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Genetic control of aspartate aminotransferase isoenzymes in *Aegilops* and *Triticum* species

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Abstract. Zymograms of the aspartate aminotransferase (AAT, EC 2.6.1.1) activity in leaf extracts from *Aegilops* and *Triticum* species revealed three AAT zones, denoted according to the decreasing electrophoretic mobility towards the anode as AAT-1, AAT-2 and AAT-3. The AAT activity zymograms of subcellular fractions isolated from *T. aestivum* seedlings made it possible to establish that the AAT-1 zone is located in the mitochondria, AAT-2 in the chloroplasts and AAT-3 in the cytoplasm. Most of the total AAT activity from wheat leaves arises from the chloroplasts and cytoplasm. The AAT-3 zone exhibited the lowest electrophoretic mobility, but 3 isoenzymes occurring within were the most visibly separated. The occurrence of a single band in this zone at the AAT-3a position (closest to the anode) for the aneuploid CS3ASDt AABBDD line (the absence of long arms of the 3rd pair of homologous chromosomes in the A genome) and at the AAT-3c position for *Ae. umbellulata* (genome UU), as well as three bands in the whole zone for *T. durum* (AABB) and *T. aestivum* (AABBDD) each, made it possible to evaluate the subunit composition of isoenzymes in the AAT-3 zone. The band at the AAT-3a position in the zymogram is formed from ββ dimers, AAT-3b from αβ and AAT-3c from αα. By comparing the distribution of isoenzyme bands intensities (the result of enzymatic activity) with the mathematical models, the frequencies of the occurrence of the α and β subunits within AAT-3 zone were evaluated. In AAT-3 from *T. durum*, α and β occurred at the ratio of 0.54 : 0.46, and in that from *T. aestivum* – 0.62 : 0.38, respectively.

Key words: *Aegilops umbellulata*, aspartate aminotransferase, isoenzymes, subcellular fractions, *Triticum aestivum*, *Triticum durum*, zymogram.

Introduction

Plant aspartate aminotransferase (AAT) catalyses the reversible transamination reaction between *L*-aspartate and 2-oxoglutarate to give oxaloacetate and *L*-glutamate. AAT is a dimer of about 90 kDa with two active sites, each formed from the amino acid residues of both subunits and containing a covalently bound pyridoxal-5'-phosphate cofactor, thus only the AAT dimer is enzymatically active (Wilkie et al. 1996).

A wide range of metabolic functions is contributed to AAT, which is the most thoroughly studied of the plant aminotransferases (Ireland and Lea 1999). Therefore, it is not surprising that many AAT isoenzymes localized in several cellular compartments were detected in different plant species. Huang et al. (1976) reported the occurrence of AAT isoenzymes in the cytoplasm, mitochondria, chloroplasts and peroxisomes from spinach leaf cells. Taniguchi et al. (1995) in millet and Schultz et al. (1998) in *Arabidopsis* localized them in the cytoplasm, mitochondria and plastid fractions of leaves, and Scandalios et al. (1975) – in the cytoplasmatic, mitochondrial and glyoxysomal fractions of maize leaves. Stephens et al. (1998) reported on five AAT isoenzymes in soybean seedlings, which were distinguishable on native PAGE. Four of them were localized in the glyoxysomes, cytoplasm, mitochondria and plastids (Stephens et al. 1998). Hart (1983)

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observed three isoenzymatic zones (groups of isoenzymes with similar electrophoretic mobility) on the AAT activity zymogram from leaf cells of hexaploid wheat (*T. aestivum*). Up to date, their subcellular localization was not established.

According to Hart (1983), AAT genes in *T. aestivum* (AABBDD, 2n = 42) occur on the long arms (L) of the 3rd pair of homoeologous chromosomes, as well as the long and short (S) arms of the 6th pair, i.e. on 3AL, 3BL, 3DL, 6AL, 6BL, 6DL, 6AS, 6BS and 6DS (Hart and Langston 1977; Hart 1983). On http://wheat.pw.usda.gov ESTs of AAT are mapped on 3AL, 3BS, 3DS and 6BS. The first three of them are identical. The zones observed by Hart (1983), i.e. AAT-1, AAT-2 and AAT-3 (numbered according to their decreasing electrophoretic mobility towards the anode) are products of genes from different loci. If a given locus controls the biosynthesis of only one type of subunits, one can record a single band in a zone. If the biosynthesis of two types of subunits is controlled by the genes from the same locus, three bands for the dimer should be visible. The AAT-3 zone in *T. aestivum* (AABBDD) is formed from two types of subunits: α and β – products of genes located in chromosome arms 3AL, 3BL and 3DL (Hart et al. 1976). It is accepted that genome A is responsible for the α subunit biosynthesis, and genomes B and D for subunit β (Hart et al. 1976). The identity of ESTs on 3AL, 3BS and 3DS precludes the bioanalytical approach to identify differences between genes coding α and β subunits, that explain differences in the electrophoretic mobility of the isoenzymes. According to Hart et al. (1976), the number of α and β subunits in the AAT-3 zone is identical and the catalytic efficiencies of particular isoenzymes are similar. If he was right, the distribution of staining intensity indirectly proving the amount of particular isoenzymes in AAT-3 would be in a ratio of 1:2:1 for *T. durum* and 1:4:4 for *T. aestivum*, respectively. An asymmetric distribution of band staining intensities in the AAT-3 zone for *T. durum* and different than expected – for *T. aestivum* were observed in our study. This raises new interesting questions on the mechanism of the formation of the isoenzymatic zones visible in a zymogram.

This report, (1) establishes the subcellular localization of three AAT zones, (2) confirms the hypothesis referring to the subunit composition of isoenzymes within the AAT-3 zone proposed by Hart et al. (1976), and (3) shows different subunit frequencies in the AAT-3 zones from *T. durum* and *T. aestivum.*

Material and methods

Plant material

Extracts from 2-week-old leaves of *Ae. umbellulata*, *T. durum*, *T. aestivum* cv. Jasna (used also for organelle isolation) and cv. Chinese Spring (CS) ditelosomic lines without long arms of the 3rd pair of homologous chromosomes in the genomes: A (3ASDt), B (3BSDt) and D (3DSDt) were used. Plants were grown in hydroponics in distilled water (seeds were pretreated with commercial fungicide Funaben (Organika-Azot SA, Jaworzno) in cuvettes kept in a growing chamber at day/night temperature 22°C/18°C (12 h photoperiod). Photon flux density was 400 μmol $m^{-2}s^{-1}$. After 7 days distilled water was replaced by medium containing 0.25% commercial fertilizer Florovit (Inco-Veritas SA, Góra Kalwaria).

Subcellular fractionation

Mitochondria

Green parts of wheat seedlings were gently ground for 5 s in a homogenizer (Ultra Turrax T25) in the proportion of 1 g of plant material and 3 mL of 30 mM morpholinepropanesulfonate buffer adjusted to pH 7.5 with KOH, containing 0.3 M sucrose, 0.2% bovine serum albumin and 0.6% polyvinylpyrrolidone, then filtered through miracloth and centrifuged at 5,000 × *g* for 2 min. The precipitate, which consisted mostly of plant tissue fragments and great amounts of chloroplasts, was discarded and the supernatant was centrifuged at 20,000 × *g* for 3 min. The precipitate was dissolved in 24 mL of sample buffer, i.e. 62.5 mM Tris-HCl pH 6.8 with 10% glycerol, then centrifuged at 20,000 × *g* for 3 min. The precipitate, i.e. the mitochondrial fraction, was dissolved in a small volume of the same buffer.

Chloroplasts

Green parts of wheat seedlings were gently ground for 5 s in the proportion: 1 g of plant material and 3 mL of 50 mM Tricine buffer, adjusted to pH 7.8 with KOH, containing 0.4 M sorbitol and 10 mM NaCl, and then filtered through miracloth and centrifuged at 4,000 × *g* for 3 min. The precipitate was dissolved in 15 mL of washing buffer (15 mM Tricine buffer adjusted to pH 7.8 with KOH, containing 10 mM NaCl and 5 mM $MgCl_2$) and centrifuged at 10,000 × *g* for 5 min. The precipitate was dissolved in a small volume of the sample buffer (see above).

Cytoplasm

Homogenate was prepared as for mitochondria isolation and then it was filtered through miracloth and centrifuged at 5,000 × *g* for 2 min. The supernatant obtained was centrifuged at 100,000 × *g* for 60 min. The supernatant was washed several times with sample buffer and then concentrated in Amicon supplied with a PM30 membrane.

Enzyme assays

The activities of the marker enzymes were determined by spectrophotometric methods: the mitochondria – citrate synthase (EC 4.1.3.7, Bergmayer 1974), the chloroplasts – alkaline C_1-fructose-1,6-diphosphatase (EC 3.1.3.11, Latzko and Gibbs 1974) and the cytoplasm – lactate dehydrogenase (EC 1.1.1.27, Bergmayer 1974). The AAT activity was assayed using the method of Bergmayer and Bernt (1974). Specific activity was expressed in μmol of the product formed per minute at 25°C (U) and per 1 mg of protein. Protein was determined according to Bradford (1976) with bovine serum albumin as a standard. The purification factor representing the quotient of the specific activity of the appropriate marker enzyme in a given fraction and the specific activity of this enzyme in the initial extract was calculated to determine the purity of each isolated subcellular fraction.

PAGE

Electrophoresis was carried out in a Mini Protean apparatus (Bio-Rad) using the native discontinuous buffer system of Laemmli (1970). The resolving gel consisted of 7.5% acrylamide, to which 10% (m/v) mannitol was added. The stacking gel was 4% acrylamide, 1 cm high. The ratio of acrylamide to bisacrylamide was 37.5 : 1. Electrophoresis was run under the constant power of 100 mV for 150 min or 300 min (to obtain a better resolution of the AAT zones).

Staining procedure for AAT activity in gel

Each gel was stained for the AAT activity by incubation in a solution composed of 0.1 M Tris-HCl buffer pH 7.5, 8 mM *L*-cysteine sulfinic acid, 0.1 mM pyridoxal-5'-phosphate, 0.5 mM 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, 5 mM 2-oxoglutaric acid and 0.16 mM 5-methyl phenazine methosulfate (Stejskal 1994).

Densitometric analysis

Gels with a dark blue insoluble formazan (the effect of isoenzyme activity) were scanned (300 pixels per inch) and analysed by a computer software package (NIH Image, http://rsb.info.nih.gov/nih-image/). The programme displays the average value (on the scale from 0 to 255) for all the pixels in the selected area. The results were the difference between the value of a selected band and the same area of the background.

Statistical approach

T. durum

Substituting various frequencies for the α and β subunits that form enzymatically active isoenzymes within the AAT-3 zone to the formula $(\alpha + \beta)^2 = \alpha^2 + 2\alpha\beta + \beta^2$, many theoretical proportions of a generalized form $\alpha^2 : 2\alpha\beta : \beta^2$ were achieved. For each densitometric measurement of the AAT-3 zone from different plants, the sum of the values obtained for single bands within the zone was calculated. Then, it was divided according to the theoretical proportions, giving a variety of theoretical distributions, i.e. expected values for particular isoenzymes in the zone. The theoretical distribution most similar to the experimental one was selected for each plant. The statistical distance between these distributions was measured using the χ^2 function: the difference between the densitometric measurement and the expected value for a single band was squared and divided by the expected value; then, the obtained values for each of the three bands were added up (χ^2). The lower χ^2, the better similarity was found between the theoretical model and the experimental results. Each selected theoretical distribution corresponded to a particular frequency of the subunit α and thus β ($\alpha + \beta = 1$).

T. aestivum

In order to achieve a variety of theoretical proportions of a generalized form $\alpha^2 : 4\alpha\beta : 4\beta^2$, the frequencies for the α and β subunits were substituted to the formula $(\alpha + 2\beta)^2 = \alpha^2 + 4\alpha\beta + 4\beta^2$. Then, the obtained theoretical proportions were respectively decreased so that the sum of the proportion components was equal to one. The further procedure was identical as in case of *T. durum*.

Results

Subcellular localization of AAT isoenzymes

The mitochondrial, chloroplast and cytoplasmatic fractions of purification factors 2.2, 2.0 and 1.7,

respectively, were obtained from leaves of *T. aestivum*. Partially purified subcellular fractions were loaded on the gel and after electrophoretic separation were stained for the AAT activity (Figure 1).

The intensities of the zones in these fractions were compared. On this basis their subcellular localization was decided. The AAT-1 zone proved to be the most intensive in the mitochondrial fraction and the AAT-2 zone in the chloroplasts. In the cytoplasm two intensive zones were observed: AAT-2 and AAT-3 (Figure 1). It was concluded that the AAT-1 zone is located in the mitochondria, AAT-2 in the chloroplasts and AAT-3 in the cytoplasm. In our opinion the quite intensive AAT-2 zone in the cytoplasm fraction (Figure 1) came with chloroplast impurities. It is worth underlining that both the chloroplast and cytoplasm zones were the strongest (Figure 1).

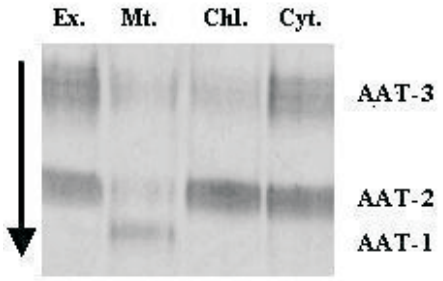

Figure 1. Subcellular localization of aspartate aminotransferase (AAT) isoenzymatic zones from *T. aestivum*. Lanes (from left to right) 1, cellular extract (Ex.); 2, mitochondria (Mt.); 3, chloroplasts (Chl.); 4, cytoplasm (Cyt.). Each lane contained equal AAT activity (0.6 U). Electrophoresis was run for 150 min.

Electrophoretic separation of isoenzymes in AAT-3 zone

In order to confirm the isoenzyme subunit composition within the AAT-3 zone, i.e. AAT-3a = ββ, AAT-3b = αβ and AAT-3c = αα, which was first proposed by Hart et al. (1976), zymograms were made of the AAT activity in the leaves of different wheat species. Figure 2 presents the zone of interest obtained after 300 min of electrophoretic separation. It is one band visible for ditelosomic line without long arms of the 3rd pair of homologous chromosomes in the A genome of *T. aestivum* cv. Chinese Spring with the genome formula: AABBDD (CS3ASDt AABBDD) in the AAT-3 zone at the AAT-3a position (AAT-3 zymograms

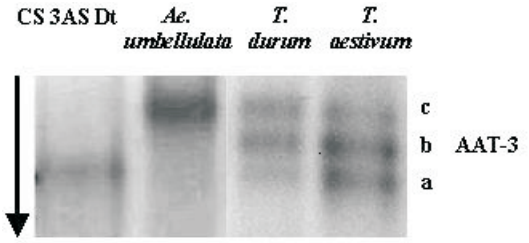

Figure 2. Aspartate aminotransferase 3rd zone (AAT-3) from different wheat species. Lanes (from left to right) 1, CS3ASDt; 2, *Ae. umbellulata*; 3, *T. durum*; 4, *T. aestivum* cv. Chinese Spring. Each lane contained equal AAT activity (0.6 U). Electrophoresis was run for 300 min.

obtained for CS3BSDt and CS3DSDt, data not shown, were identical as those for *T. aestivum* cv. Chinese Spring); for *Ae. umbellulata* (UU), the only band occurs at the AAT-3c position (Figure 2). For both *T. durum* (AABB) and *T. aestivum* cv. Chinese Spring (AABBDD), three bands in a zone are visible (Figure 2). It is worth mentioning that for *T. durum*, the greatest intensity of staining was observed for bands at the AAT-3b and AAT-3c positions, whereas for *T. aestivum* – the AAT-3a and AAT-3b bands (Figure 2).

Subunits frequencies (α and β) in AAT-3 zone for *T. durum* and *T. aestivum*

Assuming the following subunit composition of isoenzymes in the cytoplasmatic zone (AAT-3): AAT-3a = ββ, AAT–3β = αβ and AAT-3c = αα, it was attempted to evaluate the frequency of the α and β subunit occurrence within this zone. The band staining intensity columns in Table 1 present the results of the densitometric measurements of the bands within AAT-3 of randomly selected lanes in aspartate aminotransferase activity zymograms. Each lane contained different amounts of extracts from individual plants of *T. durum* or *T. aestivum*. The theoretical distribution columns in Table 1 show theoretical distributions most similar to the experimental results. The mean value of the frequency of the α subunit and the standard deviation of the arithmetic mean are also given (Table 1). The following mean frequencies of subunits were obtained: $\alpha = 0.54 \pm 0.01$ and $\beta = 0.46 \pm 0.01$ for *T. durum*; $\alpha = 0.62 \pm 0.02$ and $\beta = 0.38 \pm 0.02$ for *T. aestivum* (Table 1).

Table 1. Determination of mean α subunit frequency within aspartate aminotransferase 3rd zone (AAT-3) from leaves of *T. durum* or *T. aestivum*. The details of statistical analysis are presented in Material and methods.

Species	Band staining intensity			Statistical distance (χ^2)	Theoretical distribution			Frequency of α subunit	Mean frequency of α subunit
	AAT-3c	AAT-3b	AAT-3a		AAT-3c	AAT-3b	AAT-3a		
T. durum	8.02	9.75	5.57	0.56	7.06	11.55	4.73	0.55	0.54 ± 0.01
	14.30	17.72	12.10	1.68	11.93	22.02	10.17	0.52	
	45.28	51.25	32.29	4.95	38.97	63.77	26.09	0.55	
	15.55	19.89	12.52	1.34	28.09	49.82	22.09	0.53	
	15.85	20.52	12.99	1.36	13.86	24.59	10.90	0.53	
	25.51	30.43	20.45	3.05	21.46	38.06	16.88	0.53	
	22.83	27.11	17.44	2.44	19.65	33.47	14.26	0.54	
	8.56	9.92	6.17	0.87	7.46	12.20	4.99	0.55	
T. aestivum	8.69	14.22	12.47	1.25	7.14	17.51	10.73	0.62	0.62 ± 0.02
	10.37	17.36	14.86	1.31	8.60	21.08	12.92	0.62	
	12.30	19.13	15.63	1.59	10.42	23.45	13.19	0.64	
	29.18	48.66	43.45	4.23	23.36	59.74	38.19	0.61	
	17.77	35.55	30.30	1.42	15.36	40.96	27.30	0.60	
	15.26	26.79	23.67	1.62	12.67	32.37	20.70	0.61	
	10.09	20.70	18.85	0.94	8.35	23.99	17.37	0.58	
	18.86	25.80	23.43	3.89	15.08	33.93	19.09	0.64	

Discussion

In the present study, as in that by Hart (1983) investigations, at least three AAT isoenzymes, namely three isoenzymatic zones, were detected in hexaploid wheat (*T. aestivum*) leaves. They differ in their subcellular localization. The AAT-1 zone located in the mitochondria shows the greatest electrophoretic mobility, followed by AAT-2 from the chloroplasts, and AAT-3 from the cytoplasm as the slowest. Similar electrophoretic properties were demonstrated for three zones AAT from millet leaves (Taniguchi et al. 1995) – a plant from the same Gramineae family as wheat. However, in wheat leaves, the cytoplasm and chloroplast fractions are responsible for most of the AAT activity, whereas in millet leaves it was the mitochondrial and cytoplasmatic fractions. Perhaps these differences are associated with the different ways photosynthesis functions in millet and wheat – C_4 and C_3 plants, respectively (Drincovich et al. 2001). In millet leaves (C_4 plant), *L*-aspartate (AAT substrate) is a CO_2 transporting form (Taniguchi et al. 1995).

The zymograms obtained for the AAT-3 zones with cv. Chinese Spring (CS) ditelosomic lines without long arms of the 3rd pair of homologous chromosomes in the genomes: B (3BSDt), D (3DSDt) and with CS were the same (data not shown). In contradicted the hypothesis put forward by Hart (1983), which predicted the localization of the AAT genes on the long arms of chromosomes 3B and 3D. Our results are consistent with ESTs of AAT mapped on 3BS and 3DS (http://wheat.pw.usda.gov). The occurrence of a single band at the AAT-3a position from CS3ASDt confirmed the thesis that genes located on the long arms of the 3rd pair of homologous chromosomes are responsible for isoenzyme formation in that zone (Hart et al. 1976). The 3rd homologous chromosome pair of genome A of the studied aneuploid line has no long arms, thus the plant cannot synthesize α subunits and therefore there are no bands formed from the αβ and αα dimers occurring at the AAT-3b and AAT-3c positions, respectively (Hart et al. 1976). Furthermore, the presence of only one band at the AAT-3c position for the diploid *Ae. umbellulata* (genome UU) proves that genome U, similarly as genome A, controls the α subunit biosynthesis. Finally, it leads to the conclusion that confirms the hypothesis by Hart et al. (1976), referring to the subunit composition of particular bands: AAT-3a – ββ, AAT-3b – αβ, and AAT-3c – αα .

The applied method of comparison of the expected frequency and staining intensity distribution made it possible to state that the α-type subunits occur more often in the AAT dimers located in the cytoplasm and originating both from *T. durum* and *T. aestivum*. For the tetraploid, the α subunit frequency was 0.54, for the hexaploid – 0.62. Probably, the synthesis rate of the α subunit mRNA directed by genome A is greater than that of β subunits controlled by genome B. The experimental results of Hart (1977), who obtained the AAT activity distribution: 1:2:1 after the dissociation of the same amounts of αα and ββ dimers, and their subsequent recombination, exclude the possibility for a higher catalytic effi-

ciency of αα dimers. We consider the supposition that the type of the substrate has an impact on the isoenzyme activity distribution to be hardly possible. Using *L*-aspartate (Harris and Hopkins 1976), instead of the *L*-cysteine sulfinic acid, for activity staining the same results (data not shown) were obtained in our study.

In our opinion, the comparative method proposed by us may be applied both in studying the mechanisms of enzymatic protein biosynthesis, and for the evaluation of effects of breeding work, where isoenzymes play an important role as the most reliable single gene markers and virtually any plant tissue can be analysed for the identification of cultivars using isoenzymes (Drefahl and Buschbeck 1991; Agarwal et al. 2001; Karcicio and Izbirak 2003).

Conclusions

In hexaploid wheat (*T. aestivum*) leaf cells, three AAT isoenzyme zones differing in the electrophoretic mobility and subcellular localization were detected: AAT-1 – from the mitochondria, AAT-2 – from the chloroplasts, and AAT-3 – from the cytoplasm. Genes located on the long arm of the 3rd pair of homologous chromosomes in genome A are responsible for the formation of isoenzymes occurring within the AAT-3 zone. This locus controls the biosynthesis of the α subunit, which together with the β subunit forms active AAT dimers located in the cytoplasm. Genomes A and U govern the α subunit biosynthesis in various species from the *Aegilops* and *Triticum* genera, genomes B and D – the β subunits. In AAT-3, the AAT-3a band is formed from ββ, AAT-3b from αβ, and AAT-3c from αα dimers, respectively. It was found that two wheat species (*T. durum* and *T. aestivum*) differed from each other in terms of the occurrence frequencies of the α and β subunits within the AAT-3 zone.

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