Title: High throughput selection of antibiotic-resistant transgenic *Arabidopsis* plants

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Abstract

Kanamycin-resistance is the most frequently used antibiotic-resistance marker for *Arabidopsis* transformations, however, this method frequently causes escape of untransformed plants, particularly at the high seedling density during the selection. Here we developed a robust high-density selection method using top agar for *Arabidopsis thaliana*. Top agar effectively suppressed growth of untransformed wild-type plants on selection media at high density. Survival of the transformed plants during the selection were confirmed by production of green true leaves and expression of a firefly luciferase reporter gene. Top agar method allowed selection using a large amount of seeds in *Arabidopsis* transformation.

Keywords

Arabidopsis; Transformation; Antibiotic selection

Agrobacterium-mediated floral transformation of *Arabidopsis thaliana* has been widely used to quickly produce a population of transgenic plants, of which each individual represents a unique transformation event of the female gamete of the parental, *Agrobacterium*-treated plants [1, 2]. While the transformation methods are established, the efficiency of transformations varies around 0.5-3% depending on the ecotype, age, vigor of plants, and environmental parameters like growth temperatures [3]. Moreover, the expression level of transgene is variable among transformants transformed with the same construct [4]. Transgene can be inserted in an undesirable position like an important gene in the genomic

DNA. It is also a problem that the transgene expression often decreases in the offspring [5]. From these reasons, it is often necessary to screen a large amount of seeds in order to identify plants suitable for analysis.

Selection of transformed plants typically occurs in sterile culture where sterilized *Arabidopsis* seeds are sown on the surface of the agar medium containing antibiotics for selection. The density of seeds sown on each plate is limited because excess number of seedlings cause insufficient growth inhibition of untransformed plants by antibiotics. When kanamycin is used as a selection agent, high-density planting typically results in overgrowth of entire population and failure to distinguish true transformants.

In this study, we developed a simple and highly efficient antibiotic selection method for *Arabidopsis*, particularly suitable for kanamycin selection. We hypothesized that overgrowth of non-transformants occurs due to lifting of seedling caused by poor root penetration into the medium, and resulting poor exposure of seedlings to antibiotics. To improve root penetration, we used top agar method, instead of conventional protocol of spreading seeds on medium as suspension in 0.1% agar solution.

Dried *Arabidopsis* seeds were sterilized. Typically 0.5 ml dry volume seeds (approximately 300 mg) were treated with 10 ml of 1/10-diluted, saturated bromine solution in 15 ml falcon tube. Mixture were rotated at 18 rpm in 4 °C for overnight. It was important to ensure thorough sterilization because fungal contamination could destroy entire plate. Seeds were then washed in sterilized distilled water for 2-4 times. For kanamycin selection, agar basal medium containing 1/4x Murashige and Skoog salts, 0.2 g/L 2-(N-morpholino)ethanesulfonic acid (MES), 7 g/L agar pH 5.6-5.8 were supplemented with 50 µg/ml kanamycin and 100 µg/ml Clavamox. In case of *Agrobacterium* overgrowth, 500 µg/ml Clavamox and 500 µg/ml Claforan was used in place of 100 µg/ml Clavamox. Top agar solution contained 1/4x Murashige and Skoog salts, 0.2 g/L MES, 50 µg/ml kanamycin, 100 µg/ml Clavamox and 4 g/L agar. After autoclaving, top agar solution was kept at 45°C. To washed seeds, top agar solution was added to achieve desired density, and immediately 5 ml of suspension was spread on the surface of the selection media plates and allowed to cool and solidify.

To compare the efficacy of top agar method with the conventional method, increasing amount of Col-0 seeds were sown on selection media by each method (Fig. 1A). Plates were kept in 4°C for 2 days to stratify the seeds, and then incubated in 25°C for 10 days under 16 hours light/8 hours dark cycle. While both conventional and top agar methods inhibited growth of Col-0 plants compared to that in the absence of antibiotics (Fig. 1B), seedlings

sown by top agar method produced smaller, more chlorotic plants indicating they are more responsive to inhibition by kanamycin even at the highest density. In addition, top agar method allowed more uniform seed distribution on the medium, whereas conventional method caused seed clumping and poor seedling responsiveness to kanamycin. Overall, the results showed that top agar method using seed amount of 0.1 ml dry volume (60 mg; approximately 1200-1500 seeds) provides an effective high-density selection.

Next, kanamycin-resistant transgenic plants after floral transformation were selected by top agar method. Col-0 plants were treated with *Agrobacterium* containing pMon721 with RD29a-LUC reporter gene [6]. Seeds (100 μ l dry volume) were sterilized and sown on selection medium containing 50 μ g/ml kanamycin and 100 μ g/ml Clavamox. After stratification, plates were kept at 4°C for 2 days and then at 25°C for 10 days under 16 hours light/8 hours dark cycle. As shown in Fig. 1C (left), a group of seedlings were able to sustain growth on medium containing kanamycin. To test if they are true transformants, expression of reporter gene was observed. The plate was incubated for 2 days at 0°C to induce expression of RD29a-LUC reporter gene and plants were sprayed with 1 mM beetle luciferin. The bioluminescence was observed under electron multiplying charge coupled device (EMCCD) camera (Roper scientific). The bioluminescence was observed from 74 kanamycin resistant individuals (Fig. 1C, right) identified in this plate, establishing that the top agar method is highly reliable.

The advantage of top agar method shown here is its high capacity (1200-1500 seeds/plate as opposed to 200-300 seeds/plate using conventional method), simple preparation (top agar is the same composition as selection medium except agar concentration), and absence of extra manipulation. Other yet effective method [7] requires extra attentions for timed movement of plants between light and dark conditions to enhance selection efficiency, and has a short window of time for making effective scoring of resistance. In addition, the treatment causes etiolation of seedlings. Top agar method does not require such manipulations and is suitable for handling large number of seeds to be screened without inducing morphological changes.

Acknowledgments

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Fig. 1. Comparison of selection by conventional and top agar methods. (A) Kanamycin response of untransformed Col-0 seeds sown at indicated density using conventional and top agar methods. The medium contains 1/4x MS with 50 μ g/ml kanamycin. Plants were cultured at 25 °C for 10 days (B) Wild-type plants on 1/4x MS media without kanamycin. (C) Left, selection of transformed Arabidopsis plants by top agar method on 1/4x MS medium with 50 μ g/ml kanamycin. Right, Bioluminescence of RD29a-LUC reporter gene documented using EMCCD camera. Plants were treated at 0°C for 2 days and sprayed with 1 mM beetle luciferin. Exposure time was 5 min. Scale bar = 5mm.

ASeeds
(μ l dry vol.)Conventional
0.1% agar

Top agar

25

50

100

200

B **C**