Isozyme Variations in Fine and Aromatic Rice Genotypes

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ABSTRACT

Allozyme electrophoresis has been a reliable technique in estimating the genetic variation in crops. We surveyed isozyme variation of 24 aromatic and fine rice varieties collected from Nepal including Jetho budho landrace. Five enzyme systems were analyzed for allozyme variability that detected distinct 10 isozyme loci and 15 alleles including null alleles. The polymorphic enzyme systems, esterase revealed 8 phenotypes, 6- phosphoglutanate dehydrogenase and peroxidase each with 3 phenotypes and alcohol dehydrogenase and aspartate aminotransferase each with 2 phenotypes based on the combination of presence and absence of alleles. Dendrogram did not indicate any clear pattern of these populations into definite clusters. However 3 groups were detected at 84% of dissimilarity among 24 populations. Nepalese varieties Rato Basmati, Pahenle and Jetho Budho grouped in a separate cluster in the dendrogram.

Key words: Fine and aromatic rice, genetic diversity, isozyme

INTRODUCTION

Isozyme polymorphism in cultivated rice 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 1985). 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 tissue 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.

Rice varieties and landraces in Nepal constitute a high degree of morphological variations and are the important genetic resources for crop improvement. To optimize and accelerate the breeding process in rice, it is essential to screen, evaluate and classify the existed variability in genetic level. Thus, we focused on isozyme analysis in rice coleoptile. 24 fine and aromatic rice varieties for their significance in conservation and improvement.

MATERIALS AND METHODS

Extraction

Seed samples of fine aromatic rice were collected from different sources (Table 1). A total of 24 lines of rice varieties were considered for the isozyme analysis. Enzymes were extracted from fresh coleoptile of 7 day old seedlings grown in controlled condition of 30^{o}C in darkness. Coleoptiles from 10 seedlings from each line or variety were cut into small pieces and crushed individually in pre-chilled cavity block with 20 drops of chilled extraction buffer (L. Ascorbic acid 8.3 g, Glycerol 30 ml and pH 7.4 with NaOH, total volume 100 ml in distilled water) (GEVES 1993). Rectangular small pieces of

Whatman filter papers (wicks) were dipped in the extracted solution and then after few minutes these filter papers were stored in deep freeze (-20°C) for further process.

Gel preparation

A starch gel was prepared using the appropriate gel buffer for the specific enzymes (Table 2). A solution of hydrolyzed starch (12%) and buffer was carefully homogenized in a litter conical flask and heated with continuous swirling on a Bunsen burner flame until a clear, vigorously boiling solution was obtained. The solution was then de-aerated with membrane vacuum pump and poured into an acrylic gel mold in which electrode strips were sealed with masking tape. Solid particles and air bubbles were quickly removed with help of forceps. The gel was allowed to cool and set for approximately 30 minutes at room temperature and then it was placed for 1 h in a refrigerator for final cooling before use.

The wicks with extraction solution stored earlier in deep freeze were placed on spot plates while the gel was cooling. A slit was prepared on to cooling gel approximately 5 cm far from its cathodal end. A wick with bromophenol blue solution was inserted to serve as a tracking dye. The wicks were inserted in the slit so that they form a continuous arrangement with some gaps to avoid distortion on the sides of the papers and to facilitate comparison of migration distances among the bands produced. Intermixing between adjacent papers was avoided by removing excess extract with absorbent paper prior to insertion into the gel.

Table 1. Populations of fine and aromatic rice genotypes for isozyme analysis

SN	Genotype	Source	Origin	Remarks
1	IR 66231-127-1-3	AVT 01.7	IRRI	High yield, medium maturity (135 days), dwarf (60 cm), fine grain
2	IR 43450	AVT 01.5	IRRI	High yield, medium maturity (140 days) medium plant (110 cm.)
3	IR 43850	AVT 01.5	IRRI	High yield, medium maturity (140 days) medium plant (110 cm.)
4	IR 67015-49-2-6-3	AVT 00.3	IRRI	Medium yield, medium maturity (135 days), dwarf (70 cm), coarse grain
5	IR 65610-105-2-5-2-2-2	FFT 01.3	IRRI	High yield (4 t ha^{-1}), medium maturity (135 days), medium plant (100 cm)
6	IR 65610-38-2-4-2-6-3	CVT 01.2	IRRI	High yield, medium maturity (120 days), short plant (90 cm)
7	IR 67017-180-2-1-2	AVT 01.2	IRRI	High yield, long maturity (160 days), medium plant height (100 cm), fine grain
8	IR 43850-SKN-506	AVT 01.6	IRRI	High yield (4 t/ha), medium maturity (125 days), tall plant (130 cm.), excellent aroma, high protein content (10%)
9	IR 66231-106-1-2	AVT 00.12	IRRI	High yield medium maturity (140 days), dwarf (60 cm.), fine grain
10	IR 67017-13-3-3	AVT 00.5	IRRI	Medium yield, medium maturity (130 days), dwarf (85 cm), medium fine grain
11	IR 67417	AVT 01.4	IRRI	High yield (5 t ha^{-1}), medium maturity (135 days), medium plant height (110 cm)
12	Basmati 370	AVT 01.15	India	Fine grain, medium maturity (130 days), short plant (90 cm)
13	Basmati 385	HB-00 (Parent)	India	Fine grain, medium maturity (130 days), short plant (90 cm)
14	Rato Basmati	CVT 01.14	Nepal	Local variety of Terai, late to mature (150 days), aromatic
15	ARC 10796	HB-00 (Parent)	India	Fine grain, medium maturity (130 days), short plant height (80 cm)
16	ARC10863	HB-00 (Parent)	India	Fine grain, medium maturity (140 days), tall plant height (140 cm)
17	ARC 10679	AVT 01.3	India	High yield, long maturity period (150 days), tall plant height (150 cm.)
18	Pahenle	AVT 01.11	Nepal	Local variety of Kaski district, long maturity period (150 days), medium plant height (140 cm), aromatic
19	Pusa 834	FFT 01.1	India	High yield (5t ha^{-1}), medium maturity (135 days), medium plant height (100 cm), aromatic
20	Jetho Budho	AVT 01.12	Nepal	Local variety of Kaski district, long maturity period (150 days), tall plant height (160 cm), excellent aroma
21	FRX 92F38	AVT 01.1	India	High yield, medium maturity (140 days) short plant (90 cm)
22	Zhong Fan 11	CVT 01.4	IRRI	High yield, long duration (140 days), coarse grain, aromatic
23	PK 1501-9-2-B-1	AVT 01.8	India	High yield (5 ha^{-1}), medium maturity (135 days), dwarf plant height (90 cm), aromatic
24	CNTRL 85033-9-3-1-1	AVT 01.1	India	High yield, good aroma, medium maturity (140 days), medium plant height (120 cm)

Electrophoresis

Enzyme was separated into discrete bands by horizontal starch gel electrophoresis. The plastic film was trimmed at the edges of mold and the masking tape was peeled off to expose the gel in the electrode strips. The gel was then mounted onto the electrode trays containing appropriate tray or tank buffer in a refrigerator at about 2^{o}C (Table 2). The side where the samples were loaded was connected to the cathodal tray. A plastic bag of the ice water was placed at the gel to provide additional cooling. The appropriate wire in anodal and cathodal tray served as electrodes and was connected to the continuous current power supply. The constant parameter was the intensity, which was chosen so that the initial voltage will be about 4 volts/cm of length of the gel. The electrophoresis was stopped after 15 h.

Table 2. Gel and tank buffer system for 5-enzyme systems

Tank buffer	Gel buffer
0.400M Tris, Base = 48.46g	0.180M Tris = 10.90 g
0.105M Citric Acid H_2O = 22.06 g	0.100M L-Histamine = 7.758 g
pH adjusted to 8.0 with citric acid. Made up volume up to 1 litre in distilled water. Used without dilution.	pH adjusted to 8.0 with HCl. Made up volume up to 0.5 liter in distilled water. Used by diluting 15 ml of the above stock to 300 ml in distilled water.

Slicing

After electrophoresis, the gel was removed from the refrigerator and rectangular slabs were prepared with the anodal and cathodal parts starting from the origin of migration. A diagonal slash was 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. A slab was placed on an acrylic slicing bed, and a wire was drawn horizontally through the gel to cut a 1 mm slice. The upper part of the gel was then placed on another slicing bed and the accessible slice was transferred to a stain box. This procedure was repeated until the desired number of slices was prepared.

Staining

Zones of enzymatic activities were revealed by immersing the gel slice into a staining assay (Table 3). The stain boxes chosen were only slightly larger than the slices so that 100 ml of solution was sufficient to stain a slice. Some assays may have specific requirements such as total darkness, incubation at 40^{o}C, immediate scoring, or overnight staining. The staining solution was specific for the individual enzyme. When slice was deepen in specific staining solution, after few minutes (30 minutes, 45 minutes, 60 minutes or overnight) the bands were appeared.

Table 3. Staining solution for five enzyme systems (for 100 ml of each enzyme)

SN	Staining solution	Adh	Est	Pgd	Prx	Got
1	Distilled Water	90 ml	75 ml	95 ml	80 ml	80 ml
2	0.5M Tris, pH 8.5					20 ml
3	1M Tris, pH 8.0			5 ml		
4	1M Tris, pH 9.1	10 ml				
5	Ethanol	5 ml				
6	NAD^+	0.04 g				
7	NBT/MTT	0.02 g		0.01 g		
8	PMS	0.003 g		0.003 g		
9	0.4M Na Phosphate, pH 6.2		25 ml			
10	1% of1-Naphthyl Acetate in 50% Acetone		3 ml			
11	Fast Blue RR Salt		0.1 g			
12	Fast Blue BB Salt					0.15 g
13	6-Phosphogluconic Acid Na_3 Salt			0.04 g		
14	NADP			0.01 g		
15	Benzidine				0.1 g	
16	1M Sod. Acetate, pH 5.6				20 ml	
17	30% Hydrogen Peroxide				0.04 ml	
18	1-Ketoglutarate					0.1 g
19	Aspartic Acid					0.2 g
20	Pyridoxal-5-PO_4					0.01 g

Scoring

The most anodal zone of enzyme activity was assigned as 'locus 1', the next 'locus 2', etc. Like wise at each locus, most anodal allozyme (allele) was given alphabetical code 'a', the next 'b', etc. The zone without band was considered as 'null'. The presence of enzymes and its locus was identified according to Glaszmann et al 1988. Each band was considered as isozymic character and scored as '1' for presence and '0' for absence. Dendrogram were constructed using NTSYS pc.

RESULTS

Alcohol dehydrogenase (Adh)

Gels stained for Adh displayed two distinct bands at two activity zones (Figure 1). Band 'A' type occurred most frequently (95.8%) followed by 'B' type (4.17%) (Table 3). The bands could be corresponded to *Adh 1* with two alleles according to the migration on gel. Type 'B' allele with least frequency could be a rare allele. Type 'A' allele was common in all test except entry no. 17 (ARC 10679).

Figure 1. Types of zymogram observed in 24 populations of fine and aromatic rice for five enzyme systems

Esterase (Est)

Three loci encoded for *Est 1*, *Est 2* and *Est 9* were detected. *Est 1* exhibited two alleles including null alleles. *Est 2* coded for three alleles including null alleles. *Est 1* and *Est 2* represented anodal locus where as *Est 9* exhibited cathodal locus with three alleles including null alleles. Variety 23 (PK 1501-9-2-B-1) showed null allele in all three loci for *Est 1*, *Est 2* and *Est 9*. Zymogram showed 8 different types with highest frequency of 'C' types (47.83%) (Figure 1, Table 4).

6-Phosphoglutanate Dehydrogenase (Pgd)

A total of three alleles (excluding null allele) were revealed in population studied for Pgd encoding two loci in anodal position. *Pgd 1* was the polymorphic locus with two alleles and null allele that is quite frequent. *Pgd 2* was present in all 24 populations with single allele. Pgd zymogram showed three band types in which 'A' type occurred most frequently (66.67%) in the populations (Table 4).

DISCUSSION

Isozymes are being increasingly utilized in rice genetics and breeding. The populations under study collected from IRRI, India and Nepal exhibited wide range of agronomic characters in maturity, height, yield grain size and aroma (Table 1). The five enzyme systems resolved 18 different band types in the zymogram (Figure 1). We have resolved 10 loci in the present study such as *Adh 1, Est 1, Est 2, Est 9, Pgd 1, Pgd 2, Prx 1, Prx 2, Got 1 and Got 3* with 15 alleles excluding null alleles. All the loci except *Pgd 2, Prx 2* and *Got 3* were polymorphic. Type 'A' bands for Adh and Prx in the zymogram were more frequent. It is interesting to note that Variety ARC 10679 showed a very rare allele for *Adh 1* locus (Glaszmann et al 1988). This variety could be a marker line for *Adh 1* locus. All other loci identified in the present study were frequently occurring in the rice genome. Present study also indicated that esterase enzyme had a wide variation and could be used as genetic markers to estimate the genetic diversity (Nakagahra 1977) of cultivated fine and aromatic rice.

The variations under study did not clearly differentiated in the dendrogram (Figure 2); however, at 84% similarity level three clusters could be predicted. Cluster 1 represented 11 populations (45.8%), cluster 2 with six populations (25%) and cluster 3 with seven populations (29.2%) (Figure 2). These groups included varieties with diverse agronomic characters (Table 1), thus distinction of groups based on agronomic characters could not be established. Interestingly, the varieties in second group that also included diverse agronomic characters came out to be the origin from IRRI. Similarly, the rice varieties from Nepalese origin, *Rato Basmati*, *Pahenle* and *Jetho Budho* grouped in one cluster (Figure 2) indicating of similar genetic base for 5 enzyme systems, however, *Rato Basmati* with null allele for *Got-1* locus differed from other two varieties. This allele could also be a marker locus in *Rato Basmati*. *Pahenle* and *Jetho Budho* were local landraces originated from Pokhara valley whereas *Rato Basmati* was from Terai belt of Nepal. This showed the evidence to confirm the genetic similarity of Nepalese varieties within the group. However, some IRRI varieties were also present in groups 1 and 3. The distribution of IRRI and Indian varieties in three clusters showed their broad genetic base for the studied enzyme systems, which is understandable due to multiple crosses. The loci we investigated are only a small part of the genome. The distinction between varietal groups, we observed, did exist but they certainly did not represent all the elements of the actual genetic structure of the fine and aromatic rice.

Table 4. Frequency (%) of zymotypes for 5 enzyme systems

Adh		*Est*		*Pgd*		*Prx*		*Got*	
Zymotype	Frequency	Zymotype	Frequency	Zymotype	Frequency	Zymotype	Frequency	Zymotype	Frequency
A (23)	95.83	A (1)	4.35	A (16)	66.67	A (21)	87.5	A (14)	58.33
B (1)	4.166	B (5)	21.74	B (7)	29.17	B (1)	4.17	B (10)	41.67
		C (11)	47.83	C (1)	4.17	C (2)	8.33		
		D (1)	4.35						
		E (1)	4.35						
		F (2)	8.69						
		G (1)	4.35						
		H (1)	4.35						
		8types							

0.74 0.81 0.87 0.94 1.0

Coefficient

Figure 2. Dendrogram illustrating genetic relationship among 24 populations of fine and aromatic rice generated by the UPGMA cluster analysis

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