Chromosomal Location of Eight Isozyme Loci in Rice Using Primary Trisomics and Monosomic Alien Addition Lines

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Starch-gel electrophoresis and isoelectric focusing techniques were used to detect polymorphism and chromosomal location of isozyme loci in rice (*Oryza sativa* L.). Polymorphism for four new isozyme loci—*Mdh-3, Gdh-2, Fk-1,* and *Fk-2*—was detected. Primary trisomic analysis showed that *Dia-1* and *Fk-1* are located on chromosome 1, *Mdh-1* on chromosome 5 and *Fk-1,* and *Gdh-2* and *Tpi-1* as a cluster of genes on chromosome 11. Monosomic alien addition line analysis revealed that *Mdh-3* and *Pgd-2* are located on chromosomes 5 and 6, respectively.

Knowledge on the genetic architecture of the rice (*Oryza sativa* L., $2n = 24$) plant is essential for applying new tools of biotechnology in its improvement. Three kinds of genetic markers—morphological (plant traits), isozyme (biochemical), and restriction fragment length polymorphism (DNA)—have been used to develop genetic maps in rice. More than 175 morphological markers have been located on rice chromosomes through genetic segregation analysis (Khush and Kinoshita 1991). Various cytogenetic stocks such as primary trisomics, monosomics, and monosomic alien addition lines (MAALs) have been used to assign genes to specific chromosomes in several plant species. In rice, primary and secondary trisomics have been used extensively to develop linkage groups (Khush et al. 1984; Singh et al. 1996). The 12 linkage groups in rice have been assigned to respective chromosomes through primary trisomic analysis (Iwata et al. 1984; Khush et al. 1984). Similarly, of the 44 isozyme loci known in rice, 33 have been assigned to respective linkage groups through primary trisomic and genetic segregation analyses (Brar et al. 1991; Khush and Kinoshita 1991; Pham et al. 1990; Ranjhan et al. 1988; Wu et al. 1988). However, more isozymes need to be mapped to develop a comprehensive isozyme map and to utilize these markers in gene tagging. In this article we report the chromosomal location of seven new isozyme loci and confirmation for another locus, *Pgd-2* through primary trisomics and MAAL analysis.

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Materials and Methods

Plant Materials

The materials consisted of F_1, F_2, and BC_1 populations produced from crosses of 12 primary trisomics of rice (*Oryza sativa* L.) in the background of IR36 (Khush et al. 1984) with three isozyme tester stocks; *O. sativa* cv. Ma Hae (acc. 23754), Basmati-Fine (acc. 53642), and *O. nivara* (acc. 104443). In addition, MAALs of *O. officinalis, O. latifolia,* and *O. brachyantha* were also used for location of isozyme loci. Primary trisomics ($2n + 1$) refer to the individuals having one extra chromosome in addition to the normal chromosome complement of cultivated species. The MAALs ($2n + 1$) also have one extra chromosome, but this chromosome is from the alien (wild) species.

Starch-Gel Electrophoresis

We used starch-gel electrophoresis to assay for three enzyme systems: malate hydrogenase (MDH; EC 1.1.1.37), diaphorase (DIA; EC 1.6.4.3), and phosphogluconate dehydrogenase (PGD; EC 1.1.1.43). Four isozyme loci were analyzed using crude extracts of water soluble proteins from 5- to 6-day-old coleoptiles. Electrophoretic separations of *Mdh-1, Mdh-3, Dia-1,* and *Pgd-2* were done on horizontal starch gels using the procedures described by Delos Reyes et al. (1989), Glaszmann et al. (1988), and Second and Trouslot (1980). The allele designations for different isozyme loci are based on Glaszmann et al. 1988.

Isoelectric Focusing

Three enzyme systems: fructokinase (FK; EC 2.7.1.4), triosephosphate isomerase

Table 1. Characteristics of allozymes detected through IEF for GDH, TPI, and FK enzymes using crude extracts from 2-day-old germinating dehulled seeds

Isozyme locus	pH gradient	Allozyme	Distance from cathode (cm)	pI
Gdh-2	pH 3.5–10.0:	1	6.0	7.30
	3.5–5.0:	2	5.5	7.45
	4.0–6.0 = 1:3:2			
Tpi-1	pH 3.5–10.0:	1	5.2	7.67
	4.0–6.5:	2	4.6	7.86
	4.2–4.9 = 1:2:3			
Fk-1	pH 3.5–10.0:	1	4.3	7.39
	4.0–6.5:	2	3.7	7.78
	4.2–4.9 = 1:2:3			
Fk-2	pH 3.5–10.0:	1	6.7	6.96
	4.0–6.5:	2	6.5	7.08
	4.2–4.9 = 1:2:3			

pI = isoelectric pH measurements from five gels.

(TPI; EC 5.3.1.1), and glutamate dehydrogenase (GDH; EC 1.4.1.2) representing four isozyme loci—*Fk-1, Fk-2, Tpi-1,* and *Gdh-2*—were studied by isoelectric focusing (IEF) on a pH gradient, thin layer polyacrylamide gels (29.1:0.9) using the procedure modified from Liu and Gale (1988). Gradients were made by combining different preparations of narrow and wide pH ampholytes (Pharmacia, LKB Ampholines). Isoelectric focusing was conducted using the Multiphor II electrofocusing apparatus with 1 M NaOH as catholyte and 1 M H_3PO_4 as anolyte. Molecular separations were conducted using 2000 V, 25 mA, and 15 W for 3200 vH. Isozymes were revealed from endospermic tissues of the 2-day-old dehulled germinating seeds using the enzyme activity staining procedures described by Pasteur et al. (1988). The pH gradients used for each system and corresponding tissue specificity are given in Table 1.

Primary Trisomic Analysis

The primary trisomics for 12 chromosomes were crossed as females with representative isozyme testers exhibiting polymorphism for isozyme loci. The allelic constitution of the parents used is given in Table 2. The trisomic F_1s were analyzed for gene dosage effect based on the procedure described by Ranjhan et al. (1988) and Wu et al. (1988). The trisomic F_1s from each cross were either selfed to obtain F_2 or backcrossed to the tester to produce BC_1 populations. The segregation ratios were studied to determine the critical trisomics carrying the isozyme loci. Segregation ratios deviating from the expected disomic ratio of 1:2:1 in F_2 and 0:1:1 in BC_1 were further tested with the expected trisomic ratios of 5:7:1 for F_2 and 0:3.3:1 for BC_1, with the assumption of 30% female transmission rate of extra chromosome (Khush et al. 1984).

MAAL Analysis

Mdh-3 and *Pgd-2* were located using MAALs of *O. officinalis, O. latifolia,* and *O. brachyantha.*

Results and Discussion

Isozyme Polymorphism

Polymorphisms for three enzymes—fructokinase, triosephosphate isomerase, and glutamate dehydrogenase—were detected through IEF. Four putative loci (*Fk-1, Fk-2, Tpi-1,* and *Gdh-2*) were identified using endosperm extract from 2-day-old dehulled germinating seeds of rice. Each of the pu-

Table 2. Allelic constitution for six isozymes in IR36 and isozyme tester stocks

Material	Acc. no.	Allelic constitution[a]					
		Mdh-1	*Dia-1*	*Tpi-1*	*Fk-1*	*Fk-2*	*Gdh-2*
IR36	30416	1	1	1	1	1	1
Ma Hae	23754	1	1	2	2	2	2
O. nivara	104443	2	—[b]	1	1	1	—[b]
Basmati-Fine	53642	1	2	1	1	1	1

[a] Allele designation according to Glaszmann et al. 1988.

[b] Not analyzed.

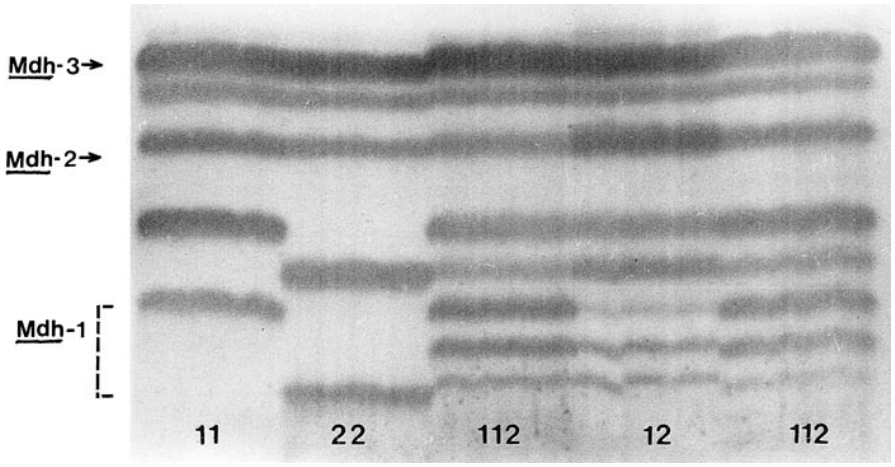

Figure 1. Zymogram showing allele dosage distribution for *Mdh-1* (alleles 112) in a critical F_1 primary trisomic-5; lanes 1–5: 1 = IR36, 2 = *O. nivara*; 3 and 5 = F_1 critical trisomic-5; 4 = F_1 (IR36 × *O. nivara*).

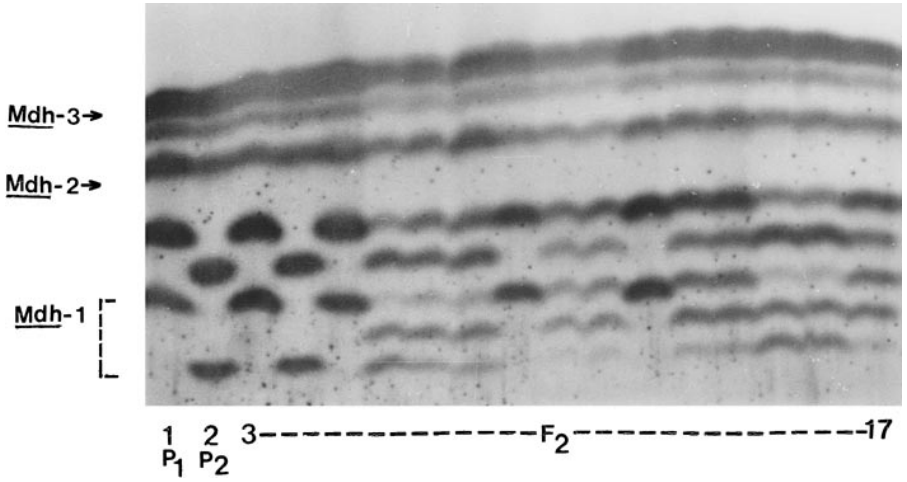

Figure 2. Zymogram showing *Mdh-1* segregation in F_2 derived from IR36 primary trisomic-5 × *O. nivara*; lanes 1–17: 1 = P1 = IR36 (alleles 11); 2 = P2 = *O. nivara* (alleles 22); 3, 5, 9, 12 (alleles 11); 4 (alleles 22); 7, 8, (alleles 12); 10, 11, 13, 14, 17 (alleles 112); 6, 15, 16 (alleles 122).

Table 3. Segregation of *Mdh-1* in progenies of IR36 primary trisomics × *O. nivara,* for *Dia-1* in progenies of IR36 primary trisomics × Basmati-Fine and for *FK-2* in the progenies of IR36 primary trisomics × Ma Hae

		Number of plants with isozyme genotype[a]											
		Mdh-1				*Dia-1*				*Fk-2*			
Primary trisomics	Progeny	11	12 112 122	22	χ^2	11	12 112 122	22	χ^2	11	12 112 122	22	χ^2
1	BC_1	0	27	32	0.42	—	—	—	—	0	50	12	23.28[b] (D)
	F_2	—	—	—	—	76	104	22	29.05[b] (D) 2.93 (T)	—	—	—	0.52 (T)
2	BC_1	0	29	24	0.48	—	—	—	—	0	28	32	0.27
	F_2	—	—	—	—	15	25	12	0.43	—	—	—	—
3	BC_1	0	37	22	3.80	—	—	—	—	0	27	31	0.28
	F_2	—	—	—	—	23	42	19	0.38	—	—	—	—
4	F_2	75	126	61	1.88	57	101	67	3.23	34	67	30	0.31
5	F_2	53	57	5	40.07[b] (D) 3.80 (T)	23	53	27	0.39	27	52	25	0.08
6	BC_1	0	46	46	0.00	—	—	—	—	0	55	48	0.48
	F_2	32	67	19	5.02	62	108	50	1.38	—	—	—	—
7	F_2	18	59	23	3.74	21	50	17	2.00	23	55	25	0.55
8	F_2	20	46	22	0.27	16	28	14	0.20	20	33	18	0.46
9	F_2	—	—	—	—	34	57	33	0.82	18	37	17	0.08
10	F_2	41	88	35	1.32	21	49	26	0.57	24	58	20	2.24
11	F_2	50	76	32	4.32	25	49	19	1.04	30	42	27	2.50
12	F_2	15	40	15	1.43	21	53	22	1.07	35	54	30	1.44

D = disomic segregation for BC_1 = 0:1:1, for F_2 = 1:2:1.

T = trisomic segregation for BC_1 = 0:3.3:1, for F_2 = 5:7:1 assuming 30% female transmission of the extra chromosome.

[a] Allele designation according to Glaszmann et al. 1988.

[b] Significant at 1% level.

tative loci exhibit two detectable polymorphic bands with distinct isoelectric points (Table 1). Of the four loci, Ma Hae acc. 23754 was found to have a second allele (designated as allele 2) which is different from the allele of IR36 designated as allele 1 (Table 2). All four loci showed three bands in F_1 heterozygotes indicating the dimeric nature of the proteins.

The polymorphism for *Dia-1* and *Pgd-2* has been reported previously (Delos Reyes et al. 1989; Glaszmann et al. 1988). None of the representative testers of *O. sativa* showed any polymorphism for *Mdh-1* and *Mdh-3* (data not shown). Hence we surveyed wild species germplasm. The wild species, *O. nivara* acc. 104443, showed polymorphism for *Mdh-1,* whereas *Mdh-3* was polymorphic in two other species, *O. officinalis* and *O. latifolia.*

Primary Trisomic Analysis

Mdh-1. Enzymes are dimers and heterozygotes exhibit a three-banded zymogram. Disomic heterozygotes show uniform intensity of the parental bands, whereas the intermediate band is twice as intense. This pattern agrees with the theoretical distribution of concentration of the various molecular forms of enzymes as described by Wu et al. (1988). All F_1 trisomics showed a three-banded zymogram with the expected intensity of three bands, indicating genotype of $Mdh\text{-}1^{12}$, except triplo-5 which exhibited allele dosage distribution in favor of IR36 (trisomic) allele ($Mdh\text{-}1^{112}$; Figure 1). Thus chromosome 5 was identified as the critical chromosome carrying the *Mdh-1* locus. Data on segregation for *Mdh-1* in F_2 and BC_1 progenies are given in Table 3. The F_2 progenies of triplo-5 also showed a segregation ratio that deviates significantly from the 1:2:1 disomic ratio but fit with the trisomic ratio of 5:7:1 (Table 3). These results were further confirmed in the F_2 of triplo-5, from trisomic F_2 plants with converse allele dosage indicating the presence of both genotypes $Mdh\text{-}1^{112}$ and $Mdh\text{-}1^{122}$ (Figure 2). The F_2 and BC progenies of all other trisomics showed expected disomic segregation ratios. Gene dosage in F_1 trisomic and genetic segregation in F_2 indicated chromosome 5 as the critical chromosome carrying the *Mdh-1* locus.

Dia-1. Enzymes are tetramers and exhibit five-banded zymograms in heterozygous condition. The five bands from a heterozygous plant appear as one thick band due to small differences in the electrophoretic mobilities of each allozyme. Because of this, it was difficult to analyze allele dosage imbalance among the different tri-

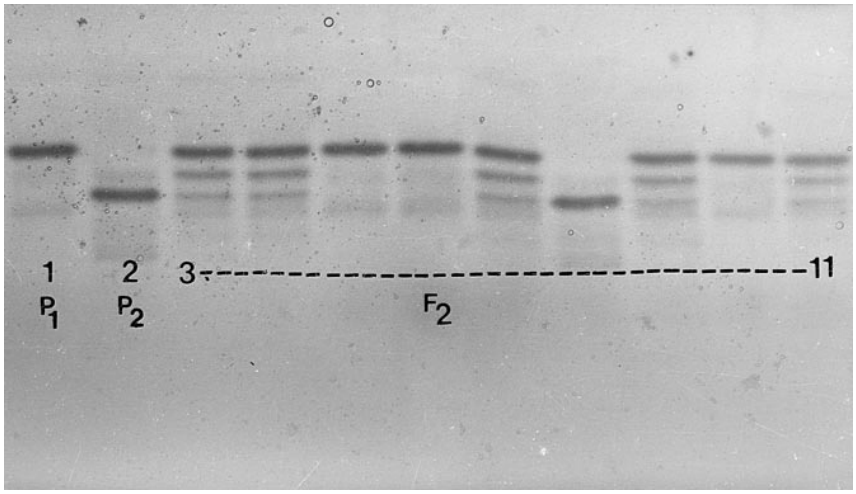

Figure 3. Zymogram showing *Fk-1* segregation in F_2 derived from IR36 primary trisomic-11 × Ma Hae; lanes 1–11: 1 = P1 = IR36 (alleles 11); lane 2 = P2 = Ma Hae (alleles 22); 3, 4, 7, 9, 11 (alleles 12); 5, 6, 10 (alleles 11); 8 (alleles 22).

Table 4. Segregation of *Fk-1*[a] in progenies of IR36 primary trisomics × Ma Hae

Primary trisomics	Progeny	Number of plants with the isozyme genotype[b] 11	12 112 122	22	χ^2
1	BC_1	0	34	28	0.58
2	BC_1	0	27	33	0.60
3	BC_1	0	30	28	0.06
4	F_2	31	63	33	0.07
5	F_2	28	53	23	0.52
6	BC_1	0	53	48	0.25
7	F_2	24	57	23	0.98
8	F_2	19	36	16	0.26
9	F_2	18	39	15	0.75
10	F_2	26	56	22	0.92
11	F_2	44	50	6	28.88[c] (D) 1.44 (T)
12	F_2	31	58	34	0.54

D = disomic segregation for BC_1 = 0:1:1, for F_2 = 1:2:1.

T = trisomic segregation for BC_1 = 0:3.3:1, for F_2 = 5:7:1 assuming 30% female transmission of the extra chromosome.

[a] Identical segregation was observed for *Tpi-1* and *Gdh-2* in this population.

[b] Allele designation according to Glaszmann et al. 1988.

[c] Significant at 1% level.

somics. However, segregation of *Dia-1* in the F_2 progenies of triplo-1 showed a significant deviation from the disomic ratio of 1:2:1 but a good fit to the expected trisomic ratio of 5:7:1 (Table 3). The rest of the 11 trisomics showed F_2 segregations that fit with the expected disomic ratios of 1:2:1. These results suggested that *Dia-1* locus was located on chromosome 1.

Fk-2. The *Fk-2* zymograms show three-banded heterozygotes indicating that the enzymes are dimers. The segregation of *Fk-2* was studied in the population derived from crosses of primary trisomics of IR36 with Ma Hae. The F_2 and BC_1 progenies of all trisomics except triplo-1 showed 1:2:1 and 0:1:1 ratios, respectively. However, BC_1 involving triplo-1 showed a good fit to trisomic ratio of 0:3.3:1 indicating the location of *Fk-2* on chromosome 1 (Table 3).

Fk-1, Tpi-1, Gdh-2. Various isozyme tester stocks were analyzed to detect polymorphism for *Fk-1, Tpi-1,* and *Gdh-2* loci. The *Fk-1, Tpi-1,* and *Gdh-2* proteins are dimers and exhibit three-banded zymograms in heterozygous condition (Figure 3). An increase in the intensity of the maternal allele was observed in all heterozygous individuals (disomic and trisomic) due to the presence of two copies of the maternal allele in the triploid endospermic tissues used in the detection of the isozymes. This made allele dosage analysis difficult. However, the segregation ratios of *Fk-1, Tpi-1,* and *Gdh-2* loci in the F_2 of triplo-11 deviated significantly from the disomic ratio but fit with the trisomic ratio of 5:7:1 (Table 4). The segregation in other trisomics agreed with the disomic ratio indicating that these three loci are indeed located on chromosome 11. Ishikawa and Morishima (1989) located *Gdh-1* on chromosome 3. Therefore we have designated this locus as *Gdh-2* which is located on chromosome 11. Furthermore, *Fk-1, Tpi-1,* and *Gdh-2* show identical segregation patterns, suggesting that these loci are tightly linked behaving as a gene cluster on chromosome 11. As seen in Table 1, differences in isoelectric points (pI) between *Tpi-1, Gdh-2,* and *Fk-1* suggest that these isozymes are products of different genes. Three genes coding for glycolytic enzymes phosphoglucomutase (PGM), alcohol dehydrogenase (ADH), and triosephosphate isomerase (TPI) occur as a cluster on the long arm of chromosome 4 in tomato (Tanksley 1979). A similar situation may exist in rice for the isozyme genes *Tpi-1, Gdh-2,* and *Fk-1.*

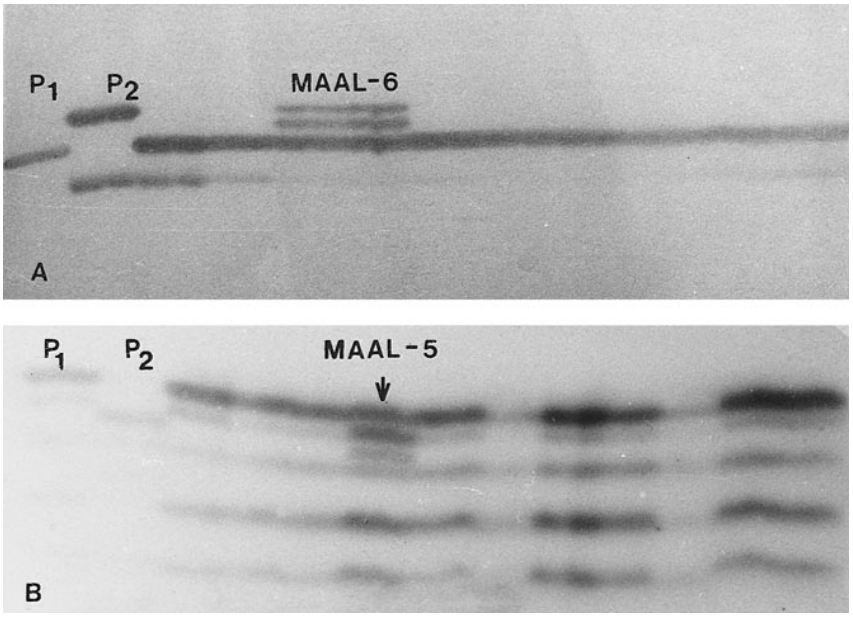

Figure 4. (A) Allele dosage distribution for *Pgd-2* in a critical MAAL-6 of *O. latifolia*: lanes 1–12; 1 = P1 = *O. sativa*; 2 = P2 = *O. latifolia*; 3, 4, 7–14 = noncritical MAALs; 5 and 6 critical MAAL-6. **(B)** Allele dosage distribution for *Mdh-3* in a critical MAAL-5 of *O. officinalis*: lanes 1–13; 1 = P1 = *O. sativa*; 2 = P2 = *O. officinalis*; 3, 4, 5, 7–13 = non-critical MAALs; lane 6, critical MAAL-5.

MAAL Analysis

The MAALs are valuable cytogenetic stocks both for alien gene transfer and mapping of isozyme loci on chromosomes of plant species. Hart and Tuleen (1983) used MAALs of wheat and related species and located several orthologous isozyme genes in four species of *Triticeae.* MAAL analysis is based on the assumption that the presence of an alien chromosome in the background of *O. sativa* results in heterozygous banding pattern for a specific isozyme locus. Furthermore, the allele dosage distribution in the critical MAAL will be in favor of the *O. sativa* allele due to the presence of two copies of that allele in contrast to a single copy of the allele from the alien chromosome.

The results of the analysis of available MAALs for *Mdh-3* and *Pgd-2* are shown in Table 5. The heterozygous banding pattern was detected for *Pgd-2* in a critical MAAL-6 of three species—*O. officinalis, O. latifolia,* and *O. brachyantha*—while all other MAALs analyzed carried only the allele of the recurrent rice parent (Table 5). Figure 4A shows allele dosage distribution in

Table 5. Chromosomal location of *Mdh-3* and *Pgd-2* through MAAL analysis

MAALs no.	*O. officinalis* (acc. 100896)		*O. latifolia* (acc. 100914)		*O. brachyantha* (acc. 101232)
	Mdh-3	*Pgd-2*	*Mdh-3*	*Pgd-2*	*Pgd-2*
4	−	−	−	−	−
5	+	−	+	−	−
6	−	+	−	+	+
7	−	−	−	−	−
8	−	−	−	−	NA
9	−	−	−	−	−
10	−	−	−	−	−
11	−	−	−	−	−
12	−	−	−	−	−

+ critical, − noncritical, NA = not available.

O. brachyantha was monomorphic for *Mdh-3.*

favor of the *O. sativa* allele in critical MAAL-6 of *O. officinalis.* Ishikawa and Morishima (1989) also reported location of *Pgd-2* on chromosome 6 of rice on the basis of linkage with *Est-2.* Similar behavior of the allele dosage distribution for *Mdh-3* was observed in MAAL-5 of *O. officinalis* and *O. latifolia* (Figure 4B). These results indicate the possible location of *Mdh-3* on chromosome 5. The location of eight isozyme loci in the present study is a valuable addition to develop a comprehensive isozyme map in rice (Khush and Kinoshita 1991). Linkage of these isozyme loci with agronomic traits would enhance the efficiency of marker-assisted selection in rice breeding.

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