ESTs analysis of the sugar beet (*Beta vulgaris* L.) responsive transcripts under salt stress

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ABSTRACT

Differential Display Reverse Transcriptase (DDRT-PCR) technique was used to analyze differentially expressed genes in sugar beet (Beta vulgaris L.*) under salt stress. Three weeks old seedlings were exposed to salt stress with 100mM and 300mM NaCl, and untreated seedlings were used as control. Thirty-three differentially expressed fragments were identified and characterized. The fragments were classified according to their time of expression after the drought stress. The significance of the function of the identified differentially expressed genes was discussed in relation to their possible roles as stress genes. Seven fragments showed no significant homology with any database sequences in the GenBank. Results of the database sequence alignment identified four fragments (Bv-1=506bp, Bv19=521bp, Bv26=899bp, and Bv-31=550bp) revealing significant homology with Expressed Sequence Tags(ESTs) from salt stressed sugar beet; twenty-one fragments showed significant sequence homology with drought and cold stress- responsive genes, as well as acetyl-CoA carboxylase and glycosyltransferases. These results implicate that several pathways are involved in the plant's response to drought stress which still needs to be elucidated further.*

Key Words: *Salinity stress, Differential Display Reverse Transcriptase (DDRT-PCR), EST, Gene expression, Beta vulgaris.*

INTRODUCTION

Sugar beet (*Beta vulgaris* L.), a species of *Chenopodiaceae* family, is one of the most important viable crops that supplies approximately 35% of the world's sugar (Liu *et al*., 2008). It is not only used in the food industry but also as a source of the clean energy *via* production of hydrogen gas and bioethanol (Dhar *et al.,* 2015). It contains a large amount of betaine and betalain metabolites. Betaine has a role in plant stress tolerance (Catusse *et al*., 2008**)**. Betalains are natural pigments that have prospective health benefits (anticarcinogenic and antioxidative). Red beet root (*Beta vulgaris* L.) is considered a cheap and rich source of betalains and is very attractive to the pharmaceutical and food industries (Wybraniec, 2005; Wybraniec *et al*., 2011 and 2013). Sugar beet needs careful agronomical practices and breeding for adaptation to biotic and abiotic stresses. It is cultivated in different ambience in Europe, North America, and increasingly in Asia, South America and lately in North Africa. Sugar beet is a biennial crop which grows a sugar-rich tap root in the first year (the vegetative stage) and a flowering seed stalk in the second year (the reproductive stage (Chen *et al*., 2016). Sugar beet is a middling salt-tolerant glycophytic (Liu *et al*., 2008) which can grow better with low concentrations of NaCl than in the absence of it (Marschner *et al*., 1981b; Heuer and Plaut, 1989 and Wu *et al*., 2013). However, growth is inhibited at higher concentrations, >150 mM NaCl (Ober and Rajabi, 2010). Salinity is considered a global problem that affects approximately 20% of global irrigated cultivated land (Flowers and Yeo, 1995). A survey conducted by FAO indicated that more than 800 million hectares of land are affected by salinity worldwide (FAO, 2008). This area is equal to more than 6% of the world's total land area (Munns and Tester, 2008). Extreme salinity is a critical environmental factor that inimically affects large agricultural land areas. Plant growth, physiological processes and metabolic processes are all affected (Magome *et al*., 2008 and Zhang *et al*., 2009). High salt levels cause ionic stress in the form of cellular Cl^- accumulation and especially Na^+ ion accumulation. Salt stress also changes the homeostasis of other ions such as Ca^{2+}, K^+, and NO_3^- (Loredana *et al*., 2011). The genome sequence of sugar beet was recently reported (Dohm *et al.* 2014), making sugar beet an excellent model for studying plant response and tolerance to salinity stress (Yang *et al*., 2012). It has been useful in characterizing and cloning of expressed sequence tags (EST) preferentially expressed in different tissues and/or under different abiotic stress conditions (Zhang *et al.,* 2005; Yong *et al.,* 2007 and Yu *et al.,* 2006).

In this study, differential display reverse transcriptase PCR (DDRT- PCR) was used to identify and isolate salt - induced transcripts from sugar beet under salt stress. Several salt stresses - responsive transcripts were isolated that had not been previously reported in association with salt (NaCl) stress, providing an initial step for identifying and characterizing novel gene(s) with regard to their regulatory elements to provide an understanding of plant adaptations to salt stress conditions.

MATERIALS AND METHODS

Plant Material and Drought Experiment

The seeds of Sugar beet (*Beta vulgaris* subsp. *vulgaris*), variety "Farida" were germinated in sand soil in the greenhouse. Five seeds were planted in each pot with three replicates each. The seeds were irrigated with tap water for one week. Then, seedlings were exposed to salt stress with 100 mM and 300 mM NaCl day and day for three weeks. Salt treated as well as the control untreated seedlings were collected, and quickly frozen in liquid nitrogen and stored at -80ºC.

RNA Extraction and cDNA Synthesis

Total RNA from about 500 mg of the frozen tissue were extracted from each of the control and the treated seedlings (100 mM and 300 mM), using TriPure Isolation Reagent (Roche Molecular Biochemicals, Mannheim, Germany). First and second cDNA strands were synthesized using ImpromTM Reverse Transcription System (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions.

Differential Display-Polymerase Chain Reaction (DD-PCR) Analysis

Differential display was carried out according to Liang and Pardee (1992) with some modifications. The amplification of cDNA was carried out with the anchor primers (**T11A**- 5'- TTT TTT TTT TTA- 3') in combination with arbitrary primers (**AP1**- 5'-AAG CTT GAT TGC C -3', **AP2**- 5'- AAG CTT CGA CTG T -3', **AP3**- 5'- AAG CTT TGG TCA G -3', **AP4**- 5'- AAG CTT CTC AAC G -3', **AP9**- 5'- AAG CTT CAT TCC G -

3', **AP15**- 5'- AAG CTT TAG AGC G -3', **AP16**- 5'- AAG CTT ACG CAA C -3'). Taq DNA polymerase (GoTaq® Flexi DNA polymerase, Promega, Madison, Wisconsin, USA, was used for amplification. The reactions of PCR involving selected DD fragments were carried out in a (GeneAmp® PCR System 9700, Applied Biosystem, USA) Separation of amplified products was carried out on 6% polyacrylamide gels using Sequi-Gen® GT Nucleic Acid Electrophoresis Cell (Bio-Rad Laboratories, Hercules, California, USA). The gels were silver stained using the silver sequence kit (Promega, Madison, Wisconsin, USA), following the manufacturer's instructions.

Isolation and re-amplification of cDNA Fragments

The differentiated bands of interest were excised from the gels using a sterile razor blade. Gel slices were incubated in 50 *µl* ddH_2O at 65°C for 30 min, and then left at room temperature for elution. The re-amplification was conducted using the same set of corresponding primers. The reactions the re-PCR involving selected DD fragments were carried out in a (GeneAmp® PCR System 9700, Applied Biosystem, USA), programmed as above. The PCR products of the re-amplifications were examined in a 2% agarose gel.

Sequencing of cDNA Fragments

The reamplified DD fragments were sequenced using ABI PRISM BigDye® terminator cycle sequencing ready reaction kit (Applied Biosystems, USA), in conjunction with ABI PRISM 3730xl DNA Analyzer (Applied Biosystems, USA), at a laboratory in South Korea (Macrogen Company). The nucleotide sequence was determined automatically by the electrophoresis of the cycle sequencing reaction product on 3730xl DNA Analyzer.

Sequence analysis

The analysis of the data was performed using the Basic Local Alignment Search Tool (BLAST) algorithm of National Center for Biotechnology Information (NCBI) database, USA (http://www.ncbi.nlm.nih.gov).

RESULTS AND DISCUSSION

Expression pattern of DD-cDNA transcripts

Differential display is a fast and widely accessible molecular biology technique described by Liang and Pardee (1992). In this study, differential display reverse transcriptase PCR (DDRT- PCR) was used to identify and isolate salt-induced transcripts from Sugar beet under salt stress. A number of salt stress-responsive transcripts were isolated that had not been previously reported in association with salt (NaCl) stress, providing an initial step for identifying and characterizing novel gene(s) with regard to their regulatory (Voelckel and Baldwin, 2003).

Functional analysis of differentially expressed fragments

In this study, mRNA differential displays were used to study sugar beet responses to salt stress. One arbitrary and seven anchored primer pair combinations were used. Thirty-two fragments were differentially over expressed, and successfully identified, due to salt treatment and/or concentration of treatment compared to the control (Fig. 1). To facilitate the subsequent analysis with the DD fragments, a specific nomenclature was adopted. The fragments named (Bv-1 to Bv-32).

Table (1): Description of DD- fragment sequences as compared to database sequences and expression patterns of differentially expressed fragments.

Fragment No.	No. of bases	Homology Accession No.	Homology	E-Value	Max. ident.
BV-1	506	EG552653.1	MM02P05_XP Sugar Beet germination cDNA library *Beta vulgaris* cDNA clone MM02_P05 5-, mRNA sequence.	1e-11	81%
BV-2	468	GR396100.1	ICC4958_CD14_C05 ICC4958 dehydration stressed root cDNA library *Cicer arietinum* cDNA clone ICC4958_CD14_C05 5-, mRNA sequence.	4e-22	69%
BV-3	503	XM_020303757.1	*Aegilops tauschii* subsp. *tauschii* K(+) efflux antiporter 2, chloroplastic-like (LOC109744604), mRNA	8e-07	80%
BV-4	497	EV209517.1	0179797 *Brassica napus* Leaf - drought stress *Brassica napus* cDNA, mRNA sequence.	1e-17	75%
BV-5	537	XM_019300280.1	*Ipomoea nil* probable beta-1, 4-xylosyltransferase IRX9H (LOC109152638), mRNA.	1e-05	95%
BV-6	553	XM_010678525.2	*Beta vulgaris* subsp. *vulgaris* biotin carboxyl carrier protein of acetyl-CoA carboxylase.	0.72	80%
BV-7	489	AM847931.1	AM847931 COL, cold stress overnight library *Nicotiana tabacum* cDNA clone nt006166095, mRNA sequence.	0.69	96%
BV-10	466	XM_010675814.2	*Beta vulgaris* subsp. *vulgaris* kinesin-like protein KIN-14I (LOC104890360), mRNA.	3e-04	86%
BV-12	552	FE897232.1	PvEST3082 Bean pod tissue cDNA Entry Library *Phaseolus vulgaris* cDNA clone BE5d-247 5-similar to F-box protein, mRNA sequence.	2e-14	77%
BV-13	525	XM_010697267.2	PREDICTED: *Beta vulgaris* subsp. *vulgaris* hypothetical protein (LOC104908181), mRNA.	1e-04	92%
BV-15	550	XM_007144919.1	*Phaseolus vulgaris* hypothetical protein (PHAVU_007G199500g) mRNA, complete cds.	0.064	70%
BV-17	494	XM_019399970.1	PREDICTED: *Nicotiana attenuate* probable glycosyl transferase At5g25310 (LOC109234119), transcript variant X1, mRNA.	2e-08	86%
BV-19	521	EG550886.1	MM01O12_RP Sugar Beet germination cDNA library *Beta vulgaris* cDNA clone MM01_O12 3-, mRNA sequence.	0.74	72%
BV-20	519	FE840556.1	DrSHF 300164 Expressed sequence tags from the Forward SSH library 30 days after water stress induction *Saccharum* hybrid cultivar Co 740 cDNA clone DrSHF 300164 similar to Hypothetical protein, mRNA sequence.	6e-08	93%
BV-21	518	BE420576.1	HWM000.D01 ITEC HWM Barley Leaf Library *Hordeum vulgare* subsp. vulgare cDNA clone HWM000.D01, mRNA sequence.	4e-10	64%
BV-23	475	DK555612.1	DK555612 full-length kale cDNA library (seedlings) *Brassica oleracea* var. *viridis* cDNA	0.016	94%

			clone KALE-105N15 3-, mRNA sequence.		
BV-24	513	XM_010695203.2	PREDICTED: *Beta vulgaris* subsp. *Vulgaris serine*/arginine-rich splicing factor.	2.3	95%
BV-25	536	GR395649.1	ICC4958_CD09_E05 ICC4958 dehydration stressed root cDNA library *Cicer arietinum* cDNA clone ICC4958_CD09_E0 5 5-, mRNA sequence.	2e-15	74%
BV-26	899	EG551811.1	Sugar Beet germination cDNA library *Beta vulgaris* cDNA clone MM03_K15 3-, mRNA sequence.	0.003	75%
BV-27	504	HS402791.1	sglf205-1h15t3 Luohanguo leaf Library *Siraitia grosvenorii* cDNA, mRNA sequence.	2e-07	72%
BV-28	637	XM_019251439.1	*Beta vulgaris* subsp. *vulgaris* probable receptor-like protein kinas.	0.020	87%
BV-29	506	XM_019250080.1	*Beta vulgaris* subsp. *vulgaris* zinc finger CCCH domain-containing protein 44.	2.3	88%
BV-30	492	DB995776.1	DB995776 Bg05 Burma mangrove cDNA library *Bruguiera gymnorhiza* cDNA clone Bg05-20_K22 5-, mRNA sequence.	0.70	88%
BV-31	550	EG549577.1	MM02A03_RP Sugar Beet germination cDNA library *Beta vulgaris* cDNA clone MM02_A03 3-, mRNA sequence.	0.064	83%
BV-32	509	KNA06354.1	Hypothetical protein SOVF_181660, partial [*Spinacia oleracea*].	8e-18	74%
BV-8	476	-	No significant homology	-	-
BV-9	536	-	No significant homology	-	-
BV-11	523	-	No significant homology	-	-
BV-14	537	-	No significant homology	-	-
BV-16	517	-	No significant homology	-	-
BV-18	502	-	No significant homology	-	-
BV-22	399		No significant homology		

The isolated cDNA fragments were analyzed using BLAST programs of the National Center for Biotechnology (NCBI) as shown in Table (1) and (Fig. 2). Scanning of fragments Bv-8, Bv-9, Bv-11, Bv-14, Bv-16, Bv-18 and Bv-22 cDNA in the GeneBank showed no homology to known genes. the transcripts labeled Bv-1, Bv-19, Bv-26 and Bv-31 had homology with the ESTs isolated from sugar beet under high salt stress (Magome *et al*., 2008). The transcripts Bv-2 and Bv-25 had high homology with the ESTs isolated under drought stress from *Cicer arietinum* root. The transcripts named Bv-4 and Bv-20 have homology with the ESTs isolated under drought stress from *Brassica napus* Leaf sugarcane leaf tissue, respectively. The transcript Bv-3 showed homology with chloroplastic $K^{(+)}$ efflux antiporter 2 which modulates monovalent cation and pH homeostasis in plastids and play a role in osmotic adjustment (Aranda-Sicilia *et al*., 2012).

The Bv-5 transcript showed homology with *Ipomoea nil* beta-1,4-xylosyltransferase involved in the synthesis of the hemicellulose glucuronoxylan, a major component of secondary cell walls (Brown *et al*., 2005). The Bv-6 transcript showed homology with *Beta vulgaris* biotin carboxyl carrier protein of acetyl-CoA carboxylase which is involved in the pathway fatty acid biosynthesis, which is part of lipid metabolism (Fall *et al.,* 1971). The Bv-7 transcript showed homology with *Nicotiana tabacum* EST under cold stress, and Bv-10 transcript showed highly homology with *Beta vulgaris* kinesin-like protein. In plants, kinesins are involved in a variety of cellular processes including intracellular transport, spindle assembly, phragmoplast assembly, chromosome motility, MAP kinase regulation and microtubule stability (Shen *et al*., 2012 and Li *et al.,* 2011) reported that mutation of rice *BC12*/*GDD1* encoding a kinesin-like protein led to dwarfism with impaired cell elongation. Nishihama *et al*. (2002) demonstrated that the expansion of the cell plate in tobacco plant cytokinesis required kinesin-like proteins (i.e., NACK1 and NACK2) to regulate the activity and localization of MAP kinase.

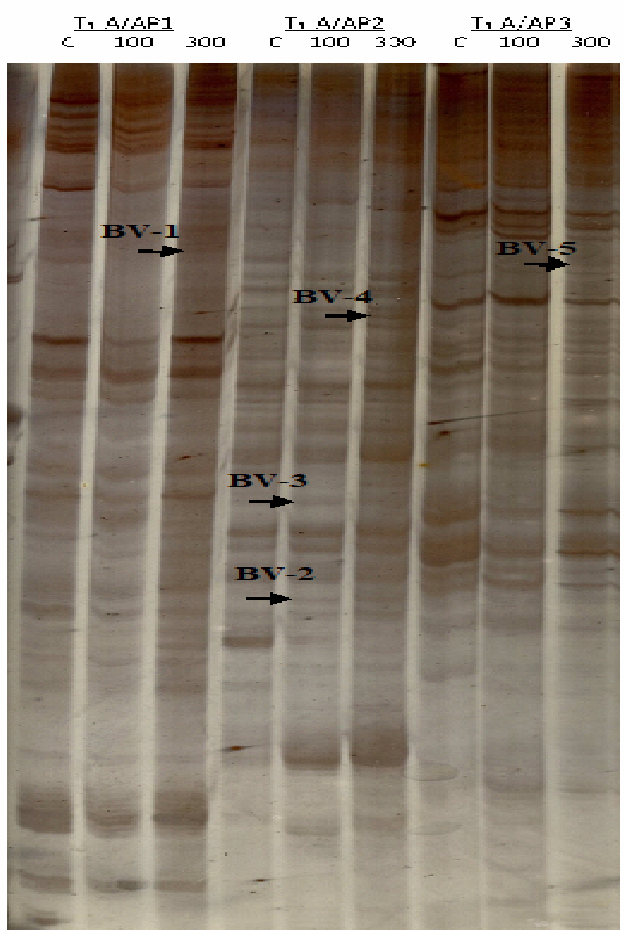

Fig. (1): DD-polyacrylamide gels of shoot cDNAs under control(c) and salt stress (100mM, and 300mM) conditions utilizing different primer combinations, (T11A with AP1, AP2 and AP3). Arrows indicate a number of differentially expressed bands on a duplicate basis.

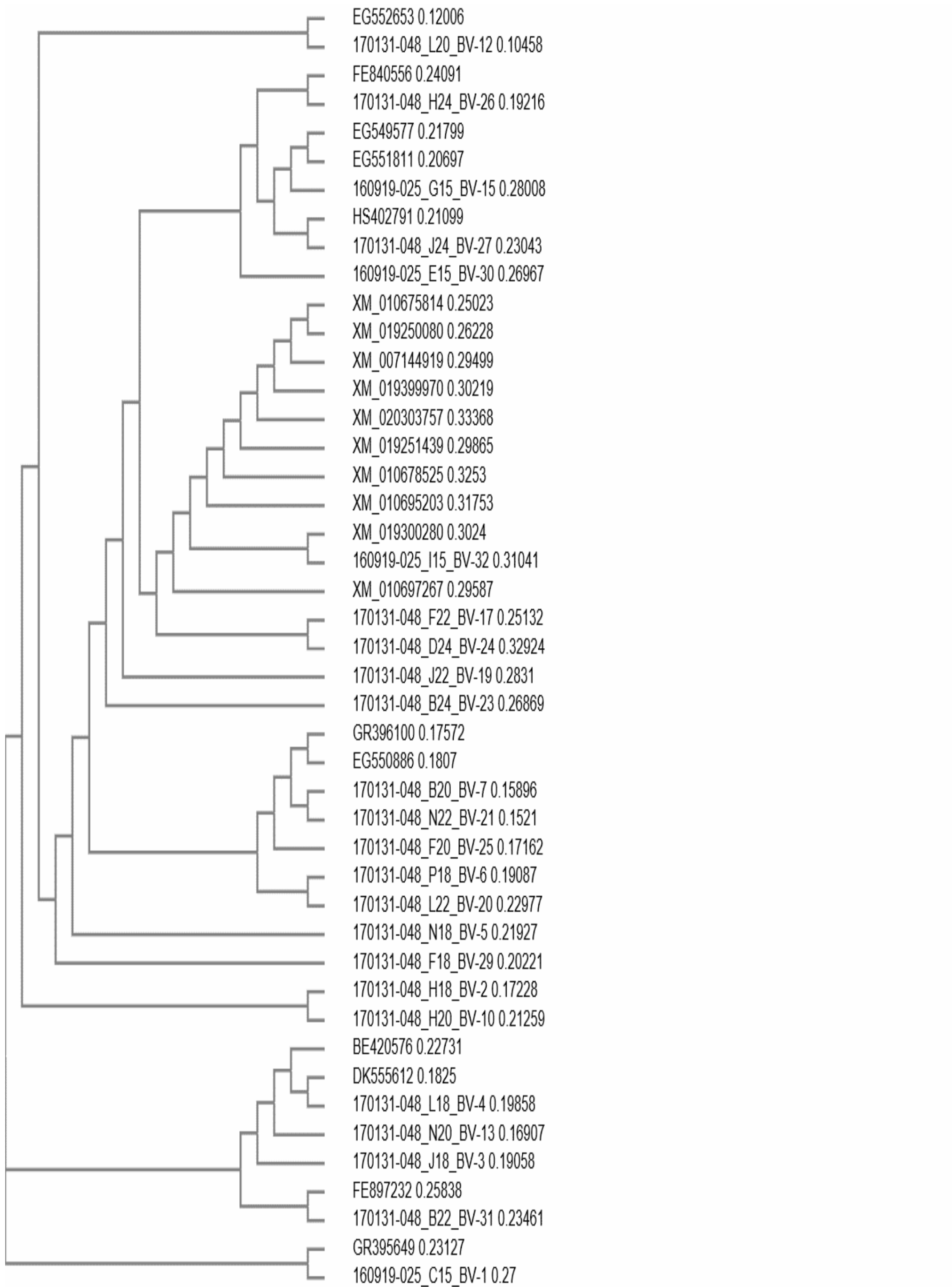

Fig. (2): Multiple alignments were performed using the default parameter of Clustal W. Phylogenetic dendrogram was generated by MEGA 6 using neighbor-joining (NJ) method with 1,000 bootstrap replicates.

The Bv-12 transcript showed high homology with *Phaseolus vulgaris* cDNA clone similar to F-box protein. The F-box protein family is involved in multiple signaling pathways for regulating root growth; the F-box protein gene reduces abiotic stress and promotes root growth in rice (Yan *et al.,* 2011). The F-box protein family in eukaryotes plays important roles in plant development and abiotic stress responses via the ubiquitin pathway (Jia *et al*., 2011).

The transcripts Bv-13, Bv-15, and Bv-32 transcripts showed homology with hypothetical proteins. The Bv-17 transcript showed homology with an enzyme of glycosyl transferases which constitute a large family of enzymes that are involved in the biosynthesis of oligosaccharides, polysaccharides, and glycoconjugates (Taniguchi *et al*., 2002). The isolated fragments were analyzed using Blast programs of the (NCBI) scanning of BV-8, BV-9, BV-11, BV-14, BV-16, BV-18 and BV-22 cDNA fragments in the gene bank showed no significant homology in BLASTn. The Bv-24 transcript showed homology with serine/arginine-rich splicing factor, members of the SR (serine/arginine-rich) protein gene family are key players in the regulation of alternative splicing, an important means of generating proteome diversity and regulating gene expression. In plants, marked changes in alternative splicing are induced by a wide variety of abiotic stresses, suggesting a role for this highly versatile gene regulation mechanism in the response to environmental cues (Duque, 2011). The transcript Bv-28 showed homology with receptor-like protein kinase. When the plants are exposed to abiotic stresses, signals are likely first sensed by receptors generally localized in the membrane, and then signals are transduced to the downstream factors and activate different stress responses. In this process, receptor-like kinase (RLK) may be the first sensor or transducer (Chang *et al.,* 2013). The transcript Bv-29 showed homology with Zinc Finger CCCH – Domain containing protein44. Znf-*CCCH* genes have been reported to play important roles in cell fate determination and hormone-regulated stress responses. To this effect, a number of members of the Znf-CCCH family have been implicated in various plant developmental and adaptation processes (Pradhan *et al.,* 2017). The enhanced expression of transporter genes in response to osmotic stress has been found for different plant species and reflects a necessary readjustment of cell water balance (Ramanjulu *et al.,* 2002). Recently the ability of sugar beet seeds to synthesize the osmoprotectant GlyBet has been demonstrated (Catusse *et al.,* 2008). GlyBet is synthesized in chloroplasts through the two-step oxidation of choline catalyzed by the two enzymes, CMO and BADH. Increased mRNA levels of both genes during germination under stress suggest an enhanced biosynthesis of GlyBet in response to stress. This finding is in agreement with the previously observed accumulation of CMO and BADH mRNAs in sugar beet leaves and roots in response to salinity and drought (McCue *et al.,* 1992 and Russell *et al.,* 1998) and proves the uniformity of the stress adaptation mechanism in sugar seedlings. At least three putative TFs belonging to AP2-EREBP, MYB and CCCH-type zinc finger families of TFs show enhanced gene expression during germination of sugar beet seeds under multi-stress conditions. Whereas, an involvement of MYB and AP2-EREBP TF families in ABA-dependent stress regulatory network is well known (Shinozaki *et al.,* 2007) An association of CCCH-type zinc finger TF family with stress response has not yet been observed in plants. Two genes related to signal transduction, serine-threonine protein kinase and sucrose non-fermenting-related protein kinase regulatory subunit and show increased mRNA amounts during germination under drought stress conditions. The sucrose non-fermenting-related kinase complex (SnRK1) of plants is a global regulator of carbon metabolism and is considered to be a crucial element of the transcriptional, metabolic and developmental regulation in response to stress (Lu *et al.,* 2007).

The Thirty-two isolated and characterized cDNA fragments were deposited in the GenBank as a result of screening for salt stress-related genes in *Beta vulgaris* L. and success in isolation of these fragments opens the door to several future aspects, like: isolation of full-length genes which have important roles to help plants survive under severe stress conditions, cDNA fragments with no significant similarities or cDNA fragments with unknown function can be used to discover new genes related to the stress response mechanisms. Moreover, transfer of the isolated genes to important crops will increase the tolerance of these plants to salt stress. This will help enhance our national program for land reclamation, by means of increasing our cultivated area with abiotic stress tolerant cultivars.

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الملخص العربي

تحليل ESTs لتتابعات النسخ الجيني المستحث لمحصول بنجر السكر(*Beta vulgaris* L.) تحت ظروف إجهاد الملوحة

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تم أستخدام تقنية تفاعل البوليميراز المتسلسل التفاضلى (DDRT-PCR) لفحص المعلمات الجزيئية للتسلسل المعبر بشكل تفاضلي (ESTs) لمحصول بنجر السكر (*Beta vulgaris* L.) تحت ظروف إجهاد الملوحة و قد تم تعريض الشتلات المنزرعة بالصوبة عمر أسبوع لظروف الأجهاد الملحى بكلوريد الصوديوم لتركيزين ١٠٠ملي مول و ٣٠٠ملي مول لمدة ثلاثة أسابيع بالمقارنة مع شتلات غير معاملة (كنترول)و تم أنتخاب عدد أثنين وثلاثين شظية وراثية متضاعفة مختلفة ناتجة بتقنية تفاعل البوليميراز المتسلسل التفاضلى (DD-PCR) و أجراء تحليل متوالية تتابعات المادة الوراثية لها وقد نتج عن دراسة التماثل و التطابق مع قاعدة بياناتBLASTn تماثل متسلسل غير معنوي لسبعة من الشظايا المعزلة ، في حين أظهرت متوالية تتابعات الشظاية الوراثية المتضاعفة المتبقية وجود تماثل و تطابق معنوى مع العديد من جينات تحمل الملوحة والجفاف التي تستجيب للإجهاد الملحى، بالإضافة إلى أسيتاليل كوانزيم كربوكسيل اكسيلاز وجليكوسيل أستيل ترانسفيراز ويمكن الأستفادة من هذه النتائج فى دراسة تحمل ضغوط الاجهاد الملحى على المحاصيل الاقتصادية.