Rapid, Efficient Production of Homozygous Transgenic Tobacco Plants with *Agrobacterium tumefaciens*: A Seed to Seed Protocol.

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Abbreviations: R0, indicates lines initially regenerated from tissueculture ; T1, plants grown from selfed R0 seeds; T2, plants grown from selfed T1 seeds.

ABSTRACT:

An optimized complete protocol was developed for *Agrobacterium tumefaciens*-mediated transformation of tobacco (*Nicotiana tabacum* L. 'SR1'), producing T1 flowering plants homozygous for the inserted T-DNA as verified by kanamycin resistance in T2 seedlings in 6 to 7 months from the time of cocultivation with *Agrobacterium*. Previous protocols require up to 9 to 12 months to obtain similar results. Procedures unique and important to this protocol include; a modified "whole-leaf" transformation coupled with a long duration of cocultivation, resulting in high rates of transformation, high levels of kanamycin in selection media resulting in few escapes, and extensive rooting of regenerants prior to a greenhouse hardening procedure. Once in the greenhouse, primary regenerants were maintained in small containers with long day photoperiod and high light levels, greatly shortening the time to seed set. Flowers from primary transformants were bagged to allow self pollination, and seed capsules harvested and dried prior to normal maturation on the plant. T2 seedlings were plated and selected on kanamycin media by an improved seed plating technique which eliminates the need for the placement of individual seeds, saving time and improving selection homogeneity. Using this protocol, over 130 independent tobacco lines from six separate gene constructs have been generated in a very short time period. Of these 130, nearly 60% segregated 3:1 for kanamycin resistance:susceptibility, indicating single transgene insertion events.

Introduction

Previous procedures for the Agrobacterium-mediated transformation of tobacco leaf disks have made the regeneration of transgenic tobacco lines a routine laboratory procedure (Horsch et al., 1985; Rogers et al., 1986; Burow et al., 1990). In performing numerous tranformation experiments we discovered several techniques which make the procedure even more timely and efficient. Using this procedure, previously inexperienced lab workers can produce and identify large numbers of homozygous T1 plants in 6-7 months from initial cocultivation with *Agrobacterium*. The protocol is based largely on the method of Burow et al. (1990) with new procedures including a modified whole-leaf transformation and long cocultivation, resulting in high rates of

transformation, high levels of kanamycin in selection media resulting in few escapes, and extensive rooting of regenerants prior to a greenhouse hardening procedure. Once in the greenhouse, primary regenerants were maintained root bound in small containers with long day photoperiod and high light levels, greatly shortening the time to seed set. Flowers from primary transformants are bagged to allow self pollination, and seed capsules harvested and dried prior to normal maturation on the plant (shortening time to harvest). T2 seedlings are then plated and selected on kanamycin media by an improved seed plating technique which eliminates the need for the tedious placement of individual seeds. In this paper we present a summary of the methods we have collected for tobacco transformation, from untransformed seed to homozygous transgenic seed. This includes many time-saving steps throughout the procedure from laboratory to greenhouse.

Materials and Methods

Equipment and Solutions

Magenta boxes (Magenta Corp., Chicago, Ill.) size GA-7 were obtained from Sigma.

Murashige and Skoog (1962) basal salts (M&S), Gamborg's B5 vitamins, Phytagel, all antibiotics, and other chemicals were obtained from Sigma. Sterile filter paper was prepared by autoclaving in aluminum foil packets. All solutions for tissue culture were autoclaved for 30 minutes at 121C.

For preparation of *Agrobacterium* strains (centrifugation and electroporation) a high speed centrifuge, and electroporation device (Hoeffer) suitable for bacterial cells, were used.

Media Recipes

YEB medium (*Agrobacterium* growth)

5.0 g sucrose
5.0 g peptone
5.0 g beef extract
1.0 g yeast extract
0.049 g $MgSO_4 \cdot 7H_20$
pH to 7.2; 15 g of agar for solid media

MSS medium (cocultivation / regeneration)

M&S Basal Salts (1 liter pkg)
1 ml of 1000x Gamborgs B5 Vitamins
30 gm Sucrose
0.5 ml of a 2.0 mg/ml solution of 6-BAP (BA)
Adjust pH to 5.6-5.8 with KOH (or NaOH)
Bring to 1000 ml
Add 2.7 g Phytagel per liter for solid media

MSr medium (seedling growth, rooting)

M&S Basal Salts (1 liter pkg)
1 ml of 1000x Gamborgs B5 Vitamins
3 gm Sucrose
7 gm Dextrose (Glucose)
Adjust pH to 5.6-5.8 with KOH (or NaOH)
Bring to 1000 ml
Add 2.7 g Phytagel per liter for solid media

Antibiotic Solutions:

Kanamycin monosulfate (Sigma K 1377) was prepared as a 50 mg/ml stock solution in distilled water and filter sterilized. Stock solution was prepared fresh for each experiment or stored at -20C for no longer than 2 weeks.

Carbenicillin (Sigma C 3416) was prepared as a 50 mg/ml stock solution in distilled water and filter sterilized. Note: Cefotaxime (Sigma C 7039) can be substituted at the same rate for Carbenicillin for some *Agrobacterium* strains which are not affected by Carbenicillin. We found also that a local University pharmacy was an inexpensive source of Cefotaxime (approx. 100 fold cheaper!).

All other antibiotic solutions were prepared in appropriate solvents (water or ethanol) and added at the concentrations described below to media which had been cooled to 55 C.

Protocol–

Transformation of Agrobacterium with plasmid constructs

As an alternative to tri-parental mating to shuttle plasmids cloned in *E. coli* into strains of *Agrobacterium*, electroporation is relatively fast and easy. Only 1pg to 1 ng of DNA of the desired construct is required along with *Agrobacterium* cells grown and prepared to be electrocompetent. Below is the procedure that is used to produce competent *Agrobacterium* (strain ABI) cells for electroporation with pMON881 constructs.

Making Competent Cells

- Start an overnight culture of *Agrobacterium* from a single colony on YEB/agar plates, using appropriate antibiotics (in this case, kanamycin, chloramphenicol). (Overnight culture in 3 ml volume, grow at **28** degrees C).
- Inoculate 1 liter of YEB liquid with 1 ml of the overnight culture.

- Grow the culture at 200 rpm, 28 C until it reaches an OD_{600} of above 0.5. (This may take several hours to overnight).
- Spin the cultures down (after cooling on ice for 10 min.) at 6000 rpm on a GSA rotor or equivalent (approx. 6000xg) for 20 minutes. (It takes longer to spin *Agro*. cells)
- Gently resuspend each pellet well in 100 ml 0.5% glycerol (sterile and ice cold) and spin down as above.
- Continue to wash and spin down as above with the following volumes:
  once with 75 ml **10%** glycerol
  once with 30 ml **10%** glycerol
- Resuspend final pellet in 1 ml total 10% glycerol (0.5 ml for each 500 ml flask).
- Aliquot into 50 to 100 ul aliquots and freeze at -80 C.
- Depending on the efficiency of transformation needed (usually very low required) these cells can be used for several months.

Electroporation procedure

- Follow manufacturer's instructions for operation of electroporation device. We have used the same conditions as those required for electroporation of *E.coli* cells with adequate results. One 50 ul aliquot of *Agro*. cells can be used for up to two electroporations.
- After electroporation, the cells are grown in 2-3 ml of YEB at 28C for 2 hrs and then plated on YEB agar plates with appropriate antibiotics. It takes about 36 to 48 hours before colonies have grown enough for analysis of results and restreaking.
- *Agrobacterium* strains are maintained aseptically by restreaking colonies on fresh plates or storing at -80 C in 15% glycerol.

Verification of plasmid constructs

- Plasmid DNA can be isolated from *Agro*. strains using the method of Lee and Rasheed (1990) with a few modifications:
- Grow 30 ml cultures of each strain for 36 to 48 hours.
- Double centrifugation times for all steps requiring precipitation of *Agro*. cells.
- If yield of plasmid DNA of a particular strain is not high enough to view by agarose gel electrophoresis, then Southern blotting of restriction digests of plasmid preparations can be used to verify the integrity of the constructs, using probes specific for the transgene.

Transformation of Tobacco

The amounts and volumes are given for a single transformation experiment consisting of a single Ti plasmid construction scaled to produce approximately 15 to 20 independent transformed plants. This involves using 20 to 30 sterile intact tobacco leaves as described below.

Preparation of a source of sterile tobacco tissue

- Sterilize seeds of *Nicotiana tabacum* L. cultivar 'SR1' by soaking in 10% household bleach, 0.1% Tween 20 for 20 minutes followed by five rinses in sterile DI H20.
- Germinate in Magenta boxes on MSr media without antibiotics and grow 3-4 weeks at 28C and 16h/8h day/night photoperiod. When plants reach the capacity of the Magenta box, upper portions of plants including the meristem and several leaves and 3-5 cm of stem can be cut and transferred to fresh MSr boxes to continue growth of aseptic tissue.

Incoculation with *Agrobacterium* and Cocultivation

- Prepare petri plates for cocultivation (100 mM, sterile) (MSS medium no antibiotics). 25 to 30 plates are sufficient for one experiment (15 - 20 independent transformants).
- Prepare 500 ml liquid MSS medium with no antibiotics for Agro-inoculation.
- Plate *Agrobacterium* on YEB medium, grow two days at 28C on agar plates, with appropriate antibiotics for strain selection (for strain ABI includes kanamycin, chloramphenicol, spectinomycin). Maintain plant tissue culture sterility levels at all stages (all bacterial transfers in sterile hood).
- In a 50 ml sterile conical tube containing 30 ml liquid MSS disperse 6-7 inoculating loops-full of *Agrobacterium* , taking loops directly from the plate.
- Using sterile large forceps, chop up media in MSS plates into approximatly 10 mm size chunks so that the media surface is very lumpy.
- From stock plants, excise healthy, green, expanding leaves of 5-7 cm in diameter. Do not use leaves crushed within the Magenta box, i.e. from plants grown too large.
- Using a razor blade, cut the underside of each tobacco leaf parallel to the midvein, leaving leaf intact but heavily cut, just prior to inoculation (Fig. 1). Do not allow leaves, after scoring, to dry out.

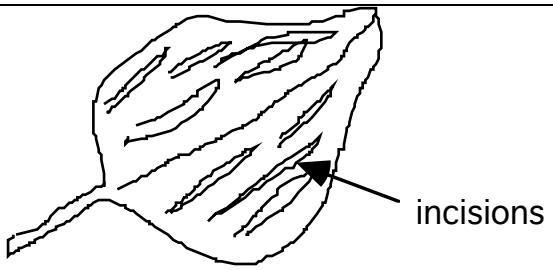

Figure 1. Multiple cut sites on tobacco leaf prior to co-cultivation.

- Disperse the *Agrobacterium* suspension and place three to four scored leaves into each 50 ml tube and incubate for approximately 10 minutes. (Five conical tubes typically produced fifteen plates for cocultivation and subsequently approximately 20

to 30 independent transformants. Five tubes are also a good number to work with given time constraints and the need for timeliness in transfer of cut leaf pieces)

- Blot excess *Agrobacterium* from leaf pieces and place onto chopped MSS plates (1-2 whole leaves per plate). Seal plates with parafilm and incubate under dim light for 24 hr (Plates can be stacked and covered with a pipet box to reduce light) and then for 5-6 days at 28C, 16h/8h day/night photoperiod in bright light (cool white flourescent , 56 micromoles/m/s). Monitor daily for signs of*Agrobacterium* overgrowth (severe overgrowth visible by eye).

Shoot Regeneration

- After 4-6 days total cocultivation (do not allow severe *Agrobacterium* overgrowth), wash leaf pieces by immersing in 250 ml liquid MSS + antibiotic (carbenicillin 200 mg/L) in a sterile 500 ml beaker, stirring vigorously with sterile forceps, not worrying about disrupting leaf intactness. Blot dry leaf pieces on sterile filter paper.
- Transfer to chopped (as before) MSS medium with selection antibiotic (300 mg/L kanamycin, 500 mg/L carbenicillin), in magenta boxes. Place leaf pieces firmly into chopped media, not worrying about submerging somewhat. Use one Magenta box per cocultivation plate, wrap with one layer of parafilm.
- Incubate in a 28 C growth chamber under 16/8 day/night photoperiod for 2 weeks.
- Transfer leaf pieces to new MSS boxes with fresh selection antibiotic, incubate an additional 10 days to 2 weeks during which the first or additional shoots should appear.

Rooting of Kan^r regenerants in tissue culture

- Transfer shoots when 1-3 cm in height by cutting cleanly away from any callus and the original leaf piece at a 45 degree angle to the newly regenerated stem. Excise bottom leaves.
- Place basal end approx. 5 mm deep into MSr media (60 ml/ box) containing 300 mg/l kanamycin in a magenta box so that the end touches bottom. Discard leaf pieces from which each shoot was chosen, as this eliminates the possiblity of selecting non-independent transformants. Number each shoot with unique number, record date and original plate for each shoot. Four to five shoots can be placed per magenta box.
- After substantial root growth (at least 3-5 cm in length and 3-5 main roots) transfer shoots to a hardening procedure prior to transfer to the greenhouse, following the method of Burow et al. (1990), as described below.

Preparation for transfer to greenhouse

- Prepare an autoclaved solution of 1/10 MS basal salts only (no vitamins, no sugars) and distribute 50 ml aliquots into magenta boxes, one for each independent rooted shoot.
- Remove plants from MSr Magenta boxes and wash off excess phytagel gently in a sink using clean but no longer sterile conditions.
- Place plants into 1/10 MS containing Magentas and let sit on lab bench with closed lid for 24 hr, then with lid ajar for an additional day.
- Transfer plants to autoclaved, pre-wetted potting mix (MetroMix 200) in 3 inch dia. plastic pots, placing plants in soil and firming in by hand.
- Water again with tap water to settle soil around roots, shaking out excess. Caution: this watering will suffice for several days in the greenhouse, tobacco plants are very susceptible to overwatering, especially at this stage.

- To prepare for greenhouse transfer, place two layers of cheesecloth over the plant and pot, secure rubber bands.

Growth in greenhouse: stress induced early flowering

- After two days under greenhouse conditions, remove one layer of cloth, remove the other after two more days. CAUTION: Water may not be necessary for at least 5 days, then water only lightly).
- After established, water stress plants by allowing plants to dry just to the wilting point between watering. Restrict root development by keeping the plants in the same 3" pots (half filled with soil) until maturity. Fertilize with 20/20/20 Peters fertilizer at one and three weeks from initial transfer to the greenhouse, all other watering with tap water only. During non-summer months, supplemental high-pressure sodium lights on a 17hr/7hr day/night photoperiod are used (one 400 watt bulb/ 40 plants, 2 m from bench height).

Controlled Pollination

- For self-pollination, waxed maize pollination bags (ear bags; Lawson #314) are placed over inflorescences beginning when corolla of first flower shows color prior to opening.
- For cross pollination (when desired for breeding multiple lines): Emasculation and pollination is done when corolla shows first color prior to flower opening. Foreign pollen is tapped on the stigma using a detached flower as source and the pollinated stigma is covered with a portion of a paper or plastic drinking straw, affixing the corolla to the straw by wrapping piano wire around the corolla. Straw is then pushed off by maturing seed capsule.

Seed harvest

- Harvest seed capsules when **first** begin to turn brown (approx. 15-20 days after pollination) or when, upon dissection, brown seeds could be seen. We use coin envelopes as containers for the pods.
- Dry seed capsules in a drying chamber consisting of a single incandescent light bulb (60W) positioned in a lab bench drawer (approx. 2-3 days).

Kanamycin Resistance Seed Germination Assay

To facilitate the identification of homozygous transgenic lines, seeds from selfed R0 plants are immediatly plated on kanamycin containing media to test for segregation of the T-DNA loci. Plants from T1 lines showing near 3:1 kan^r to sensitive ratios are then removed to the greenhouse, selfed and the resulting T2 seed tested in the kanamycin seed germination assay to identify homozygous T1 lines. To allow efficient and homogenous plating of seeds , a seed plating procedure was developed using agarose seed suspensions. Even plating of the seeds results in improved reliability of scoring for kanamycin resistance.

- Prepare solid MSr medium supplemented with kanamycin (300 mg/L) in 100 mm petri dishes (approx. 15 ml/dish, 2 dishes per seed lot).
- Sterilize and cool a solution of 0.15% (w:v) agarose (Molecular Biology Grade) in water and aliquot into 3 mls per 15 ml sterile disposable conical tube pre-labelled for each seed genotype.
- Using a sterile scalpel, and after briefly flaming each seed capsule (do not burn), cut the capsule end (only one locule necessary) and pour approx. 50-100 seeds into each 15 ml tube with agarose, using sterile technique. The goal is to pour about 50 seeds per plate, resulting in approx. 100 seeds total for each genotype.
- Mix seeds with the agarose by gently shaking for dispersal. Aseptically pour the seed/agarose mix onto the MSr/kanamycin plates. Cover and wrap with parafilm, swirl to evenly distribute seeds over plate.

- Incubate the plated seeds in a 28C growth room under constant illumination. Seeds should germinate in one week; score for kanamycin segregation after two weeks. Resistant plants will extend primary leaf primordia, sensitive plants will germinate, yellow and will not extend leaf primordia, non-germinating seeds should not exceed 10% with new seed but the percentage will increase with age. If the germination rate is significantly different from a wild type or transformed control grown under the identical conditions, an embryo lethal phenotype resulting from the transformation might be indicated.

Transplanting of seedlings from plates

- Remove seedlings after scoring and transfer to soil (Metro Mix 200) in trays with 2 inch plastic sections, (36 sections per flat) one plant per tray section. Water well and cover with plastic wrap, grow in lab for 3-4 days, then move to greenhouse. Shade plants initially with an overturned potting flat for 2-3 days, then uncover and grow to maturity under stress conditions as described above for fastest seed maturity.

Results / Discussion

Using this protocol, we have generated over 130 independent transgenic lines using sense and antisense constructs of six different cDNAs, in addition to vector-only controls. These include multiple transformation experiments that have been performed by two independent researchers. All of these transformations were performed with the *Agrobacterium tumefaciens* strain ABI and the plant binary transformation vector pMON881. Of the 130 independent transformants, nearly 60% segregated 3:1 for kanamycin resistance:susceptibility, indicating single transgene insertion events (see table I). Other transformants maintained high levels of resistance to kanamycin selection

(300 mg/ml) but segregated in ratios other than 3:1, as summarized in Table I (complete data only available for experiments with first four constructs).

This procedure is similar to that published by Burow et al. (1990) with minor yet important modifications which greatly speed up the procedure while requiring less hands-on time. We use electroporation for *Agrobacterium* transformation instead of a freeze-thaw or tri-parental mating methods. In addition, we do not use tobacco cell nurse cultures and vacuum infiltration for cocultivation. Our procedure uses a 4-5 day cocultivation using intact leaves for ease of manipulation and chopped media for good contact between the leaf piece and media. This same contact between leaf tissue and media is maintained under high levels of selection with kanamycin (300 mg/ml) during shoot regeneration. Rooting regenerated shoots in the presence of kanamycin resulted in few escapes, with kanamycin resistance remaining in nearly all lines in the next generations. Most of these lines segregated 3:1 for kanamycin resistance. Although we did not calculate our regenerants on a per calli basis, as did Burow et al. (1990), we had a success rate of one to two independent lines per small leaf cocultivated, which is comparable to their results. In one experiment using 10 to 15 intact leaves, we can reproducibly generate greater than 15 independent lines. Subsequent northern analysis for four of our constructs (over 70 independent lines) showed that approximately 60% of all homozygous lines (data not shown) showed detectable to high levels of expression of a transgene (linked to the kan^r gene).

Using this protocol we generated homozygous kan^r T2 seedlings in 6 to 7 months from the time of cocultivation with *Agrobacterium*. This is compared to previous protocols requiring up to 9 to 12 months to obtain similar results. We have collected many small techniques from laboratory to greenhouse which, when taken together, make the procedure much more efficient. Techniques important to this protocol included a

modified leaf "piece" transformation and long cocultivation, resulting in high rates of transformation as described above. High levels of kanamycin in selection media resulted in few escapes, and extensive rooting of regenerants in the presence of kanamycin prior to a greenhouse hardening procedure insured the success of each independent line. Very few, if any, lines were lost by transfer from tissue culture to the greenhouse (98% success rate). Once in the greenhouse, primary regenerants were maintained in small containers with long day photoperiod and high light levels, greatly shortening the time to seed set. Flowers from primary transformants were bagged to allow self pollination, and seed capsules harvested and dried prior to normal maturation on the plant. This allowed the screening of T1 seeds to begin at an earlier date. T1 seeds were plated and selected on kanamycin media by an improved seed plating technique which eliminated the need for the placement of individual seeds. Homozygous T1 plants were identified quickly by forced flowering in the greenhouse followed by a kanamycin seed assay. T2 plants, the third generation from tissue culture, could be grown in the greenhouse as homozygous lines expressing high levels of the transgene within 6-7 months from the initial cocultivation of leaf tissue with *Agrobacterium*.

References

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Table 1. Number of independent transformants derived from six different constructs and two separate experiments, and percentage of transformants in each segregation class (kan. resistant : kan. susceptible).

Experiment	Total Number Independent Transformants	% Segregating 3:1	% Segregating 15:1	% Segregating >3:1, <15:1	% Segregating <3:1 or >15:1
I. Constructs 1-4	70	64%	10%	18%	8%
II. Constructs 5-6	65	57%	nd	nd	nd

Table 2. Average time required for each step of the tobacco transformation protocol.

Stage	Average Time
Tissue Culture	
Leaf tissue / Agrobacterium cocultivation	**5 days**
Primary shoot selection	**20 days**
Secondary shoot selection	**10 days**
Rooting	**15 days**
Hardening for Greenhouse	**2 days**
Greenhouse	
R0 Generation	
Time till first flower	**35 days**
Time till first mature seeds	**15 days**
T1 Generation	
Time till Kan^r T1 seedlings transplanted	**20 days**
Time till first T1 flowering	**35 days**
Time till first mature T1 seeds	**15 days**
T2 Generation	
Identification of T1 homozygous line and Transplanting of T2 seedling	**15 days**
Total Time till Identification of T1 homozygous line	**187 days**