Utilize of genetic diversity and marker-trait association to improve drought tolerance in rice (*Oryza sativa* L.)

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Original article

Keywords: Rice, Water deficit, genetic diversity and phenotypic correlation

Posted Date: May 18th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-29383/v1>

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Abstract

Drought stress is one of the main problems for rice crop as it reduces the production and productivity of the grain yield significantly. In Egypt, many restrictions were made on the cultivation of rice due to its high water demand. Producing promising drought-tolerant rice cultivars in a combination with high yielding is one of the main targets for rice breeders. To address this challenge, a set of 22 highly diverse rice genotypes were evaluated under normal and drought conditions. Morphological, physiological, yield traits were recorded on each genotype. Drought susceptibility index (DSI) was estimated for six yield traits to identify the most drought-tolerant rice genotypes. High genetic variation was found among genotypes tested in the experiment. Under normal conditions, the highest phenotypic correlation was found between grain yield (GY) and sterility percentage (SP) (-0.73^{**}), while, it was among GY and chlorophyll content (CC) (0.82^{**}) under drought conditions. To identify quantitative trait loci (QTL) controlling yielding traits under drought and normal, single marker analysis was performed between all yield traits under both condition and a set of 106 simple sequence repeat (SSR) marker alleles. The genetic association analysis revealed 14 and 17 QTL under drought and normal conditions, respectively. The most drought-tolerant genotypes were selected based on DSI, the number of QTL in each selected genotypes, and the level of genetic diversity. As a result, five genotypes (Giza 178, IET1444, GZ1368-S-5-4, Nahda, Giza 14) were identified as the most promising drought-tolerant rice genotypes. Eight QTL controlling drought tolerance were identified in Giza 178, Nahda, and GZ1368-S-5-4, while, four QTL were found in IET1444. The number of different QTLs were estimated among the five selected genotypes. Giza 178 and GZ1368-S-5-4 shared the same QTLs. Seven different QTLs were found among Nahda, IET1444, GZ1368-S-5-4, and Giza 14. Combining information from phenotypic traits, genetic diversity analysis, and QTL identification was very useful in identifying the true drought-tolerant rice genotypes that can be used for crossing in the future breeding program.

Introduction

Rice is one of the most important food crops in the world and major food crop of about half of the world's population. It is the staple diet for most people living in south and south east of Asia, which is grown in 154 million hectares world-wide in a wide range of environments, (IRRI, 2004). Egypt is facing a major challenge i.e. food security strongly and directly affected by water shortage as second challenge. Where water shortage is one of the most limiting factors in more than 30% of paddy fields in Egypt, particularly in the terminal areas and the developed varieties cannot perform well under water shortage (AbdAllah 2010). Egypt has restricted the cultivation of rice areas due to its huge water demand. Thus, producing drought-tolerant genotypes is urgently needed to keep food security safe and alleviate poverty.

Environmental stresses, such as water deficit and temperature rises are major factors limiting plant growth and productivity. Yield insurance can only be attained depending on the processes determining plant development and its responses to stress. Among the crops, rice as a submerged crop, is probably more susceptible to water deficit stress than most other plant species. The shortage of irrigation water is one of the major obstacles for increasing rice production not only in Egypt but also worldwide (AbdAllah *et al.* 2013). Most of the commonly, young seedling and reproductive stages are particularly sensitive to water deficit. It has been established that water deficit stress is a very important limiting factor at the initial phase of plant growth and establishment. It affects both elongation and expansion growth (Kusaka *et al.* 2005 and Shao *et al.* 2008).

Drought tolerance is a complex trait controlled by many genes. Therefore, the genetic improvement of drought tolerance is a big challenge (Sallam *et al.* 2019a). Drought tolerance can be assessed by scoring traits that have a relation to water deficit and by which tolerant and susceptible genotypes can be discriminated. These traits can be morphological, physiological, yield traits, etc. Scoring more than trait related to drought tolerance is very helpful in identifying the true drought tolerant genotypes. Stress susceptibility index is also one of the most effective tools to discriminate the tolerant and susceptible genotypes (Lazar *et al.* 1995; Kadam *et al.* 2012; Sallam *et al.* 2015, 2019b; Sukumaran *et al.* 2018).

Drought-tolerant rice genotypes can be produced by breeding research through screening potential diverse germplasm which has genetic variation in drought tolerance. Furthermore, improving rice yield should be combined with high tolerance to drought as farmers are interested to profitably produce their crops in dry environments. The only drawback of breeding research is the cost and time consuming to achieve its goals. The recent advances in DNA technology and genomics paved the way to identify genomic regions controlling important traits such as drought tolerance in crops (Sandhu *et al.* 2014; Hussain *et al.* 2017; Eltaher *et al.* 2018; Mourad *et al.* 2018). These technologies provide DNA markers that can be combined with phenotypic data to identify genomic regions associated with target traits using genetic association analyses such as genome-wide association study, QTL mapping, single-marker analysis, etc. The genetic dissection of drought tolerance in rice depends on how many traits are planned to be scored in the experiments. The more traits recorded, the more genes controlling drought can be identified (Ovenden *et al.* 2017; Qaseem *et al.* 2018). Simple sequence repeats are one of the most useful DNA markers, after single nucleotide polymorphism, that can be used in genetic studies because they provide a high level of

polymorphism. The SSR markers were used in many studies to identify QTL associated with drought tolerance in rice (Ahmed et al. 2018; Tabkhkar et al. 2018; Verma et al. 2019; Barik et al. 2019)

The high polymorphic markers can be used in genetic diversity, QTL, gene identification studies, etc. In addition to the previous point, phenetic selection only can be misleading due to human errors or environmental factors. Instead, identifying how many QTL in each selected genotype contains can be used in deciding the best parents for crossing

Therefore, combining breeding with genetic studies such as genetic diversity and QTL analyses can be very fruitful in accelerating cultivar production with target traits through genomic selection. Extensive genetic diversity and population structure were analyzed by Salem and Sallam (2016) in a rice diverse population that was phenotyped for drought tolerance in this study. The level of genetic diversity found among the genotypes was utilized along with QTL identification and phenotypic screening for several physiological, morphological and yield traits to genetically identify the true-promising drought-tolerant rice genotypes which can be used in future rice breeding program.

Material And Methods

Plant material:

A set of 22 local and exotic rice genotypes (varieties and/or lines) representing a wide range of diversity for several agronomic and physiological characters were selected for the study. The names, pedigree and origin of these genotypes were previously described in Supplementary Table (1).

Experimental layout:

The present study was conducted at the Experimental Farm of the Rice Research and Training Center (RRTC), Sakha Agricultural Research Station, Kafr EL-Sheikh Governorate, Egypt, during the season of 2019/2020. The 22 genotypes were evaluated under normal and drought conditions during the rice growing seasons. The genotypes were grown in the nursery for 30 days and transplanted in the permanent field. Each genotype was planted in seven rows of five meters length each. Adopting spacing of 20 × 20 cm spaced plants, and seedlings of each genotype were individually transplanted in the permanent field. These materials were replicated three times in a randomized complete block design (RCBD) with the same set of genotypes in two experiments (under normal and drought conditions). Drought stress was imposed by using flush irrigation (flush irrigation is one of the surface irrigations without standing water after irrigation) every 12 days were applied after two weeks from transplanting to harvesting and all recommended cultural practices were followed.

Data of agronomic and yield and its component traits were recorded from five randomly selected representative plants in all genotypes in each replication. The methods adopted for recording the observation for each of all studied traits are presented below.

1) Agro-physiological traits

The following physiological and agronomic traits **Agronomic** were measured on each genotype; days to 50% flowering (DTF, days) was calculated as the average between numbers of days from sowing to the first panicle exertion and number of days from sowing to the last panicle emergence; plant height (Ph, cm) was measured from the soil surface to the tip of the main panicle at maturity; panicle length (PL, cm) the main panicle of each plant was measured from the base to the tip of the panicle excluding awns at complete maturity; leaf rolling (LR) was estimated by visual estimation based on methods proposed by (De Data et al., 1988), flag leaf area (FLA, cm²) the FLA of 20 leaves were measured using leaf area meter (model L1–3000 A) and chlorophyll content (CC) was measured by using chlorophyll meter (SPAD-502) Minolta Camera Co. Ltd., Japan).

2) Yield and its components traits:

Moreover, Yield and its components such as the number of panicles/plant (NOP) was counted the number of panicles/plant when all plants at the ripening stage; sterility percentage (SP, %); the unfilled grains of the main panicle were separated and counted and sterility percentage was calculated; 100-grain weight (HGW, g) was recorded as the weight of 100 random rice grains/plant and grain yield/plant (GY, g) was recorded as the weight of the individual plant grain yield and adjusted to 14% moisture content.

The drought stress was fully monitored, and the total amount of water consumed was estimated using water counters. Soil moisture content was gravimetrically determined on an oven-dry basis. At each sampling date, duplicate soil samples were taken to a depth of 60 cm using an auger. The samples were immediately transferred in tightly closed aluminum cans to the laboratory where they were weighted, oven-dried at 105 °C for 24 h, and reweighed after which their moisture content was determined. Field capacity and permanent wilting percentage were determined. The bulk density was determined using the core method to a depth of 60 cm (Klute, 1986). The soil was

clayey in texture, whereas particle size distribution was 57.1% clay, 31.5% silt, and 12.7% sand. Soil pH was 8.2, and the electrical conductivity of soil and irrigation water was 2.1.

Statistical analyses of phenotyping data:

The analysis of variation (ANOVA) was carried out by PLABSTAT software (Utz 1991) using the following model

$$Y_{ijt} = \mu + g_i + r_j + t_k + gt_{ik} + grt_{ijk}$$

where Y_{ijk} is an observation of a genotype i in a replication j tested in a treatment k (normal vs drought stress) k , μ is the general average; g_i , t_k , and r_j , refer to the effects of genotypes, treatment and replications, respectively. gt_{ik} is genotype $\times$ treatment interaction. grt_{ijk} is genotype $\times$ replications $\times$ treatment interaction (error)

Heritability of the genotypes for each trait was calculated as (h^2 = genotypic variance / phenotypic variance). The Spearman rank correlation coefficient was calculated for the phenotypic correlation between traits. All graphical presentations were performed by R software and Microsoft Excel 2016.

The drought susceptibility index was calculated for Ph, CC, FLA, HGW, SP and GY. Drought intensity (DI) was calculated according to (Lazar et al. 1995) as follow

$$DI = 1 - \frac{Y_D}{Y_N}$$

Where Y_D is the average all genotypes for the respected trait under drought stress, while, the Y_N is the average of all genotypes for the same respected trait under normal conditions. The drought susceptibility index (DSI) was estimated for each genotype and calculated according to (Lazar et al. 1995) as follow

$$DSI = \frac{1 - \frac{X_D}{X_N}}{DI}$$

Where X_D is the mean performance of each genotype for the respected trait under drought stress, while, the X_N is the mean performance of each genotype for the same respected trait under normal conditions

Genotyping and genetic analyses:

DNA extraction and simple sequence repeat marker analysis were extensively described in detail by Salem and Sallam (2016). The analysis of SSR markers on the 22 genotypes revealed 106 polymorphic alleles.

Single marker analysis (SMA) was performed using R software to test the marker-trait association between the 106 SSR markers alleles and yield traits under normal and drought conditions. The phenotypic variation explained by a marker (R^2) and allele effects of the present allele were estimated using R software. Allele effects of the present allele loci were calculated as differences between the means of the marker classes (1 vs. 0). The positive signs indicate that the present allele increases the trait, while negative signs are associated with a decrease in the trait.

Population structure and genetic diversity were analyzed by Salem and Sallam (2016) and utilized in this study to genetically select the most promising drought-tolerant genotypes.

Results

Genetic variation in drought tolerance in rice:

The analysis of variance for all traits scored in this study presented in Table 1. High significant differences between the treatments for all traits. Significant differences among replications were only found for HGW. High genetic variation was observed among all genotypes for all traits. Among all traits, PL did not have significant differences in $G \times T$. The minimum, maximum, mean, and coefficient of variation (CV) values for each traits under normal and drought conditions are presented in Table 2. The main average of all genotypes under drought conditions was lower than normal conditions for DTF, Ph, CC, FLA, PL, NOP, HGW and GY. Under normal conditions. On the other hand,

the main average under normal conditions was lower than drought conditions in LR and SP traits. The highest CV (%) was found for LR (34.19%) under N, while SP had the highest CV under D. The performance of each genotype in each trait under both treatments are presented in Supplementary Table 2. The lowest effect due to drought stress was found for CC (14.06%), while, the highest reduction occurred in GY with 39.84%. The drought had a great effect on sterility percentage with an increase of sterility reach to 79%.

Table 1
The analysis of variance for all traits scored on the 22 rice genotypes under normal and drought conditions.

AOV	d.f	DTF	Ph	CC	LR	FLA	PL	NOP	HKW	SP	GY
Treat. (T)	1	134.75**	93.43**	316.97**	416.99**	231.50**	520.28**	221.82**	186.19**	108.78**	285.26**
Rep. (R)	2	1.96	1.68	0.65	0.30	2.17	1.40	2.681	4.98*	2.13	0.38
Geno. (G)	21	401.77**	238.37**	46.18**	17.55**	44.94**	21.03**	31.01**	32.03//	181.94**	69.54**
G × T	21	38.79**	35.27**	3.07**	3.03**	13.55**	1.45	2.37**	5.49**	12.87**	13.99**
G × R	42	0.81	1.25	1.96*	0.94	1.05	0.94	0.73	1.03	0.73	1.54
G × R × T	44										
*,** significant at the 0.05 and 0.01 level of the probability, respectively.											

Table 2
Minimum, maximum, mean, and coefficient of variation (CV) for each trait scored on the 22 genotypes under normal (N) and drought (D) conditions.

Trait	Treatment		Min.	Max.	Mean	CV(%)
DTF (days)	N	1	91.67	1333.33	110.50	10.94
	D	2	78	109.67	93.53	8.54
Ph (cm)	N	3	95.73	148.87	113.20	14.99
	D	4	75.33	119.10	92.59	13.78
CC	N	5	33.59	46.91	38.01	9.28
	D	6	24.4	43.21	32.66	12.41
LR	N	7	1	3.33	2.23	34.19
	D	8	3.67	7.67	5.88	21.45
FLA	N	9	23.64	44.02	32.89	13.44
	D	10	17.26	26.94	21.40	12.64
PL	N	11	17.73	24.47	21.86	8.32
	D	12	14.08	20.76	17.33	9.57
NOP	N	13	14.63	25.3	19.04	11.58
	D	14	10.74	21.05	14.17	18.68
HKW (g)	N	15	1.99	2.99	2.41	9.49
	D	16	1.70	2.21	1.98	7.45
SP	N	17	7.32	12.84	10.24	13.95
	D	18	19.23	80.32	48.23	35.13
GY (g)	N	19	26.63	44.36	33.86	13.64
	D	20	13.51	34.93	20.37	29.76

Drought susceptibility index (DSI) was estimated for each genotype based on six important yield traits (Ph, CC, FLA, HGW, SP, and GY) to determine the most tolerant and susceptible genotypes (Supplementary Table 3). The distribution of all genotypes for each DSI is presented in Fig. 1. The number of tolerant genotypes differed by DSI. For example 12 tolerant genotypes were found based on DSI for Ph, HGW and SP while, 11 tolerant genotypes based on DSI for CC and FLA. Eight tolerant genotypes were found for GY. The tolerant genotypes for each DSI (six DSIs) were selected (Fig. 2). Two genotypes ETT1444 and Giza 178 were found to be tolerant based on all DSIs. Moreover, three genotypes (GZ1368-S-5-4, Nahda, Giza 14) were found to be tolerant to drought in five DSI. Therefore, we considered these five genotypes as the drought tolerant genotypes (Supplementary Table 3). On the other hand, three genotypes (IR74, Giza 177, and YabaniM7) were found to be tolerant in only one DSI and susceptible to five DSI. These genotypes were considered susceptible to drought stress. Furthermore, among the tolerant genotypes for each DSI, the most three tolerant (the lowest DSI values) and three susceptible genotypes (the highest DSI values) for each DSI were selected (Supplementary Table 3). As a result, IET1444 and Giza 178 were the most tolerant genotypes having the lowest values in five DSIs (CC, FLA, HGW, SP, and GY) followed by GZ1368-S-5-4 with the lowest DSI values in three indices (HGW, SP, and GY).

Phenotypic correlations among traits:

The phenotypic correlation among traits under normal and drought stress are presented in Fig. 3 and Fig. 4, respectively. Remarkably, the number of significant phenotypic correlations among traits under drought stress was higher than those under normal conditions.

Under normal conditions, the highest positive significant correlation was found between NOT and NOP ($r = 0.91^{**}$), while, the lowest positive significant correlation was between LR and Ph; NOT and GY and NOP and GY with r of 0.54^* . SP had a negative significant correlation with CC, NOT, PL, NOP and GY with a range extending from -0.49^* (NOP) to -0.73^{**} (GY).

Under drought stress, the highest positive significant correlation was again between NOT and NOP ($r = 0.96^{**}$) while, the lowest significant correlation was found between FLA and NOP ($r = 0.45^*$). SP had negative significant correlations with CC, FLA, PL, NOP and GY. Moreover, LR was negatively and significantly correlated with FLA and GY. A negative significant correlation was found between CC and LR ($r = -0.50^*$).

The phenotypic correlation among the DSIs for the seven traits is presented in Fig. 1. A positive and significant correlation was between DSI for GY and DSIs for SP, PL, FLA, and CC. DSI for SP was significantly correlated with DSIs for HGW, PL, and CC. The DSI for FLA was positively and significantly correlated with CC, PL, and HGW. A positive significant correlation was found between HGW and PL.

Single marker analysis for drought tolerance:

Marker-trait association was tested between the SSR markers and all phenotypic traits scored in this study (normal conditions and drought stress). The summary of QTL associated with traits under normal and drought stress conditions is presented in Table 3. As a total, the number of QTLs found under normal (16) was higher than those detected under drought stress (14). LR had the highest number of QTL under normal, while FLA and GY had only one QTL for each. Under drought stress CC and GY had the highest number of QTL under control conditions. NOP, Ph, and HGW had only one QTL for each. All traits had QTLs under drought stress except FLA, while three traits Ph, DTF, and HGW haven't any QTL under normal conditions.

Table 3
Number of QTLs detected
under normal and drought
conditions using single-marker
analysis

Traits	Treatments	
	Normal	Drought
CC	3	3
FLA	1	-
GY	1	3
LR	5	2
NOP	2	1
SP	2	2
Ph	-	1
DTF	-	2
HKW	-	1
Total	16	14

The details of the SMA for all traits are presented in Table 4. The highest number of QTLs detected in LR with two QTLs under drought stress and five QTLs under normal conditions. One QTL was found for DTF (D), HGW (D), FLA (N), and PL (N). Chromosome 4 had the highest number of QTLs, while, chromosome 3 had only one QTL. The phenotypic variation explained by marker (R^2) ranged from 33.34% (*qHGW_D*) to 50.21% (*qLR_D2*) under drought stress, while, it extended from 34.06% (*qLR_N3*) to 84.61% (*qLR_N5*) under normal conditions. The effect of the visible alleles for each QTL was estimated. In CC, GY, NOP, HGW and Ph all detected alleles had a positive and increasing effect on the traits. In LR one and two SRR marker alleles were found to be associated with decreased the trait under normal and drought conditions, receptively. In SP, two SSR markers alleles located on chromosome 4 were significantly associated with decreasing the trait under drought stress condition. Two marker alleles associated with earliness in flowering under drought stress. Finally, one marker allele was found to be associated with decreased FLA under normal conditions.

Table 4
Detailed single-marker analysis for each trait under normal (N) and drought (D) conditions.

Trait	Treatment	QTL	Marker ¹	Chro. ²	<i>p</i> -value	R ² ²³	AE ⁴	
CC	<i>D</i>	<i>qCC_D1</i>	Rm285-405	9	0.0030	39.40	11.56	
		<i>qCC_D2</i>	RM307-128	4	0.0030	39.45	11.56	
		<i>qCC_D3</i>	RM277-122	12	0.0074	33.52	4.871	
	<i>N</i>	<i>qCC_N1</i>	RM277-122	12	0.0044	36.97	4.410	
		<i>qCC_N2</i>	Rm285-405	9	0.0030	38.40	9.840	
		<i>qCC_N3</i>	RM307-128	4	0.0030	38.40	9.841	
GY	<i>D</i>	<i>qGY_D1</i>	Rm144-228	11	0.0050	36.12	15.632	
		<i>qGY_D2</i>	RM307-128	4	0.0039	37.79	15.990	
		<i>qGY_D3</i>	Rm285-405	9	0.0039	37.79	15.990	
	<i>N</i>	<i>qGY_N</i>	RM307-122	4	0.0044	36.97	5.710	
	LR	<i>D</i>	<i>qLR_D1</i>	Rm154-192	2	0.0021	41.66	-1.632
			<i>qLR_D2</i>	Rm433-214	8	0.0005	50.21	1.800
<i>N</i>		<i>qLR_N1</i>	Rm154-182	2	0.0062	34.75	1.291	
		<i>qLR_N2</i>	Rm154-192	2	0.0025	40.67	-0.998	
		<i>qLR_N3</i>	Rm161-185	5	0.0069	34.06	-0.957	
		<i>qLR_N4</i>	RM271-100	12	0.0014	44.10	1.20	
<i>qLR_N5</i>	Rm433-214	8	0.0006	48.61	1.096			
NOP	<i>D</i>	<i>qNOP_D</i>	Rm144-228	11	0.0020	42.05	7.497	
	<i>N</i>	<i>qNOP_N1</i>	Rm144-228	11	0.0007	47.70	6.769	
		<i>qNOP_N2</i>	Rm161-165	5	0.0065	34.47	2.560	
SP	<i>D</i>	<i>qSP_D1</i>	RM215-146	9	0.0047	36.66	23.308	
		<i>qSP_D2</i>	Rm55-214	3	0.0015	43.66	27.52	
	<i>N</i>	<i>qSP_N1</i>	RM307-122	4	0.0049	36.30	-1.648	
		<i>qSP_N2</i>	RM307-126	4	0.0029	39.79	-1.931	
DTF	<i>D</i>	<i>qDTF_D1</i>	Rm433-214	8	0.0037	32.19	-11.347	
		<i>qDTF_D2</i>	Rm154-192	2	0.0090	38.16	-10.37	
HKW	<i>D</i>	<i>qHKW_D</i>	RM307-122	4	0.0077	33.34	0.1826	
FLA	<i>N</i>	<i>qFLA_N</i>	RM215-146	9	0.0064	34.62	-6.521	
Ph	<i>D</i>	<i>qPh_D</i>	RM215-146	9	0.0072	33.80	17.689	

(¹) Maker allele refers to the name of the SSR primer followed by the band size.

(²) Chromosome number in rice genome

(³) Phenotypic variation explained by marker alleles

(⁴) allele effect of the present allele. AE referee to the effect of visible band (marker allele) of the SSR marker on the trait. Positive values increase the trait, while negative values decrease the trait.

Interestingly, a set of eight markers were found to be associated with more than one trait under normal and drought conditions (Table 4). Under normal conditions, the seven markers were associated with only one trait except RM307-122 which controlled GY and SP. Five

markers; Rm144-228, Rm154-192, Rm285-405, RM307-128, and Rm433-214 were associated with two traits under drought stress. Noticeably, some SSR markers were associated with the same trait under normal and drought conditions. For example, Rm285-405 and RM307-128 markers were associated with increased CC under normal and drought stress. The same two markers were associated with increased GY under drought stress. Rm144-228 marker was associated with increased NOP under both treatments and GY under drought stress. Rm154-192 was associated with decreased LR under both treatments, while, Rm433-214 was found to be associated with increased LR under the two treatments and DTF under drought stress. Two markers were associated with different traits under both treatments. Rm144-218 marker was associated with decreased CC under normal and PL under drought. RM215-146 was associated with decreased Ph and SP under drought stress.

By looking for the number of QTL existed in the most five drought tolerant genotypes, the highest number of total detected QTL (15) was found in Giza 178 and GZ1368-S-5-4, while IET1444 had the lowest number of detected QTLs. The five genotypes had a higher number of QTLs detected under drought compared with those detected under normal. Three drought tolerant genotypes (Giza 178, GZ1368-S-5-4 and Nahda) had eight QTLs controlling yield traits under drought stress. GZ1368-S-5-4 had the highest number of QTLs controlling yield traits under normal conditions.

The number of different significant marker alleles among the most five tolerant genotypes is presented in Table 6. Nahda had different seven marker alleles from Giza 178, IET1444, GZ1368-S-5-4. Likewise, the same results were found between Giza 14 and both Giza 178 and GZ1368-S-5-4. Giza 178 and GZ1368-S-5-4 had the same significant marker alleles.

Table 6
Number of different QTLs under drought conditions among the promising drought tolerant rice genotypes selected based of drought susceptibility index (DSI).

Genotypes	Subpop ¹ .	Giza178	IET1444	GZ1368-S-5-4	Nahda	GIZA 14
Giza178	G2		4	0	7	7
IET1444	G1			4	7	5
GZ1368-S-5-4	G2				7	7
Nahda	G1					6
GIZA 14	G2					
¹ subpopulation according to Salem and Sallam (2015)						

Use of genetic diversity analysis to improve drought tolerance:

The genetic analysis and population structure were performed on the 22 (see material and methods). The analysis of genetic diversity and population structure divided the 22 genotypes into two possible subgroups (subpopulations). The first subpopulation (G1) included AgamiM1, Arabi, Bala, IET1444, IR20, IR22, IR24, IR50, IR64, IR74, Nahda, YabaniM1, while, the second subpopulation (G2) included Yabani15, Yabanilulu, YabaniM7, Giza14, Giza171, Giza172, Giza177, Giza178, Giza181, and GZ1368-S-5-4. The mean of all genotypes in each group was calculated for each trait to study the properties of genetic diversity between the two groups in terms of yield attributes and drought tolerance (Table 7). Interestingly, genotypes in G2 performed better in six and five agronomic traits under normal and drought than those in G1, respectively. Under control and drought stress conditions and compared with genotypes in G1, genotypes in G2 were late in flowering, taller stems, higher in CC, higher in PL, higher in NOP. Moreover, genotypes in G2 were lower in SP under normal, and higher in GY under drought conditions. For DSI, genotypes in G2 had a higher level of drought tolerance (five DSIs; CC, FLA, HGW, SP, and GY) compared with genotypes in G1.

Table 7
Drought susceptibility index (DSI) and phenotypic characters under normal and drought conditions on all genotypes in both subpopulations.

	Control		Drought		DSI	
	G1 ⁽¹⁾	G2 ⁽¹⁾	G1	G2	G1	G2
DTF	108.67	112.70	90.97	96.60	NA	NA
Ph	109.41	116.47	92.38	92.77	0.95	1.00
CC	37.00	38.74	31.54	33.57	1.07	0.89
LR	1.86	2.67	5.39	6.47	NA	NA
FLA	34.23	31.85	21.20	20.99	1.05	0.94
PL	21.69	22.52	16.83	17.90	NA	NA
NOP	18.05	20.23	13.76	14.67	NA	NA
HKW	2.43	2.39	1.98	1.98	1.01	0.96
SP	10.72	9.45	44.65	52.44	0.99	0.93
GY	32.26	35.77	18.59	21.74	1.05	0.98
Number of favorable traits	4.00	6.00	4.00	5.00	2.00	6.00
⁽¹⁾ All genotypes were divided into two subpopulation according to Salem and Sallam (2016)						

Discussion

Genetic variation in drought tolerance:

The analysis of variance revealed highly significant differences between the two treatments ($p < 0.01$) for all traits scored in this study, indicating that there was an observable effect of drought on the tested genotypes. This result can be supported by estimating the effect of drought stress on the genotypes in Supplementary Table 1. Sterility percentage as the most trait affected by drought stress. At the vegetative stage, drought stress increased the sterility of spikelets by disrupting the initiation of flowers leading to a reduction of grain filling followed by a significant reduction in grain yield (Akram et al. 2013). The severe reduction in GY (40%) further supports the previous conclusion as GY was the second trait after SP affected much by drought stress. Chlorophyll content (CC) was the most trait that less affected by drought stress with a reduction reached 14%. Although drought tolerant genotypes tend to increase chlorophyll content under drought stress (Mukahirwa 2015), all genotypes in this study had a reduction in CC. The high genetic variation found among the genotypes in all trait is very useful for rice breeders to select the most promising drought tolerant genotypes.

To characterize each genotype for its ability to tolerate drought, drought susceptibility index (DTI) was calculated for six different yield traits (Ph, CC, FLA, SP, HGW and GY). Most of the earlier studies focused on calculating DSI for GY or HGW to distinguish the tolerant and susceptible genotypes. In our study, DSI for six yield traits was estimated to identify the most drought tolerant genotypes as Ph, CC, FLA and SP are also very important traits that affect the final grain yield. Giza 14 was the most tolerant for Ph, while IET4444 was the most tolerant genotypes for CC, SP, HGW and GY. As the main objective was to select the most promising drought tolerant genotypes, we selected the genotypes that were drought tolerant according to the calculated DSIs for the six traits. Five genotypes were considered the most promising drought tolerant genotypes; Giza 178, IET1444, GZ1368-S-6-4, Nahda and Giza 14. Considering more than a trait to determine the most promising genotypes are highly recommended in the selection process. Selection of the most promising genotypes based on multiple traits was also reported in wheat (Sallam et al. 2018) and faba bean (Sallam et al. 2017). The most five drought tolerant genotypes were characterized before as drought tolerant. Nahda and Giza 14 were tested for drought tolerance at the seedling stage and they had good shoot growth and root characters under drought stress (Hazman and Brown 2018). Giza 178 and IET14444 were reported as drought tolerant genotypes in (Abd Allah et al. 2010; Elshafei et al. 2019). The most susceptible genotypes in this study were the genotypes that had a susceptibility to drought in five DSIs (Giza 177, YabaniM1 and IR74). The same susceptible genotypes were also reported in other studies such as (Casartelli et al. 2018; Mohamed et al. 2019). Although the tolerant and susceptible genotype were reported in other studies, however, the characterization was at the seedling and germination stage. Here, we tested them under field conditions, confirming that these genotypes can be used in a breeding program to improve drought tolerant at different growth stages.

Phenotypic correlations:

Compared to normal conditions, GY had significant correlations with a higher number of traits. GY had positive significant correlations with CC, NOT, PL and NOP, while it had a negative significant correlation with SP. Under drought stress, GY had new significant positive correlations with FLA and HGW while, it was negatively and significantly correlated with LR. Moreover, the sizes of correlations between GY and the other traits were higher than those under normal conditions. This result indicates that rice plants make substantial physiological and morphological changes to alleviate the effect of drought. For example, GY was significantly and negatively correlated with LR under drought ($r = -0.57^{**}$), while, this correlation was non-significant under normal conditions. This indicates that the tightly rolled genotypes had a significant reduction in grain yield and low drought tolerance. Radiation on the rice leaf is reduced due to the leaf rolling resulting in the low aspiration that leads to stomatal closure. As a result, the flag leaf area is decreased. This can be observable from the positive and significant correlation between LR and FLA under only drought stress ($r = 0.69^{**}$). Therefore, the favorable genotypes tend to have a delay in leaf rolling and higher recovery rate after drought removal

Analysis of marker-trait association:

Identifying QTL associated with drought tolerance is considered one of the most important steps to genetically improve drought tolerance in rice. Many studies depend on phenotypic selection for improving drought, however, phenotypic selection could be misleading due to environmental effects or/and human errors. Therefore, it would be more useful to use the advances in DNA molecular markers and their ability to allow identifying genomic regions associated with important traits (e.g. drought tolerance). Drought tolerance is a complex trait controlled by many genes. Therefore, identifying QTL associated with target traits under drought stress environment is still the main interest for breeders and geneticists to make faster genetic improvement in rice through advanced breeding programs and marker-assisted selection. In this study, several QTLs associated with yield and its attributes under normal and drought conditions were identified using SMA. SMA is one of the QTL analyses which is used to test marker-trait association and it provides valuable information on the genetic architecture of complex traits such as a number of possible QTL, additive effects of the markers, and genetic variation explained by markers (Sallam et al. 2016; Ramchander et al. 2016). In the current study, the results of SMA found a set of 16 and 14 QTL associated with yield and its traits under normal and drought conditions, respectively.

Interestingly, eight SSR markers were associated with more than one trait under drought and control conditions (Table 5). This confirms the feature of MAS choosing highly SSR promising markers for improving yield and its traits under both conditions. These QTLs have a pleiotropic effect at one genetic locus. This will help in improving many traits by using this marker in MAS. Amar et al. (2008) and Basunanda et al. (2010) reported that QTLs governing many traits frequently occur at closely linked loci in the genome. Of the eight markers, six were associated with two QTLs under drought, while, one marker was associated with two QTLs under normal conditions. The present allele of each marker had one, and consistent, the trend regarding its effects on drought tolerance. Moreover, the allele effects pattern in different traits follows the pattern of the correlations between the traits. For instance, the correlation between GY and CC under drought stress was 0.82^{**} . In our study, these two traits shared two significant markers (RM285 and RM307) with positive allele effects. Under control, RM307-122 increased GY and decreased SP. There was a negative significant correlation between SP and GY under normal conditions ($r = -0.73^{**}$)

Table 5
Markers having significant association with more than one trait under normal and drought conditions.

Marker allele	Traits	
	Control	Drought
Rm144-228	NOP (+)	NOP-D (+), GY-D (+)
Rm154-192	LR (-)	LR-D (-), DTF (-)
RM215-146	FLA-N (-)	<i>qPh_D</i> (+), SP-D (+)
Rm285-405	CC (+)	CC-D (+), GY-D (+)
RM307-128	CC (+)	CC-D (+), GY-D (+)
Rm433-214	LR (+)	LR-D (+), DTF-D (+)
RM277-122	CC (+)	CC-D (+)
RM307-122	GY (+), SP (-)	TKW (+)

QTL validation for marker-assisted selection (MAS)

Remarkably, there some QTLs reported in this study were previously reported in some drought studies in rice. RM144-228 which associated with increased NOP under both conditions and GY under drought stress was found to be associated with an increased number of branches under well-watered conditions in Liu et al. (2010). Also, the same marker was reported with its association with early flowering under drought conditions by Yue et al. (2005). Liu et al. (2010) reported the significant association between RM161 and an increased number of branches under well-watered conditions, the same marker was found to significantly associated with increased NOP in our study. RM215 was reported with its association with early flowering under drought and drought response index by Yue et al. (2005), decreased plant height under drought by Wang *et al.* (2016), increased root length Ikeda et al. (2006). This marker was associated with decreased Ph and SP under drought in the current study. Xu et al. (2005) and Shamsudin et al. (2016) reported a significant association between RM154 grain yield under normal and drought stress, respectively. In our plant material, RM154 was found to be associated with decreased LR under both conditions. Bearing in mind that LR was significantly correlated with GY ($r = -0.57^{**}$). Furthermore, we found that the same marker was associated with early flowering under drought stress. The same significant association between the same markers and early flowering under drought stress was reported by Shamsudin et al. (2016). RM285-405 was found to be associated with increased grain yield under drought stress. The same findings were reported by Venuprasad et al. (2012). Increased CC under control and drought conditions was controlled by RM307-128 in our study. The same marker was found to be associated with a decreased number of days to leaf rolling by Yue et al. (2005). A significant negative correlation was found between leaf rolling and CC ($r = -0.50^{*}$). The same marker associated with increased grain yield under drought stress. In the study of Yue et al. (2005), a significant association between RM307 and four root traits under drought was reported. Moreover, RM307 was significantly associated with increased grain yield under drought (Yadav et al. 2019).

Use of genetic diversity analysis to improve drought tolerance in rice:

Genetic diversity of germplasm plays an important role in producing varieties with target traits. It is very crucial to understand the level of genetic diversity of germplasm before designing a breeding program to improve target traits such as tolerance to biotic stresses, abiotic stresses, high yielding attributes, etc. (Eltaher et al. 2018; Onde et al. 2019). Here, the 20 genotypes can be considered a diverse collection from different countries and their genetic diversity level was extensively studied by Salem and Sallam (2016). The information from genetic diversity was incorporated with the results of drought tolerance obtained from this study on the same set of genotypes. According to Salem and Sallam (2016), the genotypes were divided into two possible subpopulations namely, G1 and G2 (Table 6). Remarkably, as an average, the genotypes in G2 had a higher level in drought tolerance than those in G1. The genotypes in G2 was more drought tolerant in five DSIs than genotypes in G1. Moreover, G2 subpopulation had better yield attributes control condition than genotypes in G1. Under drought stress, the performance of both groups was nearly the same except NOP which could be affected by the environmental conditions. Therefore, the genotypes in both subpopulations have a good level in drought tolerance with a little bit of bias for the G2 genotypes. High genetic distance was found among all genotypes in both subpopulations ranging from 0.48 to 0.90 (Salem and Sallam 2016). Therefore, choosing genotypes from both subpopulations could be very fruitful in improving drought tolerance in rice through breeding programs. Furthermore, the genotypes of this population are *indica* and tropical *japonica*. Genotypes from both types were identified as drought tolerance in a combination with high yielding by Onde et al. (2019).

Promising drought-genotypes as candidate parents for a future breeding program:

The main objective of any plant breeders is to select suitable parents with target traits for crossing to finally produce cultivars having a combination of all target traits from the two parents. Phenotypic selection only maybe not enough and misleading due to the environmental conditions which affect the quality of phenotyping. The recent advances of DNA technology provided useful molecular markers (e.g. SSR and SNP) by which genetic diversity and QTL controlling target traits can be explored and utilized in selecting the promising parents possessing target genes controlling target traits. In the current study, we incorporate the results obtained from the phenotypic data with results of the genomic data including QTLs and genetic diversity studies to generally and phenotypically select the true promising drought tolerant genotypes. Four criteria were used, first; phenotypic selection based on DSIs for six important yield traits. As a result, two genotypes were tolerant in all DSIs and three in five DSIs (supplementary Table S3), second; a number of different QTLs each selected genotype contained (supplementary Table S4), third; the number of different QTLs between each pair of selected genotypes (Table 7), fourth; the level of genetic diversity among the five genotypes. By considering the four criteria together, Nahda is highly recommended be a candidate parent that can be crossed with Giza 14, Giza 178 and GZ1368-S-5-4 because Nahad and the other candidate genotypes are from different subpopulations meaning that the genetic similarity is very low. Moreover, Nahda had 7 different QTLs from the other genotypes. Therefore, crossing Nahda with these genotypes can be resulted in producing rice cultivars having combination of the different QTLs from both parents. Crossing between GZ1368-S-5-4 and Giza 178 may be less efficient as both parents have the same QTLs

and belong to the same subpopulation. Identified drought-tolerant rice genotypes based on phenotypic and genomic levels can be successfully used to increase the genetic diversity of drought-tolerant rice cultivars.

Conclusion

Genetic improvement of drought tolerance in rice can be achieved based on results obtained at phenotypic levels. Extensive genetic analyses including QTLs and genetic diversity were very useful in selecting the truly promising drought tolerant genotypes that can be used for crossing to produce cultivars that have higher levels of drought tolerance. Genetic identification of the number of QTLs in each selected parent confirms the results of phenotypic selection as it can be vague due to environmental conditions or human errors. Incorporating genetic analysis with phenotypic data is very important to accelerate breeding programs by selecting suitable genotypes as parents to improve target traits. Moreover, phenotypic selection based on more than one trait could also help in excluding bad performance genotypes.

Abbreviations

QTL
quantitative trait loci
SMA
single marker analysis
DTF
days to flowering
Ph
plant height
CC
chlorophyll content
LR
leaf rolling
FLA
flag leaf area
PL
panicle length
NOP
number of panicles
HGW
100-grain weight
SP
sterility percentage
GY
grain yield
G1
subpopulation 1
G1
subpopulation 2

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

All data generated or analysed during this study are included in this published article

Competing interests

The authors declare that they have no competing interests

Funding

Not applicable

Authors' contributions

MIG; conducted normal and drought experiments, and recording the phenotypic data, KFS; performed the genotyping using SSR; AS, designed the study, performed all phenotypic and genotypic analyses, wrote the whole manuscript.

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Figures

Figure 1

Phenotypic correlations and histogram analyses for the six DSI calculated for Ph, CC, FLA, HKW, SP, and GY

Figure 2

Number of DSIs in each genotype characterized as tolerant. DSIs were calculated for Ph, CC, FLA, HKW, SP, and GY. See supplementary Table 3

Figure 3

Phenotypic correlation among all traits scored under normal and drought conditions

Figure 4

Phenotypic correlation among all traits scored under normal and drought conditions

Figure 5

Number of significant markers, No. of total QTL, No. of QTLs under drought condition, and No. of QTL under normal conditions in each selected drought tolerant genotype. Selection was based on characterization of DSI (supplementary Table 3)

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