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RFLP mapping of isozymes, RAPD and QTLs for grain shape, brown planthopper resistance in a doubled haploid rice population

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Abstract

We have developed an RFLP framework map with 146 RFLP markers based on a doubled haploid population derived from a cross between an indica variety IR64 and a japonica variety Azucena. The population carries 50.2% of IR64 loci and 49.8% of Azucena loci, indicating an equal amount of genetic materials from each parent has been transmitted to the progenies through anther culture. However, some markers show segregation distortion. These distorted marker loci are located on 10 chromosomal segments. Using this map we were able to place 8 isozymes, 14 RAPDs, 12 cloned genes, 1 gene for brown planthopper (BPH) resistance, and 12 QTLs for grain length, grain width and length/width ratio onto rice chromosomes. The major gene for BPH resistance was mapped on chromosome 12 near RG463 and isozyme Sdh-1. Most of the QTLs identified for the three grain characters were closely linked on chromosomes 1, 2, 3 and 10. We concluded that the RFLP framework map presented here will be useful for mapping other genes segregating in this doubled haploid population. Thus rapid generation of doubled haploid lines and their unbiased segregation make it very attractive for gene mapping.

Introduction

Two high-density RFLP maps in rice have recently been constructed based on an interspecific backcross population [2] and an intraspecific F_2 population [16]. Based on these two maps, more than 2000 markers are available for genome study and have been used to map genes of agricultural importance. Genes for resistance to biotic stresses (blast, bacterial blight, rice tungro, green leafhopper, whitebacked planthopper, brown planthopper) and to abiotic stresses (drought, salinity, submergence) have been located via linkage to RFLP markers (see [2, 32] for reviews). Many of these studies were based on genetic analyses in F_2, F_3 or backcross populations. These are segregating generations and because they do not breed true, replicated phenotypic evaluation with identical genotypes has not been possible, limiting the exploitation of RFLP information to map other traits segregating in the same population.

Recombinant inbred lines (RIL) developed via single seed descent and doubled haploid lines (DHL) generated from pollen/anther culture are homozygous and genetically uniform within each line. Large amounts of genetically identical seeds can be produced from RIL and DHL. Thus the RFLP mapping information and the seeds can be shared among scientists to map many traits of interest [21]. Wang *et al.* [29] first used an RIL population to map genes for blast resistance in rice. The same population was then used to map genes for drought avoidance [3] and plant height [9] without the need of RFLP analysis.

Guiderdoni *et al.* [4] developed a doubled haploid population from a cross between IR64 and Azucena. Many economically important characters, including grain shape, blast resistance, bacterial blight res-

istance, brown planthopper resistance, aroma, and drought tolerance, are segregating in this population. As a first step in an international collaboration to localize genes conditioning these traits, we have constructed a framework map and used it to locate genes controlling grain shape and brown planthopper resistance. The framework map was also used to locate cloned genes, RAPDs and isozyme loci on rice chromosomes.

Materials and methods

Plant materials

The development of doubled haploid lines was described in detail [4]. Briefly, the indica rice variety IR64 was crossed to the japonica variety Azucena. Anthers from F_1 plants were cultured to produce a population of doubled haploid plants. A population of 135 doubled haploid lines was derived and used to generate the RFLP framework map. Sterility was encountered in 30% of the lines [4] making it necessary to bag panicles on the entire population to avoid cross-pollination. Only bagged seeds were used as a source of plant material.

Construction of RFLP framework map

Procedures for DNA isolation, restriction digestion, gel electrophoresis, Southern transfer and DNA/DNA hybridization followed the standard techniques [26]. Six restriction enzymes (*Dra*I, *Eco*RV, *Hin*dIII, *Sca*I, *Xba*I, *Eco*RI) were used for the parental polymorphism survey. A total of 146 RFLP markers covering all 12 rice chromosomes were scored on the doubled haploid population. The markers coded as RG, RZ, and CDO were provided by Steve Tanksley, Cornell University, USA while those coded G or C were provided by the Rice Genome Project, Tsukuba, Japan. These markers were used to construct a linkage map using the computer program MAPMAKER [18] with map distances estimated by the Kosambi function. All linked markers were identified using the GROUP command with a LOD score of 5.0 and a recombination fraction (RF) of 0.40. Ordering of markers was done with a LOD score of 4.0 and a RF of 0.40. Ripple command was used to verify the order of the markers on each chromosome. The orientation of the RFLP linkage map is based on Singh *et al.* [28].

Chromosomal location of cloned RAPDs or genes

To position the cloned RAPDs and known genes on the rice framework map, rice DNA inserts were amplified either by plasmid miniprep or by PCR using M13 Universal Primers and used as probes in Southern analysis. New marker scores were combined with the RFLP data set as the basis for linkage analysis. Genes were positioned on chromosomes with a LOD$\geq$3.0.

Chromosomal location of RAPDs

RAPD analysis was performed following the procedure of Williams *et al.* [30] with minor modifications. Amplification reactions were carried out in 25 μl containing 10 mm Tris-HCl pH 8.3, 50 mM KCl, 0.01% (w/v) gelatin, 1.9 mM $MgCl_2$, 100 μM dNTP, 40 ng of primer, 1 u *Taq* polymerase and 20 ng of rice DNA. The amplification profile was 94 °C for 2 min, followed by 45 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C with a final extension of 7 min at 72°C. Amplification products were separated in 1.5% low-melting agarose gel in 1$\times$ TAE at 5 V/cm for 5 h. The target RAPD fragments were isolated from the gel and diluted with TE buffer. The isolated fragments were used as templates for following rounds of amplification. The PCR products were analyzed by gel electrophoresis to eliminate the possibility of contaminating non-target fragments. Purified RAPDs were then used as RFLP probes for Southern analysis of the DH population.

Isozymes

Isozyme analysis was performed following the procedure of Guiderdoni *et al.* [5]. A total of 12 isozyme loci were examined and eight were found to be polymorphic (Table 1). To localize the isozymes on the RFLP map, all eight isozymes were analyzed in the DH population. MAPMAKER was used to place the isozyme markers on the RFLP map.

Mapping QTLs for grain shape

Three grain characters (grain length, grain width and the ratio of length to width) were measured under dissecting microscope according to the Standard Evaluation System of Rice [12]. From each of the 135 lines, 10 grains were randomly chosen from seed stocks. Grain length and grain width were measured at 0.1 mm precision. The means of 10 grains were used for the analysis. The ratio of grain length to grain

Table 1. Isozymes, RAPD and cloned genes placed on the framework map developed in a doubled haploid rice population from an indica-japonica cross.

Gene/ marker	Chromo- some	Marker type	Linked gene Products
A10K250	8	RAPD	
A18A1120	8	RAPD	
A3E396	8	RAPD	
A5J560	8	RAPD	
AC5	8	RAPD	xa-13
AF6	12	RAPD	Bph
AG8	8	RAPD	aroma
K5	1	RAPD	Rf-3
PGMS0.7	7	RAPD	PGMS
pRD10A	3	RAPD	PGMS
pRD10B	6	RAPD	PGMS
TGMS1.2	8	RAPD	TGMS
U10	1	RAPD	Rf-3
W1	1	RAPD	Rf-3
Adh1	11	RFLP	Alcohol dehydrogenase
Adh2	11	RFLP	Alcohol dehydrogenase
Amy1A	2	RFLP	Alpha-amylase
Amy1B	1	RFLP	Alpha-amylase
Amy1C	2	RFLP	Alpha-amylase
Amy2A	6	RFLP	Alpha-amylase
Amy3A	9	RFLP	Alpha-amylase
Amy3B	9	RFLP	Alpha-amylase
Amy3C	9	RFLP	Alpha-amylase
Amy3D	8	RFLP	Alpha-amylase
Amy3E	8	RFLP	Alpha-amylase
PalI	2	RFLP	Phenylalanine ammonia-lyase
Amp-2	8	Isozyme	Amino peptidase
Amp-3	6	Isozyme	Amino peptidase
Cat-1	6	Isozyme	Catalase
Est-2	6	Isozyme	Esterase
Est-9	7	Isozyme	Esterase
Pgi-1	3	Isozyme	Phosphoglucoisomerase
Pgi-2	6	Isozyme	Phosphoglucoisomerase
Sdh-1	12	Isozyme	Shikimate dehydrogenase

width was determined by dividing the grain length by grain width. To identify QTLs for these three grain characters, single-factor analysis (one-way analysis of variance) was conducted with Proc GLM of the Statistical Analysis System [27]. The QTLs identified with single-factor analysis were confirmed and further elaborated with interval analysis using MAPMAKER/QTL [17].

Screening of brown planthopper resistance

IR64 is resistant while Azucena is susceptible to brown planthopper [14]. To map gene(s) for brown planthopper resistance, 50 seeds from each DH line were sown in 2 replications and tested for resistance to biotype I of BPH using bulk seedling test [1]. When the susceptible checks (Azucena and TN1) were damaged, the whole population was scored for resistant and susceptible reactions. Genes for resistance were mapped with MAPMAKER [18].

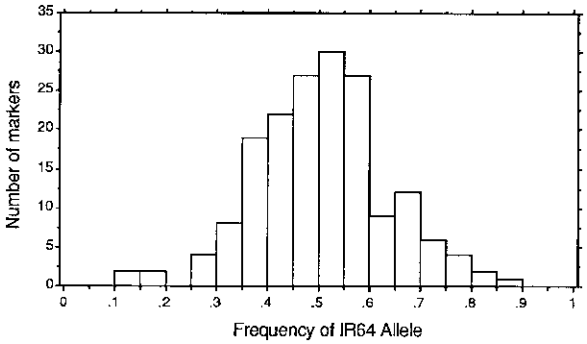

Figure 1. Distribution of IR64 allele frequencies in DH lines of the cross IR64/Azucena.

Results and discussion

Distribution of RFLP markers

A total of 175 polymorphic markers (including 146 RFLPs, 8 isozymes, 14 RAPDs and 12 cloned genes) were used to survey the 135 DHL derived from a cross between IR64 and Azucena. Because japonica rice is generally more responsive to anther culture than indica rice [4], we were interested to assess the relative frequency of IR64 and Azucena alleles in the population used in this study. The frequency of IR64 alleles for each marker was calculated and is shown in Fig. 1. The distribution was roughly symmetrical around 0.5, suggesting that there was no overall bias toward either parent. Taking the population as a whole, we determined that it carries 50.2% of IR64 loci and 49.8% of Azucena loci, indicating that an equal amount of genetic material from each parent has been transmitted to the progenies through anther culture.

As shown in Fig. 1, some markers had a higher frequency of alleles from one parent. Of the 175 markers mapped (Fig. 2), the allele frequency at 72 marker loci deviated significantly ($P < 0.05$) from the expected 1:1 ratio. There were 36 loci biased toward IR64 and the other 36 loci biased toward Azucena. These skewed marker loci were clustered on 10 chromosomal segments as shown by letter I (skewed toward IR64) and A (skewed toward Azucena) in Fig. 2. Among the distorted segments, the distortion on chromosomes 8 and 11 was most prominent as evidenced by the χ^2 values greater than 40 ($P < 0.001$).

Segregation distortion is common in indica/japonica crosses and this phenomenon has been explained genetically as the result of abortion of male and/or female gametes [11, 20]. According to this hypothesis, gametes possessing the critical combination of gametophytic alleles are sterile or inviable or have low germinability. Gametophytic (ga) genes are not only responsible for the gametic selection during fertilization but also have selective influence during *in vitro* androgenesis [6]. The frequency of marker loci closely linked to such genes would be much lower than predicted from normal Mendelian segregation. The segregation distortion on chromosomes 8 and 11 is very likely due to such segregation distortion genes. Indeed, Lin *et al.* [20] identified two ga genes through their linkage to isozyme Amp-2 (chromosome 8) and Pgd-1 (chromosome 11). Pgd-1 has been placed between RG103 and RG167 on chromosome 11 where segregation distortion is most striking in this study. Therefore the gene identified by Lin *et al.* [20] might be the same gene causing the segregation distortion in this population.

Segregation distortions are found on chromosomes 1, 4, and 7 where gametophyte development genes, sterility genes, and spikelet sterility genes have been found [15], so the distorted segregation in these four chromosomes are likely due to effects of these genes. We observed only a couple of markers showing segregation distortion on chromosome 6. This is surprising because many ga and other fertility-related genes are located in this region of the genome (see summary in [15]). It is possible that anther culture permits the plant to bypass these problems by deviating the microspores from a gametophytic pathway (with activation of ga genes) to a sporophytic pathway (with no activation of ga genes). The segregation distortion on chromosomes 5 and 2 were both towards the Azucena allele. Since no ga genes have been reported on these chromosomes and no distortion was observed in a F_2 population derived from Nipponbare/Kasalath [7], we postulate that these regions carry genes of japonica origin that are responsive to anther culture [4]. Strong segregation distortion can be a problem in gene mapping. Since the segregation distortion is minimal compared to that in other mapping population (e.g. [29]), we believe that the present DH population is suitable for map construction and gene mapping.

RFLP map construction

The 175 polymorphic marker loci were used to construct a linkage map (Fig. 2). The linkage map covered 2005 cM with an average distance of 11.5 between pairs of markers, though 6 gaps larger than 35 cM remained. Most of the RFLP markers were mapped to the same chromosomes and in the same orders as

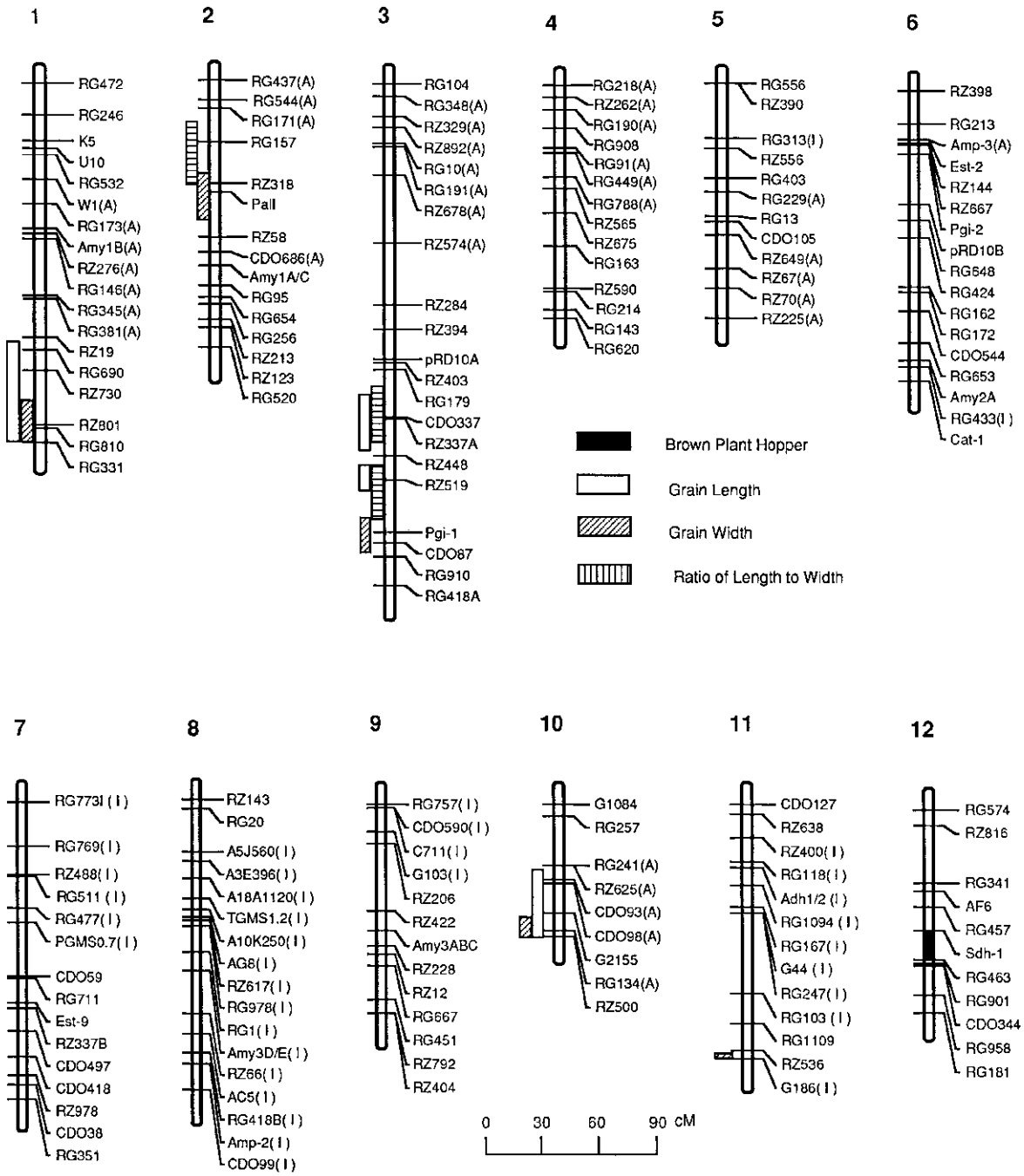

Figure 2. The RFLP framework map of doubled haploid population from IR64/Azucena. RFLP markers coded as RG, RZ, CDO were provided by Cornell University, USA and those coded as G or C were provided by the Rice Genome project of Japan. Skewed markers are indicated by (I) for IR64 and by (A) for Azucena. Mapped Bph gene is indicated by solid bar and QTLs are indicated by various bars. The length of bars indicate chromosomal segment associated with QTLs at LOD>3.

compared to Causse *et al.* [2]. However, a few markers, namely CDO93, RG190, RG218, RG241, RG418, RG649, RZ337, RZ400 and RZ892, were placed on different chromosomes. The shifts in chromosomal location for most of these markers were likely due to multiple bands detected by the RFLP probes, where the polymorphic bands were different in the DHL as compared to the BC_1 population [2]. For example, we mapped 2 loci for RG418: one on chromosome 3 as in Causse *et al.* [2], the other on chromosome 8. Another example is RG241. We mapped one of the 3 copies on chromosome 10, as previously reported in McCouch *et al.* [22], while a copy has also been reported on chromosome 12 by Causse *et al.* [2] Li *et al.* [19] mapped RG241 to both chromosomes 10 and 12. Two single-copy markers (RG190 and RG218) were placed on chromosome 4 instead of chromosome 12, a situation discussed by Causse *et al.* [2]. RG190 was also placed on chromosome 4 in a recombinant inbred population [19]. The single-copy marker CDO93 was localized to chromosome 10 instead of chromosome 4.

DHL derived from anther culture are expected to be homozygous. We, however, observed heterozygosity at 434 loci of marker/DHL combination (1.6% of total loci scored). Most of the heterozygous loci were observed in 9 DHLs, (namely, P13, P34, P78, P100, P196, P350, P355, P455, P579) and each of these lines carried more than 20 heterozygous loci. In this analysis, all heterozygous loci are treated as missing data before any analysis is performed. Heterozygous diploid lines derived from anther culture have been observed by others [24]. They proposed that the heterozygous plants are derived from regeneration of cultured somatic cells. It is not clear if the DHLs with high level of heterozygous loci derived from cultured somatic cells. Because these heterozygous plants segregate after selfing, care should be taken to eliminate DHL with a high frequency of heterozygous loci.

Mapping isozymes, RAPDs and cloned genes

The 146 RFLP markers formed a framework map which was used to place isozymes, RAPDs and cloned genes onto rice chromosomes. The availability of the framework map proved very important, especially for mapping of RAPD markers linked to agriculturally important genes. Linked RAPDs were identified using bulked segregant analysis [23] and once mapped RFLPs in the region can be used to refine the map position of the gene. One example was the mapping of AC5, a RAPD marker linked to the bacterial blight resistance gene *xa-13* [31]. Using the DHL population, we placed AC5 onto chromosome 8, linked to RZ66. RFLP markers in this area [2] were then used to map *xa-13* with greater precision. Initially, the RAPDs were cloned before mapping. Later this was found unnecessary and RAPD fragments isolated from gels were used directly as RFLP probes after reamplification. With the procedure, we were able to place several RAPD markers onto various chromosomes. These markers were linked to various genes of agricultural importance (Table 1).

Several isozyme markers segregating in this population were also mapped using the framework map. Placement of these isozymes confirmed the chromosomal assignment made previously using conventional technique and primary trisomic analysis (see [15] for a summary) and provided greater resolution of map position.

Cloned genes are very interesting per se. We have placed 12 rice genes onto the RFLP map. Mapping of Amy3D and Amy3E genes together on chromosome 8 confirmed the result obtained by chromosomal walking [10] and reported by Causse *et al.* [2]. Two genes of the Amy1 family which were previously placed on chromosome 2 based on trisomic analysis [25] and RFLP linkage analysis [2]. These two genes were located next to each other at 0 cM on the population. These two genes, Amy1A and Amy1C, appeared to be physically adjacent to each other as was the case for Amy3D and Amy3E [10]. RFLP mapping of Adh genes produced results different from those based on trisomic analysis. Ranjhan *et al.* [25] placed Adh-1 on chromosome 11 and Adh-2 on chromosome 9. Using an Adh cDNA from rice (provided by Ray Wu, Cornell University) as a probe, we mapped all hybridizing fragments on chromosome 11. Based on the banding patterns in Ranjhan *et al.* [25] these bands contained both Adh1 and Adh2. We therefore concluded that the two genes are tightly linked to each other on chromosome 11. Our result is consistent with that of Kurata *et al.* [16] who mapped 3 cDNA clones with high homology to Adh1 and Adh2 on chromosome 11.

Mapping QTLs for grain shape

The statistics of grain length, grain width and the length/width ratio are shown in Table 2. A total of 12 QTLs for the traits were located on 5 different chromosomes with LOD scores greater than 3.0 (Table 3, Fig. 2). Four QTLs were identified for grain length and they were located on chromosomes 1, 3, and 10. Five QTLs were determined for grain width and the

Table 2. Means and distribution properties for grain length, width and length/width ratio of the parents and DH lines.

	IR64	Azucena	DH population				
			mean	SD	range	skewness	kurtosis
Length (mm)	9.14 ± 0.38	9.40 ± 0.32	9.00	0.74	7.41–11.6	0.45	0.57
Width (mm)	2.77 ± 0.12	2.96 ± 0.07	2.90	0.20	2.40–3.43	0.19	0.13
Length/width	3.30 ± 0.14	3.18 ± 0.12	3.12	0.30	2.29–4.14	−0.13	0.73

Table 3. Chromosomal location and biometrical characteristics of QTL for grain traits measured in the DH population.

Character	Peak interval	Chromosome	Peak LOD	Variance%	Additive
Length	RZ730-RZ801	1	6.20	23.3	0.4013
	RZ519-RZ448	3	4.17	13.4	−0.2776
	RZ337A-CDO337	3	6.20	19.2	−0.3272
	G2155-RG134	10	5.68	17.9	−0.3246
Width	RG810-RG331	1	3.94	13.1	0.0731
	RZ318-RZ58	2	3.04	13.5	0.0809
	CDO87-Pgi-1	3	3.12	10.6	0.0661
	RG134-RZ500	10	3.06	10.1	−0.0650
	RZ536-G186	11	3.41	11.4	0.0686
Length/width	RG157-RZ318	2	4.25	16.1	−0.1319
	RZ519-Pgi-1	3	4.01	14.9	−0.1243
	RG179-CDO337	3	5.03	17.2	−0.1276

Additive: effect of Azucena alleles.

LOD scores were slightly smaller than that for grain length. Only three QTLs were identified for the ratio of grain length to width. Each of 12 QTLs explained at least 10% of the total variation. A QTL for grain length had the largest effect, explaining 23.3% of the phenotypic variation and was located in the interval RZ730-RZ801 on chromosome 1. No QTL has effect on all three characters. QTLs on chromosomes 1 and 10 have effects on both grain length and width but not their ratio. The highest LOD score for the ratio in this region is only 1.4. It is possible that this QTL has effect on grain size (weight) therefore does not affect length/width ratio. QTLs from the same parent could be opposite in effect. The major QTL for grain length on chr 1 was from Azucena while alleles from Azucena on chromosomes 3 and 10 contributed negatively to grain length. Therefore, in breeding for long slender grain, it would be wise to pyramid alleles from IR64 for QTLs on chromosomes 3 and 10 and the alleles from Azucena for QTL on chromosome 1.

Mapping the gene for brown planthopper resistance

The DHL population segregated for resistance to brown planthopper because of resistance gene(s) in IR64 [14]. IR64 shows resistance to BPH biotype 1 and 3, and intermediate resistance to biotype 2 so it is believed that IR64 has Bph 1 plus another gene or gene(s) not yet identified [14]. To map the gene(s) involved, the population (only 105 lines were used due to limited number of seeds available when the experiment was conducted) was screened for brown planthopper reaction. Using the parents as controls, 42 lines were scored as resistant and 56 lines were scored as susceptible. The remaining 7 lines were scored as missing due to inconsistent results between replications, poor seed germination or segregation within lines. The ratio of resistant to susceptible fits a 1:1 segregation indicating the presence of a major gene. Analysis with MAPMAKER showed that this gene was derived from IR64 as previously reported [14] and was located on chromosome 12 (Fig. 2). Three RFLP markers (RG463, RG901,

CDO344) and one isozyme marker (Sdh-1) showed linkage with the Bph gene at LOD>4. Recently, two Bph genes were located to chromosome 12. Bph-10(t) derived from the wild species *O. australiensis* shows resistance to all three BPH biotypes and is mapped next to RG457 [13]. Bph-1 from IR28, showing resistance to biotype 1 and 3, was located near XNpb 248 [8]. Although the pattern of resistance among three varieties are different, the gene(s) mapped are in a similar position on chromosome 12. If the gene from IR64 is Bph-1, it should be allelic to the gene (Bph-1) from IR28. Whether Bph-1 and Bph-10(t) is closely linked or allelic remains to be seen. Allelism testing is being carried out to distinguish the two possibilities.

We have developed an RFLP framework map based on a DH population derived from a modern high-yielding rice variety IR64 and a traditional tropical japonica rice variety, Azucena. The population is not biased toward either parent although some markers show segregation distortion. The mapping of isozymes, RAPDs, cloned genes and QTLs for grain characters and a gene for brown planthopper resistance proves that the RFLP framework map and the population are suitable for mapping other genes segregating in this population. Use of DH lines to map genes provides several distinct advantages over the use of recombinant inbred lines or other types of populations: (1) rapid generation of mapping DH population; (2) reduced segregation distortion as seen in this study; (3) genetically pure seeds can be produced from DH lines. These characteristics make DH lines an ideal population for international collaboration in mapping genes of interests. Gene mapping for bacterial blight resistance, blast resistance, aroma, yield components and root morphology is in progress. Furthermore, we have started to examine QTLs by environment interaction of many traits because genetically pure rice seeds are available. These studies will be reported elsewhere.

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