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Finding genomic regions and candidate genes governing water use efficiency in rice

V. ROJA[a], S. PATIL[a], D.A. DEBORAH[a], A. SRIVIDHYA[a], N. RANJITKUMAR, G. KADAMBARI, P.V. RAMANARAO, E.A. SIDDIQ, and L.R. VEMIREDDY*

Institute of Biotechnology, Acharya NG Ranga Agricultural University, Rajendranagar, Hyderabad-500030, AP, India

Abstract

Water use efficiency (WUE) is an worth attempting trait to discover the genomic regions governing it, especially in view of the diminishing water resources for the crop plants in general and rice in particular. In order to address this, the present investigation was aimed at identification of genomic regions governing WUE employing a recombinant inbred line population derived from a cross between INRC10192, a high WUE landrace, and IR64, a high yielding cultivar. A total of 36 quantitative trait loci (QTLs) were detected under control as well as drought conditions on chromosomes 1, 2, 4, 8, 9, 10, and 11. Among all, the QTLs with the marker intervals RM486-RM6703, RM6703-RM11484, RM404-RM447, RM24879-RM171, and RM229-RM332 on chromosomes 1, 8, 10, and 11 were found to govern the water use efficiency related traits such as carbon isotope discrimination, specific leaf area, leaf width, and relative water content. Nine major QTL intervals were targeted for candidate gene identification using gene ontology (GO) and transcriptome-based analyses. Overrepresented GO terms in the targeted QTLs were found to be associated with the genes/pathways controlling stomatal regulatory mechanism, stress responsive genes or transcription factors, and saccharide biosynthesis pathways under stress situation. Hence, these genes or genomic regions are potential candidates for development of high WUE rice cultivars.

Additional key words: carbon isotope discrimination, QTL mapping, recombinant inbred line, stomatal regulation.

Introduction

Moisture stress is one of the major abiotic stresses that need much attention from the scientists all over. Rice, being a semi-aquatic crop, consumes approximately 3000 to 4000 dm^3 of water to produce one kilogram of paddy. Accumulated evidence demonstrates that the water use efficiency (WUE), *i.e.*, the ratio of the biomass produced per total water transpired over a specific period of time, is a complex trait genetically controlled by many genes. The understanding of the crop WUE is very important for designing the tailor-made rice cultivars suitable to limited moisture conditions. Moreover, the existing rice germplasm contains an obvious genetic variation among accessions in relation to WUE traits. Among the different methods used to measure the WUE, carbon isotope discrimination (CID) is a widely used surrogate trait in many crops including rice. As proposed by Farquhar and Richards (1984), ^{13}C discrimination, expressed as $\delta^{13}C$ in leaf tissue is negatively correlated with WUE.

The molecular markers facilitated by the whole genome sequencing and subsequent advancements in gene discovery approaches exploiting various genomic and transcriptomic databases led to uncovering many quantitative trait loci (QTLs) in rice. However, very few attempts were made with respect to the identification and mapping WUE related traits in rice. Of them, Ishimaru *et al*. (2001) have identified six QTLs governing CID in

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Abbreviations: C - control; CID - carbon isotope discrimination; FPKM - fragments per kilobase of exon per million fragments; GO - gene ontology; LA - leaf area; LLN - leaf length; LOD - maximum likelihood ratio of odds; LWD - leaf width; NFG - number of filled grains; nsSNPs - non synonymous single nucleotide polymorphic regions; PNO - total number of panicles; QTL - quantitative trait locus; RIL - recombinant inbred line; RTLN - root length; RWC- relative water content; SHLN - shoot length; SLA - specific leaf area; SPF - spikelet fertility; T - treatment; TYLD - total yield per plant; WUE - water use efficiency.

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* Corresponding author; e-mail: drvlnreddy@gmail.com

rice. Subsequently, few more QTLs related to WUE as measured by CID have been reported (Price *et al.* 2002, Laza *et al.* 2006, Takai *et al.* 2009, Xu *et al.* 2009, This *et al.* 2010). In addition, 25 QTLs directly governing WUE related traits using pot cultures have also been identified (Zhou *et al.* 2011). In the present investigation, we made an attempt to discover some more genomic regions governing WUE traits in rice. The tightly linked flanking markers of the QTLs can be right away used for the enhancement of WUE of the local high yielding cultivars employing marker-assisted breeding. Besides, we also aimed at identification of the possible candidate genes underlying major QTLs by exploiting the publicly available rice genomic and transcriptomic databases.

Material and methods

Development of mapping population and phenotypic evaluation of recombinant inbred lines: A total of 153 recombinant inbred lines (RILs) at the F_9 generation derived from a cross involving a popular high yielding cv. IR64 and a landrace INRC10192 with a high WUE from the Assam Rice Collection (Srividhya *et al.* 2011) were used for mapping QTLs governing WUE. The RILs along with their parents were germinated in Petri plates, and 14-d-old seedlings were planted in pots (32 × 36 cm) filled with wetland soil and farmyard manure (3:1) along with urea and a 15-15-15 (N-P-K) fertilizer. At the five-leaf stage, only one plant exhibiting vigorous growth was retained in each pot. Both the genotypes were planted in two treatments, *i.e.*, the control with 100 % water supply and the treatment with a reduced amount of water (70 %) compared to normal watering. These pots were placed under a rain-out shelter with movable roofs. The plants were irrigated at certain intervals to maintain a shallow water layer and the amount of water added each time was recorded during the entire life cycle of the crop. The experiment was conducted at the Institute of Biotechnology, the College of Agriculture, ANGRAU, Hyderabad, during vegetation season 2012.

Carbon isotope discrimination: A composite leaf sample comprising 10 mature leaves representing all positions of a plant were harvested and oven dried for 3 d at 80 °C and homogenized to a fine powder with a ball mill. Carbon isotope composition was measured using an isotope ratio mass spectrometer (*Delta-plus*, *Thermo Finnigan*, Bremen, Germany) interfaced with an elemental analyzer (*NA1112*, *Carlo*, Erba, Italy) *via* a continuos flow device (*Conflo-III*, *Thermo Finnigan*) at the National Facility for Stable Isotope Studies in Biological Sciences, the Department of Crop Physiology, the University of Agriculture Science, Bangalore, India. Carbon isotope discrimination $\Delta^{13}C$ was computed using the following formula (Farquhar *et al.* 1989): $\Delta^{13}C = [\delta^{13}C_a - \delta^{13}C_{lb}] / [1+ (\delta^{13}Clb/1000)]$, where $\delta^{13}C_a$ is carbon isotope composition of the air, and $\delta 13C_{lb}$ is carbon isotope composition of the bulk leaf matter.

Relative water content: Four 6 × 2 cm portions were excised from the middle of a leaf and the fresh mass (FM) of each sample was immediately recorded. The leaf segments were immersed in pure water in sterile Petri dish and placed under dim light for 4 h to obtain the full water saturation. The samples were removed from distilled water, blotted dry and the water saturated mass (WSM) was recorded. Then, the samples were kept in a hot air oven at 80 °C overnight and the dry mass (DM) was determined. The relative water content was calculated using the formula suggested by Gonzaleg and Gonzaleg-Viar (2001): RWC [%] = (FM - DM) / (WSM - DM) × 100.

Growth parameters: The specific leaf area (SLA = LA/DM) was recorded for five leaves representing all positions of a plant collected from both the control and the treatment. The leaf area (LA) was estimated using a leaf area meter (*LICOR* model *3100*, Lincoln, Nebraska, USA) and DM as mentioned above.

The plant roots from all replications have been gently pulled out from the pots after maturity and washed thoroughly with tap water. The root length (RTLN) was measured from the crown region to the tip of the longest root using a standard measuring scale. The shoot length (SHLN) was measured from the ground level to the tip of the panicle at the maturity stage. The leaf length (LLN) was measured on the largest two leaves on each plant from the soil surface to the tip and thus included both blade and sheath portions, and an average value was used. The leaf width (LWD) was taken at the widest point (in the middle) of the same leaves. Also, the total number of panicles (PNO) per plant at maturity and the number of filled grains (NFG) per panicle were recorded, and the spikelet fertility (SPF = the ratio of filled spikelets to the total number of filled and chaffy spikelets per panicle × 100) was counted. Finally, the mass of 1 000 grains was recorded and the total yield (TYLD = the mass of filled grains per plant) was counted.

Genotyping: DNA was isolated from fresh leaf samples of INRC10192, IR64, and RIL populations using a modified protocol of the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson 1980). The PCR was performed with a 0.01 cm^3 final volume containing 25 - 50 ng of genomic DNA, 10× Taq buffer, 2.5 mM dNTPs, 0.2 μM of each forward and reverse primers, and 5 U of *Taq* DNA polymerase. The PCR was set up with an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, extension at 72 °C for 1 min, followed by a final extension at 72 °C for 7 min.

Construction of a linkage map and QTL mapping: Of 652 rice microsatellite markers and 21 indel markers used to screen INRC10192 and IR64, 110 were found to be polymorphic, and they were distributed throughout the rice genome. These polymorphic markers have been used for screening the RIL population. A linkage map was constructed using *MAPMAKER/EXP v.3*. Quantitative trait loci were identified using the simple interval mapping and composite interval mapping methods of *QTL Cartographer v. 2.5* (Wang *et al.* 2012). A maximum likelihood ratio of odds (LOD) score of 2.5 was used as a threshold for detecting a QTL.

The genes within the QTL confidence interval were studied for their differential expression within tissues like inflorescence (Davidson *et al.* 2012), shoots (Oono *et al.* 2011), and leaves (Davidson *et al.* 2012) using *Rice qTeller* (http://qteller.com/rice/index.php). The over-expressed genes were selected based on their fragments per kilobase of exon per million fragments (FPKM) values. The overexpressed genes were further analyzed using the *PosMed* (https://database.riken.jp/PosMed/) database system created by the Riken Genomic Science Center (Yokohama, Japan) for the presence of any WUE related genes in each QTL region**.** Non synonymous single nucleotide polymorphic regions **(**nsSNPs) within the predicted candidate genes were identified using *Rice Variation Map* (http://ricevarmap. ncpgr.cn/).

Gene ontology (GO) annotations were performed to identify the significant gene enrichments within the QTL interval using the plant gene set enrichment analysis *Toolkit* (Yi *et al.* 2013) (http://structuralbiology.cau.edu.cn/PlantGSEA/). Further, the significant gene sets were analyzed for the presence of any water use efficiency/stress related genes or transcription factors.

Standard *Excel* program was used to calculate mean, ranges and standard deviations of the phenotypic data collected from parents and 153 RILs. Pearson's correlation analysis between character pairs was computed at $P = 0.05$, 0.01, 0.005, and 0.001 using trait averages for yield and related traits and also for WUE related traits for both parents and the mapping population. Significance of correlation coefficients (r) at $P = 0.05$ or 0.01 is indicated by * or **, respectively.

Results and discussion

The two parents, *i.e.*, IR64 and INRC10192 differed significantly (at the 1 % level) for almost all the traits except for RWC, SPF, PNO, and NFG under the control conditions, whereas under the drought, the parents differed significantly for RWC, SLA, LLN, LWD, SHLN, and TYLD (Table 1). When tested the effect of experimental conditions, the parent INRC10192 differed significantly for the trait PNO only, whereas the parent IR64 showed significant differences for SLA, SHLN, NFG, and TYLD. The RIL population also showed significant differences in many traits except LLN, SHLN, RTLN, NFG, and TWT between the control and drought conditions. Both the parents differed significantly in CID under the control conditions. The RILs also showed segregation for this trait with the range of 17.7 - 20.9 %. Transgressive segregation and skewness were observed about 22.2 % and -0.58, respectively, in the parents for CID. In the case of yield related traits, when considered the control and drought conditions independently, the parent IR64 produced more productive tillers, NFG, and TYLD per plant (Table 1 Suppl.) and it showed a tremendous difference between the control and drought

Table 1. Significance of differences (* - at 5 %, ** at 1 % level) between treatments (control *versus* drought) in parents INRC10192 and IR- IR64 and respective RILs as well as differences between parents under control and drought conditions in water use efficiency and yield related traits. RWC - relative water content, SLA - specific leaf area, LLN - leaf length, LWD - leaf width, SHLN - shoot length, RTLN - root length, SPF - spikelet fertility, PNO - number of panicles, NFG - number of filled grains, TMT - test mass, TYLD - total yield per plant, CID - carbon isotope discrimination.

Trait	INRC10192 control/drought	IR64 control/drought	RILs control/drought	INRC/IR control	INRC/IR drought
RWC [%]	0.068	0.140	0.0002**	0.090	0.002**
SLA [$cm^2\ g^{-1}$]	0.220	0.052*	0.000**	0.030**	0.025**
LLN [cm]	0.700	0.070	0.097	0.050*	0.028**
LWD [cm]	0.120	0.120	0.009**	0.030**	0.030**
SHLN [cm]	0.140	0.015**	0.085	0.0360**	0.015**
RTLN [cm]	0.290	0.204	0.990	0.0380**	0.116
SPF [%]	0.200	0.204	0.006**	0.290	0.770
PNO	0.050*	0.250	0.001**	0.620	0.790
NFG	0.870	0.045*	0.580	0.060	0.230
TMT [g]	0.190	0.204	0.212	0.020**	0.120
TYLD [g]	0.717	0.013**	0.000**	0.030**	0.009**
CID [‰]	-	-		0.020**	-

for the trait NFG, *i.e.*, with a very low number of fertile grains under the drought and a rapid decline in TYLD compared to INRC10192 indicating that IR64 was relatively more susceptible to the stress compared to INRC10192. The mean yield reduction due to the stress as compared to the control was 55 % and 14.6 % in IR64 and INRC10192, respectively. On an average, the RILs showed 28.3 % relative yield loss under the stress when compared to the control. Frequency distribution graphs for all the phenotypic traits were drawn under both the conditions.

Significant positive correlations for the traits as SLA (0.795**), SHLN (0.425*), SPF (0.195*), and TWT (0.476**) between the control and treatment were observed (Table 2 Suppl.). Total yield per plant under the control conditions (TYLD-C) was significantly positively correlated (0.267*) with yield per plant under the treatment (TYLD-T). A significant positive correlation was also observed between LWD-T and SLA-C (0.177*), whereas significant negative correlations were observed for the character pairs RTLN-T and SLA-C (-0.177*), PNO-T and CID-C (-0.176*), and SLA-C and CID (-0.176*). Yield per plant exhibited a significant positive correlation with LWD (0.192*, 0.240*), PNO (0.286*, 0.246*), and TWT (0.299*, 0.304**) under the control as well as drought conditions, respectively. Under the control conditions, a significant positive correlation was observed in the character pairs and the correlation coefficient values between LWD and CID (0.199*), SHLN and LLN (0.200*), SPF and LLN (0.221*), and TWT and SHLN (0.245*). A significant negative correlation was also found between CID and SLA (-0.176*) under the control conditions only (Table 2 Suppl.). Under the drought, RWC showed a significant positive association with LWD (0.229*) and PNO (0.190*), however, SPF showed a significant negative

Table 2. Quantitative trait loci (QTLs) for water use efficiency and yield related traits. Chr - chromosome, LOD - maximum likelihood ratio of odds, *i.e.*, LOD score for the QTL, a0 - additive effect, PVE - phenotypic variance explained by each QTL, Al.ef. - allele effect caused by parents towards QTL, CIM - composite interval mapping; IM - interval mapping. For other abbreviations see Table 1.

No.	Trait	QTL	Chr	Position	LOD	a0	PVE	Marker interval	Al.ef.	Method
1	RWC	*qrwcc10.1*	10	156.01	3.21	3.13	12.70	RM24879-RM171	INRC	CIM & IM
2	SLA	*qslac1.1*	1	22.81	3.27	37.84	22.10	RM6703-RM11484	INRC	CIM
3	SLA	*qslac8.1*	8	412.01	2.89	27.36	40.90	RM404-RM447	INRC	CIM
4	SLA	*qslac10.1*	10	146.01	3.04	-21.58	3.46	RM24879-RM171	IR64	CIM
5	LLN	*qllnc2.1*	2	388.11	4.74	-6.43	5.14	RM263-RM279	IR64	CIM
6	LLN	*qllnc4.1*	4	49.01	3.09	-7.17	64.37	RM336-RM518	IR64	IM
7	LLN	*qllnc8.1*	8	138.31	2.68	7.02	63.07	RM1384-RM5556	INRC	IM
8	LWD	*qlwdc1.1*	1	21.81	2.52	2.79	15.60	RM6703-RM11484	INRC	CIM & IM
9	LWD	*qlwdc8.1*	8	418.01	5.60	1.79	4.61	RM404-RM447	INRC	CIM & IM
10	SHLN	*qshlnc1.1*	1	78.71	2.77	-8.04	17.00	RM488-RM11069	IR64	CIM
11	SPF	*qspfc1.1*	1	165.21	3.82	7.88	56.52	RM1-RM302	INRC	IM
12	SPF	*qspfc1.1*	1	126.01	2.85	7.09	47.10	RM11069-RM1	INRC	CIM
13	PNO	*qpnoc2.1*	2	305.21	2.87	0.88	20.50	RM12469-RM154	INRC	CIM
14	NFG	*qnfgc2.1*	2	270.91	2.90	11.37	13.50	RM341-RM12489	INRC	CIM
15	CID	*qcid1.1*	1	19.50	3.57	0.22	2.93	RM486-RM6703	INRC	CIM & IM
16	TWT	*qtwtc1.1*	1	68.71	2.99	-1.15	18.78	RM488-RM11069	IR64	IM
17	TWT	*qtwtc8.1*	8	469.91	2.71	-1.33	25.53	RM447-RM3481	IR64	IM
18	RWC	*qrwct11.1*	11	45.61	3.60	4.55	42.30	RM229-RM332	INRC	CIM & IM
19	SLA	*qslat1.1*	1	22.81	5.31	34.95	30.60	RM6703-RM11484	INRC	CIM & IM
20	SLA	*qslat8.1*	8	410.01	2.93	27.43	43.10	RM404-RM447	INRC	CIM
21	SLA	*qslat9.1*	9	23.01	2.88	37.98	37.74	RM434-RM257	INRC	IM
22	LLN	*qllnt2.1*	2	206.81	3.71	7.20	6.22	RM475-RM13530	INRC	CIM
23	LLN	*qllnt4.1*	4	91.01	3.08	7.14	6.17	RM335-RM518	INRC	CIM & IM
24	LLN	*qllnt8.1*	8	71.31	3.94	-7.20	6.21	RM1384-RM5556	IR64	CIM & IM
25	LWD	*qlwdt11.1*	11	5.01	2.57	0.86	14.12	RM1233-RM229	INRC	IM
26	LWD	*qlwdt1.1*	1	24.81	3.02	1.87	19.20	RM6703-RM11484	INRC	CIM
27	LWD	*qlwd1.2*	1	173.21	3.10	-1.57	47.00	RM1-RM302	IR64	CIM & IM
28	RTLN	*qrtlnt8.1*	8	22.51	2.71	5.86	14.60	RM3395-RM3662	INRC	CIM & IM
29	PNO	*qpnot1.1*	1	21.81	2.88	2.85	22.00	RM6703-RM11484	INRC	IM
30	PNO	*qpnot8.1*	8	21.51	3.45	2.10	23.70	RM3395-RM3662	INRC	CIM & IM
31	TWT	*qtwtt8.1*	8	331.01	2.63	0.73	8.50	RM5434-RM23362	INRC	IM
32	NFG	*qnfgt8.1*	8	399.31	2.78	18.60	22.90	RM6208-RM404	INRC	CIM
33	TWT	*qtwtt1.1*	1	83.71	3.10	-0.99	14.70	RM488-RM11069	IR64	CIM & IM
34	TWT	*qtwtt11.1*	11	61.61	4.32	1.36	27.60	RM229-RM332	INRC	CIM
35	TYLD	*qtyldt2.1*	2	207.81	2.67	-2.00	6.32	RM475-RM13530	IR64	IM
36	TYLD	*qtyldt4.1*	4	46.01	2.58	-1.99	6.28	RM335-RM518	IR64	IM

correlation with LLN (-0.186*), whereas SHLN showed a significant positive correlation (0.218*) (Table 3 Suppl.).

A total of 36 QTLs was detected for 12 traits on chromosomes 1, 2, 4, 8, 9, 10, and 11 under both the control and the treatment (Table 2). Of which, 17 QTLs were detected under the control, and 19 were under the drought conditions. The LOD score and phenotypic variation for these QTLs ranged from 2.5 to 5.6 and 2.9 to 64.3 %, respectively. Allelic effect was contributed by INRC10192 and IR64 for 25 and 9 QTLs, respectively.

Only one QTL on chromosome 1 has been identified for CID. The QTL *qcid1.1* in the marker interval RM486-RM6703 with LOD 3.5 explained 2.9 % of phenotypic variation. The INRC10192 allele contributed to the increased phenotypic effect at this QTL position. Previously, Xu *et al.* (2009) detected a QTL which accounts for a phenotypic variation of 22.5 % and has a LOD score of 8.75. Takai *et al.* (2006) reported a QTL with a phenotypic variance and a LOD score of 9.3 % and 4.0, respectively, using a RIL population derived from a cross between Milyang 23 and Akihikari.

Two QTLs for RWC were detected on chromosomes 10 and 11 under the control and drought conditions at the marker intervals RM24879-RM171 and RM229-RM332, respectively. The LOD score and phenotypic variation of these two QTLs ranged from 3.2 - 3.6 and 21.7 - 42.3 %, respectively. The allelic effect of both these QTLs was contributed by the INRC10192 parent. For the same trait, Srinivasan *et al*. (2008) mapped a QTL, *rwc11.1* on chromosome 11 at the same region mapped in the present study using double haploid (DH) population. The phenotypic variation and LOD value for the reported QTLs were 12.5 and 3.16 %, respectively. Using an RIL population developed from a cross between Azucena and Bala, QTLs for RWC were also reported previously by Price *et al.* (2002) on chromosome 10.

For SLA, a total of six QTLs has been detected on chromosomes 1, 8, 9, and 10 under the control and drought separately. The allelic effect of all these QTLs was contributed by INRC10192 except for the QTL detected on chromosome 10 wherein the allelic effect was contributed by IR64. The phenotypic variation and LOD score ranged from 3.4 to 43.1 % and 2.8 to 5.3 %, respectively. The same region on chromosome 1 in the present study has also been mapped for SLA by This *et al.* (2010). Considering the repeated linkage of the same region from two studies for SLA, wherein IR64 is a common parent, the QTL region spanning 7.0 Mb can be a potent target for candidate gene identification and further exploitation in rice breeding.

Six genomic regions effecting LLN was detected on chromosomes 2, 4, and 8. Of which, three QTLs, each under the control and drought conditions, were detected. Out of six QTLs identified, three were contributed by the parent INRC10192, whereas IR64 contributed the rest of them. The LOD values and phenotypic variation ranged from 2.6 to 4.7 % and 5.1 to 64.3 %, respectively. The marker interval RM1384-RM5556 is shown to govern LLN under both the conditions, and it explained the highest phenotypic variation of 63 %. Using backcross

Table 3 Comparison of quantitative trait loci (QTLs) identified in the present study with the previously reported ones across the *Oryza* genus. Chr - chromosome.

No.	QTLs in the present study	Chr	Marker interval	Common QTLs identified in the previous studies
1	*qslac1.1*	1	RM6703-RM11484	specific leaf area - *SLA1.1* (This *et al*. 2010)
2	*qlwdc1.1, qlwdt1.1*	1	RM6703-RM11484	leaf width - *LW1.2* (Xu *et al*. 2009)
3	*qcid1.1*	1	RM6703-RM11484	carbon isotope discrimination (Xu *et al*. 2009, Takai *et al*. 2006)
4	*qshln1.1*	1	RM488-RM11069	plant height - *qph1.1, qph1.2* (Kotla *et al*. 2013)
5	*qspfc1.1*	1	RM1-RM302	spikelet fertility - *SF1* (Lin *et al*. 1996)
6	*qtwtt,qtwtc1.1*	1	RM488-RM11069	1000 seed mass (Luo *et al*. 2001)
7	*qpnot1.1*	1	RM6703-RM11484	number of panicles (Hittalmani *et al*. 2002)
8	*qtyldt2.1*	2	RM475-RM13530	grain yield - *qgy2.1* (Zou *et al*. 2005)
9	*qpnoc2.1*	2	RM12469-RM154	number of panicles (Marri *et al*. 2005, Yuan *et al*. 2003)
10	*qllnt2.1*	2	RM475-RM13530	leaf length - *LL2.1* (This *et al*. 2010)
11	*qllnt4.1*	4	RM335-RM518	leaf length - *LL4.1* (Xu *et al*. 2009)
12	*qtyldt4.1*	4	RM335-RM518	yield under stress (Swamy *et al*. 2013)
13	*qrtlnt8.1*	8	RM3395-RM3662	root length - *qcrl8.1* (Nguyen *et al*. 2014)
14	*qlwdc8.1*	8	RM404-RM447	leaf length - *qtll-8* (Cui *et al.* 2002), flag leaf length- *qfll-8* (Mei *et al*. 2003), leaf length - *qtll-8* (Yan *et al*. 1999)
15	*qslac8.1, qslat8.1*	8	RM404-RM447	leaf area (Yan *et al.* 1999)
16	*qtwtt8.1*	8	RM5434-RM23362	1000 seed mass (Xiao *et al*. 1996, Li *et al*. 1997, Cho *et al*. 2003)
17	*qslat9.1*	9	RM334-RM257	specific leaf area - *SLA9.1* (This *et al*. 2010)
18	*qslac10.1*	10	RM24879-RM171	flag leaf length - *qfl10-1* (Yan *et al*. 1999)
19	*qrwct11.1*	11	RM229-RM332	relative water content- *rwc11.1* (Srinivasan *et al*. 2008)
20	*qtwtt11.1*	11	RM229-RM332	Grain mass - *qgw11.1* (Moncada *et al*. 2001) *qgw11* (Hua *et al.* 2003), *qgw11-1* (Cho *et al*. 2003)
21	*qlwdt11.1*	11	RM1233-RM229	leaf length - *qlt11-1* (Cui *et al*. 2002)

inbred lines derived from a cross between Nipponbare and Kasalath, Xu *et al.* (2009) discovered a QTL for LLN at the same region that was identified in the present study on chromosome 4.

Leaf width was governed by five QTLs that were dispersed on chromosomes 1, 8, and 11. Among them, two QTLs were expressed under the control conditions and remaining three under the drought. The genomic region RM6703-RM11484 governed LWD under both the conditions. The parent INRC10192 contributed to an increased phenotypic effect at this locus with a LOD value range of 3 to 5.5, explaining a phenotypic variation 4.6 to 47 %. Among the five QTLs detected for LWD, the parent INRC10192 increased the allelic effect at four loci, whereas the parent IR64 increased at one locus, *i.e.,* RM1-RM302 with a LOD value of 3.1, and the phenotypic variation explained about 47 %. Xu *et al.* (2009) also detected two QTLs for LWD on chromosome 1 at the same region between the marker interval of RM6703 and RM11484. The phenotypic variance and LOD scores were 10 - 11 % and 3.4, respectively. The leaf width QTLs detected on chromosomes 1 and 8 in the present study were also co-located with the QTLs governing SLA under both the conditions. Hence, these two traits are assumed to be controlled by the pleiotropic effect of the same gene or under thecontrol of a gene regulatory system involving one or more transcriptional factors.

Shoot length under the control conditions was influenced by one QTL on chromosome 1 at the marker interval RM488-RM11069, explaining a phenotypic variance of 17 % with a LOD score of 2.77. The parent IR64 contributed the increasing allelic effect of this locus.

Quantitative trait locus for RTLN under the drought was detected on chromosome 8 at the marker interval RM3395-RM3662, explaining a phenotypic variation and LOD score of 14.6 % and 2.7, respectively. The INRC10192 allele increased the allelic effect at this locus. Theoretically, a genotype with long roots along with a high volume can access deeper and wider soil layers and utilize water more efficiently. Since INRC10192 has a high root length and the allele effect contributed, this region can be targeted for identification of candidate genes underlying this QTL.

Spikelet fertility under the control conditions was governed by two regions on chromosome 1 at the marker intervals RM1-RM302 and RM11069-RM1, explaining a phenotypic variance and LOD score ranging from 47.1 to 56.5 % and from 3.8 to 2.8, respectively. The allelic effect of both the loci was increased by the INRC10192 parent. Lin *et al.* (1996) also reported a QTL *SF1* for SPF within the same region that has been reported in the present study.

Only one QTL controlling PNO under the control conditions was detected on chromosome 2 at the marker interval RM12469-RM154. Two QTLs were detected for PNO under the treatment situation on chromosomes 1 and 8. The phenotypic variation ranged from 20.5 to 23.7 %. The INRC10192 increased the allelic effect of the QTL. The same region has also been mapped by Hittalmani *et al.* (2002) for the PNO trait on chromosome 1 and on chromosome 2 by Marri *et al.* (2005) and Yuan *et al.* (2003).

Two QTLs were detected for this trait under the control conditions and the drought each on chromosomes 2 and 8 at the marker intervals RM341-RM12489 and RM6208-RM404, explaining a phenotypic variation and LOD score ranging from 13.5 - 22.9 % and 2.7 - 3.4, respectively. The INRC10192 caused the increased allelic effect at these loci. Using the same population in the F_6 generation, Srividhya *et al.* (2011) also reported that the marker RM404 on chromosome 8 is linked to QTLs for SPF on both primary rachii and secondary rachii and also links to QTLs for the number of chaffy grains on both primary and secondary rachii. It indicates that this genomic region harboured either a pleiotropic or tightly linked genes controlling spikelet fertility and filled grains traits.

Five QTLs influencing the mass of 1 000 grains were identified in the present study. Out of which two QTLs were identified under the control conditions on chromosome 1, and three were on chromosomes 1, 8, and 11 under the treatment. The parent INRC10192 increased the allelic effect at two loci and the other parent IR64 contributed to the increased allelic effect at the remaining three loci. Among them, the QTL *qtwtt11.1* detected under the treatment explained 27.6 % of the phenotypic variation. The LOD score and phenotypic variation caused by the QTLs controlling 1000 grain mass ranged from 2.6 to 4.3 and from 8.5 to 27.6 %, respectively. Quantitative trait locus for grain test mass under both the control and the treatment detected in the present study has also been reported by Luo *et al.* (2001) on chromosome 1, by Xiao *et al*. (1996), Li *et al*. (1997), and Cho *et al*. (2003) on chromosome 8, and by Moncada *et al*. (2001), Hua *et al*. (2003), and Cho *et al*. (2003) on chromosome 11.

Two QTLs, *qtyldt2.1* and *qtyldt4.1*, for TYLD per plant were discovered only under the drought on chromosomes 2 and 4 at the marker intervals RM475-RM13530 and RM335-RM518 with a phenotypic variance and LOD scores of 6.3, 6.2, 2.6, and 2.5, respectively. The favourable allelic effects of both the QTLs were contributed by the parent IR64. Zou *et al.* (2005) has also reported the QTL *qgy2.1* for TYLD per single plant on chromosome 2 at the same region that was identified in the present study. Using the same mapping population in F_2 and F_6 generations, Hariprasad (2003) and Srividhya *et al.* (2011), respectively, reported the *qspy2.1* QTL for single plant yield on chromosome 2. The effect of *qspy2.1* comes from INRC10192. Similarly, in the present study, a QTL for the single plant yield was identified on the same chromosome but at a different marker interval, and the favourable allelic effect for this QTL was contributed by INRC10192. This striking difference indicates the complex nature of yield due to its interaction with environment.

Generally, QTLs with major effects are more likely to be stable across multiple environments, varietal

backgrounds, and populations. Despite the present study was carried out in a single environment using a single population, together with the results of previous studies which were conducted in different environments and genetic backgrounds a total of 21 common QTLs that are associated with 11 WUE and 10 yield related traits under the moisture stress conditions were detected, and they could be considered as stable QTLs (Table 3). The stable QTLs identified under both the control and treatment conditions in the present study were *qsla1.1, qsla8.1, qlln4.1, qlln8.1, qlwd1.1*, and *qtwt1.1*. The QTLs *qpnot1.1, qtyldt2.1, qtyldt4.1, qpnot8.1, qnfgt8.1, qrtlnt8.1,* and *qtwtt11.1* were detected only under the treatment, and the QTL *qspf1.1* was detected only under the control. By pyramiding of these stable QTLs into a single high yielding genotype, it is possible to develop genotypes which give stable performance over a range of environments.

Identification of candidate gene(s) underlying major or stable QTLs is the greatest challenge in QTL mapping studies for precise introgression of the gene of interest for targeted trait improvement in breeding programmes. In order to pinpoint the exact candidate genes underlying the QTLs, it is necessary to go through the steps of fine mapping besides gene expression analysis and complementation tests. Recently, attempts have been made to predict the candidate genes underlying QTLs exploiting rice genome information, especially GO and transcriptome analyses by avoiding time consuming and laborious fine mapping steps (Monclus *et al.* 2012, Vemireddy *et al*. 2015).

In the present study, an attempt was made to identify the significantly overrepresented GO terms underlying seven major QTL intervals: RM6703-RM11484, RM488-RM11069, RM335-RM518, RM404-RM447, RM1384-RM556, RM24879-RM171, and RM229-RM332 using the plant gene set enrichment analysis tool kit. The GO annotation for the QTL intervals identified in the present study is presented in Table 4. For these QTL intervals, the ratio of annotated genes to the total number of genes ranged from 77.9 % (RM229-RM332) to 92.9 % (RM486-RM6703). The percentage of significantly overrepresented (enriched) GO terms varied between '0' for RTLN-T and PNO-T on chromosome 8 (RM3395-RM3662) to 21.05 % for LLN-C, LLN-T, and TYLD-T on chromosome 4 (RM335-RM518). No correlation was observed between the cumulated size of QTL confidence intervals on the genome for a particular trait and the number of significant GO terms in the corresponding intervals.

Table 4. Gene ontology (GO) analysis of the selected QTL regions. For abbreviations see Table 1.

Trait	Chr	Marker interval	Total number of genes	Number of genes annotated	Annotated genes [%]	Number of significant GO terms	Significant GO terms [%]
CID	1	RM486-RM6703	57	53	92.9	1	1.8
SLA-T, SLA-C, LWD-C, LWD-T, PNO-T	1	RM6703-RM11484	826	708	85.7	66	9.3
SHLN-C, TWT-C, TWT-T	1	RM488-RM11069	392	328	83.6	41	12.5
LLN-T, LLN-C, TYLD-T	4	RM335-RM518	71	57	80.2	12	21.0
SLA-C, SLA-T, LWD-C	8	RM404-RM447	837	701	83.7	74	10.5
RTLN-T, PNO-T	8	RM3395-RM3662	58	53	91.3	0	0.0
LLN-T, LLN-C	8	RM1384-RM5556	393	324	82.4	46	14.1
RWC-C, SLA-C	10	RM24879-RM171	1101	887	80.5	65	7.3
RWC-T, TWT-T	11	RM229-RM332	786	613	77.9	61	9.9

The significant GO term subtilase activity (GO: 0004289) was present in two QTL intervals; one controlling leaf traits, *viz.,* SLA, LWD, and LLN under the control and treatment, and other PNO and TYLD per plant under the treatment. The molecular function of this GO term is serine type endopeptidase activity, which has seven genes under this term. Subtilisin like serine proteases were reported to be involved in regulation of stomatal density and distribution (Berger and Altmann 2000). Another term, cellular saccharide biosynthesis (GO: 0034637), was present within the interval RM6703-RM11484 and comprised seven genes mainly involved in chemical reactions and pathways resulting in formation of sugars.

A significant GO term corresponding to protein serine/threonine kinase activity (GO: 0004674) was identified within the intervals RM6703-RM11484, RM229-RM332, and RM24879-RM171, which together comprised 33 genes. These genes were reported to be involved in regulating multiple abiotic stress tolerance traits including drought and salinity (Kulik *et al.* 2011). The significant GO term corresponding to 'response to water stimulus' (GO: 0009415) was identified within the RM229-RM332 interval only. This GO term consists of six genes that belong to the dehydrin family of proteins. Dehydrins are believed to play an important protective role in a plant cell during dehydration (Hanin *et al.* 2011). Further, two significant GO terms representing cellular reaction to stress were response to stress (GO: 0006950) identified within the RM229-RM332 interval that comprised 43 genes, and a cellular response to stress (GO: 0033554) identified within the interval RM6703-

RM11484, containing 10 genes which are involved mainly in the DNA repair system in response to stress.

An attempt was also made, in the present study, to predict candidate genes underlying major QTLs governing WUE traits by exploiting publicly available databases such as *qTeller* for transcriptome analysis and the rice variation map (*RiceVarMap*) for nsSNP to identify a genomic variation. The genes within the QTL intervals were studied for their differential expression in tissues, such as inflorescence (Davidson *et al.* 2012), shoots (Oono *et al.* 2011), and leaves (Davidson *et al.* 2012), using transcriptome analysis based on high FPKM values. In addition, we have compared the short listed genes with *PosMed* for their presence with reference to WUE. The criterion of choosing transcriptomics and nsSNPs was that a candidate gene must show variation in its gene expression and genome level in order to control a trait..

In the present study, out of nine major effect QTL intervals targeted, candidate genes for only seven QTL intervals were identified. There were no expressed candidate genes found within the QTL intervals RM335-RM518 on chromosome 4 and RM3395-RM3662 on chromosome 8.

The candidate genes that were expressed within the interval RM486-RM6703 that controls *qcid1.1*, namely, an auxin efflux carrier component (Os01g0818000), a HEAT repeat family protein (Os01g0819900) and a ubiquitin-conjugating enzyme (Os01g0819500). Among them, auxin efflux carrier component gene expression levels were higher when compared to the other genes (Table 4 Suppl.). Four nsSNPs were also identified in the gene auxin efflux carrier component. Recently, Zhang *et al.* (2012) demonstrated that the auxin efflux carrier gene has a role in drought stress response in rice besides function as an auxin polar transport.

The QTLs for SLA and LWD were found to co-locate on three regions RM6703-RM11484, RM404-RM447, and RM24879-RM171 on chromosomes 1, 8, and 10, respectively (Table 3). In total, 31 genes were found to be expressed within these leaf trait related QTL intervals. Of them, predicted candidate genes for SLA and LWD based on the common appearance of the genes in different QTL intervals controlling the same trait were a calmodulin-binding protein (Os01g0810300), an ABC transporter ATP-binding protein (Os01g0723800), trehalose-6-phosphate synthase (Os08g0445700), and lactate/malate dehydrogenase (Os08g0434300) under both the control and treatment conditions. Consistent with these results, Xu *et al.* (2011) reported that a novel rice calmodulin-like gene, OsMSR2, enhances drought and salt tolerance in *Arabidopsis*. Recently, Nguyen *et al.* (2014) reported that the ABC transporter genes are differentially regulated by drought stress. The involvement of osmolytes, especially trehalose, is pertinent in drought stress. The overexpression of trehalose-6-phosphate synthase (Os08g0445700) is widely known to enhance drought stress tolerance in rice (Li *et al.,* 2011). Based on these results, it can be presumed that the genes involved in drought stress conditions and WUE are the same. Very recently, Zhang *et al.* (2015) identified two candidate genes underlying a major QTL governing the flag leaf width in rice that encodes an RNA-binding protein (LOC_Os07g41180) and an unknown expressed protein (LOC_Os07g41200). However, for the detailed understanding of the leaf area traits and WUE at the molecular level further research is needed.

The QTL intervals that control RWC are RM24879-RM171 and RM229-RM332 on chromosomes 10 and 11, respectively. Even though no common genes between these two QTL intervals were identified, the genes that are involved in drought tolerance exist. Among them, zinc finger RING-type (Jan *et al.* 2013), water-stress inducible protein RAB21 (Todaka *et al.* 2015), and calmodulin-binding transcription activator (Xu *et al.* 2011) are already known to be involved in drought tolerance.

Conclusions

The crop plants are gifted with a natural genetic variability in the traits related to WUE. Exploiting this variability, it is time to design cultivars with a high WUE, particularly in crops like rice, that needs large amount of water. The present investigation was one of the attempts to discover genomic regions and plausible candidate genes regulating WUE traits. Among all the QTLs identified in the present study, the QTLs detected in the marker intervals RM486-RM6703, RM6703-RM11484, RM404-RM447, RM24879-RM171, and RM229-RM332 on chromosomes 1, 8, 10, and 11 were found to harbour the genes governing majority of WUE related traits, *e.g.*, CID, SLA, LWD, and RWC. Overrepresented GO terms were found to be associated with the genes/pathways controlling stomatal regulatory mechanism, expression of stress responsive genes/transcription factors, and genes of sugar biosynthetic pathways under a stress situation. Hence, these QTL regions identified are potential candidates for fine mapping and positional cloning studies. The tightly linked markers of major QTLs and functional markers derived from predicted candidate genes can be used as foreground markers for development of high WUE rice cultivars employing marker-assisted selection.

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