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BIOCHEMICAL MARKERS IN GENETIC INVESTIGATIONS OF CULTIVATED CROPS: THE PROS AND CONS

(review)

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Abstract

This literature review summarizes the accumulated knowledge and the author's own research data about suitability of seed storage proteins, allozymes and isozymes as biochemical genetic markers. These markers have a huge potential, since it allows researchers to distinguish genotypes from other in a short time. Therewith, biochemical markers are usually tissue- and organ-specific. The advantages that these markers possess over morphological markers are shown. So biochemical markers can be used on a much larger number of experimental objects than morphological ones. Protein markers are usually characterized by a greater correspondence between genotype and phenotype, and, besides, the path to the implementation of genetic differences into phenotypic ones for protein markers is much shorter than for morphological ones. In addition, metabolites (sugars, carbohydrates, secondary metabolites, etc.), which are identified biochemically after isolation from the organs or tissues of the studied organism and purification, are also referred to biochemical genetic markers. Though more than half a century has passed since the first description of biochemical markers, the physicochemical bases to their detection and identification have hardly changed methodologically. This gives some limitations on their use in genetic investigations. For example, it is shown, that plant protein polymorphisms revealed by one-dimensional electrophoresis can be subjected to quality and quantity changes because of ecological stresses such as nutrition deficiency or temperature deviations. Researchers also must take into account casual destructive changes and breaks of the analyzed molecules for various reasons, including due to non-standard conditions for protein and polypeptide extraction and purification, as well as during electrophoretic separation, which leads to non-specific electrophoretic spectra. Because of degeneracy of the genetic code and the fact that not every amino acid substitution leads to a change in charge and the molecular weight of the protein, only 30 % of nucleotide substitutions can cause electrophoretically detected protein polymorphism. Only strict observance of all methodological, biological and other restrictions, as well as established requirements, allow the correct and skillful use of biochemical markers in genetic research.

Keywords: seed storage proteins, allozymes, isozymes, polymorphism, electrophoretic patterns, biochemical markers

The search for molecular markers to solve practical tasks of plant genetics and selection began in 1960-s. At that time, DNA technologies were still absent and scientists began using protein polymorphism to evaluate and study genetic diversity. The markers based on identifying the genetic product or product of its activity the visualization of which requires biochemical analysis became known as biochemical markers. This category of markers includes not only proteins of different types (reserve, transport, construction proteins, different enzymes, etc.), but also metabolites (sugars, carbohydrates, secondary metabolites, etc.), which are identified biochemically after isolation from the organs or tissues and purification. Reserve proteins of plant seeds or enzymes are primarily used as markers. Metabolites did not gain a broad recognition as markers due to the specifics of them identifying, which require expensive and often specialized equipment (spectrophotometers, fluid analyzers and high and low pressure gas analyzers, distillation

stills, etc.). However, the application of this group of biochemical markers contains a significant potential because it allows researchers to differentiate between genotypes in a relatively short time; furthermore, metabolites, as a rule, are tissue- and organ-specific. Even in spite of the fact that they are generally all dominant, metabolites as biochemical markers are successfully used in analysis of genetic diversity of plants preserved in collections of genetic resources [1-4].

The biochemical mutations are nominally considered to belong to the marker class in question [5]. Their carriers also have specific organic molecules identified by biochemical methods; however, since manifestation of such mutations can be directly observed on plants (without resorting to biochemical manipulations), it is more properly to associate such mutant forms with a class of morphological markers identified phenotypically. We follow the rule according to which additional expensive manipulations (biochemical or molecular) should be avoided to determine variations between genotypes when simple external phenotype description suffices.

The significant breakthrough in using the marker biochemical analysis developed in the second half of 1960-s is primarily connected with a widespread distribution of electrophoretic separation of different proteins (including enzymes) in genetic studies of different objects.

The protein variations in terms of electrophoretic mobility attributable to allelic substitutions in the gene determinant can be used to analyze changes of genotypic composition of populations similar to the morphological variations related to marker loci. At the same time, protein markers, along with certain shortcomings discussed below, possess a number of advantages over common morphological markers [6]. Firstly, the electrophoretic biochemical markers can be used on a much wider number of experimental objects than morphological markers. For this, collections of marker mutants do not have to be created, which takes a long time, since numerous protein variants are much easier to detect in the available experimental material. Secondly, protein markers are usually characterized by more consistency between genotype and phenotype. Furthermore, at monolocus level homozygotes and heterozygotes are distinguishable, for instance, with the help of isoenzyme analysis, whereas morphological mutations are often recessive, less frequently they are dominant, therefore, usually it is impossible to differentiate a homozygote from a heterozygote, as has been mentioned earlier. Thirdly, the path of implementing genetic differences into phenotypic for protein markers is much shorter than for morphological trait. Fourthly and finally, the number of phenotypic classes morphologically distinguishable during hybrid segregation is determined by the number of heterozygotic marker loci (n) and with complete dominance constitutes 2^n. At the same time, genotypes with new components in the protein spectrum are sometimes observed during analysis of electrophoretic differences (allelic forms), specifically, in the progeny of remote hybrids, which are not observed in either parent forms (P_1 or P_2) or in F_1. Furthermore, the specimen are segregated, whose specter misses the lines characteristic for both parents and F_1 [7]. Therefore, the electrophoresis method allows identifying recombinants with brand new protein spectrums, which cannot be predicted because they are formed not as a result of simple combination of spectrum lines P_1, P_2 and F_1.

Whereas the modifiers do not affect the qualitative composition of proteins determined by structural genes, the modifiers alter the activity of structural genes and, therefore, the qualitative ratio between protein fractions. Registering these ratios that can be construed as common quantitative attributes allows providing a more complete characterization of the genotype. The significance of the last circumstance can be estimated based on data obtained during drosophila

tests: intensive artificial selection for increase of fly resilience against ethanol results in significant change of quantitative ratio of alcohol dehydrogenase fractions without affecting their qualitative composition [8, 9]. The authors indicate that these results are connected with the changes in different regulatory loci. Similar changes of protein spectrum during lifetime of one generation occur in case of adaptive metabolism reformation in response to stresses [7, 10].

The reserve proteins of plant seeds, allo- and isoenzymes. For the first time, the methodological approaches to study of genetic biodiversity using proteins were developed in 1960-1970s and since then have undergone almost no changes. In order to detect and describe biochemical markers associated with protein polymorphism, polyacrylamide gel electrophoresis (PAGE) with subsequent staining is most frequently used. The reserve proteins of plant seeds are primarily used for this purpose; otherwise, identification of activity of specific enzymes is performed.

The study of plant seed proteins as molecular markers, which began in 1972-1973 in Vavilov All-Union Research Institute of Plant Breeding and ISTA (International Seed Testing Association, https://www.seedtest.org/en/home.html), laid the groundwork for identification of varieties and selective registration according to electrophoretic mobility of reserve seed proteins. In 1980, 19 Congress of ISTA recommended these methods for seed farming and seed control, and in 1983 they were accepted as wheat and barley variety identification standards [11]. *Rrs14* gene responsible for resistance of barley seeds against *Rhynchosporium secalis* [12] pathogen was successfully mapped and genetic maps for *Pinus pinaster* [13-15] were constructed based on analysis of reserve seed proteins. Nevertheless, this approach did not receive a wider spread in international practice, since such markers don't encompass all linkage groups [12, 16]. During molecular-genetic identification of plant genotypes this cheap and simple express test, as a rule, is used for evaluation of large commercial and seed farming lots or for studies of parent material in seed farming and in view of specific problems of preserving plant genetic resources. Primarily, this is due to the fact that according to the central dogma of molecular biology proteins are not a direct manifestation of genes. mRNA acts as intermediary between DNA (primary carrier of genetic information) and protein (biochemical product of gene expression). Just like DNA, mRNA can be exposed to various endogenous (recombination, splicing, mutations, etc.) and/or exogenous (for instance, environmental) factors [17]. Consequently, changes in the structure and activity of translated proteins are possible, which means that proteins do not fully comply with the genetic marker requirements [18], which somewhat limits their use in this role.

The electrophoretic analysis of allozymes has been successfully used since 1960s on different groups of organisms from bacteria to many types of animals and plants [19]. The allozymes were used in physiological, biochemical, genetic and selection research to solve different tasks, including study of the structure of populations, polyploidy, hybridization and hybrid analysis, in systematics, etc. [20, 21]. The allozyme analysis is relatively simple and easy to use. As a rule, a tissue homogenate is prepared to conduct the allozyme analysis, and obtained essence is fractioned in polyacrylamide or starch gel. Furthermore, the proteins in the essence are successively divided by charges and sizes. After electrophoresis the gel is stained in accordance with the activity of the separated enzyme by adding the substrate and stain. A characteristic pattern is formed as a result of staining (in accordance with the migratory position of enzyme protein in the gel). Depending on the number of loci, their condition (homo- or heterozygosity) and enzyme molecule configuration, it can have from one to several bands.

The bands can be pleomorphic, and, therefore, informative to determine the gene loci and linkage groups.

The isoenzymes are also used for marker analysis. For instance, in tomatoes, they were used to study genetic diversity [22, 23], to localize agriculturally important genes [24], to monitor hybrid seed purity [25], to identify gene introgression and wild type chromosomes [26], to conduct pollen selection [27], and to screen haploid genotypes regenerated in cell and tissue culture in vitro [28]. However, there exist several limitations not allowing widespread usage of isoenzymes as molecular markers [29]. We have already described general molecular marker requirements [18]; the additional mandatory conditions (specifically when generating molecular genetic maps), include, firstly, the availability of a sufficient number of marker loci equally distributed in a genome at a distance of no more than 10-15 cM from each other, secondly, marker locus should be polymorphic to ensure that hybridization would identify segregation according to marker locus at the discretion of the researcher [30]. The isoenzymes do not meet these two molecular genetic marker requirements specifically. Furthermore, the purely technical inability to detect and qualitatively evaluate the activity of the enzymes due to lack of the required stains (there are significantly fewer of them than identified enzymes, and less than half of such stains are suitable for analysis of plant isoenzymes) [31].

Sometimes, the terms 'isoenzymes' and 'allozymes' are used to substitute each other, which cannot be deemed correct. The isoenzymes identify and segregate the same substrates, but are not necessarily the products of the same gene. The isoenzymes can be active in different cells, tissues and/or organelles or at different stages of organism development. An isoenzyme variant includes allozymes, which are the products of orthologic genes. Due to their allelic differences, the amino acid composition of allozymes does not coincide for one or several amino acids. Subsequently, we will be using the term 'isoenzymes' (taking into account the described differences).

Protein separation in polyacrylamide gel using one-dimensional electrophoresis method. Protein markers (along with undisputable advantages that set them apart from morphological markers) have a number of limitations. First and foremost, we will note that proteins are not classified as primary carriers of genetic information and represent products of transcription and translation of genes encoding them. Moreover, protein polymorphism identified via one-dimensional electrophoresis can be subjected both to qualitative and quantitative change due to impact of environmental stress on plants attributable, for instance, to shortage of feed elements, which is described for barley [32], peas [33], chickpea [34], soybeans [35] and other cultures [36, 37] (Fig. 1) or change of air temperature (Fig. 2) [6].

A possibility of violation of the structure and integrity of analyzed molecules due to various reasons should be taken into account [18], including failure to meet the standard conditions of polypeptide protein extraction during extraction and purification, and during electrophoretic separation, which can result in identification of non-specific electrophoretic spectra [36, 38-41]. The degeneracy of genetic code also serves a limitation because it significantly narrows the polymorphism spectrum identified at the level of amino acid sequences as compared with polymorphism of primary nucleotide sequences, and, therefore, of analysis possibilities. Furthermore, it is a known fact that just a third of the genome (or even less) encodes and expresses certain proteins. Consequently, genomic changes in the non-coding or regulatory parts of the genes (more than 70% of the genome) are overlooked during study of protein products.

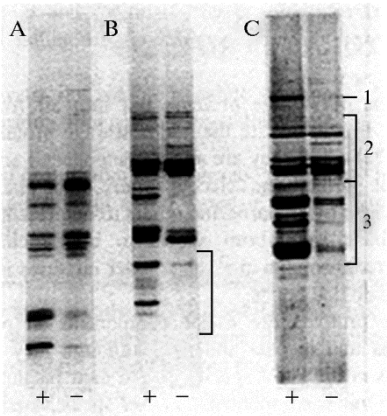

Fig. 1. The impact of sulfur shortage («–») on formation of whole proteins of colza seeds (A), sunflower seeds (B) **and barley seeds** (C)**.** Of interest is the presence of fractions of sulfur-rich low molecular weight proteins (apparently, 2S albumen) for colza and sunflower and sulfur-poor barley hordeins [36].

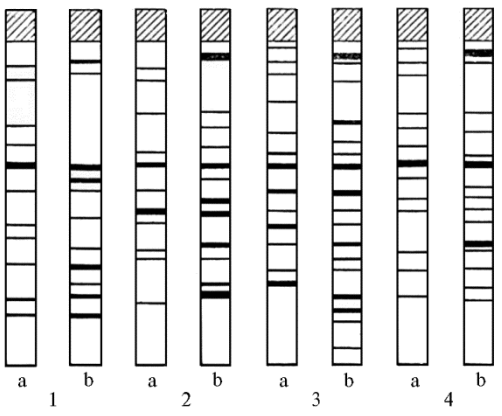

Fig. 2. The impact of temperature on the electrophoretic spectrum of leaf water-soluble proteins of seedlings of different genera of *Lycopersicon species*: 1 — *L. hirsutum* var. *glabratum*, 2 — *L. pimpinellifolium*, 3 — *L. esculentum* (Mo500) × *Solanum pinellii* (F_1), 4 — *L. esculentum* (Teplichny 200 variety); a — 25 °C, b — 40 °C [6].

Nevertheless, using proteins as biochemical markers still remains an attractive tool [11, 42-44]. This is due to three key advantages of reserve seed proteins and isoenzymes as compared with DNA markers. First, this is due to relative simplicity of protein analysis using comparatively cheap electrophoretic methods. Secondly, in a sufficiently large number of individuals (genotypes) reserve proteins or isoenzymes can be analyzed during a relatively short time. Thirdly, the isoenzymes and some reserve seed proteins are codominant markers, because both alleles in a diploid organism are usually clearly distinguishable and heterozygotes can be separated from homozygotes. As a rule, this is sufficient to determine allele frequency (specifically, in population genetics). Does the resolving power of one-dimensional electrophoretic separation method allow detecting and accurately evaluating the polymorphism of amino acid sequences constituting the basis of marker biochemical analysis?

The one-dimensional electrophoresis of protein dissociated sodium dodecyl sulfate (SDS-PAGE) belongs to the most frequently used methods of studying polymorphisma of plants using reserve seed proteins as markers [11, 12, 43, 44]. This method allows identifying variations of polypeptide molecular mass occurring as a result of indels (insertions/deletions) in the coding region of a respective gene; however, it is insensitive to the changes of polypeptide charges. Therefore, the applicability of reserve seed proteins as molecular markers is determined by indel frequency and their dimensions, which should be rather large to identify variations of molecular mass of encoded polypeptide. This problem is aggravated by heterogeneity of reserve protein polypeptides formed as a result of macroevolution of plant orders, families and genera. In other words, the task of analyzing the reserve protein intraspecific polymorphism with the help of SDS-PAGE should include the description of modification of molecular mass of each of polypeptides formed as a result of macroevolution events.

Consequently, the intraspecific polymorphism of reserve protein can be described only if evolutionary relations between the polypeptides of which it is comprised are known, at least at the level of plant families and genera. For instance, one of the studies [44] analyzed seed proteins of 11 varieties of blue lupine and seed parents of some of these varieties using a method of one-dimensional denaturating electrophoresis in polyacrylamide gel. Whereas pro-

teins from seeds were separately extracted using tris glycine electrode buffer (pH 8.3) and additional purification was not performed (using chromatography or any other method), the authors identified the heterogeneity and polymorphism not only of α- and β-conglutins, but of some other proteins as well, which have identical physical and chemical properties and are, therefore, extracted simultaneously with reserve plant seed proteins (Fig. 3, 4). Furthermore, in analyzed seed proteins polymorphism was discovered not for all varieties, and authors used microsatellite DNA analysis to clarify data of protein analysis, which showed polymorphism in varieties, for which the electrophoretic analysis of analyzed seed proteins did not give a positive result. The authors reason that during selection of parent plants the electrophoretic analysis of their proteins will allow excluding obstruction of homogenous varieties and preserving the composition and ratio of biological types for multicomponent varieties [44]. However, it is known that today the completely homogeneous varieties almost do not exist, and population varieties used in the agriculture are rather heterogeneous. The same goes for samples of genetic plant resources preserved in gene banks or primary breeding material. Nevertheless, reserve plant seed electrophoresis still remains a simple and reliable method of controlling purity of seed material lots and trademark safety, i.e., to determine the predominantly mechanical contamination of seed lots, which is declared, for instance, by ISTA (http://www.seedtest.org/en/home.html) as its goal. At the same time, global international organization Bioversity International (Italy) (http://www.bioversityinternational.org), which has studied genetic diversity since 1990-s has been recommending to perform genetic analysis with the help of molecular DNA markers [45, 46].

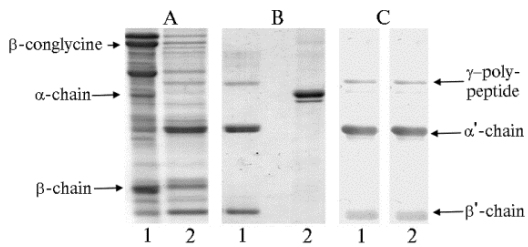

Fig. 3. Separation and purification of legumin-like protein from soybean seeds.

The partial purification of legumin-like protein by salting out using ammonium sulfate (A): 1 — summary saline essence, 2 — partially purified legumin-like preparation (fraction with ammonium sulfate concentration 30% of saturation).

The legumin-like preparation purified using chromatographic filtration via Q-sepharose (B): 1 and 2 — electropherograms of proteins dissociated with sodium dodecyl sulphate correspondingly in presence and in absence of 2-mercaptoethanol.

The immunoblotting of legumin-like preparation partially purified by salting out versus antiserum to soybean glycinin (C): 1 and 2 — antiserum dilutions, :4000 and :8000 times; similar results were obtained when using antiserums for 11S seed globulins of broad beans and oat [47].

The results identical with the data of Eggi et al. [44] were obtained in 1969-1970 and 2006-2007 by two independent groups of scientists in Russia during experiments to determine the impact of nitrogenous nutrition on blend composition of gliadine in winter wheat [17, 48, 49]. Interestingly, the cereal prolamines, which include wheat gliadine, have a rather broad polymorphic blend composition and, as a rule, belong to multigene families, which sets them apart from globulins of the dicotyledons. However, the blend composition of prolamines, as with any other plant seed protein, can be subject to change due to impact of the environment and conditions of cultivation (see Fig. 1), which was made clear by studies of the Russian scientists [17, 48, 49]. Wheat was cultivated in field conditions at different seeding rates, and with various doses of mineral fertilizers and pesticides. Significant changes in the blend composition of gliadine were observed in case of introduction of increased nitrogen doses [17, 48, 49]. The molecular genetic analysis determined that different genetically deter-

mined components in the electrophoretic spectrum of wheat gliadines have uneven response to changes of the environmental [49]. Up to 30% components manifested very high dependence of synthesis on plant cultivation environment. It is quite probable that quantitative changes in synthesis of these proteins are determined by the degree of stability of corresponding mRNA [17].

In all fairness it has to be pointed out that essentially any vegetable protein can be used as genetic marker for description of intraspecific polymorphism with the help of SDS-PAGE, if this protein satisfies the following formalized conditions: it is sufficiently conservative to be identified as a member of vegetable protein family; however, it is sufficiently variable for its microevolution changes to be identified using the SDS-PAGE method; ideally the genome should contain a single gene of potential marker protein or a small number of its clearly distinguishable variants formed as a result of the macroevolution.

It has to be made specifically clear that the required condition of applicability of SDS-PAGE method for description of polymorphism of any proteins is the purity of preparations in question (Fig. 4). As a rule, the unpurified or crudely purified preparations contain polypeptides of protein admixtures close in terms of molecular mass to studied proteins, for instance, to reserve plant seed proteins (see Fig. 4), which complicates identification of their macro- and microevolution variants.

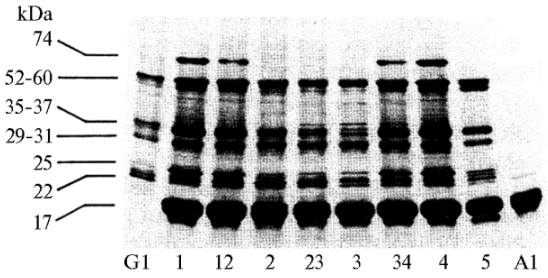

Fig. 4. Denaturating electrophoresis in polyacrylamide gel (SDS-PAGE) of salt-soluble seed proteins not purified by chromatography: 1 — cauliflower (*Brassica oleraceae*), 2 — colza (*B. napus*), 3 — turnip (*B. rapa*), 4 — cauliflower (*B. oleraceae*), 5 — forage cabbage (*B. oleraceae*); 12, 23 and 34 — mixes of the corresponding samples, G1 and A1 — purified by chromatography 12S globulin and albumen of colza seeds [40].

Rough fractionation (sedimentation and reprecipitation) by various types of salts provides only a preliminary purification (because along with the proteins in question other proteins are segregated, which are contained in plant tissues and have similar physical and chemical properties) and serve as the first stage of pure protein segregation, which should be followed by the second stage, i.e. purification by chromatographic or other methods (Fig. 5) [38-40, 50].

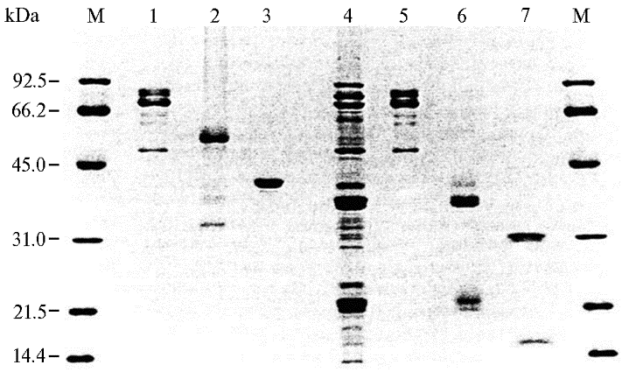

Fig. 5. Analysis of reserve soybean proteins using SDS-PAGE method: 1-3 — purified proteins in absence of 2-mercaptoethanol, 4 — crude protein (raw essence), 5-7 — purified proteins in the presence of 2-mercaptoethanol; 1 and 5 — 7S conglycinin; 2 and 6 — 11S glycinin; 3 and 7 — 8S basic globulin; M — molecular marker [51].

If purification via chromatography or another method is not performed, it is strictly necessary to provide evidence that the analyzed preparation does not contain admixtures of other proteins, or heterogeneity of protein essence being analyzed should be specified. Furthermore, keep in mind that variability of elec-

trophoretic spectrum of rough protein spectrum in case of one-dimensional electrophoresis can be the result of genotype-environment interaction [17, 34, 37, 52, 53] of varying degree of seed plumpness [54] and change of gene activity regulation [36, 49]. It has to be additionally pointed out that resolving power and specificity of bioinformatics methods used to describe protein polymorphism at the level of encoding nucleotide and amino acid sequences is higher by far compared to any electrophoretic method of their analysis [41, 55-57], which is another evidence against using the SDS-PAGE method. And finally, isoenzyme analysis is commonly used to identify an insignificant degree of variability for no more than 20-50 enzymes visualized with the help of biochemical staining [24], and common protein polymorphism, as a rule, depends on a number of endogenous and exogenous factors, which was discussed earlier, and covers only the expressed part of the genome. In some instances, it was displayed that isoenzymes vary in terms of one or several physiological properties [58] due to which they cannot be evolutionary neutral.

A more practical aspect resides in the fact that plant tissues designed for analysis of isoenzymes or other proteins immediately after collection should be used for extraction of these components, because along with the other proteins in such samples isoenzymes are usually not very stable.

In summary, a number of limitations exist that do not allow isoenzyme analysis and any other protein analysis to become universal for identifying of genetic variability in spite of its easiness of use and low cost. The new allele can be identified as polymorphic only if nucleotide replacement in DNA results in replacement of amino acid in protein, which, in turn, entails changes in electrophoretic mobility of protein molecules in question. The genetic code is degenerated and not every amino acid replacement will result in charge change and significant change of protein molecular mass; therefore, only 30 % of all nucleotide replacements are manifested as protein polymorphism detected by electrophoresis. The electrophoretic spectrum of proteins depends on genotype-environment environment and genetic interaction (for instance, on changing nutrition conditions during protein biosynthesis conditions, impact of various stresses, year, location and time of plant cultivation, etc.) and even on the terms of protein extraction and electrophoresis. Consequently, by analyzing allozymes and isoenzymes and/or other proteins it is impossible to fully determine and evaluate the genetic variability. Another problem resides in the fact that many plant species are polyploid, and, as it is known, for polyploid types the analysis of isoenzymes can be significantly complicated. Furthermore, isoenzymes can vary in terms of one or several physiological properties and in this case cannot be neutral in terms of evolution. The samples selected for analysis of protein polymorphism should be promptly used for their segregation and fractionation. Quite the opposite, the methods based on DNA analysis allow conducting research a long time after collection of plant tissues or DNA isolation due to the ability of long-term preservation of samples and preparations without property loss. If plant material collection is performed at considerable distance from the laboratory, it is quite obvious that DNA analysis is preferable.

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