

Video Article

Plant Growth and Agrobacterium-mediated Floral-dip Transformation of the Extremophyte *Schrenkiella parvula*

Guannan Wang¹, Pramod Pantha¹, Kieu-Nga Tran¹, Dong-Ha Oh¹, Maheshi Dassanayake¹

¹Department of Biological Sciences, Louisiana State University

Correspondence to: Dong-Ha Oh at ohdongha@lsu.edu, Maheshi Dassanayake at maheshid@lsu.edu

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Abstract

Schrenkiella parvula is an extremophyte adapted to various abiotic stresses, including multiple ion toxicity stresses. Despite high-quality genomic resources available to study how plants adapt to environmental stresses, its value as a functional genomics model and tool has been limited by the lack of a feasible transformation system. In this protocol, we report how to generate stable transgenic *S. parvula* lines using an Agrobacterium-mediated floral-dip method. We modified the transformation protocol used for *A. thaliana* to account for unique traits of *S. parvula*, such as an indeterminate flowering habit and a high epicuticular wax content on leaves. Briefly, *S. parvula* seeds were stratified at 4 °C for five days before planting. Plants were grown at a photoperiod of a 14 h light and 10 h dark and a 130 $\mu\text{mol m}^{-2}\text{s}^{-1}$ light intensity, at 22 °C to 24 °C. Eight to nine week-old plants with multiple inflorescences were selected for transformation. These inflorescences were dipped in an infiltration solution of *Agrobacterium tumefaciens* GV3101 carrying the *pMP90RK* plasmid. We performed two rounds of flower dipping with an interval of three to four weeks to increase the transformation efficiency. The T1 seeds were collected and dried for four weeks in a container with desiccants before germination to screen for candidate transformed lines. Resistance to BASTA was used to screen T1 plants. We sprayed the BASTA solution three times with an interval of three days starting at two week-old plants to reduce false positives. A BASTA drop test was performed on surviving individual plants to identify true positive transformants. The transformation efficiency was 0.033%, yielding 3–4 transgenic plants per 10,000 T1 seeds propagated.

Video Link

The video component of this article can be found at <https://www.jove.com/video/58544/>

Introduction

In this protocol, we describe the growth and establishment of stable transgenic lines for the extremophyte model *Schrenkiella parvula*. The availability of an efficient transformation system is a hallmark of any versatile genetic model. Plants that thrive in extreme environments, referred to as extremophytes, provide a critical resource for understanding plant adaptations to environmental stresses. *Schrenkiella parvula* (formerly *Thellungiella parvula* and *Eutrema parvulum*) is one such extremophyte model, with expanding genomic resources^{1,2,3,4,5}. However, transformation protocols have not yet been reported for *S. parvula* in published studies.

The genome of *S. parvula* is the first published extremophyte genome in Brassicaceae (mustard-cabbage family) and shows an extensive overall genome synteny with the non-extremophyte model, *Arabidopsis thaliana*¹. Thus, comparative studies between *A. thaliana* and *S. parvula* could benefit from the wealth of genetic studies performed on *A. thaliana* to make informative hypotheses on how the *S. parvula* genome has evolved and regulated differently to cope with extreme environmental stresses^{5,6,7}. *S. parvula* is one of the most salt-tolerant species (based on soil NaCl LD50) among known wild relatives of *A. thaliana*⁸. In addition to the NaCl tolerance, *S. parvula* survives and completes its life cycle in the presence of multiple salt ions at high concentrations toxic to most plants⁷. In response to the abiotic stresses prevalent in its natural habitat, it has evolved various traits, among which several have been studied at the biochemical or physiological level^{8,9,10,11}.

Since 2010, there have been over 400 peer-reviewed publications that used *S. parvula* as a target species or used it in a comparison with other plant genomes. However, a clear bottleneck could be identified with a closer look of what type of studies have been conducted. The majority of these reports discuss the potential use of *S. parvula* in future studies or use it in comparative genomic or phylogenomic studies. Due to the lack of a proof-of-concept transformation protocol established for *S. parvula*, it has not been used in functional genomic studies, despite having one of the highest quality plant genomes available to date (>5 Mb contig N50) assembled and annotated into chromosome-level pseudomolecules¹.

The Agrobacterium-mediated floral-dip transformation method has become the most broadly used method to create transgenic lines in *A. thaliana*, and the development of a reproducible system of transformation was a critical factor in its success as a genetic model^{12,13}. However, not all Brassicaceae species have been shown to be successfully transformed using the floral-dip method developed for *A. thaliana*. Specially, the Brassicaceae Lineage II species that include *S. parvula* has been recalcitrant to floral-dip based transformation methods^{14,15}.

The indeterminate flowering growth habit of *S. parvula*, combined with its narrow leaf morphology has made it challenging to adopt the standard Agrobacterium-mediated floral-dip transformation method. In this study, we report the modified protocol we have developed for reproducible transformation of *S. parvula*.

Protocol

1. Plant Growth

1. Seed sterilization (optional)

1. Prepare 50% bleach in double-distilled water (ddH₂O) with 1 or 2 drops of a non-ionic detergent (see **Table of Materials**) in a 50 mL tube. Invert the tube several times to mix the solution.
NOTE: It is preferable to conduct seed sterilization in a laminar flow cabinet with a UV sterilized surface for 15 min.
2. Add the bleach solution to ~100–200 *S. parvula* seeds in a 1.5 mL tube. Mix thoroughly and let the tube sit for 5 min.
3. Remove the bleach from the tube and add 70% ethanol. Wash the seeds by pipetting several times and then remove the ethanol solution immediately.
4. Wash the seeds in sterilized water to remove excess bleach and ethanol, then remove the water. Repeat this step 5 to 6 times.

2. Seed stratification

1. Immerse the seeds in sterilized water, and store for 5 to 7 days at 4 °C. Alternatively, sow dried unsterilized seeds directly on wet soil, and place the soil tray for 5 to 7 days at 4 °C.

3. Growing plants in preparation of transformation

1. Fill the soil mix (see **Table of Materials**) into 7 x 6 cm² pots, soak the pots in water, and spray water from the top to ensure a uniformly wet growth medium. Add 5– fertilizer beads (see **Table of Materials**) on the soil surface of each pot.
NOTE: As far as we have experienced, *S. parvula* grows well on any soil mix where *A. thaliana* can grow.
2. Using a wet toothpick, transfer 20–25 seeds per pot on the soil surface.
NOTE: A convenient practice is to put a batch of 4–5 seeds in the four corners and the center of the pot (**Figure 1**, Day 15, left panel).
3. Cover the pot tray with a clear dome to keep the seeds under high humidity during germination.
4. Keep the plant trays in a growth chamber with a light intensity set at 130 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light, 22–24 °C temperature, and 14 h per day/10 h per night cycle. Remove the domes after 7 - 10 days following germination. Add water from the bottom of the tray to keep soil moistened uniformly at a desirable level.
5. Weed out extra seedlings and leave only 4–5 healthy seedlings per pot well separated from each other (**Figure 1**, Day 15, right panel).
6. Gently water the plants every two days and fertilize with 0.2x Hoagland's solution¹⁶ once every two weeks.
NOTE: Keeping the soil moisture at a uniform level is key to growing *S. parvula* consistently and healthily.
7. Continue to grow the plants for 8–10 weeks until multiple inflorescences produce 100–150 floral buds per plant (**Figure 1**, Day 60–80). On the day planned for the floral-dip based transformation (step 4.5), remove all mature and developing siliques from the plants.

2. Cloning the Gene/Genomic Element of Interest into a Vector for Plant Transformation

1. Amplify the target DNA fragment using polymerase chain reaction (PCR)¹⁷ and isolate the PCR product using a gel extraction kit (see **Table of Materials**) according to the kit protocol or any other appropriate method to purify DNA using agarose gel electrophoresis^{17,18}. Verify the sequence of the isolated PCR product through Sanger sequencing¹⁹.
2. Clone the desired PCR product into the cloning vector and transform the cloned construct into the competent *E. coli* cells using a topoisomerase-based cloning kit (see **Table of Materials**) following manufacturer's guidelines.
3. Spread 50 μL of transformed products on Luria-Bertani²⁰ (LB) agar bacterial growth media (**Table 1**) with appropriate antibiotics, e.g., 50 $\mu\text{g}/\text{mL}$ Spectinomycin (see **Table of Materials**), and incubate at 37 °C overnight.
4. The following day, select 5–10 single colonies, inoculate into liquid LB medium with appropriate antibiotics, and incubate with gentle shaking at 37 °C overnight.
5. Isolate plasmids using a plasmid isolation kit (see **Table of Materials**) and verify through Sanger sequencing¹⁹ whether the target sequence amplified in 2.1 is properly cloned.
6. Transfer the cloned and verified PCR product to a destination vector for plant transformation compatible with recombination-based cloning (see **Table of Materials**), using a recombinase enzyme mix kit (see **Table of Materials**), following the kit manufacturer's instruction. Repeat from step 2.3 to step 2.5 to isolate and verify clones harboring proper plasmid constructs.

3. Transforming the Vector Construct for Plant Transformation into *Agrobacterium tumefaciens*

1. Transform the plasmid of the vector construct from 2.6 into the *A. tumefaciens* strain GV3101:pMP90RK²¹, which harbors a Rifampicin resistance gene for chromosomal background selection. Use appropriate antibiotics, e.g. Gentamycin or Kanamycin (see **Table of Materials**), for the selection of plant transformation construct (Ti plasmid). A brief protocol for *A. tumefaciens* transformation via electroporation is included in section 3.2.
2. ***A. tumefaciens* transformation by electroporation**
 1. Thaw the *A. tumefaciens* competent cells²² on ice. Mix 0.1–1 μg of the plasmid prepared from 2.6, dissolved in 1–2 μL of ddH₂O, with competent cells on ice. Transfer the mixture into an electroporation cuvette (see **Table of Materials**).
 2. Perform electroporation on the mixture of plasmids and competent cells from 3.2.1, using an electroporator (see **Table of Materials**) following the manufacturer's guidelines.

NOTE: Clean the surface of the cuvette before starting the electroporation.

3. Transfer the reaction mixture from the cuvette to a microcentrifuge tube that contains 1.5 mL of liquid LB and mix well with pipetting and incubate for 1 h at 28 °C with gentle shaking.

3. Inoculate the transformed *A. tumefaciens* from section 3.2 on LB plates containing appropriate selection antibiotics (e.g. Kanamycin 25 µg/mL, Spectinomycin 50 µg/mL, Gentamycin 25 µg/mL, and Rifampicin 50 µg/mL) and incubate at 28 °C for 3 days.

4. Agrobacterium-mediated Transformation of *S. parvula*

1. Inoculate the single transformed colonies from plates into 10 mL of LB liquid media containing antibiotics (the same as in 3.3) in a sterile 50 mL conical tube (see **Table of Materials**). Incubate for 24 h in a shaking incubator (see **Table of Materials**) at 250 r.p.m. at 28 °C.
2. Transfer the bacterial solution from 3.4.1 to a sterile 250 mL flask, add 40 mL of LB liquid media with appropriate antibiotics, and incubate 12 h until the optical density at 600 nm wavelength (OD₆₀₀) reaches around 2.0.
3. Centrifuge the *A. tumefaciens* culture at 3,100 x g for 10 min. Remove the supernatant and re-suspend the bacterial culture in 40 mL of *A. tumefaciens* infiltration solution (**Table 1**).
4. Dilute the resuspended *A. tumefaciens* with infiltration solution to a final OD₆₀₀ of 0.8. Add 25 µL of surfactant solution (**Table 1**) to 50 mL of diluted *A. tumefaciens* solution and mix by inverting several times.
5. Dip the inflorescence of the plants in the *A. tumefaciens* solution prepared in the section 4.4 for 20 s. Use a fresh bacterial solution after dipping inflorescence from six pots. Make sure all flowers are in contact with the solution. Pipet bacterial solutions directly onto flowers located in the lower part of the inflorescence if they cannot be dipped into the solution.

NOTE: For the first-round transformation, make sure to remove all mature and developing siliques using a sharp scalpel or small scissors. Do not remove siliques if performing transformation for the second time.

5. Post-transformation Plant Care and the Second Transformation

1. Place the floral-dipped plants horizontally in clean trays with domes to cover the plants and place in a dark growth room for 1–2 days.
NOTE: Keeping the flowers under high-humidity is important at this stage (**Figure 1**, Plants after transformation).
2. Return the plants to an upright position and transfer the plants to a growth room with a 14 h per day/10 h per night cycle, 130 µmol m⁻² s⁻¹ light intensity and 22 to 24 °C temperature.
3. Monitor the dipped inflorescences in the following week. If a significant number of flowers abort (**Figure 2**), repeat the floral dip (step 4) after about 4 weeks or after a large number of flowers have newly developed.
NOTE: Unlike the preparation step for the first transformation (step 1.3.7), do not remove pre-existing or developing siliques (**Figure 2**) before the second round of transformation.
4. Grow the plants until seeds mature and harvest seeds at ~21 weeks.
5. Dry seeds for 2–3 weeks at room temperature in an airtight container with filled with desiccants (see **Table of Materials**).

6. Selection of Positive Transformants

1. Plant the T1 seeds as described for wild type seeds in steps 1.2 to 1.3.
2. Grow the plants until the first 2 true leaves develop, approximately 10–14 days after germination.
3. **Perform the first selection for herbicide resistance (Figure 3A and 3B) as detailed below.**
 1. Dilute the glufosinate-ammonium (11.3%) herbicide (or BASTA) (**Table 1**) to 1:1,000 (v/v). Spray diluted BASTA solution on the seedlings and cover the plants with domes overnight.
 2. Repeat BASTA spraying 2–3 times every 5–7 days.
4. **Perform the second selection using a BASTA-drop test as detailed below.**
 1. Identify plants that survive after being sprayed 3–4 times with BASTA solution. Grow the plants for another 2–3 weeks until 3–5 leaves develop a relatively large surface area.
 2. Select the largest mature leaf per plant, rub the surface of the leaf gently with a finger to remove the wax layer, and place a drop of the diluted BASTA solution (from step 6.3.1).
NOTE: Mark the location of the leaf applied with the BASTA drop by placing a paper tape on the nearest stem.
 3. Monitor the leaves applied with the BASTA drop for signs of wilting for up to one week. Select the plants with leaves unaffected by the BASTA drops.
NOTE: Leaves from most false-positive plants start to wilt within two days, while leaves from true-positives are intact even after the drop of BASTA solution dries up (**Figure 3C**).
5. **Confirm positive transformants using genomic PCR.**
 1. Collect 2–3 leaves from the surviving plants at step 6.4.5.
 2. Extract genomic DNA from the leaves using the CTAB method²³ or any other appropriate DNA extraction method.
 3. Perform PCR using extracted genomic DNA samples from target plants, wild-type plants (as negative controls), and the plasmid construct from the step 3.1 (as a positive control). Use an appropriate pair of PCR primers specific to the selective marker gene, e.g., for BASTA-resistant gene (*bar*), TCAGCAGGTGGGTGTAGA (forward) and GTCAACCACTACATCGAGACAA (reverse).
 1. For the example PCR primers targeting the *bar* gene, use the following PCR conditions: the initial denaturation step at 98 °C for 30 s; followed by 30 cycles of denaturing at 98 °C for 30 s, annealing at 59 °C for 30 s, and extending at 72 °C for 30 s; and the final extension at 72 °C for 5 min.

NOTE: To ensure the insertion of the entire T-DNA, we recommend also performing genomic PCR using a PCR primer from the selective marker gene and another PCR primer specific to the target sequence cloned to the plant transformation vector at the step

4. Confirm the presence of the expected size of the amplified *bar* PCR product by agarose gel electrophoresis¹⁷ for the target samples (**Figure 4A**) as well as by sequencing the isolated PCR product¹⁹ using the same procedure as in step 2.1.

Representative Results

We developed a transformation protocol that enables harvesting of T₀ seeds within 150 days, using a floral-dip method modified from that for *A. thaliana*. **Figure 1** shows a summary of the timeline and *S. parvula* plants that represent the optimal stage for executing the transformation through floral-dip. We selected *S. parvula* plants with 70–80 flowers in multiple inflorescences at 60–80 days after germination as the target stage for transformation. A small number of pre-existing open or fertilized flowers and siliques at this stage were removed before the infiltration of *A. tumefaciens* by the floral-dip method. Infection with *A. tumefaciens* resulted in abortion of some flowers (**Figure 2**, bracket (a)). Siliques fully developed after the floral-dip are likely to contain transformed seeds (**Figure 2**, bracket (b)). Even after transformation, *S. parvula* continued to develop new inflorescences and flowers as long as the plants were kept healthy (**Figure 2**, white arrows). Due to this indeterminate flowering habit, a second round of transformation can be performed if the plant does not show signs of stress or senescence. **Figure 2A** and **2B** show examples of *S. parvula* plants after the first and second round of transformation, respectively, 25 days apart from each other. In the second transformation, existing siliques should not be removed because they may contain transgenic seeds. Also, the *A. tumefaciens* can be applied by pipetting the infiltration solution (**Table 1**) onto newly emerging flower clusters, instead of dipping the entire shoot into the solution, to minimize the damage to siliques from the first transformation.

The transformation efficiency is 0.033%, yielding 3–4 transgenic plants per 10,000 T1 seeds propagated using the current protocol. This estimate is based on ~50,000 T1 seeds tested during ten independent transformation attempts. While the efficiency is lower than that of the *Arabidopsis thaliana*, it is comparable to the transformation of another extremophyte plant *Eutrema salsugenum*²⁴ and some of the *Arabidopsis thaliana* ecotypes²⁵. The transformation efficiency may be further optimized by using alternative *Agrobacterium* strains and modifications of surfactant and infiltration solutions. The multiple BASTA spray and drop tests (steps 6.3 and 6.4) will be critical to identify true positive transformants and reduce the number of samples tested using the PCR confirmation in step 6.5 (**Figure 4A**). Further confirmation of transformation can be checked with a reporter gene expression, if the cloned sequence includes a reporter gene (**Figure 4B**).



Figure 1: Timeline of *S. parvula* transformation. Please click here to view a larger version of this figure.



Figure 2: *S. parvula* plants after transformation by floral-dip. Plants were photographed 10 days after the first floral-dip at Day 60 (A) and 25 days after the second round of floral-dip at Day 85 (B). Infiltration with *Agrobacterium* may abort silique development of flowers as shown in brackets a. Siliques fully developed after floral-dip are likely to contain transformed seeds (brackets b). White arrows indicate flowers and inflorescences newly emerged after each transformation. [Please click here to view a larger version of this figure.](#)

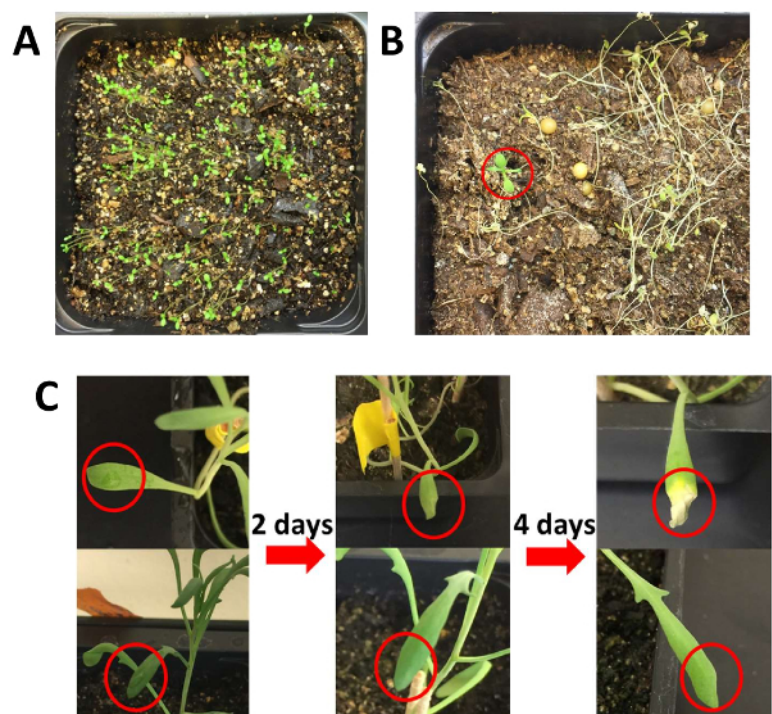


Figure 3: Selection of *S. parvula* transformants based on BASTA resistance. (A) T1 seedlings before the BASTA spray. (B) Red circle indicates a candidate transformant surviving the first-round selection by the BASTA spray. (C) The second-round selection by BASTA drop test. An example of false positives (top panel) and true transgenic plants (lower panel) are shown. [Please click here to view a larger version of this figure.](#)

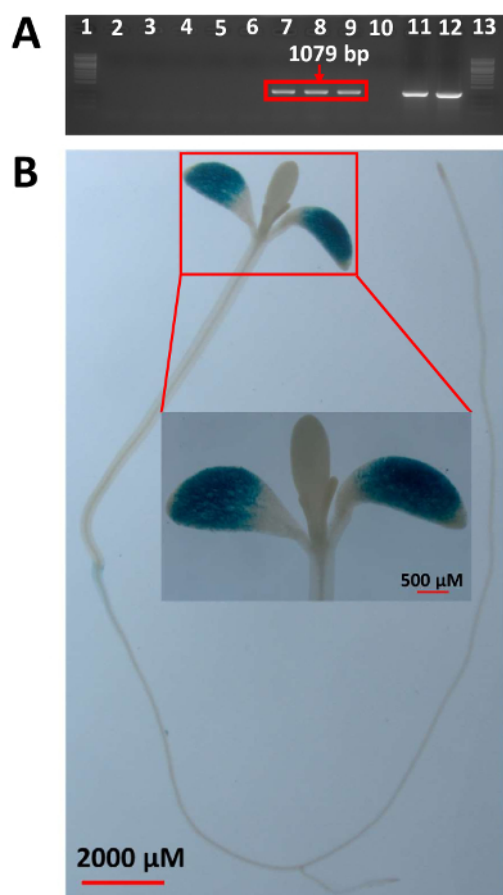


Figure 4: Confirmation of *S. parvula* transformation. (A) PCR amplification of *bar* gene from genomic DNAs extracted from *S. parvula* plants. Lane 1 and 13: size markers; Lane 2: negative control; Lane 3-5: wild-type *S. parvula* ; Lane 6-10: transgenic *S. parvula* candidates; Lane 11, 12: vector control. Lanes 7, 8, and 9 exemplify positive transformants. (B) Example of *GUS* reporter gene expression in a positive *S. parvula* transformant. [Please click here to view a larger version of this figure.](#)

Media / Solution	Reagent	Amount
Luria-Bertani (LB) bacterial growth media	NaCl	10 g
	Tryptone	10 g
	Yeast Extract	5 g
	Agar (for plates)	20 g
	ddH ₂ O	955 mL
Agrobacterium infiltration solution	MS salt (1/4x)	2.16 g
	B5 vitamins (1x)	1 mL
	Sucrose (5%w/v)	50 g
	MES	0.5 g
	N ⁶ -benzylaminopurine (BA)	10 μL
	Silwet L-77 (0.05%v/v)	500 μL
	pH	5.7

Table 1: Composition of bacterial growth media and *Agrobacterium* infiltration solution.

Discussion

The physiological state of the plant significantly influences the efficiency of transformation²⁵. The use of healthy and vigorous plants for transformation is a key requirement for successful transformation in *S. parvula*. Water or light stressed plants will have fewer flowers compared to the healthy plants ideal for transformation (Figure 1, center panel). *S. parvula* can grow at a light intensity less than 130 μmol m⁻² s⁻¹, but the plants tend to be frailer; such plants would lead to more aborted flowers following floral-dip. *S. parvula* tends to abort *Agrobacterium*-dipped flowers at a higher rate than *A. thaliana*. Therefore, every step taken to minimize aborted flowers when dipped in the *A. tumefaciens* infiltration solution contributes to a higher transformation efficiency. We recommend a light period no longer than 14 h per day. Often, transformation of *A. thaliana* is performed on plants grown in a long-day condition (e.g. 18 h light and 6 h dark) or even under continuous light. However, we found such practices result in less resilient *S. parvula* plants and lead to a low transformation efficiency.

Flower buds are continuously produced on the inflorescence axes of *S. parvula* (Figure 2, white arrows). Therefore, allowing transformation of new flowers would significantly increase the chance of getting positive transformants. A second floral-dip (step 5.3) is not essential, but strongly suggested. However, this step is relatively time consuming compared to *A. thaliana* floral dipping, because *S. parvula* produces multiple inflorescence axes.

Wild-type *S. parvula* is sensitive to BASTA, although the initial screen for positive transformants with BASTA spray (step 6.3) will leave 5–8 surviving plants out of 100 seeds germinated (Figure 3A and 3B). Most of this (>80%) will be false positives. This is largely due to the narrow leaf shape and the leaf angle of *S. parvula*, which do not provide sufficient leaf surface in an appropriate orientation to retain the BASTA solution for a sufficient duration to observe a phenotype. Additionally, due to the high wax content of the adaxial leaf surface of *S. parvula*¹⁰, it tends to create a more impervious surface for BASTA. Therefore, the second screening for positive transformants using a BASTA drop on individual leaves (step 6.4, Figure 3C) is an essential step to avoid PCR testing on hundreds of false positives (step 6.5).

The current protocol was tested with the *A. tumefaciens* strain GV3101 carrying the *pMP90RK* plasmid. The efficiency of transformation may be improved with other *A. tumefaciens* strains, including strains ABI, LMG20, and C58C1 Rif^r, with the *pMP90* virulence plasmid reported to increase transformation efficiency in *A. thaliana*²⁵. *Brassica* and *Eutrema* species are taxonomically more closely related to *S. parvula* compared to *A. thaliana*¹. Therefore, the *A. tumefaciens* strain LBA4404 that was successfully used to transform *Brassica napus* and the strain EHA105 that has been used successfully to transform *Eutrema salsugineum* may offer a higher transformation efficiency than the reported efficiency of the strain currently used^{26,27,28}.

Reducing the time and labor required by a transformation protocol is another significant factor in improving the transformation efficiency. Placing individual BASTA drops on leaves and monitoring the leaf for a week on multiple plants (step 6.4) are tedious. A future effort to increase the transformation efficiency could search for appropriate alternative selectable marker genes²⁹.

The availability of an established transformation protocol will greatly advance our ability to identify genes and novel mechanisms that allow extremophyte model plants to survive multiple abiotic stresses^{2,4}. Novel genetic variation in *S. parvula* will provide a broader pool of genetic variation that cannot be mined from the collective allelic variation identified as stress-responsive genes in the relatively stress-sensitive model, *A. thaliana* pan-genome^{5,6}. Therefore, our floral-dip based *A. tumefaciens* mediated transformation protocol developed for *S. parvula* will fill a gap for the need for such tools to perform functional genomic experiments in an extremophyte model closely related to *A. thaliana*.

Disclosures

The authors have nothing to disclose.

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