



# QTLs in salt rice variety CSR10 reveals salinity tolerance at reproductive stage

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## Abstract

Improvement in salt tolerance at reproductive stage is very important for enhancing the grain yield and highly desirable to sustain production in saline areas. In the present study, 140 F<sub>2</sub> populations derived from PS5, which is saline sensitive and CSR10, saline tolerant rice variety. We evaluated 140 F<sub>2</sub> for salinity tolerance at reproductive stage for 18 different morphological, biochemical and physiological traits. We used 436 rice HvSSR markers which covers 12 chromosomes of rice. The linkage map has been created with 100 polymorphic markers and 140 F<sub>2</sub> mapping population. We identified 39 QTLs which explained 3–45% phenotypic variance at LOD score 2.6–15.2. Significant QTLs were identified for sodium content, potassium content, sodium/potassium ratio in roots and leaves including qNaL-1.2, qNa/KL-1.3, qKR-1 and qNa/KL-1.2 with R<sup>2</sup> of 45%, 42%, 35% and 32%, respectively, on chromosome 1. We identified novel QTL *qGY-2* for grain yield on chromosome 2 along with QTL for stress tolerant index on chromosome 6, a noteworthy QTL for salt susceptible index of grain yield was recognized with 45% of phenotypic variation. We also identified four novel QTLs for chlorophyll content and one locus for proline content in leaves under salinity. Validation of these QTLs in fixed mapping population (RILs) and further fine mapping of these QTLs may be advantageous for marker-assisted breeding programs that led to development and commercial cultivation of reproductive stage salt-tolerant rice varieties.

**Keywords** Rice · QTLs · Salt · Reproductive stage

## Introduction

Rice is the most devoured cereal grain and essential wholesome nourishment for almost 50% of the human populace everywhere on the world after maize and wheat, it is the third major cereal crop to satisfy the food demand of increasing population. The total population is anticipated

to reach out 10.9 billion in 2100 (United Nations 2011). To feed such large population, the production of this important cereal crop should be enhanced by 0.6–0.9% every year until 2050 (Carriger and Vallee 2007). Hence, it needs strenuous efforts to address the need while tackling congenial climate. The abiotic stresses ominously undermine crop productivity and causes huge yield reduction in vast region (Mantri et al. 2012; Pareek et al. 2010). The environmental change has also escalated the impact of these stresses and led to enhancement of salinity and vapour pressure deficit that causes the economic loss in rice (Tack et al. 2015). Soil salinity affects about 20% of total and 50% of irrigated area throughout the world including 30% rice growing area. Hence, salinity is reckoned as one of the most predominant abiotic stresses that cause significant yield reduction (Wang et al. 2012a). The fertility of soil gets deteriorated by the soluble salts, resulting in detrimental impact on the growth and development of plants (Munns and Tester 2008). Furthermore, the irrigation with saline water and exiguous rainfall exaggerate the severity of these stresses and thus jeopardizing the crop

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productivity. Under salinity, the osmotic potential of the soil gets decreased and consequently the plant suffers the osmotic stress, resulting into reduced water uptake. This causes comparable effects those of water deficit imposed by drought. Salinity stress additionally prompts the amassing of salts in plant tissues that will ultimately increase to toxic levels, particularly in older leaves (Mitsuya et al. 2003). Accumulated salt cannot be removed back from plant system even after the irrigation of good quality water, that may cause sodium toxicity and ionic stress and leads to restricted nutrient availability to plant (Munns and Termaat 1986). The rice is fragile to salinity at both seedling and reproductive stage yet the improvement of salt tolerance level at later stage is more significant as it diminishes the grain yield more profoundly than salinity at seedling stage (Mansuri et al. 2012). The resilience at these two growth stages is not correlated with one another (Singh and Flowers 2010; Calapit-Palao et al. 2013). QTL mapping for salinity provides helpful ramifications for farming as the identification of these genomic regions facilitates to incorporate them into high yielding rice varieties through marker-assisted selection (Mauricio 2001).

For the first time, Gregorio (1997) has mapped a quantitative trait locus known as *Saltol* QTL in rice on chromosome 1 for seedling stage salinity tolerance. Extensive research has been redirected towards advancement of salt resistance; at seedling stage of rice; but not many efforts have been made to recognize QTLs related to the reproductive stage salinity (Zeng et al. 2002; Ammar et al. 2009; Mohammadi et al. 2013; Bimpong et al. 2014; Hossain et al. 2015; Pandit et al. 2010, Tiwari et al. 2016; Kumar et al. 2015; Warraich et al. 2020). In spite of QTL mapping based solely on the phenotypic performance under stress, the utilization of comparative performance of various genotypes under stress and non-stress conditions is more useful in identifying and mapping the QTLs for development of stress resistant varieties (Raman et al. 2012). This approach has useful applicability as genotypes with low yield potential in non-stress conditions generally exhibit more salt tolerance than high yielding lines. Furthermore, the mapping of QTLs for reproductive stage salinity tolerance using stress tolerance indices, have not been accounted in  $F_2$  population; however, it has been already reported in fixed populations (Pandit et al. 2010; Tiwari et al. 2016).

In the current study, we implemented the splitting method (Luna et al. 2000) by splitting the tillers and planting the identical plants of the  $F_2$  population in stress and non-stress environment, for identification of QTLs related to agronomic, biochemical and physiological traits as well as for stress indices. Identification of major QTLs responsible for salt resistance at reproductive stage will extensively accelerate the development of tolerant varieties and accordingly it will help in improving the rice production in saline areas.

## Materials and methods

### Plant materials and population development

Pusa Sugandh 5 and CSR10 rice varieties have been used to develop mapping population ( $F_2$ ). Pusa Sugandh 5 (Pusa 3A/Haryana Basmati) is a popular variety of north India that is semi dwarf, high yielding and aromatic in nature. Its main characteristics are the extra long grains having tremendous cooking quality. It is very sensitive to salinity while tolerant to shattering and resistant to gall midge and brown spot. CSR10 is a salt-tolerant rice variety, released for cultivation in sodic and saline areas by Central variety release committee. It is a short-duration (110–120 d) salt-tolerant rice variety developed from the rice line M40-431-24-114 ( $F_1$  mutant of CSR1/IR8). It is a dwarf (80–85 cm) variety with compact plant morphology that can tolerate salinity and sodicity. CSR10 is photoperiod insensitive with strong culm, highly responsive to fertilizers and possess grain yield potential of 5–6 t/ha in normal soils and 3–4.5 t/ha in highly deteriorated salt affected soils. The grains are short and bold with acceptable cooking quality. CSR10 is also used as a national check in national salinity programs.

The experiment was carried out during July 2011–November 2015. In July 2011, the parents were crossed to produce  $F_1$  seeds. In July 2012, the true  $F_1$  plants were identified by the molecular markers as well as through observing intermediate morphological characteristics. True  $F_1$ s were self-fertilized and produced  $F_2$  population. The  $F_2$  population was phenotyped for agronomic traits in microplots and the fresh leaf samples were analysed in lab for biochemical traits. At maturity, each plant was harvested and different parts of plant (root, shoot, leaf) were sampled for ionic analysis from June 2013 to July 2014. During July 2014 to November 2015, the ionic analysis of different plant parts was done along with the genotyping to find the QTLs for reproductive stage salinity tolerance in rice. Three technological replicates were used for biochemical and ionic analysis.

### Phenotypic evaluation for yield components under salinity

The phenotypic evaluation of the plants was carried out at Central Soil Salinity Research Institute-ICAR, Karnal, India, under artificially salinized concrete blocks or microplots (8 × 4.8 m and 0.5 m depth). The experiment was conducted in controlled conditions that were similar to the field conditions, with rain out shelter, which is necessary to maintain the salinity stress. The rain out shelter

has transparency sheets of 85 per cent. The temperature and humidity is almost similar to field conditions. There is no significant difference on irradiation, temperature and humidity in microplot and field condition. The 25-day-old seedlings of parents and  $F_2$  progenies were transplanted in saline plots. Each plant in  $F_2$  population is genetically different; two sets treatments were made (stress and non-stress) by splitting the tillers (Ammar et al. 2009). After 20 days of transplanting, the tillers were split into two identical sets with equal number of tillers and transplanted into two different microplots.

Saline solution was prepared by dissolving three salts, namely, NaCl,  $\text{CaCl}_2$  and  $\text{MgSO}_4$  in water to salinize the soil through irrigation. Salinity stress was imposed to the one set of  $F_2$  progenies after 15 days of 2nd transplanting till maturity by irrigating the saline water of  $\text{ECe} \sim 40$  mmol during transplanted seedlings, which increased to  $\text{ECe} \sim 80$  mmol at panicle initiation stage until maturity and another set of  $F_2$  progenies treated as control (non-stress) and irrigated with normal water.

**Morphological traits:** Standard evaluation system was followed to record the data on each  $F_2$  population along with parents for the following traits.

SES (salt evaluation score)—The plants were scored from 1 to 9 based upon the following criteria (SES 2014).

Plant height (cm)—It was measured from surface of soil to tip of the tallest panicle excluding the awns

Panicle length (cm)—It was measured in centimetres from panicle base to tip

No. of total tillers—It is a component of the culm number in which total no. of tillers were counted.

No. of productive tillers—It is the no. of tillers bearing panicles.

Biomass (g)—All living biomass above the soil including stem, leaves, threshed panicles and leaves.

Grain yield (g)—All the harvested seeds were weighted in grams.

## Physiological traits

### Estimation of $\text{Na}^+$ , $\text{K}^+$ concentration ( $\text{mmol}^{-1}\text{dwt}$ ) in root, shoot and leaf-

The  $F_2$  plants were harvest completely including roots and dried under sun. Then root, shoot and leaf were individually analysed for ionic estimation ( $\text{Na}^+$  and  $\text{K}^+$ ) using di-acid mixture ( $\text{HNO}_3:\text{HClO}_4$  3:1) on flame photometer (PFP7, Jenway, Bibby Scientific, UK).

## Biochemical traits

**Estimation of Chlorophyll content-** The chlorophyll content was measured by digesting fully expanded leaves in Dimethyl sulphoxide- DMSO (Hiscox and Israelstam 1979).

**Estimation of proline content in leaf-** Fresh leaves were used to estimate the proline content by the method described by Bates et al. (1973). The calibration curve was used to determine proline content and expressed as  $\mu\text{g}$  proline per gram fresh weight of the plant tissue.

### Estimation of salinity stress indices

The stress susceptibility index (SSI) and stress tolerance index (STI) for grain yield were calculated (Fernandez 1993) as below: -

Stress susceptibility index (SSI) =  $1 - (Y_s/Y_p)/SI$

Where  $Y_s$  is the stress yield and  $Y_p$  is the non-stress yield.

SI was calculated as:  $1 - (X_s/X_p)$

$X_s$  is the mean stress yield over genotypes and  $X_p$  is the mean non-stress yield over genotypes.

Stress tolerance index (STI) =  $(Y_p \times Y_s)/(X_p)^2$

$Y_p$ —Grain yield of a genotype under non-stress

$Y_s$ —Grain yield of a genotype under stress

$X_p$ —Mean yield of all genotypes under non-stress conditions

## Construction of a linkage map and QTL mapping

The DNA was extracted by modified CTAB method from each  $F_2$  individual (Murray and Thomson 1980). The parental polymorphism was checked using 436 rice HvSSR (Singh et al. 2010) and other 30 SSR markers. The PCR amplification was done in 10  $\mu\text{L}$  reaction mixture made up of 1.5  $\mu\text{L}$  of 10 $\times$ PCR buffer (containing 100 mM Tris-HCl, pH 8.3, 500 mM KCl and 15 mM  $\text{MgCl}_2$ ), 1  $\mu\text{L}$  of 25 ng genomic DNA, 0.5  $\mu\text{L}$  each of 5  $\mu\text{M}$  forward and reverse primers, 1.25  $\mu\text{L}$  of 1 mM dNTP, 0.5  $\mu\text{L}$  of 50 mM  $\text{MgCl}_2$ , and 0.5  $\mu\text{L}$  of 5 U/ $\mu\text{L}$  Taq DNA polymerase with 4.75  $\mu\text{L}$  sterile nanopure water. The PCR reaction was carried out in Thermal cycler by initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 45 s, extension at 72 °C for 1 min, and 30 s and final extension at 72 °C for 8 min and storage at 10 °C. The PCR was performed on 96-well plates, and the DNA fragments were separated on 2.5% agarose gels then stained with ethidium bromide dye for manual allele scoring. The linkage map was produced using the genotypic data of  $F_2$  mapping population with 100 polymorphic markers out of total 466 HvSSR and SSR markers.

## Data analysis

Phenotypic variation in the population was analysed with SAS v 9.3 (SAS institute, Cary NC). The linkage map of all 12 chromosomes was generated using Joinmap version 4.0 software (Ooijen 2006). The markers with unusual segregation were identified using the locus genotypic frequencies of Joinmap by performing Chi square test ( $p < 0.05$ ). The markers were grouped at the minimum LOD threshold of 2.5 to identify the linkage groups. The map distance was calculated in centiMorgan (cM) using the kosambi's mapping function. The ordering of markers was based upon the published marker information (Temnykh et al. 2000; Singh et al. 2010).

The QTLs was identified by composite interval mapping in Win QTL cartographer software using model 6 (Zeng 1993; Wang et al. 2007) forward and backward stepwise regression. 1000 permutations along with significance probability level of 0.05 were used to determine the thresholds (Doerge and Churchill 1996). Standard rice QTL nomenclature used for QTL naming (McCouch 2008).

## Results

### Phenotypic performance of the mapping population

The  $F_2$  population exhibited transgressive segregation in both directions, however, to various degrees. The distribution pattern of each trait also showed that these traits are

under polygenic control and both the parents, PS-5 as well as CSR10, contributed alleles for these traits. The normal distribution was observed only for frequency distribution of plant height and panicle length, whereas other traits deviated from the normal distribution and that may be due to effect of some major genes. We noticed continuous segregation for the phenotypic distributions of all studied traits with the skewness ranging from  $-0.89$  to  $2.10$ , suggesting the quantitative inheritance (Table 1). Moderate-to-high transgressive segregation was observed for mean distributions of yield components either larger or smaller than parental value (Supplementary Fig. 1).

### QTLs identification

The linkage map of 1641 cM (centimorgan) was produced with an average interval of 16.41 cM, bearing 39 QTLs for morphological, biochemical, physiological traits and stress indices (Figs. 1 and 2). The phenotypic variation of detected ranged from 3 to 45%. The list of identified QTLs along with their flanking markers, phenotypic variance, LOD values and additive effect is given in Table 2.

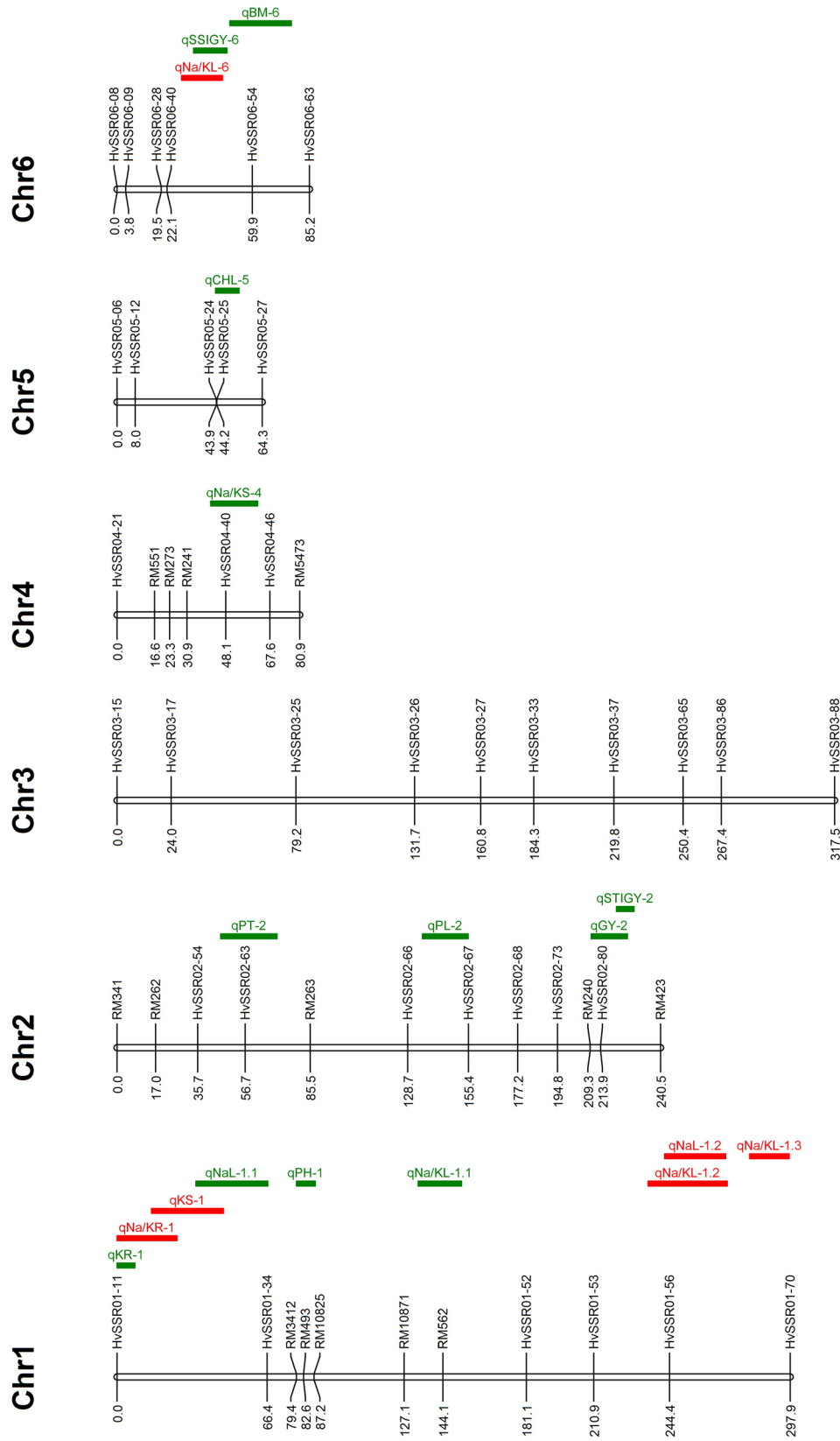
### QTLs for agronomic traits

#### SES score

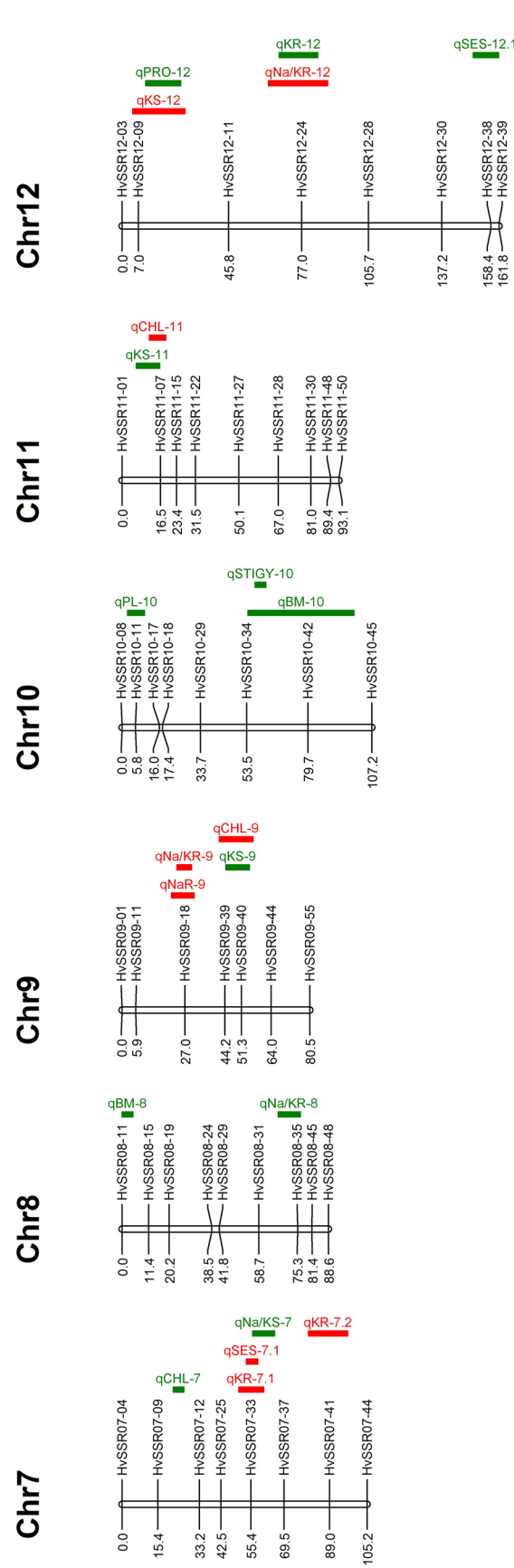
Two QTLs, qSES-7.1 and qSES-12.1, were recognized on chromosome 7 and chromosome 12 that explains 18% and 22.0% of the phenotypic variance at LOD scores 5.9 and

**Table 1** Variation for morphological traits and  $\text{Na}^+$ ,  $\text{K}^+$  ion concentrations in different tissues of rice among 140  $F_2$  progenies derived from the PS-5/CSR10 cross under salinity

S. no.	Trait	PS-5	CSR10	$F_2$ Population		
				Midparent	Range	Skewness
1	Stress evaluation score	7	3	5	3–9	0.55
2	Plant height	60	57	48.92	30–76	0.60
3	Panicle length	12	16	13.89	8–23	$-0.89$
4	No. of total tillers	6	7	5	1–12	0.76
5	No. of productive tillers	2	4	2	1–8	1.27
6	Biomass	2.50	5.50	1.64	0.50–7.30	2.22
7	Grain yield	0.75	2.30	0.91	0.09–3.58	1.27
8	Chlorophyll	0.6	2.0	0.64	0.10–4.29	2.31
9	Proline	6.0	4.8	5.23	0.20–13.87	0.61
10	$\text{Na}^+$ concentration in root	0.70	0.39	0.65	0.22–1.59	1.14
11	$\text{K}^+$ concentration in root	0.18	0.35	0.17	0.09–0.30	1.68
12	$\text{Na}^+/\text{K}^+$ ratio in root	3.97	0.96	3.73	0.83–10.18	0.72
13	$\text{Na}^+$ concentration in shoot	4.19	2.05	3.13	0.47–6.97	1.20
14	$\text{K}^+$ concentration in shoot	0.27	0.31	0.28	0.08–0.71	0.54
15	$\text{Na}^+/\text{K}^+$ ratio in shoot	15.53	6.71	13.55	1.34–34.92	1.69
16	$\text{Na}^+$ concentration in leaf	1.40	1.20	1.69	0.12–5.22	1.04
17	$\text{K}^+$ concentration in leaf	0.44	0.59	0.25	0.06–0.67	1.60
18	$\text{Na}^+/\text{K}^+$ ratio leaf	3.18	2.03	7.63	0.76–25.45	2.10



**Fig. 1** Physical map positions of QTLs identified on rice chromosomes 1-6 in the F2 population derived from PS5/CSR10. QTLs shown in green color have salt tolerant allele coming from the tolerant parent CSR10 parent and those in red color have tolerant allele contributed by the sensitive parent PS



**Fig. 2** Physical map positions of QTLs identified on rice chromosomes 7-12 in the F2 population derived from PSS/CSR10. QTLs shown in green color have salt tolerant allele coming from the tolerant parent CSR10 parent and those in red color have tolerant allele contributed by the sensitive parent PS5



**Table 2** QTLs detected for salt tolerance parameters among 140 F<sub>2</sub>s derived from the cross PS-5/CSR10 identified with QTL cartographer

Trait	QTLs	Chr	LOD	PVE (%)	Left Marker	Right Marker	Additive effect	Contributing parent	Physical position (Mb)
Stress evaluation score	qSES-12.1	12	3.9	4	HvSSR12-30	HvSSR12-39	-0.758	CSR10	22.51–22.61
	qSES-7.1	7	5.9	8	HvSSR07-25	HvSSR07-37	0.042	PS-5	17.80
Plant height	qPH-1	1	2.8	7	RM3412	RM10825	-3.370	CSR10	11.50–13.3
Panicle length	qPL-10	10	2.9	10	HvSSR10-08	HvSSR10-17	-0.820	CSR10	2.04–7.33
	qPL-2	2	3.5	24	HvSSR02-66	HvSSR02-68	-3.953	CSR10	26.1–27.06
No. of productive tillers	qPT-2	2	4.1	5	HvSSR02-54	RM263	-0.674	CSR10	21.84–25.8
Biomass	qBM-10	10	3.4	6	HvSSR10-34	HvSSR10-45	-0.419	CSR10	15.91–22.38
	qBM-6	6	3.6	7	HvSSR06-40	HvSSR06-63	-0.267	CSR10	16.48–27.83
	qBM-8	8	3.9	10	HvSSR08-11	HvSSR08-15	-0.183	CSR10	5.36–6.32
Grain yield	qGY-2	2	2.7	4	RM240	RM423	-0.288	CSR10	31.40–38.30
Chlorophyll	qCHL-11	11	3.1	5	HvSSR11-01	HvSSR11-15	0.213	PS-5	0.06–6.08
	qCHL-5	5	3.4	4	HvSSR05-25	HvSSR05-27	-0.244	CSR10	9.13–10.65
	qCHL-7	7	9.8	4	HvSSR07-09	HvSSR07-12	-1.136	CSR10	1.87–2.42
	qCHL-9	9	3.2	9	HvSSR09-39	HvSSR09-44	0.222	PS-5	17.41–19.71
Proline	qPRO-12	12	4.4	7	HvSSR12-03	HvSSR12-11	-0.317	CSR10	1.36–3.28
Na <sup>+</sup> conc.in root	qNaR-9	9	10.9	37	HvSSR09-11	HvSSR09-39	0.196	PS-5	6.94–17.41
K <sup>+</sup> conc.in root	qKR-1	1	11.3	34	HvSSR01-11	HvSSR01-34	-0.027	CSR10	6.01–10.57
	qKR-12	12	5.3	17	HvSSR12-11	HvSSR12-28	-0.017	CSR10	3.28–16.05
	qKR-7.1	7	4.5	15	HvSSR07-25	HvSSR07-37	0.016	PS-5	8.66–21.76
	qKR-7.2	7	2.8	7	HvSSR07-37	HvSSR07-44	0.018	PS-5	21.76–24.19
Na <sup>+</sup> /K <sup>+</sup> ratio in root	qNa/KR-1	1	4.1	8	HvSSR01-11	HvSSR01-34	0.436	PS-5	6.01–10.57
	qNa/KR-12	12	5.1	17	HvSSR12-11	HvSSR12-28	0.784	PS-5	3.28–16.05
	qNa/KR-8	8	3	3	HvSSR08-31	HvSSR08-45	-0.101	CSR10	17.50–24.35
	qNa/KR-9	9	15.2	32	HvSSR09-11	HvSSR09-39	1.333	PS-5	6.94–17.41
K <sup>+</sup> conc.in shoot	qKS-1	1	4.7	5	HvSSR01-11	HvSSR01-34	0.002	PS-5	6.01–10.57
	qKS-11	11	3	5	HvSSR11-01	HvSSR11-07	-0.067	CSR10	0.06–1.34
	qKS-12	12	3.3	5	HvSSR12-09	HvSSR12-11	0.060	PS-5	3.07–3.28
	qKS-9	9	3.5	5	HvSSR09-18	HvSSR09-44	-0.001	CSR10	10.40–19.71
Na <sup>+</sup> /K <sup>+</sup> ratio in shoot	qNa/KS-4	4	2.8	12	RM241	HvSSR04-46	-9.748	CSR10	26.80–30.64
	qNa/KS-7	7	5.2	6	HvSSR07-37	HvSSR07-41	-17.251	CSR10	21.76–23.79
Na <sup>+</sup> conc.in leaf	qNaL-1.1	1	3.3	6	HvSSR01-11	RM3412	-0.533	CSR10	6.01–11.50
	qNaL-1.2	1	7.7	45	HvSSR01-56	HvSSR01-70	0.716	PS-5	23.54–30.56
Na <sup>+</sup> /K <sup>+</sup> ratio in leaf	qNa/KL-1.1	1	7.6	13	RM10871	HvSSR01-52	-4.486	CSR10	14.30–20.98
	qNa/KL-1.2	1	8.1	32	HvSSR01-53	HvSSR01-70	5.099	PS-5	21.38–30.56
	qNa/KL-1.3	1	7.4	42	HvSSR01-56	HvSSR01-70	9.746	PS-5	23.54–30.56
	qNa/KL-6	6	3.4	17	HvSSR06-40	HvSSR06-63	4.052	PS-5	16.48–27.83
SSI grain yield	qSSIGY-6	6	3.2	45	HvSSR06-40	HvSSR06-54	-0.224	CSR10	16.48–24.32
STI grain yielded	qSTIGY-10	10	2.6	7	HvSSR10-08	HvSSR10-17	-0.015	CSR10	2.04–7.33
	qSTIGY-2	2	6	4	HvSSR02-80	RM423	-0.230	CSR10	32.69–38.30

3.9, respectively. The PS-5 contributed for positive alleles on chromosome 7, while CSR10 increased the phenotypic performance for QTL qSES-12.1.

### Plant height

Only one QTL (qPH-1) was identified for plant height on chromosomes 1. This locus had  $R^2$  value 7% and located in the marker interval RM3412 and RM10825 on LOD score 2.8. The alleles from CSR10 increased plant height for qPH-1 under saline stress.

### Panicle length

Two genomic regions were detected on chromosomes 2 and 10 for panicle length. A QTL (qPL-2) located between HvSSR02-66 and HvSSR02-68 markers, with a peak  $R^2$  of 24% at LOD 3.5. Another QTL (qPL-10) found between the markers HvSSR10-08 and HvSSR10-17 on chromosome 10. This QTL explained 5% of the total phenotypic variation at LOD 2.9. CSR10 alleles increased the panicle length for both the QTLs.

### No. of productive tillers

One minor QTL (qPT-2) was detected for no. of productive tillers bearing fertile spikelets, between the marker interval HvSSR02-54 and RM263 on chromosome 2. It explained 5% of phenotypic variation with LOD score 4.1 and the additive effect was exerted by CSR10 at this locus.

### Biomass

QTL mapping using CIM detected three QTLs above LOD 3 for biomass (qBM-6, qBM-8, qBM-10) on chromosome 6, 8 and 10. CSR10 alleles had a positive effect, increasing biomass at all these loci. The QTL, qBM-6 was located in the marker interval HvSSR06-40 and HvSSR06-63, controlling 7% phenotypic variation at LOD 3.6. Another QTL the qBM-8, detected in the region HvSSR08-11 to HvSSR08-15 with  $R^2$  of 10% at LOD 3.9. The biomass QTL on chromosome 10 was identified at LOD 3.4 in the marker interval HvSSR10-34 to HvSSR10-45., explained 6% of phenotypic variation.

### Grain yield

Single QTL (qGY-2) was recognized for grain yield on chromosomes 2, placed in the marker interval RM240 and RM423 at LOD score 2.7 with 4%  $R^2$  value. Peak marker for

this QTL was HvSSR02-80. Under salinity stress at reproductive stage the additive effect for grain yield was contributed by CSR 10. At the physical map, this QTL spans a region of 7 Mb on chromosome 2 between 31.40 and 38.30 Mb.

### QTLs for biochemical traits-

#### Chlorophyll

In total, four QTLs were uncovered for chlorophyll content in leaves of stressed plants. They were designated as qCHL-5, qCHL-7, qCHL-9 and qCHL-11. The QTL qCHL-5 was identified in the region of HvSSR05-25-HvSSR05-27 on chromosome 5 at LOD score 3.4. Another QTL, qCHL-7 was mapped at LOD 9.8, between HvSSR07-09-HvSSR07-12 on chromosome 7. The additive effect at these two loci was contributed by CSR 10, where each of these two QTLs described 4% of the phenotypic variance,. For the other two QTLs (qCHL-9 and qCHL-11) on chromosomes 9 ( $R^2$ -9%) and chromosome 11( $R^2$ -5%) the sensitive parent PS-5 accounted for the additive effect. The QTL, qCHL-9 was detected at LOD 3.2 between HvSSR09-39 and HvSSR09-44 markers. At LOD score 3.1, the qCHL-11 was located within the HvSSR11-01 and HvSSR11-15 region on chromosome 11.

#### Proline

A QTL for proline content in plant leaves was identified in the marker interval HvSSR12-03 to HvSSR12-11 at LOD score 4.4 on chromosome 12. This QTL explained 7.0% of the phenotypic variation One QTL (qKS-12) for potassium content in shoot is also located in the same region on chromosome 12. CSR 10 contributed the favourable alleles for proline content.

### QTLs for physiological traits

#### Na<sup>+</sup> concentration (mmol g<sup>-1</sup> dwt) in root

A significant QTL (qNaR-9) on chromosome 9 has been identified for sodium concentration in root, explaining the 37% phenotypic variation at LOD score 10.9. It was located in the marker interval HvSSR09-11 and HvSSR09-39. At this locus PS-5 alleles increased the sodium concentration in roots. Peak marker for the QTL (qNaR-09) was HvSSR09-18.

#### K<sup>+</sup> concentration (mmol g<sup>-1</sup> dwt) in root

Total four QTLs were identified for K<sup>+</sup> concentration in root. Two significant QTLs (qKR-1, qKR-12) that explained 34% and 17% of the phenotypic variation at LOD scores 11.3 and 5.3 were located on chromosome 1 and 12, respectively. The tolerant parent, CSR10 contributed additively for K<sup>+</sup>



concentration at these loci. On chromosome 7, other two QTLs, namely, qKR-7.1 ( $R^2$ - 15%) and qKR-7.2 ( $R^2$ - 7%) were found in the vicinity of markers HvSS307-37 and HvSSR07-41, respectively. PS-5 contributed additively at these loci.

#### Na<sup>+</sup>/K<sup>+</sup> ratio in root

Total four genomic regions were recognized for Na<sup>+</sup>/K<sup>+</sup> ratio in plant roots at chromosome no. 1, 8, 9 and 12. Out of these, one QTL on chromosome 9 (qNa/KR-9) accounted for 32% of phenotypic variance and co-localized with qNaR-9 in the same marker interval. The QTLs qNa/KR-1 and qNa/KR-12 have been located along with QTL for potassium content in plant roots, explaining 8 and 17% phenotypic variation, respectively. PS-5 added the positive alleles for these three QTLs. The fourth QTL (qNa/KR-8) identified in the marker interval HvSSR08-31 to HvSSR08-45 on chromosome 8 with 3%  $R^2$  value. The tolerant parent CSR10 contributed additively for this locus.

#### K<sup>+</sup> concentration (mmol g<sup>-1</sup> dwt) in shoot

Total four QTLs have been recognized for the potassium concentration of shoot and accounted for 20% of the phenotypic variation cumulatively. The QTL, qKS-1 was mapped in the region of HvSSR01-11–HvSSR01-34 located in the vicinity of QTLs qKR-1, qNa/KR-1 and qNaL-1.1 on chromosome 1. The QTLs, qKS-9 and qKS-11 on chromosome 9 and chromosome 11 co-localized with QTL for chlorophyll content (qCHL-9 and qCHL-11) in the same region. The QTL qKS-12 also mapped in the same region for the proline content QTL (qPRO-12). The PS-5 alleles at qKS-1 and qKS-12 increased K<sup>+</sup> concentration in shoot. While at qKS-9 and qKS-11 locus, CSR10 added the positive alleles.

#### Na<sup>+</sup>/K<sup>+</sup> ratio in shoot

Two QTLs (qNa/KS-4, qNa/KS-7) were identified in shoot for sodium potassium ratio. A significant QTL, qNa/KS-4, was found on chromosome 4 between the markers RM241–HvSSR04-46 and accounted for 12% phenotypic variance. Another QTL, qNa/KS-7 found on chromosome 7 flanked by HvSSR07-37 and HvSSR07-41 with  $R^2$  6%. This loci was located near the QTLs for K<sup>+</sup> concentration in root (qKR-7.1) and salt evaluation score (qSES-7.1). CSR10 allele had an additive effect for this trait at both the loci.

#### Na<sup>+</sup> concentration (mmol g<sup>-1</sup> dwt) leaf

Two QTLs were identified for Na<sup>+</sup> concentration in leaf on chromosomes 1. One QTL (qNaL-1.1) found close to RM3412 that explained 4% phenotypic variance. An

important QTL (qNaL-1.2) was recognized between HvSSR01-56 and HvSSR01-70 and accounted for 45% phenotypic variation in this trait. The additive effect was observed due to sensitive parent PS-5.

#### Na<sup>+</sup>/K<sup>+</sup> ratio in leaf

Total four QTLs were found on chromosome 1 (qNa/KL-1.1, qNa/KL-1.2, qNa/KL-1.3) and chromosome 6 (qNa/KL-6) for the ratio. The QTL qNa/KL-1.1 detected between the markers RM10871 and HvSSR01-52 and accounted for 13% of phenotypic variation. CSR10 contributed the positive alleles for this locus. Other two very significant QTLs on chromosome 1, namely, qNa/KL-1.2 and qNa/KL-1.3 located within region HvSSR01-53 to HvSSR01-70, with a peak  $R^2$  of 32% and 42%, respectively. One more QTL on chromosome 6 (qNa/KL-6) in the marker interval HvSSR06-40–HvSSR06-63, explained the 17% phenotypic variation for Na<sup>+</sup>/K<sup>+</sup> ratio in leaf.

#### QTLs for stress indices

##### SSI grain yield

One significant QTL for SSI grain yield (qSSIGY-6) was identified on chromosome 6 with tolerant alleles contributed by CSR10. It was located in the marker interval HvSSR06-40 and HvSSR06-54 explaining 45% phenotypic variation.

##### STI grain yield

Two QTLs (qSTIGY-2 and qSTIGY-10) were identified on chromosome 2 and 10 for STI grain yield. The QTL, qSTIGY-2 located within the region of QTL for grain yield (qGY-2) on chromosome 2, in the marker interval HvSSR06-80 and RM423 and explained the 4% phenotypic variance for this trait. Another QTL, qSTIGY-10 located in the marker interval HvSSR10-08–HvSSR10-17 within the region of QTL for biomass on chromosome 10, accounted for 7% phenotypic variation.

## Discussion

### Stress response of F<sub>2</sub> population

Achieving the salt resistance for reproductive phase is pivotal for yield determination in rice and hence very important in ensuring the food availability to growing population. But limited efforts has been directed towards reproductive stage salt tolerance (Mohammadi et al. 2013; Ali et al. 2013; Hossain et al. 2015; Bimpong et al. 2014; Krishnamurthy et al. 2014, 2016b) as it involves extensive and laborious

phenotyping work. It is important to study the reproductive stage salt resistance alongside seedling stage, as both are weakly associated with each other (Heenan et al. 1988; Zhu 2002). It pointed towards the involvement of different genes at different stages. The mapping population (PS-5/CSR10) exhibited significant variability at phenotypic level (Pundir et al. 2016). The mean values of most of the characteristics were more skewed towards the lower side. These results obviously showing the harming impacts of salinity on different attributes of rice and already observed in different studies (Flowers and Yeo 1981, 1995; Ismail et al. 2007; Singh et al. 2013, 2014). The  $F_2$  population exhibited wide range of grain yield response in saline environment. It has also been concluded previously that the Germplasm sources with improved salinity tolerance can be identified by selecting the surviving genotypes under extreme stress (Bimpong et al. 2014; Krishnamurthy et al. 2019a, b, c). The distribution of different traits in the  $F_2$  population suggested that PS5 and CSR10 both added the positive alleles indicating the polygenic control of these traits. The grain yield is highly influenced by salty environment for their phenotypic expression as these are controlled by polygenes (Krishnamurthy et al. 2016a, 2017). All the traits except plant height and panicle length deviated from the normal distribution. Some major genes may be responsible for this deviation (Mohammedi et al. 2013; Krishnamurthy et al. 2015, 2016c).

Salinity reduced the chlorophyll in salt sensitive  $F_2$  progenies as well as in sensitive parent PS5, whereas CSR10 and salt resistant progenies possessed the high chlorophyll level. Drying of leaves is first symptom of salinity stress that is due to reduction in chlorophyll. Reduction of chlorophyll and more occurrence of drying of leaves prolonged as salinity stress and intensity are prolonged. (Singh et al. 2012; Azooz et al. 2004; Dagar et al. 2004). Furthermore, CSR10 accumulated less proline than salt sensitive parent PS5. The  $F_2$  progenies showed normal distribution of proline with mean values skewed towards the sensitive parent. Accumulation of the proline in stress environment is a notable change in plants. In rice, the overproduction of proline under salt stress was observed by Lutts et al. (1999), as salt injury symptom instead of measure for salt tolerance

The ionic concentration (Na, K) and salt injury score of the plant provides suitable criteria for evaluation of plants under salinity (Babu et al. 2014; Krishnamurthy et al. 2014, 2016d). In current study, salinity induced an increased the  $\text{Na}^+$  concentration of all tissues, of which the roots of  $F_2$  population accumulated lowest sodium. As a result the Na, K ratio in shoot was thrice as compared to root and leaf tissues. Plants experienced the salt toxicity due to high sodium content. (Kumar et al. 2003). The rice plants tend to maintain low sodium in root zone to cope up with high saline stress. Nonetheless, keeping the low sodium concentration in cytoplasm is very crucial for a plant to survive in salty

environment (Collin et al. 2008). In response to stress, the tolerant parent CSR10 gathered very less sodium in cytosol and identified as salt excluder with low  $\text{Na}^+/\text{K}^+$  ratio. Few  $F_2$  progenies were also expressed salt tolerance as CSR10 (tolerant parent) and could have common salt tolerance mechanism as CSR10. In our study, the highest sodium accumulation and consequently highest Na/K ratio was observed in shoot trailed by leaf and root. High salinity severely affected the potassium content of  $F_2$  plants evidenced from the absence of transgressive segregants in the direction of tolerant parent for potassium concentration and  $\text{Na}^+/\text{K}^+$  ratio in root. This might be because of the absence of a viable mechanism for controlling the  $\text{Na}^+$  ions over the membranes in salt sensitive PS-5 and sensitive  $F_2$  plants. Although high amount of sodium was accumulated in the shoots of plants but few progenies were capable to keep up low  $\text{Na}^+/\text{K}^+$  proportion in shoot than tolerant parent. These transgressive segregants exhibiting salt tolerance were possibly the outcome of the gathering of QTLs conferring salt tolerance from CSR10 (Lang et al. 2000). It may be concluded that the plant leaves maintained high potassium concentration, low sodium and reduced  $\text{Na}^+/\text{K}^+$  ratio in mapping population of PS5  $\times$  CSR10. On the other hand, the shoot accumulated more sodium, and therefore, the  $\text{Na}^+/\text{K}^+$  ratio recorded highest in shoot.

#### QTLs identified at reproductive stage under salinity

It has been perceived in this study that the QTLs for SES score, chlorophyll content, potassium content and  $\text{Na}^+/\text{K}^+$  ratio in root, potassium content in shoot, sodium content and  $\text{Na}^+/\text{K}^+$  ratio in leaf gained the additive effect by PS5 as well as CSR10. The salt-tolerant parent CSR10 exhibited additive effect on the loci for productive tillers, biomass, plant height, grain yield, panicle length, proline content,  $\text{Na}^+/\text{K}^+$  ratio in shoot and stress indices (SSI grain yield and STI grain yield), whereas the additive effects in the QTLs for  $\text{K}^+$  concentration in root was due to PS-5. In this report, novel QTLs were distinguished for yield and salt resistance associated traits at reproductive stage. In this way, detection of novel QTLs for reproductive stage would give a more extensive choice for incorporating salt tolerance. QTLs for both stages into one genetic background by the using gene pyramiding methods. Seedling stage salinity tolerance QTLs (*Saltol*) has been incorporated in various rice varieties, namely, Pusa44, Sarjoo52 (Krishnamurthy et al. 2020; Bhandari et al. 2019; Singh et al. 2016), Pusa Basmati 1121 (Babu et al. 2017), ADT43 (Geetha et al. 2017), Pusa Basmati 1509 (Yadav et al. 2020). Novel high yielding, salt-tolerant varieties can be developed by the combination of genes for different growth stages (Zhu 2002). Only few studies have been reported for QTL mapping for salinity tolerance at reproductive stage (Ammar et al. 2009; Sabouri and Biabani

2009; Mohammadi et al. 2013; Bimpong et al. 2014; Hossain et al. 2015; Kumar et al. 2015; Tiwari et al. 2016, Warraich et al. 2020). In our study at reproductive stage, chromosome 1 displayed a significant role in physiological response. Eight QTLs governed the sodium, potassium and their ratio, in different plant parts, including the QTL for plant height were detected on chromosome 1 of rice. This chromosome has previously been reported to bear plant height QTLs at seedling stage (Claes et al. 1990), as well as for reproductive stage (Mohammadi et al. 2013; Bimpong et al. 2014; Hossain et al. 2015). A gene *sd1* that is known to reduce plant height in rice is also located on chromosome 1 but at the long arm. (Hedden 2003). The clustering of QTLs on short arm of chromosome 1 for sodium–potassium content (qKR-1, qNa/KR-1, qKS-1, qNaL-1.1) can be attributed to the QTLs associated with one introgression segment or due to pleiotropic effect of only one QTL that may be qKR-1 which is solely explaining the 32% of phenotypic variation. These QTLs found in the close proximity (6.6–11.50 Mb) to the position of *Saltol* QTL (11.0–12.0 Mb). This region is also known to harbour SKC1 gene that regulates the potassium homeostasis in salt-tolerant rice lines under salinity and one more gene that code for HKT type transporter. Results from our study also indicated the significance of this region in maintaining the potassium content in root and shoot at reproductive stage. We found QTLs (qNaL-1.2, qNakL-1.2 and qNakL-1.3) with high  $R^2$  value (32–45%) for  $\text{Na}^+$  in leaf and ratio of  $\text{Na}^+$  and  $\text{K}^+$  in leaf on long arm of chromosome 1 in same region (around 31.06 Mb) identified earlier (Hossain et al. 2015) in  $F_2$  population (Cheriviruppu \* Pusa Basmati 1) under reproductive stage salinity stress. Furthermore, the fine mapping of QTLs for potassium on short arm and for sodium on long arm of rice chromosome 1 may give better understanding of the likely physiological process at reproductive stage that underpin  $\text{Na}^+$ ,  $\text{K}^+$  transport and accordingly influence the salt tolerance. In our study, the occurrence of salt tolerance QTLs in *Saltol* region conflicts the earlier studies (Ammar et al. 2009; Mohammadi et al. 2013; Hossain et al. 2015). However, our results are in concordance with the GWAS study by Kumar et al. (2015) in which sodium potassium<sup>+</sup> ratio at reproductive stage reported to be associated with *Saltol* region.

Other chromosomes also possessed the QTLs for ionic parameters. A single QTL for  $\text{Na}^+$  in roots on chromosome 9 with high  $R^2$  value (37%) along with QTLs for root Na, K ratio (qNaKR-9) and potassium shoot concentration (qKS-9) identified in this study. Chromosome 9 have also been reported to bear loci for shoot potassium concentration (Koyama et al. 2001; Ahmadi and Fotokian 2011) and sodium content in roots at seedling stage (Thomson et al. 2010). This demonstrates the significant role of chromosome 9 in sodium potassium homeostasis in roots at reproductive as well as at seedling stage. Fine mapping of this chromosome may

identify the closely linked molecular markers which could be used further in molecular breeding programs. Other QTLs for ionic parameters in different tissues have been located on chromosome 4, 6, 7, 8, 11 and 12. On chromosome 4, a QTL was identified for  $\text{Na}^+/\text{K}^+$  ratio in shoot with 12%  $R^2$ . At seedling stage, Koyama et al. (2001) found QTL for shoot  $\text{Na}^+/\text{K}^+$  ratio and Wang et al. (2012b) for SKC with  $R^2$  9.6% and 18.9%, respectively, on the same chromosome. Out of four QTLs for  $\text{Na}^+$  in leaf, one was co-localized with the significant QTL for SSI grain yield on chromosome 6. In the current study, three QTLs have been identified for potassium content in root and Na, K ratio in shoot at chromosome 7. The QTLs for  $\text{K}^+$  in roots explaining 22% phenotypic variation. For the same trait, one QTL on chromosome 12 explaining 17% phenotypic variation at LOD 5.3 also identified along with QTL for  $\text{Na}^+/\text{K}^+$  ratio in root in the same region, hence these regions on chromosome 7 and 12 seems to play a significant part in maintaining the K concentration in roots along with region on chromosome 1. A very small effect QTL for Na/K ratio in root have been identified on chromosome 8 at LOD 3. At reproductive stage, Ammar et al. (2009) also recognized significant region on chromosome 2 and 8 for sodium and sodium potassium ratio in leaf in CSR27 x MI48 population. Nevertheless, in our study both these regions were devoid of any significant QTLs for ionic parameters. This could be because CSR27 is a tissue tolerant to sodium, whereas CSR 10 is a sodium excluder. Very limited studies have been reported previously for reproductive stage QTLs of ionic parameters. (Ammar et al. 2009; Hossain et al. 2015). Although these QTLs are significant in understanding the mechanism involved in salinity tolerance, however, comparison between the positions of the QTLs associated with ionic concentrations in different tissues is difficult to assess as previously detected ionic QTLs for reproductive stage are located on other chromosomes. In our study highest no. of QTLs were identified for ions in root followed by leaf and shoot. The accumulation of the lowest concentration of  $\text{Na}^+$  in roots followed by leaves and shoot under stress supported these results. Earlier studies suggested that recirculation of sodium from shoots to roots is important for detoxification of sodium (Ren et al. 2005). In this study, the higher accumulation of sodium and potassium ions had been observed in shoot as compared to leaf and root. In rice, important processes of sodium and potassium uptake occur simultaneously rather than in antagonism, hence these processes are considered to be self-governing in plant under salinity (Garcia et al. 1997; Yeo and Flowers 1982; Koyama et al. 2001).

Out of all the agronomic traits, the grain yield is highly important for reproductive stage salinity tolerance; however, it is highly influenced by other yield-contributing traits. The selection of superior genotypes based on agronomic traits and stress indices for grain yield, embarks

distinct significance in devising selection criteria for yield. An important QTL for grain yield along with the QTL for stress tolerance index (STI) were located very closely on chromosome 2 between 31 and 38 Mb region. Another QTL for STI has been located on chromosome 10. Both the QTLs for STI cumulatively explain 13% of phenotypic variation. The salt-tolerant variety CSR10 contributed for grain yield as well as for STI. In a previous study by Tiwari et al. (2016), three QTLs were mapped for SSIGY between 22.4 and 26.8 Mb position on chromosome 2 through BSA approach in CSR27 × MI48 RILs. In the current study, a significant QTL for SSI grain yield co-localized with the QTL for  $\text{Na}^+/\text{K}^+$  ratio in leaf found at chromosome 6 in the region 16.48–24.32 Mb. This region also identified to harbour SSIGY QTLs in CSR27 × MI48 RILs. Previous studies have also demonstrated the potential of chromosome 2 in controlling the agronomic traits. Mohammadi et al. (2013) reported QTLs for grain yield, panicle length and number of fertile spikelets and number of productive tillers in Sadri/FL478 population. Hossain et al. (2015) also identified a loci for grain yield on the this chromosome. The QTL for panicle length explaining 24% phenotypic variation and the QTL for number of productive tillers were been found on chromosome 2. Further fine mapping of this chromosome may lead to the detection of high effect QTLs and further transfer to other varieties. The QTLs for salt evaluation score were identified on chromosome 7 (qSES-7) and chromosome 12 (qSES-12), explaining 12% phenotypic variation cumulatively. In other studies, two QTLs have been identified for SES score on chromosomes 4 and 9 (Thomson et al. 2010) and six other QTLs were found on chromosomes 1, 3, 4 & 5 at seedling stage (Ammar et al. 2009). For SES score, three QTLs, namely, qSES5.2, qSES3.1 and qSES12.3, were also detected in earlier study on chromosome 5, 3 and 12 (Rahman et al. 2019). At reproductive stage, four QTLs for SES score, namely, qSES 1, qSES 6, qSES 10 and qSES 11 found in the rice variety NERICA-L-19 and one QTL (qSES4) was found in the BG90-2 genetic background (Bimpong et al. 2014). The novel QTL qSES-7 mapped in our study for reproductive stage salinity tolerance. We found three QTLs for biomass on chromosome 6, 8, and 10. An important QTL for biomass was identified on chromosome 8 with  $R^2$  of 10% and the increase in biomass at this locus attributed to CSR10. Hossain et al. (2015) also detected the biomass QTLs on chromosome 8 under reproductive stage salinity in  $F_2$  population. In the previous studies, chromosome 3 has known to bear significant QTL but no significant marker trait association has been found at this chromosome in the present study. It might be because of utilization of different genetic backgrounds in the different studies.

At the biochemical level, chlorophyll and proline content were utilized for the assessment of salt resistance. Munns and Tester (2008) reported that sodium accumulation in

leaf tissues and leaf fading can be observed under salinity due to osmotic stress. Consequently, chlorophyll content could be utilized as a indicator for salt resistance. Numerous QTLs have been recognized for chlorophyll content for seedling stage (Zang et al. 2008; Thomson et al. 2010; De Leon et al. 2016) but at reproductive stage the QTLs for chlorophyll and proline have never been investigated under salinity. We found four QTLs for chlorophyll explaining 21% phenotypic variation cumulatively and one QTL for proline content with  $R^2$ - 7% in leaf tissue above LOD 3. Among the four QTLs for chlorophyll content, the additive effect for two QTLs on chromosome 9 and 11 was due to sensitive parent PS-5, which accounted for 13% of phenotypic variation, whereas for other two QTLs on chromosome 5 and 7, the tolerant parent CSR10 added positive alleles and explained 8% of phenotypic variation. It indicated that, trait is controlled by many genes of small effect. No major QTL have been found for chlorophyll content may be a reason for severe impact of salinity on chlorophyll in this population. The QTLs for chlorophyll on chromosome 9 and 11 have been co-localized with QTLs for  $\text{K}^+$  in shoot showing that chlorophyll biosynthesis may have some cross converses with  $\text{K}^+$  level in shoots to combat the salty environment. These outcomes are in concordance with prior reports that showed the diminishing impact of salinity on chlorophyll (Khan et al. 2015; Nounjan et al. 2016, 2018).

## Conclusion

It can be inferred from our study that the genomic region on chromosome 1, 2 and 9 primarily influenced the salt resistance through altering the Na uptake and consequently affected the sodium potassium ratio in leaf and root and eventually the grain yield. The QTLs for SSI and STI may give new insight for selection of tolerant plants at very early stage. Co-localization of few QTLs for different traits in same region suggested their linked contribution. It is necessary to validate these QTLs in immortal mapping populations (RILs, BILs) and fine map the QTLs to use them in MABB to develop NILs. Identification of the underlying genes for these QTLs would help in understanding the mechanism for evoking the salt resistance. This could help in the development of reproductive stage salinity tolerance in rice. Pyramiding of these QTLs through molecular breeding would be advantageous for development of high yielding salt-tolerant new rice varieties in less time.

**Author contribution statement** KMSL, PCS, and AD conceptualize and planned the experiment, KMSL and PP conducted the experiments, PP isolated DNA and did molecular screening, KMSL, PP, and NMV analysed of data and



manuscript was prepared by PP, SLK, and NMV. Revision of manuscript was done by KMSL and PCS.

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## Compliance with ethical standards

**Conflict of interest** The authors declare no conflict of interest.

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