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Methods to Transfer Foreign Genes to Plants

Yoshihiro Narusaka, Mari Narusaka,
Satoshi Yamasaki and Masaki Iwabuchi
*Research Institute for Biological Sciences (RIBS), Okayama
Japan*

1. Introduction

Genome sequencing of several organisms has resulted in the rapid progress of genomic studies. Genetic transformation is a powerful tool and an important technique for the study of plant functional genomics, i.e., gene discovery, new insights into gene function, and investigation of genetically controlled characteristics. In addition, the function of genes isolated using map-based cloning of mutant alleles has been confirmed by functional complementation using genetic transformation. Furthermore, genetic transformation enables the introduction of foreign genes into crop plants, expeditiously creating new genetically modified organisms. Gene transformation and genetic engineering contribute to an overall increase in crop productivity (Sinclair et al., 2004).

This review outlines general methods for plant transformation and focuses on the development of the *Arabidopsis* transformation system.

2. Plant transformation methods

Plant transformation was first described in tobacco in 1984 (De Block et al., 1984; Horsch et al., 1984; Paszkowski et al., 1984). Since that time, rapid developments in transformation technology have resulted in the genetic modification of many plant species. Methods for introducing diverse genes into plant cells include *Agrobacterium tumefaciens*-mediated transformation (De la Riva, 1998; Hooykaas & Schilperoort, 1992; Sun et al., 2006; Tepfer, 1990; Zupan & Zambryski, 1995), recently reclassified as *Rhizobium radiobacter*, direct gene transfer into protoplasts (Gad et al., 1990; Karesch et al., 1991; Negrutiu et al., 1990; Neuhaus & Spangenberg, 1990), and particle bombardment (Birch & Franks, 1991; Christou, 1992; Seki et al., 1991; Takeuchi et al., 1992, 1995; Yao et al., 2006).

2.1 Gene transformation

Several gene transformation techniques utilize DNA uptake into isolated protoplasts mediated by chemical procedures, electroporation, or the use of high-velocity particles (particle bombardment). Direct DNA uptake is useful for both stable transformation and transient gene expression. However, the frequency of stable transformation is low, and it takes a long time to regenerate whole transgenic plants.

2.1.1 Chemical procedures

Plant protoplasts treated with polyethylene glycol more readily take up DNA from their surrounding medium, and this DNA can be stably integrated into the plant's chromosomal DNA (Mathur & Koncz, 1997). Protoplasts are then cultured under conditions that allowed them to grow cell walls, start dividing to form a callus, develop shoots and roots, and regenerate whole plants.

2.1.2 Electroporation

Plant cell electroporation generally utilizes the protoplast because thick plant cell walls restrict macromolecule movement (Bates, 1999). Electrical pulses are applied to a suspension of protoplasts with DNA placed between electrodes in an electroporation cuvette. Short high-voltage electrical pulses induce the formation of transient micropores in cell membranes allowing DNA to enter the cell and then the nucleus.

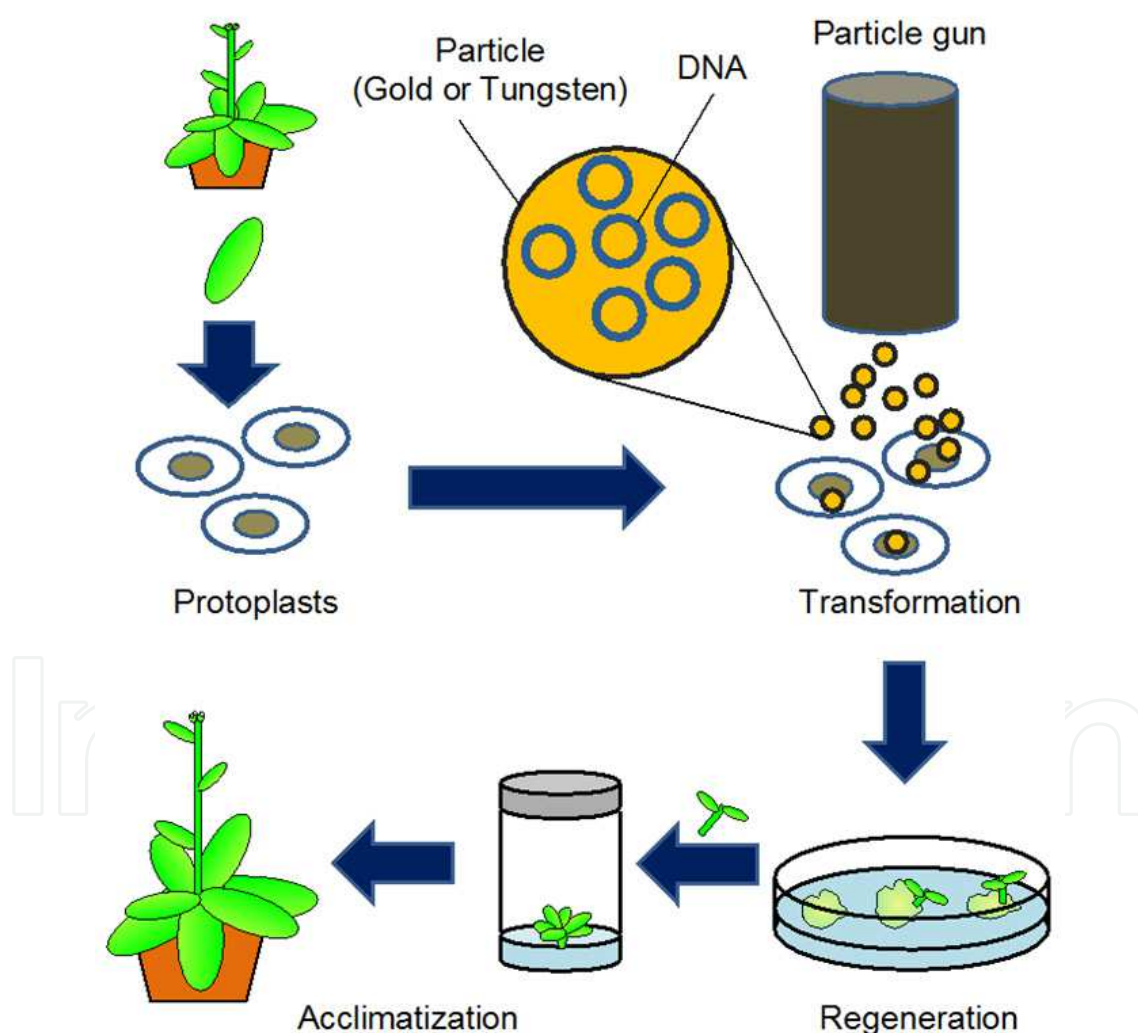


Fig. 1. Plant transformation process using particle bombardment includes the following steps: (1) Isolate protoplasts from leaf tissues. (2) Inject DNA-coated particles into the protoplasts using particle gun. (3) Regenerate into whole plants. (4) Acclimate the transgenic plants in a greenhouse.

2.1.3 Particle (microprojectile) bombardment

Particle bombardment is a technique used to introduce foreign DNA into plant cells (Birch & Franks, 1991; Christou, 1992, 1995; Gan, 1989; Takeuchi et al., 1992; Yao et al., 2006) (Figure 1). Gold or tungsten particles (1–2 μm) are coated with the DNA to be used for transformation. The coated particles are loaded into a particle gun and accelerated to high speed either by the electrostatic energy released from a droplet of water exposed to high voltage or using pressurized helium gas; the target could be plant cell suspensions, callus cultures, or tissues. The projectiles penetrate the plant cell walls and membranes. As the microprojectiles enter the cells, transgenes are released from the particle surface for subsequent incorporation into the plant's chromosomal DNA.

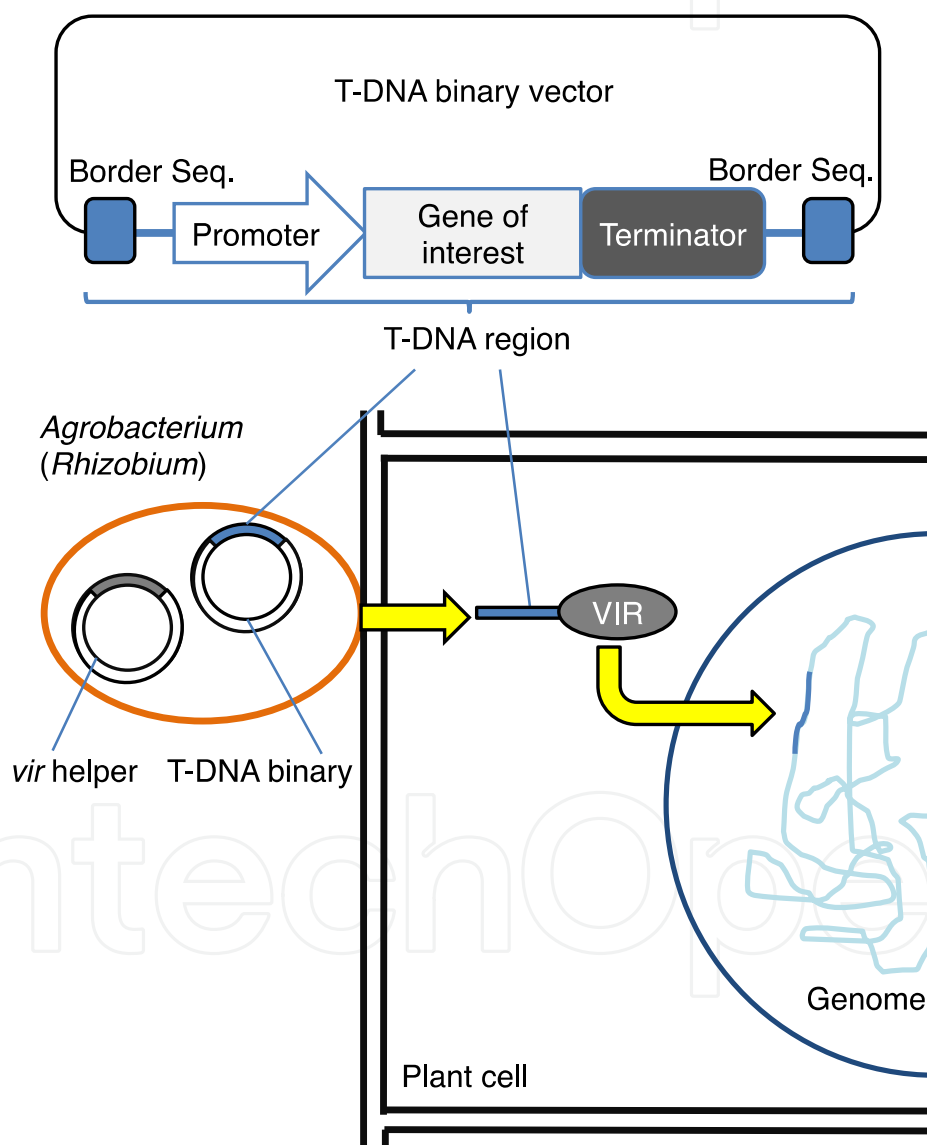


Fig. 2. The *Agrobacterium*-mediated transformation process includes the following steps: (1) Isolate genes of interest from the source organism. (2) Insert the transgene into the Ti-plasmid. (3) Introduce the T-DNA containing-plasmid into *Agrobacterium*. (4) Attach the bacterium to the host cell. (5) Excise the T-strand from the T-DNA region. (6) Transfer and integrate T-DNA into the plant genome.

2.2 Using *Agrobacterium* for plant transformation

Agrobacterium-mediated transformation is the most commonly used method for plant genetic engineering (Bartlett et al., 2008; Leplé et al., 1992; May et al., 1995; Sun et al., 2006; Tsai et al., 1994; Tzfira et al., 1997). The pathogenic soil bacteria *Agrobacterium tumefaciens* that causes crown gall disease has the ability to introduce part of its plasmid DNA (called transfer DNA or T-DNA) into the nuclear genome of infected plant cells (Figure 2) (Binns & Thomashaw, 1988; Gelvin, 2000; Nester et al., 1984; Tzfira et al., 2004; Zupan & Zambryski, 1995).

2.3 Transforming *Arabidopsis thaliana*

Arabidopsis thaliana, a small flowering plant, is a model organism widely used in plant molecular biology. The first *in planta* transformation of *Arabidopsis* included the use of tissue culture and plant regeneration (Feldmann & Marks, 1987). The *Agrobacterium* vacuum (Bechtold et al., 1993) and floral dipping (Clough & Bent, 1998) are efficient methods to generate transgenic plants. They allow for plant transformation without the need for tissue culture. The floral dipping method markedly advanced the ease of creating *Arabidopsis* transformants, and it is the most widely used transformation method. These methods were later simplified and substantially improved (Davis et al., 2009; Zhang et al., 2006), significantly reduced the required labor, cost, and time, as compared with earlier procedures.

However, these transformation methods have some problems. The floral dipping method involves dipping *Arabidopsis* flower buds into an *Agrobacterium* cell suspension, requiring large volumes of bacterial culture grown in liquid media. The large shakers and centrifuges, necessary to house the media, require sufficient experimental space. These factors limit transformation quantities. Here, we describe an improved method for *Agrobacterium*-mediated transformation that does not require the large volumes of liquid culture necessary for floral dipping.

2.3.1 Improved method for *Agrobacterium*-mediated transformation

A. thaliana can be stably transformed with high efficiency using T-DNA transfer by *Agrobacterium tumefaciens*. *Agrobacterium*-mediated transformation using the floral dipping method is the most widely used method for transforming *Arabidopsis*. We have showed that *A. thaliana* can be transformed by inoculating flower buds with 5 µl of *Agrobacterium* cell suspension, thus avoiding the use of large volumes of *Agrobacterium* culture (Narusaka et al., 2010). Using this floral inoculating method, we obtained 15–50 transgenic plants per three transformed *A. thaliana* plants. The floral inoculating method can be satisfactorily used in subsequent analyses. This simplified method, without floral dipping, offers an equally efficient transformation as previously reported methods. This method reduces overall labor, cost, time, and space. Another important aspect of this modified method is that it allows many independent transformations to be performed at once.

2.3.2 *Agrobacterium* strains

The *Agrobacterium* strain GV3101 (C58 derivative) is frequently used to transform many binary vectors, e.g., pBI121, pGPTV, pCB301, pCAMBIA, and pGreen, into *Arabidopsis*. It carries rifampicin resistance (10 mg l⁻¹) on the chromosome (Koncz & Schell, 1986). On the other hand, LBA4404 is a popular strain for tobacco transformation but is less effective for *Arabidopsis*.

2.3.3 *Agrobacterium* transformation—freeze/thaw and electroporation procedures

Agrobacterium can be transformed with plasmid DNA using the freeze/thaw (Höfgen & Willmitzer, 1998; Holsters et al., 1978) and electroporation (den Dulk-Ras & Hooykaas, 1995; Mersereau et al., 1990; Shen & Forde, 1989) procedures. The freeze/thaw procedure is very simple and does not require special equipment.

Reagents

- *Agrobacterium* strain
- 20 mM CaCl₂
- Liquid nitrogen
- Luria-Bertani (LB) agar plate
- Liquid LB medium

Equipments

- Microcentrifuge
 - Water bath
 - Eppendorf tube (1.5 ml)
1. Pellet 1.5 ml of overnight-grown *Agrobacterium* (GV3101) cells by centrifugation in an Eppendorf tube at 14,000 rpm for 1 min at 4°C.
 2. Resuspend in 1 ml of ice-cold 20 mM CaCl₂.
 3. Recentrifuge at 14,000 rpm for 1 min at 4°C.
 4. Resuspend in 200 µl of ice-cold 20 mM CaCl₂.
 5. Add binary vector DNA (500 ng or 5–10 µl from an alkaline lysis miniprep) to the suspension. Mix by pipetting.
 6. Freeze the Eppendorf tube in liquid nitrogen for 5 min and thaw at 37°C in a water bath for 5 min. Repeat two times.
 7. Cool on ice.
 8. Add 1 ml LB liquid medium to the Eppendorf tube and incubate at 28°C for 2–5 hrs with gentle agitation (150 rpm; water bath).
 9. Spread 50–200 µl of the cells onto LB agar medium containing appropriate antibiotics and incubate at 28°C for two days.

2.3.4 Selecting transformed *Agrobacterium* using polymerase chain reaction (PCR)

This method is designed to quickly screen for plasmid inserts directly from *Agrobacterium* colonies. Alternatively, the insert presence can be determined by DNA sequencing.

Reagents

- PCR components (one reaction):
 - Autoclaved, distilled water - 11.625 µl
 - 10× PCR buffer - 1.5 µl
 - 2.5 mM dNTPs - 1.2 µl
 - 10 pmol µl⁻¹ Primer #1 - 0.3 µl
 - 10 pmol µl⁻¹ Primer #2 - 0.3 µl
 - Taq* DNA polymerase (5 U/µl) - 0.075 µl
 - Total PCR master mix volume - 15.0 µl

- *Taq* DNA polymerase: Takara EX *Taq* (Takara, Otsu, Japan) (recommended)
- TBE (Tris/Borate/EDTA) buffer

Equipments

- PCR tubes (0.2 ml)
 - Thermocycler
 - Electrophoresis system
1. Prepare sufficient PCR master mix for the number of samples tested.
 2. Add 15 μ l of PCR master mix to each PCR tube.
 3. Select *Agrobacterium* colonies from the plate using a sterile toothpick or pipette tip.
 4. Insert selected colony sample into the PCR master mix and mix with a sterile toothpick or pipette tip.
(Note: Sufficient mixing results in complete cell lysis and high yields.)
 5. Briefly centrifuge tubes to collect all liquid and insert them into the PCR.
 6. Set the thermocycler conditions and start PCR.
Conditions: Preliminary denaturation at 95°C for 3 min then 40 cycles at 95°C for 20 sec, 55°C for 30 sec, and 72°C for 30 sec.
(Note: Preliminary denaturation is very important for initial cell breakage.)
 7. Run 8–10 μ l of each PCR sample on 1.0% agarose gel in 1 \times TBE buffer at 100 V for 30 min to visualize the PCR results. Stain gels according to your lab method.

2.3.5 Simplified *Arabidopsis* transformation: Floral inoculating method

Until now, a limited number of constructs could be transformed into *Arabidopsis* because of difficulty growing large volumes of *Agrobacterium*. Therefore, we focused on improvements to the floral dipping method (Figure 3) (Narusaka et al., 2010). The problem of space and volume can be solved by using a small culture volume. Each plant is transformed using only 30–50 μ l of bacteria grown in 2 ml of liquid culture. Our present method, as described below, is a simple modification of the method reported by Clough & Bent (1998).

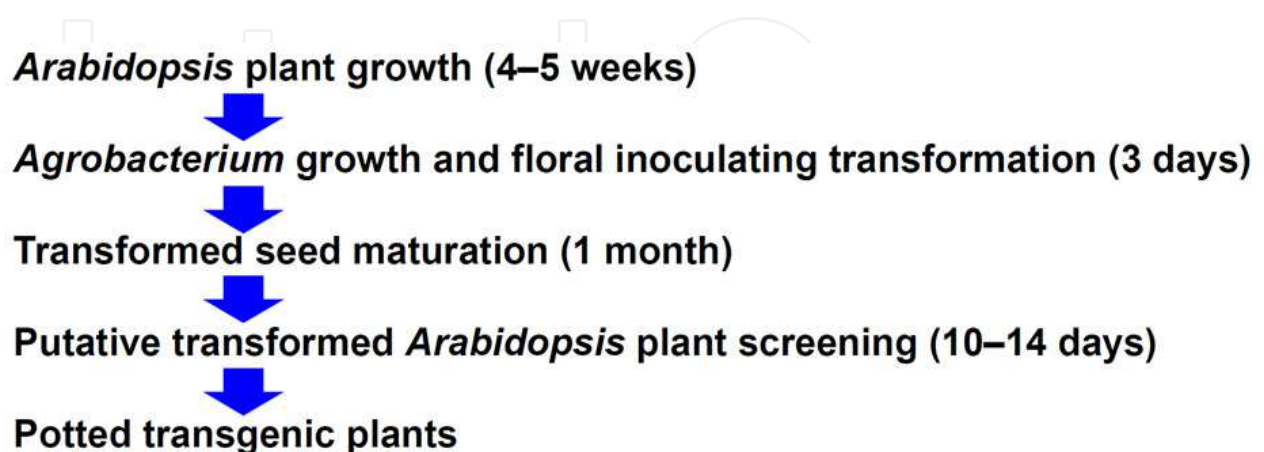


Fig. 3. Transformation using *Agrobacterium* and the floral inoculating method

Recent papers (Liu et al., 2008; Zhang et al., 2006) illustrate the floral dipping process. Clough and Bent (1998) reported that neither Murashige and Skoog (MS) salts and hormones nor optical density (OD) makes a difference in transformation efficiency. An *Agrobacterium* cell suspension containing 0.01–0.05% Silwet L-77 (vol/vol) was used in the uptake of *Agrobacterium* into female gametes, instead of vacuum-aided infiltration of inflorescences.

Reagents

- *A. thaliana*: There are marked differences in transformation efficiency between various ecotypes. For floral dipping transformation, efficiency in the Landsberg *erecta* (Ler-0) ecotype is lower than that in the Columbia (Col-0) ecotype. Transformation efficiency in Wassilewskija (Ws-0) is very high among *Arabidopsis* ecotypes.
- *Agrobacterium* strain: GV3101 (Koncz & Schell, 1986) (recommended) or others.
- 0.1% (wt/vol) agar solution
- 70% (vol/vol) ethanol
- Sodium hypochlorite solution containing 1% available chlorine and 0.02% (vol/vol) Tween 20
- Distilled water
- MS medium: 1× MS plant salt mixture (Wako Pure Chemical Industries, Osaka, Japan), 1× Gamborg's vitamin solution (Sigma-Aldrich, St. Louis, MO, USA), 1% (wt/vol) sucrose, 0.05% (wt/vol) MES, and pH 5.7 adjusted with 1 N KOH
- Bacto agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) (recommended)
- LB agar plate
- Liquid LB
- Glycerol
- Transformation buffer: 1/2× MS plant salt mixture, 1× Gamborg's vitamin solution, 5% (wt/vol) sucrose, and pH 5.7, adjusted with 1 N KOH
- 5% (wt/vol) sucrose solution
- Silwet L-77
- 6-Benzylaminopurine (BAP) (final concentration 0.01 $\mu\text{g ml}^{-1}$)
- Claforan (Aventis Pharma AG, Zürich, Switzerland) (final concentration 2 mg ml^{-1})
- Kanamycin (final concentration 30 $\mu\text{g ml}^{-1}$)
- Hygromycin (final concentration 20 $\mu\text{g ml}^{-1}$)
- Bialaphos (final concentration 7.5 $\mu\text{g ml}^{-1}$)
- Peat moss (Soil Mix, Sakata Seed Corp., Yokohama, Japan)
- Expanded vermiculite granules

Equipments

- Growth chamber
 - Plant pot (3-inch)
 - Conical tube (15 ml)
 - Eppendorf tubes (2 ml)
1. Grow *A. thaliana* plants. (Note: Plant health is an important factor. Healthy *A. thaliana* plants should be grown until they are flowering.) There are two different procedures: standard (A) and quick (B) (Zhang et al., 2006). We generally use the quick procedure, which is useful for rare seeds and seeds with low germination frequency. It is also used to retransform a transgenic line with a second construct.

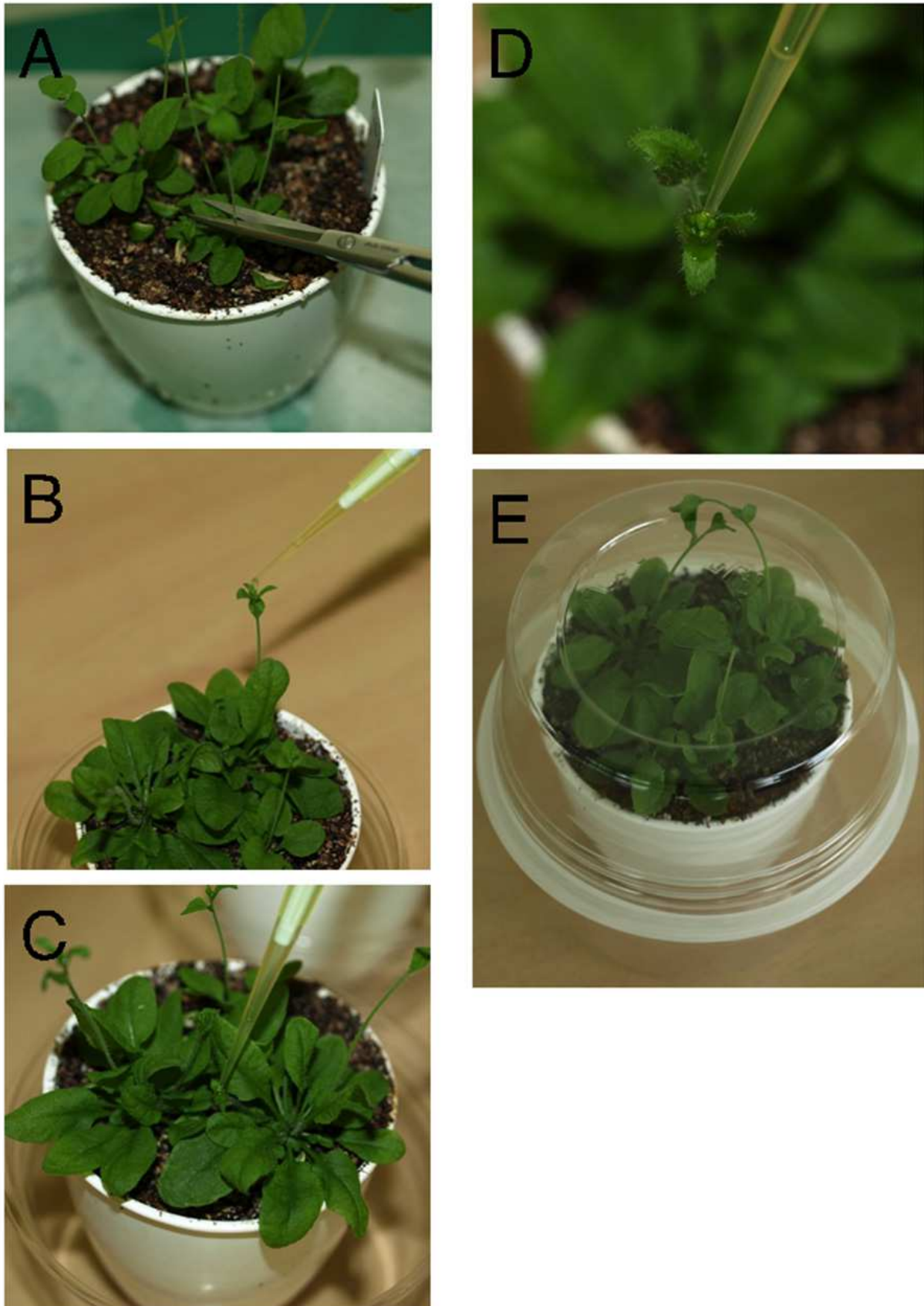


Fig. 4. Part 1.

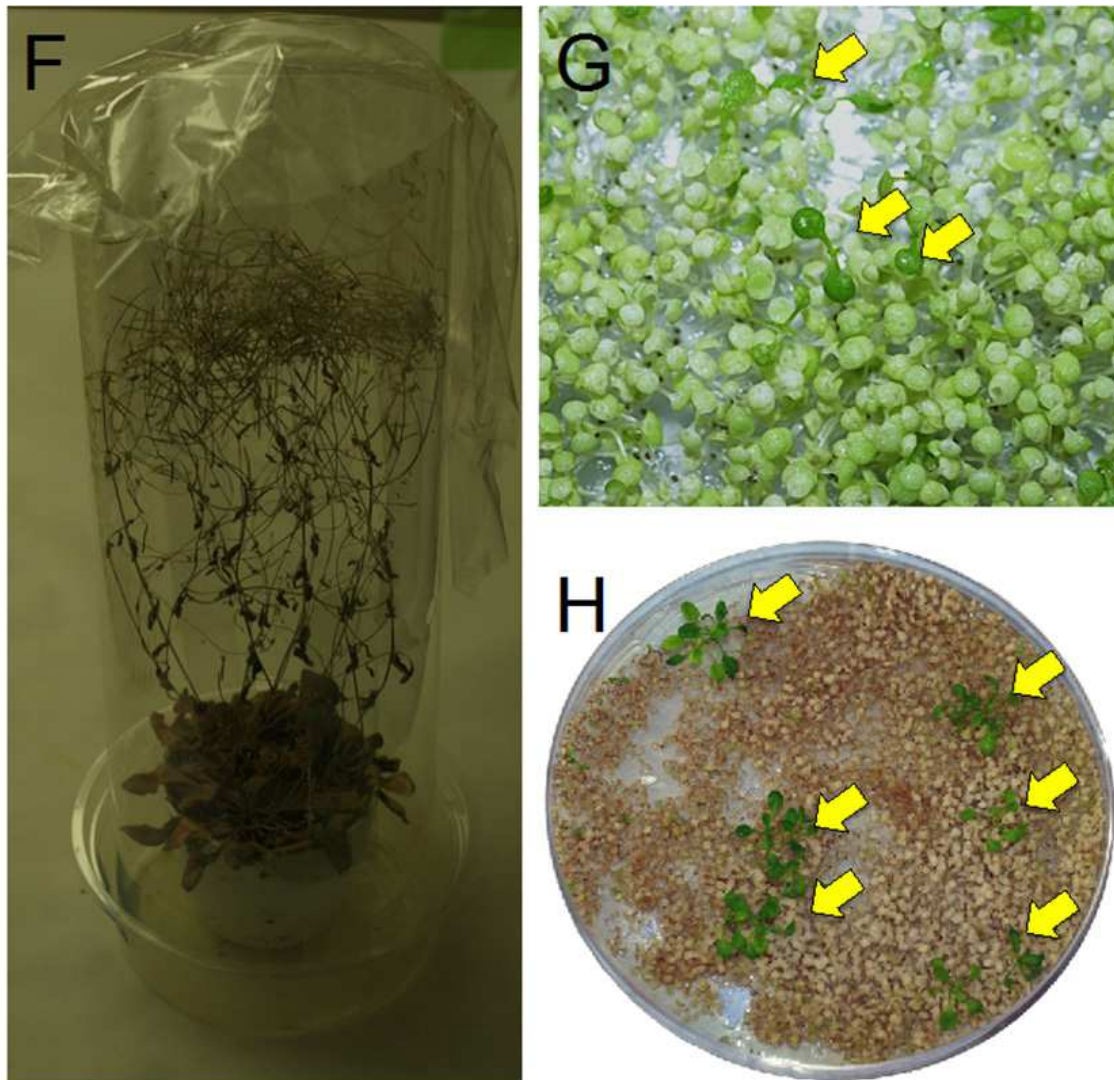


Fig. 4. part 2. Floral inoculating transformation of *Arabidopsis thaliana*. (A) Clipping primary bolts. (B, C, and D) Using a micropipette, inoculate flower buds with 5 μ l of *Agrobacterium* when plants have just started to flower after clipping primary bolts. (E) Place inoculated plants under a dome or cover for 16–24 hrs to maintain high humidity. (F) Remove the cover and grow the plants in a greenhouse or growth chamber until maturity. (G, H) Screening of putative transformed *Arabidopsis* plants. G: 10 days, H: 21 days. Arrows indicate putative transformed *Arabidopsis* plants.

- 1.a. Standard procedure (A): Suspend seeds in 0.1% (wt/vol) agar solution and keep in darkness for 2–4 days at 4°C to break dormancy. Spread seeds on wet soil (a mixture of peat moss and expanded vermiculite granules at a 1:2 ratio) in a 3-inch pot and grow under long-day conditions (16-hr light/8-hr dark) at 22°C. Thin to three seedlings per pot. Do not cover with a bridal veil, window screen, or cheesecloth.
- 1.b. Quick procedure (B): Sterilize seeds by treatment with 70% (vol/vol) ethanol for 1 min then immerse in sodium hypochlorite solution containing 1% available chlorine and 0.02% (vol/vol) Tween 20 for 7 min. Wash seeds five times with sterile distilled water. Place seeds on MS medium containing 0.8% (wt/vol) Bacto agar. Keep seeds in

darkness for 2–4 days at 4°C to break dormancy. Grow under long-day conditions (16-hr light/8-hr dark) for 3 weeks at 22°C. Transfer to pots per Step 1a. Do not cover with a bridal veil, window screen, or cheesecloth.

2. Clip primary bolts to encourage proliferation of secondary bolts (Figure 4A). Plants will be ready approximately 4–6 days after clipping.
3. Prepare the *Agrobacterium* strain carrying the gene of interest. Spread a single *Agrobacterium* colony on an LB agar plate with suitable antibiotics. Incubate the culture at 28°C for two days.
4. Use feeder culture to inoculate a 2-ml liquid culture in LB with suitable antibiotics to select for the binary plasmid in a 15-ml Conical tube at 28°C for 16–24 hrs. Mid-log cells or a freshly saturated culture (Clough and Bent 1998) can be used. (Optional: If needed, keep 500 µl of *Agrobacterium* culture in a 25% (vol/vol) glycerol stock at -80°C.)
5. Spin down 1.5 ml of the *Agrobacterium* cell suspension in 2-ml Eppendorf tubes and resuspend in 1 ml transformation buffer. OD₆₀₀ value adjustment is not required. Each small pot containing three plants requires approximately 150 µl of culture. (Optional: 5% (wt/vol) sucrose solution may be used instead of transformation buffer.)

Just before inoculation, add Silwet L-77 to a concentration of 0.02% (vol/vol) and immediately mix well. (Optional: If using transformation buffer, add 0.01 µg ml⁻¹ BAP just before transformation.)

6. Apply 5 µl of *Agrobacterium* inoculum to the flower buds (Figures 4B, C, and D), inoculating each plant with a total of 30–50 µl of inoculum.
7. Place inoculated plants under a dome or cover for 16–24 hrs to maintain high humidity (Figure 4E). Avoid excessive exposure to light. (Optional: For higher rates of transformation, inoculate newly forming flower buds with *Agrobacterium* 2–3 times at 7-day intervals.)
8. Water and grow plants normally, tying up loose bolts with wax paper, tape, stakes, twist-ties, or other means. Stop watering as seeds become mature (Figure 4F).
9. Harvest dry seeds. Though transformants are usually independent, independence can be guaranteed if seeds come from separate plants.
10. Surface-sterilize seeds by immersion in 70% (vol/vol) ethanol for 1 min, followed by immersion in sodium hypochlorite solution containing 1% available chlorine and 0.02% (vol/vol) Tween 20 for 10 min. Then, wash seeds five times with sterile distilled water.

To select for transformed plants, resuspend liquid-sterilized seeds in approximately 8 ml of 0.1% (wt/vol) agar solution containing 2 mg ml⁻¹ Claforan. Sow seeds per Step 1b in MS medium containing 0.8% Bacto agar and appropriate antibiotics or herbicide selective markers at the following concentrations: kanamycin (final concentration 30 µg ml⁻¹), hygromycin (20 µg ml⁻¹), and bialaphos (7.5 µg ml⁻¹). Claforan is necessary for *Agrobacterium* decontamination (Figures 4G and H).

11. Transplant putative transformants to soil per Step 1a. Grow, test, and use.

2.3.6 Screening of transgenic plants by PCR

Transgenes can be detected by plant genome DNA analysis with PCR (Figure 5). Although transgenes can be distinguished from their surrounding host plant genome, their presence should be determined by DNA sequencing.

PCR-based transgene detection is a simple and highly sensitive process. Subsequent PCR tests are assessed by agarose gel electrophoresis, and results are visualized by the presence or absence of the appropriately sized DNA fragment. If PCR shows a positive result, the transgene may be present. Transgene presence is confirmed by incorporating it into the genome by DNA sequencing. In contrast, a negative PCR result implies that the transgene is not present.

Simplified DNA isolation method

A small plant leaf disc (3–4 mm diameter) can be directly used as a PCR template. *Arabidopsis*, tomato, Chinese cabbage, Komatsuna (*Brassica rapa*), and tobacco leaf discs are good template candidates.

Reagents

Buffer A: 100 mM Tris-HCl (pH 9.5), 1 M KCl, 10 mM EDTA (ethylenediaminetetraacetic acid)

Equipments

- Cork borer (3–4 mm diameter)
 - Disposable blade
 - Eppendorf tube (1.5 ml)
 - PCR tube (0.2 ml)
 - Heat block
1. Cut each plant leaf disc using a cork borer (3–4 mm diameter) or disposable blade (leaf piece should be approximately 3 mm × 3 mm).
 2. Place the leaf disc into an Eppendorf tube.
 3. Add 100 µl of Buffer A.
 4. Incubate for 10 min at 95°C.
 5. Vortex thoroughly.
 6. Transfer 0.5 µl of the template DNA supernatant to a PCR tube.

PCR detection method

Reagents

- PCR components (one reaction):
 - Autoclaved, distilled water - 3.9 µl
 - 2× PCR buffer for KOD FX - 10.0 µl
 - 2 mM dNTPs - 4.0 µl
 - 10 pmol µl⁻¹ Primer #1 - 0.6 µl
 - 10 pmol µl⁻¹ Primer #2 - 0.6 µl
 - KOD FX (1.0 U/µl) - 0.4 µl
 - Total PCR master mix volume - 19.5 µl

 - Add template DNA - 0.5 µl
 - Total reaction volume - 20.0 µl

- DNA polymerase: KOD FX (Toyobo Co., Ltd, Osaka, Japan) (required)
- TBE buffer

Equipments

- PCR tubes (0.2 ml)
 - Thermocycler
 - Electrophoresis system
1. Prepare a PCR master mix for the number of samples tested.
 2. Add 19.5 μ l of PCR master mix to the template DNA and gently mix by pipetting.
 3. Briefly centrifuge tubes to collect all liquid and insert into the PCR.
 4. Set the Thermocycler condition and start PCR. Conditions: Preliminary denaturation step at 94°C for 2 min, followed by 40 cycles at 98°C for 10 sec, 55°C for 15 sec, and 68°C for 30 sec.
 5. Run 8-10 PCR samples on 1.0% agarose gel in 1 \times TBE buffer at 100 V for 30 min to visualize the PCR results. Stain gels according to your lab method.

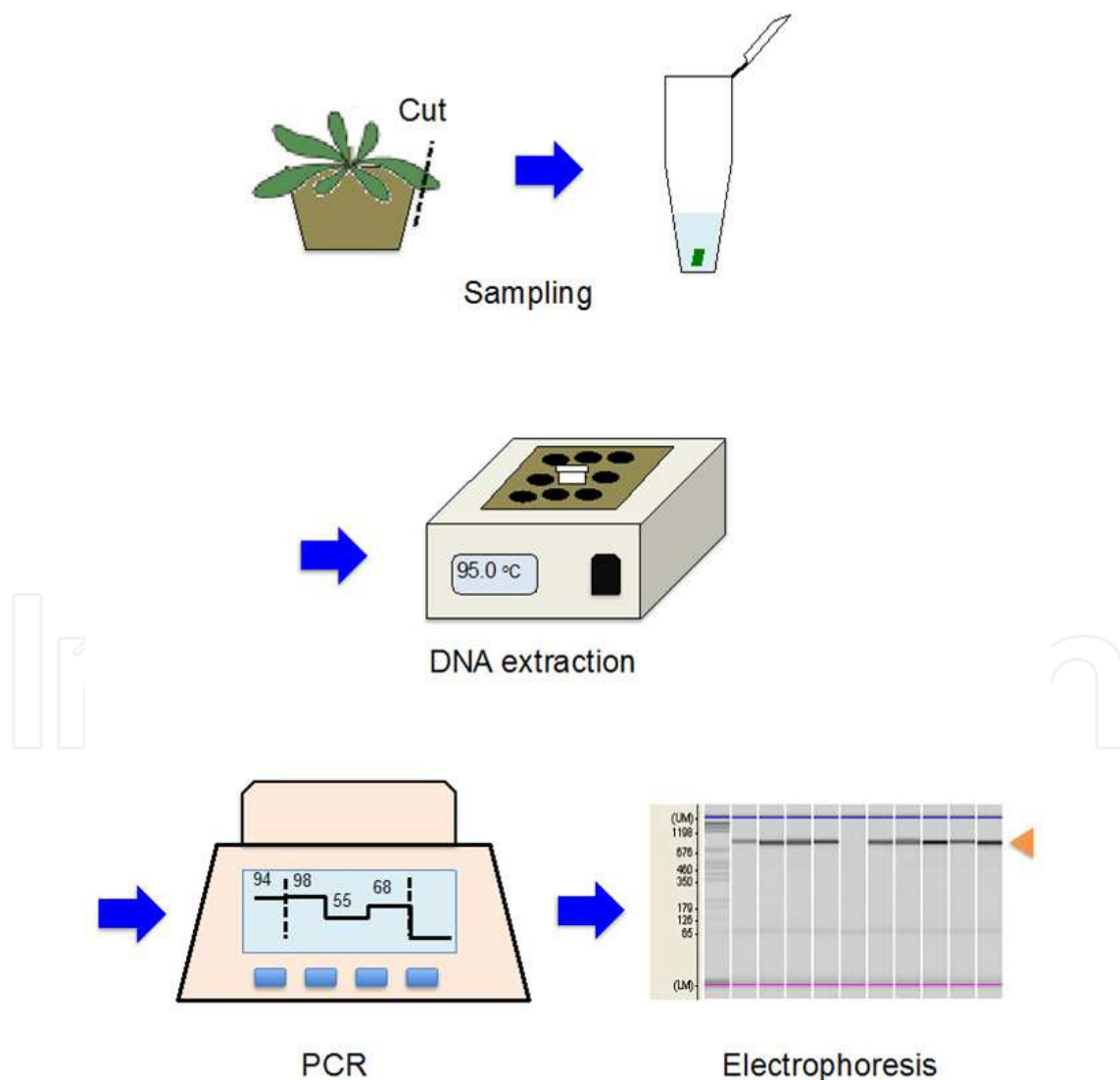


Fig. 5. Screening regimen for transgenic plants by PCR.

3. Conclusion

The floral inoculating method resulted in 15–50 transgenic plants per three transformed *A. thaliana* plants (Table 1). The method can be satisfactorily used for subsequent analyses. This simplified method does not utilize plant inversion or floral dipping, which requires large volumes of *Agrobacterium* culture. It offers equally efficient transformation as previously reported methods with the added benefit of reduced labor, cost, time, and space. Of further importance, this modified method allows many independent transformations to be performed at once.

Vector	Antibiotic marker (final concentration)	Ecotype	%Transformation ^a
pBI101	kanamycin (30 µg ml ⁻¹)	Columbia (Col-0)	0.32 ± 0.02
		Wassilewskija (Ws-0)	0.86 ± 0.12
pGWB1 ^b	kanamycin (30 µg ml ⁻¹) hygromycin (20 µg ml ⁻¹)	Wassilewskija (Ws-0)	0.31 ± 0.05

^a Values are mean ± SE.
^b Refer to Nakagawa et al. (2007).

Table 1. Transformation efficiency using floral inoculating method

4. Acknowledgment

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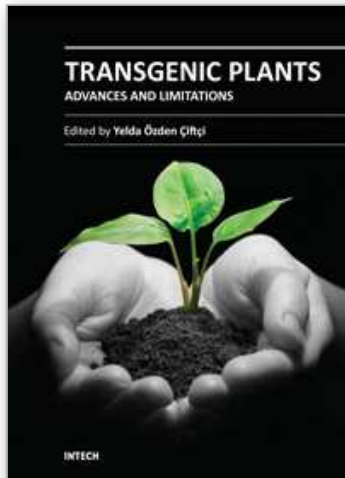
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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
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InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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