Research Paper

Identification of QTLs for yield-related traits in RILs derived from the cross between pLIA-1 carrying *Oryza longistaminata* chromosome segments and Norin 18 in rice

Emily Gichuhi^{†1,2)}, Eiko Himi²⁾, Hidekazu Takahashi³⁾, Sinhao Zhu⁴⁾, Kazuyuki Doi⁴⁾, Kazuo Tsugane⁵⁾ and Masahiko Maekawa^{*†2)}

¹⁾ Graduate School of Environmental and Life Science, Okayama University, Okayama 700-8530, Japan

²⁾ Institute of Plant Science and Resources, Okayama University, Kurashiki, Okayama 710-0046, Japan

³⁾ Graduate School of Bioresource Sciences, Akita Prefectural University, Akita 010-0195, Japan

⁴⁾ Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Aichi 464-8601, Japan

⁵⁾ National Institute for Basic Biology, Okazaki, Aichi 444-8585, Japan

To improve rice yield, a wide genetic pool is necessary. It is therefore important to explore wild rice relatives. *Oryza longistaminata* is a distantly related wild rice relative that carries the AA genome. Its potential for improving agronomic traits is not well studied. Introgression line (pLIA-1) that carries *Oryza longistaminata*'s chromosome segments, showed high performance in yield-related traits under non-fertilized conditions. Therefore, to illustrate *Oryza longistaminata*'s potential for improving yield-related traits, RILs from the F₁ of a cross between pLIA-1 and Norin 18 were developed and QTL analysis was done using the RAD-Seq method. In total, 36 QTLs for yield-related traits were identified on chromosomes 1, 2, 3, 5, 6, 7, 8, 10, and 11. Clusters of QTLs for strongly correlated traits were also identified on chromosomes 1, 3, 6, and 8. Phenotypic data from recombinant plants for chromosomes 1 and 8 QTL clusters revealed that the pLIA-1 genotype on chromosome 1 region was more important for panicle-related traits and a combination of pLIA-1 genotypes on chromosomes 1 and 8 showed a favorable phenotype under non-fertilized conditions. These results suggest that *Oryza longistaminata*'s chromosome segments carry important alleles that can be used to improve yield-related traits of rice.

Key Words: *Oryza longistaminata*, RAD-Seq, QTLs, RIL, chromosome segment.

Introduction

Rice is a major food for more than half of the world's population and a staple food in Asia. In the near future, a rapid population increase is expected, especially in Asia and Africa, which will make it necessary to further increase rice yield (Kubo and Purevdorj 2004). The increase in rice yields must take place in spite of various challenges such as water scarcity, soil salinity, climate change, reduced arable land, and high inputs prices (Khush 2005, Zhang 2007). The green revolution that began in the 1960s contributed to highly improved yield by the use of semi-dwarf stature (Tilman 1998) and the higher application of fertilizers (Khush 1995, Matson *et al.* 1997). However, overreliance

on fertilizers negatively impacts the environment over time (Tilman *et al.* 2001). Hence, the breeding of higher-yielding rice that is tolerant to low-input conditions is necessary for sustainable agriculture to meet the food demand of the growing population while conserving the environment. Recently, it has been reported that the increase in rice yield has stagnated (Zhang *et al.* 2013). The narrow genetic diversity of the parent materials of modern varieties has been suggested to be the main cause. Wild rice species are important donors for improvement in rice breeding programs (Jing *et al.* 2010). They conserve many specific genes that are presently not available or extinct in cultivated rice as well as several resistant genes (Sanchez *et al.* 2013). However, their use in breeding programs has been restricted to the introgression of major genes that control qualitative traits, such as biotic or abiotic stress resistance, due to hybrid sterility or hybrid breakdown (Nevame *et al.* 2014, Oka 1988, Tanksley and Nelson 1996). It is certain that their utilization is a promising approach for enlarging the genetic pool of

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*Corresponding author (e-mail: mmaekawa@rib.okayama-u.ac.jp)

rice and improving the varieties.

Many studies have been carried out to map and identify important QTLs for yield from inter-subspecies crosses between *japonica* and *indica* (Ando *et al.* 2008, Ashikari *et al.* 2005, Hittalmani *et al.* 2003, Kobayashi *et al.* 2004, Liu *et al.* 2009, Mei *et al.* 2005, Xing *et al.* 2008, Yagi *et al.* 2001, Zhuang *et al.* 1997). However, many desirable alleles in wild relatives have not yet been fully exploited. Only a few reports regarding the mapping and introgression of QTLs from wild species have been published. In the recent past, several QTLs for yield-related traits have been identified from *Oryza rufipogon* (Moncada *et al.* 2001, Reddy *et al.* 2007, Septiningsih *et al.* 2003, Xiao *et al.* 1996, 1998, Xiong 1999) and *Oryza glumaepatula* (Brondani *et al.* 2002). The candidate genes for some of the major QTLs for agronomic traits have already been cloned and have potential to be useful in future breeding programs as reviewed by Miura *et al.* (2011). However, yield-related traits are quantitatively inherited; hence, the cloned genes only partially explain their genetic basis. Therefore, to have a holistic understanding of the genetic regulation of yield-related traits, more QTLs and genes for yield-related traits should be identified. This can be achieved by exploring more diverse wild rice relatives.

The genus *Oryza* is comprised of 24 species of which two (*Oryza sativa* and *Oryza glaberrima*) are cultivated and 22 are wild species. The cultivated species *Oryza glaberrima* and wild species that carry the AA genome are the most accessible genetic resources for expanding the diversity of the genetic pool to improve the cultivated rice *Oryza sativa*. Of the AA genome species, *Oryza longistaminata*, grown in tropical regions of Africa, is a perennial species characterized by long anthers, strong rhizomes, leaf-blight resistance (Khush *et al.* 1991, Sacks *et al.* 2003), and a vigorous biomass under low-input conditions (Yang *et al.* 2010). Its potential in utilization for improving agronomic traits is still not clear and recently CSSLs carrying *O. longistaminata* chromosome segments were developed (Ramos *et al.* 2016). We attempted to utilize *O. longistaminata*, Mpunga wa Majani, introduced from Kenya, to breed low-input adaptable (LIA) rice lines by crossing with *Oryza sativa*, Taichung 65 (T-65). At the F₁₁ generation, six potential LIA (pLIA) rice lines were selected and characterized under non-fertilized conditions (Gichuhi *et al.* 2012).

In the present study, we first analyzed important QTLs for the high productivity of pLIA-1 in RILs developed from the cross between pLIA-1 and Norin 18 under non-fertilized conditions. We then developed two mapping populations backcrossed with Norin 18 and Koshihikari backgrounds by selecting lines that carried pLIA-1 alleles at two of the QTL clusters identified on chromosomes 1 and 8. Finally, we narrowed down the position of the target QTLs on chromosome 1 using the Koshihikari backcross and on chromosome 8 using both backcrossed populations.

Materials and Methods

Materials and trait measurement

A population of 113 RILs derived from a cross between pLIA-1 and Norin 18 grown under non-fertilized conditions was used for QTL analysis in 2014. Two backcross populations, BC₃F₂ using Norin 18 (n=243) and BC₄F₂ using Koshihikari (n=846), were developed for the fine dissection of the QTL cluster region on chromosomes 1 and 8 derived from pLIA-1. Plants were grown with a spacing of 40 cm between rows and 15 cm between plants in a non-fertilized paddy field maintained without any application of fertilizers at the Institute of Plant Science and Resources, Okayama University, Kurashiki, Japan, for more than 20 years. Five plants of each RIL were grown with two replications and the agronomic traits of 3 plants were measured. Days to heading (DH) were measured from the sowing date to the emergence of the first panicle. The culm length (CL), panicle length (PL), number of panicles per plant (NP), flag leaf length (FLL), culm-base diameter (CBD) at 5 cm above the ground, and panicle-base diameter (PBD) were measured at harvest. The panicle weight (PW), number of primary branches (PB), number of secondary branches (SB), number of spikelets per panicle (NSP), and the number of fertile and sterile spikelets were measured after drying. The percentage spikelet fertility (SF) was calculated by dividing the number of fertile spikelets by the total number of spikelets per panicle.

DNA extraction of RILs

DNA was extracted from lyophilized leaf samples using a modified Dellaporta method. The quality of extracted DNA was checked by electrophoresis on a 0.6% agarose gel in 1× Tris/Borate/EDTA (TBE; 40 mmol L⁻¹ Tris, 20 mmol L⁻¹ acetic acid, and 0.5 mmol L⁻¹ Na₂-EDTA). The QuantiFluor dsDNA System and a Quantus fluorometer instrument (Promega, USA) were used for the quantification of the extracted DNA (Dellaporta *et al.* 1983).

Library construction for genotyping by sequencing (GBS) for the RAD-Seq method

A GBS library was prepared following the protocol established by Poland *et al.* (2012). In short, (1) 200 ng (20 ng/ul × 10ul) individual samples of DNA were digested with *Pst*I (CTGCAG) and *Msp*I (CCGG), which are “rare-cutter” and “common-cutter”, respectively. (2) Digested DNA was ligated to the barcode adaptor with the *Pst*I site and the “Y”-adapter (*Msp*I site). (3) Ligated samples were pooled (multiplexed) and purified using a QIAquick PCR Purification Kit (Qiagen, Germany). (4) The pooled DNA was amplified for addition of sequences for next-generation sequencing. (5) Amplified DNA was purified by using a QIAquick PCR Purification Kit (Qiagen, Germany), quantified using the QuantiFluor dsDNA System (Promega, USA), and checked using a MultiNA electrophoresis instrument (Shimadzu, Japan). (6) The library was diluted to

10 pM and used for next-generation sequencing by MiSeq (Illumina), together with 5% PhiX control (PhiX Control v3, Illumina). Four sequencing runs were conducted using MiSeq Reagent Kits v3 (150 cycles).

Processing GBS data

TASSEL-GBS (Glaubitz *et al.* 2014) in TASSEL version 4 was used to obtain a HapMap format (hmp) file using a standard procedure of TASSEL 4. The reference genome of rice (IRGSP 1.0) was downloaded from “The Rice Annotation Project Data Base” website (<http://rapdb.dna.affrc.go.jp>) and used for the analysis. The hmp file was filtered using the GBSHapMapFilterPlugin in TASSEL-GBS using the following command line “perl run_pipeline.pl -Xmx10g -fork1 -GBSHapMapFiltersPlugin -hmp hapmap/merged/merged.chr+.hmp.txt -o hapmap/filt/filt.chr+.hmp.txt -mnMAF 0.02 -mnSCov 0.95 -mnF 0.9 -sC 1 -eC 12 -endplugin -runfork1” to remove the non-informative markers that mostly originated from sequencing errors. Then, the obtained hmp file was further filtered based on the parental genotypes (only polymorphic markers between the parents were selected) using a custom Perl script. The hmp file was converted to the “csvr” format of R/qtl (Broman *et al.* 2003) using another Perl script written by Zhu and Doi (Supplemental Text 1).

QTL analysis

A linkage map was constructed by MapDisto (Lorieux 2012). Composite interval mapping was performed for QTL analysis using the Windows QTL Cartographer 2.5 (Wang *et al.* 2007). Significant LOD scores for each trait were determined by 1000 permutations test (Churchill and Doerge 1994).

DNA extraction and genotyping in backcrossed populations

Genomic DNA was extracted from young leaf tissue using a modified procedure described by Kawasaki (1997). SSR markers and newly designed markers (Supplemental Table 1) were used for genotyping at the target regions on chromosome 1 in the BC₃F₂ population using Norin 18 and chromosomes 1 and 8 in the BC₄F₂ population using Koshihikari. The PCR reaction was prepared by mixing 3.5 µl of distilled water, 0.5 µl of 20 µM forward primer, 0.5 µl of 40 µM reverse primer, 5 µl of Quick Taq (Toyobo, Japan), and 0.5 µl of the extracted DNA. Amplification was performed in an initial denaturing step at 95°C for 7 min, then 30 cycles of 45 sec at 95°C, followed by 30 sec at 55°C, and finally, 30 sec at 72°C. Electrophoresis was done in a 3% agarose gel. The band pattern of the samples was observed in UV lighting after staining with Ethidium bromide.

Results

Parental phenotypes

As shown in Table 1, pLIA-1 showed high values in culm length, panicle length, culm-base diameter, panicle-base diameter, flag leaf length, number of primary and secondary branches, and the number of spikelets per panicle compared to those of Norin 18 and Koshihikari (2015) under both non-fertilized and fertilized conditions in 2014 and 2015. Then, pLIA-1 was characterized by a thick culm-base, long flag leaves, and a large number of primary and secondary branches that resulted in a large number of spikelets per panicle. However, the number of panicles per plant, panicle weight, and spikelet fertility were lower than those of Norin 18 and Koshihikari. The low panicle weight of pLIA-1 was likely due to low spikelet fertility. Based on the comparison of the traits between non-fertilized and fertilized conditions,

Table 1. Agronomic traits of pLIA-1, Norin 18, and Koshihikari under non-fertilized and fertilized conditions in 2014 and 2015

	Condi- tions	Culm length (cm)	Panicle length (cm)	No. of panicles	Culm-base diameter (mm)	Panicle-base diameter (mm)	Flag leaf length (cm)	Panicle weight (g)	No. of primary branches	No. of secondary branches	No. of spikelets/ panicle	Spikelet fertility (%)	Days to heading
2014													
pLIA-1	NF	92.9*	27.0*	4.8*	7.88*	2.57	39.9*	18.43	16.1	50.9	242.1	67.5*	112.3*
	% (NF/F)	94.1	103.1	66.4	89.4	99.8	91.8	98.3	97.2	103.6	105.6	117.9	102.5
	F	98.8	26.2	7.2	8.81	2.57	43.4	18.76	16.5	49.1	229.2	57.3	109.5
	NF	82.2*	20.0	9.8*	4.56*	1.64	26.1*	25.66*	11.2*	20.9	119.3	96.0*	103.4*
	% (NF/F)	94.3	98.6	63.9	96.2	101.3	87.2	72.3	94.2	100.9	97.3	98.9	99.6
	F	87.2	20.3	15.3	4.74	1.62	29.9	35.49	11.8	20.7	122.6	97.0	103.8
2015													
pLIA-1	NF	105.0*	26.8	4.8*	7.62	2.56*	41.2	20.22	14.7	54.8*	274.6*	63.8	113.7*
	% (NF/F)	92.9	100.9	83.2	100.1	104.6	108.2	112.0	101.7	117.0	117.4	110.1	99.0
	F	113.0	26.6	5.8	7.62	2.44	38.1	18.06	14.5	46.9	233.9	58.0	114.8
Norin 18	NF	82.8*	21.2*	10.0*	4.34*	1.68*	29.1	29.67*	11.7	25.7*	143.2*	92.5	105.8*
	% (NF/F)	90.8	104.7	61.4	106.6	106.2	101.5	71.4	97.9	118.2	114.3	98.9	96.6
	F	91.2	20.3	16.3	4.07	1.58	28.7	41.55	12.0	21.8	125.3	93.5	109.5
Koshihikari	NF	76.1*	20.4	13.1*	4.52*	1.62*	30.6	26.88*	11.2*	22.6*	128.7*	92.1*	79.5
	% (NF/F)	93.3	98.1	64.2	102.7	105.2	97.8	68.1	106.7	87.3	91.7	107.7	101.0
	F	81.6	20.8	20.4	4.40	1.54	31.3	39.49	10.5	25.9	140.4	85.5	78.7

NF; Non-fertilized conditions.

F; Fertilized conditions.

*, Significant at 5% level by *t*-test compared to fertilized conditions.

pLIA-1 under non-fertilized conditions showed significant decreases in culm length, number of panicles, culm-base diameter, and flag leaf length in 2014 whereas Norin 18 under non-fertilized conditions showed significant decreases in culm length, number of panicles, culm-base diameter, flag leaf length, panicle weight, number of primary branches, spikelet fertility, and days to heading in 2014. Spikelet fertility of pLIA-1 under non-fertilized conditions showed significant increases in comparison to that under fertilized conditions. On the other hand, significant decreases in culm length, number of panicles, and days to heading of pLIA-1 under non-fertilized conditions were observed in 2015. Norin 18 under non-fertilized conditions showed significant decreases in culm length, number of panicles, panicle weight and days to heading and Koshihikari showed significant decreases in culm length, number of panicles, panicle weight, number of secondary branches, and number of spikelets per panicle in 2015. Under non-fertilized conditions in 2015, pLIA-1 showed significant increases in panicle-base diameter, number of secondary branches, and number of spikelets per panicle whereas Norin 18 showed significant increases in panicle length, culm-base-diameter, panicle-base diameter, number of secondary branches, and number of spikelets per panicle and Koshihikari exhibited significant increases in culm-base diameter, panicle-base diameter, number of primary branches, and spikelet fertility compared to those under fertilized conditions. It was observed that culm length and number of panicles of pLIA-1, Norin 18 and Koshihikari

were significantly affected under non-fertilized conditions. In both years, pLIA-1 had a smaller number of reduced traits than those of Norin 18 and Koshihikari and the reduction rates of the reduced traits were also smaller than those of Norin 18 and Koshihikari. Further, pLIA-1 showed a significant increase in panicle weight under non-fertilized conditions whereas Norin 18 and Koshihikari showed significant decreases in panicle weight. Remarkably, the panicle weight of pLIA-1 under non-fertilized conditions increased compared to that under fertilized conditions. This is presumed to be caused by the increase in the number spikelet per panicle and spikelet fertility. These results suggest that pLIA-1 is tolerant to non-fertilized conditions.

RILs phenotype

All traits measured in the RIL population showed normal-curved segregation patterns (Fig. 1). Transgressive segregants were also observed in all traits except culm-base diameter and panicle-base diameter (Fig. 1). Correlations between the yield-related traits are summarized in Fig. 2. The number of primary and secondary branches per panicle was positively correlated with panicle length, culm-base diameter, panicle-base diameter, and flag leaf length, resulting in a similar correlation for the number of spikelets per panicle, an important component of yield. This is because the number of spikelets per panicle was highly correlated with the number of primary and secondary branches. However, negative correlations between the number of primary

Fig. 1. Frequency distribution of 12 yield-related traits in RILs grown under non-fertilized conditions. Black and white arrows indicate mean values of Norin 18 and pLIA-1, respectively, under non-fertilized conditions.

Fig. 2. Phenotypic correlation between agronomic traits in RILs. Solid and broken lines indicate positive and negative correlations between the traits, respectively. Thin and thick lines indicate significant correlations between the traits at 5% and 1%, respectively.

branches per panicle and culm length, spikelet fertility, or days to heading were observed. The number of panicles showed strong negative correlations with culm-base diameter, flag leaf length, number of secondary branches, and number of spikelets per panicle while the correlation with panicle weight was positive. It was observed that the panicle-development traits (number of primary branches, number of secondary branches, and number of spikelets per panicle), culm-base diameter, and flag leaf length were significantly positively correlated to each other. These correlations were consistent with those observed in the F_2 population of the same cross (Gichuhi *et al.* 2012). These results suggest that the number of spikelets per panicle is highly dependent on the size of the shoot apical meristem (SAM).

QTLs for yield-related traits

To carry out QTL analysis, the RAD-Seq method was used for analysis of RILs developed from the F_2 of the cross between pLIA-1 and Norin 18. In total, 1989 SNPs were found between pLIA-1 and Norin 18, and 479 of them were used for QTL analysis, as shown in **Fig. 3**. In the linkage map constructed, the total map length was 1160 cM which covered 74% of 1575 cM reported by Kurata *et al.* (1994). Eight large gaps were observed on chromosomes 2, 3, 4, 5, 9, 10, and 11. As indicated in pLIA-1 genotyping using SSR markers (Gichuhi *et al.* 2012), many SNPs were found to be intensively located on some chromosomes' distal regions including chromosomes 1, 2, 3, 8, 10, and 11. In chromosome 6, SNPs were well distributed in almost all regions of the chromosome. Since pLIA-1 was bred through successive selfing of more than 11 times in non-fertilized conditions, some genes adapted to this environment could possibly have been selected. As a result, a total of 36 QTLs were identified on chromosomes 1, 2, 3, 5, 6, 7, 8, 10, and 11

(**Table 2**). Specifically, most of the QTLs were detected on intensive locations of SNPs in each chromosome except for chromosome 5 (**Fig. 3**).

In total, 4 QTLs for culm length were identified on chromosomes 3, 6, and 8. The highest LOD score was found in the QTL identified on chromosome 6 which explained 24% of phenotypic variance. The pLIA-1 alleles for this QTL contributed to an increase in culm length. One QTL for panicle length was identified on chromosome 5 and it explained 23% of phenotypic variance. In total, 2 QTLs identified for the number of panicles per plant were located on chromosomes 3 and 6. The pLIA-1 allele for the QTL on chromosome 3 decreased the number of panicles per plant. Three QTLs for culm-base diameter were identified on chromosomes 1, 6, and 8. Specifically, the pLIA-1 allele for the QTLs on chromosome 8 was found to highly contribute to culm-base thickness. Three QTLs for panicle-base diameter were identified on chromosomes 1, 2, and 8. The pLIA-1 allele for the QTL on chromosome 8 had a positive contribution to thick panicle-base diameter and explained the highest phenotypic variance (42%). This QTL was found to be closely localized with the QTL for culm-base diameter on chromosome 8. Two QTLs for flag leaf length were identified on chromosome 6. The pLIA-1 alleles for one of the QTLs had a positive contribution to flag leaf length. Totally, 3 QTLs for panicle weight were identified on chromosomes 1 and 8. The pLIA-1 alleles for the QTL on chromosome 1 increased panicle weight. Although 6 QTLs for the number of primary branches were identified on chromosomes 1, 6, 7, and 8, the pLIA-1 allele for the QTL on chromosome 8 was found to markedly affect the increase in the number of primary branches. A total of 5 QTLs for the number of secondary branches were identified on chromosomes 1, 3, 6, 8, and 11. The pLIA-1 allele for the QTL on chromosome 1

Fig. 3. Location of QTLs identified in the RILs of the cross between pLIA-1 and Norin 18 under non-fertilized conditions. The abbreviations represent the following traits: Culm length (CL), panicle length (PL), no. of panicles (NP), culm-base diameter (CBD), flag leaf length (FLL), panicle weight (PW), no. of primary branches (PB), no. of secondary branches (SB), no. of spikelets per panicle (NSP), spikelet fertility (SF), 100-grain weight (WG), days to heading (DH), and grain width (GW). Triangles indicate the position of detected QTLs on the chromosomes.

contributed to increase in the number of secondary branches and explained the highest phenotypic variance of 20%. A total of 3 QTLs for the number of spikelets per panicle were identified on chromosomes 1, 6, and 8. The pLIA-1 allele for all the QTLs identified increased the number of spikelets per panicle. In addition, the pLIA-1 allele for the QTL on chromosome 8 explained the highest phenotypic variance. Because the number of spikelets per panicle showed high correlation with the number of primary and secondary branches, it is plausible that the QTLs for the number of spikelets were colocalized with the QTL for the number of

primary and secondary branches on chromosomes 1 and 8. One QTL for spikelet fertility was detected on chromosome 6, showing 13% phenotypic variation. In total, 3 QTLs for days to heading were identified on chromosomes 6, 8, and 10. The pLIA-1 allele for all the QTLs, except the QTL on chromosome 8, markedly contributed to late heading.

In this population, QTL clusters were identified on chromosomes 1, 3, 6, and 8 (**Fig. 3**) and consisted of QTLs controlling traits that were observed to be significantly positively correlated to each other (**Fig. 2**). The QTL clusters on chromosomes 3 and 8 were also observed in the F_2 population

Table 2. QTLs identified in the RILs of the cross between pLIA-1 and Norin 18 under non-fertilized conditions in 2014

Trait	Chromosome	Nearest marker	LOD	Additive effect	r ²
Culm length	3	S3_31008949	6.4	-2.96	0.10
	6	S6_24880347	12.7	4.54	0.24
	6	S6_5178450	6.5	3.01	0.10
	8	S8_16848041	3.5	-2.17	0.05
Panicle length	5	S5_6782180	4.5	1.00	0.23
No. of panicles	3	S3_27838593	5.7	-0.69	0.16
	6	S6_21539402	3.8	0.43	0.12
Culm-base diameter	1	S1_6101882	4.9	0.19	0.08
	6	S6_24880347	5.3	0.22	0.10
	8	S8_23475405	12.2	0.35	0.25
Panicle-base diameter	1	S1_6141188	3.4	0.05	0.06
	2	S2_6610143	3.6	-0.05	0.06
	8	S8_20608495	15.0	0.13	0.42
Flag leaf length	6	S6_5614177	4.2	1.61	0.11
	6	S6_10147242	3.5	-1.48	0.10
Panicle weight	1	S1_5101277	3.6	1.32	0.10
	8	S8_8615971	5.2	-1.63	0.15
	8	S8_16593699	4.1	-1.45	0.12
No. of primary branches	1	S1_38166135	6.3	-0.65	0.11
	1	S1_4264473	3.7	0.48	0.06
	6	S6_24880347	4.8	-0.51	0.07
	6	S6_3537854	4.2	0.56	0.07
	7	S7_126880	5.3	0.62	0.10
No. of secondary branches	8	S8_24854960	13.5	1.09	0.30
	1	S1_5101277	10.5	3.23	0.20
	3	S3_33394609	3.2	1.65	0.05
	6	S6_12034155	3.4	1.73	0.05
	8	S8_25097800	7.8	2.88	0.14
No. of spikelets/panicle	11	S11_21978992	4.1	1.86	0.06
	1	S1_6085421	7.4	11.41	0.15
	6	S6_12034155	4.0	8.37	0.08
Spikelet fertility	8	S8_25097800	11.2	15.26	0.25
	6	S6_13522936	4.8	-6.52	0.13
Days to heading	6	S6_9205228	19.2	3.07	0.30
	8	S8_2158357	4.2	-1.16	0.05
	10	S10_17556259	15.0	2.55	0.24

(Gichuhi *et al.* 2012). These results suggest that these chromosomal regions are major hot spots for genes that control panicle-related traits.

Backcrossed plant phenotypes

RAD-Seq analysis made it clear that the QTLs for panicle-related traits were colocalized at the distal regions of the short arm of chromosome 1 and the long arm of chromosome 8. In F₂ plants and RILs derived from the cross between pLIA-1 and Norin 18, segregations of spikelet sterility and heading date were observed. In order to reveal the clustered QTLs without interference from other background factors, backcrossed populations were developed using Norin 18 and Koshihikari. SSR markers RM8068, RM10115, RM6324, RM220, and EG03 located from 1.66 Mb to 6.09 Mb were used to introduce the chromosome 1-clustered QTL region (Fig. 6) and EM1, RM6976, EM4, EM7, EM9, EM12, and EG markers located from 22.47 Mb to 25.28 Mb

Table 3. Agronomic traits in homozygous plants carrying pLIA-1 genotype and Norin 18 (BB) genotype on chromosome 8 in back-crossed population with Norin 18

Traits	pLIA-1	Norin 18
Culm length (cm)	86.4	86.2
Panicle length (cm)	24.6*	23.7
No. of panicles/plant	9.7	10.0
Culm-base diameter (mm)	5.32*	4.49
Panicle-base diameter (mm)	1.92*	1.75
Flag leaf length (cm)	36.2*	31.3
Panicle weight (g)	33.99	31.53
No. of primary branches	16.6*	12.4
No. of secondary branches	33.9*	26.3
No. of spikelets/panicle	188.1*	148.9
Spikelet fertility (%)	93.1	92.2
Days to heading	106.8*	106.1

*; Significant at 5% level.

were used to introduce the clustered QTL region on chromosome 8 (Figs. 4, 7).

In the Norin 18-backcrossed population, only the distal region of chromosome 8 was introduced. As shown in Table 3, plants carrying the pLIA-1 genotype on chromosome 8 showed significant differences for panicle length, culm-base diameter, panicle-base diameter, flag leaf length, number of primary and secondary branches, and number of spikelets per panicle as compared to plants carrying the Norin 18 genotype on chromosome 8. Although a significant difference in days to heading was observed between pLIA-1 genotype plants and Norin 18 genotype plants, the difference was less than 1 day. The increase in the number of spikelets of pLIA-1 genotype plants was caused by the increase of the number of primary and secondary branches. Significantly longer panicles of pLIA-1 genotype plants were also possibly caused by an increased number of spikelets per panicle. The significant difference in flag leaf length between pLIA-1 genotype and Norin 18 genotype was likely brought about by a small effective QTL located in the same region of chromosome 8 since a low LOD score peak of flag leaf length was detected around 25.59 Mb of chromosome 8, although no significance was observed when using the permutation test. On the other hand, the number of panicles and panicle weight of pLIA-1 genotype plants were not significantly different from those of Norin 18 genotype plants. It is likely that the total number of spikelets per plant of pLIA-1 genotype plants was not markedly different from that of Norin 18 genotype plants though pLIA-1 genotype plants showed a larger number of spikelets per panicle than that of Norin 18 genotype plants. The total number of spikelets per plant, which is one of the important components of yield, needs to be checked. In the population backcrossed with Norin 18, some recombinant plants were obtained, as shown in Fig. 4. Although very few recombinant plants were obtained, the causal factors for culm-base diameter and number of primary and secondary branches were presumed to be located around EG and EM9-EM12, respectively, based on the comparison of a, b, c, and d recombinants plants.

Fig. 4. Agronomic traits in recombinant plants with their respective genotypes on chromosome 8 in the population backcrossed with Norin 18. Black, gray and white shadings indicate pLIA-1, heterozygous, and Norin 18 genotypes, respectively.

Fig. 5. Effect of pLIA-1 and Koshihikari alleles on 12 agronomic traits in the population backcrossed with Koshihikari. Different letters in the graph indicate significant differences at the 5% level by Tukey's test. The genotypes on the upper and lower rows shown by LL (pLIA-1) and KK (Koshihikari) on the x-axis represent chromosomes 1 and 8, respectively.

In the population backcrossed with Koshihikari, distal regions of chromosomes 1 and 8 were introduced. Then, phenotypic comparisons of plants carrying similar genotypes for chromosomes 1 and 8 and plants carrying different genotypes for each chromosome were made, as shown in

Fig. 5. It was revealed that plants carrying the pLIA-1 genotype for both chromosomes showed significant increases in panicle-base diameter, flag leaf length, and the number of primary branches. Epistatic effects were observed in panicle-base diameter, flag leaf length, and number of primary

branches. In these phenotypes, pLIA-1 genotype on either chromosome 1 or 8 does not have any effect on phenotype. On the other hand, additive effects were observed in culm-base diameter, number of secondary branches, and number of spikelets. No interaction between genotypes on chromosomes 1 and 8 was observed in these phenotypes. Culm length, panicle weight, and number of primary branches may be controlled by genes having additive and epistatic effects. As a result, the pLIA-1 genotype was revealed to be more important for panicle-related traits in the chromosome 1 region (Fig. 5) than in the chromosome 8 region (Fig. 5), and the interaction between chromosomes 1 and 8 for the pLIA-1 genotype increased the panicle-base diameter and

flag leaf length (Fig. 5). Thus, the phenotypes of recombinant genotype plants on chromosomes 1 and 8 were examined to narrow the target regions on chromosomes 1 and 8 in the population backcrossed with Koshihikari. As shown in Fig. 6, several recombinants (a1-m types) were obtained. The pLIA-1 genotype plants for RM220 or EG03 (a1, a2, e, f, g, h, k and m types) generally showed larger number of secondary branches. However, i and l types did not show an exceptionally larger number of secondary branches. It is likely that the genetic factor (s) for the number of secondary branches are located around RM220 and EG03 on chromosome 1. The genotype and phenotype of recombinant plants on chromosome 8 are shown in Fig. 7. The pLIA-1 genotype

Fig. 6. Agronomic traits in recombinant plants with their respective genotypes on chromosome 1 in the population backcrossed with Koshihikari. Black, gray and white shadings indicate pLIA-1, heterozygous, and Koshihikari genotypes, respectively.

Fig. 7. Agronomic traits in recombinant plants with their respective genotypes on chromosome 8 in the population backcrossed with Koshihikari. Black, gray and white shadings indicate pLIA-1, heterozygous, and Koshihikari genotypes, respectively.

plants for EM12 and EG (a, d, and e types) showed thick culm-base diameter. Furthermore, d and e types carrying the pLIA-1 genotype for EG had a larger number of primary and secondary branches. The phenotype of recombinant plants on chromosome 8 suggests that the region from EM9 to EG might contain genetic factors for the culm-base diameter, number of primary branches, and number of secondary branches. In this population, the order of EM9 and EM7 markers (Fig. 7) was reversed compared to that in the population backcrossed with Norin 18 (Fig. 4). This may be due to the closeness between EM9 and EM7.

Discussion

Oryza longistaminata chromosome segments-introgressed pLIA-1 under non-fertilized conditions

Wild relatives of rice have high potential for improving agronomic traits since they have extensive genetic diversity. Their continued sampling is expected to result in novel QTL/gene discoveries important for agronomic improvement. For example, a bacterial blight resistant gene, *Xa-21*, was identified from *Oryza longistaminata* and has been used to confer resistance upon elite rice cultivars (Khush *et al.* 1991, Song *et al.* 1995). However, attempts to transfer genes that control quantitative traits from wild relatives to cultivated elite varieties of rice have, generally, been limited mainly by hybrid sterility (Oka 1988). To utilize the superior characteristics of *O. longistaminata* under natural conditions, 6 introgressed lines named pLIA (potential low-input

adaptable) were selected after more than 11 times selfing of plants derived from the F₂ of a cross between MwM, *O. longistaminata* collected in Kenya, and Taichung 65 under non-fertilized conditions (Gichuhi *et al.* 2012).

Although pLIA-1, one of the lines, was characterized by superior agronomic traits (thick culm, long flag leaf, large numbers of primary and secondary branches, and a large number of spikelets per panicle) under non-fertilized conditions compared to Norin 18 and Koshihikari (Table 1), it showed very low spikelet fertility resulting from the interspecific cross. Interspecific-derived sterility problems, unfavorable linkage blocks, restricted gene combinations, and, most importantly, linkage drag problems, make it difficult to select favorable traits for breeding purposes (Brondani *et al.* 2002). Despite the overall inferior agronomic phenotypes observed in wild species, they have been a useful source of favorable genes since the beginning of modern breeding. To broaden genetic variation and overcome yield plateaus, the exploitation and utilization of favorable wild rice alleles that have been lost or weakened in cultivated rice is very important for modern breeding (Fu *et al.* 2010). For this purpose, the pLIA-1 selected in the F₅ derived from a cross between *O. longistaminata* and T-65 under non-fertilized conditions is possibly useful in a breeding program.

O. longistaminata-derived alleles for yield improvement

O. longistaminata has been studied to identify genes responsible for the rhizomatous trait and its genetic control (He *et al.* 2014, Hu *et al.* 2011, Yang *et al.* 2010, Zong *et al.*

2014). Here, we focused on identifying QTLs for yield-related traits of pLIA-1 adapted to low-input conditions. To obtain the fine locations of QTLs for agronomic traits, the RAD-Seq method was applied in RILs. Baird *et al.* (2008) demonstrated the RAD-Seq method for efficient, high-density SNP discovery and the genotyping of mapping crosses as a useful and cost-effective tool; this method was found to be highly efficient for evolutionary studies and MAS, as reviewed by Fan *et al.* (2016). Furthermore, high-density SNPs revealed by the RAD-Seq method make it extremely easy to detect QTLs for important agronomic traits and, possibly, to identify the gene of interest. Some QTLs for characteristic traits of pLIA-1 under non-fertilized conditions were detected in the F₂ of a cross between pLIA-1 and Norin 18 by using genome-wide SSR markers (Gichuhi *et al.* 2012) previously and RILs derived from the F₂ were developed for further precise QTL analysis. RILs were subjected to the RAD-Seq method and 479 SNPs were mapped, as shown in Fig. 3. As pLIA-1 had been genotyped by genome-wide SSR markers, *O. longistaminata*-derived chromosome segments were found to be unevenly distributed on 12 chromosomes: the distal region of the short arm of chromosome 1, near the centromere of chromosome 2, the distal region of the long arm of chromosome 3, most of the short arm and distal region of the long arm of chromosome 6, the semi-distal region of the long arm of chromosome 8, the centromeric region of chromosome 10, and near the centromeric region of chromosome 11 (Gichuhi *et al.* 2012). The *O. longistaminata*-derived chromosome segments examined by SSR markers were estimated to contain high-density SNPs. This result suggests that high-density SNPs might be derived from the polymorphisms between *O. longistaminata* and Norin 18. Interestingly, important QTLs for agronomic traits of pLIA-1 characteristics under non-fertilized conditions were found to be located in high-density SNP regions. In this study, a total of 36 QTLs for 12 traits were detected in RILs. The pLIA-1 alleles had a positive contribution in 25 of the QTLs identified in RILs. This accounted for more than 50% of the QTLs identified in RILs. The percentage of favorable alleles was comparable to that reported in previous studies using *Oryza rufipogon*, another wild relative of rice, where it contributed more than 50% of the beneficial alleles (Moncada *et al.* 2001, Thompson *et al.* 2003, Xiao *et al.* 1998). QTLs identified in RILs were as follows: 4 QTLs for culm length, 1 QTL for panicle length, 2 QTLs for the number of panicles, 3 QTLs for the culm-base diameter, 3 QTLs for the panicle-base diameter, 2 QTLs for flag leaf length, 3 QTLs for panicle weight, 6 QTLs for the number of primary branches per panicle, 5 QTLs for the number of secondary branches per panicle, 3 QTLs for the number of spikelets per panicle, 1 QTL for spikelet fertility and 3 QTLs for days to heading. A majority of these QTLs mapped were found to be located on the introgressed chromosome segments of *O. longistaminata* in pLIA-1 revealed in the F₂ generation (Gichuhi *et al.* 2012). To explore the genetic resources from wild rice,

several populations derived from combinations between various cultivars and wild rice have been used for QTL mapping. Numerous traits have been investigated and QTLs identified (Li *et al.* 2006, Moncada *et al.* 2001, Ramos *et al.* 2016, Yoon *et al.* 2006). In particular, Xiao *et al.* (1998) detected a total of 68 QTLs for 12 traits using a backcross population derived from a wild rice (*Oryza rufipogon*) and cultivated rice.

For sustainable agriculture, relatively high productivity of rice under low-input conditions is required. In order to reveal the genetic factors for relatively high productivity under low-input conditions, it is advantageous to evaluate the materials under non-fertilized conditions. In this study, the distal region of the short arm of chromosome 1 and the semi-distal region of the long arm of chromosome 8 specifically contained QTLs for the number of primary and secondary branches and the culm-base diameter. It was demonstrated that QTLs in the chromosome 1 region contributed to an increased number of secondary branches and QTLs in the chromosome 8 region increased the number of primary branches and culm-base diameter. To validate these results, Norin 18 and Koshihikari-backcrossed to the F₁ of the cross between pLIA-1 and Norin 18 populations were developed. The results obtained from the backcrossed populations suggests that QTLs for the culm-base diameter and number of primary branches might be located around 24.48 Mb to 25.28 Mb on chromosome 8 and the QTL for the number of secondary branches might be localized around 6.09 Mb on chromosome 1. The *OsSPL14/WFP* gene, which increases the primary branches (Miura *et al.* 2010), was reported to be located in this region of chromosome 8 and the *GN1/OsCKX2* gene, which increases the number of spikelets (Ashikari *et al.* 2005), was reported to be located on the distal region of the short arm of chromosome 1. In fact, Miura *et al.* (2010) found two major QTLs for the increased grain number of ST12 on chromosome 1 and chromosome 8. The QTL located on chromosome 1 was revealed to be *GN1A* (Ashikari *et al.* 2005). Thus, the large panicle of ST12 was caused by the interaction of *GN1A* and *OsSPL14/WFP*. Although it was likely that the QTLs for the panicle-related traits detected in this study might be attributed to *GN1A/OsCKX2* and *OsSPL14/WFP*, as the panicle phenotype of pLIA-1 grown under non-fertilized conditions was very similar to that of ST12, further fine analysis for the identification of genetic factors for panicle-related traits will be needed. Furthermore, it was revealed that this region on chromosome 8 located QTLs for culm-base diameter and flag leaf length. Notably, the pLIA-1 allele was found to increase the culm-base diameter and flag leaf length through the examination of the population backcrossed with Norin 18. Ookawa *et al.* (2010) reported that the *SCM2/APO1* gene for an increased number of secondary branches caused thick culms. This is because active cell division in the inflorescence meristem was promoted. Thus, it is likely that a genetic factor may cause the increased size of shoot apex meristem, resulting in long flag leaf length and a thick culm-

base diameter. Otherwise, two closely linked genetic factors may control each trait.

In this study, important QTL clusters on chromosomes 1, 3, 6, and 8 were observed in RILs. This phenomenon has been reported in many QTL studies of different species. In 2002, QTL clusters of domesticated-related traits of rice were reported by Cai and Morishima on chromosomes 3, 6, 8, 9, 11, and 12. Additionally, Brondani *et al.* (2002) reported that specific marker regions strongly associated with more than one trait were observed for yield-related traits including number of panicles, spikelets per panicle, spikelet fertility, 100-grain weight, grain yield per plant, and grain yield per panicle. Highly significant correlations were also observed between yield-related traits that were observed to cluster in the same chromosome locations. In previous QTL studies, it has been observed that QTLs for significantly correlated traits usually had the same chromosome location (Brondani *et al.* 2002, Hittalmani *et al.* 2003, Tian *et al.* 2006). QTLs on the same chromosome location for various traits are possibly due to either the linkage of genes or the pleiotropic effect of a single locus.

In this study, 1 QTL for spikelet fertility was detected on chromosome 6. The pLIA-1 allele reduced spikelet fertility. Chen *et al.* (2009) identified a significant QTL for pollen and spikelet fertility at the distal region of the short arm of chromosome 6 in a cross between *O. longistaminata* and *O. sativa*. In this study, some highly sterile RILs were observed as shown in the frequency distribution of spikelet fertility in Fig. 1. Since the QTL was found in the RILs bred by the SSD method, recessive factors derived from *Oryza longistaminata* are presumed to control spikelet sterility.

In conclusion, these results show that *Oryza longistaminata*'s chromosome segments carry important alleles that could be utilized for improvement of yield-related traits in rice. Similar results were also obtained using CSSLs that carry *Oryza longistaminata* chromosome segments in Taichung-65 background (Ramos *et al.* 2016). Additionally, we conclude that these important traits expressed under non-fertilized conditions were derived from *O. longistaminata* and the introgressed lines can be utilized as genetic materials adapted to low-input conditions. The identification and cloning of the genes responsible for yield-related traits especially observed in low-input conditions will be very helpful for further rice improvement as well as the conservation of the environment.

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