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An Efficient Method to Screen for Salt Tolerance Genes in Salt Cress

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1. Introduction

Arabidopsis thaliana is the most widely used model organism in plant molecular biology (Bressan et al., 2001) and it is an ideal model system for many reasons. Arabidopsis can be cultured in solid and liquid media and in soil. Greenhouses and growth chambers are suitable for Arabidopsis growth, meaning that different environmental conditions can be selected. Compared with crop plants, such as rice, wheat and tomato, Arabidopsis is more attractive due to its small size, high fecundity and short life cycle. Arabidopsis can be stably transformed using *Agrobacterium tumefaciens*-mediated transfer of T-DNA. Using the vacuum-infiltration procedure, transformants can be obtained at high efficiency. The small genome size of Arabidopsis meant that it was the first model plant to have its whole genome sequenced. Mutant lines, especially T-DNA insertion lines, of most Arabidopsis genes are obtained easily from several large seed stock libraries around the world. In conclusion, as a plant genetic model, Arabidopsis has played a significant role in characterizing the biological functions of plant genes, including salt stress-related genes.

Nevertheless, Arabidopsis is a typical glycophyte in that does not display tolerance to intense salt stress. Thus, to solve this problem, a halophytic model system needs be developed. Any new model plant must provide experimental expediency similar to that of Arabidopsis. Salt cress (*Thellungiella salsuginea*), which is closely related to Arabidopsis, has emerged as a candidate (Amtmann, 2009). Like Arabidopsis, salt cress meets certain criteria that are important for any model plant, such as small size, short life cycle, self-pollination, high seed number, small genome and efficient transformation. Salt cress can withstand dramatic salinity shocks up to 500 mM NaCl and it can grow in high salt environments that are lethal to Arabidopsis (Bressan et al., 2001). Salt cress does not produce salt glands or other complex morphological alterations either before or after salt adaptation. Expressed-sequence tag (EST) analyses of several hundred salt cress clones have shown that there is approximately 90 to 95% identity between salt cress and Arabidopsis cDNA sequences

(Zhang et al., 2008). As a result, more than 10 years ago, salt cress started to be used for studies examining the mechanisms underlying salt tolerance.

2. Importance

Forward and reverse genetic studies of salt cress were encumbered by a lack of genomic information and poor mutant line storage. So, a new method is required that combines the advantages of Arabidopsis and salt cress, while avoiding any important disadvantages. Here, an overexpression system is presented, which has been used previously in Arabidopsis (LeClere and Bartel, 2001). A similar system has been development by another group independently to mine stress tolerance genes from salt cress (Du et al., 2008). In our lab, a cDNA library of salt cress was generated from salt-treated seedlings and that was driven by double cauliflower mosaic virus (CaMV) 35S promoters. Using *A. tumefaciens*-mediated transformation, the salt cress cDNA library was randomly overexpressed in Arabidopsis. T1 transgenic plants were grown in soil, treated with NaCl and the survival rates of transgenic lines were monitored. The salt cress cDNAs expressed in these lines were identified by PCR amplification and sequencing. To confirm the initial screening results, both salt cress genes and their homologs in Arabidopsis were re-overexpressed in Arabidopsis Salt tolerance ability of the off-spring of these re-transformed lines and mutants of the homologous genes in Arabidopsis was examined. After this screening, candidate genes were chosen for further investigation of their biological functions.

3. Results

3.1 Generation of salt cress cDNA library

Binary Ti vectors are the plasmid vectors used for the *Agrobacterium*-mediated plant transformation because they are able to replicate in both the *E. coli* and *Agrobacterium* species. High level efficiency of *in vivo* recombination is needed for the construction of cDNA libraries, but the large size of the common binary Ti vectors limit the *in vivo* recombination efficiency. Moreover, the size of binary Ti vectors needs to be limited to allow for large pieces of DNA to be transferred into plants. Plasmid manipulations and cDNA library construction are also easier if the vectors replicate in *E. coli* to high copy number. A binary Ti vector with double cauliflower mosaic virus (CaMV) 35S promoter and NOS terminal, named pGreen, fulfilled these demands and was used in the present study (Hellens RP, 2000). To keep the size of pGreen to a minimum, *RepA* and *Mob*, which were necessary for plasmid replication in *Agrobacterium*, were removed. The cloning site of pGreen was based on the well known plasmid, pBluescript, meaning that it was relatively simple to rearrange selective marker and reporter genes.

For wide coverage of salt cress transcripts in relation to salt stress, whole plants were collected at different time points after NaCl treatment. Total mRNA was purified from a mixture of plant tissues and at different time points the cDNA library was constructed in pGreen. Approximately 1 x10^6 colonies were collected from LB agar for the construction of the primary library. The titer of the cDNA library in the host bacteria, *E. coli* XL1-blue MRF ' (Stratagene), was ca. 1 x10^7 colonies/μL. To analyze the insert size and recombination rate in the primary library, 100 colonies were randomly selected and the plasmids were digested by *Eco*RI/*Xho*I. Insert sizes ranged from 500 bp to 2 kb (mean size of ca. 1 kb), while efficiency of recombination was ca. 89%.

3.2 Generation of a library of Arabidopsis transgenic lines overexpressing salt cress genes

Salt cress cDNAs were constructed into pGreen and driven by the double CaMV 35S promoters. A selectable marker gene (*NPTII*) was chosen to identify positively transformed plants. As *RepA* and *Mob* were removed from pGreen, it was unable to replicate in the *Agrobacterium*. To remedy this problem, a co-resident, pSoup, was used to provide the replication function *in trans* for pGreen.

The plasmids isolated from the cDNA library were mixed with pSoup and introduced into *Agrobacterium* strain EHA105. Wild type Arabidopsis plants were transformed and T0 seeds were sown. With efficient kan resistance selection, a total of 2,000 individual transgenic lines were obtained and T1 seeds of these lines were harvested.

3.3 Screening for salt tolerant lines from the transgenic Arabidopsis library

High salt stress has multiple adverse effects on plant growth and development, such as inhibition of seed germination, retardation of plant growth and acceleration of senescence. To evaluate whether a plant is tolerant to salt, the following parameters can be monitored: seed germination, root elongation, fresh weight increase and survival rate.

Seed germination rate has been used for isolating abiotic stress tolerant or abscisic acid insensitive genes in many previous reports. However, seed quality and storage time can influence germination rate, in spite of their genetic background. In addition, fast germination is not necessary or sufficient for salt tolerance, as some lines that germinate faster than the wild type plants under high salinity conditions ultimately were found to be more sensitive to salt stress. Thus, germination rate is not sufficient for identifying salt tolerant plants. Another method for screening for salt tolerance plants is to germinate seeds on media containing salt and then monitor growth or survival rates. This method is better than the seed germination method. However it needs a consistent treatment and high quantities of seeds. Root elongation is a parameter used widely for the identification of salt stress tolerant plants. Seeds are germinated on agar plates without salt. Then, 4-days-old seedlings are transplanted onto new agar plates containing the desired concentrations of salt. Finally, root length is measured at several time points to calculate the growth rate. However, while this method is efficient for identifying salt sensitive mutants, it is not able to identify salt tolerant mutants. If using the rate of fresh weight increase as the salt tolerance parameter, the plants should be grown in liquid medium and be kept alive after being weighed. This method is time consuming and unsuitable for high-throughput screening.

Therefore, a new strategy had to be applied to avoid these disadvantages. To reduce the influence of seed quality on plant growth, plants were grown in soil before salt treatment. Among all the parameters used to evaluate salt tolerance ability, the most important one was selected in this present study, specifically survival rate. If most lines contain a single insertion, 75% of transgenic plants would contain one copy of an expressed salt cress gene. When this gene is related to salt tolerance, 75% of plants should have the phenotype. To save screening time. T1 plants were analyzed because salt cress cDNA would be expressed in approximately 75% of T1 plants.

A high-throughput screen system was set up to isolate transgenic lines with the ability to tolerate high salinity. Using wild type plants as the control, seeds of transgenic lines were germinated on MS plates and then transplanted into soil. Approximately three weeks after

germination, plants were treated with 200 mM NaCl. It was a robust screening, as most wild type plants died. The lines with a survival rate of greater than 70% were identified to be putative salt tolerance lines because about 25% of the T1 plants were wild type. We routinely screened more than 1,000 lines in soil and yielded a total of 20 candidate lines.
For confirming the salt tolerant ability of the candidate lines, the T2 seeds of each line were subjected to a secondary screen. Plasmolysis often occurs when plants are treated with high salinity without pre-conditioning. These plants tend to die of plasmolysis, rather than ionic or osmotic toxicity. This situation was avoided by adding salt in steps of 50 mM to allow the plants to adjust to the increasing salinity. Ten high salt stress tolerance lines of the 20 candidate lines were confirmed at this stage.

3.4 Isolation of the inserted salt cress cDNA expressed in the salt tolerant lines and identification of homologs in Arabidopsis

After screening, the next step was to identify the salt cress genes in these salt tolerant lines. Insert sequences were amplified by PCR and sequenced. Homologous genes in Arabidopsis were identified using BLAST.

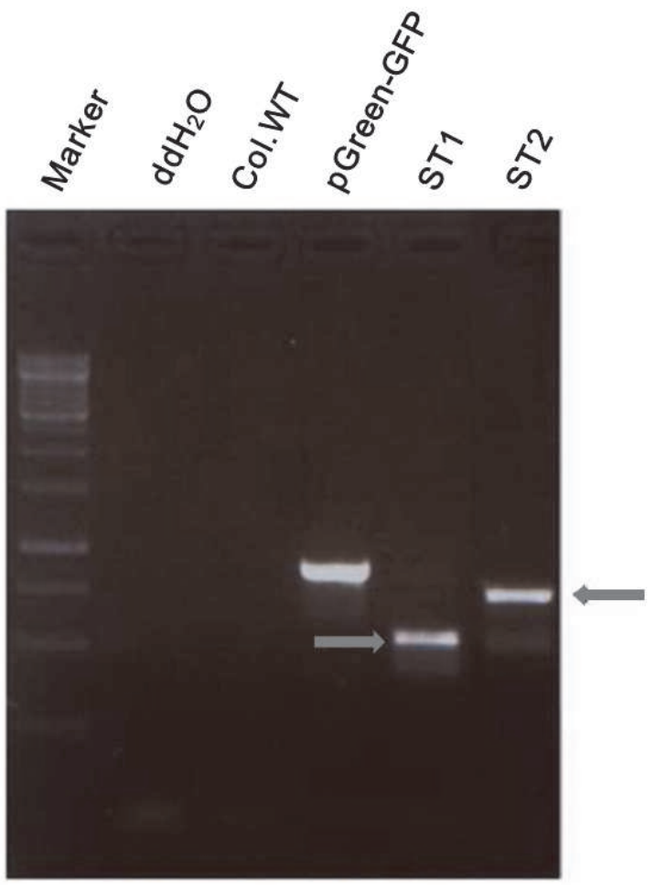


Fig. 1. Isolation of insertion sequences by PCR. ST1 and ST2 are two salt tolerant lines. The plant overexpressing pGreen-GFP was used as a positive control. Arrows indicate the amplified sequences from transgenic lines to be sequenced.

3.5 Validation of the salt-tolerance functions of the isolated candidate genes

The salt tolerant phenotype of the plants must be confirmed by other methods for the following reasons. First, environment factors, such as illumination and irrigation, are not identical for every plant, and can lead to variations among plants, even when the salt treatment is not added. Second, there is often more than one insert identified in a single transgenic line. By using the screening result alone, it is not clear which gene is responsible for the salt tolerance. Third, co-suppression can often occur. Thus, it is possible that the homologous gene in the transgenic plants is expressed at lower levels than in wild type plants, and the salt tolerant phenotype is due to reductions in the expression of these genes.

Two different strategies were adopted to solve these problems. The first approach was re-overexpression. The candidate salt cress genes and their homologs in Arabidopsis were overexpressed in the Arabidopsis wild type plants. Then the salt tolerability of these transgenic lines was checked as described above. If transgenic plants gained salt tolerant ability, the genes were considered to be related to salt stress tolerance. The second approach was to check the phenotype of homologous gene mutant lines of Arabidopsis, and RNAi lines of salt cress. If these genes were indispensable for salt tolerance, elimination or reduction of their expression level should result in increased salt sensitivity of the plants.

4. Conclusion

In this present study, a simple high-throughput method to mine salt tolerance genes from salt cress has been developed by ectopically expressing salt cress cDNA in Arabidopsis. This method does not require any genomic or cDNA sequence information for salt cress. It is convenient for gene cloning by a single PCR instead of mapping. Moreover, based on the information on homologous genes in Arabidopsis, the functional analyses of candidate salt cress genes is much simpler. Finally, gain-of-function mutants can uncover gene functions that would never be revealed by conventional loss-of-function approaches. This approach could be applied to mine genes related to interesting phenotypes in other stress or developmental conditions.

5. Materials and methods

5.1 Plant material

A. thaliana Columbia and salt cress (*T. salsuginea*) were used in this study. Seeds of Arabidopsis and salt cress were surface-sterilized with 10% bleach and then washed three times with sterile water. Sterile seeds were plated on MS medium plus 1.5% sucrose. Arabidopsis seeds were stratified in darkness for 2 to 4 d at 4 ^{o}C, while salt cress seeds were stratified for three weeks under the same conditions. Then, the seeds were transferred to a tissue culture room at 22 ^{o}C operating a 16-h light/8-h dark photoperiod. After two weeks, seedlings were potted in soil and placed in a growth chamber at 22 ^{o}C and 50% humidity operating a 16-h light/8-h dark photoperiod.

5.2 Generation of the salt cress cDNA library

For a wide coverage of transcripts related to salt stress, when salt cress plants in soil had generated four to six true leaves, they were treated with 400 mM NaCl. Leaf and root

tissues of plants were collected at 0, 15 min, 30 min, 1 h, 3 h, 6 h and 12 h. Rosettes were cut off from the plants directly and the roots were washed with water before collection. The collected tissues were mixed together, frozen in liquid nitrogen and ground to a powder. The powder was resuspended in extraction buffer (50 mM Tris-HCl, 10 mM ethylenediaminetetraacetic acid [EDTA], 2% sodium dodecyl sulfate [SDS], 10 mM LiCl; pH 6.0) and mixed with an equal volume phenol/chloroform (1:1, pH 6.0) at 65 ^{o}C. This mixture was vortexed and centrifuged at 4 ^{o}C for 15 min at 10,000 *g*. The supernatant was phenolized twice and precipitated with an equal volume of 4 M LiCl. After centrifugation, the RNA pellet was resuspended in Tris-EDTA (TE) buffer. The supernatant was phenolized again and then precipitated by adding 0.1 volumes of 3 M NaAc (pH 5.3) and three volumes of pure ethanol. The RNA pellet was washed and resuspended in TE buffer.

Total mRNA was purified, and double strand cDNA was synthesized from 5 µg of mRNA using a cDNA synthesis kit (Stratagene, CA, USA). The double-stranded cDNA, containing *Eco*RI and *Xho*I ends, was ligated into the *Eco*RI/*Xho*I-digested pGreen vector (Hellens RP, 2000). After ligation, the library was dialyzed with distilled water and electroporated into *Escherichia coli* XL1-Blue MRF' (Stratagene) .The transformants were plated on Luria Bertani (LB) agar containing 50 µg/mL kanamycin sulfate (kan).

5.3 Transformation of the cDNA library into Arabidopsis

Plasmids of the salt cress cDNA library were collected from *E. coli* XL1-Blue MRF' and electroporated into *Agrobacterium* strain EHA105 with the co-resident plasmid pSoup. The transformed agrobacteria were selected on LB agar containing 50 µg/mL kan and 100 µg/L rifampicin. All agrobacterial colonies were washed off the agar plates, resuspended and then diluted in agrobacterial infiltration medium to an OD600 of 0.7 for Arabidopsis transformation. When plants were at their peak of flowering, the Arabidopsis flowers were dipped using a vacuum infiltration method as described previously (Bechtold and Pelletier, 1998). For maintaining a high level of humidity, plants were covered for 24 h after dipping. Then, the plants were transferred to a greenhouse and allowed to grow to maturity. T0 seeds were harvested in bulk and germinated on half-strength MS medium containing 50 µg/mL kan. The positive transgenic seedlings were transplanted into soil for the production of T1 seeds.

5.4 Screening for salt tolerance lines from the transgenic Arabidopsis library

T1 seeds were surface-sterilized, germinated and cultured as detailed in the 'Plant material' Section above. For each line, 18 plants were chosen at random and transplanted into two pots. Wild type plants were used as the control. By approximately three weeks after germination, each plant had generated four to six true leaves. Then, the plants were treated with 200 mM NaCl. Normally, *A. thaliana* Columbia wild type plants are unable to withstand such a high concentration of NaCl. T1 lines with survival rates greater than 70% were chosen as salt tolerant candidates.

5.5 Confirmation of salt tolerance in the candidate lines by re-screening

To confirm the salt tolerant phenotype, T2 seeds were used. T2 seeds were surface-sterilized, germinated and cultured as detailed in the 'Plant material' Section above. Wild

type plants were used as the control. About three weeks after germination, plants had generated four to six true leaves. Then, the plants were treated with 50 mM NaCl. Salt concentration was increased by 50 mM every four days until a final concentration of 200 mM. Plants were allowed to keep on growing under these conditions. The final survival rate of salt tolerant lines would be expected to be much greater than that of wild type plants.

5.6 Isolation of the inserted salt cress cDNA and identification of homologous genes in Arabidopsis

Since salt cress cDNAs were introduced to Arabidopsis by T-DNA insertion, the flanking sequences of cDNAs were conserved in the genome of transgenic plants. Using appropriate primers (pGreen-sense: 5′-GGAACTACTCACACATTATTATGGAG-3′; and pGreen-antisense: 5′-CATTTGGAGAGGACACGCTG-3′), cDNA inserts were amplified by PCR. PCR products were cloned into T-vectors and sequenced. If more than one band emerged from the PCR as a result of multiple insertions, each band should be sequenced. According to previous studies, most salt cress genes had homologous genes in Arabidopsis. Arabidopsis homologs were identified using BLAST.

6. Acknowledgments

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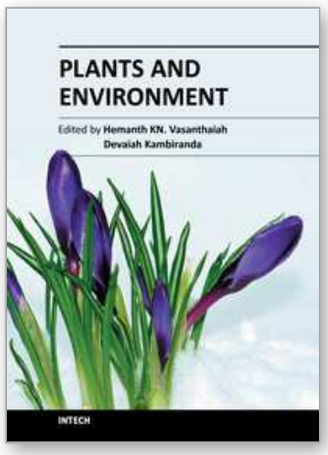
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Changing environmental condition and global population demands understanding the plant responses to hostile environment. Significant progress has been made over the past few decades through amalgamation of molecular breeding with non-conventional breeding. Understanding the cellular and molecular mechanisms to stress tolerance has received considerable scientific scrutiny because of the uniqueness of such processes to plant biology, and also its importance in the campaign â€œFreedom From Hungerâ€. The main intention of this publication is to provide a state-of-the-art and up-to-date knowledge of recent developments in understanding of plant responses to major abiotic stresses, limitations and the current status of crop improvement. A better insight will help in taking a multidisciplinary approach to address the issues affecting plant development and performance under adverse conditions. I trust this book will act as a platform to excel in the field of stress biology.

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