Genetic analysis for high productivity derived from *Oryza longistaminata* under low-input conditions in rice

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Abstract

High rice production has been achieved successfully through breeding efforts and adoption of improved cultivation techniques. Identification and utilization of high yielding genetic factors coupled with high fertilizer usage has greatly improved rice yields. However, further increase in rice yields is required to meet and sustain the food demands of an ever-increasing world population. Since the high usage of fertilizers has been shown to negatively impact the environment, a change to sustainable agriculture is needed. Therefore, breeding of higher-yielding rice that is tolerant to low-input conditions such as low application of fertilizers is necessary. Wild rice species are an important reservoir of novel traits that can be explored and utilized to breed low-input adaptable (LIA) rice. *Oryza longistaminata* is a remote wild rice that carries the AA genome whose potential for improving agronomic traits has not been well studied. To elucidate *Oryza longistaminata's* potential for improving agronomic traits, an introgression line (pLIA-1) that carries *Oryza longistaminata's* chromosome segments was bred at the Institute of Plant Science and Resources, Okayama University, Kurashiki, Japan. It showed high performance of yield-related traits under non-fertilized conditions. Thus the aim of this study is to identify QTLs for important yield-related traits under non-fertilized conditions and to investigate low-input adaptability factors in RILs and DHLs developed from the F_1 of the cross between pLIA-1 and Norin 18 and introduce the high productivity traits of pLIA-1 into Basmati to improve its yield. Through RAD-Seq and composite interval mapping, 36 QTLs for yield-related traits were identified in RILs. Clusters of QTLs for strongly correlated traits were also identified on chromosomes 1, 3, 6, and 8. Phenotypic data from recombinant plants from the Norin 18 and Koshihikari backcrosses targeting 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 brought 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. To understand the factors behind the low input adaptability of pLIA-1, Norin 18, Nipponbare and T-65 were grown and their performance compared to pLIA-1 under three different levels of nitrogen fertilizer. In comparison to the other lines, pLIA-1 showed significantly higher number of primary and secondary branches and spikelets per panicle under non-fertilizer conditions, suggesting that pLIA-1 might have a higher physiological nutrient use efficiency for development of panicle-related traits. To further examine these results, the performance of RILs and DHLs grown under fertilized and non-fertilized conditions were compared. Based on the comparison, number of panicles per plant was revealed to be highly sensitive to fertilizers. Three DH lines were presumed to be tolerant to non-fertilized condition for number of panicles per plant through GGE biplot analysis. In addition, QTL analysis in RILs grown in fertilized and non-fertilized conditions identified QTLs that increased NF/F rate derived from pLIA-1 for number of panicles per plant, number of primary branches, number of secondary branches, number of spikelets per panicle and days to heading. The pLIA-1 allele at these QTLs contributed to a small reduction of the trait values under non-fertilized conditions thereby suggesting that pLIA-1 might carry QTLs for low fertilizer tolerance. These results suggest that pLIA-1 carries important traits that can be utilized to improve modern cultivars under low-input conditions. Hence, we tried to utilize pLIA-1 in a breeding scheme for improving Kernel Basmati's panicle and culm thickness. QTL analysis in the F_2 of the cross between pLIA-1 and Kernel

Basmati identified a total of 21 QTLs for yield-related traits in 2012 and 2013. The QTL for number of primary branches was identified in the same location on chromosome 8 in both years and a QTL cluster for number of secondary branches and number of spikelets per panicle was identified on chromosome 1 in 2013. On the other hand, two QTLs for culm-base diameter were also identified in 2013. Thus, *O. longistaminata* chromosome segments of pLIA-1 were introduced into Kernel Basmati and 50 LCSILs (Longistaminata Chromosome Segment Introduced Lines) in Kernel Basmati's background were developed for utilizing the QTLs identified in the F_2 population. The promising line "Neo Basmati" carrying the thick culm and large panicle traits is expected to be bred through pyramiding and marker assisted selection.

As a result, this study shows that *Oryza longistaminata* possesses the potential to be utilized in improving agronomic traits of rice and pLIA-1 is a promising line that can be utilized for practical breeding for improved rice yields under low-input conditions.

Abbreviations

pLIA – potential Low-Input Adaptable

LCSILs – Longistaminata Chromosome Segments Introduced Lines

EtOH – Ethanol

QTL – Quantitative Trait Loci

SNP – Single Nucleotide Polymorphism

SSR – Simple Sequence Repeat

WFP – Wealthy farmer's panicle

IPA – Ideal Plant Architecture

APO1 – ABERRANT PANICLE ORGANIZATION1

MAS – Marker Assisted Selection

RILs – Recombinant Inbred Lines

OsSPL14 – Oryza sativa SQUAMOSA PROMOTER BINDING PROTEIN-LIKE14

GN1 – Grain number 1

OsCKX2 – Oryza sativa Cytokinin oxidase/dehydrogenase2

SCM2 – STRONG CULM2

DH – Doubled Haploid

NUE – Nitrogen Use Efficiency

SSD – Single Seed Decent

General introduction

Rice is a major food for more than half of the world's population. It accounts for more than 21 % of the calorific needs of the world's population (Fitzgerald *et al*., 2009). In terms of cultivated land area, rice growing field is the second largest among the cereal crops' fields in the world. However in Asians countries especially Southeast Asia it is the main staple crop and is an important economic crop for farmers and workers who grow it on millions of hectares throughout the region (Zibaee, 2013). Therefore, rice is not only important as a food but also as a source of income for a significant portion of the world population.

Improving rice yield potential has been the main breeding objective for several decades. The development of semidwarf rice (Tilman, 1998) varieties and hybrid rice (Peng *et al*., 1999) have greatly contributed to increases in irrigated rice yield during the last century. However, stagnant yield potential has recently been reported among the semidwarf varieties and their plant type were suggested to be the cause of the stagnation (Peng *et al*., 2008). Therefore, an ideotype approach to rice breeding was proposed which led to the "plant type" concept of breeding (Donald, 1968). Under this concept the New Plant Type (NPT) rice lines were developed at the International Rice Research Institute (IRRI). They were characterized by low tillering, few unproductive tillers, large panicles and resistance to lodging (Peng *et al*., 1994). They also showed higher yields but the high yields were not realised during the dry season (Peng *et al*., 2008).

To improve upland rice for growing in rain fed uplands especially in Sub-Saharan Africa, Jones *et al*. (1997) started developing New Rice for Africa (NERICA) varieties at the African Rice Centre. *Oryza glaberrima* is the main rice type grown in Africa. It exhibits

traits that are useful to survive in African environment such as weed competitiveness and tolerance to biotic and abiotic stresses, but, it is being increasingly replaced by *Oryza sativa* due to low yield performance, shattering and susceptibility to lodging (Sarla and Swamy, 2005). Therefore, the NERICA varieties were developed to combine the best traits of high yields from *Oryza sativa* and the ability to thrive under harsh environments from *Oryza glaberrima*. A total of 7 (NERICA 1-7) and 11 (NERICA 8-18) NERICA varieties were developed and released in 2000 and 2005, respectively. All the NERICA varieties released are suitable for upland rice growing in sub-Saharan Africa (Kishine *et al*., 2008).

An additional target trait in the breeding of NERICA rice varieties was the high nitrogen responsiveness of *O. glaberrima (*Fukuta *et al*., 2012*)*. Nitrogen (N) is the most limiting factor in crop production. In order to achieve higher yields, the global use of N fertilizer has increased over the years. However, plants consume less than half of the fertilizers applied (Nischal *et al*., 2012). The increased use of nitrogen fertilizer causes global warming through nitrous oxide emissions and pollution of water by nitrate leaching (Davies and Sylvester, 1995). Therefore, breeding crops that are less dependent on high N fertilizer application is essential for sustainable agriculture. The Nitrogen Use Efficiency (NUE) of crops needs to be improved. This requires development of crop varieties that have high uptake efficiency i.e. can obtain nitrogen from soils with low N concentration, and high utilization efficiency of the absorbed nitrogen within the plants for production.

Despite the above spectacular advances in improving rice yields, the looming threat to food shortage due to an explosive population growth coupled with climate change has created the need to further increase yields (Nelson *et al*., 2009). Recently, further increase

in rice yield has stagnated (Zhang *et al*., 2013). A narrow genetic diversity of the parent materials in the modern varieties has been suggested to be one of the major causing factors (De Ribou *et al*., 2013). Hence, breeding of higher yielding rice that requires low-input usage is necessary for sustainable agriculture in order to meet the food demand of the growing population harmonized with the environment.

Wild rice species are important donors for improvement in rice breeding programs (Jing *et al*., 2010). They conserve a lot of specific genes that are presently not available or extinct in the cultivated rice and several resistant genes. Their utilization in breeding programs has however been restricted to the introgression of major genes controlling qualitative traits such as biotic or abiotic stress resistance through hybrid sterility or hybrid breakdown (Tanksley and Nelson, 1996; Nevame *et al*., 2014). It is undoubted that their utilization is a promising approach to enlarge the genetic variations of rice and breed low-input adaptable rice.

Oryza longistaminata is a wild rice species that belongs to the AA genome. It is found growing in the tropical regions of Africa. It is a perennial species whose characteristics include long anthers, strong rhizomes (Sacks *et al*., 2003) and allogamy, which are important traits for hybrid seed production (Virmani *et al*., 1982). It is resistant to bacterial leaf-blight and has been utilized to confer this trait on commercial varieties (Song *et al*., 1995; Khush *et al*., 1990; Khush *et al*., 1989). Further, it has a large biomass production under low-input conditions (Yang *et al*., 2010). The large biomass of *O. longistaminata* is considered to be an important trait for breeding low-input adaptable rice. However, its utilization in breeding programs for rice improvement has been very limited, due to developed crossing barriers and hybrid sterility observed between cultivated rice and *O. longistaminata* (Chu and Oka, 1970; Chen et al., 2009). Many

researchers have been interested in the rhizomatous trait of *O. longistaminata* and its genetic control, an important trait for the perennial growth habit (Hu *et al*., 2011; He *et al*., 2014; Yang *et al*., 2010; Zong *et al*., 2014). Recently, the genome of *O. longistaminata* has been sequenced (Zhang *et al*., 2015). Deciphering the genome of *O. longistaminata* is the key to uncovering the mechanism of rhizomatous and self-incompatibility traits. It is expected to give insights into ways to improve cultivated rice. However, deciphering the genome of this species remains a major challenge due to its high heterozygosity (Zhang et al., 2015).

We previously utilized the wild relative of rice, *O. longistaminata*, locally known as Mpunga wa Majani (MwM), from Kenya to breed Low-Input Adaptable (LIA) rice lines by crossing with *Oryza sativa*, cv. Taichung 65 (T-65). At the F_{11} generation six potential LIA (pLIA) rice lines showing large biomass, tall culm, large panicle with many primary and secondary branches and thick culms were selected and characterized from a selfed progeny of the cross between MwM and Taichung 65 (T-65) under non-fertilized conditions. Out of the six pLIA lines, pLIA-1 performance was superior to that of Koshihikari, Norin 18, T-65 and Nipponbare under fertilized and non-fertilized conditions suggesting that pLIA-1's characteristics might be useful for breeding low-input tolerant varieties (Gichuhi *et al*., 2016).

Based on the above background, this study seeks to further understand the genetics behind the high performance of pLIA-1 under non-fertilized conditions. The main objectives of this study is QTL analysis for important yield-related traits including fine mapping of target QTLs on chromosomes 1 and 8 and analysis of important factors for low-input adaptable traits in pLIA-1 as follows:

First, recombinant inbred lines (RILs) and doubled haploid lines (DHLs) were developed from the F_2 and F_1, respectively, of the cross between pLIA-1 and Norin 18 and used for QTL analysis. Second, high resolution analysis of high productivity of pLIA-1 for target traits using SSR markers designed in the vicinity of the target region was done to fine map QTLs for the target traits. Third, analysis of genetic factors for high productivity of pLIA-1 under low-input conditions was done to evaluate its interaction with fertilizer. Last, we introduced chromosome segments of pLIA-1 into Basmati background with the aim of developing Longistaminata Chromosome Segment-Introduced Lines (LCSILs) for improving the low yields of Basmati rice.

Figure 1. Phenotypes of pLIA-1 (left), Norin 18 (middle) and T-65 (right) grown under non-fertilized conditions. Bar=50cm.

CHAPTER 1

QTL analysis for yield-related traits using RILs developed from the cross between pLIA-1 and Norin 18 under non-fertilized conditions

1.1 Introduction

To secure enough food without environmental degradation and high dependency on inputs, it is necessary to breed new paradigmatic varieties with comparatively high yields and adaptability to low-input conditions. This can be achieved by maximizing crops' ability to produce biomass and tolerance of various abiotic and biotic stresses through the utilization of genetic resources and distantly related wild relatives. 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 in the crosses using *Oryza rufipogon* (Moncada *et al*., 2001; Reddy *et al*., 2007; Septiningsih *et al*., 2003; Xiao *et al*., 1996; Xiao *et al*., 1998; Xiong *et al*., 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, most yield-related traits are quantitatively inherited; hence, the cloned genes only partially explain the genetic basis of traits. 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 cultivated species *Oryza glaberrima* (AA) and wild species that carry the AA genome are the most accessible genetic resources for expanding the genetic diversity to improve the cultivated rice *Oryza sativa* (AA). 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*., 1990; 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 chromosome segments substitution lines carrying *O. longistaminata* chromosome segments were developed (Ramos *et al*., 2016). We attempted to utilize *O. longistaminata*, known as Mpunga wa Majani, introduced from Kenya, to breed low-input adaptable (LIA) rice lines by crossing with *Oryza sativa*, Taichung 65 (T-65) and selecting under non-fertilized conditions. One of the bred lines, pLIA-1, showed superior performance in yield-related agronomic traits at a non-fertilized paddy field (Gichuhi *et al*., 2016) and QTLs for several pLIA-1 traits were revealed in the F_2 of the cross between pLIA-1 and Norin 18 (Gichuhi *et al*., 2016). Recently, it has been reported that RAD-Seq method using NGS is very useful in making a detailed map of QTLs (Baird et al., 2008). Thus, recombinant inbred lines (RILs) were developed from the cross between pLIA-1 and Norin 18 and subjected to QTL analysis of yield-related traits under non-fertilized conditions using the map developed by RAD-Seq method.

1.2 Materials and Methods

1.2.1 Plant materials

Recombinant inbred lines (RILs) were developed from the cross between pLIA-1 and Norin 18 through self-fertilization over generations. At the F_7 generation, 113 RILs were bred.

1.2.2 Phenotypic evaluation

The 113 RILs were grown under non-fertilized conditions in 2014. Plants were grown with a spacing of 40 cm between rows and 15 cm between plants at a non-fertilized paddy field maintained without any application of fertilizers for more than 20 years at the Institute of Plant Science and Resources, Okayama University, Kurashiki, Japan. Five plants of each RIL were grown with two replications and the agronomic traits of three plants were measured. Days to heading (DH) was calculated 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.

1.2.3 DNA extraction of RILs

The DNA of RILs was extracted from lyophilized leaf samples using a modified method of Dellaporta *et al*. (1983). 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).

1.2.4 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×10 ul) 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).

1.2.5 Processing GBS data

TASSEL-GBS (Glaubitz *et al*., 2014) in TASSEL version 4 was used to obtain a HapMap format (hmp) file by 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 "perlrun_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) by 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.

1.2.6 QTL analysis

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

1.3 Results

1.3.1 Parental phenotypes

The pLIA-1 line showed significantly higher values in culm length, panicle length, flag leaf length, culm-base diameter, panicle-base diameter, number of primary and secondary branches and number of spikelets per panicle as compared to those of Norin 18 (Table 1.1). Specifically, pLIA-1 was characterized by a thick culm-base, long flag leaves, and a large number of primary and secondary branches which resulted in a large number of spikelets per panicle (Fig. 1.1). However, the number of panicles per plant and spikelet fertility were significantly lower than those of Norin 18 (Table 1.1).

Figure 1.1. Phenotypes of pLIA-1 (right) and Norin 18 (left) panicles Bar=10cm.

Table 1.1. Mean values of agronomic traits of pLIA-1 and Norin 18 grown under non-fertilized conditions in 2014 and 2015

Line/Variety	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 spikelet/ panicle	Spikelet fertility (%)	Days to heading
pLIA-1	99.0	26.9	4.8	7.75	2.60	40.6	19.30	15.4	52.9	258.4	65.7	113.4
Norin 18	82.5***	20.6***	9.9***	4.45***	1.66***	27.6***	27.66***	11.4***	23.3***	131.3***	94.2***	104.5***

***; significant at the 1% level by Student *t*-test.

1.3.2 RILs phenotypes

All traits measured in the RILs showed normal distribution segregation patterns (Fig. 1.2). Transgressive segregations were also observed in all traits except culm-base diameter and panicle-base diameter (Fig. 1.2). Correlations between the yield-related traits were summarized in Fig. 1.3. 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 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.* 2016). These results suggest that the number of spikelets per panicle is highly dependent on the size of the shoot apical meristem (SAM).

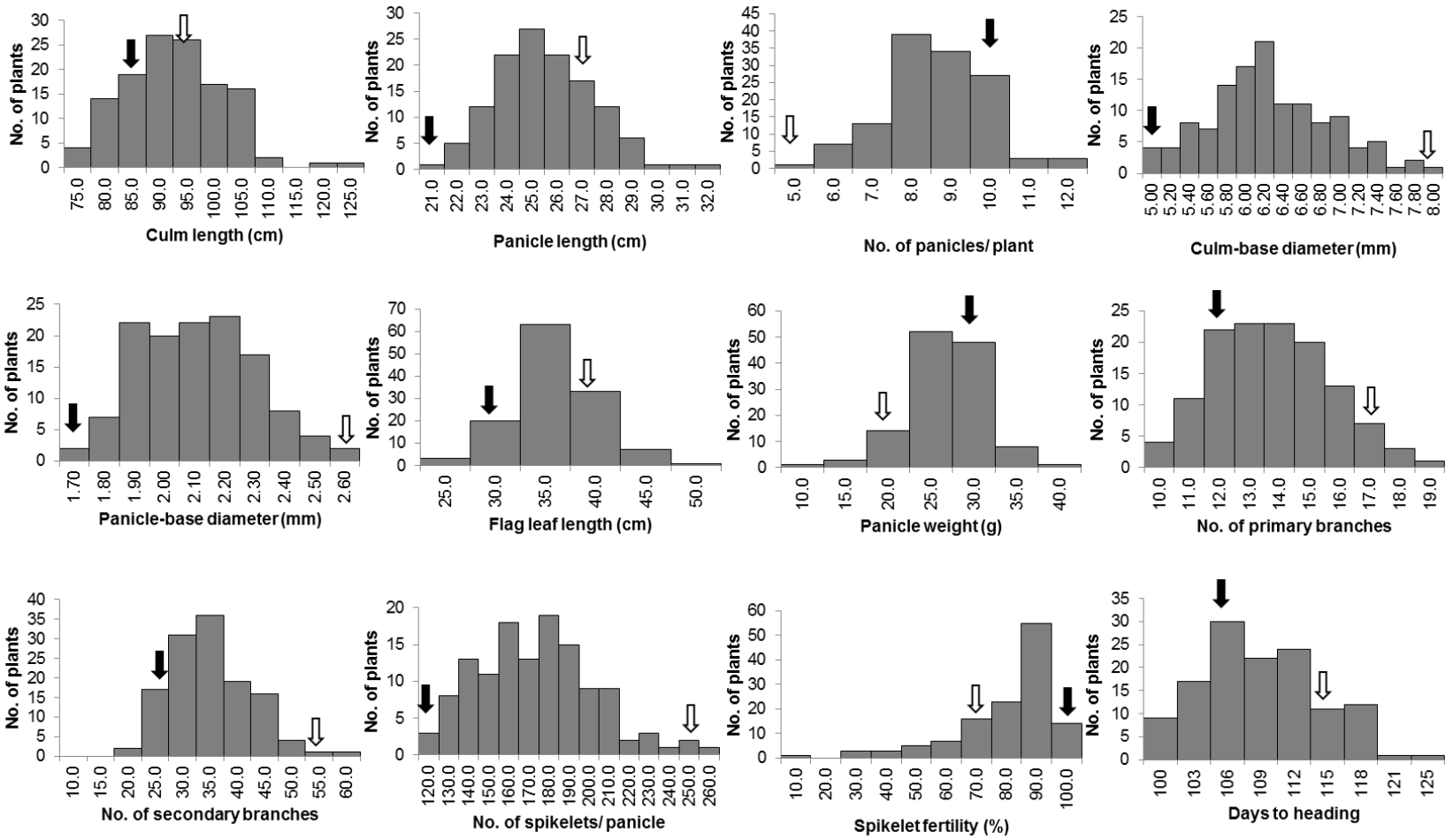


Figure 1.2. Frequency distribution of 12 yield-related traits in RILs grown under non-fertilized conditions in 2014. Black and white arrows indicate mean values of Norin 18 and pLIA- 1, respectively.

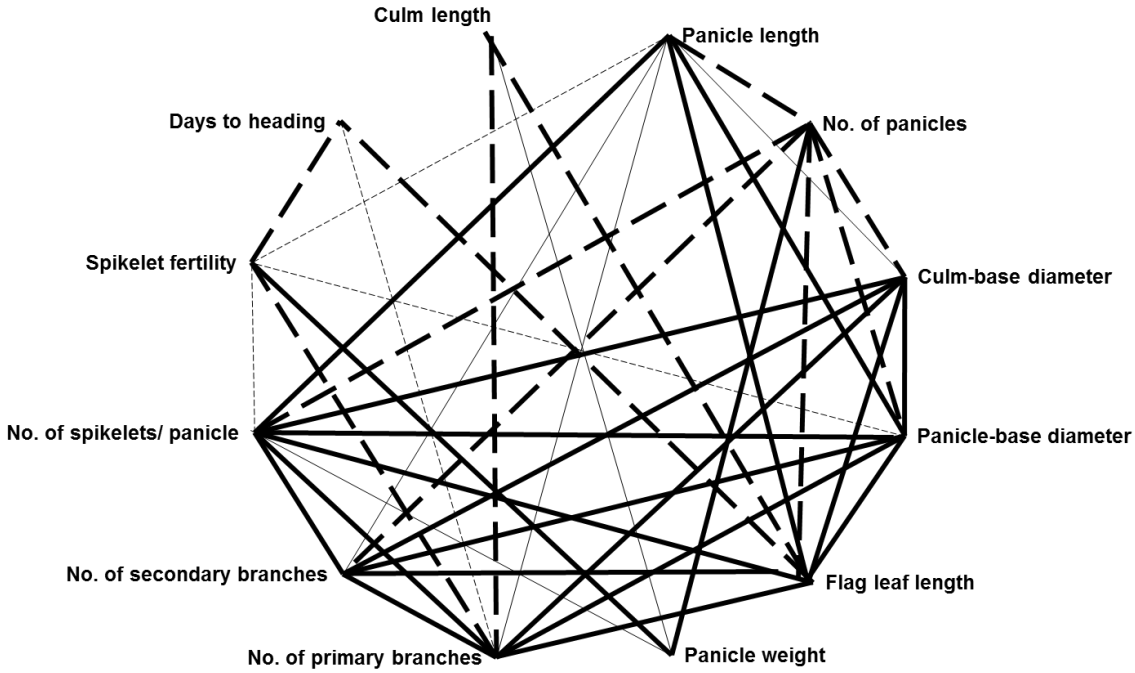


Figure 1.3. Phenotypic correlation between agronomic traits in RILs in 2014. Solid and broken lines indicate positive and negative correlations between the traits, respectively. Thin and thick lines indicate significant correlation coefficients between the traits at the 5% and 1% levels, respectively.

1.3.3 SNPs distribution and construction of the linkage map

To carry out QTL analysis, the RAD-Seq method was used for analysis of RILs developed from the cross between pLIA-1 and Norin 18. In total, 1989 SNPs were found between pLIA-1 and Norin 18 as shown in Fig. 1.4. As indicated in Fig. 1.4, 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. In the highly dense regions of SNPs, not all of the SNPs were used as markers. Instead, only a few of the SNPs nearest to the short arm side were used as markers. Therefore, 479 out of the 1989 SNPs were used for QTL analysis and construction of the linkage map (Fig. 1.5). In the linkage map constructed, the total map length was 1160 cM which covered 74% of 1575 cM reported by Kurata *et al*. (1994). On the other hand, eight large gaps>25 cM were observed on chromosomes 2, 3. 4, 5, 9, 10, and 11 (Fig. 1.5).

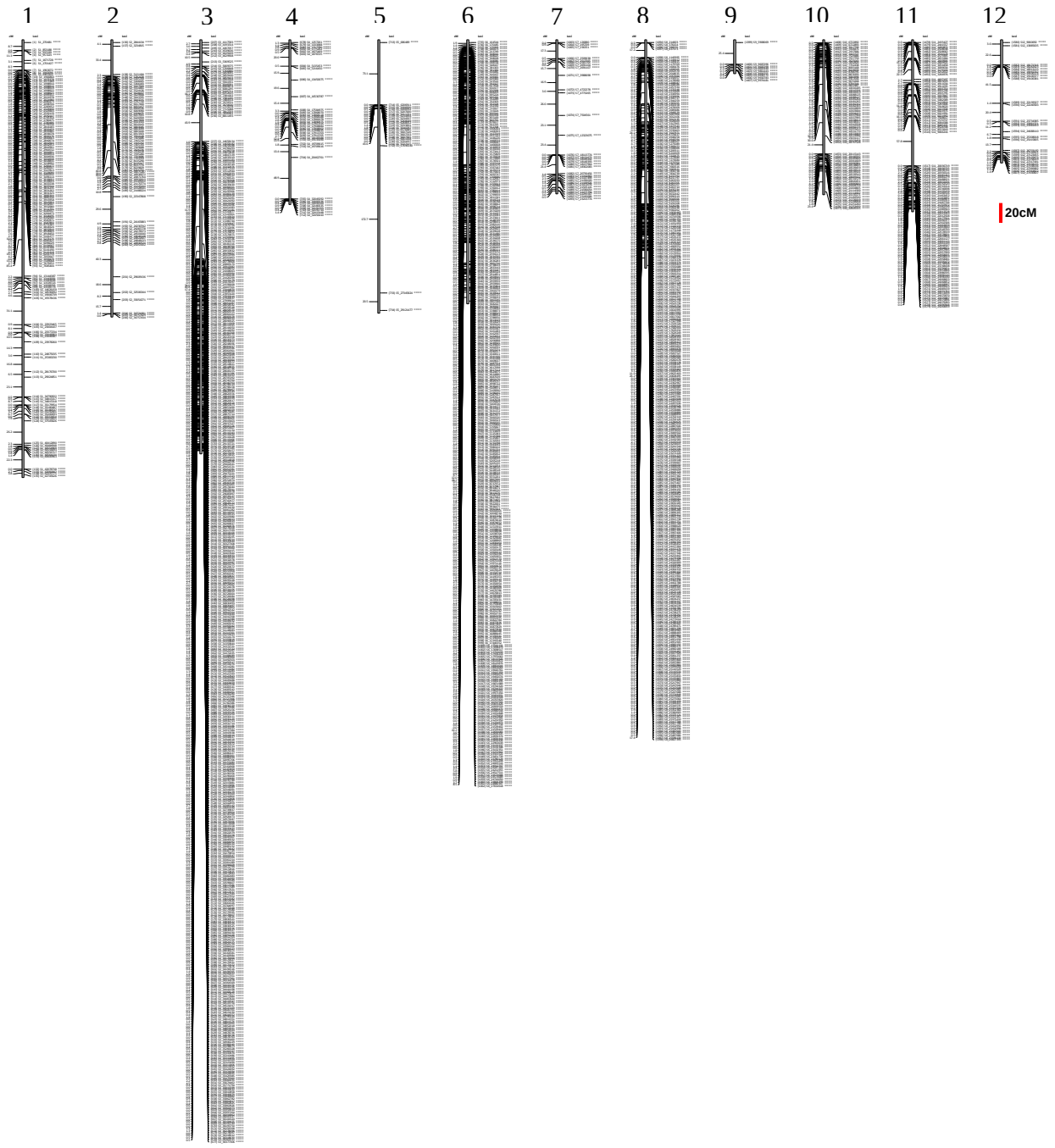
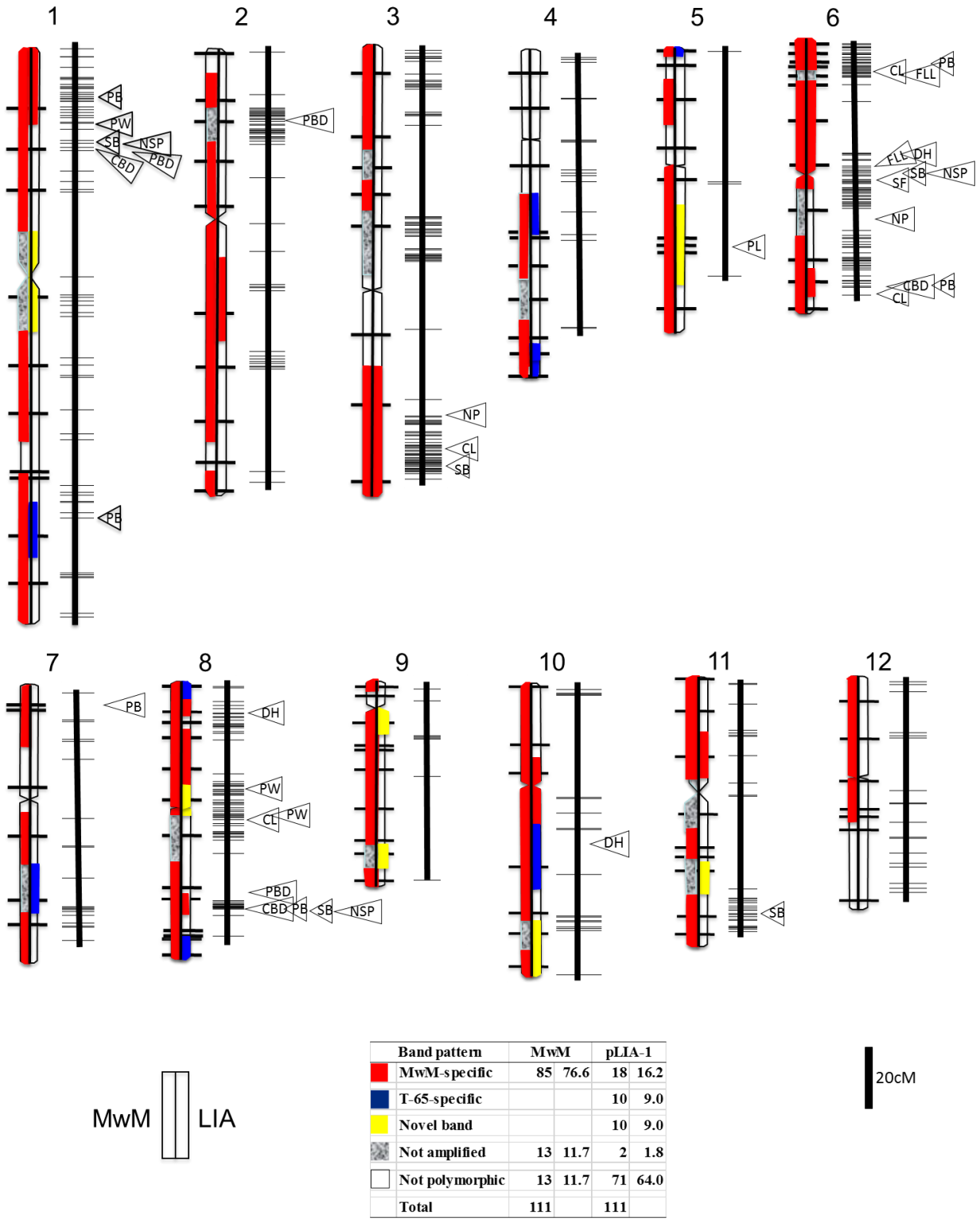


Figure 1.4. Map of SNPs identified by RAD-seq method between pLIA-1 and Norin 18.



Band pattern	MwM		pLIA-1	
MwM-specific	85	76.6	18	16.2
T-65-specific			10	9.0
Novel band			10	9.0
Not amplified	13	11.7	2	1.8
Not polymorphic	13	11.7	71	64.0
Total	111		111	

Figure 1.5. Location of QTLs identified in the RILs of the cross between pLIA-1 and Norin 18 under non-fertilized conditions in 2014. The illustration on the left side in each

chromosome represents graphical genotype (left; MwM. Right; pLIA-1) using SSR markers (Gichuhi *et al*., 2016). The right side of the chromosome shows the map with locations of SNP using RAD-Seq. The abbreviations represent the following traits: Culm length (CL), panicle length (PL), no. of panicles (P), 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), and days to heading (DH). Triangle indicates tentative position of detected QTLs on the chromosomes.

1.3.4 QTLs for yield-related traits

Overall, 36 QTLs were identified on chromosomes 1, 2, 3, 5, 6, 7, 8, 10, and 11 (Table 1.2). In particular, most of the QTLs were detected on intensive locations of SNPs in each chromosome except for chromosome 5 (Fig. 1.5).

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 majorly 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 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% of phenotypic variance. 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, highly contributed to late heading.

In this population, QTL clusters were identified on chromosomes 1, 3, 6, and 8 (Fig. 1.5) and consisted of QTLs controlling traits that were observed to be significantly positively correlated to each other (Fig. 1.3). The QTL clusters on chromosomes 3 and 8 were also observed in the F_2 population (Gichuhi *et al*. 2016). These results suggest that these chromosomal regions are major hot spots for genes that control panicle-related traits.

Table 1.2. 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	r2
Culm length (cm)	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 (cm)	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 (mm)	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 (mm)	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 (cm)	6	S6_5614177	4.2	1.61	0.11
	6	S6_10147242	3.5	-1.48	0.10
Panicle weight (g)	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
	8	S8_24854960	13.5	1.09	0.30
No. of secondary branches	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
	11	S11_21978992	4.1	1.86	0.06
No. of spikelets/ panicle	1	S1_6085421	7.4	11.41	0.15
	6	S6_12034155	4.0	8.37	0.08
	8	S8_25097800	11.2	15.26	0.25
Spikelet fertility (%)	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

1.4 Discussion

1.4.1 *Oryza longistaminata*-derived alleles for yield improvement

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*., 1990; 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 F_2 of a cross between *O. longistaminata*, MwM, collected in Kenya, and *O. sativa* cv. Taichung 65 under non-fertilized conditions (Gichuhi *et al*., 2016).

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 (Table 1.1), it showed very low spikelet fertility resulting from the interspecific cross. Interspecific-derived spikelet sterility, unfavorable linkage block, suppressed recombinations, 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 useful sources 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 F_5 derived from a cross between *O. longistaminata*, MwM, and T-65 under non-fertilized conditions was possibly useful in a breeding program.

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 SNP markers identified using the RAD-Seq method were applied in QTL analysis of 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 F_2 of the cross between pLIA-1 and Norin 18 by using genome-wide SSR markers (Gichuhi *et al*., 2016) previously and RILs derived from the F_2 were developed for further precise QTL analysis. RILs were subjected to the RAD-Seq method and 1989 SNPs were mapped, as shown in Fig. 1.4. 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 (Fig. 1.5). 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 the RILs. The pLIA-1 alleles had a positive contribution in 25 of the QTLs identified. This accounted for more than 50% of the QTLs identified for 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 the QTLs mapped were found to be located on the introgressed chromosome segments of *O. longistaminata* in pLIA-1 revealed through graphical genotyping. 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 were 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.

Spikelet fertility is an important parameter that determines grain yield in rice. Interspecific crosses using wild relatives carry several loci for sterility genes. Identification of such loci is important in order to eliminate them when breeding for higher yields using progenies derived from wild relatives. In this study, 1 QTL for spikelet fertility was detected on chromosome 6. The pLIA-1 allele reduced spikelet fertility. Similarly, 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 frequency distribution of spikelet fertility in Fig.1.2. Since the QTL was found in the RILs bred through SSD method, recessive factors derived from *Oryza longistaminata* are presumed to control spikelet sterility.

1.4.2 QTL clusters

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. QTL clusters of domesticated-related traits of rice were reported by Cai and Morishima (2012) 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 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. Ramos *et al*. (2016) also reported that some CSSLs carrying *O. longistaminata* chromosome segments in Taichung-65 background could be useful for improvement of yield-related traits. Since the QTLs were identified under non-fertilized conditions and pLIA-1 allele was found to improve yield-related parameters under these conditions, the pLIA-1 line might therefore be said to be adapted to low-input conditions. The identification and cloning of the genes responsible for yield-related traits especially observed under low-input conditions will be very helpful for further rice improvement as well as the conservation of the environment.

CHAPTER 2

High resolution analysis of QTLs for yield-related traits of pLIA-1 in the vicinity of the target regions on chromosome 1 and 8.

2.1 Introduction

To overcome the yield plateaus currently being experienced in rice breeding due to a narrow genetic basis of parental materials, many breeders have been exploiting and utilizing the favorable alleles of wild rice species, e.g., *O. rufipogon*, *O. nivara* and *O. glumaepatula* (Xiao *et al*., 1998; Brondani *et al*., 2002). Yield is a complex trait whose phenotypic differences are based on natural variation governed by several genes at quantitative trait loci and their interactions with other genome-wide loci (Matsubara *et al*., 2016). Therefore, in order to utilize the favorable alleles of wild rice, QTL mapping for traits of economic importance, precise identification of their location on the chromosome, and cloning of these QTLs are important. The pLIA-1 line selected was considered to possess characteristics comparable to the ideal plant architecture (IPA) (Gichuhi *et al*., 2016). Further, QTLs for yield-related traits under non-fertilized conditions were identified proving its potential for utilization in yield improvement of rice. Rice yield is determined by a combination of traits like number of panicles per plant, number of spikelets per panicle, spikelet fertility and grain weight. The QTLs for these yield-related traits are most often found clustered together in same chromosome locations (Brondani *et al*., 2002). As summarized in the previous chapter, 4 QTL clusters were detected on chromosomes 1, 3, 6 and 8 in the RILs. To utilize these QTLs especially observed in low-input conditions, the isolation and characterization of each QTL and identification of genes that control these traits could be very helpful for further rice

improvement as well as the sustainability of the environment. Therefore, in this chapter high resolution analysis of the QTL clusters on chromosomes 1 and 8 was done using backcrossed populations of Koshihikari and Norin 18. Backcrossed populations were used because low spikelet fertility and late heading date of pLIA-1 made high resolution analysis difficult in the F_3 and F_4 populations derived from the cross between pLIA-1 and Norin 18. Our aim was to narrow the QTL regions and understand the interactions between the QTLs controlling the different yield-related traits under non-fertilized conditions.

2.2 Materials and methods

2.2.1 Plant materials and growth conditions

Two backcross populations, BC_3F_2 using Norin 18 as a recurrent parent (n=243) and BC_4F_2 using Koshihikari as a recurrent parent (n=846), were developed for the fine dissection of the QTL cluster region on chromosomes 1 and 8 derived from pLIA-1. In the Norin 18 backcross population the target QTL cluster on chromosome 8 was introduced and in the Koshihikari backcross population both target QTL clusters on chromosomes 1 and 8 were introduced. Plants were grown with similar spacing as described in chapter 1 in the non-fertilized paddy field. A total of 12 agronomic traits were measured as previously described in chapter 1.

2.2.2 DNA extraction and genotyping

Genomic DNA was extracted from young leaf tissue using a modified procedure described by Kawasaki (1997). SSR markers and newly designed markers (Table 2.1), were used for genotyping at the target regions on chromosome 1 in the Norin 18 BC_3F_2 population and chromosomes 1 and 8 in the Koshihikari BC_4F_2 population. SSR markers RM8068, RM10115, RM6324, RM220, and EG03 located from the genome sequence 1.66 Mb to 6.09 Mb were used to introduce the chromosome 1-clustered QTL region; and EM1, RM6976, EM4, EM7, EM9, EM12, and EG markers located from the genome sequence 22.47 Mb to 25.28 Mb were used to introduce the clustered QTL region on chromosome 8 (http://rapdb.dna.affrc.go.jp/). Genotyping was done as follows: 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 ^{o}C for 7 min, then 30 cycles of 45 sec at 95 ^{o}C, followed by 30 sec at 55 ^{o}C, and finally, 30 sec at

72 ^{o}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.

Table 2.1. Additional primers used for SSR genotyping

Marker name	Chr.	Forward primer sequence	Reverse primer sequence
EM25	8	AGAGGAGAAGGGGGAGGAAT	GGAGTGCATTGGGAGGTTTA
EM21	8	CCTTGTTCTCAGGTTGCAGT	AAGACCCTGGACTCCACAAC
EM24	8	GGAGTCCAGGGTCTTGTGAG	AAATCAACCCAATCCATCCA
EM14	8	CTCGCCTCACCAATCATCAC	GACTCACCTCCTCGTCGTC
EM9	8	AGATCGGGAGGCAGAGAAG	GGAGACGGACGGCGTTTATA
EM1	8	GCCACCAAACAAGTGAACAA	CCCATGACAAACCAGCTTTT
8EM1_01	8	CCATCTTGGTCCCACTGTTCT	CGGAGAATTATTGCCAGTGAGG
EG10	8	CTCGCAGTTTACAGGCGGAAT	CTTCTTCCGACATGTAGAAATATCGTC
EM4	8	TCTTCCACATAGCACCCAGTT	TCTGGCTTGAACTGATGGTG
EM7	8	TTTTCCCTTTGGATTTTTGC	TCATGATGAAAAATGGAGTGGA
EM12	8	GATCCCTCAGCTAAGCATCG	CCAATCACTTGGCCTCTACC
EG	8	GCACTGGCACAGCTCAATTA	ATTTCCTTTACGGGCCAAA
EG03	1	ACACTAGTGCCGACATCCTCGT	CGAGCCCTACCTTAGCCCTAGTATT

2.3 Results

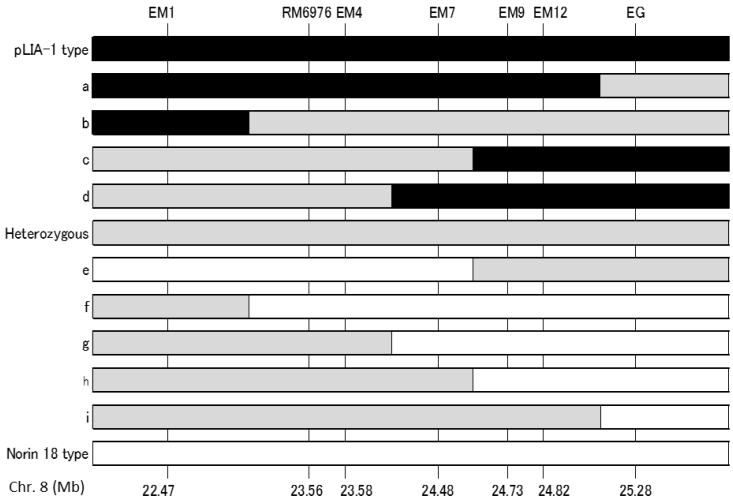
2.3.1 Norin 18-backcrossed population

In the Norin 18 backcross population, only the distal region of chromosome 8 was introduced. 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 (Table 2.2). Although a significant difference of 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 in 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 effect 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 through 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 (Table 2.2). It is likely that the total number of spikelets per plant of pLIA-1 genotype plants was not significantly 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. It will be needed to check the total number of spikelets per plant, which is one of the important components of yield. In the Norin 18 backcross population, some recombinant plants were obtained, as shown in Fig. 2.1. 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 in Fig 2.1.

Table 2.2. Agronomic traits in homozygous plants carrying pLIA-1 genotype and Norin 18 genotype on chromosome 8 in backcrossed population with Norin 18

Traits	pLIA-1 (S.E)	Norin 18 (S.E)
Culm length (cm)	86.4 (0.7)	86.2 (0.5)
Panicle length (cm)	24.6 (0.2)*	23.7 (0.2)
No. of panicles/ plant	9.7 (0.3)	10.0 (0.2)
Culm-base diameter (mm)	5.32 (0.1)*	4.49 (0.0)
Panicle-base diameter (mm)	1.92 (0.0)*	1.75 (0.0)
Flag leaf length (cm)	36.2 (0.7)*	31.3 (0.7)
Panicle weight (g)	33.99 (1.1)	31.53 (0.6)
No. of primary branches	16.6 (0.3)*	12.4 (0.1)
No. of secondary branches	33.9 (0.9)*	26.3 (0.3)
No. of spikelets/ panicle	188.1 (3.8)*	148.9 (1.4)
Spikelet fertility (%)	93.1 (0.6)	92.2 (0.6)
Days to heading	106.8 (0.2)*	106.1 (0.2)
No. of plants (n)	46.0	56.0



Culm-base diameter (mm)	No. primary branches	No. secondary branches	No. spikelets /panicle	No. of plants
5.28	16.5	33.9	187.0	51
4.76	16.0	36.0	178.0	1
4.69	13.8	27.0	158.5	4
5.61	18.0	42.0	206.0	1
5.53	15.0	37.0	192.0	1
4.90	14.0	31.6	172.1	112
4.90	14.0	35.0	186.0	2
4.41	14.0	27.0	154.5	2
4.67	13.0	29.0	160.0	1
4.46	13.0	25.0	150.0	1
4.56	13.0	33.5	182.5	2
4.50	12.5	26.8	151.0	65

Figure 2.1. Agronomic traits in recombinant plants with their respective genotypes on chromosome 8 in the population backcrossed with Norin 18. Black, gray and white rectangles indicate pLIA-1, heterozygous, and Norin 18 genotypes, respectively.

2.3.2 Koshihikari-backcrossed population

In the Koshihikari backcross population, distal regions of chromosomes 1 and 8 were introduced. Then, phenotypic comparisons of plants carrying four possible genotypes for chromosomes 1 and 8 were made, as shown in Fig. 2.2. It was revealed that plants carrying the pLIA-1 genotype for both chromosomes (LL, LL) 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 doesn't show any effects on phenotype. On the other hand, additive effects were shown in culm-base diameter, number of secondary branches, and number of spikelets. No interaction between genotypes on chromosome 1 and 8 was observed in these phenotypes. Culm length, panicle weight, and number of primary branches seem to 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 than in the chromosome 8 region, and the interaction between chromosomes 1 and 8 for the pLIA-1 genotype increased the panicle-base diameter and flag leaf length (Fig. 2.2). Then, the phenotypes of recombinant genotype plants on chromosome 1 and chromosome 8 were examined to narrow the target regions on chromosome 1 and chromosome 8 in the Koshihikari backcross population. As shown in Fig. 2.3, 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 was likely that the genetic factor (s) for the number of secondary branches were located around RM220 and EG03 on chromosome 1. The genotype and

phenotype of recombinant plants on chromosome 8 were as described in Fig. 2.4. The pLIA-1 genotype plants for EM12 and EG (a, d and e types) showed thick culm-base diameter. Further, d and e types carrying the pLIA-1 genotype for EG had larger number of primary and secondary branches. The phenotype of recombinants plants on chromosome 8 suggested that the region from EM9 to EG might locate 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. 2.4) was reversed as compared to that in the Norin 18 backcross population (Fig. 2.1). This may be due to the closeness between EM9 and EM7.

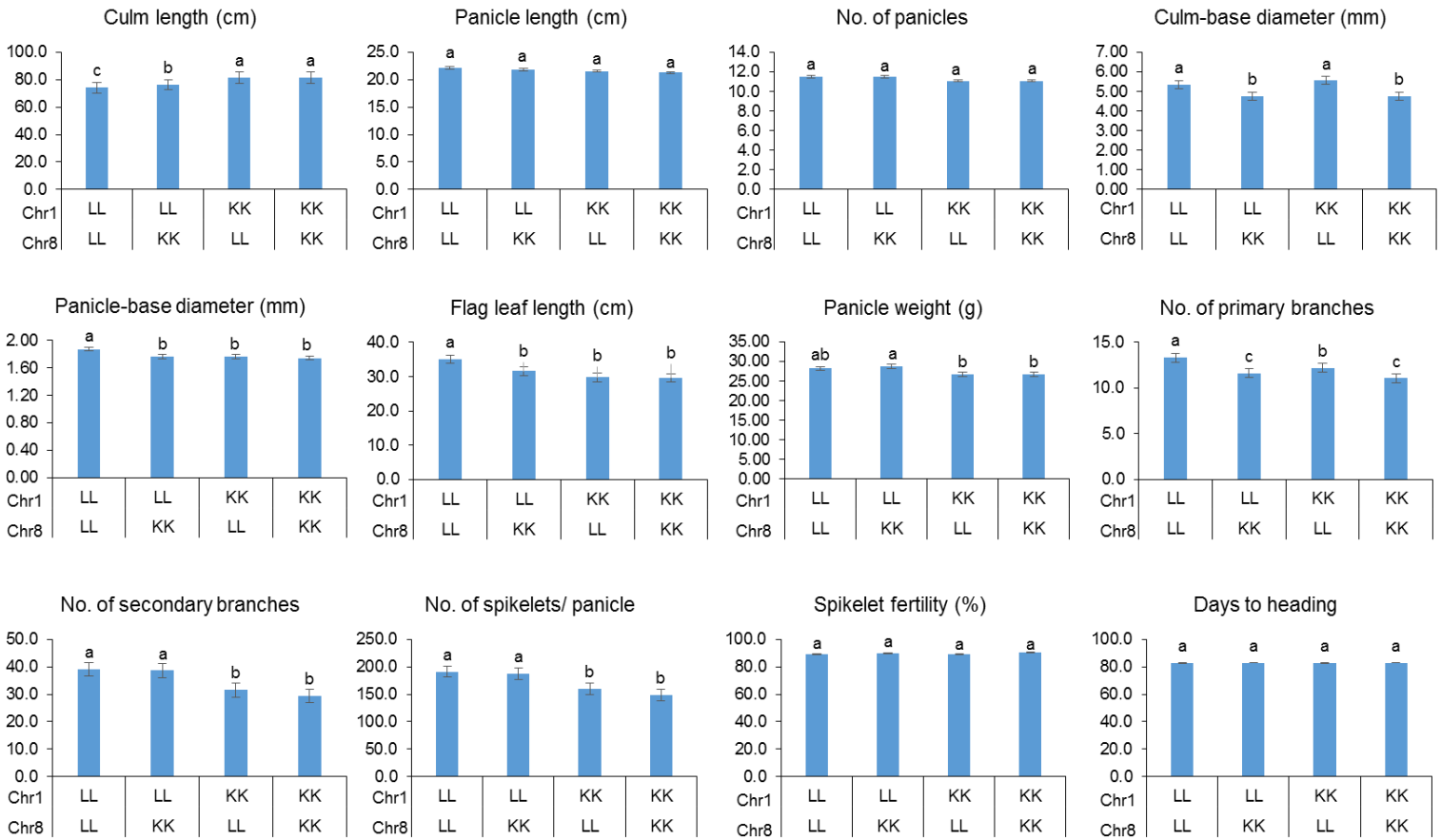
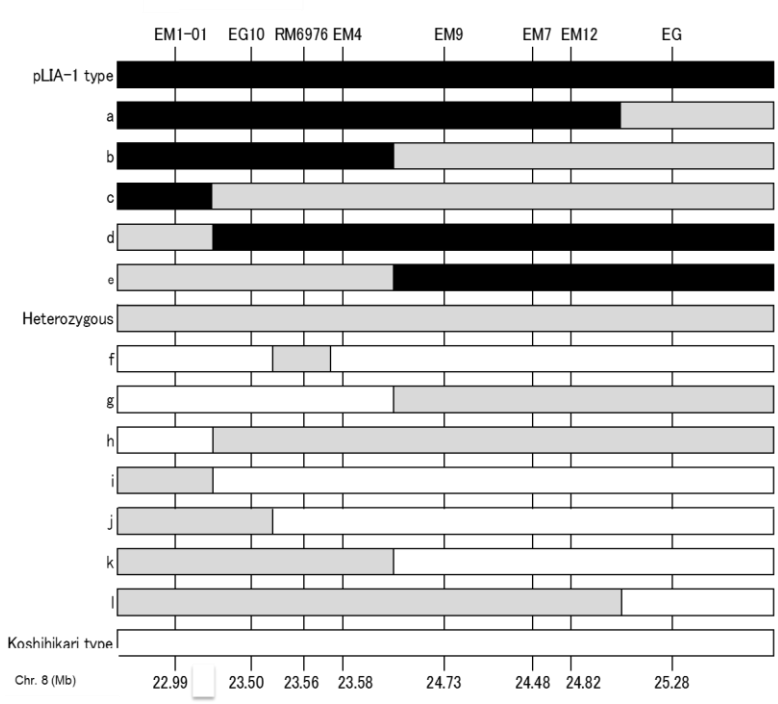


Figure 2.2. Effect of pLIA-1 and Koshihikari alleles on 12 agronomic traits in the population backcrossed with Koshihikari. Different letters in the graph indicate significant differences at the 5% level by Tukey's test. The genotypes on the upper and lower rows shown by LL (pLIA-1) and KK (Koshihikari) on the x-axis represent chromosomes 1 and 8, respectively.

	Culm-base diameter (mm)	No. primary branches	No. secondary branches	No. spikelets/ panicle	No. of plants
pLIA−1 type	4.97	12.1	38.3	185.8	142
a1	5.62	13.0	51.0	230.0	1
a2	5.00	12.2	38.4	188.7	29
b1	5.20	12.5	26.5	137.0	2
b2	4.80	11.0	36.4	172.7	9
c	5.10	12.3	41.7	179.0	3
d1	4.80	12.0	33.0	158.0	1
d2	5.15	12.4	37.0	185.0	4
e	5.01	11.4	38.2	184.7	26
f	5.25	14.0	47.0	231.0	1
g	5.17	12.4	38.4	188.4	16
h	4.73	11.0	42.0	205.0	1
i	4.97	14.0	35.0	174.0	1
j	5.10	11.5	35.5	183.0	2
k	4.90	12.7	37.3	179.9	10
l	4.51	10.0	35.0	178.0	1
m	5.28	11.0	41.0	199.0	1
Heterozygous	5.10	12.0	35.6	176.7	330
n	5.20	11.4	33.2	165.2	26
o	5.03	11.7	35.5	175.1	22
p	4.18	11.0	31.0	156.0	1
q	5.03	12.6	35.8	182.6	5
r	5.05	11.4	33.4	165.0	5
s	5.16	11.0	34.0	165.7	4
t	4.63	10.0	34.0	155.0	1
u	5.43	13.0	24.0	142.0	1
v	5.41	12.0	28.0	143.0	1
w	5.20	11.7	31.4	159.0	14
x	4.90	10.3	33.0	160.8	4
y	5.10	11.8	36.5	178.8	33
Koshihikari type	5.10	11.3	32.4	161.0	142

Figure 2.3 Agronomic traits in recombinant plants with their respective genotypes on chromosome 1 in the population backcrossed with Koshihikari. Black, gray and white rectangles indicate pLIA-1, heterozygous, and Koshihikari genotypes, respectively.



Culm-base diameter (mm)	No. primary branches	No. secondary branches	No. spikelet/ panicle	No. of plants
5.36	12.6	34.6	172.8	208
5.22	10.5	38.0	172.5	2
4.70	11.0	25.0	131.0	1
4.92	11.5	34.0	170.5	2
5.39	12.5	45.0	212.0	2
5.47	13.0	44.3	214.7	3
5.07	11.9	36.3	178.4	401
4.18	11.0	31.0	156.0	1
5.40	13.0	45.0	226.0	1
4.93	10.0	38.0	176.0	1
4.87	10.0	29.0	147.0	1
4.57	12.0	33.0	166.0	1
4.70	10.7	33.7	163.3	3
4.51	10.0	35.0	178.0	1
4.80	11.2	35.3	173.4	217

Figure 2.4. Agronomic traits in recombinant plants with their respective genotypes on chromosome 8 in the population backcrossed with Koshihikari. Black, gray and white rectangles indicate pLIA-1, heterozygous, and Koshihikari genotypes, respectively.

2.4 Discussion

QTL clusters for functionally related genes have been of great interests in crop improvement. In previous QTL analysis, 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). QTL clusters were generally presumed to result from pleiotropy of a single QTL or from tightly linked QTLs for multiple traits, either of which cases would result in genetic correlation (Jian and Zeng, 1995). 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 grown 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 dissect these QTLs, Norin 18 and Koshihikari-backcrossed to the F_1 of the cross between pLIA-1 and Norin 18 populations were developed. The results obtained from the backcrossed populations suggested 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 QTL 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 QTL 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 be causing 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 be controlling each trait.

In conclusion the location of the QTL for culm-base diameter and number of primary branches was determined to be between 24.48 Mb and 25.28 Mb on chromosome 8. On the other hand, the QTL for the number of secondary branches was localized around 6.09 Mb on chromosome 1 and showed more effect in increasing the number of secondary branches. QTLs for other traits were also identified near these regions such as the QTL for panicle-base diameter and number of spikelet per panicle. Although, we could not

narrow down their precise location, the information from these QTLs identification could be useful for yield-trait improvement in practical breeding for marker assisted selection.

CHAPTER 3

Analysis of low-input adaptable factors using RILs and DHLs derived from the cross between pLIA-1 and Norin 18

3.1 Introduction

The use of nitrogen fertilizers has been highly important for increasing crop yields and millions of tons of nitrogen fertilizer are supplied for crop production worldwide (Namai *et al*., 2009). As a result, many developed and developing countries have been able to achieve food security and keep up with the pace of an ever-increasing population. However low-N stress is a major problem in marginalized countries and regions where no or sub-optimal levels of N are supplied (Agrama *et al*., 1999) because farmers lack resources to purchase fertilizers (Ortiz-Monasterio *et al*., 2001). Recently, the increased use of nitrogen fertilizers in farming systems has been shown to produce nitrogen gases of environmental significances. Nitrous Oxide (N_2O) emissions from applied fertilizers cause global greenhouse gas accumulation and stratosphere ozone depletion while nitrate leaching degrades ground water quality (Jeffrey *et al*., 2002). Of all the nitrogen fertilizer supplied, almost half of the nitrogen is harvested with crops; the rest is lost through leaching, erosion and emissions (Charles D., 2013). It is, therefore, important to improve yields while conserving the environment by minimizing the environmental impact of N fertilizers. One solution to the above problems is to search and use genotypes that can grow and produce relatively higher yields under low-N conditions. Breeding varieties with low-N tolerance and understanding their physiological N uptake kinetics and biochemical pathway of the nitrogen absorbed from the soil will lead to increased nitrogen use efficiency (NUE).

The pLIA-1 was selected under non-fertilized conditions. Characterization data showed that pLIA-1 has potential for low input adaptability (Gichuhi *et al*., 2016). Therefore, it is expected that pLIA-1 carries traits for low-N tolerance. To ascertain this, in this chapter, the performance of pLIA-1 grown with different fertilizer levels was examined in comparison to those of Nipponbare, Norin 18 and T-65. The agronomic performance of doubled haploid lines developed from the cross between pLIA-1 and Norin 18, under both fertilized and non-fertilized conditions was also checked. Further, QTL analysis of RILs, developed in Chapter 1, grown under both fertilized and non-fertilized conditions was done.

3.2 Materials and methods

3.2.1 Phenotypic evaluation of pLIA-1, Norin 18 and T-65 at different fertilizer levels

The pLIA-1 line was grown in pots with different levels of fertilizer in a greenhouse, together with Nipponbare, Norin 18 and T-65 for comparison. Norin 18 was chosen because it showed tolerance to low input conditions in a previous study (Maekawa, 1999). Nipponbare and T-65 are reference and a parent of pLIA-1, respectively. All rice lines were grown in three levels of fertilizer treatments in 1/5000 Wagner pots, with three replicates. The fertilizer levels were full (10kg/10a each of NPK), half (5kg/10a each of NPK) and zero (no application). In each pot three 3-week old seedlings were transplanted. At the end of the 4 and 8 weeks after transplanting, three SPAD values in one plant from each pot were collected and the plant was harvested. Agronomic trait data on culm length and number of tillers were then collected and the plants were dried in a 50^{o}C oven for 4 weeks. After drying, the biomass of the plants was measured. The remaining plant was grown to maturity and the same agronomic traits data were collected. Additional data on various agronomic traits was also measured as described in Chapter 1.

Data analysis was done by ANOVA and significant differences between the means was calculated by Tukey's test using R software.

3.2.2 Development of DH lines derived from the cross between pLIA-1 and Norin 18

Doubled haploid (DH) lines were developed from the F_1 of the cross between pLIA-1 and Norin 18. At the flower initiation stage, tillers whose spikelets contained anthers with pollen at the mid-to-late uni-nucleate stage were collected. The tillers were kept at

10ºC for 5 days. Then, the anthers were picked from the young panicles, sterilized with 70% ethanol, rinsed 3 times with distilled water and cultured in a N6 medium (4 g of N6 basal salt, 10ml of N6 vitamins, 70g of sucrose, 1g of yeast extract, 20mg of glycine, 100ul of 10-3M 2, 4-D stock, 5ml of 10-3M NAA stock and 8 g of agar dissolved in 1litre of distilled water at 5.7 pH) followed by incubation at 10ºC for 5 days in the dark. The samples were then incubated at 25ºC for 35 days in the dark, for callus development. The callus was transferred into a N6 regeneration medium prepared as above, without 2, 4-D and NAA, for regeneration under 12 hours light conditions. After acclimatization, the regenerated plantlets were transferred into pots and grown in the greenhouse. The ploidy of each plant was evaluated based on spikelet size and haploid plants were selected. The haploid plants' tillers were treated with 0.1% colchicine (Fig. 3.1) to double the chromosome number and grown in the greenhouse.

Figure 3.1. Colchicine treatment of regenerated haploid plants

3.2.3 Phenotypic evaluation of DHLs and RILs under fertilized and non-fertilized conditions

The 28 DHLs and 113 RILs derived from the cross between pLIA-1 and Norin 18, were grown under two fertilization levels (fertilized conditions and non-fertilized conditions) with two replications, in 2015. Under the fertilized conditions, N, P and K at a ratio of 5-5-5 were applied at 50kg/ ha. The plants grown under non-fertilized conditions were grown in the non-fertilized paddy at IPSR. For each line, five plants in a single row were transplanted with similar spacing as described in Chapter 1. At maturity, data for 12 yield-related traits was collected from 3 plants of each line as described in Chapter 1. The GGE biplot analysis was applied for number of panicles per plant for evaluation of fertilizer responsiveness in DH lines using R software (GGEBiplotGUI package).

3.2.4 QTL analysis for yield-related traits in RILs under fertilized and non-fertilized conditions

To perform QTL analysis, the mean of the two replications under each condition was calculated. The reduction rate of the value under non-fertilized condition of the 12 yield-related traits to that under fertilized condition was also estimated by the trait value rate under non-fertilized to under fertilized conditions (NF/F rate). This parameter was used to assess the degree of the low fertilizer tolerance of the population. The genetic map was then constructed and QTL analysis was done as described in Chapter 1.

3.3 Results

3.3.1 pLIA-1 phenotype at different fertilizer levels

Fertilizer supplied treatments enhanced growth of all the lines in the vegetative stage compared to zero condition. The plants grown under full fertilizer condition had higher SPAD values, taller plant heights, more tillers and larger amounts of biomass compared to those grown under zero condition (Fig. 3.2a). The pLIA-1 line showed a significantly lower SPAD value than T-65 in every treatment. On the other hand, there were no significant differences in SPAD values of pLIA-1, Norin 18 and Nipponbare among fertilizer conditions. pLIA-1 showed significantly taller plant height than Nipponbare, Norin 18 and T-65 under zero fertilizer condition. Nipponbare, Norin 18 and T-65 showed extremely reduced number of tillers under zero fertilizer condition, whereas, pLIA-1 did not exhibit a significant reduction. There were no any differences in biomass among the lines under zero fertilizer condition.

At eight weeks after transplanting, clear differences in SPAD values were not observed among the lines except for Norin 18 in every treatment (Fig. 3.2b). Norin 18 showed a significantly higher SPAD value under the zero condition compared to that under full fertilizer condition. The pLIA-1 line did not show any differences in plant height in every treatment, however, it was significantly taller than the other lines under each condition. Although pLIA-1 did not show a significant reduction in the number of tillers in every treatment, its biomass under zero fertilizer condition was lower than that under half fertilizer condition.

At the vegetative stage, pLIA-1 had a taller plant height and no significant reduction in the number of tillers compared to the other lines under the three conditions (Fig. 3.2a, Fig. 3.2b). The reduction patterns of biomass were not different depending on fertilizer

conditions among the lines. These results suggested that a single tiller of pLIA-1 might have a relatively larger biomass due to the significant taller plant heights than the other lines observed under all three conditions.

At maturity, pLIA-1 showed no difference in SPAD values of flag leaf under the three conditions. However, in Nipponbare, Norin 18 and T-65 significant difference between zero fertilizer condition and full fertilizer condition were observed, with lower SPAD values in the full fertilizer condition (Fig. 3.3). Differential response to different fertilizer conditions was not observed in culm length, panicle length and spikelets fertility in all the lines. On the other hand, the number of tillers, number of panicles per plant and panicle weight were highly responsive to different fertilizer conditions (Fig. 3.3). Yield-related traits including number of primary branches, number of secondary branches and number of spikelets per panicle of pLIA-1 were significantly higher under zero fertilizer condition than those under the other conditions (Fig. 3.3). However, these traits were almost similar under three fertilizer conditions in Nipponbare, Norin 18 and T-65. Further, pLIA-1 showed no significant differences in panicle weight under the three conditions compared to the other lines. These results suggested that pLIA-1 may have better performance under zero fertilizer condition than Nipponbare, Norin 18 and T-65 and hence tolerant to low fertilizer condition.

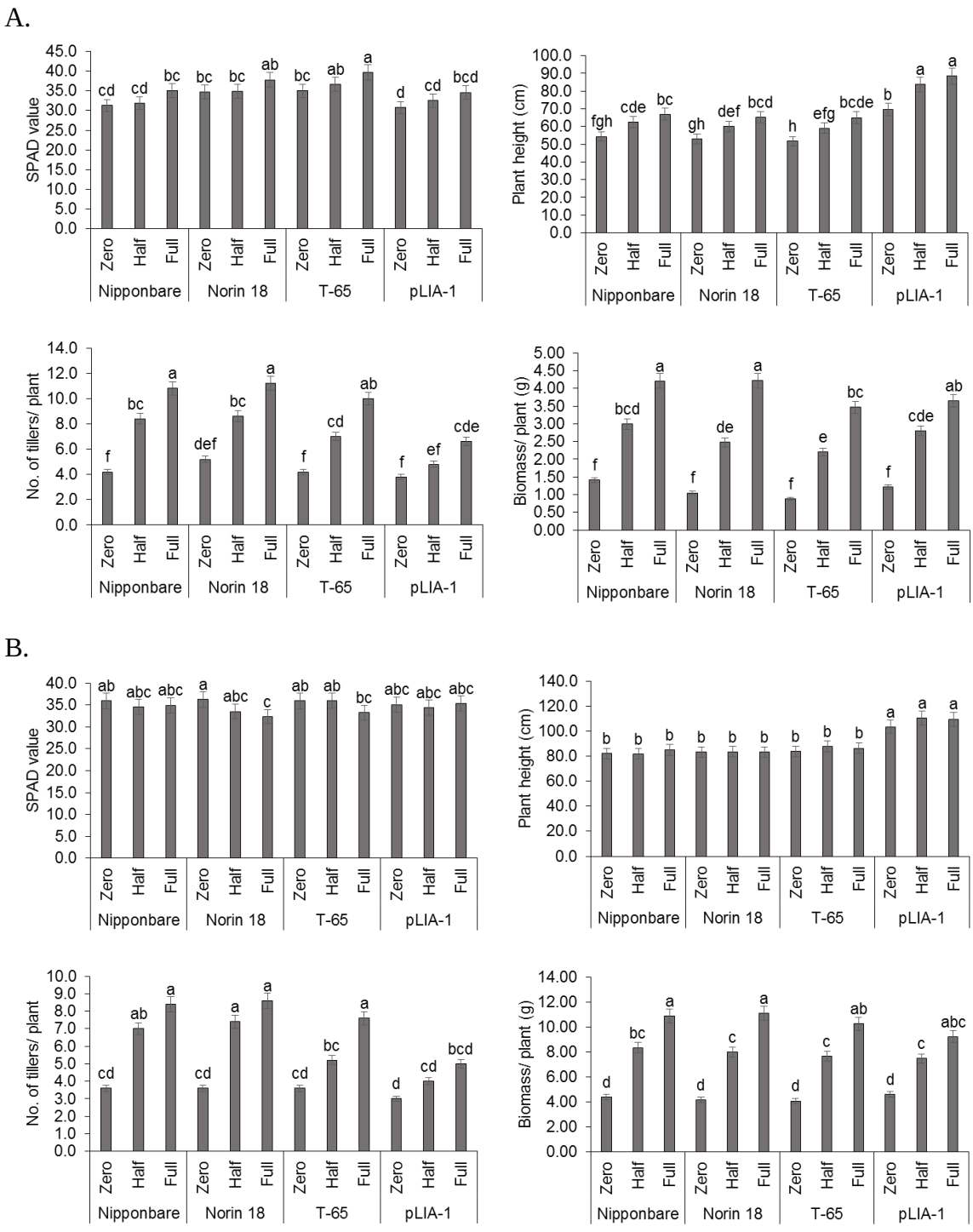


Figure 3.2. Agronomic performance of Nipponbare, Norin 18, T-65 and pLIA-1 in different levels of fertilizer application at one month (A) and two months (B) after transplanting. Different letters on top of the bars indicate significant differences at the 5% level by Tukey's test.

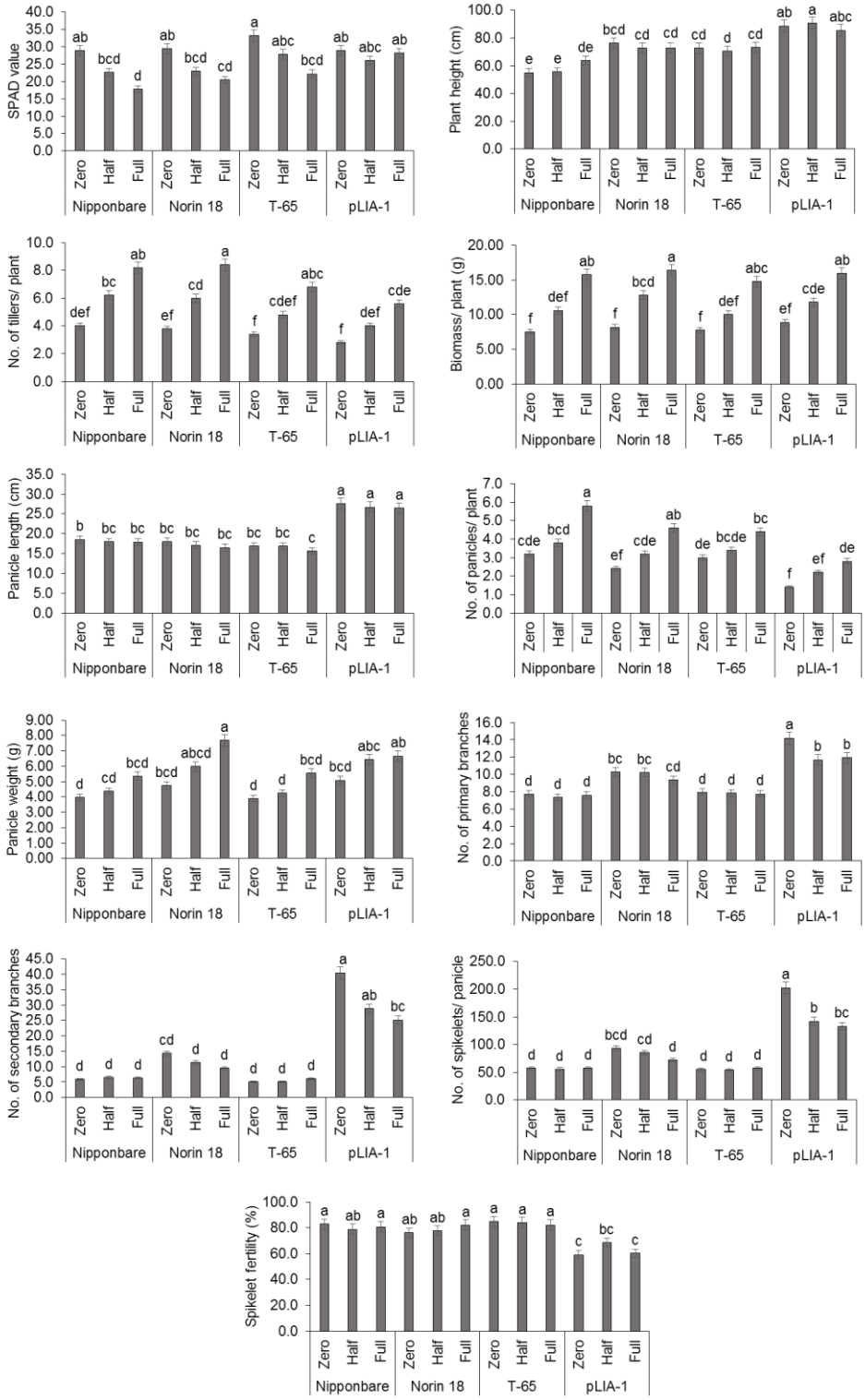


Figure 3.3. Agronomic performance of Nipponbare, Norin 18, T-65 and pLIA-1 in different levels of fertilizer application at maturity. Different letters on top of the bars indicate significant differences at the 5% level by Tukey's test.

3.3.2 Characteristics of DH lines under fertilized and non-fertilized conditions

A population of 21 DH lines developed from the F_1 of the cross between pLIA-1 and Norin 18 were evaluated and their agronomic performance were compared under both fertilized and non-fertilized conditions. Based on the comparison of the traits between non-fertilized and fertilized conditions, response patterns of traits to fertilizer were grouped into three types: fertilizer-responsive type (culm length, number of panicles per plant, culm-base diameter, flag leaf length, panicle weight and number of primary branches), fertilizer-suppressive type (number of secondary branches, number of spikelets per panicle, and spikelet fertility) and fertilizer-nonresponsive type (panicle length, panicle-base diameter, and days to heading) (Fig. 3.4). In particular, all DH lines showed more number of panicles per plant under fertilized condition than those under non-fertilized condition, suggesting that this trait was very sensitive to fertilizer (Fig. 3.4). Therefore, the relationship between number of panicles per plant under non-fertilized condition and the reduction rate of number of panicles per plant under non-fertilized condition to that under fertilized condition was checked as shown in Fig. 3.5. The DH lines seemed to divide into two groups; high reduction rate and moderate reduction rate. To further confirm this tendency, we tried to do GGE biplot analysis. PC1 and PC2 represented number of panicles per plant under non-fertilized and fertilized conditions, respectively. As shown in Fig. 3.6, three lines, #102, #103 and #104, were found to be more adaptable to non-fertilized conditions. These results suggested that reduction rate in number of panicles per plant under non-fertilized conditions might be an effective indicator for adaptability to non-fertilized conditions.

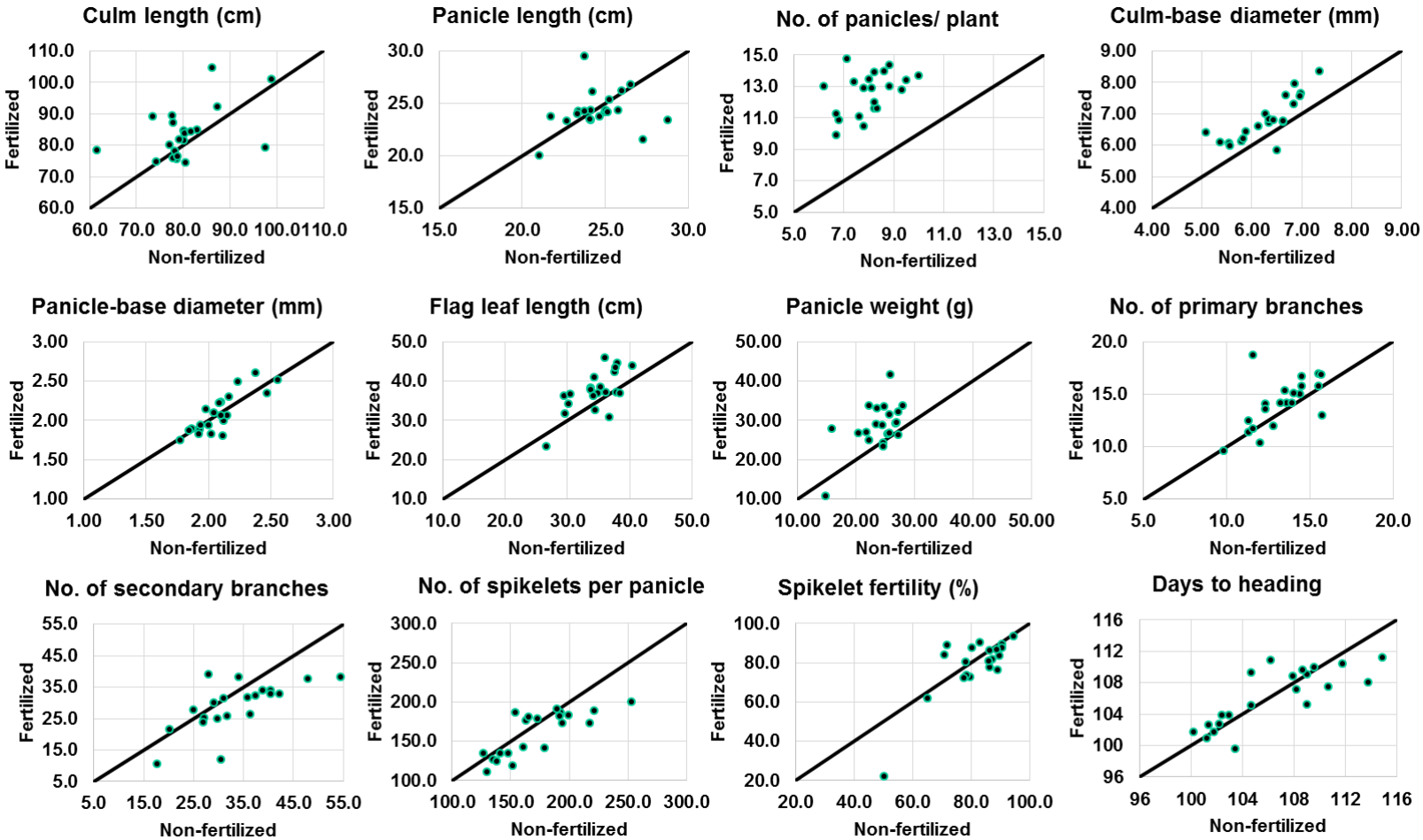


Figure 3.4. Comparison of agronomic performance of DH lines of the cross between pLIA-1 and Norin 18 grown under fertilized and non-fertilized conditions.

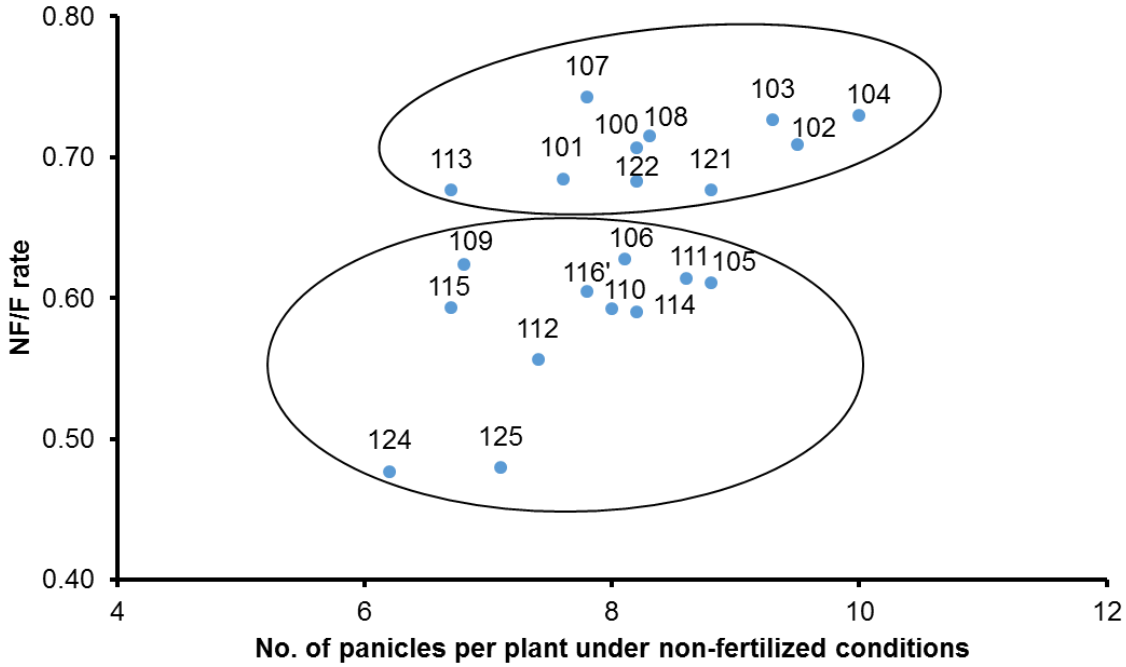


Figure 3.5. Relationship between NF/F rate and number of panicles per plant under non-fertilized conditions in DH lines.

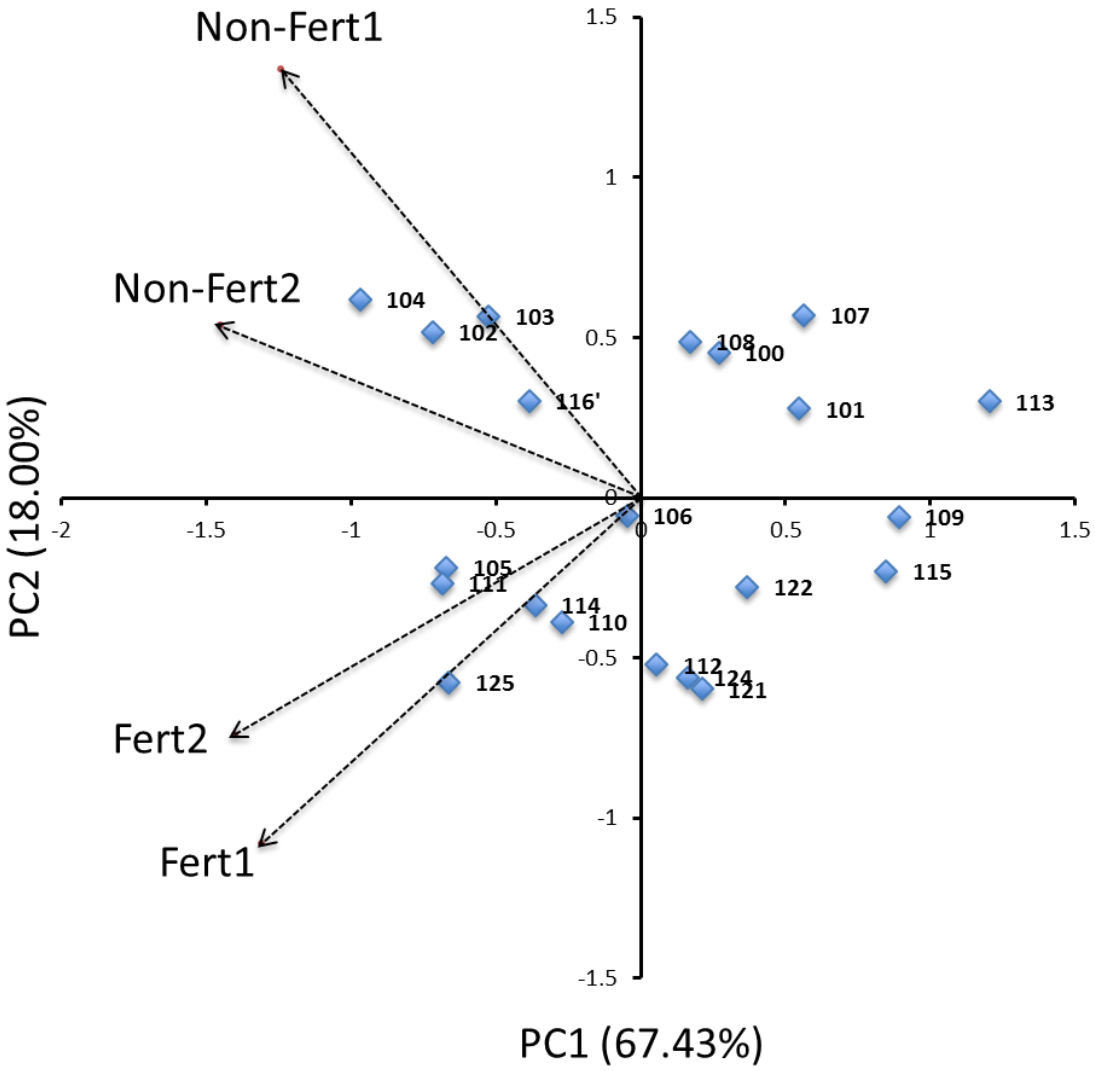


Figure 3.6. GGE biplot for number of panicles per plant of DH lines grown under fertilized (Fert1 and Fert2) and non-fertilized (Non-Fert1 and Non-Fert2) conditions. The biplot consists of PC1 scores plotted against PC2scores for both DH lines (blue diamond) and fertilizer conditions (vectors). PC1 and PC2 explained 67.43% and 18.0% of the total GGE variation, respectively.

3.3.3 Phenotypic variation of RILs under fertilized and non-fertilized conditions

To explore the factors for adaptability to low-input conditions, RILs developed from the cross between pLIA-1 and Norin 18 were grown in both fertilized and non-fertilized conditions in 2015, and subjected to QTL analysis. In the majority of the traits measured normal curve distributions were observed both under fertilized and non-fertilized conditions (Fig. 3.7). The RILs grown in non-fertilized conditions showed a narrow range of values for number of panicles per plant, culm-base diameter and panicle weight compared to those under fertilized conditions. As observed in DH lines, the frequency distribution in number of panicles per plant and panicle weight under non-fertilized conditions showed a clear shift towards lower values. On the other hand, panicle-base diameter and number of secondary branches under non-fertilized conditions tended to be distributed towards higher values than those under fertilized conditions.

The correlation coefficients between agronomic traits were then calculated under non-fertilized and fertilized conditions as shown in Table 3.1. Significant correlation coefficients between the agronomics traits under non-fertilized conditions were also observed among similar traits under fertilized conditions. In particular, significant positive correlations were observed between culm thickness traits (culm-base diameter and panicle-base diameter) and panicle-related traits (panicle length, number of primary branches, number of secondary branches and number of spikelets per panicle) in both fertilized and non-fertilized conditions. Similarly, panicle-related traits were significantly positively correlated to each other. Higher correlation coefficients were found under fertilized conditions than under non-fertilized conditions except for that between flag leaf length and number of primary branches which was lower under fertilized condition than that under non-fertilized conditions. Further, significant

correlations only under fertilized or non-fertilized conditions were observed. The correlation between culm length and either the number of panicles per plant or flag leaf length, between panicle length and number of secondary branches and between total panicle weight and number of spikelets per panicle were significant only under fertilized conditions. Days to heading showed significant negative correlation to number of panicles per plant, culm-base diameter, panicle-base diameter, number of secondary branches and number of spikelets per panicle. These significant correlations were presumed to be derived from the different distribution patterns under fertilized or non-fertilized conditions shown in Fig. 3.6, except for days to heading. On the other hand, significant correlations between spikelet fertility and either panicle-base diameter or number of spikelets per panicle were found only under non-fertilized conditions.

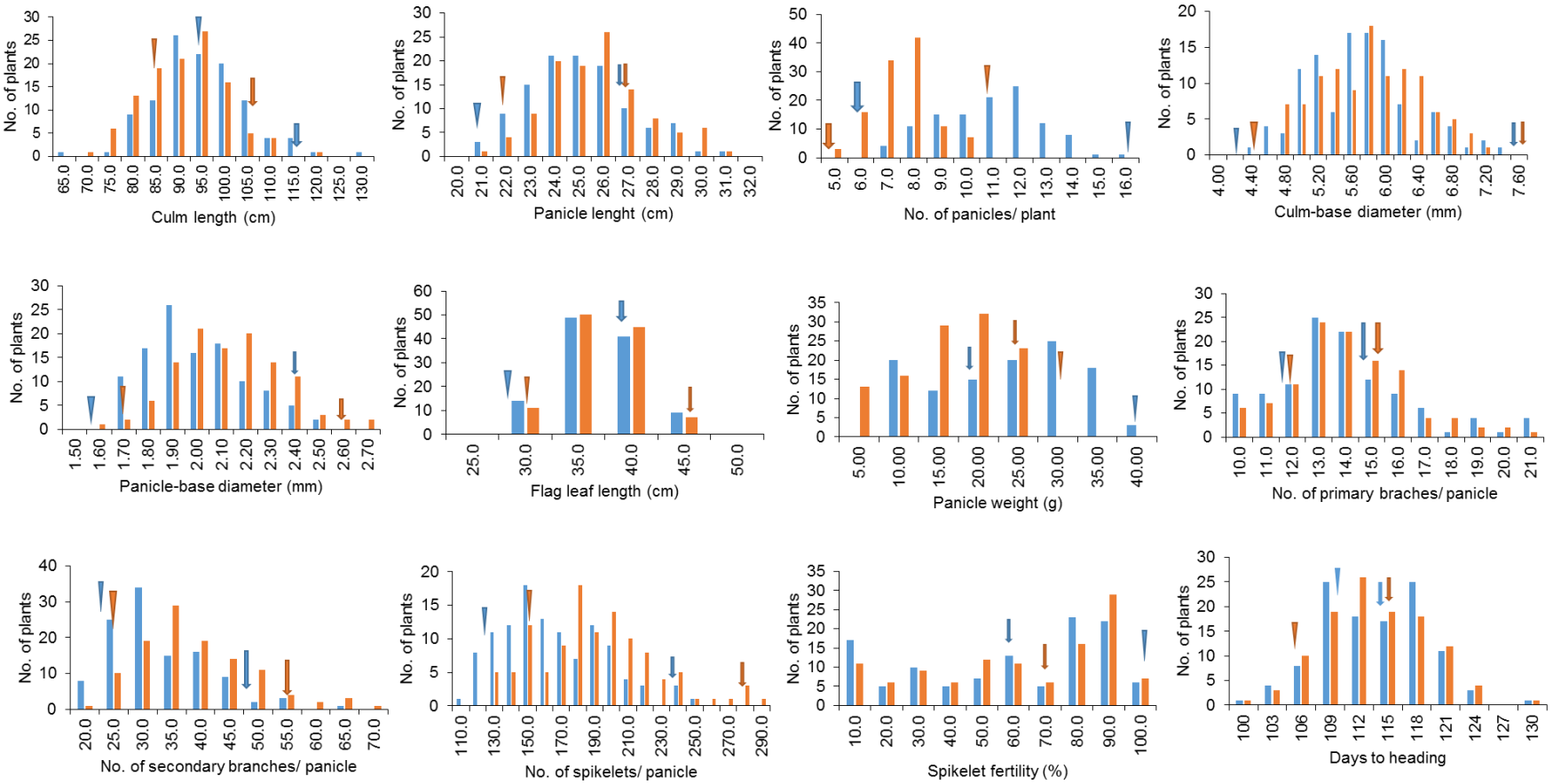


Figure 3.7. Frequency distribution of 12 yield-related traits in RILs grown in 2015. Blue and orange show fertilized and non-fertilized conditions, respectively. Triangles and arrows indicate mean values of Norin 18 and pLIA-1, respectively.

Table 3.1. Correlation coefficient between agronomic traits in RILs grown under fertilized and non-fertilized conditions in 2015

	Culm length (cm)	Panicle length (cm)	No. of panicles/ plant	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 (%)
Panicle length (cm)	0.108 0.074										
No. of panicles/ plant	0.049 0.209*	-0.020 -0.101									
Culm-base diameter (mm)	0.059 0.083	0.253** 0.353**	-0.376** -0.478**								
Panicle-base diameter (mm)	-0.124 -0.099	0.363** 0.444**	-0.422** -0.495**	0.743** 0.844**							
Flag leaf length (cm)	-0.098 -0.197*	0.353** 0.430**	-0.217* -0.265**	0.217* 0.275**	0.351** 0.412**						
Panicle weight (g)	0.242** 0.271**	0.013 0.014	0.249** 0.326**	-0.032 0.028	-0.102 -0.038	-0.117 0.011					
No. of primary branches	-0.221* -0.314**	0.228** 0.215*	-0.150 -0.097	0.362** 0.397**	0.498** 0.464**	0.307** 0.189*	0.091 0.162				
No. of secondary branches	-0.119 -0.135	0.104 0.255**	-0.364** -0.413**	0.622** 0.606**	0.694** 0.704**	0.364** 0.359**	-0.024 0.102	0.394** 0.303**			
No. of spikelets/ panicle	-0.125 -0.120	0.208* 0.361**	-0.390** -0.332**	0.643** 0.658**	0.747** 0.751**	0.391** 0.378**	-0.026 0.197*	0.573** 0.590**	0.887** 0.872**		
Spikelet fertility (%)	0.275** 0.258**	-0.051 -0.020	0.140 0.159	-0.078 0.067	-0.178* -0.010	-0.149 0.017	0.929** 0.916**	-0.030 0.040	-0.144 0.111	-0.178* 0.125	
Days to heading	-0.050 -0.138	-0.193* -0.292**	-0.168 -0.214*	-0.105 -0.214*	-0.014 -0.243**	0.133 -0.151	-0.643** -0.683**	-0.240** -0.395**	0.086 -0.200*	0.004 -0.405**	-0.577** -0.539**

* and ** indicate significant differences at the 5% and 1% levels, respectively.

Upper and lower underlined values in the same category represent correlation coefficient under non-fertilized and fertilized conditions, respectively.

3.3.4 QTLs for yield-related traits identified in RILs under fertilized and non-fertilized conditions

In total, 67 QTLs for 12 yield-related traits were detected under both fertilized and non-fertilized conditions (Table 3.2). Of these, 32 QTLs were detected under fertilized conditions, 29 QTLs under non-fertilized conditions and 6 QTLs were detected for the NF/F rate. Each of the QTLs explained 5-44% of the phenotypic variance. The QTLs detected under fertilized conditions were distributed on chromosomes 1, 3, 6, 8, 10, and 11, whereas the ones detected under non-fertilized conditions were distributed on chromosomes 1, 2, 3, 5, 6, 8, and 10. In addition, QTLs for NF/F rate were distributed on chromosome 3, 6, 10, and 11(Fig. 3.8). Among the QTLs detected, 18 consistent QTLs for the same traits located on chromosomes 1, 3, 6, 8, and 10 were detected under both conditions (Fig. 3.8). These QTLs were either detected on the same SNP in both conditions or on two different SNPs under each condition that are located close to each other (Table 3.2). On the other hand, a total of 31 differentially expressed QTLs were detected under both conditions.

Totally, 8 QTLs for culm length were detected on chromosomes 3, 6 and 8 under both conditions. Of these, 3 QTLs on chromosomes 3 and 6 were detected in similar locations under fertilized and non-fertilized conditions. The QTL at S6-24880347 showed the highest phenotypic variance under both conditions. A single QTL for panicle length was detected only under non-fertilized conditions. The pLIA-1 allele at this QTL increased panicle length. A total of 5 QTLs for the number of panicles per plant were identified under both conditions. At all the QTLs identified, pLIA-1 allele reduced the number of panicles per plant. The only QTL identified under non-fertilized conditions, was located around the same region where a similar QTL was identified on chromosome 3 under non-

fertilized conditions. In the RILs grown under fertilized conditions, 2 QTLs for culm-base diameter were identified on chromosomes 6 and 8. Similar QTLs were also identified under non-fertilized conditions located near the same SNP. The pLIA-1 alleles at all the QTLs increased culm thickness. However, the QTL on chromosome 8 showed a greater contribution to the phenotypic variance (44%) under non-fertilized conditions. In total, 5 QTLs for panicle-base diameter were identified on chromosomes 1, 2, 6, and 8 under both conditions. Of these, only the QTL on chromosome 8 was consistent under both conditions. This QTL showed the highest phenotypic variance under both conditions. The pLIA-1 allele at all the QTLs except the QTL on chromosome 2, increased panicle-base diameter. Four QTLs for flag leaf length were identified only under fertilized conditions on chromosomes 1, 6, 8, and 11. The pLIA-1 alleles at three of the QTLs increased flag leaf length. A total of 2 QTLs were identified under each condition on chromosomes 6 and 10. The QTLs were found in similar locations under both conditions. The pLIA-1 allele at all the QTLs reduced panicle weight. Totally, 4 and 3 QTLs were identified for number of primary branches on chromosomes 1, 2, 3, 6, and 8 under fertilized and non-fertilized conditions, respectively. The QTLs on chromosomes 6 and 8 were consistent under both conditions with the QTL on chromosome 8 exhibiting the highest phenotypic variance. The pLIA-1 allele had a positive contribution to the trait only at the QTL on chromosome 8. Four QTLs for the number of secondary branches were identified under each condition on chromosomes 1, 6, 8, and 11. Of these, 2 QTLs located on chromosomes 1 and 8 were consistent under both conditions and the pLIA-1 allele, only at these QTLs, increased the number of secondary branches. The QTLs on chromosomes 1 and 8 had the highest phenotypic variance under fertilized and non-fertilized conditions, respectively. A total of 6 QTLs were identified for number of

spikelets per panicle under both conditions on chromosomes 1, 6, 8 and 11. The QTLs on chromosomes 1 and 8 were consistent under both conditions. These QTLs were also found to be colocalized with the QTLs for number of secondary branches on chromosome 1 and culm-base diameter, panicle-base diameter, number of primary branches, and number of secondary branches on chromosome 8, under both conditions. The pLIA-1 allele increased the number of spikelets at all the QTLs except at the QTL on chromosome 11. The QTL on chromosome 8 showed the highest phenotypic variance under both conditions. In total, 3 QTLs for spikelet fertility were identified on chromosomes 6 and 10 under both conditions. The QTL on chromosome 10 was detected in a similar location under both conditions. At all the QTLs, the pLIA-1 allele increased spikelet sterility. A total of 3 QTLs were identified for days to heading under each condition on chromosomes 6, 8, and 10. The QTLs on chromosomes 6 and 8 were consistent under both conditions and the pLIA-1 allele at these QTLs delayed heading. The QTL on chromosome 10 had the highest phenotypic contribution under both conditions.

QTLs for NF/F rate were detected for traits of number of panicles per plant, number of primary branches, number of secondary branches, number of spikelets per panicle and days to heading (Table 3.2). The QTLs for NF/F rate for number of primary branches, number of spikelets per panicle and days to heading were mapped on chromosome 6 (Fig. 3.8). The NF/F rate QTLs for number of panicles per plant, days to heading and number of secondary branches were mapped near the QTLs for their respective traits (Table 3.2). Therefore, these results suggest that these QTLs play important roles in low fertilizer tolerance.

Table 3.2. QTLs identified in the RILs of the cross between pLIA-1 and Norin 18 grown under fertilized and non-fertilized conditions in 2015

Trait		Chr.	Nearest Marker	LOD	Additive effect	r2
Culm length (cm)	**Fertilized condition**	3	S3_32095706	5.0	-3.12	0.10
		6	S6_24880347	7.4	3.94	0.16
		6	S6_3995626	3.5	2.59	0.06
	Non-fertilized condition	3	S3_32663249	5.8	-3.09	0.11
		6	S6_24880347	7.9	3.73	0.17
		6	S6_4341166	3.6	2.48	0.07
		8	S8_9226286	3.6	-2.52	0.07
		8	S8_2410158	3.0	2.15	0.05
Panicle length (cm)	**Non-fertilized condition**	5	S5_6727115	3.2	0.69	0.11
No. of panicles/ plant	**Fertilized condition**	1	S1_6201759	4.6	-0.64	0.11
		3	S3_28369958	5.6	-0.71	0.13
		3	S3_16451181	3.4	-0.53	0.07
		8	S8_22475966	3.7	-0.55	0.08
	Non-fertilized condition	3	S3_27920637	6.1	-0.50	0.18
	NF/F rate	3	S3_31727366	3.0	2.92	0.09
Culm-base diameter (mm)	**Fertilized condition**	6	S6_24880347	5.6	0.21	0.11
		8	S8_24960189	10.8	0.33	0.26
	Non-fertilized condition	6	S6_24880347	4.1	0.15	0.06
		8	S8_24960189	19.7	0.41	0.44
Panicle-base diameter (mm)	**Fertilized condition**	6	S6_24880347	4.8	0.06	0.09
		8	S8_24960189	11.4	0.11	0.26
	Non-fertilized condition	1	S1_5275176	3.4	0.06	0.06
		2	S2_6227909	3.2	-0.05	0.06
		8	S8_24960189	11.0	0.11	0.26
Flag leaf length (cm)	**Fertilized condition**	1	S1_3915962	4.6	1.34	0.11
		6	S6_1460532	3.4	1.20	0.09
		8	S8_24706786	4.1	1.32	0.11
		11	S11_2669921	3.8	-1.20	0.10
Panicle weight/ plant (g)	**Fertilized condition**	6	S6_8948220	4.4	-3.01	0.11
		10	S10_10533086	5.3	-4.52	0.22
	Non-fertilized condition	6	S6_8948220	5.4	-2.47	0.15
		10	S10_9832487	5.0	-2.67	0.13
No. of primary branches	**Fertilized condition**	1	S1_35553655	4.8	-0.74	0.08
		3	S3_32315896	3.9	-0.68	0.07
		6	S6_24806399	6.8	-0.92	0.12
		8	S8_22475966	13.9	1.51	0.30
	Non-fertilized condition	2	S2_6803099	3.0	-0.53	0.06
		6	S6_24806399	3.5	-0.59	0.07
		8	S8_22475966	12.5	1.25	0.29
	NF/F rate	6	S6_12191074	4.8	3.09	0.13
		10	S10_21135171	3.7	-2.70	0.10

Table 3.2. Continued

Trait		Chr.	Nearest Marker	LOD	Additive effect	r2
No. of secondary branches	**Fertilized condition**	1	S1_5275176	8.0	3.59	0.17
		6	S6_6228545	4.7	-2.86	0.10
		8	S8_24706786	3.4	2.35	0.07
		11	S11_2669921	5.6	-2.99	0.12
	Non-fertilized condition	1	S1_5275176	6.0	3.49	0.12
		1	S1_13144387	3.3	2.46	0.06
		6	S6_3995626	4.1	-2.84	0.08
		8	S8_22475966	7.8	4.23	0.17
	NF/F rate	11	S11_2669921	4.0	9.15	0.13
No. of spikelets per panicle	**Fertilized condition**	1	S1_5275176	5.4	10.81	0.12
		8	S8_24706786	7.6	13.62	0.18
		11	S11_2669921	6.3	-12.14	0.15
	Non-fertilized condition	1	S1_4587893	4.2	10.55	0.08
		6	S6_19744322	3.6	9.59	0.07
		8	S8_22475966	7.7	16.42	0.20
	NF/F rate	6	S6_8948220	4.9	6.36	0.14
Spikelet fertility (%)	**Fertilized condition**	10	S10_10533086	7.8	-18.06	0.28
	Non-fertilized condition	6	S6_11515911	5.4	-10.69	0.13
		10	S10_9832487	10.2	-16.80	0.25
Days to heading	**Fertilized condition**	6	S6_8948220	8.3	2.42	0.17
		6	S6_4081636	3.4	-1.55	0.07
		10	S10_16323458	10.5	3.04	0.30
	Non-fertilized condition	6	S6_9506708	11.0	2.54	0.21
		8	S8_2410158	3.4	-1.30	0.05
		10	S10_16323458	9.8	2.49	0.22
	NF/F rate	6	S6_1460532	6.0	0.87	0.21

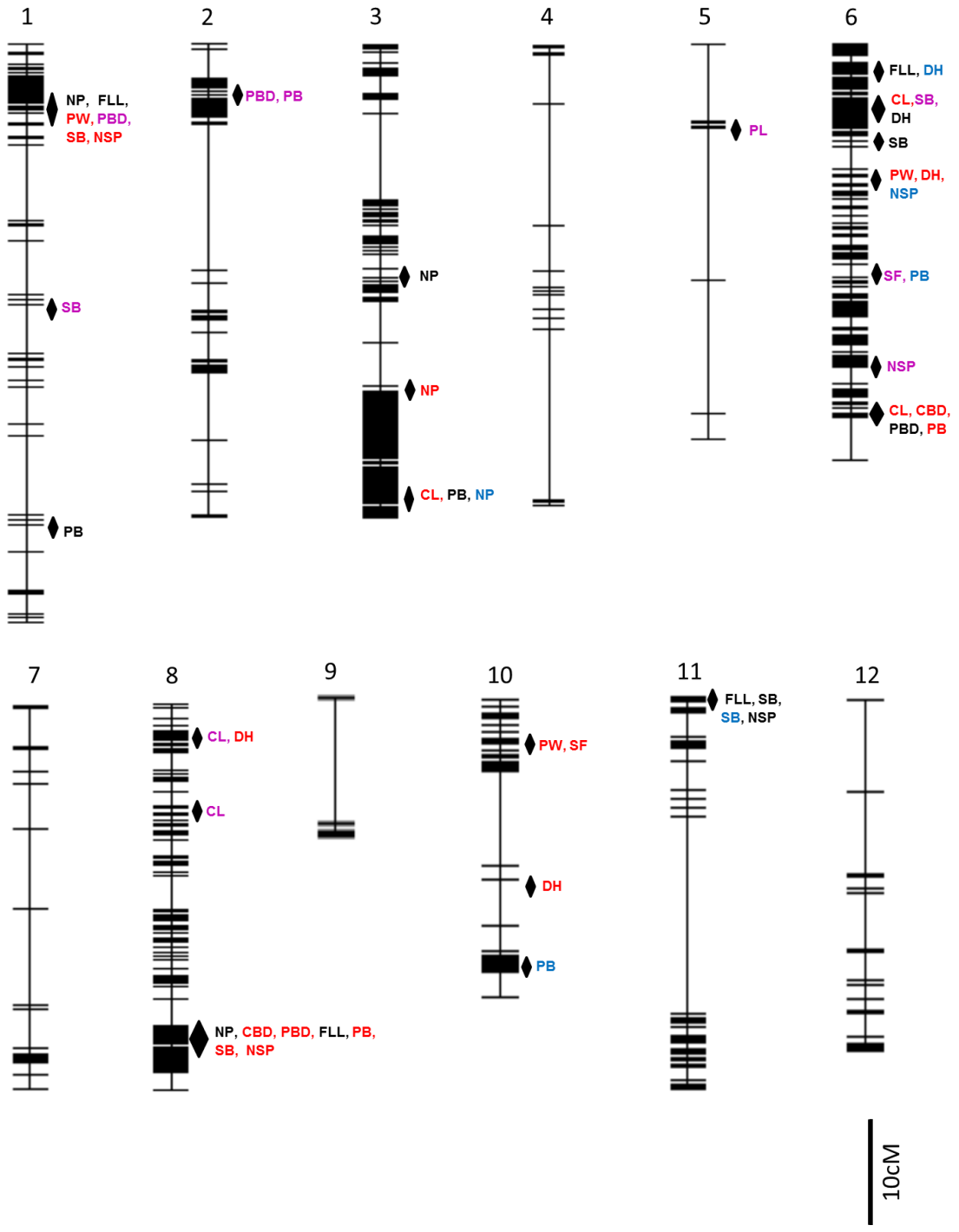


Figure 3.8. QTLs for yield-related traits identified in the RILs of the cross between pLIA-1 and Norin 18 under fertilized and non-fertilized conditions in 2015. The abbreviations represent the following traits: Culm length (CL), panicle length (PL), no. of panicles (NP), culm-base diameter (CBD), flag 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), and days to heading (DH). Diamond indicates tentative position of detected QTL on the chromosome. Black, purple and red, represent QTLs detected under fertilized conditions, non-fertilized conditions, and both conditions, respectively. QTLs identified for NF/F rate of a particular trait are shown in blue

3.4 Discussion

The final grain yield in rice is determined by many factors including environmental factors and the genotype. Fertilizer is one of the important environmental factors and plays a key role in increasing yields (Feng *et al*., 2015). In all the lines tested in this study, culm length, tiller number and biomass were the highest under high fertilizer conditions especially during the vegetative stages. This is because high fertilizer application stimulates shoot growth while low fertilizer stress results in stunted growth (Vinod and Heuer, 2012). However, responses to different levels of fertilizer differed among the 4 rice lines tested. Namai *et al*., (2009) observed similar results among 31 rice varieties at young vegetative stage. Although all the lines showed significant reduction in final biomass production when grown in half fertilizer conditions, the rate of reduction was lower in pLIA-1 and Norin 18. The pLIA-1 line developed for this study showed significantly increased number of primary branches, number of secondary branches and number of spikelets per panicle under the no fertilizer conditions. This results suggested that pLIA-1 might have high absorption rate or use efficiency of fertilizer for biomass production.

The interaction between the genotype and environment is an important factor affecting the expression of quantitative traits. In addition yield-related traits are quantitative traits that are highly influenced by environmental levels (Cho *et al*., 2007). Therefore to explore QTLs fertilizer responsiveness in pLIA-1, RILs developed from the cross between pLIA-1 and Norin 18 were grown under fertilized and non-fertilized conditions. Several studies to identify QTLs under different levels of nitrogen have been done in rice using indica and Japonica crosses (Cho *et al*., 2007; Tong *et al*., 2006; Tong *et a*l., 2011; Feng *et al*., 2015). Tong et al. (2006) reported that similar and specially expressed QTLs

under low and high nitrogen levels were identified in CSSLs derived from a cross between Teqing and Lemont rice varieties. In this study, 18 consistent and 31 differential QTLs were identified under the two conditions. These results were similar to previous studies thus suggesting the presence of interactions between QTLs for yield-related traits and fertilizer levels.

NUE is a complex quantitative trait. Recently, research in the identification of the QTLs associated with NUE to be utilized in breeding nitrogen efficient crop varieties has gained momentum (Feng *et al*., 2015). In the characterization of the DH lines developed in this study, the number of panicles per plant was identified as the most sensitive trait to low fertilizer levels. However, the NF/F rate in the number of panicles per plant differed among the DH lines. Therefore, QTL analysis was also done for the NF/F rate of each trait when grown under non-fertilized levels compared to fertilized conditions and a total of 6 QTLs were identified. The pLIA-1 allele at almost all of these QTLs increased the NF/F rate. In addition, 10 QTLs for 7 yield-related traits were only identified under non-fertilized conditions. These included QTLs for all traits except culm-base diameter, flag leaf length and panicle weight. Some QTLs identified only under non-fertilized conditions and QTLs for NF/F rate are therefore presumed to be associated with the ability to tolerate the low fertilizer stress in pLIA-1. Among the QTLs identified for NF/F rate, 2 QTLs for the NF/F rate in the number of primary branches and number of spikelets per panicle were identified on chromosome 6 in adjacent regions. Ishimaru *et al*., (2001) reported a QTL for soluble protein content at 25 days after heading around this region. Soluble protein content is related to rice nitrogen recycling (Feng *et al*., 2015). In addition 2 QTLs for the number of spikelets per panicle were identified under low N level by Feng *et al*. (2015) near the same region. On chromosome 3 a QTL

for NF/F rate for number of panicles per plant was detected. In previous reports, a QTL for NUE (Senthivel *et al*., 2008) and a QTL for number of panicles per plant under both high-N and low-N levels (Feng *et al*., 2015) were identified around this region. These results suggested that chromosomes 3 and 6 might carry key nitrogen metabolism genes in pLIA-1.

Overall, these results suggest that pLIA-1 line carries important putative QTLs for nitrogen efficiency or absorption under low fertilizer stress. These include QTLs that increased the NF/F rate of number of primary branches and spikelets per panicle and explains the significant increase observed in these traits under the no fertilizer conditions. These QTLs can be useful to breed rice varieties with improved nitrogen use or absorption efficiency for a sustainable environment.

CHAPTER 4

Introduction of high productivity of pLIA-1 into Basmati

4.1 Introduction

Through the green revolution, greater yield improvements among cereal crops including rice were obtained with the utilization of the semi-dwarf gene (*sd1*). The green revolution brought the high-yield crop varieties and high-input management practices currently utilized in agricultural systems, prevented mass starvation and improved living standards throughout the world (Brummer *et al*., 2011). However, these breakthroughs were not applicable to all commercial rice varieties. Basmati includes premium grade rice varieties that are very popular in the world. It is highly preferred among the consumers due to its characteristic fragrance, superfine long grains and excellent kernel elongation after cooking (Khush and Dela Cruz, 2002). It has a higher market price compared to other rice varieties. Hence, it is a preferred choice for production among farmers. However, it is tall, sensitive to photoperiod and susceptible to biotic stresses. It has low yields and is unresponsive to fertilizer; increased supply of nitrogen fertilizer does not translate into increase in yield (Jhang *et al*., 2006). At maturity, it is very susceptible to lodging due to thin weak stems. Attempts to improve Basmati's yield have been unsuccessful due to the complex nature of Basmati rice grain quality traits and poor combining ability with other rice genotypes (Khush and Dela Cruz, 1998). Therefore very few studies involving QTL analysis using Basmati crosses are available mainly focusing on the superior grain qualities of aroma (Li *et al*., 2004; Wan *et al*., 2006; Singh *et al*., 2012) and kernel elongation (Govindaraj *et al*., 2005). However, improving Basmati's yields is still highly important and requires extensively advanced genetics of key yield-related traits. Therefore, in this chapter the main aim was to improve Basmati's yields by introducing

the high productivity traits of pLIA-1 into Basmati. To improve the low yield of Basmati, the high number of spikelets per panicle and thick culm-base diameter of pLIA-1 line are considered to be useful. To achieve the above objective, we first crossed Kernel Basmati and pLIA-1 and performed QTL analysis in the F_2 plants. Second, we developed Longistaminata Chromosome Segments Introduction Lines (LCSILs) carrying pLIA-1 chromosome segments in Kernel Basmati's background.

4.2 Materials and methods

4.2.1 Plant materials and trait measurement

Kernel Basmati was crossed with pLIA-1 derived from F_2 of the cross between T-65 and MwM, *O. longistaminata* under non-fertilized conditions. Two F_2 populations of 55 and 80 plants of this cross were used for analysis of QTLs for several agronomic traits in 2012 and 2013, respectively. The plants were planted with a spacing of 40 cm between rows and 15 cm between plants under non-fertilized conditions at the Institute of Plant Science and Resources (IPSR), Okayama University, Japan. At heading and maturity, various agronomic traits were measured as described in Chapter 1.

4.2.2 QTL analysis

Leaf samples from seedlings of the F_2 plants were collected and dried at 50^{o}C overnight. The dried leaf samples were crushed by a Multi-beads shocker (YASUI KIKAI, Japan) and 400µl of extraction buffer (200 mM Tris-HCl (pH7.5), 250 mM NaCl, and 25 mM EDTA, 0.5% SDS) (Maekawa *et al*., 2005) was added to each sample and then strongly mixed using a microtube mixer (MT-360 Tomy) at maximum speed for 15 minutes. The mixture was centrifuged at 15000rpm at 4^{o}C for 10 minutes. After centrifugation, the supernatant transferred into a 1.5-ml tube was gently mixed with 300 µl of cold isopropanol. This mixture was centrifuged again at 15000rpm at 4^{o}C for 10 minutes. The pellet was rinsed with 500 µl of 70% EtOH. After centrifugation at 15000rpm at 4^{o}C for 5 minutes, the pellet was dried at room temperature and dissolved with 100 µl of TE.

Genotyping of the F_2 plants was conducted using 88 SSR markers distributed genome widely and found polymorphic between pLIA-1 and Kernel Basmati. The PCR reaction and electrophoresis were carried out as previously described in Chapter 2. A genetic

linkage map of 88 SSR markers was constructed using MAPMAKER 3.0. Composite Interval mapping (CIM) was performed in F_2 plants using the same procedure as described in Chapter 1.

4.2.3 Development of Longistaminata Chromosome Segments Introduced Lines (LCSILs)

The LCSILs were developed by crossing Kernel Basmati (♀) and pLIA-1 (♂) as the donor parent. The F_1 hybrids were backcrossed with Basmati to obtain the BC_1F_1 (22 plants). Successive backcrossing was then done to produce BC_2F_1 (24 plants) and BC_3F_1 (55 plants). Progenies of each backcross generation were genotyped using 85 SSR markers polymorphic between pLIA-1 and Basmati and evenly distributed across the 12 chromosomes. At the BC_3F_1 generation, backcrossed progenies carrying more than one target chromosome segments were selected and advanced by selfing to fix the introduced segments from the donor to the recurrent genome. Totally, 50 LCSILs derived from BC_3F_3, BC_3F_4 and BC_3F_5 were selected to comprise the final LCSILs set.

4.3 Results

4.3.1 Phenotypic variations

The parental plants, pLIA-1 and Kernel Basmati have tall statures and Kernel Basmati was much taller than pLIA-1 (Fig. 4.1a and 4.1b). Kernel Basmati is very susceptible to lodging at maturity. On the other hand, pLIA-1 is resistant to lodging due to a significantly thicker culm-base diameter compared to that of Kernel Basmati (Fig. 4.1b and 4.1d). In comparison to pLAI-1, Kernel Basmati's panicle was smaller in size and had few primary and secondary branches which consequently led to a significantly lower spikelet number per panicle (Fig. 4.1b and 4.1c). The above superior traits of pLIA-1 could be useful for improving Kernel Basmati's yield. Therefore, pLIA-1 was crossed with Kernel Basmati and the F_2 plants of this cross were evaluated for various agronomic traits and QTLs for yield-related traits that are important for improving Basmati's yield. Normal distribution patterns were observed in most of the traits measured in both years (Fig. 4.2 and 4.3). Transgressive segregants were observed in all of the traits recorded except for spikelet number per panicle and grain width in 2012 (Fig. 4.2) and secondary branch number and grain length in 2013 (Fig. 4.3). In both years, culm-base diameter and panicle-base diameter were significantly positively correlated to panicle length, primary branch number, spikelet number per panicle and to each other. Panicle length was significantly positively correlated to panicle-related traits (primary branch number, secondary branch number and spikelet number per panicle). The panicle-related traits were also strongly correlated to each other in both years (Tables 4.1 and 4.2).

a

pLIA-1 Kernel Basmati

b

Trait	pLIA-1 (S.E)	Kernel Basmati (S.E)
Culm length (cm)	94.3 (0.9)*	138.5 (0.8)
Panicle length (cm)	32.8 (0.7)*	25.0 (0.5)
Culm-base diameter (mm)	8.79 (0.1)*	6.72 (0.1)
Panicle-base diameter (mm)	2.73 (0.1)*	1.38 (0.0)
No. of primary branches	16.1 (0.4)*	11.1 (0.2)
No. of secondary branches	53.3 (1.9)*	3.3 (0.3)
No. of spikelets / panicle	251.8 (7.9)*	71.1 (1.8)

c

pLIA-1 Kernel Basmati

d

pLIA-1 Kernel Basmati

Figure 4.1. Morphological characteristics of pLIA-1 and Kernel Basmati. (a) pLIA-1 and Kernel Basmati at maturity. (b) Agronomic performance of pLIA-1 and Kernel Basmati. * indicates significant difference at the 5% level by *t*-test. (c) Panicles of pLIA-1 and Kernel Basmati. Bar=10 cm (d) Culm-base thickness of pLIA-1 and Kernel Basmati.

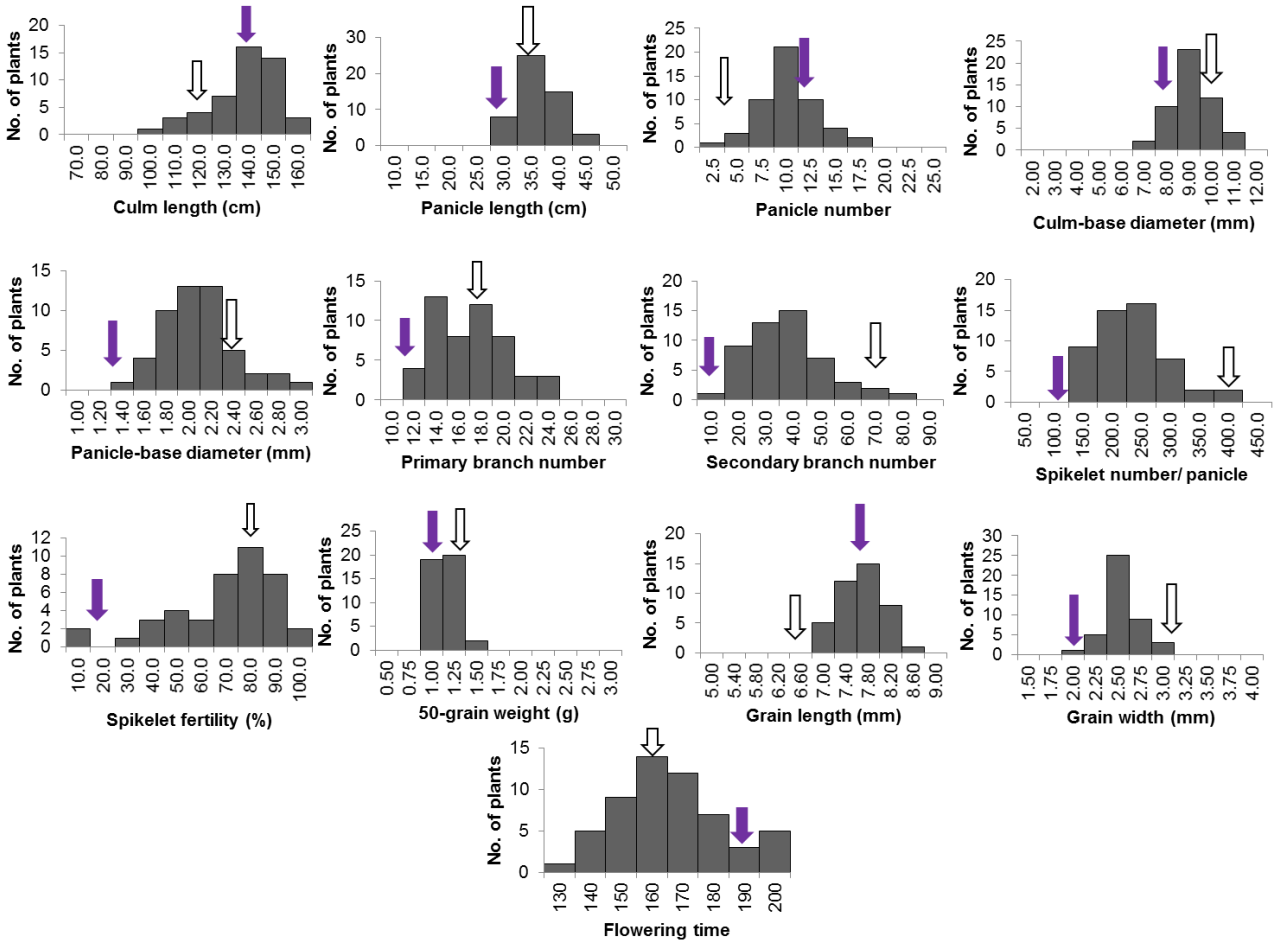


Figure 4.2. Frequency distribution of agronomic traits in F_2 of the cross between pLIA-1 and Kernel Basmati grown in 2012. White and purple arrows indicate mean values of pLIA-1 and Kernel Basmati, respectively.

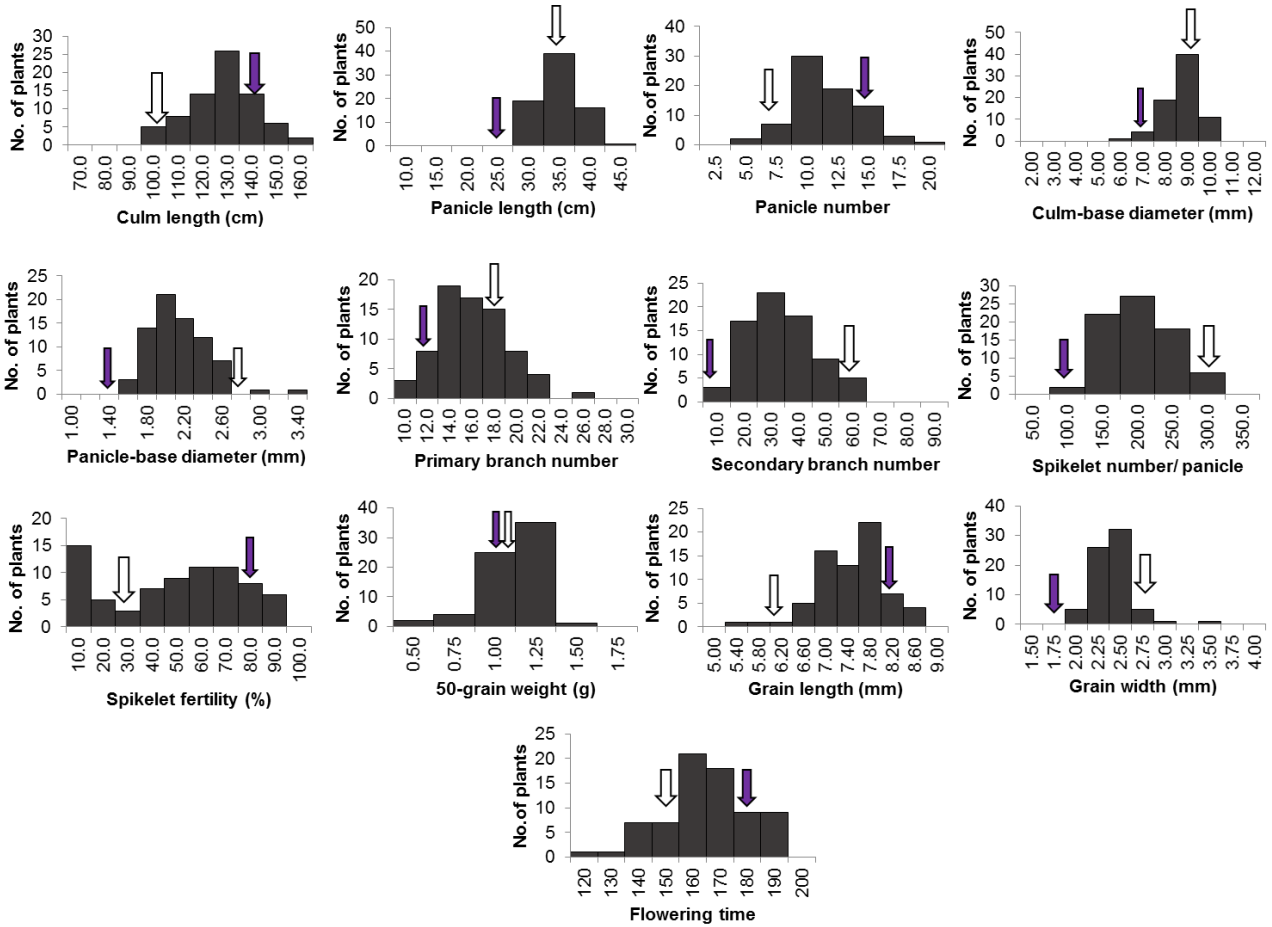


Figure 4.3. Frequency distribution of agronomic traits in F_2 of the cross between pLIA-1 and Kernel Basmati grown in 2013. White and purple arrows indicate mean values of pLIA-1 and Kernel Basmati, respectively.

Table 4.1. Correlation coefficients of yield-related traits in F_2 of the cross between pLIA-1 and Kernel basmati in 2012

	Culm length (cm)	Panicle length (cm)	No. of Panicles	Culm-base diameter (mm)	Panicle base diameter (mm)	Flag leaf length (cm)	No. of primary branches	No. of secondary branches	No. of spikelets/ panicle	Spikelet fertility (%)	50-grains weight (g)	Grain length (mm)	Grain width (mm)
Panicle length (cm)	0.334*												
No. of Panicles	0.157	-0.068											
Culm-base diameter (mm)	0.186	0.329*	0.051										
Panicle base diameter (mm)	0.147	0.466*	0.009	0.663*									
Flag leaf length (cm)	-0.015	0.063	0.013	0.136	0.180								
No. of primary branches	0.142	0.295*	0.021	0.542*	0.606*	0.203							
No. of secondary branches	0.148	0.393*	-0.116	0.430*	0.674*	0.291*	0.637*						
No. of spikelets/ panicle	0.170	0.406*	-0.058	0.493*	0.716*	0.245	0.753*	0.967*					
Spikelet fertility (%)	-0.075	0.049	-0.023	0.089	0.114	0.031	0.110	0.035	0.052				
50-grains weight (g)	0.002	-0.028	-0.073	0.210	0.038	-0.194	0.120	0.092	0.103	0.083			
Grain length (mm)	0.050	-0.063	-0.017	0.139	-0.076	0.023	-0.003	0.183	0.139	-0.031	0.562*		
Grain width (mm)	-0.014	-0.039	-0.071	0.100	-0.011	-0.186	-0.045	-0.175	-0.133	0.084	0.737*	0.030	
Days to heading	-0.295*	-0.595*	0.043	-0.048	-0.200	0.024	-0.394*	-0.389*	-0.445*	-0.090	-0.096	0.050	-0.002

* indicates significant at the 5% level.

Table 4.2. Correlation coefficients of yield-related traits in F_2 of the cross between pLIA-1 and Kernel basmati in 2013

	Culm length (cm)	Panicle length (cm)	No. of Panicles	Culm-base diameter (mm)	Panicle base diameter (mm)	Flag leaf length (cm)	No. of primary branches	No. of secondary branches	No. of spikelets/ panicle	Spikelet fertility (%)	50-grains weight (g)	Grain length (mm)	Grain width (mm)
Panicle length (cm)	0.025												
No. of Panicles	0.339*	0.139											
Culm-base diameter (mm)	-0.020	0.276*	-0.012										
Panicle base diameter (mm)	-0.109	0.449*	-0.110	0.453*									
Flag leaf length (cm)	0.105	0.200	-0.197	-0.099	0.234*								
No. of primary branches	-0.155	0.275*	0.025	0.349*	0.657*	0.151							
No. of secondary branches	0.068	0.328*	-0.048	0.211	0.757*	0.257*	0.455*						
No. of spikelets/ panicle	-0.008	0.390*	-0.020	0.319*	0.839*	0.222*	0.677*	0.943*					
Spikelet fertility (%)	0.534*	0.103	0.281*	-0.128	-0.027	0.024	-0.039	-0.015	-0.016				
50-grains weight (g)	0.183	0.154	0.123	-0.037	-0.043	-0.045	0.097	0.067	0.039	0.021			
Grain length (mm)	0.152	0.045	0.040	0.080	0.076	-0.115	0.029	0.214	0.177	-0.001	0.429*		
Grain width (mm)	-0.006	0.099	-0.100	-0.007	0.030	0.251*	0.145	-0.076	-0.019	0.064	0.021	-0.387*	
Days to heading	-0.108	-0.147	0.010	0.142	-0.059	-0.354*	-0.139	-0.078	-0.113	-0.049	-0.106	0.028	-0.258*

* indicates significant at the 5% level.

4.3.2 QTLs for yield-related traits

Polymorphisms using 115 SSR markers distributed genome-widely were checked between pLIA-1 and Basmati. Among the 115 markers used, 88 markers were polymorphic. QTL analysis was conducted for the agronomic traits measured in the F_2 of the cross between pLIA-1 and Basmati and identified 5 QTLs for 6 traits in 2012 on chromosomes 1, 6, 8 and 11 (Table 4.3) and 16 QTLs for 10 traits in 2013 on chromosomes 1, 2, 3, 4, 6, 7, 8, 11 and 12 (Table 4.3). The pLIA-1 allele improved trait performance in 5 and 8 of the QTLs identified in 2012 and 2013, respectively (Table 4.3).

One QTL for culm length was identified in 2013 on chromosome 8. The Basmati allele for this QTL increased culm length (Table 4.3). Two QTLs for number of panicles per plant were identified in 2013 on chromosomes 2 and 3. The pLIA-1 allele for the QTL on chromosome 2 increased the trait, however, the same allele decreased the number of panicles per plant for the QTL on chromosome 3 (Table 4.3). Two QTLs for culm-base diameter were mapped on chromosomes 4 and 11 in 2013. The pLIA-1 allele for both QTLs increased culm-base diameter (Table 4.3). Two QTLs for panicle-base diameter was identified on chromosomes 1 and 11 in 2012. The pLIA-1 allele for both QTLs had a positive contribution to the trait. The QTL on chromosome 1 had the highest contribution to the total phenotypic variance (Table 4.3). Totally, two QTLs for number of primary branches were detected one in each year. These QTLs were detected in similar locations in both years on chromosome 8 near RM3634 (Fig. 4.4). The pLIA-1 allele for both QTLs increased the number of primary branches. These QTLs had 37% and 32% contributions to the total phenotypic variance in 2012 and 2013, respectively (Table 4.3). One QTL for number of secondary branches was identified on chromosome 1 in 2013. The pLIA-1 allele for this QTL increased the number of primary branches (Table 4.3).

Two QTL for number of spikelets per panicle was detected in 2013 on chromosomes 1 and 7. Positive alleles for the QTL on chromosome 1 and 7 were contributed by pLIA-1 and Kernel Basmati, respectively. The QTL on chromosome 1 showed the highest LOD score (Table 4.3). Totally, two QTLs for days to heading were identified, one in 2012 and the other in 2013. The QTLs were identified in a similar location on chromosome 6 near RM190 (Fig. 4.4). The pLIA-1 allele for both of the detected QTLs shortened the time to heading (Table 4.3). A single QTL for spikelet fertility was identified in 2012 on chromosome 6. The pLIA-1 allele for this QTL decreased the number of fertile spikelets (Table 4.3). One QTL for 50-grains weight was identified in 2013 on chromosome 6. The pLIA-1 allele for this QTL increased the grain weight (Table 4.3). Totally, four QTLs for grain length were identified in both years. Three were identified in 2013 on chromosomes 3 and 6 and one in 2012 on chromosome 6. Positive alleles for two of the QTLs on chromosome 6 were contributed by pLIA-1, while Basmati contributed the positive alleles for the other two QTLs (Table 4.3). Two QTLs for grain width were detected on chromosomes 1 and 12 in 2013. The pLIA-1 allele for both QTLs was responsible for reduced grain width (Table 4.3).

QTLs for strongly correlated traits were observed to be localized near the same region on the chromosomes. These clusters of QTLs were observed on chromosomes 1, 6 and 8 (Fig. 4.4). The QTL cluster on chromosome 1 carried crucial QTLs for number of secondary branches and number of spikelets per panicle which are important parameters for rice yield.

Table 4.3. QTLs for yield-related traits identified in F_2 of the cross between pLIA-1 and Kernel Basmati in 2012 and 2013

Trait	Year	Chr.	Marker	Position (cM)	LOD	Additive effect	Dominance effect	r^2
Culm length (cm)	2013	8	RM3634	+0.0	5.2	-9.04	4.98	0.22
No. of panicles	2013	2	RM1385	+0.0	3.5	0.94	1.79	0.14
		3	RM55	+48.0	4.1	-1.46	1.15	0.20
Culm-base diameter (mm)	2013	4	RM252	+21.0	4.4	0.49	0.08	0.21
		11	RM286	+18.0	4.0	0.17	0.54	0.13
Panicle-base diameter (mm)	2012	1	RM23	+36.0	4.0	0.32	0.05	0.37
		11	RM21	+0.0	3.8	0.10	-0.31	0.20
No. of primary branches	2012	8	RM3634	+18.0	4.2	2.85	0.97	0.37
	2013	8	RM3634	+0.0	7.7	2.52	-0.39	0.32
No. of secondary branches	2013	1	RM6324	+27.0	5.7	14.28	-0.36	0.65
No. of spikelets/ panicle	2013	1	RM1331	+0.0	5.6	31.01	16.28	0.24
		7	LM7-2	+21.0	4.5	-35.05	-40.33	0.40
Spikelet fertility (%)	2012	6	LM6-7	+8.0	6.1	-26.09	14.02	0.50
50-grain weight (g)	2013	6	RM204	+0.0	11.5	0.17	0.14	0.48
Grain length (mm)	2012	6	P06	+16.0	5.2	0.42	0.06	0.49
	2013	3	RM231	+15.0	3.5	-0.32	0.22	0.14
		6	RM3	+18.0	4.9	-0.57	0.14	0.36
		6	RM204	+0.0	3.7	0.31	0.23	0.14
Grain width (mm)	2013	1	RM9	+21.0	6.4	-0.54	-0.57	0.33
		12	RM27970	+3.0	8.7	-0.54	-0.57	0.33
Flowering time	2012	6	RM190	+26.0	8.9	-14.88	-7.08	0.46
	2013	6	RM190	+3.0	12.8	-17.28	-8.66	0.50

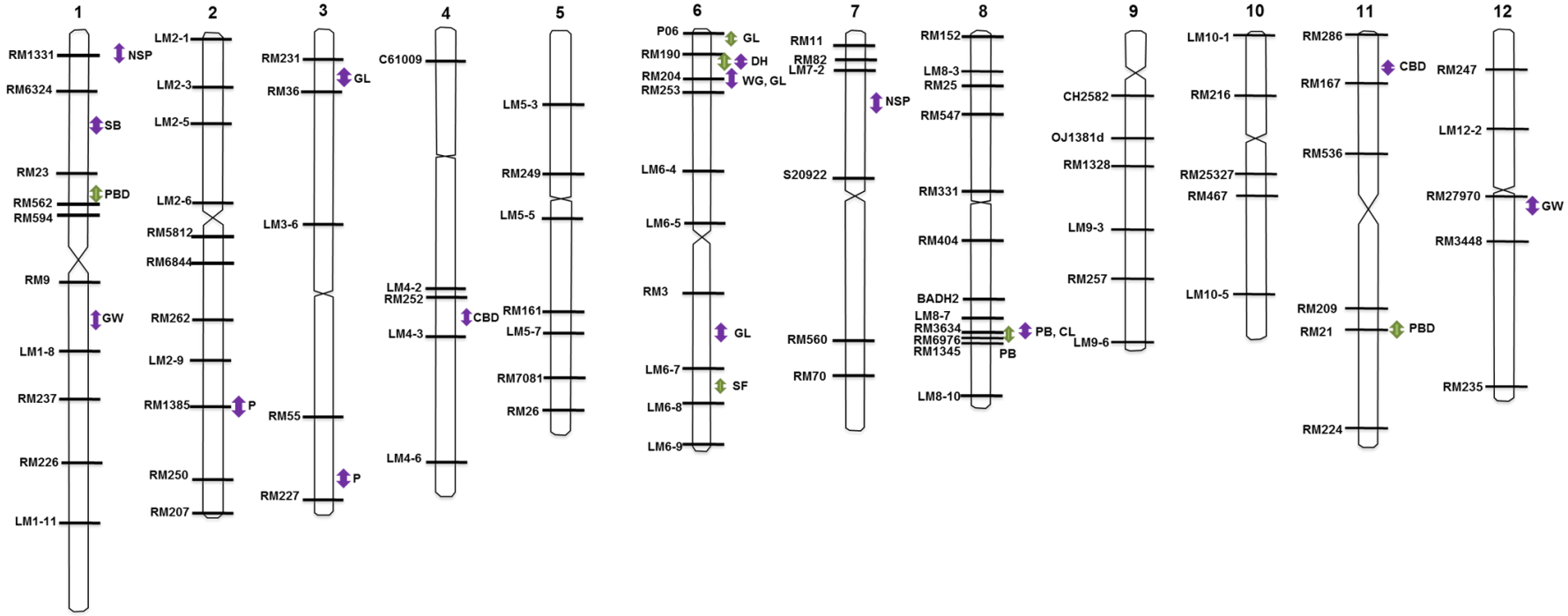


Figure 4.4. QTLs for the yield-related traits identified in F_2 of the cross between pLIA-1 and Kernel Basmati. CL; culm length. P; no. of panicles. CBD; culm-base diameter. PBD; panicle-base diameter. PB; no. of primary branches. SB; no. of secondary branches. NSP; no. of spikelets per panicle. SF; spikelet fertility. WG; 50-grain weight. GL; grain length. GW; grain width. DH; days to heading. Two headed green and purple arrows indicate tentative position of QTLs detected in 2012 and 2013, respectively.

4.3.3 LCSILs developed

A total of 50 LCSILs carrying pLIA-1 chromosome segments in Kernel Basmati background were developed as shown in Fig. 4.5. The donor genome was represented with a frequency of about 90% in the LCSILs set, based on the markers used. Chromosomes 1 and 5 of pLIA-1 were introduced in 7 and 6 LCSILs, respectively. Chromosomes 2, 4, 6, 7, 8, and 10 were each introduced in 4-5 LCSILs whereas chromosomes 3, 9, 11, and 12 were each carried by 2-3 LCSILs. In the LCSILs set, 8 chromosome segments of pLIA-1 distributed on chromosomes 3, 7, 8, and 9 were not introduced because of non-polymorphic markers.

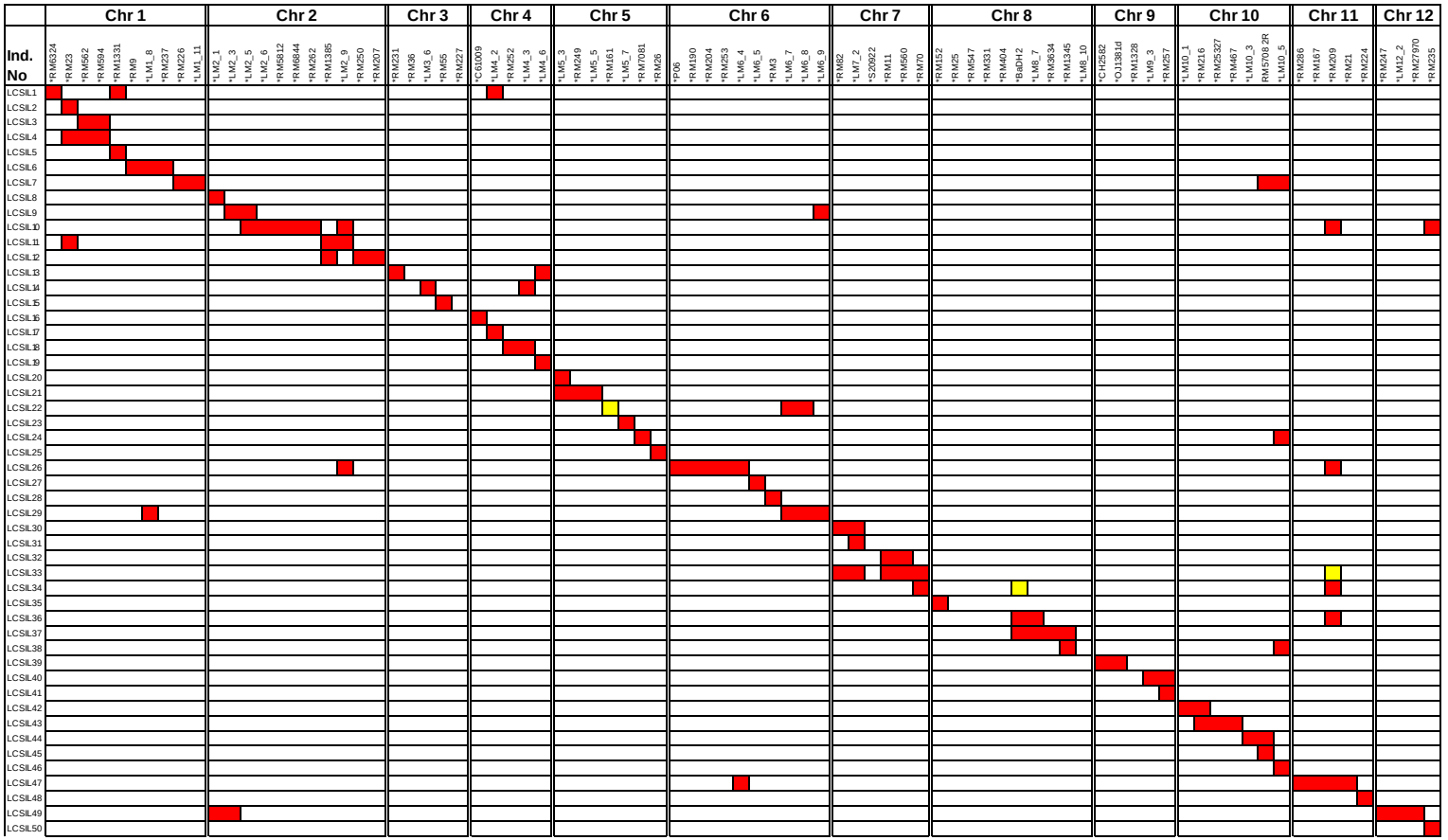


Figure 4.5. Longistaminata Chromosome Segment Introgression lines (LCSILs) carrying pLIA-1 chromosome segments in Kernel Basmati background. White, red and yellow represent genotypes of Kernel Basmati, pLIA-1 and heterozygous, respectively.

4.4 Discussion

Introgression of favorable alleles from wild relatives of *Oryza sativa* to extend its genetic diversities has been attracting increasing attention recently due to a narrow genetic diversity of modern varieties (Fu *et al*., 2010). However, attempts to transfer genes controlling quantitative traits from wild relatives to cultivated elite varieties of rice have been limited and unsuccessful. This is because such remote crosses usually bring about reproductive barriers such as hybrid sterility and most importantly linkage drag related problems. This makes it difficult to select and use superior phenotypes for breeding purposes (Brondani *et al*., 2002). Hence, introgression lines carrying wild rice relative's genome are usually developed for use in breeding programs. In comparison to Kernel Basmati, the pLIA-1 line was observed to possess superior culm thickness and large panicle size traits under non-fertilized conditions. By using a cross between the *Oryza longistaminata* introgression line (pLIA-1) and Kernel Basmati 21 QTLs controlling various yield-related traits were identified. In more than 50% of the QTLs identified in both years, the pLIA-1 allele had a positive contribution to the trait. In 2012, the F_2 population was smaller than that in 2013. Thus, fewer QTLs were presumed to be identified in 2012. Utilizing a wild rice introgression line overcame the crossing barrier and reduced hybrid sterility problems usually observed when using wild rice relatives (Chu and Oka, 1970). In the F_2 population, many transgressive segregants were observed in most of the traits measured. One of the main advantages of the remote crosses is to positively extend the genetic variation. Transgressive segregation is an important evidence of the favorable effect of such introgressions (Brondani *et al*., 2002).

Although Basmati rice is a favorite cultivar among the aromatic rice cultivars, it has poor yields especially due to its susceptibility to various biotic and abiotic stresses (Singh *et al*., 2012) and high incidences of lodging at maturity due to weak thin culms. Therefore, the thick culm of pLIA-1 is important for conferring higher lodging resistance to Kernel Basmati. Additionally, pLIA-1 is characterized by a large panicle with many primary and secondary branches and large number of spikelets per panicle which are important parameters for high yields. Among the QTLs identified, the QTL for number of primary branches per panicle was identified in similar locations on chromosome 8 in both years. This suggests that this could be a major QTL. Previously a QTL (*WFP*) encoding *OsSPL14* gene was identified near the same location and was found to regulate panicle branching (Miura *et al*., 2010). Large panicles containing a high number of primary branches is important for increasing rice yields. The QTL for days to heading was also identified in similar locations in both populations. Near this region QTLs for heading date have been identified in various mapping population involving both *Oryza sativa* varieties and wild rice. This QTL region is near the *Hd3a* and *RFT1* genes that control flowering in rice (Ogiso-Tanaka *et al*., 2013). QTL clusters were detected on chromosomes 1, 6, and 8. The QTL cluster on chromosome 1 included QTLs for number of secondary branches and number of spikelets per panicle. A very strong positive correlation was observed between these two traits. It is therefore possible that this cluster region locates a pleiotropic QTL. Overall, these results suggested that strong genetic variation exists between pLIA-1 and Kernel Basmati. Therefore, it is possible to improve Kernel Basmati by introducing the QTLs for traits governing the high productivity of pLIA-1.

Improving Kernel Basmati's yield is important since breeding high yielding varieties carrying Basmati's grain qualities has been unsuccessful (Vemireddy *et al*., 2015). Therefore, important QTLs for yield-related traits that lead to high yields should be introduced into Kernel Basmati. The QTLs for thick culms and large panicles identified in this study can be utilized to improve Kernel Basmati. Through QTL pyramiding it is possible to introduce these QTLs into Kernel Basmati to breed higher yielding Kernel Basmati cultivars that are resistant to lodging. Thus, we have been developing Longistaminata Chromosome Segment Introduced Lines (LCSILs) in Kernel Basmati background as shown in Fig. 4.5, to be used in fine mapping the location of the individual QTLs important to improve Kernel Basmati. The promising plant will be used to introduce the thick culm and large panicle traits into Kernel Basmati.

General Discussion and conclusion

Wild plant species are important reservoirs of the genetic diversity necessary to improve tolerance to biotic and abiotic stress as well as yield potential of modern cultivars. *Oryza longistaminata,* a wild species of rice, has shown tremendous usefulness in conferring biotic stress tolerance in rice (Khush *et al*., 1990; Song *et al*., 1995). However, information on *O. longistaminata*'s utilization in improving the agronomic traits of rice is lacking. This is because it is difficult to obtain crossed seeds between *O. longistaminata* and *O. sativa* (Chu and Oka, 1970) and even if crossed seeds could be obtained, the F_1 shows severe hybrid sterility (Chen *et al*., 2009). In order to utilize *O. longistaminata* as a genetic resource it is important to develop a progeny derived from a cross between *O. longistaminata* and *O. sativa*. Iwamoto et al. (1998) pointed out that *O. longistaminata* could easily be crossed with *O. sativa*. In fact, *O. longistaminata* from Kenya was able to successfully produce crossed seeds with *O. sativa* and the progeny of the cross was developed through self-fertilized and selection at a non-fertilized paddy field (Gichuhi *et al*., 2016). On the other hand, Ramos *et al*. (2016) successfully bred CSSLs of *O. longistaminata* in *O. sativa* cv. Taichung 65 background. This study aims to investigate the potential of *O. longistaminata* for improving yield-related traits in *O. sativa* especially under poor nutrient conditions using a potential low input adaptable line (pLIA-1) carrying segments of *Oryza longistaminata* (Gichuhi *et al*., 2016).

To reveal important QTLs for yield-related traits under low-input conditions, the RAD-Seq method was applied in analyzing RILs and DHLs developed from the cross between pLIA-1 and Norin 18. The RAD-Seq method is a very powerful tool and is useful for SNP detection (Baird *et al*., 2008) even in materials having highly rearranged sequences.

In fact, 1989 SNPs were detected between pLIA-1 and Norin 18 by RAD-Seq method and high-density SNPs regions were observed on chromosomes 1, 2, 3, 6, 8, 10, and 11. These high-density SNPs regions were comparable to the *O. longistaminata* chromosome segments revealed by SSR marker analysis (Gichuhi *et al*., 2016).

Several QTLs for yield related traits were identified in RILs and were found to be distributed in all chromosomes except for chromosome 9. Majority of the QTLs were detected on chromosome regions where *O. longistaminata* chromosome segments were introgressed into pLIA-1. Distal regions of chromosomes 1 and 8 were in particular QTL hot spots in RILs. Traits for which QTLs were detected in these two regions consisted of panicle-related traits and culm thickness traits. Therefore, it was suggested that these regions carry important genetic factors controlling panicle size and strong culms. Further, using backcrossed populations with Koshihikari and Norin 18, these regions were found to locate genetic factors for the number of secondary branches on chromosome 1 and culm-base diameter and number of primary branches on chromosome 8. In previous reports, QTLs for the number of primary branches have also been reported in this region (Miura *et al*., 2010; Jiao *et al*., 2010; Ando *et al*., 2008). However, this is the first time that a QTL for culm-base diameter has been reported in this region. Culm thickness is important for tolerance to lodging and to support a large panicle with many primary and secondary branches at maturity. Therefore, it is likely that these two QTLs were localized in the same region on chromosome 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 while a combination of pLIA-1 genotypes on chromosomes 1 and 8 showed a favorable phenotype under non-fertilized

conditions. Interestingly, these two QTL clusters were identified under both fertilized and non-fertilized conditions. Thus these QTL clusters are presumed to be stable QTLs and are not regulated by fertilizer levels.

In addition to the consistent QTLs identified under both fertilized and non-fertilized conditions, differentially expressed QTLs were also identified. Among these, the QTLs expressed under non-fertilized conditions are of importance as they are a reflection of differential expression of genes in pLIA-1 under low fertilizer environments compared to fertilized conditions. QTLs for NF/F rate were also detected in 5 traits. All the QTLs except one, were derived from pLIA-1 and contributed to small reductions of the trait values thereby indicating that pLIA-1 carries QTLs for low fertilizer tolerance.

One of the main goals in QTL mapping is to identify loci controlling traits of economic importance that can further be dissected and studied and eventually be utilized for improving agronomic traits such as yield (Swamy and Sarla, 2008). Several promising QTLs were identified in this study and were consistently mapped at the same chromosomal location. These QTLs can therefore be introgressed using marker assisted selection to improve commercial rice varieties. Kernel Basmati is a popular variety with very low yields mainly due to the small panicle size. Therefore, it was selected as a target for improvement by introducing the superior traits of pLIA-1 into it. To improve the low yield of Kernel Basmati, the large panicle of pLIA-1 and thick culm-base diameter is considered to be useful. QTL analysis in F_2 of the cross between pLIA-1 and Kernel Basmati provided useful information for improvement of the low yield of Kernel Basmati. Hence, Longistaminata Chromosome Segment Introduced Lines were developed through backcrossing the F_1 of the cross between pLIA-1 and Kernel Basmati with Kernel

Basmati. Promising lines from this set will be used for breeding a higher yielding Kernel Basmati.

Overall, the results presented in this study show that *O. longistaminata* has great potential to be utilized in breeding programs of rice. *O. longistaminata's* chromosome segments carry information on important alleles that could be utilized for improvement of yield-related traits. Ramos *et al*. (2016) also produced CSSLs that carry *O. longistaminata* chromosome segments in Taichung-65 background. 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 an important step towards further rice improvement while conserving the environment.

Prospects

To break the stagnant crop yields with sustainable management of cultivation practices, the utilization of wild species as important genetic resources is inevitable. However, since highly remote species develop several reproductive barriers such as hybrid weakness and hybrid sterility, it is difficult to directly utilize the wild species for practical breeding. In this study, *O. longistaminata* native to the central region of Africa was utilized in the development of pLIA-1, the selfed progeny of the cross between *O. longistaminata* and T-65, and several important QTLs for yield-related traits in pLIA-1 were detected under non-fertilized conditions. Further study of these QTLs through dissection and identifying the genes underlying them is necessary especially for resolving linkage drag. Cloning and functional characterization of these genes/QTLs will greatly strengthen our understanding of the genetic and molecular mechanisms underlying the yield-related traits governed by these QTLs under low-input conditions. This information will facilitate the breeding efforts for higher yield potential rice varieties through pyramiding of favorable QTLs under low-input conditions.

In order to utilize the superior traits of pLIA-1 to improve Kernel Basmati, LCSILs were developed. Characterization of these lines will be needed for utilization of the desirable traits to improve Kernel Basmati's yield. The selected lines will then be used to pyramid the traits into a single plant. The LCSILs are also suitable materials for precise genetic studies of the chromosome segments derived from pLIA-1, including the evaluation of gene effects, selection of enhanced molecular markers that are tightly linked with the target gene, gene expression and gene isolation.

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