

Establishment of an efficient *Agrobacterium tumefaciens*-mediated leaf disc transformation of *Thellungiella halophila*

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Abstract *Thellungiella halophila* is a salt-tolerant close relative of *Arabidopsis*, which is adopted as a halophytic model for stress tolerance research. We established an *Agrobacterium tumefaciens*-mediated transformation procedure for *T. halophila*. Leaf explants of *T. halophila* were incubated with *A. tumefaciens* strain EHA105 containing a binary vector pCAMBIA1301 with the *hpt* gene as a selectable marker for hygromycin resistance and an intron-containing β -glucuronidase gene as a reporter gene. Following co-cultivation, leaf explants were cultured on selective medium containing 10 mg l⁻¹ hygromycin and 500 mg l⁻¹ cefotaxime. Hygromycin-resistant calluses were induced from the leaf explants after 3 weeks. Shoot regeneration was achieved after transferring the calluses onto fresh medium of the same composition. Finally, the shoots were rooted on half strength MS basal medium supplemented with 10 mg l⁻¹ hygromycin. Incorporation and expression of the transgenes were confirmed by PCR, Southern blot analysis and GUS histochemical assay. Using this protocol, transgenic *T. halophila* plants can be obtained in approximately 2 months with a high transformation frequency of 26%.

Keywords *Thellungiella halophila* · Callus · Plant regeneration · Transformation

Introduction

Thellungiella halophila (salt cress; synonymous to *Thellungiella salsuginea*, Al-Shebaz et al. 1999) is an emerging model species for studies designed to elucidate molecular mechanisms of abiotic stress tolerance in recent years (Inan et al. 2004; Wang et al. 2006; Volkov and Amtmann 2006; Wong et al. 2005). The plant is a salt-tolerant relative of *Arabidopsis thaliana* and has high genetic and morphological similarity with *Arabidopsis*. *T. halophila* can complete its life cycle in the presence of 300 mM NaCl. It also has a short life cycle and high seed yields. More importantly, its genome size is small, its cDNAs share over 90% identity to the model plant *A. thaliana* (Zhu 2001). The similarity between *T. halophila* and *A. thaliana* allows for the question of differential expression of salt tolerance genes between glycophytes and halophytes to be addressed using the molecular and genetic tools available for *A. thaliana*. Several molecular tools have been created for *T. halophila* including collections of ESTs, T-DNA insertion mutants and ecotypes, as well as cDNA libraries and microarrays (Inan et al. 2004; Amtmann et al. 2005, <http://www.thellungiella.org/>).

Agrobacterium-mediated genetic transformation has become a powerful tool for functional genomics (Gelvin 2005). For unraveling the function of a gene, many basic works are needed. For example, the gene expression pattern, localization of specific proteins, phenotypes of the plants when a gene is over-expressed or knocked out. Most of these studies are dependent on an efficient and suitable transformation system. Although genetic transformation in

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most plant species needs a regeneration procedure, *Arabidopsis* can be transformed with the “floral dip” method, which is performed without the need for in vitro regeneration process (Clough and Bent 1998). This protocol has been used widely for its easy to handle and high transformation efficiency, as well as reduced occurrence of somatic mutation that is generally a problem associated with procedures requiring long-term tissue culture (Zhang et al. 2006). Similarly, the “floral dip” method was applied successfully on *T. halophila*, and a global T-DNA insertion mutagenesis of this plant is underway by using the same method (<http://www.thellungiella.org/>). However, the “floral dip” method has limitations when used for flowers with male or female sterility, as in these cases there is no seed-set for the screening of transgenic plants. This could be an obstacle for study of molecular mechanisms related to flower development and sterility. For example, it is difficult to perform genetic complementation experiments in mutants which are male or female sterile by the “floral dip” method. For the above reasons, an *Agrobacterium*-mediated genetic transformation system based on plant regeneration from somatic cells is needed as a supplementary method to the “floral dip” method.

This is the first report of establishing an *Agrobacterium*-mediated transformation method for *T. halophila* through plant regeneration from leaf explants. This will provide an alternative method for the molecular analysis of gene functions in this plant.

Materials and methods

Tissue culture conditions

Seeds of *T. halophila* (ecotype Shandong) were surface-sterilized for 30 s in 70% EtOH, transferred to 2.5% sodium hypochlorite containing 1 μ l of Tween 20 per 2 ml for 20 min, rinsed five times with sterile distilled water, and placed on Petri dishes containing MS (Murashige and Skoog 1962) solid medium to germinate. Seedlings were grown at 22°C in a 16 h light (35 μ mol m⁻² s⁻¹)/8 h dark cycle. The same growth-room conditions were used for tissue culture procedures. All plant media were adjusted with 1 N NaOH to pH 5.8, solidified with 0.8% agar, and autoclaved at 121°C for 20 min.

Callus formation, shoot regeneration and rooting

Leaves were excised from 3-week-old seedlings and placed horizontally on callus induction medium, which is MS basal medium supplemented with 3% (w/v) sucrose and different plant growth regulators. Callus induction was conducted under darkness or light. Three weeks later, the

induced calluses were transferred to fresh medium of the same composition, and incubated in the light for shoot regeneration. After another 3 weeks, the regenerated shoots were transferred to half strength MS basal medium for rooting.

Agrobacterium and plasmid

Agrobacterium tumefaciens strain EHA105 harboring the binary vector pCAMBIA 1301 was used for transformation experiments. The binary vector pCAMBIA 1301 carried both the reporter gene for β -glucuronidase (*uidA*) and the selectable marker gene for hygromycin phosphotransferase (*hpt*). The vector pCAMBIA 1301 was introduced into *A. tumefaciens* strain EHA105 by the liquid nitrogen freezing-thaw method. For plant transformation, a single colony of the bacteria was inoculated in YEB medium supplemented with kanamycin (50 mg l⁻¹) and rifampicillin (50 mg l⁻¹) and cultured overnight at 28°C. The *Agrobacterium* cells were collected by centrifugation at 10,000g for 30 s at 25°C and then resuspended in 20 ml (OD₆₀₀ = 0.4–0.5) of liquid MS medium containing 20 mg l⁻¹ acetosyringone (AS).

Transformation of *Thellungiella halophila* leaf explants

Leaves from 1-month-old seedlings were cut into 0.5 × 0.5 cm (as leaf explants) and inoculated with the *Agrobacterium* cells harboring pCAMBIA 1301 in 20 ml of liquid MS medium supplemented with 20 mg l⁻¹ AS for 15 min. Subsequently, the leaf explants were blotted on sterile filter paper to remove most of the liquid medium, and transferred to agar-solidified (0.8% w/v) MS medium supplemented with 20 mg l⁻¹ AS, 2 mg l⁻¹ benzyladenine (BA), 0.5 mg l⁻¹ 3-indoleacetic acid (IAA) for co-cultivation. After 3 days, the explants were washed with sterile water containing 500 mg l⁻¹ cefotaxime to remove *Agrobacterium* cells. Subsequently, the explants were transferred onto selection medium (MS medium supplemented with 2 mg l⁻¹ BA, 0.5 mg l⁻¹ IAA, 10 mg l⁻¹ hygromycin, and 500 mg l⁻¹ cefotaxime), and incubated in the light. Three weeks later, the leaf explants together with the calli were transferred onto the same fresh medium for shoot induction. After another 3 weeks, hygromycin-resistant shoots were separated and transferred to half strength MS basal medium supplemented with 10 mg l⁻¹ hygromycin for rooting.

PCR and Southern blot analysis

Genomic DNA was prepared from leaves of hygromycin resistant plants using the cetyltrimethylammoniumbromide (CTAB) method (Muhammad et al. 1994). Genomic DNA

from transgenic plants (one plant from each transgenic line) was used for PCR amplification of a 529 bp of *hpt* fragment using the primers of HF (5'-CGA TCT TAG CCA GAC GAG CGG GTT C-3') and HR (5'-GCT GGG GCG TCG GTT TCC ACT ATC GG-3'). To examine whether any residual *Agrobacterium* exist in the regenerated plants, *Agrobacterium VirB1* gene-specific primers VBFw 5' GAA GGC AAC AGG GCC GCT GTC 3' and VBRe 5' TCC GCC CTC CGG GGA ACG ACG C 3' were used to amplify the 630 bp fragment from the *VirB1*. The reaction mixture for PCR was incubated in a DNA thermal cycler under the following conditions: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, with a final 10 min extension at 72°C. For Southern blot analysis, 5 µg of genomic DNA was digested with the restriction enzyme *EcoRI*, separated on 0.8% (w/v) agarose gel, and transferred to Hybond N⁺ nylon membrane. The *hpt* probe was labeled with digoxigenin (DIG) by PCR method using the primers of HF and HR. Prehybridization, washing, and chemiluminescent detection of the blots were performed according to the manufacturer's instructions (Roche).

Assay for *uidA* expression in transgenic plantlet

Histochemical staining of GUS activity was performed according to Jefferson (1987). Calluses and plantlet of transgenic lines and calluses of wild type (control) were submerged into the solution containing 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) at 37°C for 3 h and then destained in 70% alcohol.

Results and discussion

Callus induction from leaf explants

Plant growth regulators are important factors, which can selectively influence the genes to trigger differentiation of cells in culture (Thorpe 1983). We examined the effects of different plant growth regulators on callus induction for leaf explants of *T. halophila*. Callus induction on MS medium supplemented with 2 mg l⁻¹ BA combined with either IAA, α -naphthaleneacetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D) at 0.5 mg l⁻¹ all resulted in a 100% induction frequency (Table 1). This indicates that BA is sufficient for the callus induction. When transferring the calluses to fresh medium of the same composition, we observed that shoot regeneration only occurred on the medium containing BA and IAA (Fig. 1a). The callus induced in the light are green (Fig. 1b) and showed higher shoot regeneration frequency (90%). While callus

induced under darkness are pale-yellow, and the regeneration frequency is obviously lower (Table 1). These indicate that light is effective to promote shoot regeneration.

We also studied the regeneration capacity of root explants, as this kind of explants were used for transformation in the earlier studies in *Arabidopsis* (Valvekens et al. 1988). We observed a similar high frequency of callus induction in these explants as that for leaf explants. However, shoot regeneration rate from these calluses was lower than that from leaf explants. The experiments conducted on leaf explants from seedlings during different development stages (2-, 4- and 6-week-old) did not exhibit significant differences in callus induction and shoot regeneration (data not shown). The regenerated shoots were finally transferred to half strength MS basal medium for rooting, and roots appeared from the cut end of the shoots within 3 weeks (Fig. 1c). The rooting rates ranged from 81 to 92%.

Influence of antibiotics on shoot regeneration from leaf

To find a suitable selectable marker for *T. halophila* transformation, we tested the sensitivity of leaf pieces of *T. halophila* to a number of antibiotics that are currently used as selection agent in plant transformation. We found that 50 mg of kanamycin, 10 mg of G418, or 10 mg of hygromycin per liter completely blocked shoot regeneration. Therefore, the antibiotics we tested could be used for selection of transformed cells in this plant. As the pCAMBIA1301 contains the *hpt* gene which confers hygromycin resistance, we selected hygromycin as selection reagent in our transformation experiments. Cefotaxime which is used for suppressing and killing *Agrobacterium*, was found to severely inhibit shoot regeneration from root explants of *Arabidopsis* (Valvekens et al. 1988). In the case of *T. halophila*, cefotaxime did not inhibit the shoot regeneration capacity of its leaf explants and, therefore, cefotaxime was used to kill *Agrobacterium* after co-cultivation with leaf explants.

Genetic transformation of leaf explants

Leaf explants were incubated with the *Agrobacterium* cells harboring pCAMBIA 1301 for different intervals (5, 10, 15, 20, 25, and 30 min). We observed that transient expression of GUS increased with the infection time. Considering the post infection process, an incubation of 15 min was selected in the subsequent transformation experiments. After 3 days of co-cultivation, the leaf explants were washed with distilled water and transferred onto the selection medium containing 10 mg l⁻¹ hygromycin, and 500 mg l⁻¹ cefotaxime. Two weeks later,

Table 1 Effects of plant growth regulators on callus induction and shoot regeneration from *Thellungiella halophila* leaf explants

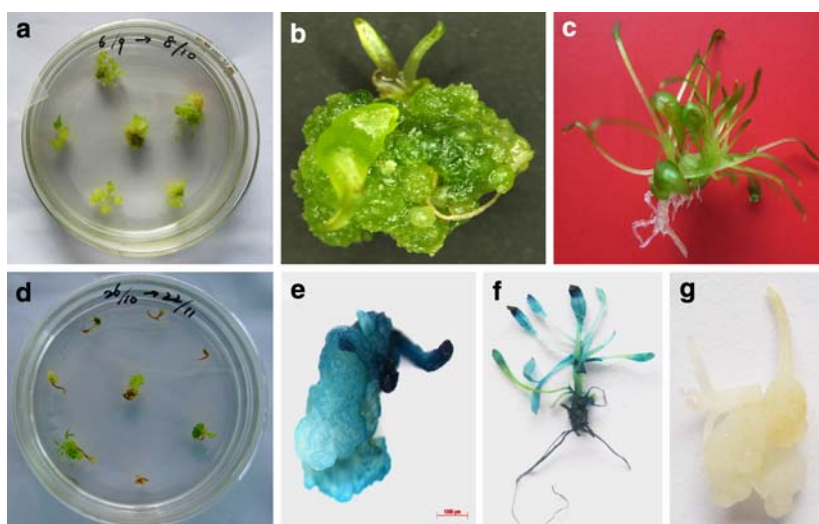
Medium	Growth regulators (mg l ⁻¹)				No. of explants	No. of exp. with calli	No. of exp. with shoots ^a
	6-BA	IAA	NAA	2,4-D			
I	2	0.5			150	150 ^b	102 (70%)
II	2		0.5		150	150 ^b	0
III	2			0.5	150	150 ^b	0
I	2	0.5			150	150 ^c	135 (90%)

^a Shoot regeneration under light for 4 weeks

^b Data were collected after callus induction for 3 weeks under dark

^c Under light

Fig. 1 Regeneration of transgenic plants from leaf explants in *Thellungiella halophila*. **a** Induction of multiple shoot/bud clumps after a 33-day culture on MS medium containing 2 mg l⁻¹ BA and 0.5 mg l⁻¹ IAA. **b** Green callus and bud formed on a single explant. **c** Regenerated plantlet after a 21-day culture on rooting medium. **d** Hygromycin resistant shoots after a 27-day culture on selection medium. **e** GUS assay of the transformed callus and buds. **f** GUS assay of transformed plantlet. **g** GUS assay of the non-transformed callus and buds



nodular green calluses grew out from the cut surface of the yellowish leaf explants. On average about 32% of the leaf explants produced hygromycin resistant calluses (Table 2). When transferring the resistant calluses to fresh medium of the same composition, shoot regeneration could be obtained in more than 85% of the green calluses (Table 2, Fig. 1d). Subsequently, more than 80% of the hygromycin

resistant shoots (one shoot from one individual resistant callus) could root on half strength MS medium containing 10 mg l⁻¹ hygromycin successfully (Table 2). Histochemical GUS assay showed that transgene expressed in calluses and in different parts of the putative transgenic plantlets (Fig. 1e, f), while there is no GUS staining in the wild-type control (Fig. 1g).

Table 2 Summary of the transformation results of *Thellungiella halophila*

Experiment	No. of explants	No. of exp. with resistant calli	No. of exp. with resistant shoots	No. of exp. with resistant plants ^a	PCR+ ^b
1	100	30	26	22	22
2	100	35	32	30	30
3	100	31	29	26	26
Total	300	96 (32%)	87 (29%)	78 (26%)	78 (26%)

^a Plants with vigorous root growth on hygromycin 10 mg l⁻¹

^b Hygromycin-resistant plants showed positive PCR result were indicated as “+”

PCR and Southern blot analysis were further used to confirm the presence and the integration of T-DNA in *T. halophila* genome, as well as the copy number integrated. As shown in Fig. 2a, the amplification of genomic DNAs from different transgenic lines with *hpt* specific primer produced the expected 539 bp band, while the same band was not detected in amplification with DNA from non-transformed plants. At the same time, the *VirB1* specific primers were also used to examine the presence of residual *Agrobacterium* in the regenerated plants. The amplification of the genomic DNA from *Agrobacterium* produced the 630 bp bands, while the amplification with DNAs from different transgenic lines did not give the same band (data not shown). These indicate that hygromycin resistant plants are truly transformed. Southern blot analysis using the *hpt*

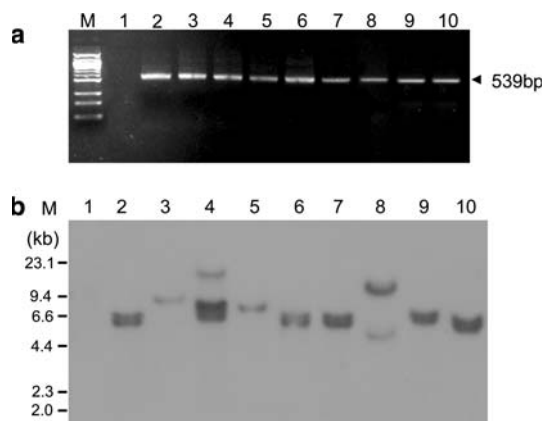


Fig. 2 PCR analysis (**a**) and Southern blot analysis (**b**) of the *hpt* gene in transgenic lines. *M* ladder, *1* wild-type plant, *2–10* different hygromycin resistant plants

probe was performed on randomly selected transgenic lines. The results showed that all the selected lines have T-DNA integrated in their genome. As the genomic DNA was restricted with *EcoRI*, which cuts only once inside the T-DNA region in pCambia 1301, thus, the number of the hybridization bands reflects the copy number of the T-DNA integrated into the *T. halophila* genome. The T-DNA copies integrated into transgenic lines ranged from 1 to 4. For example, lines 2 and 4 have single copy insertion, lines 1, 5, 6, 7, 8 and 9 have two copies, line 3 has about four copies (Fig. 2b). These results indicated that all the transgenic plants are from independent transgenic events, as they have different copy numbers of T-DNA integrated into different positions of the genome. Using this protocol, we regenerated 78 transgenic lines (each line came from one explant) in three independent experiments. On average, 26 transgenic lines (plants) were obtained from every 100 leaf explants (Table 2).

In summary, a highly efficient *Agrobacterium*-mediated leaf disc transformation method was established for *T. halophila*. This method could be used as a supplementary method to the widely used “floral dip” method, especially in complementation experiments for *T. halophila* mutants that are male or female sterile.

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