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Progeny test for generating single copy homozygous transgenic lines in Arabidopsis thaliana.

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ABSTRACT

The regenerated transgenic plants (T0) seeds were sown onto Murashige and Skoog Agar (MSA) plates containing 100µg/ml Carbenicillin antimicrobial agent, BASTA herbicide (15 µg/ml) and allowed to grow. Transgenic lines were selected by BASTA resistant (+). The genotype of T1 plants will be mixtures of homozygous (+/+), hemizygous (+/0) and homozygous (-/-). BASTA resistant T1 seedlings were allowed to grow and T2 seeds were harvested. Then those seeds were grown on MS containing BASTA (15 µg/ml) and of them, the ratio of BASTA resistant (+/+), (+/-) and susceptible (-/-) phenotype classes are 3:1.
Subsequently, T2- single copy transgenic seeds of above lines and previously created T2 – single copy transgenic line seeds were grown on MS contained BASTA (15 µg/ml). Within the survived plants, ratio of genotype (+/+) and (+/-) are 1:2. Those survived T2 seedling were allowed to grow and T3 seeds were harvested from individual plats by avoiding seed cross-contamination between plants.
Furthermore, BASTA selection was performed for those T3 seeds and plates that observed all survived seedlings selected as homozygous (+/+) transgenic lines.

GUIDELINES

Selections of the transgenic plants were carried out on plates of MSA medium supplemented with BASTA. After three days cold stratification seeds were grown approximately 10 days and healthy transgenic plants were selected and transferred to soil. Non-transgenic plants were turned in to chlorotic.

To Confirm the transgenic line we performed additional experiments;

- Transgenic plants genotyping by PCR
- Allele Sequencing
- Western blotting for screening transgene protein expression

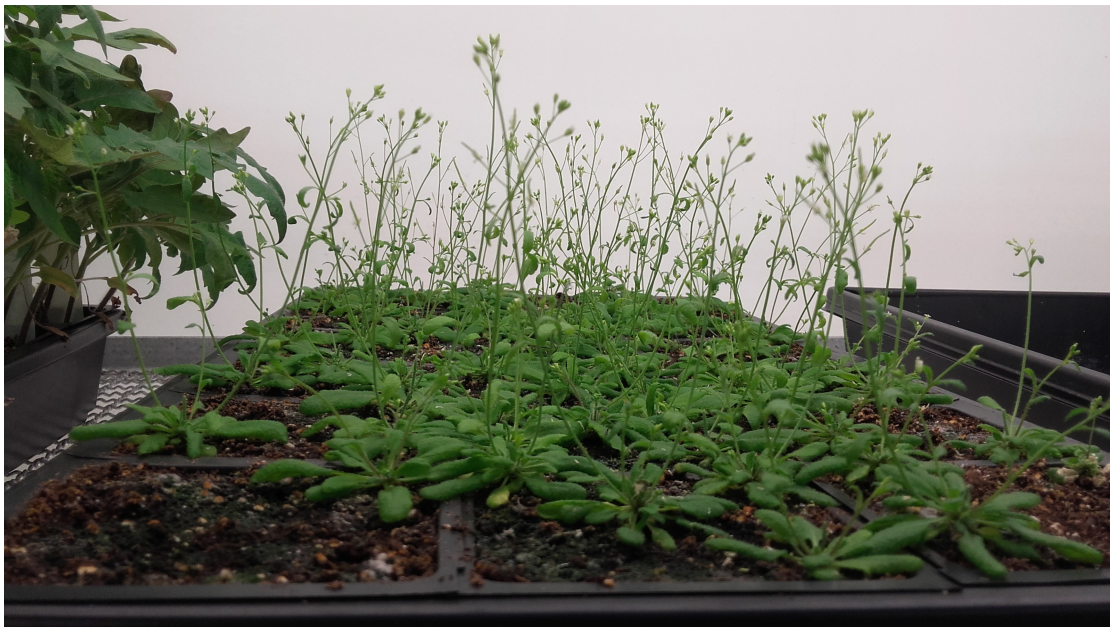
BEFORE STARTING

Transformation protocol was adapted from Zhang et al. (2006) and modified.

Zhang, X., Henriques, R., Lin, S. S., Niu, Q. W., & Chua, N. H. (2006). Agrobacterium-mediated transformation of Arabidopsis thaliana using the floral dip method. Nature protocols, 1(2), 641.

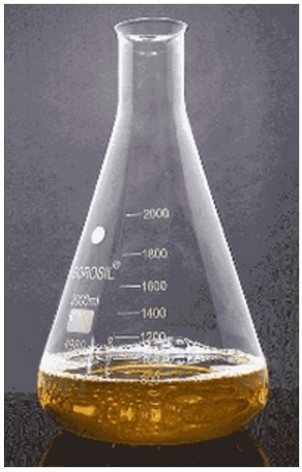
1 Plants should grow for ~4 weeks, until primary bolts appear. Then, primary bolts should be clipped to allow for the emergence of multiple secondary inflorescences.

2 When secondary inflorescences are 1 -10 cm tall (6 - 8 days after clipping) the plants can be transformed. Plan transformation at this stage.



4 weeks old *Arabidopsis thaliana* plants

3 Two days before the floral dip a single colony of transformed *Agrobacterium tumefaciens* is inoculated in 2 mL of sterilized YEP medium supplemented with Kanamycin (50 mg/ml), Rifampicin (50 mg/ml), and the selectable antibiotic characteristic for "GI" clonned plasmid. Agrobacterium is grown for 20 - 24 h at 28ºC and 250 rpm.



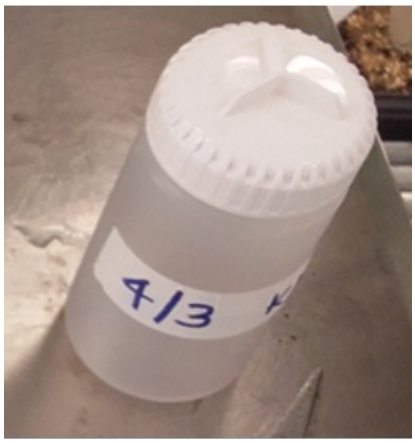
200 mL Culture + half antibiotics (100uL Kana + 100uL Ref)
~24 hours

4 The next day 1 mL of overnight culture is transferred into a 100 ml of YEP medium supplemented with the appropriate antibiotics. Agrobacterium is again grown for 20 - 24 h at 28ºC and 250 rpm, or until the OD600 has reached OD 0.8.

5 To check the OD600 for your culture use the YEP medium with rifampicin as a blank.

6 Once OD600 of 0.8 is reached, Agro cells are harvested by centrifugation (30 min at RT, at 4000 rpm).

7 The pellet is resuspended in the aqueous solution of 5% sucrose solution (Sucrose and surfactant were critical to the success of the floral dip method) and 0.05% Silwet L-77 surfactant (50 µl/100 ml) so that the final OD600 = 0.8.



Dissolve pellet in 100 mL,5% Sucrose solution

Add Silwet L-77 surfactant 50 uL

8 Plants are inverted and all the above-ground parts are dipped into this solution. 5 sec of gentle shaking of beakers is sufficient for successful transformation. Let Agro solution drip off plants for about 30 seconds, and then place plants right side up.

- Remove plant, let excess solution drip off (give a little shake if necessary)
- Lay plant horizontal in a tray, mist an opaque lid with water and cover the plants
- Incubate in the dark overnight (16-24hr)
- Return to vertical and grow plants as normal

-- for higher efficiency, repeat dip 4-5 days later with new culture.

9 Plants are covered with a tall saran-wrap dome and left in a dark cabinet for 12 - 24 h. Covering plants for 1 day to retain humidity after inoculation also raised transformation rates twofold.

You need to incubate the plants in the dark (and humid) overnight after dipping. Too close proximity can burn the flowers, also after the dipping and dark incubation. Further, do not use continuous light after dipping and growth for seed set. You will get seeds but no transformants.

10 Saran wrap is removed, and plants are placed in the low light chamber for another 3 - 5 weeks, untill T1 seeds can be collected.

11 Now we can collect T1 seeds from (T0 plants) have a single-copy primary transgenic line (T1, +/-) These seeds are hemizygous.

12 Grow these seeds and allow them to self-pollinate. These seeds contain +/+, +/- and -/- genotypes for the transgene.

13 Selecting those seeds under BASTA, (choose based on your resistance gene) you will get some survived seedlings (T2). Within the survived plants, some genotype are +/+ and some are +/-. Ratio should be 1:2 for +/+: +/- . The -/- should not be survived.

14 Grow out some of those survived T2 seedlings.

15 Harvest seeds from those individual plants. Avoid seed cross-contamination between plants.

16 Do a BASTA selection on those seeds from different individual plants. Here we should observe some plates with all survived seedlings. They are all +/+, indicating that these seeds are derived from +/+ T2 plants.

17 Keep the seedlings (T3) from those plates, as they are all +/+ (homozygous).