Conservation and Duplication of Isozymes in Plants

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Summary. Many enzymes in plants have isozymes because the same catalytic reaction is often present in several subcellular compartments, most frequently the plastids and the cytosol. The number and subcellular locations of the isozymes appear to be highly conserved in plant evolution. However, gene duplication in diploid species and the addition of genomes in polyploid species have increased the number of isozymes.

In plants, the number of isozymes of particular enzymes often reflects the number of subcellular compartments in which the same catalytic reaction is required. The best examples at the present time are the reactions of glycolysis and the oxidative pentose phosphate pathway. Both occur in the plastids and the cytosol, and an isozyme of all but one or two enzymes in these pathways has been identified in each of the two compartments (Table 1).

Evidence on the number and subcellular distribution of isozymes in plants has traditionally been obtained by fractionation of crude extracts, but only a few species such as spinach, pea, and castor bean have been examined (*1*, *2*). These investigations rarely include evidence on the genetic control of the isozymes. The studies, by population geneticists, in which the electrophoretic variability of enzymes is examined, provide a new source of information about the number and genetic basis of isozymes (*3*).

Since enzymes of carbohydrate metabolism are readily assayed, the most complete information is available for glycolysis and the pentose phosphate pathway. The fractionation studies and the electrophoretic surveys give consistent results, making it possible to formulate two hypotheses that account for isozyme number of plants: (i) The minimum number and the subcellular location of isozymes of each enzyme in higher plants are conserved, and (ii) increased numbers of isozymes in diploid species result from duplication of structural gene loci and, in polyploid species, from the addition of genomes containing homoeologous loci. In this article, I describe evidence for these proposals.

Isozyme Conservation

Of about a dozen enzymes that have been frequently examined by gel electrophoresis in conifers and many species of flowering plants (*3*), only eight (Table 2) are assayed with natural (in vivo) substrates (*4*); and, of these, glutamate dehydrogenase was the only one for which isozymes were not observed. Two isozymes were generally reported for each enzyme of glycolysis and the pentose phosphate pathway, three (or four) of aspartate aminotransferase (AAT), at least three of nicotinamide adenine dinucleotide–specific (NAD-specific) malate dehydrogenase (MDH), and two or three alcohol dehydrogenases (ADH). Several species had additional isozymes of these enzymes, but none had fewer. Both the conifers and the flowering plants, which diverged more than 100 million years ago, had the same minimum number of isozymes of each enzyme, at least in the tissue examined: green leaves in the flowering plants, and the large female gametophyte in the seed of the conifers (*5*). The conservation of the same number of isozymes in these distinct tissues of the two plant groups reflects a remarkable conservation of metabolic activities in different subcellular compartments (*6*).

Both mitochondria (*7*) and plastids in one form or another (for example, chloroplasts, proplastids, or chromoplasts) are apparently present in nearly all plant cells (*8*), and most cells also have one or several types of microbodies (*9*). As a result, isozymes of many enzymes that are distributed in two or more of these compartments are present in diverse tissues throughout the plant body (although perhaps in varying amounts). For example, both the plastid and cytosolic isozymes of phosphoglucose isomerase (PGI) (*10*, *11*) and triosephosphate isomerase (TPI) (*12*) have been identified in extracts from green leaves as well as nongreen tissues such as roots, flower petals, and pollen. These two enzymes operate only within organized pathways, which suggests that many other glycolytic isozyme pairs are also present in the same tissues. Unfortunately, the electrophoretic surveys rarely report isozyme distribution within the plant, so that it is not known whether the actual number of isozymes for many enzymes is the same as or greater than the minimum number.

The situation in animals is considerably different. In animals, the number of isozymes of particular enzymes has little apparent regularity and may vary within a phylogenetic group such as the mammals as well as from group to group. Also, in animals, many isozymes are often expressed only in certain tissues or organs or only during a few stages of development (*13*).

The subcellular location of an enzyme may have a significant influence on the amount of "permissible" variability of its coding gene. This became apparent when an analysis of published electrophoretic data in flowering plants revealed that the plastid isozyme of PGI was much less variable than the cytosolic isozyme (*14*). Not only were there fewer variants per species, but the electrophoretic mobilities of all plastid isozymes were closely similar, always migrating to the same narrow region of the gel, in contrast to those of the cytosolic isozymes, which were often grossly different. This difference between the two isozymes may be related to requirements for transporting the putative plastid precursors across the chloroplast envelope as well as to the specialized internal environment within the plastids (*15*).

Isozyme Phylogeny

Why so many plant enzymes have isozymes requires an explanation of the origin of compartmentation in plant cells. The plastids and the mitochondria of eukaryotic cells are widely believed to be descendants of prokaryotic symbionts

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(*16*), although other explanations have been proposed (*17*). The evidence is strongest for the plastids and has been based on specific comparisons of components common to them and present-day cyanobacteria. Since information on the number of enzymes with isozymes in both plastids and the cytosol has become available only recently, none of the models that discuss the origin of plastids has considered their significance.

A newly developed model includes this information, as well as other data on metabolism in plants (*18*). The model suggests that proteins now specific for plastids were once specified by the genome of the symbiont and do not reflect duplication of ancestral nuclear genes. Rather, during the evolution of the plant cell, the coding genes from the symbiont were transferred to the nucleus; yet their products are returned to the plastids after having been synthesized on cytoplasmic ribosomes. The model is based partly on the finding that both plastocyanin and plant-type ferredoxin are found in the cyanobacteria and in plant plastids, but in no other eukaryotes. Since the amino acid sequences of each of these proteins extracted from a wide variety of organisms are similar (*19*), it appears that the coding genes in the plant, which are located in the nucleus (*20*), are homologous to the coding sequences in the cyanobacteria and were derived from the endosymbiont. The Weeden model (*18*) explains the presence of a pair of isozymes for each enzyme of glycolysis and the oxidative pentose phosphate pathway in the plastid and the cytosol as a consequence of their phylogeny, and it is assumed that the symbiont and the host already possessed these (and other) pathways. Although it provides an explanation for the ancient origin of certain isozymes in plants, the model does not apply to more recent events.

Table 1. Enzymes of glycolysis and the pentose phosphate pathway that are present in higher plants as isozymes, one located in the plastids and the other in the cytosol. The evidence is based on results from fractionation of crude extracts or of protoplasts.

Enzyme	Source	Reference
Phosphoglucomutase (E.C. 2.7.5.1)	Spinach	(*68*)
	Castor bean	(*69*)
Phosphoglucose isomerase (E.C. 5.3.1.9)	Spinach	(*70*)
	Castor bean	(*71*)
	Radish	(*72*)
	Clarkia	(*21*)
Phosphofructose kinase (E.C. 2.7.1.11)	Spinach	(*73*)
	Castor bean	(*74*)
Fructose-1,6-diphosphate aldolase	Pea	(*75*)
(E.C. 4.1.2.13)	Castor bean	(*71*)
	Watermelon*	(*76*)
	Many species	(*44*)
Triosephosphate isomerase (E.C. 5.3.1.1)	Pea	(*77*)
	Castor bean	(*1*)
	Watermelon*	(*76*)
	Many species	(*44*)
Glyceraldehyde-3-phosphate dehydrogenase	Spinach	(*78*)
(E.C. 1.2.1.12)	Pea	(*78*)
	Barley	(*78*)
3-Phosphoglycerate kinase	Pea	(*79*)
(E.C. 2.7.2.3)	Castor bean	(*1*)
	Watermelon*	(*76*)
Phosphoglycerate mutase (E.C. 5.4.2.1)	Castor bean	(*69*)
Enolase (E.C. 4.2.1.11)	Castor bean	(*69*)
Pyruvate kinase (E.C. 2.7.1.40)	Castor bean	(*80*)
Glucose-6-phosphate dehydrogenase	Spinach	(*81*)
(E.C. 1.1.1.49)	Pea	(*82*)
	Radish	(*72*)
6-Phosphogluconate dehydrogenase	Spinach	(*81*)
(E.C. 1.1.1.44)	Pea	(*82*)
	Radish	(*72*)
	Castor bean	(*71*)
Ribulose-5-phosphate epimerase	Pea	(*82*)
(E.C. 5.1.3.1)		
Ribose-5-phosphate isomerase (E.C. 5.3.1.6)	Pea	(*83*)
Transketolase (E.C. 2.2.1.1)	Pea	(*82*)
	Castor bean	(*1*)
Transaldolase (E.C. 2.2.1.2)	Pea	(*82*)
	Castor bean	(*1*)
Fructose-1,6-diphosphatase	Spinach	(*84*)
(E.C. 3.1.3.11)	Castor bean	(*1*)

*Activity found in both plastid and cytosolic fractions, but isozymic nature not determined.

Increased Isozyme Number in Diploid Plants

At least 13 sets of isozymes have been identified to date as products of duplicated gene loci (Table 3). The enzymes include dehydrogenases and isomerases. They appear to have high activities in the tissues from which they were extracted, and although they catalyze essential steps in organized pathways, they are not responsible for regulating metabolic flux. All of them have been identified by gel electrophoresis, and electrophoretic variants were used to determine their mode of inheritance and linkage. The coding genes are located in the nucleus. All of the enzymes are dimeric molecules and the duplicate isozymes form catalytically active interlocus hybrid enzymes. Insofar as their subcellular locations have been examined, the members of each set of duplicated isozymes are found in a single cell compartment; plastids, mitochondria, and the cytosol are represented.

Phosphoglucose Isomerase Duplication in *Clarkia*

Phosphoglucose isomerase catalyzes the reversible isomerization of glucose 6-phosphate and fructose 6-phosphate. Plants generally have one PGI isozyme in the cytoplasm and a different one in the plastids. In the chloroplast, the PGI reaction is one of a sequence of steps that converts 3-phosphoglycerate to starch in the light. The reaction also occurs in the dark when starch is degraded to dihydroxyacetone phosphate for export to the cytosol. The cytosolic PGI isozyme functions in the pathway between the latter metabolite and sucrose. Both PGI isozymes are present in green and nongreen tissues.

Diploid species of *Clarkia* (Onagraceae), a genus of annual plants native to oak woodlands and adjacent habitats in California, either have one or two gene loci specifying cytosolic PGI isozymes (*10, 11*) in addition to the locus for the plastid isozyme (*21*). The second cytosolic PGI gene originated by a process of duplication during the evolution of the genus, since it is only found in recently evolved clarkias but not in the morphologically primitive sections of the genus or in closely related genera (*10, 11*). The subunits it specifies associate both with each other and in all possible combinations with those coded by the "ancestral" locus, indicating that they share considerable structural similarity. Plants that are heterozygous at both cod-

ing loci have as many as ten cytosolic isozymes, whereas the maximum number is three in heterozygous individuals without the duplication (Fig. 1).

The duplicated genes assort independently in the four species that have been tested (*10, 11*); this suggests they may have arisen after translocations between nonhomologous chromosomes rather than by unequal crossing-over, since the latter process yields tightly linked genes. The origin of species in *Clarkia* is consistent with this hypothesis since it involves complex and often substantial chromosomal rearrangements (*22*). Clarkias are self-compatible, making it possible to segregate a chromosomally homozygous progeny by self-pollination.

Duplications originating by chromosomal rearrangements have a high probability of being unique. Thus species with the PGI duplication presumably descended from a single common ancestor and can now be grouped into a monophyletic assemblage (*11*), whereas previously they were believed to have evolved from different stocks of ancestral clarkias (*23*).

The breeding tests in which phenotypic segregations were examined in progenies were supplemented by a new genetic technique in which the electrophoretic pattern of the isozymes in extracts from pollen is compared to that from leaf tissue of the same individual (*24*). The rationale of this procedure is that the haploid pollen of diploid species contains only one allele of each gene locus. Thus, pollen grains of heterozygotes produce one or the other PGI subunit, but not both, with the result that heterodimers formed by association of allelic products cannot be formed in them. Side-by-side comparison of the electrophoretic patterns of the two extracts reveals immediately which enzyme is absent from the pollen extract, thus identifying it as an intralocus hybrid enzyme (Fig. 2). The interlocus hybrid molecules are present in both pollen and leaf extracts.

The biochemical similarity of the duplicate cytosolic PGI's was assessed in *Clarkia xantiana* because true-breeding genetic stocks had previously been made. Since both of the coding genes were each polymorphic for three alleles in the populations examined, a number of different homodimers and interlocus heterodimers were available. All of them had similar molecular weights, suggesting they are charge isomers (*25*). They resembled each other closely in *p*H optimum, heat sensitivity, energy of activation, and apparent Michaelis constant (K_m) (fructose 6-phosphate), although

Table 2. Number of isozymes revealed by gel electrophoresis in enzymes assayed with natural (in vivo) substrates. At least one diploid species was examined in each genus listed. Species with duplicated isozymes are listed in Table 3.

Enzyme	Number of isozymes	Genus	Reference	Enzyme	Number of isozymes	Genus	Reference
Phosphoglucomutase (E.C. 2.7.5.1)	2	*Pinus*	(*85*)	Aspartate aminotransferase (E.C. 2.6.1.1)	3, 4	*Pinus*	(*93*)
	2	*Aster*	(*63*)		3	*Chenopodium*	(*86*)
	2	*Chenopodium*	(*86*)		3	*Clarkia*	(*87*)
	2	*Clarkia*	(*87*)		3	*Coreopsis*	(*65*)
	2	*Coreopsis*	(*65*)		4	*Liatris*	(*95*)
	2	*Gaura*	(*88*)		4	*Limnanthes*	(*89*)
	2	*Limnanthes*	(*89*)		4	*Lycopersicon*	(*99*)
	2	*Lycopersicon*	(*90*)		4	*Salicornia*	(*92*)
	2	*Phlox*	(*91*)		3	*Stephanomeria*	(*100*)
	2	*Salicornia*	(*92*)		3	*Tragopogon*	(*33*)
	2	*Stephanomeria*	(*45*)		3	*Xanthium*	(*101*)
Phosphoglucose isomerase (E.C. 5.3.1.9)	2	*Pinus*	(*85*)	Malate dehydrogenase (E.C. 1.1.1.37)	4	*Pinus*	(*52, 93*)
	2	*Cupressus*	(*93*)		4	*Aster*	(*63*)
	2	*Calocedrus*	(*93*)		4	*Clarkia*	(*87*)
	2	*Pseudotsuga*	(*93*)		3	*Salicornia*	(*92*)
	2	*Aster*	(*63*)		4	*Silene*	(*50*)
	2	*Chenopodium*	(*86*)	Alcohol dehydrogenase (E.C. 1.1.1.1)	2	*Carthamus*	(*37*)
	2	*Fragaria*	(*94*)		2	*Eucalyptus*	(*102*)
	2	*Liatris*	(*95*)		3	*Gaura*	(*88*)
	2	*Limnanthes*	(*89*)		2	*Helianthus*	(*103*)
	2	*Lolium*	(*96*)		2	*Hordeum*	(*98*)
	2	*Phlox*	(*91*)		3	Pearl millet	(*36*)
	2	*Salicornia*	(*92*)		2	*Stephanomeria*	(*38*)
	2	*Solanum*	(*97*)		2	*Xanthium*	(*101*)
6-Phosphogluconate dehydrogenase (E.C. 1.1.1.44)	2	*Pinus*	(*85*)	Glutamate dehydrogenase (E.C. 1.4.1.3)	None*	*Pinus*	(*85*)
	2	*Calocedrus*	(*93*)		None*	*Aster*	(*63*)
	2	*Cupressus*	(*93*)		None*	*Chenopodium*	(*86*)
	2	*Pseudotsuga*	(*93*)		None*	*Clarkia*	(*87*)
	2	*Aster*	(*63*)		None*	*Coreopsis*	(*65*)
	2	*Clarkia*	(*87*)		None*	*Hordeum*	(*98*)
	2	*Gaura*	(*88*)		None*	*Hymenopappus*	(*104*)
	2	*Hordeum*	(*98*)		None*	*Liatris*	(*95*)
	2	*Salicornia*	(*92*)		None*	*Limnanthes*	(*89*)
	2	*Silene*	(*50*)		None*	*Oenothera*	(*105*)
	2	*Stephanomeria*	(*45*)		None*	*Phlox*	(*91*)
Triosephosphate isomerase (E.C. 5.3.1.1)	2	*Aster*	(*63*)		None*	*Salicornia*	(*92*)
	2	*Lycopersicon*	(*90*)		None*	*Stephanomeria*	(*106*)
	2	*Salicornia*	(*92*)		None*	*Xanthium*	(*101*)
	2	*Stephanomeria*	(*45*)				
	2	Cultivated broccoli, celery, lettuce, parsley, spinach	(*46*)				

*Only a single enzyme was observed.

Table 3. Duplicated isozymes identified in diploid plant species.

Enzyme	Species	Reference
Cytosolic phosphoglucose isomerase (E.C. 5.3.1.9)	*Clarkia* species	(*10, 11*)
Mitochondrial malate dehydrogenase (E.C. 1.1.1.37)	Maize	(*27–29*)
Cytosolic malate dehydrogenase (E.C. 1.1.1.37)	Maize	(*27–29*)
Alcohol dehydrogenase (E.C. 1.1.1.1)	Maize	(*39, 41*)
	Pearl millet	(*36*)
	Sunflower	(*40*)
	Clarkia franciscana	(*43*)
	Stephanomeria exigua	(*38*)
Plastid triosephosphate isomerase (E.C. 5.3.1.1)	*Clarkia* species	(*46*)
Cytosolic triosephosphate isomerase (E.C. 5.3.1.1)	*Clarkia* species	(*46*)
6-Phosphogluconate dehydrogenase (E.C. 1.1.1.44)	Maize	(*47*)
Isocitrate dehydrogenase (E.C. 1.1.1.42)	Maize	(*47*)
	Soybean	(*49*)

one homodimer extracted from a self-pollinating derivative had an unusually high K_m value (*25*). Overall, the present evidence indicates little divergence has occurred among them other than that which causes their electrophoretic divergence. However, a number of other attributes, particularly the relative expression of the two genes in different tissues, requires study before the consequences of the duplication can be fully understood.

Malate Dehydrogenase

Duplications in Maize

Fractionation studies show that cells in green tissues of many diverse plants contain three MDH isozymes, one each in the mitochondria, microbodies, and cytosol (*26*). Electrophoretic surveys generally report three or four isozymes (Table 2). The mitochondrial isozyme participates in the tricarboxylic acid cycle, the microbody form in photorespiration and the glyoxylate cycle, and the cytosolic isozyme in nonautotrophic CO_2 fixation. All of the isozymes interconvert oxaloacetate and malate, but their lower K_m for the former suggests malate formation and oxidation of NADH (the reduced form of NAD) are favored (*26*). The three isozymes are dimeric molecules, but hybrid enzymes are not formed between their subunits.

In contrast to this basic system, many more than three isozymes are found in maize (*Zea mays*). The species contains three unlinked gene loci specifying the mitochondrial isozymes and two other unlinked loci for cytosolic isozymes (*27, 28*). Gene products that occupy the same compartment form both intralocus and interlocus hybrid enzymes, but such molecules are not formed between those from the different compartments. If each of the three genes coding the mitochondrial isozymes are heterozygous in a single plant, 21 different enzymes can be generated from their products (*27*). Antiserum prepared against one of the mitochondrial homodimers cross-reacts strongly with all the mitochondrial isozymes, but not with the cytosolic ones (*26, 28*). A second genetic interpretation of the subunit nature and genetic basis of the maize MDH's has also been proposed (*29*), although there appears to be no disagreement that multiple loci have evolved.

The biochemical consequences of the MDH duplications are unclear. An important finding was that it proved possible to construct plants that were homozygous for null or inactive alleles for different pairs of the loci coding mitochondrial isozymes, and that several commercial inbred lines are homozygous for such alleles at single loci (*27*). In addition, a null allele was found at one of the genes coding the cytosolic isozymes (*28*). These results suggest that the duplicated MDH isozymes have not yet acquired distinctive functions.

Alcohol Dehydrogenase Duplications

Alcohol dehydrogenase catalyzes the oxidation of alcohols and the reduction of aldehydes utilizing NAD and NADH, respectively, as cofactors, although nicotinamide adenine dinucleotide phosphate [NADP(H)] is also frequently accepted (*30*). Most plants contain two or three isozymes (Table 2), all located in the cytosol (*31*). In all species examined, ADH subunits coded by the different genes associate to form both intra- and interlocus heterodimers [reviewed in (*3*)]. The ADH isozymes show different levels of expression during development (*31–34*), may be differentially induced by growth under anaerobic conditions (*35, 36*), and are often expressed in various combinations of tissues in different species (*34, 37, 38*). They appear to represent ancient duplicated products. Well-studied examples include maize (*39*), pearl millet (*36*), and sunflower (*40*).

A number of more recent duplications of ADH loci has also been documented. The basic evidence has been the discovery of true-breeding multiple-banded electrophoretic patterns consisting of two homodimers and a heterodimer with intermediate mobility, whereas similar patterns in congeneric species exhibit genetic segregation.

This was clearly shown with the *AdhFC*^{m} compound locus in a Colombian race of maize which specifies two ADH subunits yielding three isozymes: FF, FC^m, and C^{m}C^m (*41*). It was segregating with two other alleles coding single ADH subunits that yield homodimers corresponding, respectively, to the mobilities of the *FC*^{m} products. The duplicate nature of the locus was confirmed by the induction of mutations that altered the electrophoretic mobility of either the F or the C^m subunit, but not both simultaneously (*42*).

Another ADH duplication was discovered in *Clarkia franciscana*, an annual species native to San Francisco (*43*). Branch and stem extracts of all individuals of the species have an identical true-breeding pattern for the isozymes consisting of three well-separated bands coded by two gene loci. In contrast, plants of closely related species possess a single ADH.

The ADH's of *Stephanomeria exigua*, another annual plant found in California, are specified by a small family of very tightly linked gene loci (*38*). *Adh1* is a compound locus that consists of one to several tightly linked, coordinately expressed structural genes that are inherited as a single unit. The ADH-1 isozymes are inducible in roots by flooding and are also present in thickened unflooded taproots, stems, ovaries, and seeds. The locus is tightly linked to another ADH gene that specifies a different isozyme in pollen and seeds. A particular chromosome may specify one to three ADH-1 subunits with different electrophoretic mobilities, which associate to form all possible combinations of homo- and heterodimers. Several populations were discovered in which some plants segregated chromosomes coding up to three subunits, whereas other plants had chromo-

somes that coded only one or two (*38*). The triplicate *Adh1* complexes could have originated by unequal crossovers between the genes in a duplex homozygote; if so, then the complexes that code only a single subunit may represent the reciprocal product. The polymorphism within populations for chromosomes carrying duplicated and nonduplicated segments in this population and in maize (*41*) is predicted by the general theory that differences between species arise within single populations.

Triosephosphate Isomerase Duplications

Triosephosphate isomerase catalyzes the isomerization of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, a required reaction in glycolysis, gluconeogenesis, and photosynthetic carbon dioxide formation. Isoelectric focusing of extracts from a variety of plants revealed two isozymes, one in the plastid fraction and the second in the cytosol (*44*). Electrophoretic analysis of a number of additional flowering plants also separated two TPI isozymes (Table 2).

The analysis in *Stephanomeria exigua* included genetic studies demonstrating that the isozymes are specified by two nuclear gene loci that assort independently (*45*). Heterozygotes at each locus exhibited three allozymes after electrophoresis, consistent with the dimeric subunit structure of the enzymes (Fig. 3). Hybrid enzymes were not produced between the isozymes.

In marked contrast, most diploid species of *Clarkia* have true-breeding multiple-banded electrophoretic patterns for both the plastid and the cytosolic isozymes (Fig. 3). This comes about because the TPI's in each subcellular compartment are specified by duplicated nuclear genes (*46*). Intra- and interlocus hybrid enzymes are produced within each compartment, but not between isozymes from the different compartments.

Other Duplicated Isozymes

Genetic studies of electrophoretic patterns of several other enzymes in maize have revealed isozyme configurations that are likely to represent additional duplications, although these have not yet been fully characterized. One putative duplication is that for 6-phosphogluconate dehydrogenase (6-PGD) (*47*). In most plants this enzyme is present as two isozymes, one in the plastids and the other in the cytosol (Table 2), with no hybrid enzyme formed between them. But in maize, the isozymes of 6-PGD are reported to form interlocus heterodimers (in addition to intralocus ones) (*47*); this suggests that the subunits are both present in the same compartment, although this remains to be determined. The two loci are not linked.

A similar hypothesis had been proposed to explain the finding that NADP–isocitrate dehydrogenase (IDH) in maize, presumably a cytosolic enzyme (*48*), is also specified by two unlinked gene loci whose products interact to form both intra- and interlocus hybrid enzymes (*47*). Isocitrate dehydrogenase

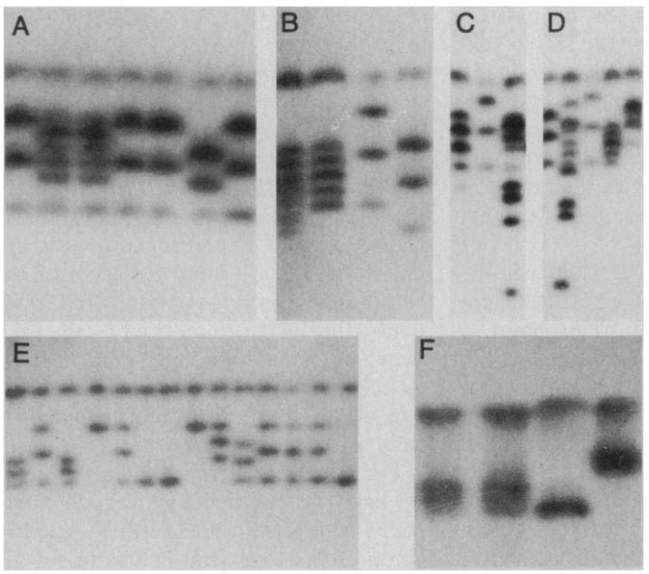
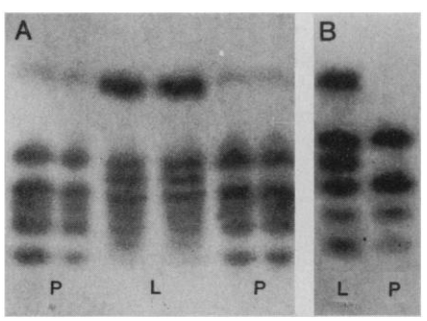


Fig. 1. Electrophoretic patterns of PGI extracted from leaves of *Clarkia* species with and without the cytosolic PGI duplication. The most anodal isozyme (top) is in the plastids. Plants with the duplication (A–D) have at least three cytosolic isozymes including two homodimers and an interlocus heterodimer. Plants with more than three isozymes are heterozygous at one or both loci. Plants that do not have the duplication (E and F) have either a single isozyme when homozygous or three allozymes when heterozygous.



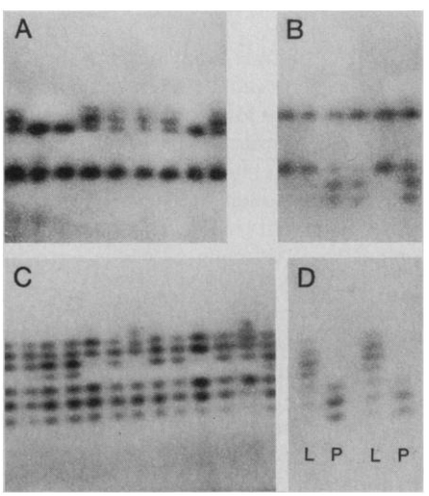


Fig. 2 (left). Electrophoretic patterns of PGI in extracts from soaked pollen (*P*) and leaf tissue (*L*) of a plant of (A) *Clarkia xantiana* and (B) *C. dudleyana*. Since an intralocus hybrid enzyme cannot be produced in the pollen (each grain contains one or the other allele of each gene locus), its absence identifies the corresponding band in the leaf extract as the intralocus enzyme. The pollen of *C. xantiana* has two missing bands because the plant was heterozygous at both genes coding the cytosolic PGI's. The *C. dudleyana* pollen has only one cytosolic band missing since this plant was heterozygous at only one locus. The most anodal band in both photos (top) is the plastid PGI isozyme. It is completely missing from the pollen extract of the *C. dudleyana* plant because only cytosolic isozymes are released from the pollen when it is soaked in buffer (*54*). The plastid isozyme may be obtained from the pollen by crushing or other disruptive processes. Fig. 3 (right). Electrophoretic patterns of TPI. *Stephanomeria exigua* (A and B) has one gene locus coding its chloroplast isozyme and a second locus coding its cytosolic one. Both isozymes are dimeric, and heterozygous individuals at each locus produce three allozymes. (A) Segregation at the locus coding the choroplast isozyme; (B) segregation at the locus specifying the cytosolic isozyme. *Clarkia* species (C) have duplicate loci for both isozymes with the result that they exhibit a minimal electrophoretic pattern of three isozymes in the plastid fraction and three additional ones in the cytosol. (D) The TPI's in *C. mildrediae* extracted from pollen (*P*) and leaf tissue (*L*). Since only the cytosolic isozymes are released from the pollen extract, the side-by-side comparison identifies the anodal triplet as the chloroplast isozymes and the less anodal triplet as the cytosolic ones. The presence of the isozyme with intermediate mobility in the pollen extract shows that it is an interlocus hybrid enzyme (see legend of Fig. 2).

is also coded by duplicated genes in various cultivars of soybean (*Glycine max*) (*49*). Only a single IDH is reported in other species (*50–52*).

Criteria to Identify Duplicated Isozymes

Evidence that two or more isozymes are products of duplicated gene loci has generally depended on the demonstration of close similarity in the amino acid sequences of their polypeptides or on the mapping of the coding genes to cytologically duplicated chromosomal material (*53*). However, concordance of certain other lines of evidence, as illustrated in the cases described above, also warrants a duplication model.

1) Related diploid species differ in the number of isozymes of a particular enzyme. Multiple-banded electrophoretic patterns are true-breeding in some of the species, but segregate genetically in other species. This observation is relevant to the requirement that the higher isozyme number resulted from an addition to the genome and not from a loss or repression of genes in the species with the lower isozyme number. The electrophoretic surveys of wild plant species showing a conserved minimum number of isozymes of many enzymes are critical in this regard.

2) The multiple isozymes are located in the same organelle or in the cytosol. The subcellular location of isozymes can often be determined by a simple comparison of electrophoretic patterns of extracts from pollen and leaf tissue (*54*) that circumvents the necessity for formal organelle partitioning of whole extracts (Figs. 2 and 3D).

3) The recognition of duplicated isozymes is greatly facilitated with oligomeric enzymes, since the subunits often associate to form electrophoretically separable interlocus hybrid molecules. The production of catalytically active hybrid enzymes is evidence of high structural similarity between the subunits coded by the different genes. When multiple forms of the same oligomeric enzymes are present in the same subcellular compartment, but hybrid enzymes are not formed, posttranslational processing rather than gene duplication may be responsible. Alternatively, the subunits may no longer be able to interact; but this has not yet been observed in plants.

The subunit composition of oligomeric enzymes can often be determined by a dissociation-reassociation test. For example, dissociation of a heterodimer is expected to yield equal numbers of each subunit. Subsequent reassociation should produce three isozymes including both homodimers and the heterodimer if reassociation is random. If the dimer is not composed of different subunits, only a single enzyme will form on reassociation. The experiment has been successfully carried out for a number of different enzymes in plants including ADH (*55*), AAT (*56*), PGI (*57*), and catalase (*58*).

Increased Isozyme Number in Polyploids

Increased numbers of isozymes are frequently observed in polyploid plants and are a predictable consequence of their mode of origin (*3*). Polyploidy characterizes about 40 percent of the higher plants and results from the doubling of chromosomes brought about by fertilizations involving unreduced gametes, thereby combining genomes carrying homoeologous loci. These loci often contain alleles that specify electrophoretically separable enzymes.

The most thoroughly studied example of isozyme multiplicity in polyploid plants is hexaploid bread wheat. The 21 chromosomes of wheat belong to seven homoeologous groups. Each group is composed of three chromosomes, one each from its three diploid genomes. The genetic basis of a number of enzymes has been examined and the coding genes mapped to specific chromosomes (*59*). The loci from each diploid species in most if not all the triplicate sets are functional, so that wheat exhibits many more isozymes than its diploid relatives.

The isozyme patterns of polyploids resemble those of heterozygous diploid individuals, but in contrast to the diploids in which the coding alleles segregate, the genes coding the multiple-banded patterns in polyploids do not segregate at meiosis and are passed to all gametes. At fertilization, the heterozygote phenotype is reconstructed. When the genes from the diploid genomes specify different polypeptide subunits of oligomeric enzymes, polyploids frequently possess hybrid enzymes that are unique and never produced in either diploid parent.

The additivity of isozymes detected simply by electrophoresis is frequently so high (*33*) that it is likely that the several diploid genomes in many polyploids are entirely expressed. However, individual gene products may be difficult to distinguish. Since polyploids are expected to have more than the minimum number of isozymes characteristic of diploids, the ploidy level of species used in studies of isozyme number must be determined.

Gene Expression in Polyploids

Little is known about the level of enzyme expression in polyploids or whether differences in the regulation of their diploid genomes are maintained when they are present together in a common tetraploid nucleus. The characteristic properties of an allotetraploid may be determined by the interaction of divergent genomes (the consequence of its hybridity), by the increase in gene dosage (the consequence of its doubled chromosomes), or by interaction between the two factors.

The problem has been studied for the ADH isozymes which predominate in dry seeds of *Tragopogon miscellus* and its diploid parents (*60*). Seeds of *T. pratensis*, one parent, contain twice as much ADH activity as those of *T. dubius*, the other parent, while *T. miscellus* is intermediate. Each diploid parent has a single ADH isozyme in its seeds, but *T. miscellus* has both of them plus a third isozyme composed of the two different subunits. The three isozymes have similar kinetic properties and highly purified proteins showed similar specific activities (*60*). Densitometry revealed that the relative activities and protein amount in *T. miscellus* averaged 1:4:4 for the DD, DP, and PP isozymes, respectively.

This ratio resulted from a twofold difference in the relative amount of the two ADH subunits produced in the tetraploid since random dimerization of a mixture of one D to two P subunits produces active enzymes in the ratio given by the binomial $(1/3\ D + 2/3\ P)^2 = 1/9\ DD + 4/9\ DP + 4/9\ PP$, exactly that observed in *T. miscellus*. The specific activity of ADH and its activity per milligram of tissue in the tetraploid are intermediate to those of the diploids because the relative expression of the ADH gene in each of its diploid genomes is not influenced by the presence of the other genome. The difference between the diploid parents in this regard has remained unaffected by the polyploidy.

Identifying Ploidy by Counting Isozymes

Gene number can be used to ascertain ploidy level. The most common chromosome number (n) in the Astereae tribe of the Compositae is nine, but the numbers vary between $n = 2$ and $n = 9$, with many species having $n = 4$ or $n = 5$. Two models have been proposed to explain the phylogenetic significance of these chromosome numbers. One suggested that $n = 9$ was the original base number of the group, and that the lower

numbers resulted from aneuploid reduction (*61*). The alternative hypothesis called attention to the rarity of species with intermediate chromosome numbers of $n = 6$ and $n = 7$, and suggested an ancestral base number of $n = 4$ or $n = 5$, so that contemporary species with $n = 9$ are allotetraploids derived by hybridization between taxa with the lower numbers (*62*).

Since the essential attribute of polyploidy is not relative chromosome number, but genome multiplication with its attendant increases in number of gene loci, gel electrophoresis was used (*63*) to determine if species with $n = 9$ have more gene loci than those with $n = 4$ or $n = 5$. The results were unambiguous; all of the species had the same number of gene loci specifying the tested enzymes, making it most unlikely that the tested species with $n = 9$ arose by allopolyploidy.

The constancy of structural gene number is not unexpected if the species with the lower chromosome numbers represent lineages that originally arose by aneuploid reduction. This process is generally conceived to involve translocation of essential euchromatin and loss only of heterochromatin and centromeres. Conservation of genes coding enzymes was also found in seven species of *Crepis* that comprise a classical aneuploid series from $n = 6$ to $n = 3$ (*64*). A similar result was found in *Coreopsis* (Compositae), a genus that includes a nearly complete aneuploid series from $n = 13$ to $n = 6$ (*65*).

Maize Duplications Reconsidered

The increase in number of isozymes brought about by polyploidy is very much greater than that resulting from specific duplications in diploid species. Thus, if many enzymes in a presumed diploid show increased isozyme number, it becomes necessary to reexamine the evidence for diploidy. From this standpoint, the numerous duplicated isozymes in maize are intriguing.

In addition to the duplicated genes described above, maize has many others that affect various morphological characteristics (*66*). Further evidence of duplication within its genome is its tolerance of chromosomal deficiencies and occasional bivalent pairing in monoploid plants (*66*). Regional chromosomal duplications may be responsible for its many gene duplications (*47*); however, an alternative hypothesis is available. Rather than being diploid, but subject to a multiplicity of specific duplications, the Maydeae tribe, which includes maize with $n = 10$, may actually be tetraploid with a chromosome number based on $n = 5$ (*66*).

The hypothesis of polyploid origin can be tested with electrophoretic data. If it is correct, the $n = 10$ relatives of maize are expected to show a similar extent of isozyme multiplicity, whereas its $n = 5$ relatives in *Coix* and *Sorghum* will have few, if any, duplicated isozymes. If gene duplication at the diploid level is responsible for the high frequency of duplicated isozymes in maize, then these should be absent from its $n = 10$ relatives. If the $n = 5$ species contain different repeated sequence DNA's, the sequences might be detectable in the maize genome by DNA hybridization, such as was recently used to identify rye-specific DNA in a wheat line that contained rye chromosomes (*67*).

Conclusions

The recognition that the minimum number and subcellular location of isozymes of many enzymes in plants are conserved organizes a substantial amount of information and leads to several predictions. (i) The presence of more than one enzyme for the same catalytic reaction in a single organelle or in the cytosol is the consequence either of gene duplication or the addition of genomes in polyploid species. (ii) The products of duplicated genes will not be present in more than one cell compartment. (iii) Hybrid enzymes will not be formed in vivo between subunits of oligomeric enzymes located in different cell compartments. (iv) Increases in the number of isozymes for many enzymes suggests a polyploid ancestry.

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4. Electrophoretic surveys frequently include many enzymes assayed with artificial substrates; for example, esterases, phosphatases, peptidases, and peroxidases. These enzymes are not discussed because their homologies cannot be determined with the present data, since their substrates in vivo and their subcellular locations are generally unknown. Differences from species to species in the number of these enzymes may have several causes, including changes in substrate specificity, reaction requirements, developmental expression, and duplication of coding genes. The relative importance of these factors cannot be evaluated with the available electrophoretic data.
5. The female gametophyte is used because it does not have the high concentrations of protein-binding phenolics and other compounds characteristic of conifer leaf tissues; and, since it is haploid, genetic analysis is simplified.
6. Several enzymes have been extracted from the needles of the conifer *Pinus ponderosa* by grinding them in liquid nitrogen followed by immersion in a specially formulated buffer. The results [J. B. Mitton, Y. B. Linhart, K. B. Sturgeon, J. L. Hamrick, *J. Hered.* **70**, 86 (1979)] show that these green tissues have two isozymes of PGI, two of phosphoglucomutase, and one of glutamate dehydrogenase, thus significantly augmenting the evidence for conservation of isozyme number.
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Darwinism and the Expansion of Evolutionary Theory

Stephen Jay Gould

Ben Sira, author of the apocryphal book of Ecclesiasticus, paid homage to the heroes of Israel in a noted passage beginning, "let us now praise famous men." He glorified great teachers above all others, for their fame shall eclipse the immediate triumphs of kings and conquerors. And he argued that the corporeal death of teachers counts for nothing—indeed, it should be celebrated—since great ideas must live forever: "His name will be more glorious than a thousand others, and if he dies, that will satisfy him just as well." These sentiments express the compulsion we feel to commemorate the deaths of great thinkers; for their ideas still direct us today. Charles Darwin died 100 years ago, on 19 April 1882, but his name still causes fundamentalists to shudder and scientists to draw battle lines amidst their accolades.

What Is Darwinism?

Darwin often stated that his biological work had embodied two different goals (*1*): to establish the fact of evolution, and to propose natural selection as its primary mechanism. "I had," he wrote, "two distinct objects in view; firstly to show that species had not been separately created, and secondly, that natural selection had been the chief agent of change" (*2*).

Although "Darwinism" has often been equated with evolution itself in popular literature, the term should be restricted to the body of thought allied with Darwin's own theory of mechanism, his second goal. This decision does not provide an unambiguous definition, if only because Darwin himself was a pluralist who granted pride of place to natural selection, but also advocated an important role for Lamarckian and other nonselectionist factors. Thus, as the 19th century drew to a close, G. J. Romanes and A. Weismann squared off in a terminological battle for rights to the name "Darwinian"—Romanes claiming it for his eclectic pluralism, Weismann for his strict selectionism (*3*).

If we agree, as our century generally has, that "Darwinism" should be restricted to the world view encompassed by the theory of natural selection itself, the problem of definition is still not easily resolved. Darwinism must be more than the bare bones of the mechanics: the principles of superfecundity and inherited variation, and the deduction of natural selection therefrom. It must, fundamentally, make a claim for wide scope and dominant frequency; natural selection must represent the primary directing force of evolutionary change.

I believe that Darwinism, under these guidelines, can best be defined as embodying two central claims and a variety of peripheral and supporting statements more or less strongly tied to the central postulates; Darwinism is not a mathematical formula or a set of statements, deductively arranged.

1) The creativity of natural selection. Darwinians cannot simply claim that natural selection operates since everyone,

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