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ONTOGENETIC VARIATION IN THE ISOFORM COMPOSITION OF SOME ENZYMES IN THE DIPLOID WHEAT

The existence of multiple molecular forms of enzymes has been observed in many organisms. At present, there is a great number of studies demonstrating the tissue and organ specificity of protein and enzyme complements in plants (Scandalios, 1964; Steward et al., 1965; Barber, Steward, 1968; Safonova et al., 1970; etc.). Furthermore, the relative abundance of each form may more or less vary during ontogenesis. Since that time, several authors have shown developmental variation in a number of isoenzyme systems during seed germination and the early growth phase (Bhatia, Smith, 1966; Macko et al., 1967; Mäkinen, 1968; Mills, Crowden, 1968; Upadhya, Yee, 1968; Bhatia, Nilson, 1969; Jaaska, Jaaska, 1969; Alexandrescu, 1970; Alexandrescu, Hagima-Călin, 1970; Mitra et al., 1970; etc.). Little is known about the biochemical changes which take place in the later stages of development of vegetative organs and during seed maturation. It is the purpose of this paper to extend these studies to the electrophoretic patterns of enzyme components during the main developmental stages of the diploid wheat, *Triticum monococcum* L.

Materials and methods

Plant material used in this investigation was a diploid spring wheat *Triticum monococcum* L. var. *flavescens*, which has been grown in the open field. Extracts were made from leaves, roots and internodes at the main stages of wheat development (seedling, sprouting, culming and forming spikes). Spikelets without reproductive structures of flowers (anthers and pistils), separately anthers and pistils were analyzed at the stage of flowering, and seeds at the milky, waxy and mature stages.

Extraction of samples was done by grinding in a prechilled mortar with acid-washed sand of 500 mg fresh weight tissue samples, using 2 ml aliquots of a cold buffer mixture at a pH of about 7.6 — 7.8, containing 50 m*M* Tris (tris-hydroxymethylaminomethane), 35 m*M* ascorbic acid, 1 m*M* EDTA and 2 m*M* $Na_2S_2O_5$. Samples were centrifuged during 30 minutes at 15,000 *g*. To the supernatants were added about 50 mg Sephadex G-200 and 200 mg sucrose, and the extracts were stored frozen, at −10 °C.

Polyacrylamide gel electrophoresis was performed using general procedures of Davis (1964), adapted by Jaaska and Jaaska (1969).

The gel was composed of 10 per cent acrylamide, 0.15 per cent N,N'-methylenebisacrylamide, 0.25 *M* Tris, 0.075 *M* HCl, 0.2 per cent triethanolamine and 0.5 mg per cent riboflavine-5-phosphate. The upper

cathode buffer contained 0.01 *M* Tris and 0.08 *M* glycine, whereas the lower anode buffer was 0.1 *M* Tris-acetate at a pH of about 8.9.

Enzyme staining. Dehydrogenases were assayed by incubating the gels in 6 ml 0.1 *M* phosphate buffer (pH 7.6) containing 0.5 ml 0.2 *M* neutralized solution of appropriate substrate, 0.5 ml 0.5 per cent NAD or NADP, as required, 0.5 ml 0.2 per cent nitroblue tetrazolium hydrochloride (NBT) and 0.5 ml 0.05 per cent phenazine methosulfate (PMS).

Acid phosphatase, esterase, leucine aminopeptidase and peroxidase activities was located as described by Jaaska and Jaaska (1969).

β-Galactosidase and β-glucosidase activity were assayed by the same way as esterase and acid phosphatase, except by using appropriate substrate, 2-naphthyl-β-galactopyranoside or 2-naphthyl-β-glucopyranoside, respectively.

Results

Peroxidase enzymograms of vegetative organs of wheat revealed the presence of a series of fractions: one slow-moving doublet, two to four, depending on the various organs and on the stages of development, minor fractions moving a little faster, one or two weak bands at the middle of the gels and one fast-moving doublet (Fig. 1*A*, *B*, *C*). The intensity of this fast-moving doublet was found to diminish as leaves increased.

Peroxidase activity depended on the internode position. Differences in this respect were already apparent in the youngest internode, i. e. the higher its position the higher was its peroxidase activity.

Qualitatively, the patterns from anthers and pistils were similar, as seen in Fig. 1*E, F*. Quantitatively, the intensity of bands was different, and the band of the lowest electrophoretic mobility had a diffuse staining in enzymograms of pistils. The spikelet tissue extracts showed the highest peroxidase activity, and, in enzymograms for spikelets, up to fourteen fractions could be distinguished.

Peroxidase patterns for seeds changed both qualitatively and quantitatively during the seed maturing. Eleven bands were observed for milky seed extracts, whereas nine were detected for waxy seeds and five for mature seeds (Fig. 1*G*). The peroxidase pattern of milky seeds was characterized by the presence of five slow-migrating isoforms with different intensity, three bands of intermediate mobility, one weak doublet and a heavy band near the anode end of gels. Some enzymatic activity remained at the site of sample application. In extracts of waxy seeds, one band of intermediate mobility, and one band of the fast-moving doublet disappeared, and the intensity of slow-migrating fractions changed. Peroxidase activity was low in extracts of mature seed. Enzymograms showed three slow-moving bands, two weak bands of intermediate mobility, and an inconsiderable activity remained at the site of origin.

Acid phosphatase. The electrophoretic patterns of acid phosphatase are shown in Fig. 2. Qualitatively, the patterns from leaves and roots were essentially similar, revealing a doublet of major isoforms of intermediate mobility and 1 or 2 minor bands. Quantitatively, they differed considerably in the intensity of individual bands relative to one another. Only minor changes were noted in acid phosphatase patterns of leaves and roots during wheat development. Enzymograms of internodes contained the same doublet as in leaves and roots, and only one slower-moving band, as seen in Fig. 2*C*.

The spikelet tissue showed the highest level of acid phosphatase activity (Fig. 2*D*). The enzymograms of the spikelet revealed the presence

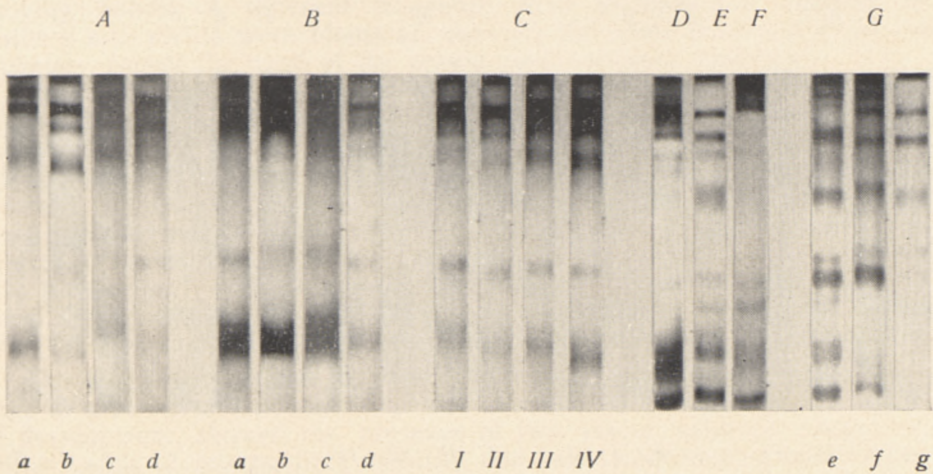

Fig. 1. Peroxidase electrophoretic patterns of wheat organs at various stages of development.
Enzymograms of (*A*) leaves; (*B*) roots; (*C*) internodes; (*D*) spikelet; (*E*) anthers; (*F*) pistils; (*G*) seeds; at stages of (*a*) seedling; (*b*) sprouting; (*c*) culming; (*d*) forming spikes; (*e*) milky seed; (*f*) waxy seed; (*g*) mature seed; (I—IV) the numbers of internode positions.

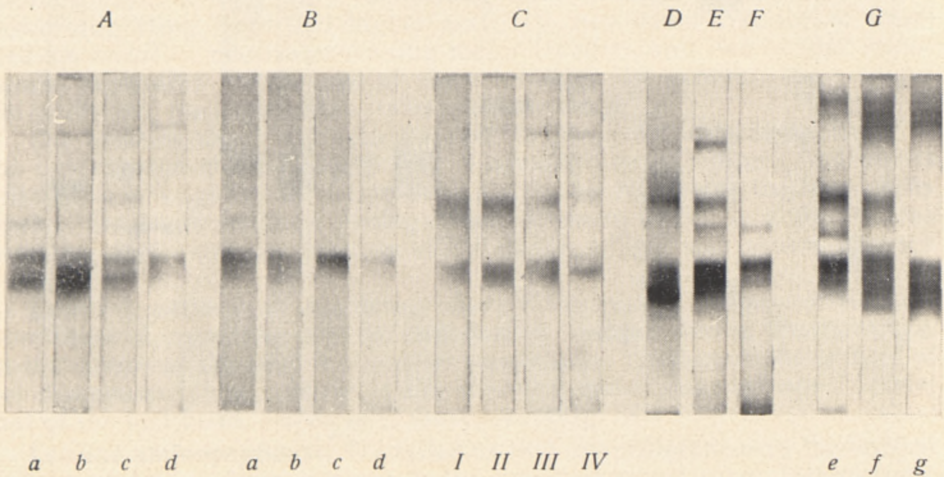

Fig. 2. Acid phosphatase electrophoretic patterns of wheat organs at various stages of development. Designations see under Fig. 1.

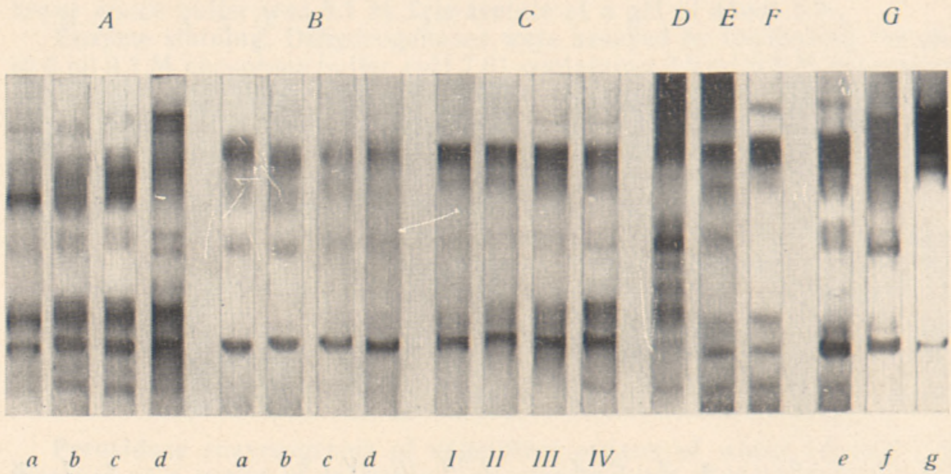

Fig. 3. Esterase electrophoretic patterns of wheat organs at various stages of development. Designations see under Fig. 1.

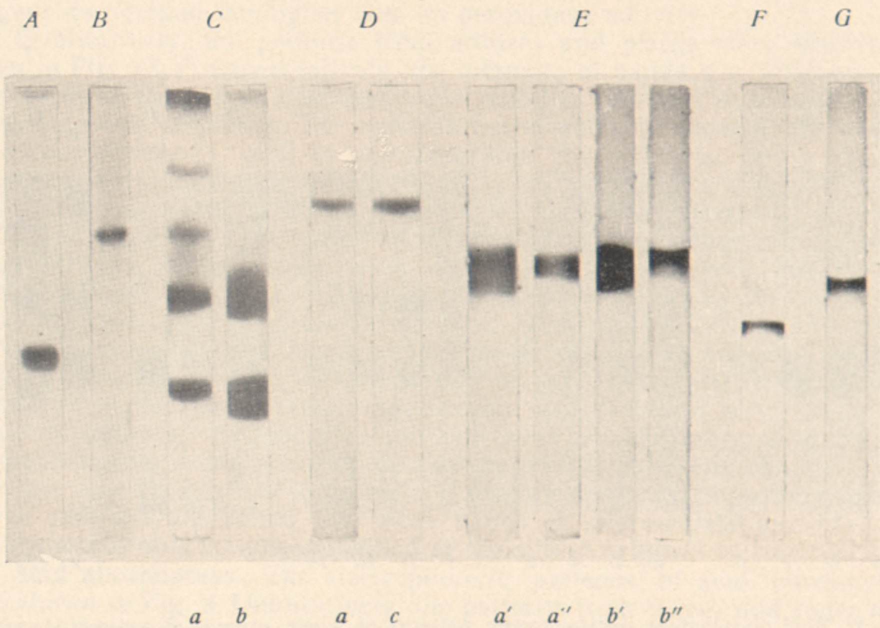

Fig. 4. Electrophoretic patterns of (*A*) leucine aminopeptidase; (*B*) β-galactosidase; (*C*) malate dehydrogenase; (*D*) glytamate dehydrogenase; (*E*) glycose-6-phosphate dehydrogenase; (*F*) 6-phosphogluconate dehydrogenase and (*G*) alcohol dehydrogenase in (*a*) leaves; (*b*) seeds; (*c*) roots; (*a′*) leaves at stage of seedling; (*a″*) leaves at later stages; (*b′*) milky seeds; (*b″*) waxy, mature seeds.

of the same heavy doublet as in the leaves and roots, and three slower-moving bands of different intensity. Acid phosphatase patterns for anthers and pistils were identical, showing the same fractions as in the spikelets, except the band of the lowest electrophoretic mobility.

Acid phosphatase patterns of seeds showed differences from those of vegetative organs. At the same time, the patterns of milky seeds and anthers (Fig. 2*E* and *G*; enzymogram *e*) were essentially similar, except for the presence of some additional activity (three bands of low electrophoretic mobility) in the milky seed extracts. After transition to the waxy seed, the enzymograms showed the presence of the same three slow-migrating bands, which now appeared more distinct and of higher intensity, and three closely-spaced faster-moving bands of similar intensity. The same fractions also persisted in mature seed tissues, except two bands near the centre of the gel, which were lacking in extracts of mature seed.

Esterase enzymograms of wheat vegetative organs revealed the presence of a series of fractions. The leaf extracts, at all investigated stages of development, showed the presence of two major esterase zones, two minor bands of intermediate mobility and two faster-moving major fractions, as seen in Fig. 3*A*. In addition, depending on the stage of plant development, some slow- and fast-moving fractions were observed in leaf extracts.

Esterase enzymograms from roots in the course of wheat development revealed, on the whole, identical patterns consisting of two slow-moving zones, one or two weak bands of intermediate mobility, one major fraction of high electrophoretic mobility, and, in addition, three weak, scarcely distinguishable closely-spaced fast-moving bands (Fig. 3*B*).

All four investigated internodes showed qualitatively identical electrophoretic patterns (Fig. 3*C*), consisting of two slow-moving zones (the slower-migrating band had higher activity), two minor fraction of intermediate mobility, one faster-moving major fraction, and two or four minor bands, depending on the age of the internodes. In general, the younger the internode, the higher was its esterase activity.

The spikelet tissue extracts showed the strongest esterase activity of the investigated wheat spike organs. Enzymograms of anthers and pistils differed only slightly. In patterns of pistils, up to six electrophoretically distinct fractions could be distinguished, whereas a diffuse staining area near the origin and one fraction at the middle of gel was absent in pistil tissues.

Esterase patterns of maturing seeds revealed only minor differences from those of vegetative organs, and showed changes during the maturing. In the course of the seed maturation process, the doublet at the middle of gel disappeared, the activity of the slowly moving zone increased, while that of the fast band, conversely, decreased (Fig. 3*G*).

Leucine aminopeptidase. We failed to find any differences in the leucine aminopeptidase isoform patterns in the course of wheat plant development. Leaf and internode extracts contained one band (Fig. 4*A*) migrating somewhat farther of the middle of gels, at all stages of development. The band intensity decreased in the order: leaves > young internodes > old internodes. No leucine aminopeptidase was noted in roots at all stages of development. The reproductive structures of flower (the anther and the pistil) and maturing seed extracts contained the same fraction which was found in extracts of overground vegetative organs.

β-Galactosidase activity was electrophoretically revealed as a distinct band only at one stage of seed development, and namely in the waxy seed (Fig. 4*B*). At the period of transition of the milky seed to waxy seed, one

distinct band appeared. It was found at all stages of waxy seed, becoming gradually weaker when going over to the mature seed, and being absent or very faint in the enzymograms of mature seed extracts.

The activity of soluble β-galactosidase was electrophoretically not found in anthers and pistils or in vegetative organs.

β-Glucosidase appeared as one light, diffuse band in roots, whereas no activity was detected in leaves at all stages of wheat development. β-Glucosidase activity was absent in the course of seed development, but revealed the presence of one fraction in extracts of mature seed.

Malate dehydrogenase. NAD-dependent malate dehydrogenase patterns of vegetative organs (leaves, roots and internodes) were essentially similar, showing the presence of two fractions and a weaker slower-moving band (enzymogram *a* in Fig. 4*C*). Some enzymatic activity remained at the site of sample application in extracts of leaves and internodes. No definite qualitative change was noted in malate dehydrogenase patterns of leaves and roots during plant development. These patterns differ only in the intensity of individual bands relative to one another.

No major qualitative difference was observed between the seeds and vegetative organs. In enzymograms of seeds, the slower-migrating minor band was absent (enzymogram *b* in Fig. 4*C*). Quantitatively, the intensity of two fractions changed during seed development. The waxy seed tissue extracts exhibited the strongest malate dehydrogenase activity, whereas the milky seed had a rather low activity, and the mature seed the lowest activity, scarcely detectable in enzymograms.

Malate dehydrogenase bands were not detected in enzymograms for anthers and pistils.

Glutamate dehydrogenase. NAD-dependent glutamate dehydrogenase in wheat vegetative (leaves, roots, internodes) and reproductive (anthers, pistils, seeds) organs appeared in enzymograms as one band at all analyzed stages of development (Fig. 4*D*). The band intensity decreased from roots to leaves and in seed extracts from milky to mature seed. In general, the roots showed the highest glutamate dehydrogenase activity, and the anthers and pistils — the lowest one of the organs examined.

Glucose-6-phosphate dehydrogenase. The first leaf tissue extracts exhibited the strongest glucose-6-phosphate dehydrogenase activity; the enzymograms showed a heavy doublet of closely-spaced bands (enzymogram *a′* in Fig. 4*E*), but upon prolonged incubation of gels in the reaction solution the doublet became fused. Enzymograms of leaves at following stages of development revealed only one of the two bands found in the first leaf (enzymogram *a″* in Fig. 4*E*). The internodes showed similar glucose-6-phosphate dehydrogenase patterns; the younger the internode, the higher was its glucose-6-phosphate dehydrogenase activity. Roots, at the stage of seedling, contained a single fraction moving a little faster than that of the leaves. At subsequent stages of development no band with glucose-6-phosphate dehydrogenase activity was found in root tissues. In general, glucose-6-phosphate dehydrogenase activity in wheat vegetative organs decreased in the course of seedling growth, showing a high activity only at early stages of development.

In the spike of wheat plant, glucose-6-phosphate dehydrogenase activity was distributed in the following way: the spikelet tissue extracts exhibited the strongest activity, the anther had a rather low activity, and the pistil the lowest one. These three organs showed a band in common with that in the leaves.

Enzymograms for milky seeds revealed the presence of two closely-

spaced major glucose-6-phosphate dehydrogenase fractions, which fused after longer reaction time (enzymogram *b'* in Fig. 4*E*).

In the course of the seed maturing, one of the two glucose-6-phosphate dehydrogenase bands of milky seeds disappeared, and, in waxy and mature seeds, only one band was observed (enzymogram *b''* in Fig. 4*E*).

6-Phosphogluconate dehydrogenase was revealed in extracts of leaves, culms, anthers and pistils at the flowering stage and in seed extracts (Fig. 4*F*). 6-Phosphogluconate dehydrogenase patterns for all investigated organs were identical, showing the presence of only one fraction of intermediate mobility. The intensity of the band varied in different organs. The 6-phosphogluconate dehydrogenase activity was strongest in the leaf tissue and lowest in the pistils. The milky seed revealed a band of 6-phosphogluconate dehydrogenase activity, whereas with transition to waxy seed this activity disappeared.

Alcohol dehydrogenase appeared as a single band in milky seeds (Fig. 4*G*). Upon prolonged incubation of gels in the reaction solution, traces of additional light bands appeared, but the background darkened. In waxy seed extracts, the same band was found. However, the intensity of the band was lower in enzymograms for waxy seeds, and no alcohol dehydrogenase was noted in extracts of the mature seed.

Homoserine dehydrogenase was detected in extracts of milky seeds as a single band at the same migration distance as the alcohol dehydrogenase band. No homoserine dehydrogenase was noted in waxy and mature seed extracts.

Discussion

The data obtained in this study suggest that several enzymes exist in multiple molecular forms in different organs of wheat. The degree of heterogeneity was found to vary considerably, depending on the kind of enzyme or wheat tissue.

No variation in different wheat organs and during development was observed in the NAD-dependent glutamate dehydrogenase pattern consisting of one band of invariable electrophoretic mobility.

The results show a similar persistant presence of an electrophoretically invariant leucine aminopeptidase fraction in all organs, except in roots, examined at various developmental stages of wheat. Leucine aminopeptidase is known to be distributed widely in both plants and animals. It is a hydrolytic enzyme that is probably of considerable importance in protein degradation during growth and development, but its specific function in the different tissues and organs is not yet understood.

Malate and glucose-6-phosphate dehydrogenase enzymograms varied slightly in different vegetative organs in the course of plant development. However, the activity of malate dehydrogenase changed considerably during seed maturation. Thus, the activity increased during seed development to a maximum in waxy seeds, and thereafter decreased to the low levels characteristic of the mature seed. Similar changes in the activity of malate dehydrogenase during seed maturation process were observed in barley grains by Duffus (1970).

The activity of glucose-6-phosphate dehydrogenase is higher in the intensive growth period. This enzyme appeared as a doublet in the first leaf tissues, remaining only as one band with lower activity at the following stages of development. A similar doublet was present in the extract of the youngest internode, whereas older internodes contained one band,

only. In immature seeds, glucose-6-phosphate dehydrogenase activity was higher than in mature seeds, and the same doublet was observed in milky seeds, whereas one band occurred in mature seeds.

6-Phosphogluconate dehydrogenase appeared as one distinct band in milky seeds and disappeared on transition to waxy seeds. Both glucose-6-phosphate and 6-phosphogluconate dehydrogenases play a role in the metabolism of glucose via the pentose shunt. This pathway is not thought to be an important contributor of energy, serving rather as a potential source of rhibosephosphate units that may be utilized in the synthesis of nucleic acids. The observation that the first leaf at the stage of seedling and the milky seed contained the highest activity is in agreement with the above views, since there is extensive nucleic acid synthesis in the young, rapidly-developing tissues.

Acid phosphatase patterns of vegetative organs proved to be different from those of maturing seeds. At the same time, the patterns of different vegetative organs (leaves, roots and internodes) were essentially similar and indicated that no great changes occur in the course of plant development. More significant shifts were observed in the isoform composition of acid phosphatase during the maturation of seeds. By visual observation, we detected that the waxy seed had the highest activity of the isoforms, while the extracts from milky and mature seeds had lower activities.

The presence of various hydrolytic enzymes (acid phosphatase, esterase, leucine aminopeptidase) has been demonstrated in various plants during seed germination and seedling development, but limited information is available concerning the developing seeds (Duffus, 1969, 1970). Their significance in the maturing of grain remains obscure.

The esterase and peroxidase data present a more heterogeneous picture in that there are several zones of enzyme activity with closely-spaced variants in each zone. Marked alteration was observed in esterase and peroxidase isoforms during seed maturation. The presence of eleven distinct peroxidase bands in milky seeds and the decrease in the number of bands and their activity during maturation process suggest that they deactivated or disappeared with seed maturation. In the absence of genetic data, it is difficult to say whether the disappearance of several bands is the result of differential deactivation of genes. The function of these enzymes in differentiation and the development of the wheat plant is unknown at the present time.

The results of the present study are in agreement with numerous similar observations that, although all the cells in different tissues of a plant carry the same genetic information, the manifestation of the phenotype as expressed in the synthesis of enzyme proteins is tissue-specific and well regulated for each stage of development.

Summary

Isoform composition of peroxidase, acid phosphatase, esterase, leucine aminopeptidase, β-galactosidase, β-glucosidase and dehydrogenases in various organs of a diploid spring wheat, *Triticum monococcum* L. var. *flavescens,* has been studied by means of polyacrylamide gel electrophoresis, during the main stages of wheat development.

Several enzymes considerably differed in isoform composition and in the degree of its variation during ontogenesis. No variation in different wheat organs and in the course of development was observed in glutamate dehydrogenase or in leucine aminopeptidase patterns consisting of one

band, apart from the fact that no leucine aminopeptidase activity was detected in roots extracts.

Glucose-6-phosphate and malate dehydrogenase patterns varied only slightly in different organs and during wheat development. More considerable shifts were detected in the isoform composition of peroxidase and esterase.

The enzymograms of vegetative organs were, for several enzymes, different from those of seeds, except certain fractions which were present in all stages of development. The composition and activity of isoforms of certain enzymes, like peroxidase and esterase, diminished during maturation of wheat grains. Activity of certain enzymes, like the enzymes 6-phosphogluconate and alcohol dehydrogenases, disappeared entirely in the extracts of mature seed.

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DIPLOIDSE NISU MÕNEDE ENSÜÜMIDE FRAKTSIOONILISE KOOSTISE ONTOGENEETILINE VARIEERUVUS

Resümee

Polüakrüülamiidgeelelektroforeesi abil uuriti diploidse nisu *Triticum monococcum* L. mõnede ensüümide (peroksüdaasid, esteraasid, happelised fosfataasid, leutsiini aminopeptidaasid, β-glükosidaasid, β-galaktosidaasid ja dehüdrogenaasid) fraktsioonilise koostise ontogeneetilist varieeruvust.

Selgus, et ontogeneesi vältel on kvalitatiivselt kõige püsivam glutamaadi dehüdrogenaas, mida kõigis nisu uuritud organeis esines vaid üheainsa fraktsioonina.

Samasugust stabiilsust täheldati ka leutsiini aminopeptidaasil nisu maapealsetes organites; juurtes tema aktiivsust ei esine.

Mõõdukaid muutusi arengu vältel tuvastati peroksüdaaside ja esteraaside fraktsioonilises koostises, kusjuures muutusid peamiselt ensüümi väikesed fraktsioonid.

Seemne ja vegetatiivsete organite ensümogrammid erinesid omavahel, välja arvatud mõned fraktsioonid, mis on ühised kõigile arenemisastmeile. Mõnede ensüümide (peroksüdaasid ja esteraasid) fraktsioonide arv ja aktiivsus vähenevad, mõnede (6-fosfoglükonaadi ja alkoholi dehüdrogenaasid) aktiivsus aga kaob seemne valmides täielikult.

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ОНТОГЕНЕТИЧЕСКАЯ ВАРИАЦИЯ ФРАКЦИОННОГО СОСТАВА НЕКОТОРЫХ ФЕРМЕНТОВ У ДИПЛОИДНОЙ ПШЕНИЦЫ

Резюме

С помощью электрофореза в полиакриламидном геле исследовался состав ряда ферментов (пероксидазы, эстеразы, кислой фосфатазы, лейцинаминопептидазы, β-галактосидазы, дегидрогеназ) в ходе онтогенетического цикла пшеницы (*Triticum monococcum* L. var. *flavescens*).

Отдельные ферменты существенно различаются между собой по степени изменчивости в ходе онтогенетического цикла. Умеренные изменения наблюдались во фракционном составе пероксидазы и эстеразы; значительно изменяются главным образом минорные фракции ферментов. Наиболее стабильной среди изученных ферментов оказалась НАД — зависимая глутаматдегидрогеназа, которая во всех органах пшеницы выявлялась как одна дискретная фракция с постоянной электрофоретической подвижностью. Такой же стабильностью обладает лейцинаминопептидаза, обнаруженная в виде одной молекулярной формы на всех стадиях развития во всех изученных надземных органах. Лейцинаминопептидазная активность отсутствует в корнях.

Энзимограммы семян и вегетативных органов значительно различаются между собой, за исключением некоторых фракций, характерных для всех органов на всех стадиях развития. Набор и активность изоформ у большинства ферментов уменьшаются при созревании семян. Об этом свидетельствует, например, исчезновение отдельных фракций пероксидазы и эстеразы. Активность некоторых ферментов в зрелых семенах полностью исчезает, что подтверждают данные о 6-фосфоглюконат- и алкогольдегидрогеназе.

Таким образом, результаты нашего исследования показывают, что в течение онтогенетического цикла фракционный состав некоторых ферментов значительно меняется, а у других остается неизменным.

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