

DATA MINING of ESTs for GENETIC IMPROVEMENT of SALT TOLERANCE in WHEAT

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ABSTRACT

A total of 4,131 expressed sequence tags (ESTs) were selected from wheat EST database (http://wheat.pw.usda.gov/cgi-bin/westsql/est_lib.cgi) to identify genic regions differing at structural and functional level between durum wheat (tetraploid genome, AABB) and bread wheat (hexaploid genome, AABBDD) cultivars in response to salt stress. Selected ESTs from salt stressed *Triticum aestivum* cDNA libraries (922 seedling, 2055 root, 1154 sheat ESTs) were used for construction of contig tags. Using contigs assembled (136 seedling, 268 root, 152 sheat) sense and antisense primers were designed to investigate the sequence and expressional differences in bread and durum wheat cultivars. The genetic information obtained from data mining of ESTs can make huge contribution to the characterization of genes in response to salinity stress, and is strongly expected to aid our understanding of the molecular mechanism of salinity stress tolerance of durum and bread wheat in particular.

Key words: *Triticum aestivum*, *Triticum durum*, contig, primer, salinity

INTRODUCTION

Excess amount of salt in the soil adversely affects plant growth and development. Nearly 20% of the world's cultivated area and half of the world's irrigated lands are affected by salinity (Baret-Lennard, 2000). Developmental processes such as seed germination, seedling growth and vigour, vegetative growth, flowering and fruit set are adversely affected by high salt concentration, ultimately causing diminished economic yield and also quality of produce (Sairam and Tyagi 2004). Salt tolerance is a complex trait involving in responses to cellular osmotic and ionic stresses and their consequent secondary stresses (e.g. oxidative stress) and whole plant coordination. The complexity and polygenic nature of salt stress tolerance are important factors contributing to the difficulties in breeding salt-tolerant crop varieties (Zhu, 2000). So far, much effort has been directed toward understanding the molecular and cellular mechanisms, which plants tolerate salinity stress with the eventual goal of improving salt tolerance of crop plants (Flowers, 2004). There is a wealth of evidence indicating that changes in gene expression occur in plants following an exposure to salt. During the last decade, a number of salt-responsive genes have been isolated and characterized. Within crop species studied, a wide range of tolerance to salinity exists, from very high (*Beta vulgaris*) to extremely low (*Citrus* spp.). There also exist salt-tolerant and salt-sensitive varieties within a given crop species. Salt-tolerant species have been frequently employed to isolate genes involved in salt-tolerance, and thereby gain an understanding of the

mechanisms that distinguish them from their salt-sensitive counterparts. On the other hand, it is surprisingly difficult to quantify differences in salt tolerance between closely related species, as the reduction in growth depends on the period of time over which the plants have grown in saline conditions. During a short time in salinity, there will be a significant decrease in growth rate, but the decrease may be the same for species that have quite different reputations for salt tolerance. For instance, durum wheat is known to be more salt-sensitive than bread wheat, and its yield is more affected (Francois et al., 1986). Munns et al. (1995) have found no differences between durum and bread wheat cultivars, nor between barley and triticale cultivars for short periods of salt exposure. They reported that there were no significant differences between the leaf elongation rate in the first 10 d of salinization of any cultivar. This could be due to the time scale and the different mechanisms that may be important in controlling growth rate at different periods of time.

A number of approaches have been undertaken to isolate genes whose expression is influenced by salinity in plants. A majority of these experimental approaches involved in screening cDNA libraries constructed from mRNA populations isolated from salt treated plants or cells (Sairam and Tyagi 2004). cDNA technology was first standardized with the human genome and over 18 millions of cDNA and expressed sequence tags (ESTs) are currently available for hundreds of organisms in public databases (www.ncbi.nlm.nih.gov) (Altschul et al., 1990). ESTs are short cDNA sequences that serve to tag the gene from which the mRNA originated. Typically, anonymous ESTs are single-pass sequenced to yield a 200-700 bp sequence that can be used to search DNA and protein databases for similar genes (Adams et al., 1991). The EST and cDNA sequences for more than 50 plant species are also available. Among plants, wheat has the largest amount of EST collection in the public database. A total of 500,898 ESTs from 38 different libraries corresponding to about 23,000 uni-genes are present in the EST database for wheat (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html). EST analysis is an effective method in mapping of many genes to chromosomal sites and discovering novel genes and investigating gene expression in different organs and tissues. Mining of EST has become a widely used approach to identify genes involved in specific biological functions (Gueguen et al., 2003). Especially obtaining ESTs from various developmental stages and in response to various biotic and abiotic stresses is an efficient and efficient approach to target the expressed portion of any genome (Randhawa, 2004). For instance, the EST approach has been used to discover novel genes and to determine the expression patterns of *Suaeda salsa* (Zhang et al., 2001), *Thellungiella halophila* (Wang et al., 2004), *Avicennia marina* (Mehta et al., 2005) and *M. crystallinum* (Kore-eda et al., 2004) in response to salinity stress.

In this study, our aim is to identify gene regions, which are differing at sequence and expressional level between durum and bread wheat genotypes in response to salt stress. For this purpose, selected ESTs from salt stressed *Triticum aestivum* cDNA libraries (seedling, root, sheat EST libraries) were used for construction of contig tags. From contigs, sense and antisense primers were designed to define the sequence differences and gene expression patterns in wheats.

MATERIALS AND METHODS

Plant material

Four bread wheat *Triticum aestivum* (Alpu01, Dağdaş, ES14 and Yıldız 98) and three durum *Triticum durum* (Ç1252, Meram and Selçuklu) wheat cultivars have been used for DNA extraction and salt stress treatments for expression analysis. Seeds of the cultivars were obtained from the Anatolian Agricultural Research Institute - Eskisehir and International Agricultural Institute – Konya, Turkey.

Analysis of wheat salt stressed ESTs

ESTs from salt stressed *Triticum aestivum* cDNA libraries (roots of seedlings, 21 days old seedling, sheath at the seedling stage) were chosen from http://wheat.pw.usda.gov/cgi-bin/westsql/est_lib.cgi. Then these EST sequences were further processed for vector contamination at the <http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html> web site and undesired vector fragments extracted from sequence lists. All processed EST sequences were compared by the Blastn algorithms using default settings in GrainGenes Blast server at Mapped wheat ESTs database section (<http://wheat.pw.usda.gov/GG2/blast.html>.) Vector NTI 9.0 contig express program, a new molecular tool from InforMax (Bethesda, MD, USA) was used for construction of contig tags from wheat salt stressed EST sequences. Unigene and singleton construction was done from unassembled ESTs and contig sequences. Alignment analyses of wheat EST sequences was performed by running the ClustalW 1.82 program in <http://www.ebi.ac.uk> web browser exposing for the conserved domains. After that, these contig assemblies were selected for further characterization and subjected to Primer 5.0 program for PCR primer designing. They were queried using BLASTX algorithm of Basic Alignment Search Tool (Altschul et al., 1990) to see functional annotation.

Genomic DNA isolation and salt stress treatment for expression analysis

Plants were grown in pots in the greenhouse. Leaves from two-week-old plants were used for DNA extraction using the method of Kidwell and Osborn (1992). Genomic DNA amplifications with the sense and antisense primers designed from contigs were performed using a PTC-100 MJ thermocycler (MJ Research, Watertown, MA) in a 25 μ l reaction volume; each reaction contained 1X PCR buffer (75 mM Tris HCl pH 9.0, 50 mM KCl, 20 mM $(\text{NH}_4)_2\text{SO}_4$), 5 mM MgCl_2 , 2.5 mM of each dNTP, 2.5 μ M of sense and antisense primers, 100 ng of genomic DNA, and 1 unit of *Taq* polymerase (Promega, USA). The thermal cycling parameters were: initial denaturation at 94°C for 2.5 min, 37 cycles of 30 s at 94°C, 30 s at 58°C and 1 min at 72°C, followed by a final extension at 72°C for 5 min. PCR products were separated in a 1.2% agarose gel, and visualized by staining with ethidium bromide and UV illumination.

Wheat seedlings were grown hydroponically in half-strength Hoagland solution circulated by air pumps in a growth chamber at 22°C, with a photoperiod of 16h light/8h dark. Thirty days after germination, gradual salt stress was imposed by adding NaCl over 3 days until a final concentration of 150 mM NaCl was achieved. CaCl_2 was added with NaCl to maintain a $\text{Na}^+:\text{Ca}^{2+}$ concentration ratio of 10:1 on a molar basis. Shoot and root samples were collected for total RNA extraction from the control plants and salt-stressed plants at three time points (3 hrs, 8 hrs, and 27 hrs) after reaching the final concentration and stored at -80° C for RNA extraction (Figure 1.)

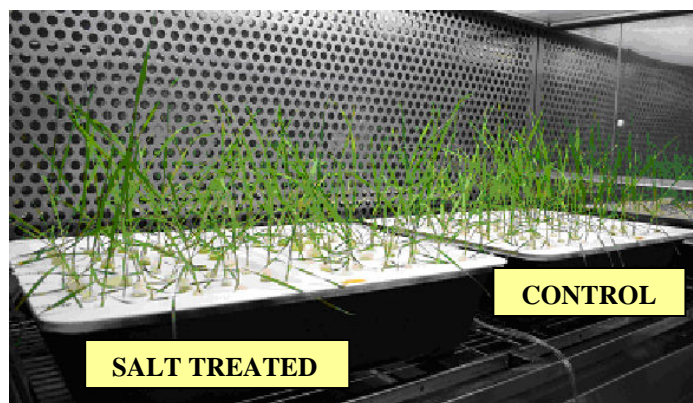


Figure 1. Salt stress treatment in hydroponic solution.

RESULTS AND DISCUSSION

Assembly of Contigs and Blast Analysis

2,055 ESTs from salt stressed root, 922 ESTs from seedling and 1,154 ESTs from sheath tissues were selected from wheat ESTs database. After extraction of undesired vector fragments from these sequences, contig tags were constructed by using Vector NTI 9.0 contig Express program. Contigs reflect differential levels of gene expression. The greater the number of constituent sequences in a contig, the more highly expressed the gene responsible for the expressed sequences. 268 contigs from root ESTs, 136 contigs from seedling ESTs and 152 contigs from sheath ESTs were assembled. Unigene and singletone construction was done from unassembled ESTs and contig sequences. % 74.01 of root ESTs, % 67.24 of seedling ESTs and % 63.26 of sheath ESTs were unigene and other general characteristics of ESTs are summarized in Table 1.

Table 1. General characteristics of salt stressed wheat ESTs

Salt stressed ESTs of root tissues from <i>Triticum aestivum</i>	
Library name	TA065E1X
Stage	Seedling
Total number of ESTs	2055
Contig number	268
Total contig size (bp)	241692
Unigene number	1521 / % 74.01
Singleton number	1253
Salt stressed ESTs of seedling tissues from <i>Triticum aestivum</i>	
Library name	TA054G1X
Stage	21 days old
Total number of ESTs	922
Contig number	136
Total contig size (bp)	81550
Unigene number	620 / % 67.24
Singleton number	484
Salt stressed ESTs of sheath tissues from <i>Triticum aestivum</i>	
Library name	TA037E1X
Stage	Seedling
Total number of ESTs	1154
Contig number	152
Total contig size (bp)	100100
Unigene number	730 / % 63.26
Singleton number	578

All ESTs were queried using BLASTN algorithm of Basic Alignment Search Tool to define “total BLASTN hit number” and “zero hit BLASTN number for wheat salt stress relation”. 21%, 22% and 57% of total blastn hit number belong to sheath, seedling and root respectively. Zero hit blastn number was the highest (44%) in root ESTs.(Figure 2a, b).

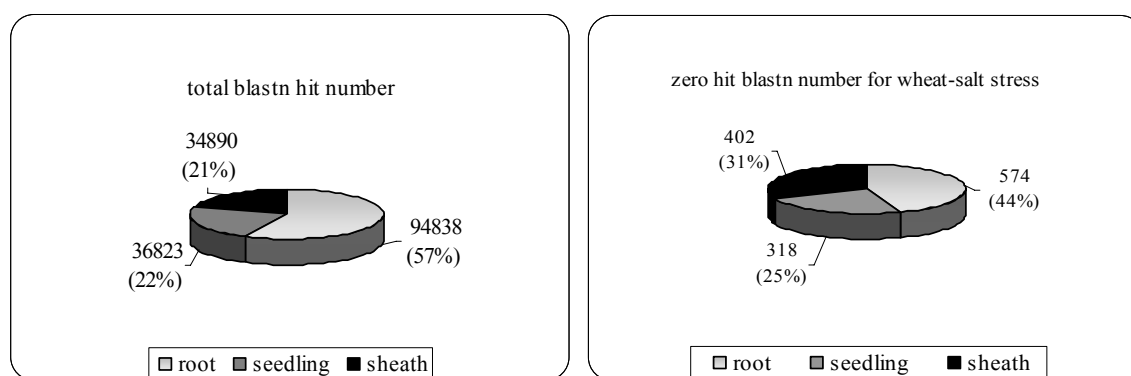


Figure 2. a) Total BLASTN hit number of ESTs, b) Zero hit BLASTN number for wheat salt stress relation.

Initially, thirty seedling contigs were selected for designing primers. Sense and antisense primers were designed to define the sequence differences and gene expression patterns in bread and durum wheat cultivars (Table 2). These contig sequences were compared by the BLASTX algorithms in the NCBI. The BLASTX translates nucleotide sequences in six reading frames and compares them against amino acid sequences. Results of the BLASTX search showed that 30% of contigs from seedling tissue have homology to genes of *Triticum aestivum*. Contig 17 did not match with any organism and functional annotation while contig 24 matching sequence had unknown function. Other contigs from seedling tissues have homology to genes of known function (Table 3).

Table 2. Primers is being used for genomic amplification and RT-PCR analysis.

Primer	Sequence (5'-3')	Primer	Sequence (5'-3')
SC1F	TTC TGC CTC GTA TTT CTG GGT C	SC 16F	TCA CCC TCA TCC GCT TCT TGC
SC1R	CGC GGC TTG CTG TCA TCT CG	SC 16R	TTG GGC AGC TCC CGC ACC TT
SC 2F	CTT CGT GTT GTC CCT CTT GC	SC 17F	AGG CTT AGG CAT CGG CTC TG
SC 2R	TTC AGT TCT CCT CCG CCT CC	SC 17R	AGC AGC AAT GGC GAG GCA CA
SC 3F	GGA TGA GGT GAT ACG GTA GAG T	SC 18F	GCT CCA CCT AGC CTC TTC CTG T
SC 3R	AGG AGA TGG CGG ACG CGA AG	SC 18R	CGT CGC CAA TGC CCT CTT CT
SC 4F	GAG CAA AGC AAG GAC GGT GAA	SC 19F	GTC GAG CGG TGC AAT GGT GT
SC 4R	CGT TGA TGC CGC TGA TGT TGA	SC 19R	GGG AAG CAG TCG TGG AAG AAG AT
SC 5F	TGT AGG TGA ACT TGA CGG TGG CC	SC 20F	TCG TCG TGC TGG GTG GAG AT
SC 5R	CAT CTT CGT CGG CGG CTT CG	SC 20R	AAG CGT CCT GAA GAG GTT GG
SC 6F	GAC AAC CAC CAT GAG CAC CG	SC 21F	CCG TCC TGC TCC TCT TGT TC
SC 6R	GTC GTC ATC CGC CAA ACC AG	SC 21R	TAG CCA GTT GTG CCC GTT GA
SC 7F	TCT TCT CCA ACT GGC TGC TC	SC 22F	TGA CCC GAC CAG CAC AGA AA
SC 7R	GCA CTT GAC CTT CTC CCA CA	SC 22R	TCG AAA TTG ATA AGC GGA GA
SC 8F	AGG ACG AAC GAG ACG GA GG	SC 23F	CTC CAA GCC ACG CGA GAC CA
SC 8R	CTC TGG GTC GCC ACG AAC AT	SC 23R	CCA TCA AGC TGC TGA ACC ATA
SC 9F	GAA CAG CGT TGA AGA CAA GGA A	SC 24F	CAG ATA ACT TCC GGC CAA ACC
SC 9R	CGA GCC CAC TGT GAT TGA TG	SC 24R	GGC TCA GCA CTC GCC ACT TC
SC 10F	TGA GCA GGT GGG ACG GAC TT	SC 25F	ATG ATG GCG TTC TCA GGG TT
SC 10R	CGT GTT CAA CGA CGC TCT GG	SC 25R	CAC CAA GGA GGG TCT GAA GC
SC 11F	TAC TCC TCG CCC AGG TCC AT	SC 26F	GGG AGG CGA GCG ACA ACA CT
SC 11R	GGT ACG TCC CGC TGA AAC AAA	SC 26R	CGG AGT ACC GCT GCT TCG TG
SC 12F	GAC GGC ATT TGC GAA ACC AT	SC 27F	TCT CCT CCT GCT TGA ATG TC
SC 12R	TAG GCA GCG GTG TTG AGC AG	SC 27R	AAT GTT ATC CGT GTT GCT CC
SC 13F	CCC CTA ATC TCA TTG CCT GTT	SC 28F	GCC ACG GCC TCA GCG AAG TA
SC 13R	CTC CCT TGT TCA TAG CTT CCT T	SC 28R	GAA GCC CAG AGC ATA GCA GAG CAG
SC 14F	TTG AGA AGA AAT ACG AAC CCA C	SC 29F	GCG AAG ACG ACG CCG ATG TT
SC 14R	CAC AAA CCC TGC ACA AGT CC	SC 29R	GAT TGC TGG AGA AAT GGA GAC GAG
SC 15F	AAG CAG GTT GGC GTC GTC GTT	SC 30F	GCA GCG GCT TTA TTA CCT ACC
SC 15R	TCC TCC TCG CTG CCT TCC AC	SC 30R	CAG CTC TGG CTT GCT CTT GC

Table 3. Abundance profile of 30 seedling contigs of *T.aestivum*.

Contigs	Functional annotation	Matched organism	E- value	Redundancy
Contig1	putative ribosomal protein L11 /NP_921904	<i>Oryza sativa</i> (japonica cultivar-group)	7E-16	1
Contig2	putative 60S ribosomal protein L18a /AAT77404	<i>Oryza sativa</i> (japonica cultivar-group)	1E-44	4
Contig3	putative 2-oxoglutarate/malate translocator	<i>Oryza sativa</i> (japonica cultivar-group)	1E-51	1
Contig4	glutamine synthetase isoform GSr2 /AAR84348	<i>Triticum aestivum</i>	1E-139	1
Contig5	CAA30379.1 protein /CAB53482	<i>Oryza sativa</i>	1E-61	1
Contig6	similar to zinc finger protein 629partial/XP_799042	<i>Strongylocentrotus purpuratus</i>	0,015	1
Contig7	phosphoethanolamine methyltransferase /AAL40895	<i>Triticum aestivum</i>	7E-134	1
Contig8	OSJNBa0027H06.13 /XP_471005	<i>Oryza sativa</i> (japonica cultivar-group)	5E-31	1
Contig9	alpha tubulin subunit /AAK81858	Rosa hybrid cultivar	3E-20	1
Contig10	O-methyltransferase /AAX94931	<i>Oryza sativa</i> (japonica cultivar-group)	1E-49	15
Contig11	Photosystem I reaction center subunit psaK, chloroplast precursor/P36886	<i>Hordeum vulgare</i>	3E-35	7
Contig12	hypothetical protein AN8496.2/XP_681765	<i>Aspergillus nidulans</i> FGSC A4	2E-53	1
Contig13	adenylate kinase b (ec 2.7.4.3)(atp-amp transphosphorylase)/AAX96124	<i>Oryza sativa</i> (japonica cultivar-group)	9E-57	2
Contig14	small Ran-related GTP-binding protein /AAM08320	<i>Triticum aestivum</i>	9E-78	1
Contig15	Hypothetical protein CBG08739 /CAE64123	<i>Caenorhabditis briggsae</i>	8,3	1
Contig16	OSJNBa0038J17.13 /NP_913315	<i>Oryza sativa</i> (japonica cultivar-group)	7E-20	1
Contig17	No significant similarity found	-	-	-
Contig18	146aa long hypothetical protein /BAA79966	<i>Aeropyrum pernix</i> K1	1,5	1
Contig19	peroxidase precursor /CAI47635	<i>Triticum aestivum</i>	7E-30	2
Contig20	putative AdoMet synthase 3/CAJ01704	<i>Hordeum vulgare</i> subsp. <i>Vulgare</i>	8E-132	1
Contig21	OSJNBb0034I13.8 /XP_474041	<i>Oryza sativa</i> (japonica cultivar-group)	0,003	1
Contig22	hypothetical protein /AAL27554	<i>Musa acuminata</i>	2E-58	3
Contig23	enolase	<i>Oryza sativa</i> (japonica cultivar-group)	1E-42	55
Contig24	unknown protein /NP_913997	<i>Oryza sativa</i> (japonica cultivar-group)	2E-65	2
Contig25	cytosolic heat shock protein 90 /AAP87284	<i>Hordeum vulgare</i>	5E-54	27
Contig26	low temperature-responsive RNA-binding protein/AAB07749	<i>Hordeum vulgare</i> subsp. <i>Vulgare</i>	1,00E-09	1
Contig26	low temperature-responsive RNA-binding protein/AAB07749	<i>Hordeum vulgare</i> subsp. <i>Vulgare</i>	1,00E-09	1
Contig27	putative mitochondrial energy transfer protein	<i>Oryza sativa</i> (japonica cultivar-group)	1E-50	1
Contig28	putative taxadien-5-alpha-ol O-acetyltransferase /NP_914499	<i>Oryza sativa</i> (japonica cultivar-group)	5,00E-67	1
Contig29	hypothetical protein /AAL27554	<i>Oryza sativa</i> (japonica cultivar-group)	7E-12	5
Contig30	glutathione S-transferase /AAK66773	<i>Triticum aestivum</i>	8E-66	24

The aim of this study is to identify the regions differing in durum and bread wheat genotypes in response to salt stress by using EST and informatics tools. With the sense and antisense primers designed from contigs DNA amplifications (Figure 3) and RT-PCR analysis are currently being used. With the advent of whole genome sequencing, it may appear that ESTs have lost some of its appeal. However, the genomes of many organisms that are important to society, including the majority of crop plants, have not yet been fully sequenced, and the prospects for large-scale funding to support the sequencing of any but a few in the immediate future is slim to none (Zheng et al., 2003).

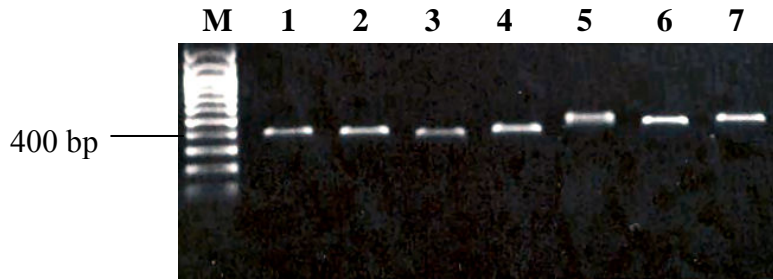


Fig 3. Genomic DNA amplification with SC1F and SC1R primer pair in wheat cultivars. M:100–1500 bp DNA ladder, 1. Alpu 01, 2. Ç1252, 3. Dağdaş, 4. ES14, 5. Meram, 6. Selçuklu, 7. Yıldız 98.

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