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ARTICLE

Functional identification of Arabidopsis stress regulatory genes using the Controlled cDNA Overexpression System

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ABSTRACT Plants respond to environmental stresses by altering the expression of many genes via a complex signalling network. We created a Controlled cDNA Overexpression System (COS) to identify genes and regulatory factors which have important role in stress tolerance. An estradiol-inducible cDNA expression library was tested in three genetic screens by selecting for salt tolerance, ABA insensitive germination and activation of a stress responsive *ADH1-LUC* reporter gene construct. Several cDNAs conferring dominant, estradiol dependent stress tolerance phenotypes were identified. Screening for enhanced salt tolerance revealed that estradiol-controlled overexpression of 2-alkenal reductase (AER) cDNA confers considerably high level of salt insensitivity. Characterization of cDNA conferring insensitivity to 3μM ABA in germination assays has identified the full-length coding region of heat shock protein HSP17.6A, suggesting its implication in ABA signal transduction. Screening for transcriptional activation of *ADH1-LUC* reporter gene has identified the ERF/AP-type transcription factor RAP2.12, which sustained high level ADH1-LUC bioluminescence and enhanced *ADH1* transcription in the presence of estradiol. Our data illustrate that application of inducible cDNA expression libraries such as the COS system provides an efficient tool for genetic identification and functional analysis of novel regulators of abiotic stress responses.
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KEY WORDS

abiotic stress,
cDNA expression library,
Agrobacterium,
novel genetic system,
estradiol-dependent cDNA overexpression

Extreme environmental conditions such as drought, low temperature and high soil salinity are among the most challenging factors for plant growth and survival. Adaptation to hostile environments requires multiple changes in metabolism, cell growth, division and differentiation, which depends on a large set of genes controlling a complex regulatory mechanism. Expression level of important regulatory genes can be so low that it is impossible to identify them using traditional molecular methods. Genetic approaches are therefore better suited to identify regulatory genes involved in plant stress responses.

We created the *Arabidopsis* Controlled cDNA Overexpression System (COS), which comprised an *Arabidopsis* cDNA expression library in the Gateway version of the pER8 plant transformation vector under the control of chemically inducible XVE expression system (Zuo et al. 2000). The expression library was introduced into *Arabidopsis* and transgenic plantlets were screened for salt tolerance, ABA insensitivity or activation of the stress-responsive *ADH1-LUC* reporter gene construct. We provide examples for each screen by describing three transgenic lines with conditional dominant stress-related phenotypes. Overexpression of the HSP17.6 gene conferred ABA insensitivity. Activation of the the 2-alkenal reductase enzyme (AER) improved salt tolerance. The RAP2.12 transcription factor was able to induce the expression of the *ADH1-LUC* reporter gene. These examples show that random cDNA transfer in a conditional expression vector, coupled with appropriate genetic screens is a powerful tool to identify novel stress genes on base of their function.

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Materials and Methods

cDNA expression library was created in a Gateway version of estradiol inducible XVE binary vector (Zuo et al. 2000) and used for *Agrobacterium* mediated *Arabidopsis* transformation. T1 seeds of infiltrated plants were screened in three genetic systems to select the transgenic lines which show estradiol-dependent stress phenotypes: 1.: selection for ABA-insensitive germination on the presence of 3 μM ABA; 2.: screening for salt tolerance in growth assay (seeds were germinated on medium containing 20 mg/l hygromycin and the resistant plantlets were transferred to medium supplemented by 225 mM NaCl and 4 μM estradiol); 3.: screening for *ADH1-LUC* reporter gene activation (*Arabidopsis* lines, carrying the stress responsive *ADH1-LUC* reporter gene were transformed with the cDNA expression library. T1 seeds were germinated on hygromycin plates, and hygromycin resistant plantlets were transferred to 1/2MS medium to assay luciferase activity with bioluminescence imaging. Plantlets showing enhanced luminescence were selected). Selected transgenic

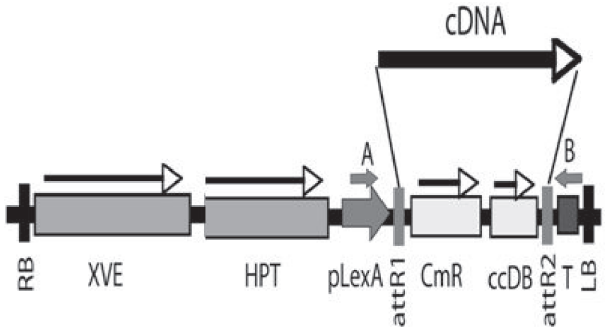

Figure 1. Schematic map of the T-DNA region of pER8GW vector. XVE: chimaeric XVE fusion gene, encoding the chimaeric transcription activator for the pLexA promoter (Zuo et al., 2000), HPT: hygromycin phosphotransferase gene, pLexA: LexA operator fused to a minimal promoter of Cauliflower Mosaic Virus 35S gene, attR1 and attR2: Gateway recombination sites, CmR: Chloramphenicol resistance gene, ccdB: suicide marker for bacterial contraselection, T: RUBISCO rbcsS3A polyA sequence; RB and LB: T-DNA left and right border sequences, respectively. cDNA: randomly inserted cDNA clone; A and B: positions of T-DNA specific PCR primers used for amplification of inserted cDNAs.

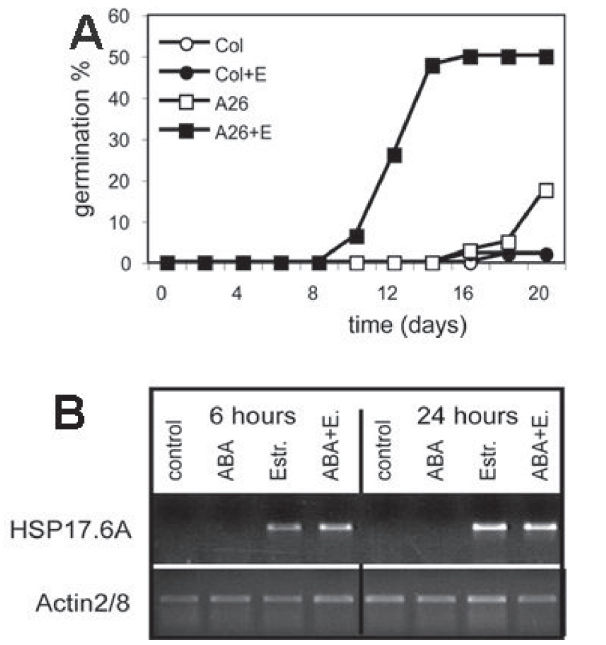

Figure 2. Characterization of line A26. A) Comparison of germination efficiencies of wild-type (Col-0) and A26 seeds in the presence and absence of estradiol on 3μM ABA-containing plates. B) Semi-quantitative RT-PCR analysis of *HSP17.6A* transcript levels. Activation of *HSP17.6A* transcription by 4μM estradiol treatment with or without 50μM ABA.

plants were allowed to flower and set seeds in the absence of estradiol. Phenotype of T2 plants was compared in the presence and absence of estradiol to determine the response to the inducer.

cDNA inserts were rescued by PCR amplification using vector specific primers (Fig. 1). 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.

Semiquantitative 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

Contruction and testing of COS cDNA library

The primary *Arabidopsis* cDNA library was constructed in the Gateway entry vector pDONR201 (Invitrogen) and subsequently transferred into the pER8GW destination vector (Fig. 1). The COS library was introduced into *Agrobacterium* by electroporation and used for large scale *in-planta* transformation of wild-type *Arabidopsis* (Col-0) plants, as well as a transgenic line carrying the *ADH1-LUC* reporter gene construct. In order to identify novel stress genes and to test the utility of the system, three screening strategies were employed: selecting for ABA insensitivity in germination screens, screening for salt tolerance on seedling growth and survival and activation of a stress-induced reporter gene construct in seedlings. The selected transgenic plants showing estradiol-dependent altered phenotypes were transferred into the greenhouse to set seeds. Phenotype of T2 plants was compared in the presence and absence of estradiol. If the altered stress phenotype was confirmed the cDNA was PCR amplified using vector specific primers (Fig. 1) and sequenced. The identity of the inserted cDNA was determined using BlastN homology search.

Identification of factors influencig ABA-sensitivity of seed germination

To identify genes which confers dominant ABA insensitive phenotype upon overexpression, T1 generation seeds of infiltrated Col-0 plants were screened for ABA insensitive germination in the presence of 3μM ABA and 4μM estradiol. These conditions block the germination of wild type seeds. 74 seedlings with open, green cotyledons and emerged radicles were selected and tested for hygromycin resistance. Progeny of the selected plants were re-tested for ABA insensitivity in the presence and absence of estradiol, and for hygromycin resistance, to estimate the number of independently segre-

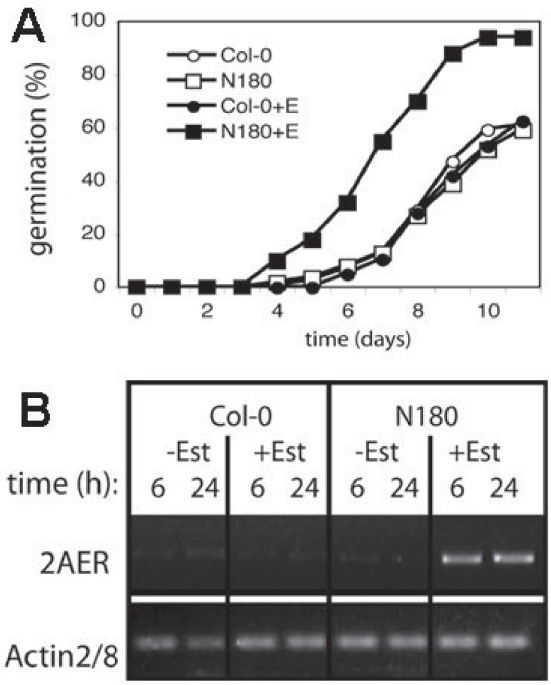

Figure 3. Characterization of line N180. A) Comparison of salt tolerance of N180 and wild-type (Col-0) seedlings germinated in the presence of 225mM NaCl with or without estradiol. B) Semi-quantitative RT-PCR analysis of 2-alkenal reductase (*AER*). Transcription of *AER* cDNA is induced by estradiol only in line N180 but not in the control wild-type (Col-0) seedlings.

gating T-DNA inserts. 19 lines showed different degree of estradiol-dependent ABA insensitive germination. To identify these genes the inserted cDNA were PCR amplified and sequenced. Sequence prediction of 11 amplified fragments showed that the cDNA encoded different classes of proteins, such as glutathione S-tranferase, small heat shock protein, SNF1-related kinase regulatory subunit, lipid transfer protein, subtilase and dehydrin type proteins.

The line A26 was chosen for further analysis. A26 seeds germinated in the presence of 3μM ABA and 4μM estradiol, while their ABA sensitivity was similar to wild-type seeds in the absence of estradiol (Fig. 2A). The hygromycin segregation ratio was 3:1 indicating the presence of single T-DNA insertion. Nucleotide sequence of the amplified PCR fragment was identical with the full length cDNA of the *At5g12030* gene and encoded the class II small heat shock protein 17.6A (HSP17.6A). Semiquantitative RT-PCR analysis confirmed that *HSP17.6A* expression in line A26 was indeed induced only by estradiol, but not by ABA (Fig. 2B).

Identification of cDNA conferring salt tolerance to *Arabidopsis*

Novel salt tolerance factors were identified by screening for enhanced growth and/or survival of 40.000 hygromycin

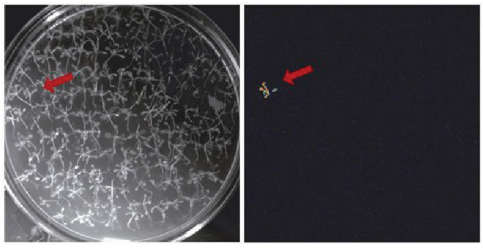

Figure 4. Identification of cDNA conferring estradiol-inducible activation of *ADH1-LUC* reporter. Image of a COS cDNA transformed ADH1-LUC seedling showing enhanced bioluminescence in the presence of 4μM estradiol (marked with red arrow).

resistant T1 generation seedlings on selective agar plates supplemented by 225mM NaCl and 4μM 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 4μM estradiol. Conditional salt tolerance was confirmed in 15 transgenic *Arabidopsis* lines, which showed at least two times higher germination rates upon estradiol treatment than Col-0 wild type seeds. The cDNA fragments, contained in the T-DNA of the salt tolerant lines were PCR amplified and sequenced. The identified genes encoded different classes of proteins such as protein kinases, various detoxifying enzymes, ribosomal proteins, a heat shock protein and several unknown proteins. Line N180 was chosen for further characterization. Segregation of hygromycin resistance in the N180 line was 3:1 suggesting the presence of a single T-DNA insert. T2 generation seeds of the N180 plant could germinate earlier in the presence of 225mM NaCl when estradiol was added, while in the absence of this inducer germination was restricted to radicle emergence, similar to wild type seeds (Figure 3A). The cDNA whose conditional overexpression conferred salt tolerance in line N180 coded for 2-alkenal reductase enzyme (AER, EC 1.3.1.74.). In semi-quantitative RT-PCR experiment enhanced *AER* transcription could be detected in estradiol-treated N180 plants when compared to non-treated N180 or Col-0 plants (Figure 3B).

Identification of stress regulatory genes controlling the expression of the ADH1-LUC reporter gene

To facilitate the identification of stress and ABA signalling factors, we constructed a stress-induced reporter gene which can be detected by non-destructive low light imaging methods when activated by different stress signals. The promoter region of the *ADH1* ((*At1g77120*) gene was fused to the promoterless firefly luciferase reporter gene and the *ADH1-LUC* construct was introduced into wild type *Arabidopsis*.

To identify *trans*-acting factors, which can induce the *ADH1-LUC* reporter gene, we re-transformed a homozygous reporter line with the COS library, and screned 20.000 hygromycin resistant T1 generation seedlings for enhanced LUC activity in the presence of 4μM estradiol. 11 plants were identified that exhibited enhanced luminescence (Fig. 4) and were transferred to greenhouse to self pollinate and to set seeds. Progeny of these plants was tested again for luciferase activity. Estradiol-dependent LUC activation was confirmed in two lines. cDNA insert was amplified from these lines. The ADH121 line was chosen for further studies because it showed reproducible, estradiol-dependent enhanced luciferase activity, and the 3:1 segregation of hygromycin resistance suggested single T-DNA insertion in this line. The conditionally overexpressed cDNA in the line ADH121 coded for RAP2.12 (*At1g53910)* transcription factor which belongs to AP2/ERF (ethylene responsive element binding factor) family of transcription factors. To confirm that the RAP2.12 transcription factor was indeed responsible for ADH1-LUC activation in ADH121 line, the amplified cDNA was cloned into pDONR201 plasmid, transferred into the pER8GW expression vector, and the contructed pER8GW-RAP2.12 vector was introduced into the ADH1-LUC line, which was originally used for the cDNA library transformation. Several ADH1-LUC/RAP2.12 transgenic lines showed estradiol-dependent luciferase activity, which was similar to the ADH121 mutant.

Discussion

The Controlled cDNA Overexpression System offers a simple technology to screen for gene functions implicated in the regulation of specific stress responses. The COS cDNA library was prepared in a chemically inducible expression vector using the Gateway technology, which offers precise transcriptional control and easy recloning of the cDNA inserts. The COS system was tested in several screening strategies, each of them aiming at a particular aspect of a stress response. Screening for salt tolerance at seedling level permitted the identification of genes which, upon overexpression, could enhance the germination rate or increase the survival of seedlings in saline environment. As an example, line N180 was characterized to show that overexpression of 2-AER cDNA confers salt tolerance to transgenic plants. Previous studies document that the 2AER enzyme has a NADPH-dependent oxidoreductase activity, which probably plays a role in the detoxification of reactive carbonyls, and hence in the protection of cells against oxidative stress (Mano et al. 2005). As high salinity and drought is accompanied by enhanced production of reactive oxygen species the functions of antioxidant enzymes, such as 2-AER, are important in mounting salt tolerance by reducing the amount of reactive radicals.

Isolation of numerous lines displaying estradiol-dependent ABA insensitivity indicates that the COS technology could also be useful for identification of novel negative regulators of ABA signaling. As an example, we showed that regulated overexpression of small heat shock protein gene *AtHSP17.6A* in line A26 conferred conditional ABA insensitivity, suggesting that this heat-shock protein is also implicated in the control of ABA sensitivity of seed germination. It is well-documented that expression of small heat-shock proteins is induced by high temperature but some of them, including *AtHSP17.6A*, are also produced in developing seeds and in response to water stress (Wehmeyer et al. 1996).

The application of luciferase reporter gene constructs driven by different stress-induced promoters facilitates non-destructive detection of gene activation in mutant screens, as well as the identification of transcription factors controlling the expression of a particular target gene. Expression of the alcohol dehydrogenase gene *ADH1* is controlled by multiple regulatory pathways, including ABA and ethylene signalling (de Bruxelles et al. 1996). Our data show that the estradiol dependent overproduction of the AP2/ERF transcription factor RAP2.12 can also activate *ADH1* expression. The AP2/ERF transcription factor family includes key regulators of abiotic and biotic stress responses.

The above described examples illustrate the COS library transformation method facilitates high-through-put screening for phenotypes conferred by inducible overexpression of *Arabidopsis* transcripts in an *Arabidopsis* genetic background.

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