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Identification of Simple Sequence Repeat (SSR) Markers for Acid Detergent Fiber in Rice Straw by Bulked Segregant Analysis

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Rice straw is a significant energy source for ruminant animals. The acid detergent fiber (ADF) content of rice straw is negatively related to intake potential of forages. Therefore, improving the digestibility of rice straw by reducing ADF content is a necessary goal in breeding programs. In the present study, simple sequence repeat (SSR) markers and the bulked segregant analysis (BSA) approach were used to identify molecular markers associated with ADF. A total of 121 BC_1F_1 plants derived from the cross of JX974 (a cultivar with high ADF, 36.6%) and Dongxiang wild rice (a wild rice with low ADF, 31.3%), with JX974 as a recurrent parent, were used to conduct BSA. Phenotypic analysis showed that ADF displayed a normal distribution in BC_1F_1 population, indicating the involvement of polygenes. A SSR marker, RM566 on chromosome 9, was identified for ADF. A small linkage map consisting of five markers was constructed by adding four other markers, and a quantitative trait locus (QTL) controlling ADF was mapped at the RM321−RM566 interval, with a distance of 3.9 cM to RM566. This QTL explained 12% of the total phenotypic variation of ADF, and its additive effect was 3%. This study is the first step to map QTL for ADF, one of the plant cell wall components in rice.

KEYWORDS: SSR markers; rice straw; acid detergent fiber (ADF); bulked segregant analysis

INTRODUCTION

Plant cell walls are major energy sources for ruminants, playing a major role in forage utilization (*1*−*3*). The plant cell wall components (CWCs) are mostly composed of cellulose, hemicelluloses, and lignins, which can be indirectly quantified as acid detergent fiber (ADF), neutral detergent fiber (NDF), and acid detergent lignin (ADL) (*4*). NDF includes all three components, and lignins are quantified as ADL. Hemicelluloses are estimated as the difference between NDF and ADF, and cellulose is estimated as the difference between ADF and ADL. ADF, NDF, and ADL have been established as important and good indicators of forage quality because they are negatively correlated with digestibility for livestock animals (*5*−*7*). In addition to digestibility, the plant CWCs may be responsible for stalk strength and resistance to insects in maize (*8*, *9*).

Rice straw makes up 50% of the dry weight of rice plants (*10*). Instead of returning it to the field as manure, rice straw can be used for various other applications (*6*, *10*). One of the most important applications is to utilize it as a source of carbohydrates for ruminants (*5*). However, it has high lignin and low nitrogen contents, which in combination are responsible for its low nutritive value. Thus, it is necessary to decrease the content of lignin to improve feed quality (*5*). For this purpose, it is necessary to understand the genetic basis of CWC in rice straw.

A number of studies showed that plant ADF, NDF, and ADL were positively correlated (*4*, *11*−*14*). ADF and NDF was consistently positively correlated in maize and barley (*4*, *11*, *13*, *14*), whereas the relationships with ADL varied in different studies (*4*, *11*−*13*). Cardinal et al. (*11*) reported that the relationships between ADF and NDF and ADL were positive, whereas Krakowasky et al. (*4*, *13*) and Mechin et al. (*15*) reported they had no relationship. In maize, however, it was found that a number of quantitative trait loci (QTLs) for ADF, NDF, and ADL were located at the same or linked marker loci no matter whether they were correlated or not (*3*, *4*, *11*, *13*). The candidate genes for cellulose and lignin biosynthesis have

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Table 1. Primer Sequences for the Five InDel Markers (*23*)

marker	chromosome	forward primer (5′ to 3′)	reverse primer (5′ to 3′)
Indel9-1	9	CTGGTTAACGTGAGAGCTCG	GCAGATCAATTGGGGAGTAC
Indel9-2	9	ACTGCTTTGATGGCTTGTG	CTCCCCAAACTGAATCC
Indel9-3	9	CTCACCTACCTAAAACCCAAC	CCACCCAAATCTGATACTG
Indel9-4	9	CTTTGGATTCAGGGGGA	AACTTGAAACGGAGGCAG
Indel9-5	9	CTATAAGACCAAAACGAAAACT	GAAAACCATTGTGTCACTGTA

been identified for some QTLs (*3*, *4*, *11*, *13*). These studies demonstrated that it is possible to select one fiber component to improve forage digestibility (*11*, *16*). For rice, it is found that ADF, NDF, and ADL were positively correlated (*12*). Analysis of the genetic control of CWC in rice will contribute to the rice breeding effort for the improvement of forage quality. Furthermore, mapping of QTLs for CWC may help to identify the candidate genes in relation to cellulose and lignin biosynthesis as demonstrated in maize (reviewed in ref *3*). However, no reports of the identification of QTLs for CWC have been reported in rice.

Bulked segregant analysis (BSA) is a rapid procedure for identifying markers in specific regions of the genome associated with a target trait (*17*). The method involves comparing two pooled DNA samples of individuals from a segregating population originating from a single cross. Within each pool, the individuals are identical for the trait or genome region of interest but are arbitrary at all unlinked regions. The procedure is rapid and requires only small amounts of DNA. BSA was initially proposed for screening qualitative traits at a single locus of large effect (*17*−*19*). With its simplicity and low cost, BSA has been used extensively as a first step for more complex traits (*20*).

Any segregating population originating from a single cross can be used to construct the bulks and conduct BSA. Backcross population may more easily detect QTL than the F_2 population (*19*, *20*), especially in case of the codominant markers used (*20*).

In this study, 121 BC_1F_1 plants derived from a backcross of JX974 and Dongxiang wild rice (*Oryza rufipogon*) were used to detect QTL controlling ADF using the BSA method. This is the first attempt to understand the genetic control of CWC in rice straw.

MATERIALS AND METHODS

Rice Materials. A population of BC_1F_1 was derived from the cross of JX974 (*Oryza sativa* L. ssp. *indica*) and Dongxiang wild rice (DWR) (*O. rufipogon*) with JX974 as a recurrent parent. A total of 121 BC_1F_1 plants and the parents were grown in 2003 at the experiment farm of the Rice Research Institute, Jiangxi Academy of Agricultural Sciences, China. The field management followed conventional practices, and all of the rice materials were harvested on time when the grains were mature.

ADF Analysis. The ADF content of straws was measured on each of 121 BC_1F_1 plant and their parents. Straws were taken only from the stems bearing an effective spike of each plant. They were first washed to remove soil from the base and then dried in a forced-air oven for 24 h at 60 °C. The sheath and leaves were removed, and only four nodes from the base up were collected. The samples were ground to pass a 1 mm sieve, then they were scanned using near-infrared reflectance spectroscopy (Foss-NIR System, Inc., Silver Spring, MD), and ADF was calculated with prediction equations developed by Kong et al. (*6*).

DNA Extraction. Roughly 5 g of fresh leaf from a single plant was collected for DNA extraction using the CTAB method (*21*).

Molecular Markers and BSA. To identify markers associated with ADF, the BSA approach was used following the method described in Michelmore et al. (*17*). Equal amounts of DNA from 30 plants with ADF <25% and another 30 individuals with ADF >33% were pooled to constitute the low ADF bulk (B1) and the high ADF bulk (B2), respectively. The two bulked pools along with parents were then subjected to molecular marker analysis. Three hundred and eighty-seven simple sequence repeat (SSR) markers were used to detect the polymorphism between the parents (*22*).

PCR Amplification and Detection. Each 20 μL amplification reaction consisted of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 0.1 mM dNTPs, 200 nM primers, 1 unit of Taq polymerase, and 100 ng of genomic DNA. All amplifications were performed on a PCR Express Thermal Cycler (Hybaid Limited, Middlesex, U.K.) under the following conditions: 5 min at 94 °C, followed by 60 s at 94 °C, 60 s at 55−60 °C (dependent on primers), and 60 s at 72 °C for 35 cycles, and 7 min at 72 °C for a final extension. The amplified products were analyzed by 2.5% agarose gel electrophoresis.

Linkage Map Construction and QTL Mapping. For construction of a small linkage map of chromosome 9, five insertion/deletion (InDel) markers designed by Shen et al. (*23*) and designated by an "Indel" prefix in this study were used. The linkage map of chromosome 9 was constructed with five markers using the Map manage QTXb20 program (*24*). Linkage was considered to be significant if $P < 0.0001$. The Kosambi mapping function was used to convert recombination frequency into map distances in centimorgans (cM). Interval mapping was used to detect QTL with $P < 0.0001$; the additive effect for QTL was also calculated (*24*).

RESULTS AND DISCUSSION

We have set up a rapid determination method using near-infrared reflectance spectroscopy for ADF, NDF, and ADL in rice straws; the prediction accuracy for ADF was highest ($r^2 = 0.96$) among all three traits (*6*). It was also found that the ADF, NDF, and ADL in rice are positively correlated (*12*), indicating that they were possibly under similar genetic control. Thus, ADF content was used in the present study to identify potential molecular markers in relation to its variation.

Variation of ADF in the BC_1F_1 Population. The ADF contents of the BC_1F_1 plants and their parents were measured by near-infrared reflectance spectroscopy (*6*). JX974 had an ADF content of 36.6%, higher than that of DWR (31.3%). The ADF in the BC_1F_1 population averaged 28.8%, ranging from 18.6 to 40.0% (**Figure 1**). A large number of the plants fell outside the range of the parents (**Figure 1**), indicating highly transgressive segregation. Transgressive variation is the appearance of individuals in segregating populations that fall beyond their parental phenotypes (*25*). The possible reason may be the fact that this BC_1F_1 population was derived from the cross between cultivated rice and wild rice species.

If the population was divided into two groups according to the midparent ADF content (the average of the parent ADF, 33.95%), that is, the ADF ≥33.95% group and <33.95% group, chi-squared (χ^2) tests indicated that the number of plants in the two groups did not accord with the expected 1:1 Mendelian ratio for the BC_1F_1 population ($\chi^2 = 21.9 > \chi^2_{(0.05,1)} = 3.84$). However, the skewness and kurtosis of the distribution of ADF content were 0.17 and −0.35, respectively, indicating a normal distribution and the involvement of several genes in the genetic control of the ADF in this population.

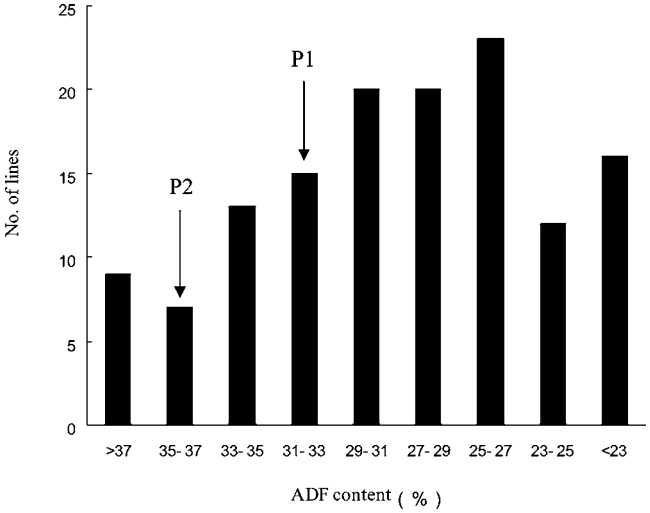

Figure 1. Frequency distribution of ADF content in the BC_1F_1 population: P1, Dongxiang wild rice (DWR); P2, JX974.

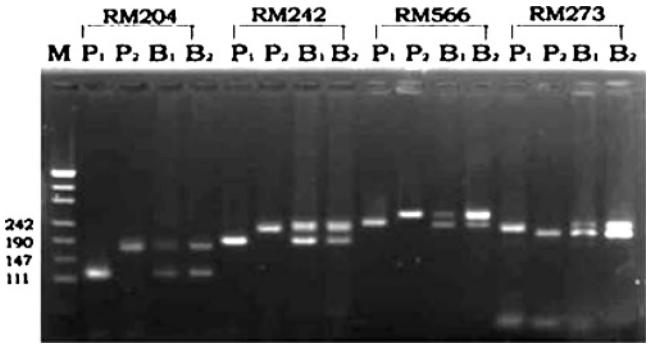

Figure 2. Screening the two bulks with some polymorphic SSR markers: B1, low ADF bulk; B2, high ADF bulk; M, marker.

Identification of SSR Markers Associated with ADF. A total of 387 SSR markers covering 12 chromosomes of rice were screened to identify polymorphic bands between the two parents. Of these, 119 markers were found to be polymorphic. These 119 polymorphic markers were used to screen the bulks. There should be two genotypes present in the BC_1F_1 plants, Aa (responsible for two bands for codominant marker) and aa (responsible for one band). In theory, there should be two bands in both bulks if the marker is not correlated to the ADF. Otherwise, there are two bands in one bulk (pool of Aa genotype), but only one band (pool of aa genotype) in the other bulk if the marker is correlated with the ADF. However, we did not find such good banding phenomena among all of the polymorphic SSRs screened (**Figure 2**); all of the polymorphic markers displayed two bands in both bulks. Moreover, most of the two bands in each bulk showed similar intensities. However, one marker, RM566, which is on chromosome 9, displayed different intensities between the two bands in the B2 bulk, the lighter band (top) matching the high ADF parent's band (P2). We suspected that this was a candidate marker in relation to ADF because the presence of another band (bottom band) might be caused by the inaccurate measurement of ADF leading to the contamination of a few low ADF plants in the high ADF bulk. It also might be caused by the fact that ADF is composed of cellulose and lignins, which possibly produced confounding effects on the marker investigation.

To confirm that RM566 was a candidate marker, the DNA concentration (100 ng/μL) of both bulks was diluted by 3, 6, 10, 12,15, and 20 times. Again, the diluted bulked samples were

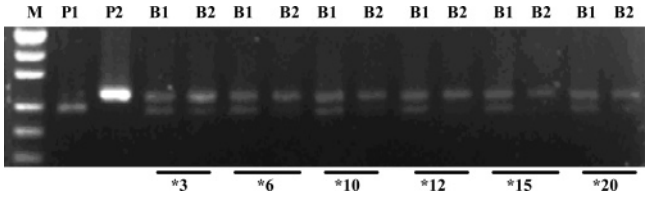

Figure 3. RM566 marker shows relationship with ADF when DNA in the two bulks was diluted at different times (the value followed by * indicates the dilution times).

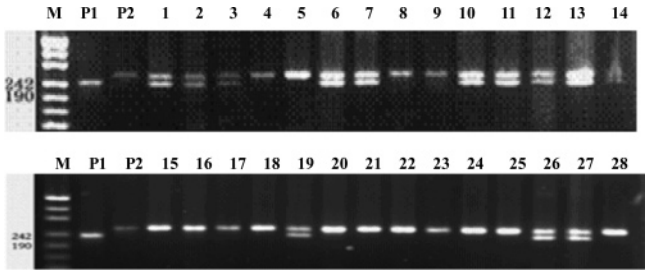

Figure 4. Segregation of RM566 in the BC_1F_1 population.

subjected to PCR analysis with RM566 primers. **Figure 3** shows that with the increased times of dilution, the bottom band of B2 bulk became less and less intense and disappeared in the 15 times dilution, indicating that the bottom band failed to be amplified by PCR due to its very low concentration of DNA in this bulk. This result further demonstrated that RM566 was a candidate marker associated with ADF.

To further confirm the RM566 associated with ADF, each of the BC_1F_1 plants was genotyped with RM566. Among 121 plants, 58 were heterozygous with two bands and 60 were homozygous with the same band pattern as parent JX974 (**Figure 4**), whereas the other 3 plants were homozygous with the same band pattern as the parent DWR; we thus discarded these three plants in further analyses. The segragation ratio of 58:60 was consistent with the expected Mendelian segregation ratio of 1:1 with $\chi^2 = 0.03$ [$\chi^2_{(0.05,1)} = 3.84$]. Analysis of variance for ADF between the two groups according to the RM566 genotypes showed that there was significant difference in ADF content between them ($P < 0.0001$). These results indicate that RM566 was associated with ADF content in the rice straw of this population.

QTL Analysis. For the mapping of QTL controlling ADF, a small linkage map of chromosome 9 was first to be constructed by adding more markers. Two other polymorphic SSR markers, RM321 and RM242, flanking both sides of RM566, were used to genotype the population. To add more markers, we used five insertion/deletion (InDel) markers reported by Shen et al. (*23*) (**Table 1**), which were designated by an "Indel" prefix. However, only Indel9-3 and Indel9-5 showed polymorphism between parents and were used to genotype the population. The other four markers also showed 1:1 Mendelian segregation in the BC_1F_1 population (data not shown). Again, the BC_1F_1 population was divided into two groups on the basis of the marker genotype, and analysis of variance for ADF showed significant difference between the two groups for three markers, RM321, RM242, and InDel9-3 (**Table 2**).

A small linkage map with these five markers was constructed using Map manager QTXb20 (*24*); the map distance between RM321 and Indel9-5 was 42.4 cM (**Figure 5**). The QTL associated with ADF was located at the RM321−RM566 interval, with a distance of 3.9 cM to RM566. This QTL explained 12% of the total phenotypic variation of ADF, and its additive effect was 3%.

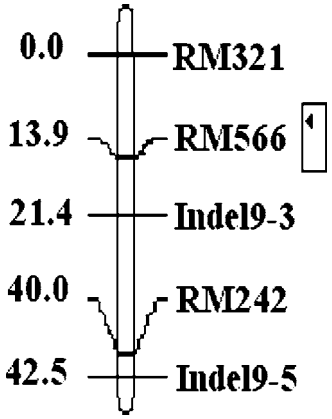

Figure 5. Linkage map for chromosome 9 with position of a QTL for ADF on the right side of the chromosome. The values on the left side indicate a locus position in centimorgans.

Table 2. Analysis of Variance for ADF According to the Marker Genotype

marker	df	SS	MS	*F*	*P* value
RM321	1	0.0240	0.0240	11.03	0.0012
RM566	1	0.0328	0.0328	15.57	0.0001
RM242	1	0.0111	0.0111	4.95	0.0280
Indel9-3	1	0.0220	0.0220	10.04	0.0020
Indel9-5	1	0.0079	0.0079	3.39	0.0683

To date, there is no report on the mapping of QTLs for CWC in rice, and it is still scarcely documented in other crops (i.e., maize and barley) and forage (*4*, *9*, *11*−*14*, *26*). In maize, a large number of QTLs were detected for ADF, NDF, and ADL in the studies of Cardinal et al. (*11*) and Krakowsky et al. (*4*, *13*), but Mechin et al. (*15*) reported only one QTL controlling NDF and ADL, respectively. In the present study, we detected only one QTL for ADF using the BSA methodology; the number of QTLs might be quite less than the real number of genes controlling this trait. However, this study is the first step to map QTL for ADF, one of the plant cell wall components in rice. To learn more about the genetic basis of CWCs in rice, it is necessary to develop a whole genetic linkage map with a larger population size. Thus, finer mapping will identify more QTLs associated with CWCs. On the other hand, ADF is a mix of two components, cellulose and lignin, which may lead to a lack of detection of QTLs. It is unknown whether the candidate genes underlying the individual QTL are genes in the cellulose or lignin biosynthesis pathways; thus, further mapping study by using cellulose and lignin contents separately will contribute to the identification of genes related to the corresponding pathway.

The SSR marker associated with ADF was developed from an interspecific cross; this marker could also be used in current rice breeding if rice materials differ in both the marker alleles and ADF content. Transfer of the marker allele from wild rice to cultivated rice is also possible, but more backcrosses should be made with cultivated rice as recurrent parent. The present study was conducted in only one environment. Further experiments should be conducted in different environments with stable mapping populations such as recombinant inbred lines and doubled-haploid populations to determine whether the CWC traits are affected by the environment and to allow dissection of the genotype × environment interaction effects at the molecular level.

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