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Transformation of *Zea mays* L. Using *Agrobacterium tumefaciens* and the Shoot Apex[1]

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ABSTRACT

Agrobacterium tumefaciens **is established as a vector for gene transfer in many dicotyledonous plants but is not accepted as a vector in monocotyledonous plants, especially in the important Gramineae. The use of *Agrobacterium* to transfer genes into monocot species could simplify the transformation and improvement of important crop plants. In this report we describe the use of *Agrobacterium* to transfer a gene into corn, the regeneration of plants, and detection of the transferred genes in the F_1 progeny. Shoot apices of *Zea mays* L. variety Funk's G90 were cocultivated with *A. tumefaciens* EHA1, which harbored the plasmid pGUS3 containing genes for kanamycin resistance (NPT II) and β-glucuronidase (GUS). Plants developed from these explants within 4 to 6 weeks. Fluorometric GUS assays of leaves and immature seeds from the plants exhibited low GUS activity. Both NOS and GUS gene fragments were amplified by polymerase chain reaction in the DNA isolated from the F_1 generations of one of the original transformed plants. Southern analysis showed both GUS and NPT probes hybridized to DNA in several of the F_1 progeny, demonstrating the incorporation of GUS and NPT II genes into high molecular weight DNA. These data establish successful gene transfer and sexual inheritance of the genes.**

Until recently, the monocotyledons and particularly the graminaceous crop species have been considered to be outside the *Agrobacterium* host range (1, 5). In the past, a general definition of host species range has been based on tumor or gall formation in inoculated plants. Gene transfer methods developed for economically important species considered to be outside of the *Agrobacterium* host range have previously been restricted to the direct transfer of DNA into protoplasts and to the few cultivars which can be regenerated from protoplasts. With the development of the particle discharge or acceleration methods of direct DNA transfer, intact cells of embryogenic callus and cell suspensions can be used. Recently, this approach resulted in the successful transformation and regeneration of corn (7, 10). This approach will be applicable to maize genotypes which form embryogenic cultures.

The host-range of *Agrobacterium* has been under continual revision since the original review by DeCleene (5). Upon reinvestigation of the host range, plants in the monocot orders Liliales and Arales were observed to produce tissue swelling in response to inoculation with virulent *Agrobacterium* and were added to the group of host species (5). The synthesis of *Agrobacterium* strain-specific opines by tissues of *Chlorophytum capense, Narcissus* (17), and *Asparagus officinalis* (14), was considered to be indicative of integration and expression of the transferred DNA. A subsequent study with *Zea mays* (8), also found strain-specific opine synthesis in extracts of seedlings inoculated with *Agrobacterium*. These studies, based on the detection of specific opines and limited cell enlargement or proliferation, raised the possibility that monocot species could be transformed by *Agrobacterium* and that gene transfer occurred in the same manner as in dicot plants. However, detection of opine synthesis may not always indicate stable transformation by *Agrobacterium* (3); evidence of the transferred DNA in the plant cells was not present in these studies.

The binding affinity of *Agrobacterium* to specific cells in vascular tissues of corn and wheat seedlings and of gladiolus disks was later reported (9). The strong tissue-specific attraction and binding of *Agrobacterium* observed in these monocots implied that monocots possessed transformable cell types. In *Z. mays*, cells in the vascular bundles of the young internodes were the tissues to which the bacteria adhered and it was proposed that these tissues contained the transformable cell population.

The demonstration of *Agrobacterium* T-DNA integration into genomic DNA of *A. officinales* (2) and *Dioscorea bulbifera* (25) showed that some monocot species could be transformed by *Agrobacterium*. The method of "agroinfection" was developed and used to study *Agrobacterium* host range in monocot species, specifically maize (11). Maize streak virus was incorporated into the *Agrobacterium* T-DNA and inoculated onto corn. Development of viral symptoms in inoculated plants implied the presence of *Agrobacterium* T-DNA in cell nuclei based on replication of the virus in the host nucleus. These events did not reflect incorporation of the T-DNA; however, this evidence indicated that *Agrobacterium* T-DNA was present in the nuclei of corn cells and that this event occurred at the same frequency as in an acknowledged host genus, *Brassica* (12). Grimsley *et al.* (13) reported that the meristem tissue of the shoot apex of *Z. mays* was the tissue most susceptible to agroinfection by maize streak virus. However, the authors felt that the meristem site preference was probably an artifact caused by the preferential replication of the virus in the meristematic tissues. Agroinfection using wheat dwarf virus was used to show that *Agrobacterium* could

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infect embryos of *Triticum aestivum, Triticum monococcum, Triticum durum*, and *Aegilops speltoides* (4). The demonstration of T-DNA integration into genomic DNA of rice, *Oryza sativa* (24), clearly showed that Graminaceous crop species can be transformed using *Agrobacterium*. Furthermore, the reports of successful gene transfer in conifer species (28) suggest an extensive rather than a limited host-range and that the previous ideas concerning host range (15) and even tissue-specific limitations should be reevaluated.

Shoot meristem and apex culture have been in use for over 20 years to obtain virus-free plants (21) and are the methods of choice for true clonal propagation in both monocotyledonous and dicotyledonous species (21, 22). Despite the notion that shoot apical meristems are inappropriate or insensitive to infection by *Agrobacterium*, little evidence exists in the literature to exclude this possibility. The same arguments which were used to exclude monocot species from *Agrobacterium* host range, *e.g.* lack of tumor formation, have also been put forward to exclude the shoot meristem or apex from tissues considered to be transformable by *Agrobacterium*.

Our success in the transformation of an elite cultivar of petunia (29) using cocultivation of isolated shoot apex explants with *A. tumefaciens*, led us to apply this procedure to a diverse group of dicotyledonous crop species. The applicability of using the apical meristem in *Agrobacterium*-mediated gene transfer has been subsequently substantiated in petunia and sunflower (PC Sijmons, Mogen International NV, personal communication) and by Hussey and colleagues (18), who reported tumor and hairy root formation in shoot apices of pea inoculated with *A. tumefaciens* and *Agrobacterium rhizogenes*. The availability of the binary vector *Agrobacterium* EHA1 containing the supervirulent pTiBo542 helper plasmid (16) led us to examine shoot apex inoculation in monocotyledonous crop species.

This report describes the transformation of corn (Z. mays L.) shoot apices with *A. tumefaciens* EHA1 containing the binary construct pGUS3, the regeneration of normal and fertile plants from shoots, and the detection of the transferred genes in F_1 progeny from two of the original transformed plants and in an F_2 from one of the original plants. To our knowledge, this is the first report to show *Agrobacterium*-mediated transformation of a monocot by inoculation of isolated shoot apices and inheritance of the transferred DNA.

MATERIALS AND METHODS

Preparation of Plant Material

The hybrid *Zea mays* L. variety Funk's G90 was used because of local availability of commercially processed seed and low incidence of seed-borne contamination after surface sterilization. To our knowledge, this hybrid is not one of the genotypes that is noted to be regenerable *in vitro* from protoplasts or callus. Seeds were rinsed 15 min in running tap water, surface-sterilized in 20% Clorox for 15 min, rinsed three times with autoclaved water, allowed to imbibe water 2 to 4 h, and placed on agar-solidified medium (1% w/v) (pH 5.7), containing 1× MS halide stock (23), to germinate. Seeds were incubated at 30°C in the dark 1 to 3 d.

Isolation of the Shoot Apex

Shoot apices were removed from the germinating embryo or seedling. Care was taken to first isolate the apex from the embryo, followed by removal of tissue proximal to the base of the meristem region. Primordial and elongating leaves were not removed (Fig. 1). The outer dimensions of the isolated apex ranged from approximately 1.0 × 0.3 mm. Because the position of the apical meristem could not be seen directly during isolation, the size range of the explants is approximate.

Bacterial Strain, Plasmid, Culture Conditions

Agrobacterium tumefaciens EHA1, a strain derived from A281 containing the supervirulent pTiBO542 plasmid (16), and the binary Ti construct, pGUS 3 (Fig. 2). The construction was a *Hin*dIII to *Eco*RI fragment from pGUS (20) placed in pARC 8 (26), by T. McKnight, Biology Department, Texas A&M University. This fragment contained CaMV 35S fused with the gene coding for GUS[2] (20) and a nopaline synthase polyadenylation site (NOS-TER). The construction of pARC8 containing the chimeric gene NOS/NPT from pNEO105 and

[2] Abbreviations: GUS, β-glucuronidase; MS, Murashige and Skoog; MUG, methylumbelliferone; NPT II, kanamycin resistance gene; PCR, polymerase chain reaction; CaMV 35S, the 35S promoter of cauliflower mosaic virus; bp, base pair; kb, kilobase pair.

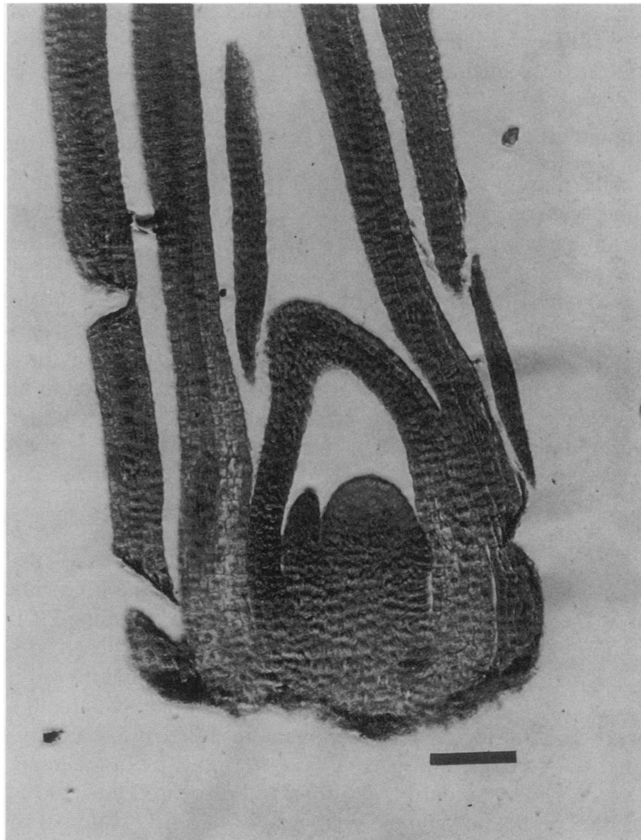

Figure 1. Thin section of the corn apex which includes the meristem and expanding leaves. Bar = 0.1 mm.

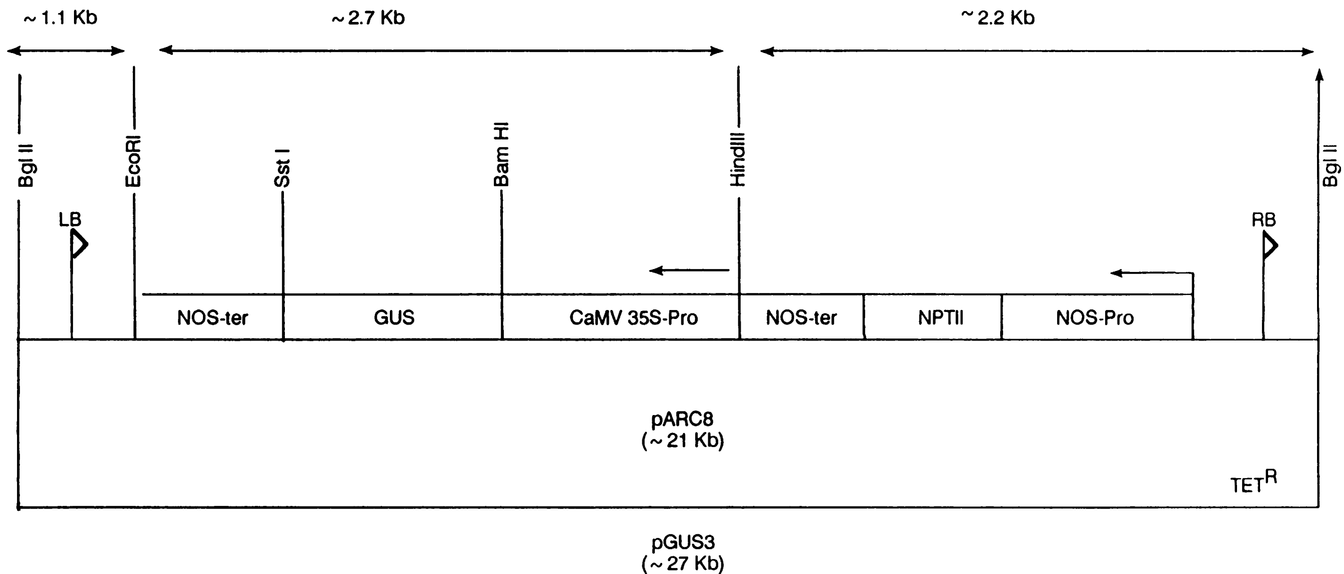

Figure 2. Map of pGUS3. The plasmid contains the 35S promoter of cauliflower mosaic virus fused with the gene coding for GUS and a nopaline synthase polyadenylation site. The construction is a *Hin*dIII to *Eco*RI fragment in pARC8 by T. McKnight, Biology Department, Texas A&M University. Both the EcoRI and HindIII sites are unique in pGUS3.

pARC4 was described by Simpson and colleagues (26). This construct was designed for efficient expression in tobacco and petunia but was employed because of availability in EHA1. The efficiency of expression of pGUS3 in monocot tissues was unknown at the time. Agrobacteria were grown on agar-solidified LB media containing 5 mg/L tetracycline.

Cocultivation and Induction of Agrobacteria

After 3 to 5 d of growth on LB media containing tetracycline, bacteria were scraped from the culture plate and a slurry was made using 0.5 mL aqueous solution of 10 mM nopaline (30) and 30 μM acetosyringone (27). The bacterial suspension was applied directly to the cut base of the shoot apex. A single invasion into the meristematic region through the inoculated shoot base with a hypodermic needle (25⅝ gauge) was performed to place bacteria in the shoot meristem. Induction of the bacteria was allowed to occur during the 2 d of contact with the plant tissue.

Plant Tissue Culture Media and Culture Conditions

The inoculated shoot apices were cultured on a basal medium containing the MS salt formulation and the following in mg/L: 0.1, kinetin; 100, *m*-inositol; 40, thiamine-HCl; 15,000, sucrose; 8,000, TC agar (Hazelton Biologicals), pH 5.7. Kinetin was included in the initial culture medium only. At specific stages in the reculture cycle, supplements to this basal medium included the following in mg/L: 7.5, kanamycin; 500, carbenicillin. Media were sterilized by autoclaving 25 min under standard conditions and dispensed into 100 × 20 mm sterile, polystyrene petri plates at 25 mL/plate; 10 to 15 explants were cultured per plate. Cultures were wrapped with parafilm, incubated under a combination of Gro-lux (GE) lights and full-spectrum Vita-lite (Duro-test), 60 to 80 $\mu E \cdot m^{-2} s^{-1}$, 16-h day at room temperatures ranging from 22 to 25°C night/26 to 28°C day.

Reculture Schedule

Inoculated shoot apices were initially placed on a medium containing 0.1 mg/L kinetin. Shoots remained on this medium in contact with *Agrobacterium* for 2 d, then were transferred to a hormone-free medium containing 500 mg/L carbenicillin. After 3 d of culture on this medium, apices were transferred to hormone-free medium containing 7.5 mg/L kanamycin and 500 mg/L carbenicillin for 7 to 10 d, then transferred to a kanamycin-free media containing 500 mg/L carbenicillin. Tissues were then recultured at least every 10 d to renew the medium and antibiotic and to exchange the culture atmosphere. Shoots rooted spontaneously on this medium within 1 to 3 weeks after isolation of the apex. Plants were transferred to soil approximately 4 weeks from date of isolation.

Transfer of Plants from Tissue Culture

Growing conditions were as follows. Plants were transferred from culture to 1 gallon pots containing Metro-mix 352 (Terra-lite), fertilized with Peat-Lite Special 15-16-17 (Peters). Plants were grown under a 16-h d regime of full spectrum fluorescent Vita-lites (Duro-test Co.) (500–700 $\mu E \cdot m^{-2} s^{-1}$) or metal halide high intensity lamps (900–1100 $\mu E \cdot m^{-2} s^{-1}$), with little natural light supplementation. Temperatures ranged from 22 to 28°C. These lighting conditions were minimal for corn but allowed the regenerated plants to grow 3 to 5 feet and flower. Male flowers matured 2 to 3 weeks before the female flowers were receptive, therefore self-pollination was impossible. Pollination was achieved using fresh

pollen from available sources (pollen and pollination, courtesy of M. Boyle and G. Cobb).

Germination of Progeny

The F_1 seed of one plant designated C_1 were disinfected as described previously. Embryos were removed from the seed to break dormancy and germinated *in vitro* using the hormone-free basal MS medium described. *In vitro* germination of progeny embryos was not necessary if senescing plants bearing seed were allowed to dry. This was the case for plant designated as C56. Seedlings were transferred to pots and grown as described. Due to plant crowding and relatively low light intensity, many of the ears of these plants were empty of seed; however, F_3 generations have been obtained from C1, and F_2 generations from C56. None of these plants could be self-pollinated because of the wide difference in maturity of tassels and ears under the existing conditions.

Identification of Transformed Plants

Putatively transformed plants were identified on the basis of the fluorescent GUS assay (20). Chloramphenicol (20 mg/L) was added to the assay buffer and assays were incubated overnight. Regenerated plants were assayed for presence of GUS activity. Leaves from different areas of these primary transformants were assayed because of the possibility that the plants were chimeric for the transferred traits. All surviving regenerated plants were hand pollinated. The progeny were assayed for GUS. Assays were not corrected for protein concentration; however, uniformly sized tissue samples were used. Assays of the leaf tissues from the F_1, F_2, and F_3 plants were also performed. Plants which did not appear to have GUS were discarded.

DNA Isolation and Analysis of Gene Incorporation

Plant tissue was frozen and stored at −20°C or −80°C. For extraction, frozen tissue was cooled with liquid nitrogen, then ground to a powder using an electric coffee mill (Braun). Ground tissue was transferred to prewarmed (65°C) extraction buffer and DNA was extracted using a procedure described by Dellaporta and colleagues (6). DNA concentrations were estimated fluorimetrically (Kontron), using Hoechst dye 33258 at 365 nm excitation and 460 nm emission wavelengths. Five to 20 μg DNA were restricted with *Hin*dIII or *Eco*RI (Promega) and separated on a 0.8% agarose gel by electrophoresis at 30 V overnight. DNA was depurinated using UV irradiation and transferred onto a nylon membrane (Gene Screen Plus, DuPont) according to manufacturer's instructions. After transfer, the membrane was NaOH treated, neutralized, air dried as directed and UV irradiated. Prehybridization and hybridization were carried out using 50% formamide at 42°C; final wash was 0.5 to 1.0 h in 0.1 × SSC and 0.2% SDS at 65°C.

PCR

Two sequences with the GUS coding region were chosen to amplify a 250 bp fragment within the gene: the 5′ primer (CTTTAACTATGCCGGAATCCATCG), located in the GUS coding region 489 bp downstream of the translation initiation site (ATG); the 3′ primer (TAACCTTCACCCGGTTGCCAGAGG), located 720 bp from the initiation site. One hundred picograms of pGUS3 were used as positive control; 200 ng of total corn DNA were used. Samples were prepared using a gene amplification kit (Promega); cycling was controlled by an Ericomp thermal cycler, programmed with the following conditions: denaturation, 94°C for 30 s; annealing, 55°C for 30 s; extension, 72°C for 60 s. Samples were subjected to 35 cycles. Amplified DNA was separated by electrophoresis in 2.0% agarose gels using Phi × 174 mol wt marker (BRL) and the identity of the major DNA fragments were determined by blot hybridization against ^{32}P-labeled GUS *Bam*HI and *Sst*I fragment. Two sequences of the NOS-NPT II gene were used: 5′primer (CCCCTCGGTATCCAATTAGAG), located in the NOS promoter region 33 bp-5′ of (ATG); the 3′primer (CGGGGGTGGGCGAAGAACTCCAG) the 3′ flanking region of the NPT II gene 150 bp-3′ of translation stop signal. The conditions used were: denaturation, 94°C for 1 min; annealing, 55°C for 1 min; extension, 72°C for 2 min. Samples were subjected to 25 cycles. Amplified DNA was separated by electrophoresis in 0.8% agarose gels using 1 kb ladder (BRL). The gel was blotted and hybridized as above. Final wash conditions were 0.1 × SSC and 0.1% SDS at 68°C for 45 min; exposure was overnight at −80°C.

Probes

The probe for GUS was made from the *Bam*HI and *Sst*I restriction fragment of the pPUR plasmid (1870 bp fragment) (courtesy T. Hodges, Purdue) and, for NPT, the neo gene (courtesy T. Hodges). DNA probes were prepared using ^{32}P and the Promega Prime-A-Gene kit. Specific activity averaged 100×10^6 counts per blot. Blots were allowed to hybridize 2 to 3 d. Film was exposed 1 to 5 d at −80°C.

RESULTS

Plant Regeneration

Shoots developed rapidly and roots formed spontaneously after 1 to 4 weeks of culture. In the course of the first two experiments described here, 25 plants were recovered from culture, of these, 15 survived to flower and produce progeny, six of these were positive for GUS (Table I). Plants were ready to transplant into soil after 4 weeks of culture. Plants exhibited normal phenotype and were fertile. Five variegated plants were produced and were transplanted to soil but did not survive.

GUS Fluorimetric Assays

Due to lower than expected GUS activity, assays of progeny leaf tissue from mature plants were performed overnight (15–18 h). Table II summarizes the data obtained from F_1 of C56 and F_2 of C1 from GUS assays, PCR gel blot and Southern analysis. The assay buffer included an antibiotic to reduce the possibility of induction of GUS in contaminating bacteria. Because of the low levels of expression which required lengthy incubation, these assays were only used to identify plants not

Table I. *Summary of GUS Activity in F_1, F_2, and F_3 of C1; F_1 and F_2 of C56*

In experiment 1, plants were cultured June 1988, and the primary transformant, C1, was hand-pollinated (Pioneer 3732 pollen) in October 1988. Experiment 2 plants were cultured November 1988, and the primary transformant, C56, was hand-pollinated (Funk's G90) in February 1989.

Expt. No.	No. Apices Inoculated	Immature Plants Recovered	Mature Surviving Plants	GUS^+/Total	Primary Transformant Designation	No. F_1 Plants	F_1 GUS^+/Total	F_1 Producing F_2[a]	No. F_2 Plants	F_2 GUS^+/Total	F_2 Producing F_3[b]	No. F_3 Plants[c]	F_3 GUS^+/Total
1	25	3	1	$1^+/1$	C1	42	$19^+/42$	C-11	2	$1^+/2$			
								C1-19	5	$2^+/5$	C1-19-176	2	$1^+/2$
								C1-20	1	$1^+/1$	C1-20-181	7	$3^+/7$
								C1-22	1	$1^+/1$	C1-22-182	7	$4^+/7$
2	55	22	14	$5^+/14$	C56[d]	37	$17^+/37$	C56-185	4	$0^+/4$			
								C56-188	89	$12^+/89$			
								C56-189	28	$8^+/28$			
								C56-191	37	$20^+/37$			
								C56-199	50	$27^+/50$			
								C56-200	15	$1^+/14$			
								C56-204	6	$4^+/6$			
								C56-207	79	$34^+/79$			
								C56-213	65	$21^+/65$			
Totals:	80	25	15	$6^+/15$									

[a] Surviving plants which produced an F_2 generation. [b] Surviving F_2 which produced an F_3 generation. [c] Surviving mature plants. [d] C-56 was the only plant of the GUS positive group in Experiment 2 that produced seeds.

to the onset of senescence, plants that appeared to have GUS activity were harvested and stored frozen at −20°C or −80°C.

PCR Amplification

DNA from six F_1 of C56 and from one F_2 of C1 were subjected to PCR amplification of a 250 bp fragment within the GUS coding region (Fig. 3A). In a separate amplification, primers for a 1000 bp fragment within the NOS/NPT II gene were used (Fig. 3B). Amplification of the expected fragments were achieved from the DNA of four, C56 F_1 as well as from the DNA of an F_2 of the C1 family. Identity of the amplified DNAs was established by blot hybridization to ^{32}P-labeled Neo or GUS probes. Both GUS and NPT II gene fragments were amplified in: C56–185, C56–188, C56–192, and C1-22–182. A positive signal with NOS/NPT II was obtained with C56–183 and C56–190 but the signal was weak when this DNA was amplified using the GUS fragment. These results indicate the presence of both GUS and NPT II in the progeny of two original transformants.

DNA Hybridizations

Two transformed plants have been identified and transformed progeny of these plants have been recovered. Data are presented in this report on one of these families, designated C56. Despite inconclusive preliminary GUS assays which showed low enzyme activity, isolated and restricted DNA from parental and F_1 plants hybridized to NPT and GUS probes. The restriction patterns of DNA with homology to NPT and GUS probes within the F_1 of C56 were identical, indicative of transformation and inheritance from a common source.

DNA extracted from the F_1 progeny of plant C56 and digested with *Eco*RI hybridized first with GUS (Fig. 4A) and then rehybridized with NPT (Fig. 4B). The restriction pattern to both probes is consistent within this progeny set and is the same as that observed in the C56 parent. The restriction pattern differs from that of the *Eco*RI and *Hin*dIII digests of genomic DNA extracted from the transforming *Agrobacterium* containing pGUS3 (Fig. 4, A, B).

Although an *Eco*RI restriction site between GUS and NPT II genes is not indicated in pGUS3 (Fig. 2) or in the *Eco*RI digest of EHA1 (Fig. 4, A, B) such a site was apparent in both C56 and F1 progeny which carry the genes (Fig. 4, A, B). The basis for this difference is not known at this time, but we do not think that this is inconsistent with a conclusion of transformation and inheritance.

Because activity of GUS was low, requiring overnight incubation the pattern of inheritance in the outcrossed F_1 progeny could not be definitively established. However, based on low GUS activity, the pattern appeared to be 1:1 in the C56 family (17 of 37 plants), and in the progeny of C1 (19 of 42) plants (Table I). This is the predicted Mendelian inheritance pattern in a heterozygous × homozygous cross. The results from GUS assays, PCR and Southern analysis are summarized in Table II.

Table II. *Assay for GUS activity and presence of GUS and NPT genes in F_1 Plants of C56 and F_2 plants of C1*

Generation Designation	Plant Designation[a]	Visual Score	Relative Fluorescence[b]	MUG[c]	PCR GUS/NPT[d]	DNA Digest and Gel Blot GUS/NPT[e]
			%	μM		
Controls	Corn Funk's G90	–	0.0	0.0	–/–	–/–
	EHA1 containing pGUS3	++++	100.0	>1.0	+/+	+/+
F_1 of C56	C56-183	+	30.5	0.3	?/+*	
	C56-184	–	–8.0	0.0	–	
	C56-185	–	–19.4	0.0	+/+*	
	C56-186	–	–15.3	0.0	–	
	C56-187	+	–18.0	0.0	+/+	
	C56-188	+++	52.8	0.53	+/+*	+/+*
	C56-189	++	14.1	0.14	?/0*	
	C56-190		–8.3	0.0	?/+*	+/+*
	C56-191	–	–12.2	0.0	–	
	C56-192	+++	141.5	1.42	+/+*	
	C56-193	–	–17.9	0.0	–	
	C56-197	–	–9.3	0.0	+/+	
	C56-199	++	51.5	0.52	+/+	–/–*
	C56-200	++	82.3	0.82		
	C56-201	–	–21.6	0.0		
	C56-202	++	44.8	0.45		
	C56-203	++	20.5	0.21	+/?	
	C56-204	+	9.0	0.90	+/+	
	C56-205		10.4	0.10	+/+	
	C56-206	+	33.9	0.34	–	
	C56-207		17.2	0.17		
	C56-208	++	95.2	0.95		
	C56-209	+	27.7	0.28	+/?	
	C56-212	–	–7.5	0.00		
	C56-213	+++	52.5	0.53	+/+	+/+*
F_2 of C1	C1-11[f]-214	++	15.5	0.15		
	C1-19-176	+++	64.4	0.64		
	C1-19-179	++	45.9	0.46		
	C1-20-181	+	91.1	0.91		
	C1-22-182	+	–11.8	0.0	+/+*	

[a] Plant designations: Plants were given unique numbers when transferred to soil. Numbers denoting the female ancestry of the plant were added. C denotes corn, therefore, C1 was the first corn plant to be transferred to soil; C56, the 56th plant to be transferred to soil; C1-19-176 indicates plant #176 is an F_1 of #19 and an F_2 of C1. [b] Values corrected for overnight incubation of negative control = 37.1%. This value was subtracted from sample fluorescence producing some negative values. [c] 100% fluorescence = 1 μM MUG; negative values converted to 0.0. [d] PCR and probe hybridization, * = Figure 3. [e] DNA digest and gel blot, * = Figure 4. [F] F_1 plants, C1-11, C1-19, C1-20 and C11-22, were positive for GUS and NPT after DNA digest and gel blot.

DISCUSSION

Transgenic corn plants have been produced using *Agrobacterium*-mediated DNA transfer to cells within the isolated shoot apices. Some of the outcrossed progeny of these plants carry the transferred DNA. The cultivar of corn used in this study, the hybrid Funk's G90, was chosen only because of a low level of seed-borne contamination and local availability, not out of consideration of tissue culture potential. These studies have focused on pairing an almost universal method of plant regeneration, which is not limited to genotype or cultivar (21, 22), with *Agrobacterium*-mediated plant transformation. Utilization of the disarmed *Agrobacterium* EHA1 helped to circumvent the problems concerning selection of appropriate *Agrobacterium* strains and the effect of endogenous plant hormone interactions with *Agrobacterium* encoded hormones and tumor phenotype. Two enhancers of transformation, acetosyringone (27) and nopaline (30), were used; however, the role of these compounds in the success or efficiency of the transformation is not known at this time.

The construct pGUS3 was used because of availability in EHA1 during the summer and fall of 1988 when these experiments were underway. Since that time, constructions using promoters isolated from maize have improved gene expression in corn. The first intron of maize alcohol dehydrogenase 1 (Adh 1) placed adjacent to the 35S promoter was used by

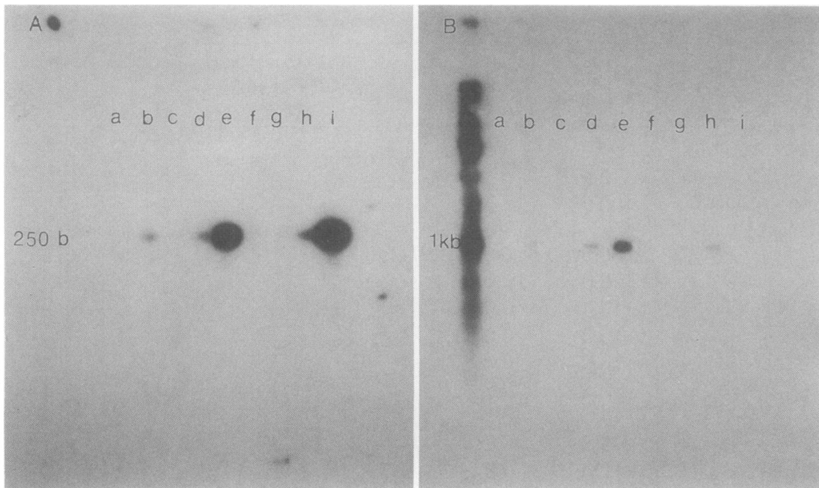

Figure 3. A, Blot transfer of DNA from six F_1 progeny of C56 and one F_2 of Cl after PCR amplification of a 250 bp GUS fragment and gel electrophoresis. DNA was transferred and hybridized to ^{32}P-labeled GUS 1.8 kb probe. DNA fragments of the predicted 250 kb size hybridized with the GUS probe in five of the eight samples. Mol wt marker Phi × 174 is not shown. Lane a, untransformed corn DNA; b, C1–22–182, an F_2 of Cl; lanes c–h, F_1 of C56; c, C56-183, d, C56-185, e, C56-188, f, C56-189, g, C56-190, h, C56-192; lane i, 1.8 kb GUS fragment is a positive control. B, Blot transfer as in A after PCR amplification of a 1000 bp fragment containing nos promoter and 3′ flanking region of the NPT II gene. DNA was hybridized to ^{32}P-labeled neo probe. Mol wt 1 kb marker is shown at left. Fragments of the predicted 1000 bp size hybridized with the neo probe in five of the seven samples. Lanes are as in A except that lane i, 1.8 kb GUS fragment, is the negative control.

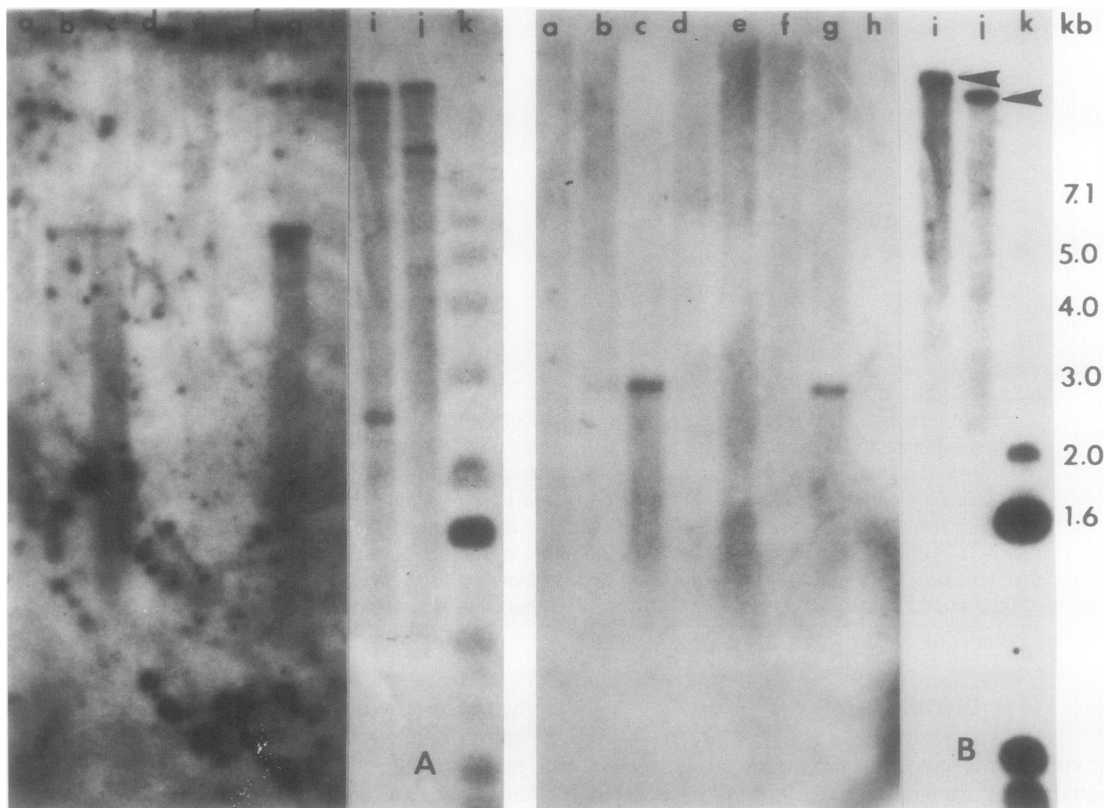

Figure 4. A, Southern blot of F_1 progeny of plant C56; GUS probe. Detection of DNA fragments containing GUS coding sequences after *Eco*RI digestion of transformed and untransformed corn DNA. Lane a, untransformed corn DNA; lane b, C56-188 DNA; lane c, C56-190 DNA, lanes d–f, 5, 10, 13 μg DNA from C56-199; lane g, C56-213 DNA; lane h, untransformed corn DNA; lane i, *Eco*RI digest of EHA1 DNA containing pGUS3; lane j, *Hin*dIII digest of EHA1 DNA containing pGUS3, lane k, BRL 1 kd ladder. B, Southern blot of F_1 progeny of plant C56; NPT probe. Rehybridization of blot in A. Detection of fragments containing NPT coding sequences after EcoRI digestion of DNA from: lane a, untransformed corn; lane b, C56-188, lane c, C56-190; lanes d–f, C56-199; lane g, C56-213; lane h, untransformed corn; lane i, EHA1 containing pGUS3; lane j, *Hin*dIII digest of EHA1 containing pGUS3; lane k, BRL 1 kb ladder. The two high mol wt bands are the predicted fragments; the lower mol wt fragments were not expected.

both DeKalb and USDA/Monsanto groups (7, 10), as well as other sequences, to enhance gene expression. Since our construct had not been modified for corn, efficiency of gene expression and the function of the 35S and nos promoters in corn tissues and the meristem was not known. Under these conditions, our concern was that a rigorous antibiotic selection would obscure a successful transformation event by killing the meristem. The assumption that transferred genes were not efficiently expressed was also the basis for accepting overnight incubation times for GUS and relatively low levels of activity (when compared to transformed petunia, data not shown) to indicate potential transformants. The burden of proof under these circumstances rested in detection of the transferred genes in the genome of the regenerated plants and in the progeny. Due to this inherent experimental restriction, questions as to sectoring in the meristem, developing plants, ears and tassels could not be addressed in this study. Because of low GUS activity and the destructive nature of the assay, we were unable to track chimeras in regenerating plants. However, the first of these plants to produce progeny (C1) appeared to have GUS activity only in the lower nodes which included the node from which the ear developed. The second of these plants (C56), was probably also chimeric. This assumption is based on the low signal obtained after hybridization with the GUS probe (data not shown). It is impossible for us to speculate at this time on the different chimeras that could develop, especially in a plant as developmentally complex and genetically active as maize. Irish and Nelson (19) reported that after isolation and culture of shoot apices of corn, the original number of nodes normally present in the mature embryo were regenerated by the isolated apices. This determinant aspect of corn development may play an important role in the use of the shoot apex for gene transfer in maize.

Although *Agrobacterium*-mediated transformation using the method described may be coincidentally limited to the cultivar used in this study, we believe that this possibility is unlikely. We propose that this simple approach can be used to transform other corn cultivars and, with modification, other monocot species. In summary, this method has produced transformed plants and transgenic F_1 plants of *Z. mays* using *Agrobacterium*. Plant regeneration was from the preexisting shoot apical meristem and sufficiently rapid to allow transfer to soil approximately 4 weeks from isolation and inoculation.

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