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The Use of Isozymes as Biochemical Markers in Rice Research

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ABSTRAK

Penggunaan Isozim sebagai Markah Biokimia dalam Penelitian Padi. ***B. Abdullah.*** Isozim adalah berbagai bentuk molekuler suatu jenis enzim dari jaringan suatu organisme yang mempunyai daya katalisis sama. Produksi isozim dikontrol oleh gen yang berbeda yang mengontrol suatu aktivitas metabolisme. Isozim dapat dideteksi dan diisolasi, sehingga dapat digunakan sebagai markah biokimia untuk membedakan makhluk hidup. Pada padi, fungsi isozim telah diteliti dan dipelajari. Hubungan yang erat antar isozim telah diketahui, demikian juga hubungan antara beberapa isozim dengan sifat-sifat penting padi. Isozim mempunyai beberapa kelebihan dibandingkan dengan markah morfologi, sehingga dapat digunakan sebagai markah biokimia pada pemuliaan padi. Isozim juga telah digunakan untuk mengklasifikasi plasma nutfah padi. Namun demikian, isozim belum digunakan oleh pemulia padi secara intensif, karena jenis atau jumlah isozim yang berhubungan erat dengan sifat-sifat penting padi masih terbatas.

Kata kunci: Isozim, markah biokimia, padi

The success of a plant breeding program is dependent on two major factors. Firstly on the availbilty of genetic variability of traits of interest, and secondly on the availbilty of efficient markers for selecion of the traits. Traditional plant breeders use morphological charcters as markers in selecting the desirable traits. However, morphlogical markers have several disadvantages when used as markers in a plant breeding program. For examples, recessive alleles of genes for morphological characters may be deleterious when homozygous and epistatic and/or pleotropic effects of such genes limit the number of markers. In the last decade, molecular markers are available, but they were very expensive and need sophisticated instruments.

Isozymes refer to multiple molecular forms of an enzyme sharing a catalytic activity derived from a tissue of single organism (Markert and Moller, 1959). They are generally made up of subunits, and the various subunits give rise to enzymes with the same catalytic activity. This enzyme subunits maay be isolated and separated by modern methods of protein chemistry, such as electrophoresis. Major functions of isozymes are controlling metabo-lic activities of the organism. Differ-ent genes code for isozymes, while each gene may have different alleles at the same locus, coding for slightly modified proteins as a subclass of the isozyme called allozymes.

The differences in size, configuration, and ionic charges among the isozymes allow them to be detec-ted and resolved by various separa-tion procedures. These procedures included electrophoresis, which was combined with appropriate histochemical processes and ion exchange chromatography. Various electrophoresis techniques existed, such as starch gel electrophoresis, polyacrylamide gel electrophoresis (PAGE), sodium dodecyl sulfate (SDS) PAGE, and isoelectric focusing.

Since the amino acid sequence of proteins are determined by nucleotide sequences of structural gene loci, "the analysis of protein structure using electrophoresis is a first approximation analysis of a gene" (Gottlieb, 1977). The number of polymorphic bands in a zymogram is dependent on the number of loci, the number of alleles per locus, and the quarternary structure of the enzyme (Simpson and Withers, 1986). Gene mutations that cause substitution, deletion or addition of amino acids within the structure of an enzyme may be detected if they affect electrophoretic migration.

Studies on the detection of isozyme variation using electrophoresis have been done extensively. Isozyme variation that is revealed in characteristic banding patterns called zymogram can be detected by proper staining. Zymograms can be interpreted genetically.

Isozymes are the products of genes through transcription and translation processes, therefore their expressions depend on the stage of plant growth as well as plant tissues. Being gene products, isozymes show band intensity that is proportional to the dosage of the encoding gene. In a diploid, heterozygous for an isozyme locus, the two allozymes will have balanced band intensity (Wu, 1987). In contrast, a trisomic heterozygote will show higher band intensity for the allozym encoded by the allele present in two dosages. Using trisomic, analysis by dosage effect permits chromosomal location of isozyme loci (Nielsen and Frydenberg, 1971). However, isozyme markers are very limited than that of molecular markers.

As biochemical markers, isozymes have advantages compared to morphological markers. The alleles (allozymes) at most isozyme loci are co-dominant. This co-dominant cause no deleterious changes in plant phenotype

through recessiveness or pleotrophy and allows heterozygous to be distin-guished from homozygous. iso-zymes rarely exhibit epistatic inter-action so that a genetic stock con-taining an infinite number of mark-ers could be constructed (Tanksley and Rick, 1980). The process is non-destructive since only small amounts of plant tissue are needed. Any plant tissue can be used as samples, including leaves, roots, pollen, and callus, so that the technique is very versatile. It is also possible to screen plant at seedling stage and retains only desirable genotypes, therefore, save time and money. Isozymes have been used as biochemical markers in plant and animal breeding (Edwards *et al*., 1987; Geldermann, 1975). Isozyme marker technique can be carried out on crude extracts, there-fore, purification of the enzymes is not required and greatly simplifying matters, and can be done in all types of laboratories unlike mol-ecular marker techniques. How-ever, compare to molecular mark-ers isozyme markers have some disadvantages.

ISOZYME STUDIES IN RICE

Chromosome Location of Isozyme Genes

A rapid progress in the location of isozyme loci on the rice chromosomes through linkage and trisomic analyses. Rice geneticists first used linkage analysis with morphological markers. Nakagahara and Hayashi (1976) located *Est-2* locus to chromosome 3. Sano and Mori-shima (1984) located *Pg1-2* and *Est-2* on chromosome 3 by linkage with a waxy marker, *wx*. The recombination values were 35% and 22%, respectively. They also confirmed linkage between *Acp-1* and *Pox-2*. Sano and Barbier (1985) located *Amp-3* on chromosome 3, and demonstrated its linkage to *Est-2* with a recombination value of 1.4%.

The use of trisomic analysis for isozyme gene location in rice was first demonstrated by Ranjhan *et al*. (1988). Detecting gene dosage effects on starch gel electrophoresis, they assigned *Adh-1* to chromosome 11, *Est-8* to chromosome 7, *Sdh-1* to chromosome 6 and *Pgi-1* to chromosome 4. Inspired by the efficiency of this method, Wu *et al*. (1988) set out to locate 10 isozyme loci on the chromosomes. Their study resulted in the mapping of *Got-1*, *Est-5*, and *Icd-1* to chromosome 1; *Amp-1* to chromosome 2; *Cat-1* and *Pox-5* to chromosome 3; *Acp-1*, *Acp-2*, and *Pox-2* to chromosome 6; *Amp-2* and *Amp-4* to chromosome 8, and *Pgd-1* to chromosome 1. De Los Reyes *et al*. (1989) reported the location of *Got-3* to chromosome 2. Summary of chromosome locations of 21 isozyme loci is already known (Table 1).

A number of the remaining known isozyme loci have not been mapped because they are monomorphic, lacking allelic variants. These monomorphic loci are *Gdh-A*, *Mdh-B*, *Mdh-C*, and *Pgm-A* (Second, 1982). Some loci have a null allele as the only alternative allele, such as *Pox-C*, *Est-A*, *Est-G* (Second, 1982), *Enp*-1 and *Est*-1 (Glaszmann *et al*., 1988), which precludes mapping by trisomic analysis.

Linkage of Isozyme Genes to Rice Traits

The ability to breed for a trait, without having to score directly for the trait is often desirable. If the gene of interest is recessive, isozyme-based selection is particularly useful because the recessive gene can be followed without having to do progeny test.

Once linkage of the individual genes to isozyme markers is established, progeny testing to distinguish among individuals having the same phenotype but different numbers of independent genes controlling the character, may omitted. Indirect selection through isozymes can be of value in gene pyramiding,

Table 1. Summary of the known chromosomal location of isozyme loci in rice

Enzyme	Locus	Chromosome	Reference
Acid phosphatase	*Acp*-1	3	Sano and Morishima, 1984
	Acp-2	6	Ranjhan *et al*., 1988
Alcohol dehydrogenase	*Adh*-1	11	Ranjhan *et al*., 1988
Aminopeptidase	*Amp*-1	2	Wu *et al*., 1988
	Amp-2	8	Ranjhan *et al*., 1988
	Amp-3	3	Sano and Barbier, 1985
	Amp-4	8	Wu *et al*., 1988; Ranjhan *et al*, 1988
Catalase	*Cat*-1	3	Wu *et al*., 1988
Esterase	*Est*-2	3	Nakagahara and Hayashi, 1976; Sano and Morishima, 1984; Sano and Barbier, 1985
	Est-4	1	Wu *et al*., 1988
	Est-5	1	Wu *et al*., 1988
	Est-8	7	Ranjhan *et al*., 1988
Glutamate oxaloacetate transferase	*Got*-1	1	Wu *et al*., 1988
	Got-3	2	De Los Reyes *et al*., 1989
Isocitrate dehydrogenase	*Icd*-1	1	Wu *et al*., 1988
Phosphogluconatedehydrogenase	*Pgd*-1	1	Ranjhan *et al*., 1988
Peroxidase	*Pox*-2	3/6	Sano and Morishima, 1984; Ranjhan *et al*., 1988
	Pox-5	3	Wu *et al*., 1988
Phosphoglucose isomerase	*Pgi*-1	4	Ranjhan *et al*., 1988
	Pgi-2	3	
Shikimate dehydrogenase	*Sdh*-1	6	Ranjhan *et al*., 1988

i.e. for the incorporation of two or more independent genes, which give a similar phenotype. Several studies have determined linkage of isozyme loci to morphological, physiological, and quantitative traits in rice

Sato *et al*. (1987) established the linkage of *Est-2* and *Pgi-2* to locus controlling the length of apiculus hair, *Ap*h, in chromosome 6. Weng and Chen (1987) reported the isolation of esterase gene encoding the E-l band with high photosynthetic ability. Pham (1988) determined the linkage of *Est-1* and *Pgi-2* to quantitative trait loci for plant height and spikelet fertility.

Some rice scientists conducted studies on rice isozyme in relation to heterosis. Gupta and Singh (1977) studied esterase isozyme pattern of F_1 hybrids and parents and found out that four cathodal bands were correlated with grain weight. Yi (1984) also studied esterase using acrylamide gel of 114 combinations of male sterile lines, maintainer and their F_1 hybrids. The results showed that hybrids, which have 6A and 5A bands, comple-ment to each other showed eco-nomic superiority.

Peng (1986) examined 100 restorer and maintainer lines on six enzymes and considered 11 loci. The yield performance of F_1 hybrids generated from selected 18 lines was surveyed by means of diallelic design. No clear relationship between isozyme data and heterosis could be determined. It was concluded that performance of the hybrids could not be predicted based merely on the isozyme pattern.

Lopez (1989) studied the association of some isozyme loci with some quantitative traits of rice. They found that *Amp*-3 and *Est*-2 are associated with grain yield per plant. The two enzymes, *Amp*-3 and *Est*-2, and *Pgi-2*, which are located on chromosome 3, were found to be associated with number of spikelet per panicle. *Est-2* was found to be associated in most of the quantitative traits studied name-ly grain yield, number of spikelets per plant, number of primary and secondary branches per panicle, panicle length and leaf length. While *Pgi-2* was found to be associ-ated with two important yield com-ponents, number of spikelets per panicle and spikelets fertility. *Pgd-1* was found to be associated with spikelet fertility. This isozyme is not yet known its location. *Est-9* (chromosome 7) and *Amp-1* (chromosome 2) were found to be associated with tiller number and plant height, re-sceptically.

The F_1 hybrid sterility in *indicia*/*japonica* crosses is the major problem in producing hybrids between these two subspecies. The sterility is caused by an allelic interaction at a locus between *S*-5^i allele of the *indica* and *S*-5^j allele of *japonica*. Some tropical *japonica* is known to have neutral allele, *S*-5^n. This donor of *S*-5^n is known as a wide compatibility variety (WCV). Malik and Khush (1996b) were able to identify WCV using isozyme markers. They found that all WCVs have allele 2 and all non-WCVs have allele 1 of aminopeptidase (*Amp-1*). They also found that WC is linked to *Amp*-3 and *Est*-2, which are located on chromosome 6.

The use of Isozymes in Rice Breeding

Isozyme techniques have been used in plant genetic and breeding. As biochemical marker, isozyme can be used for germplasm classification, gene mapping, selection, monitoring genetic segregation and recombination in distance crosses, characterization F_1 hybrid nature of calli and somatic hybrid plants, identification of sexual hybrid, identification of plants from pollen or anther wall, variety/hybrid purity, and determination of phylogenic relationship in plant. Once isozyme genes are mapped, they can be utilized efficiently as biochemical markers to map other genes such as morphological and physiological genes or otherwise classical linkage methods are utilized (Tanksley and Rick, 1980).

In rice, isozyme has provided data relevant to several lines of research, including gene mapping, gene regulation, developmental genetics and evolution (Endo and Morishima, 1983. More than fifteen enzymes have been detected in starch gels. These enzymes are alcohol dehydrogenase (ADH), lactase dehydrogenase (LDH), malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH), 6-phosphogluconate dehydrogenase (6PGH), glutamate dehydrogenase (GDH), catalase (CAT), peroxidase (POX), phosphoglucomutase (PGM), aspartate aminotransferase (AAT), glutamate oxaloacetate transaminase (GOT), esterase (EST), acid phosphatase (ACP), aminopeptidase (AMP), and phosphoglucose isomerase (PGI) (Endo and Morishima, 1983). Many enzymes have also been studied using other techniques, i.e. polyacryl-amide gel media. Tissue specificity of 13 enzymes has been deter-mined (Second and Trouslot, 1980; Second, 1982).

CLASSIFICATION AND SELECTION

Isozyme variations provide a useful clue for the estimation of population structures and phylogenetic relationships, because the zymographic pattern directly reflects a particular gene system (Shahi *et al*., 1969). Based on the proportion of loci that have more than a single allele, the number of alleles per locus and mean proportion of genes which is heterozygous

per individual, isozyme data can provide estimates of genetic variation within natural populations (Allard and Kahler, 1971; Brown, 1978). This property has been extensively exploited in rice. Studies of isozyme polymorphism contributed immensely to the elucidation of the evolution and subspecies classification of *O. sativa*.

Chu (1967) found that a peroxidase band was related to differentiation of rice ecotypes because this band existed in most *indica* rice but was absence in *japonica* rice. Nakagahara *et al*. (1975) were able to relate four main esterase banding patterns to four variety types from 776 Asian native varieties, namely *indica* (from India), *sinica* (South China), *japonica* (Japan) and *javanica* (Indonesia).

Worldwide collection of 973 rice varieties was classified by Fu and Pai (1979). Based on peroxidase and acid phosphatase isozyme that was determined through starch gel electrophoresis. Their isozyme-based classification is in agreement with conventional classification, i.e., based on plant morphology, physiology, and hybrid sterility.

The use of a multiple isozyme system was described by Second (1982). He surveyed 40 presumed loci coding 13 isozymes in 1948 strains of cultivated rice (*O. sativa* and *O. glaberrima*) and the wild forms of African *O. breviligulata*. He found that the mean gene diversity index and number of alleles detected at a single locus differed between species. Calculations of genetic distances showed that *O. glaberrima* and *O. breviligulata* formed a genetic group from *O. sativa*. Within *O. sativa*, the varieties tended to cluster into two groups, which corresponded, to the so-called indicas and japonicas. His finding further suggested that *O. glaberrima* was domesticated from *O. brevigulata* independently from *O. sativa*. While *O. sativa* originated from two independent domestic-ation in Asia, and the weed forms of *O. breviligulata* were derived from introgression of *O. sativa* into *O. glaberrima*.

Glaszmann *et al*. (1988) surveyed 14 polymorphic loci in *O. sativa*. They found strong differentiation of the varieties into the *indica* and *japonica* types. From the isozyme data, they concluded the *javanica* varieties and typical upland rice from Southeast Asia belonged to the *japonica* group. Further he examined 1688 varieties of the Asian cultivated rice at 15 to 21 presumed loci. According to the enzymatic variation, he further divided the cultivated varieties into six groups. Groups I, II, III, IV, and V consisted of rices usually classified as *indica*. Group VI encompassed the classical *japonica* and *javanica* types (Glaszmann 1986). Using Glaszmann's classification Malik and Khush (1996a) also classified Philippines and Thailand rice germ plasm. They detected variability of isozyme for 16 isozymic loci in 6532-rice germplasm. Based on isozyme analysis, they found that 99% of the germplasm belongs to only two groups, group I (*indica*) and group VI (*japonica*) in both countries.

Two isozyme markers, *Amp-3* and *Est-2* have been used in marker-based selection program for developing breeding lines with wide compatibility at IRRI (Malik and Khush, 1996b).

Monitoring Gene Introgression

Isozymes can be used as tools for detecting alien introgression from wild germplasm into cultivated rice (Tanksley and Rick, 1980). When isozyme polymorphism is observed between wild species and cultivated species, isozyme analysis can be used to detect the introgression genes from wild germplasm and recovery of the recurrent parent background. Pro-gress in return to the recurrent genotype is indicated by a predominance of recurrent parent isozyme genotypes.

Relative genetic contribution of wild species, *O. minuta* (AABB) and cultivated rice (AA) was studied in their hybrids and backcross deriva-tives using isozyme markers (Romero *et al*., 1993). They found that the isozyme content of F_1 hybrids reflected its triploid, while that of the backcross progenies paralleled the duplication of the genome and the gradual loss of *O. minuta* chromosomes during the backcrossing process.

An isozyme, *Sdh-1* was used to identify interspecific hybrids between two-bulu rice and their six wild relatives at seedling stage. Out of 35 hybrids produced, 33 were proved to be true interspecific hybrids while the other two were selfed seedlings of bulu rice (Abdullah *et al*., 2000a). Fifteen isozymes were used to monitor gene introgression from two accessions of *O. minuta* into an elite new plant type of rice. Six isozymes (*Amp-4*, *Got-1*, *Got-3*, *Pgd-1*, *Pgd-3* and *Sdh-1*) were found to be polymorphism between *O. minuta* and the rice. *Got-1* and *Sdh-1* showed polymorphism between the two accessions, Acc. 101089 and 101141 of *O. minuta*, therefore, these two isozymes were used to identify and differentiate progenies from the two different accessions (Abdullah *et al*., 2000b). IRRI is also using isozymes to monitor gene introgression from wild species into cultivated rice in its wide hybridization program.

Future Application of Isozymes

Isozymes could be produced from crude extract of plant tissues

such as coleoptiles and young leaves; therefore, the isozyme technique is simpler than that of molecular techniques. As direct products of genes, isozymes would be better makers than that of molecular markers that are not the genes of interest. A number of isozymes known to be associated or linked with agronomic characters, however, limited number of isozymes hinders the use of isozymes in rice research. Tremendous progress in research on molecular markers has been made and different types of molecular markers, more simple techniques and procedures have made molecular markers widely used in rice research. Therefore, research in isozymes must be done more intensively to increase the number of isozymes that would enhance the use of isozymes in rice research.

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