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**Research paper**

# Development of EST-SSR molecular markers in rice (*Oryza sativa* L.) under salinity stress and identification of key genes

**Alireza Tarinejad*, Mohammad Reza Hoseinzadeh, Sedigheh Soltanpour, and Mohammad Majidi**

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Department of Biotechnology, Faculty of Agriculture, Azarbaijan Shahid Madani University, Tabriz, Iran
*Corresponding author; Email: tarinejad@yahoo.com

**Abstract**
Expressed sequence tags of simple sequence repeats (EST-SSRs) are used to investigate genetic diversity and develop molecular markers for plants under biotic and abiotic stresses. However, there are a limited number of molecular markers based on the ESTs in rice to cope with the abiotic stresses including salinity, for use in breeding programs. Among 8299 ESTs, available in the NCBI database for salinity stress, 525 contigs, and 1139 singleton sequences were obtained. Twenty EST-SSR markers could be introduced for the selection of tolerant varieties to salt stress in rice by analysis of contigs and singletons. In contigs and singletons, significant common gene ontology terms are mainly related to the single-organism cellular process and response to abiotic stresses. The highest percentage of transcription factors for contigs and singletons was related to ERF, Dof, MYB, C2H2, BBR-BPC, bZIP, and WRKY. Moreover, the HSP81-2 (heat shock protein 81-2) and regulators of complement activation were identified as proteins of hub genes that were related to the salt stress tolerance mechanisms. Three uncharacterized hub genes (*OS02T0161900-01*, *OsJ_19443*, and *OsJ_04035*) including EST-SSRs for function identification were investigated by the 3D protein structure homology-modeling. *OS02T0161900-01* as tetra ubiquitin, *OSj-19443* as a serine/threonine-protein kinase/endoribonuclease, and *OSj-04035* as a triosephosphate isomerase were identified. Most of the hub genes were related to environmental stresses and our findings provided candidate genes and transcription factors involved in salinity stress. The development of functional markers associated with abiotic stress tolerance will be helpful to facilitate rice breeding programs. However, before using these markers, laboratory confirmation is necessary.

**Keywords:** abiotic stress, contig, gene discovery, *Oryza sativa* L., salt, singleton, yield

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## Introduction

Cereals such as rice, corn, wheat, barley, and sorghum are used in human nutrition and animal feed worldwide, and rice is one of the main sources of global food security (Chen *et al.* 2012). Rice productivity has greatly improved during the Green Revolution through the development of new cultivars, irrigation infrastructure, new management techniques, and synthetic fertilizers and pesticides. In general, scientists have tried to develop and improve seed quality through classical breeding as well as biotechnology, but many factors including planting time, irrigation, fertility, variety, harvesting conditions, and biotic and abiotic stresses

affect the quality and quantity of rice (Siebenmorgen *et al.* 2013).

Biotic and abiotic stresses cause different stresses in plants, so plants respond to these stresses with different strategies. Common reactions of plants to avoid or tolerate abiotic stresses include stomatal closure, reduction of photosynthesis, increase of active oxygen scavenging activity, reduction of leaf growth, and increase of root length. Many of these responses are coordinated by plant hormones such as abscisic acid and jasmonic acid as critical regulators of tolerance to abiotic stresses (Cohen and Leach 2019). Salinity is a complex trait that affects plant growth and physiological and morphological traits. The regulation of many genes or gene families may be involved in responding to this abiotic stress (Emon *et al.* 2015).

The knowledge of the salinity effects on rice seedling growth and yield will improve management practices in fields and increase our understanding of the mechanisms of salinity tolerance in rice (Mohammadinezhad *et al.* 2010). Therefore, evaluating the genetic diversity of rice genotypes can provide valuable information for the genetic improvement of salinity-tolerant rice (Negrão et al. 2011; Emon *et al.* 2015).

Molecular markers can improve the efficiency of breeding programs by developing genetic maps and increasing information about genetic diversity in plant germplasm. SSR markers with a high degree of polymorphism, high reproducibility, co-inheritance, and specificity are considered one of the most widely used markers in the field of genetic diversity (Powell *et al.* 1996). If an EST marker is found contiguous with the desired trait, this marker can provide access to the gene and gene tracing and increases the role of genetic markers in evaluating changes in gene transcripts and known gene activities (Yu *et al.* 2004).

The advent of the genomics era has led to the generation of an ever-expanding collection of DNA sequence data, including vast collections of ESTs. These ESTs are potentially valuable sources of gene-based SSR markers for population genetic analysis. EST-SSRs have several advantages, including rapidness, cost-effectiveness, and a high level of portability between taxa. EST-SSRs can often be transferred over relatively large taxonomic distances, spanning species within the same genus, and in some cases multiple genera within the same family. This type of transferability is unique to EST-SSRs (Ellis and Burke 2007).

Physiological assessment based on molecular markers provides information on the amount of genetic diversity. In recent years, scientists have been looking for genetic solutions to introduce varieties of rice that can maintain the quantity and quality of yield under abiotic stresses, including salinity, by

using the EST-SSR markers (Sabouri *et al.* 2009; Sarkar *et al*. 2019; Al Azzawi *et al.* 2020).

Microsatellites or simple sequence repeats (SSRs), characterized by high polymorphism, wide distribution in genomes, co-dominance, reproducibility, and high information content, have been frequently used for genetic analyses. Though SSRs are markers of choice in many plant species, only a very limited number of SSR markers are publicly available for rice. Due to the rapid increase of sequencing information, the generation of EST-derived microsatellite markers becomes an attractive alternative to complement existing SSR collections. In this regard, the use of EST or cDNA-based SSRs has been reported for several species including grape, sugarcane, durum wheat, rye, etc. (Thiel *et al.* 2003).

The present study was designed assuming that there are SSR molecular markers in ESTs under salinity stress in rice. In particular, the study aimed to develop SSR molecular markers based on the ESTs information of the genes associated with salinity stress. In addition, we focused on the identification of transcription factors (TFs), and hub genes with regard to the response of rice to salt stress. Also, it was attempted to understand the molecular basis of salt tolerance in rice based on ESTs under salinity stress.

## Materials and Methods

### *Bioinformatic analysis of EST sequences*

The main workflow of bioinformatis analysis is depicted in Figure 1. The EST sequences were downloaded from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Screening out the contaminations including removing vector sequences (vector clipping), repeat sequences (poly A tail residue repeats masking), and low complexity sequences was performed by the software VecScreen (http://www.ncbi.nlm.nih.gov/VecScreen), and Repeat Masker (http://ftp.genome.washington.edu/RM/RepeatMasker.html). The contigs and singletons were determined by EGassembler software (Masoudi-Nejad *et al.* 2006) in the FASTA format. The EST-SSR sequences were identified by SSRLocator software (https://microsatellite.org/ssr.php). Primers were designed for the EST sequences containing SSR. The sequences were blasted to discover whether the used EST sequences are related to a specific protein or not and identify new genes. Ensemble plants (https://plants.ensembl.org/index.html) were used to determine the chromosomal location of contigs and singletons. The primer blast web program was used to control the produced fragments.

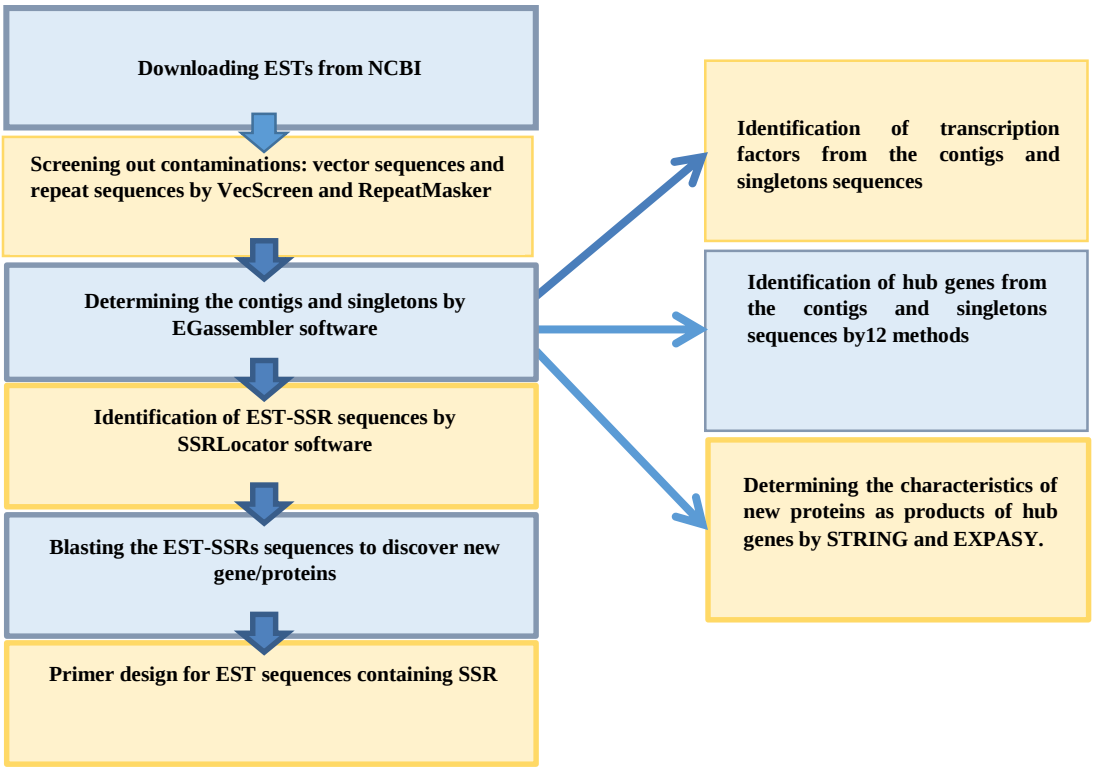



Figure 1. The workflow was used for data exploring, development of EST-SSR markers, and identification of hub genes from rice.

***Gene set enrichment analysis***

Gene set enrichment analysis was conducted by the Plant Reg Map site (http://plantregmap.gao-lab.org/) for contig and singleton sequences containing SSR markers. The biological process categories of Gene Ontology (GO) annotation were also characterized.

***Identification of transcription factor in the promoters of meta-DEGs***

To identify TFs, the genomic sequences of 1500 bp upstream from contig and singleton sequences containing SSR markers were downloaded from the Biomart website (http://plants.ensembl.org/biomart/martview/) This set of sequences was analyzed for conserved promoter elements, including the transcription factor (Bhargava *et al.* 2013) by PlantPAN 2.0 website (PlantPAN; http://PlantPAN2.itps.ncku.edu.tw).

***Identification of Hub genes***

Protein-Protein Interaction (PPI) network was drawn by Cytoscape software version 3.9.1 and information was derived from the STRING website (https://string-db.org). Hub genes were also characterized using the CytoHubba plugin in Cytoscape (Ram 2013). Construction of the PPI network and identification of contigs and singletons with regard to salt stress candidate hub genes with

12 methods was achieved. The top ten nodes are presented with a color scheme from red (highly essential) to yellow (essential). The top ten genes in the PPI network were calculated by MCC, DMNC, EPC, MNC, Degree, Bottel Neck, EcCentrity, closeness, Radiality, Betweenness, Stress, and Clustering Coefficient methods.

***Homology modeling of three uncharacterized hub genes***

The interaction of uncharacterized hub genes with other rice proteins was investigated by the Coexpression site. Then three-dimensional protein structures of identified hub genes were modeled through the Swiss Model, an automated protein homology modeling server (http://swissmodel.expasy.org).

**Results**

***Analysis of SSRs in contig sequences of ESTs under salt stress***

Out of 8299 EST expressed in NCBI about salinity stress in rice, 525 contigs were selected after removing poly A tail residue and other contaminations. Accordingly, most of the protein functions in the obtained contigs were related to salinity tolerance, biological stress tolerance, transcription factors, and heat shock proteins.

Table 1. The sequences of the SSRs in relation to salt stress were obtained from the EST contigs.

| No. | Contigs | SSR sequence | Structure | Start of SSRs in the sequence | End of SSRs in sequence the |
|---|---|---|---|---|---|
| 1 | Contig6 | (GTA)6 | P | 1062 | 1079 |
| 2 | Contig17 | (GCG)6 | P | 475 | 492 |
| 3 | Contig19 | (CCG)6 | P | 310 | 327 |
| 4 | Contig32 | (CCG)6 | P | 100 | 117 |
| 5 | Contig54 | (GA)7 | P | 141 | 154 |
| 6 | Contig58 | (AG)7 | P | 78 | 91 |
| 7 | Contig67 | (CAC)6 | P | 110 | 127 |
| 8 | Contig104 | (GT)8 | P | 459 | 474 |
| 9 | Contig113 | (CGC)6 | P | 62 | 79 |
| 10 | Contig118 | (TC)9 | P | 394 | 411 |
| 11 | Contig123 | (GT)7 | P | 466 | 479 |
| 12 | Contig133 | (GCG)6 | P | 101 | 118 |
| 13 | Contig152 | (CTC)6 | P | 57 | 74 |
| 14 | Contig175 | (TC)7 | P | 31 | 44 |
| 15 | Contig176 | (GCA)6 | P | 44 | 61 |
| 16 | Contig190 | (TC)8 | P | 23 | 38 |
| 17 | Contig190 | (TG)8 | P | 397 | 412 |
| 18 | Contig236 | (TC)8 | P | 23 | 38 |
| 19 | Contig236 | (TG)8 | P | 503 | 518 |
| 20 | Contig237 | (AC)7 | P | 113 | 126 |
| 21 | Contig265 | (GA)7 | P | 42 | 55 |
| 22 | Contig270 | (TC)8 | P | 162 | 177 |
| 23 | Contig314 | (AG)7 | P | 79 | 92 |
| 24 | Contig381 | (TCT)6 | P | 201 | 218 |
| 25 | Contig473 | (CT)7 | P | 67 | 80 |

P: Perfect

The analysis of SSRs in the contigs sequence of ESTs in connection with salt stress is represented in Table 1. Among the identified microsatellites, the most common repeated motifs in SSRs are related to the sequence of TC with five repetitions (20%) and GCG,

CCG, GA, AG, GT, and TG sequences, each with two repetitions (8%). In total, 25 contig sequences containing SSR markers were extracted from the analysis of 525 contigs related to 8299 EST sequences, of which 10 of these markers had three nucleotides and 15 of them had two nucleotides.

Table 2. Protein characteristics of expressed contigs containing SSR markers in relation to salt stress.

| No | Contigs | Accession number | Protein name | Function | Biological process | Ligand |
|---|---|---|---|---|---|---|
| 1 | Contig6 | AB753859.1 | Rice salt sensitive 1 | Uncharacterized | | |
| 2 | Contig17 | D12633.1 | 40S subunit ribosomal protein | Ribonucleoprotein, ribosomal protein | Translation | |
| 3 | Contig19 | XR_003243819.1 | Uncharacterized LOC9266083, transcript variant X3 | | | |
| 4 | Contig32 | XM_015783913.2 | 60S ribosomal protein L15 | Ribonucleoprotein, ribosomal protein | Translation | |
| 5 | Contig54 | XM_015764584.1 | Hydrolase activity, V-type proton ATPase 16 kDa proteolipid subunit-like | Hydrolase activity, V-type proton ATPase 16 kDa proteolipid subunit-like | | |
| 6 | Contig58 | XM_015767242.2 | Uncharacterized protein LOC4329444 | | | |
| 7 | Contig67 | XM_015793079.2 | Histone H2B.2 | DNA binding protein heterodimerization activity | Nucleosome assembly | |
| 8 | Contig104 | AY491534.1 | Metallothionein-like protein | Metal ion binding, reactive oxygen species metabolic process | | Metal-binding, metal-thiolate cluster |
| 9 | Contig113 | EF575872.1 | 60S ribosomal protein l39 | Ribonucleoprotein, ribosomal protein | Translation | |
| 10 | Contig118 | AC121363.2 | Hypothetical protein | | | |
| 11 | Contig123 | XM_015764750.1 | metallothionein-like protein 4C | Metal ion binding | Stress response | Metal-binding, metal-thiolate cluster |
| 12 | Contig133 | AB110175.1 | Hypothetical protein | Protein self-association, protein transmembrane transporter activity | Mitochondrion and organization, protein transport, transport | |
| 13 | Contig152 | XM_015770371.1 | Serine/arginine-rich splicing factor RSZ23 isoform X2 | | | |
| 14 | Contig175 | KP401713.1 | NBS-LRR-like resistance protein | ATP binding | Plant defense | ATP-binding |
| 15 | Contig176 | XM_015766313.1 | V-type proton ATPase subunit E | Proton-exporting ATPase activity, phosphorylative mechanism, structural constituent of ribosome | Hydrogen ion transport, ion transport, transport | |
| 16 | Contig190 | EF576366.1 | Metallothionein-like protein 1 | Stress response | | Metal-binding, metal-thiolate cluster |
| 17 | Contig190 | No significant | | | | |
| 18 | Contig236 | EF576366.1 | Metallothionein-like protein 1 | | | |
| 19 | Contig236 | No significant | | | | |
| 20 | Contig237 | XM_015761751.1 | Metallothionein-like protein 1A | Metal ion binding | Stress response | Metal-binding, metal-thiolate cluster |
| 21 | Contig265 | XM_026024055.1 | Uncharacterized protein | | | |
| 22 | Contig270 | AY445626.2 | chlorophyll a/b binding protein presusor | | | |
| 23 | Contig314 | AF010584.1 | Water-stress inducible protein | Rsponse to water deprivation, response to abscisic acid | Stress response | |
| 24 | Contig381 | XM_015759459.1 | Protochlorophyllide reductase B, chloroplastic | Protochlorophyllide reductase activity | Chlorophyll biosynthesis, photosynthesis | NADP |
| 25 | Contig473 | EF575776 | Senescence associated protein | | | |

***Protein characteristic of expressed contigs containing SSR markers under salt stress***

Table 2 shows the included enzymes and proteins as follows: histone H2B.2 (DNA binding), hypothetical protein, water-stress inducible protein (in response to water

shortage), protochlorophyllide reductase B, chloroplastic (protochlorophyllide reductase activity), ribosomal proteins, and proteins related to salinity stress which can play a role in inducing tolerance or sensitivity to salinity. Due to the presence of SSR markers on the genes encoding these enzymes and proteins, they could be used as selectable markers in rice breeding programs under salt stress. The designed primers for the contig sequences are shown in Table 2. The sequence of primers and the size of the produced parts along with the annealing temperature of each primer is shown in Table 3.

***Grouping of contigs effective in salt stress tolerance***

To understand the genetic affinity of the identified contigs effective in salinity-stress tolerance. The grouping results using the average neighbor integration method based on Euclidean distance with Mega version 5 software, are shown in Figure 2. The contigs could be classified into five groups. The first group included contigs 236, 190, 123, 104, 237, and 19. The second group included contigs 113 and 314. The third group comprised of only contig 6. In the fourth group, contigs 67, 381, 54, 473, 17, 175, 32, 58, and 270 were included. Finally, the fifth group includes contigs 133, 265, 176, 118, and 152. The contigs located in a cluster probably have similar protein blocks concerning their function.

Table 3. Reverse and forward primers of expressed contigs in relation to salt stress based on SSR markers.

| Contig | Accession number | Forward primer | Reverse primer | A (F) | A (R) | SPF |
|---|---|---|---|---|---|---|
| Contig6 | AB753859.1 | ACCTCAGAACAGTGCTATTA | CAGCAGAACATCTAGGATAC | 49.92 | 49.79 | 111 |
| Contig17 | D12633.1 | CCAGGTAAAACTAGTAGCAA | TCTTCAGATACTACTTGCGT | 50.05 | 50.12 | 432 |
| Contig19 | XR_003243819.1 | GTACAGAAACATACCAGGAA | GTAAATAAGCCATTCAACAG | 50.02 | 50.16 | 486 |
| Contig32 | XM_015783913.2 | GACACTTGACATGGACTG | CAAAGTACTTGTAGGTGGAG | 49.49 | 49.90 | 486 |
| Contig54 | XM_015764584.1 | GGACTGAAGGAGTAGAAACT | GAAGAGGTAGTAGGGCTTG | 50.03 | 51.59 | 463 |
| Contig58 | XM_015767242.2 | TTCATTTTAGTACACCCATC | GAGAGAATCAACCAATACAA | 50.07 | 50.03 | 506 |
| Contig67 | XM_015793079.2 | CAATCTCCAAATAGAAACAC | CACCAAACACCTACTACACT | 49.74 | 49.96 | 560 |
| Contig104 | AY491534.1 | CTGCATCTACCATCTATCAT | AACCAATAACATAGCACATC | 49.99 | 50.09 | 144 |
| Contig113 | EF575872.1 | GACATGGACTGAAGAGTAGA | TAAGCCTGTGTTTTAACTTC | 49.77 | 49.97 | 480 |
| Contig118 | AC121363.2 | GAAACATTCATAACCAAAAG | TTTTTGAAACCCTAGACG | 49.92 | 51.21 | 307 |
| Contig123 | XM_015764750.1 | TTTCAATCTCTCATTTCAT | TTTTCACATACACAAAACAA | 50.03 | 49.95 | 548 |
| Contig133 | AB110175.1 | AAGGAGTATTTAAGGCAAAT | CTTTCATAAGCTTCCTTGTA | 50.24 | 50.05 | 364 |
| Contig152 | XM_015770371.1 | CTTGGACTTAAACTCTTCC | ATAGCACTTCATGTCAGAAC | 48.90 | 49.99 | 403 |
| Contig175 | KP401713.1 | ATAATCCTTATCCCTTCAAC | GAGGTCATCTTACCTTCTCT | 50.04 | 49.89 | 254 |
| Contig176 | XM_015766313.1 | TACCACTCCCGTGGAC | TTCTTTCTAACTTCGACTTG | 52.90 | 49.95 | 302 |
| Contig190 | EF576366.1 | AATTGCACTCATCTCAA | ATAGTTGCTGAAGTGTTTGT | 46.94 | 49.83 | 197 |
| Contig190 | No significant | TCTCTTTGCACTACATTCTT | GTCTGTAAAAACTTCAGGTG | 50.07 | 49.82 | 531 |
| Contig236 | EF576366.1 | AATTGCACTCATCTCAA | ATAGTTGCTGAAGTGTTTGT | 46.94 | 49.83 | 197 |
| Contig236 | No significant | TCTCTTTGCACTACATTCTT | AGACACCTCACAATATTCAC | 50.07 | 49.58 | 555 |
| Contig237 | XM_015761751.1 | TTTCACAAACAACATTTACA | ATCTCAAAAGCTCTTCTTCT | 49.95 | 50.13 | 519 |
| Contig265 | XM_026024055.1 | GAAGACTACTCGTCTGCTC | TAGGTTTTCTTTTGTTGTGT | 49.62 | 50.00 | 274 |
| Contig270 | AY445626.2 | GTACATCAGAAATTCACCAC | TAAGATGTTAATGAATTGGG | 50.09 | 50.33 | 153 |
| Contig314 | AF010584.1 | GGAGTAGTAAAATCACCACA | TAATTAATCCTGCTACGAAC | 50.02 | 49.94 | 421 |
| Contig381 | XM_015759459.1 | GAGTGATGTGCTATTGATTT | GCTAAGTAATTCGTGAGAAA | 50.04 | 50.02 | 121 |
| Contig473 | EF575776 | AACTCTCTCTCTCTCCTACC | GAAGAACTTGTTGATAGCTG | 49.06 | 50.07 | 325 |

Annealing temperature (A), Forward (F), Reverse (R), Size of the produced fragment (SPF).

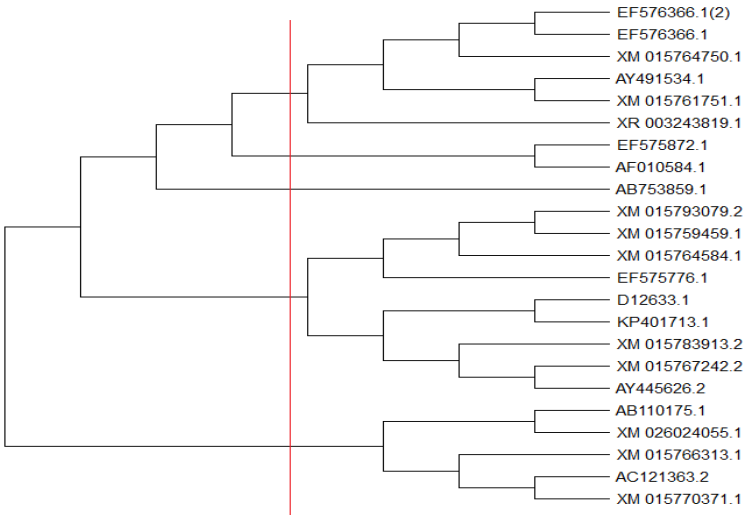



Figure 2. Grouping of the identified contigs containing SSR markers effective in salt stress tolerance based on the DNA sequence.

***Analysis of SSRs in the singleton sequence of ESTs in relation to salt stress***

Out of 8299 ESTs expressed in NCBI in relation to salinity stress in rice, 1139 singletons were selected after removing poly A tail residues and other contaminations. The SSR analysis in the singletons sequence of ESTs with regard to salt stress is shown in Table 4. The highest percentage (20%) of SSR markers related to GA sequences had four repetitions, followed by TC with three (15%), and TG and AG with two repetitions (10%). In total 20 markers related to SSRs in the sequences were detected. Seven of these motifs had three nucleotides (35%) and the others had two nucleotides (65%).

***Protein characteristic of expressed singletons containing SSR markers under salt stress***

The protein characteristic of singletons expressed under salt stress containing SSR markers were as follows (Table 5): F-box protein PP2-A13 (as a ubiquinone protein), SNARE 11 (SNAP receptor activity and SNARE binding), water-stress inducible protein (response to water deprivation, response to abscisic acid, and inducible proteins under water stress) as well as ribosomal proteins. Due to the presence of SSR markers on the genes encoding these enzymes and proteins, these markers can be used as selectable markers in rice breeding programs under salt stress conditions if a visible band is produced on the agarose gel. Based on this principle, primers were designed for each of the sequences mentioned in Table 4. The sequence of forward and reverse primers, as well as the size of the produced fragments, along with the annealing temperature and chromosome number of the marker are shown in Table 6.

Table 4. The SSR sequence that was obtained from the singletons ESTs with regard to salt stress.

| No. | Contig | No of loci | SSR sequence | Structure | Start of SSRs in the sequence | End of SSRs in the sequence |
|---|---|---|---|---|---|---|
| 1 | XM_015786461.2 | 1 | (GA)8 | P | 60 | 75 |
| 2 | BQ619484.1 | 1 | (ACG)6 | P | 154 | 171 |
| 3 | AJ853695.1 | 1 | (TA)7 | P | 552 | 565 |
| 4 | CV724719.1 | 1 | (GCG)6 | P | 172 | 189 |
| 5 | CV728146.1 | 1 | (AGG)6 | P | 218 | 235 |
| 6 | CV727278.1 | 1 | (TC)8 | P | 21 | 36 |
| 7 | CV727970.1 | 1 | (TC)8 | P | 60 | 75 |
| 8 | CV727970.1 | 1 | (TG)7 | P | 434 | 447 |
| 9 | CV725469.1 | 1 | (GA)8 | P | 24 | 39 |
| 10 | CV726281.1 | 1 | (TGG)6 | P | 151 | 168 |
| 11 | CV725757.1 | 1 | (GA)9 | P | 100 | 117 |
| 12 | CV725354.1 | 1 | (AG)7 | P | 64 | 77 |
| 13 | CV726269.1 | 1 | (GA)8 | P | 40 | 55 |
| 14 | CV725405.1 | 1 | (TC)9 | P | 63 | 80 |
| 15 | CV725741.1 | 1 | (GGC)6 | P | 49 | 66 |
| 16 | CV727310.1 | 1 | (CT)9 | P | 64 | 81 |
| 17 | CV725895.1 | 1 | (AG)7 | P | 62 | 75 |
| 18 | CV726505.1 | 1 | (TG)8 | P | 386 | 401 |

Table 5. Protein characteristics of the expressed singletons containing SSR markers in relation to salt stress.

| No. | Contigs | Accession number | Protein name | Function | Biological process | Ligand | Notes |
|---|---|---|---|---|---|---|---|
| 1 | XM_015786461.2 | XM_015786461.2 | Protein RICE SALT SENSITIVE 3-like | DNA binding | | | bHLH-MYC and R2R3-MYB transcriptio Pfam14215 |
| 2 | BQ619484.1 | XM_015756771.2 | F-box protein PP2-A13 | Pathway: protein ubiquitination | Ubl conjugation pathway | | , F-box domain; Pfam00646 Phloem protein 2; Pfam14299 |
| 3 | CV724719.1 | XM_015763355.2 | Ubiquitin-conjugating enzyme E2 27 | | | | Ubiquitin-conjugating enzyme; Pfam00179 |
| 4 | CV728146.1 | XM_015777559.1 | Uncharacterized protein LOC4333744 | | | | |
| 5 | CV727970.1 | XM_015777559.1 | Uncharacterized protein LOC4333744 | | | | |
| 6 | CV727970.1 | EF576366.1 | Metallothionein-like protein 1 | Metal ion binding | Stress response | Metal-binding, metal-thiolate cluster | Metallothionein; Pfam01439 |
| 7 | CV726281.1 | AY740014.1 | SNARE 11 | SNAP receptor activity, SNARE binding | Protein transport, Transport | | t-SNARE complex subunit, syntaxin |
| 8 | CV725757.1 | XM_015784703.2 | 60S ribosomal protein L29-1 | Structural constituent of ribosome | Cytoplasmic translation | | Ribosomal L29e protein family; Pfam01779 |
| 9 | CV725354.1 | AF010584.1 | Water-stress inducible protein | Response to water | Stress response to water deprivation, response to abscisic acid cold acclimation | | |
| 10 | CV725405.1 | AB018376.1 | Early nodulin | Electron transfer activity | | | Early nodulin 93 ENOD93 protein; Pfam03386 |
| 11 | CV725741.1 | AF189365.1 | D-ribulose-5-phosphate 3-epimerase | Ribulose-phosphate 3-epimerase activity, isomerase | | | |
| 12 | CV727310.1 | AF039532.1 | Harpin induced gene 1 homolog | | | | Late embryogenesis abundant protein; cl12118 |
| 13 | CV725895.1 | D26538.1 | Protein induced by water stress | Metal ion binding | | | |
| 14 | CV726505.1 | EF576366.1 | Metallothionein-like protein 1 | Metal ion binding | Stress response | Metal-binding, metal-thiolate cluster | Metallothionein; Pfam01439 |
| 15 | CV725284.1 | XM_015755553.2 | Uncharacterized protein LOC4326979 | | | | |

***Clustering of singletons effective in salt stress tolerance***

To understand the genetic affinity of the identified singletons effective in tolerance to salinity stress, their grouping by average neighbor integration method based on Euclidean distance using Mega5 software is shown in Figure 3. The identified singletons were classified into five groups. The first group consisted of singletons CV726505.1, CV725354.1, CV725895.1, and CV727970.1. The second and third groups, each had only one singleton (CV725757.1 and CV724719.1, respectively). The fourth group included XM_015786461.2 and CV727310.1, and the fifth group comprised BQ619484.1, CV725405.1, CV725741.1, CV726281.1, and CV725284.1.

Table 6. The sequencing of reverse and forward primers of the expressed singletons in relation to salt stress based on SSR markers.

| | Contigs | Accession number | Forward primer | Reverse primer | A (F) | A (R) | SPF |
|---|---|---|---|---|---|---|---|
| 1 | XM_015786461.2 | XM_015786461.2 | CTCTCATTCTCTGTGGTGGC | GTAGACCCACTGGGAGTCGT | 58.37 | 59.02 | 388 |
| 2 | BQ619484.1 | XM_015756771.2 | GGAGGAGAACCGAAGGTGTA | GCGCGTACAGTTCTTTCTTG | 59.13 | 58.72 | 631 |
| 3 | CV724719.1 | XM_015763355.2 | AAATCGGCGTTGTTAGTTCC | CCGTCTTCAATGTAAGGGCT | 59.09 | 59.19 | 452 |
| 4 | CV728146.1 | XM_015777559.1 | CAGGGTAGCAGCTTCCTTTC | TACGCCATCATCTGATCCAT | 59.07 | 58.90 | 227 |
| 5 | CV727970.1 | XM_015777559.1 | CTTGGACCTGACATGGACTG | ACACACACACAGGAGCCATT | 59.09 | 59.02 | 438 |
| 6 | CV727970.1 | EF576366.1 | CTGGCAGAGAAGACCACAAA | CACAATATTCACAGGCCACA | 59.00 | 57.96 | 311 |
| 7 | CV726281.1 | AY740014.1 | GCTGTGTCGGAGACCACC | TGACAACTCGCTCAAAGTCC | 60.26 | 59.01 | 432 |
| 8 | CV725757.1 | XM_015784703.2 | CTTGGACCTGACTGGACTGA | CTGAGGAACTTTGGGTCCAT | 58.80 | 58.98 | 244 |
| 9 | CV725354.1 | AF010584.1 | TTAGCAATCCATTCCGATCC | CTGCATCACGTATTGCACAG | 59.86 | 58.89 | 331 |
| 10 | CV725405.1 | AB018376.1 | TGCCGCTACACTCGATCTAC | GAGAGGATCTTCTTGTCGGC | 59.05 | 58.96 | 386 |
| 11 | CV725741.1 | AF189365.1 | ACCCACACTATCGGAATTGG | AGGCACCAGCTTTTGCTAAT | 59.67 | 59 | 310 |
| 12 | CV727310.1 | AF039532.1 | CCTCCTCCTCCACTTGTTGT | GTTGGTGGAGAGGGTGAACT | 59.14 | 59 | 308 |
| 13 | CV725895.1 | D26538.1 | TTAGCAATCCATTCCGATCC | TACAATCTTGTGCTCGCCTC | 59.86 | 59.02 | 131 |
| 14 | CV726505.1 | EF576366.1 | CAATCCCTGCAACTGCTAAA | CAGGATGAAGAACACAAGCTG | 58.91 | 58.51 | 144 |
| **15** | CV725284.1 | XM_015755553.2 | AGAAGAAGAAGAAGAGCCGC | CTCTCCTCCTCCTCATCCAC | 58.02 | 58.767 | 178 |

Annealing temperature (A), Forward (F), Reverse (R), Size of the produced fragment (SPF).

***Gene ontology enrichment analysis of contigs and singletons***

The GO enrichment analysis of contigs and singletons was done to identify the significantly enriched GO terms in the biological process. The 10 top terms were GO: 0050896- response to stimulus, GO: 0044699- single-organism process, GO: 0044763- single-organism cellular process, GO: 0006950- response to stress, GO: 0009628- response to abiotic stimulus, GO: 0042221- response to chemicals, GO: 0010035- response to an inorganic substance, GO: 1901564- organonitrogen compound metabolic process, GO: 0015979- photosynthesis, GO: 1901700- response to oxygen-containing compound (Table 7). Therefore, most of the gene ontologies are involved in the response to internal and external abiotic stress tolerance, and metabolic and photosynthetic processes.

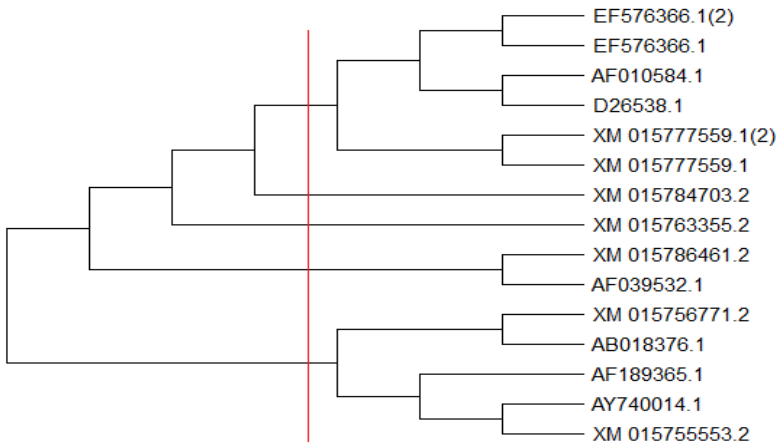



Figure 3. Grouping of identified singletons containing SSR markers effective in salt-stress tolerance based on DNA sequences.

***Identification of TFs for contigs and singletons analysis of ESTs***

The contigs and singletons sequence of ESTs has been obtained by SSRLocator software, Contigs and singletons containing SSR markers were analyzed through PlantPAN 3.0 to find transcription factors expressed under salinity. The thirty-eight TF families were categorized. Most of the TFs belonged to ERF (29%), Dof (8%), MYB (6.4%), C2H2 (5.7%), BBR-BPC (5.11%), bZIP (4.54%), and WRKY (3.85%) (Fig 4). Other studies have proven these transcription factors can play a key role in stress tolerance.

Table 7. Top 10 biological process categories of gene ontology (GO) annotation of contigs and singletons in Rice. The GO enrichment analysis of contigs and singletons was implemented by PlantRegMap. GO terms with p≤ 0.01 were adopted in this study.

| GO ID | GO Term | Number of ESTs[+] | p-value |
|---|---|---|---|
| GO:0050896 | Response to stimulus | 160 | 1.5e-18 |
| GO:0044692 | Single-organism process | 267 | 2.6e-17 |
| GO:0044763 | Single-organism cellular process | 218 | 8.6e-17 |
| GO:0006950 | Response to stress | 107 | 1.6e-16 |
| GO:0009628 | Response to abiotic stimulus | 71 | 9.7e-15 |
| GO:0042221 | Response to chemical | 85 | 8.4e-14 |
| GO:0010035 | Response to inorganic substance | 42 | 1.4e-13 |
| GO:1901564 | Organo-nitrogen compound metabolic process | 84 | 1.6e-11 |
| GO:0015979 | Photosynthesis | 25 | 2.4e-11 |
| GO:1901700 | Response to oxygen-containing compound | 49 | 1.3e-10 |

[+]Number of ESTs that belong to each gene ontology term.

***Identification of hub gene contigs and singletons analysis of ESTs***

For identifying the biological relationships between the genes, the PPI network was drawn. The hub genes were identified using the CytoHubba plugin in Cytoscape 3.9.1 with twelve methods (Figure 5). HSP81-2 (Heat shock protein 81-2) with eight methods, RCA

(Ribulose bisphosphate carboxylase/ oxygenase activase) with seven methods, osj-04035 (Os01g0841600), osj-09272 (Glycera - adehyde-3-phosphate dehydrogenase), RPS15 (30S ribosomal protein S15) with six methods and OS02T0161900-01 (Os02g0161900), OSj-19443 (Os05g0549100), Hsp81-3 (Heat shock protein 81-3), Osj-04024 (70 kDa heat shock protein) with five methods were presented as candidate hub genes in Fig 5 and Table 8. Screening genes by five to ten methods confirmed the high importance of these genes in response to salt stress.

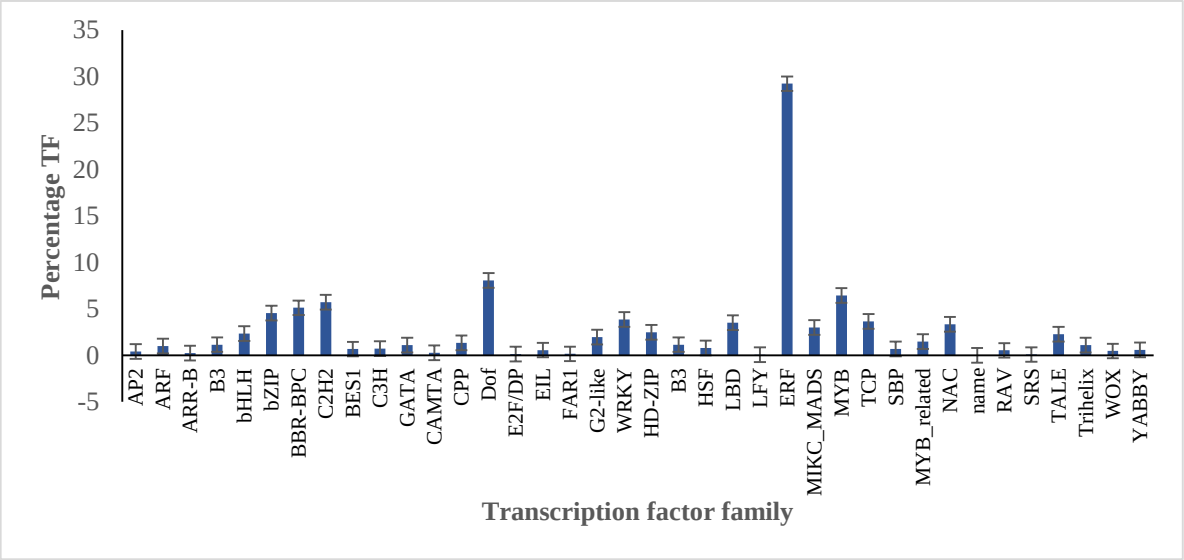



Figure 4. Diagram of the transcription factors (TFs) percentage of EST salt-responsive contig and singleton genes of *Oryza sativa* L. based on PlantRegMap. Promoter sequences were downloaded from Biomart and TFBS scanned in the promoter sequence from PlantRegMap. The Y-axis shows the percentage of TFs.

***Homology modeling and domain-based analysis of the three uncharacterized hub genes***

Using the online STRING tools (functional protein association network), the interacting partners predicted in *OS02T0161900-01*, *OSj-19443*, and *OSj-04035* are shown in Figure 6. The functional partners observed in the STRING network of OS02T0161900-01 protein were OS04T0647300-01, OS07T0661300-01, PBD1, OsJ_04611, OsJ_04022, OS04T0429200-01, OsJ_00593, OsJ_07567, OsJ_15184, and OsJ_07348. The functional partners observed in the STRING network of OSj-19443 were OS11T0603200-01, OsJ_23259, OsJ_000491, OS01T0552300-01, OsJ_06259, WEE1, OsJ_08777, CDC6, CDKD-1, and CKS1. The functional partners observed in the STRING network of OSj-04035 were OsJ_04614, OsJ_15048, OsJ_22300, OsJ_20876, OS10T0442100-00, OS05T0496200-01, OS01T0800266-00,

OsJ_09272, OsJ_05543, and OsJ_05558. These interaction networks give some insights into as protein-protein interactions and the proteins involved in salt stress tolerance either directly or indirectly.

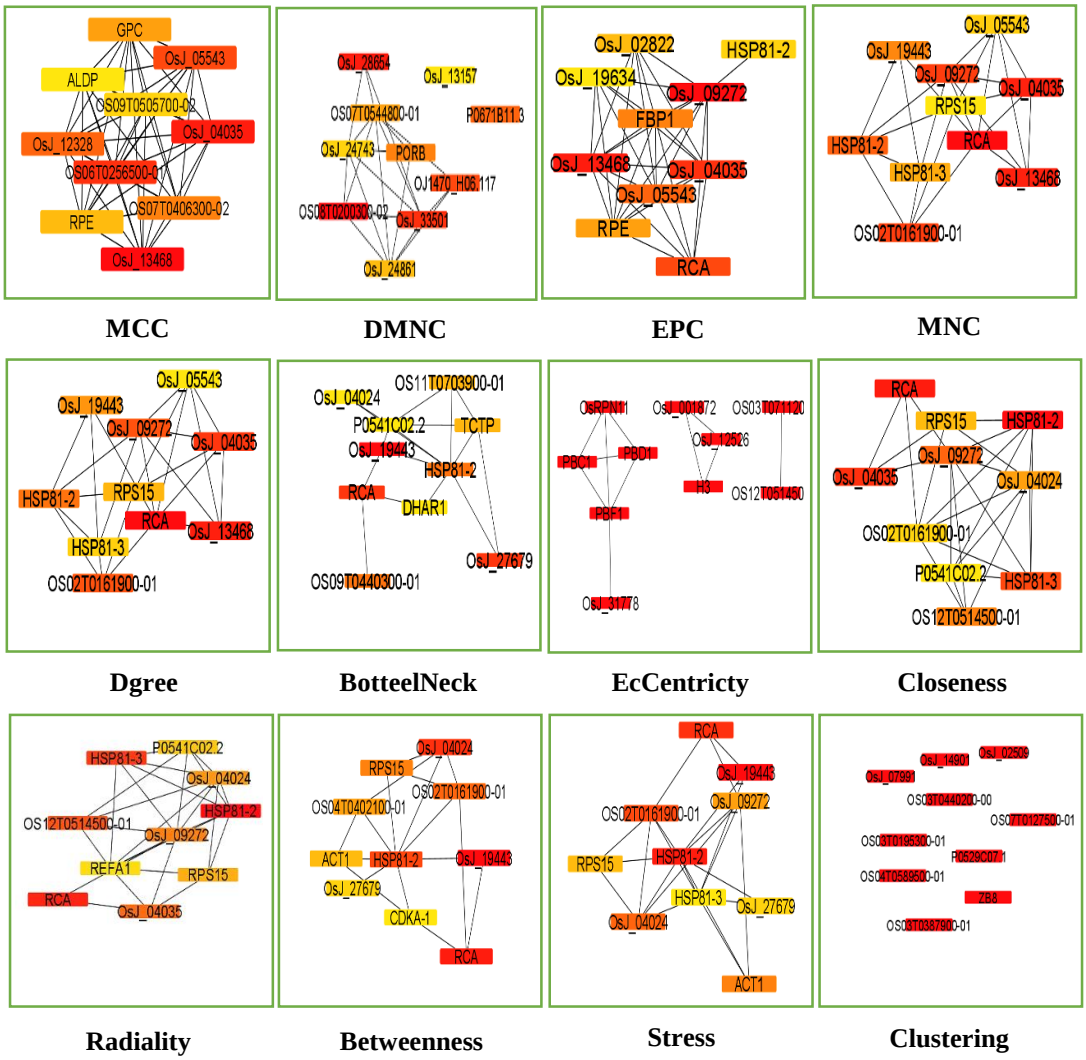



Figure 5. Construction of protein-protein interaction (PPI) network and identification of contigs and singletons in relation to salt stress candidate hub genes with 12 different methods to identify the hub genes. The top 10 nodes are presented with a color scheme from red (highly essential) to yellow (essential). Top 10 genes in the PPI network were calculated by MCC, DMNC, EPC, MNC, Degree, Bottel Neck, EcCentrity, closeness, radiality, betweenness, stress, and clustering coefficient. HSP81-2 with eight methods, RCA with seven methods, osj-04035, osj-09272, and RPS15 with six methods, and OS02T0161900-01, OSj-19443, Hsp81-3, and Osj-04024 with five methods were identified as products of candidate hub genes.

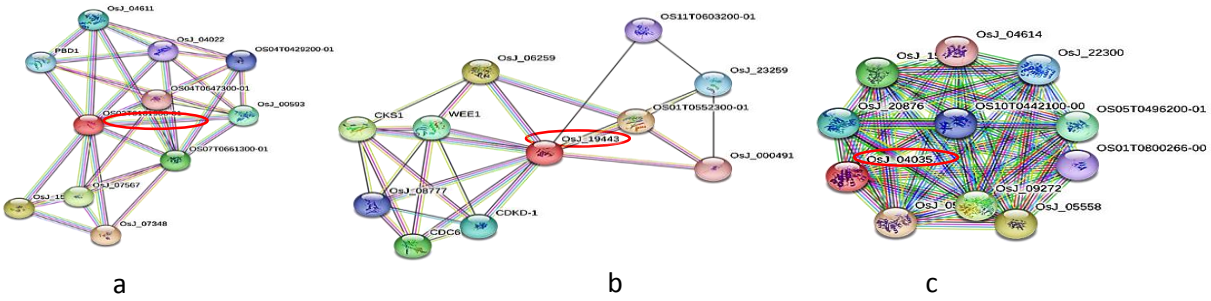



Figure 6. Coexpressed network representing the predicted functional partners of the proteins (a) OS02T0161900-01, (b) OSj-19443, and (c) osj-04035.

Table 8. List of 10 top contig and singleton-ranked nodes scored by 12 methods in Cytohubba in relation to salt stress in rice.

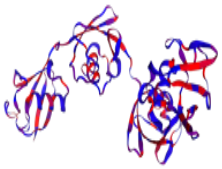
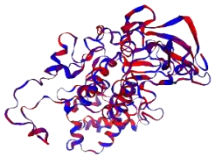
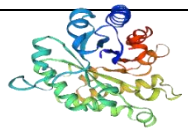
| Method | Gene name |
|---|---|
| MCC | OsJ_13468, OsJ_04035, OS06T0256500-01, OsJ_05543, OsJ_12328, OS07T0406300-02, GPC, RPE, OS09T0505700-02, ALDP |
| MNC | RCA, OsJ_04035, OsJ_13468, OS02T0161900-01, OsJ_09272, HSP81-2, OsJ_19443, HSP81-3, OsJ_05543 RPS15 |
| Degree | OsJ_13468, OsJ_04035, OS02T0161900-01, OsJ_09272, HSP81-2, OsJ_19443, RPS15, HSP81-3, OsJ_05543 |
| Closeness | HSP81-2, RCA, OsJ_04035, HSP81-3, OsJ_09272, OS12T0514500-01, OsJ_04024, RPS15, OS02T0161900-01, P0541C02.2 |
| Bottle neck | OsJ_19443, OsJ_27679, RCA, OS09T0440300-01, HSP81-2, TCTP, OS11T0703900-01, OsJ_04024, DHAR1, P0541C02.2 |
| Stress | OsJ_19443, HSP81-2, RCA, OS02T0161900-01, OsJ_04024, ACT1, OsJ_09272, RPS15, OsJ_27679, HSP81-3 |
| DMNC | OS08T0200300-02, OsJ_28654, OsJ_33501, OJ1470_H06.117, P0671B11.3, PORB, OS07T0544800-01, OsJ_24861, OsJ_24743, OsJ_13157 |
| Ec centricity | OsRPN11, OsJ_001872, PBD1, OsJ_31778, H3, OS03T0711200-01, OsJ_12526, PBC1, PBF1, OS12T0514500-01 |
| EPC | OsJ_09272, OsJ_13468, OsJ_04035, RCA, OsJ_05543, FBP1, RPE, OsJ_02822, HSP81-2, OsJ_19634 |
| Radiality | HSP81-2, RCA, HSP81-3, OS12T0514500-01, OsJ_04035, OsJ_09272, OsJ_04024, RPS15, P0541C02.2, REFA1 |
| Betweenness | OsJ_19443, RCA, OsJ_04024, HSP81-2, OS02T0161900-01, RPS15, OS04T0402100-01, ACT1, OsJ_27679, CDKA-1 |
| Clustering coefficient | P0529C07.1, OsJ_02509, OS03T0195300-01, ZB8, OS07T0127500-01, OS03T0387900-01, OsJ_07991, OS04T0589500-01, OS03T0440200-00, OsJ_14901 |

Homology modeling was done to predict the 3-D structures of *OS02T0161900-01*, *OSj-19443*, and *OSj-04035* based on the template structure from rice at a resolution of 1.6Å to 2.6 Å deposited in PDB. Unspecified hub genes had the same pattern as the template proteins. It characterized template proteins such as tetra ubiquitin with 96.05% identity to OS02T0161900-01, serine/threonine-protein kinase/endoribonuclease with 22.64% identity to *OSj-19443*), and triosephosphate isomerase with 78.74% identity to *OSj-04035* (Table 9).

## Discussion

Rice production in different regions is often affected by biotic and abiotic stresses, which reduce the yield of this important crop (Kumar *et al.* 2016). Salinity stress is the second major problem after drought (Hosseini *et al.* 2018). Approximately 30% of the areas under rice

Table 9. The modeled 3D structure, homology modeling analysis, and domain of three uncharacterized hub genes

| Uncharacterized hub gene | Length | Domains in Pfam+ | 3D protein structure homology-modelling | Templates matching by target sequence | Method | Sequence identity |
|---|---|---|---|---|---|---|
| OS02T0161900-01 (Os02g0161900) | 484 aa | Ubiquitin_2 |  | Tetra ubiquitin crystal structure of optineurin UBAN in combination with linear ubiquitin | X-ray, 2.69 Å | 96.05% |
| OSj-19443 (Os05g0549100) | 559 aa |   |  | Serine/threonine-protein kinase/endoribonuclease IRE1 IRE1 ALPHA in combination with with imidazo[1,2-b]pyridazin-8-amine compound 2 | X-ray, 2.14 Å | 22.64% |
| osj-04035 (Os01g0841600) | 255 aa |   |  | Triosephosphate isomerase, cytosolic crystal structure of *Arabidopsis thaliana* cytosolic triose phosphate isomerase | X-ray, 1.6Å | 78.74% |

*Pfam is a comprehensive collection of protein domains and families.

cultivation in the world are affected by salinity (Takehisa *et al.* 2004).

Of the 8299 ESTs available in the databases for salt stress, 525 contigs, and 1139 singletons were obtained, and 25 contigs and 20 singletons, including SSR markers, have been selected to produce bands on agarose gel electrophoresis. There are an estimated 5700–10000 microsatellite sequences with different di-, tri-, and tetra-nucleotide repeat units in the rice genome that can be potentially used to construct a genetic map based solely on microsatellite markers (Temnykh *et al.* 2000). In the present study, the most common repeated motifs in EST containing SSRs were related to TC and GA sequences with 20% frequency, followed by GCG, CCG, GA, AG, GT, and TG sequences with 8-10% frequency. From these markers, 35-40% contained three nucleotides and 60-65% had two nucleotides. In the study of the rice genome, McCouch *et al.* (2002) reported the highest proportion of SSRs in polyGA motifs (36%), followed by polyAT motifs (15%), and polyGCCG motifs (8%), in line with the current study.

GO enrichment analysis of contigs and singletons showed that most of them are involved in the response to internal and external abiotic stress tolerance and metabolic and photosynthetic processes. Most of the TFs expressed under salinity stress belonged to ERF, Dof, MYB, C2H2, BBR-BPC, bZIP, and WRKY. These results showed that a particular set of TFs regulated the expression of genes in

response to salinity stress. Other studies have proven that these transcription factors can play a key role in stress tolerance. According to Kwon *et al.* (2018), bZIP-TFs are downstream factors in the abscisic acid signal pathway and the salt stress response and improve biomass and lipid productivity under salinity stress in *Nannochloropsis salina*. Guo *et al.* (2021) reported that bHLH can regulate the plant's adaptive response to abiotic stresses besides having vital roles in reproduction such as in flower and fruit development and in the biosynthesis of secondary metabolites such as anthocyanin. The large family of MYB has also important roles in primary and secondary metabolism and response to abiotic stresses, especially drought and salinity stress (Tang *et al*. 2019).

HSP81-2 with eight methods, RCA (ribulose-1,5-bisphosphate carboxylase/oxygenase) with seven methods, osj-04035, osj-09272, RPS15 with six methods, and OS02T0161900-01, OSj-19443, Hsp81-3, Osj-04024 with five methods were identified as candidate hub genes. HSP81-2, Hsp81-3 and Osj-04024 as HSP proteins have been reported in response to abiotic stresses. HSPs play a role in maintaining cellular homeostasis, as well as stabilizing protein folding and preventing polymerization. With the help of some HSPs, denatured or misfolded proteins are further resolubilized, followed by refolding or degradation by proteases (Wu *et al.* 2022). Also, salinity reduced ribulose diphosphate carboxylase/oxygenase (Rubisco) activity in tomato plants, thereby altering biochemical reactions that regulate stomatal exchange (Yang *et al.* 2022). Kumari and Verma (2020) reported that Rubisco activity and photosynthetic characteristics such as chlorophyll, photosystems, and net photosynthesis rate decrease under salinity stress.

Three hub genes (OS02T0161900-01 as tetra ubiquitin, OsJ_19443 as a serine/threonine-protein, and OsJ_04035 as a triosephosphate isomerase) were identified by 3D protein structure homology-modeling. OS02T0161900-01 protein was associated with OS04T0647300-01 (similar to peptidase C19 family), OS07T0661300-01 (ubiquitin carboxyl-terminal hydrolase), PBD1 (proteasome subunit beta type-2-A), OsJ_04611 (E2 ubiquitin-conjugating enzyme), OsJ_04022 (putative ubiquitin carrier protein UBC7), OS04T0429200-01 (Os04g0429200), OsJ_00593 (ubiquitin carboxyl-terminal hydrolase), OsJ_07567 (putative PRT1 protein), OsJ_15184 (Os04g0476800) and OsJ_07348 (putative ubiquitin-associated (UBA) protein). OS02T0161900-01 plays a role in the ubiquitination pathway. The ubiquitin (Ub)-26S proteasome system is a regulatory mechanism for critical eukaryotic cellular processes such as cell cycle progression, cell

signaling, DNA repair, and biotic and abiotic stress responses (Kim and Kim 2013). The ubiquitin-proteasome system (UPS) facilitates the reduction of the harmful effects of environmental stress and acts as a negative and/or positive regulator of responses to biotic and abiotic stresses (Stone 2022).

In protein-protein interaction, OsJ-19443 was related to OS11T0603200-01 (ABC transporter family protein), OsJ_23259 (putative SHOOT1 protein), OsJ_000491 (probable protein phosphatase 2C 1), OS01T0552300-01 (probable protein phosphatase 2C 5), OsJ_06259 (protein-serine/threonine phosphatase), WEE1 (WEE1-like protein kinase), OsJ_08777 (proliferating cell nuclear antigen), CDC6 (cell division control protein 6 homolog), CDKD-1 (cyclin-dependent kinase D-1), and CKS1(cyclin-dependent kinases regulatory subunit 1).

OSj-19443 is a putative serine/threonine-protein kinase/endoribonuclease. Under salinity stress, ion transporters, and related regulatory gene products allow plants to mitigate salinity stress at the cellular and/or molecular level. In plants, various protein kinases are involved in the integration of different stress signaling pathways and are responsible for combating the harmful effects of salinity. The mitogen-activated protein kinase (MAPK) cascade is one of the main pathways involved in salinity-induced osmotic stress and its downstream transmission (Shah *et al.* 2021).

OsJ_04035 **(**Os01g0841600) was related to OsJ_04614 (transaldolase), OsJ_15048 (glyceraldehyde-3-phosphate dehydrogenase), OsJ_22300 (phosphoglycerate kinase), OsJ_20876 (putative fructose/tagatose bisphosphate aldolase), OS10T0442100-00 (phosphoglycerate kinase), OS05T0496200-01 (phosphoglycerate kinase), OS01T0800266-00 (phosphoglycerate kinase), OsJ_09272 (glyceraldehyde-3-phosphate dehydrogenase), OsJ_05543 (phosphoglycerate kinase), and OsJ_05558 (glyceraldehyde-3-phosphate dehydrogenase). OsJ_04035 plays a role in the carbohydrate biosynthesis pathway.

**Conclusions**

In this study, we developed microsatellite markers in rice with different di- and tri-nucleotide repeats based on the screening of EST databases. The present study determined forty-five ESTs-SSR markers for salinity stress by the bioinformatics approach to use in breeding programs for selecting tolerant cultivars under salinity stress. Among the ESTs, a total of 10 hub genes, including SSR markers, were identified that can be used in breeding programs via gene transfer or gene pyramiding by conventional breeding methods. Given that EST-SSRs are based on highly conserved exon sequences, they have a

high likelihood of being transferable to other species. Therefore, these EST-SSRs probably will be suitable for other *Gramineae* species.

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**Conflict of Interest**

The authors declare that they have no conflict of interest with any organization concerning the subject of the manuscript.

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# توسعه مارکرهای مولکولی EST-SSR در برنج (*Oryza sativa* L.) تحت تنش شوری و شناسایی ژن‌های کلیدی

**علیرضا تاری نژاد*، محمدرضا حسین‌زاده، صدیقه سلطانپور و محمد مجیدی**

گروه بیوتکنولوژی کشاورزی، دانشکده کشاورزی، دانشگاه شهید مدنی آذربایجان، تبریز
*مسئول مکاتبه؛ Email: tarinejad@yahoo.com

**چکیده**

در گیاهان EST-SSRs یا توالی‌های ساده تکراری بیان شونده برای بررسی تنوع ژنتیکی و توسعه مارکرهای مولکولی مرتبط با تنش‌های زیستی و غیرزیستی استفاده می‌شوند. با وجود این، در برنج مارکرهای مولکولی محدودی در رابطه با EST برای سازش به تنش‌های غیرزیستی از جمله شوری برای استفاده در برنامه‌های اصلاح نباتات وجود دارد. از میان ۸۲۹۹ عدد EST قابل دسترس در پایگاه داده NCBI برای تنش شوری در برنج، ۵۲۵ contig و ۱۱۳۹ singleton به دست آمد. برای گزینش ارقام متحمل به تنش شوری در برنج ۲۰ مارکر مولکولی EST-SSR از طریق آنالیز این contigها و singletonها تعیین شد. آنتولوژی ژن مشترک و معنی‌دار برای contigها و singletonها عمدتا مرتبط با فرایند سلولی و پاسخ به تنش‌های غیرزیستی بود. بیشترین درصد فاکتورهای رونویسی برای contigها و singletonها با ERF، Dof، MYB، C2H2، BBR-BPC، bZIP و WRKY مرتبط بود. علاوه بر این، پروتئین شوک حرارتی HSP81-2 و تنظیم‌کننده‌های فعال‌سازی مکمل (RCA) به‌عنوان محصول‌های ژن‌های هاب شناسایی شدند که با مکانیسم‌های تحمل به تنش شوری مرتبط بودند. سه ژن hub ناشناخته (*OS02T0161900-01*، *OsJ_19443* و *OsJ_0403*) حاوی مارکرهای EST-SSR برای شناسایی عملکردشان از طریق مدل سازی همسان سازی ساختار پروتئین سه بعدی مورد بررسی قرار گرفتند و محصول *OS02T0161900-01* به عنوان تترا یوبیکوئیتین، *OSj-19443* به عنوان سرین/ترئونین پروتئین کیناز/اندوریبونوکلئاز و *OSj-04035* به عنوان ایزومراز تریوزفسفات شناسایی شد. اکثر ژن‌های hub به دست آمده در تحقیق حاضر مرتبط با تحمل به تنش‌های غیرزیستی بودند که می‌توان آن‌ها را به عنوان ژن‌های کاندید و فاکتورهای رونویسی درگیر در تحمل به تنش شوری منظور کرد. توسعه مارکرهای مولکولی عملکردی می‌تواند در تسهیل برنامه‌های اصلاح برنج موثر واقع شود. باوجوداین، قبل از استفاده از این نشانگرها تائید آزمایشگاهی آن‌ها لازم است.

**واژه‌های کلیدی:** برنج، تنش غیرزیستی، شناسایی ژن، شوری، عملکرد، contig، singleton