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ARTICLE

Identification of *Arabidopsis* and *Thellungiella* genes involved in salt tolerance by novel genetic system

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ABSTRACT High salinity is a major constraint to plant growth and development. Plants respond to environmental stresses by altering gene expression pattern via a complex signaling network. We developed a novel genetic system based on conditional cDNA overexpression to isolate genes involved in plant salt tolerance. Transformation-ready Arabidopsis and Thellungiella cDNA libraries cloned in a plant expression vector under control of an inducible promoter were used to transfer into *Arabidopsis*, where activation of the inserted cDNA can lead to conditional phenotypes. Transgenic lines were tested in different screens (germination assay, growth-survival test). Our genetic system was suitable to identify not only well-known genes coding for proteins involved in stress tolerance, but several novel regulatory genes were discovered. Line N33 shows estradiol-dependent salt tolerant germination. It has a single T-DNA insertion; the full length cDNA encodes an unknown protein. This gene was designated as *Novel Salt Tolerance* (*NSTO*). The *Thellungiella* library allowed large scale random interspecific gene transfer and subsequent identification of novel regulatory genes which control stress tolerance in halophyte species. Our data illustrate that application of inducible cDNA expression libraries provides an efficient tool for genetic identification and functional analysis of novel positive or negative regulators of plant salt tolerance.
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The terrestrial plants' growth, development and yield are greatly affected by unfavorable environmental conditions. Salinity stress is one of the most important stress factors causing severe agronomical yield losses. High salinity can disturb the ion- and osmotic balances of the plant cells, causes intracellular water loss and increased production of reactive oxygen species, decreased photosynthetic activity and inhibited shoot and root growth. World population is growing at an alarming rate and production of enough food has become a challenge. Therefore better understanding of mechanisms involved in plant salt tolerance is crucial. Multiple changes in metabolism, cell growth, division and differentiation are required for adaption to adverse environmental conditions. Expression level of important regulatory genes can be so low that it is impossible to identify those using traditional molecular methods. Genetic approaches are therefore better suited to identify regulatory genes involved in plant stress responses.

We developed a novel genetic system to identify genes involved in salt tolerance. It is based on conditional cDNA overexpression. Arabidopsis and Thellungiella cDNA libraries were cloned by Gateway™ technology into the chemically inducible XVE expression cassette of pER8 plant transformation vector (Zuo et al. 2000; Papdi et al. 2008). The cDNA libraries were introduced into wild type Arabidopsis by Agrobacterium-mediated gene transfer. Infiltrated seeds were tested for altered salt tolerance in different selection systems. In the T2 generation the phenotype was re-tested. In selected lines, overexpressed cDNAs were amplified by polymerase chain reaction using two vector specific primers and sequenced. Identity and exact length of the inserted cDNA was determined by sequence homology searches.

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Materials and methods

Transformation-ready *Arabidopsis* and *Thellungiella* cDNA libraries were cloned and tested as previously published (Papdi et al. 2008), and used for Agrobacterium-mediated Arabidopsis transformation. T1 seeds of infiltrated plants were screened in three selection systems to identify transgenic lines which show estradiol-dependent stress phenotypes:

Identification of salt tolerant lines in growth-survival test: infiltrated seeds were germinated on hygromycin and the resistant 10-12 days old plants (approximately 40.000) were transferred to media containing 200 mM NaCl + 4 μM 17-β-estradiol. 2 weeks later the turgescent green plants were planted the greenhouse to set seeds. The phenotype was re-tested in T2 generation.

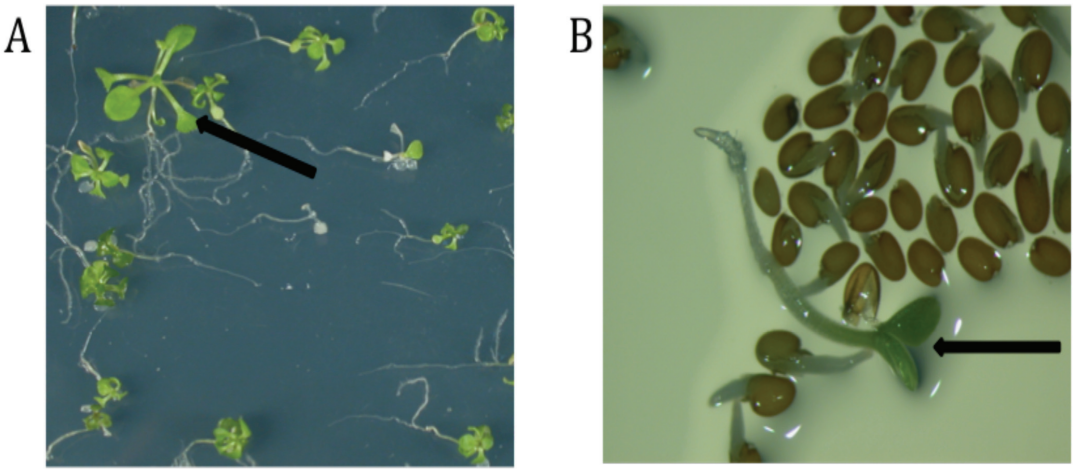

Figure 1. Identification of salt tolerant plants. A) Selection of salt tolerant plant in growth-survival test. 10-12 days old hygromycin resistant seedlings were transferred to media containing 200 mM NaCl + 4 μM estradiol. Turgescent, green plant (indicated by arrow) was selected and transferred to the greenhouse. B) Identification of salt tolerant plant in germination assay. Infiltrated seeds were germinated on 225 mM NaCl + 4 μM estradiol and seedling with opened, green cotyledons was selected (indicated by arrow). Picture was taken at 8th day of germination.

Identification of salt sensitive lines in growth-survival test: infiltrated seeds were germinated on hygromycin and resistant plants (10-12 days old) were transferred to media containing 130 mM NaCl + 4 μM 17-β-estradiol. 3 weeks later the small, chlorotic plants were transferred to the greenhouse to set seeds.

Identification of lines showing altered salt sensitivity in germination test: infiltrated seeds were germinated on media containing 225 mM NaCl and 4 μM 17-β-estradiol. Seedlings which could germinate (have opened green cotyledons at 7th-8th day of germination) were tested for hygromycin resistance and transferred to the greenhouse. Media supplemented with 130 mM NaCl+ 4 μM 17-β-estradiol was used to identify lines showing salt sensitive germination. Selected plants were transferred to normal seed germination media to recover before hygromycin selection. Antibiotic resistant seedlings were planted to the greenhouse.

cDNA inserts were rescued by PCR amplification using vector specific primers (Papdi et al. 2008). Identity and exact length of the inserted cDNA was determined by sequence homology searches. In order to verify the phenotype conferred by estradiol-inducible cDNA overexpression, the amplified PCR fragment was cloned into pDONR201 vector (Invitrogen), using Gateway™ BP Clonase reaction (Invitrogen) and its nucleotide sequence was verified. Cloned cDNAs were transferred into pER8GW plant expression vector using the Gateway LR Clonase reaction (Invitrogen) and used for plant transformation.

Semi-quantitative RT-PCR was used in gene expression studies. Total RNA was isolated from *in vitro*-grown seedlings or from different organs of greenhouse-grown plants. The DNase-treated RNA was used for cDNA synthesis (High capacity cDNA synthesis kit, Applied Biosystems).

Results

Identification of salt tolerant lines in growth-survival test

Approximately 40.000 hygromycin resistant 10-12- days old seedlings were transferred to media containing 200 mM NaCl + estradiol. 2 weeks later the turgescent green plants were planted the greenhouse to set seeds (Figure 1, left panel). The phenotype was re-tested in T2 generation and lines showing salt tolerance in estradiol-dependent manner were selected. The overexpressed cDNAs were PCR amplified and sequenced. Homology searches revealed that besides cDNAs coding for well-known stress related proteins (i.e. glutathione-S-transferase, small heat shock proteins, etc), novel factors (*i.e.* 2-alkenal reductase) involved in salt stress response were discovered, too.

Identification of salt sensitive lines in growth-survival test

Infiltrated seeds were germinated on hygromycin and resistant plants (10-12 days old) were transferred to media containing 130 mM NaCl + estradiol. 3 weeks later the smaller, chlorotic plants were transferred to the greenhouse to set seeds. In re-tested, selected lines the overexpressed cDNAs code for members of NAC (NAM, ATAF1,2, CUC2) transcription factor family and an AGAMOUS-like protein.

Identification of lines showing altered salt sensitivity in germination assays

In order to identify salt tolerant lines infiltrated seeds were germinated on media containing 225 mM NaCl and 4 μM 17-β-estradiol. 434 seedlings which could germinate (have

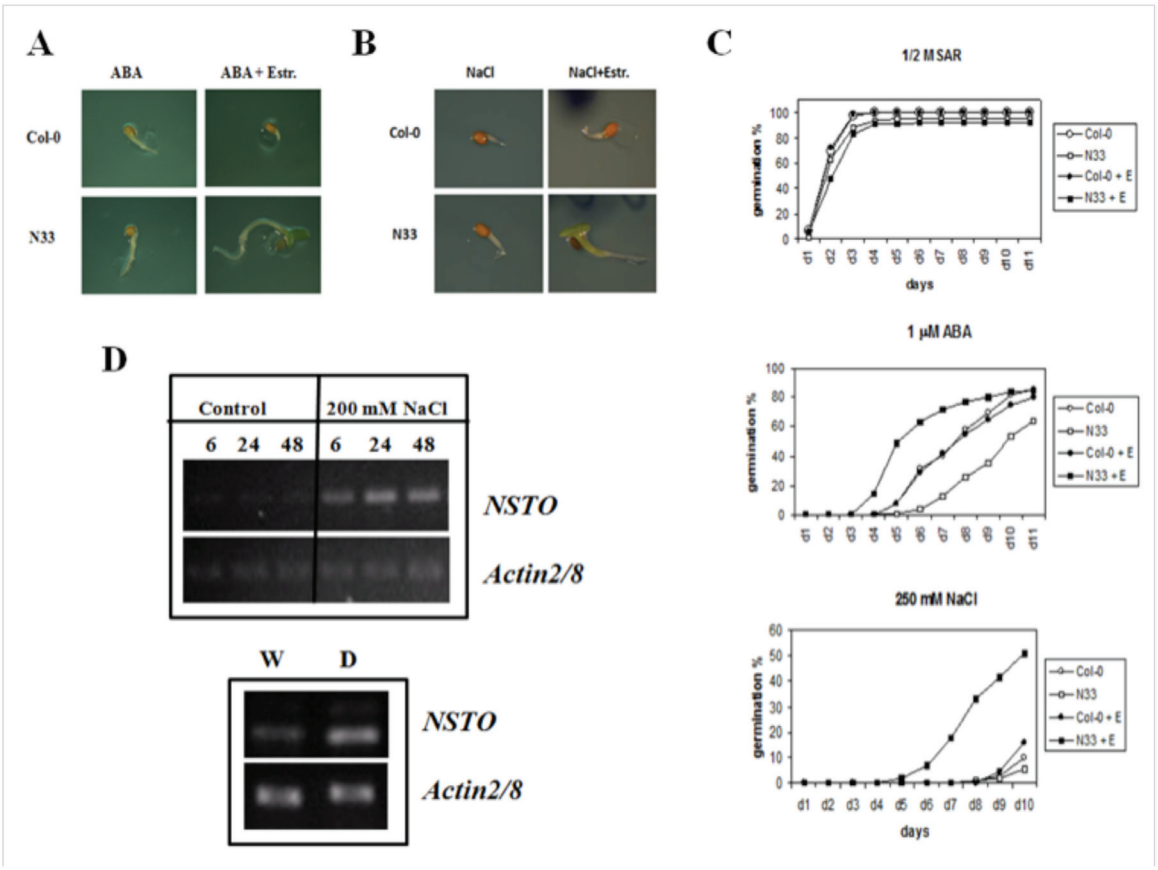

Figure 2. Characterization of NOVEL SALT TOLERANCE (NSTO) gene. A) Estradiol-dependent overexpression of NSTO gene confers ABA insensitive germination. Col-0 and N33 line (carrying full length *NSTO* cDNA) were germinated on ½ MSAR medium containing 1 µM ABA in the presence or absence of 4 µM estradiol. Pictures were taken 6 days after sowing. B) Line N33 shows estradiol-dependent salt tolerant germination. Wild type (Col-0) and N33 seeds were germinated on medium containing 250 mM NaCl with or without 4 µM estradiol. Pictures were taken at 9th day. C) Quantitative analysis of germination tests described at A) and B). D) Semi-quantitative RT-PCR analysis indicates the upregulation of *NSTO* gene expression during salt stress and under water deficit. 2 weeks old wild type seedlings were treated with 200 mM NaCl for 6, 24 and 48 h (upper panel). 4 weeks old greenhouse-grown wild type plants were watered regularly (W) or subjected to drought stress (D) for 1 week (bottom panel).

opened green cotyledons at 7th-8th day of germination; Figure 1, right panel) were selected as putative salt tolerant plants and their hygromycin sensitivity was tested. 95 antibiotic resistant seedlings were planted the greenhouse to produce seeds. Estradiol-dependent salt tolerant germination phenotype of 8 lines was confirmed in T2 generation.

Some part of infiltrated seeds was germinated on media supplemented with 130 mM NaCl and estradiol. The 437 putative salt sensitive plants (showing delayed germination and chlorosis) were transferred to normal germination media in order to recover before hygromycin selection and 69 antibiotic resistant seedlings were transferred to the greenhouse. 7 lines showed the salt sensitive germination phenotype in the T2 generation.

In several lines showing altered salt sensitivity during germination, the overexpression of NAC transcription factors can be responsible for the phenotype. Besides NAC factors, we could identify cDNAs coding for bZIP, ERF (Ethylene Responsive Element Binding Factor), Zn finger and SCARECROW transcription factors, too.

Thellungiella cDNA library transformation

Thellungiella salsuginea (*halophila)* is a close halophyte relative of Arabidopsis. It can tolerate extreme salinity, drought and cold. Because of several advantageous properties (*i.e.* short life cycle, self-pollination, small genome size, high sequence homology to Arabidopsis, easy genetic transformation) Thellungiella has become a model system in salt tolerance reserch (Amtmann 2009). In order to explore genetic diversity for stress tolerance, cDNA library of Thellungiella was introduced randomly into Arabidopsis plants. By screening for salt tolerance of 20.000 such transformed seedlings, twenty salt tolerant lines have been isolated. Inserted cDNA clones of these lines were PCR amplified and sequenced to determine the identity of the encoded proteins. Conditional overexpression of putative translation initiation

factor, heat shock proteins, peroxidase, Ca^{2+} -binding protein, Zn finger protein could lead to increased salt tolerance. It was confirmed by repeated germination and growth assays in five lines.

Characterization of Novel Salt Tolerance gene (NSTO)

Line N33 was chosen for detailed characterization. It shows high degree of estradiol-dependent salt tolerant and ABA insensitive germination (Figure 2A, B, and C). Hygromycin segregation data revealed that a single T-DNA insertion can be found in this line and the full length cDNA coding for an unknown protein. We designated this gene as *Novel Salt Tolerance* (*NSTO*). The cDNA was re-cloned into pER8 vector and transgenic Arabidopsis lines were generated. Transgenics show estradiol-dependent salt tolerant germination indicating that really the conditional overexpression of the *NSTO* is responsible for the phenotype. We got a T-DNA insertion line from publicly available mutant collection (http://signal.salk.edu/cgi-bin/tdnaexpress) where mutation affected the *NSTO* gene. Knock out mutation of this gene resulted in salt sensitive germination. The *NSTO* gene was characterized at transcriptional level in semi-quantitative RT-PCR experiments. Expression level of the gene is low, but we could detect it in every organ and tissue. *NSTO* transcript level is increased by drought and salt stress (Figure 2D). For intracellular localization, *35S::NSTO-YFP* gene construct was introduced into wild type Arabidopsis. The fusion protein was detected in root tip of transgenics and showed nuclear and cytoplasmic localization. This observation was confirmed by biochemical method also.

Discussion

In higher plants responses to environmental stresses are controlled by a complex web of ABA dependent and independent signaling pathways. To perform genetic screens for identification of novel Arabidopsis and Thellungiella genes involved in the control of salt stress responses, cDNA expression libraries were created in a Gateway version of a plant expression vector under control of a chemically inducible promoter. It allows the controlled activation of the inserted cDNA, and the generation of conditional, dominant phenotypes. Constitutive overexpression of regulatory genes can be deleterious for the plant development and productivity. Controlled expression of the inserted cDNA can avoid these difficulties.

Identity of the inserted cDNA was determined by direct sequencing of the PCR fragment and sequence homology search of the public sequence databases. As the cDNA insert was flanked by Gateway™ recombination sites, the PCR fragment could easily be re-cloned.

Novel salt tolerance factors were identified by screening for enhanced growth and/or survival of 40.000 hygromycin resistant T1 generation seedlings on selective agar plates supplemented with 200 mM NaCl and 4 μM estradiol. Most plantlets bleached on such high salt medium with the exception of 177, which remained green and survived .The selected plantlets were transferred to greenhouse to flower and produce seeds. Salt tolerance of T2 generation seedlings was subsequently tested in germination and growth assays in the presence or absence of 4 μM estradiol. Conditional salt tolerance was confirmed in 15 transgenic Arabidopsis lines. The identified genes encoded different classes of proteins such as protein kinases, various detoxifying enzymes, ribosomal proteins, a heat shock protein, 2-alkenal reductase and several unknown proteins.

In several lines, conditional overexpression of members of NAC transcription factor family can be responsible for altered salt sensitivity during germination and seedling growth. NAC proteins constitute one of the largest families of plant-specific transcription factors and have been implicated in abiotic stress responses, developmental programs and defense reactions (Olsen et al. 2005).

In several lines (showing altered salt sensitivity during germination) cDNAs coding for bZIP (basic region/leucine zipper motif) transcription factors were discovered. In plants, bZIP proteins regulate processes including pathogen defense, light and stress signaling, seed maturation and flower development.

Our conditional cDNA overoxpression system was suitable to identify well-characterized stress-related genes and novel factors. Estradiol-dependent overexpression of *NSTO* (coded for an unknown protein) conferred high degree of salt tolerant germination. However, more detailed characterization of NSTO is necessary to determine its function and mechanism via overexpression of NSTO leading to salt tolerant germination.

Our novel genetic system provides a simple and powerful technology to screen for gene functions implicated in the regulation of specific stress responses. Results obtained from *Arabidopsis* model system can be used to improve abiotic stress tolerance of crop plants (*i.e.* oilseed rape).

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