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NOTE

Analysis of Salt-Induced mRNA Transcripts in Tunisian Local Barley (*Hordeum vulgare*) Leaves Identified by Differential Display RT-PCR

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Introduction

Salinity is one of the most serious factors limiting the productivity of agricultural crops, with adverse effects on germination, plant vigor, and crop yield (Munns and Tester 2008). Salinization affects many irrigated areas mainly due to the use of brackish water. Worldwide, more than 45 million ha of irrigated land have been damaged by salt, and 1.5 million ha are taken out of production each year as a result of high salinity levels in the soil (Munns and Tester 2008). High salinity affects plants in several ways: water stress, ion toxicity, nutritional disorders, oxidative stress, alteration of metabolic processes, membrane disorganization, reduction of cell division and expansion, and genotoxicity (Munns 2002; Zhou et al. 2012). Together, these effects reduce plant growth, development, and survival.

The adaptation of plants to salt stress (i.e., resumption of growth after exposure to high soil salinity) requires cellular ion homeostasis involving net intracellular Na^+ and Cl^- uptake and subsequent vacuolar compartmentalization without toxic ion

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accumulation in the cytosol (Agarie et al. 2007; An et al. 2007; Abuqamar et al. 2009). In most plant species capable of growing in saline environments, Na^+ appears to reach a toxic concentration before Cl^- does; consequently, most studies have focused on Na^+ exclusion and the control of Na^+ transport within the plant (Munns and Tester 2008). Compatible solutes are fundamental for a plant's osmotolerance by protecting enzymes from denaturation, stabilizing membranes or macromolecules or by playing mediating osmotic adjustment, giving the plant a more adaptive advantage (Ashraf and Akram 2009; Petronia et al. 2011). Such compatible solutes include proline and glycine betaine (GB) whose content increases greatly under salt and drought stress (Munns 2002; Sakamoto and Murata 2002; Fariduddin et al. 2013) and are the major metabolites found in durum wheat (other cereals and members of the Poaceae) under salt stress (Sairam and Tyagi 2004; Ashraf and Akram 2009).

The use of differential display reverse transcriptase polymerase chain reaction (DDRT-PCR) has often been described as an efficient method for the identification of stress induced under specific conditions. It also provides rapid and multiple comparisons of plant responses to stress durations and intensities (Ingram and Bartels 1996; Xu et al. 1996; Guo et al. 2007). In this report, DDRT-PCR (Harkamal et al. 2007) was used to isolate salt-induced transcripts from barley leaves exposed to high salt stress (200 mM NaCl). Thirteen cDNA fragments differentially expressed under salt stress were isolated, sequenced, and analyzed. Their possible function during this stress is discussed.

Materials and Methods

Plant Growth Conditions

Kebilli II was selected after examination of several morphological, physiological and agronomic traits which demonstrated its tolerance to a high level of salinity (180 mM NaCl) when grown in vitro. Seeds of barley Kebilli II were germinated on wet filter paper, transferred to hydroponic culture with one quarter-strength Murashige and Skoog (MS) medium, and grown in a growth chamber (16 h light/8 h dark photoperiod and 60 ± 10% relative humidity) (Benderradji et al. 2011) for 1 week prior to initiation of the salinity stress assay. Ten plants were maintained on the same medium (control treatment), and for a second batch of 10 plants, the growth medium was replaced with one quarter-strength MS medium with 200 mM NaCl. Plants were grown for 3 days under these conditions and 5 repetitions were performed for each treatment.

Extraction and Preparation of RNA

When the plants reached 15 cm or longer and after 7 days of treatment, terminal leaves were collected from control and salt-stressed plants (Brini et al. 2007), frozen in liquid nitrogen, and reduced to a powder with a mortar and pestle. Total RNA was extracted by the guanidinium thioisocyanate method (Golldack et al. 2002).

Before cDNA synthesis, the RNA sample was treated with RNase-free DNAase (Promega, Toulouse, France) for 1 h at 37°C, to eliminate any contamination with genomic DNA. For densitometric analyses the spectrophotometer (Cecil, Munchen, Germany) was used.

cDNA Synthesis

First-strand cDNA synthesis was performed using 2 μg of total RNA with M-MLV Reverse Transcriptase (Promega), six HAP primer combinations, and an oligo (dT) primer (Life Technologies, New York, USA). The PCR mixture contained 40 ng cDNA, 2 μM HAP primers, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin and 1 U of AmpliTaq DNA polymerase (Perkin-Elmer, New York, USA) in a final volume of 20 μl. Amplification was performed using a 45-cycle PCR reaction (30 s at 95°C, 60 s at 40°C, 30 s at 72°C followed by 5 min at 72°C). A 519-bp fragment of the barley *alpha-tubulin 2* gene was used as a control by amplification with the following two primers: TUB5′ (5′-GTGATC TCAGCTGAGAAGGC-3′) and TUB3′ (5′-CCTCATCACCGTCCTCGCC-3′) (Jarošová and Kundu 2010), in a 30-cycle PCR (30 s at 95°C, 30 s at 60°C, 60 s at 72°C). Selective amplification products were denatured in 50% formamide at 95°C and separated by electrophoresis on a polyacrylamide gel (6%) containing urea and TBE according to Sambrook et al. (1989). Bands were stained with ethidium bromide and visualized and quantified with an image analyzer (Biorad, GelDoc XRS, France).

Transcript-Induced Fragment Isolation

After viewing the gel under the UV lamp, the bands of interest were identified and cut out from the gel, soaked in 100 μl of water, boiled for 20 min, precipitated with ethanol and resuspended in 11 μl of nuclease-free water. The protocol adopted Promega's Wizad SV Gel and PCR Clean-Up System. The resulting purified bands were reamplified for 36 cycles using the same primer combination adopted for selective amplification.

Sequence Analysis

The reamplified PCR products were sequenced via an automated sequencer at the Pasteur Institute, Tunis (2 repetitions per sample). The fragments were sequenced in both directions. The sequencing reaction used the primer extension reaction (PCR) as cycle sequencing. AmpliTaq polymerase extended the primer to incorporate the dideoxynucleotides labeled with a fluorophore. In each cycle, a template strand was copied. Amplification of 25 cycles was linear. Similarity searches of expressed sequence cDNA (query) were compared with sequences already studied and collected in specialized databases such as Genbank under the existing barley DB (http://www.shigen.nig.ac.jp/barley) and other databases such as NCBI. Comparisons were made with Blast (BlastN and BlastX) software, an algorithm-based statistical model that applies to sequence comparisons without insertions-deletions.

Results

Transcripts involved in the response to salt stress in barley were identified by DDRT-PCR in plantlets subjected to 200 mM NaCl for 2 weeks. As shown in the example of the obtained expression profile (Fig. 1), about 100 novel cDNA fragments were induced by 200 mM NaCI, whereas others were repressed by the same treatment. About 2,000 cDNA bands were generated, 134 of which were upregulated and 89 were downregulated.

The length of the amplified cDNA fragments varied from 300 to 900 bp, depending on the primer combination used. We selected about 13 cDNA fragments that were reproducible with the six primers used and corresponded to the most abundant transcripts induced by salt detected on polyacrylamide gels. These cDNA fragments were named cDNA salt stress response (cDSSR). After purifying and sequencing these fragments, a first sequence comparison using the BlastN program showed that some of the selected cDNAs were homologous to each other. This was the case for cDSSR2 and cDSSR3, as well as for cDSSR1 and cDSSR4, which are contig sequences. cDSSR6 and cDSSR10 share a homologous internal sequence of about 100 nucleotides, suggesting that they may correspond to the same gene family involved in the salt stress response.

cDNA expression in barley tissues was analyzed by DDRT-PCR on total RNA extracted from leaves of plants cultivated in vitro in the absence of salt or in the presence of 200 mM NaCl. The expression of cDSSRs varied in this study: 45% (cDSSR A) were upregulated de novo by salt stress whereas 25% (cDSSR B) were mainly induced by 200 mM NaCl. In contrast, 30% of cDSSR C showed basal expression under control conditions, which significantly increased in response to salt stress (Fig. 2). Despite the repeated attempts to study the expression pattern of cDSSR1, cDSSR6 and cDSSR10 cDNA, we were not able to demonstrate the accumulation of the corresponding RNA transcripts.

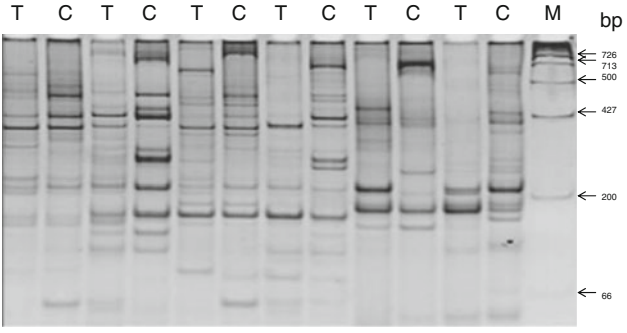

Fig. 1 Example of the obtained expression profile on 6% polyacrylamide gel. Transcripts involved in the response to salt stress in barley (Kebilli II accession) were identified by DDRT-PCR in plantlets exposed to 200 mM NaCl for 1 week. *Lanes C* control; *T* treated; *M* ϕ × 174 DNA/*Hin*fl markers (Promega, France)

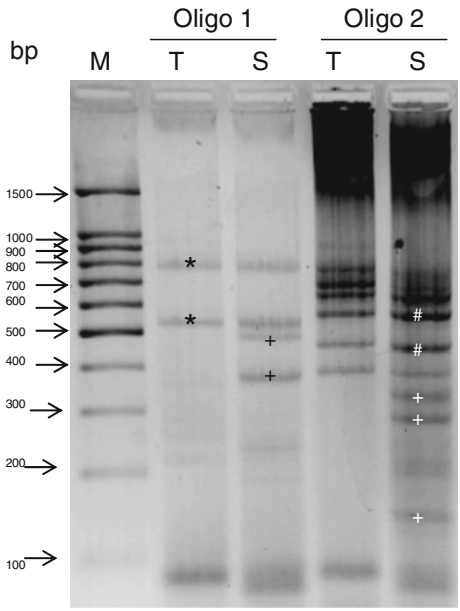

Fig. 2 Profile of electrophoretic reamplified cDNA for tolerant accession (Kebilli II) in 6% acrylamide gel using the primer combined with Oligo1 [HAP3 oligodT11 (G)] and primer combined with Oligo2 [HAP1 oligodT11 (C)]. **cDRRs A* cDNA with constitutive expression. *#cDRRs B* cDNA upregulated by salt stress. *+cDRRs C* cDNA induced by 200 mM NaCl. *Lane M* 100 bp ladder (Promega)

Discussion

In this study, we demonstrated that regulation of gene expression in the salt-tolerant and salt-sensitive genotypes of barley is distinctly different. Salinity treatment triggered differential gene expression responses in two barley genotypes: Kebilli II and Kelibia I. Despite only using 10 primer combinations, several salt-induced transcripts were identified by DDRT-PCR, showing the power of detection of this method. A databank search revealed similarities with wound, salt and other biotic and abiotic stress-responsive transcripts; 75% of the cDSSRs analyzed were homologous to ESTs isolated from other barley species (*Hordeum murinum*, *H. bulbosum*) submitted to biotic stresses, a virus and bacteria (40%: cDSSR2, cDSSR10, cDSSR11), or abiotic stresses: salt, cold and drought (60%: cDSSR1, cDSSR3, cDSSR7 and cDSSR10). A number of the cDSSR fragments were also homologous to ESTs expressed in different plant species in response to biotic (cDSSR4 and cDSSR6: tobacco and *Capsicum annuum*) or abiotic stress (cDSSR4 and cDSSR13: *Hordeum vulgare* and *Vitis infesta*). These data confirm that plants often use correlated ways to respond to various environmental biotic and abiotic stresses (Lu et al. 2005; Hossain et al. 2006; Soliman et al. 2009). The role of calcium, activated oxygen, abscisic acid (ABA) and ethylene in a signaling network has been suggested for both kinds of stress (Furihata et al. 2006; Fujii et al. 2007). The different salt-induced cDNA fragments identified in this study belong to five different classes, assessed relative to the literature:

Class 1: cDNA fragments related to salt and drought stress-responsive sequences. Three cDSSR fragments are closely related to ESTs induced by salt stress, the first formed by halophytic plants such as ice plant (*Lamprantus* sp.) (cDSSR1 and cDSSR13). On the other hand, cDSSR1, which seems to be expressed in response to

a variety of abiotic stresses (salt, drought, temperature), may encode a putative β-galactosidase. This protein plays a role in cell wall turnover (James et al. 2006; Liu et al. 2010), is proposed to act in the release of stored energy for rapid growth and in the release of free galactose during normal growth (Schmidt and Stougaard 2010), and is also supposed to be able to degrade cell wall components during senescence (Kim et al. 2007). cDSSR4 and cDSSR13 encode a putative senescence-associated protein and a proline-rich cell wall structural protein (Irshad et al. 2008). The cDSSR7 fragment encodes a NADP glyceraldehyde dehydrogenase which is involved in membrane structure and shares common domains with several other dehydrogenases, and is an essential membrane enzyme, functioning at the central junction at respiration, glycolysis and phospholipids biosynthesis. Its critical role is indicated by the multi-tiered regulatory mechanisms that stringently control its expression and function (Yeh et al. 2008).

Class 2: cDNA fragments related to wound stress response. Besides its similarity with the EST induced by osmotic stress, this cDSSR12-derived putative protein is homologous to wound-induced proteins. Such an interaction between wound and salt/drought stress response was previously described for the facultative halophyte, rice (*Oryza sativa*) (Talamè et al. 2007; Armengaud et al. 2009). On the other hand, cDSSR8 encodes a serine proteinase inhibitor which is a known wound-inducible protein (Hamida et al. 2005; Groppa and Benavides 2008). Serine proteinase inhibitor is supposed to function in the protection of plants against protein degradation occurring during physical damage imposed by either environmental factors or pathogens (Zhou et al. 2012); this protease seems to be activated to ensure the integrity of signaling components (Gibon et al. 2006; Zhao and Bughrara 2008).

Class 3: cDNA fragments homologous to biotic stress-responsive ESTs or proteins. cDSSR2, cDSSR4, cDSSR5 and cDSSR11 are homologous to ESTs upregulated by different biotic and abiotic stresses in a variety of *Hordeum vulgare* species (Nakashima et al. 2007; Hu et al. 2008). Some of these EST seem to be related to the hypersensitive reaction (HR) involved in plant resistance to pathogens (Fricke et al. 2006; Elvira et al. 2008). The cDSSR5 fragment is homologous to pathogen resistance genes identified in *H. vulgare* and the *Solanum* genus. On the other hand, a putative PR4 protein (chitinase) involved in fungi resistance (Bertini et al. 2009) seems to be encoded by the cDSSR8 fragment. Thus, different signal transduction pathways exist in plants that are activated by both biotic and abiotic stresses (Caporale et al. 2004; Li et al. 2009).

Class 4: cDNA fragments are involved in housekeeping. The putative cDSSR3-encoding protein shares a sequence common with barley (*H. vulgare*) photoperiod responsive (Phor1) protein that has a function in gibberellic acid (GA) signaling (Roessner et al. 2006). The GA family reportedly has an inhibitory effect on potato tuber induction (Hamida et al. 2005; Sutton et al. 2007).

Class 5: cDNA fragments encoding senescence-related proteins cDSSR4 and cDSSR13 seem to encode a putative senescence-associated protein resembling that of pea (*Pisum sativum*), which corroborates previous data indicating that senescence can be induced by different biotic and abiotic stresses (Lutts et al. 1996; Gagneul et al. 2007).

cDRR1 (class I) represent a gene *NHX1*, which encodes a protein acting as a antiporter Na^+/H^+ and which allows sequestration of sodium within the vacuole of barley. This function is supported by several authors. Indeed, analysis of membrane transporters and previous biochemical studies have shown that Na sequestration in the vacuole is governed by antiporters (Na^+/H^+) localized in the vacuolar membrane (An et al. 2008). Similarly, according to Yokoi et al. (2002) and Zhou et al. (2012), sequencing of the *Arabidopsis* genome has led to the identification of a gene controlling the AtNHX1 antiporter (Na^+/H^+) and subsequently five additional homologous genes of AtNHX1 sequestering sodium in the vacuole. Confirming these results, Soliman et al. (2009) overexpressed AtNHX1 in tobacco, which led to improved salt tolerance of tobacco. Their overexpression in new varieties may improve salt stress tolerance in barley or other plants (Isayenkov et al. 2010; Zhou et al. 2012).

Another point that also needs focused research is the use of Na^+/H^+ antiporter genes from halophytes. Thus, most of the transgenic experiments utilized Na^+/H^+ antiporters from glycophytes, mostly from *Arabidopsis*, rice and other species (Zhang et al. 2012). Halophytes have well developed salt tolerance mechanisms and the high salt tolerance of these plants might partly be due to the high efficiency of their membrane and vacuolar Na^+/H^+ antiporter genes (Muscolo et al. 2011). Therefore, research should be focused on the isolation of Na^+/H^+ antiporters from various halophyte species that can survive under highly saline conditions. Introduction of these antiporters into crop plants may enhance their salt tolerance by many fold (Gill et al. 2013). Therefore the future transgenic development for salt tolerance should use antiporters from halophytes (Ruan and Teixeira da Silva 2011). In addition to this, prior to the introduction of halophytic Na^+/H^+ antiporters into crop plants, the salt tolerance mechanism of both the recipient and donor plants should be understood. It is also important to know in which part of the plant the gene is to be targeted and expressed, because the recipient and donor may differ in localization and expression of the antiporter gene in different organs such as roots or shoots (Khan 2011).

Moreover, the Na^+/H^+ exchange activity of vacuolar antiporters is relatively low and limits their use in future molecular breeding programs. It is recommended that future research should focus on modification of the function of vacuolar antiporters by improving its Na^+/H^+ exchange activity (Zhong et al. 2012). This can be achieved through DNA shuffling technology in which mutations are generated in the gene structure, which are then recombined to produce new versions of the gene. Zhong et al. (2012) applied this technology to the *Arabidopsis* vacuolar *NHX1* gene and generated a new gene designated *AtNHXS1* using a yeast complementation system. Expression of this gene in yeast improved the tolerance to salt stress.

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