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An Immunochemical Approach to Species Relationship in *Triticum* and Some Related Species

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Summary. An immunological reaction, precipitation in gel, was produced using a rabbit antiserum directed to a specific protein constantly present in bread wheats (*T. aestivum*, genome AABBDD), but absent in *durum* wheat (*T. durum* Desf., genome AABB). This protein was isolated in the soluble-protein fraction of bread wheat caryopses by combined biochemical and immunological techniques.

The availability of such a specific anti-bread wheat serum made possible the analysis of a series of varieties and species of wheat and of some closely related (*Secale, Aegilops*) and less closely related (*Hordeum, Haynaldia*) taxa to determine whether the protein was present or absent.

Hordeum vulgare, Haynaldia villosa, Triticum monoccocum and *Triticum turgidum* gave a negative result, while positive results were obtained in *T. aestivum, T. timopheevi, T. zhukovskyi, Secale cereale, Aegilops speltoides, Ae. mutica, Ae. comosa, Ae. caudata, Ae. umbellulata, Ae. squarrosa*, and also in the artificial amphiploids (*Ae. speltoides* × *T. monococcum*) and (*Ae. caudata* × *T. monococcum*).

It is concluded that these results agree closely with the classification of *Triticum* proposed by MacKey in 1966. The investigated protein not only permits the differentiation of *T. aestivum* from *T. turgidum*, but also *T. turgidum* from *T. timopheevi* at tetraploid level and *T. monococcum* from all the diploid species of *Aegilops*.

Introduction

Serodiagnostic methods have been used for several decades to elucidate some problems of plant taxonomy, but with rather inconclusive results. This partial success is probably due to the technique used in antiserum preparation. Often, total protein extracts have been inoculated into the animal, so that the consequent immunological reaction was directed towards a large, and often very wide, protein spectrum. It is evident that this method has a precise value only when rather general systematic relationships are being studied (Jensen, 1968). When relationships among species within the same genus or among closely related genera are being considered, a preliminary biochemical characterization of the protein becomes necessary: screening is then based on a reduced number of immunological reactions by the animal and allows the preparation of antisera highly specific for only one or a few proteins.

Recently, Cantagalli et al. (1969) described an immunological method to detect the illegal mixture of *durum* wheat with *Triticum aestivum* for macaroni production. This method is based on an immunological reaction of precipitation in gel obtained with a rabbit antiserum directed to a specific protein which is constantly present in bread wheats (*T. aestivum*, genome AABBDD), but which is absent in macaroni wheats (*T. durum*, genome AABB) (Piazzi and Cantagalli, 1969).

This protein has been isolated in the soluble protein fraction of bread wheat seeds (caryopses) by biochemical techniques (Cantagalli and Piazzi, 1968).

The availability of this specific antibread wheat serum made possible an analysis of some closely related (*Secale, Aegilops*) and less related (*Hordeum, Haynaldia*) species, to ascertain the presence or absence of the protein.

Its presence could be taken as evidence of a close taxonomical and genetic relationship. Moreover, comparison of these data with the knowledge of wheat phylogenesis based on other information would be interesting, both to eventually obtain further evidence and for assessing the value of the immunochemical method.

Material and Methods

a) Species analyzed

Caryopses, freed of glumes, were ground, including the embryo and pericarp, and soluble proteins were extracted from the flour obtained using a phosphate buffer ($Na_2HPO_4 \cdot 2\,H_2O$: 0,025 M; KH_2PO_4: 0,041 M; NaCl: 0,48 M) pH 6.6, following the method already reported (Cantagalli et al., 1969). Species analyzed are classified according to MacKey (1966) for *Triticum* and according to Kihara (1949) for *Aegilops*. Species and varieties analyzed are listed in tables 1 and 2.

b) Protein extraction and preparation of the specific antiserum

Bread wheat flour obtained from the *T. aestivum* variety Rex (200 g) was mixed with 400 ml of phosphate buffer pH 6.6. The suspension was left for 24 hours at 4 °C and then centrifuged to eliminate the insoluble fraction.

The extract was filtered through Sephadex G 100 (Cantagalli and Piazzi, 1968) and the solution containing the protein was concentrated, sterilized by filtration and inoculated into rabbits, using several injection cycles.

Table 1. *Synthesis of the positive or negative immunoreactions with specific rabbit anti-bread wheat serum on species closely related or less closely related with Triticum*

Group	Genus	Species	Subspecies	Variety	Cultivar	Genome	Reaction
Diploids	*Hordeum*						
(2 *n* = 14)	*Hordeum*	*vulgare*			Leonessa		−
	Haynaldia						
	Haynaldia	*villosa*					−
	Secale						
	Secale	*cereale*				RR	+
	Aegilops						
	Aegilops	*speltoides*	*ligustica*			SS	+
	Aegilops	*speltoides*	*aucheri*			SS	+
	Aegilops	*mutica*				M^tM^t	+
	Aegilops	*comosa*				MM	+
	Aegilops	*caudata*				CC	+
	Aegilops	*umbellulata*				C^uC^u	+
	Aegilops	*squarrosa*				DD	+
	Triticum						
	Triticum	*monococcum*				AA	−
	Triticum	*monococcum*		*flavescens*		AA	−
	Triticum	*monococcum*		*macedonicum*		AA	−
	Triticum	*monococcum*	*boeoticum*	*thaoudar*		AA	−
Tetraploids							
(2 *n* = 28)	*Triticum*						
	Triticum	*turgidum*	*dicoccoides*		a	AABB	−
	Triticum	*turgidum*	*dicoccoides*		b	AABB	−
	Triticum	*turgidum*	*dicoccum*		*vernal*	AABB	−
	Triticum	*turgidum*	*carthlicum*			AABB	−
	Triticum	*turgidum*	*turgidum*		*plinianum*	AABB	−
	Triticum	*turgidum*	*turgidum*	*durum**		AABB	−
	Triticum	*turgidum*	*turgidum*	*polonicum*		AABB	−
	Triticum	*turgidum*	*turgidum*	*polonicum*	*ispahanicum*	AABB	−
	Triticum	*timopheevi*	*araraticum*		a	AAGG	+
	Triticum	*timopheevi*	*araraticum*		b	AAGG	+
	Triticum	*timopheevi*	*timopheevi*			AAGG	+
Hexaploids							
(2 *n* = 42)	*Triticum*						
	Triticum	*zhukovskyi*				AAGGA'A'	+
	Triticum	*aestivum*	*vulgare**			AABBDD	+
	Triticum	*aestivum*	*sphaerococcum*			AABBDD	+
Artificial (*Ae. speltoides* × *T. monococcum boeoticum*)						SSAA	+
Amphidiploids (*A. caudata* × *T. monococcum boeoticum*)						CCAA	+

* See Table 2

The serum obtained at the end of the treatment was absorbed repeatedly with *durum* wheat flour. Control of this serum was made by immunodiffusion and immunoelectrophoretic techniques (Piazzi and Cantagalli, 1969).

c) Immunodiffusion and immunoelectrophoretic analysis

The immunodiffusion analysis was carried out according to a slight modification of Flandre's and Damon's micromethod (1961). Two ml of 1% "agarose" solution in barbital buffer, pH 8.2. IS. 0,05, was stratified on microscopic slides. After "agarose" gelification a series of holes were made according to the best requirements for analysis. After removing the agarose from the holes, these were filled with antiserum and the samples to be analyzed.

The immunoelectrophoretic analysis was carried out according to Scheidegger's micromethod (1955) on identical "agarose" slides. An electric field of 4 mA/cm (50 min.) in barbital buffer, pH 8.2 IS. 0.1, was used for the antigen migration. After electrophoresis, antiserum was added in linear holes. The slides were maintained in diffusion at room temperature in a damp environment for 48—72 hrs. Washing was carried out in barbital buffer solution (barbital buffer pH 8.2, IS. 0.1, 20% distilled water 80% and KCl 0.68% g/v added) with several changes of solution, for 3—4 days. Before staining, the slides were placed in a 1% tannic acid solution for 10 min., dried at room temperature, and stained with 0.1% Coomassie blue solution (10% acetic acid, 50% methanol, 40% distilled water). Destaining was carried out by washing several times in the following solution: 10% acetic acid, 50% methanol, 10% glycerol and 30% distilled water. Positive antigen-antibody reactions, showing the presence of specific bread wheat soluble-protein, were represented by clear lines (or arcs) of precipitation in the gel.

Experimental Results

Pictures showing some typical results of immunodiffusion and immunoelectrophoretic analysis are

Table 2. *Synthesis of the positive or negative immunoreactions with specific rabbit anti-bread wheat serum on different varieties of Triticum durum and Triticum aestivum*

T. durum Cultivars (negative reaction)	*T. aestivum* Cultivars (positive reaction)	
	Italian lines	Foreign lines
1) Cappelli	1) Brescia	1) Admonter
2) Castelporziano (Cp B 132)	2) Campodoro	2) Bezostaja 1
3) Castelfusano (Cp C 48)	3) Chianti	3) Brucker Harrach
4) Garigliano	4) Combine	4) Clédor
5) Capeiti	5) Diamante	5) Dankowska Selekcyjna
6) Maliani 1D	6) Elia	6) Fehmarn Weiss
7) Maliani 8D	7) Farnese	7) Novi Sad
8) Sincape 9	8) Fiorello	8) Odesskaja
9) Aziziah	9) Flaminio	9) Probus
10) SS O 111	10) Fontarronco A	10) Rekord
11) Casteldelmonte (Gr A 145)	11) Fortunato	11) Rex
12) Lakota	12) Funo	12) Turmalin
13) B 52 (Grifoni)	13) Funello	13) Zenith
	14) Funone	
	15) Funotto	
	16) Gagliardo	
	17) Lama	
	18) Leonardo	
	19) Leone	
	20) Lepre	
	21) Libellula	
	22) Lince	
	23) Lontra	
	24) Lucciola	
	25) Lupo	
	26) Madif 21	
	27) Mara	
	28) Mentana	
	29) S. Pastore	
	30) Strampelli	

shown in figs. 1 and 2, respectively. Though some slight differences in the intensity of precipitation of the protein are evident, the reactions are always clear, making evaluation (positive or negative) very easy. The immunoelectrophoretic analysis demonstrates that the isolated protein migrates towards the anode. From table 1, which presents a summary of results, it is clear that, with the exception of *Hordeum vulgare, Haynaldia villosa, Triticum monococcum* and *Triticum turgidum*, all the other species, varieties and artificial amphiploids give a positive reaction. In all these lines, therefore, the protein isolated from bread wheat is present. Also all the diploid species of *Aegilops* have the synthesis of this specific protein in common.

Discussion

The data obtained in this research are in close agreement with the classification of *Triticum* proposed by MacKey (1966). The investigated protein not only permits easy differentiation between *T. aestivum* and *T. turgidum*, but also distinguishes, at the tetraploid level, *T. turgidum* from *T. timopheevi*, and, at the diploid level, *Triticum monococcum* from all the *Aegilops* species. Although certainly not conclusive, these data are contrary to the taxonomic revision of *Triticum* and *Aegilops* proposed by Bowden (1959) and recently supported by Sears and Morris (1968).

From a rapid comparison of data, it is evident that the presence of this protein in *T. aestivum* should be related to the presence of the D genome (from *Aegilops squarrosa*). The presence of the protein in *T. timopheevi* and related wild types (*T. araraticum*) and its absence in the whole *T. turgidum* group underline a clear diphyletic origin; *T. timopheevi* has retained the genetic information coming from the *Aegilops* donor species. The positive serological response of the artificial amphiploids (*Ae. speltoides* × *T. monococcum thaudar*) and (*Ae. caudata* × *T. monococcum*), resulting from the union of a negative (*Triticum monococcum*) with two positive species (*Ae. speltoides* and *Ae. caudata*), seems to indicate epistasis of the protein forming ability; this is demonstrated even further by *T. zhukovskyi*. The absence of the protein in the *T. turgidum* group remains, however, an unsolved problem.

The universal response of many taxa of *T. turgidum*, of such different ecogeographical origins, points towards a monophyletic origin for this group, as already hypothesized by Giorgi and Bozzini (1969a) on cytological grounds. Genetic information leading to the synthesis of the investigated protein should have been lost — if we accept the *Aegilops* origin of the B genome in wheat — in the early phases of

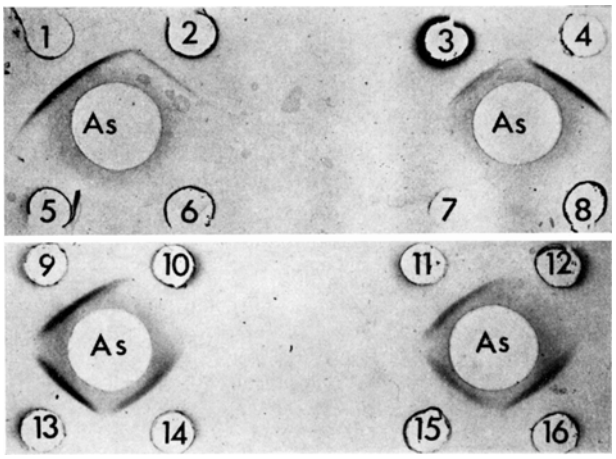

Fig. 1. Immunodiffusion analysis of soluble proteins of *Triticum* varieties and of related and unrelated species (1···16).

As = Specific rabbit anti-bread wheat serum;
1 = *Aegilops squarrosa*;
2 = *Aegilops umbellulata*;
3 = *Aegilops speltoides aucheri*;
4 = *Triticum aestivum sphaerococcum*;
5 = *Hordeum vulgare*;
6 = *Haynaldia villosa*;
7 = *Triticum ispahanicum*;
8 = *Triticum polonicum*;
9 = *Triticum aestivum* cv. Probus;
10 = *Triticum durum* cv. Cappelli;
11 = *Triticum aestivum* cv. Cledor;
12 = *Triticum durum* cv. Lakota;
13 = *Triticum aestivum* cv. Mentana;
14 = *Triticum aestivum* cv. Funo;
15 = *Triticum aestivum* cv. Bezostaja 1;
16 = *Triticum aestivum* cv. S. Pastore

speciation of *T. turgidum*, thus giving indirect evidence for the hypothesis proposed by Giorgi and Bozzini (1969b) on the possible mechanism of synthesis and speciation of tetraploid wheats.

The negative response of *Hordeum* and *Haynaldia* is also interesting, as it shows that this protein is not common to many unrelated genera. The presence of the protein in *Secale* is in line with the affinity of *Secale* with *Triticum*: it is well known that they can easily cross and produce viable amphiploids (*Secalotricum* and *Triticale*).

In conclusion, the data submitted gives much information which integrates well with the present knowledge on the speciation of *Triticum* and *Aegilops*. The value of the immunological technique in plant taxonomy seems to be confirmed by the similarity of its results to data obtained by cytogenetical and morphological analyses. With particular reference to wheat speciation, this method could integrate with, and also give impulse to, the recently developed biochemical methods based on protein (Johnson et al., 1965, 1968) and enzyme (Bhatia, 1968) electrophoresis, because of its higher specificity and sensitivity.

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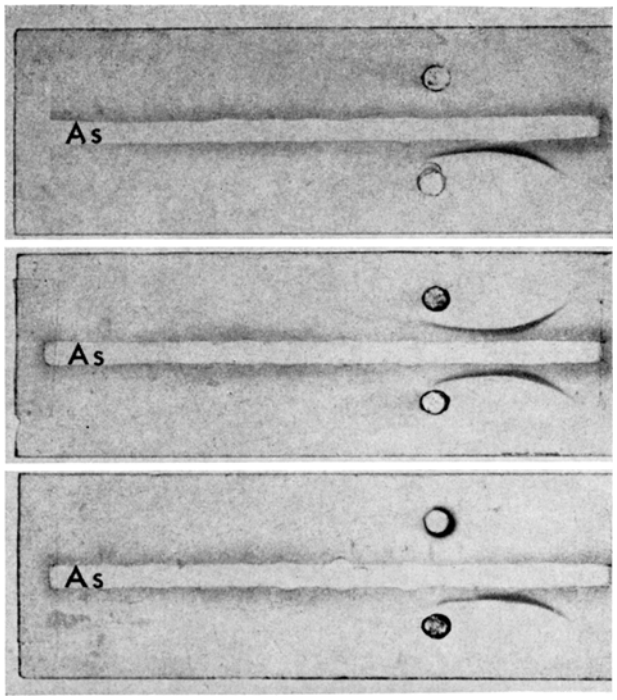

T. durum cv. Cappelli

T. aestivum cv. Funo

T. aestivum cv. Probus

T. aestivum cv. Cledor

T. durum cv. Lakota

T. aestivum cv. Bezostaja

Fig. 2. Immunoelectrophoretic analysis of soluble proteins of *Triticum durum* and *Triticum aestivum* varieties carried out with the specific anti-bread wheat serum (As)

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