Recovery of Primary Transformants of Valencia-type Peanut Using *Agrobacterium tumefaciens*[1]

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ABSTRACT

Four regenerable seedling explants of peanut cv. New Mexico Valencia A, two different strains of *Agrobacterium tumefaciens*, and two transformation protocols were used in peanut genetic transformation experiments. The putative transformation-competent cell types were identified by transient expression of the β-glucuronidase (GUS) reporter gene, and were compared to the regeneration-competent cell types identified histologically in the four explant systems. One primary transformant plantlet and two primary transformant shoots were recovered from petiolule-with-blade-attached explants inoculated with *Agrobacterium* strain CKS (A208:pTi37ASE X pEMZ) following a long co-cultivation time on the regeneration medium and using low selection pressure for kanamycin resistance. The leaves of the primary transformants expressed nopaline accumulation used as a marker gene, and the engineered 35S-15kD zein protein coding sequence as determined by western blot. The results from these experiments may be useful for developing reliable methods of genetic transformation for valencia-type peanut.

Key Words: Peanut, transformation, *Agrobacterium tumefaciens*, zein protein, recombinant DNA.

The production of transgenic plants using *Agrobacterium* vectors has been achieved in a number of crop species (Gasser and Fraley, 1989). However, despite the success with soybean (Hinchee *et al.*, 1988), and recovery of transformed calli of pea (Lulsdorf *et al.*, 1991), *Vigna unguiculata* (Garcia *et al.*, 1986) and *Vicia narbonensis* (Pickardt *et al.*, 1991), the grain legumes generally remain recalcitrant to transformation by this method. Reasons for this recalcitrance may be that the *in vitro* regeneration systems are not suitably efficient, or that the regeneration-competent cells in the treated explants are not competent for transformation.

Identification and characterization of regeneration-competent cells and transformation-competent cell types in different regenerable explants may be useful for predicting transformation efficiency using *Agrobacterium* vectors. Several reliable regeneration systems in peanut have been established in recent years. Somatic embryogenesis was obtained from immature zygotic embryos (Hazra *et al.*, 1989; Ozias-Akins, 1989; Sellars *et al.*, 1990) and mature embryo explants (McKently, 1991). Shoot organogenesis was achieved using immature leaflets (Pittman *et al.*, 1983, 1984; McKently *et al.*, 1991; Cheng *et al.*, 1992), and from various seed and seedling explants (McKently *et al.*, 1990; Cheng *et al.*, 1992). However, regeneration-competent cell types within peanut explants have been characterized only with immature leaflet explants (Pittman *et al.*, 1983). Few studies have been conducted to identify transformation-competent cell types within regenerable explants using a reporter gene, such as β-glucuronidase (GUS) gene expression (Janssen and Gardner, 1989).

There has been limited study of gene transfer into peanut using *Agrobacterium*. Lacorte *et al.* (1991) induced hormone-autonomous tumors on peanut seed and seedling explants by *Agrobacterium* strain A281. These tumors were opine positive, contained DNA homologous to the T-DNA of the inciting strain, and expressed the binary vector reporter genes encoding GUS and neomycin phosphotransferase II (NPT II). However, there were no transgenic plants recovered from these transformed tumor tissues. Transgenic plants have been obtained from runner-type peanut following microprojectile bombardment of embryogenic callus (Ozias-Akins *et al.*, 1993).

In this report, we characterize the regeneration-competent and putative transformation-competent cell types in several regenerable seedling explants of peanut, used in part with the aim of developing suitable protocols for efficient transformation and regeneration of valencia-type peanut plants. We also report the production of transgenic peanut shoots and plantlet using *A. tumefaciens*, as evidenced by the expression of transgenes in the primary transformants.

Materials and Methods

Plant Material and Explant Preparation. Peanut cultivar New Mexico Valencia A (*Arachis hypogaea* L.) was used in all experiments. Seed germination, explant preparation, and shoot organogenesis procedures were as described by Cheng *et al.* (1992). The four regenerable seedling explants used were sections of immature leaflets, petioles and epicotyls, and petiolules-with-blade-attached.

Histological Analysis of Regeneration-Competent Cell Types. Histological procedures to identify regeneration-competent cell types in the four seedling explants were based on Wagley *et al.* (1987). Samples selected from 6, 12, or 20 d regenerating explant cultures were fixed in formalin acetic acid (FAA) (95% ethanol 50 mL, glacial acetic acid 5 mL, formalin 10 mL, and H_2O 35 mL) for 24 h, and then infiltrated through an ethanol series (60, 50, 45, and 25%) and a t-butanol series (5, 10, 35, 55, 75, and 100%). Dehydrated samples were embedded in paraplast and serially sectioned at a thickness of 10 µm. Sections were mounted on glass slides, stained with 1% toluidine blue, washed serially in ethanol and xylene, and mounted with permount.

Agrobacterium *Strains.* *Agrobacterium tumefaciens* strain C58C1:pGV3850X pGS (provided by J. Kemp, Plant Genetic Engineering Laboratory [PGEL], NMSU) was used in transient GUS assay experiments to identify putative transformation-competent cell types in the four seedling explants. The GUS coding sequence (Jefferson, 1987) was inserted into pMON316 (Rogers *et al.*, 1987; gift from S. Rogers, Monsanto Co., St. Louis, MO) between the CaMV 35S promoter and the NOS 3' termination region. This construct was chosen because the GUS gene exhibited very low expression in the bacterium.

Two strains of *A. tumefaciens* were used in stable transformation experiments. The first strain was C58C1:pGV3850 X pSF22 (gift from Susan Fender, PGEL). pSF22 was derived from pMON316, and included a hygromycin resistance gene inserted between the 35S promoter

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and the NOS 3' termination region, and the entire β-phaseolin gene (Reichert, 1989) inserted behind the NOS 3' sequence. The second strain was CKS (A208:Ti37ASE X pEMZ). pEMZ was derived from pMON316 (Bagga *et al.*, 1995). The 15-kD zein coding sequence (provided to J. Kemp, PGEL, from Mycogen, Inc.) was cloned between the 35S promoter and the NOS 3' termination region.

Agrobacterium *Culture and Co-cultivation.* The different strains of *Agrobacterium* were cultured with vigorous agitation overnight at 28 C in liquid LB medium supplemented with different antibiotics: strain C58C1:pGV3850 X pSF22 with 10 mg/L rifamycin, 100 mg/L ampicillin, 25 mg/L spectinomycin, and 25 mg/L streptomycin; strain CKS with 50 mg/L spectinomycin, 20 mg/L kanamycin and 25 mg/L chloramphenicol; strain C58C1:pGV3850 X pGS with 50 mg/L ampicillin, and 25 mg/L spectinomycin. Explants were immersed in *Agrobacterium* suspensions for 10 min, then blotted dry with sterile filter paper. Except where noted otherwise, treated explants were placed on semi-solid Murashige and Skoog (1962) basal medium (MS) without hormones, and incubated at 28 C with a 16 hr photoperiod (15 μmol/m²/s). After 2 d co-cultivation, explants were transferred to fresh MS medium with 400 mg/L claforan to eliminate the *Agrobacterium*. Explants were washed in 200 mg/L claforan solution for 30 min every 2 d.

Transient Assays to Localize Putative Transformation-Competent Cell Types. Histochemical localization of GUS expression in peanut explants following co-cultivation with *Agrobacterium* was performed as described by Jefferson (1987). Explants cultured for a total of 4 d (2 d after a 2-d co-cultivation) were sampled for transient GUS assays. Sampled explants were fixed in 0.3% formaldehyde for 10 min, then submerged for 8 hr in 1 mM 5-bromo-4-chloro-3-indolyl-β-glucuronide (X-Gluc) solution including 50 mM sodium phosphate (pH 7). Samples for embedding were subsequently dehydrated through ethanol and t-butanol series as above. Embedded samples were sectioned to 10-12 μm thicknesses, dried, deparaffined, and mounted with permount.

Transformation, Selection and Plant Regeneration. Two protocols were tested in the stable transformation experiments. The first procedure utilized a general transformation protocol as developed for tobacco and tomato (Gasser and Fraley, 1989), characterized as having a short co-cultivation time and stringent selection. The second protocol involved a long co-cultivation time on the callusing medium or the regeneration medium and used low selection pressure. This alternate transformation protocol was developed after a series of preliminary experiments directly comparing high vs. low selection pressures and short vs. long co-cultivation times.

With the general transformation protocol, the inoculated explants were co-cultivated for 2 d on MS medium. Explants were transferred to regeneration media (MS + 10 mg/L N^6-benzyladenine [BA] + 1 mg/L 1-naphthaleneacetic acid [NAA]) where high selection pressure was imposed (>90% of the nontransformed explants were suppressed from regenerating in control experiments). Selection consisted of 60 mg/L kanamycin or 5 mg/L hygromycin for petiolule-with-blade-attached explants, 80 mg/L kanamycin or 6 mg/L hygromycin for epicotyl explants, and 100 mg/L kanamycin or 7 mg/L hygromycin for petiole explants. Responsive tissues were transferred to fresh selection media monthly for three to four cycles of selection.

In the alternate transformation protocol, the inoculated explants were co-cultivated in callusing medium (MS + 2 mg/L NAA + 0.5 mg/L BA) or regeneration medium (MS + 10 mg/L BA + 1 mg/L NAA) for 6 d, then transferred to media of the same composition where low selection pressure was imposed (70% of the nontransformed explants were suppressed from callusing or regenerating in control experiments). Selection consisted of 50 mg/L kanamycin or 3 mg/L hygromycin for petiolule-with-blade-attached explants, 60 mg/L kanamycin or 3 mg/L hygromycin for epicotyl explants, and 80 mg/L kanamycin or 5 mg/L hygromycin for petiole explants. After 1 mo of culture, the responsive explants were transferred to fresh media for further selection. After 2 mo of selection, responsive tissues were transferred to MS + 10 mg/L BA + 1 mg/L NAA + 2 mg/L gibberellic acid (GA) + 2 mg/L thidiazuron (TDZ) for an additional selection cycle during shoot regeneration and development. After a total of 3-4 mo of selection, responsive cultures were transferred to MS + 1 mg/L NAA for further shoot development and rooting.

Nopaline Assay and Western Blot Analysis. Nopaline synthesis was detected as described by Reichert (1989), using leaflet tissues at least once from each recovered shoot or plantlet. Western blot analysis was based on Reichert (1989) with a modification of the protein extraction (Bagga *et al.*, 1995). Young leaves were ground with a plastic rod in the presence of phosphate-buffered saline (PBS: 523 mg/L K_2HPO_4, 408 mg/L KH_2PO_4, 7 mg/L NaCl) in microfuge tubes. The suspension was centrifuged at 15,000 rpm for 15 min at 4 C. The supernatant was discarded and the pellet resuspended in 1 mg samples/10 mL zein cocktail (70% ethanol, 1% 2-mercaptoethanol, and 0.1% Triton X-100). The suspension was incubated at 65 C for 30 min, centrifuged and the supernatant air dried at room temperature. The proteins were subjected to electrophoresis in a 15% sodium dodecyl sulfate/polyacrylamide gel, electroblotted onto nitrocellulose, and subjected to immunoblot analysis with polyclonal antiserum to zein.

Results and Discussion

Comparison of Regeneration-Competent and Putative Transformation-Competent Cell Types. Selection of regenerable explants showing a good correspondence between regeneration-competent cell types and transformation-competent cell types may be important in the development of efficient *Agrobacterium*-mediated transformation systems. The regeneration-competent cell types in New Mexico Valencia A petiolule-with-blade-attached explants were found in this study to include primarily epidermal and cortical cells at the cut end of the explant, and vascular tissue cells adjacent to the cut end of the explant (Fig. 1). In epicotyl sections, shoot organogenesis arose primarily from epidermal cells and from small regions of the cortical cells and vascular tissue (data not shown; see Cheng, 1993). Adventitious buds developed primarily from epidermal cells and from some of the cortical cells near the surface of petiole explants, and from epidermal cells only in the case of leaflet explants.

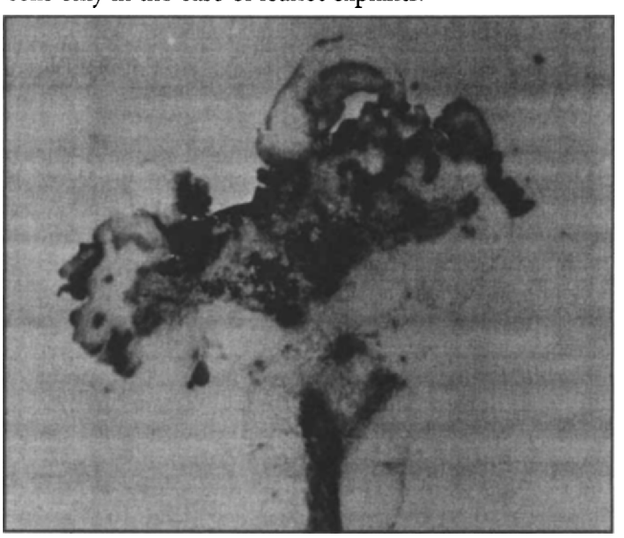

Fig. 1. Adventitious bud primordia formed from epidermal cells, cortex cells and a portion of the vascular tissue cells of a petiolule-with-blade-attached explant of peanut cv. New Mexico Valencia A, after 13 d culture on 25 mg/L BA + 1 mg/L NAA.

Transformation-competent cell types were identified putatively by using transient GUS expression in regenerable explants. This identification was tentative because it is recognized that there may not be a strict correspondence between transient and stable gene expression in host plant cells (Potrykus, 1990). The identifications made in our study were safeguarded by the use of a bacterial vector that showed very low bacterial expression of the GUS reporter gene. Putative transformation-competent cell types in cv. New Mexico Valencia A petiolule-with-blade-attached explants included the vascular cambium, part of the

surrounding phloem and part of the inner layer of the cortical cells (Fig. 2). Epicotyl explants exhibited transient GUS expression in some cortical and pith cells near the vascular tissues (data not shown; see Cheng, 1993). Transient GUS expression was found in a region of the outer layer of cortical cells and a small region of the epidermal cells in both petiole and leaflet explants.

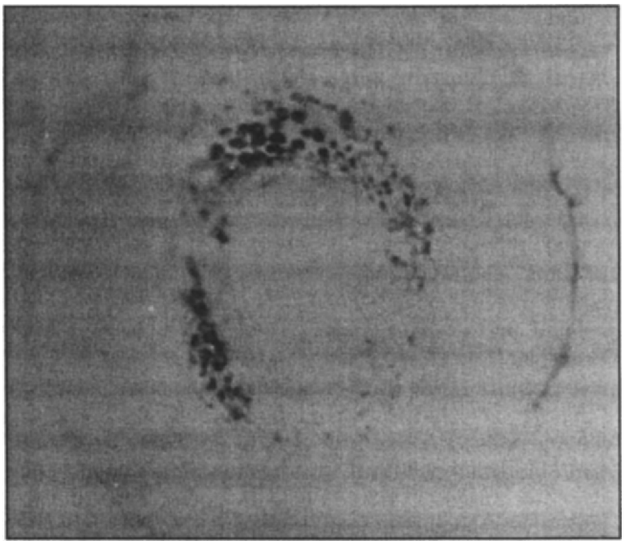

Fig. 2. Transient GUS expression in the region of the vascular cambium cells in a petiolule-with-blade-attached explant of peanut cv. New Mexico Valencia A, after 4 d total culture following co-cultivation with *Agrobacterium*.

These results indicated that different peanut explants exhibit different regeneration and transformation potentials. The correspondence between regeneration-competent and putative transformation-competent cell types showed limited overlap in all four explants, but perhaps was best in petiolule-with-blade-attached explants. Accordingly, stable transformation efforts were focused on the former explant system.

Stable Transformation Experiments. The results of the stable transformation experiments are summarized in Table 1. About 1850 explants were used in the general transformation protocol experiment. While numerous shoots and plantlets were recovered following selection, none of them proved to be transformed according to nopaline assays. However, it is possible that an insufficient number of explants were tested in this experiment to assure the recovery of a rare transformant.

About 5500 explants were used with each *Agrobacterium* strain in the protocol involving long co-cultivation time in callusing or regeneration medium with low selection pressure (Table 1). No transformants were recovered using strain C58C1:pGV3850 X pSF22, according to nopaline assays. Three primary transformants were obtained using petiolule-with-blade-attached explants treated with strain CKS. All three primary transformants were recovered following kanamycin selection; however, kanamycin and hygromycin selection were not compared directly in this experiment. The three primary transformants included one plantlet and two shoots identified initially by nopaline synthesis (Fig. 3). The positive control was derived from a

Table 1. Number of peanut cv. New Mexico Valencia A explants co-cultivated with two Agrobacterium strains, number of shoots or plants regenerated, and number of primary transformants obtained.

Explant type	Strain	Explants	Shoots	Plants	Trans-formants
		----------no.----------			
General protocol					
Petiolule + blade	C58C1	589	24	17	0
	CKS	603	51	14	0
Epicotyl	C58C1	167	19	15	0
	CKS	145	41	16	0
Petiole	C58C1	184	18	12	0
	CKS	162	21	14	0
Long co-culture time					
Petiolule + blade	C58C1	3200	21	16	0
	CKS	3410	32	7	3
Epicotyl	C58C1	610	27	10	0
	CKS	593	18	4	0
Petiole	C58C1	1230	19	11	0
	CKS	1301	28	8	0
Leaflet	C58C1	459	20	21	0
	CKS	442	19	7	0

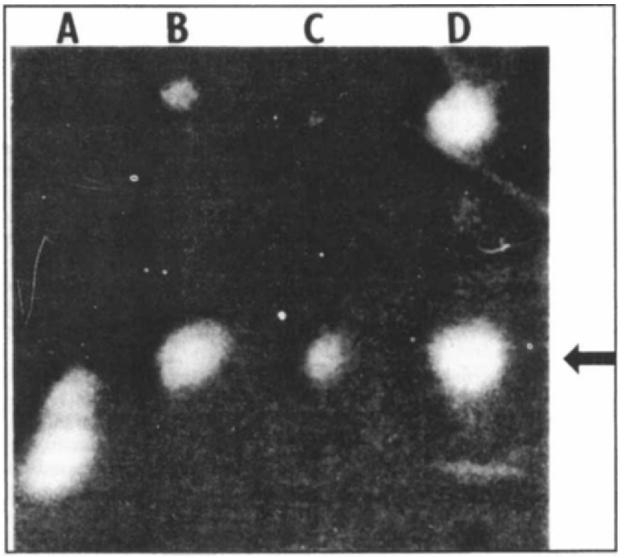

Fig. 3. Nopaline assays of regenerated peanut cv. New Mexico Valencia A shoots or plantlet transformed with *Agrobacterium* strain CKS using the procedure involving long co-cultivation time on callusing or regeneration medium and low selection pressure (A: positive control, stored analytical reagent grade nopaline; B, C: transformed shoots; D: transformed plantlet).

solution of authentic nopaline stored in the refrigerator. Following storage the nopaline reagent yielded two spots in this assay that migrated closely together, while the nopaline produced by the transgenic tissue yielded a single spot that migrated to a slightly different position. The negative controls (nontransformed plantlets; not shown) all showed only the upper spot in the figure, which is not related to nopaline.

The three primary transformants were confirmed by assay for zein protein synthesis in leaflet tissues using dot blot analysis. All three primary transformants were positive for zein gene expression (data not shown; see Cheng, 1993). No cross-reaction was detected from the nontransformed leaflet tissue controls; the positive control included con-

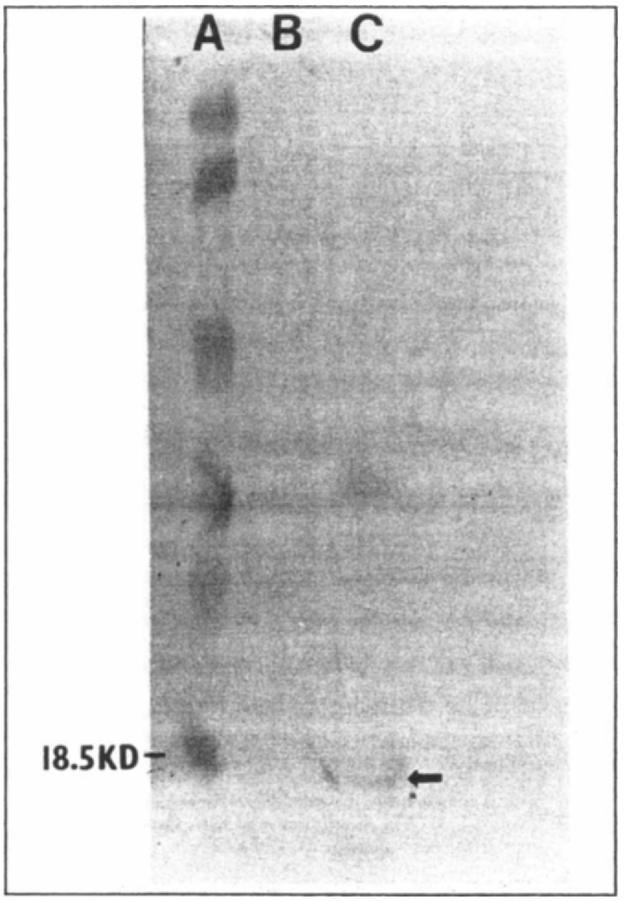

Fig. 4. Western blot assay for detection of zein protein expression in a leaflet of the transformed plantlet of peanut cv. New Mexico Valencia A (arrow identifies the 15-kD zein location; A: molecular weight markers; B: negative control, nontransformed plantlet; C: transformed plantlet).

firmed transgenic tobacco leaves expressing the zein gene. The primary transformant plantlet was further assayed for zein expression in the leaflet using western blot analysis (Fig. 4). There was no signal present in nontransformed control peanut leaflets; the positive control (not shown) consisted of confirmed transgenic tobacco leaves expressing the zein protein. A 15-kD zein protein was detected in the tested leaflets of the primary transformant. A polypeptide with higher molecular weight than geniune 15-kD zein but recognized by anti-zein sera was also detected in the primary transformant. A similar additional polypeptide recognized by anti-zein sera also was reported in tobacco seeds transformed with the 15-kD zein gene (Hoffman *et al.*, 1987).

The primary transformants were recovered from only one of the combinations of three factors under study involving four explants, two *Agrobacterium* strains and two transformation protocols. This result suggests that there is a complex interaction among these three factors. This interaction may be explained partially by the relationship between regeneration-competent and putative transformation-competent cell types in the four regenerable explants. The combination of petiolule-with-blade-attached explant + *Agrobacterium* strain CKS + long co-cultivation time and low selection pressure protocol should be further tested and optimized for transformation of valencia-type peanut.

A nearly linear relation between transformation frequency and duration of co-cultivation in pea was reported (De Kathen and Jacobsen, 1990). Our results also indicated that a longer co-cultivation time increased the transformation efficiency when the explant was cultured in phytohormone-containing medium. Preculture conditions also can be critical for improving transformation efficiency. Chyi and Phillips (1987) compared several treatments that favored the recovery of transformed tomato plants. One of the best treatments involved the addition of kinetin into the co-cultivation medium. Similarly, flax explants precultured on shoot regeneration medium showed improved transformation efficiency compared to nonprecultured flax explants (Dong and McHughen, 1993).

In summary, this study documented the production of stably transformed valencia-type peanut shoots and plantlet using an *Agrobacterium* vector. We also presented preliminary results documenting the relationship between regeneration-competent cell types and putative transformation-competent cell types in several regenerable explant systems. Finally, an engineered zein gene was expressed in the primary transformant leaf tissues. However, because of a catastrophic fire in our laboratory, all the primary transformants and cultures were lost before molecular evidence of stable integration into the genome of the primary transformants or heritability data could be obtained. Nevertheless, the information presented in this report represent significant progress toward the development of a useful transformation system for valencia-type peanut.

We have initiated experiments to repeat and to further optimize the protocol presented here. To date we have recovered additional primary transformants with strong marker gene expression from petiolule-with-blade-attached and leaflet section explants using the long co-cultivation protocol and low kanamycin selection pressure. Southern analysis has confirmed the stable incorporation of foreign DNA into the genome (data not shown). One long-term goal of our program is to introduce the zein gene into this crop as a means to develop germplasm with nutritionally balanced protein content.

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