Chapter 22

Soybean [*Glycine max* (L.) Merr.]

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Abstract

In this chapter we describe an *Agrobacterium tumefaciens* transformation method of soybean that utilizes mature half seeds and regeneration from the cotyledonary node region. This method results in fertile transformed soybean plants and transgenic seed in approximately 9 months. Using mature half seeds as starting material has proven to be a reliable method that does not require additional wounding for infection to occur. We have continued to make improvements in the protocol, resulting in an efficient plant regeneration system.

Key words *A. tumefaciens*, Cotyledonary node, Glufosinate, Half seed, Soybean, Transformation

1 Introduction

Soybean is an economically important crop with many agronomic and industrial applications. Processed soybeans continue to serve as a major source of animal feed and vegetable oil and have new potential use in the bio-diesel sector. Biotechnologists have successfully transformed soybean since 1988 when the first reports using particle bombardment [1] and *Agrobacterium*-mediated cotyledonary node transformation [2] were published. Soybean genetic and plant breeding research and development programs now routinely include transformation technology; however, the genotypes that are amenable to transformation and the low transformation frequency limit its potential. Several recent publications have reviewed the history of soybean transformation discussing the range of explants, selectable markers, and technologies used [3, 4]. Several factors discussed in these review papers were important in our protocol development and improvement. For example, the use of the *bar* gene in the binary vectors combined with ammonium glufosinate selection decreased many of the chimeric selection issues previously associated with kanamycin selection [5, 6]. The addition of the antioxidant L-cysteine and thiol compound dithiothreitol to the cocultivation media have also increased the transformation efficiency [7, 8].

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This chapter focuses on the *A. tumefaciens*-mediated cotyledonary-node transformation using half seed of mature soybean seeds as the explant source. This method was first reported in 2006 [9]. The transformation process involves *Agrobacterium* infection on the half-seed explants obtained from overnight-imbibed seeds. The major advantage of this method [10] compared to the previously reported cotyledonary node method [2, 7] is that the deliberate manual wounding of the adaxial side of the cotyledonary node is omitted. As the result, the method is less labor intensive and more reproducible in our hands. We have further streamlined and improved the method since it was first published by simplifying the cocultivation step and lowering the level of chimeric plants being transferred to soil (*see* **Note 1**). While the efficiency of this method remains relatively low at ~5 % (5 herbicide-resistant T1 events per 100 infected and shoot-forming half-seed explants), the method requires no special wounding techniques other than dissecting the seed into two intact half seeds and trimming the embryonic axis. The process from seed dissection to transgenic soybean seed requires approximately 9 months.

2 Materials

2.1 A. tumefaciens Strains and Vectors

A. tumefaciens strain EHA101 [11] has been used routinely with this protocol. We use the standard binary vector pTF101.1 [12], which is a derivative of the pPZP binary vector [13]. The vector contains a selectable marker gene (*aad*A gene for resistance to antibiotics spectinomycin and streptomycin) for bacterium, origins of replication for *E. coli* and *Agrobacterium*, and right and left border fragments of *A. tumefaciens* T-DNA. The plant selectable marker gene cassette consists of double 35S CaMV promoter [14], a tobacco etch virus translational enhancer [15], the phosphinothricin acetyltransferase (*bar*) gene [16], and a soybean vegetative storage protein terminator [17]. There is also a multiple cloning site for subcloning the gene of interest between the right border region and the plant selectable marker cassette (*see* **Note 2**).

2.2 Plant Materials

Field-grown mature soybean seed cultivars Thorne, Williams, Williams 79, and Williams 82 have been successfully used as the mature half-seed explants for this method. These genotypes are from maturity group III.

2.3 Media Stock Solutions

All stocks are made with double-distilled water (ddH_2O), filter sterilized using a 0.22 μm cellulose acetate (CA) membrane filter (with the exception of acetosyringone), and aliquoted. Storage temperatures are listed.

2.3.1 Phytohormones

1. 6-Benzyl-aminopurine (BAP, 1 mg/ml): Dissolve 0.05 g BAP in 1 ml of 1 N NaOH. Bring up to 50 ml final volume with ddH_2O. Store at 4 °C.
2. Gibberellic acid (GA3, 1 mg/ml): Dissolve 0.05 g GA3 in 1 ml 95 % EtOH. Bring up to 50 ml final volume with ddH_2O. Store at 4 °C.
3. Zeatin riboside (ZR, 1 mg/ml): Dissolve 0.05 g ZR in 0.5 ml 1 N NaOH. Bring up to 50 ml final volume with ddH_2O. Store at 4 °C.
4. Indole acetic acid (IAA, 1 mg/ml): Dissolve 0.05 g IAA with 1.0 ml 1 NaOH. Bring up to 50 ml final volume with ddH_2O. Store at –20 °C.
5. Indole-3 butyric acid (IBA, 1 mg/ml): Dissolve 0.05 g IBA in 0.5 ml 1 N KOH. Bring up to 50 ml final volume with ddH_2O. Store at –20 °C.

2.3.2 Antibiotics and Selective Agents

1. Ammonium glufosinate (20 mg/ml): Dissolve 0.250 g ammonium glufosinate in 12.5 ml ddH_2O. Store at –20 °C.
2. Cefotaxime (100 mg/ml): Dissolve 1.0 g cefotaxime in 10 ml ddH_2O. Store at –20 °C.
3. Timentin (100 mg/ml): Dissolve 1.0 g timentin in 10 ml ddH_2O. Store at –20 °C.
4. Vancomycin (50 mg/ml): Dissolve 1 g vancomycin in 20 ml ddH_2O. Store at –20 °C.
5. Herbicide solution (232 ppm ammonium glufosinate): Add 1.25 ml Liberty™ herbicide (Bayer Crop Science, LP) (18.9 % ammonium glufosinate) to 800 ml ddH_2O in a 1-l spray bottle. Add 1 ml Tween 20 and bring final volume to 1 l with ddH_2O. Store at room temp.

2.3.3 Other

1. Asparagine/pyroglutamic acid solution: Dissolve 0.5 g L-asparagine and 1.0 g L-pyroglutamic acid in 80 ml ddH_2O. Add ddH_2O to 100 ml final volume.
2. Acetosyringone (AS, 100 mM): Dissolve 0.196 g of acetosyringone in 10 ml ETOH. Store 1 ml aliquots at –20 °C.
3. L-Cysteine: Dissolve 400 mg (for each liter of CC media) in 5 ml ddH_2O.
4. Dithiothreitol (DTT): Dissolve 154 mg (for each liter of CC media) in 5 ml ddH_2O.

2.4 Culture Media for A. tumefaciens

1. YEP medium: 5 g/l Yeast extract, 10 g/l Bacto peptone, and 5 g/l NaCl. Adjust pH to 7.0 with 1 N NaOH. Appropriate antibiotics should be added to the cool medium prior to inoculation. For pTF101.1 in EHA101 this is 50 mg/l kanamycin and 100 mg/l spectinomycin.

2.5 Soybean Tissue Culture Media

1. Cocultivation medium (CCM): 1/10 strength B-5 media with Gamborg Vitamins (Phytotechnology labs product G398; *see* **Note 3**), 3.9 g/l 2-(N-morpholino)ethanesulfonic acid (MES), 30 g/l sucrose, 1.67 mg/l BAP, adjust pH to 5.4 with 1 N KOH. Add 4.25 g/l Noble Agar (Difco). Autoclave, cool to 55 °C, then add cysteine (400 mg/l), DTT (154.2 mg/l), and acetosyringone to 200 μM. Pour into sterile 100 × 15 mm plates (~10 ml/plate).
2. Infection medium (IM): 1/10 strength B-5 media with Gamborg Vitamins (Phytotechnology labs product G398; *see* **Note 3**), 3.9 g/l MES, 30 g/l sucrose, 1.67 mg/l BAP adjust pH to 5.4 with KOH. Autoclave and cool to 55 °C. Add acetosyringone to 200 μM just before using.
3. Shoot induction medium (SIM): Full-strength Gamborg B5 Medium with Gamborg Vitamins (Phytotechnology labs product G398; *see* **Note 3**), 1.67 mg/l BAP, 0.59 g/l MES, 30 g/l sucrose. Adjust pH to 5.6 with KOH and add 7 g/l Phyto Agar (Plant Media-BioWorld). Autoclave and cool to 55 °C. Add 100 mg/l timentin, 100 mg/l cefotaxime, 50 mg/l vancomycin, and 8 mg/l glufosinate. Pour into sterile 100 × 20 mm plates (~35 ml/plate).
4. Shoot elongation medium (SEM): Full-strength MS Medium with Gamborg Vitamins (Phytotechnology labs product M404; *see* **Note 3**), 0.59 g/l MES, 30 g/l sucrose, adjusted pH to 5.6 with KOH and add 7 g/l Phyto Agar. Autoclave and cool to 55 °C. Add 50 mg/l asparagine, 100 mg/l L-pyroglutamic acid, 0.1 mg/l IAA, 0.5 mg/l GA_3, 1 mg/l ZR, 100 mg/l timentin, 100 mg/l cefotaxime, 50 mg/l vancomycin, and 8 mg/l glufosinate. Pour into 100 × 25 mm petri dishes (~35 ml/plate).
5. Rooting media (RM): 1/2 strength MS Medium with Gamborg Vitamins (Phytotechnology labs product M404; *see* **Note 3**), 20 g/l sucrose, adjusted pH to 5.6 with KOH and add 7 g/l Phyto Agar. Autoclave and cool to 55 °C. Add 1 mg/l IBA and 3 mg/l glufosinate. Dispense into sterile 25 × 150 mm vial (10 ml/vial).

3 Methods

3.1 Seed Sterilization

1. Place mature soybean seeds in 100 × 15 mm Petri plates in a single layer (about 120 seeds per plate).
2. Arrange up to five open Petri plates in a bell jar desiccator in such a way that all interior plate surfaces are exposed and allow enough space to accommodate a 250 ml beaker. Place lids upright along the inside wall of the desiccator. Place desiccator in an exhaust (chemical fume) hood.

3. Place a 250 ml beaker with 100 ml of commercial bleach (6 % sodium hypochlorite) in the center of the desiccator. Slowly add 3.5 ml of concentrated (12 N) HCl dropwise along the side of the beaker.
4. Seal the desiccator immediately and let stand overnight (16–20 h).
5. Open desiccator, place lids on Petri dishes, and transfer plates to a laminar flow hood. Remove the lids and allow the seeds to air out for at least 30 min to remove the excess chlorine gas.
6. Store sterile seeds in sealed Petri dishes under room temperature (22 °C) until use.

3.2 A. tumefaciens Infection Medium Preparation

1. Bacteria cultures for experiments are initiated from −80 °C glycerol stocks 2 days prior to an experiment. Start a 2 ml culture of *Agrobacterium* strain carrying a binary vector by inoculating the YEP media containing appropriate antibiotics (here we add 100 mg/l spectinomycin and 50 mg/l kanamycin) with a loop of bacteria from the glycerol stock. Allow the culture to grow for 12–20 h at 28 °C in a shaking incubator (~250 rpm).
2. Approximately 20 h prior to the experiment transfer ~250 μl starter culture to 250 ml YEP containing antibiotics in a 1 l flask.
3. Allow the culture to grow at 28 °C, 250 rpm, to $OD_{620} = 0.8–1.0$.
4. Collect *Agrobacterium* culture by centrifuging at 5,180 × *g* for 20 min at ambient temperature (~22 °C).
5. Resuspend the pellets in infection medium (IM) by gently pipetting with a transfer pipet. Bacterial pellets are resuspended to 50 % the original volume (pellet from a 50 ml liquid culture is resuspended in 25 ml IM).
6. Allow the resuspended culture to shake gently at ~60 rpm for at least 30 min at room temperature before use.

3.3 Seed Imbibition

1. Approximately 16 h prior to the infection experiment, add sterile ddH_2O to the sterilized seeds until the seeds are 2/3 covered. This is approximately 25 ml of water for 120 seeds in a 15 × 100 mm plate (*see* **Note 4**).
2. Cover the plates with aluminum foil to block out the light and place in a 24 °C incubator overnight.

3.4 Explant Preparation and Inoculation

1. Transfer ~10 imbibed seeds to a sterile paper towel for dissection.
2. Using forceps to hold an individual seed and a #15 scalpel blade to make a longitudinal cut along the hilum to separate the cotyledons. Gently peel off the seed coat. Trim the emerging embryonic axis to no less than 3 mm (Fig. 1a).

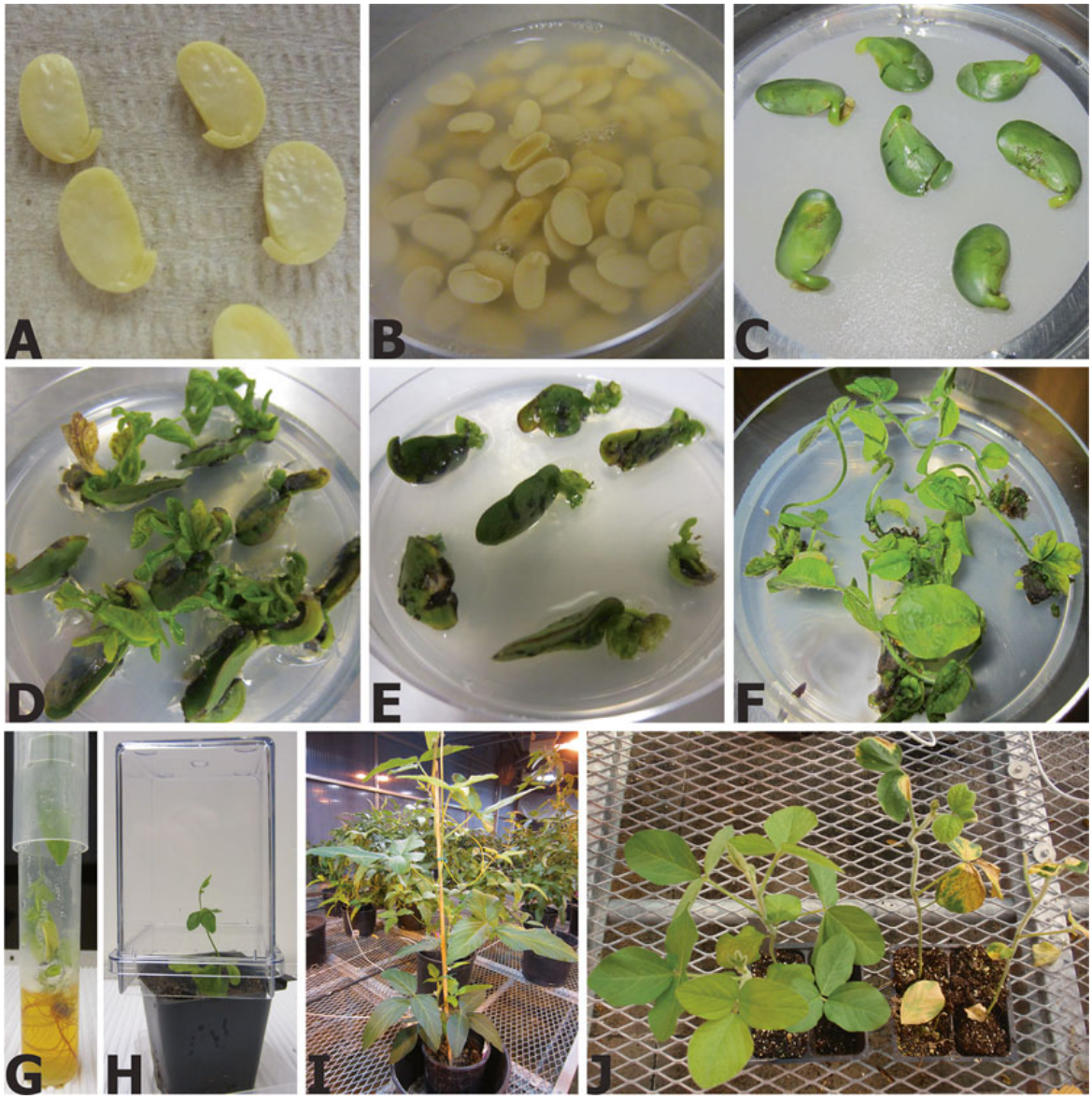

Fig. 1 Soybean transformation using *Agrobacterium tumefaciens* and the mature half-seed cot-node method. (**a**) Explants prepared by dissecting imbibed mature seeds and trimming embryonic axis. (**b**) Inoculation of explants with IM/*Agrobacterium*. (**c**) Cocultivation of half seeds for 3–5 days on CCM. (**d**) Shoot initiation from cot-node and apical meristem after 2 weeks on SI medium. (**e**) Second transfer to SI media. Shoot tissue from cot node and apical meristem is cut off. (**f**) Elongation of shoots on SE medium after 1 month. (**g**) Shoot that has produced roots on RM medium. (**h**) Transplanting of rooted plants to soil and covered to initially keep humidity high. (**i**) Transplanting to larger container for seed production. (**j**) Progeny testing of R1 seed. Putative transgenic plants (*left*) remain green after screening with ammonium glufosinate herbicide selection

3. Dissect approximately 40–50 seeds (80–100 half seeds) and transfer them to a 20×100 mm sterile plate. Add 25 ml of *Agrobacterium* infection medium. Gently swirl the covered dishes to ensure that all explants are in contact with the infection media. Allow the explants to incubate for 20–30 min at room temperature (Fig. 1b).

4. After inoculation, transfer the half seeds to sterile paper towel adaxial (flat) side up to drain away excess *Agrobacterium* culture.
5. Transfer the half seeds to CCM (ten per plate) so that the flat, adaxial side is in contact with the filter paper.
6. Wrap individual plates with parafilm or place plates in plastic bags with venting slits.
7. Incubate the plates at 24 °C with an 18:6 photoperiod (140 μM/m²/s) for 3–5 days (Fig. 1c).

3.5 Selection and Plant Regeneration

3.5.1 Shoot Induction

1. After 3–5 days of cocultivation, remove the half seeds from CCM and place briefly on sterile paper towel flat side (adaxial) down.
2. Transfer the explants onto SIM, 6–8 explants per plate. Orient the half seed so that the nodal end of the cotyledon is imbedded in the medium with the flat side facing up at a 30°–45° angle. Transfer only the seeds that have an intact embryonic axis.
3. Wrap each plate with vent tape and incubate at 24 °C, 18:6 photoperiod (140 μM/m²/s) for 14 days (Fig. 1d).
4. Transfer explants to fresh SIM after 14 days. Trim shoot growth from the cot-node and apical area until flush with cotyledon; also trim back the embryonic axis to 3 mm (Fig. 1e).
5. Maintain cultures in the incubator as before for 14 additional days.

3.5.2 Shoot Elongation

1. After 28 days on SIM (2 transfers), remove the regenerated shoot tissue from the cot-node area of the explants by cutting away the majority of the cotyledon. At the base of each shoot is a swollen area called the shoot pad. Be sure to leave part of the shoot pad attached to the explant. Remove necrotic brown tissues on shoot pad by making a fresh cut at the base of the explant shoot pad that will be in contact with the medium. Slightly imbed the explants into the media (six per plate).
2. Transfer the tissue to fresh SEM every 2 weeks. At each transfer be sure to make a fresh cut at the base of the shoot pad. Allow up to 8 weeks (four transfers) for shoot elongation (Fig. 1f).

3.5.3 Rooting of Transgenic Plants

1. When elongated shoots are at least 3 cm long, excise them close to the shoot pad.
2. Transfer the shoots into a 25 × 150 mm vial containing RM by embedding the shoot approximately 1/2 cm into the media.
3. Incubate at 24 °C, 16:8 photoperiods (140 μM/m²/s) for up to 3 weeks (*see* **Note 5**).

3.6 Transplanting and Greenhouse

1. After 2–3 weeks, many of the elongated shoots will develop normal, healthy-appearing roots (Fig. 1g). These plantlets can be transplanted to soil. Carefully remove the plant from the rooting media (*see* **Note 6**).
2. Gently remove the agar medium from the roots by gently rinsing with lukewarm tap water.
3. Place plantlet into an individual 2.5-in. plastic pot containing moistened soil mix (Redi-Earth Peat-Lite). After transplanting, water once with 1/4 strength 20-20-20 (N-P-K) Liquid Plant Fertilizer.
4. Cover the plantlets to keep the humidity high. Grow plants in a greenhouse or a growth chamber with 24 °C temperature and 16:8 photoperiods (Fig. 1h).
5. Once plants begin actively growing in soil, the herbicide solution may be applied to confirm the *bar* gene resistance. Apply herbicide solution (ammonium glufosinate 232 ppm) to the upper surface of one leaf twice over a 3-day period.
6. After 1 week the plants with leaves resistant to ammonium glufosinate herbicide screening may be immediately transplanted for seed production.
7. Fill a 1-gal nursery pot with soil (MetroMix 900). Add one tablet of controlled-release fertilizer with trace elements (Osmocote Plus15-8-11) (N-P-K) 7.5 g per 1-gal pot.
8. Transplant herbicide-resistant plant to the middle of the 1-gal pot. The soil should cover all the roots.
9. Fill with water to the top edge of the pot. Let it drain completely and water once more until the water reaches the top. After this time water as needed.
10. The greenhouse conditions are set to a 28 °C (day) and 21 °C night with a 16:8-h light:dark photoperiod 500 μM/m^2/s.
11. Marathon (insecticide) may be added 1 week after transplanting for aphid control. Yellow sticky traps may also be used to reduce or eliminate white flies or gnats.
12. Plants usually require staking for support. Loosely bind main stem and branches to a long bamboo stake using twist ties (Fig. 1i).
13. After pods are fully filled out and plant leaves begin to die, remove all leaves from the plants. Discontinue watering. Allow seeds to dry down.
14. The soybean pods will dry at variable rates. To prevent pod shatter and consequent seed loss, remove mature seedpods immediately and store them in a labeled paper bag until all pods on the plant are harvested (*see* **Note 7**).

3.7 Progeny Analysis of R_1 Generation

1. Plant ~8 seeds from R_0 plants in soil to test for the *bar* gene resistance. Include a positive control (expressing the *bar* gene resistance to the ammonium glufosinate herbicide spray), as well as a negative control (wild type).
2. After the first trifoliate has fully emerged (approximately 2 weeks after planting, spray plants with herbicide solution (ammonium glufosinate @ 232 ppm)). Spray once, and then 2 days later spray again.
3. Record the number of Liberty-resistant plants in approximately 7 days (Fig. 1j).

4 Notes

1. We have eliminated washing the half seeds between the transfer from CCM to SIM [6]. We now include glufosinate selection in the RM medium which eliminates chimeric plants.
2. Most of our experience with this protocol is with pPZP 200-derived vectors. The majority of our *Agrobacterium* strain experience is with EHA101, but we have successfully used LBA4404 and EHA105RecA strains with this protocol.
3. We use commercial media from Phytotechnology Laboratories for convenience. The complete description is currently available at www.phytotechlab.com. Other commercial or homemade media using the same recipe should work. Gamborg B-5 Basal Medium (Phytotechnology Laboratories product G398) contains full-strength B5 major and minor salts, full-strength B5 vitamins, 28 mg/l ferrous sulfate, and 38 mg/l disodium ethylenediaminetetraacetate dihydrate Na EDTA. MS Modified Basal Medium with Gamborg Vitamins (product M404) contains full-strength major and minor salts, full-strength B5 vitamins, 29 mg/l ferrous, and 38 mg/l Na EDTA.
4. To avoid having seeds split and/or crack during the 16-h imbibition prior to dissection it may help to partially submerge the seeds rather than covering completely with water. Additional water can be added immediately prior to dissection if seed coats appear dry.
5. Cultures are moved from 18-to-6 photoperiod setting for shoot elongation to 16–8 setting for rooting to acclimate plantlets to the greenhouse photoperiod, which is 16–8 setting.
6. Do not use forceps to remove plants from rooting tubes, as the roots will often break off. Instead remove the entire agar plug from the tube using a small chemical spatula.

7. The seed set for R_0 plants is quite variable and plant maturity times vary considerably over the year with photoperiod length and light intensity. Seed yields can vary from 1 to 250 seeds per plant and 3–6 months of greenhouse time to acquire mature seed.

References

1. Christou P, McCabe DE, Swain WF (1988) Stable transformation of soybean callus by DNA-coated gold particles. Plant Physiol 87:671–674
2. Hinchee MAW, Connor-Ward DV, Newell CA, McDonnell RE, Sato S, Gasser CS, Fischoff DA, Re DB, Fraley RT, Horsch RB (1988) Production of transgenic soybean plants using agrobacterium-mediated DNA transfer. Nat Biotechnol 6:915–922
3. Homrich MS, Wiebke-Strohm B, Weber RL, Bodanese-Zanettini MH (2012) Soybean genetic transformation: a valuable tool for the functional study of genes and the production of agronomically improved plants. Genet Mol Biol 35:998–1010
4. Hyeyoung Lee, So-Yon Park and Zhanyuan J. Zhang (2013). An Overview of Genetic Transformation of Soybean, A Comprehensive Survey of International Soybean Research - Genetics, Physiology, Agronomy and Nitrogen Relationships, Prof. James Board (Ed.), ISBN: 978-953-51-0876-4, InTech, doi: 10.5772/51076. Available from: http://www.intechopen.com/books/a-comprehensive-survey-of-international-soybean-research-genetics-physiology-agronomy-and-nitrogen-relationships/an-overview-of-genetic-transformation-of-soybean
5. Zhang Z, Xing A, Staswick P, Clemente T (1999) The use of glufosinate as a selective agent in *Agrobacterium*-mediated transformation of soybean. Plant Cell Tissue Organ Cult 56:37–46
6. Zeng P, Vadnais DA, Zhang Z, Polacco JC (2004) Refined glufosinate selection in *Agrobacterium*-mediated transformation of soybean [Glycine max (L.) Merrill]. Plant Cell Rep 22:478–482
7. Olhoft P, Somers D (2001) L-Cysteine increases *Agrobacterium*-mediated T-DNA delivery into soybean cotyledonary-node cells. Plant Cell Rep 20(8):706–711
8. Olhoft P, Lin K, Galbraith J, Nielsen N, Somers D (2001) The role of thiol compounds in increasing Agrobacterium-mediated transformation of soybean cotyledonary-node cells. Plant Cell Rep 20:731–737
9. Paz MM, Martinez JC, Kalvig AB, Fonger TM, Wang K (2006) Improved cotyledonary node method using an alternative explant derived from mature seed for efficient Agrobacterium-mediated soybean transformation. Plant Cell Rep 25:206–213
10. Paz M, Wang K (2009) Soybean transformation and regeneration using half-seed explants. US Patent #7,473,822, 6 Jan 2009
11. Hood EE, Helmer GL, Fraley RT, Chilton MD (1986) The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. J Bacteriol 168:1291–1301
12. Paz MM, Shou H, Guo Z, Zhang Z, Banerjee AK, Wang K (2004) Assessment of conditions affecting Agrobacterium-mediated soybean transformation using the cotyledonary node explant. Euphytica 136(2):167–179
13. Hajdukiewicz P, Svab Z, Maliga P (1994) The small versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. Plant Mol Biol 25:989–994
14. Odell JT, Nagy F, Chua NH (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313:810–812
15. Carrington JC, Freed DD (1990) Cap-independent enhancement of translation by a plant potyvirus 5′ nontranslated region. J Virol 64:1590–1597
16. White J, Chang SY, Bibb MJ (1990) A cassette containing the bar gene of Streptomyces hygroscopicus: a selectable marker for plant transformation. Nucleic Acids Res 18:1062
17. Mason HS, Dewald D, Mullet JE (1993) Identification of a methyl jasmonate-responsive domain in the vspB promoter. Plant Cell 5(3):241–251