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Investigation into the Arabidopsis transformant selection time and escapes frequency reduction

Ali DEHESTANI[1,2,3], Gholamreza AHMADIAN[2], Ali Hatef SALMANIAN[3], Nadali Babaeian JELODAR[1], Mehdi SEYEDI[3] and Kamal KAZEMITABAR[1]*

1. Agronomy and plant breeding department, Sari Agriculture and Natural Resources University, Sari, Iran
2. Molecular genetics department, National Institute for Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran
3. Plant biotechnology department, National Institute for Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran
* Corresponding Author: P.O. Box: 578, Sari, Iran; Fax: (+98)1513822715; Tel: (+98)9111116203; Email: kazemi_ka@yahoo.com

Abstract. Selection of putative transformants after Arabidopsis in planta transformation and the main factors involved in are comprehensively discussed. Different antibiotic concentrations, basal medium composition, incubation condition of selection plates and various selection criteria were investigated. Results showed that although kanamycin concentrations from 35 to 75 mgl^{-1} could discriminate transformants from non-transformed seedlings, optimum selection was carried out on plates with 75 mgl^{-1} kanamycin. This concentration of kanamycin along with three days dark treatment before light incubation of selection plates resulted in etiolated seedlings which were distinguished quickly by their small dark green secondary leaves. Secondary leaves development was demonstrated to be the most reliable criteria for rapid and accurate selection of transformants with a selection efficiency of 97.5%. In contrast, variations in root length and seedling colour complicated the selection process resulting in more wrong selections. Addition of sucrose to selection medium deferred the selection procedure by repressing antibiotic toxicity; while sucrose omission accelerated discrimination of transformants form bleached non-transformed seedlings. We strongly recommend use of 75 mgl^{-1} kanamycin supplemented medium, 3 day dark treatment before light incubation of plates, and selection of transformants using secondary leaves development criteria for rapid and accurate selection of transformed seedling. These modifications would eliminate false positive transformants while reducing selection period to 6 days.

Key words: In planta transformation, Secondary leaves, seedling morphology; selection efficiency, Arabidopsis, Antibiotic concentration.

Introduction

Heterologous expression of different genes and study of their effects on plant biology has provided plant biologists with an excellent tool for their research experiments. Various strategies have been used for plant transformation resulting in permanent or transient expression of the transgene. *Agrobacterium tumefaciens*-mediated gene transfer is undoubtedly one of the most frequently used techniques for stable or transient transformation of many plant species (Weising et al. 1988, Hooykaas & Shilperoort 1992).

To date, most of the fundamental molecular biology studies have been focused on two major plant models: Tobacco (*Nicotiana tabacum*) and Arabidopsis (*Arabidopsis thaliana*) due to their intrinsic properties which made them more suitable for molecular biology experiments (Newell 2000). The latter, Arabidopsis, have gained more attention recently, after its complete genome was sequenced (The Arabidopsis Genome Initiative 2000) and subsequently, a wide array of information such as complete contig maps, YAC clones, cDNA libraries and EST sequences have been available for this species. Furthermore, T-tagging of the Arabidopsis genome (Feldmann 1991) has facilitated reverse-genetic study of Arabidopsis genes function and accelerated isolation and cloning of various genes (McKinney et al. 1995). Various Agrobacterium-mediated transformation techniques for Arabidopsis have been established, among them, so called in planta methods are most frequently used due to their apparent advantages over other methods including: omission of complicated tissue culture procedures, genetically uniform progenies, high rate of transformants obtained within a quite short time, and minimal labour and reagents required for transformation (Bent 2000). After the first report of Arabidopsis transformation through in planta method (Feldmann and Marks 1987), several in planta procedures for Arabidopsis transformation were designed, including: clip and squirt method (Chang et al. 1994, Katavic et al. 1994) vacuum infiltration (Bechtold et al. 1993), floral dip (Clough & Bent 1998), floral spray transformation (Chung et al. 2000).

The time required for transgenic line production is a major issue in most experiments. Furthermore, designing a selection criteria for identification of true transformants in selection process is very important to save time and greenhouse space. Different parameters determine seedling morphology in in-vitro selection procedures e.g. basal medium composition, antibiotic concentration, in vitro growth conditions (dark/light regime, temperature etc.). For rapid selection of transformed seedlings with high accuracy all of theses parameters should be considered. Kanamycin resistance gene or Neomycin phosphotransferase (nptII), which confers resistance to the aminoglycoside antibiotics, e.g. kanamycin is present in most plant transformation vectors and has been one of the most common used dominant selectable marker genes in plant transformation procedures (Steinbiss & Davidson 1989). Green seedlings with developing true leaves are determined as putative transformants, while non-transformed plants will bleach gradually. The time for this process is 7-10 days for routine Arabidopsis transformant selection (Bechtold et al. 1993, Clough and Bent, 1998). A modified form of selection was recently reported, which accelerated the selection process allowing transformant selection in a 3.25 days period. The selection plates were subjected to a light/dark regime of 4–6 h light, 48 h dark and 24 h light (3.25 d) and transformants were determined by their green expanded cotyledons (Harrison et al. 2006). Variable concentrations of kanamycin were used for selection process. While most of methods used 50mgl^{-1} kanamycin for selection procedure (Bechtold et al. 1993, Clough and Bent 1998, Zhang et al. 2006) others implemented higher (Bechtold et al. 2000, Hudson et al. 1999) or lower (Neff et al. 1999, Spiegelman et al. 2000) concentrations of kanamycin in selection medium. The basic medium itself also seemed to be effective in distinguishing putative transformants. Harrison et al. reported that a longer selection period was required for clear identification of transformants when 2% sucrose was added to selection medium, while addition of vitamins had no effect on transformant selection (Harrison et al. 2006). Different criteria for selection of kanamycin resistant plants have been proposed. Commonly, sharply green colour of seedlings, expanded cotyledons or lengthy roots alone or in combination were used for putative transformant selection but there is no proof which one is more reliable. This is more complicated especially when different factors like light/dark treatments, agar concentrations, medium contents, sucrose and kanamycin concentration changes seedlings' morphology (Clough & Bent 1998, Bent 2000, Harrison et al. 2006).

This study was designed to investigate critical factors affecting kanamycin selection of transformed Arabidopsis seeds, aimed to shortening the time required for identifying transformed seedling and enhancing accuracy of selection process by use of reliable morphological selection criteria. Parameters like kanamycin concentration, light or darkness incubation of plates, selection medium composition and morphological criteria for transformant selection were investigated thoroughly.

Materials and Methods

Plant growth

Arabidopsis thaliana (Columbia) seeds were vernalized by keeping on moistened filter papers in Petri dishes at 4°C for 2 days and were then sown in individual pots in flats and grown under greenhouse conditions at 22°C with a photoperiod regime of 16 hours light and 8 hours dark (long days). When reached 2 to 5 cm, the primary inflorescences were clipped for stimulation of secondary bolts emergence. Infiltration experiments were performed when most of secondary inflorescences were about 5 to10 cm with several young floral buds.

Binary vector construction

The recombinant binary vector used for Arabidopsis in planta transformation experiments, pBI121-ChiS, is a derivative of pBI121(Clontech, USA), which its β-glucuronidase (uidA) gene sequence was replaced by a 1.7 kb fragment encoding a bacterial Chitinase (ChiS) from B. Pumilus SG2. The orientation and correct alignment of the fusion of the fragments in these constructs were verified by digestion with restriction enzymes and was successfully expressed in tobacco (unpublished results). This recombinant binary vector was introduced to *Agrobacterium tumefaciens* strain GV3101 (Koncz & Schell 1986) by electroporation.

Agrobacterium culture and Vacuum Infiltration

A single colony of Agrobacterium was used for inoculation of a 5 ml starter culture including YEP medium (10 gl^{-1} Bacto peptone, 10 gl^{-1} Yeast extract and 5 gl^{-1} NaCl) supplemented with 25 mgl^{-1} Gentamycin and 30 mgl^{-1} Kanamycin, incubated overnight at 28°C with vigorous shaking (180 rpm). Overnight starter culture was used to inoculate a new 400 ml YEP medium prepared and grown as above. Overnight cultured Agrobacterium cells (with an OD_{600} of 1.6 to 2) were centrifuged at 5000 g for 15 min and the pellet was resuspended in 2 volumes of Infiltration medium (IM) containing 1/2 MS salts (Murashige & Skoog 1962) and 5% sucrose. The IM suspension was then incubated for a further 2-3 hours at 28°C with vigorous shaking.

Vacuum infiltration was performed according to Bechtold et al. with some modifications (Bechtold et al. 1993). In a vacuum chamber (large glass desiccator) plastic vessels were filled with IM containing 0.01% Tween-20 added just before infiltration. Plant inflorescences were immersed in horizontally at a position allowing maximum number of inflorescences submerged by IM. Using a Pump, vacuum was gradually

applied until air bubbles emerged on the surface of IM and the vacuum was held for a further15-20 minutes. The vacuum was then released quickly to force the Agrobacterium cells into the plant tissue. The infiltrated plants were briefly blotted on filter papers for seconds and were then horizontally placed in trays wrapped with transparent plastic covers to maintain high humidity. The trays were kept at greenhouse at a low light atmosphere for 24 h and were then set upright and grown at normal greenhouse conditions for a further 4 weeks. Siliques that have been developed before transformation were removed 2 days after infiltration. When the siliques were dry, seeds of all plants were harvested in bulk and stored for selection on selection plates.

Selection of putative transformants

For selection of transformants, harvested seeds were surface sterilized by immersion in 75% ethanol for 1 min, then immersed in 20% commercial bleach (1% active sodium hypochlorite) solution containing 0.05% Tween-20 for 12 min, followed by four rinses in sterile distilled water before being placed on selection plates. Selection of transformants, transplantation of putative transformed seedlings and plant growth was performed according to routine procedures (Zhang et al. 2006). Seeds of fifty T_1 plants (T_2 seeds) were harvested in bulk and were used as a homogeneous material for main optimization experiments. The high percentage of transformants within seed lot makes possible a more precise evaluation of parameters affecting selection efficiency. Hereafter, different parameters including: basal medium composition, kanamycin concentration, light or dark incubation of plates and transformant selection criteria were analyzed using this homogeneous seed lot for comparison of the treatments. For basal selection medium, MS salts (Murashige & Skoog 1962) at half strength with or without B_5 vitamins (Gamborg et al. 1968) and two concentrations of sucrose (15gl^{-1} or no sucrose) were assessed. Five concentrations of kanamycin monosulfate (25, 35, 50, 75 and 100 mgl^{-1}) were analyzed for optimum selection pressure. Disinfected seeds were plated on kanamycin selection plates using a Pasteur pipette, stratified for 3 days at 4°C and then transferred to 24°C growth chamber. Two light regimes for selection plate incubation were assessed: 3 days dark followed by a photoperiod of 16 h light and 8 h dark (50-100 microEinsteins), or continuous photoperiod of 16 h light and 8 h dark without any dark day treatment. There different selection criteria: root length, seedling colour and secondary true leaves development were used for putative transformant identification and their credibility was evaluated. There were 5 replications (selection plates) for each treatment. Selected putative transformants were transplanted into pots containing a water saturated soil mixture (peat: perlite: vermiculite, 1:2:1) for further analysis by polymerase chain reaction (PCR). The PCR reactions for each treatment were carried out using 30 seedlings selected as transformants. Furthermore, for 75 and 100 mgl^{-1} kanamycin supplemented medium, to determine whether selection pressure at concentrations higher than 50 mgl^{-1}would cause wrong elimination of transformants or not, 25 seedlings which were not considered as trasnformants were analyzed through PCR using a pooling strategy. Transformant Identification Coefficient (TIC) for each treatment was calculated as follows: (number of identifiable kanamycin resistant seedlings/total number of germinated seeds in each plate) × 100. Real Transformation Efficiency (RTE) was calculated by dividing number of PCR positive seedlings/total number of seedlings tested by PCR) × 100.

Polymerase chain reaction

Polymerase chain reaction (PCR) was performed for assessing the selected putative transformants to make certain of the T-DNA region integration into the plant genome. Small leaf segments were collected from plants two weeks after transplantation and Genomic DNA was isolated using a quick DNA Miniprep procedure (Weigel & Glazebrook 2002). Some young seedlings from selection plates were directly used for DNA extraction. About 1 µl of the isolated DNA (5-10 ng) was used as template in PCR reactions.

PCR was performed to amplify an internal 380 bp fragment of nptII gene in the T-DNA region. The primers were as follows: Kan-F (5'-ATGAGCCATATTCAACGGGAA-3') and Kan-R (5'-TAAGAAAAACTCATCGAGCAT-3'). PCR reactions were prepared in a total volume of 25 µl containing 5-10 ng of DNA template, 1× reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM of dNTPs (Cinnagen, Iran), 0.25 pmol each of primers and 1.5 U of Taq DNA polymerase (Cinnagen, Iran). The following conditions were used for PCR: 94°C for 5 min and 30 cycles of 94°C for 45 s, 54°C for 1min and 72°C for 1 min; followed by a final extension at 72°C for 8 min. A wild type Arabidopsis DNA sample was included as negative control. PCR products were loaded on 1.5% agarose gel and stained with ethidium bromide and then photographed by a UVP gel documentation system (UVP, England).

Data analysis

The data were analyzed using Microsoft Office Excel and MSTATC. Statistical estimations and mean comparisons were performed using the LSD test at $p<0.05$.

Results

Continuous light versus dark/light treatment

Two different light treatments resulted in a high variation in seedlings morphology. Putative transformants grown under continuous light condition had more expanded cotyledonary leaves and shorter hypocotyls and roots compared to those of dark/light grown under dark/light condition. The secondary true leaves in dark/light grown seedlings were early emerged, while the primary leaves were still small. In contrast, light grown seedlings didn't produce secondary leaves before primary leaves expansion.

After 3 days incubation in dark, germinated seeds were etiolated with long hypocotyls and a white to pale green colour. After 2-3 days incubation in light, the secondary leaves on putative transformants became visible as dark green small specks surrounded by light green primary leaves (Figure 1, right). They had long roots with fewer branches connected to upper segments by pale green, long, thin hypocotyls. On the other hand,

plates incubated in continuous light regime contained green short seedlings which their secondary leaves emerged 5-6 days after incubation.

They were hardly distinguishable before 2-3 days, until they were more developed and secondary leaf growth of non-transformed seedlings was arrested. Commonly, light grown putative transformed seedlings were shorter, with green short hypocotyls and more branched roots (Figure 1, left).

Different kanamycin concentrations

Assessment of plants survived in each kanamycin concentration was performed 8 days after incubation. Average Transformant identification coefficient (TIC) and real transformation efficiency (RTE) for each concentration was calculated as described above. The results are summarized in Table 1. In medium supplemented with 25 mgl^{-1} kanamycin TIC and RTE were estimated as 76.4 and 46.66 respectively. For 50 mgl^{-1} kanamycin medium a TIC of 29.8 and RTE of 93.3 were observed. TIC and RTE of 35 mgl^{-1} kanamycin were calculated as 30.6 and 90, respectively. In 75 mgl^{-1} kanamycin containing medium a TIC of 30.1 and a RTE of 100 were recorded. In 100 mgl^{-1} kanamycin selection plates calculated TIC and RTE were 21.6 and 83.3, respectively. These results indicated apparent low selection pressure for medium with 25 mgl^{1} kanamycin which caused high frequency of escapes. Various medium with 35, 50 and 75 mgl^{-1} kanamycin didn't show any significant differences, while medium containing 100 mgl^{-1} kanamycin exhibited lower TIC indicating high selection pressure of this medium which might have eliminated some putative transformants. Figure 2 shows TIC and RTE variation with different kanamycin concentration.

Furthermore, 25 seedlings from 75 and 100 mgl^{-1} kanamycin supplemented mediums which were not considered as transformants were analyzed by PCR to demonstrate they were not erroneously considered as non-transformants due to high selection pressure of the medium.

DNA pools were created for each 5 seedlings (total of 25 seedlings were arranged as a 5×5 square) such that each seedling was included in 2 separate pools. When both pools containing a given seedling were PCR positive, the seedling was considered as transformant and was further analyzed in a separate PCR reaction. The DNA pool of seedlings from medium with 75 mgl^{-1} kanamycin produced no band indicating that no true transformant was eliminated by mistake. In 100 mgl^{-1} kanamycin medium, 3 out of 25 seedlings were proved to be true transformants by PCR, notwithstanding they didn't seem to be transformed in selection medium. Analysis of average transformant number for 35, 50 and 75 mgl^{-1} kanamycin mediums revealed no significant difference between treatments, while the number of transformants from 25 and 100 mgl^{-1} kanamycin supplemented medium showed a significant difference (Table 1).

Basic medium composition

Selection plates containing B_5 vitamins didn't show any meaningful difference when compared to those without vitamins. Growth rate, seedling development or seedling colour weren't affected by this parameter. Addition of sucrose, in contrast deferred differentiation of both transformed and non-transformed seedlings. Putative transformant seedlings grown on selection plates with sucrose could be identified 1-2 days later than those grown on plates without sucrose. This was more significant for the plates incubated under continuous light condition. In these plates, transformed and non-transformed plats retained their green colour similarly and detection was generally carried out upon secondary true leaves expansion.

Seedling morphology and selection criteria

Three different criteria for transformant selection were investigated throughout all treatments (light regime, kanamycin concentration and basal medium composition). A total of forty seedlings were selected from differently treated plates according to each selection criteria and were analyzed through PCR. Figure 3 shows PCR amplification of a 380 bp partial fragment of nptII gene from seedlings selected by secondary leaves development criteria. The first group selected by their sharp green colour, contained 36 PCR positive seedlings of 40 seedlings tested. Out of 40 seedlings selected via root length characteristic, 25 were PCR positive. Finally, 39 seedlings out of 40 selected seedlings according to secondary true leaves development were PCR positive. Selection of plants using a combination of seedling colour and secondary leaves development criteria resulted in 100% agreement with PCR analysis, while use of root length as selection criteria was misleading. A considerable number of seedlings with a short root were proved to be true transformant when tested by PCR, while some with longer roots didn't produce a band in PCR.

Discussion

Selection of putative transformant seedlings in Arabidopsis in planta transformation experiments is a critical step for identification of true transformed seedlings at a short period of time with minimal labour. Several selection strategies have been developed that are

Figure 1. Seedlings morphology influenced by different light treatments. Left, Putative transformants grown under continuous light condition were shorter, with more developed cotyledons and green short hypocotyls and short roots while dark/light treated seedlings had longer hypocotyls and roots and small cotyledons. Right, Transformants grown on 75mgl-1 kanamycin selection plates and under dark/light condition had small, dark green secondary leaves surrounded by pale green primary leaves.

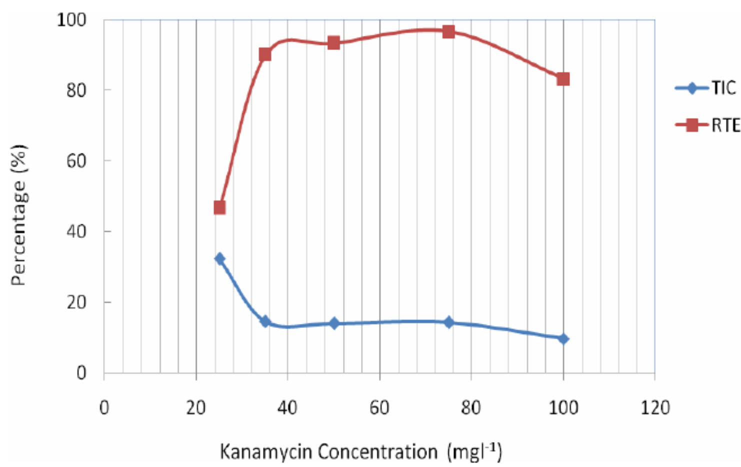

Figure 2. Transformant Identification Coefficient (TIC) and Real Transformation Efficiency (RTE) variations with different kanamycin concentrations.

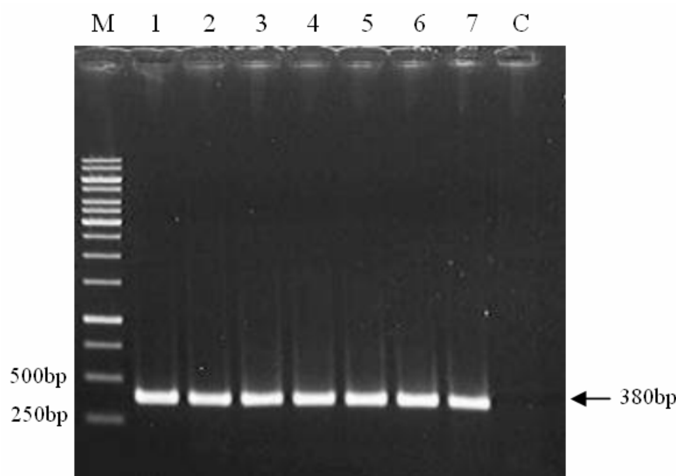

Figure 3. PCR amplification of a 380 bp fragment of *nptII* gene from seedlings selected by secondary leaves development criteria. M: molecular weight marker, 1-7 selected transformants, C: wild type control.

Table 1. Average Transformant Identification Coefficient (TIC) and Real Transformation Efficiency (RTE) for each kanamycin concentration. Means with the same letter did not differ significantly at $p<0.05$.

Kanamycin Concentration (mgl^{-1})	Total analyzed seedlings	Identifiable putative transformants	TIC	Total tested seedlings	PCR positive seedlings	RTE
25	1618	524	32.4a	30	14	46.6a
35	1344	196	14.6b	30	27	90bc
50	1510	213	14.1b	30	28	93.3b
75	1417	204	14.4b	30	29	96.6b
100	1048	102	9.8c	30	25	83.3c

mostly based on use of antibiotic or herbicide resistance genes, while other strategies including fluorescent screening selection (Wei et al. 2004) are not very common. Kanamycin selection is undoubtedly the most used selection procedure for transformant screening. The nptII gene is a constitutive element of most Agrobacterium binary vectors (Steinbiss & Davidson 1989). Although selection process is well described in early works (Bechtold et al. 1993, Clough & Bent 1998) various modifications of the basic methods have been reported for better selection results. Due to these diverse methodologies, some aspects of selection procedure may be highly ambiguous, among them selection medium composition (Zhang et al. 2006, Harrison et al. 2006), kanamycin concentration (Hudson et al. 1999, Spiegelman et al. 2000, Zhang et al. 2006), light or dark treatment of the plated seeds (Zhang et al. 2006, Harrison et al. 2006) have been prone to modifications in different selection methods.

In this study three major factors influencing the selection efficiency have been investigated thoroughly. Different treatments of selection medium, kanamycin concentration and light/dark incubation of plates were tested to determine the optimum requirements of selection process. Furthermore, in order to reduce false positive selections, and for rapid and accurate selection of putative transformants, three selection criteria were analyzed.

It is highly recommended to stratify plated seeds for 3 days at 4°C. A 3 days cold treatment instead of 2 days cold treatment prior incubation at growth chamber helped synchronized germination and more reliable subsequent selection.

Antibiotic concentration was proved to be a critical factor influencing the selection efficiency. Wei and colleagues reported lower transformation efficiency through kanamycin selection compared to a fluorescence selection. They suggested that this could result from insufficient expression level of the NPTII in some of the transgenic plants due to position effect of the T-DNA insertion site. Selection frequency was 3.1% ± 0.34% for the fluorescence selection method, while kanamycin resistant selection resulted in only 0.56% ± 0.13% using the same seed batch (Wei et al. 2004). There was no coincidence between our results with these interpretations. Comparison of TIC in 35 mgl^{-1} kanamycin medium with 50 or 75 mgl^{-1} kanamycin mediums revealed no significant difference between these selection pressures (at $p<0.05$).

At very low concentrations of kanamycin (25 mgl^{-1}) the selection pressure was too low for identifying true transformants. Most of plants retained their normal green colour and started to produce secondary leaves in both light and dark/light conditions. At selection mediums supplemented with 35 or 50 mgl^{-1} kanamycin, due to light green colour of seedlings, selection process was deferred up to one week after incubation. This time was shorter for plates incubated at dark/ light conditions, after 5 days, transformants were distinguishable by their dark green secondary leaves between pale green primary leaves. On medium with 100 mgl^{-1} kanamycin, plants were small with undeveloped leaves which made the selection process more troublesome. TIC of 21.6 implies inappropriate selection pressure, which is more emphasized by revealing that 3 out of 25 seedlings assumed to be non-transformed were true transformant indicating 12% loss of transformant selection. Furthermore, selection mediums supplemented with 75 mgl^{-1} kanamycin were suitable for transformant selection. After 5 days, plants grown under light or light/dark conditions were easily detectable. Normal green colour and darker colour of small secondary leaves of transformed plants were obviously different from pale green leaves of non-transformed seedlings. Wei and colleagues reported more seedlings in dark/light treated plates, when all plants were etiolated with a faint green colour, transformed ones were identified by their dark green secondary leaves starting to develop. In this medium all of the selected putative transformants were demonstrated to be true transformants (RTE of 100). Moreover, selection pressure was proved to be

appropriate and none of 25 non-transformed seedlings tested were PCR positive.

According to this study omission of sucrose from selection medium would accelerate the selection process. It was previously reported that sucrose and vitamins deferred the selection process (Harrison et al. 2006).

In this study addition of vitamins caused no significant difference. Among three selection criteria investigated, secondary leave development was proved to be most effective. Basal medium composition, light or dark treatment has little effect on this characteristic, while seedling colour or root length showed more variability in different growth conditions.

This study showed a wide range of kanamycin concentrations 35 - 75 mgl^{-1} could be used for Arabidopsis transformant selection. It was proved that dark/ light treatment will decrease the selection time, while increasing selection efficiency and accuracy. Dark/light incubation in combination with 75 mgl^{-1} kanamycin selection plates helped for a more rapid selection. High concentration of kanamycin deferred chlorophyl formation of etiolated non-transformed plants in comparison with transformed ones, allowing a more quick selection. It also favoured seedlings with longer hypocotyls which were easily selected and transplanted. We strongly recommend a 75 mgl^{-1} kanamycin concentration and dark/light treatment of plates for optimum selection. This combination will help for more accurate and rapid selection of transformants in Arabidopsis transformation experiments.

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References

Bechtold, N., Ellis, J., Pelletier, G. (1993): In planta Agrobacterium-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. Comptes Rendus de l`Academie des Sciences 316: 1194–1199.

Bechtold, N., Jaudeau, B., Jolivet, S., Maba, B., Vezon, D., Voisin, R., Pelletier, G. (2000): The Maternal Chromosome Set Is the Target of the T-DNA in the in Planta Transformation of *Arabidopsis thaliana*. Genetics 155: 1875–1887.

Bent, A. (2000): Arabidopsis in Planta Transformation, Uses, Mechanisms, and Prospects for Transformation of Other Species. Plant Physiology 124: 1540–1547.

Chang, S.S., Park, S.K., Kim, B.C., Kang, B.J., Kim, D.U., Nam, H.G. (1994): Stable genetic transformation of *Arabidopsis thaliana* by *Agrobacterium* inoculation in planta. Plant Journal 5: 551–558.

Chung, M.H., Chen, M.K., Pan, S.M. (2000): Floral spray transformation can efficiently generate *Arabidopsis* transgenic plants. Transgenic Research 9: 471–476.

Clough, S.J, Bent, A. (1998): Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant Journal 16: 735–743.

Feldmann, K.A., Marks, M.D. (1987): *Agrobacterium*-mediated transformation of germinating seeds of *Arabidopsis thaliana*: a non-tissue culture approach. Molecular and General Genetics 208: 1–9.

Feldmann, K.A. (1991): T-DNA insertion mutagenesis in *Arabidopsis*: mutational spectrum. Plant Journal 1: 71–82.

Gamborg, O.L., Miller, R.A., Ojima, K. (1968): Nutrient requirements of suspension cultures of soyabean root cells. Experimental Cell Research 50: 151–158.

Harrison, S.J., Mott, E.K., Parsley, K., Aspinall, S., Gray, J.C., Cottage, A. (2006): A rapid and robust method of identifying transformed *Arabidopsis thaliana* seedlings following floral dip transformation. Plant Methods 2:19. Doi: 10.1186/1746-4811-2-19.

Hodson, M., Ringli, C., Boylan, M.T., Quail, P.H. (1999): The FAR1 locus encodes a novel nuclear protein specific to phytochrome A signaling. Genes and Development 13: 2017-2027.

Hooykaas, P.J.J., Shilperoort, R.A. (1992): Agrobacterium and plant genetic engineering. Plant Molecular Biology 19: 15-38.

Katavic, V., Haughn, G.W., Reed, D., Martin, M., Kunst, L. (1994): In planta transformation of *Arabidopsis thaliana*. Molecular and General Genetics 245: 363–370.

Koncz, C., Schell, J. (1986): The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimeric genes carried by a novel type of *Agrobacterium* binary vector. Molecular and General Genetics 204: 383-396.

McKinney, E.C., Ali, N., Traut, A., Feldmann, K.A., Belostotsky, D.A., McDowell, J.M., Meagher, R.B. (1995): Sequence-based identification of T-DNA insertion mutations in *Arabidopsis*: actin mutants act2-1 and act4-1. Plant Journal 8: 613–622.

Murashige, T., Skoog, F. (1962): A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiologia Plantarum 15: 473–479.

Neff, M.M., Nguyen, S.M., Malancharuvil, E.J., Fujioka, S., Noguchi, T., Seto, H., Tsubuki, M., Honda, T., Takatsuto, S., Yoshida, S., Chory, J. (1999): BAS1: A gene regulating brassinosteroid levels and light responsiveness in Arabidopsis. Proceedings of the National Academy of Sciences 96 (26): 15316-15323.

Newell, C.A. (2000): Plant transformation technology Development and application. Molecular Biotechnology 16:53-65.

Nichols, K.W., Heck, G.R., Fernandez, D.E. (1997): Simplified selection of transgenic *Arabidopsis thaliana* seed in liquid culture. Biotechniques 22: 62-63.

Spiegelman, J.I., Mindrinos, M.N., Fankhauser, C., Richards, D., Lutes, J., Chory, D., Oefnera, P.J. (2000): Cloning of the Arabidopsis RSF1 Gene by Using a Mapping Strategy Based on High-Density DNA Arrays and Denaturing High-Performance Liquid Chromatography. Plant Cell 12 (12): 2485–2498.

Steinbiss, H.H., Davidson, A. (1989): Genetic manipulation of plants: from tools to agronomic applications. Science Progress 73: 147–168.

The Arabidopsis Genome Initiative (2000): Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408: 796-815.

Wei, S., Bravdo, B.A., Shoseyov, O. (2004): Fluorescent Screening of Transgenic Arabidopsis Seeds without Germination. Plant Physiology 135 (2): 709–714.

Weigel, D., Glazebrook, J. (2002): Arabidopsis: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Weising, K., Schell, J., Kahl, G. (1988): Foreign genes in plants: transfer, structure, expression and applications. Annual Review of Genetics 22: 421–477.

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): 641-646.

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