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ORIGINAL RESEARCH ARTICLE

Physiological Response of Selected Rice Accessions to Salinity and *In Silico* Analysis of *DREB1A* Gene Among Diploid *Oryza* Species

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ABSTRACT

The responses of selected rice accessions to variant salt concentrations and *in-silico* analysis of *DREB1A* gene among diploid *Oryza* species were evaluated. Ten (10) rice accessions were selected based on their popularity in farmer's fields. Seedlings of each variety (one per pot) were watered with variant salt concentrations of 0mM, 100mM and 200mM for 21 days. The morpho-physiological characters (plant height, number of tillers, root length and dry weight) were evaluated using a standard evaluation system for rice. The reference sequences of *OsDREB1A* and *AtDREB1A* were used as queries to search against the 10 diploid *Oryza* species in the BLASTN of the PlantEnsembl database to reveal *DREB1A* orthologs. The retrieved *DREB1A* orthologs were used to compute the physicochemical properties of their proteins, gene motifs, intron-exon architecture and phylogenetic relationship. The studied accessions showed significant differences ($p < 0.05$) in morpho-physiological responses to salinity. The accessions *Zaqama*, *Yar-Garnaki*, *Yar-Yuti*, *Samira* and *Chana-Beru* performed better under salt stress and there was no significant difference ($p > 0.05$) between the control and salt-treated groups. Additionally, the *in-silico* analysis of *DREB1A* gene identified 10 orthologs with conserved single transcript, AP2 domain and unstable protein (characteristics of TFs) across the 10 diploid *Oryza* species. Phylogenetic analysis revealed 3 clusters of African rice and its progenitor, Asian rice and their relatives and *O. brachyantha*/*O. punctata* complex, similar to the evolution of rice species. Conclusively, salt stress affects rice in a concentration-dependent manner and *DREB1A* gene is a conserved plant transcription factor (TF) across diploid *Oryza* species.

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Salinity, Rice, DREB1A, Diploid *Oryza*, Transcription factor

INTRODUCTION

Cultivated rice species consist of *Oryza glaberrima* Steud. (African rice) and *Oryza sativa* L. (Asian rice), both of which have undergone independent domestication during their evolution. African rice, endemic to West Africa has domesticated from its putative progenitor *O. barthii* about 3,500 years ago (Wambugu *et al.*, 2019). These two cultivated species play an essential role in enhancing food security in sub-Saharan Africa where rice is becoming more popular as a staple food (Seck *et al.*, 2010). Achieving self-sufficiency requires significant yield increases to bridge the gap that exists between current and potential yields (Van-Ittersum *et al.*, 2016). However, climate change is projected to be a major threat likely to hinder the achievement of yield improvements in sub-Saharan Africa (Van-Oort and Zwart, 2018).

With the world population projected to reach 9.6 billion by 2050 (UNFPA, 2014), crop production will

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need to increase by about 44 million tons annually to meet the needs of the growing population. However, the limited extent of arable land, coupled with the emergence of unpredictable environmental conditions and abiotic stressors related to climate change, pose serious challenges to meeting global food production demands (Eckardt, 2009; FAO, 2009, 2012; Cominelli *et al.*, 2013; Islam *et al.*, 2015a, 2015b). Rice is sensitive to many abiotic stresses such as cold, salinity, drought and submergence (Lafitte *et al.*, 2004). Under these stresses, salinity is a serious limiting factor for rice production and yield stability in rain-fed fields (Upadhyaya *et al.*, 2009). It is estimated that more than 30% of the world's food is produced on irrigated land, but at least 20% of irrigated land is susceptible to high salinity and a further 50% of irrigated land is also affected by moderate or secondary salinity (Munns, 2002; Ruan *et al.*, 2010). Rice is glycotropic (salt-sensitive), and excess salt is associated with all major metabolic activities, including cell wall damage, accumulation of electron-dense protein particles, protoplasmolysis, cytoplasmic lysis, and damage to the ER. Improving salt tolerance is therefore a promising approach to meet increasing dietary needs (Munns, 2002). Wild rice germplasm is considered to be a valuable genetic resource for improving rice varieties (Quan *et al.*, 2017). However, information on improving salinity tolerance in rice grown with wild relatives is limited (Munns, 2002).

Dehydration response element binding proteins (DREBs) are plant-specific transcription factors that specifically bind to DRE/CRT elements in response to abiotic stresses such as salinity, drought, and cold (Liu *et al.*, 1998). It belongs to the AP2 superfamily and contains a highly conserved APETELA2 (AP2) domain with seven key amino acids (four R residues, two W residues and one V residue) that play important roles in the binding of CRT/DRE elements (Chen *et al.*, 2007). A valine (V) at amino acid position 14 is a key interaction site and is characteristic of the subfamily (Cao *et al.*, 2001). The DREB genotypes (DREB1 and DREB2) belong to the ethylene response element-binding factor/APETELA2 (ERF/AP2) domain gene family and bind to the dehydration response (DRE) cis-element (TACCGACAT) of the RD29A promoter to regulate drought, salinity, and induction of ABA-independent responses to cold (Yamaguchi-Shinozaki and Shinozaki, 1994; Sakuma *et al.*, 2006). Conserved sequence analysis across the rice genome indicates the presence of DREB homologs. The frequency and distribution of the DREB genes vary greatly between plant species and families due to gene expansions triggered by whole genome duplication (WGD) events (Wang *et al.*, 2019). Despite the conserved nature of DREB1A gene across plants, many plant species vary greatly in their abilities to tolerate salinity as typically observed in diploid rices (Gumi et al., 2018). Though there are reports on *DREB1A* gene in Asian rice (Cao *et al.*, 2001; Dubouzet *et al.*, 2003; Filiz and Tombuloglu, 2014) to date, even with the completion of the whole genome sequence of African rice, little has been done on *DREB* genes in African rice (Gumi *et al.*, 2018). The aim of this study was to evaluate the physiological responses to the salinity of selected rice lines and to analyze the DREB1A gene in diploid rice species using an *in silico* approach.

MATERIALS AND METHODS

Germplasm Collection

In total, ten (10) rice accessions were used for this study. Nine (9) rice accessions were purchased from Hamdana Farms Limited and the check/control variety (FARO-44) was obtained from IFAD Sokoto (Table 1). All accessions were selected based on their current popularity and adoption in farmer's fields.

Table 1: Rice Accessions collected at Hamdana Farms

S/N	Accessions	Species Name	Place of Collection
1	*Sufi*	*O. glaberrima*	*Hamdana Farms*
2	*Dogo-Dogo*	*O. glaberrima*	*Hamdana Farms*
3	*Jamila*	*O. glaberrima*	*Hamdana Farms*
4	*Dogon-Kade*	*O. glaberrima*	*Hamdana Farms*
5	*Samira*	*O. glaberrima*	*Hamdana Farms*
6	*Yar-Ganraki*	*O. glaberrima*	*Hamdana Farms*
7	*Chana-Beru*	*O. glaberrima*	*Hamdana Farms*
8	*Zakama*	*O. glaberrima*	*Hamdana Farms*
9	FARO-44	*O. sativa*	*IFAD, Sokoto*
10	*Yar-Yuti*	*O. glaberrima*	*Hamdana Farms*

Seed Planting and Experimental Design

Seeds (approximately 40-50) of each rice accession were carefully selected and surface-sterilized with 4% commercial bleach for 10 mins and rinsed with water five times. Each seed was then air-dried on Whatmann's filter paper and used for seed germination. Seeds of each accession were germinated in plastic containers separately for 15 days and uniformly germinated seedlings were selected and used for phenotyping under salt stress conditions. The containers were laid out in a completely randomized design and replicated three times. For salt stress treatment, laboratory-grade NaCl was weighed and dissolved in distilled water to make variant concentrations of 0mM (as control), 100mM and 200mM of salt concentrations which were used to irrigate the plants. Each treatment was replicated 3 times and each replicate consisted of 1 plant. The seedlings were exposed to salt stress by watering with saline water of 100mM and 200mM concentrations. The seedlings were watered with 0.5 liters of the irrigation solution to maintain uniform concentration for 21 days. After 21 days of salt stress episode, vegetative characters (plant height, number of tillers, root length, dry weight of shoot and roots) were evaluated. For plant height (cm), the individual length of each plant was measured and the average of the 3 replicates was used as the mean height of the treatment. For root length (cm), the rhizosphere of each plant was carefully washed in water and air-dried using blotters for 30 minutes. The length of each root was measured using a ruler across its vertical length. For the dry weight of root and shoot, after measuring plant height, tillers and root length, each plant was dissected into 2 portions (root and shoot) using sterile blade and the separated plants were kept in an oven at 65^{0}C for 48 hours (until uniform weight was observed).

Sequence Retrieval and Identification of *DREB1A* Orthologs

To identify the nucleotide and protein sequence of *DREB1A* genes among the 10 diploid *Oryza* species, we first identified Os*DREB1A* sequences of *O. sativa* japonica that was already annotated and characterized from Plant Transcription Factor Database: PlantTFDB v.4.0, http://planttfdb.gao-lab.org (Jin *et al.*, 2017) and used as queries to search against the whole genome of the other 9 diploid species using the BLAST tools of Gramene database-http://gramene.org by adopting the cut off value of $E < 10^{-4}$ and percentage identity of > 80% (Kersey *et al.*, 2016; Ganie *et al.*, 2017). Next, all *DREB1A* protein sequences obtained were searched for the best length and sequence homology to further confirm their identity as *DREB1A* genes. For all the identified DREB1A proteins, the compute pI/MW tool of the ExPASy server (http://www.expasy.org) was used to calculate the physical and chemical properties of the proteins, such as the molecular weight (MW), GRAVY, pH and theoretical isoelectric point (pI).

Identification of Conserved Motif, Subcellular Localization and Gene Structure

To identify conserved motifs, the multiple EM for motif elicitation (MEME) suites- http://meme-suite.org/ (Bailey *et al.*, 2009) was used to identify motifs within the identified *DREB1A* sequences of diploid species, with the following parameters: any number of repetitions, maximum of 10 mismatches, and an optimum motif size of 6–70 amino acid residues. For *DREB1A* genes architecture, exon–intron structures of the identified *DREB1A* genes were determined by comparing CDS sequences and the corresponding genomic sequences using the Online Gene Structure Display Server- GSDS 2.0: http://gsds.cbi.pku.edu.cn/ (Hu *et al.*, 2015) and were checked using TBtools (https://github.com/CJChen/TBtools). The Subcellular localization of *DREB1A* proteins was predicted using plant subcellular localization integrative predictor (PSI-Predictor; http://bis.zju.edu.cn/psi) using P value of <0.05 as described by Liu *et al.* (2013).

Phylogenetic Analysis

The amino acid sequences of the identified *DREB1A* orthologs of 10 diploid species were imported into MEGAX and multiple sequence alignment was performed using ClustalW Omega with a gap open and gap extension penalties of 10 and 0.1 respectively (Tamura *et al.*, 2011). The aligned sequences were then used to construct an unrooted phylogenetic tree based on the neighbor-joining (NJ) ML Method using bootstrap values of 1,000 replicates.

Statistical Analysis

The results on vegetative characters of selected rice accessions under different concentrations of salt were expressed as mean + Standard Deviation (SD) of three replicates and the data were subjected to two-way analysis of variance (ANOVA) and the significant difference ($p < 0.05$) between means were determined by Duncan's Multiple Range test. Statistical Package for Social Science (SPSS) Version 24 was used for the analysis.

RESULTS

Effects of Salinity on the Growth and Development of Rice

A number of rice accessions were affected by increasing concentration of salt. The effect of salt stress was concentration dependant across the varieties. Under control conditions, *Jamila* and *Dogo-Dogo* varieties have the highest plant height of 74.30cm and 72.00cm respectively. In the 100mM treatment, *Dogon-Kade* and *Chana-Beru* have the highest plant height of 42.8cm and 38.0cm respectively. In the 200mM treatment, *Zakama* and *Samira* recorded the highest plant height of 45.80cm and 42.0cm respectively (Figure 1A). However, plant height is significantly different ($p < 0.05$) across salt treatments and rice varieties evaluated. The root length of rice varieties as affected by salt stress are significantly different ($p < 0.05$) across rice varieties and salt concentrations. Under control conditions, *Dogo-Dogo* and *Jamila* had the longest roots of 26.60cm and 23.00cm, respectively. In *Samira, Zakama,* and *Yar-Yuti*, the 100mM treatment has the longest roots compared to the rest of the treatments, while in *Faro-44, Chana-Beru, Yar-Garnaki* and *Sufi*, the 200mM treatment has the longest roots compared to the control and 100mM treatments (Figure 1B). The number of tillers per plant decreased significantly ($p < 0.05$) with increasing salt concentrations. Under control conditions, all the evaluated rice varieties (except *Yar-Garnaki*, *Zakama* and *Faro-44*) have the highest number of tillers per plant than the salt-treated groups (100 and 200 mM). The varieties *Dogo-Dogo, Dogon-Kade* and *Jamila* have 3.66, 3.33 and 3.00 tillers per plant, respectively in the control category (Figure 1C). The dry weight (g/plant) evaluated decreased with increasing concentrations of salt stress. The control groups have a higher dry weight per plant than in salt-stressed groups (100mM and 200mM) in *Jamila, Dogo-Dogo, Dogon-Kade, Samira, Yar-Yuti* and *Yar-Garnaki*. In contrast, *Faro-44, Sufi, Zakama* and *Chana-Beru* have a higher dry weight in salt-stressed groups (100mM and 200mM) than the control (Figure 1D). However, there is a significant difference ($p < 0.05$) in dry weight between rice varieties and salt stress treatments.

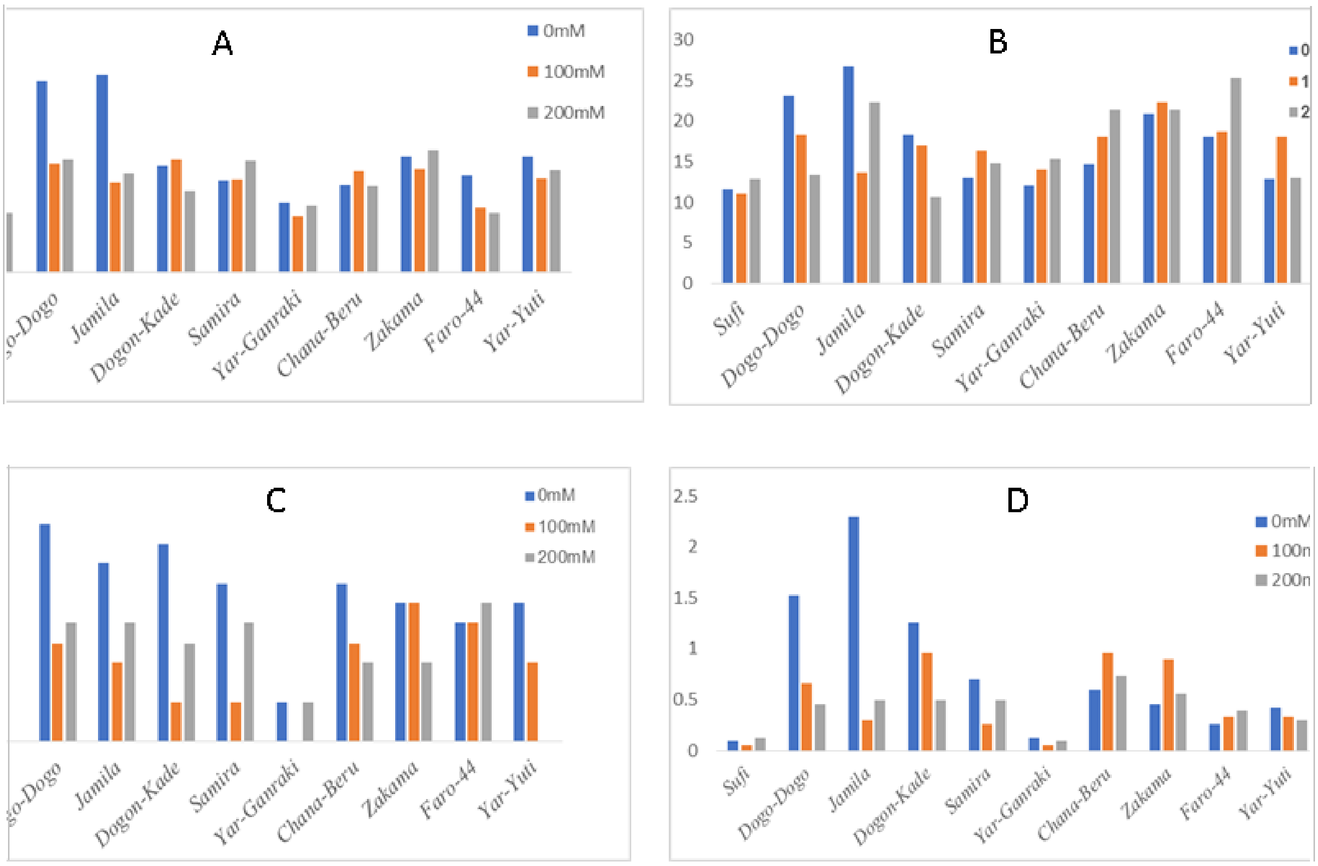

Figure 1: The response of selected rice varieties to different concentrations of salt. (A) Plant height (cm), (B) Number of tillers per plant, (C) Length of root (cm) and (D) Dry weight (g/plant) of the selected rice accessions under control (0mM), 100mM and 200mM salt concentrations.

Phylogenetic Analysis for *DREB1A* Protein Sequence

Multiple sequence alignment and consensus sequence for the AP2 domain of DREB1A protein across the rice species revealed sequence conservation. The phylogenetic tree generated for different *DREB1A* genes identified in 10 diploid *Oryza* species revealed a total of 3 clusters or sub groups (Figure 3). The African rice (*O. glaberrima*) and its progenitor *O. barthii* formed a separate clade while the Asian rices (*O. sativa* spp Indica and Japonica) along with their ancestors (*O. nivara* and *O. rufipogon*) occupied another separate clade. Additionally, the distant species of *O. brachyantha* and *O. punctata* occupied a separate clade in conformity with rice's evolution.

Analysis of Motif and Intron-Exon Architecture of *DREB1A* Orthologs

For conserved motif analysis, a total of 10 conserved motifs were identified in the *DREB1A* genes using MEME software. The AP2 domain designated as Motif 1 (with a size of 50 amino acids residues) occurred in all the identified *DREB1A* genes which affirmed its conserved nature in *DREB1A* proteins. Motifs 2 and 3 also occurred in all the identified *DREB1A* genes, affirming their conserved nature. The only non-significant Motif 8 (with the size of 6 amino acids residues) was found in three *DREB1A* proteins of *O. barthii*, *O. brachyantha* and *O. punctata*. For genes architecture, the exon-intron regions of the *DREB1A* genes were examined by comparing CDS sequences and the corresponding genomic sequences which revealed an intron less *DREB1A* genes among the diploid *Oryza* species except in the wild species of *O. barthii* and *O. brachyantha* with 1 intron each (Figure 4). The Sub cellular localization of *DREB1A* proteins as revealed by PSI-Predictor indicated that all the orthologous DREB1A proteins were localized in the cell's nucleus. This affirmed their role as transcription factors located in the cell's nucleus.

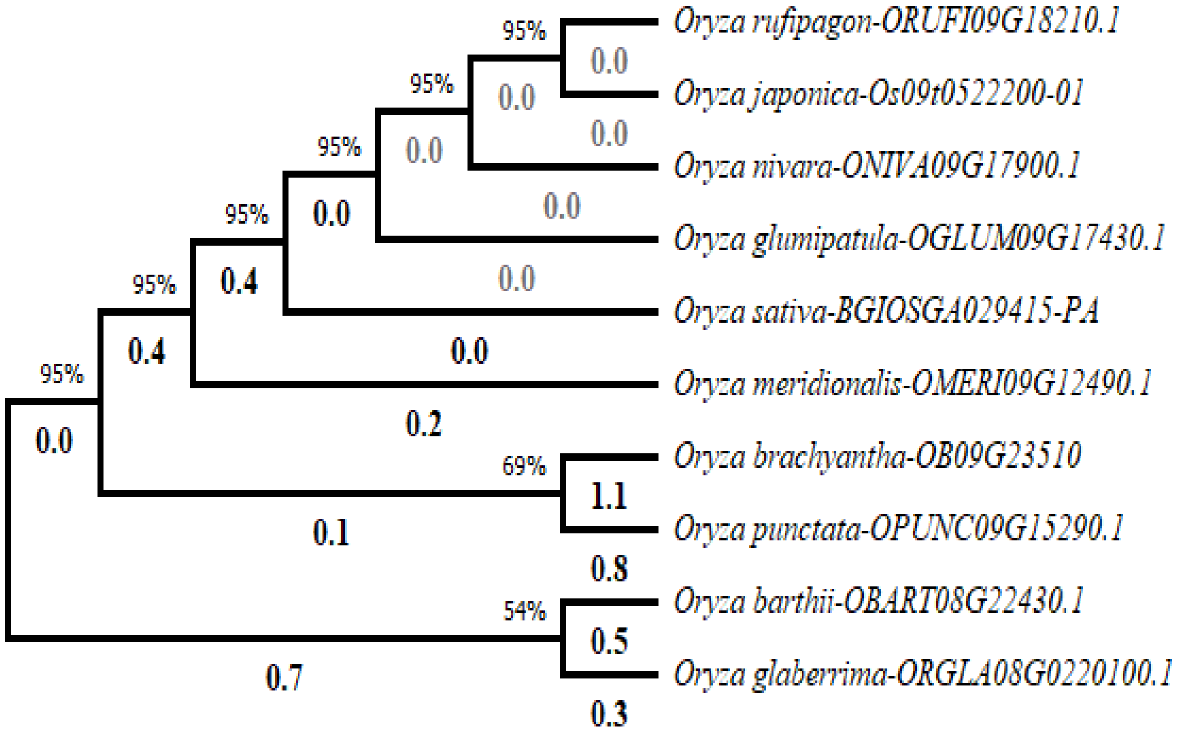

Figure 3: Phylogenetic Analysis for *DREB1A* Protein Sequence

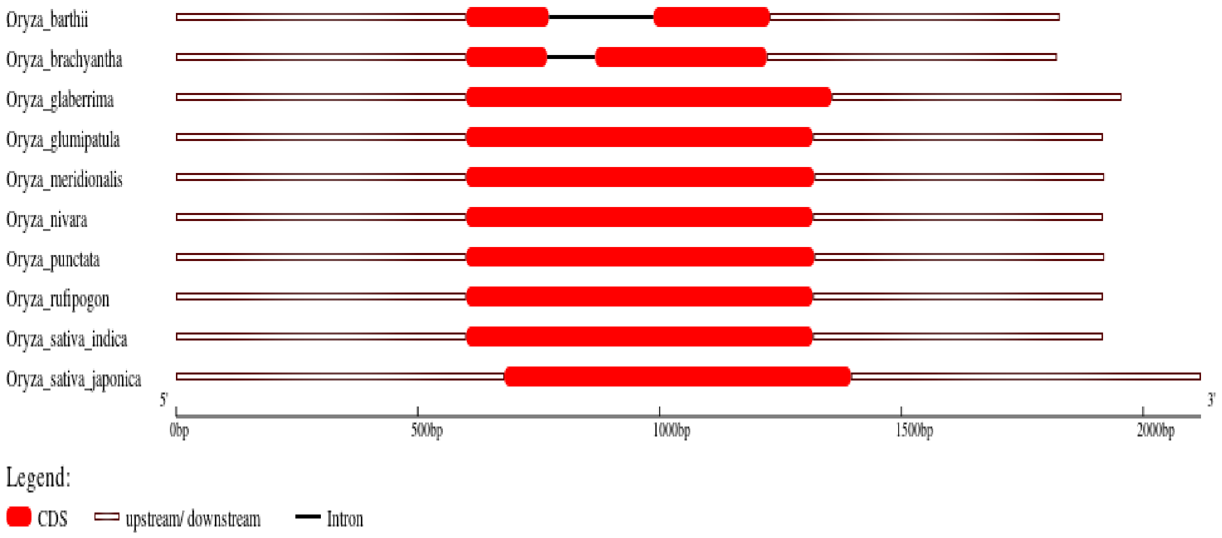

Figure 4: Analysis of Intron–exon architecture of *DREB1A* orthologs.

Physicochemical Properties of *DREB1A* Orthologous in Selected Diploid *Oryza* species

The physicochemical properties of the DREB1A proteins from the identified species revealed that the molecular weight of the proteins varies across the 10 diploid *Oryza* species with the wild species *O. glaberrima* encoding the largest protein (26795.7 Da) while *O. barthii* has the lowest protein weight of 14436.58 Da. The cultivated *O. sativa* Indica and its progenitor *O. nivara* had proteins with molecular weight of 25,404.14 and 25,390.11 Da respectively. The species of *O. sativa* Japonica, *O. rufipogon* and *O. glumipatula* has similar protein with molecular weight of 25390.11 Da. The instability index among the 10 identified proteins ranges from 60.81 in *O. brachyantha* to 65.85 in *O. glaberrima* confirming the unstable nature of the proteins across all the 10 identified species. The negative GRAVY values across all the 10 species (*O. sativa Indica* -0.39, *O. barthii* -0.517, *O. brachyantha* -0.475, *O. glaberrima* -0.28, *O. glumipatula* -0.39, *O. meridionalis* -0.416, *O. nivara* -0.39, *O. punctata* -0.379, *O. rufipogon* -0.39 and *O. sativa japonica* -0.39) and their positive theoretical PI which ranges from 4.11 in *O. barthii* to 5.06 in *O. sativa indica* affirmed their characteristics as unstable proteins typical of plant transcription factors (Table 5).

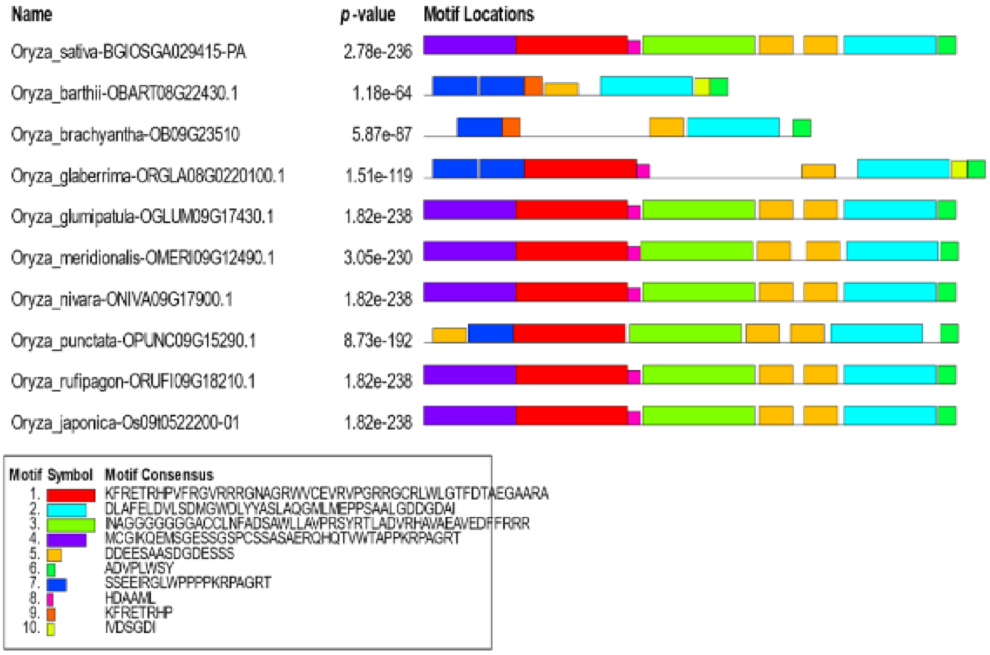

Figure 5: Conserved motif analysis of *DREB1A* ortholog

Table 5: Physicochemical Properties of *DREB1A* Orthologues in Selected Diploid *Oryza* Species.

S/N	Species	Gene ID	A. A	M.W	T.P. I	(+) Res	(-) Res	I.I	S. I	A. I	GRAVY	C.D
1	*O. sativa indica*	BGIOSGA029415-TA	238	25404.14	5.06	25	33	63.37	unstable	63.32	-0.39	AP2
2	*O. Barthi*	OBART08G22430.1	136	14436.58	4.11	9	25	64.76	unstable	58.24	-0.517	AP2
3	*O. Brachyantha*	OB09G23510.1	173	18459.21	4.53	15	28	60.81	unstable	63.35	-0.475	AP2
4	*O. Glaberrima*	ORGLA08G0220100.1	251	26795.7	4.64	25	39	65.85	unstable	70.56	-0.28	AP2
5	*O. glumipatula*	OGLUM09G17430.1	238	25390.11	5,05	25	33	62	unstable	63.32	-0.39	AP2
6	*O. meridionalis*	OMERI09G12490.1	239	25577.27	4.9	25	35	62.87	unstable	63.05	-0.416	AP2
7	*O. nivara*	ONIVA09G17900.1	238	25390.11	5.05	25	33	62	unstable	63.32	-0.39	AP2
8	*O. punctata*	OPUNC09G15290.1	239	25478.09	4.87	22	33	62.7	unstable	61.05	-0.379	AP2
9	*O. rufipogon*	ORUFI09G18210.1	238	25390.11	5.05	25	33	62	unstable	63.32	-0.39	AP2
10	*O. sativa Japonica*	Os09t0522200-01	238	25390.11	5.05	25	33	62	unstable	63.32	-0.39	AP2

KEY: **A.A:** Amino Acid
C.D: Conserved Domain
M. W: Molecular Weight
T.P.I: Theoretical Isoelectric Point
(+) Res: Total No of (+) residues (Asp + Glu)
(-) Res: Total No of (-) residues (Arg + Lys)
I.I: Instability Index
A.I: Aliphatic Index
S.I: Stability Index
GRAVY: Grand average Hydropathy

DISCUSSION

The response of rice varieties to varying salt concentrations revealed that plant height (cm), root length, and the number of tillers are affected negatively by increasing salt concentrations. Rice is sensitive to various abiotic stresses, including salinity, drought, submergence and cold (Lafitte *et al.,* 2004). Among these stresses, salinity is a serious limiting factor to rice production and yield stability (Upadhyaya, 1996). Soil salinity is among the major abiotic stresses affecting crop productivity worldwide (Zhu, 2001). In general, salinity affects plant height in rice in a concentration-dependent manner as reported by Rashid *et al.,* (2017), Fathelrahman *et al.,* (2015) and Hussain *et al.,* (2005). Root elongation during stress benefits plants by restoring contact with water below the saline soil. This interesting phenomenon might suggest the root absorbed much water to dilute the large amounts of Na^+ in its tissues, which is another effective approach to cope with salt stress.

The protein sequences of the DREB1A orthologs identified were aligned and the evolutionary relationship of *DREB1A* genes among the diploid *Oryza* species was revealed. The evolution of *DREB1A* genes among the diploid *Oryza* species agrees with the evolution of rice species. The cultivated rice species (*O. glaberrima* and *O. sativa*) are derived from wild species of *O. barthii* and *O. nivara,* respectively. Other wild species such as *O. brachyantha* and *O. punctata* followed different patterns of evolution. Multiple sequence alignment of *DREB1A* sequences of *Oryza* species showed a high level of sequence conservation in the AP2 domain while comparatively less sequence conservation with other wild species like *O. brachyantha.* Evolutionary analyses revealed a phylogenetic tree that precisely dissects the functional groups within each subfamily according to *Oryza* AP2/ERF genes. However, the clustering of *O. glaberrima DREB1A* to that of *O. barthii* suggest that the *DREB1A* protein sequences between these two species are closely related in accordance with the fact that O. *glaberrima* has been domesticated from its progenitor O. *barthii* (Sweeney and McCouch, 2007). The analysis of the intron–exon architectures of the *DREB1A* gene sequences among the diploid *Oryza* species suggested that the length of the coding regions varied from 635bp to 1,450bp and intron length varied from 850bp to 975bp. However, 80% of the *DREB1A* genes in diploid *Oryza* species were intron-less. The only intron gene was observed in *O. barthii* (progenitor of African rice) and *O. brachyantha* (a distant wild species). This phenomenon suggested that *DREB1A* orthologs with introns evolved earlier than the intron-less genes. Previously, DREB genes were believed to be intron-less until recently when many DREB genes containing introns were found especially in DREB2A of rice (Matsukura *et al.*, 2010; Gumi *et al.*, 2018) and other cereals like maize (Qin *et al.*, 2007; Liu *et al.*, 2013) and barley (Guo *et al.*, 2016). Many reports have shown that alternative splicing plays an important role in the differential expression of stress responsive genes in plants at either protein or transcriptional level of modifications (Matlin *et al.*, 2005; Szakonyi and Duque, 2018). Motif analysis among the *DREB1A* protein sequences of diploid *Oryza* species indicates a conserved array of motifs across all of the diploid *Oryza* species, which indicates that the motifs are essential for the proper functioning of the protein (Wuchty *et al.*, 2003). The *DREB1A* gene of *O. glaberrima* and various *Oryza* species have shown a high degree of similarity with other *DREB1A* of rice relatives and have a signature of the AP2 domain.

CONCLUSION

Conclusively, the studied rice accessions showed differential responses to salinity with markedly observed variation across varieties and salt concentration. Additionally, the *in silico* analysis of *DREB1A* gene among the diploid *Oryza* species revealed that all the orthologs have conserved single transcript, AP2 domain and unstable protein characteristics of plant transcription factors. Phylogenetic analysis revealed the clustering of *Oryza glaberrima DREB1A* to that of *Oryza barthii* and the cultivated Asian sub species to *O. nivara* and *O. rufipogon* which suggest the fact that *Oryza glaberrima* has been domesticated from its progenitor *Oryza barthii* and Asian rice domesticated from *O. nivara*

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