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A CANDIDATE GENE RESPONSE TO DROUGHT STRESS CONDITION IN RICE (Oryza sativa L.)

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ABSTRACT

Drought is the most important constraint reducing rice yield in rainfed areas. The understanding of molecular basis of genes tolerance to drought stress will help to breed elite rice cultivars under lower water requirements. To offer some lights on candidate genes response to drought stress, we conducted the experiment in IR64 rice cultivar under artificial drought stressed-condition. Isolating and sequencing of candidate gene tolerant to drought was performed. The analysis of phylogenetic relationship with identified candidate genes in different species was also investigated. The results showed that isolated gene can responses to drought stress; and involves in the regulation of salt stress. It provides an overview and to shorten the list of genes response to drought stress condition. Looking forward in the future analysis, it needs to investigate the expression of this gene at different stages under drought stress conditions; and then an appropriate strategy to be required to introgress this gene into promising rice varieties for developing drought-tolerance rice varieties. Gene isolated in this study was submitted to GeneBank with Accession number GU269889.

INTRODUCTION

Rice is the world's most important food crop and a primary source of food for more than half the world's population. Rice production increased by 130% from 257 million tons in 1996 to 600 million tons in 2000 (Khush, **2005**). Large areas of rice are grown under lowland and upland rainfed conditions. These areas respectively occupy 31% and 11% of the global rice-growing area (IRRI, **2001**). Global reduction in rice production due to drought averages 18 Mt annually was reported (Evenson et al., **1996**). This abiotic stress is therefore a major constraint to rice production in water-limited environments (Bernier et al., **2008**). Early droughts often result in delayed sowing or transplanting. Yield reductions from early droughts are minimal and result mainly from a reduction in tiller numbers. Breeding varieties suited to these conditions is an important element in reducing risk and increasing productivity in drought-prone environments, but progress in breeding for drought tolerance in rice has been slow (Venuprasad et al., **2009**). In recent years, the use of genotypic variation for genomic research on drought tolerance mechanisms has been enhanced by the development of introgression lines from drought tolerant donor cultivars into promising cultivars and the selection of drought tolerant backcross populations (Li et al., **2005**; Lafitte et al., **2006**). The genotypic variation in drought tolerance together with the genetic tools available for rice, such as marker maps, sequence information, and microarrays (Matsumoto et al., **2005**; Rensink and Buell, **2005**) and the possibility to test the agronomic relevance of a scientific discovery (Xu et al., **2006**), make rice a most interesting model system for research in drought tolerance of grass crops. Rice plants may achieve their adaptation to drought by complex mechanisms in both physiology and phenology (Lafitte et al., **2002**). As tremendous efforts over the past decade, drought tolerance in rice was dissected by using the QTL mapping approach and a few general results in have been obtained regarding the genetics of drought tolerance in rice (Fu et al., **2007**). The number of loci affecting drought tolerance and each of its components is very large and widely distributed across the rice

genome, but only a few QTLs are detectable in any specific population/environment. Therefore, the understanding of genes underlying drought tolerance depends on the cloning and characterization of QTL genes, which remain the most challenging tasks in plant biology because of difficulties in determining and identifying QTL candidate genes, and characterizing their underlying gene networks and metabolic pathways (Li et al., **2005**). At molecular level, large numbers of genes are known to be involved in plant responses in drought. These include genes involved in signal transduction and transcriptional regulation (Xiong and Zhu, **2002**; Oh et al., **2005**; Fujita et al., **2005**), biosynthesis of osmotic and other protectors. Beside, the availability of rice genome sequence will permits now the identification of the function of each of rice genes through functional genomics, was published in 2002, serving as a gold standard for all future investigation. To bring some light on functional gene response to drought stress, the present study was aiming cloning and sequencing to identify candidate gene response to drought stress condition and determining their relationship with identified candidate genes, providing some insights into the molecular basis of gene tolerant to drought in rice.

MATERIALS AND METHODS

Plant material

Seeds of Indica rice cultivar, IR64, which were obtained from Genebank of Cuulong Delta Rice Research Institute (CLRRI), were grown directly into the trays under natural environmental conditions in the greenhouse during **2009 dry season**. After 14 days, the seedlings of IR64 rice cultivar were transferred into the pots with three seedlings per one pot. The seedlings were then treated with non-well watered condition. The survival of seedlings was evaluated based on the observation that growing seedlings were determined to be survivors and the non-growing and wilted seedlings were determined to be nonsurvivors.

DNA extraction

The leaves of survived seedlings were harvested, packaged into alumminium foil and placed in liquid nitrogen immediately to avoid degradation, and then stored at -80^{0}C until to be used. The CTAB method was used for DNA extraction the following some modification by Dr. Lang (2000). In briefly, the leaves were placed in mortar and pestle and ground in liquid nitrogen. The powdered leaves were then mixed with CTAB extraction buffer with the total of volume 1L containing 20g CTAB, 81.82g NaCl, 100ml 1M Tris pH 8.0, 40ml 0.5M EDTA pH 8.0 and sterile distilled water) and incubated at 65^{0}C in the water bath for 15 min. Chloroform:isoamylalcohol (24:1) was added to the mixture. The mixture was then put on a shaker at room temperature and incubated at 65^{0}C for 15 min. The samples were centrifuged and the supernatant was transferred to 1.5mL clean tubes. To precipitate DNA at the bottom of the tube, samples were mixed with 80% ethanol and placed in the freezer at -20^{0}C for 1h or overnight and centrifuged to pelletize DNA. DNA pellet was the rinsed twice with 70% ethanol. The ethanol was drained off and DNA pellet was dried at the room temperature. DNA was dissolved in 50µl TE buffer and stored at 4^{0}C until ready to use. The quantification and quality of DNA was checked by spectrophotometer; and to be visible on 0.8% agarose gel stained with ethidium bromide.

PCR amplification, Cloning and DNA Sequencing

Two degenerate primers designed based on high conserved regions of reported drought-induced genes in rice Os01g48190, Os01g73960, Os02g20170, Os04g14150, Os05g01730, Os05g28980, Os05g48800 and Os12g36900 to amplify coding region of drought-induced genes. The sequence of two primers is the following as: forward-5'ADR SAH HYD NRV HCA YWT 3' and reverse- 5'GHN MYK NHA VVH DRG NNT 3'. The PCR reaction was conducted in a final volume of 25 µl containing 5 µl 5X buffer with $MgCl_2$, 2 µl of 10 mM dNTP, 1 µl of each 5 mM primer, 1 µl genomic DNA and 0.1 µl of 5U/ µl Tag polymerase (Promega GoTag). The reaction conditions for PCR included a denaturing step of 94^0 for 5 min followed by 32 cycles of 30 sec at 94^{o}C, 30 sec at 59^{o}C and 1 min 30 sec at 72^{o}C, ending with an elongation step of 5 min at 72^{o}C. After agarose gel 1.2% electrophoresis, PCR

products was purified by using QIAquick gel Extraction Kit (QIAGen, USA) and cloned into M13 . Recomination plasmids were isolated from transformed cells with QIAGen Plasmid Mini Kit (QIAGen, USA). The inserted PCR fragment was sequenced on an Applied Biosystem 3130 DNA sequencer and analyzed by Applied Biosystem 3130 analyzer software as it is suggested by manufacturer.

Bioinformatic and sequence analysis

The prediction and identification of protein sequence was performed through FGENESH **program** (http://mendel.cs.rhul.ac.uk/mendel.php?topic=fgen). Pfam available online (http://pfam.sanger.ac.uk/) was used to predict the protein function, while isoelectric point stability, amino acid length was analyzed by Protparam in Expasy Website (www.expasy.ch). To determine the cellular location of protein and terminal peptide signal, pSORT program (http://wolfpsort.seq.cbrc.jp) and SignalP available online (http://www.cbs.dtu.dk/services/SignalP) was used, respectively. HNN program (Combet et al., **2000**) in Expasy (www.expasy.org) was used to predict the secondary structure of selected protein sequences. The tertiary structure prediction of protein was predicted using PHYRE (http://www.sbg.bio.ic.ac.uk/phyre/) (Kelley and Sternberg et al., **2009**). For analyzing phylogenetic relationship, drought-induced sequences was derived from the TIGR, Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/). Information about chromosome location, coding region sequence and amino acid length, cDNA, protein length was retrieved from TIGR. To consider relationship between candicate genes tolerant to drought, comparative genomics in plant website (http://bioinformatics.psb.ugent.be/plaza/) was also used. Searching BLASTp was also performed by querying against Swissprot protein sequences for obtaining additional protein sequences. As a selection criteria, a protein satisfied E value $\leq 10^{-4}$ it is selected as a candidate protein. Multiple protein sequence alignment was conducted by using CLUSTALX version 2.0.12. The Neighbor-Joining method in MEGA version 4.0 was used to construct phylogenetic tree based on Poisson-corrected paorwise distances between protein sequences and node robustness was assessed using bootstrap approach for 1000 resampling (Tamura et al., **2007**). Multiple Em (Expectation Maximization) for Motif Elicitation (MEME) program (Bailey and Elkan, **1994**) (http://meme.sdsc.edu/meme) was used to identify conserved protein motif in protein sequences and TMHMM tool integrated in InterProScan program (Mulder and Apweiler, **2007**) was employed to predict trans-membrane segments in protein sequences. Protein sequences were submitted to PLACE (Plant *cis*-acting regulatory DNA elements) (http://www/dna.affrc.go.jp/PLACE/) to identify all possible promoter/cis-element. Chromosome locations of drought-induced genes were estimated in accordance to genetic markers assigned to the BAC/PAC clones. The site used for genetic distance identification was http://rgp.dna.affrc.go.jp/cgibin/statusdb/stattable.pl?chr=X&lab=RGP. Chromosomal mapping for all the genes was carried out using MAPINSPECT software. Finally, nucleotide sequence sequenced from BAC13A9 was submitted to GeneBank through BankIt program with accession number GU269889.

RESULTS AND DISCUSSION

Overview for drought-induced genes in rice

The total of 9 genes found under drought stress condition, which distributed on 5 different chromosomes. Three of them located on chromosome 5, 2 genes in chromosome 1 and 2, and 2 gene located in chromosome 4 and 12 (Figure 1). The length of amino acid sequences among them range from 121 amino acid (aa) to 247 amino acid (aa). These proteins were induced under drought condition and play an important role in response to abiotic stresses.

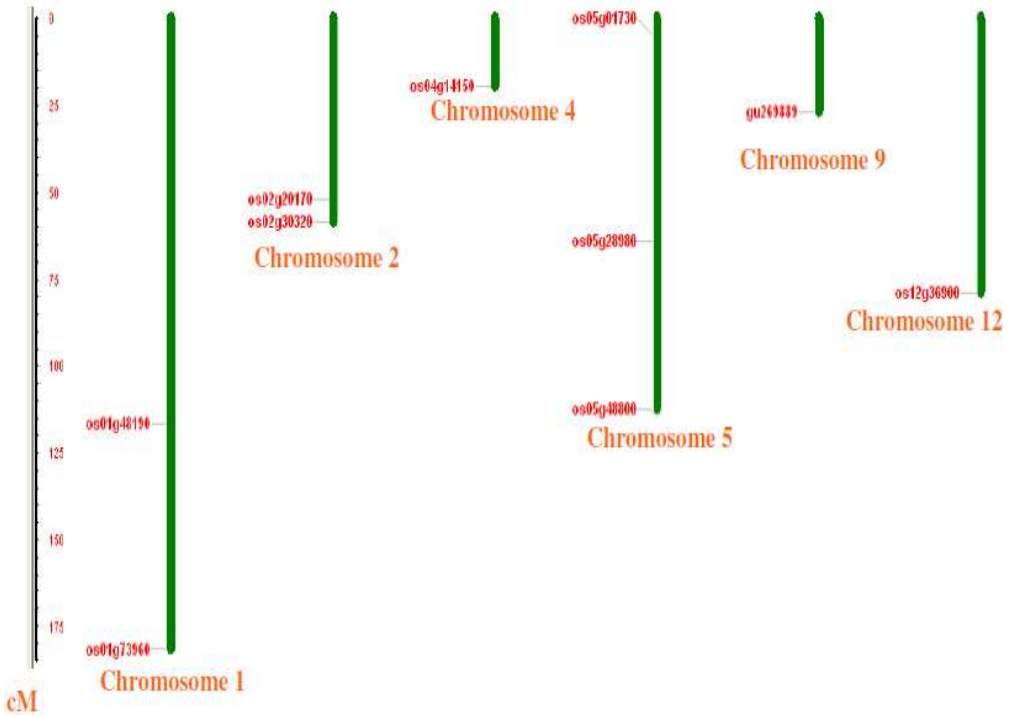

Figure 1: The location of drought induced protein on the chromosome by MAPINSPECT software. All the position in Centi Morgan unit (cM) was estimated according to genetic markers assigned to BAC/PAC clones related to predicted sequences

Table 1. Locus name and information of drought induced protein sequences

Locus name	Chr	Acc number	AA Length	pI	Mol. Weight	BAC/PAC clone	cM
Os01g48190	1	AP003223	247	4.4205	27802.9	P0007F06	116.5
Os01g73960	1	AP003277	203	5.0678	23132.9	P0518C01	181.8
Os02g20170	2	AP005009	246	7.0688	27683.7	P0572A04	51.9-52.2
Os02g30320	2	AP005066	121	9.3452	12109.7	P0047E05	-
Os04g14150	4	AL662948	-	-	-	OSJNBa0060B20	19.6
Os05g01730	5	AC129716	209	4.5028	23649.2	OSJNBa0068N01	4.6
Os05g28980	5	AC104272	234	5.1504	25351.1	OJ1045_C06	64.1
Os05g48800	5	AC113333	227	5.3281	25302.2	OJ1115_B06	112.4-115.7
Os12g36900	12	AL845342	219	4.2739	24433.8	OSJNBa0027H05	78.9-86.5
-	9	GU269889	156	9.46	17605.8	-	-

Sequence analysis

Protein-encoding nucleotide sequence (accession number GU269889 in Genebank) was identified by FGENESH, which locates at position from 629bp to 1137bp in the nucleotide sequence, encoding a 156 amino acid sequence (aa) in length. This protein is similar to methyltransferase family with accession number DUF248. In biochemical characteristics, protein sequence that has molecular weight and isoelectric point, is 17605.8 and 9.46 respectively. The essential amino acid present in the protein sequence is leucine approximately for 15.4%, subsequently glycine and arginin (7.7%). The amount of negative protein residues (Asp and Glu) is 9 while that of positve protein residues (Arg and Lys) is 16 in combination with instability index (53.48). These results indicated that protein encoded from

nucleotide sequence is instable. The location of protein found in the chloroplasts, stromal side of the thylakoid membrane in the vicinity of the photosystem I in the non-stacked and fringe portion of the membrane. The secondary structure of protein is composed of 46.79% helices. Extended strands form 13.46% of the residues while the remaining 39.74% comprise the random coil. The tertiary structure of protein sequence is similar to GUANINE-N(7)-)-METHYLTRANSFERASE. The degredation of Guanine-N(7)-methyltransferase involve to the pathway of rapidly tRNA degredation.

Phylogenetic analysis

Based on phylogenetic relationship between sequences, 10 genes including GU269889 divided into three functional groups: group 1 contains 2 genes which belong to methyltransferase family (GU269889, LOC_Os04g14150), group 2 has 6 genes (LOC_Os01g73960, LOC_Os05g01730, LOC_Os01g48190, LOC_Os05g48800, LOC_Os02g20170, LOC_Os05g28980, LOC_Os12g36900) of Di 19 protein family and remaining 1 gene (LOC_Os02g30320) in the group 3 belongs to Zinc finger domain (Figure 2). Di 19 protein has been found strongly expressed in both the roots and leaves during progressive drought (Gosti et al., **1995**) while the function of Di 1 protein is still unknown.

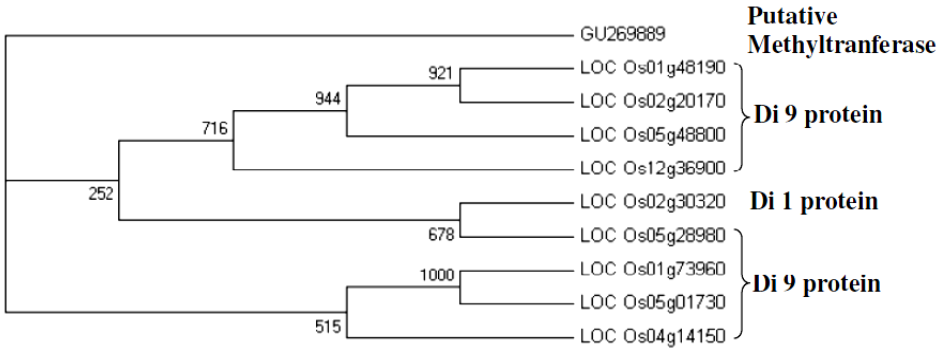

Figure 2: Phylogenetic relationship between protein sequences including GU269889 by ClustalW (http://www.ebi.ac.uk/Tools/es/cgi-bin/clustalw2). The tree was generated by using MEGA method. The nucleotide sequence that encoded a 156 amino acid sequence, was sequenced from BAC13A9 and submitted to GeneBank with accession number GU269889 through BankIt program. The annotation of these genes is shown at the right of figure.

To consider phylogenetic relationship between drought-induced genes in different species, the phylogenetic tree of 74 genes in three species was built using MEGA version 4.0 (Figure 3). Drought tolerance is considered as a complex trait, it is not surprise that there are many genes involving for tolerance to this trait. The results indicated that correlation relationship between genes can be classified into six groups in which the group 5 contains 31 genes including GU269889. In this group, GU269889 gene has to be closest relationship with genes namely: AT2G38880, AT4G15910, AT5G48870, AT5G43260, SB04G020580, LOC_Os02g30320, SB05G004100, AT3G24500, AT2G45640, AT2G40350, AT2G41430. Gene, namely AT2G38880, encodes a transcription factor from the nuclear factor Y (NF-Y) family, AtNF-YB1, conferring drought tolerance (Nelson et al., **2007**) while AT4G15910 gene encodes a gene whose transcript level in root and leaves increases to progressive drought stress (Vogel et al., **2005**). The transcript level is also affected by changes of endogenous or exogenous abscisic acid level. It appears to be a member of plant-specific gene family that includes late embryo-abundant and zinc- IAA-induced proteins in other plants. It is very interesting that gene AT5G48870 gene contain gene SAD1 which encodes a polypeptide similar to multifunctional Sm-like snRNP proteins that are required for mRNA splicing, export, and degradation. Mutation in this gene increases plant sensitivity to drought stress and ABA in seed germination, root growth, and the expression of

some stress-responsive genes (Verslues et al., **2006**). Also in Arabidopsis, gene AT3G24500 is one of three genes, encoding multiprotein bridging factor 1, a highly conserved transcriptional coactivator. It serves as a bridging factor between a bZIP factor and TBP. Its expression is specifically elevated in response to pathogen infection, salinity, drought, heat, hydrogen peroxide, and application of abscisic acid or salicylic acid. Constitutive expression enhances the tolerance of transgenic plants to various biotic and abiotic stresses. In comparison with 11 genes in this group, GU269889 gene shows the closest relation with AT2G45640 and AT2G40350. It suggests that GU269889 is not only response to drought stress but also involving in the regulation of salt stress (Lim et al., **2006** and Song et al., **2006**).

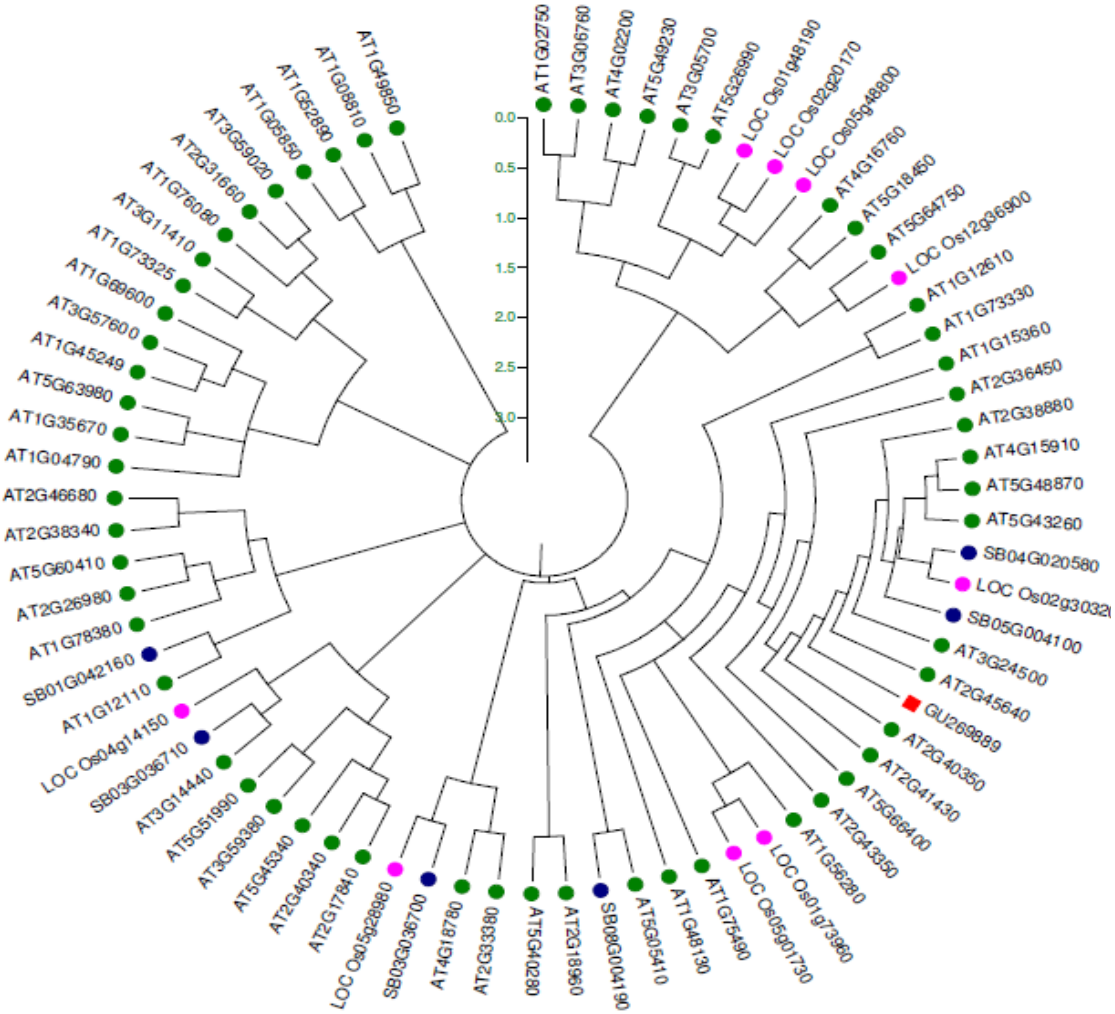

Figure 3: Phylogenetic relationship between candidate genes in different species. The color denotes the following: green-*Arabidopsis thaliana*, pink-*Oryza Sativa*, blue- *Sorghum bicolor*, red-gene sequenced from BAC13A9. The phylogenetic circular was inferred using UPGMA method. The bootstrap consensus tree inferred from 1000 replicates. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from dataset. Phylogenetic analysis was conducted in MEGA version 4 (Tamura et al., **2007**).

Motif analysis

The motifs present among the protein sequences were analyzed using MEME. The results of analysis showed that a total four of 25 motifs including motif 1, 2, 5, 7 contain the functional Di 19 protein domain. Motif 4, HRCEMKFVLLEMDRILRPTGYAIIRENAYFLDSV , is only found in 2 genes GU269889 and LOC_Os04g14150 which locate on chromosome 9 and 4, having the function of methytransferase. The remaining motifs did not define any known functional domain.

Promoter/cis-element analysis

The identification of cis-acting regulatory elements can provide important key for understanding spatial and temporal expression of protein sequences. It can also provide insights on the physiological and molecular processes that involved the action of drought-induced genes. To date, there are not been any report in the identification of essential promoters in these protein sequences. These protein sequences were used as queries for signal scan search for promoter through PLACE. Only three among 10 protein sequences was identified in which two of them DOFCOREZM, TAAAGSTKST1 found in GU269889 and one promoter TATABOX4 found in LOC_Os02g20170 gene. DOFCOREZM provide the binding sites for DOF proteins in maize (Yanagisawa and Schmidt, **1999**) while TAAAGSTKST1 provide target site for trans-acting StDOF1 protein controlling guard cell-specific gene expression (Plesch et al., **2001**) and TATABOX4 found in the 5' upstream region of sweet potato sporamin A gene (Grace et al., **2004**).

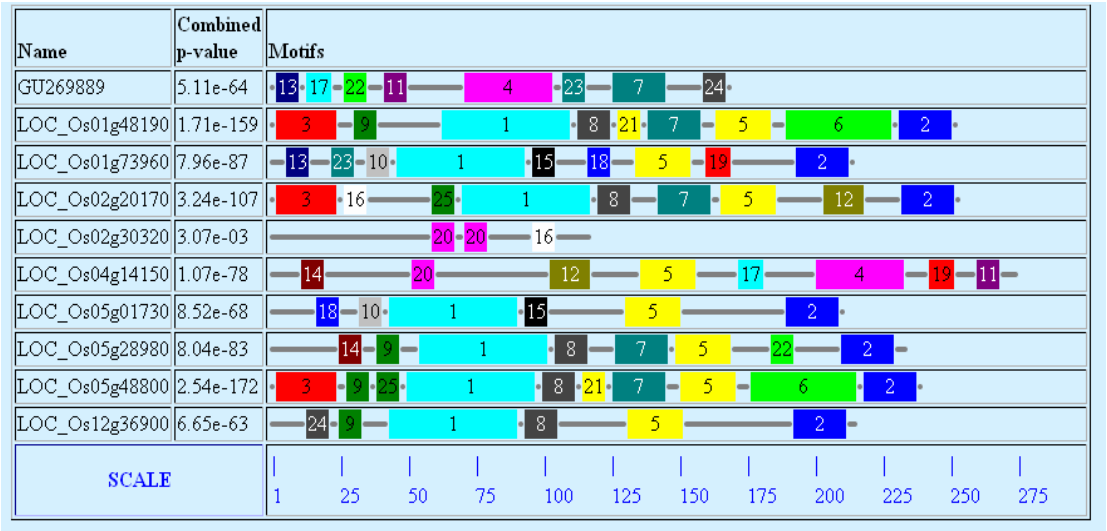

Figure 4: Protein motifs in 10 protein sequences. These motifs were analyzed and identified by MEME (http://meme.sdsc.edu/meme/intro.html).

CONCLUSION

To the drought-induced genes, many of which are functionally annotated, the genes with the highest repression factors were mostly of unknown or putative function. These genes could be as relevant for drought-tolerance as the highly induced genes, however, they are obviously much more difficult to explain and much more time consuming to study functionally. In the here presented study, we indicated that gene GU269889 has a potential functional for tolerance to drought stress, even salt stress through the analysis of phylogenetic relationship. It provides an overview and to shorten the list of genes response to drought stress condition. Looking forward in the future analysis, it needs to investigate the expression of GU269889 gene at different stages under drought stress conditions; and then an appropriate strategy to be

required to introgress this gene into promising rice varieties for developing drought-tolerance rice varieties.

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