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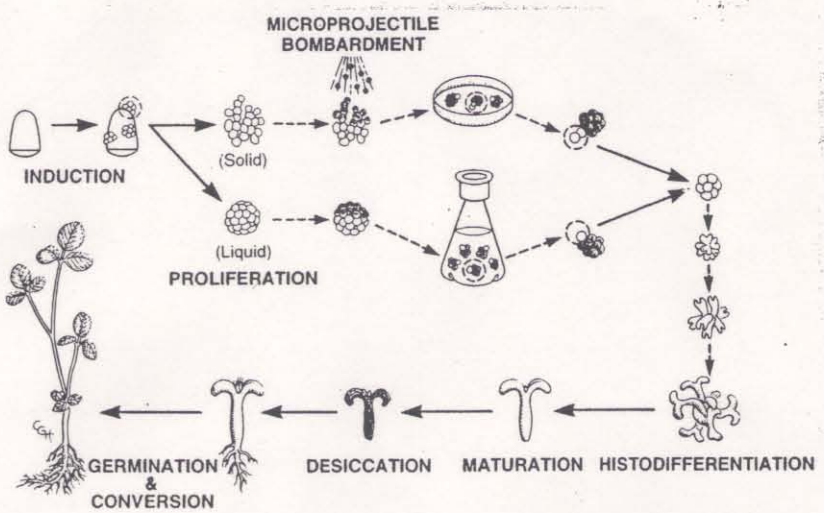

The International Association for Plant Tissue Culture

Recent advances in soybean transformation

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Introduction

Transformation of soybean (*Glycine max* [L.] Merr.) has been far from routine. The first two reports of soybean transformation used two very different methods to transform soybean: Hinchee *et al.* (1988) used *Agrobacterium*-mediated transformation of cotyledonary nodes while McCabe *et al.* (1988) used particle bombardment of shoot meristems. Soybean transformation reports following these initial papers have been limited and the transformation efficiency for soybean has remained low. In this paper, we will identify the major challenges to soybean transformation, review recent advances, and give protocols of some of the procedures used by our laboratories.

For any method of transformation to be successful, cells that are regeneration-competent must also be accessible and transformation-competent. Transformation will not be successful if either transformation or regeneration is inefficient, or if transformation and regeneration are "uncoupled." Knowledge of both processes and the ability to bring them together will result in recovery of transgenics.

There are several methods to genetically transform plants, such as *Agrobacterium*-mediated transformation of excised plant tissues (Horsch *et al.*, 1985), particle bombardment (Sanford, 1988), electroporation (Fromm *et al.*, 1985), silicon carbide fibers (Kaeppler *et al.*, 1990), liposome-mediated transformation (Caboche, 1990) and *in planta Agrobacterium*-mediated transformation via vacuum infiltration of whole plants (Bechtold *et al.*, 1993). The first two methods are the most commonly used for soybean transformation. The other methods have not been optimized for soybean, and are therefore less efficient and not often used.

Tissue culture and target tissues for soybean transformation

One of the critical prerequisites for all current soybean transformation procedures is the ability to manipulate plant tissue *in vitro*. For soybean regeneration, two principal methods have been identified: somatic embryogenesis and shoot morphogenesis. Each of these systems presents both advantages and disadvantages for production of transformed plants, and each can be used with both of the predominant transformation systems.

Somatic embryogenesis

Somatic embryogenesis is the process whereby embryos develop from either microspores or somatic tissues. Somatic embryos have both shoot and root axes and form whole plants upon germination. Although somatic embryogenesis in soybean has been reported by a number of laboratories (Christianson *et al.*, 1983; Lippmann and Lippmann, 1984; Lazzeri *et al.*, 1985; Ranch *et al.*, 1986; Parrott *et al.*, 1988), most of the protocols described in these early studies have not been successfully used for transformation work. This limitation was overcome when Finer (1988) described a somatic embryogenesis system in which embryos were induced from immature soybean cotyledons by placing the explant on high levels of 2,4-D (40 mg/l). Embryogenic tissue could be proliferated by subculture to either the induction medium (Finer, 1988) or a liquid suspension culture medium containing lower levels of 2,4-D (Finer and Nagasawa, 1988). Histological analysis of proliferating embryos indicated that new somatic embryos were initiated at or near the surface of the older embryos (Finer, 1988; Finer and McMullen, 1991). With this system, the surface origin of new embryos makes this tissue a suitable target for transformation.

Shoot morphogenesis

Shoot morphogenesis is the process of shoot formation and development. Shoots, which can form from a number of different tissues, are excised and rooted to generate new plants. For transformation, foreign DNA can be introduced into pre-existing shoot meristematic tissue or tissue that could potentially give rise to shoots. Shoot morphogenesis for soybean was first reported by Wright *et al.* (1986) using the cotyledonary nodes of seedlings, and Barwale *et al.* (1986) using the cotyledonary nodes of immature embryos. When placed on medium containing 6-benzyl-aminopurine (BAP), shoots formed *de novo* from subepidermal tissue. The main advantage of this method is that proliferating shoots can form rooted plants in less than three months, whereas plant recovery from embryogenic cultures can take 4 months or more. Loss of fertility in regenerated plants is therefore less likely to occur with this system. Another advantage of shoot morphogenesis is that the explants are derived from seedling explants, and thus there is no need to maintain a constant source of fruiting plants for explants as in the somatic embryo system.

Methods of soybean transformation

Agrobacterium-*mediated transformation*

Agrobacterium-mediated transformation utilizes *Agrobacterium* as the biological vector to introduce a portion of its DNA into the plant genome, resulting in production of transformed plants (Zambryski, 1988; Hooykaas and Schilperoort, 1992). Wounded plant tissues give off specific phenolic compounds which induce *Agrobacterium* to express a set of *vir* genes (Hooykaas and Beijersbergen, 1994). Expression of the *vir* genes results in the production of single-stranded DNA which is transferred and integrated into the plant genome. The major problem with this method of transformation is the host- and tissue-specificity associated with biological vectors. Although soybean was initially not considered to be susceptible to *Agrobacterium* (DeCleene and DeLay, 1976), it has since been determined that soybean can be a suitable host for *Agrobacterium* (Pederson *et al.*, 1983). However, there is still some degree of cultivar specificity (Owens and Cress, 1985; Hinchee *et al.*, 1988; Parrott *et al.*, 1989; McKenzie and Cress, 1992; Bailey *et al.*, 1994; Droste *et al.*, 1994; Mauro *et al.*, 1995) and transformation efficiencies for regenerable tissue such as somatic embryos and cotyledonary nodes can be very low. Transformation inefficiencies can be partially overcome by the addition of the chemical inducer, acetosyringone, to induce expression of the *vir* genes (Stachel *et al.*, 1985; Delzer *et al.*, 1990) or by the use of highly virulent *Agrobacterium* strains which constitutively express the *vir* genes (Hansen *et al.*, 1994). One major advantage of using *Agrobacterium* is that, depending on the strain, lower copy numbers of DNA can be integrated into the plant genome (Tinland and Hohn, 1995). In contrast, direct DNA uptake methods can result in introduction of multiple copies, which can be fragmented or recombine in an uncontrolled fashion (Hadi *et al.*, 1996).

Recovery of the first transgenic soybean plants using *Agrobacterium*-mediated transformation was reported in 1988 (Hinchee *et al.*). Cotyledonary node explants from the cultivar "Peking" were inoculated with a disarmed strain of *Agrobacterium* which conferred β-glucuronidase (GUS) expression and resistance to kanamycin and glyphosate. The explants were placed on a medium containing BAP for shoot induction, and kanamycin along with other antibiotics to remove residual *Agrobacterium*. After a few months, plantlets were recovered and tested for GUS expression and/or glyphosate resistance. Only 6% of the shoots that survived selection were transgenic. Eight plants were recovered and segregation data from these plants indicated that they had single inserts. Even though the transformation was not very efficient, this report did confirm that it was possible to transform regeneration-competent cells of a soybean cultivar susceptible to *Agrobacterium* infection.

Townsend and Thomas (1993) used a similar procedure to obtain transgenic plants of cultivar "Pioneer 9341". Factors important to their success were: (1) the use of acetosyringone, (2) the use of cocultivation temperatures between 18 and 28°C, (3) the inoculation with *Agrobacterium* at 10^8 to 3×10^9 cells ml^{-1}, and (4) the use of pyroglutamic acid in the regeneration medium.

The *Agrobacterium*/cotyledonary node procedure was also recently used to introduce the Bean Pod Mottle Virus coat protein into soybean (Di *et al.*, 1996). Six independent transformants were recovered after several hundred explants were inoculated with *Agrobacterium*, confirming both the potential use of the *Agrobacterium*/cotyledonary node procedure as well as its low efficiency.

Another *Agrobacterium*-based procedure that resulted in the production of primary transgenic plants utilized immature cotyledonary tissue to produce somatic embryos (Parrott *et al.*, 1989). Three transgenic plants containing the introduced 15 kD zein gene were recovered. Unfortunately, these primary transformed plants were chimeric, and no progeny that contained the 15 kD zein gene were recovered. Theoretically, problems with chimerism can be overcome through the use of repetitive embryogenesis.

Recently, a new and potentially more efficient method was developed for introduction of *Agrobacterium* to plant target tissues. This new technique, called "Sonicated Assisted *Agrobacterium*-mediated Transformation" (SAAT) involves subjecting the plant tissue to brief periods of ultrasound in the presence of *Agrobacterium* (Trick and Finer, in press). Scanning electron and light microscopy revealed that the SAAT treatment produced thousands of small channels throughout the tissue, permitting *Agrobacterium* to infect and subsequently transform the tissues. The use of SAAT on embryogenic suspension cultures has resulted in the recovery of several stably transformed, non-chimeric clones. The utility of this technique for efficient delivery of *Agrobacterium* holds great promise to increase the efficiency of the *Agrobacterium*-mediated transformation methods described above and expand the use of *Agrobacterium* on other target tissues.

Particle bombardment

Particle bombardment technology is based on the acceleration of DNA-coated particles towards a plant cell with such a force as to penetrate the cell wall and membrane. Once inside the cell, the DNA disassociates from the particles and is integrated into the plant genome. The major advantage of particle gun over *Agrobacterium* is the removal of biological incompatibilities. Intact plant tissue, such as meristems, can potentially be used as a target for DNA transformation. However, particle bombardment does have its limitations and drawbacks. Unless devices can be made to precisely deliver DNA-coated particles deep within the target meristematic tissue (McCabe *et al.*, 1988), transformation will continue to be limited to cells at or near the surface. Analysis of transgenic tissues obtained via particle bombardment shows either simple DNA integration events (Christou *et al.*, 1989; Parrott *et al.*, 1994; Stewart *et al.*, 1996) or high copy number events with high levels of recombination in the resulting inserts (Hadi *et al.*, 1996). This type of integration event is known to affect transgene expression (Jorgensen *et al.*, 1996).

The first report of particle gun-mediated transformation of soybean used shoot meristems as the target tissue (McCabe *et al.*, 1988). The putative transformed shoot apices were induced to form multiple shoots prior to whole plant regeneration. All of

the transgenic plants described in this first report were chimeric. In subsequent studies, non-chimeric plants were obtained through the use of screening methods for selection of plants that contained transgenic germ-line cells (Christou *et al.*, 1989; Christou, 1990; Yang and Christou, 1990). However, using shoot tips as a source tissue for particle bombardment is very labor-intensive, because each regenerated plant must be screened for transgenic sectors that could potentially give rise to a transgenic flowering shoot. Specifically, it is the L2 and L3 layers that need to be transformed if transgenic progeny are to be recovered (Christou and McCabe, 1992). For this reason, this method can only be successful if the bombardment device can produce enough force for the particles to penetrate past the L1 layer and into the L2 layer (Sato *et al.*, 1993).

Somatic embryos offer an alternative target tissue for particle bombardment. Recovery of transgenic soybean plants using this target tissue was first reported by Finer and McMullen (1991). This system has since been used by numerous laboratories to produce transgenic soybean (Browse *et al.*, 1993; Sato *et al.*, 1993; Parrott *et al.*, 1994; Hadi *et al.*, 1996; Stewart *et al.*, 1996; Liu *et al.*, 1996). Proliferating somatic embryos are ideal for the particle gun- and *Agrobacterium*-mediated transformation, because the origin of the proliferating embryos is at or near the surface of the older embryos. In addition, these embryogenic initials are numerous in these cultures and selection for the transformed embryogenic tissue grown in liquid medium is more stringent when the appropriate selection agent is applied.

When embryogenic suspensions were directly compared to shoot apices as the target tissue for particle bombardment, embryogenic suspensions appeared to be the more efficient of the two methods (Sato *et al.*, 1993). Bombardment of the shoot apex with the GUS gene resulted in sectored GUS activity in 30% of the bombarded shoots; however, none of these shoots generated a transgenic plant. In contrast, bombardment of somatic embryogenic suspensions produced four GUS-positive clones, each of which were able to regenerate into non-chimeric transformed plants.

However, the use of embryogenic suspensions does suffer from limitations. The major limitations of this method are the efforts required to establish liquid cultures and problems with sterility of plants regenerated from old embryogenic cultures (Hadi *et al.*, 1996). This fertility problem appears to be a function of the tissue culture process, because non-transformed plants regenerated from older established cultures usually exhibit partial or full sterility, as well as other morphological abnormalities (Liu *et al.*, 1992; Liu *et al.*, 1996). Therefore it is imperative to establish and transform young (<1 year old) cultures. Aside from this drawback, these cultures have several advantages as targets for transformation. Embryogenic tissue is generally very responsive to transformation (Finer and McMullen, 1991; Finer and McMullen, 1990; Fromm *et al.*, 1990), and embryogenic soybean is no exception. Many independent clones can be recovered from each experiment, and many plants can be regenerated from each clone. Another advantage of using embryogenic suspension cultures is that chimeric plants should not be recovered if embryos are allowed to proliferate under selection for a period of time after the transformation. The introduced trait should therefore be in germ-line tissue and be passed on to the progeny.

In planta methods of transformation

In perhaps the simplest of all techniques for soybean transformation, the axillary region of germinating seeds were inoculated with *Agrobacterium*. Of the seeds inoculated, 0.07% were reported to be transgenic, and to produce transgenic progeny (Chee *et al.*, 1989). A tissue culture-free procedure such as this one could be very attractive if the frequency of success was higher.

Electroporation of intact nodal meristems is another transformation method recently reported which circumvents the soybean tissue culture process completely (Chowrira *et al.*, 1995). This process involved removing the fully expanded leaves to expose the terminal bud from 7–10-day-old seedlings. The introduced DNA was injected with a syringe into the terminal bud in a solution containing lipofectin. The apical meristem was then electroporated in a circular electrode and the plant was grown to maturity with no selection. This procedure was performed on several meristems and some chimeric plants were obtained. This method is intriguing but, without more supportive data and optimization, it is too early to determine its ultimate usefulness for soybean transformation.

Finally, transformation of soybean chloroplasts was reported after DNA was injected directly into soybean ovaries. The reported transformation rate of 24% was high, but transgenic chloroplasts were not consistently transmitted to the progeny (Liu *et al.*, 1989; 1990).

Future direction of soybean transformation

Soybean transformation has been reported by a small number of university and industrial laboratories. Unfortunately, all of the procedures described in the literature suffer from problems of low efficiency, poor reproducibility and limited cultivar specificity. The Soybean Center for Tissue Culture and Genetic Engineering was established in 1993 to aid and assist academic and industrial scientists in the area of soybean transformation. The goals of the center are to find efficient and reliable methods to transform soybean and to transfer this technology for soybean improvement. This center is comprised of laboratories from the following universities: University of Kentucky, The University of Georgia, and The Ohio State University. Due to this collaboration, there is optimism that soybean transformation will become routine in the near future. Described below in detail are four methods that The Soybean Center for Tissue Culture and Genetic Engineering is using to transform soybean. These methods include use of the particle gun to introduce foreign DNA into either embryogenic suspension tissues or embryogenic tissue on semi-solid medium and use of *Agrobacterium* with either embryogenic suspension cultures or cotyledonary nodes. A protocol is also included for regeneration of soybean plants from somatic embryos. Between the three laboratories that make up The Soybean Center for Tissue Culture and Genetic Engineering, we are refining and optimizing these methods as well as developing new methods for soybean transformation. Additional information and updates to protocols may be obtained at http://mars.cropsoil.uga.edu/homesoybean/.

Protocol 1: Particle bombardment of soybean embryogenic suspension cultures using the Particle Inflow Gun (PIG) and the Biolistic PDS-1000 (Fig. 1)

Initiation and proliferation of embryogenic cultures

Initiate and maintain soybean embryogenic cultures using the procedure of Finer and Nagasawa (1988) as modified by Bailey *et al.* (1993). Aseptically remove immature seeds (4–6 mm) of soybean from sterilized pods. Cut off and discard the end of the seed/embryo containing the embryonic axis. Push out and separate the two cotyledons from the seed coat and place on D40 induction medium (MS salts, B5 vitamins, 6% sucrose, 40 mg/l of 2,4-D, 0.2% Gelrite™, pH 7.0) (Bailey *et al.*, 1993). Culture explants on induction medium with the abaxial side facing the medium (Hartweck *et al.*, 1988). Accelerate the induction of somatic embryos by wounding the initial explant with a scalpel (Santarem *et al.*, in press). After 3–4 weeks on D40 medium, transfer the embryo-induced cotyledons to D20 medium (D40 medium with concentration of 2,4-D and sucrose reduced to 20 mg/l and 3%, respectively; pH 5.8) (Wright *et al.*, 1991) for proliferation. Selectively subculture high quality tissue every two to four weeks. Initiate liquid suspensions by placing compact, small-lobed embryogenic tissue in FN medium (Finer and Nagasawa, 1988). Selectively subculture high quality tissue every 10–14 days.

Pretreatment of tissue

For each bombardment, place 0.5 g of tissue (previously subcultured 5–10 days before bombardment) in the center of a sterile Petri dish (100×15 mm) and spread out in a uniform layer of embryogenic "clumps". Remove excess medium with a sterile pipette and pre-dry the samples uncovered in a laminar-flow hood for 15 min prior to bombardment (Vain *et al.*, 1993). After pretreatment, cover samples with a Petri dish lid until bombardment. Generally, bombardment should take place within 30 min after pretreatment.

Preparation of tungsten and DNA for particle bombardment (PIG)

Sterilize 50 mg of tungsten particles (M10, Sylvania) in a 1.5 ml microfuge tube with 500 μl 95% ethanol for 20 min. Briefly pellet particles by centrifugation and resuspend in 500 μl sterile deionized water. Pellet and wash particles five more times with sterile deionized water. After last wash, resuspend tungsten particles in 500 μl sterile deionized water at a concentration of 100 mg/ml. Five minutes prior to bombardment, precipitate DNA onto the tungsten particles. For DNA precipitation, rapidly mix together 25 μl of resuspended particles, 5 μl of DNA (1 μg/μl), 25 μl 2.5 M $CaCl_2$, 10 μl 100 mM spermidine and precipitate on ice for 5 min.[1] Gently tap the microfuge

[1] Sterile 2.5 M $CaCl_2$ can be stored at room temperature for at least one year. The 1.0 M spermidine stock is stored at −20°C and is stable for 6 months. This stock solution is diluted with water to 0.1M

tube on the surface of the bench to form a defined particle/solution interface and carefully remove and discard 50 μl of the liquid.

Preparation of gold and DNA for particle bombardment (PDS-1000)

Add 12 mg of 1 μm gold particles to 1 ml 100% ethanol in a 1.5 ml microfuge tube.[2] Sonicate for 10 sec to suspend the gold particle and place on ice for 30 sec. Repeat sonication/ice two additional times and spin down the gold particles. Remove the ethanol and add 1.0 ml sterile distilled water. Vortex to resuspend particles. Spin down and wash two additional times with sterile water. After the final spin, resuspend gold particles in 200 μl sterile distilled water and fix the microfuge tube to the vortexer. When the DNA preparation is ready, withdraw 50 μl of the gold particle suspension to a new microfuge tube. Add 5 μg DNA (1 μg/μl), 50 μl of 2.5 M $CaCl_2$ and 20 μl of 100 mM spermidine to 50 μl gold suspension and vortex for 3 min. Microfuge for 10sec and remove the supernatant. Add 400 μl of 70% Ethanol, vortex, spin and remove supernatant. Add 40 μl 100% Ethanol, sonicate three times for 1 sec, and spread 5 μl of DNA/gold suspension on each of eight microcarriers and allow to dry.

Bombardment with a particle inflow gun (PIG)

Resuspend the DNA-coated tungsten particles and load a 2 μl aliquot into a sterile syringe filter unit[3]. Immediately attach the syringe filter to the leur-lock adapter at the top of the PIG chamber, place the target tissue 15 cm from the syringe filter, place a 500 μm mesh baffle[4] over the tissue and apply vacuum to the PIG. When the vacuum is around -100 kPa, apply one 50 msec burst of helium (60 psi) to the apparatus, delivering the coated particles to the target tissue. Following bombardment, vent the chamber and remove the target tissue.

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spermidine prior to use. DNA is stored at a concentration of 1 μg/μl in TE at -20°C. For co-transformation, an equimolar amount of each plasmid is used. To enhance transformation of one plasmid over another, increase the molar ratio to 5:1 or 10:1. If precipitated particles are not used within 20 min, they may be difficult to resuspend due to agglomeration.

[2]Prepare gold just prior to each use. Do not store or freeze. We normally use 1 μm gold particle size. Other sized particles are available.

[3]Particles are typically resuspended by either vortexing or "wash boarding" the microfuge tube over a microfuge tube holder a few times. Once particles are resuspended, it is critical to load the 2 μl droplet into the syringe filter and bombard as soon as possible. If particles fail to resuspend it is best to re-precipitate another sample. Often the cause is either a DNA sample contaminated with bacterial chromosomal DNA or ethanol contaminants from the particles. To correct these problems, be sure to thoroughly rinse the tungsten particles and use a high quality DNA preparation.

[4]The baffle is made by cutting the top and bottom off a 400 ml plastic beaker so the height is about 8 cm. A 500 μm nylon or steel mesh is then melted on the bottom side of the cut beaker. This baffle serves two functions: it more evenly distributes the particles and also contains tissue displacement within the Petri dish during bombardment.

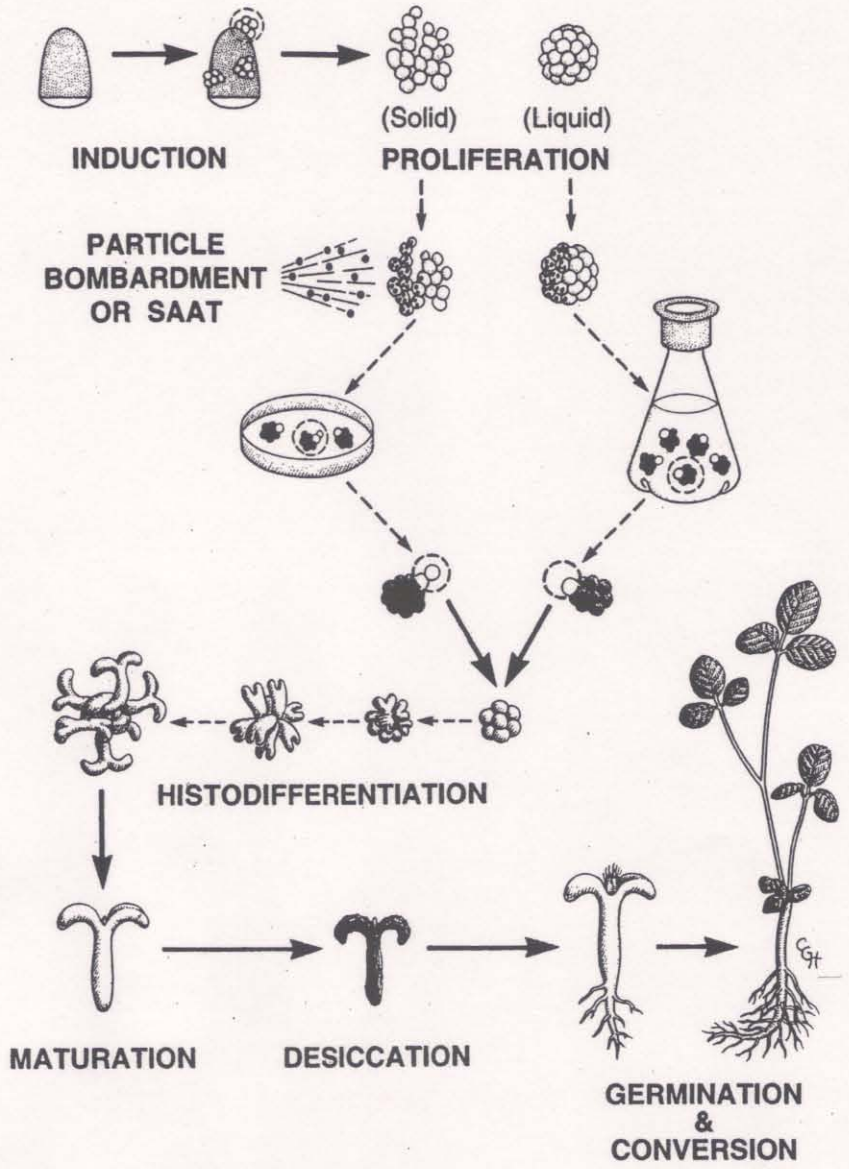

Fig. 1. Scheme for genetic transformation of soybean (*Glycine max* (L.) Merr.) embryogenic cultures and regeneration of transgenic plants. Embryogenic clusters derived from the culture of immature cotyledons on auxin-containing medium are subjected to particle bombardment. Following selection on medium containing an appropriate selective agent, repetitive embryos undergo histodifferentiation, maturation and desiccation prior to regeneration of plants. The particle bombardment stage can be replaced with SAAT.

Bombardment with a DuPont/BioRad Biolistic PDS1000

The prepared tissue is centered under the "target" zone for the Biolistic Particle Delivery System (PDS-1000) with a helium accelerator, and manufacturers procedures are followed with the instrument.[5] We normally use 26–27 inches of Hg, 650 psi, with the target tissue placed 9 cm under the rupture disk.

Post-treatment of tissue and selection of tissue

Allow the target tissue to recover in the covered Petri dish for 30–90 min after bombardment. Place the embryogenic tissue in 30 ml liquid FN medium (Finer and Nagasawa, 1988) and culture on an orbital shaker (150 rpm) in light (30 $\mu Em^{-2}s^{-1}$) at 27°C. Select transformed tissue by the addition of a selection agent to the medium seven to 14 days after bombardment. Use a concentration of 20–50 mg/l hygromycin or G418 depending on the selectable marker used. Replenish medium weekly and isolate transgenic clones 4–6 weeks after bombardment. Regenerate plants as described in Protocol 5.

PROTOCOL 2: *Agrobacterium*-mediated transformation of soybean embryogenic suspension cultures using SAAT

Tissue preparation

Initiate and maintain soybean embryogenic suspensions as described in Protocol 1.

Agrobacterium *preparation*

Grow *Agrobacterium* EHA105 (Hood *et al.*, 1993) containing a binary vector under selection of 50 mg/l rifampicin and the appropriate antibiotic for retention of the binary vector in a modified Luria broth (LB) medium containing 5 g NaCl and 5 g sucrose to log phase. Pellet the bacteria at 1500 *g* for 10 min, resuspend in FN medium, re-pellet as above, and finally resuspend in FN medium to an OD_{600nm} between 0.01 and 0.5.

Tissue sonication and coculture

Transfer ten clumps of highly embryogenic soybean suspension culture tissue (2–4 mm in diameter) to sterile 13×100 mm borosilicate glass tubes. Add 1 ml of diluted *Agrobacterium* suspension to the clumps and sonicate the tissue 0–60 sec using a bath sonicator, Model PC5 (L & R Manufacturing Co., Kearny, New Jersey, USA). Blot the tissue on filter paper to remove residual *Agrobacterium* and transfer to baffled 125 ml flasks with 30 ml FN medium containing 100 μM AS. After 2 days of coculture, replace medium with fresh FN medium containing 400 mg/l Timentin™. Select and

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[5]Vacuum, helium pressure and rack placement in the PDS1000 can be varied and the best results determined empirically. Additional methods have also been used to concentrate the DNA target zone by the use of barrel placed over the tissues (see Torisky *et al.*, 1996).

maintain stably transformed cultures as described in Protocol 1 with the addition of 400 mg/l Timentin™ to the FN medium. Regenerate plants as described in Protocol 5.

PROTOCOL 3: Particle bombardment of D20 proliferating embryo cultures (Fig. 1)

Tissue preparation

Initiate embryogenic cultures and maintain cultures on D20 as per Protocol 1. Use freshly-subcultured, high quality cultures for bombardments.

Particle bombardment

For each bombardment, place 10 embryogenic clumps from D20-maintained cultures (about 100 mg tissue, total) in the center of a 100×15 mm Petri dish containing D20 medium. Dry the tissue (on the medium) uncovered in a laminar flow hood for 10 min prior to bombardment. Precipitate DNA onto particles and perform bombardment as described in Protocol 1. Bombard tissue in each plate twice. Between bombardments, rotate the plate 180°. Ten days after bombardment, subculture tissue on D20 medium. Start selection for transformed tissue using lower levels of selective agent and then increase after the first month (Stewart *et al.*, 1996). For hygromycin selection, transfer the tissue to D20 medium containing 25 mg/l hygromycin 14 and 24 days after bombardment. Thereafter, subculture or transfer tissue to D20 medium containing 50 mg/l hygromycin. Throughout selection, subculture or transfer bombarded tissue every seven to ten days. Isolate transgenic clones 2–3 months after selection. Regenerate plants as described in Protocol 5.

PROTOCOL 4: *Agrobacterium*-mediated transformation of soybean cotyledonary nodes (Fig. 2)

Explant preparation

Surface sterilize and germinate soybean seeds on a medium containing half-strength B5 salts and vitamins (Gamborg *et al.*, 1968) 30 g/l sucrose, 6 g/l agarose, pH 5.5) for 1–2 days. Prepare the cotyledonary nodes by the complete removal of the seed coat, the removal of all but 4 mm of the hypocotyl, and the bisection of the remaining hypocotyl to yield two identical explants still attached to the cotyledon (Fig. 2). Expose explants, adaxial side up, and macerate with a fine scalpel through the meristem and cotyledonary node. This maceration disrupts primary shoot morphogenesis and provides an entry route for the *Agrobacterium*. Place explants in a 16× 125 mm borosilicate glass tube (~10 explants/tube) and immerse in a medium containing B5 salts and vitamins, 9.9 mg/l BAP, 0.2 mg/l indole butyric acid (IBA), 100 μM AS, 30 g/l sucrose, pH 5.5 (co-cultivation medium) until all the explants are prepared for *Agrobacterium* inoculation.

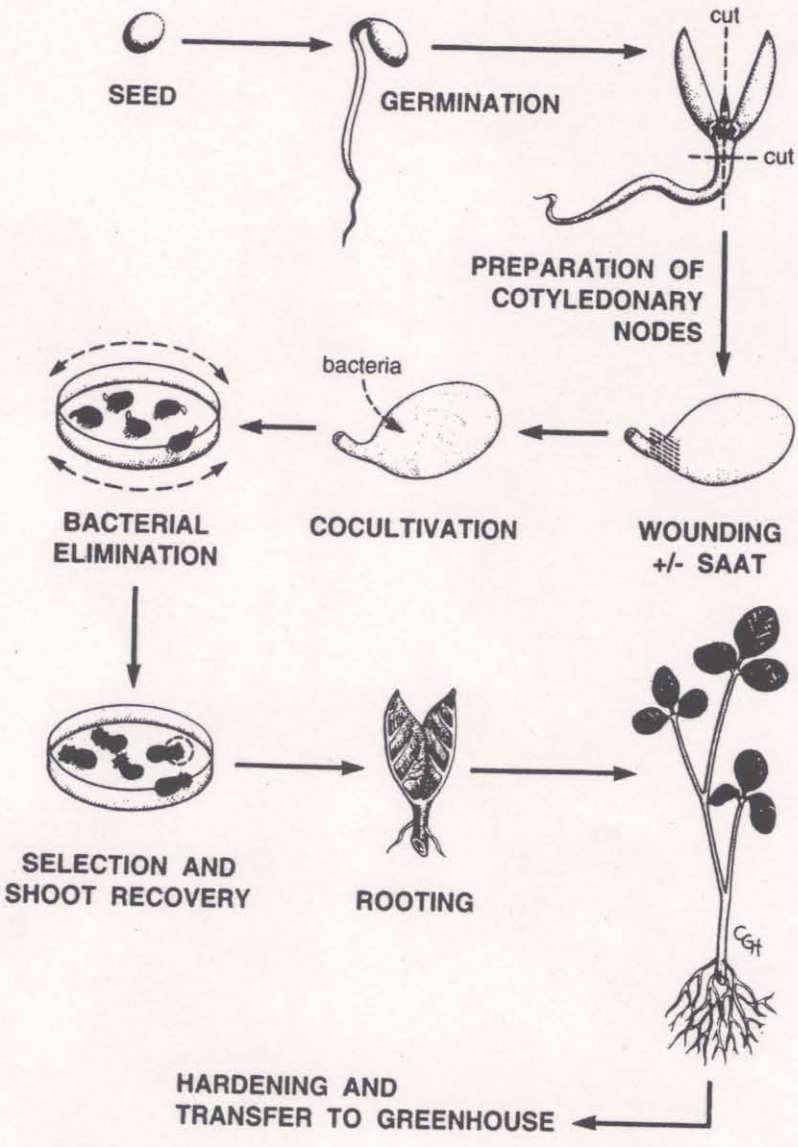

Fig. 2. Scheme for genetic transformation of soybean (*Glycine max* (L.) Merrill) cotyledonary nodes using *Agrobacterium tumefaciens* and recovery of transgenic plants. Excised cotyledonary nodes are wounded and then co-cultivated with *Agrobacterium*, after which the bacterium is eliminated with antibiotic. Following culture of the cotyledons on medium containing an appropriate selective agent, regenerated shoots are rooted and acclimated in the greenhouse. The use of SAAT prior to cocultivation may enhance the process.

clusters on this medium up to 4 weeks, or until individual embryos elongate sufficiently to be separated easily. Transfer individual differentiated embryos to MSM6 medium (same as MSM6AC, except for omission of activated charcoal) to promote maturation. Optimal density of individual embryos is 25 per 100×15 mm Petri dish. Maintain the temperature and light intensity as above. Embryo maturation should occur within about 4 weeks, although embryo appearance is a more reliable indicator of maturity. Immature embryos are a uniform green, but as they reach maturity, the cotyledons will become pale and eventually develop a cream color, which indicates attainment of physiological maturity. Desiccate mature embryos in 100×15 mm Petri dishes at a density of 25 embryos per dish and seal with Nescofilm™ (Karlan Research Products Corporation, Santa Rosa, CA). A small piece (approximately 1 cm^3) of MSM6 medium can be placed inside the dish to promote gradual desiccation of embryos over a period of 5–7 days.

Germination and conversion

Germinate desiccated embryos on MSO medium (MS salts, B5 vitamins, 3% sucrose, 0.2% Gelrite™, pH 5.8) in 100×20 mm Petri dishes at 26°C, a light intensity of 60–80 $\mu Em^{-2}s^{-1}$ and a 23-h photoperiod (Parrott et al., 1988). Within 3–4 days, some embryos should begin to develop roots and pubescent shoots. Embryos capable of conversion will germinate within 3–4 weeks. Transfer plantlets with healthy branched roots and an elongating shoot to Magenta GA-7 boxes (Magenta Corp., Chicago, IL) containing 50–60 ml of MSO "Lite" medium (MSO with 1.5% sucrose). A maximum of four or five plantlets of similar size in each box is recommended. These can be allowed to grow for 2–3 weeks. Maintain the temperature, light intensity and photoperiod as above. Transfer plants to small pots (e.g., 6.35–cm) containing a 1:1 mixture of sand and Hyponex potting soil (Hyponex Corp., Maryville, OH), and place inside a pair of GA-7 boxes joined with a coupler. The soil is placed in the pot which is placed in the GA-7 box and autoclaved. Alternately, pots can be placed together in a clean plastic box, and covered to maintain high humidity. The soil mix should be saturated with water and these plants can be placed in a growth chamber at 25°C± 2°C, with 75% relative humidity, a 23-h photoperiod, and a light intensity of 200 $\mu Em^{-2}s^{-1}$. After 1–2 weeks, the plant coverings can be gradually removed over a 2- to 7-day period to induce acclimatization, and the plants can then be manipulated like normal seedlings and transferred to large pots in a greenhouse. Maintain the extended photoperiod until plants reach the desired size, at which point the photoperiod is reduced to induce flowering.

Conclusions

In this report, we present extensive and detailed protocols for transformation of soybean. Although particle bombardment of embryogenic suspension cultures (Protocol 1) and *Agrobacterium*-mediated transformation of cotyledonary nodes (Protocol 4) are the primary procedures used by our laboratories at present, the other

Agrobacterium *preparation and inoculation*

Grow *Agrobacterium* containing the gene of interest overnight in LB with appropriate antibiotics. The day prior to explant inoculation, re-inoculate 50 ml LB with appropriate antibiotics with 5 μl of overnight growth, shake for approximately 24 hr at 28°C and 200 rpm until OD_{600nm} is approximately equal to 0.5. Harvest and wash *Agrobacterium* as described in Protocol 2, except resuspend the bacteria in co-cultivation medium at a final concentration of 1.0 OD_{600nm}. Add equal volumes of bacteria to the explants, both in co-cultivation medium. SAAT-treat 30–60 sec using a bath sonicator as described in Protocol 2 and/or vacuum-infiltrate for 10 sec at 27 mm Hg. The duration of sonication and the duration and amount of vacuum applied are based on our best results to date but should be determined empirically for each tissue. After SAAT and/or vacuum treatment, remove explants to solid co-cultivation medium with 6 g/l agarose and place in the dark at 21°C for 48 hr.

Selection and plant regeneration of inoculated explants

Transfer co-cultured explants to liquid selection containing B5 salts and vitamins, 1.1 mg/l BAP, 0.2 mg/l indole butyric acid (IBA), 30 g/l sucrose, pH 5.7, augmented with 200 mg/l vancomycin, 100 mg/l Timentin™, 500 mg/l Mefoxin®, 50 mg/l kanamycin or 30 mg/l hygromycin (depending on selectable marker) and culture at 27°C, 150 rpm for 3 days with daily medium changes. After 3 days, transfer explants to solid selection medium (6 g/l agarose) and subculture every 10 days. After two subcultures, separate and discard cotyledons from the shoot morphogenic tissue. Transfer remaining explants to fresh medium with antibiotic selection (kanamycin or hygromycin) and subculture bi-weekly until shoots are detected. Remove and subculture new shoots emerging from the necrotic tissues for 8–12 weeks and then place on a medium containing half-strength B5 salts and vitamins, 2 mg/l IBA, 500 mg/l Mefoxin® and 6 g/l agarose for rooting. Once plantlets begin to root, transfer to sterile potting soil mix and acclimate to the greenhouse.

PROTOCOL 5: Regeneration/conversion of plants from embryogenic cultures (Fig. 1)

Soybean embryogenic cultures may be maintained on solid D20 (Wright *et al.*, 1991) or liquid FN (Finer and Nagasawa, 1988) media. Embryogenic clusters from either of these systems can be converted into plants using the following protocol, which is adapted from Bailey *et al.* (1993).

Histodifferentiation, maturation and desiccation

Transfer 20–25 embryogenic clusters derived from either D20 or FN media to 20–25 ml of MSM6AC medium (MS salts, B5 vitamins, 6% maltose, 0.5% activated charcoal (added after pH adjustment), 0.2% Gelrite™, pH 5.8) in 100×15 mm Petri dishes. Maintain cultures at 26°C and at a light intensity of 10 $\mu Em^{-2}s^{-1}$. Maintain

procedures are being evaluated as they do have good potential. For successful soybean transformation, the procedures that we present should be followed closely and the response of the cultures to the various manipulations should be noted. Often, tissues respond differently under different environments and the timing of a procedure must be adjusted accordingly. Soybean transformation in our laboratories is still not as efficient as we would like. Large efforts in our laboratories are still ongoing to make soybean transformation more efficient for us and others.

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