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ORIGINAL PAPER

Starch-bound 2S proteins and kernel texture in einkorn, *Triticum monococcum* ssp *monococcum*

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Abstract The starch granule proteins from 113 einkorn wheat (*Triticum monococcum* ssp *monococcum*) accessions were analyzed by acidic, polyacrylamide gel electrophoresis (A-PAGE), and two-dimensional A-PAGE x SDS-PAGE. All accessions were confirmed to contain equal amounts of two polypeptide chains corresponding to puroindoline B (Pin-B), as well as a prominent component plus a faint band corresponding to puroindoline A (Pin-A). When compared with soft-textured common wheat, "monococcum" accessions showed an increase of 3.2- and 2.7-fold in Pin-A and Pin-B levels on the starch granules, respectively. In addition, all accessions contained a novel component of the 2S super-family of seed proteins named Einkorn Trypsin Inhibitor (ETI), which was found to be encoded as a pre-protein 148 residues long. Wild-type ETI encoded by allele *Eti-A^{m}1a* and "valine-type" ETI encoded by allele *Eti-A^{m}1b*, which occurred in 107 and six einkorn accessions, respectively, were found to accumulate on starch granules as a mature protein of 121 amino acids with a hydrophobic central domain. The einkorn accessions exhibited an average SKCS index as low as -2.05 ± 11.4, which is typical of extra-soft kernels. The total surface area of starch granules in "monococcum" wheat, as determined by visual assessments in counting chambers, was estimated at 764 mm^2/mg of starch, and was about 1.5 times higher than that for common wheat. The results are discussed in relation to the identification of factors that cause the extra-soft texture of einkorn kernels.

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Introduction

For millennia, A-genome diploid wheat *Triticum monococcum* ($2n = 2x = 14$) has played a relevant role in the human diet. Its presence as a wild food resource was detected in archaeological remains dating back to 23,000 years before the present (BP) on the shores of the Sea of Galilee. Domesticate einkorn wheat with a non-scattering ear rachis spread from the so-called Levantine Corridor, a narrow strip of land sandwiched between the Mediterranean Sea and the inland desert steppe, throughout adjacent Mediterranean countries in the early pre-Pottery Neolithic age, approximately 9,000 years BP (Peltenburg et al. 2001). The finely processed einkorn wheat bran found as the main food remain in the colon of the mummy of the Tyrolean Iceman, who lived in the Eastern Alps around 5,300 years BP (Oegll 2000), demonstrates the great importance of this wheat for the European agricultural communities during the Neolithic and Calcolithic ages. Since the Bronze age, approximately 3,800 years BP, "monococcum" wheat has been gradually replaced by more productive tetraploid or hexaploid wheat species *T. turgidum* and *T. aestivum*, its cultivation being now confined in a few hundred hectares in Europe (Nesbitt and Samuel 1996). However, after centuries of oblivion, einkorn is currently being reconsidered as a food crop because of the high amounts of protein, zinc, iron, and antioxidant

compounds such as lutein, tocopherols, and tocotrienols accumulated in its kernels (Hidalgo et al. 2006; Hidalgo and Brandolini 2008). Moreover, the reduced toxicity, if any, of "monococcum" flour against the intestinal mucosa of celiac patients (Pizzuti et al. 2006), and the identification of einkorn accessions with good bread-making quality coupled with resistance to leaf rust and powdery mildew (Saponaro et al. 1995) greatly increased the economical prospects of *T. monococcum*.

On the other hand, einkorn accessions were found to produce extra-soft kernels with SKCS (Single Kernel Characterization System) values as low as −5 to 11, which are infrequent amongst the cultivated wheats (Pogna et al. 2002). As a consequence, "monococcum" kernels are quite prone to damage during mechanical harvesting, which results in reduced grain germination and viability. The principal determinant factors of kernel texture are puroindoline A (Pin-A) and puroindoline B (Pin-B), two α-helical, tryptophan- and cysteine-rich isoforms belonging to the 2S super-family of seed proteins, which occur in the starchy endosperm of the *Triticeae* and *Aveneae* tribes (Morris 2002). Pin-A and Pin- B are basic proteins, approximately 13 KDa in size, which can be easily separated from each other by acidic, polyacrylamide gel electrophoresis (A-PAGE) at pH 3.1 (Corona et al. 2001). Puroindolines occur at high amounts on the surface of starch granules from soft wheat compared with those from hard wheat (Corona et al. 2001). Furthermore, they show foaming properties and permeabilizing effects on bacterial and fungal membranes (Jing et al. 2003), and affect crumb structure, rheological properties of wheat dough and starch granule integrity during milling (Giroux et al. 2000). In common wheat and D-genome wheat species *Aegilops tauschii*, Pin-A and Pin-B are encoded by genes *Pina-D1* and *Pinb-D1* at the Hardness (*Ha*) locus on the short arm of chromosome 5D (Giroux and Morris 1997). The orthologous *Pina-A^{m}1* and *Pinb-A^{m}1* genes from *T. monococcum* were found to be 0.14 cM apart in the distal region of the short arm of chromosome 5A (Tranquilli et al. 1999). A space of about 32 kb has been found between these genes in a BAC clone of *T. monococcum* (Chantret et al. 2004). *Pina-A^{m}1* and *Pinb-A^{m}1* revealed a high degree of similarity (>94%) with their counterparts in *T. aestivum* (Tranquilli et al. 1999; Simeone et al. 2006).

Here an attempt is made to disclose the genetic and biochemical bases of the extra-soft texture of einkorn kernels by comparing 113 accessions of different geographic origins for their kernel texture characteristics and starch-bound protein patterns, as determined by SKCS analysis and A-PAGE fractionation, respectively. PCR amplicons obtained with primers specific for puroindoline-encoding genes from einkorn accessions with contrasting A-PAGE patterns were sequenced as well.

Materials and methods

Plant material and grain hardness

Grains of 113 *T. monococcum* spp *monococcum* accessions of different geographic origins (Table 1) kindly provided by Dr. A. Brandolini (CRA-SCV, S. Angelo Lodigiano, Italy) were used in the present study. Common wheat cvs Bolero, Chinese Spring, Leone, Libellula and Wisdom 400 were analyzed as well. Einkorn and common wheat were grown at the CRA-QCE experimental station in Rome in single 1 m-long rows, 30 cm apart, under the same agronomical conditions. Hardness readings of 200-dehulled kernels from each "monococcum" accession were obtained with the Perten Model SKCS 4100 (Perten Instruments Springfield, IL, USA).

Extraction, fractionation, and immunoblotting of starch-bound proteins

Starch-bound proteins were extracted for 1 h at room temperature with 50 mM NaCl and 50% (v/v) propan-2-ol from 50 mg of air-dried starch granules, as described previously (Corona et al. 2001). After centrifugation at 5,000×*g* for 5 min, proteins in the supernatant were precipitated with two volumes of acetone at −20°C overnight and air-dried. Proteins were suspended in 25 µl of 8.5 mM sodium lactate buffer (3.4 g/l of 97% NaOH adjusted to pH 3.1 with lactic acid) and mixed with 25 µl of 50% (v/v)

Table 1 Einkorn accessions analyzed and their origin

Origin	Nomenclature
Albania	108, 331, 529, 1632, 1636 (*c, c*, –)*, 1641, 1643 (*a, g*,–) 1649, 1650, cv. Monlis (*c, c, a*)
Austria	194, 195, 196, 197, 300, 566, 567, 568
Bulgaria	233, 234, 249, 322, 341(*c, c*, –) 342, 343, 476
Germany	147, 150, 151, 156, 241, 481(*c, c*, –) 565, 1341
Greece	335, 336, 338, 339, 340, 432, 515, 517
Hungary	398, 522, 523, 524, 525, 533, 534, 535
Italy	1135, 1335, 1605, 1606, 1607, 1658
Yugoslavia	375, 574 (*b, c, b*), 471, 514
Morocco	1395, 1396, 1506, 1507, 1508, 1510, 1511, 1513, 1514
Near East	27, 106 (*c, c*, –) 353, 576 (*c, c*, –)
Rumania	276, 357, 358, 539, 541 (*c, c*, –) 542, 543, 1134
Spain	330, 467, 508 (*b, a*, –), 509, 510, 1157, 1389, 1623
Switzerland	193, 205, 247, 251, 265, 314, 315
Turkey	5, 7, 14, 127, 415, 492, 496, 505
Ex USSR	2, 3 (*c, c*, –), 121(*c, c, b*), 544, 584, 1358 (*c, c*, –), 1397, 1516, Sal 98-38

* Alleles at the *Pina-A^{m}1, Pinb-A^{m}1*, and *Eti-A^{m}1* loci, respectively, as determined by direct sequencing of PCR amplicons

glycerol, containing 0.1% (w/v) pyronine γ. Aliquots of 3 µl (for einkorn wheat) and 10 µl (for common wheat) of this solution were used for A-PAGE fractionation.

A-PAGE at pH 3.1 and two-dimensional A-PAGE x SDS-PAGE of starch-bound proteins were carried out as described by Gazza et al. (2006). A-PAGE fractionations were scanned with the UVIpro Gel Analysis System Software 1.2 (Uvitec, UK), and puroindolines were quantitatively assessed by calculating their pixel volumes in nine einkorn accessions and bread wheat cv. Bolero (two replications). Immunoblotting with a polyclonal anti-Pin-A antiserum was performed as described previously (Gazza et al. 2006).

Protein sequencing and mass spectrometric analysis

For *N*-terminal sequencing, starch-bound proteins fractionated by A-PAGE were transferred onto a 0.2 µm PVDF membrane (Sequi-Blot^{TM}, BioRad, USA). Gel and membrane were pre-equilibrated for 20 min in a transfer CAPS buffer, pH 11.0, containing 10% (v/v) methanol in distilled water, transferred between two double layers of 3 MM chromatography paper (Whatman,UK) and electro-blotted at 50 V for 90 min in a wet apparatus Transfer Cell (Bio-Rad, USA). After the transfer, membranes were stained for 10 min in a solution containing 50% methanol and 16% Coomassie Brillant Blue R250 and destained with a solution of 50% methanol in distilled water. *N*-terminal sequencing of the first six amino acids of selected protein bands was performed by Primm Srl (Milan, Italy) using the Applied Biosystems Model H49 Procise Protein Sequencer. Selected protein bands were excised from the gel, subjected to in-gel digestion with trypsin (modified porcine trypsin, Promega, USA) or Glu-C (*Staphilococcus aureus* strain V8, Aldrich, USA) and analyzed by MALDI-TOF MS (Voyager DE-PRO, Applied Biosystems, USA) and RP-HPLC/n-ESI-MSMS (LTQ, Thermo Fischer Scientific, USA) as reported previously (Fontanini et al. 2007).

DNA extraction, PCR amplification, and gene sequencing

Genomic DNAs were isolated from young leaves using the CTAB (CetylTrimethylAmmonium Bromide) method. PCR amplifications of puroindoline genes were performed with the primer pairs described by Gautier et al. (1994). Reactions were performed in a 50 µl volume containing 200 ng of genomic DNA, 20 pmol of each primer, 200 µM of each dNTP, 2 U RedAccutaq (SIGMA), 1× Taq buffer. Samples were denaturated at 98°C for 3 min and then submitted to 35 cycles of 1 min of denaturation at 94°C, 1 min annealing at T_m and 1 min elongation at 68°C. A final cycle with an extension of 7 min at 68°C completed the reactions. The PCR products were analyzed on 1.8% agarose gels, stained with ethidium bromide and visualized under UV light. PCR fragments were eluted from the agarose gel with the Nucleospin-Extract kit (Machery-Nagel, Germany) and sequenced on the ABI 3730 DNA sequencer.

RACE (Rapid amplification cDNA ends)

Total RNA was extracted with TRIZOL reagent (Invitrogen, USA) from 70 mg of seeds collected at 14 and 21 days post anthesis from einkorn cv. Monlis. Ten micrograms of RNA were subjected to 3′ and 5′ RACE performed with the Invitrogen RACE System kits according to manufacturer's instructions. cDNA samples were denaturated at 94°C for 3 min before the addition of 2.5 U of Go Taq DNA polymerase (Promega, USA), and then submitted to 35 cycles of 1 min of denaturation at 94°C, 1 min annealing at T_m and 1.5 min elongation at 72°C, with final extension of 7 min at 72°C.

Results

Fractionation of starch-granule proteins

A-PAGE fractionation of starch-granule proteins of 113 "monococcum" accessions revealed six prominent bands in the cathodic region of the gel (Fig. 1, bands *b1*, *b1**, *b2*, *b2**, *b3*, and *b3**). In the same gel region, common wheat cv. Bolero exhibited three strong bands (Fig. 1, *lane 6*, *arrows*) corresponding to Pin-A and Pin-B encoded by wild-type alleles *Pina-D1a* and *Pinb-D1a* (Corona et al. 2001), Pin-B occurring as a pair of bands. Bands *b1* + *b1** appeared in all the 113 einkorn accessions analyzed. On the contrary, proteins *b2* and *b2** occurred as alternative bands

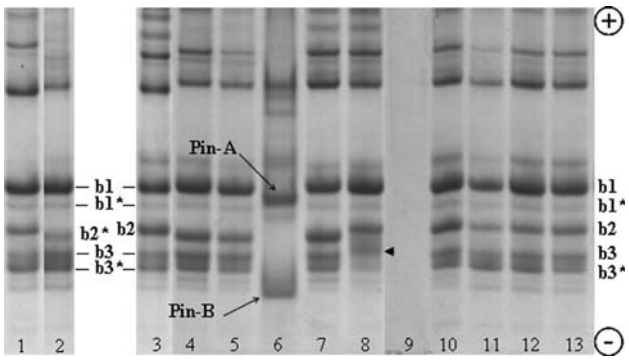

Fig. 1 A-PAGE fractionation of starch granule proteins from einkorn accessions (1) 1134, (2) 574, (3) 576, (4) 492, (5) 496, (7) 505, (8) 508, (10) 515, (11) 514, (12) 510, and (13) 509. *Lanes* 6 and 9 show starch granule proteins from common wheat cv. Bolero and durum wheat cv. Colosseo, respectively. Principal bands are lettered. Puroindolines (Pin-A and Pin-B) are marked. *Arrowhead* indicates the slow-moving doublet *b3* + *b3** in accession 508

in 107 and six accessions, respectively (Fig. 1). Finally, band pair *b3* + *b3** was found at the same position in the A-PAGE patterns of all the einkorn genotypes analyzed except accession 508, which exhibited a slow-moving *b3* + *b3** doublet (Fig. 1, *lane 8, arrowhead*).

When fractionated by two-dimensional A-PAGE x SDS-PAGE (Fig. 2), proteins *b1*, *b1**, *b2*, *b3*, and *b3** in einkorn cv. Monlis appeared as five spots in the 13–15 KDa region, components *b2*, *b3*, and *b3** being slightly faster in the second dimension than proteins *b1* and *b1**. The apparent molecular weight of protein *b2** in the A-PAGE x SDS-PAGE pattern of accession 574 was the same as protein *b2* in cv. Monlis (data not shown).

Analysis of band pairs *b1* + *b1** and *b3* + *b3**, and their encoding genes

The polyclonal antiserum developed against the 16-mer DRASKVIQEAKNLPPR sequence in the *C*-terminal region of mature Pin-A (Krishnamurthy and Giroux 2001) reacted with proteins *b1* and *b1** in the A-PAGE pattern of einkorn cv. Monlis (Fig. 3, *lane 4*), suggesting that these polypeptides correspond to Pin-A. Direct sequencing of the 447 bp PCR amplicons obtained from 13 accessions with the primer pair specific for the coding DNA sequence (CDS) of gene *Pina-D1a* (Gautier et al. 1994), revealed the presence of non-synonymous Singe Nucleotide Polymorphisms (SNPs). In particular, cv. Monlis and nine other accessions were found to share allele *Pina-A^{m}1c* (GenBank accession AJ242715) with five amino acid changes with respect to wild-type allele *Pina-D1a* in cv. Chinese Spring (CS). Alleles *Pina-A^{m}1b* (AY622786) and *Pina-A^{m}1a* (AY302092) with six or four amino acids substitutions with respect to CS, were found in two and one einkorn

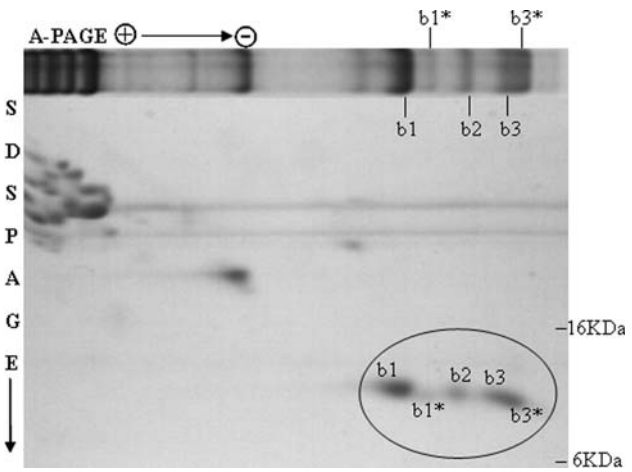

Fig. 2 Two-dimensional A-PAGE x SDS-PAGE fractionation of starch granule proteins from einkorn cv. Monlis. Letters indicate prominent bands and spots. Molecular weights are shown at the right-end side

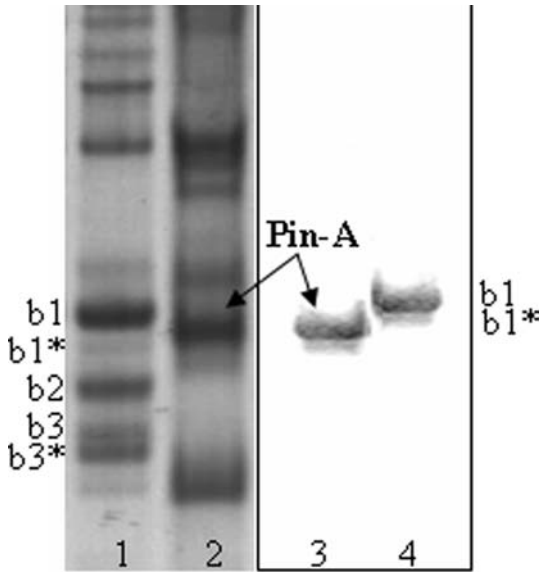

Fig. 3 A-PAGE fractionation and immunostaining of starch granule proteins with the polyclonal antiserum anti-Pin-A. 1, 4 Einkorn cv. Monlis and 2, 3 common wheat cv. Bolero

accessions, respectively (Table 1). These substitutions increased the net negative charge of einkorn Pin-A at pH 3.1, resulting in band pair *b1* + *b1** with a reduced mobility compared with Pin-A in bread wheat (Fig. 1).

The 447 bp amplicons obtained in 13 einkorn accessions with the primer pair specific for the CDS of common wheat Pin-B (Gautier et al. 1994) showed nine (allele *Pinb-A^{m}1c,* AY622797*)* to 10 (allele *Pinb-A^{m}1g*, AY622799) amino acid substitutions with respect to wild-type Pin-B in CS. Pin-B encoded by these alleles exhibited an increased negative charge, which is in accordance with the slow A-PAGE mobility of band pair *b3* + *b3** as compared with that of the Pin-B doublet in common wheat cv. Bolero (Fig. 1). In addition, accession 508 was quite unique in showing the change from N to D at position 29 of Pin-B. This amino acid substitution introduced an additional negative charge into band pair *b3* + *b3**, which exhibited an unusual slow mobility upon A-PAGE (Fig. 1, *lane 8, arrowhead*).

Analysis of band pairs *b2* + *b2** and their encoding genes

Bands *b2* and *b2** were never observed in the A-PAGE pattern of starch-granule proteins extracted from bread wheat (Pogna et al. 2002; Gazza et al. 2006). Sequencing of the first six amino acids at the *N*-terminal end of band *b2* from einkorn cv. Monlis revealed the SVGDQC sequence. Direct sequencing of the cDNA amplicons obtained by 3′ RACE with a mix of primers designed on sequence SVGDQC revealed the CDS of a protein with 97.5% similarity with the rye trypsin inhibitor (RTI) described by Lyons et al. (1987). Therefore, protein *b2* was named einkorn trypsin

inhibitor (ETI), and its coding gene, *Eti-A^{m}1a* (FJ985717). The 5′ RACE with primers designed on the 3′-terminal end of *Eti-A^{m}1a* revealed the presence of 23 amino acids upstream of the SVGDQC sequence (Fig. 4). In order to confirm these findings, protein *b2* of einkorn cv. Monlis was excised from an A-PAGE gel, digested with trypsin or Glu-C and submitted to mass spectrometric analyses. The MALDI-TOF mass spectra and the MSMS spectra obtained by RP-HPLC/n-ESI-MSMS of the resulting peptide mixtures revealed a mature *ETI* polypeptide with 121 amino acid residues and three amino acid substitutions at positions 4 (from G to D), 70 (from Q to P), and 106 (from P to H) with respect to RTI. Four amino acids at the *C*-terminal end of native ETI were absent in the mature form of this protein.

Compared with *Eti-A^{m}1a,* gene coding for ETI in einkorn accessions 574 from Yugoslavia and 121 from USSR with band *b2** showed the A to V substitution at position 11 of the mature protein. This novel allele was named *Eti-1A^{m}b* (FJ985718), and its encoded protein,"valine-type" ETI. A-PAGE patterns of starch-bound proteins suggest the occurrence of allele *Eti-1A^{m}b* in accessions 492, 505, and 496 from Turkey, and accession 342 from Bulgary as well (Fig. 1).

Extraction of Pin-A, Pin-B, and ETI by methanol

Air-dried starch granules from either cv. Monlis, which contains wild-type ETI (band *b2*), or accession 574, which contains "valine-type" ETI (band *b2**), were suspended in 85% aqueous methanol for 5 min and then centrifuged at 8,000×*g* for 10 min. A-PAGE fractionation of proteins in the supernatant revealed the presence of either protein *b2* or *b2** in the absence of Pin-A (bands *b1* + *b1**) or Pin-B (*b3* + *b3**) (Fig. 5, *lane 1*). Subsequently, the starch granules in the pellet were re-suspended in a fresh 85% methanol solution for 30 min and then centrifuged at 8,000×*g* for 10 min. No traces of bands *b1, b1*, b2, b2*, b3,* and *b3** were found in the A-PAGE pattern of proteins in the supernatant of this second extraction (Fig. 5, *lane 2*). On the contrary, the starch granules in the pellet released bands *b2* or *b2** along with Pin-A and Pin-B when re-suspended in 50 mM NaCl and 50% (v/v) propan-2-ol (Fig. 5, *lane 3*).

Kernel hardness and puroindoline content

The einkorn accessions exhibited an extra-soft grain texture (mean SKCS index = −2.0 ± 11.4 SE) as compared with soft-textured common wheat cv. Bolero (mean SKCS value = 33.5 ± 14.5). The six einkorn accessions with "valine-type" ETI showed a mean SKCS index (−7.4 ± 12.7) not statistically different from the general mean. On the contrary, there was a highly significant inverse correlation ($r = -0.56$, $P < 0.01$) between kernel hardness and kernel weight in the 113 accessions analyzed. As observed in common wheat (Gazza et al. 2008), variation in kernel weight was found to explain approximately 31% of the phenotypic variation for kernel hardness in einkorn wheat, as calculated by the coefficient of determination (r^2). Puroindolines were quantified by densitometric scanning of bands *b1, b1*, b3,* and *b3** in the A-PAGE fractionation of a 3 µl solution of starch-bound proteins from bread wheat cv. Bolero and nine einkorn accessions (481, 492, 496, 505, 508, 509, 510, 514, and 515), and the amounts of Pin-A and Pin-B were expressed as pixel volumes. "Monococcum" wheat accumulated significantly high amounts of both Pin-A and Pin-B on the starch granules compared with bread wheat (Table 2). However, no significant correlation was found between puroindoline content and kernel hardness in einkorn wheat.

Discussion

In bread wheat, kernel hardness has a major impact on many aspects of milling such as energy to grind, amount of tempering water, particle size, and flour yield. In addition, grain texture is a key trait to the end-use quality because a high level of damaged starch granules is produced during milling of hard kernels as compared with soft kernels. The water absorption and the rheological characteristics of wheat dough depend on the proportion of damaged starch, which also exerts a strong influence on yeast growth during fermentation, crumb softness, and bread-keeping characteristics. As a consequence, many countries generally use

M	A	F	K	H	Q	L	I	L	W	A	A	V	M	L	A	I	L	A	A	20
A	S	A	S	V	G	D	Q	C	V	P	G	L	A*	M	P	H	N	P	L	40
G	A	C	R	T	Y	V	V	S	Q	I	C	H	V	G	P	R	L	F	T	60
W	D	M	K	R	R	C	C	D	E	L	L	A	I	P	A	Y	C	R	C	80
E	A	L	R	I	L	M	D	G	V	V	T	P	Q	G	V	F	E	G	G	100
Y	L	K	D	M	P	N	C	P	R	V	T	Q	R	S	Y	A	A	T	L	120
V	A	P	H	E	C	N	L	H	T	I	H	G	S	P	Y	C	P	T	L	140
Q	A	G	Y	G	V	V	F													

Fig. 4 Amino acid sequence of Einkorn Trypsin Inhibitor (ETI) encoded by allele *Eti-A^{m}1a* in cv. Monlis. The parts underlined are absent in mature ETI, as determined by MS/MS analysis. Star at position 11 of mature ETI indicates the A residue that is replaced by the V residue in "valine-type" ETI encoded by allele *Eti-A^{m}1b*

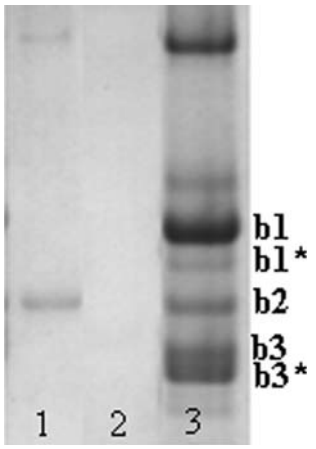

Fig. 5 A-PAGE fractionation of einkorn starch granule proteins. 1 Extraction with 85% methanol for 5 min, 2 re-extraction with fresh 85% methanol for 30 min, and 3 re-extraction with 50 mM NaCl and 50% isopropanol for 1 h

Table 2 Mean SKCS index and mean levels of Pin-A and Pin-B accumulated on starch granules from common wheat cv. Bolero and nine einkorn accessions

Material	SKCS index	Puroindoline level[a]		
		Pin-A	Pin-B	Total
Cv. Bolero	33.5 ± 14.5[b]	22.2 ± 6.0	19.5 ± 7.4	41.6 ± 13.1
Einkorn	−7.4 ± 8.0	70.4 ± 13.6	53.1 ± 9.1	123.5 ± 20.2
r		−0.25 ns	−0.54 ns	−0.41 ns

r = coefficient of correlation between SKCS index and puroindoline level in einkorn

ns not significant

[a] Number of pixels × 1000

[b] SD

soft-textured cultivars in cookies and cakes, and hard-textured cultivars in pan or flat breads (Morris and Rose 1996). Einkorn kernels exhibit an extra-soft texture (Pogna et al. 2002, and present data), which often results in negative SKCS values, quite infrequent amongst the other cereal crops except oats. In this latter species, kernel breakage during mechanical harvesting or dehulling was found to be correlated with the extra-soft characteristics of its kernels (Doehlert and McMullen 2000; Peltonen-Sainio et al. 2001). A similar correlation likely exists in einkorn wheat, as suggested by the high rates (≥30%) of damaged kernels observed here during dehulling of einkorn grain using an impact dehuller that eject kernels through a spinning rotor causing them to collide with the walls of a drum. Wounded caryopses exhibit reduced germination and viability, and attract fungus invasion, raising the risk of mycotoxins when grain is consumed for food or feed.

The mechanism by which kernel texture is determined seems to be similar in both "monococcum" and common wheat, and implicates specific puroindoline genes. In the A-PAGE patterns of einkorn and common wheat, Pin-B occurred as a pair of bands (*b3* and *b3**) with similar intensity, which could correspond to different protease cleavage of a native Pin-B, as highlighted previously by mass spectrometry (Blochet et al. 1993). Similarly, Pin-A in "monococcum" wheat appeared as a major component (*b1*) and a minor faint band (*b1**), which are probably formed from a single native component through in vivo post-translational cleavages. As expected, these polypeptides and their counterparts in common wheat cv. Bolero reacted with the anti-Pin-A antibody (Fig. 3). The primary structures of einkorn Pin-A and Pin-B were found to be in accordance with their reduced mobility in A-PAGE as compared with Pin-A and Pin-B in common wheat. In particular, Pin-B in einkorn accession 508 revealed an unusual slow mobility and the change from N to D at position 29 due to a Pin-B encoding allele never described before in *T. monococcum* ssp *monococcum*, but already observed (Genbank accession ACA60818) in its wild relative *T. beoticum* (=*T. monococcum* ssp *aegilopoides*). This indicates that this allele appeared before the domestication of einkorn wheat.

A major difference between einkorn and common wheat was the relative abundance of Pin-A and Pin-B on the starch granules, as quantified by densitometric scanning of A-PAGE gels. On average, einkorn accessions showed an increase of 3.2- and 2.7-fold in Pin-A and Pin-B levels, respectively, when compared with cv. Bolero (Table 2).

Common wheat and "monococum" wheat share a trimodal size distribution of their starch granules, i.e., large (A-type), medium (B-type), and small (C-type) granules. However, the average size of einkorn granules was found to be smaller than that of bread wheat (Stoddard 1999). To gain a more detailed information in this aspect, A-type (≥10 µm in diameter) and small granules (B- plus C-type, <10 µm) from four soft bread wheat genotypes (cvs Bolero, Leone, Libellula, and Wisdom 400) and four einkorn wheat genotypes (cv. Monlis and accessions 331, 1395 and Sal 98-38) were compared for their diameter and surface area. In particular, an aliquot (50 µl) of a 5 ml suspension of 100 mg of air-dried starch granules in distilled water was injected into a counting chamber with the Thoma ruling (0.2 mm cell depth) (Fig. 6). The total number N of A-type and small granules in the starch suspension injected into 20 random-chosen Thoma cells (50 × 50 µm^2) was determined with a Leica DMLB100T optical microscope. Moreover, the average diameter D of A-type and small granules was calculated using the printed photos of the starch granules from 15 random-chosen Thoma cells, and the total surface area of A-type and small granules was then estimated as πND^2 (Table 3). In both wheat species, the small granules made the largest contribution (approximately 92%) to the total number of endosperm starch granules. However, einkorn wheat exhibited about twice as much large and small

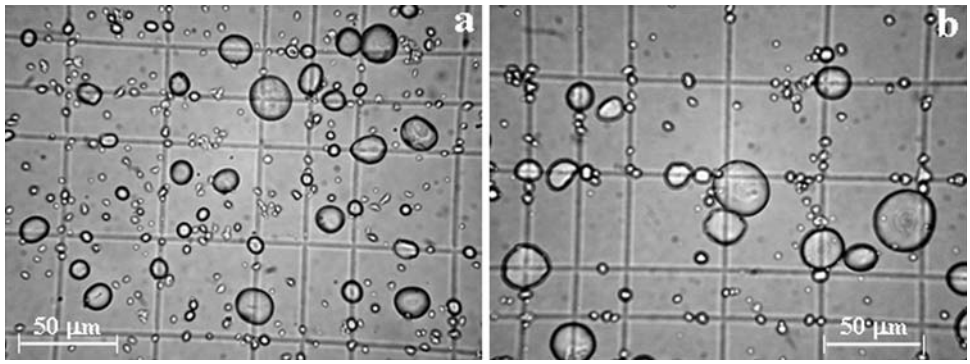

Fig. 6 Microscopic image of two counting chambers with starch granules from **a** *T. monococcum* accession 331 and **b** *T. aestivum* cv. Bolero. *Bar* = 50 µm

Table 3 Number, mean diameter, and total surface area of starch granules in 1 mg of starch from four common wheat cultivars and four einkorn accessions

Material	No. of starch granules ($\times 10^4$)			Mean granule diameter (µm)		Total granule surface (mm^2)		
	A-type	Small type[a]	Total	A-type	Small type	A-type	Small type	Total
Einkorn wheat	50.8 ± 6.6[b]	574 ± 23	625 ± 28	17.8 ± 3.5	3.8 ± 0.4	504 ± 228	260 ± 47	764 ± 188
Common wheat	21.4 ± 0.8	242 ± 19	264 ± 19	22.2 ± 1.4	5.0 ± 0.6	331 ± 51	189 ± 52	520 ± 89

[a] B-type plus C-type granules; [b] SE

granules as common wheat. By contrast, the average diameters of A-type and small granules in einkorn accessions were 25–30% shorter than those of common wheat cultivars. As a consequence, the total surface area of granules in "monococcum" wheat was estimated at 764 ± 188 mm^2/mg of starch, and was about 1.5 times higher than that for common wheat. Thus it is concluded that the high amount of puroindolines accumulated on the starch granule in einkorn wheat could be partly due to the high bonding surface area in this diploid wheat species as compared with common wheat. However, the difference in the total starch surface area, although statistically significant, was much less than the difference in puroindoline accumulation (about 3 to 1) between the two wheats.

Another distinctive trait of einkorn wheat is the presence of ETI in the A-PAGE pattern of starch-bound proteins. No traces of this polypeptide were found in both common and durum wheat, as well as in *Aegilops tauschii*, the D-genome donor to common wheat. The CDS of this protein was found to contain 444 bp coding for an ETI pre-protein 148 residues long. Mature ETI, 13,323 Da in size, was shown to be a polypeptide chain of 121 amino acids that starts with the SVG tripeptide and ends with a Y residue. A signal peptide with 23 residues and a hydrophobic domain was observed upstream of the SVG tripeptide at the *N*-terminal end of the ETI precursor. The cysteine content and the CC and CRC motifs of mature ETI proved to be identical to those of the 2S super-family of seed proteins that includes α-amylase/trypsin inhibitors, grain softness proteins (GSP), puroindolines, and tryptophanins. However, ETI lacks the unique amphiphilic tryptophan-rich lipid binding domain, has a calculated isoelectric point of 7.5 and exhibits a hydrophobic central domain at positions 45 to 78.

Whereas starch granules exposed to 85% aqueous methanol for 5 min gave a faint ETI band (Fig. 5), those re-exposed to a fresh 85% methanol solution did not release any trace of this protein. On the contrary, re-suspension of starch granules in 50 mM NaCl and 50% (v/v) propan-2-ol resulted in a prominent ETI band in combination with Pin-A and Pin-B. Our conclusion from this experiment is that ETI occurs in the einkorn endosperm as a free or loosely bound polypeptide promptly extracted with 85% methanol. In addition, a certain amount of ETI interacts tightly with the starch granules and displays some common features with puroindolines. In particular, ETI and puroindolines could interact in reducing the strength of the adhesion between the starch granule surface and the surrounding matrix, and work as the actual casual agents of the extra-soft texture of einkorn kernels. Moreover, interaction of these 2S components could account for the high amount of puroindolines accumulated on the starch granules of einkorn wheat. The *Eti-A^{m}1a* gene is currently being transferred to durum wheat cv. Svevo and common wheat cv. Bolero by a transgenic procedure to test its effect on kernel texture.

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