Puroindoline polymorphism and kernel texture in einkorn (*Triticum monococcum*)

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ABSTRACT

When fractionated by A-PAGE, starch granule proteins from 113 'monococcum' wheat (*Triticum monococcum* ssp. *monococcum*) accessions appeared as three major components (bands *b1*, *b2* and *b3*) in the cathodic region of the gel. All three proteins are approximately 13kDa in size, as determined by two dimensional A-PAGE x SDS-PAGE. Bands *b1* and *b3* were shown to correspond to puroindolines A (Pin-A) and B (Pin-B), respectively, whereas protein *b2* was quite different in having the N-terminal SVGDQC sequence, which has a high similarity with that of an α-amylase/trypsin inhibitor described in rye. Ten accessions exhibited a fast-moving version of protein *b2* as well as softer kernels, as compared to einkorn genotypes with wild-type *b2*. Moreover, accession ID 508 was found to possess a slow-moving Pin-B because of an N to D amino acid substitution at position 29 of the Pin-B sequence. Ecotilling and PCR amplification revealed several non-synonymous SNPs in the coding regions of Pin-A and Pin-B. All the einkorn accessions analysed here exhibited an extra-soft grain texture (mean SKCS value = -2.5), variation being as high as -23.9 to 21.5. A highly significant inverse correlation was observed between SKCS index and kernel weight, the difference in this latter trait explaining approximately 32% of the phenotypic variation for kernel hardness.

INTRODUCTION

Puroindolines A (Pin-A) and B (Pin-B) are two α-helical, tryptophan- and cysteine-rich isoforms occurring in the starchy endosperm of the *Triticeae* and *Aveneae* tribes (Morris, 2002). They are basic proteins, approximately 13 kDa in size, which show affinity for polar lipids, foaming properties and permeabilizing effects on bacterial and fungal membranes (Jing *et al.,* 2003). Variation in puroindoline composition was shown to affect grain hardness, crumb structure and rheological properties of wheat dough (Giroux *et al.,* 2000). Puroindolines occur at high amounts on the surface of starch granules of soft bread wheat as compared with those from hard bread wheat (Corona *et al*., 2001). A clear separation of puroindolines can be obtained by acid polyacrilamide gel electrophoresis (A-PAGE) at pH 3.0 (Corona *et al*., 2001). In commom wheat (*Triticum aestivum* L., $2n = 6x = 42$, genome AABBDD) and *Aegilops tauschii* (Coss.) ($2n = 2x = 14$, genome DD), Pin-A and Pin-B are encoded by genes at the *Pina-D1* and *Pinb-D1* loci, respectively (Giroux and Morris, 1997). These loci occur at the *Hardness* (*Ha*) locus on the short arm of chromosome 5D (Giroux and Morris 1997). In *Triticum monococcum*, a diploid wheat (2n=2x=14) with genome AA, the *Pina-A^m1* and *Pinb-A^m1* genes coding for Pin-A and Pin-B respectively, were found to be closely linked to each other in the distal region of the short arm of chromosome 5A . Genes at these loci show a high degree of sequence similarity (>94%) with their counterparts in *T. aestivum*. In the present work, 113 'monococcum' accessions of different geographic origins are compared for their kernel texture and starch-bound protein pattern, as determined by A-PAGE fractionation. Moreover, PCR amplicons obtained with primers specific for puroindoline-encoding genes of 'monococcum' accessions showing contrasting A-PAGE patterns, were sequenced and compared with those of bread wheat cultivars.

MATERIALS AND METHODS

Plant material and grain hardness

Grains of 113 *T. monococcum* accessions of different geographic origins kindly provided by Dr. A. Brandolini (CRA, S. Angelo Lodigiano, Italy), were used in the present study. Common wheat cvs. Bolero and Chinese Spring were analysed as well. Single grain hardness readings were obtained by analysing 10-kernel samples of each accession with the Perten Model SKCS 4100.

A-PAGE and Western Blotting

Puroindolines were extracted from 3mg of air-dried starch granules as described previously (Corona *et al*., 2001). A-PAGE fractionation of starch-bound proteins was carried out at pH 3.1 as described by Gazza *et al*., (2006) in a 10cm x 10cm electrophoresis apparatus. Immunoblotting with a polyclonal anti-Pin-A antiserum was performed as described previously (Gazza *et al*. 2006), except for the use of CAPS as a transfer buffer and a wet apparatus (Biorad, USA) for protein blotting.

DNA extraction, PCR amplification and gene sequencing

Genomic DNAs were isolated from young leaves using CTAB **(**CetylTrimethylAmmonium Bromide). PCR amplifications of puroindoline genes were performed with the primer pairs described by Gautier *et al*. (1994). Reactions were performed in 50µl volume containing 100ng of genomic DNA, 20pmol of each primer, 200µM of each dNTP, 2U of RedAccutaq (SIGMA), 1Xtaq 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 used an extension of 7 min at 68°C. The PCR products were analyzed on 1.8% agarose gels, stained with ethidium bromide and visualized under UV. PCR fragments were eluted from the agarose gel with the Nucleospin-Extract kit (Machery-Nagel) and sequenced on the ABI 3730 DNA sequencer.

Ecotilling

Genomic DNA from cv. Monlis, the sole 'monococcum' wheat registered in Italy, has been used as a reference. Amplicons of puroindoline genes were obtained through two PCR amplifications. The first PCR amplification was performed on 100 ng of genomic DNA in a final volume of 25 µl using the Optimase Polymerase conditions (Transgenomic, USA). DNA was first submitted to a denaturation step of 5 min at 94 °C followed by 35 cycles of 30 seconds of denaturation at 94 °C, 2 min annealing at 52°C, 2 min extension at 72 °C for, and 5 min extension at 72 °C. Pin-A-encoding gene was amplified with primer pair *Prompina2* (CTTGAACAACCTGCACA) and *Pin-A low* (Gautier *et al*. 1994), whereas gene encoding for Pin-B was amplified with primer pair *Prompinb3* (ACAGAAAACCACGGCTAGA) and *Pin-B low* (Gautier *et al*. 1994). A 1:100 dilution of the first PCR solution was used as a template for the second PCR reaction with the primer pairs specific for the DNA sequences coding for Pin-A and Pin-B (Gautier *et al*., 1994) in a final volume of 50 µl. Amplification following the Optimase Polymerase conditions was performed as described above except for an annealing step at 58 °C for Pin-A and at 54 °C for Pin-B.

Equal amounts (~750 ng) of Pin-A and Pin-B fragments from test ('monococcum' accession) and reference (cv. Monlis) were mixed in a final volume of 30 µl, heated at 95 °C for 10 min and cooled at 25 °C for 20 min. A volume (15 µl) of this mixture was digested with 1 µl of Nuclease S1 and 1 µl of Enhancer S (Transgenomic, USA) for 20 min at 42°C. The digested products (500 ng) were fractionated on a 2% TransOneK^{TM} agarose gel.

RESULTS AND DISCUSSION

Electrophoretic separations of starch granule proteins

Upon A-PAGE fractionation, proteins extracted from starch granules of all the 'monococcum' accessions investigated here appeared in the cathodic region of the gel as three prominent components (*b1*, *b2* and *b3*, Fig. 1), the faster *b3* band being a doublet. In the same gel region, puroindolines Pin-A and Pin-B encoded by wild-type alleles *Pina-D1a* and *Pinb-D1a* in common wheat cv. Bolero (Corona *et al*., 2001), occurred as two strong bands (Fig.1, lane 5). As expected, no protein was found in the A-PAGE pattern of durum wheat cv. Colosseo (Fig. 1, lane 11).

The polyclonal antiserum developed against the 16 mer DRASKVIQEAKNLPPR sequence in the C-terminal region of mature Pin-A of common wheat reacted strongly with protein *b1*, suggesting that this polypeptide corresponds to puroindoline Pin-A encoded by the *Pina-A^{m}1* gene in *T. monococcum*.

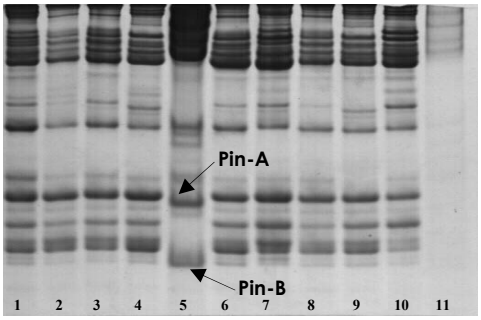

Figure 1. Fractionation by A-PAGE of starch-bound proteins from different "monococcum" accessions (lanes 1-4; 6-10). Lane 5: common wheat cv. Bolero. Lane 11: durum wheat cv. Colosseo**.**

When fractionated by two dimensional A-PAGE x SDS-PAGE electrophoresis (Fig. 2) proteins *b1* (=Pin-A), *b2* and *b3* appeared as three major spots in the Mw 13 KDa region, protein *b3* being slightly faster than Pin-A and protein *b2* in the second dimension. Because of its electrophoretic behaviour, the *b3* doublet was tentatively assumed to correspond to Pin-B encoded by the *Pinb-A^{m}1* locus.

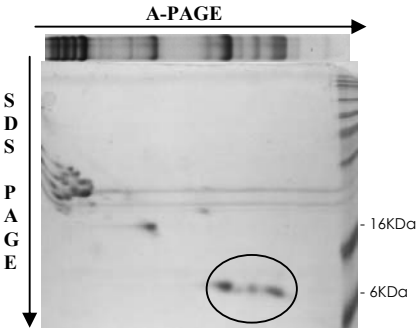

Figure 2. Two dimensional A-PAGE x SDS-PAGE electrophoresis of starch-bound proteins of einkorn cv. Monlis. *b1, b2* and *b3* proteins occur in the oval**.**

No significant variation in mobility of Pin-A was observed amongst the 113 'monococcum' accession analysed here. On the contrary, Pin-B and protein *b2* showed two different A-PAGE patterns (Fig.3). In particular, the A-PAGE pattern of accession ID 508 from Spain was quite unique in showing an unusual slow-moving Pin-B (Fig.3, lane 3), whereas the A-PAGE pattern of accession ID 505 from Turkey (Fig.3, lane 2) exhibited a strong *b2* band slightly faster that its counterparts in all 'monococcum' accessions showed in Figure 3. This fast-moving band *b2* was observed in 10 'monococcum' accessions from Turkey, Bulgaria and Yugoslavia. The einkorn genotypes analysed here showed similar amounts of Pin-A as well as of Pin-B. On the contrary, there was a significant variation in the amount of protein *b2* (Fig. 3).

PCR amplification of puroindolines

The amplicon obtained in cv. Monlis with the primer pair for the coding region of Pin-A (Gautier *et al.*, 1994) was mixed with its counterpart produced by each 'monococcum' accession, heated at 95 °C, cooled at 25 °C and digested with Nuclease S1. The presence of two or more bands in the agarose gel (ecotilling) of the digested DNA mixture was assumed to be an evidence of sequence polymorphism between the reference cv. Monlis and the test accession, whose Pin-A amplicon

was then sequenced on the ABI 3730 DNA sequencer. Non-synonymous single nucleotide polymorphisms (SNPs), which resulted in 5-6 amino acid changes with respect to the *Pina-D1a* allele of bread wheat cv. Chinese Spring were obtained in cv. Monlis and accessions ID 1643 and ID 508 (Fig.4). An ecotilling test was also performed using the primer pair specific for the coding region of Pin-B (Gautier *et al.,* 1994). When compared to wild-type allele of *Pinb-D1a* in cv. Chinese Spring, the 447bp Pin-B amplicons from einkorn showed several non-synonymous SNPs that changed 9 to 11 amino acids in the different 'monococcum' accessions analysed (Fig.4). Interestingly, one of these SNPs changed N (asparagine) to D (aspartic acid) at position 29 in the Pin-B sequence of accession ID 508 (Fig.4). This amino acid substitution could account for the reduced mobility of protein *b3* in the A-PAGE pattern of accession ID 508 (Fig.3, lane 3), supporting the hypothesis that the *b3* doublet corresponds to Pin-B.

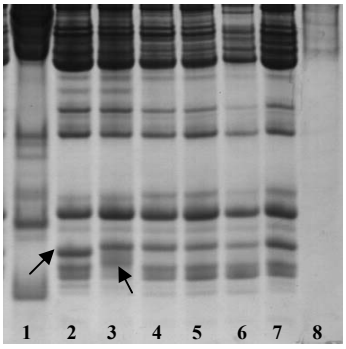

Figure 3. A-PAGE fractionation of starch-bound proteins. Lanes: (1) common wheat cv. Bolero; (2) monococcum accession ID505; (3) monococcum accession ID 508; (4-7) monococcum accessions; (8) durum wheat cv. Colosseo.

The starch granule protein b2

The *b2* polypeptide was never observed in the A-PAGE pattern of starch granule proteins extracted from bread wheat cultivars and several cereal species (Corona *et al*., 2001; Gazza *et al*., 2006). When the A-PAGE fractionation of starch granule proteins was western-blotted using CAPS as a transfer buffer at pH 11, this protein migrated to the anodic PVDF membrane, whereas the other starch granule proteins moved to the opposite direction (Figure 5). N-terminal part of *b2* was found to begin with the SVGDQC sequence, which shows a high similarity with that of an α-amylase/trypsin inhibitor described in rye. In this context it is note-worthy that puroindolines and α-amylase/trypsin inhibitors both belong to the 2S, cysteine-rich, protein family, approximately 13 KDa in size.

Kernel hardness as determined by SKCS

All the 'monococcum' accessions analysed here exhibited an extra-soft grain texture (mean SKCS value = -2.5), variation being as high as -23.9 to 21.5. A significant ($P<0.01$) inverse correlation was observed between SKCS value and kernel weight ($R=-0.56$) confirming previous findings in common wheat kernels (Gazza *et al*., 2008). The difference in kernel weight explained approximately 32% of the phenotypic variation in SKCS index, as calculated by the coefficient of determination R^2. The 'monococcum' accessions possessing the fast-mowing protein *b2* produced softer kernels (mean SKCS value = -7.1) compared to those with the wild-type version of this band. However, this difference was not statistically significant, and requires further investigation. Accession ID 508 possessing the slow-moving Pin-B showed a relatively high SKCS index (3.8), which was, however, lower than those of other 26 'monococcum' accessions with wild-type Pin-B.

Puroindoline A

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CS       MKALFLIGLLALVASTAFAQYSEVVGSYDVAGGGGAQQCPVETKLNSCRNYLLDRCSTMK 60
Monlis   ************************I***************L*******************
ID 1643  ************************I***************L*******************
ID 508   ************************I***************L*******************

CS       DFPVTWRWWKWWKGGCQELLGECCSRLGQMPPQCRCNIIQGSIQGDLGGIFGFQRDRASK 120
Monlis   ***************L********Q*********************S*************
ID 1643  ***************L********Q*********************S*************
ID 508   ***************L********Q*************************************

CS       VIQEAKNLPPRCNQGPPCNIPGTIGYYW 148
Monlis   ****************************
ID 1643  **********K*****************
ID 508   ****************************
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Puroindoline B

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CS       MKTLFLLALLALVASTTFAQYSEVGGWYNEVGGGGGSQQCPQERPKLSSCKDYVMERCFT 60
Monlis   ********************************A********L*******************
ID 1643  ********************************A********L*******************
ID 508   ****************************D***A********L*******************

CS       MKDFPVTWPTKWWKGGCEHEVREKCCKQLSQIAPQCRCDSIRRVIQGRLGGFLGIWRGEV 120
Monlis   *************************Q***************GM***K****F*****D*
ID 1643  *************************Q***************GM***K***F*****DA
ID I508  *************************Q***************GM***K****F*****DA

CS       FKQLQRAQSLPSKCNMGADCKFPSGYYW 148
Monlis   ***I************************
ID 1643  ***I************************
ID 508   ***I************************
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Figure 4. Amino acid substitution in Pin-A and Pin-B of monococcum accessions when compared to *T. aestivum* cv. Chinese Spring (CS).

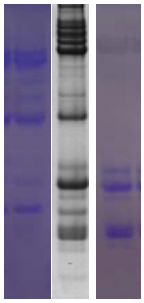

Figure 5. A-PAGE fractionation (middle) and Western blotting on PVDF membranes (left: anodic region; right :cathodic region) of starch granule proteins from 'monococcum' cv. Monlis.

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