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Publish by Abstract

Adaptation of Cotton Shoot Apex Culture to *Agrobacterium*-Mediated Transformation

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Abstract. A protocol is presented for rapid genotype-independent transformation and regeneration of cotton (*Gossypium* spp.) from shoots isolated from germinating seedlings. Isolated shoots are inoculated with a super-virulent strain of *Agrobacterium tumefaciens*, subjected to a mild antibiotic selection, and directly regenerated as shoots *in vitro*. Shoots do not dedifferentiate and mutation rates are low. Rooted shoots can be obtained within 6–10 weeks of isolation and inoculation depending on the cotton cultivar.

Key words: *Agrobacterium*, cotton, *Gossypium*, shoot apex, transformation

Introduction

The*Agrobacterium*-mediated transformation method described here was developed from the genotype-independent shoot apex plant regeneration method we originated for cotton (Gould and Smith, 1988; Gould et al., 1991a). This transformation and regeneration approach (Smith et al., 1992) has been used in *Agrobacterium*-mediated transformation of petunia (Ulian et al., 1988), maize (Gould et al., 1991b) and rice (Park et al., 1997). A more efficient and detailed procedure is described here. Changes to earlier procedures include use of precultured shoots in the inoculation step to insure activation of cell division in apical meristematic tissues, and inclusion of cytokinin in the culture medium during selection to promote shoot growth.

Use of the *Agrobacterium* vector is technically simple and gene transfers are often low copy, permanent and heritable. In this method, genetic transformation occurs following inoculation of dividing meristematic cells in a shoot apex with a supervirulent strain of *A. tumefaciens*. Hormonal manipulation is kept to a minimum to permit the native developmental program

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in the apex to control plant regeneration, allowing regeneration to be plant-driven and, therefore, genotype-independent. Plants generate directly from inoculated shoots on a simple MS-based medium (Murashige and Skoog; 1962; Shabde and Murashige, 1977) that has been used to regenerate many cultivars of *Gossypium* (Gould et al., 1991a), maize (Gould et al., 1991b) and other cereals. No other hormonal manipulations are necessary unless shoot multiplication is desired (Hemphill et al., 1998).

Shoot meristem and apex cultures became popular in the ornamental nursery industry after the discovery that rapidly growing shoots of many virus-infected clones could be free of virus and used to produce virus-free germplasm (Morel and Martin, 1952). Over time, it was observed that the incidence of genetic mutations and somaclonal variation was low in plants regenerated from shoots. One of the reasons for this low mutation frequency may be the absence of tissue dedifferentiation steps that are common in the initiation of callus and somatic embryo cultures. This event is known to trigger retrotransposon activity in cultured plant tissues and produces permanent mutations (Hirochika, 1993).

The procedure described here uses transformation-competent cells in the shoot apex of germinating seedlings for *Agrobacterium*-mediated transformation and for plant-driven regeneration. Mature seed is used because it is readily available and easily germinated when needed. Isolation and inoculation of shoots is followed by regeneration of normal, fertile plants that flower and set viable seed. An overall transformation efficiency of 5–10% or greater can be expected.

Materials and Methods

Plant material and culture media

Start with fresh, de-linted seed of any desired variety of cotton (*Gossypium* spp.). Culture medium is made from pre-mixed Murashige & Skoog, 1962 (MS) inorganic salt formulation and minimal organics that includes myo-inositol and thiamin (can be purchased through Sigma (M-9274), or Gibco BRL, Grand Island NY).

Seed germination medium (SG)

Eight grams of Bacto Agar in 1 liter of water, dispense SG into sterile petri-plates @ 25 ml/plate, or $\frac{1}{2}$ pint glass canning jars, or baby food jars at approx. 20 ml/jar. Cover jars with inverted petri dish.

Plant culture media (MS)

All plant media are based on the **MS** formulation (Murashige and Skoog, 1962) (M-9274 Sigma), solidified with 8 g/L agar, sterilized by autoclaving, and dispensed into sterile plastic petri dishes (deep dishes are best).

MS+Kin: MS + kinetin 0.1 mg/L (Gould et al., 1991a; Shabde and Murashige, 1977).
MS+C: MS+carbenicillin (Sigma) 500 mg/L (or Clavamox® 250 mg/L).
MS+Kin+C+Kan: MS+kinetin 0.1 mg/L, carbenicillin, 500 mg/L (or Clavamox®, 250 mg/L) + kanamycin 30–50 mg/L.
MS+AC: MS + 0.5–1% charcoal (activated and neutralized).
LB: Bacterial culture media is solidified using 15 g/L agar (Sambrook et al., 1989).
LB+Kan: LB + kanamycin, used with *A. tumefaciens* EHA105 strain and most other *Agrobacterium* vectors, except EHA101.
LB+Kan+X: LB + kanamycin, 50 mg/L + X (appropriate antibiotic), for the EHA101 strain (which has native chromosomal kanamycin resistance) containing a binary vector carrying resistance to a different specific antibiotic (X) (i.e., pGA482, tetracycline 5–10 mg/L).
Vir **IM**: *Vir* Introduction medium is seventy-five mM MES pH 5.4 (Wayne Barnes, personal communication), 2% glucose (Fullner et al., 1996), and acetosyringone 30–100 μM (Veluthambi et al., 1989). We use 50 μM acetosyringone, octopine 100 mg/L (Veluthambi et al., 1989; Raineri et al., 1990), Kinetin 100 mg/L.

Potting soil

2 portions peat: 1 washed river sand: 2 vermiculite, coarse grade (2:1:2). If 1 part = 1 cu. ft., then add: 500 ml dolomite, 250 mL gypsum and slow-release macro- and micronutrients (Courtesy of Joe Thaxton, USDA, Southern Crops Regional Facility). Cotton needs a neutral pH and is sensitive to calcium deficiency (use 1 or 2 cherry tomato plants as indicator plants for calcium deficiency).

Bacteria and culture media

A. tumefaciens, EHA101 (Hood et al., 1986), EHA105 or other super-virulent strain. Strike out a fresh plate of *Agrobacterium* two days prior to shoot inoculation. Grow bacteria for two days on agar-solidified **LB** medium containing selective antibiotic on desk or bench top, 20–27 °C. Do not exceed 28 °C.

A. tumefaciens is a soil bacterium and virulence can be lost when incubated at 28 °C or higher.

Virulence induction medium **IM** contains activators and protectors of the *Agrobacterium* virulence genes, which can increase transformation rate. After two days growth on **LB**, described above, scrape bacteria from one culture plate and mix with 1 ml *vir***IM** to make a thick bacterial suspension. Vortex briefly and use immediately.

Binary vectors

A binary vector carrying the desired genes must be transferred to *A. tumefaciens* (Mersereau et al., 1990). The binary vectors pGA482 (An, 1987) carrying tetracycline resistance, or pPZP 200 series (Hajdukiewicz et al., 1994) carrying spectinomycin resistance are suitable for use with EHA101. The binary vectors pGA482, pPZP 100 series, pBI101 series including pBI121, or pBIN19 can be used with EHA105.

Method

1. **Seed sterilization.** Cotton seed can be difficult to decontaminate and a number of approaches work; however, only one is presented here. Wrap de-linted seeds in cheesecloth and soak in water 30 min. Remove water and soak seeds in H_2O_2 (30%) for 30 min. Rinse 3X with sterilized water. Soak seeds in 50% Chlorox solution for 30 min, change the solution every 10 min. Remove Chlorox and rinse seeds 4X in sterilized water. Leave seeds in final rinse 30 min–1 h.
2. **Seed germination.** Squeeze seeds out of the seed coat and culture onto **SG** medium to germinate 7 days, in light, at 27–30 °C. Germination will take longer if the temperature is lower. Removing the seed coat prior to germination reduces contamination.
3. **Shoot isolation and preculture.** The seedling shoot is embedded in the stem between the cotyledons. Remove one cotyledon by pushing down on it until it snaps off (Figures 1 and 2). This exposes the shoot apex (described in Gould et al., 1991a). The shoot (epicotyl) is removed from the seedling and cultured in **MS+Kin** for 3–5 days, 5 shoots/plate. Remove shoots if they are contaminated. This step helps initiate cell division in the apex and allows identification of contaminated shoots prior to inoculation.
4. **Inoculation**. Two approaches to shoot inoculation are described. Alternative approaches can be used as long as bacteria are applied to meristem regions (Figure 3).

Table 1. Inoculation and schedule – cotton shoot apex transformation

Procedure	Time	Media and Components
Seed germination	5–7 days in light	Seed Germination Medium (**SG**)
Shoot isolation and pre-culture	3–7 days	**MS**(Sigma M-9274)+ 0.1 mg/l **kinetin** **(MS+kin)**
Agrobacterium culture	1–2 days 20–24 °C	EHA101(pTi) **(LB+Kan+Tet)** EHA105(pTi) **(LB+Kan)**
Shoot apex isolation and inoculation	$\frac{1}{2}$ day	Scrape new growth of bacteria from overnight culture. Mix bacteria with 1 ml *vir* **Induction Medium** (*vir* **IM**): 75 mM MES pH=5.4; 100 uM, acetosyringone, 100 mg/L octopine, 2% glucose, 10mg/100ml kinetin.
Co-cultivation	3–7 days	**MS** + 0.1 mg/l **kinetin (MS+kin)**
Reculture	1 week	**MS** + **carbenicillin** 500 mg/l **(MS+C)**
Selection	1–2 weeks	**MS** + **carbenicillin** 500 mg/l+0.1 mg/l **kinetin** + **kanamycin** Test: 10–50 mg/l (**MS+C+K+Kin**)
	1–2 weeks	Selection can be repeated 1 week.
Rooting	1–4 weeks 2 weeks	**MS** + **Charcoal 3%** (**MS+AC**) If roots don't form, transfer to **MS+Kin** and allow shoots to enlarge. If shoots do not root, try options **a.** or **b**. **a.** Transfer large non-rooted shoots to soil following treatment of stem with rooting hormone powder (Rootone). Maintain high humidity and light. **b.** Graft non-rooted shoots onto germinated cotton seedlings.

I. Place shoot tissue shoot upright and 'shave' the shoot on two opposing sides creating a wedge of tissue with the shoot meristem in the center.

II. Shoots are bisected from apex to base producing two asymmetrical 'halves'. Inoculate and culture both halves although only the larger half will grow. Inoculate wound with *A. tumefaciens* mixed 1:1 in virulence induction medium (*vir* **IM**).

In both I and II, remove excess tissue from shoot base. Do not remove leaf primordia or elongating leaves, since these structures supply hormones and other growth factors that aid development of the shoot on the simple medium used (Shabde and Murashige, 1977). Place approximately 5 inoculated shoots onto 1 plate of **MS+Kin**.

5. **Co-cultivation (MS+Kin)**. Once all shoots have been prepared, inoculate shoot region generously with *Agrobacterium* mixed 1:1 with *vir* **IM**. Vacuum infiltrate 5 min. Keep the cultures in light on desk or bench top. Incubate 19–22 °C if possible to promote formation of bacterial pili (Fullner, 1996). Do not put the cultures under direct light, such as in a culture room, because the interior of the culture can become too hot. If condensation forms inside the culture plate, the culture is too hot. During cocultivation, check for bacterial overgrowth and move shoots to a clean spot on the plate if overgrowth occurs. Co-cultivate for 3–7 days. This amount of time is sufficient for genomic incorporation, transcription and accumulation of transcripts to occur, to aid survival during selection.
6. **Culture (MS+C)**. Transfer shoots to **MS+C** for 7 days. In **MS+C** and in **MS+Kin+C+Kan** we use Clavamox® (amoxicillin plus clavulanic acid, an inhibitor of penicillinase activity, Smith/Kline Bechamp, Veternary) in place of carbenicillin. It is relatively inexpensive and it is effective in killing *Agrobacteria* at low concentrations (250 mg/L), yet is not toxic to plant tissues at high concentrations (10,000 mg/L). It can be obtained through a veterinary pharmacy or a Veterinarian. Clavamox®, comes as sterile individually packaged tablets that are dissolved and suspended in 5 ml sterile water and added directly to cooled autoclaved media.
7. **Selection (MS+Kin+C+Kan)**. Transfer to **MS+Kin+C+Kan** selection medium containing 30–50 mg/L kanamycin and 500 mg/L carbenicillin (or Clavamox®) for 7 days to 2 weeks, at the rate of 5 shoots/plate. Small shoot explants of cotton are sensitive to kanamycin because of the prevalence of cell division. In this meristem-based method, if the meristem is killed, the procedure fails. One of the current problems in the shoot apex method is that the promoters used to drive *neo* (in many cases it is the *nos* promoter in *npt*II), may not be efficiently used in plant

meristem. Under these circumstances, the selection pressure used must be low and escapes occur; however, transformed shoots are not killed. If most shoots appear to be dying after 7 days to 2 weeks, transfer to rooting medium **MS+AC** (described below). However, if most shoots look robust after 7 days to 2 weeks of culture, reculture onto **MS+Kin+C +Kan** for an additional 2 weeks.

8. **Rooting (MS+AC)**. Surviving shoots will root spontaneously on **MS+AC** in 2 to 4 weeks. Culture 3 shoots/plate. Shoots that do not root should be returned to **MS+Kin** for 2–4 weeks, then transferred to MS+AC. Allow shoots to grow and root. Remove rooted plants from agar, rinse well, treat with fungicide (Subdue+Benlyate) and transfer to hydrated Jiffy 7 peat pellet contained in a 1 pint canning jar or magenta box. Place under high light, cover with a lid from a plastic Petri plate and keep watered. Once the root system becomes established, leaves will enlarge and plants will begin to grow vigorously. If shoots haven't rooted, there are two options:

   I. Dip the shoot stem in rooting powder, transfer to hydrated Jiffy 7 pellets and maintain under relatively high light. Water rooted shoots, treat with fungicide and keep in a sealed zip-lock bag until shoot begins to grow.

   II. Another alternative is to graft the shoot onto a germinated seedling of the same variety (Luo and Gould, unpublished).

9. **Flowering and pollination**. Flowering of the R_0 plants occurs within 20 days depending on the amount of light the plants receive and the temperature. In the field, cotton is insect pollinated. Cotton plants grown in a greenhouse are self-pollinated. Plants may need to be hand pollinated to insure self-pollination when determining segregation patterns.

10. **Progeny analyses.** Collect seed (R_1 , R_2... generation) and delint. Greenhouse-grown seeds are relatively free of contaminating organisms, therefore, sterilization does not need to be intensive. Surface sterilize de-linted seeds with 20% fresh bleach for 25 min, rinse 3X in sterile water, and germinate in water/agar (**SG**), or Jiffy peat pellets. Screen seedlings using GUS activity (Jefferson, 1988), and PCR amplification for the transferred genes. Transfer to soil and to the greenhouse. Under greenhouse conditions, cotton is self-pollinated and the transferred genes should segregate in the R_1 in a 3:1 pattern (75% will be positive). If more copies have been transferred, the ratio of plants carrying the gene will be greater. If regenerated plants are chimeric, any pattern of transformation in the R_1 can be expected.

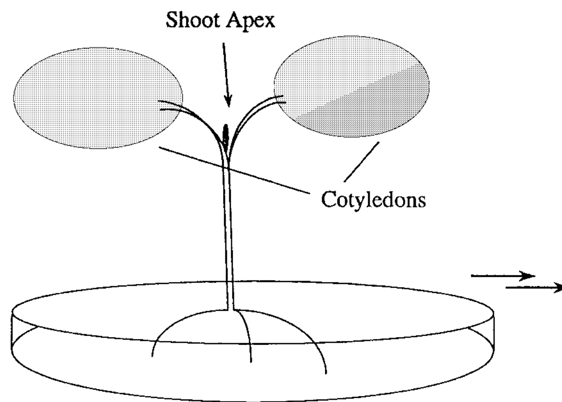

Figure 1. The seedling shoot is embedded in the stem between the cotyledons.

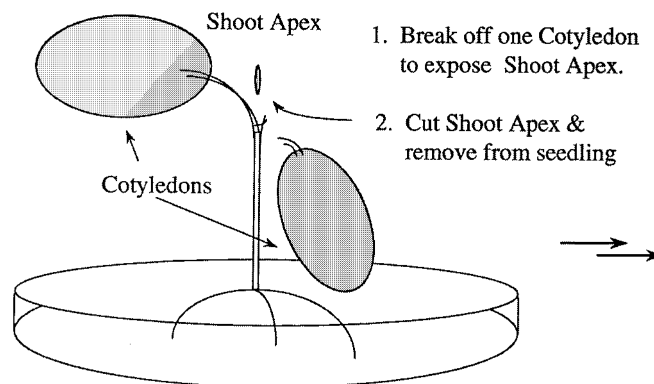

Figure 2. Remove one cotyledon by pushing down until it snaps off. This exposes the shoot apex. The seedling shoot apex, or epicotyl, is removed from the seedling and cultured in **MS+Kin** for 3–5 days.

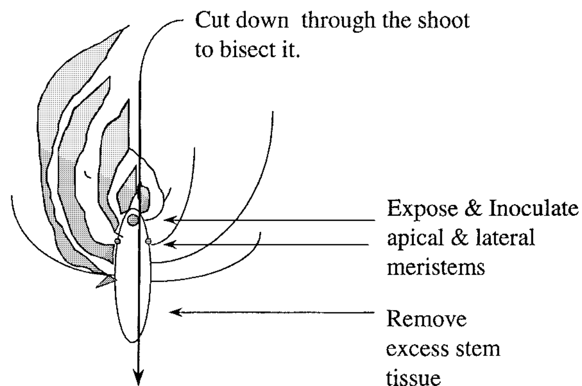

Figure 3. A lateral section of the cotton shoot is removed to expose the meristems in the apical region. This area is then inoculated with *Agrobacterium* and recultured.

Discussion

Three factors are important in *Agrobacterium* mediated transformation:

I. Presence of actively dividing cells (transformation-competent cells) in the plant apex.

II. Use of a 'super-virulent' strain of *A. tumefaciens* and induction of virulence.

III. An antibiotic selection level tailored to the level of promoter activity in the inoculated apical meristem and explant size, to avoid killing transformed tissues.

1. Transformation competent cells. In the procedure outlined here, isolated and inoculated seedling shoot apices are immediately cultured on **MS+Kin** to promote cell division in the apex and aid regeneration of the apical meristem. Inclusion of cytokinin in the culture medium helps to insure that some cells in the apex will be transformation competent.

2. Bacterial virulence. The super-virulent and broad host-range *A. tumefaciens* strains (EHA101, EHA105 and similar strains) are important for successful transformation of cotton shoots. Virulence can be induced by a variety of methods to insure activation of *vir* gene transcription. We have used *vir* **IM,** acetosyringone, nopaline or octopine in MES buffer (Veluthambi et al., 1989; Rainieri et al., 1990; Fuller et al., 1996; Wayne Barnes, personal communication).

3. Selection protocol. At this time, a permissive selection protocol is important in the practice of this method because plant regeneration is dependent on vitality of the meristem. The promoter used with the selectable resistance gene such as *neo*, must be active in the meristem for selection to work as one would normally expect. If cells in the meristem die, the organization inherent in the meristem is destroyed and regeneration from the meristem is abolished. In *nptII*, the *nos* promoter drives *neo*. If the *nos* promoter is used, protection to 10–30 mg/L of kanamycin can be expected from small, recently inoculated shoots. The CaMV 35S promoter is not active in plant meristem or shoot apex and if used with the antibiotic resistance gene, conferres *no* protection to this vital region. The amount of selection tolerated also depends on the size and preparation of the shoot explant, which is operator dependent and highly individualistic. Ten mg/L is recommended for the first selection passage. If too many escapes are produced following this procedure, increase the level of selection.

Note

Regenerated plants (R_0) should be considered chimeric. Upon analysis, transfered genes may be present in less than single copy, or present as multiple insertion events. For Southern blot

analysis, it is best to use the progeny (R_1, R_2, etc.) of self pollinated or outcrossed regenerated individuals. The transgenic progeny will not be chimeric and will reflect the transformation event that was present in the germ line.

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