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CHROMOSOMAL ANALYSIS OF ISOZYME LOCI AND THE ALLELIC EXPRESSION AT CELLULAR LEVEL IN RICE

—Genetical studies on rice plants, XCVII[1]—

R. ISHIKAWA, H. MORISHIMA*, K. MORI and T. KINOSHITA

Plant Breeding Institute, Faculty of Agriculture, Hokkaido University, Sapporo 060 JAPAN

* National Institute of Genetics, Mishima, 411 JAPAN

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Introduction

Isozymes have been extensively used for population and evolutionary genetics and plant breeding in various crops.[32] It is also known that the use of biochemical markers such as isozymes and RFLPs (restriction fragment length polymorphisms) have an important role in genetics and plant breeding.[33]

Since the position of *Est-2* has been decided in the first linkage group,[22] nearly 20 loci of various isozymes were allocated in the respective chromosomes and used as markers at whole plant level. Polymorphisms of about 40 isozymes were studied in rice hitherto and used for the studies on the evaluation of germ plasm and the phylogenetic relationship of species.[10,11,31]

Isozyme patterns also seem to be useful genetic markers in parasexual hybrids and *in vitro* selection. In rice, however, isozymes have been scarcely used as such genetic markers because of there being little knowledge about their expression in callus level, and the pattern of their expression in callus should be verified and compared with that in the original plants. Therefore we investigated allelic expression of 14 isozyme loci in seed calli derived from parental strains and their F_1 plants.

This is a collaborative research project which has been carried out in both the National Institute of Genetics and Hokkaido University to develop biochemical markers located on rice chromosomes.

Materials and Methods

Trisomic Analysis: A series of primary trisomics with the genetic background of *Oryza sativa* cv. Nipponbare (Japonica) was developed in Kyushu University.[14]

1) Contribution from the Plant Breeding Institute, Faculty of Agriculture, Hokkaido University, Sapporo, JAPAN

TABLE 1. Nine isozyme loci examined and allelic constitutions of four parental strains used in the trisomic analysis

Locus	Enzyme species	Allelic constitution			
		Nipponbare	Acc001	W628	W1653
Acp-1	Acid phosphatase	+9	−4	+9/−4*	+9
Amp-2	Aminopeptidase	1	2	1/2*	1/2*
Cat-1	Catalase	2	1	1/2*	2
Est-2	Esterase	0	2	1	0
Pgd-1	Phosphogluconate dehydrogenase	1	1	2/2*	1
Pgi-1	Phosphoglucose isomerase	2	1	1/2*	2
Pgi-2	do.	1	2	1/2*	1
Pox-2	Peroxydase	0	4C	0/4C*	0/4C*
Sdh-1	Shikimate dehydrogenase	2	2	4	1

*: Hetrozygous loci.
Nipponbare (Japonica), Acc001 (Indica), W628 and W1653 (*O. rufipogon*).

Among them, eight trisomic types were used as female for crossings to a Vietnamese strain, Acc001 (Indica) and two wild strains, W628 and W1653 (perennial lines of *O. rufipogon*). It is known that Acc001 and the wild rice strains carry isozyme alleles different from Japonica trisomic lines. Isozyme loci investigated and the allelic composition of the parental strains are given in Table 1 and Plate 1. There were some problems related with Indica-Japonica crossings such as F_1 hybrid sterility and F_2 segregation distortions. It was difficult to obtain enough seeds to examine F_2 populations in some cross combinations. On the other hand, wild strains, *Oryza rufipogon* were used for supplementary crosses to examine the isozyme loci which did not indicate a segregation in Japonica-Indica crosses. F_1 hybrids were grown in 1985 and 1986 and the trisomic plants were screened depending on morphological characters and chromosome counting. Both disomic and trisomic segregation ratios were examined in F_2 segregations of the crosses by the χ^2 test. As for the effect of the segregation distortion, it was possible to discriminate the trisomic ratios from the distorted disomic ratios because of the prominent decrease of the alleles derived from Indica parents in the trisomic segregation.

Linkage analysis: Recombination value between two loci was calculated from the maximum likelihood method given by ALLARD.[1]

Isozyme analysis: Plumules of F_2 seedlings were mainly used as materials for isozyme analysis. Two buffer systems modified in accordance with the methodology of CARDY *et al.*[7] were used for electrophoresis (Table 2). The respective species of isozymes were prepared with the method as shown in Table 3. Gels for continuous and discontinuous buffers contained 13% and 14% (W/V) starch, respectively (ratio of Connaught starch and self-made starch was 2 : 1). Sac-

TABLE 2. Continuous and discontinuous buffer system

Buffer system	Contents
Continuous buffer	Gel buffer: 15 mM histidine-HCl titrated to pH 7.6 with tris. Electrode buffer: 0.4 M tris titrate to pH 7.6 with citric acid.
Discontinuous buffer	A: 0.05 M tris titrated to pH 8.1 with citric acid B: 0.19 M boric acid titrated to pH 8.1 with NaOH Gel buffer: mixture of 9 parts of A solution, 1 parts of B solution and 2.5 mg saccharose. Electrode buffer: Only B is used.

TABLE 3. Appropriate buffer system for various enzymes

Enzyme	Buffer system
Aminopeptidase (AMP)	Continuous buffer system
Esterase (EST)	do.
Phosphogluconate dehydrogenase (PGD)	do.
Shikimate dehydrogenase (SDH)	do.
Acid phosphatase (ACP)	Discontinuous buffer system
Alcohol dehydrogenase (ADH)	do.
Catalase (CAT)	do.
Esterase (EST)	do.
Phosphoglucose isomerase (PGI)	do.
Peroxydase (POX)	do.

charose for discontinuous buffer, gel buffer and starch were mixed and heated in a hot bath. They were poured into gel trays and cooled before use. For extraction for isozyme assay, plumules and calli were crushed with a few drops of demineralized water. The filter paper which absorbed the extraction was inserted into gel for electrophoresis.

Continuous and discontinuous gels were run with their electrode buffer at 60 mA in a refrigerator. After one hour, only the discontinuous gel was electrophoresed at a constant voltage of 170 V. Both of them were run continuously for 5 hours. Then, gels were sliced and divided for staining of each isozyme species. Stains buffer and substrates for each isozyme are shown in Table 4.

Tissue culture: Three parental strains, H-79 (Japonica), Acc224 (Japonica) and IR36 (Indica) and their hybrids were used for the tissue culture. Calli derived possibly from scutellum of seeds were grown on MS medium at 25°C under light. They were subcultured every month on the fresh medium. Zymograms of 14 isozymes were investigated in the calli induced one month later from the plating

TABLE 4. Staining solution and buffer used for iozyme assays

Enzyme	Contents
ACP	75 ml 0.1 M CH_3COONa-acetic acid pH 5.0 buffer 100 mg Fast Garnet GBC 36 mg 1-naphthyl phosphate disodium salt
ADH	75 ml 0.1 M Tris-HCl pH 8.5 buffer 5 ml ethanol 5 mg NAD 5 mg MTT 1.5 mg PMS
AMP	75 ml 0.1 M Tris-maleic acid pH 6.9 buffer 12.5 mg Fast Black K salt 50 mg DL-alanine-2-naphthylamide (for *Amp-2*) 10 mg L-leucyl-2-naphtylamide (for *Amp-3*)
CAT	90 ml distilled water 5 mg $FeCl_3$ 5 mg $K_3Fe(CN)_6$ Gels were incubated in 3% H_2O_2 solution for a few minutes, then it was rinced with distilled water before staining.
EST	40 ml distilled water 50 ml 0.2 M NaH_2PO_4 10 ml 0.38 M Na_2HPO_4 2.5 ml N-propanol 50 mg Fast Blue RR
	15 mg 1-naphthyl acetate 20 mg 2-naphthyl acetate
PGD	75 ml 0.1 M Tris-HCl pH 8.0 buffer 5 mg 6-phosphogluconic acid trisodium salt 5 mg NADP 5 mg MTT 1.5 mg PMS
PGI	75 ml 0.1 M Tris-HCl pH 8.0 buffer 40 mg D-fructose-6-phosphate barium salt 5 mg NADP 5 mg MTT 1.5 mg PMS 10 units glucose-6-phosphate dehydrogenase
POX	0.05 M CH_3COOK-acetic acid pH 5.0 buffer 10 mg 3-amino-9-ethylcarbazole 2 ml 0.1 M $CaCl_2$ 1 ml 3% H_2O_2 and 1% acetic acid solution 2 drops of eugenol
SDH	75 ml 0.1 M Tris-HCl pH 8.5 buffer 10 mg shikimic acid 5 mg NADP 5 mg MTT 1.5 mg PMS

(initial stage). After three or four subcultures. zymograms of calli at the later stage were examined and compared with those at the initial stage.

Results

1. Loci of six isozyme genes

It is known that an Indica strain, Acc001 possesses variant alleles of seven isozyme species. 56 combinations between seven isozyme genes and the eight trisomic types were used for trisomic analysis. As shown in Table 5, χ^2 test indicated a significant deviation from the disomic ratios (1 : 2 : 1 or 1 : 3) in the 31 combinations. They were divided into two groups, an increased type of Indica alleles (II) or homo- and hetero-zygote of Indica alleles (II+IJ) and a decreased

TABLE 5. Segregations of isozyme loci in the 56 trisomic-isozyme gene combinations of the crosses between eight primary trisomics and an Indica strain, Acc001

Primary trisomic	*Acp-1*			*Amp-2*			*Cat-1*			*Est-2*		*Pgi-1*			*Pgi-2*			*Pox-2*	
	JJ	IJ	II	JJ	IJ	II	JJ	IJ	II	JJ	IJ+II	JJ	IJ	II	JJ	IJ	II	JJ	IJ+II
A	48	119	4**	9	27	12	33	50	26	4	44**	10	24	24	7	23	21*	58	117*
B	11	77	35**	26	75	31	46	67	17**	21	118**	16	48	32	14	69	13**	16	123**
C	10	22	24**	17	40	15	13	48	17	8	64**	26	39	16	23	25	33**	8	64**
D	38	82	60*	69	77	8**	21	72	56**	23	158**	36	103	36	37	94	50	28	153**
E	6	33	32**	16	37	25	13	47	15	8	64**	21	43	15	9	28	41**	10	71**
F	12	29	37**	13	40	27	15	37	23	10	70**	18	46	16	11	45	21	11	69**
G	20	76	54**	32	70	49	34	82	34	20	112**	36	61	45	23	80	49**	24	128**
H	7	22	13	13	20	10	6	26	11	1	42**	7	20	16	6	26	11	3	40**

J and I indicate alleles derived from Japonica and Indica parents, respectively.
**, *: Significant deviation from the disomic ratios (1:2:1 or 1:3) at the 1 and 5% levels, respectively.

TABLE 6. Segregations of isozyme loci in the six trisomic-isozyme gene combinations in the crosses between four trisomics and two strains of wild rice

Primary trisomic	*Pgd-1*			*Sdh-1*		
	J J	J W	WW	J J	J W	WW
A	—	—	—	12	10	3*
C	14	20	9	9	21	13
G	37	50	4**	21	52	19
H	—	—	—	56	90	30

J and W indicate alleles derived from Japonica trisomics and wild rice disomic strains, respectively.
**, *: Significant deviation from the disomic ratio (1:2:1) at the 1 and 5% levels, respectively.

type of II or II+IJ. There was a tendency that *Acp-1*, *Est-2*, *Pgi-2* and *Pox-2* show the former type of segregation irrespective of the trisomic types. There is a possibility that the segregation distortion is caused by the gametophyte genes[15,19,21] and/or the close linkage with F_1 sterility gene.[23,24] In contrast with this, the four combinations underlined indicated the later type of the segregation, and the participation of trisomic segregation was suggested. Therefore, the observed segregations were compared with those expected from the various ratios of segregations expected in F_2 of trisomic of *AAa* or *Aaa* genotypes[16] (Table 7 a).

Although the fitness of the observed segregations was inadequate in some combinations, it was plausible to estimate the trisomic segregation affected by the

TABLE 7. F_2 segregations of six isozyme genes in the progenies of trisomic F_1 plants derived from the crosses between the trisomics and Acc001 (a) or wild rice strains (b)

a. Cross; Trisomic × Acc001

Disomic or trisomic ratio	*Acp-1* JJ	IJ	II	χ^2	*Amp-2* JJ	IJ	II	χ^2	*Cat-1* JJ	IJ	II	χ^2	*Pox-2* JJ	IJ+II	χ^2
O	48	119	4		96	77	8		46	67	17		58	117	
D	42.8	85.5	42.8	48.9**	45.3	90.5	45.3	85.66**	32.5	65	32.5	13.06**	43.8	131.3	6.19*
T-1	57.0	104.5	9.5	6.62*	60.3	110.6	10.1	31.72**	43.3	79.4	7.2	15.35**	58.3	116.7	0.003(n.s.)
T-2	61.8	97.4	11.9	13.09**	65.4	103.1	12.6	22.62**	46.9	74.0	9.0	7.73*	63.2	111.8	0.67(n.s.)

b. Cross; Trisomic × W628 or W1653

Disomic or trisomic ratio	*Pgd-1* JJ	JW	WW	χ^2	*Sdh-1* JJ	JW	WW	χ^2
O	37	50	4		12	10	3	
D	22.8	45.5	22.8	28.42**	6.3	12.5	6.3	7.48**
T-1	30.3	55.6	2.25	2.25(n.s.)	8.3	15.3	1.4	5.31(n.s.)
T-2	32.9	51.8	51.8	1.44(n.s.)	9.0	14.2	1.7	3.16(n.s.)

O: Observed numbers, D: Theoretical numbers expected from the disomic segregation, 1:2:1 or 1:3 for *Pox-2*, T-1: Theoretical numbers expected from the randam chromatid segregation including both disomic and trisomic plants (50% transmission of the extra chromosome through the female), 6:11:1 or 1:2 for *Pox-2*, T-2: Theoretical numbers expected from the maximum equational segregation including both disomic and trisomic plants (50% transmission of the extra chromosome through the female) 26:41:5 or 13:23 for *Pox-2*.

**, *: Significant at the 1 and 5% levels, respectively.

segregation distortion. Thus, it was estimated that *Acp-1*, and *Pox-2* are located on trisomic A (linkage group *d-33*), *Amp-2* on trisomic D (linkage group *sug*) and *Cat-1* on trisomic B (linkage group I).

On the other hand, the locations of *Pgd-1* and *Sdh-1* were analyzed in the crossings with *O. rufipogon*. As shown in Table 6, the segregation ratio showed a significant deviation from 1:2:1 (disomic ratio) in the two combinations, between trisomic G and *Pgd-1*, and between trisomic A and *Sdh-1*. Both segregation ratios fitted into the trisomic ratios (Table 7 b). Thus it is demonstrated that *Pgd-1* is located on trisomic G (linkage group VIII) and *Sdh-1* belongs to trisomic A (linkage group *d-33*).

Triallelic heterozygotes: It is known that the allele dosage combinations are clearly identified in the locus of monomeric enzyme, such as *Sdh-1*. In this experiment, trisomic plants selected from trisomic A×W1653 showed one dose

of allele 1 from W1653 and two doses of allele 2 from trisomic A. Then the plant was crossed with an Indica strain, AG414 having allele 4. Out of eleven plants of B_1F_1, two plants showed the three different allelic bands (Plate 2-1). Therefore the results proposed evidence that *Sdh-1* is located on trisomic A (linkage group *d-33*).

Allele dosage effects: Gene dosage effects are expressed in trisomic and tetrasomic plants. If the isozyme locus is located on the critical trisomic plant, the one that has three copies of the relevant gene, the trisomics in F_2 should display an altered banding pattern reflecting the presence of two maternal alleles and one paternal allele. In F_1 and F_2 plants derived from trisomic G×Acc001, *Pgd-1* locus showed a dosage effect (Plate 2-2). Similar effects were detected in the locus of *Amp-2* and *Sdh-1* in the crossings with the critical trisomics, D and A respectively. In these cases, the bands derived from the trisomic parents were more intensified than those from diploid parents, while the reverse case showing the dark stained band of diploid allele appeared in some F_2 plants. It is probable that the recombinants having two doses of Indica allele and one dose of Japonica allele were produced among the trisomic plants.

2. Linkage relations

A linkage relation was proposed between the loci of *Adh-1* and *Pgd-1* in the linkage group VIII. As shown in Table 8, the recombination value was calculated as 19.4±0.03%.

Another linkage relation was detected between the loci of *Gdh-1* and *Pgi-1* as shown in Table 8. *Gdh-1* enzyme which was newly analyzed may be more than a trimeric form and the zymograms are shown in Plate 2-3. Only Acc259 indicated a variant having allele 2, while the other strains such as Taichung 65 and Acc001 and IR36 possessed the allele 1. Linkage analysis showed the

TABLE 8. F_2 segregation data showing linkage relations

a. Acc224 (*Adh-1*^{1}, *Pgd-1*^{1}) × Acc647 (*Adh-1*^{2}, *Pgd-1*^{2})

		Adh-1 1/1	1/2	2/2	Recombination value (%)
	1/1	21(14.9)	9(7.2)	1(0.9)	
Pgd-1	1/2	9(7.2)	25(31.6)	5(7.2)	19.4±0.03
	2/2	1(0.9)	6(7.2)	15(14.9)	

χ^2 for independence = 90.59**, $p<0.01$
χ^2 (recom. value = 19.4%) = 5.69, $p=0.5-0.7$

b. Acc001 (*Pgi-1*^{1}, *Gdh-1*^{1}) × Acc259 (*Pgi-1*^{2}, *Gdh-1*^{2})

		Gdh-1 1/1	1/2	2/2	Recombination value (%)
	1/1	33(35.5)	7(6.5)	0(0.3)	
Pgi-1	1/2	5(6.5)	76(71.5)	5(6.5)	8.4±0.02
	2/2	1(0.3)	8(6.5)	34(35.5)	

χ^2 for independence = 188.07, $p<0.01$
χ^2 (recom. value = 8.4%) = 3.52, $p=0.7-0.9$

Parenthesis means the theoretical numbers based on the respective recombination values estimated (19.4% or 8.4%).

recombination value 8.4±0.02% between *Gdh-1* and *Pgi*-1. Both genes belong to linkage group XI.

3. Expression of isozymes at cellular level

Allelic constitution of the materials are given in Table 9. The expression

TABLE 9. Allelic constitution of the isozyme loci in the three parental strains used for F_1 seed production

Locus	Allelic constitution			Locus	Allelic constitution		
	H-79	Acc224	IR36		H-79	Acc224	IR36
Acp-1	+9	+9	−4	*Est-2*	0	1	2
*Adh-1**	2	1	2	*Est-5*	2	2	2
*Adh-2**	1	1	1	*Est-9*	1	1	2
Amp-1	1	1	1	*Pgd-1*	1	1	2
Amp-2	1	1	2	*Pgi-1*	2	2	1
Amp-3	1	2	1	*Pgi-2*	1	1	2
Cat-1	2	2	1	*Sdh-1*	2	2	1

*: Enzyme species; Alcohol dehydrogenase.

TABLE 10. Expression of 14 isozyme genes in the various tissues and stages of whole plants and calli

Locus	Whole plant			Callus stage	
	Young root	Plumule	Mature leaf	Initial	Later
Acp-1	+	+	+	+	−
Adh-1	+	+	+	+	+
Adh-2	+	−	−	+	+
Amp-1	+	+	(+)	+	(+)
Amp-2	+	+	−	+	+
Amp-3	+	+	(+)	+	+
Cat-1	+	+	−	+	+
Est-2	+	+	+	+	+
Est-5	+	+	+*	+*	+*
Est-9	+	+	−	+	−
Pgd-1	+	+	−	−	−
Pgi-1	+	+	+	+	+
Pgi-2	+	+	+	+	+
Sdh-1	+	+	+	+	+

+: Expression present.
(+): Expression present showing a broad band.
+*: One of the bands is absent or shows a weak intensity.
−: Expression absent.

of 14 isozyme genes both at plant and cellular levels is shown in Table 10.

Acp-1: In the initial stage of callus, *Acp-1* expresses three bands as well as those in plumule.[9] However, the number of the bands gradually increased near the three bands resembling those in the mature leaf and their expression in calli became obscure after the second subculture.

Adh-1: Mostly stable throughout the plant and callus levels. Band expression in calli is intensified in comparison with those in plumule and mature leaf.

Adh-2: Expressed both at the initial and later stages of callus, though the enzymatic activity was slightly reduced at the later stage. It is known that *Adh-2* is expressed in young root and plumule grown only under anaerobic condition.

Amp-1: Expression in the later stage of calli was obscured and it was difficult to discriminate the genotypes owing to the expression of a broad band.

Amp-2: Stable expression throughout plant and cellular levels except mature leaf. Although the new bands appeared in the callus stages, the genotypes can be identified in the callus.

Amp-3: A new band appeared in the anodal side of the major bands in calli. Otherwise the expression is similar to those in plumule.

Cat-1: Most stable expression throughout all stages in both young plant and callus. It is known that *Cat-1* is expressed even in pollen grains.

Est-2: Band pattern by the use of the substrate, 2-naphthyl acetate is prominent and stable throughout all developing stages of plant and callus.

Est-5: A pair of bands was expressed in young root and plumule, while the absence of the band located on anodal side and a new band by the use of the substrate, 1-naphthyl acetate occurred below the cathodal band both in mature leaf and calli at the later stage.

Est-9: Band pattern by the use of the substrate, 2-naphthyl acetate is prominent in young root, plumule and calli at the initial stage. However, the band expression disappeared after the second subculture.

Pgd-1: The stable expression is restricted only in young root and plumule. No expression in mature leaf and calli at all stages.

Pgi-1: The expression is stable throughout all stages of both plant and callus.

Pgi-2: The same expression with *Pgi-1*.

Sdh-1: Expressed throughout all stages both in plant and callus. Sometimes a new band is recognized in the cathodal side.

Expression in F_1 hybrids

As shown in Table 11 and Plates 3 and 4, the expression of eleven kinds of isozyme genes was investigated in both parents and their F_1 hybrids. Among them, three genes, *Adh-1*, *Cat-1* and *Est-2* showed strong activity and their genotypes were clearly identified in the calli of the parents and their F_1s. In the cases of *Amp-2*, *Amp-3* and *Sdh-1*, one or two new bands appeared besides the two allelic bands in the calli of the parents and their F_1s. Both *Pgi-1* and

TABLE 11. Expression of 11 isozyme genes in the calli derived from the crossed seeds (F_1)

Locus	Expression in calli		Locus	Expression in calli	
	Initial	Later		Initial	Later
Acp-1	+	−	*Est-9*	+	−
Adh-1	+	+	*Pgd-1*	−	−
Amp-2	+	+	*Pgi-1*	+	+
Amp-3	+	+	*Pgi-2*	+	+
Cat-1	+	+	*Sdh-1*	+	+
Est-2	+	+			

+ : Heterozygous genotypes were identified.
− : Unidentified.

Pgi-2 showed normal expression throughout all materials in the parents and their F_1s, while their activity was reduced slightly in the calli at the later stage.

Discussion

As we reported earlier,[12,13,30] our trisomic analysis of isozyme genes indicated that *Cat-1* belongs to linkage group I, *Pgd-1* to linkage group VIII, *Acp-1*, *Pox-2* and *Sdh-1* to linkage group *d-33*. In addition, two linkage relationships were demonstrated between *Adh-1* and *Pgd-1* and between *Gdh-1* and *Pgi-1* with the recombination values, 19.4% and 8.4%, respectively. These relations coincided with those obtained by RANJHAN *et al.*[29] and WU *et al.*[35] As TANKSLAY and RICK[32] stated, isozyme markers are suitable for F_2 linkage studies of self pollinated crops because of their co-dominant nature. The consistency of the recombination values and chromosomal allocation between different authors indicates the reliability of the genetic analysis.

In tomato, 9 of the 12 chromosomes were marked in at least one position by using 34 genes.[32] After that, saturated linkage maps were constructed based on isozyme and random cDNA clones derived from mRNA. In rice, a RFLP genetic map containing 144 loci was constructed by using randomly selected single copy DNA clones.[34] Chromosomal location of isozyme genes can be effectively used for such mapping. We are also carrying on the mapping of isozyme genes in the conventional linkage maps which include 139 loci of morphological markers and useful genes for plant breeding.[17]

For their uses, 20 isozyme genes are assigned to the eight linkage groups quoting the previous publications (Table 12). As the isozyme gene mapping expands, various possibilities emerge for the use of gene engineering and plant breeding such as gene tagging, monitoring and screening of the genes responsible for agronomic traits.

Since isozymes are the direct end product of gene expression, character

TABLE 12. Chromosomal allocation of 20 isozyme genes for eight linkage groups

Linkage group	Chromosome (1)	(2)	Trisomic (3)	Genes	Reference
I	6	3	B	*Amp-3, Cat-1, Est-2 Pgi-2, Pox-5*	13, 20, 22, 30, 35
III	3	1	O	*Est-5, Got-1, Icd-1*	35
IV	10	7	F	*Est-9 (Est-8)*	29, 30
VIII	9	11	G	*Adh-1, Pgd-1*	12, 29, 35
X	8	2	N	*Amp-1*	35
XI+XII	5	4	M	*Gdh-1, Pgi-1*	29
d-33	4	6	A	*Acp-1, Acp-2, Pox-2 Sdh-1*	12, 13, 28, 29, 35
sug	12	8	D	*Amp-2, Amp-4*	12, 35

(1), (2), (3): Naming by NISHIMURA, SHASTRY *et al.* and IWATA and OMURA, respectively (Ref. 17).

expression is relatively stable. However, some parameters such as modification genes, growth environment, organ age and tissue specificity can induce changes of zymograms.[26,27,28] At the cellular level, only a few studies have dealt with the isozyme gene expression during *in vitro* culture in some plant species such as common[4] and bush beans[2,3], tobacco[5] and celery.[25]

We have planned on the use of isozyme markers for *in vitro* genetic selections and rapid assays for agronomic traits which require cellular expression. Our studies indicated that four out of the 14 isozyme loci, *Est-2, Pgi-1, Pgi-2* and *Sdh-1* showed a quite stable expression in all parts including callus, while the others showed a stage or organ-specific expression. Expression of seven loci, *Adh-1, Adh-2, Cat-1, Est-2, Pgi-1, Pgi-2* and *Sdh-1* remained in a stable pattern in both the initial and the later stages of seed calli. Therefore these loci can be safely used as a marker of chromosomes or chromosome segments at cellular level.

On the other hand, the expression of *Acp-1, Amp-1, Amp-2, Amp-3, Est-5* and *Est-9* showed an alteration during subcultures. In addition, *Pgd-1* was not expressed in either the initial or the later stage of calli. *Cat-1* was expressed in plumule but not in aged root at plant level. However, root-derived calli indicated the *Cat-1* band at cellular level. It was suggested that calli should tend to lose tissue specificity during subcultures.

Tissue culture might give rise to somaclonal variability and, in combination with *in vitro* selection, variants exhibiting specific resistance or tolerance can be isolated. The use of isozyme markers are also important in production of parasexual hybrids by cell fusion.[8] The development of new *in vitro* techniques with the aid of molecular markers such as isozyme and RFLPs are well advanced and can be used for the improvement of important cereal crops such as rice.[18]

Summary

Of the 9 isozyme loci studied, 6 have been assigned to 4 chromosomes through trisomic analysis. Primary trisomics with Nipponbare background were crossed with an Indica tester, Acc001 from Vietnam which carries variant alleles at several isozyme loci as compared with the trisomic series. Plumules of F_2 seedlings were used for isozyme assay and F_2 segregations were examined for fitness with the expected disomic ratio (1 : 2 : 1 or 3 : 1).

As to the *Acp-1*, *Amp-2*, *Cat-1* and *Pox-2* loci, a trisomic ratio was satisfied showing that homozygotes for the allele from the trisomic parent are significantly larger than those for the allele from the Indica tester. The segregation distortion caused by the gametophyte gene and F_1 sterility gene or genes was observed in some Indica-Japonica crosses. An increase of the Indica allele over those from the trisomic parents in Indica-Japonica crosses was clearly distinguished from the trisomic segregation which shows an opposite type of segregation in most cases.

Besides that, the loci of *Pgd-1* and *Sdh-1* were determined from the crosses, the trisomics × wild rice (*O. rufipogon*) strains. Thus, it was estimated that *Cat-1* is located on chromosome 6 (linkage group I), *Pgd-1* on chromosome 9 (linkage group VIII), *Acp-1*, *Pox-2* and *Sdh-1* on chromosome 4 (linkage group *d-33*) and *Amp-2* on chromosome 12 (linkage group *sug*).

Two linkage relations were presented between *Adh-1* and *Pgd-1* and between *Gdh-1* and *Pgi-1* with the recombination values, $19.4 \pm 0.03\%$ and $8.4 \pm 0.02\%$ respectively. Therefore a new gene, *Gdh-1* belongs to linkage group XI.

Allele expression of 14 isozyme loci were studied in calli derived from 3 parental strains and their hybrids. Four out of 14 isozyme loci, *Est-2*, *Pgi-1*, *Pgi-2* and *Sdh-1* showed a quite stable expression but the others showed a stage- or organ-specific expression. Expressions of seven loci, *Adh-1*, *Adh-2*, *Cat-1*, *Est-2*, *Pgi-1*, *Pgi-2* and *Sdh-1* were stable in both the initial and later stages of the seed calli. These loci can be safely used as a marker of specific chromosome or chromosome segment at cellular level. During the subcultures, phenotypes of *Acp-1*, *Amp-1*, *Amp-2*, *Amp-3*, *Est-5* and *Est-9* were modified or disappeared. *Pgd-1* was not expressed in either initial or later stages. *Cat-1* was expressed in plumule but not in old root tissue. However, root-derived calli showed *Cat-1* band. It was suggested that calli should tend to lose tissue specificity during subcultures. The development of new *in vitro* techniques with the aid of molecular markers (isozyme and RFLPs) both at plant and cellular levels are important for the varietal improvement of rice.

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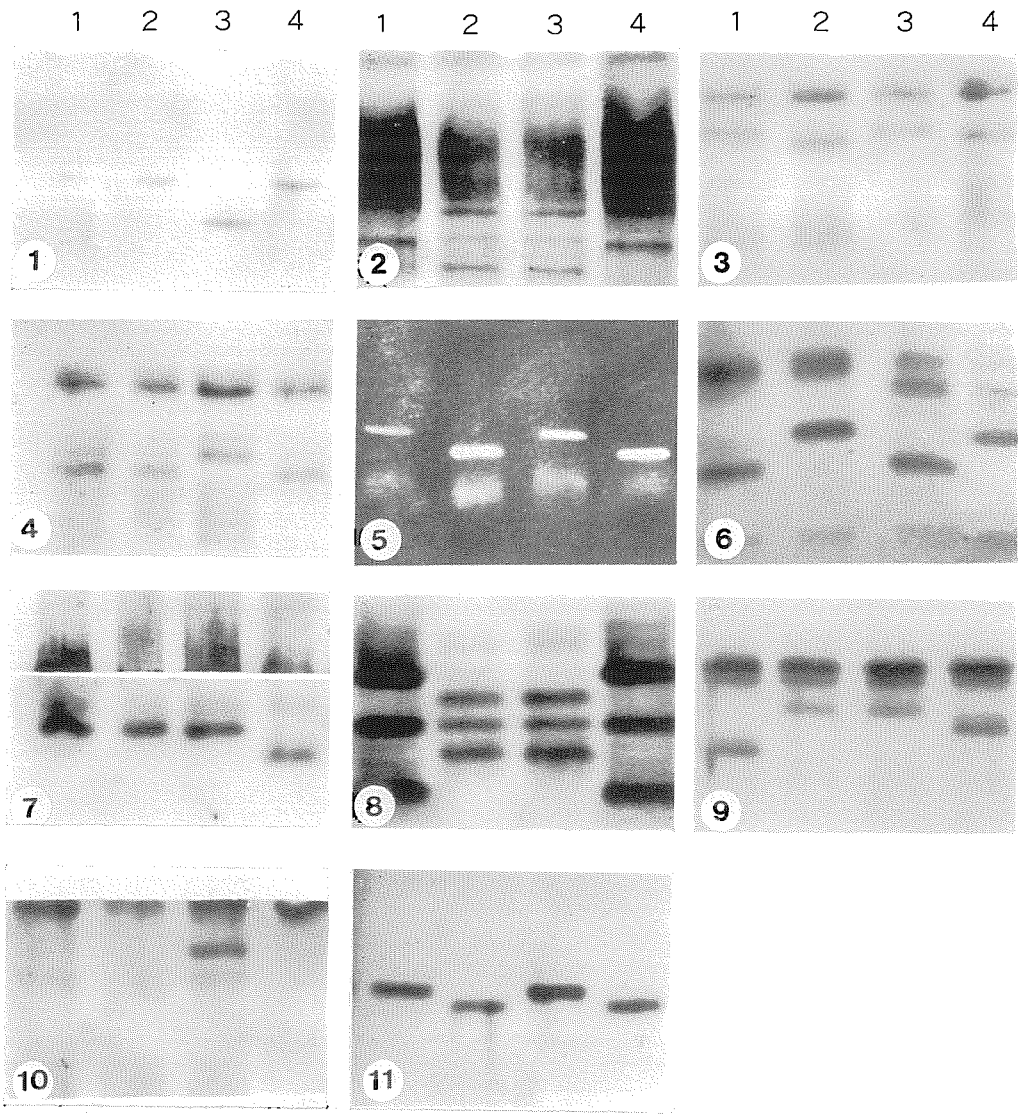
Explanation of Plates

Explanation of Plates

Plate 1. Zymograms showing 11 isozyme genes.

No.	Isozyme		Lane 1	Lane 2	Lane 3	Lane 4
1.	Alcohol dehydrogenase	*Adh-1*	2	2	1	2
2.	Acid phosphatase	*Acp-1*	−4	+9	+9	−4
3.	Aminopeptidase[1]	*Amp-2*	2	1	2	1
4.	Aminopeptidase[2]	*Amp-3*	1	1	2	1
5.	Catalase	*Cat-1*	2	1	2	1
6.	Esterase	*Est-2*	1	2	1	2
7.	Esterase	*Est-9*	1	1	1	2
8.	Phosphoglucose isomerase	*Pgi-1*	1	2	2	1
		Pgi-2	2	1	1	2
9.	Phosphogluconate dehydrogenase	*Pgd-1*	1	2	2	1
10.	Peroxidase	*Pox-2*	0	0	4C	0
11.	Shikimate dehydrogenase	*Sdh-1*	2	1	2	1

1) Substrate; DL-alanine-2-naphthylamide.
2) Substrate; L-leucyl-2-naphthylamide.

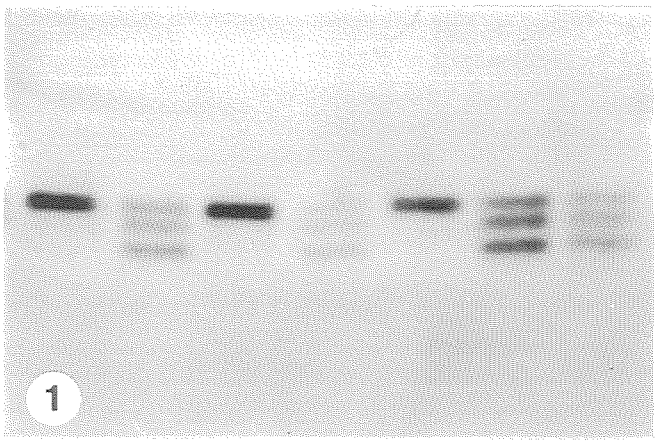

1 2 3 4
1 2 3 4
1 2 3 4
1
2
3
4
5
6
7
8
9
10
11

Explanation of Plates

Plate 2. 1: Triple heterozygotes at *Sdh-1* locus segregated in BF_2 population.

2: Allele dosage effects at *Pgd-1* locus.
Lane 3 and 4: Plants having two doses of allele 1 and one dose of allele 2.

3: GDH-1 zymograms of parents and their F_1s.
Lane 1 and 2: Parental strains, Acc259 and Acc001 respectively.
Lane 3 and 4: Heterozygotes in F_2
Lane 5, 6 and 7: Homozygotes in F_2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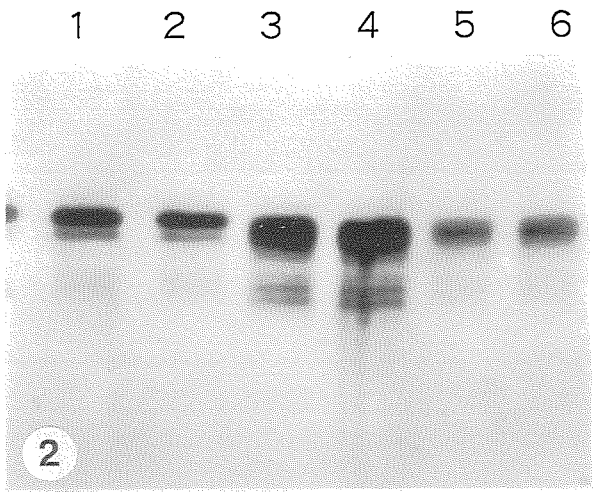
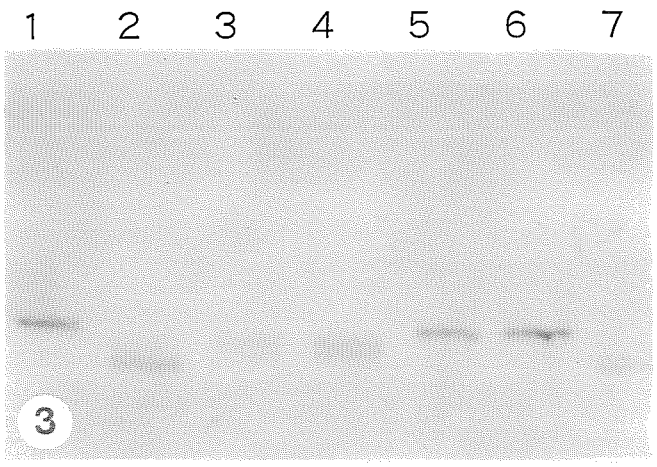
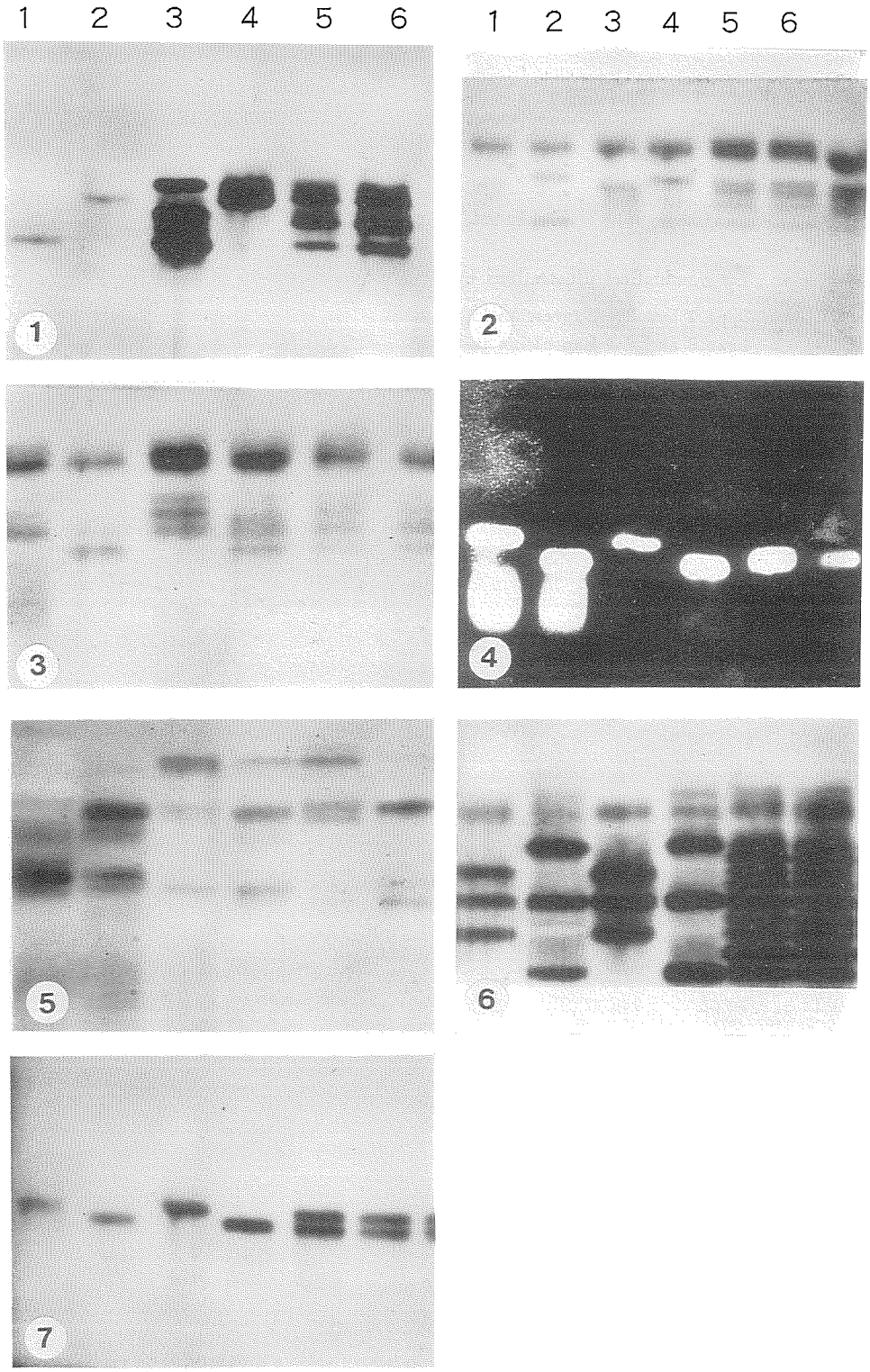

1 2 3 4 5 6
2


1 2 3 4 5 6 7
3

Explanation of Plates

Plate 3. Zymograms of 11 isozyme loci both in plant and callus.

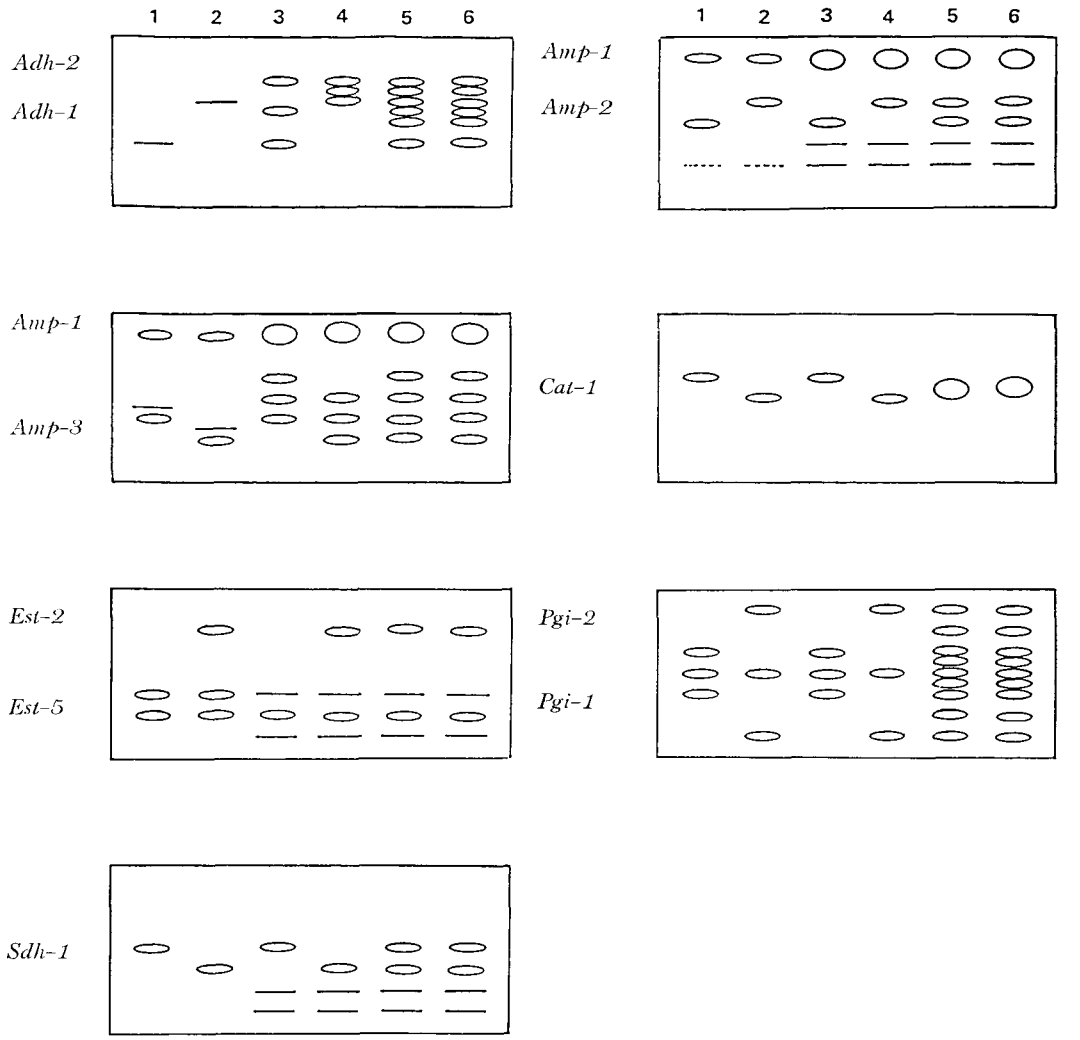
1: Alcohol dehydrogenase-1, 2.
2: Aminopeptidase-1, 2.
3: Aminopeptidase-1, 3.
4: Catalase.
5: Esterase-2, 5.
6: Phosphoglucose isomerase-1, 2.
7: Shikimate dehydrogenase.


1 2 3 4 5 6
1 2 3 4 5 6
1
2
3
4
5
6
7

Explanation of Plates

Plate 4. Diagrammatical illustrations of the zymograms shown in Plate 4.

Lane 1 and 2: Expression in plumule.
Lane 3 and 4: Expression in calli.
Lane 5 and 6: Expression in calli induced from the crossed seeds (F_1).


1 2 3 4 5 6
Adh-2
Adh-1
1 2 3 4 5 6
Amp-1
Amp-2
Amp-1
Amp-3
Cat-1
Est-2
Est-5
Pgi-2
Pgi-1
Sdh-1