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Genetic variability of leaf esterases in *Triticum aestivum* L. 2n = 6x = 42

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Summary. Starch gel electrophoresis with two different buffer systems and several substrates and inhibitors have been used to study the electrophoretic variability of esterases in leaves of cultivars of *Triticum aestivum*. Each one of the buffer systems showed different levels of variability, according to the electrophoretic patterns. At the same time green and etiolated leaves showed different patterns in each buffer system. The variability was dependent upon the developmental stage of the leaves. According to the results from chromosomal location, the genes controlling esterases in green leaves were located in homoeology group 3, while the genes controlling esterases in etiolated leaves were in homoeology group 6. But both esterase isozymes showed a similar electrophoretic migration and a similar reponse to substrates and inhibitors. The possible origin of both sets of genes due to an interchromosomal duplication is discussed.

Key words: Leaf esterases – Genetic variability – *Triticum aestivum*

Introduction

Isozymes have been revealed as practical and useful genetic and biochemical markers, as well as good estimators of the genetic variability in populations. The importance of obtaining knowledge of the total variation of isozymatic systems and their genetic control are therefore essential, and hundreds of studies have been devoted to that task.

It is known that a single electrophoretic method is not able to detect all the variability of an isozymatic system. Several techniques, including sequential electrophoresis, heating denaturalization, electrofocusing, and the use of different substrates and inhibitors, have been described as increasing the amount of the observed variability (Coyne 1982).

Esterases (EC 3.1.1.–) include a large number of different carboxylic ester hydrolases. Some of these have a very wide substrate specificity and are able to hydrolize both endogenous and exogenous esters of differing structures (Walker and Mackness 1983). The fact that enzymes that show esterase activity can hydrolize non-ester bonds as well raises the question of what the normal physiological role of these esterases is.

Esterases are one of the most variable isozymatic systems in plants, both for the number of loci and alleles controlling it and for the different isozyme patterns observed in different organs and tissues. Due to this variability, esterases are one of most widely used isozymatic systems in genetic studies and in the characterization of crop plant cultivars (Tanksley and Orton 1983), but the results obtained can be different depending upon the electrophoretic technique used, the plant tissue studied, and the developmental state of the plant.

Esterases of hexaploid wheat (*Triticum aestivum* L.) have been widely studied. According to the results from electrophoretic techniques, esterase systems show great variability and they have been described as monomers or dimers (Barber et al. 1968, 1969; May et al. 1973; Jaaska 1980). Genes for esterases of wheat mature seeds have been located in genes of homoeology group 3 (Cubadda et al. 1975; Nakai 1976; Ainsworth et al. 1984; Rebordinos and Perez de la Vega 1989). The genes controlling wheat leaf esterases have been located in homoeology groups 3 and 6 (Barber et al. 1968, 1969; Bergman 1972; May et al. 1973; Nakai 1976; Jaaska 1980).

Our aims in this work were to study and characterize the extent of variability of both green and etiolated leaves

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Table 1. Esterase patterns observed in seedlings of different cultivars of hexaploid wheat

Cultivar	TRIS maleic EDTA		Poulik			
	Green	Etiolated	Green		Etiolated	
			1	2	1	2
Anza	A	A	F	M	Q	Q
Avocet	B	H	B	L	V	S
Banks	A	A	H	J	U	V
Bastion	A	A	H	J	U	Z
Bindawarra	B	A	H	J	Q	Q
Canaleja	ND	ND	C	K	U	X
Capitole	ND	ND	F	M	U	Z
Chinese Spring	A	C	D	L	U	Y
Chris	A	A	H	J	R	R
Condor	A	C	J	P	U	P
Cook	A	A	J	P	Q	Q
Gamenya	B	H	C	K	T	Y
Halberd	B	A	H	J	U	Z
Isis	E	A	A	A	T	Y
Jabiru	E	I	ND	ND	ND	ND
Jacup	D	H	C	K	U	X
Jupateco	A	A	H	J	U	X
Kite	B	H	H	J	V	S
Lance	ND	ND	B	L	Q	Q
Magdalena	A	C	H	J	R	R
Mara	C	C	H	J	R	R
Millewa	E	C	ND	ND	ND	ND
Nava	A	A	ND	ND	ND	ND
Pane 247	F	I	H	N	T	T
Shortim	B	L	B	B	ND	ND
Songlen	E	J	F	M	F	F
Tincurrim	A	C	A	A	R	R

ND – Not determined

of hexaploid wheat cultivars by means of starch gel electrophoresis. Two different systems of buffers as well as several substrates and inhibitors have been used to study the first and second leaves of germinating seedlings. Furthermore, the nullitetrasomic and ditelosomic series of Chinese Spring wheat have been used to relate different isozyme esterases to specific chromosome arms.

Materials and methods

Wheat cultivars

Hexaploid wheat *Triticum aestivum* L. (2n = 6x = 42) cultivars used are listed in Table 1.

Aneuploid lines

The nullisomic-tetrasomic (NT) and ditelosomic (DT) lines of Chinese Spring wheat, kindly supplied by Prof. E.R. Sears, were used to locate the chromosomes that control leaf esterases. All the available nullitetrasomic lines (lacking the NT *2A–2B, 2A–2D, 4A–4B, 4A–4D,* and *4D–4B*) for the seven homoeology groups and the ditelosomic for groups 3 and 6 were screened. The nomenclature of NT means that, for instance, *3A–3B* is an individual lacking the two *3A* homologous chromosomes but having four *3B* homologous chromosomes, chromosomes *3A* and *3B* being homologous. Ditelosomic nomenclature means that, e.g., *3AL* is an individual telocentric for the long arm of the two *3A* chromosomes, thus lacking the short arms of both *3A* chromosomes.

Germination

Seeds were germinated and grown until the 16th day at a controlled temperature of 21° ± 1 °C. Green seedlings were kept under a 12-h photoperiod, and etiolated seedlings were maintained in a dark room.

Enzyme extraction

Leaves from 16-day-old seedlings were excised and crushed. The crude extract was absorbed onto paper wicks (Whatman 3M, 10 × 5 mm) and inserted in gels. In each wheat cultivar the first and the second leaves were analyzed independently.

Electrophoretic procedures

Paper wicks soaked with the crude extract were inserted 4.5 cm from the cathode in 170 × 140 × 10 mm, 12% w/v horizontal starch gels. Two different buffer systems were used: the Poulik system (modified from Poulik 1957), and the TRIS maleic EDTA (modified from Brown et al. 1978). The Poulik buffer system was: electrode buffer, 0.3 *M* boric acid, 0.1 *M* NaOH, pH 8.6, and gel buffer 0.015 *M* TRIS, 0.003 *M* citric acid, pH 8.1; in this system gels were first electrophoresed at 21 $V cm^{-1}$ for 10 min, then the wicks were removed from the gel, and the electrophoresis continued at 13 $V cm^{-1}$ for 4.5 h. The TRIS maleic EDTA system was: electrode buffer 0.1 *M* TRIS, 0.1 *M* maleic acid, 0.01 *M* EDTA, 0.01 *M* $MgCl_2$, pH 7.4, and the gel buffer was a one-tenth dilution of the electrode buffer; electrophoresis was carried out at 4 °C, 15 $V cm^{-1}$ for 5.5 h, and wicks were removed after the first 10 min.

Staining and inhibition

Gels were stained for esterase activity using a method described by Ainsworth et al. (1984); 100 mg of Fast Blue RR Salt and 50 mg of α-naphthyl acetate were dissolved in 5 ml of acetone and made up to 100 ml with 0.05 *M* phosphate buffer, pH 7.5. Starch gels were horizontally sliced on four slices; one of them was stained as mentioned above at 30 °C until brown bands appeared; it was then washed and fixed in a solution of 7% acetic acid. The other three slices were stained with different substrates or inhibitors.

In addition to α-napthyl acetate, three other substrates were used: β-naphthyl acetate, α-napthyl propionate, and α-naphthyl butirate.

Three reported inhibitors of esterases were employed: parahydroximercuribenzoate (pHMB), iodoacetamide, and EDTA. The concentrations and the procedure for the use of the substrates and inhibitors have been previously described (Rebordinos and Perez de la Vega 1989).

Nomenclature

Each isozymatic activity zone was named with the abbreviation of the enzymatic system in capital letters (EST) followed by a number, 1–4, from faster to slower migration. Isozymes were also numbered from faster to slower migration in gels.

Results

TRIS maleic EDTA

Using this system of buffers, no differences were observed between the isozyme patterns of the first and second leaves, either from green or etiolated seedlings.

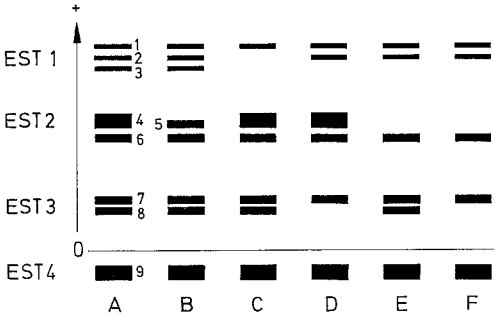

Fig. 1. Green leaf esterase patterns obtained using the TRIS maleic EDTA buffer system

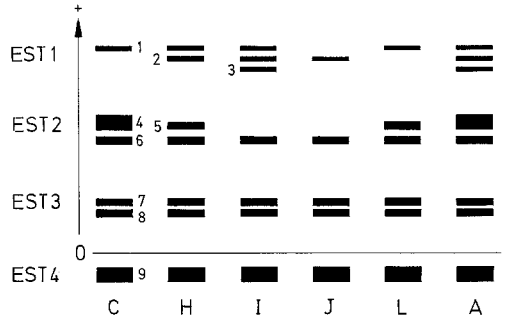

Fig. 2. Etiolated leaf esterase patterns obtained using the TRIS maleic EDTA buffer system

The patterns observed for green and etiolated seedlings in each cultivar are shown in Table 1. Three anodic zones of activity (EST1, EST2, and EST3) and one cathodic (EST4) were observed (Figs. 1 and 2). Isozymes 1, 2, and 3 were included in EST1, isozymes 4, 5 and 6 in EST2, isozymes 7 and 8 in EST3 and, finally, isozyme 9 in EST4. Isozymes had the same mobility in green and etiolated seedling leaves, and a total of ten different patterns was observed (Figs. 1 and 2); some of these patterns were seen exclusively in green or etiolated seedlings, and most of the cultivars from green seedlings showed an isozyme pattern different from the one in etiolated seedlings (Table 1).

The use of the nullitetrasomic and ditelosomic lines of Chinese Spring wheat allowed us to relate the green seedling isozymes 1, 2, 3, and 8 to chromosome arm *3AL*, and isozyme 7 to *3DL*, while isozyme 1 of etiolated seedlings was related to *6AL*. No other isozyme could be chromosomically located, since they were present in all the aneuploid lines tested (Table 2).

The effects of substrates and inhibitors were the same on esterases of green and etiolated seedlings. β-Naphthyl acetate stained isozymes of the EST1 and EST3 zones red, α-naphthyl propionate did not stain any isozyme of the EST1 zone; α-naphthyl butirate stained no band; pHMB inhibited the isozymes of EST1 and EST4 zones and partially inhibited the ones of EST3; iodoacetamide and EDTA decreased the staining level of all isozymes, but none of them disappeared.

Poulik system

The use of this system of buffers revealed three anodic zones of activity (EST1, EST2, and EST3). We have only

Table 2. Chromosomal location of esterase isozymes of Chinese Spring using TRIS maleic EDTA system

Green seedlings									Etiolated seedlings						
Aneuploid lines	Isozymes								Aneuploid lines	Isozymes					
	1	2	3	4	6	7	8	9		1	4	6	7	8	9
3A-3B	−	−	−	+	+	+	−	+	*6A-6B*	−	+	+	+	+	+
3A-3D	−	−	−	+	+	+	−	+	*6A-6D*	−	+	+	+	+	+
3AL	+	+	+	+	+	+	+	+	*6AL*	+	+	+	+	+	+
3AS	−	−	−	+	+	+	−	+	*6AS*	−	+	+	+	+	+
3B-3A	+	+	+	+	+	+	+	+	*6B-6A*	+	+	+	+	+	+
3B-3D	+	+	+	+	+	+	+	+	*6B-6D*	+	+	+	+	+	+
3BL	+	+	+	+	+	+	+	+	*6BL*	+	+	+	+	+	+
3BS	+	+	+	+	+	+	+	+	*6BS*	+	+	+	+	+	+
3D-3A	+	+	+	+	+	−	+	+	*6D-6A*	+	+	+	+	+	+
3D-3B	+	+	+	+	+	−	+	+	*6D-6B*	+	+	+	+	+	+
3DL	+	+	+	+	+	+	+	+	*6DL*	+	+	+	+	+	+
3DS	+	+	+	+	+	−	+	+	*6DS*	+	+	+	+	+	+
All other lines	+	+	+	+	+	+	+	+		+	+	+	+	+	+

− = isozyme absent; + = isozyme present

Fig. 3. Green leaf esterase patterns obtained using the Poulik system

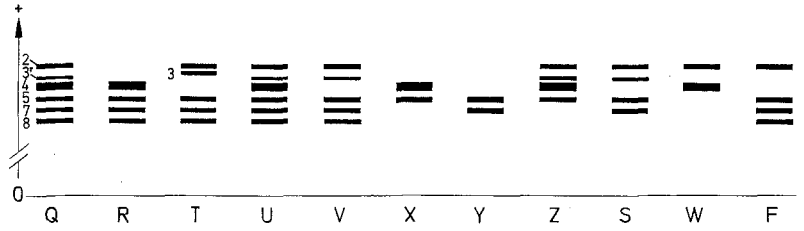

Fig. 4. Etiolated leaf esterase patterns obtained using the Poulik system

Table 3. Chromosomal location of esterase isozymes of first leaf of Chinese Spring with Poulik system

Green seedlings						Etiolated seedlings						
Aneuploid lines	Isozymes					Aneuploid lines	Isozymes					
	1	2	4	7	8		2	3′	4	6	7	8
3A-3B	−	−	+	+	+	*6A-6B*	−	−	+	+	+	+
3A-3D	−	+−	+	+	+	*6A-6D*	−	−	+	+	+	+
3AL	+	+	+	+	+	*6AL*	+	+	+	+	+	+
3AS	−	−	+	+	+	*6AS*	−	−	+	+	+	+
3B-3A	+	+	+	+	+	*6B-6A*	+	+	+	+	+	+
3B-3D	+	+	+	+	+	*6B-6D*	+	+	+	+	+	+
3BL	+	+	+	+	+	*6BL*	+	+	+	+	+	+
3BS	+	+	+	+	+	*6BS*	+	+	+	+	+	+
3D-3A	++	−	+	+	+	*6D-6A*	+	+	+	+	+	+
3D-3B	+	−	+	+	+	*6D-6B*	+	+	+	+	+	+
3DL	+	+	+	+	+	*6DL*	+	+	+	+	+	+
3DS	+	−	+	+	+	*6DS*	+	+	+	+	+	+
All other lines	+	+	+	+	+		+	+	+	+	+	+

− = isozyme absent; + = isozyme present; +− = isozyme present with low intensity; ++ = isozyme present with higher intensity than normal

studied the fastest zone (EST1) in detail since the other two zones showed poor resolution. Twelve isozymatic patterns with a total of eight isozymes were seen in this EST1 zone in green seedlings, in comparison with the three isozymes observed in EST1 when the TRIS maleic EDTA buffer system was used (Figs. 1 and 3). The same zones of activity were seen in etiolated seedlings, and for the EST1 zone a total of seven isozymes and 11 patterns was observed (Fig. 4). Isozymes with the same number in green and etiolated seedling patterns (except isozyme 3′) coincided in migration in gels, but only two patterns (F and P) coincided in both green and etiolated seedlings in all the sets of cultivars tested (Table 1). On the other hand, when this buffer system was used, most of the cultivars showed an esterase pattern in the first leaf different from the pattern of their second leaf, both in green and etiolated seedlings (Table 1).

The effects of substrates and inhibitors on green and etiolated leaves were similar. β-Naphthyl acetate stained the EST1 and EST3 zones red; α-naphthyl propionate did not stain isozymes 1 and 8 in green seedlings and 2 and 8 in etiolated ones; α-naphthyl butirate stained no band; pHMB inhibited all isozymes of the EST1 zone except 4, 5, and 6 in green leaves and 4 and 5 in etiolated ones. Isozyme 7 of Pane 247 was specifically not inhibited; this fact suggests that isozyme 7 of Pane 247 is a different isozyme from the isozyme with the same migration observed in the remainder of the cultivars assayed. Iodoac-

etamide and EDTA decreased in general the staining level of all isozymes, but none was completely inhibited.

Table 3 shows the results of the chromosomal location of Chinese Spring EST1 isozymes. In green seedlings, isozyme 1 was related to arm *3AL*, isozyme 2 to arms *3AL* and *3DL*, while isozymes 2 and 3′ of etiolated seedlings were related to *6AL*. No other isozyme could be chromosomically located.

Discussion

In a review about the resolution of electrophoretic techniques, Coyne (1982) pointed out that there is not a single electrophoretic technique able to detect all the variability and, therefore, the best way to study isozymatic variability is to use more than one technique. This statement seems to be especially suitable for plant esterases. As previous (Nakai 1976; Hvid and Nielsen 1977; Tanksley and Rick 1980; Schmidt-Stohn and Wheling 1983; Ainsworth et al. 1984; Rebordinos and Perez de la Vega 1989) and present results show, the pattern and the number of zones and bands of plant esterases that can be studied vary widely depending upon the particular electrophoretic technique, the plant tissue studied, and the developmental state of the plant.

In our study, the TRIS maleic EDTA buffer system revealed four esterase zones, while the Poulik system failed to reveal the cathodic zone EST4; on the other hand, while the first system showed a total of three different isozymes in the EST1 zone, the second system showed a total of nine isozymes in this zone but a very poor resolution in other zones. The differences in the number of isozymes should be mainly due to co-migrating bands.

The second origin of variability we observed is the developmental state of the leaves. Most of the cultivars showed different esterase patterns between their first and second leaves after 16 days of germination (Table 1), both in green and etiolated ones, when the Poulik system was used (differences have also been observed by electrofocusing; unpublished results). These differences were correlated with the size of the leaves: in cultivars whose first and second leaves had a similar size on the 16th day, the pattern of both leaves was the same, while in those cultivars in which the first leaf was more developed than the second, the patterns were different.

These differences between first and second leaves show that results regarding the variability of esterases among wheat cultivars and their identification could be different depending upon the tissue and its development. For instance: cultivar Canaleja has pattern C (isozymes 1, 2, 3, 6, 7, and 8) in its first leaf, and pattern K (isozymes 2, 4, and 6) in its second one (Fig. 3), but if both leaves are crushed together the pattern would be A (1, 2, 3, 4, 6, 7, and 8). Patterns H and J together would give a pattern with isozymes 1, 2, 4, 6, 7, and 8, not observed in our single-leaf analyses.

Some other conclusions can be drawn about the patterns of first and second leaves.

(1) Some patterns have only been observed in the first or in the second leaf (Table 1).

(2) There is no correspondence between the patterns in first and secondary leaves, if they are different. For instance, Avocet, Chinese Spring, and Lance have pattern L in green secondary leaves, whereas Chinese Spring has pattern D in its first leaf and Avocet and Lance have B.

(3) Although some of the second-leaf patterns could be an intermediate step to reach the pattern of the older leaf (i.e., pattern L with isozymes 2, 5, and 6 could become pattern B with 2, 5, 6, and 8 after new genetic activity appears during leaf development), others do not fit this model since most isozymes are different (i.e., pattern J has isozymes 2, 4, 6, and 7 while pattern H has 1, 7, and 8; Table 1).

These results raise the question as to whether the differences observed between wheat cultivars are due not only to differences in the esterase genotypes but also to a tissue dependent on differential expression of the genes controlling esterases during development. Likewise, no correspondence between the patterns of green and etiolated leaves was found in either buffer systems (Table 1).

The use of different inhibitors and substrates shows that the isozymes observed in green and etiolated leaves are similar, although the chromosomal location data show that at least some of them are coded by different genes.

The chromosomal location data indicate the following.

(1) Most of the isozymes observed must be coded by two or more genes, probably in homoeologous chromosomes, since they are present in all the NT and DT lines tested.

(2) Previous works reported that genes controlling wheat green leaf esterases were located in the chromosomes of homoeology group 3 (Barber 1968, 1969; Bergman 1972) and those controlling etiolated leaf esterases were located in group 6 (May et al. 1973; Jaaska 1980). Our simultaneous study with green and etiolated seedlings confirms these results, since the isozymes we have been able to locate were related to chromosomes of groups 3 and 6. Nakai (1976) also located some genes for isozymes from green leaves in homoeology group 6, but all of them except one were in different chromosome arms than the ones located for etiolated leaves, therefore, they must be different genes.

(3) There are some genes that are expressed in green leaves but not in etiolated leaves and vice versa. When the TRIS maleic EDTA buffer is used, the fastest isozyme 1 of green seedlings is related to a gene located in chro-

mosome arm *3AL*, while the fastest isozyme of etiolated seedlings, similar at least in electrophoretic mobility, is related to *6AL*. The same is true for isozymes numbered 2. When the Poulik buffer system is used, the one in green leaf is located in *3AL* and *3DL*, while that of etiolated leaf is in *6AL*.

The question still remains as to whether the genes located in chromosomes of homoeology group 3 are only expressed in green seedlings and those in the homoeology group 6 only in etiolated ones. Since for each buffer system most of the isozymes are observed in both green and etiolated leaves, another question is whether esterase genes in both homoeology groups 3 and 6 have the same origin, i.e., whether they are the result of an interchromosomal duplication that occurred in a common ancestor diploid species.

Esterases are useful tools in genetically based studies, since they show a high degree of genetic variability in plant species, but our work reemphasized previous results, pointing out that much of the variation observed in esterases is due not only to different electrophoretic techniques, but also to differences in plant development and to environmental factors. Therefore, special attention should be directed to control these factors and to use a tissue or organ in a specific developmental condition when esterases are studied.

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