Progress in the Development of Tissue Culture and Transformation Methods Applicable to the Production of Transgenic Peanut

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ABSTRACT

Genetic engineering is a tool for crop improvement that extends our access to beneficial traits beyond sexually compatible crosses. Genes from virtually any organism can be cloned and introduced into peanut. Gene function can be influenced by the regulatory elements used to control expression as well as the genome context of the integration site(s) where one or multiple copies of the transgenes are inserted. Methods for the production of transgenic peanut (*Arachis hypogaea* L.) that are based on biological or direct DNA transfer have been developed over the last decade. The most reliable method for the introduction of foreign DNA is microprojectile bombardment of embryogenic tissue cultures. With the use of a selectable marker gene for hygromycin resistance, transgenic plants can be recovered in 12-14 mo. Transgenic peanuts resistant to the lesser cornstalk borer have been produced with the objective of reducing aflatoxin contamination by decreasing insect damage to developing pods. The future application of this tool to increase pest resistance and enhance quality traits in peanut has enormous potential.

Key Words: *Arachis hypogaea*, groundnut, genetic engineering.

Arachis hypogaea L. is one of the principal leguminous crops globally valued for its protein content and oil quality. Crop improvement has been based on conventional breeding methods where crosses between two parents with desirable traits are followed by generations of selfing and selection in this naturally inbreeding species (47). Much of the breeding effort has been focused on enhancing disease resistance, particularly to fungal and viral pathogens. Sources of resistance in the gene pool of *A. hypogaea* are limited, however, and resistance genes

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present in related species are difficult to transfer by sexual hybridization because of incompatibility and ploidy barriers. In addition, breeders are faced with market standards that require a well-defined plant architecture and pod morphology. These traits vary primarily with botanical types of cultivated peanut which include *A. hypogaea* ssp. *hypogaea* var. *hypogaea* (runner/virginia market types), *A. hypogaea* ssp. *fastigiata* var. *fastigiata* (valencia market type), and *A. hypogaea* ssp. *fastigiata* var. *vulgaris* (spanish market type). Ideally, the introduction of novel traits should be accomplished without altering the specific market characteristics of a desirable genotype. Genetic engineering offers an unequaled opportunity to introduce previously inaccessible genes into existing varieties that have outstanding yield potential.

Efficient regeneration of highly totipotent cells is an essential component of genetic engineering systems, whether the regenerated plant results from a preformed shoot meristem or an undifferentiated callus cell or embryo initial that is competent to express a morphogenic program. Establishment of an efficient regeneration system in peanut has not been trivial and has been built on decades of research beginning with the regeneration of plants from *in vitro*-cultured de-embryonated cotyledons (40). Recently, an array of regeneration protocols have been published that describe plant development through somatic embryogenesis or shoot morphogenesis. Genetic transformation of regeneration-competent cells quickly followed and will be the main topic of this review with a focus on significant advances in the last decade of the millennium.

Regeneration- and Transformation-Competent Tissues

Successful gene transfer relies on the acquisition and integration of foreign DNA by regeneration-competent cells. In peanut, terminally differentiated cells such as mature leaf mesophyll rarely express totipotency after dedifferentiation and cell division. This limitation was particularly obvious with mesophyll cell (41) and mesophyll protoplast (67) cultures of *A. hypogaea*, where the only regenerable protoplast cultures reported thus far have been derived from immature cotyledons (51). Juvenile leaves, on the other hand, are much more regeneration-competent *in vitro*; however, the competent cells are localized primarily to the region surrounding the central vein and not throughout the leaf lamina (1, 14, 82).

Cells which are susceptible to *Agrobacterium* infection also appear to be restricted to certain positions within the leaves. In peanut leaves that are not completely expanded, the susceptible cells are localized primarily to tissue near the central vein, but the susceptibility is highly dependent upon leaf age/stage of development (58). Although the spatial distribution of *Agrobacterium*-susceptible and regeneration-competent cells in leaves appears to overlap (15), fully substantiated recovery of transgenic plants from *Agrobacterium*-treated leaf explants has been limited to a single peanut genotype, New Mexico Valencia A (16, 17). Although transformation of other var. *fastigiata* genotypes using leaf explants may have been accomplished, stable gene transfer was incompletely documented (28). The number of regeneration-competent cells in a leaf explant may be highly genotype dependent since the valencia-type peanuts tested showed a higher frequency of plant regeneration from leaf explants than spanish or runner/virginia-type peanuts (14, 22, 28). Transfer of foreign DNA to regeneration-competent cells of incompletely expanded leaves by microprojectile bombardment was not efficient enough to allow recovery of transgenic plants, even from a var. *fastigiata* genotype (22). Perhaps the regeneration-competent region is too restricted in size to receive an adequate number of "hits" with DNA-coated gold particles in order to compensate for the low frequency of stable transformation expected.

Most seed and seedling tissues of peanut, except for the radicle, can be used to establish regeneration-competent tissue cultures (Tables 1 and 2). A complex explant that consists of immature leaves and stem tissue is the zygotic embryo axis. Both immature and mature embryo axes are competent for shoot regeneration either through development of preexisting apical or axillary shoot meristems (78), de novo formation of shoot primordia (Table 2), or proliferation of somatic embryos (Table 1). Tissue proliferation occurs from the epicotyledonary region of the stem and the proximal region of immature leaves.

The cotyledons of the immature zygotic embryo also serve as another suitable explant for the initiation of embryogenic cultures. The proximal end of the excised cotyledon, when cultured at the appropriate stage of development on auxin-containing medium, will rapidly form somatic embryos that undergo repetitive growth (9, 69, 70). Such repetitive embryogenic cultures that have a high fraction of dividing, totipotent cells are ideal targets for transformation since the small fraction of stably transformed cells have a greater chance of being recovered during selection than would a single transformed cell in a terminally differentiated organ. The repetitive growth of transformed somatic embryos on selective media also further reduces the probability of recovering chimeric plants. Regardless of the means of transformation, actively dividing cells are required for the integration of foreign DNA.

For any explant in tissue culture, the pathway to differentiation is dependent primarily on the growth regulators incorporated into the culture medium. In general, compounds with cytokinin activity such as benzylaminopurine (BAP), kinetin (K), and thidiazuron (TDZ) promote shoot initiation and development, and auxins such as 2,4-D (2,4-dichlorophenoxyacetic acid) and picloram induce somatic embryogenesis. Other culture variables that have been shown to influence the magnitude of response rather than the response pathway are basal medium, light, and temperature (73). Aside from culture conditions, genotype of the explant tissue plays a major role in culture response. The rate of initiation and maintenance of embryogenic cultures clearly is influenced by peanut genotype (69).

Transformation efficiencies frequently are directly

Table 1. Conditions for initiation of somatic embryogenesis from cultured explants.

Explant type	Growth regulators	Response frequency[a]	Somatic embryos per explant[b]
	mg/L	%	no.
Immature cotyledon	0.5-2 picloram[68][69][71]	60[68] 58[71] 55[69]	1[68]
	7.5 2,4-D[5]	98	7
	10 2,4-D[34]	18	3
	20 2,4-D[26][29]	49[26][29]	9[26][29]
	40 2,4-D[25]	nr[d]	2
	50 2,4-D[8]	46	12
Cotyledon from seeds germinated on 2.2 TDZ	2.2 TDZ[36]	40	29
Mature cotyledons	0.5 NAA + 5 BAP[86]	80	52
Immature embryo axis	0.5 picloram[69]	85	3
	3 2,4-D[37]	100	15
	10 2,4-D[26][29][34]	100[26][34] 90[29]	10[26][34] 28[29]
Immature embryo	0.02 picloram[c] [79]	73	3
	10 2,4-D[34]	14	2
Mature embryo epicotyl	30 2,4-D[6]	100	11
	30 picloram[54]	>90	18
	36.5 centrophenoxine[54]	>90	17
Mature embryo axis	3 picloram[61][60]	58[61] 52[60]	4[61] 29[60]
	10 2,4-D[34]	50	3
Bissected leaves from seeds germinated on 2.2 TDZ	2.2 TDZ[36]	>90	7
Leaflet segments	40 2,4-D + 0.2 K[7][10]	14[7]67[10]	4[7]6[10]
Young leaflets	20 2,4-D[21][20]	79[21] 76[20]	nr
	20 2,4-D + 0.5 BAP[90]	69	35

[a]Best response frequency determined by the percentage of explants producing somatic embryos in combination with the number of somatic embryos per explant.

[b]The number of somatic embryos is difficult to compare among studies unless it is clearly stated how fasciated/fused embryos are counted and whether the number per explant represent all explants or only responding explants.

[c]Medium also included adenine, abscisic acid, and casein hydrolysate.

[d]nr = not reported.

related to the tissue culture response; highly regenerative cultures are often transformation competent. However, there also is a genotypic effect on transformation frequency that may be related to developmental/physiological differences between explants or cultures. Susceptibility to *Agrobacterium* infection also varies greatly among genotypes of peanut (49). Tissue culture protocols have been tested with many different genotypes because research groups focused on peanut transformation are usually interested in applying protocols to regionally adapted cultivars (Table 3).

In general, transformation systems can utilize primary cultures (if the regeneration rate is sufficiently high) where shoot primordia or somatic embryos are formed directly from the explant, dedifferentiated callus cultures (as long as there is a sustained capacity for plant regeneration), or repetitive cultures. All three types of culture systems have been developed in peanut, although it is prudent to keep the tissue culture phase as abbreviated as possible to reduce somaclonal variation (50) and detrimental effects on regeneration or reproductive capacity (70, 92). Consequently, even though suspension cultures (25, 84) provide a constant source and large quantity of morphogenic units, their rapid initiation may be genotype dependent and the recovery of fertile plants may be more difficult than from repetitive cultures maintained on semi-solid medium.

Delivery of Foreign DNA

Genes from distantly related or unrelated organisms can be introduced into actively dividing peanut cells using the biologically based method of *Agrobacterium* infection or the direct DNA delivery methods of electroporation or microprojectile bombardment. Each method has limitations with regard to the competent target tissues and genotype. Since *Agrobacterium* infection relies on biochemical factors within the plant, it is the more genotype-dependent method. Likewise, electroporation requires the DNA to be in contact with the plasma membrane of competent cells and is most effective with protoplasts, plant cells that have had their cell walls removed (51). Tissue electroporation, al-

Table 2. Conditions for initiation of shoot morphogenesis from cultured explants. GA_3, gibberellic acid; NOA, naphthoxyacetic acid; IAA, indole-3-acetic acid.

Explant type	Growth regulators	Response frequency	Buds or shoots per explant
	mg/L	%	no.
Whole seed	50 BAP[23]	80	nr
Immature cotyledon + axis	2.2 TDZ → 1.1 TDZ → 0.5 K + 0.5 GA3[35] callus → shoots	100 → 100	3
Mature cotyledon + axis	25 BAP[63][73]	52[63]; 94[73]	12[63]; 18[73]
Mature cotyledon	25 BAP[63][73]	56[63]; 40[73]	2[63]; 14[73]
Mature cotyledon, halved	4.5 BAP + 2.2 2,4-D[80]	95	8
Epicotyl	0.2 K + 2.2 2,4-D → 6.7 BAP + 5NOA[74]	80	35
Hypocotyl + cotyledon	2.2 TDZ[53]	nr[a]	85
Hypocotyl	30 TDZ[43]	60	27
Cotyledon from 8-d-old seedlings	30 TDZ[43]	60	16
Cotyledon from 7-d-old seedlings	0.5 K+ 2 NAA → 2 BAP + 0.5 NAA[89] callus → shoot primordia	94 → 89	32
Cotyledonary node from 7-d-old seedlings	0.5 K+ 2 NAA → 2 BAP + 0.5 NAA[89] callus → shoot primordia	86 → 100	35
Cotyledonary node + hypocotyl from 10-d-old seedlings	10 TDZ[44]	nr	18
Leaf from 7-d-old seedlings	0.5 K+ 2 NAA → 2 BAP + 0.5 NAA[89] callus → shoot primordia	100 → 100	45
Leaf from 8-d-old seedlings	30 TDZ[43]; 5 BAP + 1 NAA[64]	35[43]; 38[64]	17[43]; 1-3[64]
Leaf from 10-d-old seedlings	5 BAP[73]	43	10
Leaflet from 4-8-d-old seedlings	4 BAP + 2 NAA[22]	15	12
Leaflet from imbibed seeds	4 BAP + 2 NAA[82]; 5 BAP + 4 NAA[18]	92[82]; 90[18]	37[82]; 15[18]
Leaflet from unimbibed seeds	4 BAP + 2 NAA[82]; 3 BAP + 1 NAA[55]	94[82]; 66[55]	53[82]; 13[55]
Leaflet + petiolule (8-11-d-old)	25 BAP + 1 NAA[14]	62	27
Leaf sections from 7-d-old seedlings	4 BAP + 0.5 IAA[75]	62	nr
Leaf sections from 6-8-d-old seedlings	1 TDZ + 1 NAA[1]	34	5
Leaf sections from 10-12-d-old seedlings	2.2 BAP + 0.1 IAA[27]	33	7
	25 BAP + 1 NAA[14]	50	56

[a]nr = not reported.

though successful with some species (24), has not been reported for peanut. The least genotype-dependent method thus far reported for DNA delivery in peanut is microprojectile bombardment where virtually any tissue can serve as a target.

Delivery of foreign DNA can be monitored by transient expression; however, there often is no clear correlation between transient expression and stable transformation (2). Transient expression in peanut has been monitored most frequently with the β-glucuronidase (GUS or *uidA*) reporter gene. Quantitation of transient expression usually is achieved by counting the number of GUS-positive foci per unit area, either manually or using computer-based image analysis procedures (4, 45, 92). Transient expression can be detected within a few hours of free-DNA uptake when the gene is under the control of an appropriate promoter. Transient assays after *Agrobacterium* infection/coculture also can be conducted; however, they are likely to produce artifactual results if the GUS gene does not contain an intron or other modification that prevents either expression or processing in prokaryotic cells (83). Since staining for GUS activity is a destructive process and the product of the assay can diffuse out of the expressing cell, other nondestructive, vital assays of fluorescent or luminescent reporter genes [such as green fluorescent protein (GFP) and luciferase] have the advantage of allowing real-time monitoring of transient expression at the cellular level (55, 93).

Selectable Marker Genes

Since only a small fraction of cells to which DNA has been delivered actually integrate the DNA into their genomes, an efficient selection scheme is central to the recovery of stable transformants. Typically chosen are selectable marker genes that confer resistance to a toxic compound such as an antibiotic or herbicide. Expression of such genes allows the growth of transformed cells in an environment that is lethal to untransformed cells. Alternatively, reporter genes can be used to screen nonselected, but putatively transformed tissues for expression patterns that would be expected for a stable transformant. Reporter gene-based screening techniques alone, particularly when destructive and applied after plant regeneration, are too labor intensive and costly for most academic labs to implement, but can result in the recovery of transformed plants (12). Of the selectable marker genes available, hygromycin phosphotransferase (*hph*) and neomycin phosphotransferase II (*nptII*), enabling selection on hygromycin (10-20 mg/L) or kanamycin (50-125 mg/L), respectively, are used the most widely in peanut. Kanamycin resistance is more readily displayed during shoot morphogenesis (16, 80) than during embryogenesis where hygromycin selection is very efficient (56, 70). Selection within a short time span subsequent to DNA delivery and selection in liquid medium both appear to enhance the potential for recovery of transgenic

Table 3. Released genotypes tested in tissue culture.[a]

Cultivar	Market type	Reference	Cultivar	Market type	Reference
AT108	runner	6	Spancross	spanish	68,69
AT120	runner	54,57	Dixie Spanish	spanish	68,69
AT127	runner	5,7,14,25,53,94	GAT-2741 (released as Georgia Browne)	runner/spanish	71
GK3	virginia	6	Toalson	spanish	69,70,81
GK7	runner	6,10,54	Spanco	spanish	14,61
GK17	runner	6	Tamnut	spanish	22,61
GK19R-1	spanish	6	Chico	spanish	1,20,61,63,64
F435AT	spanish	6	Pronto	spanish	14,53
VC1	virginia	54,57	EC-5	spanish	53
NC6	virginia	53	Starr	spanish	14,53
NC7	virginia	6,14,22,53,55,56,61,63,64,79,82	Gajah	spanish	55,56
Florigiant	virginia	12,14,53,60,62,63,64	Comet	spanish	79
Tifrun	runner	68,69	OAC Ruby	valencia	36
Florunner	runner	6,12,61,63,64,68,70,81,92,95	OAC Garroy	valencia	36
MARC I	runner	81,92,95	TMV 2	spanish	20,76,86,87
Georgia Runner	runner	6,53,71,92,95	JL 24	spanish	18,19,20,34,80
Sunbelt Runner	runner	68,71	Arkansas Valencia	valencia	6
Virginia Runner G-26	runner	68,69,71	Georgia Red	valencia	14,69
GA119-20	runner	68	McRan	valencia	36,79
Sunrunner	runner	61,63,64,69	New Mexico Valencia	valencia	14,15,16,17,30,43,44,52
Okrun	runner	61,74	Tennessee Red	valencia	53
Georgia Green	runner	35	UPL PN4	valencia	22
SER 56-15	runner	68	Tatu	valencia	73
Tifton-8	virginia	68			

[a]Other Indian cultivars tested include: SB11, TG26, DRG12, TAG24, KOYANA, RHRG13, RHRG16, TG3, TG9, TG17, TG19A, TG27, RHRG50, RHRG93, ICGS11, M13, JLM1, TMV7, and VRI2 [20, 26, 27, 28, 29, 34, 37, 75, 85, 88, 89, 90].

plants (92).

Regulatory Elements

The promoter is the most critical regulatory element for achieving gene expression for genes of selectable markers or other traits. Several heterologous promoters have been shown to function in peanut. The frequently used CaMV35S promoter acts constitutively in peanut as in many other plants (66). The plant expression vector, pBI121, which contains a *uidA* gene driven by the CaMV35S promoter and the *nptII* gene driven by the nopaline synthase promoter, has been most commonly used in reports of *Agrobacterium*-mediated stable transformation of peanut (16, 17, 28, 80, 85, 87). In instances where hygromycin has been used for selection, the CaMV35S promoter has been used universally to control the *hph* gene (56, 57, 70, 81, 92, 95). There are numerous versions of the CaMV35S promoter that differ in length and may be tandemly duplicated with or without leader sequences from other viral genes such as the cucumber mosaic virus (95), alfalfa mosaic virus (12, 52), or tobacco etch virus (80). Transient expression has been documented for the two monocot promoters *Emu* and actin 2 (45) and the promoter from the 2S albumin gene from Brazil nut (48); and GUS expression was reported from the mannopine synthase promoter, although no quantitative data were shown (62). The most thorough analysis of the pattern of expression of a heterologous promoter in peanut has been with the soybean vegetative storage protein B promoter fused with *uidA* (92). GUS expression under the control of the *vspB* promoter was strong in stems, pod walls, and anthers; moderate in leaves; and weak in roots. Activity was increased upon incubation of tissues with jasmonic acid, a response that was predicted based on previous analysis of the soybean promoter (59). We have begun similar analyses of other promoters in peanut including the ubiquitin 3 promoter from potato (33) and the actin 2 promoter from *Arabidopsis* (3). The ubiquitin promoter confers high levels of expression in actively dividing tissues, including embryogenic cultures. The actin 2 promoter confers expression in leaves and roots, but not in embryogenic tissues; thus, the actin 2 promoter is not useful in fusions with selectable marker genes when transgenic embryogenic lines are being selected (authors' unpubl. results).

Molecular Analysis of Stable Transformants

Integration of foreign DNA into putatively transformed tissues/plants must be confirmed by molecular analyses. The polymerase chain reaction (PCR), when used to amplify a specific fragment of the foreign DNA, is often useful as a preliminary screen but is not a definitive test of integration. PCR from samples derived from *Agrobacterium*-cocultured tissues is discouraged

since only a few residual bacterial cells in the plant tissues may result in amplification of the target gene and the production of artifactual evidence for transformation. Southern blot analysis can produce the most conclusive data for DNA integration if the restriction enzymes chosen to digest the genomic DNA are appropriate to demonstrate junction fragments between the plasmid DNA and the host genomic DNA. Independent transformants then can be distinguished since the junction fragments will be variable in size, and often in copy number (81).

Analysis of transgene expression in the primary transformant and its progeny provides further evidence for stable transformation. Expression at the RNA level can be documented by Northern blot analysis and at the protein level by activity or immunological assays (77). Immunological assays such as ELISA (enzyme-linked immunosorbent assay) or Westerns require the availability or production of antibodies. Such antibodies for *cryIA*(c) and the tomato spotted wilt virus nucleocapsid protein can be acquired commercially and used for analysis of transgenic peanut (57, 95). It is relatively common to find transgenic lines where transgene integration can be detected by Southern analysis, but transgene expression is undetectable (95). Although this observation may be due to transgene disruption, many nonexpressing transgenics contain intact transgenes and the lack of expression is a result of epigenetic silencing that may be related to the plant's defense mechanisms (31).

Reported Transformation Systems for Peanut

Although some evidence for *Agrobacterium*-mediated transformation was published in the early 90s (28, 62), the first thoroughly documented *Agrobacterium*-mediated transformation of peanut was reported in a series of publications using the cultivar, New Mexico Valencia A (16, 17, 52). Li *et al.* (52) unequivocally demonstrated foreign gene integration in multiple transgenic events derived from this cultivar. The transformation system was built upon a regeneration protocol where multiple shoots were induced from cultured leaf sections on 25 mg/L BAP and 1 mg/L NAA (naphthaleneacetic acid). *Agrobacterium* cultures were treated with wounded tobacco leaf extract to enhance *vir* gene induction prior to coculture with peanut leaf sections. Transformed shoots were selected on kanamycin at a frequency of 0.2-0.3% of the inoculated explants. The same cultivar has been used by other groups for transformation studies (30). It appears that this transformation method is highly genotype dependent, probably due to the lower regeneration response from leaflet explants of many runner cultivars (A. Xing, pers. commun.).

Coculture of the cotyledonary node, cotyledon, or hypocotyl with *Agrobacterium* reportedly yielded a high frequency of transformants (> 20%) from Indian cultivars VRI-2 and TMV-7 based on kanamycin resistance (85). Genomic DNA from only two putative transformants was analyzed by Southern blot hybridization and both appeared to show integration although the proper plasmid control was not presented for comparison. The same group also reported that the cotyledon from germinated seeds of cultivar TMV 2 could be efficiently transformed by cocultivation and selection on kanamycin under culture conditions that would promote somatic embryogenesis (87). The Southern blot, however, was of poor quality and thus was uninterpretable. Rohini and Rao (76) reported a 3% transformation frequency using a nontissue-culture-based, *Agrobacterium*-mediated transformation method with genotype TMV 2. The protocol utilized treatment of *Agrobacterium* with wounded tobacco leaf extract as in Cheng *et al.* (16) and wounding/cocultivation of the embryo axis attached to one cotyledon similar to that used in the study of McKently *et al.* (62). A Southern blot showing apparent integration of the *uidA* gene in the T1 generation was shown.

Using the mature cotyledonary "seed" leaves, *Agrobacterium*-mediated transformation recently has been accomplished at a reported efficiency of 55% (80). The transformation system appears very straightforward, neither requiring *vir* gene-induction treatments nor a lengthy tissue culture phase. Two cultivars, one spanish (JL-24) and one virginia type (ICGS-44), reportedly could be transformed at high efficiency, although integration patterns in progeny of transgenic plants were shown for only JL-24. If *Agrobacterium*-mediated transformation of organogenic cotyledon cultures proves to be broadly applicable in peanut, success likely will be dependent upon the ability to induce the required regeneration response as shown with JL-24 where 95% of the explants produced 4-10 shoots each. Other research using the cotyledon as an explant did not achieve the same frequency or magnitude of regeneration response as demonstrated by Sharma and Anjaiah (80). For example, a comparable explant of de-embryonated cotyledon (cotyledon with embryo axis surgically excised) that had been longitudinally bisected showed only 15% response frequency and four shoots per responding explant (63). The main differences, respectively, between these two regeneration studies were genotype (JL-24, although five others were tested and gave a similar response frequency, vs. Florigiant), culture medium (4.5 mg/L BAP + 2.2 mg/L 2,4-D vs. 25 mg/L BAP), light (constant vs. photoperiodic), and perhaps orientation (cut proximal end in contact with medium vs. unspecified).

Direct DNA transfer methods such as microprojectile bombardment can circumvent the genotype dependency of *Agrobacterium* infection. Stable transformation of peanut has been accomplished by microprojectile bombardment of embryogenic cultures and selection on hygromycin (70) or bombardment of apical and lateral meristems on the embryo axis followed by screening for reporter gene activity (12). Bombardment of embryogenic cultures and selection on hygromycin appears to be the most widely applicable technology since it has been used by at least three different groups to transform multiple cultivars including runner, virginia, and spanish market types (56, 57, 70, 81, 92, 95). Variations in published protocols include bombardment pressures,

osmotic treatment, and time of initiation of selection, among other parameters that may or may not influence transformation efficiency. The limitations of this technique are primarily the maintenance of regeneration capacity and recovery of fertile plants from the embryogenic cultures as well as the greater frequency of multiple copy insertions that result when compared with *Agrobacterium*-mediated transformation (although it may be possible to decrease this frequency by bombarding with only the linear gene cassettes free of the plasmid backbone (32). The essential steps of the process involve initiation of repetitive embryogenic cultures (3-6 mo), bombardment, selection (2-3 mo), increase of tissues (1-3 mo), plant regeneration (2-4 mo), and acclimitization and seed maturation (4-6 mo) for a minimum time frame of 12 mo from initiation of cultures to recovery of transgenic seed.

As indicated earlier, the culture period for repetitive embryogenic cultures should be kept to a minimum to facilitate the recovery of fertile plants (92). Although the use of repetitive embryogenic cultures for transformation is frequently criticized as being fraught with regeneration problems and too lengthy, we have observed that at least 80% of hygromycin-resistant embryogenic lines can regenerate plants over a period of 3-4 mo. In addition, a single bombardment can yield more than three independent transformants, and more than 12 bombardments can be carried out per day (92). If the components of the transformation experiments are optimized (most importantly the quality and age of the embryogenic cultures), then more than 100 transgenic lines could be generated from 1 wk of bombardment experiments.

Enhancing Beneficial Traits in Peanut Through Genetic Engineering

In addition to the selectable marker and reporter genes described above, genes for insect and virus resistance have been introduced into peanut (12, 52, 57, 80, 81, 95). Genetic engineering for insect resistance has been focused on the insecticidal crystalline proteins from *Bacillus thuringiensis* (72). In peanut, insect resistance *per se* has not been the primary target, but rather the reduction of aflatoxin produced by *Aspergillus* fungal invasion of lesser cornstalk borer-damaged tissues (81). Virus diseases are prominent targets of peanut disease research, not only because of their prevalence in most peanut-growing regions of the world, but also because of ineffective control measures. Tomato spotted wilt virus has become a major and often devastating disease in the southeastern USA. Partial disease control can be achieved by following prescribed cultural practices and selecting less susceptible genotypes. The potential exists to enhance host-plant resistance through pathogen-derived genes. Introduction of the nucleocapsid protein gene from the tomato spotted wilt virus into peanut (12, 52, 57, 95) may eventually allow the recovery of highly resistant genotypes; however, durable and high levels of resistance have not been achieved to date (52, 57).

Finally, there are numerous traits that potentially could be manipulated with single or few gene introductions to produce more pest-resistant, healthier, higher quality peanuts. These include oil quality such as a high oleic acid (42), reduced allergenicity by down regulation of highly allergenic peanut proteins (13), herbicide tolerance (46), insect resistance using genes other than *Bt* (38), fungal resistance (11, 65), nematode resistance (91), and nutrient composition (39). Such traits collectively would benefit growers, manufacturers, and consumers thus resulting in increased marketability of peanuts as a commodity and wholesome, healthy food.

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