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Research Article

# SEQUENTIAL VARIATIONS OF ABIOTIC STRESS TOLERANCE RESPONSIVE GENES IN RICE (*Oryza sativa* L) WITH GENE BASED MARKER APPROACHES

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**Abstract-** Rice (*Oryza sativa* L) is one the most important and widely cultivated food crops of the world, particularly for South Asian countries. Considering the detrimental effect of drought, salinity and low/ high temperature stress on rice yield, efforts have been initiated in the direction of developing abiotic stress tolerant crops through a combination of conventional and modern genomic technologies to sustain rice production in these adverse environmental conditions. The present study, focused on the identification tolerance genes and allelic variations of the candidate genes, will be of great use in management of stresses in rice. Through a comprehensive literature search of NCBI and RAP-DB databases, 12 abiotic stress tolerance genes were selected and all the genes under study were functionally validated genes and reported to play significant role in conferring tolerance to drought and salinity stresses in rice. Based on phenotypic characterization in drought and salinity stress environments, 14 rice accessions were selected for sequencing analysis and the polymorphism of these selected genes was studied using Nippon bare sequence as reference. In PCR assay, polymorphism was detected with six gene based marker while with other six markers, no polymorphism was seen. Sequence alignment analyses indicate differences at nucleotide level at different positions of these stress tolerance genes. The phylogenetic tree generated based on nucleotide sequences, clearly established the evolutionary relationships between the tolerant and susceptible genotypes could be of great help in the development of functional markers for use in MAS programs designed to enhance the level of tolerance against different stresses in rice.

**Keywords**- Rice, Polymerase Chain Reaction, Candidate genes, Sequence alignment.

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## Introduction

Rice (*Oryza sativa* L.), a diploid species having 24 (2n) chromosomes, belongs to the genus Oryza of Gramineae family (Poaceae). It is the primary food crops nearly half of the world's population. It is a self pollinated crop having a genome size of ~430 Mb, and both japonica (Nippon bare) and indica (93–11) were sequenced[1-2]. The production of rice constitutes more than 30% of the world cereal production today. In 2025, there will be 4.6 billion people depend on rice for their daily nourishment as it is an important source of carbohydrate. In Asia, more than 90% of the world's rice is grown and consumed [3]. For this reason, rice has become a highly strategic and priority commodity for food security in the world [4]. In India, the coverage of the rice crop is >44 mha, the largest in the world and is grown under extremely diverse environments. By 2050, India's population is expected to reach 1.6 billion from the current level of 1.2 billion and there will be greater demand for food. The cereal requirement of India by 2020 will be ~ 257 Mt depending on income growth [5-6]. The demand for rice is expected to be 122 Mt and this will have to be produced from the existing area. With shrinking land resource and under adverse climatic conditions, it is a challenging task.

The productivity of rice is severely affected by various abiotic stresses such as drought, salinity, high/low temperature and submergence [7]. Every year up to 82% of crop yield is lost due to abiotic stress and the amount of available, productive arable land is continuously decreasing [8]. The environment related abiotic factors are responsible for 40-50% reduction in yield in rice [9]. Different types of abiotic stress factors affect either individually or in combination through alteration of plant growth, development and through changes in their metabolism. Considering the effect of drought, salinity and cold on rice grain yield, it is very important to intensify work in the direction of development of tolerant crops through utilization of landraces and improved rice genotypes. Identification of novel gene and alleles responsible for the crop yield under adverse environmental conditions is a necessity and modern biotechnological tools can be of help in this regard.

The necessity to develop abiotic stress tolerant crops is intensifying due to increasingly limited water supplies for irrigation, accumulation toxic ions and high concentration of salts in saline soils. In addition, other stresses imposed by global climate changes in arid and semi-arid areas and season oriented dry coastal areas also influence yield [10-11]. Serraj et al. [12] revealed the importance of abiotic stresses that alter the signalling pathways depends on the stage of plant growth and duration period. In the rice cultivars, the drought and salinity stresses induce accumulation of various metabolites and alter the physiological traits [13] and morphological traits such as shoot and root length, weight vary between the tolerant and susceptible genotypes.

The drought and salinity tolerance related physiological and biochemical traits such as proline accumulation, chlorophyll and carotenoids contents, relative water content, antioxidants, stomatal conductance and transpiration rate, accumulation of toxic ions and reactive oxygen species [14-19] in plants growing under water-deficit and saline conditions have been investigated in many plant species such as wheat [20], Arabidopsis [21], pearl millet [22], sorghum [23-24], maize [25],tomato [26], cowpea [27], barley [28] and rice [29-32].

The tolerance to drought and salinity is complex and controlled by several genes (or) QTLs and is often confounded by differences in plant's phenology [33-34]. To address these complexities of plant responses to drought and salinity is vital to understand the physiological and genetic basis of response. The development of drought and salinity tolerance in plants through e genetic engineering [35-38], physiological [39-40] and genomics approaches [19, 41-43] have been studied in various crops. The genome mapping, high through put sequencing, functional

genomics and transcriptome analysis have provided powerful tools for molecular dissection of drought and salinity tolerance in various crops [28].

Recently, a number of potential candidate genes involved in the adaptive responses to abiotic stresses in cereal crops have been identified through transcriptomic and transgenic approaches [44-47]. These candidate genes (CGs) are sequenced and associated with known biological function of metabolic and physiological trait expression in biotic and abiotic environments [48-50]. Thus, understanding the morphological and physiological traits associated with drought and salinity tolerance genes in rice is a major requirement for the development of a broad range of tolerant cultivars as a feasible solution to climate change adaptation strategy. The molecular, physiological and bioinformatics technologies have provided new insights into drought and salinity stress tolerance, thus providing plant breeders with greater knowledge of the gene pathways and provided new tools for enhancing the crop yields. Keeping this in view, the present study was an effort to identify suitable genotypes having multiple abiotic stress tolerance from a germplasm set, collected from different eco-geographic regions of India.

## Material and Methods

### Plant materials

Fourteen rice accessions, collected from diverse eco-geographical regions of India, were selected from different screening experiments against drought, salinity and osmotic stress and possess different degrees of stress tolerance (high tolerance, moderate tolerance and susceptible) under drought, salinity and osmotic stress treatments [Table-1].

### Genomic DNA isolation

The rice seeds were germinated in dark on moistened filter papers for 2 days at $30^0$C in a plant growth chamber. The seedlings were used extraction of genomic DNA. DNA isolated from rice leaves as per Dellaporta et al. [51]. The extracted genomic DNA was checked on 1 % agarose gels for quality.

### Literature search for abiotic stress tolerance genes

To find the putative genes expressed under different under drought, salinity condition, public databases and literature can provide comprehensive information. In the present study, NCBI public database (http://www.ncbi.nlm.nih.gov/) was used to retrieve the sequence of the known abiotic stress tolerance genes and their sequence homology was studied with Nippon bare in Rice Genome Annotation Project Blast search program (RGAP Release-7 (blastn) (http://rice.plantbiology.msu.edu) [Table-2].

**Table-1** *List of selected fourteen rice accessions including tolerant and susceptible cultivars*

| S. No | Rice accessions | IC [a]/ AC No[b] | Sources of the genotypes | Status of Tolerance [a]/ Susceptible [b] level | No. of traits | Selected for gene based markers |
|---|---|---|---|---|---|---|
| 1 | N22(TC) | 41151[b] | NRRI | DSR, RWC%, Proline, SPAD.PEG [a] | 5 [a] | |
| 2 | CR-143-2-2(TC) | NAA | NRRI | DSR, RWC%, Proline, SPAD [a] | 4 [a] | |
| 3 | IR20(SC) | 75515[a] | NRRI | DSR, RWC%, Proline, SPAD.PEG [b] | 5 [b] | |
| 4 | BAM 8 | 124238[a] | CTG | DSR, Proline, RWC% [b] | 3 [b] | |
| 5 | BAM 28 | 390641[a] | CTG | DSR, RWC%, PEG [b] | 3 [b] | OsRacB |
| 6 | BAM 47 | 390317[a] | CTG | DSR, Proline, SPAD, PEG [a] | 4 [a] | OsCDPK7 |
| 7 | BAM 83 | 390725[a] | CTG | DSR, RWC%, Proline [b] | 3 [b] | OsERF3 |
| 8 | BAM 290 | 123518[a] | CTG | DSR, RWC%, SPAD [b] | 3 [b] | OsGRF8 |
| 9 | BAM 295 | 125623[a] | CTG | DSR, RWC%, SPAD [b] | 3 [b] | OsDREB1 |
| 10 | BAM 731 | NAA | MGL | DSR, Proline, SPAD [a] | 3 [a] | OsCam |
| 11 | BAM 859 | 309044[a] | AP | DSR, RWC%, Proline, SPAD [a] | 4 [a] | |
| 12 | BAM 1243 | 375802[a] | AP | DSR, RWC% [b] | 2 [b] | |
| 13 | BAM 3252 | 268284[a] | ND | DSR, RWC%, SPAD, PEG [a] | 4 [a] | |
| 14 | BAM 4060 | 393076[a] | ND | DSR, RWC%, Proline [a] | 3 [a] | |

**IC-**Indigenous collection**, NRRI-**National Rice Research Institute, **CTG**- Chhattisgarh**, MGL**- Meghalaya, **AP**- Andhra Pradesh, **ND**- New Delhi, **NAA**-Name not available, **TC-** tolerant control, **SC**- susceptible control

**Table-2** *Details of nucleotide and amino acid sequence similarity analysis with Nippon bare genome sequences in RGAP databases*

| S. No | Genes/ transcription factor | Chromosome | CDS-Coordinates (5'-3') | Gene length | | RGAP BLASTn statistics | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Nucleotide length | Amino acid length | Hit score | Mol. Weight | Sequence Identity | E-Value |
| 1 | OsDREB1 | 9 | 20404332-20403149 | 717bp | 239 | 5920 | 25390.1 | 100% | 2.3e-262 |
| 2 | OsCDPK7 | 4 | 29531223-29536492 | 1656bp | 552 | 4517 | 60966.2 | 99% | 0. |
| 3 | OsERF3 | 6 | 3376679-33768085 | 708bp | 236 | 5535 | 24270.5 | 100% | 6.0e-245 |
| 4 | OsGRF8 | 11 | 20521765 - 20525236 | 1230bp | 410 | 18005 | 43896.9 | 100% | 0. |
| 5 | OsCam3 | 1 | 9887626 – 9889328 | 450bp | 150 | 6661 | 16831.7 | 99% | 5.4e-296 |
| 6 | OsRacB | 2 | 1084283 – 1080513 | 594bp | 198 | 14584 | 21624 | 99% | 0. |

**Table-3** *PCR analysis of abiotic stress inducible genes/transcription factor specific markers*

| S. No | Genes/ transcription factor | Locus/ Gene bank ID | Traits | Forward Primer(5'-3') | Reverse Primer(3'-5') | References |
|---|---|---|---|---|---|---|
| 1 | OsDREB1A | LOC_Os09g35030 [a] | Drought cold and salinity | CGGTAATGTGATGGAACAAGTTG | TCGTGCAGAAACAATACTGTCAAG | [52] |
| 2 | OsCDPK7 | AB042550 [b] | Drought, Cold and salinity | GATGTATGGACTGCAGGTGTC | TGCATCCATAAGATCACGAA | [53] |
| 3 | OsERF3 | LOC_Os01g58420 [a] | Drought and Submergence | CAGCAATAGCACGGTAGACA | AGGAGTCGGAGTCACTTTGT | [54] |
| 4 | OsGRF8 | BK004863 [b] | Drought and Osmotic stress | GAGTGCCAATGCTGAGCTCTTGTG | GTGCTTGTAGATCAGTGCCTGGTG | [55] |
| 5 | OsCam3 | Z12828 [b] | Drought and salinity | TCGTCTAGGCAAGAAGATGAA | TGGGTAGGCTTACCTCCTTT | [56] |
| 6 | OsRacB | AY579208 [b] | Drought and salinity | GCATTATGCACCTGGTGTG | AGGTAGAAACACCAGCCAAA | [57] |

[a] MSU/TIGR locus name (Nipponbare, japonica); [b] EMBL/Genbank locus name

**Primer designing**

For PCR assay, gene specific primers were designed with Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) for known six abiotic stress tolerance genes such as OsCDPK7 [53], OsDREB1 [52], OsCam [56], OsERF3 [54], OsGRF8 [55] and OsRacB [57] [Table-3] and their efficiency was analyzed in Fast PCR program. These primers were further used for the amplification in the PCR.. All the markers and PCR reagents were from Sigma Aldrich, India.

**PCR amplification for the abiotic stress tolerance genes**

All the rice accessions includes highly tolerant, moderate tolerant and susceptible genotypes were used for the PCR based amplification using the designed gene specific primers. The PCR reaction mixture contained 30 to 50ng templates DNA, 10pmol/µl of each of the primers, 2.5mMdNTPs, PCR buffer (10mMTris /HCl, pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, and 0.01 mg gelatin/ml) and 0.5unit Taq DNA polymerase in total volume of 10µl reaction. The PCR amplification conditions were carried out by 94°C for 4 min; followed by 35 cycles at 94°C for 30 s, at 55°C for 30 s, and 72°C for 1 min; with a final extension at 72°C for 7 min (PTC-200 Thermo cycler; Bio-Rad, Germany). The PCR products were detected using a 1% agarose gel electrophoresis and observations were recorded with a gel documentation system (Alpha Innotech, USA). The amplified PCR product of all the six candidate gene derived from 14 rice accessions were sequenced specific (Bioserve, Hyderabad, India)

**Sequence alignment and Phylogenetic tree analysis**

The nucleotide sequences were analysed and edited using with BioEdit software and converted into FASTA format. The sequences of reference genome of Nippon bare and different stress tolerance levels of the rice genotypes were used for multiple sequence alignment in ClustalW software (http://www.ebi.ac.uk/Tools/msa/clustalw2/) [58]. Outputs of the multiple sequence alignment (MSA) file were taken as input for phylogenetic tree construction using the Neighbor-Joining method [59] and Neighbor-Joining algorithm implemented in the Molecular Evolutionary Genetics Analysis software version 5.0 (MEGA 5.0) [60]. Bootstrapping (500 replicates) was used to evaluate the degree of support for a particular grouping pattern in the phylogenetic tree. Branch lengths were assigned by pair wise calculations of the genetic distances and missing data were treated by pair wise deletions of the gaps.

**Results**

A total of twelve abiotic stress tolerant genes were selected and sequences of them were obtained from the NCBI database. The sequence homology was studied using Nippon bare as reference genome. MSU RGAP Release-7 (blastn) was used to identify and predict the genes in rice. All twelve abiotic stress responsive candidate genes were partially amplified in 14 rice accessions. Among the total 12 STS, six markers as OsCDPK7 (*Calcium dependant protein kinase*) [53], OsDREB1 (*Dehydration-responsive binding element*) [52], OsCam (*Calmodulin*) [56,65], OsERF3 (*Plant-specific ethylene response factors*) [57], OsGRF8 (*Growth regulating factor*) [55] and OsRacB (Small *guanosine triphosphate (GTP)-binding proteins*) [57], showed polymorphism between tolerant and susceptible rice genotypes which were characterized for their tolerance levels against abiotic stress conditions. In the earlier reports, the functions of all these genes was validated by various researchers and known to play significant role in altering the ability of the plant to survive under abiotic stress conditions [66-68]. We had tested the selected rice accessions with these known 12 abiotic stress tolerance genes and analysis performed on the sequence generated from the data obtained from the six genes for which polymorphism was seen in these 14 rice accessions [Fig-1].

The nucleotide sequence information of the amplified PCR products was obtained from Bioserve, Hyderabad, India. The sequences from all the fourteen rice accessions were aligned using ClustalW software (http://clustalw.genome.jp/) with reference genome sequences of Nippon bare. It revealed that, compare to known abiotic stress tolerance controls and Nippon bare, significant nucleotide variations were observed at different positions.

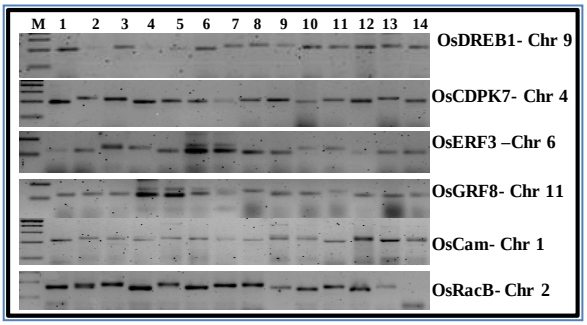
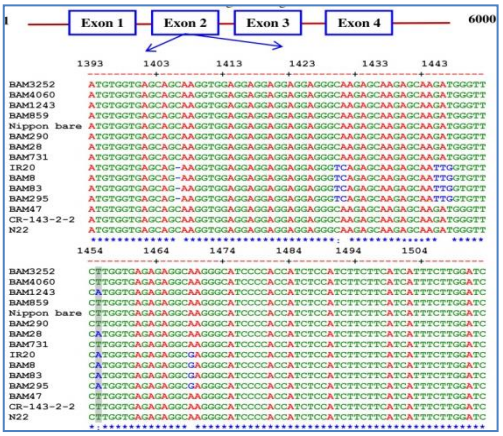
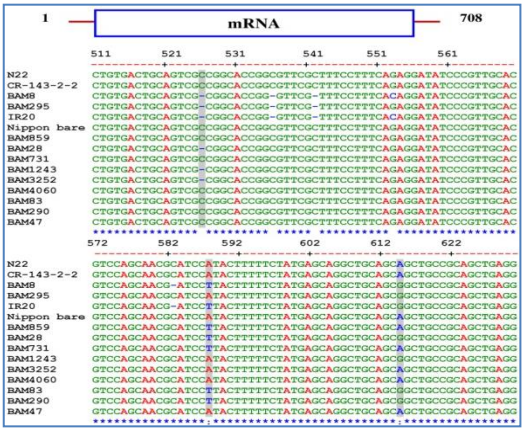



**Fig-1 PCR screening with six gene specific markers**

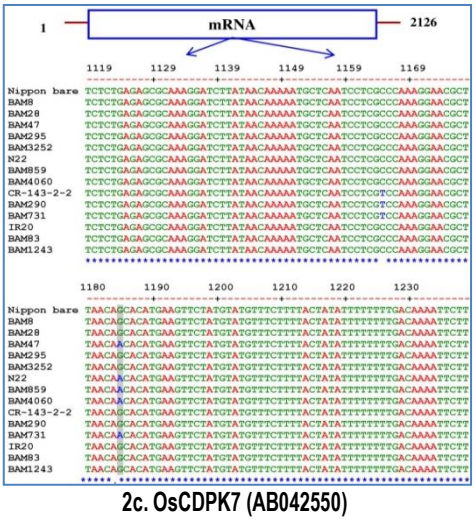
**Sequencing of putative candidate genes**



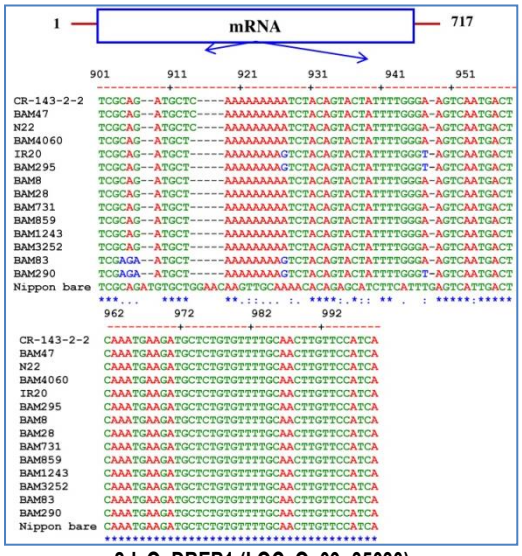

**2a. OsGRF8 (BK004863)**



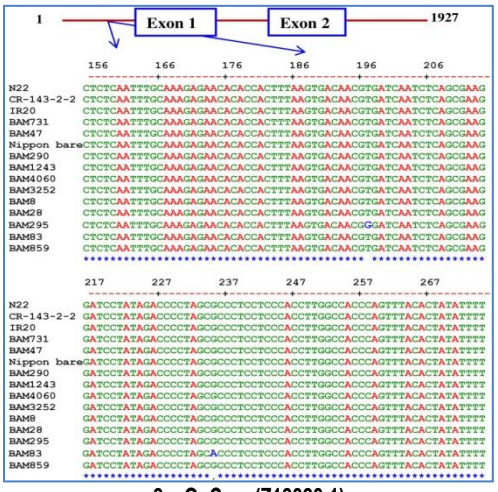

**2b. OsERF3 (LOC_Os01g58420)**



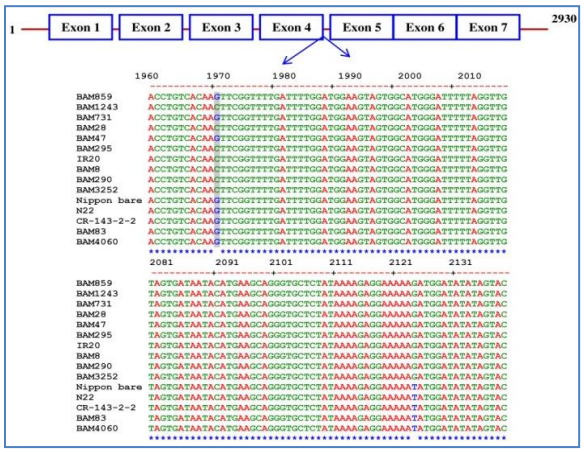

**2c. OsCDPK7 (AB042550)**




**2d. OsDREB1 (LOC_Os09g35030)**




**2e. OsCam (Z12828.1)**




**2f. OsRacB (AY579208.1)**

**Fig-2 Multiple Sequence alignment (MSA) of nucleotide sequences with six genes in rice**

**Sequence alignment**

The sequence alignment results are indicate nucleotide changes in different position having InDels and SNPs in known abiotic stress tolerance genes as OsCDPK7, OsDREB1, OsCam, OsERF3, OsGRF8 and OsRacB. Among the six gene sequence alignments, allelic variation was observed in four genes (OsCDPK7, OsERF3, OsGRF8 and OsDREB1) in the exon sequences while in the other two genes i.e. OsCam, and OsRacB, the changes (allelic variation) were in the intron region of genes in comparison to reference genome [Fig-2a-f].

**Sequential variations at exon regions**

**OSGRF8:**

In IR20, the susceptible control and BAM8 (susceptible), BAM83 (susceptible), BAM295 (susceptible) had "TC". In BAM47, BAM731, BAM4060, BAM3252, BAM290, BAM1243, Nippon bare and tolerant controls N22, CR-143-2-2 had "CA". In IR20, the susceptible control and BAM8 (susceptible), BAM83 (susceptible), BAM295 (susceptible) had "TTG" while in BAM47, BAM731, BAM4060, BAM3252, BAM290, BAM1243, Nippon bare and tolerant controls N22, CR-143-2-2 had "GAT" [Fig-2a]. This alleles having TC and TTG in these positions is related to susceptibility while change to CA and GAT is associated with tolerance. SNPs showing "A" at the position 1456 in the susceptible controls IR20 and BAM1243 (susceptible), BAM28 (susceptible), BAM8 (susceptible), BAM83 (susceptible) and BAM295 (susceptible). It appears that the change observed at 1456 position to A might be associated susceptibility.

**OsERF3:**

Variations in the form of SNPs and InDels at different positions in the gene sequences were in the exon regions. It appears that from the change at the 588th position, clear conclusion is not evident as from this change, the tolerant and susceptible genotypes could not be identified. In the 615th position, tolerant controls N22, CR-143-2-2, and reference genome Nippon bare, BAM859 (tolerant), BAM731 (tolerant), BAM3252 (tolerant), BAM4060 (tolerant) and BAM47(tolerant) having "A "while nucleotide changing to "G" in susceptible genotypes such as IR20, BAM8 (susceptible), BAM295 (susceptible), BAM28 (susceptible), BAM1243 (susceptible), BAM83 (susceptible) and BAM290 (susceptible) genotypes [Fig-2b]. This change appears to be associated with tolerance as genotypes having A are tolerant while the genotypes with G are susceptible

**OsCDPK7:**

The variations were located in the exon regions. At the position of 1185, SNPs (A/G) was observed. In tolerant rice accessions such as BAM47 (tolerant), BAM859 (tolerant), BAM4060 (tolerant), and BAM731 (tolerant) and tolerant control N22 had "A" allele. The remaining genotypes had "G" allele including one of the control CR-143-2-2,IR20, BAM8, BAM28, BAM83, BAM290, BAM295, BAM1243, BAM3252 and reference genome Nippon bare [Fig-2c]. The changes at position do not provide any basis for distinguishing the tolerant and susceptible genotypes.

**OsDREB1:**

The variations were located in the exon regions. At the position of 927 (G/A) and 947 (A/T) SNPs were observed in only in susceptible control IR20 and phenotypic susceptible rice accessions such as BAM83 (susceptible), BAM290 (susceptible), BAM295 (susceptible). It appears that the change at positions 927 (to G) and 947 (to A) are associated with susceptibility. [Fig-2d]. The change at 904-907 (AGA/CAG) was observed in BAM83 (susceptible) and BAM290 (susceptible) accessions having "AGA" and remaining all BAM accession and tolerant controls N22, CR-143-2-2, and susceptible control IR20 had "CAG" nucleotide sequence. The change is does not appear to be related to either tolerance or susceptibility.

**Sequential variations at intron region**

**Calmodulin**

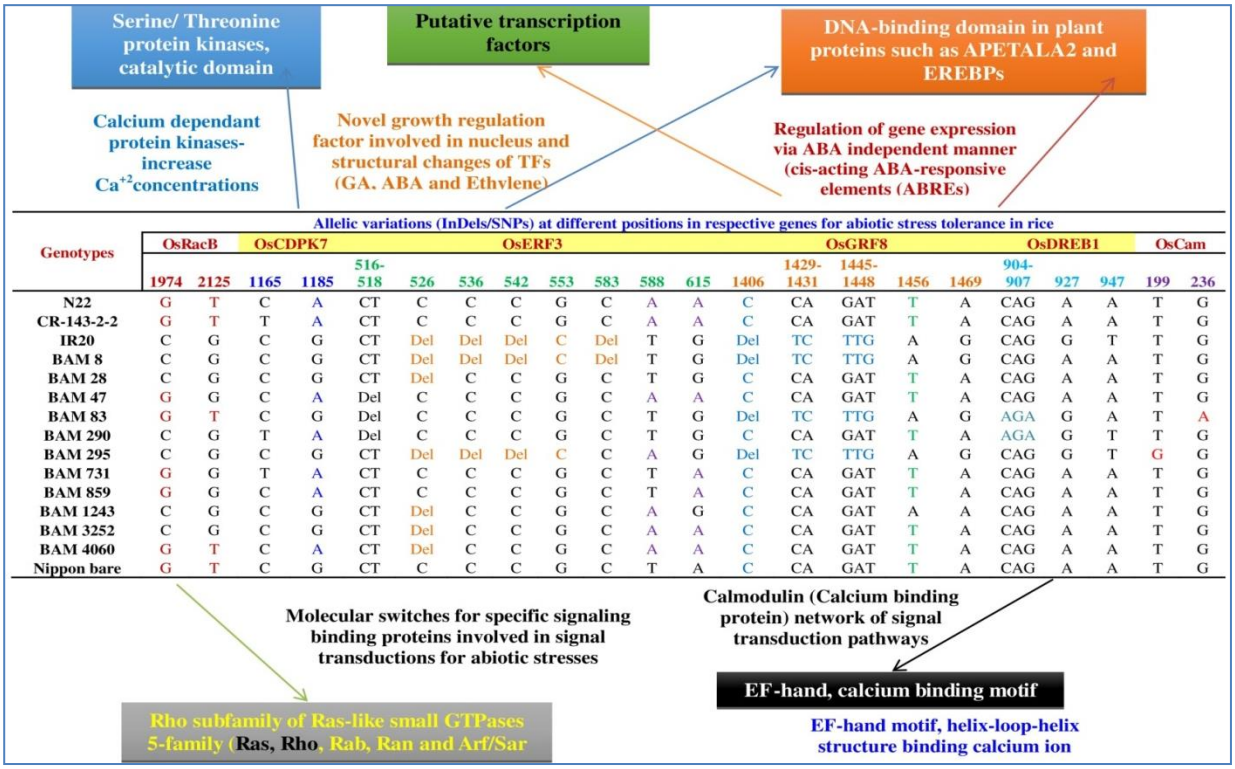
The SNPs were located in the intron region of the nucleotide sequence of the gene. Oscam gene does not affect the tolerance/susceptible genotypes, but these SNPs are located in two genotypes only [Fig-2e].

**OsRacB:**

These variations were located in the intron region. A single SNPs was identified at the position of 1974 (G/C). In tolerant rice accessions such as BAM47 (tolerant), BAM83 (susceptible), BAM731 (tolerant)), BAM859 (tolerant), BAM4060 (tolerant), and CR-143-2-2, N22 tolerant controls and Nippon bare had "G" allele while all

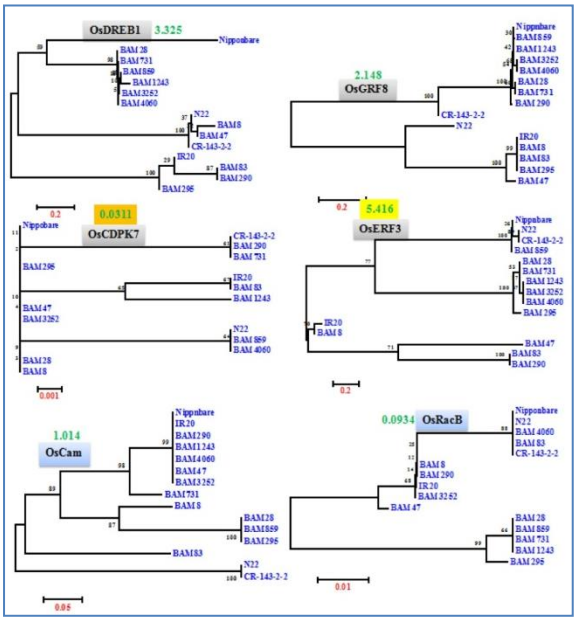
susceptible BAM accessions and IR20 had "C" at the position [Fig-2f]. The sequence of Nippon bare has similar sequence pattern as that of the tolerant controls and tolerant BAM accessions. Though a relationship could be established, since the change is at the intron region, it may not be of practical value.

Significant expression analysis of these six abiotic stress tolerance genes has been reported in rice accessions under drought, salinity and cold condition. From the sequential analysis results, it is possible to differentiate tolerant accessions the tolerant accessions from the susceptible accessions by following the alleles of three genes OsCDPK7, OsERF3 and OsGRF8 that are reported to confer tolerance to drought and salinity. From the sequence pattern, six genotypes i.e. BAM47, BAM290, BAM731, BAM859, BAM3252 and BAM4060 were similar to N22, CR-143-2-2, the tolerant controls [Fig-3]. But, all phenotyping data suggest that four rice accessions i.e. BAM47, BAM731, BAM859, and BAM4060 were tolerant similar to N22, CR-143-2-2, the tolerant controls indicating that some of these reported genes, may not be involved in conferring tolerance. This evident from data obtained from accessions like BAM 290 and BAM3252 which possess sequences similar to the tolerant controls, but they are not highly tolerant to stress.




| Genotypes | OsRacB | | OsCDPK7 | | OsERF3 | | | | | | | | OsGRF8 | | | | OsDREB1 | | | | OsCam | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1974 | 2125 | 1165 | 1185 | 516-518 | 526 | 536 | 542 | 553 | 583 | 588 | 615 | 1406 | 1429-1431 | 1445-1448 | 1456 | 1469 | 904-907 | 927 | 947 | 199 | 236 |
| N22 | G | T | C | A | CT | C | C | C | G | C | A | A | C | CA | GAT | T | A | CAG | A | A | T | G |
| CR-143-2-2 | G | T | T | A | CT | C | C | C | G | C | A | A | C | CA | GAT | T | A | CAG | A | A | T | G |
| IR20 | C | G | C | G | CT | Del | Del | Del | C | Del | T | G | Del | TC | TTG | A | G | CAG | G | T | T | G |
| BAM 8 | C | G | C | G | CT | Del | Del | Del | C | Del | T | G | Del | TC | TTG | A | G | CAG | A | A | T | G |
| BAM 28 | C | G | C | G | CT | Del | C | C | G | C | T | G | C | CA | GAT | T | A | CAG | A | A | T | G |
| BAM 47 | G | G | C | A | Del | C | C | C | G | C | A | A | C | CA | GAT | T | A | CAG | A | A | T | G |
| BAM 83 | G | T | C | G | Del | C | C | C | G | C | T | G | Del | TC | TTG | A | G | AGA | G | A | T | A |
| BAM 290 | C | G | T | A | Del | C | C | C | G | C | T | G | C | CA | GAT | T | A | AGA | G | T | T | G |
| BAM 295 | C | G | C | G | CT | Del | Del | Del | C | C | A | G | Del | TC | TTG | A | G | CAG | G | T | G | G |
| BAM 731 | G | G | T | A | CT | C | C | C | G | C | T | A | C | CA | GAT | T | A | CAG | A | A | T | G |
| BAM 859 | G | G | C | A | CT | C | C | C | G | C | T | A | C | CA | GAT | T | A | CAG | A | A | T | G |
| BAM 1243 | C | G | C | G | CT | Del | C | C | G | C | A | G | C | CA | GAT | A | A | CAG | A | A | T | G |
| BAM 3252 | C | G | C | G | CT | Del | C | C | G | C | A | A | C | CA | GAT | T | A | CAG | A | A | T | G |
| BAM 4060 | G | T | C | A | CT | Del | C | C | G | C | A | A | C | CA | GAT | T | A | CAG | A | A | T | G |
| Nippon bare | G | T | C | G | CT | C | C | C | G | C | T | A | C | CA | GAT | T | A | CAG | A | A | T | G |

**Fig-3 Nucleotide changes in selected 14 rice genotypes in different positions of abiotic stress tolerance genes**




(The Neighbor- Joining (NJ) algorithm analyses using percentage identities was constructed based on a multiple sequence alignment generated with the program MEGA4 (Scale represents percentage substitution per site)

**Fig-4 Phylogenetic tree representing alignment of specific gene sequences with different tolerant levels of rice accessions.**

However, significant novel allelic variation was observed in OsERF3 at the position of 588th (A/T) and 615th (A/G), in OsCDPK7 at the position of 1185th (G/A) and in OsGRF8 at the position of 1455th (T/A) at single nucleotide level and these variations can differentiate the tolerant and susceptible genotypes. Among the six genes, novel allelic changes were identified at functional nucleotide sequences (exons) of OsERF3 and OsGRF8. These superior alleles can be transferred into in high yielding rice varieties utilizing the MAS approaches.

**Phylogenetic tree analysis**

This analysis can reveal evolutionary relationships predicted from the multiple sequence alignment. The length of nucelotide sequences of each two branches can represents the distance between the sequence of rice genotypes and bottom of the units indicate the number of substitution events in tree structure. Distance-matrix methods of phylogenetic analysis clearly rely on a measure of genetic distance between the sequences being classified. Results of phylogenetic tree [Fig-4] showed that, susceptible control of IR20 is closely related with BAM83, BAM290 and BAM295 and this group of tree also includes BAM47, BAM8 and N22 and CR-143-2-2 in OsDREB1 gene. In case of OsCDPK7 and OsCam genes, susceptible genotypes like BAM8, BAM28, BAM83, BAM295 and BAM1243 are similar to Nippon bare while tolerant accessions are in a separate group. In case of OsRacB and OsGRF8, the Nippon bare sequence is similar tolerant genotypes while susceptible genotypes had another tree structure. In case of OsERF3, Nippon bare sequence is similar to tolerant genotypes like BAM290, BAM731, BAM3252 and BAM4060, while susceptible genotypes like BAM8 showed the IR20 pattern.

## Discussion

Rice (*Oryza sativa* L) is a most important and widely cultivated food crop all over the world and for many South Asian countries, it is the primary source of food. To meet the needs of the growing population, the estimated demand of rice production for the year 2025 for India stands at 140 million tons. However, the production and productivity remained low in India due to diverse ecosystems and different stresses that affect the rice production significantly.

Development of abiotic stress tolerant rice genotypes using the modern biotechnological tools needs a thorough understanding of the mechanism of tolerance to drought and salinity which can lead to identification of set of candidate genes or specific alleles controlling the morphological and physiological traits associated with abiotic stress tolerance in rice. The genes related to drought and salinity tolerance can be identified by thorough understanding of abiotic stress responses in plants using gene expression profiling tools. In rice, several studies on whole-genome gene expression and transgenic expression in response to drought and salinity have been reported earlier [51, 52, 62-64] and found major differences between susceptible and tolerant genotypes.

Transcription factors and protein kinases are important components of signal amplification and transduction networks conveying diverse signals to specific responses. The DREBs are members of the ERF family of transcription factors and follow ABA-independent signal transduction pathway. The two subclasses of DREBs, DREB1 and DREB2 are separately involved in cold and dehydration stress, respectively [69]. Ras /Rho family of Ras-related plant-specific signaling molecules plays important roles in plant growth, development and acting as key regulators in responses to environmental changes in plants [70,71]. OsRacB encoded a putative protein of 197 amino acids and transcription unit was 2930 bp in length, consisting of seven exons and six introns. Guet al. [70] and Berken [72] revealed that RopGTPase are master regulator involved in the negative regulation of abscisic acid (ABA) signaling and influencing to adaptation of plants to various environmental situations. The expressions of Ras/Rho genes occurred in whole leaf and single cells of Arabidopsis [73], Auxin signaling pathways [74] and Salt tolerance [57].

Calmodulin is a part of the network of signal transduction pathways centered on calcium ions as second messenger in eliciting responses to different signals, including many biotic and abiotic stress signals [75]. It's playing important roles in the structural integrity of the cell wall and intracellular regulator in plant growth and development during the stress responses [65]. There is ample evidence for the involvement of Ca2+ signaling in abiotic stress responses including, Mechanical stimulation [76], Drought [21, 77], Osmotic [78] and Salinity [79], Cold [80], Flooding [81] and Heat shocks [77]. OsCDPK is a class of plant protein kinases that contain a kinase domain and Ca2+binding domain is involved in regulating multiple cellular responses, genetic fundamental processes and majorly plant defense response in cold, salinity, and drought [82].

Based on the above data, selected genotypes having different levels of tolerance and amplified selected six genes using designed primers. The sequence alignment of nucleotide sequences using with Nippon bare as the reference genome revealed nucleotide variations in the form of InDels in the exon regions of OsCDPK7, OsERF3, OsGRF8 and OsDREB1 and for OsCam and OsRacB genes, the variation observed was in the introns. The sequential variations could be associated with the tolerance and susceptibility of the rice genotypes under study.

From the phenotypic and genotypic association results indicated that among the total rice accessions, four rice accessions as BAM 47, BAM 859, BAM731 and BAM 4060 which showed high levels of tolerance in all phenotypic experiments has the similar sequence patterns at the molecular level. It was observed that these rice accessions have the same alleles that are present in the known tolerance controls (N22 and CR-143-2-2). However, the specific significant novel allelic variation was observed in OsERF3 at the position of 588$^{th}$ (A/T) and 615$^{th}$ (A/G), in OsCDPK7 at the position of 1185$^{th}$ (G/A) and in OsGRF8 at the position of 1455$^{th}$ (T/A) at single nucleotide level and these variations can differentiate the tolerant and susceptible genotypes. Among the six genes, novel allelic changes were identified at functional nucleotide sequences (exons) of OsERF3 and OsGRF8. These novel superior alleles can be transferred into in high yielding rice varieties utilizing the MAS approaches for abiotic stress tolerance.

## Conclusion

Drought and salinity, two of the most important stresses that seriously affect crop cultivation and productivity in the worldwide. This problem attracted the attention of biotechnologist and plant breeders and efforts are through development of crops incorporated with stress tolerance to abiotic stresses. The present study was undertaken with an objective to identify superior alleles of abiotic stress tolerance genes in rice. For this purpose, twelve previously validated abiotic stress responsive candidate genes known previously for their significant role in the tolerance responses against drought, osmotic and salinity stress in rice were selected. Gene specific primers were designed and the amplified of rice genotypes were sequenced after purification. High-quality sequences were generated from the 14 selected rice accessions and Nippon bare, the reference genotypes and sequence alignment analysis was performed using MSA. It is interesting to see that sequential variations (InDels/SNPs) observed could clearly distinguish the tolerant from susceptible genotypes. Comparison of phenotypic and molecular data, BAM 47, BAM 971 and BAM 4060 accessions showed highly tolerant reaction and the sequence pattern observed in these accessions was similar to that of N22, the tolerant control. Therefore, present study provides basic information about some abiotic stress responsive genes followed allelic variations with respective rice genotypes and subsequently in breeding superior rice varieties giving better yield under abiotic stresses conditions by pyramiding favorable alleles in single variety using modern molecular breeding approaches.

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**Conflict of interest** Authors declare that they have no conflicting interest.

## Author Contribution

AM and GJN conceived the idea and designed the experiments. Performed the experiments by AA. The article written by GJN and AA.

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