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Genetic basis of drought tolerance in Iranian rice (*Oryza sativa* L.) recombinant lines at vegetative and reproductive stages

Original paper

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Abstract: To evaluate the genetic basis of drought tolerance in rice, an experiment was conducted using 120 recombinant inbred lines (RILs) derived from Neda × Ahlamitarom cross. A factorial experiment based on completely randomized design with three replications was used under greenhouse conditions setting at Gonbad Kavous University. In this study, Polyethylene Glycol (PEG) 6000 was employed to induce osmotic stress (at levels of −4.5 and −9 bar) during both vegetative and reproductive stages. In addition to assessing root and shoot morphological characters, genetic linkage map was constructed using Simple Sequence Repeat (SSR), Inter Primer Binding Site (iPBS), Inter-Retrotransposon Amplified Polymorphism (IRAP), and Inter Simple Sequence Repeats (ISSR) markers. Sixteen Quantitative Trait Loci (QTLs) were identified during vegetative growth stage, while twenty QTLs were identified during the reproductive stage. Through a comparative analysis of the three evaluated treatments, the qRN-12, qRS-11, and qRV-12 lines were determined as stable QTLs suitable for the selection of drought-tolerant lines at the vegetative stage under varying conditions. Several new alleles associated with drought-tolerant QTLs were identified in this study. Notably, in two distinct environmental conditions, crucial QTLs, such as qNTF-12 and qNL-3, related to the numbers of fertile tillers and leaves, were identified as stable QTLs at the reproductive stage. The QTLs identified at vegetative and reproductive stages in this study can serve as stable and major QTLs for selecting drought-tolerant lines in marker-assisted selection (MAS).

Keywords: major QTL, osmotic stress, quantitative trait loci, rice.

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Introduction

Rice (*Oryza sativa* L.) is a staple food consumed regularly and plays a crucial role in ensuring the food security over half the world's population. Global rice production is projected to increase significantly, with estimates ranging from 58 to 567 million tons (Mt) by 2030 (Mohidem et al., 2022). Rice constitutes the primary food source for overhalf of the population in 60% of the world (Mohidem et al., 2022), with its derivatives contributing to 70% of their energy requirements.

Drought stands out as the major environmental constraint to rice production and yield stability in rainfed areas. Asia emerged as the most drought-affected region from 2003 to 2013, experiencing a 40% of total crop and livestock production resulting in losses totaling 28 billion USD (Rajurkar et al., 2021). The imperative to improve rice adaptation to drought and drought- resilient varieties is growing in significance, especially in light of the diminishing agricultural water resources worldwide (Pandey and Shukla, 2015). Drought is the foremost environmental factor that often negatively affects cereal yield. Therefore, improving drought tolerance becomes a crucial objective in cereal breeding programs (Aliyu et al., 2011; Giasi Oskoei et al., 2014). As outlined by Kramer and Boyer (1995), water scarcity can manifest during the early growing season or at any point from flowering to the grain filling, with stress severity contingent upon the duration and frequency of stress occurrences. A study indicates that stress application causes changes in plant roots, promoting increased water absorption. During drought stress exposure, water is typically scarce in the soil surface layer, prompting the the main plant root to grow deeper to cope with drought stress and enhance water absorption. On the other hand, lateral root development is inhibited under drought stress (Zhang et al., 2010).

The detrimental effect of drought stress is directly associated with reduced biomass production (Ji et al., 2012). Drought also caused significant reductions in seedling wet and dry weights. Jongdee (2001) observed that drought stress reduced leaf development and tillering, thereby lowering the photosynthetic rate and leaf surface area because of early aging. All these factors reduce grain yield in drought conditions (Price and Courtois, 1999; Fischer et al., 2012). Drought stress inflicts irreparable damage to rice at both vegetative and reproductive stages. Furthermore,, the genotype × environment (GE) relationship is a challenge for producers and has been reported to to hinder progress in quantitative trait selection. Due to the GE relationship, QTL that are important in an environment may not be important in phenotype determination in another environment (Sarayloo et al., 2015; Shirmohammadli et al., 2018). Accordingly, QTLs showing the quantitative GE relationship among a series of environments were reported to be appropriate in MAS programs (Zhang et al., 2010; Noryan et al., 2021). Three QTLs associated with yield, biomass, and reduced harvest index under drought stress were identified in pot experiments conducted over two consecutive years (Wang et al., 2007).

Multiple QTLs have been reported for rice yield and yield components under a variety of drought stress (Lilley et al., 1996; Price and Courtois, 1999; MacMillan et al., 2006). Drought tolerance is a complicated feature associated with many genes affected by the responses of interested components, which may be different in interaction with types, severity, and duration of water shortage. Furthermore, most crop traits are expressed differently in normal and stressful conditions and are influenced by environmental factors (Lilley et al., 1996; Aliyu et al., 2011; Babu et al., 2014). Until recently, breeding programs have focused on above-ground traits and direct selection for yield per se, while the crop's "hidden-half," i.e., the roots have been largely overlooked. Plant roots are important organs in determining grain productivity driven by water uptake and nutrient acquisition (Alahmad et al., 2019).

A qLA-4 for seed toxicity on chromosome 4 in a RIL produced from IR26/Jiucaiqing in drought stress and non-stress conditions detected (Price and Courtois, 1999; Zhang et al., 2010). Five stable QTLs among 24 cases were identified for drought-related traits at the reproductive stage of rice under the stress of PEG and flooded conditions (Sabouri, 2007).

Wang et al. (2005) identified stress-tolerant QTLs in rice mainly at the reproductive stage. However, limited reports are available on stress-tolerant QTLs

at the germination stage of rice seeds. MacMillan et al. (2006) found Six QTLs for root traits of rice in four different treatments. Most of the QTLs were the same for a single trait in four different environments. QTLs were reported for rice yield and yield components under various types of drought stress in numerous experiments. Babu et al. (2014) identified five QTLs for grain yield in a population of two CT9993/IR62266 varieties under drought stress in southern India.

The rise in abiotic stresses such as drought, salinity, and submergence significantly hinders increases in rice. Developing a rice variety with inherent tolerance against these major abiotic stresses will help achieve a sustained increase in rice production under unfavorable conditions (Muthu et al., 2020). The current study aimed to evaluate the drought tolerance of two genotypes derived from Neda × Ahlamitarom varieties, identify drought tolerance-related QTLs, and pinpoint the locations of molecular markers linked to QTLs controlling rice traits at vegetative and reproductive stages in drought stress and normal conditions.

Materials and Methods

Phenotypic evaluation

In this study, the plant material was 120 F_8 RILs derived from Neda × Ahlamitarom varieties (Ahlamitarum and Neda cultivars are tolerant and sensitive to salinity, drought stress, and deficiencies of some mineral elements. These cultivars were selected to undergo different biotic and abiotic stresses during different breeding programs of the Iranian *O. sativa* germplasm at Gonbad Kavous University.) to evaluate root and seedling morphological characters under osmotic stress treatment at the vegetative and reproductive stages. In the present study, two independent trials were carried out as follows. In the first experiment, the stress was applied until the end of the vegetative stage and before the reproductive stage, so the plants were harvested and the traits were recorded. In the second experiment, the plants were subjected to stress from the maximum tillering stage until ripening. Both experiments were conducted using a factorial experiment based on the completely randomized design with three replications in the greenhouse of Gonbad Kavous University. The two factors included in this study were lines and stress status.

During the vegetative stage, the grains of experimental lines initially germinated in Petri dishes. Subsequently, germinated seedlings were transferred to pots with 40 cm height and 30 cm diameter, with three seedlings planted per pot. Each line was evaluated in three replications of three treatments, control (EC = 0.850 dS/m) and two drought treatments (−4.5 and −9 bar). Osmotic stress was applied using a solution of water and PEG 6000. The PEG concentration used to produce the required potential was obtained from Eq. (Michel and Kaufmann, 1973).

$$\varphi = -(1.18 \times 10\text{-}2)\, C - (1.18 \times 10\text{-}4)\, C2 + (2.67 \times 10\text{-}4)\, CT + (8.39 \times 10\text{-}7)\, C2T$$

In this equation, φ is the osmotic potential, C is PEG 6000 concentration (g/l), and T is the temperature (°C).

During the reproductive stage, the seeds of experimental lines initially germinated in Petri dishes. Subsequently, germinated seedlings were transferred to pots, three seedlings planted per pot. Each line was evaluated in three replications of two treatments, control (EC = 0.850 dS/m) and drought treatments (−9 bar).

Both treatments underwent nutritional operations and the control of pests and diseases. Each pot was fed with 1 g of a rice-specific complete fertilizer (N:15%, P_2O_5 15%, K_2O 30%, Mg 1%, and Fe 1 mg with chelated Fe) three times at the beginning of growth, 3 weeks after planting seedlings, and the beginning of heading. Diazinon 10% G (1 g per pot) was used for pest control. Other crop operations, including fertilizing, weed control, and pest/disease control, were carried out at appropriate times. Plant morphologic traits were recorded from the beginning of the reproductive period. The studied traits were stem diameter, total panicle weight, flag leaf width, flag leaf length, fertile tiller number, total tiller number, panicle length, stem wet weight, root wet weight, root volume, drought number, root area, root area density in both stress and non-stress environments (Golesorkhy et al., 2016). Plant leaf chlorophyll content was measured three times by a chlorophyll content meter (CL-01). To compare osmotic stress tolerance in the lines, phenotypic scores were recorded after the application of stress conditions (Lanceras et al., 2004) every 4 days until

harvest time (Table 1). Final firing symptoms for leaf drought resistance at the vegetative stage (Loresto, 1981) were used for determination of plant reaction against osmotic stress. Collected data were analyzed using SAS Ver. 9.1 software.

Genotypic evaluation

DNA was extracted from fresh leaves of both lines and parents using the CTAB method (Saghai Maroof et al., 1994). SSR markers on chromosome 12 were specified from the related website (http://www.gramene.org). The polymerase chain reaction (PCR) at a volume of 10 µl was performed for each DNA sample (Zahedi et al., 2019). For this test, 2 µl of diluted genomic DNA was first partitioned in each PCR tube. Then, 8 µl of the PCR solution (other than genomic DNA) was added to each tube and shaken gently. To prepare the reaction mixture, double-distilled water, a stock solution (containing 0.04 unit/µl of *Taq* DNA polymerase, PCR buffer at 1xfinal concentration, 50 mM of $MgCl_2$, 10 mM of dNTPs, and 0.5-0.75 ng of diluted DNA), and 60 ng of each of forward and reverse primers were added to a 1.5 ml microtube. The reaction mixture was then centrifuged and partitioned in DNA-containing tubes. Finally, 4 µl of inorganic oil was poured into each tube to prevent the evaporation of contents. PCR tubes were placed in a thermocycler (iCycler, BIORAD, USA). Temperature cycles for SSR markers included initial denaturation (at 95 °C for 2.5 min), 35 cycles with denaturation (at 95 °C for 1 min), primer annealing at their specific temperatures (for 30 sec), elongation (at 72 °C for 30 sec), and final amplification (at 72 °C for 5 min). Temperature cycles for ISSR, iPBS and IRAP markers consisted of initial denaturation (at 95 °C for 5 min), denaturation (at 95 °C for 1 min), 10 cycles of primer annealing (at 42-54 °C for 1 min), elongation (at 72 °C for 1 min), 25 cycles of primer annealing at their specific temperatures (for 45 sec), and final amplification (at 72 °C for 5 min). PCR products were separated using 6% denaturing polyacrylamide gel electrophoresis. The bands were visualized by the rapid silver staining (Caetano-Anollés and Gresshoff, 1994).

Linkage map and analysis of QTLs

A genetic map obtained from SSR and ISSR markers was produced using QTXB17 Map Manager software (Shirmohammadli et al., 2018). The genetic map was constructed by genotyping 120 genotypes of the F_8 generation from the Neda and Ahlamitarom varieties using 30 SSR (McCouch, 1997) and 15 ISSR (Mohd Ikmal et al., 2021; Noryan et al., 2021) markers. An expected ratio of 1:1 was created for the segregation of tested lines for the employed markers using the χ^2 test by Map Manager Software. To assign each marker to the relevant chromosome, the obtained linkage groups were compared with genetic maps proposed Chen et al. (2007) and McCouch (1997). The recombinant ratios between the markers were converted to the map unit (cM) using a previous map function (Chen et al., 2007). QTLs were detected using composite interval mapping (CIM) with a LOD score threshold of 3.0 using QTL Cartographer (Noryan et al., 2021).

Table 1. Scores and firing symptoms for leaf drought resistance at the vegetative stage.

Scores	Reaction	Leaf firing
0	Highly resistant	No symptoms of stress
1	Resistant	Slight leaf tip drying
3	Moderately resistant	Leaf tip drying extended to ¼ in the top three leaves
5	Intermediate	Half of the younger leaf blades dried, and all lower leaves dried
7	Susceptible	¾ of younger leaf blade dried
9	Highly susceptible	All leaves dried

Results

ANOVA and correlations at the vegetative stage

The results of analysis of variance (ANOVA) at the vegetative stage under flooded conditions and osmotic stress levels of −4.5 and −9 bar indicated the significance of all studied traits at the 1% level (Table 2).

The maximum and minimum coefficients of variation (CV) for root volume (49.354) and root area (1.636), respectively, belonged to the −9 bar level. Different reactions of rice genotypes under osmotic stress were also investigated by some other researchers reported that most of the traits studied in osmotic stress and temperature conditions showed significant differences at the 1% level in ANOVA, indicating variation between studied lines for the measured traits (Lanceras et al., 2004; Iravani et al., 2008).

Table 2. Analysis of variance of the examined traits at the vegetative stage under normal and osmotic stress conditions.

Source of variation	df	Mean square					
		Chlorophyll content (mg/gr)	Stem length (cm)	Root length (cm)	Root number (cm)	Leaf length (cm)	Leaf width (cm)
Normal							
Line	119	0.058**	18.713**	11.694**	5.181**	31.564**	0.771**
Error	240	0.013	1.984	1.996	1.320	19.797	0.168
CV		44.517	7.410	15.699	17.818	30.527	13.471
-4.5 bar stress							
Line	119	0.252**	20.985**	11.213**	5.130**	21.852**	0.834**
Error	240	0.174	3.044	2.048	1.594	11.329	0.253
CV		25.998	8.798	15.394	18.834	22.296	19.199
-9 bar stress							
Line	119	0.361	56.622**	13.629**	6.645**	10.468**	0.581**
Error	240	0.332	45.487	2.123	0.895	1.738	0.0798
CV		17.536	37.119	16.013	17.307	9.571	13.519

Source of variation	df	Mean square						
		Stem wet weight	Root wet weight	Root volume	Drought number	Root area	Root area	Root area density
Normal								
line	119	0.001**	0.000**	0.001**	6.557**	0.027**	0.001**	1.020**
Error	240	0.000	0.000	0.000	0.022	0.006	0.000	0.164
CV		11.763	4.609	27.038	3.752	3.786	14.804	17.096
-4.5 bar stress								
Line	119	0.000**	0.000**	0.001**	10.105**	0.011**	0.000**	0.997**
Error	240	0.000	0.000	0.000	0.222	0.002	0.000	0.098
CV		12.233	4.835	6.908	7.834	2.375	7.549	8.222
-9 bar stress								
Line	119	0.000**	0.000**	0.0008**	6.595**	0.006**	0.000**	1.109**
Error	240	0.000	0.000	0.000	0.011	0.001	0.000	0.220
CV		33.607	6.166	49.354	4.299	1.636	20.291	23.723

*,**Significant at p = 0.05 and 0.01, respectively.

Baraty et al. (2014) found significant differences at the 1% level for the number of tillers, number of nodes, plant height, panicle length, awn length, flag leaf length, peduncle length, number of rows, 1000-grain weight, and yield between lines. Similarly, our data showed that the lines were significantly different for all traits at the 1% level, except for plant height, which corresponds to the aforementioned study, except for plant height.

Linkage map

The linkage map prepared based on 30 SSR and 15 ISSR markers (with 60 replicated polymorphic alleles) on 120 individuals of the F_8 population assigned the markers to 12 linkage groups belonging to 12 rice chromosomes with a map distance based on the Kosambi (1944) function equal to 1411.3 cM and an interval of 15.34 cM between two flanking markers. Maximum (156.3 cM) and minimum (81.3 cM) linkage lengths were determined on chromosomes 11 and 10, respectively.

QTLs identified under osmotic stress and normal conditions at the vegetative stage

Normal conditions

One QTL for the root number was located at the IRAP-5–RM111 marker distance on chromosome 12, with a LOD of 2.842, and could explain 10.3% of phenotypic changes. Our estimation revealed that the reducing additive effect was inherited from the Ahlamitarom. For the root area trait, one QTL was also found on chromosome 11 at the IRAP23-1 - IRAP23-7 genomic distance. qRS-11 was determined as a large-effect QTL with a coefficient of determination (R^2) of 13.

-4.5 bar stress conditions

To explain changes in the chlorophyll content trait, one QTL was identified on chromosome 2. An R^2 of 14.5 was obtained for qCL-2. The study of the additive effect of this QTL revealed that chlorophyll content was reduced by the Ahlamitarom alleles. For the stem length trait, three QTLs were located at ISSR21-2 – ISSR4-3, ISSR37-3 – ISSR8-6, and ISSR29-2 - IRAP17-1 genomic distances on chromosomes 5, 6, and 7. qSL-7 was detected as a large-effect QTL with a greater R^2 (13.4%) than the other QTLs. For the root volume trait, one QTL was located on chromosome 12 at ISSR15-3 - RM12 genomic distances. The additive effect and R^2 for this QTL were 0.01 and 13.6%, respectively. The positive additive effect and Neda alleles caused an increase in this trait. For the root diameter trait, two QTLs with additive effects were determined on chromosomes 2 and 5. Neda parental alleles contributed to the elevated root diameter trait. R^2 values for these two QTLs were 12.9% and 14%, respectively.

–9 bar stress conditions

For the traits evaluated under osmotic stress, seven QTL-containing distances were identified that controlled five traits (Table 3). Two QTLs for the root number were located on chromosome 12 in 50 and 32 positions at ISSR30-1 – RM277 and ISSR15-3 – RM12 genomic distances, respectively. The reducing additive effect for this trait was derived from the Ahlamitarom. R^2 values for these two QTLs were 12.8% and 13.2%, respectively.

For root wet weight, one QTL was recognized on chromosome 8 at the iPBS2074-3 – IRAP-4 genomic distance with an R^2 of 13.5%. For the root volume trait, two QTLs were identified on chromosome 12 in 32 and 50 positions at ISSR15-3 – RM12 and RM277 – IRAP-1 genomic regions, with R^2 values of 15.1% and 13.7%, respectively. For the root area trait, one QTL was also found on chromosome 11 at the IRAP23-1 - IRAP23-7 genomic region. The additive effect for this QTL was derived from the Neda. For the root diameter trait, one QTL was determined on chromosome 12 at the RM277 – IRAP-1 genomic distance with an R^2 value of 12.9%.

The descriptive statistical analysis of data in normal and low-irrigation conditions indicated the presence of variance required for multivariate analyses. The results showed the normal distribution of all evaluated traits. The average values of all traits are represented in Table 3.

Reproductive stage

Before any analysis, Bartlett's test results indicated the homogeneity of the test error variance. Accordingly, the results of ANOVA and correlation for the traits in osmotic stress and normal conditions are presented in Tables 4 and 5, respectively. According to the results, all evaluated traits were significantly different at the 1% level in the two experimental conditions at the reproductive stage.

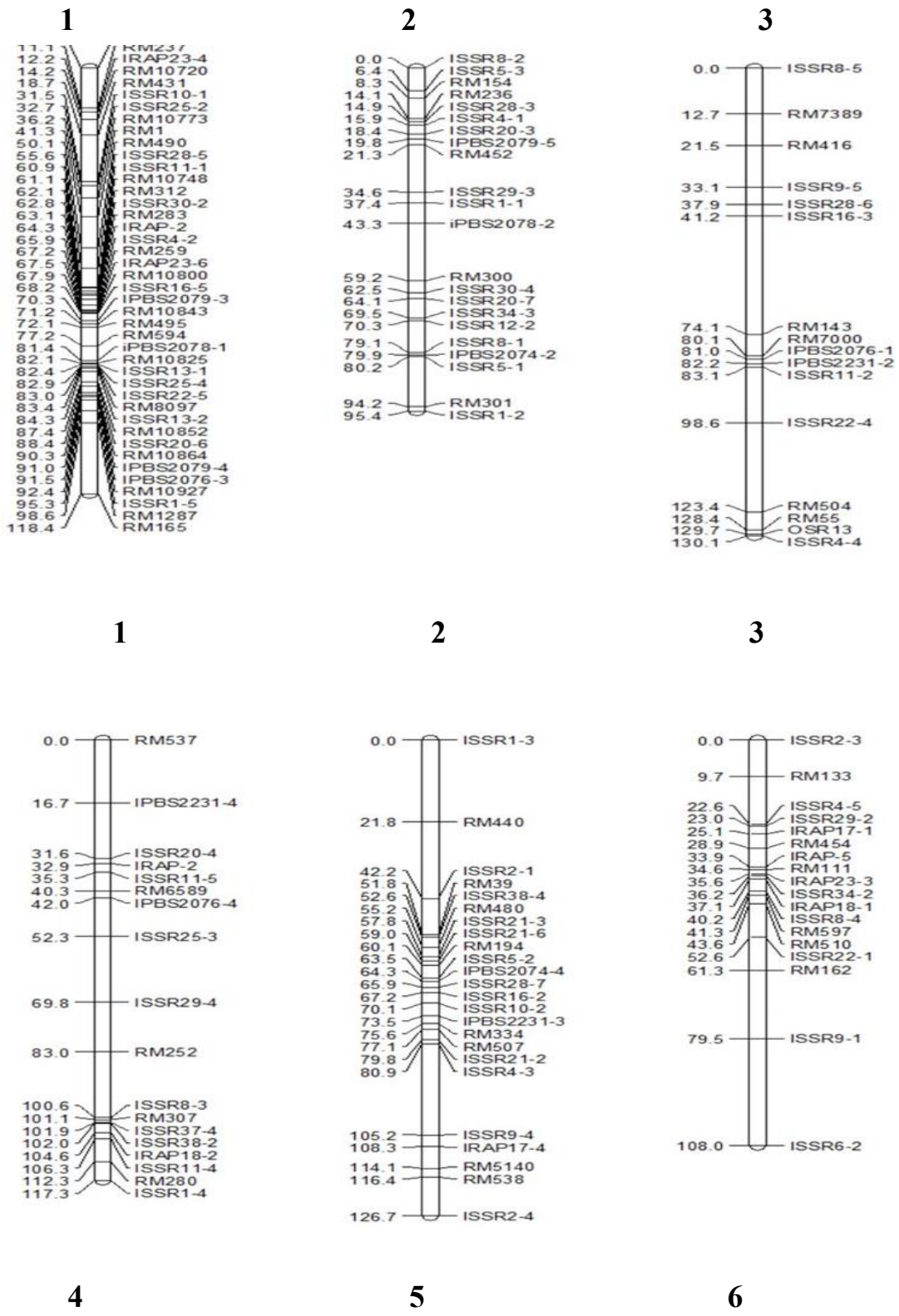
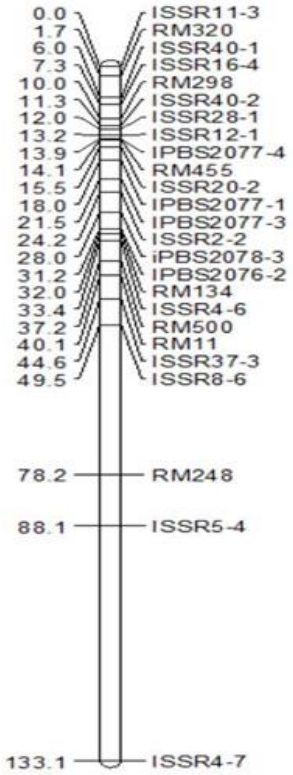


Figure 1. SSR, ISSR, iPBS and IRAP linkage map in the F8 population resulting from the crossing of Taremmahali and Neda. The names of the markers are shown on the right side of the linkage groups and the genetic distance between the markers based on the Kosambi (1944) function is shown on the left side.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11.3 ISSR40-2
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13.2 ISSR12-1
13.9 IPBS2077-4
14.1 RM455
15.5 ISSR20-2
18.0 IPBS2077-1
21.5 IPBS2077-3
24.2 ISSR2-2
28.0 iPBS2078-3
31.2 IPBS2076-2
32.0 RM134
33.4 ISSR4-6
37.2 RM500
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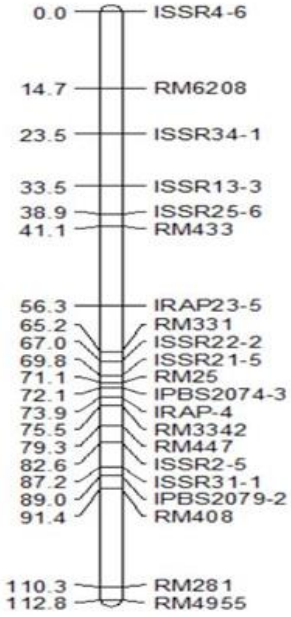
49.5 ISSR8-6
78.2 RM248
88.1 ISSR5-4
133.1 ISSR4-7

7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38.9 ISSR25-6
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65.2 RM331
67.0 ISSR22-2
69.8 ISSR21-5
71.1 RM25
72.1 IPBS2074-3
73.9 IRAP-4
75.5 RM3342
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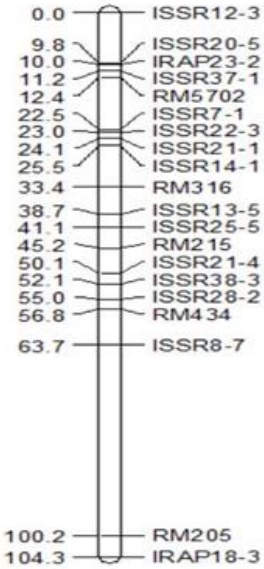
8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33.4 RM316
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41.1 ISSR25-5
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50.1 ISSR21-4
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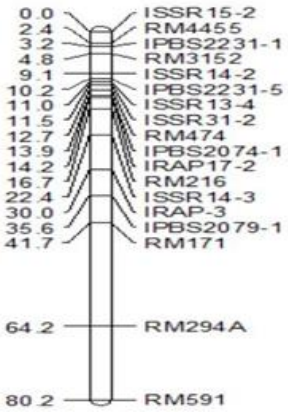
9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12.7 RM474
13.9 IPBS2074-1
14.2 IRAP17-2
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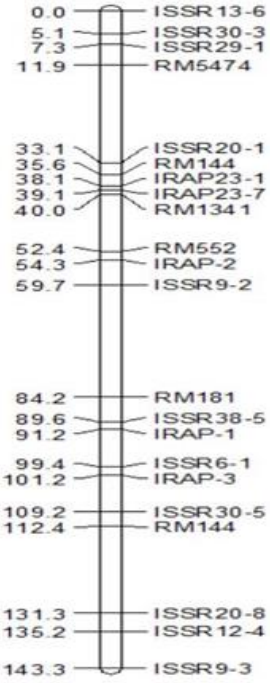
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59.7 ISSR9-2
84.2 RM181
89.6 ISSR38-5
91.2 IRAP-1
99.4 ISSR6-1
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135.2 ISSR12-4
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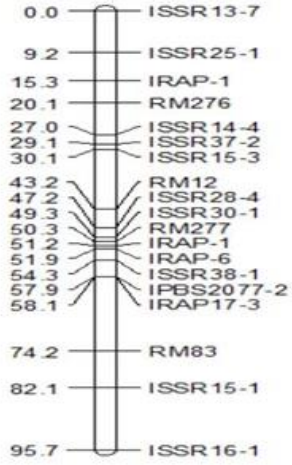
11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47.2 ISSR28-4
49.3 ISSR30-1
50.3 RM277
51.2 IRAP-1
51.9 IRAP-6
54.3 ISSR38-1
57.9 IPBS2077-2
58.1 IRAP17-3
74.2 RM83
82.1 ISSR15-1
95.7 ISSR16-1

12

Table 3. Identified QTLs for investigated traits at the vegetative stage under normal and osmotic stress conditions (-4.5 and -9 bar stress).

Trait	QTL	Chromosome	Position (cM)	Flanking markers	LOD	Additive effect	R^2	Allele direction
Normal								
Root number	qRN-12	12	50	ISSR30-1 – RM277	2.842	-0.463	12.5	Ahlamitarom
Root area	qRS-11	11	76	IRAP23-1 – IRAP23-7	2.85	-0.248	13	Neda
-4.5 bar stress								
Chlorophyll content	qCL-2	2	80	iPBS2074-2 – ISSR5-1	3.468	-44.424	14.5	Ahlamitarom
Stem length	qSL-6	6	24	ISSR29-2 - IRAP17-1	3.302	45.18	12	Neda
	qSL-7	7	48	ISSR37-3 – ISSR8-6	2.841	-1.98	13.4	Ahlamitarom
	qSL-5	5	80	ISSR21-2 – ISSR4-3	2.746	-4.692	12	Ahlamitarom
Root volume	qRV-12	12	32	ISSR15-3 – RM12	2.950	0.01	13.6	Ahlamitarom
Root diameter	qRD-5	5	52	ISSR38-4 – RM480	2.913	0.003	14	Neda
	qRD-2	12	6	ISSR8-2 – ISSR5-3	2.841	0.039	12.9	Neda
-9 bar stress								
Root number	qRN-12	12	50	ISSR30-1 – RM277	2.712	-0.586	12.8	Ahlamitarom
	qRN-12	12	32	ISSR15-3 – RM12	2.315	-0.695	13.2	Ahlamitarom
Root wet weight	qRFW-8	8	72	iPBS2074-3 – IRAP-4	2.123	0.028	13.5	Neda
Root volume	qRV-12	12	32	ISSR15-3 – RM12	2.58	-0.007	15.1	Ahlamitarom
	qRV-12	12	50	RM277 – IRAP-1	2.068	-0.006	13.7	Ahlamitarom
Root area	qRL-11	11	38	IRAP23-1 – IRAP23-7	2.189	0.322	12.7	Neda
Root diameter	qRD-12	12	50	RM277 – IRAP-1	2.042	-0.006	12.9	Ahlamitarom

The statistically significant differences between the genotypes represent a high variance between the evaluated plant material and probably their different mechanisms in response to osmotic stress, which can be used in good genotype selection. The highest correlation (0.393**) was observed between the filled grain weight and flag leaf length under normal and stress conditions. Leaf area was positively and significantly correlated with total panicle weight (0.380**), stem diameter (0.265**), flag leaf length (0.275), and filled grain weight (0.356**). Positive and significant correlations were also observed between the number of fertile tillers, the number of total tillers (0.322**), and the number

of fertile tillers (0.388**). The main panicle weight was positively and significantly correlated with total panicle weight (0.319**) and full grain weight (0.334**). There were also positive and significant correlations between root dry weight (0.261**), leaf number (0.306**), and leaf area (0.379**).

QTLs identified under osmotic stress and normal conditions at the reproductive stage

The results of QTLs analyzed under osmotic stress and normal conditions are shown in Table 6.

One QTL was located for the number of total tiller trait on chromosome 12 in normal conditions, and 15.6% of the total phenotypic changes in this trait was explained by qNTT-12, which was positively affected by the Neda alleles.

In stress conditions, one QTL was determined for the number of fertile tiller trait on chromosome 12. qNTF-12 was recognized in similar stress and normal conditions and showed more effect in stress conditions. Similar QTLs at the ISSR16-1-ISSR15 distance could explain 12.7% and 12.8% of the phenotypic variance of these traits under stress and normal conditions. The QTLs also represented a dominant effect. One QTL was identified for the panicle weight trait on chromosome 6 in normal conditions. qLSP-6 was recognized with LOD 2.12 and 12.9% phenotypic variance. This trait was increased by the Neda alleles.

Table 4. Analysis of the variance of evaluated traits under normal and drought stress conditions at the reproductive stage.

Mean square							df	Source of variation
Stem diameter	Total panicle weight	Flag leaf width	Flag leaf length	Fertile tiller number	Total tiller number	Panicle length		
Normal								
1.654**	3.870**	12.321**	85.153**	2.360**	3.433**	74.332**	119	Line
0.191	1.769	0.631	2.089	0.394	39.359	26.927	240	Error
10.500	6.325	10.709	6.684	19.184	17.028	5.454		CV
Drought								
0.705**	21/752**	4.411**	86.868**	2.628**	0.818**	18.524	119	Line
0.115	2.645	0.421	3.886	0.460	0.764	25.055	240	Error
10.794	7.804	9.473	9.657	20.024	2557.1	5.739		CV

Continiued Table 4. Analysis of the variance of evaluated traits under normal and drought stress conditions at the reproductive stage

Mean square								df	Source of variation
Root dry weight	Leaf area	Filled grain weight	Number of filled grain	Main panicle weight	Total weight of panicles	Plant dry weight	Number of leaves		
Normal									
7.906**	55.687**	0.442	767.996**	0.414**	3.893**	10.477**	1.089**	119	Line
0.127	4.291	0.036	6.515	0.002	0.508	0.687	0.246	240	Error
8.688	17.422	24.066	23.602	4.673	27.573	15.764	10.936		CV
Drought									
7.337**	25.380**	3.219**	703.002**	0.411**	2.374**	13.436**	1.144**	119	Line
0.133	2.360	0.014	40.558	0.002	0.286	1.251	0.260	240	Error
7.740	14.883	11.455	31.040	6.701	32.680	21.254	9.896		CV

*,**Significant at p = 0.05 and 0.01, respectively.

Table 6. Putative QTLs for reproductive traits in F8 population under osmotic stress and non-stress conditions.

Trait	QTL	Markers interval [a]	Chr.	Position (cM) [b]	LOD	Additive effect(A)	R^2 (%)[c]	DPE[d]
Non-stress								
Total number of tillers	qNTT-12	ISSR15-1-ISSR16-1	12	82	2.99	-2.072	15.6	Neda
Number of fertile tillers	qNTF-12	ISSR16-1-ISSR15-1	12	110	2.13	-0.38	12.7	Ahlamitarom
Panicle length	qLSP-6	IRAP-5 – RM454	6	32	2.12	-0.022	12.9	Neda
Main panicle weight	qWSPM-8	ISSR4-6-RM6208	8	8	2.18	-0.18	13	Ahlamitarom
Number of leaves	qNL-3	ISSR11-2–ISSR22-4	3	86	2.428	0.156	12.9	Neda
Root dry weight	qWDR-5	ISSR21-6 – ISSR21-3	5	58	2.288	-0.988	13.2	Ahlamitarom
	qWDR-9	ISSR28-2 – ISSR38-3	9	54	2.708	-0.359	14.8	Neda
Osmotic stress								
Flag leaf length	qLLF-1	RM165- RM1287	1	112	2.328	0.117	13	Neda
Number of fertile tillers	qNTF-12	ISSR16-1-ISSR15-1	12	76	2.434	-0.444	12.8	Ahlamitarom
Flag leaf width	qWLF-8	ISSR4-6-RM6208	8	2	2.23	-0.62	13.2	Ahlamitarom
Stem diameter	qSTD-11	ISSR20-8-ISSR12-4	11	134	2.121	-0.464	11.8	Neda
Number of leaves	qNL-3	ISSR11-2–ISSR22-4	3	62	2.428	-0.958	12.5	Ahlamitarom
Plant dry weight	qWDP-8	RM447 – ISSR2-5	8	82	2.686	0.191	11.3	Neda
	qWDP-1	IRAP2-RM283	1	64	2.157	1.153	10.8	Neda
Total weight of panicles	qWTS-12a	ISSR15-1 – ISSR16-1	12	72	3.254	-0.113	13.1	Neda
	qWTS-12b	IRAP17-3-RM83	12	64	2.617	-0.804	12	Neda
	qWTS-2	RM154-RM236	2	12	2.115	-0.361	11.5	Neda
Filled grain weight	qWGRF-9	ISSR22-3 – ISSR21-1	9	24	2.123	-2.28	13.2	Neda
Leaf area	qLA-1	RM1287-RM165	1	110	2.481	1.446	11	Neda
	qLA-8	ISSR4-6-RM6208	8	0	2.371	-1.757	11.7	Ahlamitarom

[a] Underlined markers are more closer to QTL; [b] QTL position from the nearest flanking marker (cM); [c] Phenotypic variance explained by each QTL. [d] DPE, Direction of phenotypic effect.

In normal conditions, one QTL was detected for the main panicle weight trait on chromosome 8. qWSPM-8 represented 13% of the total phenotypic variance. Neda alleles positively influenced qGN13-8. In stress conditions, one QTL was identified for the number of leaves trait on chromosome 3. In normal conditions, qNL-3 between ISSR11-2 and ISSR22-4 on chromosome 3 could explain 12.9% of the total phenotypic variance. The additive allele of these QTLs could be explained by the Ahlamitarom variety.

In normal conditions, two QTLs for root dry weight were determined on chromosomes 5 (ISSR21-6 - ISSR21-3) and 9 (ISSR28-2 - ISSR38-3), representing 13.2% and 14.8% of the total phenotypic variance, respectively. This trait was increased by Ahlamitarom alleles on chromosome 5 while Neda alleles on chromosome 9 reduced this trait. In stress conditions, one QTL was located for the flag leaf length trait on chromosome 1. The phenotypic variance of this trait (13%) was explained by qLLF-1 with a loading level of 2.328. This increase was mediated by the Neda parental effect. In osmotic stress conditions, one QTL was recognized for the flag leaf width trait on chromosome 8 (ISSR4-6-RM6208), which explained 13.2% of the total phenotypic variance. In normal conditions, one QTL was detected for the stem diameter trait on chromosome 11 located between ISSR20-8 and ISSR12-4 markers, explaining 11.8% of the total variance. In osmotic stress conditions, two QTLs were found for the panicle dry weight trait on chromosomes 8 and 1. One QTL was located on chromosome 8 at the RM447 - ISSR2-5 distance, which explained 11.3% of the total phenotypic variance. Neda alleles caused the increase in this trait. In osmotic stress conditions, three QTLs for the total panicle weight trait were discovered on chromosomes 12 and 2. Two QTLs (qWTS-12a and qWTS-12b with LOD values of 3.254 and 2.617) on chromosome 12 explained 13.1% and 12% of the phenotypic variance, respectively. The increasing effect of the alleles in these QTLs was attributed to the Neda parent. In osmotic stress conditions, one QTL for the filled grain weight trait was found on chromosome 9 (ISSR22-3 - ISSR21-1) and explained 13.2% of the total phenotypic variance. The increasing effect of the allele in this QTL could be explained by the Neda parent. In osmotic stress conditions, two QTLs for the leaf area trait were identified on chromosomes 1 (RM1287-RM165) and 8 (ISSR4-6-RM6208), which explained 11% and 11.7% of the total phenotypic variance. Neda parental alleles were effective in increasing the QTL on chromosome 1 whereas Ahlamitarom alleles in the QTL on chromosome 8 reduced this trait.

4. Discussion

Based on the results, all evaluated traits at the vegetative stage were significantly different at the 1% level in the three experimental conditions at the reproductive stage. Paralleled to our findings, Bhandari et al. (2023) reported that drought stress negatively impacts the general morphology of rice plants, including plant growth, plant biomass, yield, roots, and grain development.

In terms of morphology (Mohd Ikmal et al., 2021), plants under water stress exhibit decreased germination, leaf size, leaf area, leaf number, biomass, cell growth, and elongation due to low or insufficient water flow onto the xylem or neighboring cell. The statistically significant differences between the genotypes indicate a high variance between the evaluated plant material and probably their different mechanisms in reaction to osmotic stress, which can be used in the selection of good genotypes. The comparison of QTLs located in three flooded, −4.5 bar, and −9 bar conditions showed that two, seven, and seven QTLs were respectively identified in these treatments. In −4.5 bar osmotic stress conditions, two large-effect QTLs (qCL-2 and qRD-5) for chlorophyll content and root diameter were found on chromosomes 2 and 12, compared to one large-effect QTL (qRV-12) detected on chromosome 12 in −9 bar osmotic stress. Among rice chromosomes, most of the traits were identified on chromosome 12 and its genomic regions. According to the comparison of three treatments, qRN-12 and qRS-11 on chromosomes 12 and 11 in flooded conditions and −9 bar osmotic stress, respectively, as well as qRV-12 on chromosome 12 in −4.5 and −9 bar osmotic stress, were jointly located regarding the chromosome and genomic loci. These QTLs are stable and are not influenced by environmental factors. The mentioned QTLs controlled root number, root area, and root volume traits, playing an important role in these traits. Kamoshita et al. (2002) found only two stable QTLs among 31 cases identified for seven traits related to root morphology in two non-stress and −6 bar conditions. These results demonstrate that the identified QTLs and the large-effect QTL with a high R^2 can be jointly effective in two different conditions without environmental effects using the MAS method to produce and improve drought-tolerant rice varieties. Similar to our results, Lilley et al. (1996), Zhang et al. (2005), and Kato et al. (2007) identified QTLs mostly associated with root

traits (length, area, volume, and dry weight) at the vegetative stage.

Horii et al. (2006) recognized two QTLs on chromosomes 1 and 9 (marker intervals RM243-RM23 and RM257-RM258, respectively) for root diameter at the rice vegetative stage; these QTLs do not match with those identified in the present study. Babu et al. (2003) investigated the relationship between the secondary traits and yield of the rice plant using the QTL analysis in osmotic stress conditions. They found 47 QTLs for different traits that explained 5-59% of changes. Regions on chromosome 4 included a large-effect QTL controlling plant height and root traits, which does not correspond to our results. The present findings are in line with those of Sabouri (2007), who determined two QTLs on chromosomes 1 and 8 for rice root wet weight in osmotic stress conditions. They reported additive effects (−1.01 and −1.04) and phenotypic explanations (22.44 and 22.97) for the located QTLs.

Mardani et al. (2014) detected QTLs associated with drought tolerance in an $F_{2:4}$ population by employing 105 AFLP and 131 microsatellite markers, during germination and vegetative stages in rice. They located three, two, and two QTLs for germination rate, germination percentage, and root number, respectively, on chromosome 12. Long-Zhi et al. (2006) studied QTLs controlling root wet and dry weights in a population derived from an Indica and Japonica rice cross under salinity and osmotic stress. They determined QTLs on chromosomes 8 and 12 for root wet weight, which explained total phenotypic variance with R^2 values of 21 and 12.3%, which is inconsistent with our results. Consistent with this study, Khalili et al. (2017) discovered four loci on chromosomes 1H, 2H, 5H, and 6H for leaf chlorophyll content. The discrepant results obtained in the present and other investigations may be attributed to the number and type of evaluated markers, population type, the number of individuals in the population, the type of examined parents, and environmental conditions.

In this research, QTLs were recognized at the reproductive stage, and genetic relationships between traits were compared in two different conditions. Of 20 QTLs detected in both conditions, 13 and 7 QTLs were associated with osmotic stress and normal conditions. Under osmotic stress, three large-effect QTLs (qNTF-12, qWTS-12a, and qWDP-8) were found on chromosomes 8 and 12 in comparison to two large-effect QTLs (qNNT-12 and qWDR-9) identified on chromosomes 9 and 12 in normal conditions. In the comparison of the two treatments, only two QTLs (qNTF-12 and qNL-3) on chromosomes 3 and 12 were determined in osmotic stress and normal conditions. The mentioned QTLs are stable and are not affected by environmental factors. In this investigation, qNTF-12 and qNL-3 QTLs were found for NTF and NL under both conditions, which may play an important role in NTF and NL. The QTLs were located at intervals of ISSR15-1 and ISSR11-2 markers on chromosomes 3 and 12. Wang et al. (2007) determined seven QTLs for these traits on chromosomes 3, 7, and 10 using a RIL (F2:9) population, and qNL-3 with LOD = 2.6, which explained 9% of the total phenotypic variance, is similar to the QTL recognized in our study. The regional and phenotypic variance with the explained QTL (qNL-3) is also similar to QTLs identified by Teng et al. (2001).

In the current study, two QTLs for plant dry weight (WDP) on chromosomes 1 and 8 were found in normal conditions, which disagrees with other studies (Redona and Mackill, 1996; Zhang et al., 2005). They studied different traits and phenotypic methods. One QTL for WDP on a similar region on chromosome 8 was reported in normal conditions by Ji et al. (2012). Similarly, the ISSR16-1-ISSR15-1 area on chromosome 12 contains one QTL for NTF, which was reported earlier by many researchers. For example, drought-tolerance QTLs in the same area were reported by Price and Courtois (1999), Lafitte et al. (2002), and Wang et al. (2007). Therefore, this region seems to be a good candidate for improving drought-tolerant varieties through marker selection as well as map saturation and local gene formation. The current investigation is the first to identify three QTLs for the WTS trait on chromosomes 2 and 12, which is not in agreement with other studies. Some of the 20 identified QTLs are reported for the first time. In this study, most of the identified QTLs show a wide range of inadequate effects, suggesting the complexity of the studied traits. In our investigation, the F8 population derived from an Ahlamitarom × Neda cross is a new source of novel drought-tolerant QTLs. These results demonstrate that these QTLs

were effective for the production and improvement of drought-tolerant rice varieties using the MAS technique. Such varieties provide yield sustainability to farmers in regions impacted by drought and submergence (Mohd Ikmal et al., 2021) while serving as important genetic materials for future breeding programs.

Conclusion

In this study, we identified QTLs associated with drought-tolerance particularly on chromosome 12. Our findings underscore the variability of rice RILs across all traits in both environments. In drought stress conditions, the F8 population derived from the Ahlamitarom × Neda cross serves as a novel reservoir of identified QTLs at both vegetative and reproductive growth stages. Thus, these QTLs can be effectively utilized as reliable and stable QTLs for the identification of drought-tolerant lines at all stages of plant growth using the MAS approach.

Supplementary Materials

No supplementary material is available for this article.

Author Contributions

Conceptualization, M.N. and H.S.; methodology, M.N.; software, H.S; validation, M,N., H.S., and I.M.H; formal analysis, M.N.; investigation, M.N.; resources, H.S.; data curation, M.N.; writing—original draft preparation, M.N.; writing—review and editing, H.S.; visualization, M.N.; supervision, H.S. ; I.M.H; and F.D.K project administration, H.S.; funding acquisition, H.S. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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مقاله پژوهشی

ساختار ژنتیکی تحمل به خشکی در لاین‌های نوترکیب برنج ایرانی (*Oriza sativa*) در مراحل رویشی و زایشی

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چکیده: به منظور بررسی ساختار ژنتیکی تحمل به خشکی در برنج، آزمایشی با استفاده از ۱۲۰ لاین نوترکیب (ندا × اهلمی طارم) به صورت فاکتوریل در قالب طرح کاملاً تصادفی با سه تکرار در گلخانه دانشگاه گنبدکاووس انجام شد. در این تحقیق برای القای تنش اسمزی (۴/۵- و ۹- بار)، از PEG 6000 در مراحل رویشی و زایشی استفاده شد. علاوه بر اندازه‌گیری صفات مورفولوژیکی ریشه و اندام هوایی، از نشانگرهای SSR، iPBS، IRAP و ISSR برای ایجاد نقشه پیوستگی ژنتیکی استفاده شد. به ترتیب در مراحل رویشی و زایشی ۱۶ و ۲۰ QTL شناسایی شدند. در مقایسه سه تیمار ارزیابی شده، qRN-12، qRS-11، و qRV-12 به عنوان QTLهای پایدار مناسب برای انتخاب لاین‌های متحمل به خشکی در مرحله رویشی در شرایط مختلف تعیین شدند. چندین آلل جدید مرتبط با QTLهای تحمل به خشکی در این مطالعه شناسایی شدند. در دو شرایط محیطی، QTLهای مهم شناسایی شده، مانند qNTF-12 و qNL-3، متعلق به تعداد پنجه‌های بارور و برگ‌ها بودند که به عنوان QTL های پایدار در مرحله زایشی تعیین شدند. در این مطالعه، QTLهای شناسایی شده در مراحل رویشی و زایشی می‌توانند به عنوان QTLهای پایدار و اصلی برای انتخاب لاین‌های متحمل به خشکی در انتخاب به کمک نشانگر مورد استفاده قرار گیرند.

کلمات کلیدی: QTL بزرگ اثر، تنش اسمتیک، مکان های ژنی کنترل کننده صفات کمی، برنج.

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