



A simple, highly efficient *Agrobacterium tumefaciens*-mediated moss transformation system with broad applications

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Received: 8 January 2024 / Accepted: 20 June 2024 / Published online: 19 July 2024

Abstract Mosses, particularly desiccation-tolerant (DT) species, are important model organisms for studying genes involved in plant development and stress resistance. The lack of a simple and efficient stable moss transformation system has hindered progress in deciphering the genetic mechanisms underlying traits of interest in these organisms. Here, we present an *Agrobacterium tumefaciens*-mediated transformation system for DT mosses that uses *Agrobacterium* strain EHA105 harboring the binary vector pCambia1301-GUS. This system achieved transformation efficiencies of 74% and 81% in *Physcomitrium patens* and *Bryum argenteum* protonemata, respectively, without the need for culture and callus formation prior to regeneration. We detected GUS enzyme activity in the regenerated transgenic moss via histochemical staining. Southern blot, PCR, and RT-qPCR analyses confirmed the presence of the *GUS* gene. In addition, we successfully used this system to transform wild DT *Syntrichia caninervis*. Furthermore, *P. patens* and *B. argenteum* transformed using this system with the stress resistance gene *EsDREB* from the desert plant *Eremosparton songoricum* (Litv.) exhibited improved salt tolerance. We thus present an efficient tool for the genetic analysis of DT moss species, paving the way for the development of stress-resistant crop cultivars.

Keywords *Agrobacterium tumefaciens*, Highly efficient, Moss, Transformation

INTRODUCTION

As a close relative of early vascular plants, the model moss *Physcomitrium patens* is a useful tool for

bioinformatics and molecular biology studies of plant development and stress resistance. The *P. patens* genome resembles that of *Arabidopsis thaliana* in its size and high frequency of homologous recombination, but resembles that of yeast in its inefficiency of gene targeting and haploid-dominant life cycle. These features make *P. patens* an interesting and useful tool for

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molecular genetic studies in plants (Lang et al. 2008; Schaefer 2002; Schuette et al. 2015, 2009). The most common methods to transform this moss are through particle bombardment and polyethylene glycol (PEG)-mediated DNA uptake, specifically PEG-mediated transformation of individual cells that are then regenerated into whole plants (Rensing et al. 2020). Although many species have been transformed in this manner, these methods remain challenging, particularly for *P. patens*, because they rely on the moss protonemata being at a specific stage of differentiation. Moreover, it is challenging and time consuming to extract protoplasts from protonemata and to manage their subsequent differentiation (Newell 2000).

Upon rehydration, desiccation-tolerant (DT) bryophytes can fully recover after losing virtually all their free intracellular water (Proctor et al. 2007). The DT moss *Bryum argenteum*, an important biotic component of ecosystems in northwestern China (Zhang et al. 2007), is emerging as a useful organism for studying the molecular, structural, and ecological aspects of vegetative desiccation tolerance in plants (Gao et al. 2015; Li et al. 2014a; Stark et al. 2010). The DT moss *Syntrichia caninervis* Mitt. (Pottiaceae), a biotic component of North American and Asian dryland ecosystems (Silva et al. 2021; Stark and Brinda 2015), has also emerged as a model plant for studying desiccation tolerance, dehydration, and rehydration (Buitink et al. 2000; Gao et al. 2014). Genetic transformation systems have not been developed for *B. argenteum* or *S. caninervis*, limiting the analysis of gene function in DT mosses and, more generally, evolutionary developmental biology studies of land plants.

Agrobacterium tumefaciens-mediated transformation has long been used for the genetic manipulation of a broad variety of plants, because it does not require expensive equipment and leads to the stable expression of foreign genes (Takata and Eriksson 2012). *Agrobacterium*-mediated stable transformation can yield high expression of target genes in monocotyledons, such as rice (*Oryza sativa*) (Raineri et al. 1990) and maize (*Zea mays*) (Ishida et al. 1996), dicotyledons, such as *A. thaliana* (Zhang et al. 2006), and the liverwort *Marchantia polymorpha* (Tsuboyama and Kodama 2014), but this method has not yet been reported in moss. This approach has several advantages over other transformation methods, such as the ability to integrate a small number of transgene copies into the plant genome and to transfer relatively large DNA segments and integrate intact transgenes into the genome (Frangedakis et al. 2021).

In this study, we developed the first successful *Agrobacterium*-mediated transformation system for DT

mosses. Our method is based on the *Agrobacterium*-mediated transformation of protonemata or gametophytes. We first optimized our protocol for mosses using protonemata as starting material because of their rapid growth and then extended the method to gametophytes from the wild moss *S. caninervis*. The transformants regenerated directly from transformed gametophytes without the need for culture and callus formation prior to regeneration. Finally, we introduced an abiotic stress-resistance gene from *Eremosparton songoricum* (Litv.) (*EsDREB*), a flowering pioneer plant adapted to desert environments that is used in the Gurbantunggut Desert of Xinjiang to protect the local ecosystem from desertification, into the two moss species to verify that the system can be used for the functional study of DT genes (Li et al. 2014b).

RESULTS

In our effort to develop a simple and reliable stable transformation system for mosses, we tested the potential of *Agrobacterium*-mediated gene delivery to generate stable transgenic moss lines. Several factors determine the efficiency of *Agrobacterium*-mediated gene delivery: the antibiotic concentration used to select transgenic lines, the preculture time before infection, the sucrose concentration in the growth medium, the *Agrobacterium* strain, and the infection period. We examined each of these factors in this study.

To achieve stable moss transformants, we used the pCambia1301 vector containing the *GUS* reporter gene. We also used the pBI121-GFP overexpression vector for transformation of moss with *EsDREB*. This gene was chosen because *EsDREB2B* transcript accumulation is induced in *E. songoricum* by a variety of abiotic stresses, including drought, salinity, cold, heat, heavy metals, mechanical wounding, and oxidative stress. The transgenic gametophytes or protonemata obtained using this protocol can be cultivated in various media tailored to specific requirements, accommodating diverse starting materials and expediting the acquisition of transgenic material.

Selection of the appropriate antibiotic concentration

A selectable marker gene is required for the efficient recovery of stable transgenic lines following *Agrobacterium* transformation. We chose the hygromycin resistance gene as the selectable marker gene. To identify the appropriate hygromycin concentration for *P. patens* and *B. argenteum*, we treated untransformed *P. patens* and *B.*

argenteum protonemata with concentrations ranging from 0 to 100 mg/L. A 3-week incubation with 75 mg/L hygromycin was sufficient to inhibit the growth of untransformed protonemata of both *P. patens* and *B. argenteum*, with survival rates of 9% and 20%, respectively (Fig. 1A). The sensitivity of the

protonemata to hygromycin did not differ appreciably between the two mosses.

We also tested the antibiotic concentrations on transformed plants, which we produced using *Agrobacterium* strain EHA105, media with no sucrose, preculture of 6 days, *Agrobacterium* antibiotic of OD₆₀₀ = 0.6, 30 min infection time, and a transformation duration of 30 min. The transformation efficiency did not significantly increase at a hygromycin concentration of 100 mg/L compared to 75 mg/L (Fig. 1B). Thus, we selected 75 mg/L as the appropriate hygromycin concentration.

Selection of transgenic plantlets

For each transformation, we added hygromycin screening solution (75 mg/L) to *P. patens* and *B. argenteum* protonemata in a culture dish, cultured them for 1 month, and then selected healthy protonemata for further investigation (Fig. S1). To confirm the successful integration of the transgene into the surviving protonemata, we performed GUS staining to examine GUS activity throughout the moss life cycle (Fig. 2). GUS activity was observed in the selected protonemata, gametophytes, and regenerated gametophytes.

For plants that tested positive for GUS activity, we confirmed the integration of the transgene through PCR amplification of the *GUS* gene followed by DNA purification and gel electrophoresis. The expected band size of 823 bp from amplification of a fragment of the *GUS* gene was amplified from the DNA derived from the regenerated plant lines (Fig. S2). These results demonstrated that the regenerated lines indeed represented transgenic plants.

To confirm the stable integration of the transgene in PCR-positive plants, we performed Southern blot analysis. To this end, we digested genomic DNA with *Hind*III, which cuts once within the T-DNA, excising the *GUS* gene along with part of the genomic DNA at the integration site. Plants with a single integrated T-DNA should produce a single band. We detected the resultant fragment by hybridization to a 1,029-bp radiolabeled probe specific for the *GUS* gene from pCambia-1301. Samples from both mosses contained the *GUS* transgene, thus confirming the stable integration of the transgene (Fig. 3). For the *GUS*-positive samples, digestion with *Hind*III resulted in two clear bands, indicating the presence of two copies of the T-DNA in the sample.

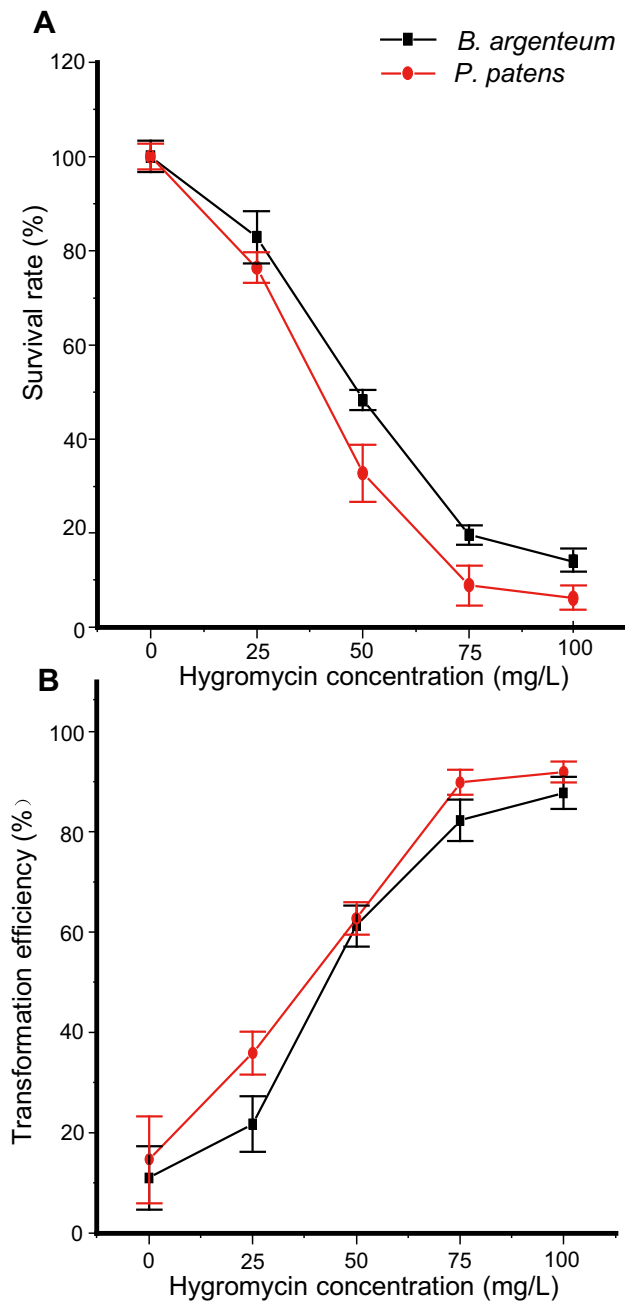


Fig. 1 Recovery of stable transgenic *P. patens* and *B. argenteum* protonemata using hygromycin as the selectable marker. **A** Survival rate of moss protonemata incubated with the indicated hygromycin concentrations for 15 days. **B** Transformation efficiency of moss protonemata incubated with the indicated hygromycin concentrations for 15 days. Data are mean $\pm$ SEM (n = three independent sets of experiments)

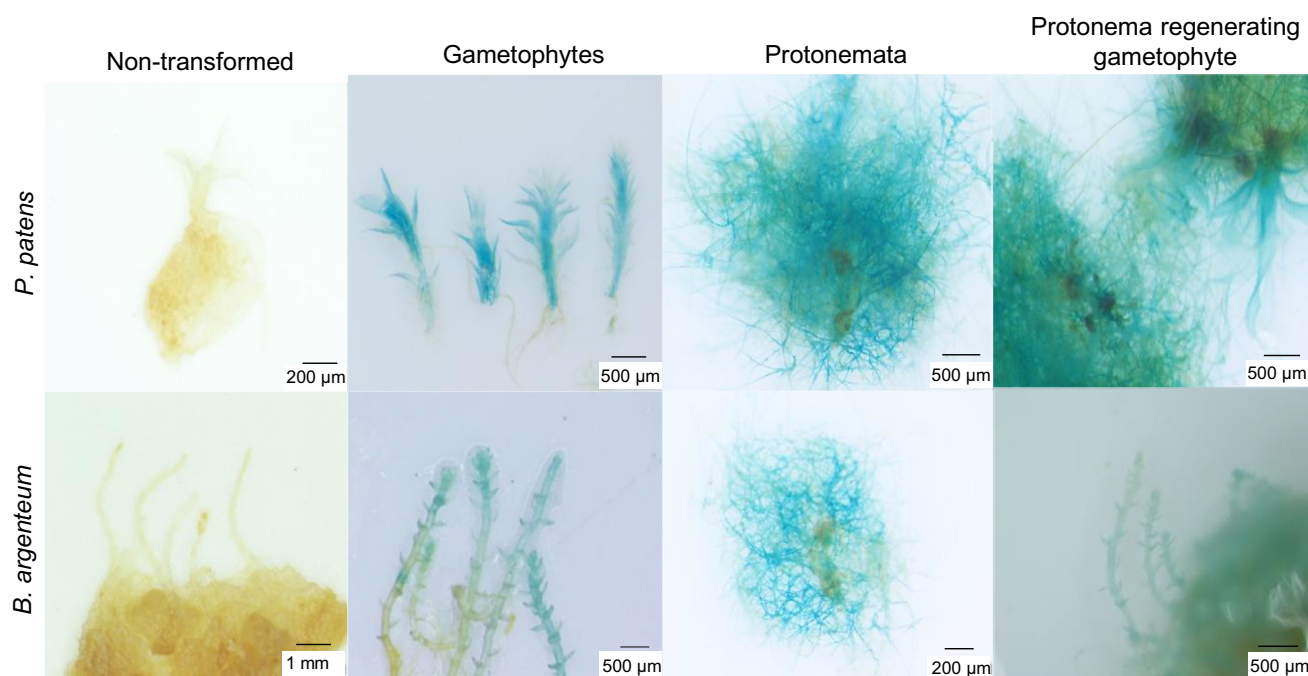


Fig. 2 Histochemical assay of *GUS* expression in moss transformed with *Agrobacterium* harboring pCAMBIA1301-*GUS*

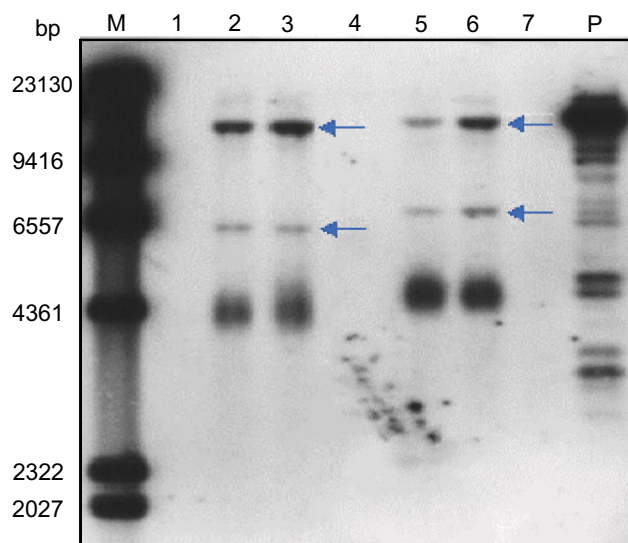


Fig. 3 Southern blot analysis of transgenic *P. patens* and *B. argenteum* with *GUS* gene. M: DNA size markers; P: pCAMBIA1301-*GUS*, positive control; 2, 3: transformed *P. patens*; 5, 6: transformed *B. argenteum*; 1, 4: digested DNA derived from non-transformed *P. patens* and *B. argenteum*, respectively; 7: blank control. The arrows indicate inserted copy of the *GUS* transgene

Optimization of factors influencing *Agrobacterium*-mediated transformation

We evaluated the effects of sucrose concentration, *Agrobacterium* cell densities, *Agrobacterium* strain, infection period, and duration of preculture, on the transformation efficiencies and survival rates of *P.*

patens and *B. argenteum* protonemata (Fig. 4). Following transformation, the mosses were grown on selection medium, for 30 days, to identify transformants, allowing us to calculate their survival rates and transformation efficiencies.

Sucrose concentrations of 0%, 1%, 2%, 5%, and 10% in the transformation buffer resulted in median *B. argenteum* transformation efficiencies of 81%, 55%, 45%, 42%, and 18%, and median *P. patens* transformation efficiencies of 74%, 50%, 52%, 45%, and 17%, respectively (Fig. 4A). Thus, including sucrose in the transformation buffer decreased the transformation efficiency in a dose-dependent manner. Indeed, sucrose in the medium led to the browning of the moss plants and the entrance of *Agrobacterium* into the transition growth phase, which would be expected to reduce the transformation efficiency.

The amounts of *Agrobacterium* relative to moss protonemata, the *Agrobacterium* strain, and the infection period also influenced the transformation efficiency. For *B. argenteum*, we observed a significant increase in transformation efficiency with increasing *Agrobacterium* cell densities from OD₆₀₀ 0.1 to 0.6, followed by a subsequent significant decrease in efficiency and the overgrowth of *Agrobacterium* at OD₆₀₀ > 0.6 (Fig. 4B). *P. patens* showed a similar trend, but with the peak transformation efficiency seen at an *Agrobacterium* OD₆₀₀ of 0.8. Among the five *Agrobacterium* strains tested, EHA105 exhibited the highest transformation efficiency for both *B. argenteum* and *P. patens*

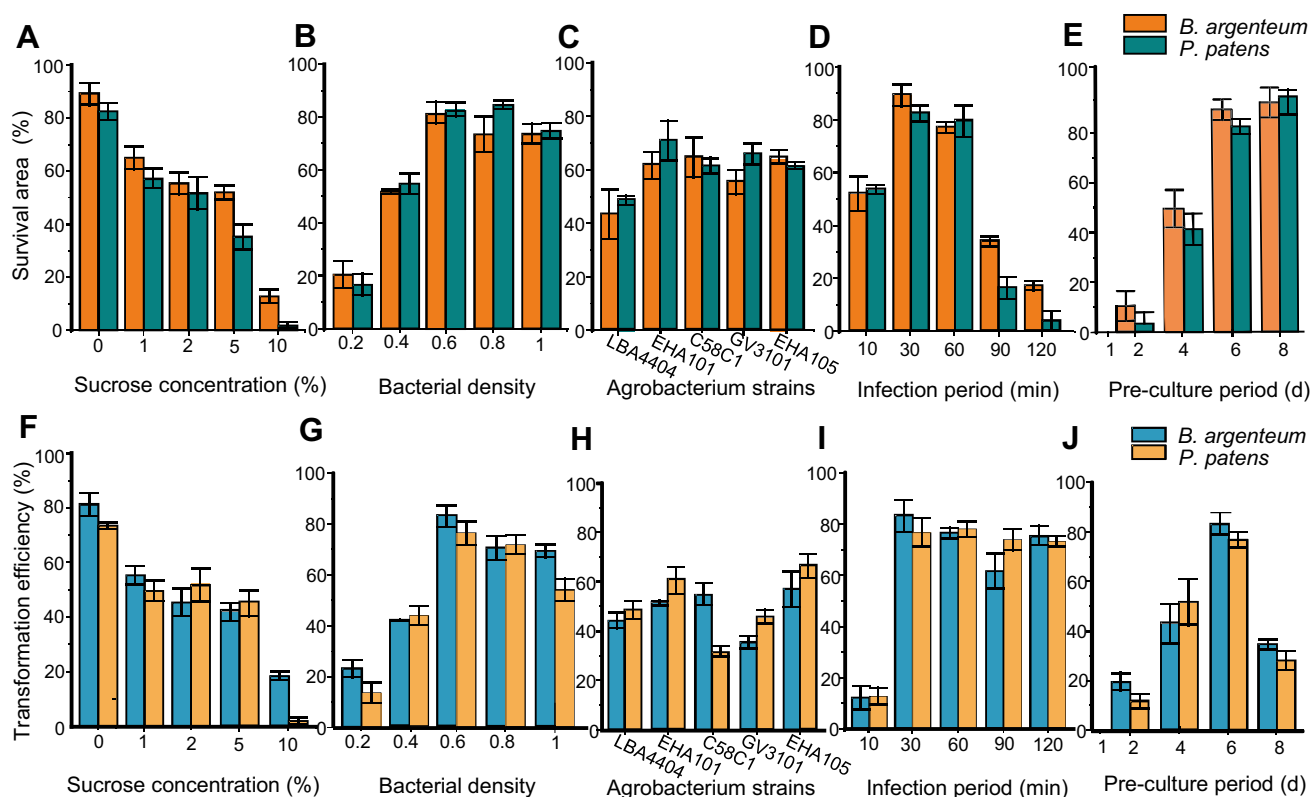


Fig. 4 Effects of different factors on protonemata survival rate and transformation efficiency. **A** and **F** Survival rate and transformation efficiency of transformed *P. patens* and *B. argenteum* under different sucrose concentrations. **B** and **G** Survival rate and transformation efficiency using different *Agrobacterium* cell densities (OD₆₀₀ values of 0.2, 0.4, 0.6, 0.8, and 1.0). **C** and **H** Survival rate and transformation efficiency using the indicated *Agrobacterium* strains. **D** and **I** Survival rate and transformation efficiency after the indicated infection periods. **E** and **J** Survival rate and transformation efficiency following the indicated pre-culture period. Each treatment comprised samples on five petri dishes and was performed in triplicate. Data are mean $\pm$ SD

(Fig. 4C). In terms of the infection period, either an increase or a decrease from our original protocol of 30 min significantly decreased the percentage of surviving moss protonemata in both moss species (Fig. 4D).

We also investigated whether preculturing the moss affected the transformation efficiency. Preculturing the protonemata on PpNH4 medium for 1, 2, 4, 6, or 8 days, in a petri dish sealed with cellophane led to median *B. argenteum* transformation efficiencies of 0%, 19%, 44%, 81%, and 77%, and median *P. patens* transformation efficiencies of 0%, 12%, 51%, 74%, and 58%, respectively (Fig. 4E). These results indicated that a preculture period of 6 days was optimal.

Testing transgene inheritance

We next assessed whether the transgene could be stably inherited into moss throughout its life cycle. Following regeneration of the transgenic lines, the generation of gametophytes and the expression of the *GUS* gene in the regenerated gametophytes indicated that the transgene

had been successfully passed on to the next generation. Moreover, we tested transgene inheritance for at least 10 generations (Fig. S3) and determined that it was still stably inherited.

Application of the transformation system to other mosses

To determine whether our method could be used for transformation of other moss species, we tested it on the typical desert moss, *S. caninervis*. Seven days after transformation, we detected GUS activity in the *S. caninervis* gametophytes, and after 7 days of co-culture, we also detected GUS activity in the regenerated moss plants. After 15 days of continuous selection, the T₁ plants were green, and subsequent GUS assays yielded blue-stained regenerated moss plants (Fig. 5A–D). The final percentage of GUS-stained plants among the regenerated plants was 45%. The relative expression level of *GUS* was detected in the transgenic lines (Fig. 5E). These results demonstrated that this method

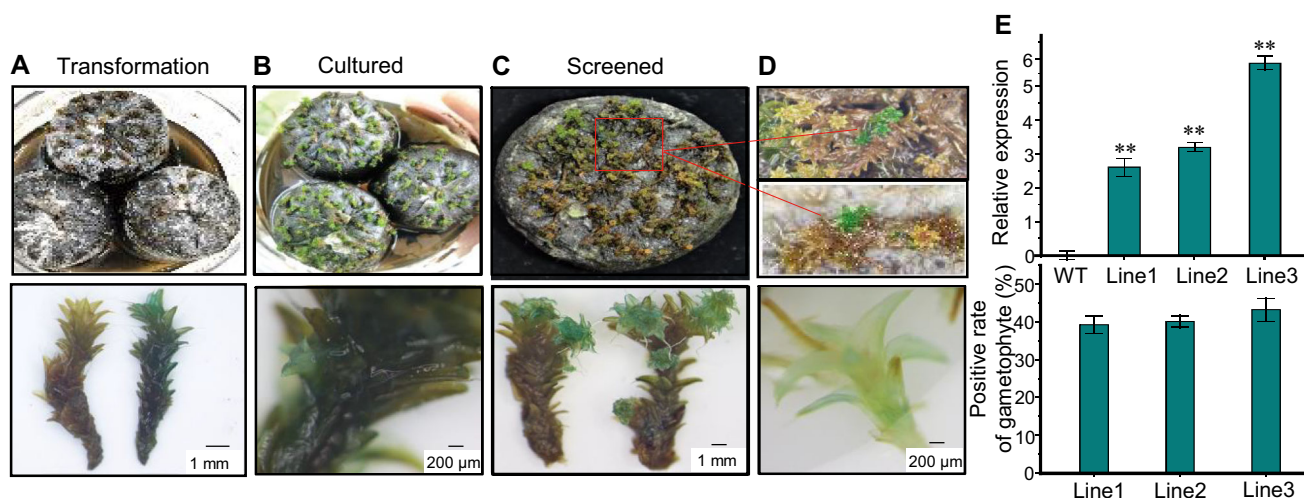


Fig. 5 Histochemical assay of *GUS* expression in *S. caninervis* transformed using *Agrobacterium*. **A, B** Phenotype and histochemical staining was performed after 0 and 7 days of transformation, respectively. **C** Phenotype and GUS staining was performed after a 15 day selection period. **D** Phenotype and GUS staining in regenerated gametophytes. **E** Statistical analysis of relative *GUS* expression levels and regenerated gametophytes positivity rate. Data are mean $\pm$ SD of three biological replicates; asterisks indicate significant differences from WT (** $P < 0.01$, Student's *t* test)

can be successfully applied to *S. caninervis* and that the transgene can be stably inherited.

Engineering stress tolerance in mosses

To further assess our stable transformation method, we used it to introduce the stress resistance gene *EsDREB* into *P. patens* and *B. argenteum*, which we then treated with different salt concentrations (100 and 200 mM NaCl). In both species, the growth rates and extension of protonemata produced by these transgenic mosses, after 30 days of growth on PpNH4 moss growth medium (without added NaCl), were greater than those for wild-type *P. patens* and *B. argenteum* (Fig. 6). When grown in the presence of 100 mM NaCl, the protonemata of both the transgenic and wild-type mosses had a smaller growth area than those grown in the absence of added NaCl, but the areas of the transgenic protonemata were significantly larger than those of the wild-type mosses. Treatment with 200 mM NaCl had similar, but stronger, effects on growth, with the protonemal growth areas of the transgenic mosses again being significantly greater than those of the wild-type plants of each species. These results indicated that the *EsDREB* transgene improves the growth of moss protonemata under salt stress and confers salt stress tolerance. Therefore, our method can be successfully used to verify the function of genes from other plants.

DISCUSSION

Agrobacterium-mediated transformation has been used in various plant species (Frangedakis et al. 2021; Nishimura et al. 2006; Olhoft et al. 2004). Traditional *Agrobacterium*-mediated genetic transformation requires the establishment of tissue culture systems, which are time-consuming and result in low transformation efficiencies. Although several transformation methods not requiring tissue culture have recently been described, they are not compatible with all types of plants (Cao et al. 2023a, b).

An *Agrobacterium*-mediated stable transformation method has not previously been established for moss. Historically, gene targeting was accomplished in *P. patens* through PEG-mediated transformation of protoplasts, a method widely used in plants. For more efficient gene manipulation in *P. patens*, RNA interference, CRISPR/LbCas12a, and particle bombardment methods have been developed and applied (Pu et al. 2019; Bezanilla et al. 2003; Cho et al. 1999). However, genetic transformation systems for DT mosses, such as *S. caninervis*, *B. argenteum*, and *Syntrichia ruralis*, have not been reported. This lack of molecular genetic methods for mosses limits studies of DT mosses and the exploration and utilization of genes conferring desiccation tolerance.

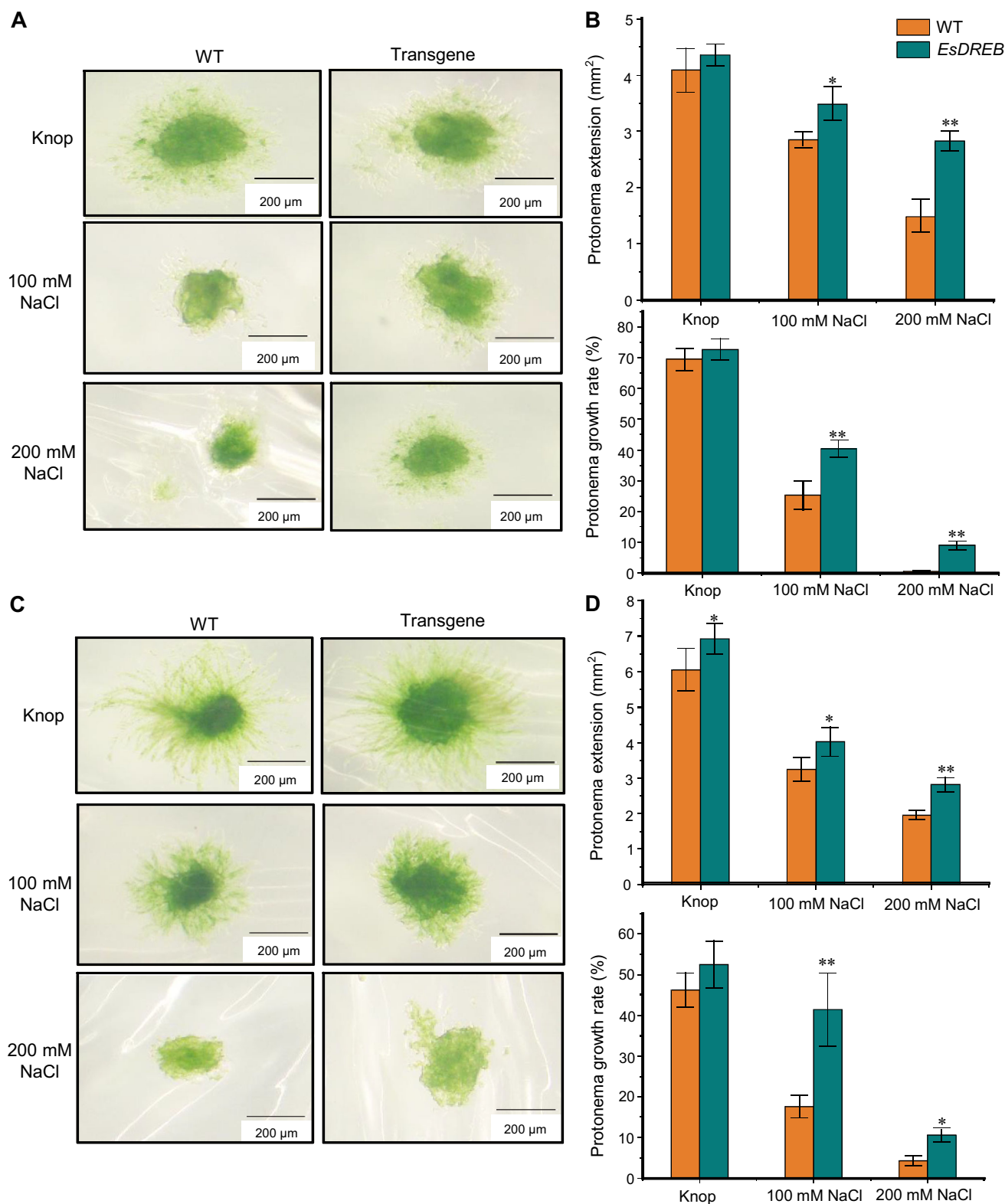


Fig. 6 Functional verification of *EsDREB* in salt stress tolerance through heterologous expression in *P. patens* and *B. argenteum*. **A** Growth phenotype of *P. patens*. **B** Protonemata extension and growth rate of *P. patens*. **C** Growth phenotype of *B. argenteum*. **D** Protonemata extension and growth rate of *B. argenteum*. Protonemata growth rate = (growth area of protonemata before treatment)/(growth area of protonemata after treatment) × 100%. Each treatment comprised samples on five petri dishes and was performed in triplicate. Data are mean ± SD (each treatment comprised five petri dishes and was performed in triplicate)

Comparing PEG- and *Agrobacterium*-mediated transformation methods

We compared the outcomes of classical transformation (PEG-mediated transformation of protoplasts) with those of *Agrobacterium*-mediated transformation in *P. patens*. In PEG-mediated transformation, protonemata cultured for 6 days are processed into protoplasts, which are subsequently transformed and screened, and transgenic protonemata are obtained within 10–50 days with a transformation efficiency of 0.5–1 transformants/ μg DNA. This technique yields $\sim 10^6$ protoplasts per 90-mm-diameter plate of tissue, with relative transformation frequencies of 1 to 22 integrative transformants per 10^5 regenerated plants, depending on the vector (Cove et al. 2009; Schaefer and Zryd 1997). Thus, a 90-mm culture dish can produce up to 22 transformants following protoplast transformation.

In our *Agrobacterium*-mediated transformation method, protonemata cultured for 6 days are directly transformed and subsequently screened, resulting in transgenic lines within 37 days. We obtained a positive transformation rate of 73%, which should yield an even greater number of transgenic protonemata. The duration required to obtain transgenic protonemata differs only slightly between the two methods. However, *Agrobacterium*-mediated transformation is significantly more efficient than PEG-mediated transformation, as the former directly targets protonemata, enabling the rapid generation of a substantial number of transgenic strains. In addition, protonemata can be transferred to Knop medium during transformation, allowing the direct acquisition of transgenic gametophytes (Fig. S4).

Optimization of factors influencing *Agrobacterium*-mediated transformation

In the current study, a straightforward, effective *Agrobacterium*-mediated transformation protocol was devised for mosses using protonemata or gametophytes. In our approach, *Agrobacterium* directly infects protonemata or gametophytes, eliminating the need for protoplast preparation or regeneration to yield intact transgenic plants. To improve the efficiency of moss transformation, we optimized several factors, including the sucrose concentration, *Agrobacterium* cell concentration, bacterial strain, infection duration, and preculture duration.

The *Agrobacterium* cell densities used is a critical factor influencing transformation efficiency (Amoah et al. 2001). Elevated bacterial cell density may result in the overgrowth of *Agrobacterium* on the medium, thereby impeding the survival of mosses and

diminishing the transformation efficiency. Conversely, lower concentrations of *Agrobacterium* can lead to reduced transformation efficiency. The optimal transformation density of *Agrobacterium* varies based on the species and strain of *Agrobacterium* used. For instance, for *Marchantia polymorpha*, an *Agrobacterium* density corresponding to $\text{OD}_{600} = 0.5$ might already be saturating for the transformation of sporelings (Tsuboyama and Kodama 2014). In our method, the *Agrobacterium* density yielding the highest transformation efficiency was an OD_{600} of 0.6.

The infection duration affects transformation efficiency (Manders et al. 1994). Here, we obtained the greatest transformation efficiency with an incubation period of 30 min. The *Agrobacterium* strain used also affects plant transformation efficiency (Azizi-Dargahlou and Pouresmaeil 2023; Frangedakis et al. 2021), which we confirmed in our system, finding that strain EHA105 exhibited the highest transformation efficiency in both moss species.

The state of the plant material also affects the transformation efficiency, as the plant susceptibility to *Agrobacterium* infection varies at different stages of culture. In *M. polymorpha*, preculture for 7 days led to the highest transformation efficiency (Ishizaki et al. 2008). In the present study, the most effective period of protonemal preculture was 6 days. Furthermore, the optimal infection duration varies by plant species (Jha et al. 2011; Kumar et al. 2019; Yang et al. 2016). When the infection duration is too short, the probability of exogenous DNA insertion into the moss genome is low, and when the infection duration is too long, destruction of protonemata can easily occur. We established that, for the mosses we studied, the transformation efficiency was greatest after 30 min of infection, with shorter or longer periods being less effective.

Application of the transformation system to other mosses

Both *B. argenteum* and *S. caninervis* are DT mosses that lack established transformation systems. The absence of established transformation systems hinders studies of gene function in these species. We applied our transformation system to field materials of *S. caninervis* and directly obtained regenerated T_1 generation gametophytes via re-culturing in peat pellets (Liu et al. 2021). Moreover, heterologous expression of a gene that functions in stress resistance in mosses resulted in enhanced salt stress resistance. Therefore, our method can be used to verify gene function in mosses.

In summary, we developed a rapid and simple transformation method for DT mosses using

Agrobacterium strain EHA105. Optimized transformation conditions were as follows: the bacterial density was $OD_{600} = 0.6$; samples were precultured for 6 days; 200 μ M acetosyringone was used as an inducer; and transformants were selected using 75 mg/L hygromycin. Under these optimized conditions, the transformation efficiency was almost 80%. Stable transgenic plants that expressed *GUS* were regenerated from protonemata or gametophytes. *Agrobacterium*-mediated transformation of protonemata or gametophytes enables the rapid generation of a relatively large number of transgenic plants and is amenable to scaling up. Moreover, this transformation system is well suited for transforming DT field moss materials, establishing a crucial foundation for identifying candidate genes associated with stress tolerance.

MATERIALS AND METHODS

Plant materials and treatments

B. argenteum and *P. patens* gametophytes were grown on Knop medium for 20 days and transformed as described below. *P. patens* and *B. argenteum* protonemata were grown for 1, 2, 4, 6, or 8 days on solid PpNH4 medium prior to transformation (Lopez-Obando et al. 2021).

Plasmid construction and *Agrobacterium* culture

The plant binary vector pCAMBI1301 was introduced into *Agrobacterium* strains EHA105, LBA4404, GV3101, EHA101, and C58C1 via the freeze/thaw method.

A modified version of the AGROBEST protocol was performed, which involves antibiotic treatment of *Agrobacterium* cultures for 3 days followed by a 2 day co-culture period with moss (Wu et al. 2014). A single *A. tumefaciens* colony from a fresh plate was inoculated into 5 mL liquid LB medium supplemented with 50 mg/L kanamycin and 25 mg/L rifampicin (Solarbio, China) and incubated with shaking (220 rpm) at 28 °C for 20–24 h. To increase the number of *Agrobacterium* cells for the second activation, 1 mL of the bacterial suspension was inoculated into 50 mL of LB liquid medium supplemented with the appropriate antibiotics and incubated with shaking (220 rpm) at 28 °C for 12–16 h. The *Agrobacterium* cells were collected by centrifugation, resuspended in 50 mL AB:MES salts (17.2 mM K_2HPO_4 , 8.3 mM NaH_2PO_4 , 18.7 mM NH_4Cl , 2 mM KCl, 1.25 mM $MgSO_4$, 100 μ M $CaCl_2$, 10 μ M $FeSO_4$, 50 mM MES, 2% glucose [w/v], and 200 μ M acetosyringone, pH 5.5), and grown overnight. Before being incubated with

moss, the culture was again centrifuged and resuspended to an OD_{600} of 0.2, 0.4, 0.6, 0.8, or 1.0 in a 50:50 (v/v) mixture of AB:MES salts and half-strength Knop liquid moss growth medium (pH 5.5).

Agrobacterium-mediated moss transformation

To simplify the *B. argenteum* and *P. patens* protonemal infection process, 5 mL of *Agrobacterium* culture was directly added to each culture dish. After 30 min of treatment, the bacterial solution was removed with a sterile pipette, and the culture plate was sealed with Parafilm. For gametophyte transformation, the gametophytes were placed in a 50-mL centrifuge tube, 20 mL of infection solution was added to the tube, and the mixture was returned to the culture plate after 30 min. The culture plates were sealed with cellophane. The plates were cultured in the dark for 3 days, and the cultures were transferred to standard culture conditions for *P. patens* for further cultivation. One month after screening and culture, validation was carried out.

For *S. caninervis* transformation, moss cultivated on peat pellets was directly inoculated by immersion for 30 min and then placed back onto the peat pellets. After 3 days of cultivation in the dark, the same light conditions as used for *P. patens* were resumed. Transformants were selected after 15 days of cultivation.

Culture screening

After 7 days of co-cultivation, 2 mL of sterile water supplemented with 75 mg/L hygromycin was added to the moss culture dish. During the co-cultivation screening period, if *Agrobacterium* overgrowth occurred, the plants were washed twice with sterile water, after which screening solution was added.

For *S. caninervis* selection, *S. caninervis* cultivated on peat pellets was sprayed with distilled water containing 75 mg/L of hygromycin daily for 15 days.

Plant RNA extraction and quantitative RT-qPCR

Total RNA was extracted from the samples using an EZNA Plant RNA Kit (OMEGA, USA). Total RNA (1 μ g) was used to synthesize first-strand cDNA with One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen, China). Quantitative PCR was performed with 2 $\times$ SYBR Premix Ex TaqTM (TaKaRa, Japan). The *P. patens* *Actin* gene (AW698983) (Okada et al. 2009), *B. argenteum* *Actin* gene (TR77358|c0_g1_i4) (Gao et al. 2017), and *S. caninervis* α -tubulin2 gene (Li et al. 2015) were used as internal controls.

DNA extraction and PCR analysis

Genomic DNA was isolated from the samples using an EZNA® Plant DNA Kit (OMEGA, USA). *NPTII* (GenBank: AF234297.1) was used to verify T-DNA insertion events. The PCR primer set was designed to specifically amplify the DNA fragment of the *GUS* gene based on the nucleotide sequence of pCAMBIA1301 (accession number: AF234316). Phanta Max Super-Fidelity DNA Polymerase (Vazyme, China) was used according to the manufacturer's instructions. The PCR procedure was as follows: initial denaturation at 95 °C for 30 s; 30 cycles at 95 °C for 10 s, 52 °C for 15 s, and 72 °C for 20 s; and a final extension step at 72 °C for 5 min.

GUS staining assays

The transgenic protonemata or gametophytes were submerged in GUS staining solution containing 50 mM sodium phosphate (pH 7.0), 0.5 mg/L 5-bromo-4-chloro-3-indolyl- β -glucuronide, 0.1% Triton X-100, 0.5 mM $K_3[Fe(CN)_6]$, and 0.5 mM $K_4[Fe(CN)_6]$ overnight in darkness at 37 °C. The samples were washed several times in 75% ethanol to remove the chlorophyll (Jefferson et al. 1987). Tissues that turned blue harbored the *GUS* gene.

Southern blot analysis

A 1- μ g aliquot of genomic DNA was digested with *Hind*III at 37 °C. The digested DNA was subjected to electrophoresis on a 1% agarose gel and transferred to a Hybond-N⁺ nylon membrane (GE Healthcare) using a vacuum transfer device (BIO CRAFT). The DNA was hybridized with a radiolabeled probe comprising a random primer of a 1,052-bp fragment obtained by PCR amplification of pCAMBIA1301 with GUS primers (F: CGAAGTCACAGCCAAAAGCC; R: GCGAAATATTCCTCGTGCACC).

Statistical analysis

Statistical analysis of the survival rates and GUS staining frequencies of the mosses was performed by Student's *t* test ($P < 0.01$). The survival rate of protonemata (%) = area of surviving protonemata/total area evaluated. The protonemal transformation efficiency (%) = (transgene-positive protonemal samples/surviving protonemata after screening $\times 100\%$). Each experiment was independently repeated three times.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s42994-024-00174-4>.

Author contributions X.L, D.Z and Y.Z designed the study; P.Z, L.Y and X.L. performed the experiments; P.Z. and X.L. analyzed and wrote the data; P.Z. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding This work was supported by the Leading Talents in Technological Innovation programme (2022TSYCLJ0049), the Second Tibetan Plateau Scientific Expedition and Research (STEP) program (2019QZKK0502030403).

Data availability All data generated during this study are included in the article and its supplementary information files.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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