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Transcript profiling of salt tolerant tobacco mutants generated via mutation breeding

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ABSTRACT

The main aim of the study is to identify the genes differentially, predominantly or specifically expressed in salt tolerant tobacco mutants, improved from Akhisar 97 and İzmir Özbaş varieties via mutation breeding, with respect to unstressed control plants. Seven tobacco mutants which have different salt tolerance capacities were evaluated by Gene Fishing analysis. Under stress conditions differentially expressed 100 reproducible bands were identified (74 of up-regulated and 20 of down-regulated while 6 were unknown). 75 of differentially expressed genes (DEGs) were successfully extracted from the gel and sequence analyses were performed. Functional annotation of the DEGs was performed against Blastn by interrogating their sequences. The 65 salt-regulated differentially expressed genes showed similarity with known genes, while 6 of DEGs didn't show any genetic similarities with known genes. DEGs were classified in eleven functional categories involving the abiotic stress response, biotic stress response, energy metabolism, cellular transport, catalitic activity, protein modification, amino acid metabolism and transcription factors. All the mutants were evaluated for their regulatory mechanisms against salt stress. The current data reveal that these six DEGs should be identified by next generation sequencing techniques and functional analysis should be design to understand the role of these six differentially expressed genes of tobacco mutants in further studies to improve new genetic resources.

1. Introduction

Salinity is one of the most common and important abiotic stress factors in the agricultural land that affects productivity and yield quality via osmotic and toxic effects (Türkan and Demiral, 2009). Adverse effects of saline soil decrease agricultural productivity and economy of the countries remarkably. The sustainability of agriculture in saline lands depends on the selection of tolerant species and/or breeding of existing species. Therefore, it is getting more and more important to increase the tolerance potentials of the existing species and consequently, increasing the productivity of the yield in recent studies.

High salt concentrations in soil disturb homeostasis in plants and cause various other stresses (Munns and Tester, 2008; Zhu, 2002). Eventhough Na^+ cations are the main toxicity agents over plants, some species present more sensitivity to Cl^- anions (Türkan and Demiral, 2009). Higher accumulation of Na^+ ions in soil changes the osmotic equillibrium between the soil and the roots of the plants and causes the phenomenon called physiological drought.

Increase of world population in contrary to decrasing agricultural productivity requires developing new breeding strategies against salt and other stresses to satisfy the global food requirements (Chaves et al., 2009; Passioura, 2007). Therefore investigating physiological, biochemical and molecular responses of plants against abiotic stresses are the most important topics in breeding studies.

There are various salt tolerance mechanisms in plants as maintaining the ionic and osmotic homeostasis, controlling and restoring the hazardous effects caused by the stress and regulation of growth (Zhu, 2002). Alterations on physiological, biochemical and molecular basis occur via major changes in gene expressions in response to salinity in plants (Xu et al., 2013). Salt tolerance of the plants is in relation with complex molecular mechanisms such as sensing of the stress by the plant, signal transportation, stress-related gene expressions and production of osmoprotectant metabolites. There are various studies to identify these mechanisms in several plants (Fan et al., 2013; Munns and Tester, 2008; Parida and Das, 2005; Zhang et al., 2016).

The methods focusing on solving the soil salinity problem cost excessive time and money. Therefore, recent scientific studies are focusing on producing more practical solutions against the problem. The main aim of these studies is to select and improve salt tolerant plants. For this

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purpose, cloning approach of the genes that play role in antioxidative mechanisms, accumulation of osmolytes or selective accumulation of ions are the center of interest recently.

Mutation breeding is an useful alternative method to improve tolerant plants. Radiation treatment is one of the most widely used methods to induce gene recombinations among plant genome. It gives opportunity to increase the frequency of the mutations that happen spontaneously. The important point of mutation breeding studies is to determine the appropriate radiation doses to improve new plants with desired characteristics while maintaining the other agronomic properties. In this stage, molecular analysis in selection studies are gaining importance recently (Ahloowalia and Maluszynski, 2001; Patade et al., 2006).

Annealing control primer (ACP)-based differentially expressed gene (DEG) analysis system which is simple and rapid has been widely used to discriminate the genes which are responsible for a specialized function under a certain stage or a condition. This method is preferred in transcriptomic studies for its specifity and sensitivity (Ki-Won et al., 2015; Kim et al., 2011; Qu et al., 2015).

Tobacco (*Nicotiana tabacum* L.) which belongs to Solanaceae family is an annual diploid plant. It is frequently used in molecular analysis as a model plant. Although its genome has been completely sequenced, there is no transcriptomic data of tobacco in any means.

In this study, we used 250 mM NaCl tolerant M_3 generation mutant tobacco plants which were derived from two oriental tobacco varieties (Akhisar-97 ve İzmir Özbaş). Transcriptomic data were obtained by ACP-based DEG PCR analysis of these mutants under salt stress and compared against control plants. The obtained data were evaluated through bioinformatic tools. This novel study on identifying the transcriptional differences of the salt tolerant mutants will contribute to literature in this field.

2. Material and methods

2.1. In vivo salt tolerance capacity analysis

The seeds belonging to M_3 generation of Akhisar-97 and İzmir Özbaş controls and mutant plants, which were selected due to their NaCl tolerance capacities at 250 mM NaCl (Celik and Atak, 2015), were germinated on perlite under controlled conditions as 26 °C/18 °C and 16 h light/8 h dark day/night period in a climate room. Seedlings were watered by Hoagland solution (Hoagland and Arnon, 1950). On 14th day of normal growth, salt stress tolerance upper-limit detection analyses of M_4 generation were initiated by adding 0, 250, 300 and 325 mM NaCl to Hoagland solution.

The higher salt concentrations and the periods of their tolerances were morphologically evaluated against control group for each mutant tobacco plant. Each mutant were selected due to the growth profile of their positive and negative control groups and were analyzed. The tolerant mutant tobacco plant codes and their tolerance limits (concentration and period) were given in Table 1. 30 single seeds were used for each treatments. In the experiments, each treatment was performed as triplicate. The growth profiles of the mutants were photographed and presented in Fig. 1.

Table 1
The tolerance limits of NaCl stress tolerant tobacco (*Nicotiana tabacum* L.) mutants generated by mutation breeding.

Mutant Code	Origin	NaCl Concentration (mM)	Day
1.1	Akhisar-97	325	10
1.3	Akhisar-97	300	8
1.10	Akhisar-97	250	12
1.17	Akhisar-97	250	12
3.3	İzmir Özbaş	300	9
3.25	İzmir Özbaş	300	12
3.31	İzmir Özbaş	250	12

2.2. Salt stress application for differential expression analyses

Two different trials were set for gene expression analyses. The control and salt stress tolerant tobacco seeds of Akhisar-97 and İzmir Özbaş varieties were sown into perlite and grown for 14 days by adding Hoagland solution. 14-days-old seedlings were subjected to NaCl stress under determined duration and concentrations which plants presented tolerance in preliminary studies. At the end of the stress period, plants were harvested and the leaves were stored at −80 °C until analyses. For each treatment, 10 seeds were used. Second experimental design was set up as described except salt treatment. Leaves were also harvested and stored until they were used as negative control. As control plants, seeds of non-mutated and mutated tobacco plants were used to discriminate the differences among the mother plant and the mutants both under non-stressed and NaCl stressed conditions.

2.3. RNA isolation, cDNA synthesis and ACP-based Gene Fishing PCR reactions

Total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manual instructions. Quality of RNA was confirmed by gel electrophoresis and the concentration was calculated due to the spectrophotometric measurements at 260/280 nm of spectrophotometer.

3 µg RNA for each salt stressed, non-stressed control and mutant plants were used during subsequent DEG analysis. First strand cDNA synthesis was performed by using a Gene Fishing™ DEG Premix Kit (Seegene, Rockville, MD) as recommended by the manufacturer. In briefly, 3 µg of RNA representing each treatment group reverse-transcribed to first-strand cDNA at 42 °C for 90 min in a reaction mix (20 µL) containing 1x reaction buffer, 2 mM dNTP mix, 20 U RNase inhibitor (Promega), 2 µL of 10 mM (dT)15-ACP1 (Seegene), and 1 µL Moloney murine leukemia virus transcriptase (200 U/µL, Promega). The first-strand 5x diluted cDNAs were used for further experiments.

Second-strand cDNA was synthesized by first-stage PCR followed by a second-stage PCR in a final reaction volume of 20 µL containing 50 ng of the diluted first-strand cDNA, 1x SeeAmp ACP™ master mix (Seegene), 1 µL of 10 mM dT-ACP2, and 2 µL of 5 mM arbitrary ACPs. The reaction mixture was placed in a pre-heated (94 °C) thermal cycler for the first-stage PCR (one cycle at 94 °C for 1 min followed by 50 °C for 3 min and 72 °C for 1 min). The second-stage PCR amplification profile contains 40 cycles of 94 °C for 40 s followed by 65 °C for 40 s, 72 °C for 40 s, and a 5 min final extension at 72 °C. In the present study, 120 ACP primers were used during the second-stage PCR to capture the DEGs. The amplified PCR products were resolved in a 2% agarose gel.

2.4. Cloning and sequencing of DEGs

100 DEGs were excised from the gel due to their intensity or presence/absence between the leaf samples of stressed and non-stressed/stressed non-mutated control and non-stressed/stressed mutant tobacco plants under different concentrations of NaCl and stress durations. DEGs were extracted by using Gel DNA recovery kit (Zymoclean, USA). 25 DEGs were not successfully exctracted from the gel due to low concentration and excluded from the sequencing analysis. 75 DEGs were cloned into Topo TA Cloning Kit for Sequencing (Invitrogen, USA) according to the recommendation of the manufacturer (Seegene DEG Fishing). Positive colonies from 75 DEGs were selected and independent clones were single-pass sequenced with M13F/R primers in an ABI 3730x1 Genetic Analyzer (Applied Biosystems, Foster City, CA). DNA sequences were processed manually to remove the vector backbone and the poly (A) tail. Functional annotation of the DEGs was performed against Blastn by interrogating their sequences (Effendy et al., 2013).