

***Ensifer*-mediated *Arabidopsis thaliana* Root Transformation (E-ART): A Protocol to Analyse the Factors that Support *Ensifer*-mediated Transformation (EMT) of Plant Cells**

Dheeraj Singh Rathore^{1,2}, Fiona M Doohan² and Ewen Mullins^{1,*}

¹Teagasc, Dept. of Crop Science, Oak Park, Carlow, R93 XE12, Co. Carlow, Ireland; ²Earth Institute and School of Biology and Environmental Sciences, University College Dublin, Belfield, Dublin 4, Ireland

*For correspondence: ewen.mullins@teagasc.ie

[Abstract] *Ensifer adhaerens* OV14, a soil borne alpha-proteobacteria of the Rhizobiaceae family, fortifies the novel plant transformation technology platform termed '*Ensifer*-mediated transformation' (EMT). EMT can stably transform both monocot and dicot species, and the host range of EMT is continuously expanding across a diverse range of crop species. In this protocol, we adapted a previously published account that describes the use of *Arabidopsis thaliana* roots to investigate the interaction of *A. thaliana* and *Agrobacterium tumefaciens*. In our laboratory, we routinely use *A. thaliana* root explants to examine the factors that enhance the utility of EMT. In addition, the E-ART protocol can be used to study the transcriptional response of *E. adhaerens* and host plant following exposure to explant tissue, the transformability of different *Ensifer adhaerens* strains/mutants as well as testing the susceptibility of *A. thaliana* mutant lines as a means to decipher the mechanisms underpinning EMT.

Keywords: *Ensifer adhaerens* strain OV14, Plant transformation, *A. thaliana*, Root assay, Transient GUS expression

[Background] The advancement of *Ensifer*-mediated transformation (EMT) technology to successfully transform dicots viz., *Arabidopsis thaliana*, *Solanum tuberosum*, *Nicotiana tabacum*, *Manihot esculenta*, *Brassica napus*, and the monocot; *Oryza sativa*, has been previously reported (Wendt *et al.*, 2012; Zuniga-Soto *et al.*, 2015; Chavarriga-Aguirre *et al.*, 2016; Rathore *et al.*, 2016). Additionally, genomic analysis of *E. adhaerens* OV14 by Rudder *et al.* (2014) revealed the bacterium possessed a 7.7 Mb genome comprised of two circular chromosomes (3.96 Mb and 2.01 Mb) and two plasmids (1.61 Mb and 125 Kb). A comparative analysis of the genome of *E. adhaerens* OV14 with *Agrobacterium tumefaciens* C58 (classic genetic engineer) and *Sinorhizobium meliloti* 1021 (a rhizobia with a propensity for low rates of genetic transformation; Broothaerts *et al.*, 2005), highlighted that both *E. adhaerens* OV14 and *S. meliloti* 1021 contain homologs to several chromosomal-based genes essential for *Agrobacterium*-mediated transformation (AMT). However, a suite of genes which positively influence the successful transformation of a plant genome were found to be only present in the genome of *E. adhaerens* OV14 but absent from *S. meliloti* 1021 (Rudder *et al.*, 2014). Overall, the sequence analysis of the *E. adhaerens* OV14 genome has significantly expanded the knowledge base describing the genetic systems that regulate the transformation of plant genomes via EMT.

To date several transient transformation systems have been proposed to study gene function in plants in regard to the use of *A. tumefaciens* (Wroblewski *et al.*, 2005; Gelvin, 2006; Bhaskar *et al.*, 2009; Li *et*

al., 2009; Van Loock *et al.*, 2010; Hwang *et al.*, 2013; Krenek *et al.*, 2015). Whereas, the stable transformations of any plant species are lengthy processes to test the utility of a bacterial transfection system to transform the plants. The advantages of transient transformation methods include rapid production of results, functional genomic studies and recombinant protein production (Van Loock *et al.*, 2010; Krenek *et al.*, 2015). The model plant *A. thaliana* is a powerful research tool with which to study the molecular, genetic and biochemical processes that support genetic transformation of somatic tissues as well as *in planta* (Provar *et al.*, 2016). In contrast to the several months typically required for the transformation of primary crop species, these investigations require a rapid, reproducible and easy quantification method to determine the rate of transient transformation. Previously, Gelvin (2006) reported an efficient and reproducible quantitative assay for *Agrobacterium*-mediated Transformation using *A. thaliana* roots. This assay has well served the purpose of testing either *Agrobacterium* strains or *A. thaliana* ecotypes/mutants in the authors' laboratory for more than two decades, reflecting the significance and competency of the assay in publications such as Shi *et al.* (2014). Conversely, a transient transformation method to facilitate comparable studies via EMT is not available. In response, the *Ensifer*-mediated *A. thaliana* Root Transformation (E-ART) protocol presented here is designed to address this deficit so that specific genetic and microbiological factors that support/enhance EMT can be identified to support the application of the technology to agronomically important crop species. The protocol is a modified version of an existing AMT based quantitative *A. thaliana* roots assay (Gelvin, 2006). While developing the E-ART protocol, we learnt that it is possible to improve transient GUS expression in *A. thaliana* root segments by adjusting several experimental factors (*e.g.*, time of co-cultivation, acetosyringone concentrations, *etc.*) involved in the early stages of *E. adhaerens* transfection. E-ART, being the first quantitative method of transient gene expression for EMT will facilitate the rapid evaluation of novel *E. adhaerens* strains in plant transformation while also providing a platform to assess the genetic response of plants to EMT.

Materials and Reagents

1. 2 ml centrifuge tubes
2. Square Petri-dishes (Greiner Bio One International, catalog number: 688161)
3. Parafilm (Bemis, catalog number: PM992)
4. 50 ml Falcon tube
5. Scalpel blades (NO. 10A, Swan Morton, catalog number: 0302)
6. Sterile filter papers (GE Healthcare, catalog number: 1004-090)
7. Petri dish 92 x 16 mm w/o cams (SARSTEDT, catalog number: 82.1472)
8. *A. thaliana* seed (in this case ecotype Columbia, Col-0)
Note: A. thaliana seed is no more than 6 months old being stored at 4 °C.
9. *E. adhaerens* strain OV14 harbouring plasmid of choice (in this case pCambia5105/pCambia5106 plasmids [Jefferson *et al.*, 2006])
10. 70% ethanol

11. Distilled sterile water (DSW)
12. Bleach (5% sodium hypochlorite; final concentration used is 50% Bleach, *i.e.*, 1:1 Bleach:water)
13. Tween-20
14. Agarose (0.1%, Sigma-Aldrich, catalog number: A9539-500G)
15. Antibiotics for bacterial selection: kanamycin, streptomycin, spectinomycin (Duchefa Biochemie)
16. Sodium chloride (0.9% NaCl solution)
17. Acetosyringone (Sigma-Aldrich, catalog number: D134406)
18. Cefotaxime sodium (Duchefa Biochemie, catalog number: C0111.0005)
19. Tryptone (Oxoid, catalog number: LP0042)
20. Yeast extract (Oxoid, catalog number: LP0021)
21. Calcium chloride dehydrate (Duchefa Biochemie, catalog number: C0504)
22. Agar No. 1 (Oxoid, catalog number: LP0011)
23. MS basal salts (Duchefa Biochemie, catalog number: M0221)
24. Sucrose (Duchefa Biochemie, catalog number: S0809)
25. 2,4-Morpholino-ethane sulfonic acid (MES monohydrate) (Duchefa Biochemie, catalog number: M1503)
26. Myo-inositol (Duchefa Biochemie, catalog number: I0609)
27. Nicotinic acid (Duchefa Biochemie, catalog number: N0611)
28. Pyridoxine (Duchefa Biochemie, catalog number: P0612)
29. Thiamine-HCl (Duchefa Biochemie, catalog number: T0614)
30. D-Glucose monohydrate (Duchefa Biochemie, catalog number: G0802)
31. Indole-3-acetic acid (IAA) (Duchefa Biochemie, catalog number: I0901)
32. 2,4-Dichlorophenoxyacetic acid (2,4-D) (Duchefa Biochemie, catalog number: D0911)
33. Kinetin (Duchefa Biochemie, catalog number: K0905)
34. X-GlcA cyclohexylammonium salt (Duchefa Biochemie, catalog number: X1405)
35. Dimethyl sulfoxide (DMSO) (Duchefa Biochemie, catalog number: D1370)
36. Teagasc-Tryptone Yeast-extract (TTY) medium (Rathore *et al.*, 2015) (see Recipes)
37. MS based media (see Recipes)
 - a. Seed germination media (SGM)
 - b. Co-cultivation media (CCM)
 - c. Callus Induction media (CIM)
 - d. Vitamin stock
38. X-GlcA solution (see Recipes)
39. Histochemical GUS stain solution (Jefferson *et al.*, 1987) (see Recipes)

Equipment

1. Pipettes (P1000, P100, P10)
2. Controlled environment room/chamber to grow healthy *A. thaliana* plants (24 °C, 16 h light, 8 h dark), abbreviated as CT room
3. Conical flasks (250 ml, sterile)
4. Centrifuge
5. Incubator (28 °C and 37 °C)
6. Shaker incubator (28 °C, 220 rpm)
7. Fridge (4 °C) and freezers (-20 °C and -80 °C)
8. Laminar flow to perform aseptic work
9. Bead sterilizer/flame to sterilize forceps
10. Scalpel blades handles (No.7 S/S, Swan Morton, catalog number: 0907)
11. Autoclave (15 min at 121 °C and 15 psi)
12. pH meter
13. Weighing balance
14. NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, Thermo Scientific™, model: NanoDrop™ 2000)
15. Elga water purifier (Veolia Water Solution & Technologies, model: PURELAB® OPTION-R 7, catalog number: OR007BPM1)
16. Stereo-microscope

Software

1. SAS system (Version 9.3, copyright 2002-2010 by SAS Institute Inc., Cary, NC, USA)

Procedure

A. Seed sterilization and germination

1. Surface sterilize *A. thaliana* seeds (~5 mg) in a 2 ml tube with 70% ethanol (EtOH) by vortexing the tube for 2 min. Discard the EtOH with an adjustable micropipette (1,000 µl) and wash seeds twice with DSW. All the steps were performed under a laminar flow hood to ensure aseptic conditions throughout.
2. Add 50% Bleach containing 0.1% Tween-20 to the seeds and sterilize for 10 min with vigorous shaking. Once complete, decant Bleach with a 1,000 µl micropipette (Figure 1A).
3. Rinse the seeds for five times with DSW to remove any residue of Bleach.
4. Post-sterilization process, add 0.1% agarose (previously autoclaved for 15 min at 121 °C) to seeds for ease of handling.

5. Using a micropipette (1,000 μ l), place ~4 seeds per drop aligned in a row leaving ~1 inch distance from the top end of square Petri-dishes (Figures 1B and 1C) containing MS based seed germination medium (SGM, see Recipes). Double wrap the Petri-dishes using Parafilm.
6. Incubate the Petri-dishes vertically in a stand (to ensure elongated root formation) in a CT (controlled temperature) room set at 24 °C, 16 h light ~4,000 lux photoperiod and 8 h dark for 15-20 days (Figure 1D).
7. Root harvesting should be achieved before roots reach the end of dish, at which stage ~20 segments of < 0.5 cm length each should be obtained per plant.

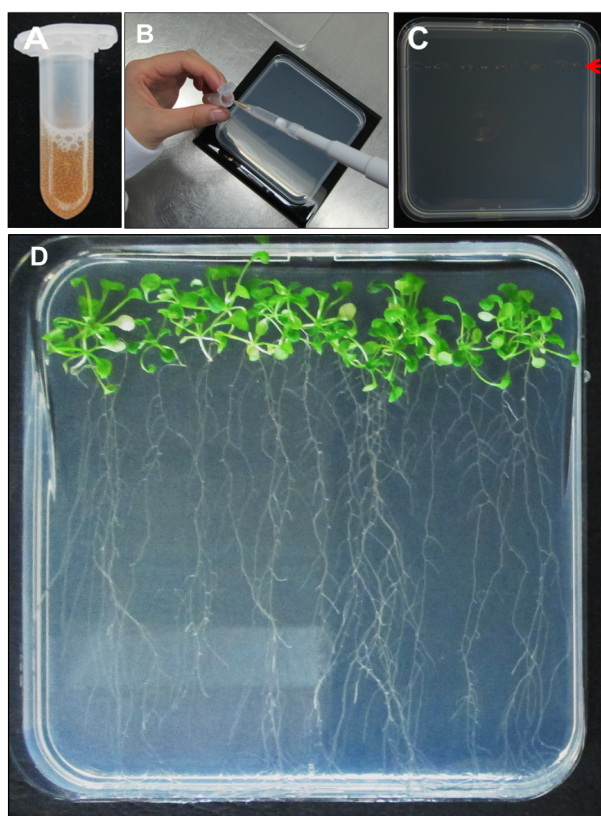


Figure 1. *Arabidopsis thaliana* seed sterilisation and germination. A. The *A. thaliana* ecotype Col-0 in 50% Bleach in a 2 ml centrifuge tube; B. Placing sterilised seeds on square Petri-dishes leaving ~1 inch top space for plants to grow; C. Plated seeds on SGM; D. Vertically aligned Petri-dishes in CT room (at 24 °C, 16 h photoperiod) showing plantlets with roots.

B. Preparation of *Ensifer* inoculums

1. Grow *E. adhaerens* OV14_pCambia5105 (here onwards E5105) on a TTY agar (see Recipes) containing 100 mg/L kanamycin, 200 mg/L streptomycin and 200 mg/L spectinomycin to select for the unitary plasmid containing T-DNA as well as virulence (*vir*) genes, overnight at 28 °C (Figure 2A).

2. Pick a single colony to inoculate 10 ml TTY broth containing appropriate antibiotics (100 mg/L kanamycin, 200 mg/L streptomycin and spectinomycin) in a sterile conical flask and grow overnight at 28 °C and 220 rpm to reach 0.8 OD_{600 nm} (Figures 2B and 2C).
3. Pellet the cells in 50 ml Falcon tubes by centrifugation and wash them using 0.9% NaCl to remove any antibiotic residues. Centrifuge bacterial cultures at 3,750 x g (rcf) for 25-30 min for the inoculum preparation. Re-suspend the cells in 0.9% NaCl to maintain 0.8 OD_{600 nm} (Figure 2D).
4. Add 200 μM acetosyringone and incubate at 28 °C, 220 rpm for additional 1.5 h to induce virulence.

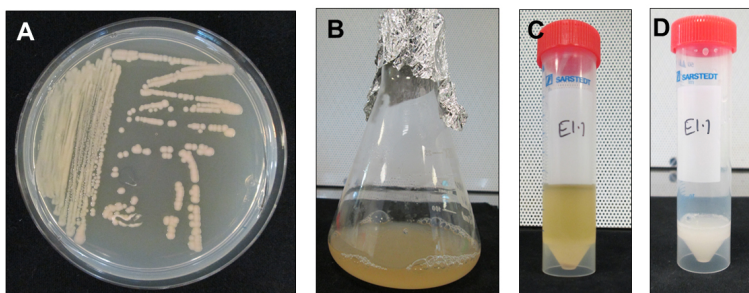


Figure 2. Steps involved in preparing E5105 cultures for transfection of root segments.

A. Overnight grown E5105 on TTY agar containing 100 mg/L kanamycin + 200 mg/L streptomycin + 200 mg/L spectinomycin to obtain single colonies. B. Single colony derived E5105 culture in TTY broth with appropriate antibiotics. C. E5105 culture in a 50 ml Falcon tube for centrifugation to remove TTY broth + antibiotics. D. E5105 resuspended in 0.9% NaCl and supplemented with 200 μM acetosyringone for *vir* induction at 28 °C, 1.5 h.

C. Preparation and transfection of *A. thaliana* root segments

1. Separate the *A. thaliana* roots from plants using a sterile scalpel blade before laying roots down on a Petri-dish containing a small amount (~500 μl) of DSW to ensure adequate root hydration (Figures 3A and 3B).
2. Align roots together and cut into 0.3 to 0.5 cm long segments (Figure 3C). Place bundles of ~30 root segments on MS based co-cultivation medium (CCM, see Recipes) containing 200 μM acetosyringone.
3. Using a P1000 pipette place 2-3 drops of E5105 on each the *A. thaliana* root bundle and allow a transfection time of 10 min (Figure 3D). Untreated root segments (no bacteria) should be used as a negative control.
4. Remove any excess bacterial solution after the 10 min transfection time using the P1000 pipette. Double wrap the Petri-dishes with Parafilm.
5. Co-culture the bacteria and *A. thaliana* roots at 20 °C, in the dark for 5 days.

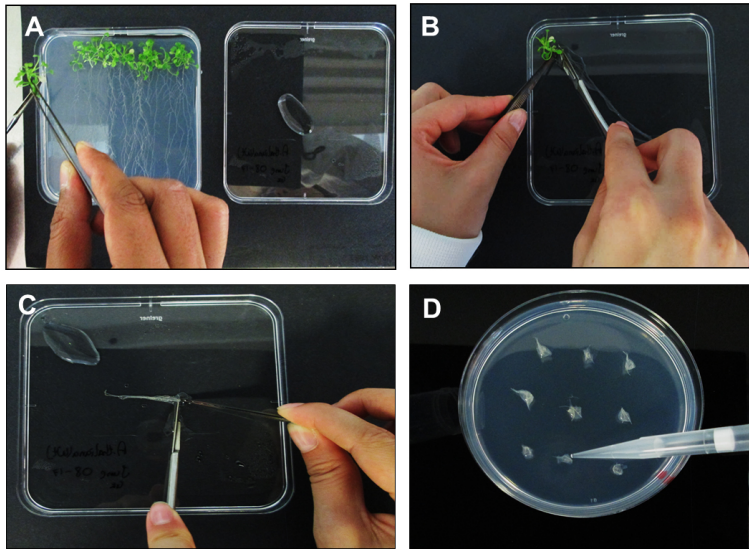


Figure 3. Processing of *Arabidopsis thaliana* plants and inoculations. A. Moving *A. thaliana* plants from SGM to square Petri-dish lid containing DSW; B. Separating the roots from the shoots; C. Cutting *A. thaliana* roots into ~3 mm segments; D. Inoculating the root segments with E5105 post 1.5 h *vir* induction.

D. Transient GUS expression assay

1. Post 5 days co-cultivation; wash treated/untreated root segments in DSW containing 500 mg/L cefotaxime to mitigate bacterial overgrowth.
2. GUS staining: The activity of β -glucuronidase in putative transgenic root segments is performed as described by Jefferson *et al.* (1987) using one of two ways:
 - a. For faster results, the washed roots are treated in GUS stain solution (see Recipes) with an overnight incubation at 37 °C. To remove the excess stain, wash root segments in 90% (v/v) ethanol (Figures 4A and 4B).
 - b. To allow further proliferation of root segments and to facilitate ease of handling, transfer the roots onto callus induction medium (CIM, see Recipes) containing 250 mg/L cefotaxime. Seal the plates with Parafilm and incubate for additional 5-6 days at 24 °C in the dark, before staining in GUS stain solution, overnight at 37 °C (Figures 4C and 4D).

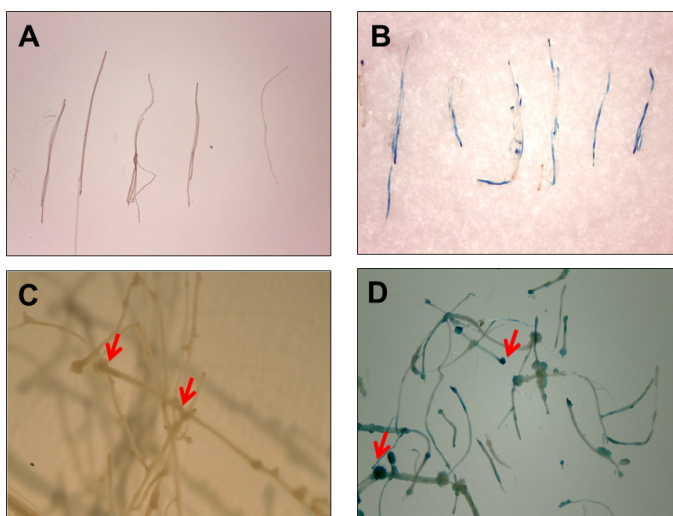


Figure 4. Histochemical GUS activity via E-ART. Root segments were immediately transferred to GUS stain post 5 days co-cultivation (A) untreated control and (B) treated with E5105. GUS staining of root segments showing callus formation (indicated with red arrows) when segments were transferred to CIM containing 250 mg/L cefotaxime for 6 days post co-cultivation (C) untreated control and (D) treated with E5105.

Data analysis

Perform all experiments in triplicate with three technical replicates (one Petri-dish containing 9 bundles of root explants was treated as one technical replicate). The transient expression of GUS in *A. thaliana* roots treated with E5105 (*E. adhaerens* OV14 carrying pCAMBIA5105) is measured based on GUS activity *i.e.*, blue colouration. Count blue roots/spots containing root segments for each treatment in each replicate under a stereo-microscope and record in MS Excel (Microsoft, USA). For statistical significance, analyse data using the General Linear Model (GLM) in the SAS system (Version 9.3, copyright 2002-2010 by SAS Institute Inc., Cary, NC, USA). The GLM procedure enables *t*-test (LSD) and Tukey's studentized Range (HSD) test using the SAS system.

Notes

1. Store seeds in Falcon tubes, properly labelled for the ecotype at 4 °C.
2. All steps are strictly performed under aseptic conditions in a laminar flow hood. All media are autoclaved (15 min at 121 °C) and media supplements such as growth hormones, antibiotics, are filter sterilised and added after autoclaving.
3. Seal the Petri-dishes with a double layer of Parafilm to maintain humidity to grow healthy plants. It is primarily important for high transient GUS expression that the plants are young to provide healthy roots quickly.
4. Always process the *A. thaliana* plants for obtaining root explants before a flower bolt emerges as older plants will lead to lower transformation rates as detailed by Gelvin (2006).

5. More importantly, always use fresh bacterial cultures to obtain higher rates of transformations. Do not allow bacterial cultures (E5105) to grow over 0.8 OD_{600 nm}. Higher OD cultures will overgrow bacteria during co-cultivation that will negatively influence the GUS expression.
6. The transfection of roots should be performed within 30 min of cutting the root segments. Leaving cut root segments for a longer time will affect the transfection and transformation negatively.
7. Always include untreated root explants (no bacterial inoculum) as a negative control in the experiment.
8. Current work shows that a combination of 5 days co-cultivation time and 200 µM acetosyringone improved rate of transient GUS expression in the *A. thaliana* root segments.

Recipes

Note: The following media are prepared, autoclaved and stored at room temperature.

1. Teagasc-tryptone yeast extract (TTY) medium
 - 1.0% tryptone
 - 0.5% yeast extract
 - After autoclaving add 20 ml of 1 M sterile CaCl₂ per litre of medium
 - To make TTY agar, add 1.5% agar to TTY broth prior to autoclaving

2. MS based media (Table 1)

Note: The MS based media are poured in Petri-dishes and can be stored at 4 °C for up to 15 days. Prepare vitamin stock, filter sterilise and store aliquots of 1 ml at -20 °C for up to 1 month.

Table 1. Media recipe used for *Ensifer*-mediated *Arabidopsis thaliana* root transformation (E-ART)

Media	Components (per litre)	Add after sterilization (per litre)
Seed germination medium (SGM)	2.2 g MS basal salts 10 g sucrose 10 g agar (pH 5.8)	
Co-cultivation medium (CCM)	4.32 g MS basal salts 0.5 g 2,4-morpholino-ethane sulfonic acid (MES) 10 ml myo-inositol solution 10 g sucrose (pH 5.7) 7.5 g agar	1 ml Vitamin stock (0.5 mg/ml nicotinic acid, 0.5 mg/ml pyridoxine, 0.5 mg/ml thiamine-HCl)
Callus induction medium (CIM)	4.32 g MS basal salts 0.05 M MES 10 ml myo-inositol solution 20 g glucose (pH 5.7) 7.5 g agar	1 ml Vitamin stock 5 µg/ml IAA 0.5 µg/ml 2,4-D 0.3 µg/ml kinetin

3. X-GlcA solution

To prepare 1 M of X-GlcA solution:

Add 521.8 mg of X-GlcA to 10 ml DMSO and vortex to dissolve

Aliquot 1 ml in centrifuge tubes to store at -20 °C for long-term use

Note: This substance is hygroscopic and light sensitive.

4. Histochemical GUS stain solution

Prepare GUS solution by adding below listed chemicals to reach the final concentrations as:

50 mM sodium phosphate buffer pH 7.0

1 mM EDTA

0.1 mM of ferri- and ferro-cyanide, individually

0.1% SDS

40 mM X-GlcA

Store aliquots at -20 °C

Acknowledgments

This research work was supported by the Teagasc Walsh Fellowship Scheme which funded DSR. Authors gratefully acknowledge Prof. Stanton B. Gelvin for the *Agrobacterium* transformation of *Arabidopsis thaliana* Roots: A Quantitative Assay (2006) published as a chapter in Methods in Molecular Biology, *Agrobacterium* protocols, which was adapted to develop E-ART protocol.

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