in maize tissues

(*Zea mays*/*Agrobacterium*/transformation)

WEN-HUI SHEN, JESÚS ESCUDERO*, MICHAEL SCHLÄPPI†, CYNTHIA RAMOS, BARBARA HOHN‡, AND ZDENA KOUKOLÍKOVÁ-NICOLA

Friedrich Miescher-Institut, P.O. Box 2543, CH-4002 Basel, Switzerland

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ABSTRACT ***Agrobacterium tumefaciens* is routinely used to engineer desirable genes into dicotyledonous plants. However, the economically important graminaceous plant maize is refractory to tumor induction by inoculation with virulent strains of *A. tumefaciens*. Currently, the only clearcut evidence for transferred DNA (T-DNA) transport from *Agrobacterium* to maize comes from agroinfection. To study T-DNA transfer from *Agrobacterium* to maize cells in a virus-free system, we used here the β-glucuronidase (GUS; EC 3.2.1.31) gene as a marker. GUS expression was observed with high efficiency on shoots of young maize seedlings after cocultivation with *Agrobacterium* carrying the GUS gene. *Agrobacterium* virulence mutants, incapable of transferring T-DNA to dicot tissue, were shown to be deficient in eliciting GUS expression in maize. Hence, expression of the T-DNA-located GUS gene in maize cells is strictly dependent on *Agrobacterium*-mediated DNA transfer. Histochemical staining of maize shoots revealed GUS expression located mainly in the leaves and the coleoptile.**

Agrobacterium tumefaciens, a Gram-negative soil bacterium, is able to transfer and to integrate part of its DNA, the transferred DNA (T-DNA), into the nuclear genome of receptive plant cells (1, 2). The successful invasion of *Agrobacterium* results in the formation of crown gall tumors on the plant. This unique system of *Agrobacterium*-mediated plant transformation has been adapted to engineer desirable genes into plants. However, the host range of *Agrobacterium*, as judged by tumor formation, is limited to most dicotyledonous plants (3), precluding the use of this system for the economically important graminaceous plants.

Zea mays had been considered a nonhost of *Agrobacterium* since it does not develop tumors after inoculation with *A. tumefaciens*. However, opines, amino acid derivatives that are synthesized upon expression of T-DNA-specific genes, have been detected in maize seedlings inoculated with *Agrobacterium* (4). T-DNA transfer from *Agrobacterium* to the maize cell has been clearly demonstrated by agroinfection (5). In this system, partially or completely duplicated genomes of maize streak virus have been introduced into the T-DNA and the resultant *A. tumefaciens* strain has been used to inoculate maize seedlings. DNA transfer was monitored by the appearance of viral symptoms on the recipient maize plant. This technique has been used to test tissue specificity of *Agrobacterium*-mediated gene transfer in maize. It was shown that the tissue at or near to the shoot apical meristem of maize seedlings is most susceptible to agroinfection (6) and that competence of immature maize embryos for agroinfection is developmentally regulated (7). In this agroinfection system, a successful infection requires a compatible interaction not only between *Agrobacterium* and a maize cell but also between the virus and the maize cell. Thus, the tissue specificity observed in agroinfection may not faithfully reflect the direct interaction between *Agrobacterium* and maize. In addition, because the virus spreads systemically, it has not been possible to analyze which maize cells are the primary recipients for the T-DNA.

In an effort to explore the direct interaction between *Agrobacterium* and maize cells, we used an improved β-glucuronidase (GUS; EC 3.2.1.31) gene as well as an intron containing GUS gene (intron-GUS) that represent tight markers for gene expression in the plant. We introduced these genes into the T-DNA and investigated T-DNA transfer by detecting histochemically the GUS activity in plant tissues. After cocultivation with *Agrobacterium*, GUS staining was observed most frequently in the leaves and also in the coleoptiles of the shoots of young maize seedlings. This GUS-marked T-DNA transfer was shown to depend on the maize genotype used as recipient and to require active virulence genes of the *Agrobacterium* strains used for the transfer.

MATERIALS AND METHODS

Plasmid Constructions and Bacterial Strains. A GUS gene with its initiation codon embedded in a sequence of eukaryotic origin was originally constructed by Schultze *et al.* (8). The plasmid pGUS23, containing this GUS gene in the pUC7 vector, has been described (9). pBG5 was constructed by cloning the GUS gene-containing *Eco*RI fragment of pGUS23 into the *Eco*RI site of the binary vector pBIN19 (10). The *Hin*dIII fragment containing the same GUS gene of pGUS23 was also cloned into the *Hin*dIII site of the binary vector pCGN1589 (11), resulting in pCG5. pIG221, which contains an intron-GUS gene in the binary vector pBIN19, has been described (12). Plasmids were maintained in *Escherichia coli* strain DH5α and isolated as described (13).

pBG5, pCG5, and pIG221 were introduced into different *A. tumefaciens* strains using the electroporation (14) or the triparental-mating method (15). Table 1 lists *Agrobacterium* strains used.

Preparation of Maize Shoots. Maize lines Golden Cross Bantam (GB) and A188 have been described (5–7, 19). Line K55 was provided by V. Walbot (Department of Biological Sciences, Stanford University). GB seeds and immature kernels of A188 and K55 harvested 14–17 days after pollination were surface sterilized in 1.4% sodium hypochlorite/

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Abbreviations: T-DNA, transferred DNA; GUS, β-glucuronidase; X-Gluc, 5-bromo-4-chloro-3-indolyl glucuronide; AS, acetosyringone.

*Permanent address: Instituto Nacional de Investigaciones Agrarias, Apartado 8111, 28080 Madrid, Spain.
†Present address: Department of Embryology, Carnegie Institution of Washington, 115 West University Parkway, Baltimore, MD 21210.
‡To whom reprint requests should be addressed.

Table 1. Characteristics of *Agrobacterium* strains used for cocultivation with maize shoots

Strain	Chromosome	Virulence genes	Binary vector
C58C1 (pBG5)	Nopaline-type C58C1 (ref. 16)	None	GUS gene in pBIN19
C58C1 (pTiC58, pBG5)	id	pTiC58: a nopaline wild-type Ti plasmid	id
C58C1 (pIG221)	id	None	Intron-GUS gene in pBIN19
C58C1 (pTiC58, pIG221)	id	pTiC58: a nopaline wild-type Ti plasmid	id
C58C1 (pTiC58, pCG5)	id	id	GUS gene in pCGN1589
LBA4301 (pJK270, pCG5)	Octopine-type Ach5 derivative (ref. 17)	pJK270, containing the full set of C58 virulence genes (ref. 18)	id
LBA4301 (pJK190, pCG5)	id	pJK190, differing from pJK270 by a polar Tn*5* insertion in *vir*B4 (ref. 18)	id
LBA4301 (pJK210, pCG5)	id	pJK210, differing from pJK270 by Tn*5* insertion in *vir*B11 (ref. 18)	id

id (idem), Property of strain identical to the one noted a line above.

0.05% SDS for 20 min and washed three times for 5 min each in sterile water. Seeds were germinated on water-wet filter paper at 28°C in the dark. Embryos were isolated from immature kernels and germinated on agar plates made from MS medium containing 3% sucrose and 1 mg of thiamine hydrochloride per liter (7) in a phytotron under a regime of 16-hr light (20,000 lux) and 8 hr of dark, at 25°C. Shoots were isolated from seedlings by cutting just below (about 1–3 mm) the coleoptilar node, where the shoot meristem is located.

Growth of *Agrobacterium* Strains for Cocultivation Experiments. Strains were grown in shaking liquid cultures at 28°C for 48 hr in YEB medium (20) supplemented with appropriate antibiotics. They were subcultured in the same medium following a 1:20 dilution and grown for a further 20 hr, reaching a final titer of 1–2 $\times$ 10^9 cells per ml. Cells were then harvested by centrifugation, washed with 10 mM $MgSO_4$, and resuspended in 10 mM $MgSO_4$ to a final titer of 1–2 $\times$ 10^{10} cells per ml. Acetosyringone (AS; Aldrich Chemie, Steinheim, F.R.G.) was added at a final concentration of 200 μM to the *Agrobacterium* suspension, just before the cocultivation with maize shoots.

Cocultivation of Maize Shoots with *Agrobacterium*. The shoots were dipped into the *Agrobacterium* suspension and subjected to vacuum infiltration [-0.4 to -0.6 atm (1 atm = 101.3 kPa)] for 5 min. The infiltrated maize shoots were cultured on agar plates containing MS medium with 3% sucrose and 1 mg of thiamine hydrochloride per liter, supplemented with 200 μM AS, in the phytotron, under the same conditions as for the germination of immature embryos. The shoots were collected for the GUS staining assay 3 days later.

GUS Assay. Maize shoots were soaked with 0.052% 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) (Biosynth, Staad, Switzerland) in 100 mM NaH_2PO_4 (pH 7.0) in the presence of 0.1% sodium azide. After 10 min of vacuum infiltration, the reactions were continued at 37°C for 2 days in the dark. Shoots were destained for chlorophyll by rinsing with ethanol (70–90%).

RESULTS

Activity of GUS in Maize Tissue Is a Consequence of T-DNA Transfer. Our initial studies employed a GUS gene in which the sequence coding for the first 29 amino acids of the open reading frame V of the cauliflower mosaic virus was fused to the GUS structural gene (8). This GUS fusion gene has a much higher post-transcriptional expression efficiency in plant cells than the original one (8). The improved GUS gene, driven by the 35S promoter and terminated by the nopaline synthase terminator, was inserted into the T-DNA of *Agrobacterium* binary vectors pBIN19 and pCGN1589, resulting in pBG5 and pCG5, respectively (Table 1). Unlike the original GUS gene, this improved GUS gene did not express detectable (above background) levels of GUS activity in *Agrobacterium* cells (data not shown).

In some studies we used an intron-GUS gene constructed by the insertion of a modified intron of the castor bean catalase gene into the N-terminal part of the GUS coding sequence (12). It has been shown that this intron-GUS did not express GUS activity in *Agrobacterium* cells (12) and that the GUS activity in rice cells was dramatically enhanced by the presence of this intron (21).

Three-day-old seedlings grown from mature seeds of the maize line GB have been shown to be highly competent for agroinfection (5, 6, 19). Shoots excised from 3-day-old seedlings were cocultivated with *Agrobacterium* strain C58C1 (pTiC58, pBG5) for 3 days (25°C, 16 hr of light and 8 hr of dark) and stained with the GUS substrate X-Gluc. After destaining with ethanol (70–90%) to remove chlorophyll, about 10% of shoots tested exhibited blue spots indicating GUS expression (data not shown).

To increase the number of seedlings tested, but minimize the expense of X-Gluc, maize immature embryos were tested as a source of plant material. Immature embryos and seedlings germinated from them are much smaller than in the case of mature seeds. More importantly, from our greenhouse-grown maize, they are usually sterile, thus avoiding endogenous GUS activity of contaminating microorganisms. It has been shown previously that immature embryos of maize line A188 are highly competent for agroinfection (7). However, direct injection of *Agrobacterium* C58C1 (pTiC58, pBG5) into immature embryos produced blue staining only on few A188 immature embryos (22). Therefore, we used the shoots isolated from plantlets germinated from A188 immature embryos for cocultivation with *Agrobacterium*. As shown in Table 2, group A, after cocultivation with strain C58C1 (pTiC58, pBG5), blue spots indicating GUS activity were observed on about 30% of the shoots isolated from plantlets 5 and 8 days after germination of the embryos, which were harvested 14 days after pollination. The observed blue spots covered more than one cell (Fig. 1). This may be due to the enzyme and/or to the noncolored intermediate cleavage product diffusing from the original GUS-positive cells into the adjacent ones. The number of blue spots was estimated by counting under a stereomicroscope. Only spots (small or big) that were well separated from each other were counted as individuals. The number of dark blue individual spots forming large continuous blue sectors was estimated. With *Agrobacterium* strain C58C1 (pTiC58, pBG5), the percentage of shoots showing blue spots and the average number of blue spots per shoot were increased when older immature embryos (17 rather than 14 days after pollination) were used (Table 2). *Agrobacterium* strain C58C1 (pTiC58, pIG221), which contains the intron-GUS gene, also produced a high number of blue spots on a high percentage of shoots (Table 2, group B).

Table 2. GUS expression detected on shoots of maize plantlets germinated from immature embryos, after cocultivation with *Agrobacterium*

Group	*Agrobacterium*	Maize	No. of shoots tested	No. of shoots showing blue spots	% of shoots showing blue spots	Total no. of blue spots	Average no. of blue spots per shoot
A	C58C1 (pTiC58, pBG5)	A188, 14 DAP					
		5 DAG	41	13	32	266	6.4
		8 DAG	62	18	29	263	4.2
		K55, 14 DAP, 5 DAG	54	0	0	0	0
	C58C1 (pBG5)	A188, 14 DAP					
		5 DAG	37	0	0	0	0
		8 DAG	60	0	0	0	0
B	C58C1 (pTiC58, pBG5)	A188, 17 DAP, 5 DAG	26	13	50	959	36.9
	C58C1 (pTiC58, pIG221)	id	56	46	82	1304	23.3
	C58C1 (pIG221)	id	56	0	0	0	0
C	C58C1 (pTiC58, pBG5)	A188, 17 DAP, 5 DAG	33	24	73	1031	31.2
	C58C1 (pTiC58, pCG5)	id	43	14	32	236	5.5
	LBA4301 (pJK270, pCG5)	id	54	7	13	96	1.8
	LBA4301 (pJK190, pCG5)	id	56	0	0	0	0
	LBA4301 (pJK210, pCG5)	id	55	0	0	0	0

DAP, days after pollination; DAG, days after germination; id (idem), maize material identical to the one noted a line above.

To test whether the blue spots observed on maize shoots cocultivated with *Agrobacterium* strains C58C1 (pTiC58, pBG5) and C58C1 (pTiC58, pIG221) were T-DNA transfer-specific, GUS assays were performed on shoots of maize A188 immature embryos cocultivated with different *Agrobacterium* strains (Table 1). We never observed any blue staining on shoots cocultivated with the Ti plasmid-deficient *Agrobacterium* strains C58C1 (pBG5) and C58C1 (pIG221) (Table 2, groups A and B). To confirm that the *Agrobacterium* strain C58C1 (pBG5) still contained a functional pBG5 plasmid, we isolated the pBG5 plasmid from this *Agrobacterium* strain and introduced it into *Agrobacterium* strain C58C1 (pTiC58). The resultant *Agrobacterium* strain was T-DNA transfer-competent, as tested by the GUS activity assay (data not shown).

To establish that our observed GUS-positive maize shoots result from *vir*-gene-mediated T-DNA transfer, two helper plasmids with known *virB* mutations were tested: LBA4301 (pJK190, pCG5) and LBA4301 (pJK210, pCG5) (Table 2, group C). No blue spots were observed. In contrast, the

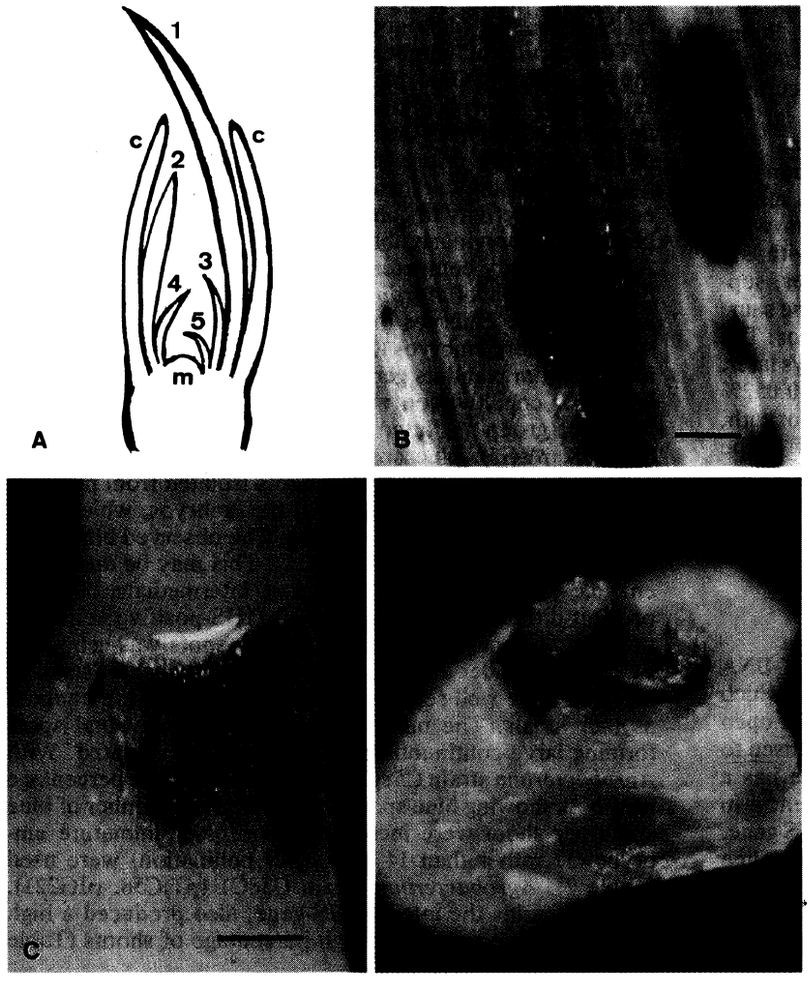

FIG. 1. T-DNA transfer to maize shoots, as tested by histochemical analysis of GUS expression. (*A*) Schematic representation of a maize shoot (5 days after germination of 17-day-old embryo) containing apical meristem (m), leaves (1–5), and coleoptile (c). (*B* and *C*) GUS expression in a leaf and in a coleoptile, respectively. (*D*) A sectioned shoot apex showing GUS expression. (Bars = 0.2 mm.)

corresponding wild-type strain LBA4301 (pJK270, pCG5) proved to be transfer-proficient, although at a significantly lower level than the strain C58C1 (pTiC58, pBG5) (Table 2, group C). This appears to be a consequence of a different chromosomal background as well as the use of a different binary vector (Tables 1 and 2, group C). A similar decrease in efficiency of these *Agrobacterium* strains was also observed in tobacco (data not shown). The blue spots we observed on the maize shoots thus correlate with the T-DNA transfer capacity of the *Agrobacterium* strain used (Table 2).

Competence of Different Maize Lines and Different Tissues of Maize Seedlings for *Agrobacterium*-Mediated T-DNA Transfer. Interestingly, the maize line K55 was not competent for T-DNA transfer using otherwise identical conditions (Table 2, group A). This observation is in agreement with the results of agroinfection experiments, where only <10% of the K55 plants inoculated at the 3-day-old seedling stage showed viral symptoms, as compared to 80–100% for maize line A188 (data not shown).

The shoots used in our experiments contained the shoot apical meristem, the leaves, and the coleoptile (Fig. 1*A*). The fourth and sometimes the fifth leaf of sectioned shoot apices could be seen clearly under the stereomicroscope. During cocultivation with *Agrobacterium*, enlargement of the preformed leaves was observed. The blue spots we observed after cocultivation of the shoots with *Agrobacterium* were mainly distributed on the leaves, especially at their veins (Fig. 1*B*), and also quite frequently on the coleoptile (Fig. 1*C*). Although less frequently, blue staining at the coleoptilar node, which contains the shoot meristem, was also observed (Fig. 1*D*). As the contact between *Agrobacterium* and maize cells of different types of tissue may not have been equally facilitated by our experimental procedure, and as the total number of cells in these different tissues varies, we cannot compare quantitatively the competence of different maize tissues for *Agrobacterium* T-DNA transfer. However, qualitatively, it is clear from our results (Fig. 1) that tissues of the leaf, the coleoptile, and possibly also the apical meristem are competent for *Agrobacterium*-mediated T-DNA transfer. In contrast, cocultivation of maize roots or scutellum with *Agrobacterium* never led to GUS expression (data not shown).

Effects of AS and Wounding on *Agrobacterium*-Mediated T-DNA Transfer. AS is known to induce the expression of the *Agrobacterium vir* genes responsible for the initiation of processes leading to transformation (23). Although maize was shown to produce its own substances capable of virulence induction of *Agrobacterium* (refs. 24 and 25; W. S. Chilton, personal communication), in all experiments described so far, AS was applied as an external inducer to increase the efficiency of T-DNA transfer. To test whether AS played an important role, cocultivation of maize shoots with *Agrobacterium* was performed in the absence of AS. Omission of AS from the cocultivation indeed decreased the efficiency of T-DNA transfer, as judged from the total number of blue spots and the percentage of shoots showing blue spots (Fig. 2). Wounding of plant tissue is known to be required for *Agrobacterium*-mediated T-DNA transfer. In presence of AS, extra-wounding with forceps through the whole surface of the maize shoots just before cocultivation increased the number of blue spots per positive shoot, although the fraction of positive shoots did not change significantly compared to shoots without the extra-wounding treatment (Fig. 2). In the absence of AS, a similar enhancement by extra-wounding was also observed (data not shown). On each shoot (with or without extra-wounding treatment) showing GUS expression, the number of blue spots could vary from 1 up to >100. We do not know the origin(s) of this big variation of competence among different individuals in a population of maize plantlets.

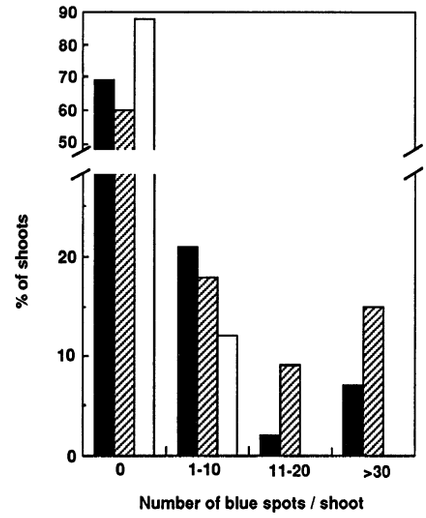

FIG. 2. Effects of AS and wounding on the efficiency of T-DNA transfer from *Agrobacterium* to maize. The black columns represent the results obtained with the standard conditions in which the maize shoots were cocultivated with *Agrobacterium* strain C58C1 (pTiC58, pBG5) in the presence of AS as an external inducer. The shaded columns represent the results obtained after wounding with forceps through the whole surface of the maize shoots just before cocultivation under the standard conditions. The results obtained by excluding AS from the standard conditions are represented by the white columns. Twenty-five to 30 shoots were used in each case. Essentially identical results were obtained in three independent experiments.

DISCUSSION

Activity of GUS in Maize Tissue Is Due to *Bona Fide* T-DNA Transfer. We have used two GUS constructs, one a fusion with cauliflower mosaic virus open reading frame V (8) and one bearing an intron in the N-terminal part of the GUS coding sequence (12). These GUS genes did not lead to detectable enzyme activity in *Agrobacterium* cells (ref. 12; data not shown). After cocultivation with *Agrobacterium* carrying these GUS constructs, a high percentage of maize shoots exhibited blue spots (Fig. 1, Table 2).

Several lines of evidence clearly indicate that we are detecting *bona fide* T-DNA transfer: (*i*) AS, a known virulence gene inducer, increased the number of blue spots (Fig. 2); (*ii*) increased wounding, a prerequisite for T-DNA transfer in dicots, increased the number of blue spots (Fig. 2); (*iii*) the presence of a Ti plasmid in the *Agrobacterium* vector strain was absolutely required for production of blue spots (Table 2); (*iv*) agroinfection of maize has been shown to require *virA*, *-B*, *-D*, and *-G* (24). In the present study, mutations in *virB*, a virulence gene absolutely required for T-DNA transfer (2), totally eliminated formation of blue spots (Table 2, group C). Since *virB* mutants are proficient for T-DNA excision (26), this finding rules out any T-DNA processing-dependent DNA rearrangement in the bacterium that could lead to GUS activity. We conclude that the observed GUS-positive spots in our experiments are a result of T-DNA transfer to maize cells.

Efficiency of T-DNA Transfer to Maize Cells Is Influenced by Maize Genotype, *Agrobacterium* Strain, Induction, and Wounding. We observed a high frequency of T-DNA transfer to shoot tissues with maize line A188 but none with line K55, at least under the conditions used (Table 2, group A). Agroinfection has been found to be similarly maize line dependent (refs. 7 and 27; unpublished data). The correlation between the two assays strengthens the conclusion that GUS-positive spots seen here are indeed a result of T-DNA transfer.

Maize plant material used in *Agrobacterium* transformation is an important factor in efficiency. A lower percentage of shoots from seedlings gave GUS-positive spots than did shoots from immature embryos. Exactly comparable studies have not been made in agroinfection experiments. In our studies, soft shoots from immature embryos may afford greater sensitivity to wounding and entry of *Agrobacterium* than do the more rigid structures of seed-derived shoots.

For producing GUS-positive spots, *Agrobacterium* strain C58C1 is a more successful T-DNA donor than is LBA4301 (Table 2, group C). Further, T-DNA vector pBG5, with a replication origin from pRK2 and nopaline Ti borders, is more efficient than pCG5, with origin from an Ri plasmid (pRiHRI) and octopine Ti borders (Table 2, group C). The basis of this difference has not been explored further, but the two vectors have a similar difference in efficiency for tobacco seedling transformation (data not shown).

In the present study, addition of AS as inducer of *vir* genes greatly enhances the percentage of transformed shoots and the number of spots per shoot (Fig. 2). In agroinfection, in contrast, there is no effect of AS on the percentage of infected plants (24). The plant material and transformation regimes are different in the two studies, but in both cases T-DNA transfer can occur without AS, consistent with findings that maize plants produce a *vir* gene inducer (refs. 24 and 25; W. S. Chilton, personal communication).

All shoots in the present study are wounded by the excision process and possibly also by vacuum infiltration with *Agrobacterium*. Further wounding with forceps increased the frequency of GUS-positive spots per shoot. Because this was observed either with or without addition of AS, the mechanism is presumably not just an increased release of maize *vir* gene inducer substance. Wounding may favor the penetration of bacteria into the maize shoots or access to susceptible cells in the shoot.

T-DNA Transfer Can Occur in Several Types of Maize Tissues. The GUS reporter constructs employed in this study allow analysis of the maize cells that can act as recipients for T-DNA transfer; this has not been possible by the agroinfection approach (1, 27) because the assay is systemic infection of the plant. Meristematic cells are believed to be most susceptible to agroinfection (6), but it is possible that meristematic cells favor steps after T-DNA transfer, such as recombinational excision, replication, and spread of maize streak virus. Maize streak virus is believed to be phloem limited (28). In the present study, blue spots were observed primarily on leaves and coleoptile and also in the area of shoot apical meristems (Fig. 1). We never observed GUS-positive spots on roots or scutellum (data not shown).

GUS-positive spots observed in this study often covered multicellular areas, but this may be due to diffusion of enzyme products from a single cell. Microprojectile bombardment of maize tissue with DNA containing a GUS gene has been found to produce diffuse spots (29). In crown gall disease, actively dividing host plant cells are required for tumor formation, and integration of T-DNA into the plant nuclear genome is observed in transformed cells (30). In contrast, GUS expression following *Agrobacterium* inoculation may only require entry of T-DNA into the plant cell nucleus and could be independent of integration. Thus, nondividing cells, such as most of the maize coleoptile and maize leaf cells, even if they lack the enzymatic machinery for integration, could still have means of getting T-DNA into the nucleus and transcribing it. Alternatively, T-DNA could integrate into the chromosomal DNA of nondividing and/or dividing cells and give rise to GUS-positive spots. The present study does not allow us to distinguish among these interesting possibilities.

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