

# Haplotype distribution and association of candidate genes with salt tolerance in Indian wild rice germplasm

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## Abstract

**Key message** The association of natural genetic variations of salt-responsive candidate genes belonging to different gene families with salt-tolerance phenotype and their haplotype variation in different geographic regions.

**Abstract** Soil salinity covers a large part of the arable land of the world and is a major factor for yield losses in salt-sensitive crops, such as rice. Different gene families that respond to salinity have been identified in rice, but limited success has been achieved in developing salt-tolerant cultivars. Therefore, 21 salt stress-responsive candidate genes belonging to different gene families were re-sequenced to analyse their genetic variation and association with salt tolerance. The average single nucleotide polymorphism (SNP) density was 16 SNPs per kbp amongst these genes. The identified nucleotide and haplotype diversity showed comparatively higher genetic variation in the transporter family genes. Linkage disequilibrium (LD) analysis showed significant associations of SNPs in *BADH2*, *HsfC1B*, *MIPS1*, *MIPS2*, *MYB2*, *NHX1*, *NHX2*, *NHX3*, *P5CS1*, *P5CS2*, *PIP1*, *SIK1*, *SOS1*, and *SOS2* genes with

the salt-tolerant phenotype. A combined analysis of SNPs in the 21 candidate genes and eight other HKT transporter genes produced two separate clusters of tolerant genotypes, carrying unique SNPs in the ion transporter and osmoticum-related genes. Haplotype network analysis showed all the major and few minor alleles distributed over distant geographic regions. Minor haplotypes may be recently evolved alleles which migrated to distant geographic regions and may represent recent expansion of Indian wild rice. The analysis of genetic variation in different gene families identified the relationship between adaptive variations and functional significance of the genes. Introgression of the identified alleles from wild relatives may enhance the salt tolerance and consequently rice production in the salinity-affected areas.

**Keywords** Wild rice · Salt stress · Association · Haplotype

## Introduction

Globally, about thirty percent of the irrigated agricultural land is affected by salinity (<http://www.ars.usda.gov/Main/docs.htm?docid=10201&page=1>). Human population is projected to be 9.2 billion by the year 2050, and hence, the agricultural production must be doubled to ensure adequate food supply (FAO, UN 2009). Rice is one of the most important food crops, which fulfils approximately 20 % of the global food requirement, and hence, it could act as a model crop for global food security (Maclean et al. 2013). However, during the last decade, the growth in rice productivity has reached a plateau, possibly due to intensive agriculture practices, changes in climatic conditions, and/or associated abiotic stresses (Mishra et al. 2016). The changing climate, frequent intermittent flooding, drought,

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and reduced freshwater availability have led to enhanced salt concentration in soil (Turrall et al. 2011). Continuous climatic fluctuations challenge the society to develop climate-resilient agricultural crops. Plants are sensitive to climatic variations and environmental stresses, which create an alarming situation for sustaining genetic diversity of crops and world food security (Mercer 2010). New adaptive mutations would be insufficient to endure such rapid climatic fluctuations. Hence, existing wild rice genetic resources, with well-documented adaptive variations in different genes, may have retained potential genes to withstand such stressed conditions (Shaw and Etterson 2012). Landraces are known to contain higher genetic diversity than the modern high-yielding varieties of rice; however, inadequate sources of salt tolerance in these require the exploration of a novel genetic variation present in the wild rice germplasm.

Natural populations of A-genome wild rice, the progenitors of cultivated rice, are a rich source of genetic diversity. Hence, in this study, we analysed Indian wild rice accessions collected from different regions of India including the stress hotspots for sequence variation in salt-tolerance candidate genes and phenotype. Different stress tolerance genes have already been exploited from the wild rice germplasm, including the male sterility gene found in *Oryza rufipogon* (Yuan et al. 1993), *Bph 19(t)* gene from *O. rufipogon* (Li et al. 2010), and the grassy stunt virus-resistance gene from *Oryza nivara* (Khush and Ling 1974). For salinity-stress tolerance, inositol methyl transferase was identified and L-myoinositol 1-phosphate synthase was introgressed from the wild rice *Oryza coarctata* into *Oryza sativa* Indica cultivar (Das-Chatterjee et al. 2006; Sengupta et al. 2008). The beneficial alleles derived from wild rice have been transferred into elite genetic backgrounds, which have led to enhanced yield and trait performance of rice varieties. Identification of such allelic variants and exploiting candidate gene diversity from wild rice is in its nascent stage. In this context, a genome-wide survey of candidate genes responding to stress may spur on the case for identifying adaptive variations, conducting evolutionary studies, and ultimately result in crop improvement. The present molecular breeding strategies for the improvement of cultivars depend upon the availability of tightly-linked markers, or functional genic markers based on the sequence variation in the causal gene for the agronomic traits, or both (Xu et al. 2012).

Salinity imposes both osmotic and ionic stresses, and results in the induction of a network of complex physiological responses, which is regulated by a plethora of genes (Kumar et al. 2013). The effect of osmotic stress is alleviated by the production of cellular osmolytes (proline, glycine betaine, manitols, etc.), whose synthesis

is regulated by the genes of metabolic pathways (Singla-Pareek et al. 2008). Ion transporters are another group of genes, pivotal for ion homeostasis of the system, which is further controlled by signalling genes and transcription factors (Serrano and Rodriguez-Navarro 2001; Zhu 2003). Many significant genes of essential biochemical pathways responding to salt stress were selected for the present study. The regulatory genes of the osmolyte biosynthesis pathways are key to their production, such as *P5CS1* (Jacobs et al. 2003) and *P5CS2* (Hur et al. 2004) for proline, *BADH1*, *BADH2*, *CMO* for glycine betaine (Nakamura et al. 1997), and *MIPS* for myoinositol (Das-Chatterjee et al. 2006). Among the transporter gene families, *HKT* (Ren et al. 2005; Horie et al. 2007), *NHX* (Fukuda et al. 2011), *SOS1* (Martinez-Atienza et al. 2007), and *PIP1;1* (Liu et al. 2013) genes were selected for the study. Important genes of the signalling components regulating ionic homeostasis, namely, *SIK1* (Ouyang et al. 2010), *SOS2*, and *SOS3* (Martinez-Atienza et al. 2007) as well as transcription factors, such as *bZIP23* (Xiang et al. 2008), *bZIP71* (Liu et al. 2014), *HsfC1B* (Schmidt et al. 2012), and *MYB2* (Yang et al. 2012) were also analysed in the study. The extent of vulnerability to salinity and other abiotic stresses depend upon the specific growth stage of the crop, wherein the flowering stage is the most susceptible, as it leads to greater yield losses (Ghomi et al. 2013; Singh et al. 2015a). The identification of functional allelic variants of these candidate genes will contribute towards designing climate-resilient rice varieties that are salt tolerant.

In the present study, 21 genes responding to salinity stress and belonging to different functional categories, such as osmolytes, transporters, and transcription factors, were selected for allele mining. The genes were PCR amplified, re-sequenced, and analysed in the representative sample, comprising 103 accessions of wild and cultivated rice.

## Results

### Variation for salt tolerance among the wild rice germplasm

A set of 299 wild rice accessions were phenotyped for seedling stage salinity tolerance. Tolerance was measured as standard evaluation score (SES) and four tolerance-associated traits, namely, relative water content (RWC), chlorophyll content, total biomass, and sodium and potassium ion concentrations. From the overall screening, we found two accessions highly tolerant, 14 tolerant, 28 moderately tolerant, 69 sensitive, and 186 highly sensitive to salt stress (Mishra et al. 2016).

### Sequence diversity in salt stress-responsive candidate genes

Twenty-one salt-tolerance candidate genes belonging to different functional categories were PCR amplified and re-sequenced from 103 selected accessions of wild rice showing varying levels of salt tolerance. These included regulatory genes of major osmolyte synthesis pathways, transporter gene families, transcription factors, signalling components regulating ions, and anti-oxidative stress-responsive genes. Sequencing covered entire genes, including promoter, coding and noncoding regions, and 5' and 3' UTR. A total of 125.5 kb sequenced region covering all the 21 genes identified 2043 SNPs with an average density of 16 SNPs per kb. Among the osmotic-stress-regulating genes, *MIPS1* had the lowest eight segregating sites (S) and 0.00024 nucleotide diversity ( $\pi$ ), while *BADH1* had the highest 50 segregating sites and *BADH2* had the highest nucleotide diversity 0.0004 (Table 1). The number of haplotypes ranged from 7 in *MIPS1* to 27 in *BADH1*, and haplotype diversity ranged from 0.2 in *MIPS1* to 0.736 in *P5CS2*. The signalling genes regulating ion concentration, such as *SIK1*, *SOS2*, and *SOS3* had 13, 18, and 11 haplotypes and 0.481, 0.44, and 0.405 haplotype diversity, respectively. Among the transporter family genes sequenced, the highest nucleotide diversity of 0.00212 was observed for the *NHX2*, while the largest number of 42 haplotypes was for *NHX2* followed by 25 for *SOS1* gene. Maximum haplotype diversity of 0.888 was found for *NHX2* gene, while the lowest value was 0.146 for *NHX4* gene. Among the four transcription factor genes involved in the regulation of different pathways, *MYB2* was absolutely conserved with no segregating sites in 103 wild rice genotypes, while *bZIP71* and *hsfC1B* were monomorphic in the coding region. The observed number of haplotypes and haplotype diversity for *bZIP23*, *bZIP71*, and *hsfC1B* genes were 5, 5, and 7, and 0.182, 0.00158, and 0.217, respectively (Table 1).

### Association of SNPs in the candidate gene with salt-tolerance phenotype

LD-based association analysis showed a variable number of SNPs in 14 of the 21 genes having significant associations with the SES score for salt tolerance in the wild rice accessions (Table 2). Overall, the highest number of 14 SNPs in *PIP1* gene showed association with the salt-tolerance phenotype. Among seven genes belonging to osmolyte biosynthesis pathways, one SNP each in *MIPS1*, *MIPS2*, and *P5CS1* and two SNPs each in *BADH2* and *P5CS2* gene showed significant association with the SES score. Among the signalling pathway genes, five SNPs in *SIK1* and two SNPs in *SOS2*; while among transporter

family genes, one SNP each in *SOS1*, *NHX1*, *NHX3*, nine SNPs in *NHX2* and 14 SNPs in *PIP1* genes were significantly associated with the SES score. Out of four transcription factor genes studied, one SNP in *hsfC1B* and two SNPs in *MYB2* gene were also associated with the SES score. While most of the associated SNPs were found in the 5' upstream, and 3' downstream noncoding or intronic regions, *SIK1* and *MYB2* genes had SNPs from the exonic region associated with the salt-tolerance phenotype (Table 2).

### Identification of salt-tolerant groups of genotypes based on 29 candidate genes

Apart from these 21 candidate genes for salt tolerance, sequence data for eight *HKT* transporter genes (Mishra et al. 2016) were also used to prepare a concatenation of all the 29 genes for the grouping of wild rice genotypes. To exclude rare alleles, a filter was set at a minimum count of six genotypes per SNP site. A neighbour joining (NJ)-based dendrogram was generated using SNPs from the promoter, coding, and noncoding regions of all the 29 genes (Fig. 1). This grouped the genotypes into three clusters, i.e., cluster-I, II, and III. Cluster-I had only one wild rice accession NKSZR238, and cluster-II consisted of 12 wild rice accessions. Cluster-III was the major cluster and was further divided into two sub-clusters, such as clusters-IIIA and IIIB. Cluster-IIIA had only wild rice accessions which was further subdivided into two minor groups. The cluster analysis revealed a random distribution of tolerant accessions among different groups, except for two clusters of four and six accessions which were all of tolerant type. This suggested that certain combinations of alleles of these candidate genes are associated with the salt-tolerance phenotype. From the two minor clusters, sub-cluster-IIIA-1-2 has only tolerant wild rice accessions. In addition, sub-cluster-IIIB-3 also grouped only tolerant accessions. The remaining tolerant accessions were randomly dispersed across different sub-clusters. Seven cultivated rice varieties of *O. sativa*, including both salt tolerant and sensitive cultivars, were grouped together in a separate sub-cluster-IIIB-5. To identify the differentiating SNPs and corresponding gene(s), genomic sequences of each tolerant cluster were separately aligned with five each of tolerant and susceptible accessions from neighbouring clusters (Table 3). For cluster-IIIA-1-2, SNPs differentiating the tolerant and sensitive accessions were found in *HKT2;3*, *NHX1*, *SOS1*, *BADH1*, and *P5CS2* genes. Similarly, for cluster-IIIB-3, differentiating SNPs were found in *HKT1;3*, *P5CS2*, and *BADH1* genes. SNP annotation from individual tolerant sub-clusters showed one SNP each of *BADH1* and *NHX1*, four SNPs each of *HKT2;3* and *P5CS2*, and 14 SNPs of *SOS1* in cluster-IIIA-1-2 and additionally one SNP

**Table 1** Nucleotide and haplotype diversity and tests of neutrality for functionally different class 21 salt stress-related genes

Gene name	Region	S	Pi	Theta $w$	$D$	$D^*$	$F^*$	H	HD
<i>BADH1</i>	Coding	4	0.00012	0.00059	-1.58076	-1.48067	-1.77986	5	0.095
	Noncoding	46	0.00048	0.00188	-2.34498	-3.52147	-3.65153	26	0.564
	All	50	0.0004	0.00157	-2.37276	-3.56838	-3.69553	27	0.564
<i>BADH2</i>	Coding	1	0.00012	0.00122	-1.02456	-2.03876	-2.02071	2	0.019
	Noncoding	8	0.00157	0.00429	-1.53287	-0.36347	-0.90502	7	0.391
	All	9	0.00113	0.00335	-1.6501	-0.94646	-1.41199	8	0.406
<i>CMO</i>	Coding	2	0.00031	0.0006	-0.73471	0.68311	0.28689	3	0.196
	Noncoding	14	0.00041	0.00152	-1.98486	-1.30324	-1.84738	7	0.235
	All	16	0.00038	0.00127	-1.94489	-0.99043	-1.60015	8	0.36
<i>MIPS1</i>	Coding	2	0.00006	0.00039	-1.29845	-1.07813	-1.34084	3	0.057
	Noncoding	35	0.00038	0.00179	-2.43042	-4.23509	-4.21886	17	0.367
	All	8	0.00024	0.00068	-1.55022	-1.98405	-2.17491	7	0.2
<i>MIPS2</i>	Coding	3	0.00007	0.00042	-1.48762	-2.00936	-2.16438	4	0.095
	Noncoding	35	0.00038	0.00179	-2.43042	-4.23509	-4.21886	17	0.367
	All	38	0.00029	0.00142	-2.45914	-4.37198	-4.32638	19	0.412
<i>P5CS1</i>	Coding	2	0.00005	0.00022	-1.15145	-1.07813	-1.28773	3	0.094
	Noncoding	40	0.00064	0.00247	-2.30248	-5.17175	-4.82236	20	0.64
	All	42	0.00043	0.00165	-2.3137	-5.13595	-4.79734	21	0.651
<i>P5CS2</i>	Coding	9	0.00028	0.001	-1.79187	-1.70083	-2.05523	9	0.345
	Noncoding	28	0.00065	0.00168	-1.83882	-0.90548	-1.51875	16	0.652
	All	37	0.00052	0.00144	-1.97325	-1.37759	-1.93615	19	0.736
<i>SIK1</i>	Coding	5	0.00005	0.00039	-1.86262	-3.23234	-3.28129	5	0.095
	Noncoding	20	0.00025	0.00087	-2.06598	-0.95106	-1.63773	11	0.453
	All	25	0.00017	0.0007	-2.22413	-1.9716	-2.47587	13	0.481
<i>SOS2</i>	Coding	3	0.00005	0.00053	-1.60439	-3.42618	-3.34496	4	0.058
	Noncoding	21	0.00037	0.00147	-2.19218	-3.87596	-3.86839	17	0.425
	All	24	0.00029	0.00122	-2.27815	-4.44799	-4.32219	18	0.44
<i>SOS3</i>	Coding	2	0.0001	0.00065	-1.29845	-1.07813	-1.34084	3	0.057
	Noncoding	12	0.00049	0.00148	-1.76168	-2.94468	-3.0017	9	0.36
	All	14	0.00038	0.00125	-1.88536	-3.01428	-3.10424	11	0.405
<i>SOS1</i>	Coding	15	0.00017	0.00083	-2.22377	-3.60076	-3.69196	11	0.332
	Noncoding	55	0.0002	0.00103	-2.56582	-4.86016	-4.70144	20	0.426
	All	70	0.00019	0.00097	-2.59796	-5.08939	-4.86017	25	0.524
<i>NHX1</i>	Coding	0	0	0	0	0	0	1	0
	Noncoding	11	0.00019	0.00063	-1.82078	-2.57511	-2.74439	10	0.316
	All	11	0.00013	0.00044	-1.82078	-2.57511	-2.74439	10	0.316
<i>NHX2</i>	Coding	7	0.00031	0.00132	-1.77694	-0.56381	-1.15222	5	0.165
	Noncoding	26	0.00384	0.00483	-0.61337	0.08543	-0.22178	40	0.887
	All	33	0.00212	0.00311	-0.98025	-0.12381	0.5544	42	0.888
<i>NHX3</i>	Coding	0	0	0	0	0	0	1	0
	Noncoding	32	0.00036	0.00131	-2.21475	-2.16709	-2.61926	21	0.628
	All	32	0.00027	0.001	-2.21475	-2.16709	-2.61926	21	0.628
<i>NHX4</i>	Coding	3	0.00037	0.00365	-1.60439	-3.42618	-3.34496	2	0.019
	Noncoding	5	0.00142	0.00662	-1.66519	-3.23234	-3.20494	3	0.146
	All	8	0.00087	0.00504	-2.00028	-4.41492	-4.25174	3	0.146
<i>NHX5</i>	Coding	4	0.00006	0.00063	-1.77339	-3.89949	-3.78141	5	0.077
	Noncoding	22	0.00033	0.00094	-1.90709	-3.24287	-3.27157	13	0.457
	All	26	0.00027	0.00087	-2.06039	-4.07601	-3.94815	15	0.485
<i>PIP1;1</i>	Coding	0	0	0	0	0	0	1	0
	Noncoding	17	0.00058	0.00233	-2.11859	-3.35957	-3.46217	10	0.347
	All	17	0.00041	0.00167	-2.11859	-3.35957	-3.46217	10	0.347

**Table 1** continued

Gene name	Region	S	Pi	Theta $w$	$D$	$D^*$	$F^*$	H	HD
<i>bZIP23</i>	Noncoding	8	0.00023	0	0	0	0	5	0.182
<i>bZIP71</i>	Coding	0	0	0	0	0	0	1	0
	Noncoding	6	0.00007	0.00057	−1.97331	−3.68494	−3.67914	5	0.095
	All	6	0.00006	0.00047	−1.97331	−3.68494	−3.67914	5	0.095
<i>hsfC1b</i>	Coding	0	0	0	0	0	0	1	0
	Noncoding	5	0.00036	0.00132	−1.53655	−1.09903	−1.46503	7	0.217
	All	5	0.00034	0.00124	−1.53665	−1.09903	−1.46503	7	0.217
<i>MYB2</i>	Coding	0	0	0	0	0	0	1	0
	Noncoding	0	0	0	0	0	0	1	0
	All	0	0	0	0	0	0	1	0

$S$  segregating sites,  $\pi$  nucleotide diversity,  $\theta_w$  nucleotide diversity with Watterson’s estimator,  $D$  Tajima’s  $D$  statistic,  $D^*$  Fu and Li’s  $D^*$  statistic,  $F^*$  Fu and Li’s  $F^*$  statistic,  $H$  number of haplotypes,  $HD$  haplotype diversity

**Table 2** Association of SNPs in selected salt stress response genes with standard evaluation score (SES) (at  $R^2 > 5\%$  and  $p < 0.01$ )

Gene	SNP	$p$ value	Marker $R^2$	Region	Gene	SNP	$p$ value	Marker $R^2$	Region
<i>BADH2</i>	S1278	0.00238	0.12738	5’UTR	<i>SIK1</i>	S220	0.00616	0.12674	Syn
	S1442	0.00424	0.11788	5’UTR		S6835	0.00653	0.10504	NC
<i>HsfC1b</i>	S2433	0.00287	0.12318	NC		S6863	0.00653	0.10504	NC
<i>MIPS1</i>	S4315	0.00424	0.13324	5’UTR		S6897	0.00653	0.10504	NC
<i>MIPS2</i>	S164	0.00695	0.1214	5’UTR		S7059	0.00665	0.10466	NC
<i>MYB2</i>	S288	0.00584	0.08661	NC	<i>PIP1;1</i>	S14	1.75E−04	0.18752	5’UTR
	S1033	0.00978	0.0989	Syn		S152	5.93E−04	0.15895	5’UTR
<i>NHX1</i>	S5432	0.00327	0.1222	5’UTR		S1111	4.63E−04	0.1647	Int
<i>NHX2</i>	S308	5.78E−04	0.1389	NC		S1121	4.63E−04	0.1647	Int
	S774	0.0011	0.11206	Int		S1479	0.00659	0.10474	Int
	S775	0.002	0.13124	Int		S2325	1.55E−04	0.19766	Int
	S776	0.0011	0.11206	Int		S2708	9.24E−04	0.15491	Int
	S777	0.0011	0.11206	Int		S2734	0.0017	0.14048	Int
	S778	0.00346	0.11898	Int		S2768	3.44E−05	0.23815	Int
	S1087	8.79E−04	0.1166	Int		S2770	7.78E−05	0.21728	Int
	S1088	6.99E−04	0.12134	Int		S2771	0.00946	0.10152	Int
	S1089	5.14E−04	0.12776	Int		S3153	1.91E−04	0.19387	Int
	<i>NHX3</i>	S6670	0.00391	0.11629	5’UTR		S3155	0.00213	0.13552
<i>P5CS1</i>	S4957	0.0039	0.11632	Int		S3276	0.00683	0.10694	Int
<i>P5CS2</i>	S15	0.00155	0.14502	NC	<i>SOS1</i>	S888	0.00485	0.11161	NC
	S33	0.00506	0.1139	NC		<i>SOS2</i>	S4429	0.0045	0.12134
						S4496	1.65E−04	0.18881	5’UTR

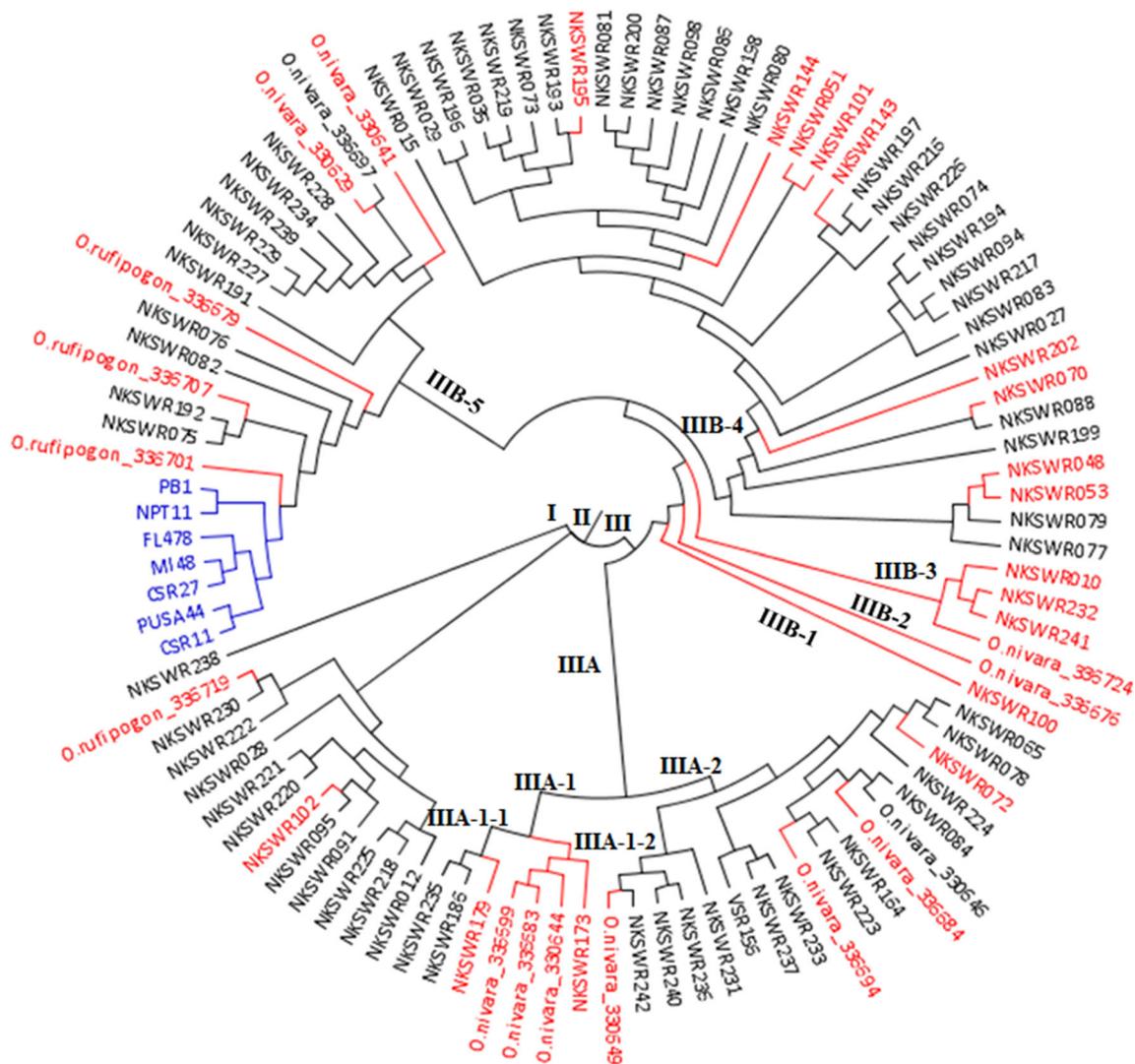
NC noncoding, Int intron, Syn synonymous, UTR untranslated region

each from *P5CS2* and *BADH1* and three SNPs from *HKT1;3* in cluster-IIIB-3 differentiating the clusters (Fig. 1; Table 3).

**Geographical distribution of the haplotypes of salt-responsive gene**

The set of 103 wild rice accessions belonging to Indo-Gangetic plains, Western Himalayas (Himachal Pradesh), and the West Coast (Gujrat and Maharashtra) were genetically structured into three sub-populations by genome-wide SNP markers (Mishra et al. 2016). All the genes had a

major haplotype present in a large number of accessions and a few minor haplotypes. These haplotypes were distributed randomly across three genetically structured populations (Fig. 2). Minor haplotypes with a maximum of seven accessions (five from the West Coast and two from lower Gangetic plain region) were found in 13 genes. Among them, *SIK1* and *P5CS2* further differentiated these accessions of Gujrat from those of Indo-Gangetic plains into two separate haplotypes. However, *SOS1*, *SOS2*, and *SOS3* genes differentiated these groups of accession into two haplotypic groups randomly but not by geography. Seven accessions in H4 haplotype of *CMO* gene with



**Fig. 1** Neighbour joining tree-based on SNPs of all the 29 genes. The genes were concatenated based on their position in the genome, aligned, and cluster was made using SNPs. Red lines indicate tolerant

wild rice lines and blue lines indicate *O. sativa* cultivars and black line indicate intolerant wild rice (colour figure online)

NKSWR186 in the group being an exception, and haplotypes H5 and H6 with three and eight accessions, respectively, of *SOS3* gene and haplotype H3 with five accessions of *PIPI1* gene belonged specifically to upper and middle Indo-Gangetic plains (Fig. 2). Cultivated rice varieties were grouped with major haplotype within most of the genes, or they were distributed across the minor haplotypes for some of the genes.

## Discussion

Salinity imposes substantial yield losses in rice, but to date, only a limited number of tolerance sources have been identified and that too with only partial success towards the development of salt-tolerant cultivars. To identify new

sources of salt tolerance, studies have been conducted on rice landraces, but greater genetic variations in wild rice germplasm remain unexplored. Therefore, in this study, we attempted to identify the genetic potential of Indian wild rice by analysing sequence variations in 21 salt stress-responsive candidate genes. It was found that the transporter genes had the highest average nucleotide variations per gene, followed by osmotic stress and signalling intermediates, while transcription factors had the lowest variations. This indicated that genetic variation has a significant relationship with adaptive variation and functional significance of the genes (Storz and Wheat 2010). However, number of haplotypes and haplotype diversity were broadly similar for all classes of genes except for transcription factors, which were significantly lower. This study also indicated differences between nucleotide and haplotype

**Table 3** Unique SNPs in the accessions belonging to tolerant clusters

Cluster	Gene	Region (change)	Position in gene	
Cluster-IIIB-3	HKT2;3	E (A/I)	1339	
		E (A/I)	1340	
		E (Syn)	1853	
		E (Syn)	2053	
	NHX1	5'UTR	259	
	SOS1	3'UTR	493	
		3'UTR	742	
		3'UTR	760	
		3'UTR	764	
		3'UTR	765	
		3'UTR	819	
		3'UTR	850	
		3'UTR	857	
	BADH1	I	2279	
		P5CS2	I	2349
	P5CS2	E (Syn)	2447	
I		2552		
I		4758		
CLUSTER-IIIA-1-2		SOS1	3'UTR	298
			3'UTR	554
	3'UTR		1034	
	3'UTR		1591	
HKT1;3	5'UTR	744		
	E (V/E)	1367		
	E (Syn)	2347		
BADH1	5'UTR	5925		
	P5CS2	5'UTR	7068	

*E* exonic, *Syn* synonymous SNP, *I* intron, *UTR* untranslated region

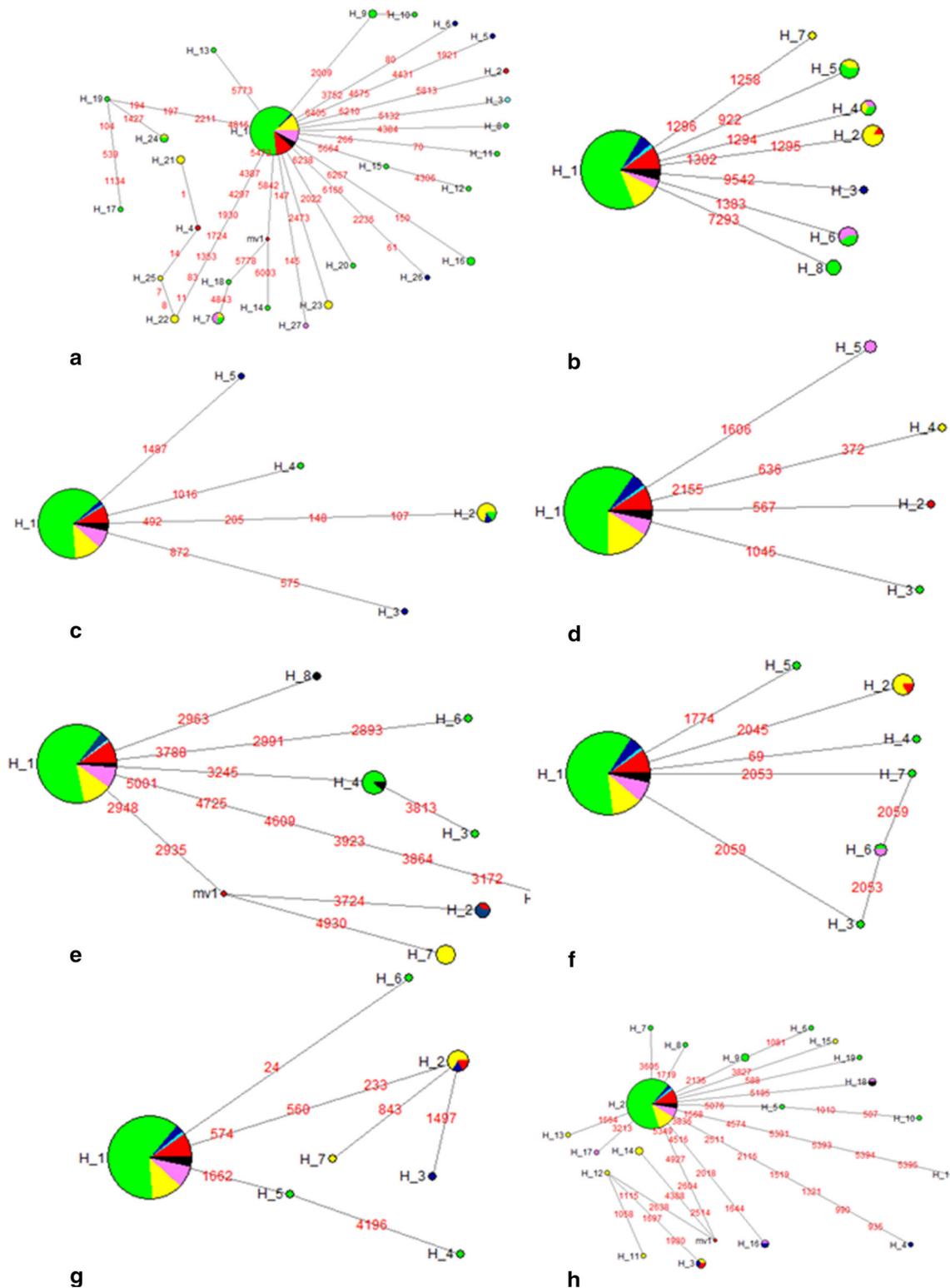
diversity as well as the nature of variation across different gene families. Differences between nucleotide and haplotype diversity have been measured across different genes in wild and cultivated rice, such as sucrose synthase 3 gene (Lestari et al. 2011), *OsDREB1F* gene (Singh et al. 2015b), *OsSNAC1* gene (Singh et al. 2015c), and across *HKT* gene family (Mishra et al. 2016).

Association analysis showed that while most of the trait-associated SNPs were present in the promoter regions, only a few were present in the exonic regions. Mutations in the regulatory region are usually responsible for altered gene expressions (Stern and Orgogozo 2008). SNPs in the promoter regions of different genes were analysed, and an associated W-box region of *WRKY* transcription factor binding site was identified in the *BADH2* gene. The role of *WRKY* transcription factors in both biotic and abiotic stresses tolerance has been reported (Eulgem et al. 2000). *BADH2* regulates the synthesis of betaine from choline,

which is an osmoprotectant that allows normal growth under salinity stress (Chen et al. 2008). Another associated gene *SOS2* although regulates ionic stress response, its role in regulating dehydration stress response has also been identified through transcriptional activation of abscisic acid signalling (Abe et al. 2003). An *MYB* transcription factor regulates transcriptional activation of dehydration responsive genes. Its recognition site has been identified in the promoter region of *SOS2*, and hence, it can be inferred that the dehydration response is also a part of the salt-tolerance mechanism. In response to dehydration, an interaction between *SOS2* protein and *ABI2* (abscisic acid-insensitive2), a protein phosphatase has been identified in *Arabidopsis*, showing cross talks with the ABA signalling pathway (Ohta et al. 2003). It further corroborates the role of regulatory networks, including plant hormone and different downstream gene families, in both ionic and osmotic homeostasis for salt tolerance in rice.

None of the haplotypes of analysed genes had only tolerant accessions suggesting that no single gene was potent enough to impart complete salt tolerance. As salt tolerance is governed by multiple genes, the SES score is a measure of cumulative action of all the genes/QTLs responding to salt stress. The previous studies conducted on haplotype-based association analysis have rarely shown association with SES score; however, this study did shows association with SES and other salt-related traits, such as  $\text{Na}^+$  concentration in shoot (Platten et al. 2013). Hence, an authentic differentiation of tolerant alleles of a gene could be done by a combination of two techniques: namely, a precise phenotypic measurement, and second, a molecular or genetic technique that is able to dissect the role of a particular gene in an interactive metabolic pathway governing salt tolerance. In this study, none of the haplotypes of the 21 genes had grouped all the tolerant accessions, which were distributed in different haplotype group. Negrão et al. (2013) also found similar results, where most of the tolerant accessions were not grouped into haplotypes.

Salt tolerance is governed by multiple gene families. Transporters are important regulators of ion homeostasis, while, osmolytes are equally important to maintain osmotic potential and the functional integrity of biomolecules. Transcription factors are the master regulators of gene expression. In this study, we have identified haplotypes for different gene families, and improved salt-tolerant varieties can be developed by assembling superior alleles of different classes of genes into a high-yielding genetic background. Kurokawa et al. (2016) have used haplotype information of 14 useful alleles for the construction of SNP array, which could be used in haplotype-based selection for pyramiding of important genes in rice. Transgenic tobacco plants, having a bicistronic transcriptional unit for



**Fig. 2** Haplotype network of 20 genes and their distribution in seven geographic locations. **a** *BADH1*, **b** *BADH2*, **c** *bZIP23*, **d** *bZIP71*, **e** *CMO*, **f** *hsfC1b*, **g** *MIPS1*, **h** *MIPS2*, **i** *NHX1*, **j** *NHX2*, **k** *NHX3*, **l** *NHX4*, **m** *NHX5*, **n** *P5CS1*, **o** *P5CS2*, **p** *PIP1*, **q** *SIK1*, **r** *SOS1*,

**s** *SOS2*, **t** *SOS3*. Each circle represents a haplotype, and the size of the circle is proportional to haplotype frequency. Colour coding represents geographic location of the accessions. The numbers on the branch indicate the position of mutation in the sequenced region

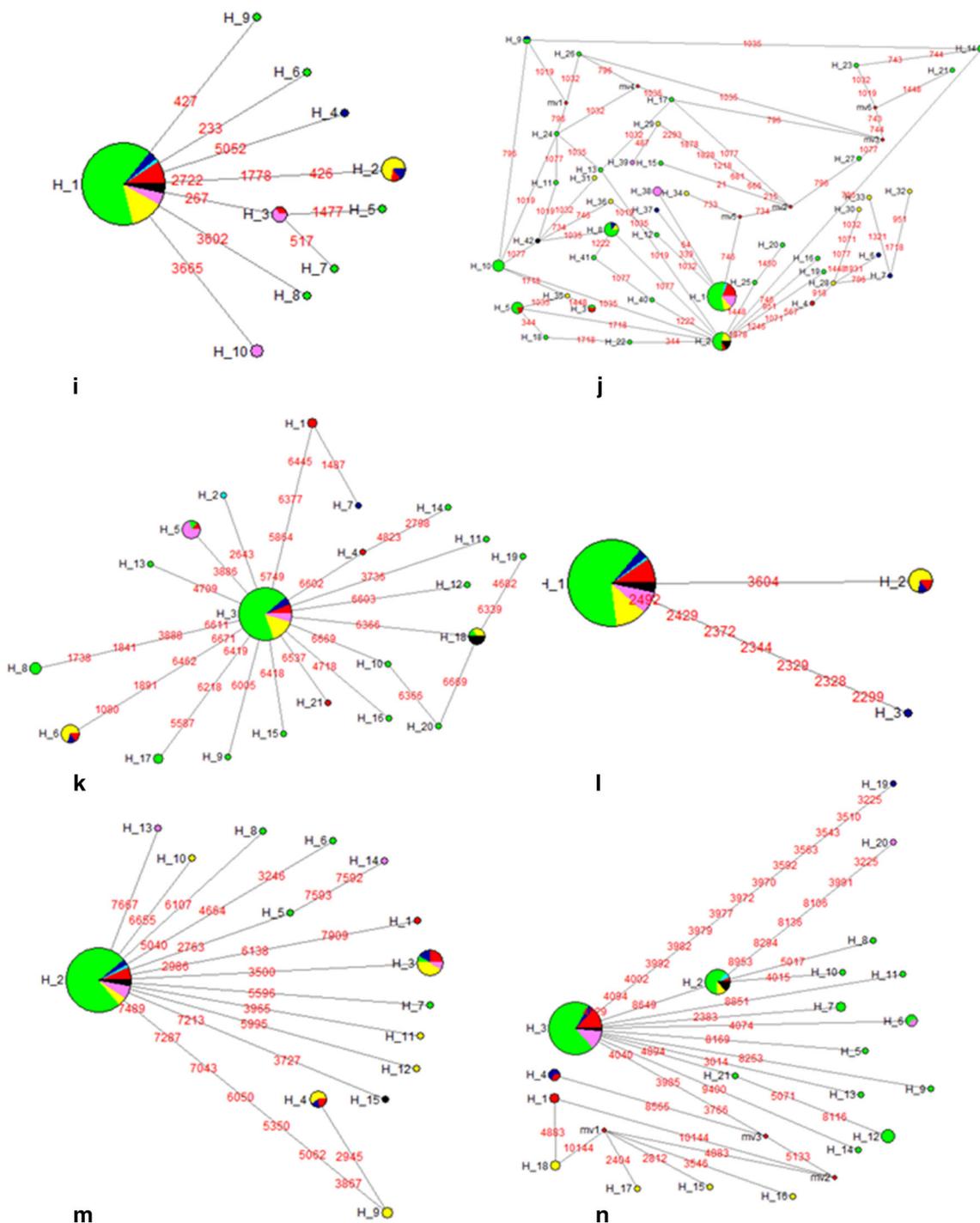


Fig. 2 continued

expressing a wheat antiporter and an H<sup>+</sup> pyrophosphate, were salt tolerant (Gouiaa et al. 2012). Seven different QTLs for drought tolerance are being transferred into submergence tolerant varieties having *Sub1* gene (Singh et al. 2015d). Hence, it is concluded that a single candidate gene may not provide an adequate level of salt tolerance.

Therefore, cumulative action of multiple genes may be exploited for developing salt-tolerant cultivars.

Clustering of genotypes based on the concatenation of 21 gene sequences was used to assess if any particular combination of alleles of these genes led to salt-tolerant phenotype (Fig. 1). Clustering showed an even distribution of

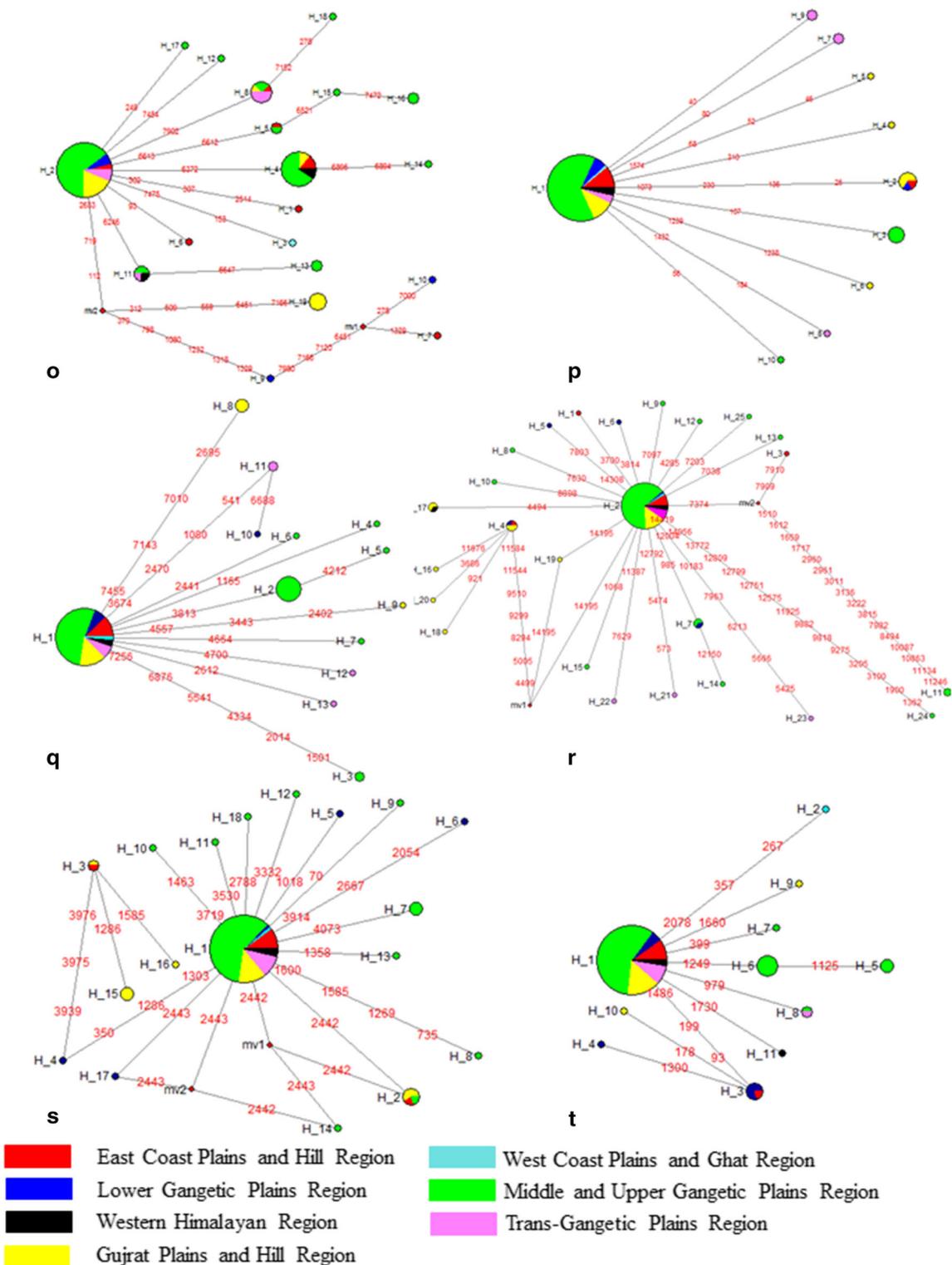


Fig. 2 continued

tolerant accessions among different clusters. This indicated the difference in genetic potential of the accessions and the presence of various tolerant alleles of the candidate genes. Earlier, Platten et al. (2013) identified seven haplotypes for

the *HKT1;5* gene in seven different clusters. Furthermore, analysing epistatic interaction, two tolerant sub-cluster, viz. sub-cluster-IIIA-1-2 and sub-cluster-IIIB-3 grouped all the tolerant accessions. This is likely due to different molecular

mechanisms for salt tolerance, or the presence of different interacting sets of multiple genes in each group. It is possible that the clustering of tolerant accessions in these subgroups is due to their close phylogeny and underlying genetic control of salt tolerance may be simple. However, this can only be deciphered by inheritance analysis after crossing the tolerant accessions with closely related sensitive accessions. We identified the SNPs which differentiated the tolerant clusters, and both the clusters showed variations in the transporter family and osmolyte biosynthetic pathway genes. Hence, the phenotypic difference was perhaps due to the regulation of both osmolyte accumulation and ionic concentrations. Phylogenetic analysis, based on Nei coefficient, revealed separate grouping of cultivated rice varieties which may be due to their phylogenetic distance from the wild rice accessions. It also shows that the cultivated varieties have retained only a fraction of the wider genetic variation, because domestication of cultivated rice is based on a particular group of wild rice. It is a well known fact that elite modern rice varieties represent only a fraction of the total genetic variations present in their wild relatives (Brar and Khush 2003; Londo et al. 2006; McNally et al. 2009). Extensive analysis is required to identify the ancestral population from which the cultivated rice might have evolved.

In neutral allele model, new alleles are supposed to be produced by mutations and the most frequent allele is inferred to be the oldest one (Watterson and Guess 1977). In the present study, major haplotypes of all the genes are present in accessions from each of the three genetically structured sub-populations of wild rice. Similar results were obtained by Singh et al. (2015e). This indicates ancient origin of these haplotypes, which is the reason for their even distribution in wild rice from across multiple geographic regions of India. The haplotype network analysis of some genes showed a different eco-geographical distribution of haplotypes. In particular, minor haplotypes, representing the same seven accessions from two geographically different salt-affected areas, were found across the haplotype network of seven genes. It is supposed that this is due either to adaptive variations acquired by the accessions, or to the migration of the accessions between geographic regions. Moreover, in six other genes, the cluster of these seven accessions was further divided into two subgroups, but not by geography. These can be considered as recent haplotypes that originated in Gujrat Hills and Plains region (Fig. 2). It could represent a recent expansion of Indian wild rice, and it may, therefore, be inferred that minor haplotypes play a significant role in studying evolution and the domestication history of rice. Furthermore, the probability of adaptive variations across multiple genes was quite low, and hence, it may be concluded that directional expansion of wild rice may be a

primary reason for such common variations. Population expansion has also been supported by significant negative tests of selection and neutrality (Tajima's  $D$  and Fu and Li's  $D^*$ , Singh et al. 2015b). In cultivated rice, we observed that the haplotype of one gene was distributed into multiple haplotypes of other genes. Londo et al. (2006) also measured such variations between the haplotype networks of different genes. They inferred that the differences were due to the diverse evolutionary history of different types of genes. In addition, they mentioned that such variations could represent lineage sorting, or multiple origins of the *O. sativa indica* lineage; however, this study was conducted on wider geographic regions.

The wild progenitors of cultivated rice are a rich source of genetic variations; however, the level of variations is different across different gene families. Unravelling the genetic potential residing within individual gene families may indicate their acquired function. Existence of high genetic variations among transporter genes signifies the relationship between adaptive variations and functional significance, which has further been validated by candidate gene-based association analysis. Genetic interaction between different genes following overlapping tolerance mechanism may be responsible for tolerance among the studied set of wild rice. The identified minor haplotypes may play a significant role in analysing evolutionary studies of crop. The measuring of phenotypic variation and exploiting of sequence variations of the candidate gene may enhance our understating towards salt tolerance and also help in the development of new salt-tolerant cultivars.

## Materials and methods

### Plant material and their salt-tolerance phenotypes

A total of 299 accessions of wild rice (*O. nivara* and *O. rufipogon*) collected from different geographical regions of India were used. Of these, 95 wild accessions and eight cultivated rice varieties were used for targeted re-sequencing of the candidate genes for salt-tolerance. Phenotyping for all the accessions was conducted as per IRRI's SES protocol with minor modifications (Gregorio et al. 1997), at the National Phytotron Facility, IARI, New Delhi, India for three consecutive years as published elsewhere (Mishra et al. 2016).

### PCR amplification and re-sequencing

Genomic DNA was extracted from the leaf tissues using the CTAB method described by Murray and Thompson (1980). All of the 21 genes were PCR amplified using the primer walking method (Supplementary Table 1). The

nucleotide sequence of the genes was retrieved from NCBI database (<http://www.ncbi.nlm.nih.gov/>), and primers were designed using the Primer3 software (<http://www.genome.wi.mit.edu>). Specific amplification and validation of the primers was done by NCBI Primer BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) against *Oryza* taxon and high stringency conditions during PCR amplification. The PCR amplifications were carried out in 25  $\mu$ l reactions consisting of 1 unit of SpeedStar™ HS DNA polymerase from TAKARA BIO INC, 1 $\times$  Fast Buffer, 2  $\mu$ l of dNTP mixture, 0.5 Picomoles each of the two primers, and 80 ng of template DNA. The PCR reaction was carried out in a Bio-Rad Thermal cycler with initial denaturation at 98 °C for 3 min followed by 38 cycles of denaturation for 10 s at 98 °C, annealing for 1 min at 64 °C and extension for 1 min at 68 °C, and final extension for 10 min at 68 °C. The amplified products were further checked by electrophoresis in 1 % agarose gel in 1 $\times$  TBE buffer.

PCR products were directly sequenced by Ion Torrent PGM sequencer (Life Technologies) after fragmentation, library preparation, purification, and cycle sequencing according to the manufacturer's instructions. Briefly expounded, the amplicons of different genes of the same genotype were pooled in equimolar concentration, sheared to a size of  $\sim$ 200 bp and then barcoded to identify individual accessions. The sheared and barcoded products were then size-selected, pooled together in equimolar ratios, and then PCR-sequenced. At each step, purification was done using Agencourt AMPure XP reagent using Kingfisher Flex. Ion OneTouch2™ was used for emulsion PCR to clone the library on the beads and thereafter for enrichment. The enriched library was loaded onto the Ion PGM 316 chip, and sequencing was performed on Ion PGM™ sequencer.

### Sequence data analysis

Coverage analysis and variant-caller plugins were run, and the sequences were viewed in IGV (Robinson et al. 2011; Thorvaldsdóttir et al. 2013). SamTools mpileup command was used to generate consensus sequences, and alignment was done against the reference sequence of respective genes by ClustalW (Thompson et al. 1994) in BioEdit (Hall 2011). Nucleotide polymorphisms were analysed using the DnaSP software version 5.10 (Rozas et al. 2003). The level of silent-site nucleotide diversities per site ( $\pi$ ) (Nei 1987) and population mutation parameter ( $\theta$ ) (Watterson 1975) was estimated. The sliding window analysis was performed to examine nucleotide polymorphism across the genes in all accessions using the DnaSP software. Statistical tests of neutrality, such as Tajima's  $D$  (Tajima 1989), Fu, and Li's  $D^*$  and  $F$ , were calculated to examine the selection pressure at this locus. A haplotype network was constructed for

the comparison of genealogical relationships among the haplotypes using the Network software (Bandelt et al. 1999) (<http://www.fluxus-engineering.com>).

### SNP-trait association analysis

Association analysis was performed with the MLM model, considering both kinship ( $K$ ) and population structure ( $Q$ ), implemented in the TASSEL software. The kinship ( $K$ ) and population structure ( $Q$ ) have been generated for this set using a genome-wide 48-plex Illumina GoldenGate SNP genotyping assay (Mishra et al. 2016). For association mapping, filtered sites within genes were used to determine linkage disequilibrium (LD) by correlation between alleles at two loci in the TASSEL 5.0 (Bradbury et al. 2007) software and the significance of LD among SNPs was determined by Fisher's exact test. The mixed model showed least deviation of observed  $P$  values from expected  $P$  values in  $Q-Q$  plot when compared with that of  $Q$  (population structure) or  $K$  (kinship) model only. A probability value of 0.01 was used as the threshold for the significance of SNP–trait associations. Functions of associated sites in the promoter region were identified by PLACE (Higo et al. 1999). For a combined analysis of 29 candidate genes (21 genes from this study and the eight HKT transporter genes published earlier, Mishra et al. 2016), sequences of all the genes were concatenated according to their position in the genome using TASSEL and UPGMA tree was constructed. The tree was edited using FigTree v 1.4.2.

**Author contribution statement** SM: design and conduct of experiments, sequencing, data analysis, and manuscript preparation; BS: collection and characterization of wild rice, and conduct of experiments and manuscript drafting; PM: manuscript editing; VR: planning of experiment, and manuscript editing; NKS: wild rice collection, planning and supervision of the experiments, and manuscript editing and finalization.

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### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

### References

Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB)

- function as transcriptional activators in abscisic acid signalling. *Plant Cell* 15:63–78
- Bandelt H-J, Forster P, Röhl A (1999) Median-joining networks for inferring intraspecific phylogenies. *Mol Biol Evol* 16:37–48
- Bradbury PJ, Zhang Z, Kroon DE, Casstevens TM, Ramdoss Y, Buckler ES (2007) TASSEL: software for association mapping of complex traits in diverse samples. *Bioinformatics* 23:2633–2635
- Brar D, Khush G (2003) Utilization of wild species of genus *Oryza* in rice improvement. Science Publishers, Enfield
- Chen S, Yang Y, Shi W et al (2008) Badh2, encoding betaine aldehyde dehydrogenase, inhibits the biosynthesis of 2-acetyl-1-pyrroline, a major component in rice fragrance. *Plant Cell* 20:1850–1861
- Das-Chatterjee A, Goswami L, Maitra S, Dastidar KG, Ray S, Majumder AL (2006) Introgression of a novel salt-tolerant  $\gamma$ -myo-inositol 1-phosphate synthase from *Porteresia coarctata* (Roxb.) Tateoka (PcINO1) confers salt tolerance to evolutionary diverse organisms. *FEBS Lett* 580:3980–3988
- Eulgem T, Rushton PJ, Robatzek S, Somssich IE (2000) The WRKY superfamily of plant transcription factors *Trends. Plant Sci* 5:199–206
- FAO, UN (2009) How to feed the world in 2050. In: High-Level Expert Forum, Rome
- Fukuda A, Nakamura A, Hara N, Toki S, Tanaka Y (2011) Molecular and functional analyses of rice NHX-type  $\text{Na}^+/\text{H}^+$  antiporter genes. *Planta* 233:175–188
- Ghomi K, Rabiei B, Sabouri H, Sabouri A (2013) Mapping QTLs for traits related to salinity tolerance at seedling stage of rice (*Oryza sativa* L.): an agrigenomics study of an Iranian rice population. *OMICS* 17:242–251
- Gouiaa S, Khoudi H, Leidi EO, Pardo JM, Masmoudi K (2012) Expression of wheat  $\text{Na}^+/\text{H}^+$  antiporter TNHXS1 and  $\text{H}^+$ -pyrophosphatase TVP1 genes in tobacco from a bicistronic transcriptional unit improves salt tolerance. *Plant Mol Biol* 79(1–2):137–155
- Gregorio GB, Senadhira D, Mendoza RD (1997) Screening rice for salinity tolerance. International Rice Research Institute discussion paper series, Metro Manila
- Hall T (2011) BioEdit: an important software for molecular biology. *GERF Bull Biosci* 2:60–61
- Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant cis-acting regulatory DNA elements (PLACE) database. *Nucleic Acid Res* 27:297–300
- Horie T, Costa A, Kim TH et al (2007) Rice OsHKT2; 1 transporter mediates large  $\text{Na}^+$  influx component into  $\text{K}^+$ -starved roots for growth. *EMBO J* 26:3003–3014
- Hur J, Jung K-H, Lee C-H, An G (2004) Stress-inducible OsP5CS2 gene is essential for salt and cold tolerance in rice. *Plant Sci* 167:417–426
- Jacobs M, Angenon G, Hermans C, Thu TT, Roosens NH (2003) Proline accumulation and  $\Delta$  1-pyrroline-5-carboxylate synthetase gene properties in three rice cultivars differing in salinity and drought tolerance. *Plant Sci* 165:1059–1068
- Khush GS, Ling K (1974) Inheritance of resistance to grassy stunt virus and its vector in rice. *J Hered* 65:135–136
- Kumar K, Kumar M, Kim S-R, Ryu H, Cho Y-G (2013) Insights into genomics of salt stress response in rice. *Rice* 6:27
- Kurokawa Y, Noda T, Yamagata Y et al (2016) Construction of a versatile SNP array for pyramiding useful genes of rice. *Plant Sci* 242:131–139
- Lestari P, Lee G, Ham T-H et al (2011) Single nucleotide polymorphisms and haplotype diversity in rice sucrose synthase 3. *J Hered* 102:735–746
- Li R, Li L, Wei S et al. (2010) The evaluation and utilization of new genes for brown planthopper resistance in common wild rice (*Oryza rufipogon* Griff.). *Mol Entomol* 1:1–7
- Liu C, Fukumoto T, Matsumoto T et al (2013) Aquaporin OsPIP1; 1 promotes rice salt resistance and seed germination. *Plant Physiol Biochem* 63:151–158
- Liu C, Mao B, Ou S et al (2014) OsbZIP71, a bZIP transcription factor, confers salinity and drought tolerance in rice. *Plant Mol Biol* 84:19–36
- Londo JP, Chiang Y-C, Hung K-H, Chiang T-Y, Schaal BA (2006) Phylogeography of Asian wild rice, *Oryza rufipogon*, reveals multiple independent domestications of cultivated rice, *Oryza sativa*. *Proc Natl Acad Sci USA* 103:9578–9583
- Maclean J, Hardy B, Hettel G (2013) Introduction and setting. Rice Almanac: source book for one of the most important economic activities on earth, 4th edn. GRiSP, IRRI, USA, pp 1–14
- Martinez-Atienza J, Jiang X, Garcíadeblas B, Mendoza I, Zhu J (2007) Conservation of the SOS pathway for salt-tolerance in rice. *Plant Physiol* 143:1001–1012
- McNally KL, Childs KL, Bohnert R et al (2009) Genomewide SNP variation reveals relationships among landraces and modern varieties of rice. *Proc Natl Acad Sci USA* 106:12273–12278
- Mercer J (2010) Disaster risk reduction or climate change adaptation: are we reinventing the wheel? *J Int Dev* 22:247–264
- Mishra S, Singh B, Panda K et al (2016) Association of SNP haplotypes of HKT family genes with salt tolerance in Indian wild rice germplasm. *Rice* 9:1
- Murray M, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acid Res* 8:4321–4326
- Nakamura T, Yokota S, Muramoto Y, Tsutsui K, Oguri Y, Fukui K, Takabe T (1997) Expression of a betaine aldehyde dehydrogenase gene in rice, a glycinebetaine nonaccumulator, and possible localization of its protein in peroxisomes. *Plant J* 11:1115–1120
- Negrão S, Cecília Almadanim M, Pires IS et al (2013) New allelic variants found in key rice salt-tolerance genes: an association study. *Plant Biotechnol J* 11:87–100
- Nei M (1987) Molecular evolutionary genetics. Columbia University Press, New York
- Ohta M, Guo Y, Halfter U, Zhu J-K (2003) A novel domain in the protein kinase SOS2 mediates interaction with the protein phosphatase 2C ABI2. *Proc Natl Acad Sci USA* 100:11771–11776
- Ouyang SQ, Liu YF, Liu P et al (2010) Receptor-like kinase OsSIK1 improves drought and salt stress tolerance in rice (*Oryza sativa*) plants. *Plant J* 62:316–329
- Platten JD, Egdane JA, Ismail AM (2013) Salinity tolerance,  $\text{Na}^+$  exclusion and allele mining of HKT1; 5 in *Oryza sativa* and *O. glaberrima*: many sources, many genes, one mechanism? *BMC Plant Biol* 13:1
- Ren Z-H, Gao J-P, Li L-G et al (2005) A rice quantitative trait locus for salt tolerance encodes a sodium transporter. *Nat Genet* 37:1141–1146
- Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, Mesirov JP (2011) Integrative genomics viewer. *Nat Biotechnol* 29:24–26
- Rozas J, Sánchez-DelBarrio JC, Messeguer X, Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* 19:2496–2497
- Schmidt R, Schippers JH, Welker A, Mieulet D, Guiderdoni E, Mueller-Roeber B (2012) Transcription factor OsHsfC1b regulates salt tolerance and development in *Oryza sativa* ssp. *Japonica*. *AoB Plants* 2012:pls011
- Sengupta S, Patra B, Ray S, Majumder AL (2008) Inositol methyl transferase from a halophytic wild rice, *Porteresia coarctata* Roxb. (Tateoka): regulation of pinitol synthesis under abiotic stress. *Plant Cell Environ* 31:1442–1459
- Serrano R, Rodriguez-Navarro A (2001) Ion homeostasis during salt stress in plants. *Curr Opin Cell Biol* 13:399–404

- Shaw RG, Etterson JR (2012) Rapid climate change and the rate of adaptation: insight from experimental quantitative genetics. *New Phytol* 195:752–765
- Singh B, Bohra A, Mishra S, Joshi R, Pandey S (2015a) Embracing new-generation ‘omics’ tools to improve drought tolerance in cereal and food-legume crops. *Biol Plant* 59:413–428
- Singh BP, Jayaswal PK, Singh B et al (2015b) Natural allelic diversity in *OsDREB1F* gene in the Indian wild rice germplasm led to ascertain its association with drought tolerance. *Plant Cell Rep* 34:993–1004
- Singh BP, Singh B, Kumar V, Singh PK, Jayaswal PK, Mishra S, Singh NK (2015c) Haplotype diversity and association analysis of *SNAC1* gene in wild rice germplasm. *Indian J Genet Pl Br* 75(2):157–166
- Singh R, Singh Y, Xalaxo S, Verulkar S et al (2015d) From QTL to variety-harnessing the benefits of QTLs for drought, flood and salt tolerance in mega rice varieties of India through a multi-institutional network. *Plant Sci* 242:278–287
- Singh N, Jayaswal PK, Panda K, Mandal P et al (2015e) Single-copy gene based 50 K SNP chip for genetic studies and molecular breeding in rice. *Sci Rep* 5:11600
- Singla-Pareek SL, Yadav SK, Pareek A, Reddy M, Sopory S (2008) Enhancing salt tolerance in a crop plant by overexpression of glyoxalase II. *Transgenic Res* 17:171–180
- Stern DL, Orgogozo V (2008) The loci of evolution: how predictable is genetic evolution? *Evolution* 62:2155–2177
- Storz JF, Wheat CW (2010) Integrating evolutionary and functional approaches to infer adaptation at specific loci. *Evolution* 64:2489–2509
- Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* 123:585–595
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acid Res* 22:4673–4680
- Thorvaldsdóttir H, Robinson JT, Mesirov JP (2013) Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Brief Bioinform* 14:178–192
- Turrall H, Burke J, Faures JM (2011) Climate change, water and food security. FAO
- Watterson G (1975) On the number of segregating sites in genetical models without recombination. *Theor Popul Biol* 7:256–276
- Watterson G, Guess H (1977) Is the most frequent allele the oldest? *Theor Popul Biol* 11:141–160
- Xiang Y, Tang N, Du H, Ye H, Xiong L (2008) Characterization of *OsZIP23* as a key player of the basic leucine zipper transcription factor family for conferring abscisic acid sensitivity and salinity and drought tolerance in rice. *Plant Physiol* 148:1938–1952
- Xu X, Liu X, Ge S et al (2012) Resequencing 50 accessions of cultivated and wild rice yields markers for identifying agronomically important genes. *Nat Biotechnol* 30:105–111
- Yang A, Dai X, Zhang W-H (2012) A R2R3-type MYB gene, *OsMYB2*, is involved in salt, cold, and dehydration tolerance in rice. *J Exp Bot* 63:err431
- Yuan S-C, Zhang Z-G, He H-H, Zen H-L, Lu K-Y, Lian J-H, Wang B-X (1993) Two photoperiodic-reactions in photoperiod-sensitive genic male-sterile rice. *Crop Sci* 33:651–660
- Zhu J-K (2003) Regulation of ion homeostasis under salt stress. *Curr Opin Plant Biol* 6:441–445