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Polymorphism and chromosomal location of endogenous α-amylase inhibitor genes in common wheat

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Summary. Polymorphism of an endogenous α-amylase inhibitor in wheat was studied using isoelectric focusing followed by monoclonal antibody – based immunoblotting. Ten isoforms of the inhibitor detected in common wheat and its wild counterparts were assigned to five homoeologous loci. Three α-amylase inhibitor loci (*Isa-1*) were identified in common wheat and located on the long arms of chromosomes 2A, 2B and 2D. In a sample of 27 bread wheats, eight durum wheats, and 12 diploid wheat relatives, amphiploids and triticales, a high resolution isoelectric-focusing separation demonstrated two active and one *null* allele at the *Isa-A1*, two alleles at the *Isa-B1*, one allele at the *Isa-D1*, four alleles at the *Isa-S1*, and one allele at the *Isa-G1* locus. The most frequent electrophoretic pattern of common wheat cultivars consisted of two isoforms, encoded respectively by the *Isa-B1b*, *Isa-D1a* alleles and the *Isa-A1null* allele. All the durum wheats had only one inhibitor form controlled by allele *Isa-B1b*, which was accompanied by the *null* allele at the *Isa-A1* locus.

Key words: *Triticum* sp. – *Aegilops* sp. – α-amylase inhibitor – Polymorphism – Monoclonal antibodies

Introduction

The bifunctional proteinaceous α-amylase/subtilisin inhibitor, which inactivates the α-AMY-1 group of cereal α-amylases and the bacterial serine protease, subtilisin, has been identified and characterized in wheat (Warchalewski 1976, 1977; Mundy et al. 1984), barley (Mundy et al. 1983; Weselake et al. 1983a, b; Hejgaard et al. 1984a), rye (Hejgaard et al. 1984b) and triticale (Zawistowska et al. 1989). The role of this inhibitor in regulating endogenous α-amylase activity during preharvest sprouting of grain has been the subject of numerous studies (Hill et al. 1988; Masojć and Larsson-Raźnikiewicz 1991). Although it was shown that application of the pure barley α-amylase inhibitor improved the baking quality of sprout-damaged wheat flour (Zawistowska et al. 1988), direct involvement of the inhibitor in controlling grain sprouting is still not clear due to insufficient knowledge of its genetic variation amongst cultivars.

The studies of Hejgaard et al. (1984a, b) showed that the inhibitor from barley and rye is encoded by a single structural gene located on chromosomes 2H and 2R, respectively. The α-amylase/subtilisin inhibitor locus in cereals has been assigned the symbol *Isa-1* (McIntosh 1988). Consequently, the encoded polypeptide is designated ISA-1 (Hart and Gale 1988). Further studies (Masojć and Gale 1990) revealed that the *Isa-1* locus is located on the long arm of chromosome 2 in rye and *Aegilops umbellulata* and that it is highly polymorphic. As many as ten alleles were found at the *Isa-R1* locus (Masojć 1991) by means of isoelectric focusing (IEF).

Genetic variation for the endogenous α-amylase inhibitor in wheat (*Triticum aestivum* L.) is not known. Sadowski et al. (1986) detected only one inhibitor form in wheat cultivars using polyclonal antibodies and immunoblotting. Moreover, IEF analysis of the inhibitor, followed by staining for subtilisin, has also shown no polymorphism for this protein in common wheat (Konarev 1986). In triticale two isoforms of

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ISA-1 have been reported; one inherited from rye the other from wheat (Zawistowska et al. 1989).

The existence of only one isoform of the inhibitor in an allohexaploid wheat containing A, B and D genomes seems unlikely and may be a result of the separation methods not being sensitive enough to reveal ISA-1 polymorphism. Therefore, the aim of the present investigation was to study polymorphism of the endogenous α-amylase inhibitor in wheat and its wild counterparts using high resolution IEF coupled with monoclonal antibody-based immunoblotting.

Materials and methods

Plant material

The forty seven genotypes, involving common and durum wheat, wild relatives of wheat and triticales, used in this study (see Table 1) were from the Agriculture Canada Research Station, Winnipeg, Manitoba, Canada. The Chinese Spring nulli-tetrasomic and ditelosomic lines used for chromosomal location studies were obtained from the Cambridge Laboratory, Norwich, UK. Synthetic tetraploids of an AADD genome composition were developed by crossing *T. aegilopoides* with *Ae. squarrosa* (Aung and Kerber, unpublished).

Monoclonal antibodies

Monoclonal antibodies (Mab 9A11, IgG_1 isotype) specific to α-amylase inhibitor from wheat and barley were prepared by Zawistowski (unpublished).

Isoelectric focusing

The crude inhibitor extracts were prepared from sound grain by briefly grinding a single kernel in a mortar with 0.1 ml of distilled water. The resultant suspension was centrifuged for 10 min at 10,000 g to yield a clear supernatant. Whole and quarters of sample applicators (Pharmacia) were soaked in the supernatant and applied to the anodal end of the IEF gel.

Isoelectric focusing was performed in the Multiphor II electrophoresis apparatus (LKB, Bromma, Sweden) connected to a circulating cooling bath at 3 °C. Polyacrylamide gels (0.3 mm thick, T – 7.5%, C – 3%), containing 8% v/v Pharmalyte pH 5–8 and 10% v/v glycerol, were cast according to the method outlined in the LKB manual (1986). Glutamic acid, 0.04 M (anode) and 1 M NaOH (cathode) were used as electrolytes. The distance between electrodes was 23 cm. Focusing conditions were determined by setting the constant power supply to 3,000 V, 25 mA and 20 W. The separation time was 6 h.

Protein staining

Gels were stained with Coomassie Brilliant Blue R-250 (CBB) according to LKB (1977).

Immunoblotting

After electrophoresis, proteins were transferred from the gel to a nitrocellulose membrane (0.45 μm) by diffusion in which the membrane was placed on top of the gel followed by three layers of filter paper (Bowen et al. 1980). Transfer was carried out in 0.1 M Tris-HCl buffer (pH 8.5) for 45 min. Nitrocellulose membranes were rinsed with distilled water, air dried and then used for immunostaining. After five washes with Tris-buffered saline (TBS), pH 8.0, membranes were incubated in 5% w/v skim milk in TBST buffer (TBS containing 0.05% Tween 20) for 2 h with gentle agitation, washed four times for 5 min in TBST and incubated with monoclonal antibodies (Mab 9A11) diluted (1:100) in Blotto (TBST containing 1% w/v skim milk) for 1 h. The blots were washed with five changes of TBST for 5 min each and incubated with goat anti-mouse IgG alkaline phosphatase conjugate (BioRad, Richmond, Calif.) diluted in Blotto (1:3000) for 1 h. After washing with three changes of TBST for 5 min each, followed by a last wash in 0.1 M Tris-HCl buffer (pH 9.5) containing 0.1 M NaCl and 5 mM $MgCl_2$, membranes were developed by incubating in substrate solution containing nitroblue tetrazolium chloride and 5-bromo-4-chloro-3 indolyl phosphate p-toluidine salt for 1 h. The substrate solution was prepared as suggested by the manufacturer (Sigma Chemical Co., St. Louis, Mo.). All steps were carried out at room temperature.

Results

IEF separation

Figure 1 shows the focusing pattern of proteins extracted from wheat and triticale cultivars and either stained with Coomassie Brilliant Blue (Fig. 1A) or probed with monoclonal antibodies (Fig. 1B). The

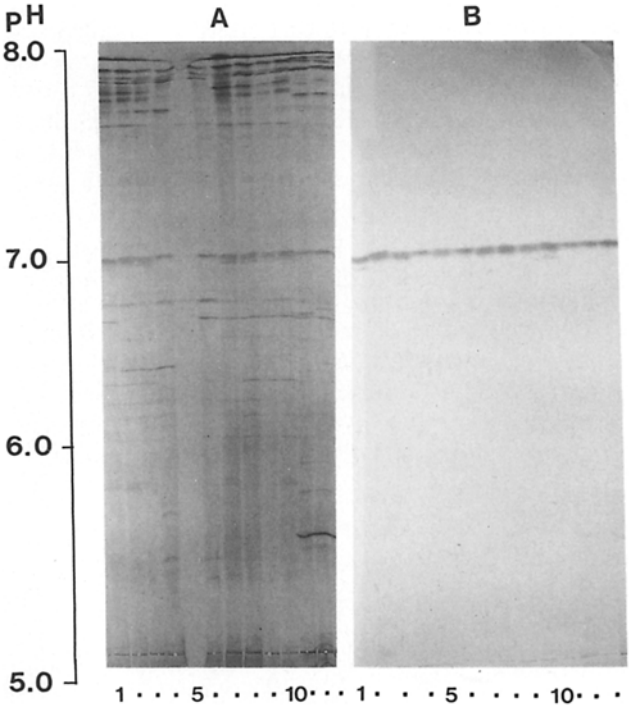

Fig. 1. IEF patterns of the endogenous α-amylase inhibitor from wheat (*1–10*) and triticale (*11–13*) cultivars after protein staining (*A*) and immunoblotting (*B*). Lanes: (*1*) Chinese Spring, (*2*) RL 4555, (*3*) Prelude, (*4*) Tetra-Prelude, (*5*) Canthatch, (*6*) Tetra-Canthatch, (*7*) Biggar BSR, (*8*) Columbus, (*9*) BW 121, (*10*) Bihar, (*11*) Wapiti, (*12*) Frank, (*13*) Carman. The samples used for each gel were from the same extraction. On the *A* and *B* gels, 20 and 5 μl of each sample were applied respectively

antibodies reacted specifically with wheat ISA-1 polypeptides and did not bind to other proteins, including inhibitor polypeptides of rye origin present in triticale. Staining the gel for inhibitor activity (data not shown) confirmed the high specificity of Mab 9A11. However, use of this technique resulted in band diffusion and it precluded photographing the gels in situ.

Focusing of wheat endogenous α-amylase inhibitor was performed on a gel with a 23-cm separation distance. The longer distance than that commonly used (around 10 cm) improved the resolution of proteins, yielding several inhibitor bands. Moreover, using the monoclonal antibody probe, inhibitor polypeptides at concentrations in the gel below the detection limit of protein staining with CBB, were clearly visualised on immunoblots (Fig. 1A, B, lane 5). The ISA-1 forms were the predominant polypeptides with pIs in the proximity of pH 7.0 (Fig. 1B); however, the differences of their isoelectric points were too small to be precisely determined.

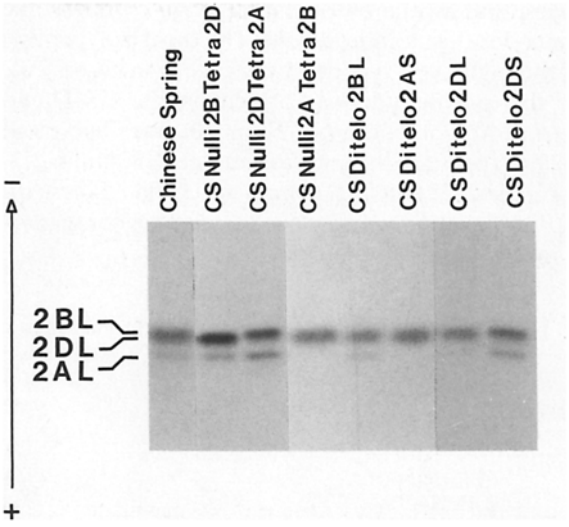

Fig. 2. Immunoblots of the endogenous α-amylase inhibitor from Chinese Spring nulli-tetrasomic and ditelosomic lines

Chromosomal location

The banding patterns of inhibitor polypeptides from Chinese Spring (CS) are shown in Fig. 2. The study of nulli-tetrasomic and ditelosomic lines shows that the polypeptide with the lowest pI is encoded by a gene on the long arm of chromosome 2A since this form was absent in CS Nulli 2A and CS Ditelo 2AS lines. Moreover, Mab 9A11 bound stronger to this polypeptide in the CS Nulli 2D-Tetra 2A line than in the parental CS. Of the two adjacent polypeptides with higher pIs than the 2AL allelic form, one with the lowest pI was absent in CS Nulli 2D and CS Ditelo 2DS lines. This polypeptide was present, however, in the CS Ditelo 2DL line and its presence was even more pronounced in the CS Nulli 2B-Tetra 2D line

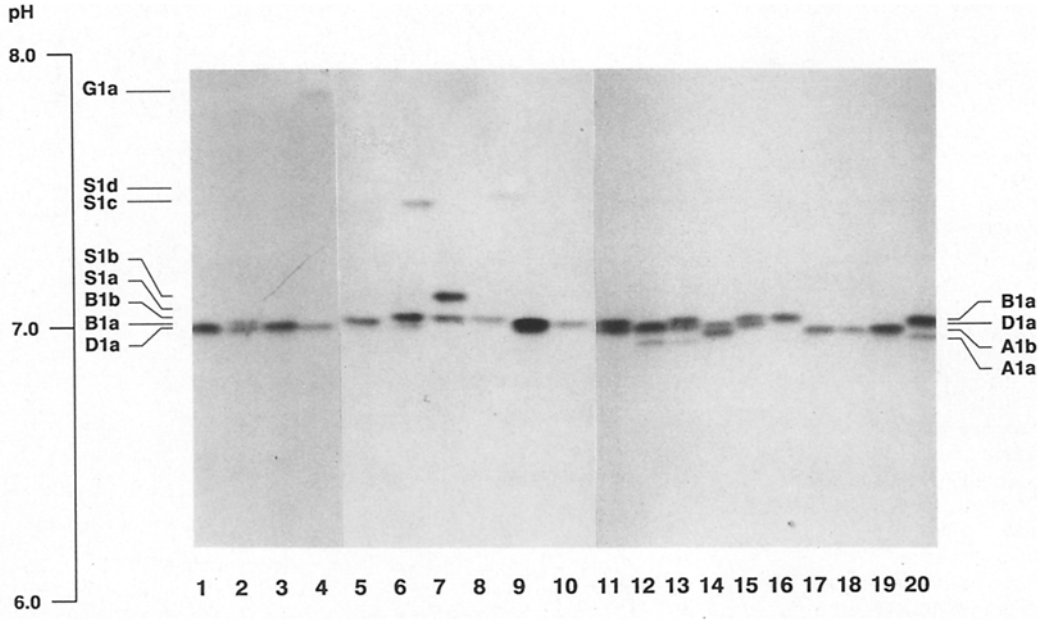

Fig. 3. Immunoblots of selected genotypes representing all isoforms and patterns of the endogenous α-amylase inhibitor found within the materials studied. *Lanes:* (*1*) Owens, (*2*) Monopol, (*3*) Inia 66, (*4*) Timgalen, (*5*) Stewart 66, (*6*) *Ae. speltoides* 85-611, (*7*) *Ae. speltoides* 90-507, (*8*) *Ae. speltoides* 85-622, (*9*) RL 5869, (*10*) Tetra-Prelude, (*11*) Columbus, (*12*) Chinese Spring, (*13*) Bihar, (*14*) *T. aegilopoides*/*Ae. squarrosa* 5, (*15*) Hercules/*Ae. squarrosa*, (*16*) Hercules, (*17*) *T. monococcum* 1, (*18*) *T. monococcum* 2, (*19*) *T. boeoticum*, (*20*) Chinese Spring

showing that a gene on the long arm of chromosome 2D encodes this inhibitor form. The third polypeptide with the highest pI is coded by a gene located on 2BL since this polypeptide was present in the CS Ditelo 2BL, and absent in the CS Nulli 2B, line. The band intensity appeared to increase in the CS Nulli 2D-Tetra 2A and CS Ditelo 2DS lines which did not contain the 2BL allelic form, suggesting a possible compensation mechanism.

Allele identification

Analysis of a sample of 36 common and durum wheat cultivars indicates that the allelic forms of inhibitor are different in wheat cultivars and cv Chinese Spring (Table 1, Fig. 3). In the majority of cultivars the polypeptide encoded by 2AL is missing. Protein staining and immunoblotting confirmed that the lack of this isoform is apparently caused by a null allele

Table 1. *Isa-1* alleles found on homoeologous chromosomes of group 2 in common and durum wheat and related species

No.	Cultivar/species/cross	Genome composition	*Isa-1* allele at chromosome		
			2AL	2BL	2DL
1	Chinese Spring	AABBDD	*a*	*a*	*a*
2	BAU 84015	AABBDD	*a*	*a*	*a*
3	Cajeme 71	AABBDD	*null*	*a*	*a*
4	Casavant	AABBDD	*null*	*a*	*a*
5	Bihar	AABBDD	*a*	*b*	*a*
6	Columbus	AABBDD	*null*	*b*	*a*
7	Neepawa	AABBDD	*null*	*b*	*a*
8	Biggar BSR	AABBDD	*null*	*b*	*a*
9	BW 121	AABBDD	*null*	*b*	*a*
10	RL 4555	AABBDD	*null*	*b*	*a*
11	RL 4137	AABBDD	*null*	*b*	*a*
12	RL 5869	AABBDD	*null*	*b*	*a*
13	Monopol	AABBDD	*null*	*b*	*a*
14	Sinton	AABBDD	*null*	*b*	*a*
15	Owens	AABBDD	*null*	*b*	*a*
16	Wildcat	AABBDD	*null*	*b*	*a*
17	Inia 66	AABBDD	*null*	*a*	*a*
18	Timgalen	AABBDD	*null*	*G1a**	*a*
19	Gabo	AABBDD	*null*	*b*	*a*
20	Oxley 19	AABBDD	*null*	*b*	*a*
21	Little Club	AABBDD	*null*	*b*	*a*
22	Laval 19	AABBDD	*null*	*b*	*a*
23	ST 6	AABBDD	*null*	*b*	*a*
24	Prelude	AABBDD	*null*	*b*	*a*
25	Tetra-Prelude	AABB	*null*	*b*	–
26	Canthatch	AABBDD	*null*	*b*	*a*
27	Tetra-Canthatch	AABB	*null*	*b*	–
28	Hercules	AABB	*null*	*b*	–
29	Hercules/*Ae. squarrosa*	AABBDD	*null*	*b*	*a*
30	Vic	AABB	*null*	*b*	–
31	Berillo	AABB	*null*	*b*	–
32	Ward	AABB	*null*	*b*	–
33	Stewart 63	AABB	*null*	*b*	–
34	Langdon	AABB	*null*	*b*	–
35	Stewart 63/RL 5261	AABBDD	*null*	*b*	*a*
36	*Triticum monococcum* 1	AA	*b*	–	–
37	*Triticum monococcum* 2	AA	*b*	–	–
38	*Triticum boeoticum*	AA	*b*	–	–
39	*Aegilops speltoides* 85-622	SS	–	*S1a,d*	–
40	*Aegilops speltoides* 90-507	SS	–	*S1a,b*	–
41	*Aegilops speltoides* 85-611	SS	–	*S1a,c*	–
42	*T. aegilopoides/Ae. squarrosa* 5	AADD	*b*	–	*a*
43	*T. aegilopoides/Ae. squarrosa* 6	AADD	*b*	–	*a*
44	*T. aegilopoides/Ae. squarrosa* 8	AADD	*b*	–	*a*
45	Triticale cv Wapiti	AABBRR	*null*	*b*	–
46	Triticale cv Frank	AABBRR	*null*	*b*	–
47	Triticale cv Carman	AABBRR	*null*	*b*	–

* *Isa-1* gene probably transferred from *T. timopheevi*

(*Isa-A1null*). However, two *Isa-A1* alleles of the A genome, encoding the inhibitor polypeptide, were found in several cultivars. The allele designated *Isa-A1a* was found in Chinese Spring, BAU 84015 and Bihar, while *Isa-A1b* was found in the diploid species *Triticum monococcum* and *T. boeoticum*. The inhibitor polypeptide encoded by the *Isa-A1b* allele had a higher pI than that coded by the *Isa-A1a* allele, and the ISA-A1b band was more intense, similar to that exhibited by the *Isa-B1* and *Isa-D1* alleles in tetraploid and hexaploid wheats.

The *Isa-D1* locus on the long arm of chromosome 2D was found to be monomorphic in the genotypes examined. The allele *Isa-D1a* detected in cv Chinese Spring (Fig. 3, lanes 12 and 20) was present in all wheat cultivars and in crosses involving *Aegilops* species, while it was absent in durum wheat and triticale cultivars which lack the D genome (Fig. 3 and Table 1).

Two *Isa-B1* alleles were identified among bread and durum wheats. Allele *Isa-B1a*, detected in Chinese Spring, was also present (Fig. 3, lanes 1 and 3) in five other cultivars (BAU 84015, Cajeme 71, Casavant, Owens and Inia 66). The allele present in the majority of hexaploid wheats and triticales (Fig. 3, lanes 5, 10 and 16) was designated *Isa-B1b*. The inhibitor polypeptide with the unusually high pI value, as compared to those encoded by *Isa-B1*, was found in the cv Timgalen derived from crosses involving *T. timopheevi*. The variant allele was most likely from the tetraploid; therefore, it was assigned to the homoeologus *Isa-G1* locus and designated *Isa-G1a* (Fig. 3, lane 4). Neither of the AA genotypes nor the AADD synthetic tetraploids had any of the *Isa-B1* alleles, showing that none of the ISA-1 isoforms are the result of AA and DD ISA-1 subunit combinations (Table 1). Four alleles at the homoeologous *Isa-S1* locus were found in the three *Ae. speltoides* accessions (Fig. 3, lanes 6, 7 and 8), encoding polypeptides with higher pIs than those of *ISA-B1* forms. Polypeptides with pIs in ascending order are coded by alleles designated *Isa-S1a*, *Isa-S1c* and *Isa-S1d*.

Discussion

The IEF separation of the previously reported single wheat ISA-1 polypeptide (Konarev 1986; Sadowski et al. 1986; Zawistowska 1989) into several isoforms was achieved in this study by increasing the distance of the IEF resolution to 23 cm. In addition, a monoclonal antibody (9A11) against endogenous α-amylase inhibitor proved to be beneficial in discerning wheat ISA-1 polypeptides with similar pI values.

The analysis of Chinese Spring nulli-tetrasomic and ditelosomic lines showed that the *Isa-1* locus is located on the long arm of each of the group 2 chromosomes, which confirms earlier findings in barley (Hejgaard et al. 1984a), rye and *Ae. umbellulata* (Masojć and Gale 1990). In addition, two homoeologous *Isa-S1* and *Isa-G1* loci with four alleles and one allele, respectively, were identified, which suggests that the high polymorphism for *Isa-1* might be expected among species closely related to the donors of the B genome. Two alleles were found at the *Isa-B1*, whereas only one allele was present at the highly conserved *Isa-D1*, locus. Of the three alleles found at the *Isa-A1* locus, *Isa-A1null*, which did not produce the inhibitor protein, was the most common. *Isa-A1b* was present in *T. monococcum* and *T. boeoticum*, whereas *Isa-A1a* was found only in three cultivars of Asiatic origin (Chinese Spring, BAU 84015, and Bihar). The reason for the lack of expression of the *Isa-A1null* allele in most bread and durum wheat cultivars is not clear. The situation parallels that found for HMW glutenin subunits of the 1A group, in which absence in hexaploid wheat results from the presence of a terminating sequence inside the transcribed portion of the gene (Forde et al. 1985). Apparently, it seems to be the evolutionary tendency in common wheat to silence the expression of functionally equal structural genes. The inhibitor isoforms encoded by *Isa-B1* and *Isa-D1* may play an important role in grain biochemistry as null alleles were not found at either loci.

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