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cDNA-AFLP analysis of salt-inducible genes expression in *Chrysanthemum lavandulifolium* under salt treatment

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ABSTRACT

Chrysanthemum lavandulifolium (Fisch. ex Trautv.) Makino is a halophyte species that belongs to the *Asteraceae* family, and the genus *Chrysanthemum*. It is one of the ancestors of *C.* × *morifolium* Ramatella. Understanding the tolerance mechanism associated with salt stress in *C. lavandulifolium* could provide important information for explaining the salt tolerance of higher plants and could also help enhancing breeding programs of cultivated Chrysanthemum. In this study, cDNA amplified fragment length polymorphism (cDNA-AFLP) was used to detect differential gene expression in leaves of *C. lavandulifolium* in response to NaCl treatment. The determination of membrane permeablility, peroxidase activity (POD), malon-dialdehyde (MDA), as well as proline and leaf chlorophyll contents under different NaCl concentrations showed that a 200 mM NaCl treatment was an optimal condition for the cDNA-AFLP experiment. Using this concentration during different times (0, 3 h, 12 h, 24 h and 48 h), we obtained 1930 cDNA fragments using 64 primers. After sequencing 234 randomly chosen cDNA clones and BLASTx analyzing, we got 129 expressed sequence tags (ESTs) which had no significant homology with other sequences, 85 ESTs were homologous to genes with known functions, whereas the rest of ESTs showed homology to unclassified or putative proteins. 25 ESTs that were similar to known functional genes involved in several abiotic and biotic stresses were confirmed by semi-quantitative RT-PCR and qRT-PCR. The expression patterns of these salt-responsive genes not only responded to salt stress but also to plant hormones, such as abscisic acid (ABA), and to other abiotic stresses such as drought and cold. These results indicate an extensive cross-talk among several stresses. Our results provide interesting information for further understanding the molecular mechanisms of salt tolerance in *C. lavandulifolium*.

Introduction

Salinity is one of the major abiotic stresses that adversely affect crop productivity and quality. About 20% of irrigated agricultural land is negatively affected by salinity (Flowers, 2004). A high concentration of salt causes ion cytotoxicity and hyperosmotic stress in plants. Likewise, it results in nutrient constraints of farmlands (Katiyar-Agarwal et al., 2005). In contrast to plant resistance to biotic stresses, which is mostly dependent on monogenic traits, the genetically complex responses to salt stresses are multigenic and controlled by cascades of molecular networks. Thus, it is more difficult to be modified by genetic engineering.

Under high salt conditions, a variety of genes can be induced to expressions that contribute to molecular, physiological and morphological tolerance in response to stress. These genes include three major categories: (1) genes encoding regulatory proteins involved in signaling cascades, such as genes of mitogen-activated protein (MAP) kinases, calcineurin B-like protein (CBL), protein phosphatase, and encoding regulatory protein factors such as gene families of CBF/DREB (C-repeat-binding factor/dehydration-responsive binding protein), NAC (NAM, ATAF, and CUC transcription factors) and WRKY; (2) genes encoding proteins that function directly to protect membranes, such as chaperones, late embryogenesis abundant proteins (LEA), detoxification enzymes, and enzymes controlling the biosynthesis of compatible solute such as betaines, fructan, mannitol, proline, sorbitol, trehalose, D-ononitol and several types of proteases (Motoaki et al., 2007; Chen and Murata, 2002); and (3) genes involved in water and ion uptake and transport. Understanding the mechanisim of these categories could provide clues to improve crop tolerance to salt stresses.

Chrysanthemum × *morifolium* is one of the world's major ornamental plant genera. Flower breeders have created numerous genotypes during millennia of breeding, which has enabled its establishment in ten cuts, potted flowering, and garden crops

Abbreviations: cDNA-AFLP, cDNA amplified fragment length polymorphism; ESTs, expressed sequence tags; RT-PCR, reverse transcription-polymerase chain reaction; qRT-PCR, quantitative real time polymerase chain reaction.

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worldwide (Teixeira da Silva, 2003). However, the planting areas of chrysanthemums are limited due to its sensitivity to drought and salt loading stress. Because its allopolyploid ($2n = 6x = 54$) and aneuploid nature, genetic improving has been complicated due to inbreeding depression and genetic load (Anderson, 2007). Similarly, it is difficult to analyze its molecular mechanisms under abiotic stress.

Several studies have shown that wild ancestors of cultivated plants are one of the major genetic resources of plant tolerance to abiotic and biotic stresses (Baisakh et al., 2006; Fu et al., 2005; Rodriguez-Uribe et al., 2011; Si et al., 2009; Wu et al., 2010). *Chrysanthemum lavandulifolium* (Fisch. ex Trautv.) Makino is closely related to cultivated chrysanthemums and has a relatively small diploid genome ($2n = 2x = 18$). It is widely distributed in slopes, rocks, valley, banks, unclaimed lands and loess hilly lands of North China. Apparently, this species may have unique mechanisms enabling its survival in salty and dry conditions (Lin and Shi, 1993). Understanding these mechanisms could help in finding useful gene resources to improve the ability of cultivated chrysanthemums to grow under abiotic stress conditions. Here, we report a first step towards recognizing the genetic basis of salt tolerance mechanisms of *C. lavandulifolium* at genomic level using cDNA amplified fragment length polymorphism (cDNA-AFLP).

Materials and methods

Plant material, growth, and treatments

Seeds of *Chrysanthemum lavandulifolium* were obtained from the nursery of Beijing Forestry University. Seeds were germinated and plants were grown under greenhouse conditions with 12 h light period (300 μE m^{-2} s^{-1}, 21–25 °C) and 12 h dark period (15 °C). A group of plants (at eight leaves stage) were treated with 100, 200 or 300 mM NaCl during 0, 3, 6, 9 or 12 d to determine the physiological index; other seedlings were treated with 200 mM NaCl during 0, 3, 12, 24 and 48 h, respectively; then, their leaves and roots were frozen in liquid nitrogen and conserved at −80 °C for cDNA-AFLP and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analyses. Because roots largely lack some major metabolic pathways in response to abiotic stresses, such as photosynthesis, flavonoids and anthocyanin biosynthesis (Jiang and Deyholos, 2006), we chose leaves for the initial cDNA-AFLP analysis to obtain as many genes involved in several pathways as possible.

For the RT-PCR and quantitative real time polymerase chain reaction (qRT-PCR) analyses, test plants were carefully removed from the soil and were left to dry in a growth chamber for 12 h. Leaf water potential under normal conditions and drought stress were measured by a Scholander pressure chamber method (Ribeiroa et al., 2009) to indicate the level of drought stress in leaves; *C. lavandulifolium* seedlings were placed in a growth chamber at 4 °C during 24 h for a cold treatment; seedlings were irrigated with 150 μM ABA during 24 h for an ABA treatment, and all excised leaf samples of different treatments were immediately used for RNA extraction or quickly frozen in liquid nitrogen and stored at −80 °C.

Determination of membrane permeability, POD, MDA, proline and chlorophyll of leaves

The procedure used for determining membrane permeability (also called relative conductance rate) was based on a method of Yan et al. (1996). Total peroxidase activity (POD) was measured according to Rivero et al. (2001). The malon-dialdehyde (MDA) content was estimated using a procedure described by Heath and Packer (1968). Free proline content was measured as described by Bates et al. (1973). For chlorophyll estimation, leaves were ground in 80% chilled acetone; then, they were centrifuged at 4 °C and 4000 rpm for 5 min; the supernatant was taken, and its absorbance was read at 663, 645 and 480 nm; values were calculated according to Samir et al. (2010). These experiments were repeated in triplicates to ensure the reproducibility of results.

cDNA-AFLP

Total RNA was isolated from *C. lavandulifolium* leaves, which were treated during 0, 3, 12, 24 and 48 h using a Trizol method and purified by PolyATtract mRNA isolation system I (Promega, USA). Synthesis of cDNA was performed with Superscript II Reverse Transcriptase, RNase H, DNA PolymeraseI, and *Escherichia coli* ligase (Invitrogen, Carlsbad, CA, USA). Double-stranded cDNA was digested with restriction enzymes *Eco*RI and *Mse*I. The digested products were ligated to adapters with sequences as follows: EcoRI adapter, 5′-CTC GTA GAC TGC GTA CC-3′, 3′-CTG ACG CAT GGT TAA-5′; MseI adapter, 5′-GAC GAT GAG TCC TGAG-3′, 3′-TACTCA GGACTCAT-5′. The ligated products were pre-amplified with corresponding primers (EcoRI: 5′-GAC TGC GTA CCA ATT C-3′, MseI: 5′-GAT GAG TCC TGA GTA A-3′). Following the pre-amplification step, products were diluted (30×) with TE buffer, and 2 μl were used for the final selective amplifications using a combination of different *Eco*RI and *Mse*I primers and a touchdown PCR amplification program (Vos et al., 1995). PCR products were identified on 6% polyacrylamide sequencing gel at 70 W for 1 h. Fragments of cDNA were then visualized by silver staining.

Isolation and sequencing of cDNA-AFLP fragments

Polymorphic expressed sequence tags (ESTs) based on absence or differential intensity were cut from the gel with a sharp razor blade; then, they were put into a 0.5 ml centrifuge microtubes with 40 μl distilled water and placed in boiling water for 10 min; the supernatant was used as a template for further PCR reactions. PCR products were recovered from agarose gels, cloned into T-easy vector (Tiangen, China) and sequenced. After the removal of vectors, sequence database searches were performed. Nucleotide and translated sequences were analyzed for their homology with nucleotide sequences, respectively, by using publicly available nonredundant genes/transcripts in the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/BLAST) using BLASTX algorithms.

RT-PCR

Semi-quantitative RT-PCR was used to characterize the expression of 25 salt-induced ESTs with known functions. A total of 2 μg of total RNA was used in RT-PCR using cDNA Synthesis Mini Kit according to the manufacture's instruction (Promega, USA) to produce total cDNA. *ClActin* served as endogenous control sequence. DNA bands on ethidium bromide stained gels were quantified using BioRad Imaging System. The expression analysis of each cDNA sequence was repeated three times.

qRT-PCR

According to our RT-PCR results, we chose seven ESTs (CL6, −44, −80, −49, −20, −13, and −7) for further expression analysis by qRT-PCR, which were performed according to Jayaraman et al. (2008).

Results

Physiological response to salt stress in C. lavandulifolium

Comparison of physiological parameters in leaves of five control plants and salt treated plants of *C. lavandulifolium* showed that