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A large insert *Thellungiella halophila* BIBAC library for genomics and identification of stress tolerance genes

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Abstract Salt cress (*Thellungiella halophila*), a salt-tolerant relative of *Arabidopsis*, has turned to be an important model plant for studying abiotic stress tolerance. One binary bacterial artificial chromosome (BIBAC) library was constructed which represents the first plant-transformation-competent large-insert DNA library generated for *Thellungiella halophila*. The BIBAC library was constructed in *Bam*HI site of binary vector pBIBAC2 by ligation of partial digested nuclear DNA of *Thellungiella halophila*. This library consists of 23,040 clones with an average insert size of 75 kb, and covers 4× *Thellungiella halophila* haploid genomes. BIBAC clones which contain inserts over 50 kb were selected and transformed into *Arabidopsis* for salt tolerant plant screening. One transgenic line was found to be more salt tolerant than wild type plants from the screen of 200 lines. It was demonstrated that the library contains candidates of stress tolerance genes and the approach is suitable for the transformation of stress susceptible plants for genetic improvement.

Keywords BIBAC library · Salt cress · Salt tolerance · *Thellungiella halophila*

Abbreviations

BIBAC	Binary bacterial artificial chromosome
T. halophila	*Thellungiella halophila*
MS	Murashige and Skoog
PFGE	Pulsed-field gel electrophoresis
GUS	Glucronidase
HMW	High-molecular-weight
LMP	Low-melting-point
DTT	Dithiothreitol

Introduction

Abiotic stresses, such as drought, salinity, extreme temperatures, chemical toxicity and oxidative stresses are serious threats to agriculture and result in the deterioration of the environment. Salinization is becoming one of the primary causes of crop loss worldwide. In order to survive stresses, different plant species adapt multiple stress-related signaling pathways that activate gene transcription and its downstream machinery, involving responses to cellular osmotic and ionic stresses and their consequent secondary stresses (Zhu 2001, 2003; Xiong and Zhu 2002). Most of the researches on plant salt tolerance have been done in the model plant *Arabidopsis*, however, *Arabidopsis* as glycophyte for study salt tolerance is limited for its low survival capacity under salt stress.

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Thellungiella halophila (*T. halophila*), a close relative of *Arabidopsis*, is a halophyte which can survive sea-water-level salinity and complete its life cycle in presence of 500 mM NaCl far beyond the capacity of *Arabidopsis* (Inan et al. 2004). It is becoming a genetic model system for the salinity tolerance study because it is similar to *Arabidopsis* in terms of growth, development and also DNA sequences. Furthermore, like *Arabidopsis*, transformation of *T. halophila* can be accomplished by the floral dipping method (Bressan et al. 2001; Zhu 2001; Inan et al. 2004; Wong et al. 2005). Using a comparative genomic approach, gene expression profiles of salt cress were analyzed with a full-length *Arabidopsis* cDNA microarray. The results indicated that many previously characterized salt-stress genes of *Arabidopsis* were differently regulated in salt cress and only a few salt cress genes were salt inducible. Salt tolerance of *T. halophila* may be due to constitutive expression of many genes that function in stress tolerance but they are stress inducible in *Arabidopsis* (Taji et al. 2004, 2008; Kant et al. 2006). *T. halophila* has specific root ion-channel features, and accumulates less sodium and retains more potassium than *Arabidopsis* (Volkov and Amtmann 2006). And the population of *T. halophila* transport genes is approximately 1.5 times the size of those in *Arabidopsis*, regulating a unique ion transportation system (Taji et al. 2008). Improving salt tolerance of plants by genetic means has been an important aim of modern agricultural development (Yamaguchi and Blumwald 2005). Heterologous expression of *T. halophila* H^+*-PPase* or *ThCYP1* improved the salt tolerance of tobacco plants (Gao et al. 2006; Chen et al. 2007; Duan et al. 2007). Overexpression of an H^+*-PPase* gene from *T. halophila* in cotton can enhance plants tolerance to salt and improve growth and photosynthetic performance (Lv et al. 2008).

Despite these achievements, our knowledge about the genetics and genomics of salt cress is still limited, restricting our ability to dissect the molecular mechanisms underlying salt tolerance of salt cress.

BIBAC system with high-molecular-weight DNA had been developed not only for genome research as a BAC library, but also for facilitating gene discovery and functional studies by direct transformations of BIBACs carrying genes or gene clusters into plants via *Agrobacterium* (Hamilton et al. 1996; Hamilton 1997; Tao et al. 2002). BIBAC libraries for many plant species have been established (He et al. 2003; Song et al. 2003; Lichtenzveig et al. 2005; Ortiz-Vazquez et al. 2005; Feng et al. 2006; Li et al. 2007; Vega et al. 2008). In order to identify key salt tolerance-related genes, a BIBAC library was constructed which was the first large-insert library of *T. halophila*. Here we present the construction and characterization of the plant-transformation-competent *T. halophila* BIBAC library. BIBAC clones which have inserts over 50 kb were selected and transformed into *Arabidopsis*. Transgenic plants were obtained for salt tolerant analysis. We propose clusters of salt stress-related genes of *T. halophila* may confer salt tolerance to transgenic *Arabidopsis*.

Results

T. halophila is extremely salt tolerant

For the functional genomic purpose, we have tested the salt tolerance of *T. halophila* compared with *Arabidopsis* under plate growth condition. Three-week-old seedlings of *T. halophila* and *Arabidopsis* germinated on Murashige and Skoog (MS) medium were transferred to the same medium plus 500 mM NaCl. After 5 days of treatment, complete chlorosis was observed in all *Arabidopsis* seedlings. In comparison, salt cress seedlings were not significantly affected (Fig. 1). This indicated that *T. halophila* plants could be tolerant to higher salt stress than *Arabidopsis* on

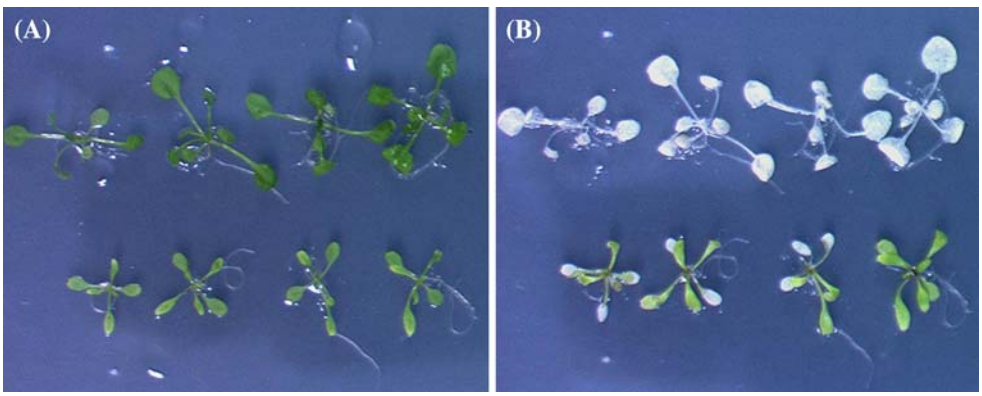

Fig. 1 Salt tolerance assay of *Arabidopsis* and *T. halophila*. Three-week-old seedlings of *Arabidopsis* (*upper part*) and *T. halophila* (*lower part*) seedlings in MS medium were transferred to same medium plus 500 mM NaCl to test salt tolerance. **a** Before transferring. **b** Five days after transferring

plate and this may provide a good condition for genetic analysis of *T. halophila* plants.

Construction and characterization of *T. halophila* BIBAC library

A large-insert BIBAC library was constructed from *Bam*HI partially digested nuclear DNA of *T. halophila* in the binary vector pBIBAC2. Transformation efficiency was estimated to have 1×10^5 clones per microgram of ligated DNA, using self-made electrocompetent cells *E. coli* DH10B. A total of 23,040 clones were duplicated and arrayed in 60 × 384-well plates and maintained in −80°C. To estimate the insert size of *T. halophila* BIBAC library, more than 1000 clones were randomly selected and analyzed by *Not*I digestion and pulsed-field gel electrophoresis (PFGE) (Fig. 2b). Over the 1000 clones analyzed, 331 clones were found to have no inserts, suggesting that 67% (approximately 15,436) of 23,040 clones in the library have DNA inserts. For further analysis of the insert size, fifty of those clones with insert were digested with *Bam*HI and separated by a 1% agarose gel in 1× TBE buffer (Fig. 2c). The insert size of clones was estimated using the lambda DNA and cloning vector fragment as the molecular-weight standards. Majority of clones had inserts size ranged from 50 to 100 kb and average size is about 78 kb, while most of clones contain insert size are between 81 and 90 kb (Fig. 2d). The genome size of *T. halophila* with 7 chromosomes is approximately twice of *Arabidopsis* (∼300 Mb), thus by calculation, our BIBAC library represents a genome coverage of 4× equivalent of the *T. halophila* haploid genome.

Stability of large-insert BIBAC clones in *E. coli* host is critical for long-term maintenance and use of a library for genome research and transformation. The intact recombinant plasmid is necessary for plant transformation as deletion of any functional fragments in BIBAC plasmid will affect or abolish the successful transformation. To test the stability of *T. halophila* BIBAC library, three random selected clones has been maintained for 7 days in *E. coli*. DNA was isolated from each clone on 1st, 2nd and 7th days of growth, digested with *Bam*HI, and fractionated on agarose gel. No restriction pattern changes were observed with all three tested BIBAC clones (Fig. 3). This indicates that the large-insert BIBAC2 clones are highly stable in *E.coli* host cells.

Transferring large DNA fragments of *T. halophila* to *Arabidopsis*

One of important purposes is directly transferring large insert DNA of *T. halophila* to other stress sensitive plants to screen the stress tolerant gene(s). We choose

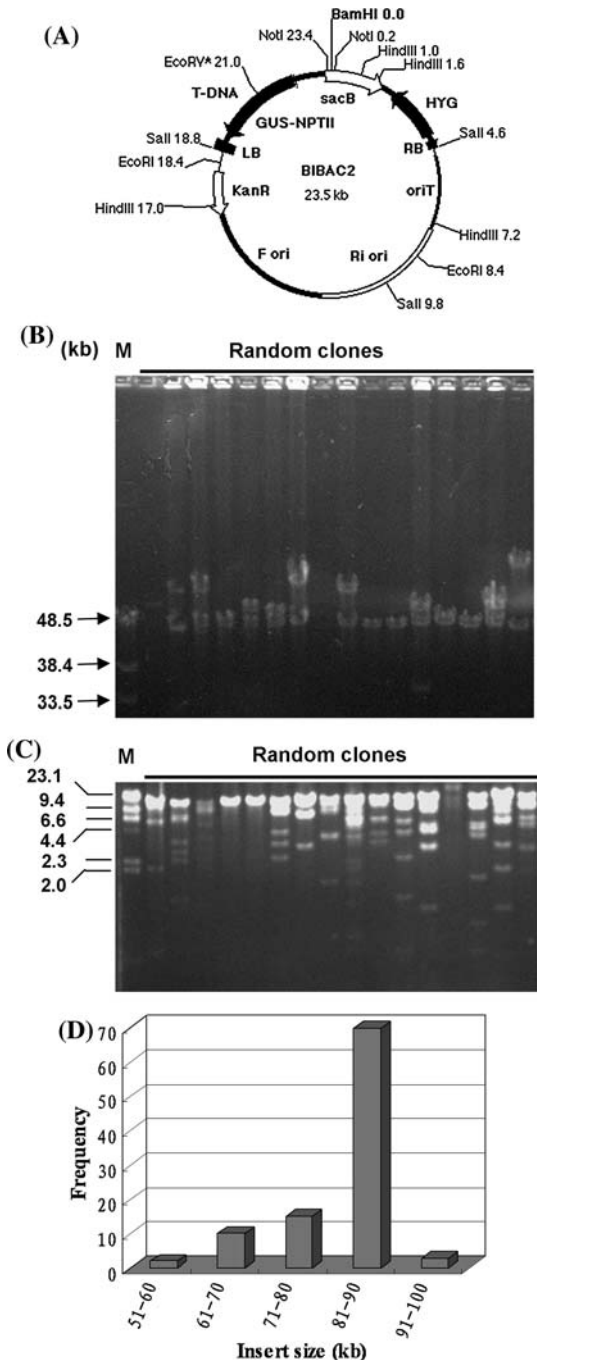

Fig. 2 Analysis of insert size of randomly selected clones from the *T. halophila* BIBAC library. **a** Schematic representation of BIBAC2 vector. **b** BIBAC clones digested with *Not*I and separated in pulsed-field gel, stained with ethidium bromide and photographed. **c** BIBAC clones digested with *Bam*HI and separated by a 1% agarose gel. The band(s) appearing in all *lanes* was from the cloning vector-BIBAC2 and the remaining bands in a *lane* were inserts. **d** Insert size distributions of 50 clones randomly selected from the BIBAC library

Arabidopsis as a host plant to be transformed and screened since *Arabidopsis* is well known in genetics and easy to be transformed. Floral dipping method has been widely used

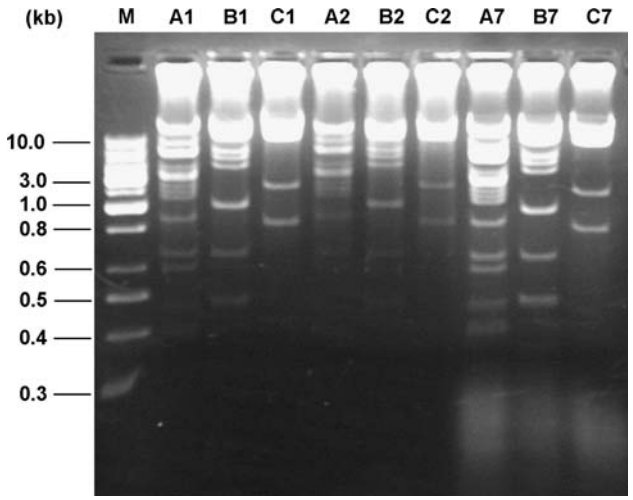

Fig. 3 Stability of *T. halophila* BIBAC clones. DNA isolated from 1-day (1), 2-day (2) and 7-day (7) cultures of each clone was digested with *Bam*HI and fractionated on an agrose gel. *A*. *B* and *C* indicates different clone. *M* indicates 1 kb DNA marker

to transform binary vectors less than 30 kb to *Arabidopsis*, but no reports on transferring the larger T-DNA fragment by this method. To test whether the floral dipping method can be used, the BIBAC clones confirmed to have inserts ranged from 50 to 100 kb were introduced into *A. tumefaciens* EHA105 for *Arabidopsis* transformation. T_1 generation seeds were collected and transformants were selected on MS agar plates containing kanamycin (Fig. 4a), since the kanamycin selection marker gene *NPTII* is adjacent to the left border. For each transformation, one set of 12 *Arabidopsis* plants was used. Of a total 210 BIBAC clones transformed to *Arabidopsis*, kanamycin-resistant lines had been obtained from 185 clones (~88%).

The transformation efficiency for single BIBAC clone is variable from 0.05 to 0.12% of total T_1 seeds, which is much lower than that of binary vector transformation by floral dipping method in *Arabidopsis* (Clough and Bent 1998). It was demonstrated previously that positive PCR assays with two flanking markers *(NPTII* and *hpt*) were a good indicator of complete T-DNA transfer (Frary and Hamilton 2001). The *sacB* gene which located near the right border and the *NPTII* gene which located near the left border were amplified successfully from all kanamycin resistant plants, respectively (see below). The backbone of BIBAC vector also contains *Glucronidase* (*GUS*) gene flanking to *NPTII*, thus transgenic plant should have GUS activity if T-DNA stably integrates into *Arabidopsis* genome. Transgenic plants grown in selection medium were tested for GUS activity and GUS expression was detected in all tissues of all kanamycin-resistant transgenic plants (Fig. 4b). So we conclude this method is useful to transfer larger DNA fragment to *Arabidopsis*.

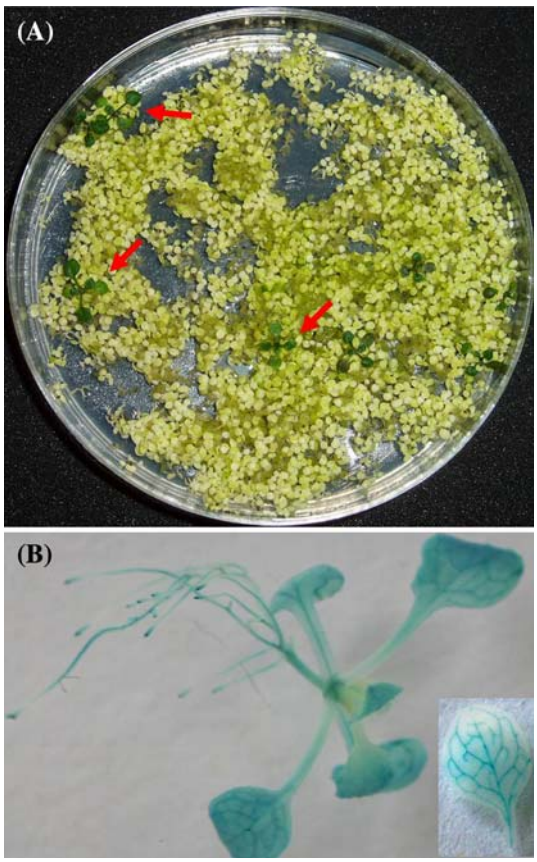

Fig. 4 Selection of BIBAC *transgenic lines*. **a** T_1 seeds were germinated on MS medium plus 50 mg/l kanamycin. Photographed after 3 weeks of post-germination. Those green leave seedlings indicated by arrows are putative transgenic plants. **b** GUS expression pattern in transgenic *Arabidopsis* seedlings

Salt tolerance screening of *Arabidopsis*-BIBAC transgenic plants

To explore salt tolerance-related gene(s) of *T. halophila*, we examined whether BIBAC clones can improve salt tolerance of transgenic plants. For each BAC clone we keep at least 5 independent T_1 transgenic plants to collect seeds for further analysis. The T_2 *Arabidopsis* transgenic seedlings, from at least 5 individual lines, were grown on MS plates containing 175 and 200 mM NaCl, respectively. Of total 200 BAC clones screened, all 5 lines obtained from a BIBAC clone cataloged as BAC256, about three-quarter of seedlings (follow Mendel segregation ratio) from each of 3 lines proved to be more salt tolerant than wild type plants at two different concentration of NaCl tested. For the other two lines, most seedlings (>90%) of each line showed similar higher tolerance to salt. This could be due to multiple copies of T-DNA insertion in transgenic lines. Thus we tested kanamycin resistance of all 5 lines, the percentage of kanamycin resistant seedlings is correlated very well to that of salt tolerance phenomenon observed in

each line. This result indicated each of the three lines contains one T-DNA insertion and the other two lines might contain at least two insertions and the insert of the BAC256 might contribute the salt tolerance.

Next the salt tolerance assay was done using seeds of one of the homozygous line plants. As shown in Fig. 5a, under normal growth condition, no difference was found between BAC256 and wild type plants. However, germination rates of BAC256 were higher than those of wild type plants in medium plus different concentration NaCl (Fig. 5a). Furthermore, 2-week-old BAC256 seedlings showed enhanced salt tolerance compared with wide-type plants and another transgenic line BAC27 (Fig. 5b). When the treatment of the transgenic plant with NaCl in soil was done, leaves of wild type plants become yellow earlier, and there are higher rate of sensitive plants (56.1%) of wild type plants than that of BAC256 (29.5%) (Fig. 6). The results of both on plate and in soil treatment indicated that BAC256 contains salt tolerance-related gene(s). The above results also demonstrated that BIBAC clones of *T. halophila* can improve salt tolerance of plants.

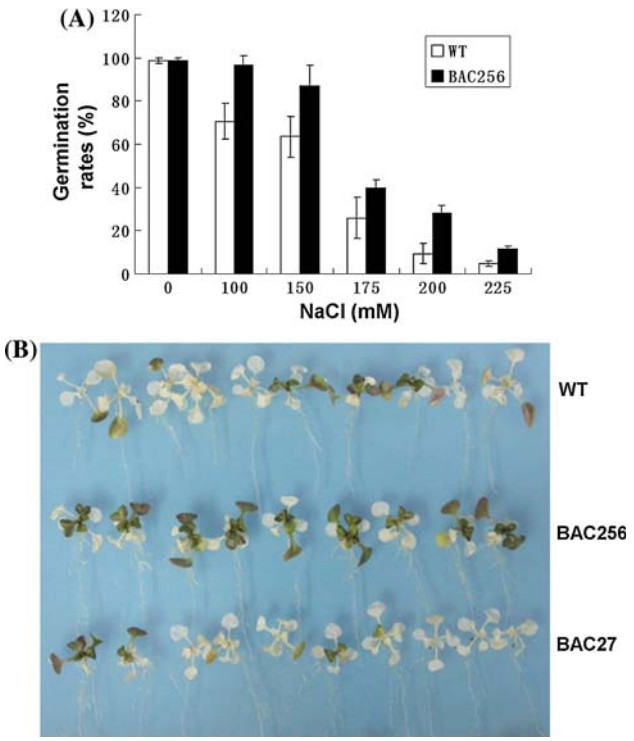

Fig. 5 Salt-treatment assay of wild-type and transgenic BIBAC *Arabidopsis*. **a** Germination rates (radical emergence) of wild type and BAC256 on MS medium containing 100, 150, 175 or 200 mM NaCl. Percentages are means ($n = 60$ to 90 each) of three repeats ± SD. **b** Growth phenotype of BAC256, wild type plants and another BAC transgenic plant BAC27 with 200 mM NaCl treated. Two-week-old plants were transferred to MS medium added with NaCl for 1 week and photograph was taken

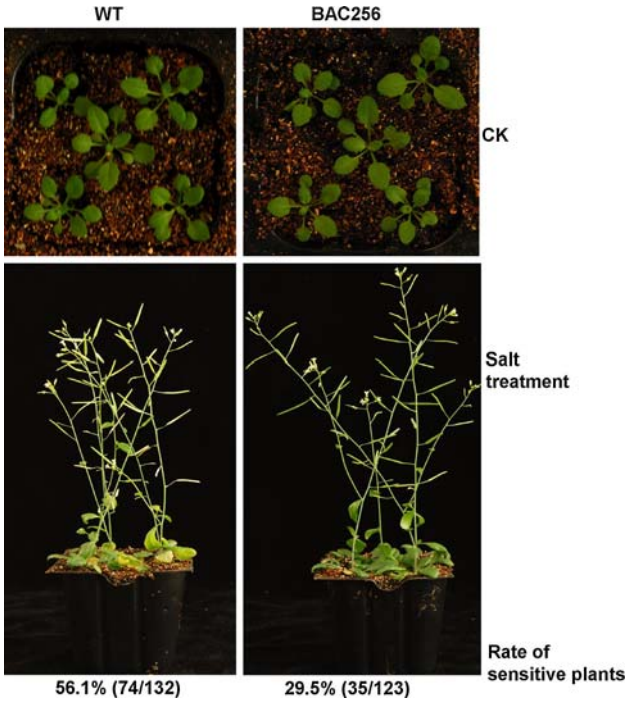

Fig. 6 Phenotype comparison of BAC256 and wild type plants with NaCl treated in soil. Plants with withered and yellow leaves are considered sensitive plants. CK, 3-week-old plants under standard growth condition. Salt treatment, plants treated with gradient NaCl (50-100-150-200 mM)

Restriction enzyme analysis disclosed the BIBAC clone Th00256 (BAC256) containing DNA fragment about 120–130 kb. Transgenic lines from BAC256 were analyzed to examine whether large T-DNA was integrated into the plant genome by PCR and genomic Southern blot assay. Expected PCR products, the *sacB* gene which located near the right border and the *NPTII* gene which located near the left border were successfully amplified (Fig. 7a). Genomic DNA from transgenic line BAC256 or BAC27 was isolated, digested with *Eco*RI, and hybridized with the probe generated from the insert of BAC256 or BAC27. Different BIBAC-probe specific hybridized bands were observed in both transgenic lines (Fig. 7b, c). The result indicated that the insert was transferred into the *Arabidopsis* genome. Both PCR and Southern blot analyses demonstrated the salt tolerant transgenic *Arabidopsis* contain large DNA fragment from *T. halophila*. The different transgenic lines from the same BIBAC clone all showed salt tolerance, which excludes the possibility that salt tolerance effect was produced by T-DNA insertion mutation.

Discussion

BIBAC libraries have been reported for many plant species, including tomato, wheat, soybean, *Brassica spp.*, rice,

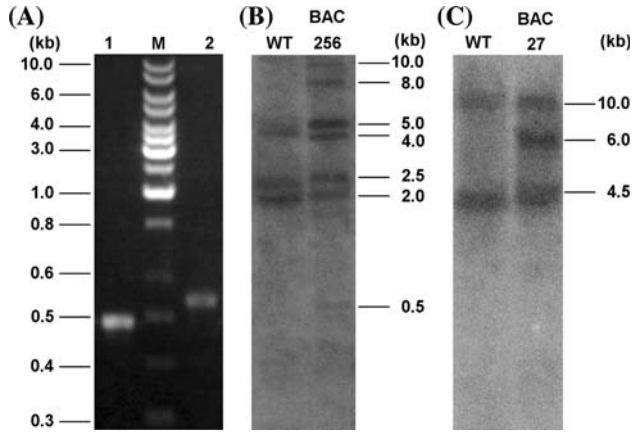

Fig. 7 Transgenic plants assayed by PCR and Southern blot analysis. **a** PCR analysis of genomic DNA to detect the presence of the *sacB* and *NPTII* genes. *M* Molecular weight markers. *1*. *sacB* gene. *2 NPTII* gene. **b** Genomic DNA from wild type plants and BAC256 were digested with *Eco*RI, the insert was used as probe for Southern hybridization. **c** Genomic DNA from wild type plants and BAC27 were digested with *Eco*RI, the insert was used as probe for Southern hybridization

Arabidopsis, potato, banana, chickpea and maize. Here we constructed a BIBAC library for the *T. halophila* Shandong ecotype in the *Bam*HI site of BIBAC2 and successfully transferred large inserts into *Arabidopsis* for screening salt tolerant plants. BIBAC2 was designed to transform high-molecular-weight DNA into plants, facilitating map-based gene cloning, functional analysis of genomic sequence and genetic engineering (Hamilton et al. 1996; Hamilton 1997). To construct the BIBAC library, nuclei from plant tissue were isolated using method described by Zhang et al. (1995), which can decrease the abundance of small DNA fragments and contamination of organelle DNA. This library consists of 23,040 clones with an average insert size of 75 kb, and covers 4× *Thellungiella halophila* haploid genomes. Furthermore, the large-insert BIBAC2 clones are highly stable in *E.coli* host cells, which is very critical of long-term maintenance and use of a library.

This is the first time to screen salt tolerant plants by transforming large DNA fragment using functional genomics approach and BAC256 plants showed higher salt tolerance than control plants. At the genetic level, salinity tolerance has been considered to be a quantitative trait (Flowers et al. 2000; Koyama et al. 2001; Quesada et al. 2002; Kumari et al. 2009). A cluster of genes may be in the same signal pathway and regulate in a coordinate mechanism (Staskawicz et al. 1995; Ning et al. 2008). Using this library, we may not only find salt-related gene(s) or genes in cluster, but also we can clone salt-related key genes using map-based cloning. *T. halophila* and *Arabidopsis* are all belonged to Cruciferae family, and very good colinearity has been found to exist between several Cruciferae members. From some BIBAC clones sequence result (Deng et al. 2009), we also found that *T. halophila* and *Arabidopsis* have high degree of gene sequence identity and microsynteny. Using *Arabidopsis* genome sequence and many polymorphic DNA markers, we can clone key salt-related loci by map-based cloning way dependent on comparative genomics. Furthermore, using this BIBAC library to map salt-related genes is very useful because it does not require precise salt-related genes positions. The BIBAC-based contigs can cover relatively wide chromosome region with just a few clones. Once a salt-related gene is mapped, the BIBAC clones in the same contig could be transformed individually and find complementing clone easily.

In a word, using the BIBAC library, we could identify more important salt-related genes in *T. halophila* by functional complement and comparative genomics in the future.

Materials and methods

Preparation of BIBAC2 vector and high-molecular-weight (HMW) DNA from nuclei

The BIBAC2 vector was purified by the alkaline-lysis method, followed by using Qiagen Max-Prep Kits. The vector DNA was completely digested and dephosphorylated as described previously (Zhang et al. 1996). Twenty grams of tissue were collected from the aerial part of 20-day-old *T. halophila* plants, frozen in liquid nitrogen, and stored at −80°C. The tissue was grinded in liquid nitrogen, nuclei were isolated, and HMW nuclear DNA was prepared and purified in 1% low-melting-point (LMP) agarose plugs (Zhang et al. 1995).

BIBAC library construction

Eight 1% plugs containing HMW DNA were sliced into small piece of equal size at nine pieces per plug. The plug slices were equilibrated in 24 ml of 1× reaction buffer 3 (Life Technologies, USA) containing 2 mM of spermidine and 1 mM of dithiothreitol (DTT) on ice for 1 h, with one change after 30 min. The plug slices were then transferred into 24 1.5-ml tubes, three slices per tube, each tube containing 170 µl fresh 1× reaction buffer containing 0.5 mg/ml of BSA, 2 mM of spermidine and 1 mM of DTT. The 24 tubes were divided into three groups on ice, eight tubes per group, and to each group, 0.4, 0.6 and 0.8 units of *Bam*HI were added per tube, respectively. The tubes were incubated on ice for 60 min to allow the enzyme access to the DNA in the plug slices. The digestion was conducted at 37°C for 8 min and stopped by adding one tenth volume of

0.5 M EDTA, pH 8.0. DNA fragments ranging from 50 to 100 kb were selected by pulsed-field gel electrophoresis (PFGE) using the CHEF DRIII apparatus (BioRad, USA). The size-selection was conducted on a 1% agarose gel in 0.5× TBE buffer with a 90-s pulse time at 6 V/cm, 14°C for 24 h. The agarose-gel zones containing DNA fragments from 50 to 100 kb were excised. The DNA was eluted from the gel slices into dialysis tubing by PFGE at 6 V/cm, 40-s pulse time and 14°C for 4 h, followed by reversing the polarity of the currency for 60 s. The DNA was collected carefully with wide-pore tips. The eluted DNA was dialyzed in the same tube in 0.5× TE buffer (5 mM of Tris–HCl, pH 8.0, 0.5 mM of EDTA, pH 8.0) at 4°C for 4 h, with one buffer change per hour. The DNA was collected and ligated to the vector DNA at a molar ratio of 1:4 at 16°C for 18 h using T4 DNA ligase (Life Technologies, USA). The recombinant DNA was used to transform into *Escherichia coli* DH10B competent cells using the Cell Porator and Voltage Booster electroporation system (Life Technologies, USA) using 375 V, 330 uF capacitance, low ohms impedance and a fast charge rate, and 4 kΩ resistance. The electroporated cells were incubated in 1 ml of SOC medium at 37°C for 1 h, plated onto LB agar medium containing 50 mg/l of kanamycin and 5% (w/v) sucrose, and then incubated at 37°C for 24 h. Clones were arrayed in 384-well microtiter plates containing a mixture of LB medium and 1× freezing buffer [36 mM of K_2HPO_4, 13.2 mM of KH_2PO_4, 1.7 mM of Na citrate, 0.4 mM of $MgSO_4$, 6.8 mM of $(NH_4)_2SO_4$, 4.4% (v/v) glycerol] with 50 mg/l of kanamycin. The clones were incubated at 37°C for 20 h and then stored at −80°C.

Analysis of BIBAC clones

BIBAC clones were randomly selected from the library and grown in LB medium containing 50 mg/l of kanamycin at 37°C overnight. DNA was isolated and some insert DNA was released from the vector by digestion with *Not*I (New England Biolabs, USA) and subjected to PFGE on 1% agarose gels in 0.5× TBE at a 5-s initial pulse time, a 15-s final pulse time, at 14°C, and 6 V/cm for 18 h. The others were digested by *Bam*HI (New England Biolabs, USA) and run on 1% agarose gels in 1× TBE.

Plant transformation with BIBAC clones

Plant seeds (*Arabidopsis* ecotype Columbia) were surface-sterilized and placed on agar plates containing 1/2 MS salts, B5 vitamins and 1.5% sucrose. The plates were kept in the dark at 4°C for stratification for 2 days and then transferred to 22°C with 16/8 h light/dark cycle. *Agrobacterium*-mediated transformation was performed via floral dip of flowering *Arabidopsis*. Transgenic plants were selected by placing seeds of the infected plants on plates containing 1/2 MS salts, B5 vitamins, 1.5% sucrose and 50 mg/l kanamycin. Plants were subsequently selected with kanamycin for two generations to identify homozygous plants.

Genomic DNA analysis

Potential transgenic plants were initially tested by PCR using primers for the *NPTII* and *sacB* genes. Primers used were as the following: *NPTII* forward, 5′-TCGGCTAT GACTGGGCACAACA-3′ *NPTII* reverse, 5′-AAGAAG GCGATAGAAGGCGATGCG-3′; *sacB* forward, 5′-GGTC GGCGACAACTCAATCCACAG-3′; *sacB* reverse, 5′-GC GGCACTGCGAAGTGAGAGTAAG-3′. Plants that were tested positive for the BIBAC T-DNA by PCR were all verified by Southern analysis using the whole insert. For Southern hybridization, approximately 10 μg of genomic DNA was digested with *Eco*RI. Digested DNA was separated on 1% agarose gels with 1× TBE buffer. DNA was transferred to Hybond N^+ membrane using the alkaline transfer protocol provided by the manufacturer (Amersham). The probe was prepared using Redprimer II kit (Amersham).

Histochemical assays for GUS enzyme activity

Histochemical assays for GUS enzyme activity were performed as described by Jefferson (Jefferson et al. 1987). Plants was incubated in GUS assay solution (100 mM sodium phosphate [pH 6.8], 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide), 10 mM EDTA, 0.1% triton X-100, 20% methanol and 1 mM 5-bromo-4-chloro-glucuronide) at 37°C for 8–16 h. Pigments in the tissue were removed by 70% ethanol for photographic analysis.

NaCl tolerance assay

Seeds were sterilized and suspended in 0.15% agar and plated on MS medium supplemented with 1.5% sucrose, then stratified in darkness at 4°C for 3 days and transferred to growth chambers. To conduct germination rate assay, seeds were plated on MS medium without or with NaCl 100, 150, 175, 200 and 225 mM). For salt stress assay of seedlings, 3-week-old seedlings of both *T. halophila* and *Arabidopsis* ecotype germinated on MS plates were transferred to the same medium plus 500 mM NaCl or 2-week-old seedlings were transferred to MS medium added with 200 mM NaCl. For salt stress assay of 3-week-old plants in soil, the gradient NaCl (50-100-150-200 mM) was added to soil every 5 days.

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