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Evaluation of Genetic Diversity of *Cis*-acting Elements of Abscisic Acid Responsive Element Binding Protein (*ABRE-BP*) in Selected Sri Lankan Rice Varieties

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ABSTRACT . *Salinity is a major abiotic stress that affects rice cultivation. Osmotic stress caused by salinity activates tolerance mechanisms in rice. The Abscisic Acid Responsive Element Binding Protein (ABRE-BP), transcribes for a bZIP family transcription factor that binds to the cis-regulatory element Abscisic Acid Responsive Element (ABRE) at the promoter of downstream salinity responsive genes and regulates their expression. Hence, the study was focused on analyzing the nucleotide diversity of a region in ABRE-BP promoter. Analysis of the ABRE-BP sequences of 47 Sri Lankan rice varieties and two reference varieties retrieved from a public database revealed many insertions and deletions (INDELs) and single nucleotide polymorphisms (SNPs) in the promoter. Eleven, abiotic stress related cis-elements were identified, but none of the INDELs spanned over them. However, SNPs either deleted existing cis-elements or created new cis-elements. The salinity stress responsive elements, MYBCORE and GT1GMSCAM4 were detected in few varieties, and were not associated with salinity tolerance based on current available salinity ratings. The presence of SNPs in cis-elements clustered them to nine groups at 68% similarity. The DNA polymorphisms on stress responsive cis-acting elements did not show a strong association with the known salinity ratings of many varieties. Nevertheless, the presence of INDELs affects the relative distance between elements, and thus may alter the expression of the ABRE-BP that regulates downstream stress responsive genes.*

Keywords*: ABRE-BP, ABRE, Cis- elements, Salinity, Sri Lankan rice varieties*

INTRODUCTION

Rice (*Oryza sativa* L.) is cultivated in regions with diverse environmental conditions. In some parts of the world, rice is grown under various abiotic stress conditions such as: drought, submergence, frost prone conditions, saline, acidic and toxic soils (Sankar *et al.,* 2011). These conditions directly affect the plant growth and its productivity. Out of the persisting abiotic stress factors, drought and salinity are the major contributors to crop failure in rice cultivations (Bartels and Sunkar, 2005). Due to the climatic changes, rising of sea levels, geo-chemical weathering of rocks and improper drainage in cultivated lands, rapid expansion of salinity affected land is expected (El-Swaify, 2000). It is hard to treat and amend a salinity affected soil and thus, only the crops that are tolerant to such adverse conditions can be cultivated (Pitman and Lauchli, 2002). Due to the fact that 20% of irrigated

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lands under cultivation are affected by soil salinity, development of rice cultivars that are tolerant to salinity is a priority in rice breeding programs all over the world (Pitman and Lauchli, 2002).

When a plant is under stress, the stress signal gets transmitted to responsive sites *via* many pathways including regulation of ion homeostasis *via* salt overly sensitive (SOS) regulatory pathway for ions and hormones induce signal transduction pathways such as abscisic acid (ABA)–dependent pathway (Sairam and Tyagi, 2004). Increasing soil salinity changes the osmotic potential of the plant cell and creates a water deficit condition, a potential stressful condition triggering the activation of salinity tolerance response gene cascades (Shinozaki and Yamaguchi-Shinozaki, 1997). The activation of the synthesis of the plant stress hormone ABA, in turn activates a cascade of stress responsive genes to deliver plant tolerance/resistance responses (Nakashima *et al*., 2009). The ABA inducible stress responsive genes such as, *salt, GST* and *rab 16-A* delivering salinity tolerance have been reported in rice (Mundy and Chua, 1988; Garcia *et al.,* 1998; Taji *et al.*, 2002; Rabbani *et al*., 2003; Walia *et al.,* 2005; Jain *et al.,* 2010).

The abscisic acid responsive element (ABRE) is a commonly found *cis*-element in stress responsive genes (Uno *et al.,* 2000). A bZIP-family transcription factor *ABRE-BP* binds to the ABRE *cis*-element(s) and induces the expression of ABA-inducible salinity responsive genes in rice (Nakashima *et al.,* 2009; Todaka *et al.*, 2015). Thus, the expression of the salinity responsive genes in *ABRE-BP* regulon is dependent upon the level of expression of the *ABRE-BP*. The *cis*-elements found in the promoter of *ABRE-BP* and the distance between those elements are crucial in mediating its expression. To our knowledge, the *ABRE-BP* promoter region of different rice varieties has not been characterized. Identifying and comparing the *cis*-elements, their relative distance and DNA polymorphisms may shed insight on the contribution of these elements for the salinity responses in these varieties. The salinity responses of most Sri Lankan varieties are not well characterized and of those that have been characterized the actual salinity ratings reported in previous work have been at times contradictory (de Costa *et al*., 2012; Pradheeban *et al.,* 2014). However, varieties such as *Pokkali* and *Kuruluthudu* are well known for their salinity tolerance.

In the current *in silico* analysis, sequences covering the upstream region of *ABRE-BP* of 47 Sri Lankan rice varieties and two reference varieties (cultivar 93-11(*indica*) and Nipponbare (*japonica*)) were used to detect DNA polymorphisms of the *ABRE-BP* promoter region, specially at *cis*-elements related to abiotic stresses and their possible contribution to salinity responses.

METHODOLOGY

DNA resources

For the current study, a 800 bp sequence upstream of the transcription start site (TSS) in *ABRE-BP*, of 47 Sri Lankan rice varieties were retrieved from the Rice SNP-Seek Database (http://oryzasnp.org/iric-portal/) using the locus position of *ABRE-BP* in *O. sativa* subsp. *japonica* (OS06G0211200; chromosome 6 position 5,676,157 to 5,682,033) (Table 1). Sequences of reference varieties *O. sativa* subsp. *japonica* cv. Nipponbare (Os06g0211200) and *O. sativa* subsp. *indica* cv. 93-11 (BGIOSGA022536) were retrieved from the Gramene database (http://www.gramene.org/).

Table 1. Selected Sri Lankan rice varieties and their accession numbers

Rice Variety	Accession Number
105	IRGC 40896-1
3210	IRGC 116950-1
A 69-1	IRGC 55305-1
Alagusamba	IRGC 8944-2
Balasuriya	IRGC 66509-1
BW 295-5	IRGC 63098-1
Chandina	IRGC 36420-1
Galawaka Handeran	IRGC 31381-1
Godawel	IRGC 15750-1
H 6	IRGC 157-1
Halsudu Heenati	IRGC 15599-1
Heendikwee	IRGC 15587-2
Herath Banda	IRGC 67630-1
Hodarawala	IRGC 67631-1
Honderawala	IRGC 47372-1
Kahatawee	IRGC 12004-1
KaluIlankalayan	IRGC 36270-1
Karutha Seenati	IRGC 15515-2
Kotteyaran	IRGC 47383-1
Kula Karuppan	IRGC 55328-1
Kurukaruppan	IRGC 15449-1
Kurulu Wee (white)	IRGC 66518-1
Kurulutudu	IRGC 36304-1
Matholuwa	IRGC 8901-1
Moddai Karuppan	IRGC 15465-1
Mudaliga Wee	IRGC 74706-1
Murunga	IRGC 15428-1
Muttu Samba	IRGC 36333-1
Nalumoolai Karuppan	IRGC 8993-1
Pachchaperumal	IRGC 3474-1
Pannithi	IRGC 51049-1
PeriyaVellai	IRGC 15475-1
PodiHeenati	IRGC 36345-1
Podiwee	IRGC 11938-1
Pokkali	IRGC 8948-1
Puttu Nellu	IRGC 55346-1
Race Perumal	IRGC 55347-1
Rangoon Samba	IRGC 11940-1
Ranruwan	IRGC 36360-1
Samba	IRGC 11993-1
Sayam	IRGC 31538-2
Sigardis	IRGC 15555-1
SinnaSitira Kali	IRGC 51064-1
Sithaiyan Kottai Samba	IRGC 50155-1
SuduKarayal	IRGC 15348-1
Vellai Kolomban	IRGC 15517-1
WIR 1391	IRGC 51605-1

Sequence analysis

The retrieved *ABRE-BP* promoter sequences of 47 Sri Lankan varieties and two reference varieties, were aligned using ClustalW (cost matrix: IUB, gap open cost: 15 and gap extend cost: 6.66) in Geneious v7.1.3 (Biomatters Ltd., New Zealand) with manual editing. The nucleotide polymorphisms of the promoter region were analyzed using DnaSP v5.10.1 (University of Bacelona, Spain). The *cis*-elements present in the promoter of the *ABRE-BP* were identified and was annotated using the PLACE database (https://sogo.dna.affrc.go.jp; Higo *et al.*, 1999). A cluster analysis (complete linkage method and euclidean distance) was carried out considering the single nucleotide polymorphisms (SNPs) present within the identified *cis*-elements, using Minitab v15 (Minitab Inc., USA).

RESULTS

The retrieved 800 bp promoter regions of the *ABRE-BP* sequences, revealed six INDELs at the positions, 787 bp (INDEL 1), 757 bp (INDEL 2), 696 bp (INDEL 3), 695 bp (INDEL 4), 572 bp (INDEL 5) and 336 bp (INDEL 6) upstream of the TSS (Fig. 1). The INDEL 1 with a 25 bp region was the longest INDEL. The varieties *Puttunellu* and cultivar 93-11 lacked sequence at INDEL1. The sequence spanning INDEL 2, INDEL 3, and INDEL 5 was rare among the selected varieties (of the varieties, 93% (INDEL 2), 73% (INDEL 3), and 36% (INDEL 5), did not consist sequences spanning the given INDELs). Further, the sequences spanning INDEL1, INDEL4 and INDEL6 were often present. Out of the varietal panel 4% did not consist sequences spanning over the INDEL 1, 4% spanning the over INDEL 4, and 16% spanning over the INDEL 6, respectively.

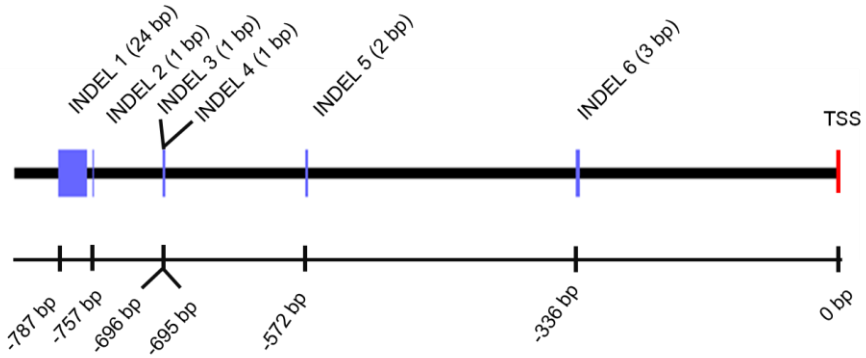

Fig. 1. Illustration of the size and the positions of six insertions/deletions (INDEL) starting from the transcription start site (TSS) in the promoter region of abscisic acid responsive element binding protein

The *in silico* analysis revealed presence of 57 SNP sites among the 49 varieties (data not shown). The frequency of occurrence of all SNP sites in the considered region was 7.4%. Out of that, 6.6% (51 SNPs) were parsimony informative sites and 0.8% (six SNPs) were singleton variables. Out of the 51 parsimony informative sites, 42 sites lead to transition mutations and nine to transversion mutations. From the six singletons, five lead to transition mutations and one to a transversion mutation.

In the 800 bp region of the *ABRE-BP* promoter considered in the current study, the detected SNPs created and deleted *cis*-elements. Considering all 49 varieties, a total of 14 abiotic stresses related *cis*-elements were identified (Table 2; Fig. 2). Three of the *cis*-elements ACGTATERD1 (involved in expression of early response to dehydration), DPBFCOREDCDC3 (bZIP transcription factor binding sequence present in ABA inducible genes) and MYCCONSENSUSAT (present in dehydration responsive genes) were conserved across all 49 varieties. Based on the remaining 11 *cis*-acting elements related to abiotic stress responses that showed SNP variations, the 49 varieties were clustered in to nine groups at a similarity percentage cut -off of 68 % (Fig. 3).

Table 2. Sequences of abiotic stress responsive *cis*-acting elements present in an 800 bp upstream region of the abscisic acid responsive element binding protein (Source: PLACE database)

***C*is-acting element**	**Sequence (5' – 3')**	**Remarks**
ASF1MOTIFCAMV	TGACG	Biotic and abiotic stress differentially stimulate the activation sequence-1 element (AS-1) (Després *et al.,* 2003)
MYCCONSENSUSAT	CATATG	An MYC recognition site found in the promoters of the dehydration responsive gene – *rd22* and many other genes in *Arabidopsis* (Abe *et al.,* 1997)
ACGTATERD1	ACGT	Required for the etiolation–induced expression of *erd1* (early responsive to dehydration) in *Arabidopsis* (Simpson *et al.,* 2003)
DRE2COREZMRAB17	ACCGAC	DRE2 is found in ABA and drought inducible genes (Kizis and Pages, 2002)
DRECRTCOREAT	ACCGAC	A dehydration responsive *cis*-acting element (Yamaguchi-Shinozaki and Shinozaki, 1994; Dubouzet *et al.,* 2003)
LTRECOREATCOR	CCGAC	A low temperature stress responsive element which related to ABA stress response pathway. It contains the same core sequence as DRE (Basu *et al.,* 2014)
CBFHV	CCGAC	A C-repeat binding factor. Dehydration responsive element (Nakashima *et al.,* 2009)
MYBCORE	CCGGTTA	Involved in water stress responses (Urao *et al.,* 1993)
GT1GMSCAM4	GAAAAA	Salinity stress responsive element (Park *et al.,* 2004)
ACGTATERD1	ACGT	Element involved in the expression of early response to dehydration (Simpson *et al.,* 2003)
DPBFCOREDCDC3	ACACCTG	bZIP transcription factor binding sequence present in ABA inducing genes (Kim *et al.,* 1997)
MYCCONSENSUSAT	CACCTG	An MYC recognition site present in dehydration responsive genes (Abe *et al.,* 1997)

Among the analyzed sequences there were two entries from the variety *Hondarawalu* (accessions IRGC 67631 and IRGC 47372). Accession level differences involving INDELs and SNPs were detected in the promoter region of *Hondarawalu* including, four INDELs and 14 SNPs. The variations detected among the *Hondarawalu* accessions indicated heterogeneity of the germplasm.

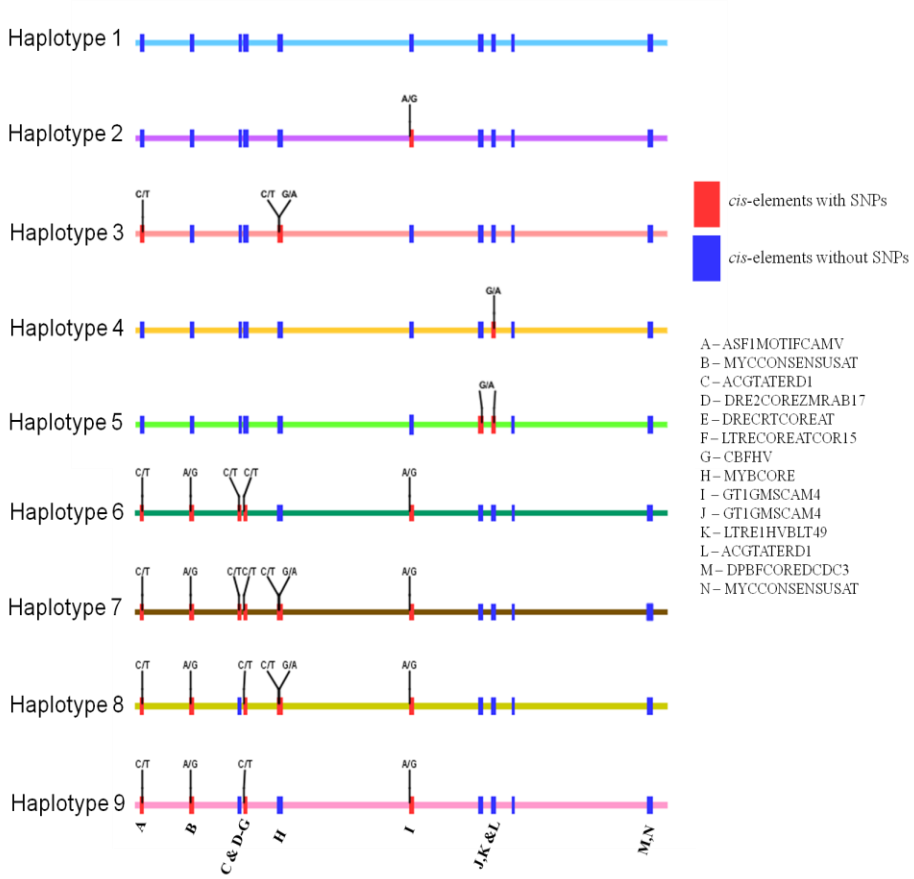

Fig. 2. Illustration of haplotypes based on abiotic stress related *cis*-elements and their single nucleotide polymorphisms in the promoter region of the abscisic acid responsive element binding protein among the selected Sri Lankan rice varieties

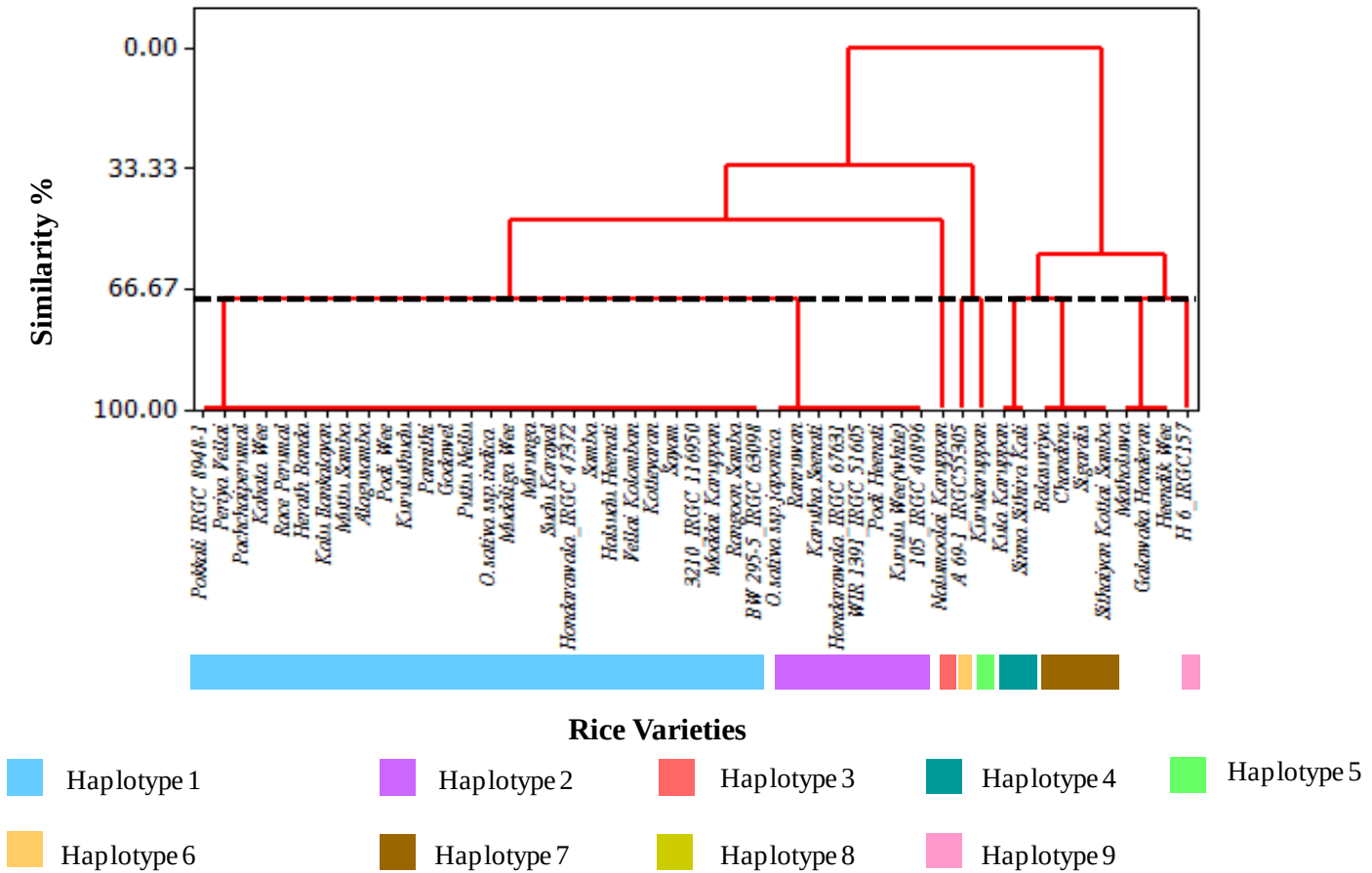

Fig. 3. Cluster analysis of selected 49 rice varieties based on the single nucleotide polymorphisms in *cis*-elements of abscisic acid responsive element binding protein. Colored bars indicate the haplotype groups based on the clustering at a 68% similarity

The tolerant variety *Pokkali* carried nine abiotic stress response related *cis*-elements and did not carry the stress responsive elements GT1GMSCAM4 (two copies found in other varieties) and the element LTRE1HVBLT49D found in other varieties. Further, five of the identified abiotic-stress related elements in *Pokkali* (ACGTATERD1, DRE2COREZMRAB17, DRECRTCOREAT, LTRECOREATCOR15 and MYBCORE) were deleted in some other varieties.

Based on the current salinity ratings surveyed through available literature, the varieties *Pokkali, Kuruluthuda, Kahata wee,* and *Kotteyaran* has been reported as tolerant (de Costa *et al.,* 2012; Pradheeban *et al.,* 2014) and *Nipponbare* and *Hondarawalu* as susceptible (de Costa *et al*., 2012). The rating of *Pachchaperumal* remained ambiguous as de Costa *et al*., (2012) reported it as susceptible and Pradheeban *et al.,* (2014) as tolerent at seedling stage. To our knowledge the remaining varieties were not evaluated for salinity responses.

DISCUSSION

Osmotic stress responses in plants controlled by different regulons involves a cascade of genes that get up-regulated and down-regulated based on environmental cues (Nakashima *et al*., 2009). The stress responses can get delivered *via* an ABA dependent or independent signal transduction pathway. These pathways involve specific *cis*-elements such as: dehydration responsive element (DRE), C- repeat (CRT), and ABRE that are responsible for regulating the expression of osmotic stress responsive genes. The DRE and CRT are known to be involved in both cold and drought stresses, while ABRE is mainly engaged with salinity and drought stress (Shinozaki and Yamaguchi-Shinozaki, 2000). Phytohormone ABA produced under osmotic stress is recognized by ABA receptors in the cell and the signaling components induce the ABA-dependent gene *ABRE-BP* (Sairam and Tyagi, 2004). The *ABRE-BP* codes for a bZIP family transcription factor which binds to the ABRE elements in the promoter of stress responsive genes up-regulating their expression (Chaves *et al.,* 2003; Tuteja, 2007; Nakashima *et al*., 2014). The *ABRE-BP* acts as an early responsive gene in ABA dependent stress responsive regulon (Sairam and Tyagi, 2004) and thus, the *ABRE-BP* acts as a master regulator in ABA dependent stress response. The *cis*-elements present, their relative distance and DNA polymorphisms involved in the promoter region could alter the expression of a gene (Dubouzet *et al.,* 2003). Thus, such variations in the *ABRE-BP* could alter its expression and affect the expression of the down-stream genes that are under its regulation. Hence, in the current study the diversity of *cis*-elements of *ABRE-BP* were evaluated.

The analysis of the *ABRE-BP* promoter sequences of 49 rice varieties retrieved from public databases showed several INDELs and SNPs. The sequence over the major INDEL (INDEL1- 25 bp) was found to be absent only in two varieties, *Puttu Nellu* and cultivar 93-11 (*indica* reference). However, the salinity ratings of *Puttu Nellu* and cultivar 93-11 are not known. Among the varieties that have a sequence over the major INDEL, varieties such as *Pokkali, Kuruluthudu, Kahata Wee* and *Kotteyaran* were already reported as salinity tolerant and *Hondarawalu* as a susceptible variety (de Costa *et al.,* 2012). Thus, the INDEL1 did not show a direct association to the currently reported salinity tolerance ratings. Similarly, none of the other INDELs and SNPs identified in the current study showed strong associations with available salinity ratings. Salinity is not under the control of a single gene; rather, it is controlled by several quantitative trait loci (QTL; *Saltol* (Mohammadi-Nejad *et al*., 2008; Thomson *et al*., 2010) and *qDW1.1* in chromosome 1, *qDW2.1* and *qDW2.2* in chromosome

2, *qDW6.1* in chromosome 6 (Bimpong *et al*., 2014), *qSNC3* in chromosome 3, *qSNC9* in chromosome 9 and *qSNC11* in chromosome 11 (Wang *et al*., 2012) and *rab-16A* (Mundy *et al.,* 1990)). Thus, the overall salinity response is a cumulative effect of the responses coming from many QTL/genes. However, only the genes carrying the ABRE *cis-e*lements are under the regulation of *ABRE-BP*. Only the promoter region of the gene *rab16A* has been characterized to carry ABRE elements (Mundy *et al.,* 1990). It is not known if the other QTL/genes responsible for salinity tolerance are carrying ABRE *cis*-elements or not.

Among various regulatory elements found in a promoter region, *cis*-elements play a key role in regulating gene transcription. The *cis*-elements get regulated by the status of various cellular responses, including signal molecules related to abiotic stresses (Chaves *et al.,* 2003). The six INDEL sites detected in the current study did not fall over any *cis*-elements that were previously known to be associated with abiotic stress tolerance. However, an INDEL can cause a change in the relative positions of the adjacent *cis*-acting elements and thus could have an effect on the overall expression rate of the gene. Hence, it is recommended that an expression analysis be carried out for *ABRE-BP* of different varieties to see if these INDELs have an effect on the relative expression of the gene. Out of the identified 11 abiotic stress responsive *cis*-elements, the elements MYBCORE (CNGGTTA) and GT1GMSCAM4 (GAAAAA) are known to be related to salinity stress responses. These *cis*-elements are present in the C3HC4-type RING finger gene family, which plays a key role in growth and development, and abiotic stress responses (Ma *et al.,* 2009). The c*is*-element MYBCORE is also known to be involved in stress response regulation in rice plants under dehydration, salinity, drought and low temperature stress (Ma *et al.,* 2009). Further, the c*is*-element GT1GMSCAM4 is also known to be related to salinity stress and biotic stress tolerance (Park *et al.,* 2004; Ma *et al.,* 2009; Trivedi *et al*., 2013). In stress responsive genes a GT-1 like transcription factor binds to the *cis*-element GT1GMSCAM4 and regulates responses under salinity stress (Park *et al.,* 2004). Previous studies indicates the presence of GT1GMSCAM4 in most of the highly expressing genes in rice sperm cells (Sharma *et al*., 2011). The GT1GMSCAM4 element is found in known susceptible varieties such as Nipponbare and *Honderawala* and several other varieties with unknown salinity ratings, but not in known salinity tolerant varieties such as *Pokkali* (IRGC 8948-1), *Kuruluthudu* (IRGC 36304-1), *Kahata Wee* (IRGC 12004-1), *Kotteyaran* (IRGC 47383-1) and in several varieties with unknown salinity responses. Similarly a direct association could not be established with respect to the c*is*-element MYBCORE, due to lack of information related to salinity stress responses.

Based on the abiotic stress related *cis*-elements carrying SNP sites (11 sites; Fig. 2), the studied rice varieties were clustered into nine clusters (Fig. 3). The salinity tolerant variety *Pokkali* was clustered into haplotype 1 with tolerant varieties *Kuruluthudu, Kahata Wee, Kotteyaran* and moderately salinity tolerant variety *Samba* (de Costa *et al.,* 2012; Pradheeban *et al.,* 2014). However, within the same haplotype were the salinity susceptible variety *Honderawala* (de Costa *et al.,* 2012) , *Pachchaperumal* with an ambiguous salinity rank (de Costa *et al.,* 2012; Pradheeban *et al.,* 2014) and 20 other varieties of which the tolerance level is unknown. Thus, the SNPs in these elements are not strongly associated with salinity responses of these varieties.

Even though this *in silico* study revealed the nucleotide diversity of the *cis*-elements present in *ABRE-BP* promoter region, the association of the detected polymorphisms to salinity tolerance ratings was not possible as the salinity responses of many of the tested varieties were not available. Therefore, it is recommended that a systematic phenotypic study be

carried out to clearly identify the salinity rank of the used accessions at different life stages. The current study is an initial step to identify the nucleotide diversity of the *cis*-elements present in *ABRE-BP* among a panel of Sri Lankan rice varieties, and based on the already available salinity ratings preliminary inferences were made on the associations of the DNA polymorphisms detected in the promoter region of *ABRE-BP* with emphasis on salinity stress response related *cis*-elements.

CONCLUSIONS

The nucleotide diversity analysis of the *ABRE-BP* promoter region revealed DNA polymorphisms among the studied rice varieties, and some of these polymorphisms were found within the *cis*-elements. A total of 11*cis*-elements related to abiotic stress responses were detected and the identified SNPs deleted existed *cis*-elements or generated new *cis*-elements. No INDELs fell on the *cis*-elements. The groups created based on a cluster analysis of these SNPs indicated that the SNPs in *cis*-elements of the *ABRE-BP* may not be a strong regulator of salinity tolerance. However, the INDELs changed the relative distance between *cis*-elements and their position from the TSS, imposing possible effects on the expression of the *ABRE-BP* that regulate stress responsive genes enriched with ABRE element(s).

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