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TRANSCRIPTIONAL CHANGES IN SALT-RESPONSIVE GENES OF BARLEY SUBJECTED TO SALT STRESS

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ABSTRACT: Salinity is one of the major constraints for crop yield because it limits plant growth and reduces both the crop yield and the use of agricultural land. Increased salt tolerance requires new genetic sources of salt tolerance, and more efficient techniques for identifying salt-tolerant germplasm.

The DD-RT-PCR technique (Differential Display-Reverse Transcription- Polymerase Chain Reaction) is one of these techniques, which are able to compare and identify changes in gene expression at the mRNA and the cDNA levels between any pair of contrasting genotypes. It was performed using mRNA extracted from the aerial part of two contrasting Tunisian barley genotypes (Sabra: tolerant and kelibia: sensitive accessions) subjected or not to salt stress.

In this study, we have used this technique (RT-PCR) to identify cDNAs corresponding to transcripts up- or down-regulated by salt stress in barley. Within 18 primer combinations (3 Oligod(T) x 6 arbitrary primers), we have identified a total of 58 differential display products which are over-expressed or disappeared in stressed samples indicating a qualitative and quantitative difference in the gene expression.

***The up-expressed fragment were eluted and sequenced at the Pasteur Institute of Tunis and then compared to those of the bank data (Genbank Barley) to determine sequences having an optimal alignment with the query.

The result was the identification of many salt-responsive transcripts fragments corresponding to hypothetic proteins (T17F15.140-like and Mei2-like), to some proteins involved in oxidative and heat stress (GAD1= Glutamic Acid Decarboxylase) or to proteins involved in the resistance to pathogens (ß-1,3-glucanase) and to anionic flux resistance. But the most important finding is the identification of genes encoding the Na^+/H^+ antiporter which sequestrate sodium into vacuole in the tolerant barley genotype.

These two last transcripts encoding the sequestration of Na could be used as markers for selecting salt tolerant genotypes in the program of varietals improvement to salt stress tolerance.

KEYWORDS: *Hordium vulgare*, barley, differential display, salt response, gene expression, mRNA product.

1 INTRODUCTION

Agricultural productivity, in arid zone, frequently faces unfavorable growth conditions such as extreme temperatures, drought and salt stress. These adverse conditions can disrupt cellular structures and produce high levels of reactive oxygen species (ROS) that could lead to oxidative damage of structures.

Some plant species are able to survive and produce although under harsh environmental conditions, which are harmful for other plants [1]. This variable species behavior reflects a many different stress-response mechanisms which occur at all

levels of plant organization [2]. Many changes are known on response to environment stress including osmotic adjustment, modification of the plant architecture, decreases in water uptake by cytoplasmic accumulation of additional solutes through the process of osmotic adjustment [1]; [3], hydraulic limitations to leaf growth by inducing regulated decreases in root hydraulic conductivity [4], cell wall rigidity, growth reduction, leaf senescence, and other agronomic traits [5], [6].

At the molecular level, gene expression is modified upon stress [7] and epigenetic regulation plays an important role in the regulation of gene expression in response to environmental stress [8]. This regulation is characterized by a number of biochemical changes that ultimately result from a selective increase or decrease in the biosynthesis of a large number of proteins.

Several genes have been described that respond to salt stress at the transcriptional level in a variety of plant species [9], [10], [11].

Some of those genes are involved in cell structure protection such as OtsA gene (Operon trehalose synthase) which is responsible for trehalose synthesis (osmoprotectant) and which in turn, allows the proteins to retain their native configuration under environment stress without denaturation, others are implicated in ion transport or ion sequestration [12] and others are also implicated in transcription factors, protein kinases, and phosphatases [13].

Barley is one of the oldest cultivated crops in the world. It is the fourth most important cereal crop. It is used as feed of livestock, as human food and as malts or cosmetic product [14].

In Tunisia, as well as in the other African countries, barley is one of the most important cereal crop cultivated and occupies between 34% and 38% of the cereal area. Taking into account its importance and the fact that it is generally cultivated in marginal zones, it is important to analyze salt-responsive gene expression under saline and non saline conditions, as it may increase our understanding of the molecular mechanism of salt stress and the role of differential gene expression in salt tolerance genotypes.

The aim of the present study was to examine the differential expression of transcripts under salt stress and favorable conditions. We have focused on isolating and identifying the over expressed genes in saline conditions. The expression and the identification of these up-regulated transcripts in a salt tolerant genotype compared to those of susceptible ones could be used as molecular markers in screening and selecting barley genotypes for salt tolerance or as an efficient marker-assisted selection in breeding program.

2 MATERIAL AND METHODS

2.1 PLANT MATERIAL, STRESS INDUCTION AND RNA ISOLATION

To identify the most tolerant genotypes to salt stress conditions, we have carried out a trials in pots under greenhouse and at germination stage (Figure 1). All the seedling genotypes were watered regularly, every two days, with distilled water (control) or with a solution containing 200mM NaCl, during 21 days, since the sowing date.

According to some parameters (vigor, establishment rapidity, chlorophyll index, exhibition of necrosis symptoms, …), two contrasting barley genotype (“Sabra”: tolerant and “Klibia”: sensitive accessions) were selected for the molecular study.

Figure 1: Behavior of different barley genotypes under salt stress (A) or under favorable conditions (B). The circled genotype is the most tolerant to salt stress and on which the molecular study was conducted

After 21 days of sowing date, leaves were collected from salt-stressed and irrigated plants. The total RNA was isolated from 500mg of fresh leaf tissue according to Chomczynski and Sacchi, (2006)[15] method. Leaves were crunched in liquid nitrogen and homogenized in 4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, 0.1 M ß-mercaptoethanol, and then extracted with an equal volume of phenol: chloroform (1:1).

The RNA is subsequently precipitated by adding a double volume of absolute ethanol and centrifugation at 12,000 rpm for 10 minutes. The pellet was washed with 1 ml of ethanol 75% DEPC-treated (diethyl pyrocarbonate). After that, a second centrifugation was performed under the same conditions; the pellet was dried quickly in the air and then dissolved in 50 µl of DEPC-treated water.

The concentration and purity of RNA samples were determined by spectrophotometer at 260 and 280 nm wave lengths and then the ratio (OD_{260}/OD_{280}) was calculated. The total RNA was homogenized at 0.2 µg/µl before their use and their integrity was checked again using formaldehyde-agarose gel electrophoresis.

2.2 DIFFERENTIAL DISPLAY

Differential display of cDNA was performed using Gene hunter Differential Display Kit following the manufacturer's protocol.

To obtain the cDNA of the two barley genotypes subjected or not to salt stress, the RNA was reverse-transcribed (MMLV reverse transcriptase) using three types of anchored oligo-dT and six arbitrary primers HAP (Table 1). These three one-base anchored oligo-dT primers with 5' HindIII sites were used in combination with a series of arbitrary 13mers (also containing 5' HindIII sites) to reverse transcribe and amplify the barley RNAs.

Table 1: Primers used in the differential display technique.

Primers	Sequence
5' Arbitrary primers	
HAP1	5'AAGCTTGATTGCC-3'
HAP2	5'AAGCTTCGACTGT-3'
HAP3	5'AAGCTTGGTCAG-3'
HAP4	5'-AAGCTTCTCAACG-3'
HAP5	5'-AAGCTTAGTAGGC-3'
HAP6	5'-AAGCTTGCACCAT-3
3' Anchored oligo-dT primers	
HT11(G)	5'-AAGCTTTTTTTTTTTG-3'
HT11(C)	5'-AAGCTTTTTTTTTTTC-3'
HT11(A)	5'-AAGCTTTTTTTTTTTA-3'

The PCR was performed in 20µl mixture in a thermal cycler (Biometra UNO II). The reaction system was: 2 µl of 2 µM anchored oligo-dT primer HTllM (M=A, G or C), 0.2 µg total RNA, 1.6 µl of 250 µM dNTP in a volume of 20 µl for denaturation 5 min at 65°C and the incubation for 10 min at 37°C was followed, then the MMLV (100µM) reverse transcriptase was added for 5 min at 37°C and 5 min at 72°C.

Three independent reactions, using HTllM (M = G, A, C) as primers, were performed according to what was used by [16], [17]) and [18]. The cDNA were then amplified by PCR. The reaction mixtures (20 µl) included 2.0 µl of resultant cDNA, 2.0 µl of 10 X PCR buffer, 1.6 µl of dNTP (25 µM), 2.0 µl of HAP primer (2.0 µM), 2.0 µl of HTllM primer (2.0 µM), 0.2 µl of AmpTaq, and dH_20 10.2 µl.

The samples were subjected to 40 cycles of PCR using the following parameters: 94°C for 30 sec, 42°C for 2 min, and 72°C for 30 sec; the last cycle was followed by a 5 min extension at 72°C. The PCR products were fractionated by electrophoresis in denaturing 6% polyacrylamide gel. At the end of electrophoresis, the banding is done by soaking the gel in ethidium bromide buffer (10 mg/ml) and the photography was done in a digital documentation system (Gel-Doc).

The differentially expressed gene fragments were excised from denaturing 6% polyacrylamide gels, soaked in 100 µl dH_2O for 10 min, and then boiled for 15 min. After centrifugation for 2 min at 12000 rpm to pellet any solid debris, the supernatant was removed to another tube. The cDNA was recovered by ethanol precipitation in the presence of 10 µl of sodium acetate (3M) for overnight at -80°C and then centrifuged at 12000 rpm and 4°C for 10 min and finally washed with 95% ethanol, dissolved in 10 µl of dH_2O, kept at -20°C until use.

The eluted fragments were re-amplified again in 25 µl PCR mixture using the same set of arbitrary and anchored primers that generated the differential product. Re-amplified PCR fragments (10 µl) were resolved on a 1.5% agarose gel.

2.3 SEQUENCING OF DIFFERENTIALLY EXPRESSED TRANSCRIPTS UNDER SALT STRESS

Twelve (12) up-regulated fragments on fifty eight (58) differentially displayed bands, were isolated from the gel as indicated above, re-amplified and dissolved in a TE buffer [10:1 mM (pH 8.0)] for being sequenced and compared to a query sequence with that of database, using BLAST algorithm (**B**asic **L**ocal **A**lignment **S**earch).

3 RESULTS

3.1 DIFFERENTIAL cDNA DISPLAY BETWEEN CONTROL AND SALTED BARLEY PLANT

The differential display technique (Figure 2) showed some unique bands in both salted or control samples. However the number of bands varies according to primer combinations. For example, in the OligodT_{11}(C)/HAP4 combination (Figure 2), we observed disappearance (down regulated) of some cDNA bands in sensitive genotype subjected to salt stress conditions. In the opposite, some cDNA bands were induced (up-regulated) in tolerant genotype under salt stress conditions and some other bands are of higher intensity (Figure 3).

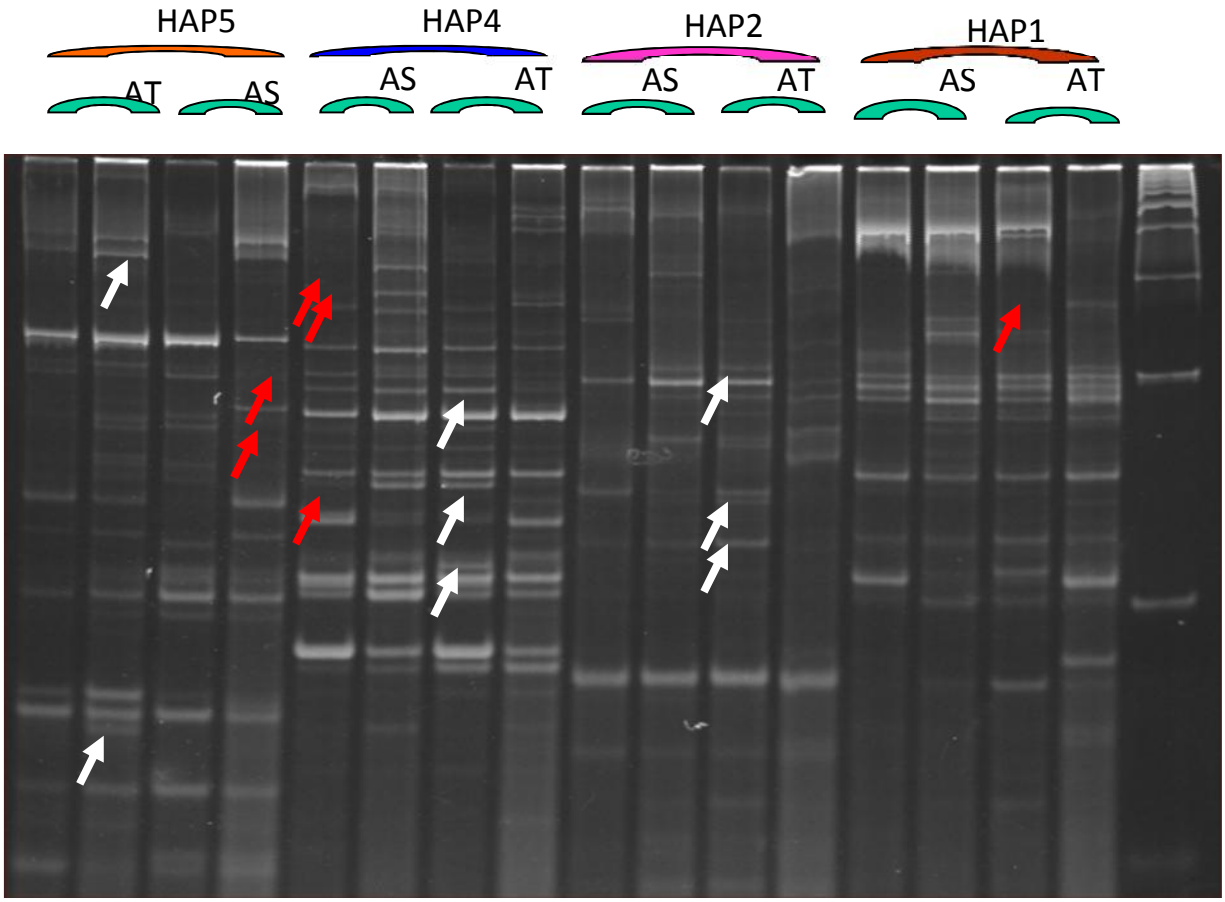

Figure 2: CDNA profile of salt-stressed and control barley genotype plants subjected or not to 200 mM NaCl and using OligodT_{11}(C) combined to 4 arbitrary primers.

M= 100pb ladder (Promega); TA: Salt-tolerant genotype (Sabra); SA: Salt-sensitive genotype (Klibia), S= Stressed and C= Control

: Indicate down-regulated transcripts under salt stress

: Indicate up-regulated or new activated transcripts under salt stress.

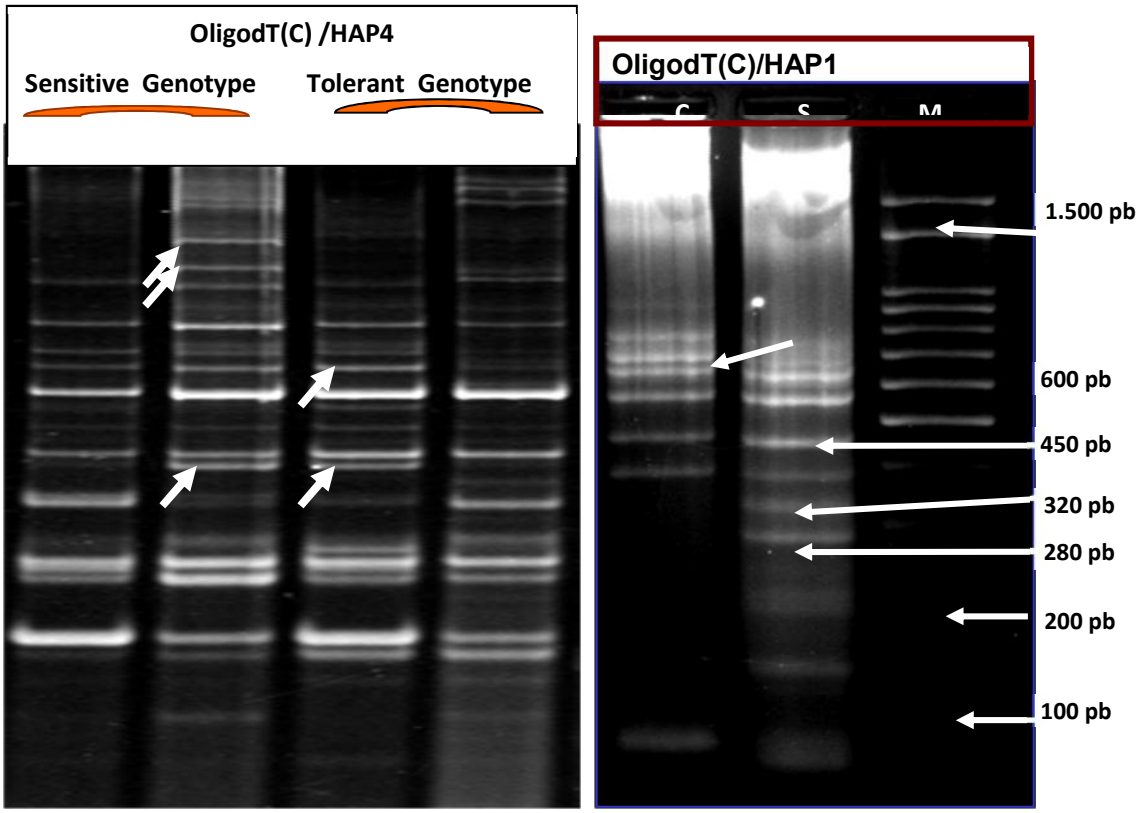

Figure 3: cDNA profile of salt-stressed and control barley genotype plants subjected or not to 200 mM NaCl and using $OligodT_{11}$(C) combined to HAP4 or $OligodT_{11}$(C) combined to HAP1.

Salt-tolerant genotype is (Sabra); Salt-sensitive genotype is (Klibia)

C = Control

S = Salted

M = 100pb marker

The size of amplified cDNA bands vary from 200 to 800 pb according to the primer combination. The comparison of band intensity between control and salted barley plants in all primer combination revealed that some bands were of lighter intensity in stressed samples as compared to their levels in control. However, salt stress induces several new transcripts as it was shown in Figure 3.

3.2 COMPARISON OF UP-REGULATED CDNA FRAGMENTS TO THAT OF DATABASE

The up-regulated cDNA fragments were sequenced and compared with that of database, using BLAST algorithm (Basic Local Alignment Search). The alignment of transcript fragment (Table 2) has revealed a common field between the different sequences which may reveal a functional significance. It classifies the up-regulated fragments into 5 functional groups, including mineral nutrition, cell defence against pathogens, production of secondary metabolites, Na anti-transportation and unknown functions

The first group revealed the presence of sequence contig which is a set of overlapping DNA segments derived from a single genetic source.

The second group contained the genes encoding certain enzymes like polygalacturonase (PG1, PG3, PG4) or proteins with unknown functions. These type of enzymes has been shown, in tomato, by [19] who suggest also that these polygalacturonases (PG) may exist in different forms (PG1, PG2a, PG2b, and PGx) commonly referred to as PG isoenzymes.

The third group contained STS (Sequence Tagged Site) which represent, according to the BLAST alignment, a flow of anionic resistance and mineral nutrition genes.

The fourth group is represented by proteins belonging to the serine carboxypeptidase family, encoding diverse group of enzymes involved in protein degradation or secondary metabolites production [20].

Finally the fifth group represented the NHX1 genes encoding proteins acting as an antiporter Na^+/H^+ and allowing the sodium sequestration into the barley vacuole.

Globally, the result obtained allowed us to identify many salt-stress responsive genes, from which most of them is unknown genes being involved in salt-stress response but some genes corresponded to that controlling the plant nutrition or Na sequestration in the barley vacuole (Table 2). This finding was supported by [21] and [22] for which Na^+ can move symplastically into an adjacent cell via plasmadesmata; or it can be transported into the vacuole through vacuolar Na+/H+ antiporters such as *NHX1.*

Tableau 2: Alignment of sequences experimentally obtained with those of the bank data "Barely Genbank DB".

Sequence Number	Sequence identification (optimal alignment)	Protein name	Gene name	Score (bit)	E-Value	Identity (%)
G1	AF474982	Hypothetic protein (T17F15.140-like and Mei2-like)		30	0.79	100
G3, G4	L44097 STS mRNA		Gene of Almt Gene of resistance to anionic flux and mineral nutrition	32	0.19	100
G5	AF474982	hypothetical protein T17F15.140-like protein, hypothetical protein, Mei2-like protein		30	0.81	100
G8	AF521177	HGA4, PG3, GAD1, PG1, PG4, HGA1	HGA5_1, HGA5_2, HGA4, rh2, pg3, gad1, pg1, pg4, hel1, HGA2, HGA1	30.2	1.4	100
G9	AY184476	Na+/H+ antiporter	NHX1	743	0.0	100
K4	AJ234533 Genomic fragment (clone MWG0604)			30	1.8	100
K7	L44097 STS mRNA		Almt	32	0.18	100
K8	Y09602	serine carboxy peptidase II, CP-MII	Cxp; 2	30	0.70	100
K13	AJ234533 DNA fragment (clone MWG0604)			30	1.5	100
K14	X00408	ATPase, beta subunit, ATPase, subunit E	atpB, atpE, tRNA2-MET	406	e-113	96
K15	AX356916,AX356915 Clone 001-044-A10					

4 DISCUSSION

The reverse transcription of mRNA has revealed many cDNA bands with variable intensity especially in the stressed plant where some bands have lighter or higher intensity as compared to their levels in control samples. This indicates that salt stress modified some mRNA synthesis both qualitatively and quantitatively, reducing overall protein synthesis, as reported in

soybean by [23] and [2]. As well, salt stress activated several new transcripts indicating variation in gene expression at transcription levels. This would suggest down regulation and up-regulation of these genes.

According to [24], the sequence contig observed corresponds to regions involved in regulation of gene expression or encoding protein called hypothetical protein (T17F15.140 and Mei2-like-like) with unknown role or with function not yet demonstrated experimentally. These regions may be involved in nutrient metabolism as it was demonstrated by [25] or in various physiological events as it was suggested by [26].

The genes encoding proteins (PG1, PG3, PG4, HGA1, HGA4, GAD1= Glutamic Acid Decarboxylase) correspond to an unidentified proteins where some of them may play role in pectin-degrading as it was suggested by [19] (PG1, PG3 and PG4). They might correspond also to pathogenicity related proteins involving the ß-1 ,3-glucanase which is the first line of defense of barley against fungal pathogens according to that was demonstrated by [27] (HGA1 and HGA4).

The GAD1 is a glutamic acid decarboxylase enzyme which is involved in the degradation process of glutamate [28] and plays an important role in the process of oxidative stress tolerance. The tissues, in which this pathway is blocked, are more sensitive to oxidative stress [28]. It was previously demonstrated [29] that salt stress induced many oxidative compounds which were prevented by an efficient antioxidative system (SOD, APX, catalase, tocopherol,…) in plant tolerant.

The gene encoding the serine carboxypeptidase's family mediates some biochemical reactions to maintain the osmotic and ionic intracellular medium stable. The omnipresence of these proteins also suggests an important role in the physiology of the cell as it was suggested by [30]. This role may be an osmotic adjustment mechanism which allows plants to reduce their leaf water potential, maintain their cells turgor, and preserve their membrane integrity and then their photosynthetic activity as it was suggested by [31]. Furthermore, this gene may play an important role in protein degradation or secondary metabolites production as it was recently demonstrated by [20].

The most important finding in this study is genes NHX1 which encode proteins acting as an antiporter (Na^+/H^+). This achievement 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 [32].

Similarly, according to [33], sequencing of the Arabidopsis genome has led to the identification of genes controlling the AtNHX1 antiporter (Na^+/H^+) and subsequently five additional homologous genes of AtNHX1 sequestering sodium in the vacuole. To confirm these results [34] have over-expressed the AtNHX1 in tomato and led to improve salt tolerance of tomato. Also, [35] have identified, in rice, a NHX1 homologous (OsNHX1), who's over expression conferred an increase of salt tolerance in rice.

5 CONCLUSION

This study has shown mRNA and then cDNA differentially displayed in barley genotypes subjected or not to salt stress.

The identified genes were mainly related to plant defense (ß-1 ,3-glucanase), to oxidative stress response (glutamic acid decarboxylase), to proteins acting as an antiporter (Na^+/H^+) and to other proteins with function not yet fully demonstrated experimentally.

Our results provide evidence suggesting that tolerance to salt stress in barley and especially in "Sabra" variety is carried out mainly by enhancing the expression of many physiologically important mechanisms in which the sequestration of sodium into vacuole. The ability of the vacuole to sequestrate sodium is a key factor for this variety tolerance but it appears conceivable that more than one mechanism is responsible for gene activation under salted conditions. These genes could be involved in various physiological functions of major importance for barley tolerance to salt stress.

The cDNA fragment and their corresponding genes could serve as useful markers for the identification of salt tolerant genotype and they could be used also as an efficient marker-assisted in breeding program.

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