



Expressed sequence tag-based prediction of putative genes responsive to drought tolerance in rice (*Oryza sativa*) using *in silico* approach

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ABSTRACT

In present genomic era, rapid genetic gains can be achieved by exploitation of novel genes associated with the trait of interest employing molecular breeding and genetic engineering. In the present study genes responsible for drought stress in rice 10746 expressed sequence tags (ESTs), expressed under drought stress condition were retrieved from the NCBI. The downloaded ESTs were clustered and assembled into 1120 contigs and 5559 singletons using CAP3 programme. The contigs were further subjected to identification of transcription factor, a total of 62 putative transcription factors were identified and sorted into 17 putative TF families. The contigs were subjected to BLASTX in NCBI to identify unique sequence which were further aligned to *Oryza sativa* Indica Group (ASM465v1) in gramene database using BLAT to retrieve the upstream and downstream sequences for putative gene identification. The retrieved sequences were analysed for transcription start site, PolyA tails and coding sequences which are essential features of gene using online tool fsgene. The present study found that, 46 contigs out of 1120 contigs has key gene structure and was considered as putative novel genes which may contribute to the drought tolerance in indica rice. These genes may be useful in development of drought tolerant varieties through smart breeding.

Introduction

Rice is a major cereal crop and a staple food for 1/3rd of the world's population, accounting for 76 percent of calorie intake in Southeast Asia. (Miura *et al.*, 2011). In relation to production, more than 90% of the rice is being produced from china, India, Bangladesh, Indonesia, Vietnam and Thailand. China being the largest population country, it has produced 146.7 million metric tons of milled rice for the 2019-2020. India followed the second place with 118.9 million metric tons of milled rice (Statista, 2021). The rice yield are being significantly affected by biotic and abiotic factors among which drought stress is one of the most serious threat, resulting in a considerable yield drop (Swamy and Kumar, 2013;

Sahebi *et al.*, 2018). The drought situation may be depicted as low rainfall or higher evaporation rates leading to stunted crop growth and reduced yield (Rollins *et al.*, 2013). The extent of yield loss depends on the drought intensity which intern depends on frequency and duration of rainfall, evaporation and soil moisture content (Oladosu *et al.*, 2019). It is estimated that, about 42 million ha of rice cultivation is under water scarcity (Yang *et al.*, 2019) and this raised the yield gap between the actual (4 t/ha) and potential (10 t/ha) (Oladosu *et al.*, 2019). To support the needs of growing population by 2050, it is prerequisite to develop high yielding rice varieties with multiple beneficial traits along

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with drought stress (Chukwu *et al.*, 2019). Breeding rice cultivars tolerant to drought is a cost-effective and long-term solution for increasing rice yields in marginal lands (Pandey and Shukla, 2015). Several researchers have attempted to transfer drought tolerant traits in the past through traditional breeding methods, but the success has been slow due to a lack of acceptable donors with high levels of tolerance, trait's polygenic nature, and lack of feasible screening procedures (Pandey and Shukla, 2015). This stagnant improvement has paved the way for exploitation of genetic engineering, which involves introduction of novel foreign genes into popular cultivar, thereby increasing the tolerance level (Yamaguchi and Blumwald, 2005). For this, identification of critical genes and pathways involved in the tolerance is pre requisite (Sanchez *et al.*, 2011). Plants being sessile developed various tolerance mechanisms at genic, biochemical, anatomical and phenotypic level which are under genetic control to overcome the menace of drought stress (Gupta *et al.*, 2020). Generally, drought stress involves up regulation and down regulation of hundreds to thousands genes which can be categorized into membrane transport genes, signalling genes and transcriptional regulatory genes (Upadhyaya and Panda, 2019). At molecular level, gene expression follows ABA-dependent and ABA-independent regulatory mechanisms (Gupta *et al.*, 2020). Out of variable gene products, transcription factors (TFs) are prime and has significant role in expression of drought tolerance genes (Nuruzzaman *et al.*, 2013). TFs control gene expression by interacting with cis-regulatory elements in the promoter region of stress genes (Wang *et al.*, 2016). Due to this, stress responsive TFs have got lot of attention and genetic engineering of crop plants with overexpression of a single TF may lead to over expression of multiple downstream genes.

With the advent of next generation DNA sequencing technologies, the complete genome sequence of japonica (Nipponbare) and indica cultivars (93-11) were successfully sequenced in 2002 (Goff *et al.*, 2002; Yu *et al.*, 2002). More importantly, the sequence of expressed genes were generated from cDNA libraries in the form of short nucleotide sequences (200–800 bases) known as expressed sequence tags (ESTs) which were deposited in the repository. This breakthrough has led to the access

for complete gene sequences, discovery of genes and their interactions across tissues as well as development stages. ESTs are prime choice of researchers due to following reasons *viz.*, gene discovery, genome annotation, mapping, polymorphism analysis, gene prediction, gene structure identification, and expression. The ESTs were utilised to find novel genes in wheat that were responsive to drought and salt stress and also salt stress responsive genes in rice (Nahas *et al.*, 2019; Bhati *et al.*, 2016). The comprehensive analysis of drought tolerance ESTs would give a clearer picture of transcriptional mechanisms to drought stress, aids in identification of critical genes and discovery of stress responsive promoters and the cis elements. The above utilities can be used in genetic engineering/cisgenic approach for improving/incorporating drought resistance genes into popular rice cultivars. In view of this, the present study was attempted to identify putative candidate genes expressed under drought conditions based on ESTs.

Material and Methods

A total of 10,746 ESTs or cDNA of *Oryza sativa* on date 5.4.2021 that have been expressed under drought conditions were downloaded from the EST database of NCBI (www.ncbi.nlm.nih.gov). Later, redundant ESTs, low complexity sequence, vector sequence, genomic repeats were masked by EGGASSEMBLER (Guo *et al.*, 2015, Masoudi-Nejad *et al.*, 2006). The CAP3 programme was used to aggregate the processed EST sequences into clusters (Huang & Madan 1999). The clustering yields singletons and contigs in which singletons are the ESTs having low nucleotide similarity with other ESTs. Whereas, contigs are formed due to overlapping of the ESTs with similar sequences. All assembled contigs were further analyzed to find the transcription factors using the PlantTFcat online tool (<http://plantgrn.noble.org/PlantTFcat>).

EST contigs annotation and identification of putative genes

The nucleotide sequence of EST contig were compared with the nr protein database of NCBI by using BLASTX programme with default options. Here, the nucleotide sequences were first translated into reading frames and compared with the proteins

to assign function. The EST contigs with no BLAST hit were aligned on the reference sequence of *Oryza sativa* Indica Group (ASM465v1) in gramene data base using BLAT with 97% similarity (Kent, 2002). The length of EST contigs aligned on the rice genome increased by 1 kb upstream and downstream to predicting the structure of genes with transcription initiation sites (TSS), extreme polyA tails, and coding sequences (CDS). The FGENESH program is used for the above analysis (Salamov & Solovyev, 2000).

Promoter analysis of candidate genes

The above candidate genes were validated by examining the promoter region of the putative predictive gene for cis regulatory elements using PlantPAN website (<http://PlantPAN2.itps.ncku.edu.tw>). The server contains the information regarding the experimentally validated transcription factor binding sites which will be used for prediction of cis regulatory sequence in the putative genes (Chow *et al.*, 2015).

Results and Discussion

In our study, A total of 10746 EST sequences related to indica rice drought tolerance were downloaded from Gene Bank of NCBI. The online server EGAssembler was used to process the EST into contigs and singletons for further analysis (Bhati *et al.*, 2016). Out of total ESTs, 149 ESTs with low quality, short sequences are eliminated in the first step of EG assembler. The remaining 10597 EST sequences were then further analysed to look for repetitions and low-complexity sequences (Bhati *et al.*, 2016). As a result, the total elements masked and cut are 139711 bp (2.50 percent) of the query sequence's overall length. The remaining EST sequences were then assembled into 1120 contigs and 5559 singletons using the CAP3 programme, which is part of the same programme (Table 1). The clustering of EST sequences reveals the number of genes, their content, and the number of gene families implicated in stress reactions (Bhati *et al.*, 2016; Nahas *et al.*, 2019). Assembled ESTs account for only 10.42% of the total ESTs utilized which are in accordance with earlier reports (Bhati *et al.*, 2016; Nahas *et al.*, 2019). Singleton represents expressed transcripts that cannot be assembled into larger

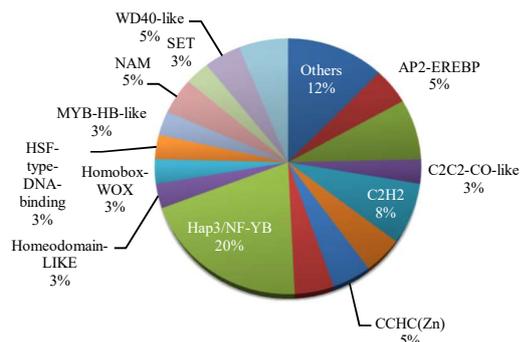
contigs to absence of overlapping sequences (Nahas *et al.*, 2019). These singletons are the product of only one mRNA of a gene therefore not considered for further analysis (Nahas *et al.*, 2019). Remaining 1120 contigs were submitted to online tool PlantTFcat (<http://plantgrn.noble.org/PlantTFcat>) to find the transcription factors (TF) responsible for activation of downstream genes responsible for drought tolerance (Nahas *et al.*, 2019). From the above analysis, Sixty two contigs were found to be putative transcription factors and sorted into 17 putative TF families. Among the 17 TF families, TF Hap3/NF-YB constitute nearly 20 % followed AUX-IAA (8 %), C2H2 (8 %), Znf-LSD (6 %), C3H (5%), CCHC(Zn) (5%) and GRAS (5%) (Figure 1) TF plays important role in gene expression of downstream genes upon signal perception under abiotic stress (Yuan *et al.*, 2016).

Because transcription factors operate as master regulators directing the expression of multiple target genes, they are the most promising candidates for unravelling the molecular mechanism underpinnings of abiotic stress responses (Nakashima *et al.*, 2009). The present study has found AUX-IAA transcription factor involved in drought stress. A recent study has revealed that auxin play major role in abiotic stress response by signal transduction and modulating the expression of multiple abiotic stress-related genes through interactions with auxin responsive factor and altered antioxidant enzyme activities (Shani *et al.*, 2017; Luo *et al.*, 2018). It was also found that the Aux/IAA rice OsIAA6 and OsIAA20 gene is regulated under water stress and its overexpression in transgenic rice improved drought tolerance through up regulation of auxin biosynthesis (Zhang *et al.*, 2021).

The utilization of these genes will be of prime importance in breeding drought stress tolerance in rice. The present study also found genes encoding for NUCLEAR FACTOR Y (NF-Y) transcription factors which are found in higher eukaryotes and belong to the CCAAT-binding factor (CBF) family and also known as the heme-activating protein (HAP) family. Evidence suggests that NFY subunits are important regulators of abiotic stress in plants (Nelson *et al.*, 2007). The overexpression of gene *GmNF-YA3* and *NFYA5* in *Arabidopsis thaliana* enhanced drought tolerance

Table 1: Summary of EST analysis conducted with EGAssembler

Feature	Numbers
Total number of ESTs	10746
EST total nucleotides (nt)	5580409 bp
Singleton	5559
Contig	1120
Average GC content (%)	47.95

**Figure 1: Distribution of TFs in the EST-contigs**

(Ni *et al.*, 2013). Similarly, in another study, transgenic maize and Arabidopsis expressing the gene NFYB1 showed significantly improved drought tolerance and yield under drought stress conditions (Nelson *et al.*, 2007). Recently, Su *et al.* (2018) identified that interaction of ZmNF-YA3 to the promoter region of ZmMYC4, ZmBHLH92 and ZmFAMA in maize improves temperature and drought tolerance. C2H2 zinc finger proteins play a role in plant response low temperatures, salt, drought, and oxidative stress through ABA-dependent and ABA-independent pathways (Kim *et al.*, 2013, Muthamilarasan *et al.*, 2014). SCOF-1, a soybean cold-inducible C2H2 zinc finger transcription factor operates under low-temperature tolerance (Kim *et al.*, 2001). Furthermore, C2H2 proteins improved rice drought resistance by modulating ROS-scavenging activities, proline, H₂O₂ and other cellular components. The ZFP245 a C2H2-type zinc finger protein was expressed in the roots, stems, leaves and panicles of rice under drought stress and believed to play big role in tolerance (Huang *et al.*, 2005). In soybean over expression of *GmGRAS37* in root hairs has improved the resistance to drought and salt stresses (Wang *et al.*, 2020). PAT1, a GRAS member from wild grape, improved Arabidopsis abiotic stress

tolerance (Czikkell and Maxwell, 2007). Likewise, GRAS23 regulates stress related gene expression in rice, resulting in drought resistance and oxidative stress tolerance, (Xu *et al.*, 2015). The transcription factors found in present study could be an excellent candidates for breeding transgenic rice cultivars tolerant to abiotic stress. Moreover, genetic engineering of multiple stress regulatory TF genes would be more rewarding for enhancement of stress tolerance in plants compared to focusing on a single individual gene.

Candidate gene prediction from EST-contigs:

Of the 1120 EST-contigs, 37 contigs with low nucleotide number i.e less than 300 were discarded from analysis. The remaining 1083 contigs were subjected to BLASTX in NCBI database of non-redundant proteins for functional annotation. The analysis showed that 63 contigs have no BLASTX hit and which were used for prediction of candidate genes responsible drought tolerant in *oryza sativa*., these 63 contigs were aligned against the genome sequence of *Oryza sativa* Indica Group (ASM465v1) in gramene database using BLAT at 97% similarity to find the genomic position and chromosomal location. The results showed that, six contigs could not be aligned onto the reference genome were excluded from further analysis. The remaining 57 contigs that were aligned with the reference genome and 1 kb upstream and downstream of the aligned sequence were retrieved for gene prediction and promoter analysis. Most of the contigs were located on chromosome 5 followed by chromosome 2, 7, 9 and least were present on chromosome 11 followed by 10 (Figure 2). Bhati *et al.* (2016) also found the similar results where in the chromosome 5 has more number of genes. The online tool FGENESH was used to predict the gene structure with transcription start site, PolyA tails at the extremes and coding sequence. The analysis showed 11 contigs viz., 118, 234, 248, 347, 381, 396, 429, 441, 841, 893 and 937 had no potential gene features and which were discarded from further analysis. Rest forty six contigs out of 1120 contigs were considered as putative novel genes which may contribute to the drought tolerance in indica rice. The coding position of new genes was presented in Table. 2. These identified genes may be mined for presence of cis regulatory elements and after which the genes are validated by designing new primers.

Table 2: The coding position of new genes

Contig Id	Chr. No.	Start (bp)	End (bp)	Length	Strand
Contig 2	4	23978692	23983123	4431	-
Contig 21	8	26499274	26501606	2332	-
Contig 74	7	17603491	17606469	2978	+
Contig 104	2	2142088	2144469	2381	-
Contig 137	5	5775957	5778539	2582	+
Contig 203	6	2645280	2647846	2566	+
Contig 205	9	17328039	17330067	2028	+
Contig 288	7	27754522	27757192	2670	+
Contig 294	6	23347486	23349696	2210	-
Contig 296	12	13766817	13769009	2192	+
Contig 324	5	8054078	8056647	2569	+
Contig 420	6	2180471	2183793	3322	+
Contig 464	2	6289181	6291445	2264	+
Contig 467	2	24254842	24256863	2021	+
Contig 481	9	15833257	15835862	2605	+
Contig 549	5	27764216	27766696	2480	+
Contig 571	4	32070044	32072546	2502	+
Contig 613	5	8053336	8055955	2619	-
Contig 620	3	1037582	1040151	2569	+
Contig 629	2	867386	869776	2390	+
Contig 678	3	4747269	4749892	2623	+
Contig 686	2	3849536	3851979	2443	-
Contig 710	2	26687427	26689869	2442	+
Contig 717	5	24238623	24241051	2428	-
Contig 718	4	27115876	27118418	2542	+
Contig 753	2	10305179	10307640	2461	+
Contig 776	6	17705156	17707616	2460	+
Contig 808	7	16778769	16781223	2454	+
Contig 812	5	549129	551455	2326	-
Contig 819	2	1197427	1199789	2362	+
Contig 823	2	1880223	1882592	2369	+
Contig 875	1	35680354	35682715	2361	+
Contig 880	5	3523583	3525971	2388	+
Contig 886	5	24047816	24050142	2326	-
Contig 888	12	20813518	20815830	2312	-
Contig 913	3	34335432	34337804	2372	-
Contig 920	9	17365261	17367637	2376	-
Contig 941	10	6254250	6256940	2690	+
Contig 950	1	3021484	3023781	2297	+
Contig 963	9	863254	865573	2319	+
Contig 967	8	26472569	26474872	2303	+
Contig 973	9	3131850	3134173	2323	+
Contig 976	5	5805432	5807745	2313	-
Contig 1063	5	22565074	22567427	2353	+
Contig 1080	3	37596768	37599101	2333	+
Contig 1082	7	20368825	20371141	2316	+

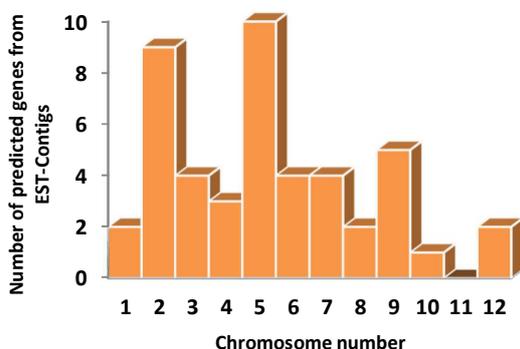


Figure 2: Number of predicted gene on different chromosome of rice using FGENESH.

Conclusion

Rice being a model crop, large sets of data is being generated on various omic approaches like genomics and proteomics. The generated data is being maintained in various databases like Gramene, NCBI, Phytosome etc., which can further

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utilized for in silico mining to discover new putative genes and so on for understanding the gene regulatory network and also for development of stress tolerant crops. The current investigation was focused on identification of novel putative drought responsive genes in rice using in-silico approach. The present study identified forty six putative genes with unknown functions which may directly and indirectly involved in the drought stress tolerance mechanisms. There is a need to find the functions of the newly identified putative genes with knockout technology to have comprehensive knowledge about the candidate genes and to understand the molecular mechanisms underlying the stress tolerance which in turn helps in breeding rice crop tolerant to drought stress through genetic engineering.

Conflict of interest

The authors declare that they have no conflict of interest.

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