Selecting Homozygous Transformed Plants

Monday, May 06, 2013
4:08 PM

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Materials:
Agrobacterium transformed seed (T0 generation)
Sterile 1.5 ml microfuge tubes
Sterile 15 ml falcon tubes
50% Bleach solution
Sterile dH_2O
Sterile 0.1% Agarose
Sterile pipette tips 1 ml and 10 µl
10 mg/ml Kanamycin
Kanamycin selection plates
Parafilm
Laminar flow hood (for 0.5 hours)
Pots, soil and covers
1/4 strength Hoagland's solution

Procedure:
T1 Generation

1. Take each of the 6 independent lines of your T1 seeds and measure out 100 µl (≈ 2500 seeds) in a 2 ml microfuge tube. (100 µl is just below the first mark at the bottom of the tube)
2. Pipette in ≈ 2 ml 50% bleach solution. Mix by inversion for 5 - 7 minutes
3. Carefully pour off beach solution and add sterile dH_2O, mix for 1 minute
4. Repeat step 3 more times
5. Resuspend seeds in 2 ml sterile 0.1% Agarose solution, add 5 µl 10mg/ml Kan
6. Pipette seeds onto Kanamycin selection plates evenly distribute seeds using pipette tip (easiest done by dotting the seeds on using a Pasture pipette)
7. Leave plates open a crack in laminar flow hood for ≈ 15 minutes to allow excess moisture to soak in and/or evaporate
8. Cover plates and seal with micropore
9. Cover plates with foil and place in 4°C room for 2 days to stratify
10. After stratification place plates in the light (80 – 200 µmol $m^{-2}s^{-1}$) for 6 hours
11. After light treatment place plates in the dark at room temp for 2 days
12. Place plants in the light (80 – 200 µmol $m^{-2}s^{-1}$) for 5 - 7 days incubation in the light (light does not need to be continuous)
13. After light treatment, pick plants that have green expanded cotyledons. You will want to pick about 5 resistant plants for each of the 6 independent lines
14. Pick plants with forceps by gently scraping away seedlings that are Kan susceptible, they will be yellow in color with very small cotyledons. Then very gently remove the Kan resistant plant by picking up by one of the cotyldeons with forceps
15. Transfer the plant to soil watered with 1/4 strength Hoaglands and keep in tray with cover on in high humidity.
16. Grow plants up for seed, the seed will be the T2 generation and will be hemizygous (multiple tDNA's in the genome) for the insert.

17. While plants are growing up for seed do a PCR with primers in the Kan resistance gene or your transgene to make sure they are really transformed and not just freak Kan resistant plants

T2 Generation

1. From the 30 plants that you have harvested seed from, we will use 10 plants but be sure to keep seed from all 30 plants, in case you don't find any hits in the first batch
2. Put ≈ 100 seeds (doesn't have to be exactly 100 but you need to count to know how many) on small Kan selection plates made with agar and without vancomycin
3. Cover plates with foil and place in 4°C room for 2 days to stratify

4. After stratification place plates in the light (80 – 200 μmol $m^{-2}s^{-1}$) for 6 hours
5. After light treatment place plates in the dark at room temp for 3 days

6. Place plates in the light (80 – 200 μmol $m^{-2}s^{-1}$) for 5 - 7 days incubation in the light (light does not need to be continuous)

7. After light treatment you want to find a plate with a 3:1 ratio of resistant plants i.e. 3 plants alive for every one plant that is dead.
   From the plants on these plates 1/2 will be heterozygous for the insert, 1/4 will be homozygous for the insert (what you want), and 1/4 will be WT (you also want this for your azygous control, see method below)
   Note: If you have a 15:1 ratio it means you have 2 inserts of your gene
8. Pick about 16 resistant plants with forceps by gently scraping away seedlings that are Kan susceptible, they will be small and largely rootless. Then very gently remove the Kan resistant plant by picking up by the hypocotyl with forceps
9. Transfer the plant to soil watered with 1/4 strength Hoaglands and keep in tray with cover on in high humidity.
10. Grow plants up for seed, the seed will be the T3 generation

Note: You can do experiments with the T2 generation but the progeny will not be stable

Zygous, WT control Selection from T2 Generation

1. Take T2 seed (only needed from 1 construct since we are looking for a WT) and plate out ≈ 100 seeds on MS plates without Kan.
2. From these plants test at least 16 with PCR for the Kan gene and find a plant that does not have the Kan gene, this will be your zygous control

T3 Generation

1. From each of the 16 plants that you have harvested seed from put ≈ 100 seeds (doesn't have to be exactly 100 but you need to count to know how many) on Kan selection plates made with agar and without vancomycin
2. Cover plates with foil and place in 4°C room for 2 days to stratify

3. After stratification place plates in the light (80 – 200 μmol $m^{-2}s^{-1}$) for 6 hours
4. After light treatment place plates in the dark at room temp for 3 days

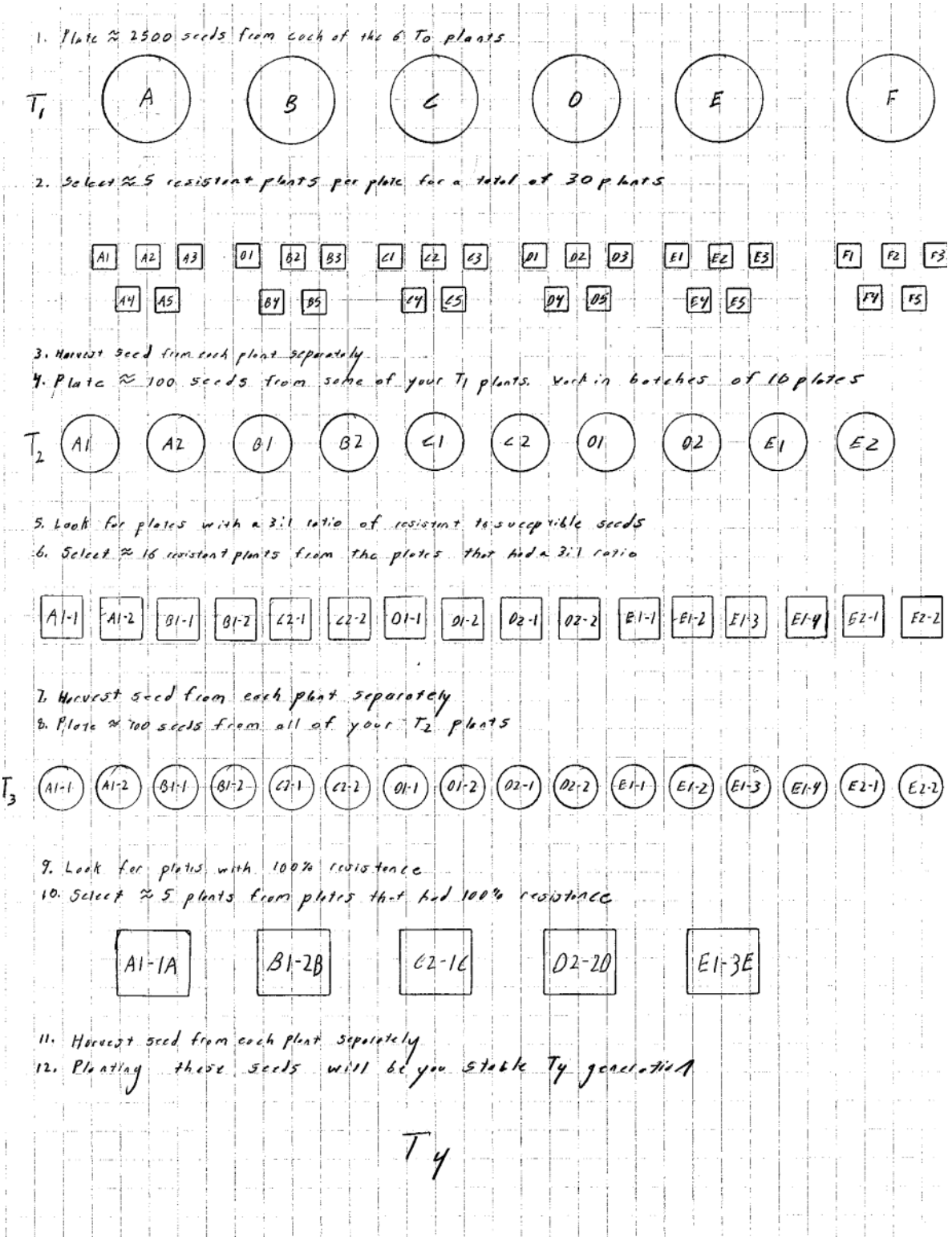
5. Place plates in the light (80 – 200 μmol $m^{-2}s^{-1}$) for 5 - 7 days incubation in the light (light does not need to be continuous)

6. After light treatment you want to find a plate with 100% resistance, these will be your homozygous plants for the insert
   Note: If you have a 3:1 ratio of resistant plants it means the line is heterozygous
7. Pick 2 - 5 resistant plants (in case one dies, and to find a varity of RNAi knock down

levels) with forceps by gently scraping away seedlings that are Kan susceptible, they will be small and largely rootless. Then very gently remove the Kan resistant plant by picking up by the hypocotyl with forceps

8. Transfer the plant to soil watered with 1/4 strength Hoaglands and keep in tray with cover on in high humidity.
9. Grow plants up for seed, the seed will be the T4 generation and should be the stable homozygous transformants!

Naming Policy and Visual Plan For Selecting Transgenic Plants



Example name of a T_4 plant: 35SGPT2 A1-1A

This Method was adapted from:

Harrison SJ, Mott EK, Parsley K, Aspinall S, Gray JC, Cottage A (2006) A rapid and robust method of identifying transformed *Arabidopsis thaliana* seedlings following floral dip transformation. *Plant Methods* 2:19

Danhoff L, Green lab protocol for vacuum infiltration transformation of Arabidopsis. Used in Ken Keegstra's lab

Plant Selection Reagents

50% Bleach Solution with 0.02% Triton X-100, 250 ml
In 125 ml dH_2O
+ 125 ml commercial bleach (Clorox, Meijer brand etc)
+ 50 µl Triton X-100, 0.02%

Sterile 0.1% Agarose, 100 ml
In a 250 ml bottle
+ 100 ml dH_2O
+ 0.1 g Agarose
Autoclave

Kanamycin 10mg/ml, 10 ml
In 10 ml sterile dH_2O
+ 0.1 g Kanamycin sulfate (Roche 10 106 801 001)
Once Kanamycin is dissolved place in syringe and filter sterilize using a 0.45 µm syringe filter, as you push Kanamycin solution through filter aliquot into sterile 2 ml tubes. store in -20 to -80° C

Gentamicin 10mg/ml
Comes as a stock liquid from Invitrogen cat# 15710-064
Make sure that master stock stays sterile

Kanamycin or Gentamicin Selection Plates, 1 L
In 1L beaker of H_2O,
+ 4.3 g MS salts (Caisson Laboratories)
+ 10 g sucrose, 1%
+ 0.5 g MES
pH to 5.7 w/KOH

In 2L Erlenmeyer flask,
+ pHed solution from 1L beaker
+ 6 g phytoblend, 0.8% (Caisson Laboratories)
+ stir bar

Cover with double layer of aluminum foil
Autoclave for 40 minutes; put the 2L of solution in a smaller autoclave container, half filled with water and then put this into a larger container to autoclave. This ensures most solid is incorporated into the solution.
Stir cool ≈ 2 hours (this ensures phytoblend goes into solution, while also allowing the temperature to drop to a level where antibiotics will not be degraded while preventing

solidification of the media)

+ 5 ml 10mg/ml kanamycin stock (50 μg/ml final concentration for plant selection)
+ 1.5 ml 10mg/ml gentamicin (15 μg/ml final concentration for plant selection)
+ 500 mg vancomycin (optional, to control bacterial growth)

Pour into 150x25mm plates (each holds approximately 67ml or 1 L media will do 15 plates)