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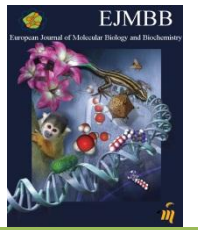

IN-SILICO PREDICTION AND FUNCTIONAL ANALYSIS OF SALT STRESS RESPONSIVE GENES IN MAIZE (*Zea mays*)

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ABSTRACT
Maize is frequently impacted by salt stress condition. Therefore, the present work emphasises on reconstruction and annotation of salt stress genes. In order to identify genes responsible for salt stress in Maize 116258 expressed sequence tags (ESTs), expressed in salinity stress condition, were mined from the different web recourses. The downloaded ESTs were clustered and assembled into 11042 contigs. Biological functions were obtained only for 6448 out of 11042 contigs through Gene Ontology (GO). The remaining contigs were used for reconstruction, validation and annotation of salt stress genes. These contigs were mapped on to Maize genome and full length gene sequences were designed. These designed candidate genes were further validated by means of promoter analysis. The study claims the possible involvement of the predicted genes in salt stress mechanism and may be useful in molecular breeding programme in Maize salinity research.

INTRODUCTION

Maize (*Zea mays*) is most important cereal crop grown widely throughout the world, ranking first in world production. India ranks sixth in global Maize production, contributing to 2.4% of world population. Growth rate of Maize production in India is not keeping in pace with the global production in spite of technological advancements. Also it faces even greater challenges due to various abiotic stresses such as salinity, drought, high radiation and extreme temperatures in changing climatic conditions. Among these stresses, soil salinity has greater impact for Maize productivity as Maize is a highly salt-sensitive crop [1]. Soil salinity is one of the most serious problems for irrigated agriculture, which drastically affect crop productivity throughout the world. This is mainly due to low precipitation and high transpiration causing disturbance in salt balance in the soil; this also renders ground water brackish and affects plant growth adversely [2, 3]. During last decades the problem of salt salinity in India is gradually increasing over time due to many factors including climate change, rise in sea levels, excessive irrigation without proper drainage etc. Therefore, it is important to develop salt tolerant varieties of Maize in order to increase Maize productivity in the saline areas and extent its area of cultivation.

Development of salt tolerant varieties is one of the important challenges of traditional Maize breeding-programs in the recent past. The main problem in the traditional breeding approach is lack of understanding of genetic mechanisms for salt tolerance. Salinity reduces water potentials in the leaves of Maize and length and dry mass of the stem [4], and affects leaf elongation and water transport in xylem vessels in Maize, as well as length and conductivity of the root in Maize [5, 6]. Therefore, it is important to study the salt stress mechanism at molecular level and identify novel genes based on its expression under soil salinity condition for the development salt tolerance in Maize varieties. The identification of highly expressed genes under salinity conditions provides a more

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comprehensive understanding of the transcriptional responses to salinity stress and aids in the identification of stress responsive promoters and responsible cis elements within them. These identified genes will provide the means of improving/incorporating salt resistance mechanism in Maize through genetic engineering.

In the present study an attempt has been made to identify putative candidate genes expressed under soil salinity conditions based on EST. These candidate genes were further in-silico validated based on examination of cis regulatory elements in the promoter region. In order to identify role in salt stress mechanism functional characterization has been performed. The finding of the study will be useful in development of salt resistant varieties of Maize and other similar crops.

MATERIALS AND METHODS

In this study 116258 salt related ESTs in Maize were downloaded from NCBI (www.ncbi.nlm.nih.gov). These ESTs were masked to eliminate sequence parts that would cause incorrect clustering [7]. These ESTs were masked for genomic repeats, vector sequence, low complexity sequence (including poly-A tails), and sequencing artifacts. The processed ESTs sequences were grouped into clusters using CAP3 software [8] of EGAssembler software [9]. The sequences which cannot be grouped due to their low similarity to other ESTs results in singletons. These singletons may represent genes where only single mRNA has been collected for the expressed gene or may be a result of contamination, were not considered for further analysis in this study.

Functional analysis of EST-contigs

Functional analysis of the EST-contigs was performed using Blast2GO v 2.5 [10]. Blast2GO is a Gene Ontology (GO) based annotation tool and found to be effective in the functional characterization of plant sequence data [10]. Further the assembled EST-contigs were first translated in all reading frames and compared against the protein sequence database (NCBI nr) to identification of potential translation products using BLASTX. The EST-contigs homologous with annotated proteins in nr database were selected for functional characterisation. The EST-contigs sequences were then categorized according to the GO vocabularies into three categories i.e. molecular function, biological process and cellular component. Remaining ESTs-contigs, having no BLAST hit and GO prediction were analysed for identification of novel candidate genes related to salt stress in Maize.

Gene Identification from assembled EST-contigs

The EST-contigs for which GO terms were not assigned were aligned on genome of Zea mays using BLAT [11]. The length of the aligned EST-contigs on Maize genome was further extended upto ~3 kb up and down stream. These aligned extended sequences were used to predict the structure of gene with TSS (transcription start site), PolyA tails at the extremes and CDS (coding sequences) in between by a gene prediction program FGENESH [12]. Further an in-silico analysis was carried to validate the above candidate genes by examining the cis regulatory elements in the promoter region of the predicted genes.

RESULTS

Assembling of ESTs into Contigs

A total of 116258 EST sequences related to salt stress tolerant of Maize were pertaining to different tissues and stress levels downloaded from GenBank. The average length of theses ESTs is 669 base pairs. Out of these 116258 EST sequences, 732 reads were trimmed and 87 sequences discarded through the sequence cleaning process. Further during masking process 8 LINE, 215 LTR elements belonging to the retroelements group and 9 belonging to the DNA transposons group, were identified and removed. Therefore, total repetitive elements which were masked 2229424 bp i.e. about 2.87% of the total size of query sequence. The remaining ESTs sequences for Zea mays were assembled into 11042 EST-contigs. Most of these contigs consists of two or three ESTs. These assembled EST account for only 9.5 % of the size of total ESTs. Less abundant or lowly expressed transcripts could not be assembled into larger contigs and remained as 18582 singletons.

Functional Annotation of EST-contigs

In order to functional characterized of 11042 assembled and translated EST-contigs were compared against NCBI nr database. Out of 11042 EST contigs, 11002 contigs were selected based on homology search. These 11002 EST contigs were further subjected to GO functional classification. Out of 11002 EST contigs GO terms were available only for 6448 EST contigs. It has been noticed that overall 36787 GO terms were retrieved which means on an average 6 GO terms per contigs were obtained. Maximum 29 GO terms for one contigs and minimum one GO term for 1462 contigs were retrieved. The distribution of GO terms per contigs is given in the Figure. 1. In general it is observed that number of contigs decreases with increasing number of GO terms due to obvious reasons. Again EST-contigs sequences were categorized according to the GO vocabularies i.e. Molecular Function (Figure. 2), Cellular Component (Figure. 3) and Biological Process (Figure. 4) with obtained number of GO terms as 14042, 11335 and 11410 respectively.

Candidate Gene prediction from EST contigs

Out of 11042 the remaining 40 EST-contigs for which no BLASTX hit and GO terms were found were considered for candidate gene prediction of the salt stress tolerant genes. These EST-contigs were aligned on genome of Zea mays using BLAT. The score range of the

alignments of these EST-contigs on Maize genome is given in the Table 1. Perusal of table 1 reveals that 2 out of 40 contigs have not aligned on Maize genome. Rest 38 contigs that aligned on genome with different score ranges were further extended up to 1kb up and down stream on the genome. Altogether, 17 of such genomic region were obtained, with TSS (transcription start site), PolyA tails at the extremes and CDS (coding sequences) in between, as novel candidate genes. Further it was found that these novel candidate genes were distributed among Chromosome number 1 to 10 where chromosome number 1 contains maximum 7 genes, chromosome 3, 7, 9 contains 2 genes each whereas chromosome number 2, 5, 6, 10 contains minimum gene that is one each.

Promoter analysis of novel candidate genes

The objective of promoter analysis is to identify the possible cis-acting DNA sequences that may be responsible for the regulation of candidate gene expression. Cis-acting sequences are the regulatory sequences that are part of the gene, which influence only the expression of the gene that contains them. Although, these sequences mostly found just upstream of the TSS but they can also be present much further upstream, or on the 3' end of the gene, or even within the introns and exons of a gene. Thus, the cis regulatory elements in the promoter region of the candidate genes were obtained from PLACE (http://www.dna.affrc.go.jp/PLACE/), i.e. a database of motifs found in plant cis-acting regulatory DNA elements all from previously published reports. In contrast, salt stress responsive cis elements of the promoter regions in cereals were collected from the publish literature. The collected salt stress responsive cis elements viz. ABRE, ARR, DOF, DRE, MYB, MYC, NOD, RAV and WRKY along with their conserved cis motif sequences are given in the Table 3. Table 4 provide the list of all reported salt responsive cis elements present in candidate genes. Perusal of Table 4 reveals that contigs 11, 574, 641, 711, 921, 923, 924, 5818, l5822, 5879, 6234, 6296, 6306 and 6397 having all the nine salt stress responsive reported cis elements whereas contigs 138, 294 and 6298 has eight among their cis elements present in promoter region. The presence of these cis elements in the promoter region of candidate genes indicates their possible involvement in salt stress mechanism of Maize.

Table 1. Score wise categorization of 184 EST-Contigs after BLAT search

Score Range	Number of EST-Contigs	Id of predicted gene structures
401 - 500	3	138, 5822, 6981
501 - 600	2	294, 574
601 - 700	2	296, 6700
701 - 800	8	11, 708, 753, 921, 5818, 6234, 6416,
801 - 900	3	924, 6296, 6391
901 - 1000	3	922, 6270, 6397
> 1000	17	162, 388, 479, 641, 711, 914, 923, 1104,
No alignment	2	

Table 2. Coding position of the Insilco validated candidate genes

Contig ID	Chr #	Start Position	End Position	Length	TSS	CDS Type	CDS Start	CDS End	PolyA
Contig11	3	213786682	213789112	2431		CDSl	546	716	88
						CDSi	803	943	
						CDSi	1518	1595	
						CDSi	2033	2147	
						CDSi	2260	2431	
Contig138	1	3280241	3281683	1443	225	CDSf	311	515	1404
						CDSl	604	1007	
Contig294	9	19393255	19397673	4419		CDSl	575	634	138
						CDSi	829	852	
						CDSi	943	1141	
						CDSi	1254	1297	
						CDSi	1384	1561	
						CDSi	1665	1849	
						CDSi	1934	1995	
						CDSi	2070	2160	
						CDSi	2278	2370	
						CDSi	3887	3982	
						CDSi	4071	4114	
						CDSf	4198	4264	
Contig574	3	156748254	156751006	2753		CDSl	219	842	149
						CDSf	2234	2689	

Contig641	7	140007059	140008884	1826		CDSl	467	1213	218
						CDSf	1330	1767	
Contig711	1	171230125	171235960	5836		CDSf	398	627	5363
						CDSi	843	908	
						CDSi	1426	1579	
						CDSi	1735	1827	
						CDSi	1914	1977	
						CDSi	2183	2274	
						CDSi	2632	2759	
						CDSi	3554	3825	
						CDSl	4732	5309	
Contig921	1	175792223	175794059	1837	1638	CDSo	719	1120	561
Contig923	5	196034987	196036365	1379		CDSf	24	203	1274
						CDSl	299	913	
Contig924	1	38311142	38315820	4679	238	CDSf	599	838	4352
						CDSi	945	1019	
						CDSi	1101	1231	
						CDSi	1373	1427	
						CDSi	1522	1632	
						CDSi	2985	3053	
						CDSi	3132	3039	
						CDSi	3563	3631	
						CDSi	3719	3773	
						CDSl	3926	4128	
Contig5818	7	167840148	167841845	1698		CDSl	576	699	419
						CDSi	784	896	
						CDSi	980	1065	
						CDSi	1167	1288	
						CDSi	1366	1425	
Contig5822	1	292530755	292534307	3553	3376	CDSo	2344	2871	1392
Contig5879	2	184179143	184180578	1436	1273	CDSo	208	1134	114
Contig6234	10	73127721	73131636	3916	105	CDSf	382	677	3840
						CDSi	780	862	
						CDSi	2501	2595	
						CDSi	2699	2772	
						CDSi	3041	3123	
						CDSl	3246	3427	
Contig6296	9	150151435	150158686	7252	2785	CDSl	621	751	172
						CDSi	915	1101	
						CDSi	1206	1403	
						CDSi	1496	1758	
						CDSf	2518	2665	
Contig6298	1								
Contig6306	6	154310896	154314065	3170	83	CDSf	423	1211	3104
						CDSi	1873	2046	
						CDSi	2121	2525	
						CDSl	2607	2954	
Contig6397	1	84413044	84415111	2068	516	CDSf	814	892	2004
						CDSi	1285	1781	
						CDSl	1947	1964	

*CDSf – First Coding segment, CDSi – Internal Coding segment, CDSl – Last Coding segment, CDSo – one Coding segment

Table 3. Cis-element and conserved cis motif sequences related to salt stress gene expression in Rice from publish literature

Cis elements	Conserved cis motif sequence	References
ABRE	ACGTG	Choi et al., 2000
ARR	NGATT	Sakai et al., 2001
DOF	AAAG	Kang and Singh, 2000
DRE	RCCGAC	Qiang et al., 2000; Dubouzet et al., 2003
MYB	WAACCA/YAACKG/CNGTTR/AACGG/GGATA	Dai et al., 2007; Yang et al., 2012
MYC	CATGTG/CACATG/CANNTG	Liu et al., 2007
NOD	CTCTT	Kim et al., 2006
RAV	CAACA	Sohn et al., 2006
WRKY	TGAC	Chen et al., 2011

Table 4. Presence of salt stress cis-element in the candidate genes

Contig Id	Cis-acting elements	Number of cis Element
Contig11	ABRE, ARR, DOF, DRE, MYB, MYC, NOD, RAV, WRKY	9
Contig138	ABRE, ARR, DOF, MYB, MYC, NOD, RAV, WRKY	8
Contig294	ABRE, ARR, DOF, MYB, MYC, NOD, RAV, WRKY	8
Contig574	ABRE, ARR, DOF, DRE, MYB, MYC, NOD, RAV, WRKY	9
Contig641	ABRE, ARR, DOF, DRE, MYB, MYC, NOD, RAV, WRKY	9
Contig711	ABRE, ARR, DOF, DRE, MYB, MYC, NOD, RAV, WRKY	9
Contig921	ABRE, ARR, DOF, DRE, MYB, MYC, NOD, RAV, WRKY	9
Contig923	ABRE, ARR, DOF, DRE, MYB, MYC, NOD, RAV, WRKY	9
Contig924	ABRE, ARR, DOF, DRE, MYB, MYC, NOD, RAV, WRKY	9
Contig5818	ABRE, ARR, DOF, DRE, MYB, MYC, NOD, RAV, WRKY	9
Contig5822	ABRE, ARR, DOF, DRE, MYB, MYC, NOD, RAV, WRKY	9
Contig5879	ABRE, ARR, DOF, DRE, MYB, MYC, NOD, RAV, WRKY	9
Contig6234	ABRE, ARR, DOF, DRE, MYB, MYC, NOD, RAV, WRKY	9
Contig6296	ABRE, ARR, DOF, DRE, MYB, MYC, NOD, RAV, WRKY	9
Contig6298	ABRE, ARR, DOF, MYB, MYC, NOD, RAV, WRKY	8
Contig6306	ABRE, ARR, DOF, DRE, MYB, MYC, NOD, RAV, WRKY	9
Contig6397	ABRE, ARR, DOF, DRE, MYB, MYC, NOD, RAV, WRKY	9

Figure 1. Distribution of number of EST-contigs vs number of GO terms obtained

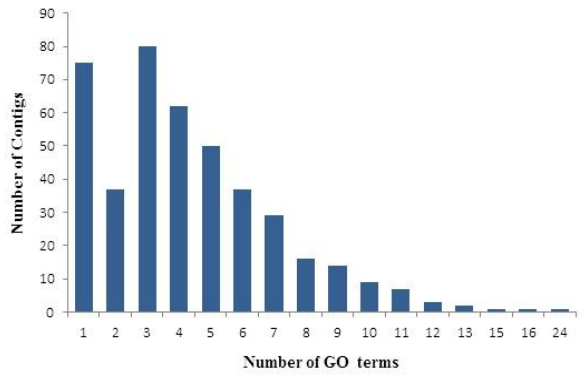


Figure 2. Distribution of GO terms in the Molecular Function category

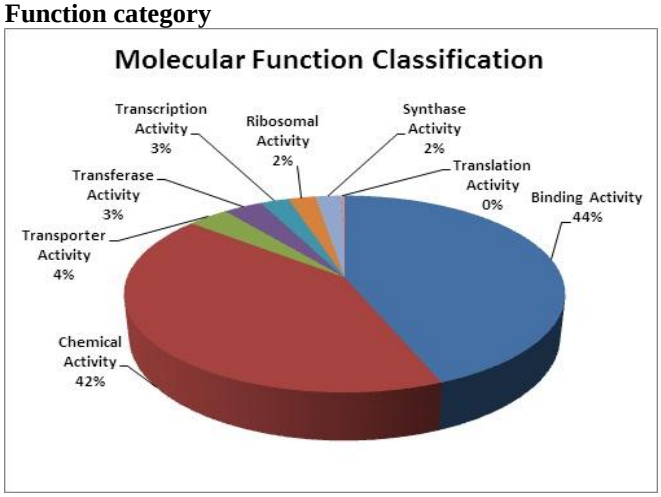


Figure 3. Distribution of GO terms in the Cellular Component category

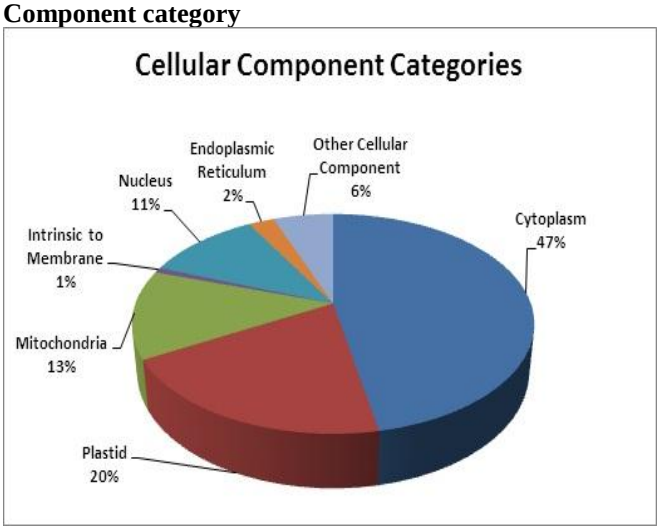
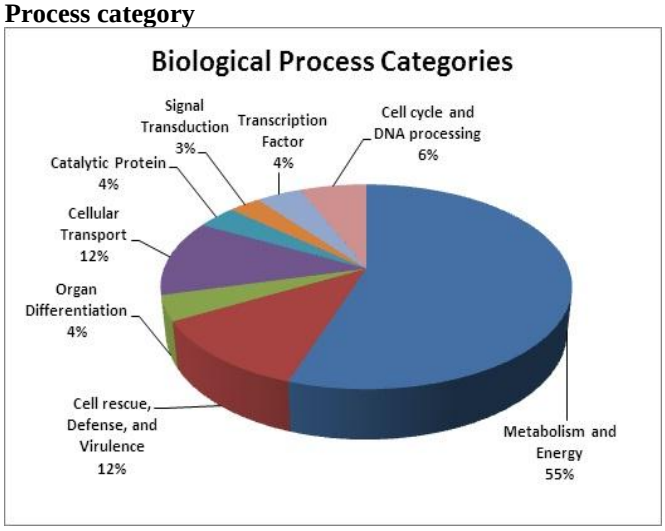


Figure 4. Distribution of GO terms in the Biological Process category



DISCUSSION

Development of salt tolerant varieties is one of the important challenges of Maize breeding-programs in the recent past. An important genomic approach to identify salt stress related genes is based on ESTs generated from different cDNA libraries representing stress treated tissues collected at various stages of development. Putative functions are assigned to such stress-responsive genes by sequence comparison to the protein database. This type of analysis provides valuable information regarding a gene associated with stress. Analyzing the various EST collections enabled us to find stress-regulated genes.

Further these EST contigs were classified with respect to different molecular functional activities. It has been observed that 86% of the total EST contigs belongs to binding activity (44%) and chemical activity (42%). Distributions of molecular functional activities were presented in Figure 2. In various plant species, it has been observed that the binding activity has been closely associated with regulation of salt-stress-modulated gene expression [13]. It has also been stated that proteins involved in various chemical activities like kinases, pyro phosphatases, and hydrogenases play significant roles in detecting and relaying salt stress signals for the regulation of specific genes and thus mediate cellular responses to salt stress [14]. Different molecular functional activities which are responsible for salt stress mechanism such as transporter activity (4%), transferase (3%), transcription (3%), ribosomal activity (2%), synthesis (2%) and translation (0.4%) and are observed in other EST contigs. *Arabidopsis thaliana* glutathione S-transferase U17 (AtGSTU17) plays role in adaptive responses to salt stresses by reverse functioning of stress-mediated signal transduction pathways [15]. Ribosomal protein S4 homologue to *Chrysodidymus synuroideus* [16] was detected to be up-regulated under salt stress.

It was also observed that overexpression of helix-loop-helix (bHLH) encoding gene, OrbHLH2, localized in the nucleus, conferred increased tolerance to salt and osmotic stress, and the stress - responsive genes DREB1A/CBF3, RD29A, COR15A and KIN1 were up regulated in transgenic plants [17].

Out of GO term pertaining to cellular component category 80 % belongs to cytoplasm (47%), Plastid (20%), mitochondrion (13%) and nucleus (11%) (Figure. 3). It has been already reported that these four cellular components are related to salt stress in plant. Localization of gene AtLecRK2 to cytoplasm is induced by salt stress in *Arabidopsis* [18]. It has been reported that plastid-expressed choline monooxygenase gene improves salt tolerance through accumulation of glycine betaine in tobacco [19]. Higher levels of *Arabidopsis thaliana* uncoupling proteins (AtUCP1) improves tolerance to salt stress, so the cellular localization of the EST-contigs in the mitochondria confers the putative role of these contigs in salt stress [20]. A stress-responsive cyclophilin gene (CYP1, enriched significantly in the nucleus) expression is highly inducible by salt in *Thellungiella halophila* [21]. The other less prominent cellular locations were endoplasmic reticulum (2%), intrinsic to membrane (1%), and others organelles (6%). Many rice heat shock proteins (HSPs) have been reported to be regulated by salt stress and localized in the endoplasmic reticulum [22].

Out of GO terms pertaining to biological process category 55% belongs to metabolism and energy (Figure. 4). It has been reported that many genes, involved in various metabolism and energy processes such as bioenergetics, secondary metabolism, lipid metabolism, amino acid metabolism, nucleotide metabolism, under salt stress [14]. A total of 12.54% of the EST-contigs were found to be actively involved in the cellular transport. Vitart et al. [23] reported that the activity of vacuolar type H+-ATPase and vacuolar pyro phosphatase (responsible for cellular transport of ions) is increased by salt treatment and induced gene expression for the upregulation. There were many other biological process found amongst the EST-contigs involved in cell defense (12%), DNA processing (6%), catalytic activity (4%), presence of transcription factors (4%) and signal transduction (3%) and all these has some or other role in salt stress responses [24-28].

CONCLUSION

Maize, being an agriculturally important crop worldwide is severely affected by salinity. The advance biotechnological methods need to be revolutionized to develop salt-tolerant Maize varieties. Current study focuses on this aim to provide in-silico analysed salt-related genes in Maize. We have reported 18 putative salt-related genes in Maize which can be further validated by breeders and used in Maize breeding programmes.

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