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Electrophoretic variation of isozymes in plumules of rice (*Oryza sativa* L.)— a key to the identification of 76 alleles at 24 loci

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J.C. Glaszmann, B.G. de los Reyes, and G.S. Khush[1]

ABSTRACT

A simple and efficient method has been developed for studying isozyme variation in rice. It involves starch gel electrophoresis of crude rice plumule extracts, followed by staining of 13 enzymes. It permits monitoring the variation at 24 polymorphic loci distributed on at least 8 chromosomes. Technical procedures are described, and the zymograms obtained from materials containing all known alleles are shown. A total of 76 alleles can be readily identified.

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ELECTROPHORETIC VARIATION OF ISOZYMES IN PLUMULES OF RICE (*ORYZA SATIVA* L.) — A KEY TO THE IDENTIFICATION OF 76 ALLELES AT 24 LOCI

Isozyme polymorphism in Asian cultivated rice (*Oryza sativa* L.) has received much attention in recent years. A major input was the demonstration by Second and Trouslot (1980) that considerable variation can be revealed by starch gel electrophoresis. Some extensive studies have involved up to 25 genes expressed at tillering and flowering (Second 1982), and 21 genes in coleoptiles a few days after germination (Glaszmann 1985a). At present, at least 36 polymorphic loci can be surveyed during the development of the rice plant. Location of isozyme loci on the rice chromosomes has progressed simultaneously, by linkage analysis (Glaszmann 1985b, Nakagahra and Hayashi 1976, Pai et al 1975, Sano and Barbier 1985, Sano and Morishima 1984) and by trisomic analysis (Ishikawa et al 1986, Ranjhan et al 1986, Wu 1987). The former has resulted in the precise mapping of 3 loci, while the latter has associated 16 genes with their respective chromosomes.

Knowledge of the extent of polymorphism and the chromosomal location of genes encoding isozymes makes them helpful as genetic markers in rice. These genes present various advantages (Tanksley and Rick 1980), such as stable expression in a wide range of environments; the absence of epistatic interrelationships, which permits surveying many genes simultaneously; and, usually, codominance, which permits determining the exact genotype. The use of tissues of young plants simplifies the manipulation of the materials, permits early determination of their enzymatic characters, and thus makes the possible associated screening procedures more efficient.

We have focused on isozymes in rice plumules. In this paper, we review procedures for enzyme extraction, electrophoresis, and staining, and we give precise descriptions of the zymograms obtained and discuss their genetic control.

PROCEDURES

Materials

The varietal materials that were subjected to isozyme analysis to produce the zymograms described below are listed in Table 1. They were selected from the world collection maintained at the International Rice Germplasm Center (IRGC) at the International Rice Research

Table 1. Enzyme variation observed in plumules and coleoptiles of *O. sativa* rices.

Enzyme[a]	Locus[b]	Allele	Frequency[c]	Marker accession[d]
Phosphoglucose isomerase (PGI)	*Pgi-1*	1	F	6274, 27748, 43400
		2	F	12880, 43675, 33888
		3	VR	CNPAF 800157
		4	VR	19906
	Pgi-2	1	F	6274, 27595
		2	F	29726, 41048
		3	R	6245, 6538
		4	R	12880, 9158
Glutamate oxaloacetate transaminase (GOT)	*Got-1*	1	VF	6274, 6538, 27748
		2	VR	6245
		3	VR	42469
	Got-3 (t)	1	VF	8896, 9145, 27748
		2	VR	6304
Shikimate dehydrogenase (SDH)	*Sdh-1*	1	F	6245, 12880, 6538
		2	F	23754, 27748, 43675
		3	VR	53950
		4	VR	53642, 6274
Alcohol dehydrogenase (ADH)	*Adh-1*	0	VR	NIG 624
		1	VF	6274, 27748, 32301
		2	R	23754, 43675
		3	R	6245

Continued on next page

Table 1 continued

Enzyme[a]	Locus[b]	Allele	Frequency[c]	Marker accession[d]
Isocitrate dehydrogenase (ICD)	*Icd-1*	1	VF	6274, 53642, 37901
		2	R	27642
		3	VR	9158
Phosphogluconate dehydrogenase (PGD)	*Pgd-1*	1	F	6538, 12880
		2	R	7755
		3	F	6274, 6245
	Pgd-2 (t)	1	VF	6274, 6245, 6538
		2	VR	43675
Malic enzyme (MAL)	*Mal-1*	1	F	12880
		2	F	27748, 6274
Aminopeptidase (AMP) Leu-NAm Arg-NAm Ala-NAm substrates	*Amp-1*	0	VR	29040
		1	F	27595, 6274, 43675
		2	F	12880, 6245, 6538
		3	R	23754
		4	R	33888
		5	VR	27748
		6	VR	9011
Aminopeptidase (AMP) Ala-NAm substrate	*Amp-2*	1	F	12880, 19906
		2	F	6274, 6245
		3	VR	6434
		4	VR	32301
Aminopeptidase (AMP) Leu-NAm substrate	*Amp-3*	0	R	12880, 33888
		1	F	6274, 43675
		2	F	6245, 27748, 6434
		3	VR	43400, 32575
		4	R	33888, 9158
		5	VR	33153
		6	R	6538, 27595
Aminopeptidase (AMP) Arg-NAm substrate	*Amp-4*	1	VF	6274, 12880, 6245
		2	R	33191, 23754
		3	VR	33688
Endopeptidase (ENP)	*Enp-1* (t)	0	?[e]	9145
		1	VF	8896, 27748, 27762
Esterase (EST)	*Est-1*	0	F	43675, 23754
		1	F	6274, 33888, 27748
	Est-2	0	F	43675, 12880, 27748
		1	F	37901, 23754, 6434
		2	F	6274, 33153, 53642
	Est-5	0	VR	37801
		1	VF	6274, 32301, 6434
		2	R	6538, 27595
	Est-9	1	F	12880, 6245
		2	F	6274, 6538
Acid phosphatase (ACP)	*Acp-1*	1	F	6245, 6538
		2	F	12880, 43675
		3	VR	27595
	Acp-2	0	F	6245, 6538
		1	F	43675
	Acp-4	1	F	23754, 6274
		2	F	6245, 6538
Catalase (CAT)	*Cat-1*	1	F	6274, 6245, 6538
		2	F	23754, 32301
		3	VR	43675
Peroxidase (POX)	*Pox-5*	1	F	6274, 6245, 27748
		2	F	6538, 12280

[a] Leu-NAm, Arg-NAm, and Ala-NAm stand for leucyl-β-naphthylamide, arginyl-β-naphthylamide, and alanyl-β-naphthylamide, respectively. [b] (t) stands for tentative: no allelism test has been conducted yet. [c] 0% < VR (very rare) < 1% < R (rare) < 10% < F (frequent) < 90% < VF (very frequent) < 100%. [d] These are accessions of the International Rice Germplasm Center, except one from the Centro Nacional de Pesquisa-Arroz, Feijão, Golania, Brazil, and one from the National Institute of Genetics, Misima, Japan. [e] Fewer than 50 accessions have been analyzed.

Institute (IRRI), with the exception of one line from the Centro Nacional de Pesquisa-Arroz, Feijão (CNPAF), Goiania, Brazil, and one from the National Institute of Genetics (NIG), Misima, Japan. This sample contained all the alleles observed in a survey of more than 3,000 accessions from 6 continents.

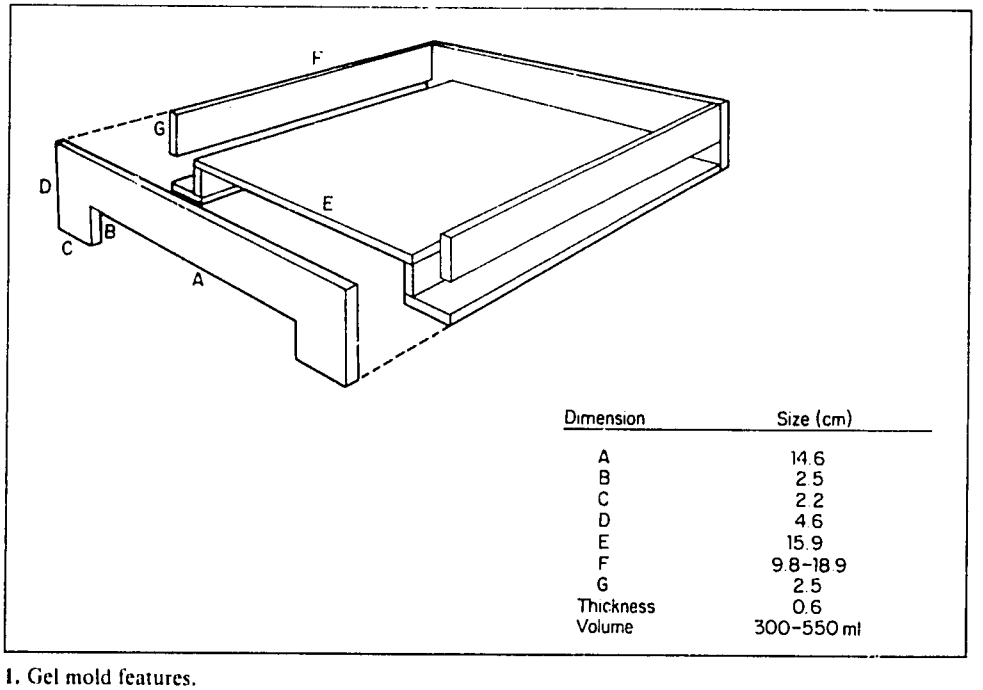
Gel and sample preparation

A starch gel is prepared using the appropriate gel buffer for the specific enzymes under investigation (Table 2). A solution of hydrolyzed starch (14%) and buffer is carefully homogenized in a 1-liter erlenmeyer flask and heated with continuous swirling on a magnetic stirrer hotplate until a clear, vigorously boiling solution is obtained. The solution is then deaerated with a tap aspirator and poured into an acrylic gel mold (Fig. 1) in which electrode strips have been sealed with masking tape. Solid particles and air bubbles can be quickly removed with forceps. The gel is allowed to cool and set for approximately 30 min at room temperature and is then covered with a plastic film

Table 2. Buffer systems for electrophoresis (see Appendix 1 for the chemicals).

Enzyme[a]	Buffer system	Stock solutions		
		Composition	Amount	Directions for using
PGI SDH ADH ICD AMP EST	I. Gel Tris (0.009 M) Histidine (0.005 M) pH 8.0	Trizma base (Tris) Histidine mono HCl Completed with H_2O	10.40 g 9.60 g 0.5 liter	Dilute 20 times before use
CAT (GOT)	I. Electrode Tris (0.400 M) Citrate (0.105 M) pH 8.0	Trizma base (Tris) Citric acid, H_2O Completed with H_2O	96.92 g 44.33 g 2.0 liter	Ready for use
GOT PGD ME ACP	II. Gel Tris (0.076 M) Citrate (0.006 M) pH 8.8	Trizma base (Tris) Citric acid, 1 H_2O Completed with H_2O	46.05 g 5.85 g 0.5 liter	Dilute 10 times before use
POX (PGI) (ADH) (CAT)	II. Electrode Na-borate (0.30 M) pH 8.2	Sodium hydroxyde pellets Borid acid Completed with H_2O	4.80 g 37.10 g 2.0 liter	Ready for use

[a]Without parentheses = currently used in our laboratory, in parentheses = can be used efficiently.



1. Gel mold features.

to prevent excessive dehydration. It is placed for 20 min in a freezer (-15 °C) or for 1 h in a refrigerator for final cooling before use.

The plumules and coleoptiles at 4-10 d after germination are used for enzyme extraction. They are placed on spot plates while the gel is cooling. When the gel is cold, a slit is prepared approximately 6 cm from its cathodal end; a bromophenol blue solution is applied to serve as a tracking dye. The plant tissues are ground with a small amount of cold distilled water. Filter paper wicks (whatman no. 3) are used to absorb the extracts. Their sizes can be adjusted to the specific requirements of the experiment. They are inserted in the slit so that they form a continuous arrangement, to avoid distortion on the sides of the papers and to facilitate comparison of migration distances among the bands produced. Intermixing between adjacent papers is avoided by removing excess extract with absorbent paper prior to insertion into the gel.

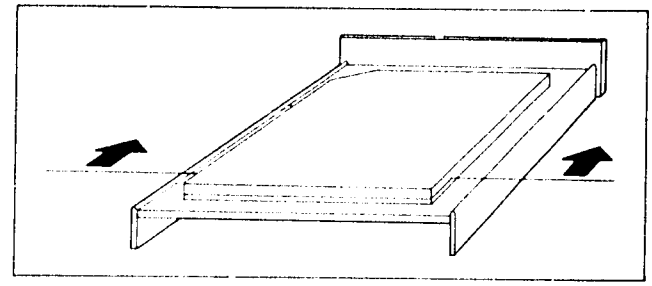
Adding a small amount of an antioxidant (e.g., 0.1% mercaptoethanol) to the water used for extraction can improve the quality of the zymograms for peroxidase and alcohol dehydrogenase. However, most enzymes can be satisfactorily investigated with pure water.

Electrophoresis

The plastic film is trimmed at the edges of the mold, and the masking tape is peeled off to expose the gel in the electrode strips. The gel is then mounted onto the electrode trays containing the appropriate tray buffer in a refrigerator at about 2 °C. The side where the samples were loaded is connected to the cathodal tray. A plastic bag of ice water is placed atop the gel to provide additional cooling. Platinum wire in the anodal tray and ordinary stainless steel wire in the cathodal tray serve as electrodes and are connected to a continuous-current power supply. A potential difference is applied through the gel. The constant parameter is the intensity, which is chosen so that the initial voltage will be about 10 volts/cm of length of the gel. The electrophoresis is stopped after 4 h.

Slicing

After electrophoresis, the gel is removed from the refrigerator, and rectangular slabs are prepared with the anodal and cathodal parts starting from the origin of migration. A diagonal slash is made on the upper right corner of the anodal slab and the lower right corner of the cathodal slab to later trace back the initial arrangement of the samples. Several slices can be prepared from the slabs to stain several enzymes. A slab is placed on an acrylic slicing bed (Fig. 2), and a wire is drawn horizontally through the gel to cut a 1-mm slice. The upper part of the gel is then placed on another slicing bed, and the accessible slice is transferred to a stain box. This procedure is repeated until the desired number of slices is prepared.



2. Gel slicing procedure.

Staining

Zones of enzymatic activity are revealed by immersing the gel slice into a stain assay (Table 3). The stain boxes chosen are only slightly larger than the slices so that 50 ml of solution is sufficient to stain a slice. Some assays may have specific requirements such as total darkness, incubation at 40 °C, immediate scoring (catalase), or overnight staining (acid phosphatase), which are summarized in Table 3.

In markedly disjointed activity zones, an individual slice can be used to reveal several enzymes: 1) by cutting several portions in the slice (e.g., catalases from 0 to 4 cm, alanine aminopeptidases from 4 to 8 cm, slow-moving acid phosphatases from 0 to 3 cm, and esterase from 3 to 8 cm); 2) by staining an enzyme, scoring it, then adding the substrate for a second enzyme (e.g., shikimate dehydrogenases followed by isocitrate dehydrogenases); or 3) by using the agar layer method for an enzyme and reusing the same slice by immersing in another assay solution (e.g., phosphoglucose isomerases followed by alcohol dehydrogenases).

For cases 1 and 2, the possibility of unexpected alleles must be kept in mind, requiring special attention.

Table 3. Staining procedures (see Appendix 1 for the chemicals and Appendix 2 for the buffers).

Phosphoglucose isomerase (PGI)		
Fructose-6-phosphate	50 mg/ml	1 ml
Tris-HCl buffer, 0.5 M, pH 8.5		20 ml
$MgCl_2$ solution, 0.1 M		2 ml
β-nicotinamide adenine dinucleotide phosphate (NADP)	5 mg/ml	1 ml
Glucose-6-phosphate dehydrogenase	10 units/ml	1 ml
Just before mixing with agar solution, add:		
Nitroblue tetrazolium salt (NBT)	10 mg/ml	1 ml
Phenazine methosulfate (PMS)	1 mg/ml	1 ml

Mix with 25 ml 2% agar (0.5 g/25 ml) formerly brought to 80 °C (boiling) and kept at 60 °C. Immediately pour into the stain box. The starch gel is later placed on the agar layer.

Protect from light. Incubate for 30 min at 40 °C.

Glutamate oxaloacetate transaminase (GOT)	
DL-aspartic acid	100 mg

Continued on opposite page

Table 3 continued

α-ketoglutaric acid		50 mg
Tris-HCl buffer, 0.5 M, pH 8.5		20 ml
Pyridoxal 5-phosphate		1 mg
	Water	50 ml
Just before use, add:		
Fast blue BB salt (75%)		40 mg

Protect from light. Incubate for 30 min at 40 °C.

Shikimate dehydrogenase (SDH)

Shikimic acid	25 mg/ml	1 ml
Tris-HCl buffer, 0.5 M, pH 8.5		10 ml
NADP	5 mg/ml	1 ml
	Water	50 ml
Just before use, add:		
NBT	10 mg/ml	1 ml
PMS	1 mg/ml	1 ml

Protect from light. Incubate for 30 min at 40 °C.

Alcohol dehydrogenase (ADH)

Ethanol, absolute		0.25 ml
Tris-HCl buffer, 0.5 M, pH 8.5		5 ml
β-nicotinamide adenine dinucleotide	10 mg/ml	1 ml
	Water	50 ml
Just before use, add:		
NBT	10 mg/ml	1 ml
PMS	1 mg/ml	1 ml

Protect from light. Incubate for 20 min at 40 °C.

Isocitrate dehydrogenase (ICD)

DL-isocitric acid	100 mg/ml	1 ml
Tris-HCl buffer, 0.5 M, pH 8.5		10 ml
NADP	5 mg/ml	1 ml
	Water	50 ml
Just before use, add:		
NBT	10 mg/ml	1 ml
PMS	1 mg/ml	1 ml

Protect from light. Incubate for 30 min at 40 °C.

Arginine aminopeptidase (AMP)

L-arginyl-β-naphthylamide	5 mg/ml[a]	5 ml
Fast black K salt		15 mg
Tris-maleate buffer, 0.2 M, pH 3.3		25 ml
NaOH solution, 0.1 M		20 ml

Incubate for 30 min at 40 °C.

Alanine aminopeptidase (AMP)

DL-alanyl-β-naphthylamide	10 mg/ml[a]	5 ml
Fast black K salt		15 mg
Tris-maleate buffer, 0.2 M, pH 3.3		25 ml
NaOH solution, 0.1 M		20 ml

Incubate for 60 min at 40 °C.

Endopeptidase (ENP)

N-α-benzoyl-DL-arginine-β-naphthylamide	5 mg/ml[a]	5 ml
Fast black K salt		15 mg
Tris-maleate buffer, 0.2 M, pH 3.3		25 ml
NaOH solution, 0.1 M		20 ml

Incubate for 30 min at 40 °C.

Esterase (EST)

α-naphthyl acetate	50 mg/ml	1 ml
β-naphthyl acetate	25 mg/ml in N-propanol or acetone	1 ml

Continued in next column

Phosphate buffer, 0.1 M, pH 6.5		50 ml
Fast garnet GBC salt		15 mg

Incubate for 45 min at 40 °C.

Phosphogluconate dehydrogenase (PGD)

Phosphogluconic acid	10 mg/ml	1 ml
Tris-HCl buffer, 0.5 M, pH 8.5		10 ml
$MgCl_2$ solution, 0.1 M		2 ml
NADP	5 mg/ml	1 ml
	Water	50 ml
Just before use, add:		
NBT	10 mg/ml	1 ml
PMS	1 mg/ml	1 ml

Protect from light. Incubate for 45 min at 40 °C.

Malic enzyme (MAL)

DL-malic acid		250 mg
Tris-HCl buffer, 0.5 M, pH 8.5		20 ml
$MgCL_2$ solution, 0.1 M		1 ml
NADP	5 mg/ml	2.5 ml
	Water	50 ml
Just before use, add:		
NBT	10 mg/ml	1 ml
MPS	1 mg/ml	1 ml

Protect from light. Incubate for 2 h at 40 °C.

Leucine aminopeptidase (AMP)

L-leucyl-β-naphthylamide	5 mg/ml[a]	5 ml
Fast black K salt		15 mg
Tris-maleate buffer, 0.2 M, pH 3.3		25 ml
NaOH solution, 0.1 M		20 ml

Incubate for 45 min at 40 °C.

Acid phosphatase (ACP)

α-naphthyl acid phosphate		50 mg
Acetate buffer, 1 M, pH 4.65		10 ml
$MgCl_2$, 0.1 M		1 ml
Fast garnet GBC salt		25 mg

Incubate at 40 °C. Some bands appear quickly; some will need overnight incubation.

Catalase (CAT)

(1) H_2O_2 solution, 0.7%, (1 ml conc./50 ml) Pour onto the gel and wait until bubbles appear in the gel (1-3 min). Rinse.	50 ml
(2) KI solution, 1.5% (0.75 g/50 ml) Pour onto the gel. Bands appear as white on a blue background. Score quickly because they disappear after a few minutes.	50 ml

For gels at pH 8.8, it is necessary to add 1 drop of acetic acid to solution 2.

Peroxidase (POX)

3-amino-9-ethyl carbazole		20 mg
N, N-dimethyl-formamide		2.5 ml
Acetate buffer, 1 M, pH 4.65		5 ml
$CaCl_2$ solution, 0.1 M		1 ml
	Water	50 ml
Just before use, add:		
H_2O_2 solution 0.7% (1 ml conc./50 ml)		1 ml

Leave for 60 min.

[a] To prepare the stock solution, the substrate is first dissolved in a few drops of N, N-dimethyl-formamide.

Preservation

Gels can be preserved after rinsing with water and fixing in a methanol:water:acetic acid (5:5:1) solution.

An alternative method facilitating storage is gel drying; gels are rinsed with water, treated with a few drops of glycerol on both sides, placed between 2 wet cellophane paper sheets, clamped on a glass plate, and dried at 60 °C for 2 h.

ZYMOGRAMS AND GENETIC INTERPRETATIONS

Zymograms are presented separately for each enzyme as a photograph and a diagrammatic interpretation (Fig. 3-19). They display all bands encountered so far in *O. sativa*. Figure 20 shows all the zymograms observed in heterozygous materials.

The genetic interpretations are based on reports of Pai et al (1975), Nakagahra (1977), Second and Troaslot (1980), Sano and Morishima (1984), Glaszmann et al (1984), Sano and Barbier (1985), and many unpublished data obtained at the Office de la Recherche Scientifique et Technique Outre-Mer, Centre International de Recherche Agronomique pour le Developpement, and IRRI. The chromosomal locations of the genes were established by linkage analysis for *Est-2*, *Amp-3*, and *Pgi-2* and by trisomic analysis for the other 15 loci.

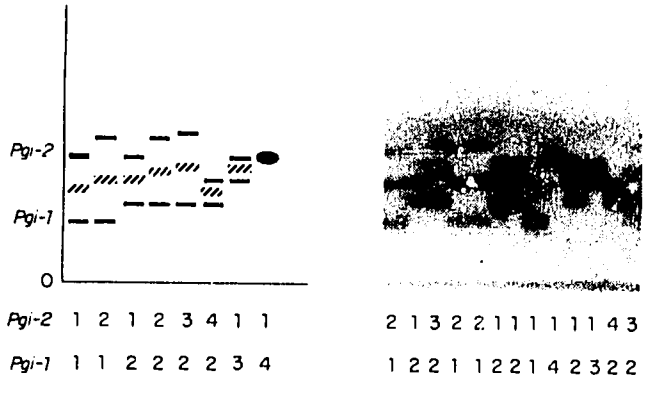
Phosphoglucose isomerase (PGI)

Zymograms show three bands in homozygous materials (Fig. 3). The enzymes are dimers, and the three bands correspond to the three combinations of the two protomers encoded at two loci, *Pgi-1* and *Pgi-2*.

Four alleles have been identified at each locus.

Single heterozygotes show 6 bands, whereas double heterozygotes show as many as 10 bands when there is no overlapping.

Pgi-1 is located on chromosome 4 and *Pgi-2* on chromosome 3.



3. Zymogram and diagrammatic interpretation of phosphoglucose isomerase (PGI).

Glutamate oxaloacetate transaminase (GOT)

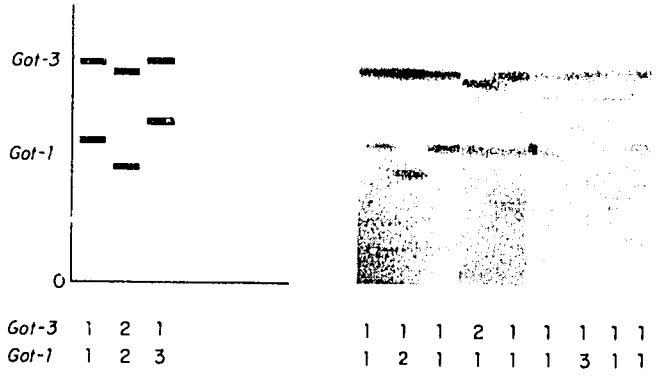
GOT zymograms usually show two bands (Fig. 4). The lower band is encoded at locus *Got-1*. GOT-1 enzymes are dimers, and thus heterozygotes produce three bands. Three alleles are known. *Got-1* is located on chromosome 1. The upper band is less variable. Two accessions with a slightly lower band have been observed. No hybrid having these has been produced yet. However, interspecific hybrids show three bands, which suggests that these enzymes are dimers. They are here considered as coded by the *Got-3* (t) gene (t for tentative).

Shikimate dehydrogenase (SDH)

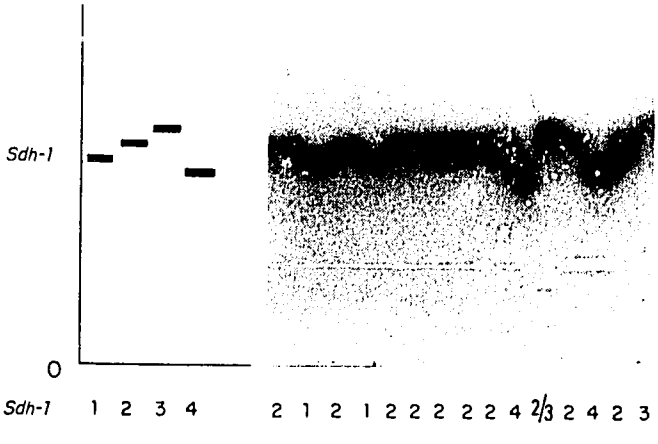
Homozygous materials are characterized by a single band, encoded by *Sdh-1*, which is located on chromosome 6 (Fig. 5). Four alleles are known. Isozymes are monomers, and hybrids produce two bands corresponding to the parental types.

Alcohol dehydrogenase (ADH)

Four types of zymograms were found, three showing a single band and one showing no band, corresponding to three active alleles and one silent allele at a single locus,



4. Zymogram and diagrammatic interpretation of glutamate oxaloacetate transaminase (GOT).



5. Zymogram and diagrammatic interpretation of shikimate dehydrogenase (SDH).

Adh-1 (Fig. 6). The enzymes are dimers, and heterozygotes show typical three-banded zymograms. *Adh-1* is located on chromosome 11.

Isocitrate dehydrogenase (ICD)

ICD zymograms consist of a major band sometimes accompanied by a slightly slower and faint secondary band (Fig. 7). There are three variant types, corresponding to three alleles at a single *Icd-1* locus located on chromosome 1. Heterozygotes display three major bands, typical of a dimeric enzyme, often accompanied by a slower secondary band.

Phosphogluconate dehydrogenase (PGD)

PGD zymograms comprise two zones of activity (Fig. 8). The lower zone consists of a single major band, sometimes accompanied by a faint, slightly faster secondary band. There are 3 migration rates, which correspond to the 3 alleles of the *Pgd-1* locus on chromosome 11. The heterozygotes display three bands, indicating a dimeric structure. The higher zone of activity consists of a single band, with two types, the faster one being very rare. Hybrids display three bands. This may correspond to a *Pgd-2* (t) gene encoding dimeric enzymes.

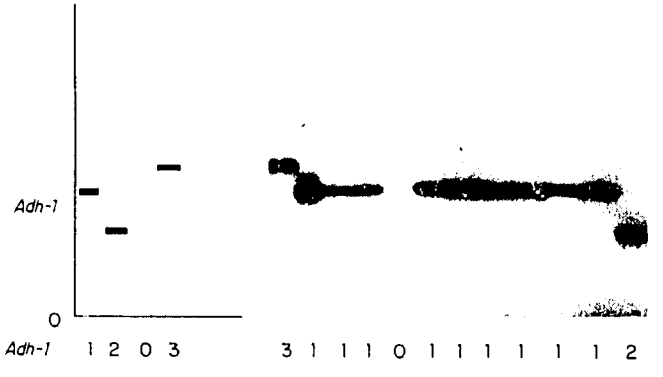
Malic enzyme (MAL)

MAL zymograms display two zones of high activity (Fig. 9). Only the higher zone shows repeatable variation. Two types are distinguished, one consisting of a single fast band, the other having three barely separated, slightly slower bands. The heterozygotes display a wide band that encompasses both parental types. Although the zymograms do not shed any light on the quaternary type of the enzyme, the variation clearly corresponds to polymorphism of a single gene, *Mal-1*.

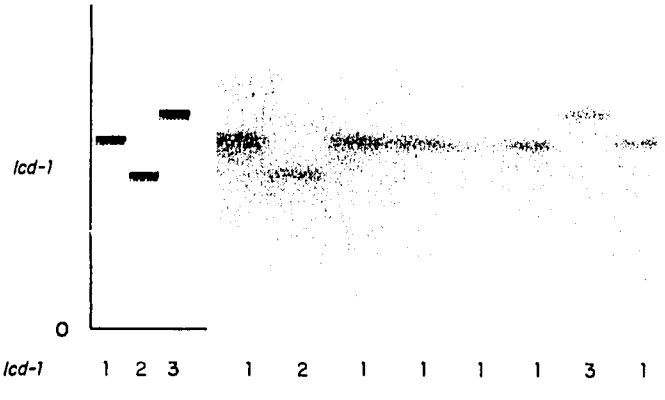
Aminopeptidases (AMP)

Aminopeptidases constitute a complex, with various forms contrastingly reacting with various substrates. Separate stainings using L-leucyl-β-naphthylamide (Leu-NAm), L-arginyl-β-naphthylamide (Arg-NAm), and DL-alanyl-β-naphthylamide (Ala-NAm) permit a clear understanding of the whole genetic control.

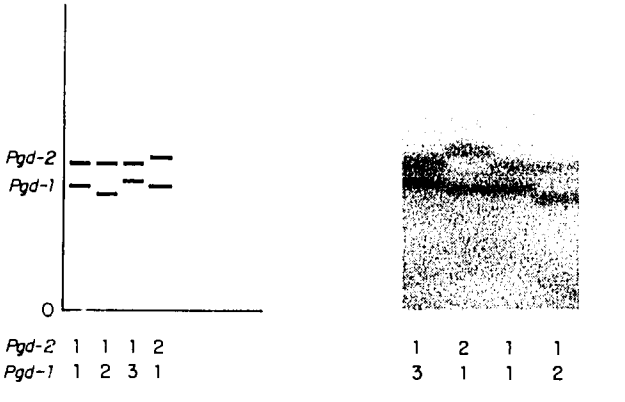
One gene, *Amp-1*, codes for enzymes active with the three substrates, which correspond to the bands observed with all three staining solutions (Fig. 10-13). Four migratory classes are distinguished, and one case of absence of band. The most frequent migratory class can be further split into three specificity classes (Fig. 13); band AMP-1[1] appears similarly intense with all substrates; band AMP-1[5] is faint with all substrates; band AMP-1[6]



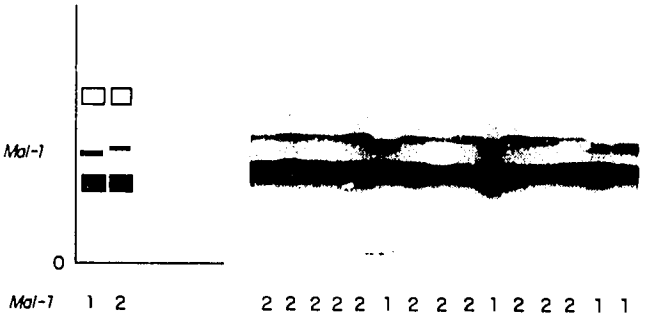
6. Zymogram and diagrammatic interpretation of alcohol dehydrogenase (ADH).



7. Zymogram and diagrammatic interpretation of isocitrate dehydrogenase (ICD).



8. Zymogram and diagrammatic interpretation of phosphoglucomate dehydrogenase (PGD).



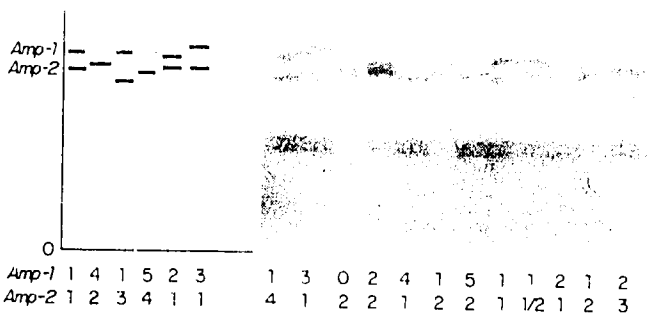
9. Zymogram and diagrammatic interpretation of malic enzyme (MAL).

has an intensity intermediate between those of the other two with Leu-NAm and Arg-NAm and is very faint with Ala-NAm. A similar pattern of specificity is observed for AMP-1[4]. Thus *Amp-1* has 7 alleles. It is located on chromosome 2.

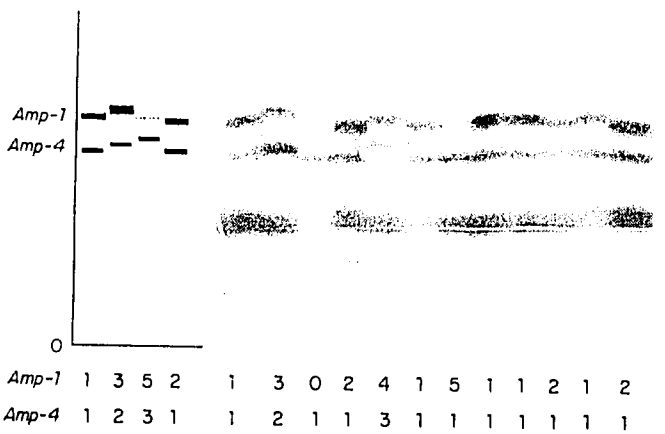
One gene, *Amp-2*, encodes enzymes most active with Ala-NAm (Fig. 10, lower bands). Four migratory classes are distinguished, corresponding to four alleles. *Amp-2* is on chromosome 8.

One gene, *Amp-4*, codes for enzymes most active with Arg-NAm (Fig. 11, lower bands). It has three alleles 1, 2, and 3. Band AMP-4[2] is more intense. *Amp-4* is on chromosome 8, but independent from *Amp-2*.

One gene, *Amp-3*, codes for Leu-NAm-specific enzymes (Fig. 12, lower bands). The corresponding bands appear quickly after the gel is put into the solution. Scoring should be done within 20 min, because then bands corresponding to AMP-2 and AMP-4 enzymes start to appear. As a matter of fact, bands AMP-1, -2, -3, and -4 can be seen on a gel after overnight staining. The zymograms are then very complex. There are seven *Amp-3* alleles. One is a silent allele. All active alleles are identified based on the migration rate of their bands; however, alleles 1, 2, and 3 produce very intense bands, while alleles 4, 5, and 6 produce faint bands.



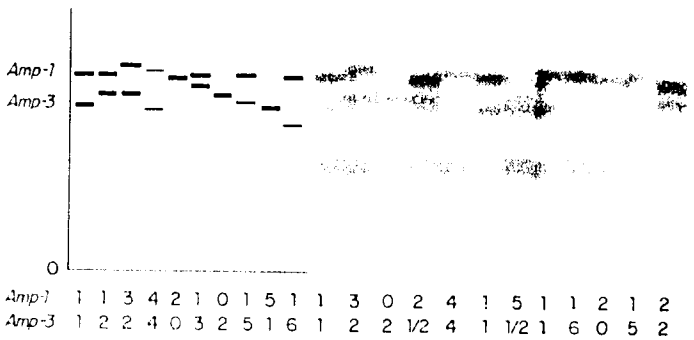
10. Zymogram and diagrammatic interpretation of aminopeptidase (AMP), alanine substrate.



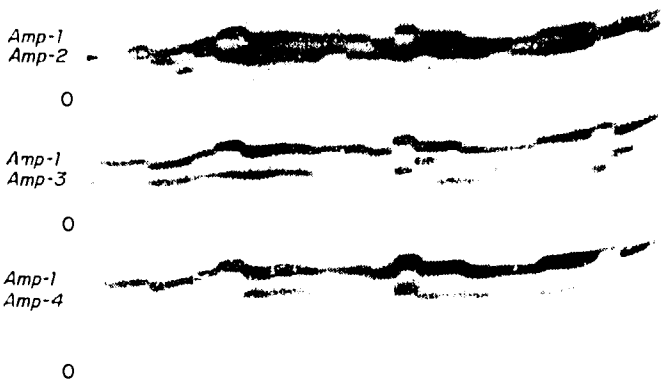
11. Zymogram and diagrammatic interpretation of aminopeptidase (AMP), arginine substrate.

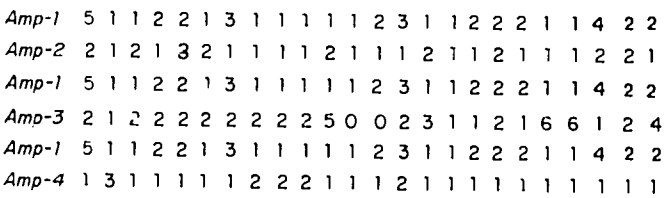
Endopeptidase (ENP)

ENP zymograms exhibit a single band (Fig. 14). One case of absence of band was also found. The variation is tentatively interpreted as a single gene, *Enp-1* (t).

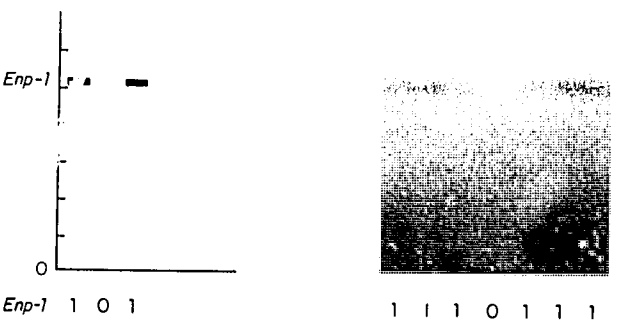


12. Zymogram and diagrammatic interpretation of aminopeptidase (AMP), leucine substrate.

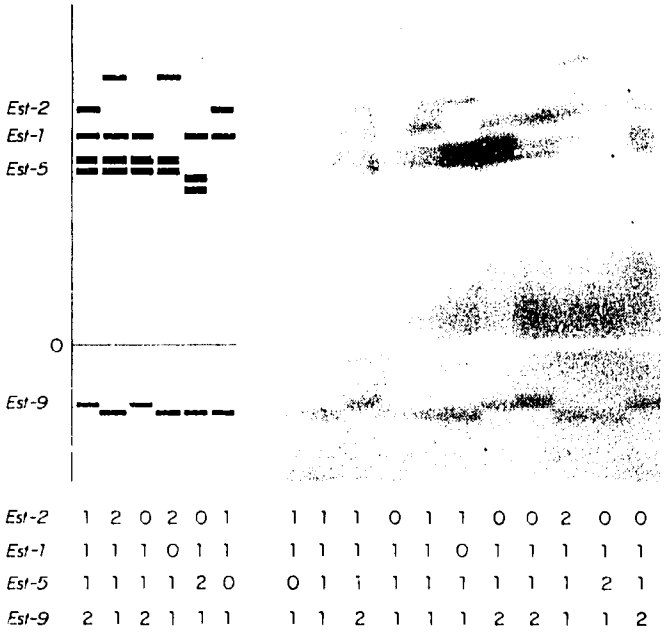




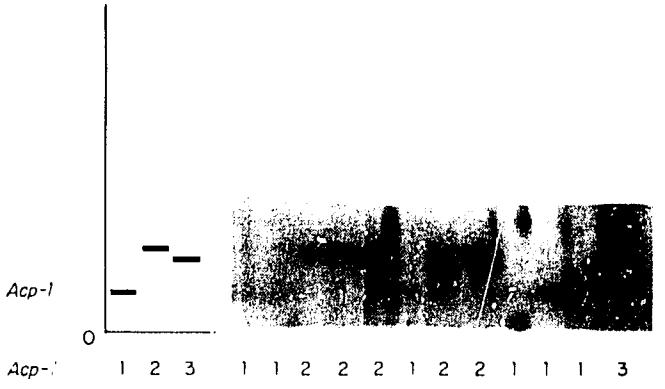
13. AMP zymograms comparing AMP-1 bands in the three substrates.



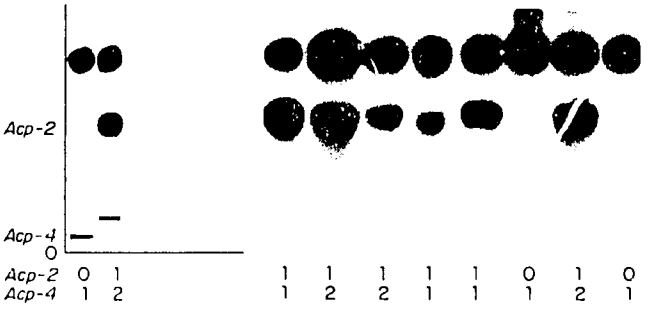
14. Zymogram and diagrammatic interpretation of endopeptidase (ENP) (scale in cm).



15. Zymogram and diagrammatic interpretation of esterase (EST).



16. Zymogram and diagrammatic interpretation of acid phosphatase (ACP-1, migration buffer I).



17. Zymogram and diagrammatic interpretation of acid phosphatase (ACP-2 and ACP-4, migration buffer II).

Esterase (EST)

Variation for EST in plumules is due to polymorphism at four loci (Fig. 15).

Est-9 encodes enzymes that migrate on the cathodal side. These are β-naphthylacetate (β-NAc)-specific (red color). There are two alleles, and the hybrid has the three bands typical of a dimeric enzyme.

Est-5 is responsible for a double black (α-NAc-specific) band with two levels of migration corresponding to two active alleles. One silent allele is also encountered. The heterozygotes have only the parental bands, which indicates a monomeric structure. *Est-5* is located on chromosome 1.

Est-1 codes for a major red (β-NAc-specific) band accompanied by a faint faster band. One active allele and one silent allele are known. The gene is more active in the green part of the plumule.

Est-2 codes for a major brown (α- and β-NAc-specific) band. It has two active alleles and one silent allele. The heterozygotes have only the parental bands, indicating the monomeric structure of the enzyme. *Est-2* is located on chromosome 3 and is very tightly linked to *Amp-3*.

Acid phosphatase (ACP)

The variation of acid phosphatases in plumules is related to three polymorphic genes—*Acp-1*, *Acp-2*, and *Acp-4*.

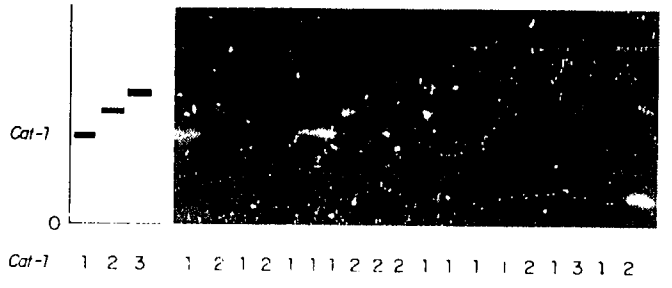
Acp-1 is consistently expressed in green plumules (i.e., after exposure to light). It has three alleles, each responsible for a single band in homozygotes (Fig. 16). When two alleles are combined in a heterozygote, they produce three bands, indicating a dimeric structure. *Acp-1* is located on chromosome 6.

Acp-2 has one allele responsible for a strong band (Fig. 17) in green leaves, and one null allele. Its expression, however, is not consistent in plumules, and the absence of the band does not imply the presence of the null allele. Thus, the presence of the band, but not its absence, provides insight into the *Acp-2* genotype.

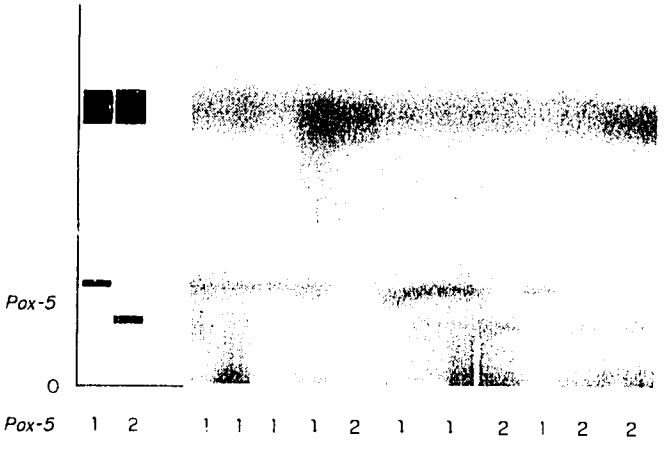
Acp-4 has two active alleles responsible for slow-migrating bands. In green tissues, superimposition of various acid phosphatases makes it difficult to distinguish ACP-4 allozymes. Band resolution is best in white, non-chlorophylliferous tissues (Fig. 17). The heterozygotes have two bands, showing the monomeric structure of the enzyme. In some gels, these bands cannot be seen; this is due more likely to an uncontrolled technical fluctuation than to a regulatory process.

Catalase (CAT)

CAT in plumules is encoded by the single gene *Cat-1*, which has three alleles corresponding to three bands differing in migration rate (Fig. 18). The enzyme is a tetramer, as indicated by a five-band pattern in heterozygotes. In routine electrophoresis, these five bands are not fully separated, and they appear as a single, thick, diffuse band of intermediate mobility. *Cat-1* is located on chromosome 3.



18. Zymogram and diagrammatic interpretation of catalase (CAT).



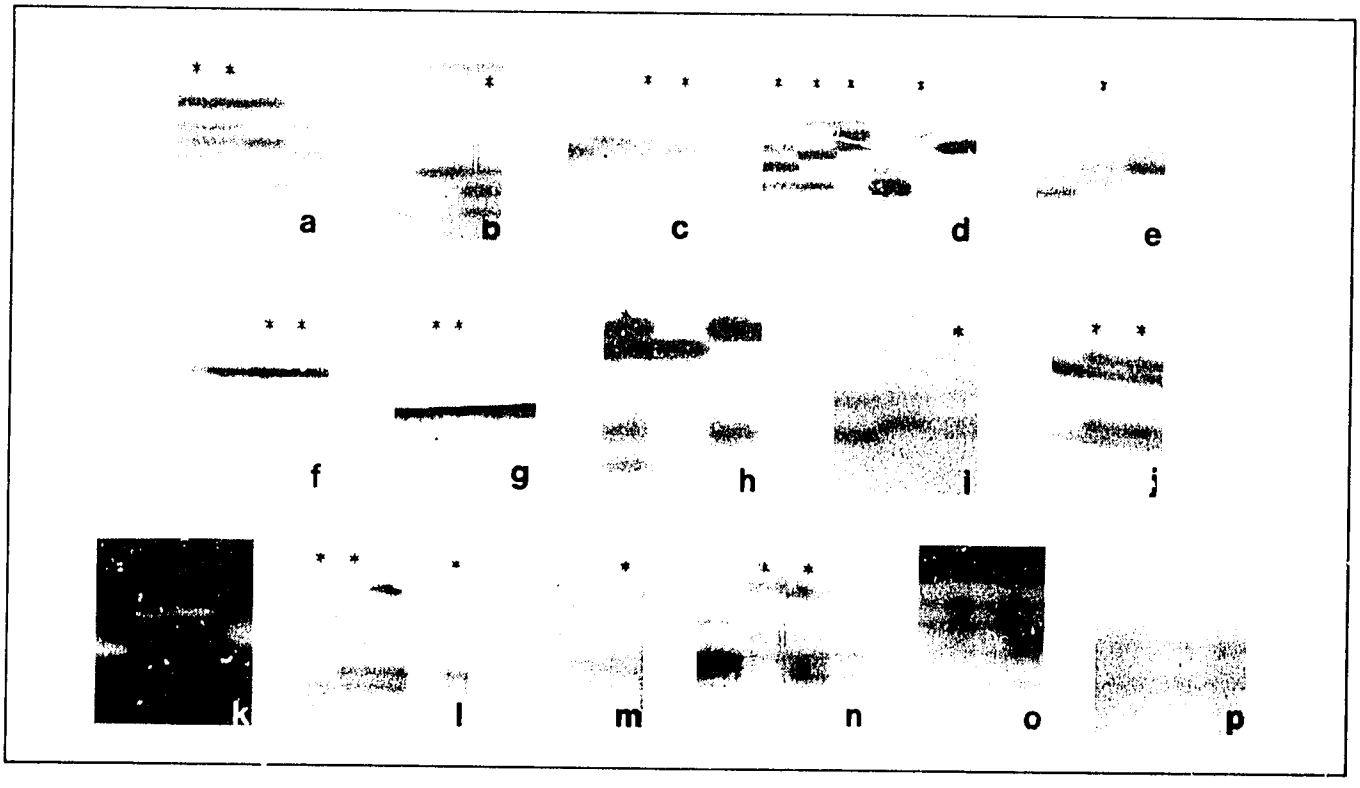
19. Zymogram and diagrammatic interpretation of peroxidase (POX).

Peroxidase (POX)

Reproducible anodal POX variation in plumules is controlled by a single gene, *Pox-5*. Two active alleles are known. The heterozygotes display only the parental bands, showing the enzyme is a monomer (Fig. 19). *Pox-5* is located on chromosome 3.

DISCUSSION

Isozymes are being increasingly utilized in rice genetics and breeding. The use of various electrophoretic techniques and various materials in several laboratories has resulted in the existence of various nomenclatures, correspondence among which is not always clear. The Rice Genetics Cooperative established in 1985 (Swaminathan 1985) has promoted consultation among scientists, and a common nomenclature for isozyme loci has been proposed (Morishima and Glaszmann 1986), with indication of the published synonyms. Table 4 gives these synonyms for the loci considered in the present paper. Nomenclature of alleles is more difficult to finalize, because minor differences in the electrophoretic procedures may drastically affect the migration patterns of the allozymes. Moreover, comparison of results among different laboratories is made difficult by the frequent polymorphism observed within varieties and lines. We have developed a set of marker lines purified for isozymes that possess all



20. Zymograms of heterozygous materials (*). a) PGI-1, b) GOT-1, c) SDH, d) ADH, e) ICD, f) PGD-1, g) MAL, h) AMP-1 (upper) and AMP-3 (lower), i) AMP-2 (lower), j) AMP-4 (lower), k) CAT, l) EST-5 (first sample from the left) and EST-2 (second and fifth samples), m)EST-9, n) ACP-1, o) ACP-4, p) POX.

Table 4. Correspondence between the loci described and published synonyms.

Locus symbol	Enzyme (enzyme code no.)	Published synonyms (reference)		
		(1)	(2)	(3)
Acp-1	Acid phosphatase	*Acp-B (Pac-AMC)*		
Acp-2	(E.C. 3.1.3.2.)	*Acp-C (Pac-Fa/Sa)*	*Pac-2*	
Acp-4			*Pac-1*	
Adh-1	Alcohol dehydrogenase (E.C. 1.1.1.1.)	*Adh-A*		
Amp-1	Aminopeptidase	*Lap-E*	*Lap*	
Amp-2	(E.C. 3.4.11.-)		*Aap*	*Amp-1*
Amp-3				*Amp-2*
Cat-1	Catalase	*Cat-A*	*Cat*	
Est-1	Esterase	*Est-D*	*Est-3*	
Est-2	(E.C. 3.1.1.-)	*Est-E*	*Est-4*	
Est-5		*Est-B*		
Est-9		*Est-Ca*	*Est-1*	*Est-c1*
Got-1	Glutamate oxaloacetate transaminase	*Got-A*		
Got-3	(E.C. 2.6.1.1.)	*Got-C*		
Icd-1	Isocitrate dehydrogenase (E.C.1.1.1.42)	*Icd-A (Idh-A)*		
Pgd-1	Phosphogluconate	*Pgd-A*		
Pgd-2	dehydrogenase (E.C.1.1.1.43)	*Pgd-B*		
Pgi-2	Phosphoglucose isomerase (E.C.5.3.1.9)	*Pgi-A* *Pgi-B*		
Pox-2	Peroxidase (E.C.1.11.1.7)			*Px-2*

(1) Second and Trouslot 1980; Second 1982, 1985.
(2) Glaszmann et al 1984.
(3) Pai et al 1973, 1975; Pai and Fu 1977; Morishima and Sano 1984; Sano and Barbier 1985.

known alleles at 24 loci, and we keep their seeds available to rice scientists (see Table 1). We hope that the present paper, together with the availability of our marker lines, will stimulate interest in isozymes in rice research and promote their wider use.

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Appendix 1. Chemicals used.

Chemical	Company and catalog no.[a]	Unit
Buffers		
Acetic acid, glacial	FC A-38	2.5 liter
Borid acid	BC 0084-1	500 g
Citric acid, monohydrate	BC 0110-1	500 g
L-histidine hydrochloride, monohydrate	SC H8125	100 g
Hydrochloric acid	FC A-144	2.5 liter
Maleic acid	SC M0375	500 g
Potassium phosphate, monobasic	BC 1-3246	500 g
Sodium hydroxyde pellets	FC S-318	500 g
Sodium phosphate, dibasic, 7-hydrate	SC 1-3824	500 g
Trizma base (Tris)	SC T1503	5000 g
Substrate		
D-Fructose-6-phosphate, disodium salt	SC F3627	10 g
DL-aspartic acid	SC A9006	100 g
α-ketoglutaric acid	SC K1750	25 g
Shikimic acid	SC S5375	5 g
Ethanol, absolute	M 983	2.5 liter
DL-isocitric acid, trisodium salt	SC I1252	5 g
6-phosphogluconic acid, trisodium salt	SC P7887	1 g
DL-malic acid	SC M0875	500 g
L-leucine-β-naphthylamide, hydrochloride	SC L0376	1 g
L-arginine-β-naphthylamide, hydrochloride	SC A6512	1 g
DL-alanyl-β-naphthylamide, hydrochloride	SC A2503	1 g
α-naphthylacetate	SC N8505	5 g
β-naphthylacetate	SC N6875	5 g
α-naphthyl acid phosphate	SC N 7125	5 g
N-α-benzoyl-DL-arginine-β-naphthylamide, hydrochloride	SC B4750	5 g
Hydrogen peroxide, 30%	FC H-325	500 ml
Staining salts, cofactors, enzymes		
Magnesium chloride, 6-hydrate	BC 2444-1	500 g
β-nicotinamide adenine dinucleotide (NAD)	SC N8881	1 g
β-nicotinamide adenine dinucleotide phosphate (NADP)	SC N0505	5 g
Glucose-6-phosphate dehydrogenase (G6PDH)	SC G5760	1000 units
Nitroblue tetrazolium salt (NBT)	SC N6876	5 g
Phenazine methosulfate (PMS)	SC P9625	0.5 g
Pyridoxal-5-phosphate	SC P9255	0.1 g
Fast blue BB salt (75%)	SC F3378	5 g
β-nicotinamide adenine dinucleotide	SC N8881	1 g
Fast black K salt	SC F7253	25 g
Fast garnet GBC salt	SC F0875	25 g
Potassium iodide	BC 1-3165	500 g
3-amino-9-ethylcarbazole	SC A5754	10 g
Calcium chloride	BC 1332-1	500 g
Solvents		
Acetone	FC F-18	4 liter
Methanol absolute	BC 9070-3	4 liter
N, N-dimethyl-formamide	FC D-119	946 ml
1-propanol	FC A-414	4 liter
Starches		
Connaught starch-hydrolyzed	FC S-676	2 kg
Starch, hydrolyzed	SC S-4501	2 kg
Miscellaneous		
Glycerol, anhydrous	BC 2136-1	500 ml
Agar	SC A7002	250 g
Bromphenol blue	SC B6131	10 g
2-mercaptoethanol	K	25 g

[a]SC = Sigma Chemical, FC = Fisher Chemicals, BC = Baker Chemicals, M = Merck, K = Kansai Reagent Co. Mention of company or trademark name does not imply approval to the exclusion of other products that might also be suitable.

Appendix 2. Buffer composition for the enzyme assays.

PGI, GOT, SDH, ADH, ICD, PGD, ME:	
Tris-HCl buffer, 0.5 M, pH 8.5	
Trizma base (Tris)	121.1 g
Water	
Hydrochloric acid	27.0 ml
Water	
	2000 ml
AMP	
Tris-maleate buffer, 0.2 M, pH 3.3	
Trizma base (Tris)	24.2 g
Water	
Maleic acid	23.2 g
Water	
	1000 ml
NaOH solution 0.1 M	
Sodium hydroxide pellets	4.0 g
Water	
	1000 ml
EST	
Phosphate buffer, 0.1 M, pH 6.5	
Sodium phosphate, dibasic, 7 H_2O	3.87 g
Potassium phosphate, monobasic	9.89 g
Water	
	1000 ml
ACP, POX	
Acetate buffer, 1.0 M, pH 4.65	
Sodium hydroxide pellets	8.0 g
Water	
Acetic acid, glacial	30 ml
Water	
	500 ml

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