



Research Article

Single marker analysis for leaf gas exchange traits from RILs of RD 23 (*O. sativa* L.) and *O. longistaminata*

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ABSTRACT

Rice is frequently affected by drought. However, economic water usage by the crop less impacted the stress. Its improvement should thus rely on assessing and utilizing the genetic bases of Carbon balance and water use efficient traits. These days, sequence based analysis is widely used to identify the associated hotspot loci to a given trait of interest. For two cropping seasons, 135 *Oryza sativa* L./*Oryza longistaminata* RILs were phenotyped to four leaf physiological traits and single marker analysis was integrated to identify consistently and significantly correlated SNPs. Through the RADseq technique, 20,014 SNPs were identified from the phenotypically diversified lines and in particular, 20 SNPs were defined as significantly associated hotspot loci. This study therefore, implicated marker-trait associations for leaf physiological traits. And such significantly associated loci can be used as tools for marker assisted selection of the relatively drought tolerant and highly photosynthetic lines of perennial rice.

1. Introduction

Rice (*Oryza sativa* L.) is the widely consumed staple food for a large part of the world's human population [1]. In the past decade however, climatic change was found to be one of the most devastating factors that limits rice yield [2]. Plants can respond to water deficit via regulating stomatal closure and density [3]. Since grain yield is derived from carbon assimilation, enhancing photosynthetic efficiency along with a profitable water use potential should be the concern of modern rice breeders.

These days, the narrow genetic basis of cultivated rice further impeded the yield potential [1]. Thus, the untapped genetic resource of wild rice species can be a valuable resource and option for the genetic improvement of cultivated rice [4]. For instance, assessing photosynthetic and water use efficiency within the genus *Oryza* is essential for identifying potential donors, use them in a wide crossing program and developing high yielding rice varieties even under harsh environment [5]. According to [6], the light-saturated assimilation rate in wild rice species was higher than cultivars of *O. sativa*. Exploiting the diversified photosynthetic potential in wild rice gene pool could therefore enhance

rice yield [7]. Other than its promising perennial and blight resistance traits, the African wild rice (*O. longistaminata*) was reported as superior for its efficient water use and carbon balance system [8].

From recombinant populations, genetic basis of yield related traits can be characterized through mapping the corresponding chromosomal regions [9]. Efforts on precise genetic dissection of agronomic traits via gene pyramiding and rational design have been made for rice yield improvement [9, 10, 11]. However, associated genes of photosynthetic and gas exchange indices have not been efficiently screened even by conventional methods [12]. Therefore, applying the current sequence based genotyping methods are useful to offer a resolved QTL with a few or even a single candidate gene [9]. Restriction Associated DNA Sequencing (RADSeq) is the one among the most efficient and cost-effective whole-genome sequencing approaches [13]. In particular, a single marker analysis from such sequence reads can precisely detect and/or locate a hotspot-SNP via its informative statistical approaches such as ANOVA [14].

To better understand the genetic basis of the photosynthetic and leaf gas exchange traits of elite restorers such as *O. longistaminata*, a set of recombinant inbred lines (RILs) must be assessed. Hence, this study used

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Table 1

Range of the four leaf physiological traits along with the F test values from the 135 RILs and their parents.

Leaf physiological Trait	Minimum	Maximum	Mean	Std Dev	MSE (136)	F test (136)
Pn	1.55	28.76	11.18	5.61	24.89	1.52**
Gs	0.04	1.27	0.23	0.14	0.015	0.003**
Ci	10.98	372.71	248.34	75.27	2681.67	3.22***
E	1.35	14.82	5.88	2.61	4.49	2.02***

Where; Pn (photosynthetic rate), gs (stomatal conductance), Ci (Intercellular CO₂ concentration), Tr (Transpiration rate), MSE (Mean square error) and numbers in parenthesis represent degree of freedom

*** (highly significant at p<0.001), ** (significant at p<0.01)

Table 2Correlation coefficients among the four leaf physiological traits of the 135 RD23 variety of *O. sativa* L. X *O. longistaminata* RILs.

	Pn	Gs	Ci	Tr
Pn	1.00	0.44***	-0.24***	0.25**
Gs		1.00	0.36***	0.55***
Ci			1.00	0.11
Tr				1.00

Key; Pn (photosynthetic rate), gs (stomatal conductance), Ci (Intercellular CO₂ concentration), Tr (Transpiration rate)

** Significant at p<0.01; *** Highly significant at p<0.001

a single marker analysis system on 135 RILs derived from (*Oryza sativa* L./*Oryza longistaminata*) for mapping hotspot regions of the photosynthetic and Water Use potential traits.

2. Materials and methods

2.1. Plant cultivation and Leaf physiological traits measurement

In the wet seasons of 2017 and 2018, 135 RILs and their two parental lines (RD 23 and *O. longistaminata*) were grown in Jinghong, China. Randomized block design was used for the experiment and all plant materials were grown in paddy fields following normal field management practices.

At the panicle initiation stage, four leaf physiological traits (Photosynthetic rate, stomatal conductance, Transpiration rate and intercellular CO₂ concentration) for all RILs and their parents were measured from fully expanded flag leaves by the portable photosynthesis measuring system (LI6400XT LI-COR, Lincoln, NE, USA) on a sunny and windless days from 10:00 a.m. to 1:00 p.m.

Table 3Year wise significant (p-value < 0.01) and consistent SNPs from the genome-wide analyses of the RD 23 *O. sativa* L. and *O. longistaminata* RILs.

Leaf Physiological trait	SNP position	Chromosome no	P value in 2017	LoD value in 2017	P value in 2018	LoD value in 2018
Photosynthetic rate (Pn)	21109027	8	0.008	2.10	0.011	1.96
	21109028	8	0.008	2.10	0.011	1.96
	21109029	8	0.008	2.10	0.011	1.96
	21109039	8	0.009	2.05	0.009	2.05
Stomatal conductance (Sc)	30849707	1	0.006	2.22	1.99E -06	5.70
	3110922	6	0.001	3	0.012	1.92
	4233115	6	0.010	2	0.003	2.52
	7845707	6	0.008	1.1	2.07E -05	4.68
Intercellular CO ₂ (Ci)	9691659	8	0.010	2	0.014	1.85
	20930186	8	0.001	3	0.010	2
	20931661	8	0.008	2.1	0.002	2.70
	15184833	11	0.008	2.1	0.002	2.70
	319600	8	0.006	2.22	0.014	1.85
	11510283	8	0.007	2.15	0.012	1.92
	11974960	8	0.011	1.96	0.003	2.52
	12082201	8	0.011	1.96	0.003	2.52
Transpiration Rate (Tr)	12400036	8	0.006	2.22	0.003	2.52
	15474378	8	0.011	1.96	0.003	2.52
	22428157	4	0.012	1.92	0.006	2.22
	22468083	4	0.012	1.92	0.006	2.22

2.2. RAD-seq library preparation and single marker analysis

The DNA from two parental lines and RILs was extracted following the CTAB procedure [15]. DNA quality was determined on a 1.0% agarose gel and normalized to a 50 ng/ul concentration.

The 150 bp long reads of each individual were obtained according to the identified taq sequences. Such reads were aligned to the Nipponbare reference genome (version BROADs1, Ensembl release 64) via the Bawtie2 Alignment Tool version 0.11.3 [16]. From the Stacks package, consensus sequences for each individual were extracted and merged in to a catalog [17]. Based on the Bayesian estimation of site frequency at each location, SNP calling was performed. The likelihoods of genotypes for each individual were integrated and sites with a probability of over 0.95 were accepted as candidate SNPs [18]. Markers were filtered with designated missing values (>20%) and common SNPs with a P-value < 0.01 to both cropping seasons were defined as significantly associated markers [19].

3. Results

3.1. Variation of leaf physiological traits and their correlations

The grand mean and standard error of the four traits; Photosynthetic rate, stomatal conductance, intercellular CO₂ concentration and transpiration rate for the 135 RILs and their parental lines (RD23 of *O. sativa* and *O. longistaminata*), were 11.18 ±5.61; 0.23 ±0.14; 248.34 ±75.27 and 5.88 ±2.6 respectively (Table 1).

A considerable variation for the four leaf physiological traits was observed among the recombinant inbred lines (Table 1). In particular, the two leaf physiological traits called intercellular CO₂ concentration and Transpiration rate depicted highly significant differences (p<0.001). A remarkably significant differences at p<0.01 were also reported for the remaining two traits called photosynthetic rate and



Fig 1. Manhattan plots displaying the genome-wide association based on singlemarkers analysis to the four leaf physiological traits; Pn (A), gs (B), Ci (C) and Tr (D). Each dot represents a SNP and genomic order or SNP position on the chromosome was indicated on the x-axis. The value on the y-axis represents the $-\log_{10}$ of the P-value. The contrasting colors represent the 2017 and 2018 cropping seasons. Commented [h5]: All of this is removed.

stomatal conductance (Table 1).

Through regressing the values from lines of this study, a Pearson correlation was evaluated for one of the leaf physiological trait over the other (Table 2). Except the correlation among Pn and Ci (-0.24), all the rest combinations showed positive associations. For instance, highly significant ($P < 0.001$) correlations were observed between stomatal conductance and the rest three leaf physiological traits; Transpiration rate ($r = 0.55$), photosynthetic rate ($r = 0.44$), and Intercellular CO₂ concentration ($r = 0.36$). Likewise, a positive and significant correlation ($P < 0.01$) was reported among photosynthetic rate and Transpiration rate ($r = 0.25$). Exceptionally, a positive but insignificant correlation was showed among the intercellular CO₂ concentration and Transpiration rate ($r = 0.11$).

3.2. Detection of loci associated with leaf physiological traits

Using the RAD-seq approach, a total of 20,014 high quality SNPs were identified from the genome of 135 RILs and their parents. Based on the phenotyped leaf physiological traits of the two cropping seasons, a single marker based analysis detected twenty significantly correlated and consistently associated loci (Table 3). These loci were distributed over chromosome 1, 4, 6, 8 and 11. In particular, chromosome 8 was the most saturated chromosome with 13 significantly associated SNPs. Chromosome 4 and chromosome 6 had a respective 2 and 3 hotspot SNPs. Whereas, chromosome 1 and 11 had the least (1 linked SNP in each). Based on p-values of the significant SNPs, the LOD values were ranged from 1.1 to 5.7. Number and distribution of the loci was also varied depending on type of leaf physiological traits. For instance, majority (75%) of the loci were associated with Transpiration rate and intercellular CO₂ concentration (Table 3). Besides, four loci that were linked to chromosome 8 were significantly correlated with photosynthetic rate. The only loci residing to chromosome 1 was found to be significantly associated with stomatal conductance.

After filtering the whole genome sequence data of the RILs and their parents, 20,014 SNPs were retained for the subsequent Genome Wide Association Studies (GWAS). The single-marker analysis from the whole sequence dataset revealed association of the 4 leaf physiological traits with each SNP loci at significance levels.

4. Discussion

Leaf physiological traits are key traits to improve rice yield productivity [20]. Hence, rice breeding programs need to incorporate efficient water use potential and superior photosynthetic characters from potential donors such as; *O. longistaminata* [21]. The diversified genetic backgrounds of RILs can provide useful insight into the genetic basis of yield formation [22]. Unlike conventional molecular markers, recombination bins defined by SNPs from whole genome sequencing allow accurate representation of recombination events across the RILs in the entire genome [9]. From a highly photosynthetic and water conserving RILs, single-marker analysis could therefore detect hotspot-SNPs to desirable leaf physiological traits, design selective markers and simultaneously develop promising breeding materials into varieties.

4.1. Analysis of critical leaf physiological traits

The 135 RILs assessed in this study indicated a statistically significant difference for the four leaf physiological traits (Table 1). Such perennial rice lines can thus have a chance to be developed in to diversified varietal types. In different rice improvement programs, a correlation study is fundamental for breeders to have selection and understand major traits [20, 23, 24]. Correlation analysis among the four leaf physiological traits indicated a significant and positive correlation of gs with Pn, Tr and Ci (Table 2). According to [25], highly significant and positive trait to trait correlations revealed a common genetic base. Thus, the highly significant associations among the

assessed traits implicate necessity of such RILs for the evaluation of an efficient water use and Carbon balance potential [26]. The other significant correlation was detected between pn and Tr. This observation could implicate pliotrophy and the promising future of such RILs to be developed in to highly photosynthetic and drought resistant perennial rice varieties. Other than pleiotropic effect or linkage of the genes, undetected SNPs or loci could likely be responsible for the pn and Tr correlation [9]. To the contrary, the only negative but significant association was recorded among pn and Ci. This report might be from the higher level of Carboxylation efficiency which favors CO₂ fixation [27, 28]. In other sense, a strongly significant but negative correlation between pn and Ci could be due to the magnificent kinetics of RUBISCO [29, 30].

4.2. Single marker analysis for identifying associated SNPs of leaf physiological traits

In the RILs population, level of explained variance might be correlated with a single marker [19]. Hence, stably associated markers with target trait can be useful for breeding with broad adaptability to diversified environmental conditions [31]. In the present study, 20 hotspot-SNPs located on chromosomes 1, 4, 6, 8 and 11 were identified as stable over the 2 cropping seasons (Table 3). According to [20], association of a given loci to several traits has an immense value in the tasks of rice breeding and marker assisted selection. For instance, 65% of the detected hotspot-SNPs of this study were residing on chromosome 8 (Table 3). Due to the molecular complexity of structural genes, many loci for different traits co-localize in a given chromosomal region [32]. Here, the single marker analysis revealed association of chromosome 8 to the three leaf physiological traits (Pn, Tr and Ci). [22] also reported association of linked loci of this chromosome to many yield related leaf characteristics. Besides, chromosome 8 was suggested as a hot spot for alleles with positive effect on drought recovery traits [32].

According to [33], significantly associated SNP loci exhibiting minimum *P*-value could explain the maximum phenotypic variation. Other than the minimum *P* values, significant SNP peak markers can precisely confirm positions of hotspot loci for the introgression of a target trait [20]. Thus, significant and stable genetic loci across the two cropping seasons (2017 and 2018) can implicate inheritance of useful alleles from *O. longistaminata* to the RILs population. Additionally, the hotspot SNPs can serve as a foundation for further marker-assisted selection of highly photosynthetic and a relatively drought tolerant perennial rice varieties (Fig 1).

Author Contributions

GF, ZS and FH designed and performed the experiments. GM wrote the manuscript. BR and EH analyzed the sequence data. ML revised the manuscript. All authors read and approved the final manuscript.

Declarations of Competing Interest

The authors have no conflict of interest.

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