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APPLICATION OF ISOZYME TECHNOLOGY TO CROP IMPROVEMENT

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ABSTRACT

There are many instances where individual marker genes are of value in breeding programs or genetic studies, or where multiple markers are useful even if their relative positions on a linkage map are not known. In these cases, isozyme markers are of value because they can be detected relatively easily and inexpensively, as well as at an early stage of plant development. Most isozyme polymorphisms have been shown to have a simple genetic basis. Allozymes, the isozyme bands that denote allelic polymorphism at a single locus, display codominant expression, so that plants heterozygous at an isozyme locus may be distinguished from either homozygote. These qualities of isozymes make them useful for the confirmation of hybridity and other forms of pedigree analysis. Isozymes have also been used to tag genes of agricultural importance. Examples of these uses of isozymes will be presented.

INTRODUCTION

The most commonly used biochemical markers are isozymes. In this presentation, I intend to briefly discuss isozyme methodology and the advantages of biochemical markers. I will then discuss what I believe are the most beneficial uses of isozymes. The focus will be on uses of isozymes for genetic improvement. Most examples will involve perennial fruit crops, since these are the kinds of plants with which I work. Most of this material is covered in greater length in a chapter of a book that is soon to be published (Moore and Durham, in press).

Isozymes as Marker Genes

Markert and Moller (1959) coined the term isozyme (isoenzyme) to describe multiple molecular forms of an enzyme, derived from the same individual, that share a catalytic activity. A number of biochemical techniques have been used to distinguish and characterize isozymes, but by far the most common is horizontal starch gel electrophoresis followed by histochemical staining of the gel. Widespread use of isozymes in plant science begins in the 1960s. Much of the early work in plant systems was descriptive, describing polymorphism, or variability, in isozyme banding patterns, but providing no genetic analysis for the polymorphism. Genetic characterization of plant isozyme loci became common in the late 1970s. The

general uses and advantages of isozymes have been reviewed many times (Tanksley and Rick, 1980; Tanksley and Orton, 1983; Weeden, 1989).

The equipment required for isozyme analysis is relatively inexpensive and the techniques involved are fairly simple. Sample preparation is usually accomplished by grinding a small amount of tissue in a buffered solution. The samples are then absorbed into filter paper wicks which are inserted into a starch gel. Starch gels are simple to prepare and the starch is non-toxic. Electrophoresis can be carried out using buffers with a wide range of concentrations and pHs and requires only an inexpensive power supply unit. After electrophoresis, the gels can be sliced horizontally into four or five slices and stained for different enzymes, so that a great deal of data can be collected from a single gel. During enzyme activity staining, all necessary substrates and cofactors for enzyme detection, including some type of indicator compound, are added in solution to the gel and isozymes are detected by the formation of a chromic region in the gel.

The simplicity of this procedure allows it to be used by researchers that have a program that is primarily field-oriented, such as plant breeders. This simplicity also allows the procedure to be scaled up so that relatively large-scale screening of populations can be done. Although there are exceptions, many isozymes are consistently expressed in different plant tissues and under different environmental conditions. Thus, seeds or seedling tissue can often be used for analysis. This is especially valuable in perennial fruit crops where juvenile periods of five to seven years are common.

Many isozyme polymorphisms have been shown to have a simple genetic basis. Isozyme bands that denote allelic polymorphism at a single locus (often termed allozymes) display codominant expression, so that plants that are heterozygous may be distinguished from either homozygote. At monomeric enzyme loci, where the enzyme is composed of a single polypeptide, homozygotes will display one band and heterozygotes will produce two isozyme bands. Homozygotes at a dimeric enzyme locus will produce only one subunit form, which will homodimerize to produce one isozyme band. Heterozygotes produce two subunit forms at the locus, each of which homodimerize; the subunits also heterodimerize to form an intermediately-migrating heterodimer and thus a three-banded isozyme banding pattern. This codominant expression makes it possible to easily distinguish hybrids if the parents differ for alleles at a locus.

In enzyme staining systems where specific substrates are used, there are usually a limited number of loci present. However, some assays, such as that for peroxidase, use nonspecific substrates not present in plant cells, and in this

case a larger number of loci may be visualized. When several enzyme staining systems are used, it is possible to characterize a number of isozyme loci. Generally, isozyme polymorphisms are phenotypically neutral and epistatic or pleiotropic interactions between loci do not occur. Thus it is possible to examine a number of loci in a single segregating population and to construct populations that should segregate at many loci.

Once a putative isozyme locus is identified, genotypes at the locus are determined in members of a segregating progeny, usually a backcross or F_2. Chi square analysis is then performed to test whether alleles at the locus are segregating in the expected Mendelian fashion. A nonsignificant chi square value indicates that segregation at the locus is as expected.

Genetic analyses of isozyme loci in fruit crops were not common until the last decade. When the literature was reviewed by Torres in 1983, isozyme loci had been analyzed by progeny tests in only five fruit crops and the numbers of loci characterized were limited. Since that time, genetic characterization of isozyme loci has occurred in essentially all of the major fruit crops. Particular progress has been made in Malus, Prunus, and Vitis species. Frequently now, there are more genetically characterized isozyme loci than morphological characters in fruit crop species.

While a single plant will display a maximum of two isozyme alleles at a locus, the most useful isozyme loci are those where a number of alleles exist within a given taxa. The degree of polymorphism present varies between species and may be correlated with the breeding system of the species, i.e. whether it is self-pollinated versus outcrossing. Some species, such as Citrus, have been found to be highly polymorphic, with many alleles present at a locus. For example, Torres et al. (1985) listed 12 loci in citrus with a total of 39 alleles; allele numbers ranged from two to five at an individual locus. Other species are much less polymorphic. Little polymorphism has been found in peach in spite of extensive surveys of various enzyme staining systems, electrophoretic conditions, and cultivars and accessions (Arulsekar et al., 1986; Durham et al., 1987; Mowrey et al., 1990).

Once individual isozyme loci have been genetically characterized in a segregating population, the recombination rates of alleles at each pair of loci can be determined using classical linkage analysis. The loci can then be ordered into a linkage map and distances between loci can be expressed as recombination units, usually given in centiMorgans (cM) with one cM equal to 1% recombination. Once a sufficient number of markers is mapped, the number of linkage groups should equal the n number of chromosomes in the organism. Linkage maps based on isozymes have been produced for some species, for example tomato (Tanksley and Rick, 1980).

However, there are difficulties in using isozymes for the construction of linkage maps. The primary disadvantage is that it has only been possible to develop a relatively few isozyme markers, even within the best characterized species. Although a large number of potentially useful enzyme staining systems have been developed (Vallejos, 1983), generally only a much smaller subset of those systems has been found to give well-resolved banding patterns in a given species. A still smaller number of systems will be amenable to genetic analysis. The maximum number of available loci is usually not much more than 20 in perennial fruit crops; not all of these loci may be useful for mapping if polymorphism is not present in the analyzed population. The low number of markers has led to the identification of few linkage groups in fruit crops. Three linkage groups have been identified in grapes (Weeden et al., 1988), four linkage groups were identified in apple (Manganaris and Alston, 1987; Weeden and Lamb, 1987), two linkages have been found in Citrus (Torres et al., 1985), and one instance of linkage has been observed in avocado (Torres et al., 1986).

Thus, it may not be advantageous to use isozymes exclusively for studies that require a genetic map containing many marker loci. Restriction fragment length polymorphisms (RFLPs), markers that detect polymorphism at the DNA level, are better for this purpose. Although the initial steps in the detection of RFLP markers as opposed to isozyme markers are more labor intensive and expensive, the power of the technique may override these considerations. For example, Tanksley and Rick (1980) reported the genetic analysis of 27 isozyme markers in tomato. Not all of the markers were equally useful because of tissue specificity and lack of polymorphic alleles. In 1986, Bernatzky and Tanksley (1986) produced a linkage map in tomato using more than 100 polymorphic loci of which the majority were RFLP markers. By 1988, the number of RFLP markers that had been mapped by Tanksley and associates was greater than 300 (Young et al., 1988) and in 1989, 350 markers had been mapped (Ganal et al., 1989).

However, there are a number of instances where individual marker genes may be of value in breeding programs or genetic studies, or where multiple markers are useful even if their relative positions on a linkage map are not known. It is in these instances that isozymes are most valuable. Isozymes are frequently superior to morphological markers in these cases because they are not tissue or developmental stage specific and because heterozygotes can be easily distinguished from homozygotes. Thus isozyme loci have been very useful in breeding and genetics studies in fruit crops, as is discussed below.

Isozymes in Fruit Crop Improvement

Genetics and breeding studies are difficult in perennial fruit crops. The origins of many fruit crop species and

cultivars are lost in antiquity, so that little may be known about taxonomic relationships within species or genera. In almost every fruit crop, some aspect of reproductive biology complicates hybridization; examples are self-incompatibility and apomixis. Additionally, there may be characters that are of horticultural value that complicate fruit crop breeding; examples are seedlessness in grapes and early-ripening in peach (where the fruit ripens before embryos mature). Many fruit crops are highly heterozygous. This heterozygosity, along with the lack of genetic knowledge in many of these species, makes progeny characteristics following hybridization unpredictable. Undoubtedly, however, the most serious impediment to fruit crop breeding is the long juvenile period of many of these crops.

Hybrid Identification

Isozymes have had great utility for hybrid identification in fruit crops. A major advantage of using isozymes as opposed to morphological markers for hybrid identification is that the technique can be employed at a very early stage in the plant's development. This is particularly advantageous with perennial crops.

Confirmation of hybridity with marker genes when making crosses in a breeding program is often useful. For example, isozyme analysis is routinely used in some citrus breeding programs to screen progenies for hybrid seedlings (Roose, 1988). Many citrus types are polyembryonic; they produce seeds that can contain both zygotic embryos and apomictic embryos that arise from nucellar (maternal) tissue in the seeds. When a polyembryonic maternal parent is used in a cross, many (or even all) of the seedlings produced may be nucellar in origin, and so genetically identical to the maternal parent. Further, it may not be possible to identify hybrids by morphological characteristics until the trees fruit, which can take up to ten years. The use of isozymes to distinguish nucellar and zygotic seedlings was proposed a number of years ago (Iglesias et al., 1974), but in early studies the genetic basis of the isozyme banding patterns observed was not known. Once a number of polymorphic isozyme loci were genetically characterized (Torres et al., 1978, 1982; 1985), studies on the production of zygotic versus nucellar seedlings could be done with a high degree of accuracy (Soost et al., 1980; Torres et al., 1982).

The opposite problem arises in the production of citrus rootstocks. Citrus rootstocks are propagated from seed via nucellar embryony; one criterion for the selection of citrus rootstock cultivars is that they produce a high frequency of nucellar seedlings. Zygotic seedlings are produced, but are hopefully eliminated by the visual scoring and roguing of off-type seedlings in citrus nurseries. However, until isozyme markers became available, the efficiency of the visual roguing process could not be tested. The frequencies at which

zygotic seedlings are produced in populations of several different rootstock cultivars have now been estimated using isozymes (Hirai et al., 1986; Ashari et al., 1988; Khan and Roose, 1988; Moore and Castle, 1988; Xiang and Roose, 1988; Anderson et al., in press).

Factors that influence these frequencies were identified in this research and the characteristics of the zygotic seedlings that were produced were studied. Zygotic seedlings detected by isozyme analysis were frequently smaller than nucellar seedlings, but varied in size so that height alone could not be used as a criterion for selection. Many, but not all, of the zygotic seedlings identified via isozyme analysis could be distinguished from nucellars on the basis of other morphological characteristics, but the ability to do this appeared to be cultivar dependent. One interesting discovery was that nearly all of the zygotic seedlings that were produced appeared to arise via selfing, except where budded trees in established groves or rootstock trials were examined. This may be because self-pollinated plants are likely to be less vigorous and to be rogued in the nursery. Existing budded trees in the field have been examined using bark tissue (Anderson et al., in press; Roose and Traugh, 1988). Roose and Traugh (1988) found that some trees on zygotic rootstocks were smaller and lower-yielding than their nucellar equivalents, but many were similar and a few yielded significantly more.

Isozymes have also been useful for confirming hybridity following wide crosses. For example, this laboratory was involved in a study where interspecific hybrids between peach [Prunus persica (L.) Batsch] and almond (P. amygdalus Batsch) were desired (Chaparro et al., 1987). Three isozyme loci were identified that could be used to verify the identity of the hybrids, as opposed to plants produced via accidental self-pollination. Nondestructive samples could be taken for analysis from cotyledons or primary leaves, so that hybrids could be verified at a very early stage of development.

The difficulties with hybridization in fruit crops often lead to nontraditional plant breeding approaches such as embryo rescue and protoplast fusion. Again, in these kinds of studies, isozymes can be useful for hybrid identification. Several examples can be given from research in which this laboratory was involved. Isozyme banding patterns were used to determine the nature of somatic embryos derived from papaya (Carica papaya L.) ovules following controlled pollination with the related but sexually incompatible species C. cauliflora Jacq. (Moore and Litz, 1984). Plantlets produced from the embryos were found to be most likely hybrid. The genetic origins of young grape seedlings resulting from the ovule culture of monoembryonic and polyembryonic ovules from seedless x seedless cultivar crosses were determined using isozyme genotypes (Durham et al., 1989). Most seedlings that were produced from monoembryonic ovules via

embryo rescue could be shown to be zygotic in origin. The multiple seedlings that arose from polyembryonic ovules also appeared to be mainly zygotic in origin. Isozyme genotypes have been used to verify the production of interspecific somatic hybrids in citrus following protoplast fusion (Grosser et al., 1989). The isozyme banding pattern of the tetraploid somatic hybrid was an additive combination of the two parental patterns.

Cultivar Identification

Possibly the largest number of studies of isozyme polymorphism in fruit crops fall into this category. In some cases, there is only a description of the polymorphism observed, while in other cases the genetic basis of the polymorphism has been known or elucidated. Some studies have been done for the stated purpose of distinguishing cultivars (e.g. DeWald et al., 1988) while in other instances such surveys have been done not for the primary purpose of cultivar identification, but in the course of genetic or taxonomic studies (e.g. Durham et al., 1987).

The usefulness of these kinds of studies is often not clear. It has been suggested that isozymes could be used for the verification of the identity of fruit crop cultivars in the nursery. This would be desirable because fruit crops are often purchased as rooted or grafted plants, and there may be a long maintenance period before they go into production. Thus the purchase and growing of nursery stock may represent a large investment of time and money and verification that the cultivar is the correct one would be valuable. However, we know of no case where this is done in practice.

Also, there are several difficulties in using isozymes for cultivar identification. A major problem is that there may not be sufficient isozymic polymorphism for unambiguous identification of individual cultivars. This may be because of an inherent lack of polymorphism, due to the nature of the species or to the lack of genetic variability in the group of cultivars examined. An additional limitation to the use of isozymes for cultivar identification in many fruit crops is that valuable cultivars have arisen by sporting, or mutation, and isozymes have been of little use in identifying such sports, as would be expected. The same problems of obtaining sufficient variability to uniquely identify any cultivar make it doubtful that isozymes would be of great use in plant varietal patenting or protection.

Pedigree Analysis

Isozymes have frequently been employed for this purpose in fruit crops, often in conjunction with cultivar characterization. In particular, isozyme genotypes of parents and putative progeny have been compared to determine whether a

plant had actually arisen as surmised. For example, isozymes were used in this laboratory to investigate the origin of several pineapple cultivars, especially as to whether they arose via hybridization or mutation (DeWald et al., 1988).

Other Uses of Isozymes

Isozymes have been used to analyze genetic relationships among populations and taxa. For example, isozymes have been used by researchers to make conclusions about species numbers and relationships in Citrus (Torres et al., 1978; Potvin et al., 1983). Citrus taxonomy is complicated by apomixis and the ancient origin of the crop and a number of taxonomic systems have been proposed. Previous taxonomic studies based on morphological characters suggest that there are three basic affinity groups in Citrus (Barrett and Rhodes, 1976). Isozyme and other biochemical data in general support this concept (Potvin et al., 1983; Roose, 1988) and indicate that other Citrus taxa may have been produced by hybridization, originally between these ancestral species, and later through other hybridizations. However, taxonomic studies may be hindered by the scarcity and nonrandom distribution of isozyme loci (Roose, 1988). RFLPs are increasingly being used for such studies.

Many researchers are interested in finding marker genes that are tightly linked to genes that code for agriculturally important traits. The classic example of tagging a qualitative trait with a marker gene was the discovery in tomato of the very tight linkage between an isozyme locus, APS-1, and Mi, the gene determining root-knot nematode resistance in tomato (Rick and Fobes, 1974). This relationship has been widely used by tomato breeders, who can screen for the isozyme marker as opposed to doing laborious tests to evaluate nematode resistance of individual plants (Rick, 1988). A limited number of reports of isozyme markers linked to economically important traits in other species have been published; these were recently reviewed by Weeden (1989). However, the number of isozymically tagged useful traits is small. Relatively few isozyme markers are available in any species, so that it is serendipitous when one is identified that is tightly linked to a useful trait. Also, there may not be sufficient polymorphism at the marker locus for the locus to be effectively used as a tag. In addition, all such isozyme markers thus far identified tag single gene traits and most agriculturally important traits are polygenic in nature. RFLP markers should overcome many of the limitations of isozymes for tagging useful genes. With sufficient research, a much larger number of RFLPs can be generated. In addition, RFLPs can be used to detect, and so tag, loci that influence the expression of quantitative traits (Tanksley et al., 1989).

Isozymes have also been suggested for use as marker genes to reduce the number of generations needed in various plant breeding schemes, particularly in backcross breeding (Tanksley

and Rick, 1980). Isozyme markers dispersed throughout the genome would allow the more rapid obtention of the recurrent parent genotype. This use of isozymes may be of value for some plant breeders. However, the advent of RFLP mapping has allowed this technique to be greatly refined (Tanksley et al., 1989).

CONCLUSIONS

Isozymes have many uses in plant breeding and genetics studies. The simplicity of the technique and the ease of analysis makes isozymes valuable for studies that involve mass screening and allows them to be used in programs where the research is primarily field-oriented. There will continue to be a place for isozyme techniques in these cases. However, for genetic studies that require a well-populated linkage map, the use of isozymes will be increasingly supplemented with, or surpassed by, the use of RFLPs.

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