Journal of Visualized Experiments

Plant growth and Agrobacterium-mediated floral-dip transformation of the extremophyte, Schrenkiella parvula

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58544R1
Full Title:	Plant growth and Agrobacterium-mediated floral-dip transformation of the extremophyte, Schrenkiella parvula
Keywords:	extremophyte; Schrenkiella parvula; Thellungiella parvula; Eutrema parvulum; floral-dip; plant transformation; Agrobacterium; selection of transformants
Corresponding Author:	Maheshi Dassanayake Louisiana State University Baton Rouge, LA UNITED STATES
Corresponding Author's Institution:	Louisiana State University
Corresponding Author E-Mail:	maheshid@lsu.edu
Order of Authors:	Maheshi Dassanayake
	Guannan Wang
	Pramod Pantha
	Kieu-Nga Tran
	Dong-Ha Oh
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	A155 Life Sciences Building, Baton Rouge, LA, 70803

TITLE:

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

AUTHORS & AFFILIATIONS:

Guannan Wang[1], Pramod Pantha[1], Kieu-Nga Tran[1], Dong-Ha Oh[1], Maheshi Dassanayake[1]

[1]Department of Biological Sciences, Louisiana State University, Baton Rouge, LA, USA

Corresponding Authors:

Maheshi Dassanayake (maheshid@lsu.edu)

Dong-Ha Oh (ohdongha@lsu.edu)

Email Addresses of Co-authors:

Guannan Wang (gwang23@lsu.edu)
Pramod Pantha (ppanth1@lsu.edu)
Kieu-Nga Tran (ktran39@lsu.edu)

KEYWORDS:

Extremophyte, *Schrenkiella parvula, Thellungiella parvula*, *Eutrema parvulum*, floral-dip, plant transformation, Agrobacterium, selection of transformants

SUMMARY:

Agrobacterium-mediated transformation using a floral-dip method can be successfully employed to create stable transgenic lines of the extremophyte model *Schrenkiella parvula*. We present a protocol modified from that for *Arabidopsis thaliana*, considering different growth habits and physiological characteristics of the extremophyte.

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-hour light and 10-hour dark and a 130 µmol m^{-2} 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.

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[1–5]. 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[5–7]. *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 plants[7]. 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 [8–11].

Since 2010, there have been over 400 peer-reveiwed 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 pseudomolecules[1].

The Agrobacterium-mediated floral-dip transformation method has become the most broadly used method to create trasngenic 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

1.1. Seed sterilization (optional)

1.1.1. Prepare 50% bleach in double-distilled water (ddH_2O) 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 minutes.

1.1.2. 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 minutes.

1.1.3. Remove the bleach from the tube and add 70% ethanol. Wash the seeds by pipetting several times and then remove the ethanol solution immediately.

1.1.4. Wash the seeds in sterilized water to remove excess bleach and ethanol, then remove the water. Repeat this step 5 to 6 times.

1.2. Seed stratification

1.2.1. 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.

1.3. Growing plants in preparation of transformation

1.3.1. Fill the soil mix (see **Table of Materials**) into 7 x 6 cm^2 pots, soak the pots in water, and spray water from the top to ensure a uniformly wet growth medium. Add 5-6 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.

1.3.2. 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).

1.3.3. Cover the pot tray with a clear dome to keep the seeds under high humidity during germination.

1.3.4. Keep the plant trays in a growth chamber with a light intensity set at 130 µmol m^{-2} s^{-1} light, 22 – 24 °C temperature, and 14-h-day/10-h-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.

1.3.5. Weed out extra seedlings and leave only 4-5 healthy seedlings per pot well separated from each other (**Figure 1**, Day 15, right panel).

1.3.6. Gently water the plants every two days and fertilize with 0.2x Hoagland's solution[16] once every two weeks.

Note: Keeping the soil moisture at a uniform level is key to growing *S. parvula* consistently and healthily.

1.3.7. 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

2.1. 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 sequencing[19].

2.2. 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.

2.3. Spread 50 µL of transformed products on Luria-Bertani[20] (LB) agar bacterial growth media (**Table 1**) with appropriate antibiotics, *e.g.,* 50 µg/ mL Spectinomycin (see **Table of Materials**), and incubate at 37 °C overnight.

2.4. 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.

2.5. Isolate plasmids using a plasmid isolation kit (see **Table of Materials**) and verify through Sanger sequencing[19] whether the target sequence amplified in 2.1 is properly cloned.

2.6. 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*

3.1. Transform the plasmid of the vector construct from 2.6 into the *A. tumefaciens* strain GV3101:pMP90RK[21], 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.

3.2. *A. tumefaciens* transformation by electroporation

3.2.1. Thaw the *A. tumefaciens* competent cells[22] on ice. Mix 0.1 – 1 µg of the plasmid prepared from 2.6, dissolved in 1-2 µL of ddH_2O, with competent cells on ice. Transfer the mixture into an electroporation cuvette (see **Table of Materials**).

3.2.2. 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.

3.2.3. 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 hour at 28 °C with gentle shaking.

3.3. 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*

4.1. 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 hours in a shaking incubator (see **Table of Materials**) at 250 r.p.m. at 28 °C.

4.2. 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 12-36 h until the optical density at 600 nm wavelength (OD_{600}) reaches around 2.0.

4.3. Centrifuge the *A. tumefaciens* culture at 3100 x g for 10 min. Remove the supernatant and re-suspend the bacterial culture in 40 mL of *A. tumefaciens* infiltration solution (**Table 1**).

4.4. Dilute the resuspended *A. tumefaciens* with infiltration solution to a final OD_{600} 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.

4.5. Dip the inflorescence of the plants in the *A. tumefaciens* solution prepared in the section 4.4 for 20 seconds. 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

5.1. 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).

5.2. Return the plants to an upright position and transfer the plants to a growth room with a 14-h-day/ 10-h-night cycle, 130 µmol m^{-2} s^{-1} light intensity and 22 to 24 °C temperature.

5.3. 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.

5.4. Grow the plants until seeds mature and harvest seeds at ~21 weeks.

5.5. 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

6.1. Plant the T1 seeds as described for wild type seeds in steps 1.2 to 1.3.

6.2. Grow the plants until the first 2-3 true leaves develop, approximately 10 - 14 days after germination.

6.3. Perform the first selection for herbicide resistance (**Figure 3A and 3B**) as detailed below.

6.3.1. Dilute the glufosinate-ammonium (11.3%) herbicide (or BASTA) (**Table 1**) to 1:1000 (v/v). Spray diluted BASTA solution on the seedlings and cover the plants with domes overnight.

6.3.2. Repeat BASTA spraying 2-3 times every 5-7 days.

6.4. Perform the second selection using a BASTA-drop test as detailed below.

6.4.1. 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.

6.4.2. 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.

6.4.3. 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**).

6.5. Confirm positive transformants using genomic PCR.

6.5.1. Collect 2-3 leaves from the surviving plants at step 6.4.5.

6.5.2. Extract genomic DNA from the leaves using the CTAB method[23] or any other appropriate DNA extraction method.

6.5.3. 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).

6.5.3.1. For the example PCR primers targeting the *bar* gene, use the following PCR conditions: the initial denaturation step at 98 °C for 30 seconds; followed by 30 cycles of denaturing at 98 °C for 30 seconds, annealing at 59 °C for 30 seconds, and extending at 72 °C for 30 seconds; and the final extension at 72 °C for 5 minutes.

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

6.5.4. Confirm the presence of the expected size of the amplified *bar* PCR product by agarose gel electrophoresis[17] for the target samples (**Figure 4A**) as well as by sequencing the isolated PCR product[19] using the same procedure as in step 2.1.

REPRESENTATIVE RESULTS

We developed a transformation protocol that enables harvesting of T_0 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 salsugenium*[24] and some of the *Arabidopsis thaliana* ecotypes[25]. 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 AND TABLE LEGENDS

Figure 1: Timeline of *S. parvula* transformation.

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.

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.

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.

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

DISCUSSION

The physiological state of the plant significantly influences the efficiency of transformation[25]. 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 hours per day. Often, transformation of *A. thaliana* is performed on plants grown in a long-day condition (*e.g.* 18-hour light and 6-hour 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 Rifr, 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[26–28].

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 genes[29].

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*.

ACKNOWLEDGMENTS

This work was supported by a National Science Foundation award MCB 1616827.

DISCLOSURES

The authors have nothing to disclose.

REFERENCES

1. Dassanayake, M. *et al.* The genome of the extremophile crucifer *Thellungiella parvula*. *Nature Genetics* **43** (9), 913–918 (2011).
2. Oh, D.-H., Dassanayake, M., Bohnert, H.J., Cheeseman, J.M. Life at the extreme: lessons from the genome. *Genome Biology* **13** (3), 241 (2012).
3. Whited, J. The Next Top Models. *Cell* **163** (1), 18–20 (2015).
4. Dassanayake, M., Yun, D.O.D., Bressan, R.A., Cheeseman, J.M., Bohnert, J.H. The scope of things to come: New paradigms in biotechnology. *Plant Biotechnology and Agriculture: Prospects for the 21st Century*. 19-34 (2009).
5. Dittami, S.M., Tonon, T. Genomes of extremophile crucifers: New platforms for comparative genomics and beyond. *Genome Biology* **13** (8), 166 (2012).
6. Amtmann, A. Learning from evolution: *Thellungiella* generates new knowledge on essential and critical components of abiotic stress tolerance in plants. *Molecular Plant* **2**

(1), 3–12 (2009).
7. Oh, D.-H., Hong, H., Lee, S.Y., Yun, D.-J., Bohnert, H.J., Dassanayake, M. Genome structures and transcriptomes signify niche adaptation for the multiple-ion-tolerant extremophyte *Schrenkiella parvula*. *Plant Physiology* **164** (4), 2123–2138 (2014).
8. Orsini, F. *et al.* A comparative study of salt tolerance parameters in 11 wild relatives of *Arabidopsis thaliana*. *Journal of Experimental Botany* **61** (13), 3787–3798 (2010).
9. Uzilday, B., Ozgur, R., Sekmen, A. H., Yildiztugay, E., Turkan, I. Changes in the alternative electron sinks and antioxidant defence in chloroplasts of the extreme halophyte *Eutrema parvulum* (*Thellungiella parvula*) under salinity. *Annals of Botany* **115** (3), 449–463 (2015).
10. Teusink, R.S., Rahman, M., Bressan, R.A., Jenks, M.A. Cuticular waxes on *Arabidopsis thaliana* close relatives *Thellungiella halophila* and *Thellungiella parvula*. *International Journal of Plant Sciences* **163** (2), 309–315 (2002).
11. Jarvis, D.E., Ryu, C.H., Beilstein, M. A., Schumaker, K.S. Distinct roles for SOS1 in the convergent evolution of salt tolerance in *Eutrema salsugineum* and *Schrenkiella parvula*. *Molecular Biology and Evolution* **31** (8), 2094–2107 (2014).
12. Clough, S.J., Bent, A.F. Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant Journal* **16** (6), 735–743 (1998).
13. Koornneef, M., Meinke, D. The development of Arabidopsis as a model plant. *Plant Journal* **61** (6), 909–921 (2010).
14. Bai, J., Wu, F., Mao, Y., He, Y. *In planta* transformation of *Brassica rapa* and *B. napus* via vernalization-infiltration methods. *Protocol Exchange* **10**, 1028 (2013).
15. Sparrow, P.A.C., Goldsack, C.M.P., Østergaard, L. Transformation technology in the Brassicaceae. *Genetics and Genomics of the Brassicaceae*. 505–525 (2011).
16. Hoagland, D.R., Arnon, D.I. The water-culture method for growing plants without soil. *California Agricultural Experiment Station Circular*. **347** (347), 1–32 (1950).
17. Saiki, R. *et al.* Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239** (4839), 487–491 (1988).
18. Sun, Y., Sriramajayam, K., Luo, D., Liao, D.J. A Quick, cost-free method of purification of dna fragments from agarose gel. *Journal of Cancer*. **3**, 93–95 (2012).
19. Sanger, F., Nicklen, S., Coulson, A.R. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74** (12), 5463–5467 (1977).
20. Bertani, G. Studies on Lysogenesis I. The mode of phage liberation by lysogenic *Eschericia coli*. *Journal of Bacteriolgy* **62** (3), 293–300 (1951).
21. Koncz, C., Martini, N., Szabados, L., Hrouda, M., Bachmair, A., Schell, J. Specialized vectors for gene tagging and expression studies. *Plant Molecular Biology Manual*. 53–74 (1994).
22. Weigel, D., Glazebrook, J. Transformation of *Agrobacterium* using electroporation. *Cold Spring Harbor Protocols* **2006** (30) (2006).
23. Murray, M.G., Thompson, W.F. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research* **8** (19), 4321–4326 (1980).
24. Inan, G. Salt cress. a halophyte and cryophyte Arabidopsis relative model system and its applicability to molecular genetic analyses of growth and development of extremophiles. *Plant Physiol*. **135** (3), 1718–1737 (2004).
25. Ghedira, R., De Buck, S., Nolf, J., Depicker, A. The efficiency of *Arabidopsis thaliana* floral

dip transformation is determined not only by the *Agrobacterium* strain used but also by the physiology and the ecotype of the dipped plant. *Molecular Plant-Microbe Interactions*. **26** (7), 823–832 (2013).

26. Shaohong, F.U., Xianya, W.E.I., Yingze, N.I.U., Shixing, G.U.O. Transformation of Brassica napus with the method of floral-dip. *Biotechnology: Genomics and Its Applications*. 45–49 (2005).

27. Li, J., Tan, X., Zhu, F., Guo, J. A rapid and simple method for *Brassica napus* floral-dip transformation and selection of transgenic plantlets. *International Journal of Biology* **2** (1), 127 (2010).

28. Li, H.Q., Xu, J., Chen, L., Li, M.R. Establishment of an efficient *Agrobacterium tumefaciens*-mediated leaf disc transformation of *Thellungiella halophila*. *Plant Cell Reports* **26** (10), 1785–1789 (2007).

29. Wu, G., Rossidivito, G., Hu, T., Berlyand, Y., Poethig, R.S. Traffic lines: New tools for genetic analysis in *Arabidopsis thaliana*. *Genetics* **200** (1), 35-45 (2015).

Figure 1

Click here to download Figure cut_JoVE_Figure1_300dpi.tif

Figure 2

Click here to download Figure cut_JoVE_Figure2_300dpi.tif

Figure 3

Click here to download Figure cut_JoVE_Figure3_300dpi.tif

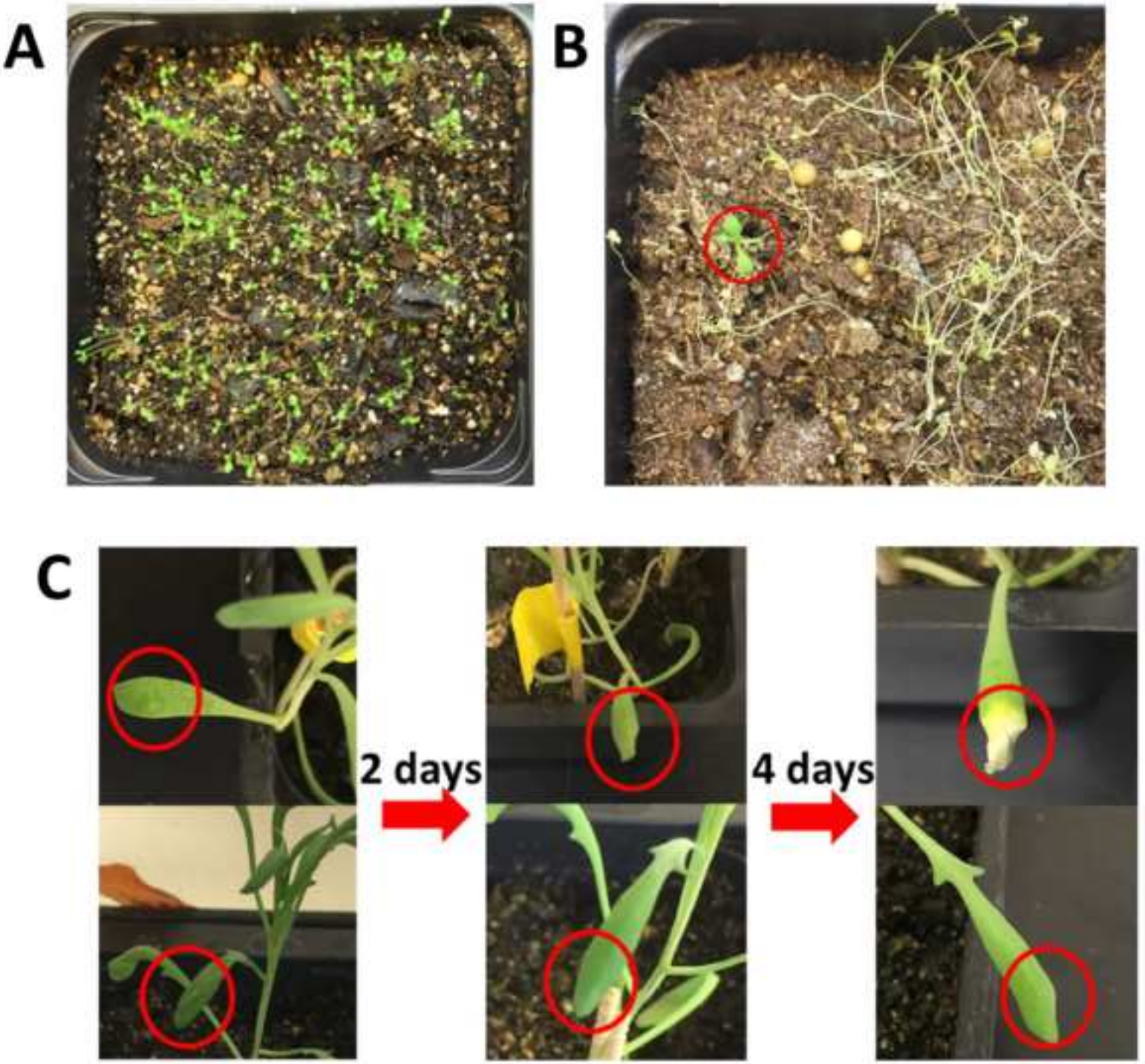


Figure 4

Click here to download Figure cut_JoVE_Figure4_300dpi.tif

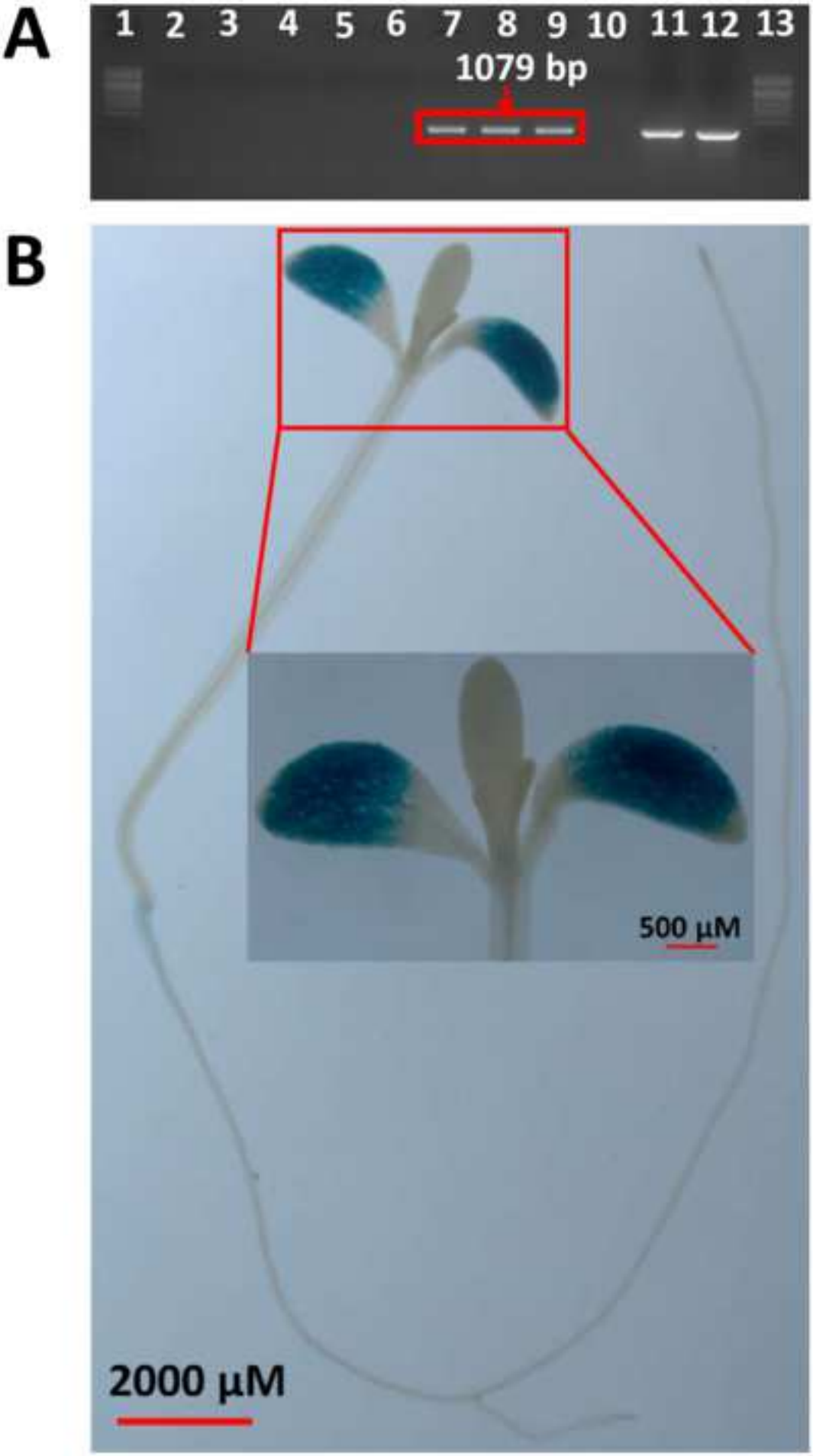


Table 1

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
	ddH2O	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 of Materials

Click here to download Table of Materials JoVE_List of Materials 072718.xls

Name	Company	Catalog Number
Agar	VWR International, Radnor, PA	90000-762
B5 vitamins	Sigma-Aldrich, St. Louis, MO	G1019
Desiccant	W A Hammond Drierite, Xenia, OH	22005
Destination vector for plant transformation	TAIR	Vector:6531113857
Electroporation cuvette	USA Scientific	9104-5050
Electroporator	BIO-RAD Laboratories, Hercules, CA	1652100
Fertilizer beads	Osmocote Garden, Marysville, OH	N/A
Gel extraction kit	iNtRON Biotechnology, Boston, MA	17289
Gentamicin	Sigma-Aldrich, St. Louis, MO	G1914-5G
Glufosinate-ammonium (11.3%) herbicide (BASTA)	Bayer environmental science, Montvale, NJ	N/A
Kanamycin	VWR International, Radnor, PA	200004-444
MES	Bioworld, Dublin, OH	41320024-2
MS salt	MP Biomedicals, Santa Anna, CA	092621822
N6-benzylaminopurine (BA)	Sigma-Aldrich, St. Louis, MO	B3274
NaCl	Sigma-Alrich	S7653
Non-ionic detergent	Sigma-Aldrich, St. Louis, MO	9005-64-5
Plasmid isolation kit	Zymo Research, Irvine, CA	D4036
Recombinase enzyme mix kit	Life Technology	11791-020
Rifampicin	Sigma-Aldrich, St. Louis, MO	R3501-1G
Shaking incubator	ThermoFisher Scientific, Waltham, MA	SHKE4450
Soil mix	Sun Gro	SUN239223328CFLP
Spectinomycin	VWR International, Radnor, PA	IC15206705
Sterile 50ml conical tubes	USA Scientific, Ocala, FL	1500-1811
Sucrose	VWR International, Radnor, PA	57-50-1
Surfactant solution	Lehle seeds, Round Rock, TX	VIS-02
Topoisomerase-based cloning kit	Life Technologies, Carlsbad, CA	K240020
Tryptone	VWR International, Radnor, PA	90000-282
Yeast Extract	VWR International, Radnor, PA	90000-722

Comment
Bacto Agar Soldifying Agent, BD Diagnostics
Gamborg’s Vitamin Solution
Indicating DRIERITE 6 mesh

pKGWFS7
Electroporation cuvette, round cap, 0.2 cm gap
MicroPulser Electroporator
Osmocote Smart-Release Plant Food Flower & Vegetable
MEGAquick-spin Total fragment DNA purification kit
Gentamicin sulfate
FINALE herbicide
Kanamycin monosulfate
MES, Free Acid
Hoagland's modified basal salt mixture
6-Benzylaminopurine solution
Sodium chloride
TWEEN 20
Zyppy Plasmid Kits
Gateway LR Clonase II Enzyme mix
Rifampicin, powder, >= 97% (HPLC)
MaxQ 4450 Benchtop Orbital Shakers
Sun Gro Metro-Mix 360 Grower Mix

50 ml conical screw cap tubes, copolymer, racks, sterile
Sucrose, ACS
Silwet L-77
pENTR/D-TOPO Cloning Kit, with One Shot TOP10 Chemically Competent E. coli
BD Bacto Tryptone, BD Biosciences
BD Bacto Yeast Extract, BD Biosciences

JoVE JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Plant growth and Agrobacterium mediated floral-dip transformation of the extremophyte, Schrenkiella parvula

Author(s): Guanran Wang, Pramod Partha, Kieu-Nga Tran, Dong-Ha Oh, Maheshi Dassanayake

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☒ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

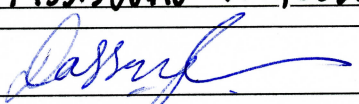
12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Maheshi Dassanayake
Department:	Biological Sciences
Institution:	Louisiana State University
Title:	Assistant Professor
Signature:	
Date:	5/31/2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We proofread and revised the manuscript as instructed.

2. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .svg, .eps, .psd, or .ai file.

We uploaded all figures as 300-dpi .png files as instructed.

3. Please upload each Table individually to your Editorial Manager account as an .xls or .xlsx file.

We converted Tables into separated .xlsx files and uploaded as instructed.

4. Please remove the titles and Figure Legends from the uploaded figures. The information provided in the Figure Legends after the Representative Results is sufficient.

We removed figure title and legends from uploaded figures.

5. Please shorten the figure legends. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.

We revised figure legends to be more concise, as instructed. If the figure is self-explanatory (e.g., Figure 1), we removed the legend entirely and instead added references in the appropriate places in the Protocol.

6. The current Abstract is over the 150-300 word limit. Please shorten it.

The Abstract in the original submission contained 292 words. We have further shortened it to 266 words in this revision.

7. Please define all abbreviations before use.

We spelled out all abbreviations at their first appearance in the revision.

8. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Osmocote, Scotts Miracle-Gro Co., MEGAquick-spin, iNtRON biotechnology, Gateway cloning technology, Invitrogen Co., Zymo Research, Gateway LR ClonaseII, USA Scientific, MicroPulser, BIO-RAD, Silwet L-77, etc.

We replaced all commercial product names with generic terms as instructed. We added to the information on generic terms and their respective commercial products used in to the

experiment, to the "List of Materials". Where the generic term of a commercial product was used in the revised text, we added references to the list of materials as [see Materials].

9. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Please revise 2.6, 4.1, etc. accordingly.
We revised the text as instructed.

10. 2.7: Please write the text in the imperative tense. Any text that cannot be written in the imperative tense may be added as a "Note."
We revised the text as instructed.

11. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please revise 2.1, 6.5.3-6.5.5 accordingly.
We revised the text as instructed, by adding references or providing more details.

12. 2.1: Please provide more details or add a reference for gel extraction of PCR product.
We added the information on the gel extraction kit, as well as a couple references.

13. 6.5.3: Please list PCR conditions and primers.
We added PCR conditions and primer sequences for the amplification of the *bar* gene.

14. Please reference Figure 1 in the Protocol section.
We added references to Figure 1, as notes to the step 1.3.2 and 5.1.

15. Please reference Table 1 in the Protocol section for composition of growth media and infiltration solution.
We added references to Table 1 at steps 2.3 and 4.3.

16. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
We highlighted from the step 4 to step 6.4.3 by adding a gray shade to the text. We believe these steps are crucial because they contain the necessary modifications from conventional flower-dipping methods used for *Arabidopsis thaliana*.

17. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.
Please see our response to the comment #16.

18. Discussion: Please also discuss any limitations of the technique.
The transformation efficiency lower than the most popular model plant *Arabidopsis thaliana* (ecotype Col-0) can be considered as a limitation. We added a more detailed discussion on this point.

19. References: Please do not abbreviate journal titles.
We revised the references as instructed.

...

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This submission describes a plant transformation protocol that is suitable for use with Schrenkiella parvula, a highly stress tolerant extremophile relative of the far less stress tolerant genetic model plant, Arabidopsis thaliana. To fully exploit a species as a genetic model, it is essential to have a way of manipulating genes to validate their role(s) in generating a particular phenotypic response. An impediment to exploiting S. parvula has been the virtual absence of a transformation protocol. One commonly used approach that works well for Arabidopsis involved dipping flowers into a solution containing Agrobacterium carrying a cloned gene of interest. As the authors point out, not only has the conventional approach for transforming S. parvula been problematic, the plant continues to flower after the dipping is done and the screening for successfully transformed T0 plants is complicated by a screening strategy that leaves many false positives among very few true transformants. These are all deterrents to using S. parvula for finding genes associated with stress tolerance despite the extraordinary physiological stress tolerance features that this plant displays and the excellent genomic tools available for its study. The solutions to these concerns that this protocol provides make sense and are laid out clearly: multiple dipping treatments works well with a plant that is difficult to transform and has indeterminate flowering (and more chances to generate transgenic seeds) and considering the narrow leaves and wax as impediments to the penetration of a selection agent are factors that should be obvious...but no doubt also easily overlooked. The protocol will offer suggestions to those working with other extremophytes that suffer from poor transformation and/or

selection efficiencies and this should increase the research promise of other plants highly adapted to environmental stress.
The protocol is well written, the authors highlight steps that require extra precautions and they provide reasons why following the precautions yields better results. The title describes the contents of the manuscript well. It might be advantageous for the authors to include a former name in the keywords, for example, as changes in the taxonomy for several extremophytes has made for confusion in the literature. The illustrations are also clear and support the text well.
Thank you for the comments. Per reviewer's suggestion, we included two former names of the extremophyte, *Eutrema parvulum* and *Thellungiella parvula*, among the keywords.

Major Concerns:
No major concerns. The protocol is detailed and the results are described well. Although a recovery of 3 or 4 transformants from 10,000 seeds may seem problematic, this low recovery approximates the rates obtained for some Arabidopsis ecotypes that are also considered recalcitrant to transformation (eg. C24). It may well be that some of the floral features that make S. parvula resistant to transformation could be some of the same features that allows the plant to resist extreme stresses. However, that consideration cannot be addressed without having at least some means of generating transgenics in the first place. Finally, this submission also provides an important benchmark against which researchers can compare differences related to the use of other strains/plasmids/treatment conditions to devise an improved recovery of transformants in the future.

Minor Concerns:
There is a lot of good information and detail on how to propagate healthy plants. In that regard, it would be useful if the authors named the soil they used in the text as it only seems to be named in the list of supplies. Can any soil be used equally well or is the one given the only suitable substrate?
We appreciate the reviewer's comment. However, we were advised not to use commercial names in the main text, hence the reference to the exact soil mix was only in the list of supplies. In our experience, any soil mix where *Arabidopsis thaliana* can grow will also work for *S. parvula*. We added this information as a note to the step 1.3.1

…

Reviewer #2:
Manuscript Summary:
The manuscript by Dassanayake et al. focuses on a transformation protocol for Schrenkiella

parvula, a halophytic relative of Arabidopsis thaliana. Along with the related Eutrema salsugineum, S. parvula has been developed as a powerful tool for comparative physiological, biochemical and molecular analysis of adaptive mechanisms that allow plants to cope highly saline conditions. Such are the importance of these two halophytes, that they have been declared as new model organisms (Zhu et al., 2015, The next top models. Cell 163: 18-20). The development of functional genomics tools and protocols is essential to further the development of these model halophytes, and therefore this manuscript is not just another transformation protocol for an extra plant species but a critical platform to further our understanding of salt tolerance mechanisms in halophytes.
Overall the protocol described in the manuscript can be clearly followed and the figures nicely augment the written protocol to provide an excellent visual aid as well as confirming transformation.

Major Concerns:
None.

Minor Concerns:
1. My only question is whether PCR of the Bar gene alone is sufficient to prevent false positives, as plants can sometime be transformed with the selective marker but not with the target gene (or the target gene can suffer deletions/insertion etc). Would it not be wise to add that PCR with target gene-specific primers should be performed on the plants exhibiting the presence of the Bar gene?

We appreciate the reviewer's comment. We have also confirmed our transformants using one primer from the Bar gene and another from the target sequences. We added this as a recommendation in the note to the step 6.5.3 in the revised manuscript.

2. There are numerous English grammatical mistakes and erroneous use of terminology. While I understand that the manuscript will be copy-edited, I want to point out the terminological errors:
(a) Lines 140, 181 and 190: Bacteria are not "poured" onto plates or "dissolved" in liquid media. The plates or liquid are "inoculated" with bacteria.
(b) Lines 197-198: Bacterial pellets are not "dissolved" in a solution. They are "re-suspended".
We thank the reviewer for pointing out these. We have revised those two points as well as the entire procedures to replace casual languages to more appropriate scientific terms.