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ORIGINAL PAPER

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Genetic and biochemical analysis of common wheat cultivars lacking puroindoline a

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Abstract Puroindoline a (Pin-a) and puroindoline b (Pin-b), two basic isoforms encoded by the *Pina-D1* and *Pinb-D1* loci respectively, involved in controlling grain texture in wheat, were isolated from starch granules of soft wheat cultivars using three different extraction procedures, and fractionated by acidic polyacrylamide gel electrophoresis (A-PAGE). Tris buffer containing 1% Triton X-114 extracted Pin-a and small amounts of Pin-b, whereas 1% SDS preferably extracted Pin-b. Large amounts of both puroindolines were isolated by a solution containing 50% propan-2-ol and 50 mM NaCl. This solution extracted reduced amounts of Pin-b and no traces of Pin-a from starch granules of 20 hard common wheats containing the null allele *Pina-D1b*. The absence of Pin-a was confirmed by immunostaining with an anti-Pin-a antiserum. With the exception of two cultivars, null Pin-a cultivars gave no PCR fragment with three primer pairs specific to either the coding region or the promoter region of *Pina-D1a*, suggesting that major changes had occurred at the *Pina-D1* locus in these genotypes. Cultivars Fortuna and Glenman were unique in giving size-specific PCR fragments with all primer pairs for the allele *Pina-D1a* and showed a cytosine deletion at position 267 in the coding region of the Pin-a gene, which resulted in a TGA stop codon at position 361. However, there was no evidence of a mutated protein in the A-PAGE or SDS-PAGE patterns of Fortuna and Glenman. The novel gene, provisionally named *Pina-D1c*, is the first null allele due to a point mutation that has been identified at the *Pina-D1* locus.

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Introduction

Wheat (*Triticum aestivum* L.) grain texture is controlled by the *Ha* locus on the distal end of the short arm of chromosome 5D (Mattern et al. 1973; Law et al. 1978). This locus is inseparably linked to the *Pina-D1* and *Pinb-D1* genes coding for puroindoline a (Pin-a) and puroindoline b (Pin-b), respectively (Greenwell and Schofield 1989; Giroux and Morris 1997). Pin-a and Pin-b are the predominant components of friabilin, a group of different polypeptides 13–15 kDa in size, which occur in higher amounts on the surface of starch granules of soft wheats as compared to those from hard wheats (Greenwell and Schofield 1986; Blochet et al. 1993; Gautier et al. 1994; Morris et al. 1994; Pogna et al. 2002). In fact, puroindolines are claimed to be the principal determinant factors of endosperm texture (Giroux and Morris 1998), which strongly affects flour yield, starch damage and water absorption of dough. *Pina-D1* and *Pinb-D1* are absent in AB-genome durum wheat (*T. turgidum* spp. *durum*), which is characterized by extra-hard kernel texture (Gautier et al. 1994; Pogna et al. 2002). In mature kernels, puroindolines are located in the starchy endosperm and in the aleurone cells (Dubreil et al. 1998). They are basic isoforms, 60% identical in their amino acid sequences, with a relatively high number of cysteine and lysine residues. Moreover, they share a characteristic tryptophan-rich amphiphilic domain, which makes them unique among plant proteins, and confers them a strong affinity to lipids (Gautier et al. 1994). In particular, variation in endosperm texture was correlated with the affinity of the tryptophan-rich domain of puroindolines for polar lipids in the amyloplast membranes (Giroux and Morris 1997). Furthermore, the lipid-binding properties of puroindolines has been assumed to account for the significant influence of these proteins on dough quality

(Dubreil et al. 1997; Igrejas et al. 2001) and plant resistance to pathogens (Dubreil et al. 1998; Krishnamurthy et al. 2001).

A-PAGE fractionation of proteins associated with starch granules provided a clear separation of Pin-a and Pin-b compared with SDS-PAGE, and showed that hard wheat cultivars have faint Pin-b bands as compared to those of the soft cultivars, suggesting that grain texture is associated with the amount of Pin-b on starch granules (Corona et al. 2001a, b; Pogna et al. 2002). On the contrary, the intensity of Pin-a in several hard cultivars is similar to that of the soft cultivars. The reduced amounts of Pin-b in the starch extracts of hard cultivars was found to be correlated with point mutations from the wild-type *Pinb-D1a* allele coding for Pin-b in soft cultivars (Giroux and Morris 1998; Lillemo and Morris 2000; Corona et al. 2001a, b). Several hardness mutations at *Pinb-D1* have been described (Lillemo and Morris 2000).

A null mutation for Pin-a (allele *Pina-D1b*), with no protein or mRNA present (Giroux and Morris 1998), was also found to drastically reduce the amount of Pin-b associated with starch granules (Corona et al. 2001a; Pogna et al. 2002). The occurrence of the wild type *Pinb-D1a* allele in hard wheat cultivars containing allele *Pina-D1b* suggested that interaction of Pin-b with starch granules is prevented by the absence of Pin-a (Giroux and Morris 1998; Corona et al. 2001a). The very hard grain texture characteristics of cultivars lacking Pin-a indicate that this protein may have a direct influence on kernel hardness as well.

Here, bread wheat cultivars lacking Pin-a are investigated for their genetic compositions at the puroindoline-coding genes, and for their electrophoretic patterns after extraction of starch granule proteins with different solvents. The present study was aimed at investigating the molecular basis of the mutations in the *Pina-D1* genes, as well as at acquiring additional evidence of the ability of puroindolines to modulate grain texture.

Materials and methods

Plant material

Samples of grain from bread wheat (*Triticum aestivum* L.) cultivars Amidon (K62515), Fortuna (K46963) and Glenman (K59555) obtained from USA collections were used in the present study. The Chinese Spring cultivar (CS) and the other bread wheat cultivars used were grown at the Istituto Sperimentale per la Cerealicoltura, Rome, Italy (Table 1). The CS Nulli 5D/Tetra5B line, Langdon 5D(5B) chromosome substitution line and the durum wheat cv. Langdon were also used.

Extraction of proteins and electrophoretic analyses

Puroindolines were extracted from either whole crushed kernels or starch granules. Extraction from crushed kernels (0.100 g) was performed with 0.4 ml of Tris buffer containing 1% Triton X-114 according to the extraction protocol described by Giroux and Morris (1998). Proteins in the detergent-rich phase were precipitated with two volumes of acetone at −20°C.

Three different procedures were used to extract puroindolines from 50 mg of air-dried starch granules obtained as described previously (Corona et al. 2001a). In the first procedure, starch granules were mixed with 0.5 ml of Tris buffer containing 1% Triton X-114 and extracted as described above. In the second procedure, starch granules were suspended in a solution containing 50 mM NaCl and 50% (v/v) propan-2-ol. After sonication for a few seconds, the suspension was kept at room temperature for 1 h and then centrifuged at 8,000 *g* for 10 min. Proteins in the supernatant were precipitated with two volumes of acetone at −20°C overnight and then air-dried. In the third procedure, starch granules were mixed with 0.5 ml of a solution containing 1% (w/v) sodium dodecyl sulfate (SDS) and sonicated for a few seconds. After extraction for 30 min at 50°C, the suspension was centrifuged at 8,000 *g* for 10 min and the supernatant was dialyzed against 0.1 M NaCl overnight at 4°C. Proteins were then precipitated with two volumes of acetone at −20°C overnight and air-dried.

Before loading for A-PAGE fractionation, proteins were suspended in 50 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 a half volume of 50% (v/v) glycerol, containing 0.1% (w/v) pyronine Y. Electrophoresis was performed at pH 3.1 in a Hoeffer SE 600 apparatus (Amersham Pharmacia Biotech) using 17% acrylamide gels run at 430 V until the dye reached the bottom of the gel. The separating gel (16 cm×18 cm, 1.5 mm thick) was prepared by mixing 1.8 g of urea, 34 ml of 30% acrylamide stock solution ($T = 30\%$ and $C = 1\%$), 15 ml of ascorbic acid solution and 10 ml of distilled water. The ascorbic acid solution contained 0.4 g ascorbic acid, 6 mg ferrous sulfate, 40 ml sodium lactate buffer

Table 1 Allele compositions at the puroindoline loci *Pina-D1* and *Pinb-D1* in common wheat cultivars used in the present study

Pina-D1b (null allele) *Pinb-D1a* (wild-type allele)	*Pina-D1a* (wild-type allele) *Pinb-D1a*	*Pina-D1a Pinb-D1b* (serine-type allele)
Amidon, Barra, Ciano, Dorico, Eridano, Falcon, Fortuna, Glenman, Golia, Guadalupe, Inia 66, Idice, Kalyansona, Manital, Mendos, Padus, Prinqual, Sibilla, Super X, Yecora	Centauro, Chinese Spring, Leone, Leopardo	Francia, Marberg, Mieti, Newana

brought to 100 ml with distilled water. After cooling at 4°C, the acrylamide solution was mixed with 5 µl of 30% hydrogen peroxide and immediately poured into the gel cassette. The 5% acrylamide stacking gel (35 ml) was prepared as described above with appropriate modifications and polymerized with 5 µl of 30% hydrogen peroxide. The electrophoretic buffer was 0.17 g/l of 97% NaOH adjusted to pH 3.1 with lactic acid.

SDS-PAGE fractionation of puroindolines was performed in a Mini-Protean II cell (Bio-Rad) using a 15% polyacrylamide gel. Proteins were suspended in 25 µl of a solution containing 5% 2-mercaptoethanol, 25 mM Tris, 0.12% (w/v) SDS, 10% (w/v) glycerol and 0.2% (w/v) pyronine Y, incubated at 100°C for 5 min and fractionated at 140 V until the tracking dye reached the bottom of the gel. A 0.25% (w/v) solution of Coomassie Brillant Blue R250 in 6% trichloracetic acid was used to fix and stain both the A-PAGE and SDS-PAGE gels.

Western blotting and spot-blotting

Western blotting was performed in a Bio-Rad semi-dry transfer cell using nitrocellulose membranes (Immobilon-NC, Millipore). SDS-PAGE gels were equilibrated for 30 min in a transfer buffer, pH 9.2, containing 80 mM Tris, 13 mM glycine and 20% (v/v) methanol in distilled water, transferred between two double layers of 3 MM chromatography paper (Whatman), pre-equilibrated in the same transfer buffer, and electro-blotted at 14 V for 40 min. Before the transfer, A-PAGE gels were equilibrated for 1 h in the transfer buffer with 2% SDS added. After the transfer, membranes were maintained for 1 h in PBS buffer containing 3% (w/v) commercial fish gelatin as a blocking agent, and incubated for 16 h in the same buffer containing 1% (w/v) commercial fish gelatin and the anti-friabilin antiserum. Three antisera were used. The monoclonal antiserum conjugated to horseradish peroxidase of the Durotest S kit (Rhone-Poulenc Diagnostic Technologies) was diluted 1:500, whereas the polyclonal Pin-a or Pin-b-specific antisera developed by Krishnamurthy and Giroux (2001) and kindly provided by M.J. Giroux, Department of Plant Sciences, Montana State University, Bozeman, USA, were diluted 1:250. After incubation with a 1:250 dilution of a goat anti-rabbit horseradish peroxidase conjugate (Sigma), blots were stained with 4-chloro-1-naphthol and hygrogen peroxide.

The monoclonal antibody of the Durotest S kit was also used in spot-blotting immunoreactions. Cracked grains (100 mg) were suspended in 500 µl of 1% (w/v) SDS at 50°C for 30 min and centrifuged at 5,000 *g* for 10 min. An aliquot (5 µl) of the supernatant was then spotted onto a nitrocellulose membrane (Immobilon-NC). The membrane was maintained for 30 min in PBS buffer containing 3% (w/v) commercial fish gelatin, incubated for 3 min in the same buffer containing 1% (w/v) commercial fish gelatin and the Durotest S antiserum, and then stained as described above.

DNA isolation and PCR amplification

Genomic DNAs were isolated from young leaves using the procedure of DellaPorta et al. (1983). The sense strand primer 5′-ATGAAGGCCCTCTTCCTCA-3′ and the antisense strand primer 5′-TCACCAGTAATAGCCA-ATAGTG-3′ were used to amplify the Pin-a gene (Gautier et al. 1994). Amplification of the Pin-b gene was performed with the sense strand primer 5′-AT-GAAGACCTTATTCCTCCTA-3′ and the antisense strand primer 5′-TCACCAGTAATAGCCACTAGG-GAA-3′ (Gautier et al. 1994).

Amplification of the promoter region of allele *Pina-D1a* was performed using either 5′-CTTGAACAAC-CTGCACA-3′ (calculated by Bioinformatics Bielefeld and named prom-A1) or 5′-CCTCGGACACCTTG-TTAA-3′ (named prom-A2) as the sense strand primer, coupled with the antisense primer 5′-TCACCAGTAA-TAGCCAATAGTG-3′ (Gautier et al.1994). The promoter region of *Pinb-D1* was amplified using the sense strand primer 5′-CACATGATTCTAAATAC-3′ coupled with the antisense strand primer 5′-TCACCAGTAA-TAGCCACTAGGGAA-3′.

Reactions were performed in 50 µl volumes containing 300 ng of genomic DNA, 10 pmol of each primer, 200 µM of each dNTP, 1x *Taq* DNA polymerase buffer (Invitrogen), and 2.5 U of *Taq* DNA polymerase (Invitrogen). The samples, which were denaturated at 94°C for 5 min before the addition of *Taq*, were submitted to 35 cycles of 1 min of denaturation at 94°C, 1.5 min annealing at T_m and 1.5 min elongation at 72°C. A final cycle with an extension of 7 min at 72°C completed the reactions. The PCR products were analyzed on 1.8% agarose gels, stained with ethidium bromide and visualized under UV.

DNA sequencing

The PCR fragments were eluted from the agarose gel with the Nucleospin-Extract kit (Macherey-Nagel) and sequenced on a Perkin Elmer ABI Prism 377 DNA sequencer, using the dideoxynucleotide chain termination method.

Results

A-PAGE fractionation of starch granule proteins extracted with propan-2-ol

Upon A-PAGE, puroindolines a and b extracted from the starch granules of the Langdon 5D(5B) substitution line using 50 mM NaCl in 50% propan-2-ol appeared as

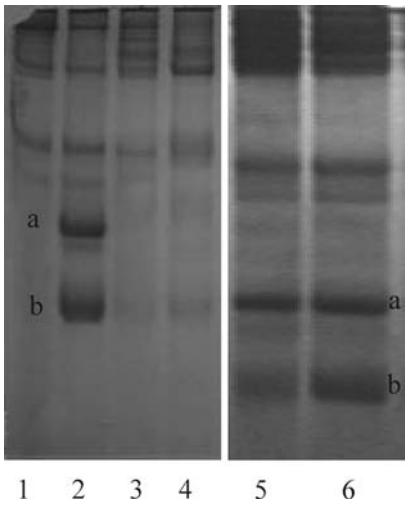

Fig. 1 A-PAGE fractionation of starch granule proteins extracted with propan-2-ol/NaCl from durum wheat cv. Langdon (*lane 1*), chromosome substitution line Langdon 5D(5B) (*lane 2*), cv. Glenman (*lane 3*), cv. Fortuna (lane *4*), cv. Mieti (*lane 5*), and cv. Chinese Spring (*lane 6*). Puroindolines a and b are marked by *a* and *b*, respectively

two prominent bands (Fig. 1, lane 2). This line has chromosome 5D of cv. Chinese Spring substituted for chromosome 5B of durum wheat cv. Langdon. As expected, both proteins were absent in the A-PAGE pattern of cv. Langdon (Fig. 1, lane 1). Strong puroindoline bands were also obtained from starch granules of soft bread wheat cultivars containing wild-type alleles *Pina-D1a* and *Pinb-D1a* (Fig. 1, lane 6). No traces of Pin-a, together with reduced amounts of Pin-b (Fig. 1, lanes 3 and 4) were found in the starch extracts of the 20 hard wheat cultivars carrying the null allele *Pina-D1b* listed in Table 1. Finally, the hard wheat cultivars carrying allele *Pinb-D1b* coding for serine-type Pin-b exhibited a strong Pin-a band coupled with a faint Pin-b band (Fig. 1, lane 5).

A-PAGE fractionation of puroindolines extracted with 1% SDS or Triton X-114

When proteins extracted from starch granules of Langdon 5D(5B) using 1% SDS were dialyzed against 0.1 M NaCl and fractionated by A-PAGE, puroindoline b appeared as an intense band (Fig. 2, lane 2), whereas Pin-a appeared as a faint band compared with its counterpart extracted with propan-2-ol (Fig. 2, lane 4). Preferential extraction of Pin-b by sodium dodecyl sulfate was confirmed in all soft wheat cultivars analyzed (data not shown).

The A-PAGE pattern of proteins extracted from crushed seeds of Langdon 5D(5B) using 1% Triton X-114 according to the sequential extraction protocol described by Giroux and Morris (1998) showed a strong Pin-a band coupled with a very faint Pin-b band (Fig. 3, lane 2). The intensity of the Pin-a band

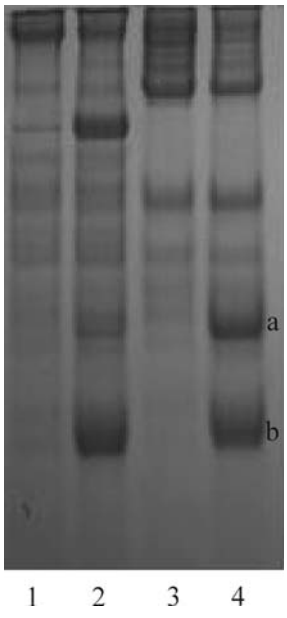

Fig. 2 A-PAGE fractionation of starch granule proteins extracted with 1% SDS (*lanes 1 and 2*) or propan-2-ol (*lanes 3 and 4*) from durum wheat cv. Langdon (*lanes 1 and 3*) and Langdon 5D(5B) (*lanes 2 and 4*). *a* Pin-a, *b* Pin-b

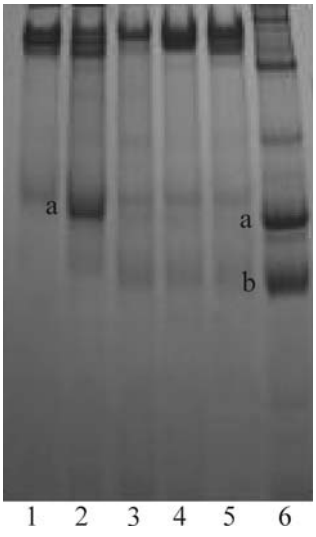

Fig. 3 A-PAGE fractionation of proteins extracted with Triton X-114 from seeds of durum wheat cv. Langdon (*lane 1*), Langdon 5D(5B) (*lane 2*), cv. Glenman (*lane 3*), cv. Barra (*lane 4*), and cv. Fortuna (*lane 5*). *Lane 6* shows puroindolines a and b extracted with propan-2-ol from starch granules of Langdon 5D(5B)

was similar to that of its counterpart extracted with propan-2-ol (Fig. 3, lane 6). Hard wheat cultivars carrying the null allele *Pina-D1b* exhibited a faint Pin-b band and no traces of Pin-a in their seeds extracted with Triton X-114 (Fig. 3, lanes 3–5). Preferential extraction of Pin-a by Triton X-114 was also observed in A-PAGE fractionations of puroindolines from starch granules (data not shown).

Immunological tests

The monoclonal antiserum of the Durotest S kit (Rhone-Poulenc Diagnostic Technologies) was tested against the starch granule proteins extracted with propan-2-ol/NaCl and fractionated by A-PAGE. The antiserum recognized Pin-a in Langdon 5D(5B) and gave a weaker reaction with Pin-b as well (Fig. 4, lane 2). On the other hand, the polyclonal antiserum to Pin-a developed by Krishnamurthy and Giroux (2001) reacted strongly with a component of the SDS-PAGE pattern of proteins extracted with Triton X-114 from wheats containing the wild-type allele *Pina-D1a* (Fig. 5, lanes 3 and 11, arrows), whereas it gave no reaction with extracts from cultivars lacking Pin-a (Fig. 5, lanes 2 and 4–10). The polyclonal antiserum to Pin-b reacted with a 14 kDa starch granule protein extracted with propan-2-ol/NaCl and fractionated by SDS-PAGE (Fig. 6, lanes 1 and 3, arrows). The antiserum reacted with all the cultivars analyzed, irrespective of their grain texture; however, the reaction was strong in wheats possessing wild-type alleles *Pina-D1a* and *Pinb-D1a* for soft texture (Fig. 6, lane 3), and comparatively weak in hard cultivars (Fig. 6, lane 1). Finally, the proteins extracted from cracked grains of soft or serine-type hard wheat cultivars analyzed here (Table 1) showed strong spot-blotting immunoreactions with the Durotest S antiserum, whereas null Pin-a cultivars exhibited very weak reactions (data not shown).

Molecular characterization of *Pina-D1* in bread wheat cultivars lacking Pin-a

Except two cultivars, all hard wheat cultivars lacking Pin-a analyzed here (Table 1) gave no PCR amplification product using the terminal primers specific to *Pina-D1a* (Gautier et al. 1994) (Fig. 7 lanes 2, 3 and 7). On the contrary, the two exceptional cultivars Fortuna and Glenman (Fig. 7, lanes 4 and 5) gave a 447 bp product identical in size to that obtained from cultivars possessing wild-type allele *Pina-D1a* (Fig. 7, lanes 1, 6, and 8–10). In addition, cultivars Fortuna and Glenman differed from the null Pin-a cultivars in that they gave a PCR product of the expected size when amplified with the sense strand primer prom-A1 located 290 bp upstream from the ATG start codon of *Pina-D1a* (Fig. 8, lanes 7 and 8).

The PCR fragments obtained by amplification of cultivars Fortuna and Glenman using the prom-A2 sense strand primer specific for the promoter region of *Pina-D1a* were eluted from the agarose gels and sequenced using the dideoxynucleotide chain termination method. No nucleotide variation with respect to the

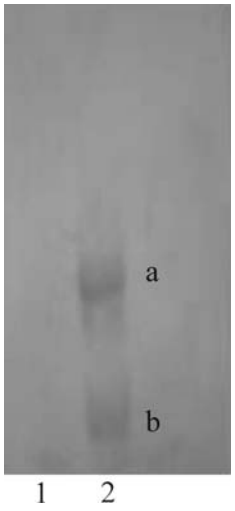

Fig. 4 Starch granule proteins extracted with propan-2-ol, fractionated by A-PAGE and immunostained with the monoclonal antiserum of the Durotest S kit. *Lane 1* Durum wheat cv. Langdon, *lane 2* Langdon 5D(5B). *a* Pin-a, *b* Pin-b

Fig. 5 Reaction of anti-Pin-a antiserum against proteins extracted with Triton X-114 from crushed kernels and fractionated by SDS-PAGE. *Lane 1* Molecular weight markers, *lane 2* cv. Amidon, *lane 3* cv. Chinese Spring, *lane 4* cv. Falcon, *lane 5* cv. Padus, *lane 6* cv. Prinqual, *lane 7* cv. Super X, *lane 8* cv. Manital, *lane 9* cv. Barra, *lane 10* durum wheat cv. Langdon, *lane 11* Langdon 5D(5B). *Arrows* indicate a polypeptide of approximately 14 kDa in size

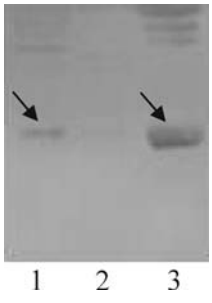

Fig. 6 Reaction of anti-Pin-b antiserum against proteins extracted with propan-2-ol from starch granules and fractionated by SDS-PAGE. *Lane 1* Glenman, *lane 2* durum wheat cv. Langdon, *lane 3* Langdon 5D(5B). *Arrows* indicate a polypeptide of approximately 14 kDa in size

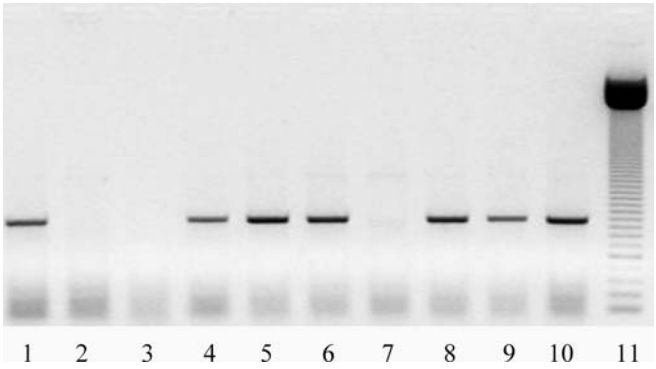

Fig. 7 PCR amplification products obtained with Pin-a specific primers (Gautier et al. 1994). *Lane 1* cv. Chinese Spring, *lane 2* cv. Barra, *lane 3* cv. Eridano, *lane 4* cv. Glenman, *lane 5* cv. Fortuna, *lane 6* Langdon 5D(5B), *lane 7* cv. Amidon, *lane 8* cv. Centauro, *lane 9* cv. Leone, *lane 10* cv. Leopardo, *lane 11* DNA size markers (50 bp ladder)

wild-type *Pina-D1a* allele was found in the 857 bp region upstream from the ATG codon. On the contrary, sequencing of the PCR amplification product obtained from Fortuna and Glenman with the terminal primers specific to the coding region of *Pina-D1a* revealed a single point mutation at position 267. This mutation consisted of deletion of a single cytosine, which resulted in a TGA stop codon at position 361 because of a shift in the reading frame (Fig. 9a). The putative product of this mutated gene is 10 kDa in size and 28 amino acids shorter than wild-type Pin-a. Because of the sequence difference in the C-terminal domain with respect to Pin-a (Fig. 9b), the calculated isoelectric point (9.1) of the mutated protein is remarkably lower than that (10.5) of puroindoline a (Gautier et al. 1994).

The sense-strand primer specific for the wild-type allele *Pina-D1a* (Gautier et al. 1994) and the antisense strand primer 5′-ATTGCGGTGGCATTTGCC-3′ specific for the mutated *Pina-D1* allele in cultivars Fortuna and Glenman were used in a PCR test performed on genomic DNAs from the cultivars listed in Table 1. A single 279-bp size-specific product was obtained from cultivars Fortuna and Glenman (Fig. 10, lanes 8–11), whereas the remaining cultivars gave several fragments of larger sizes (Fig. 10, lanes 1–5 and 7). These fragments were not specific to chromosome 5D, as demonstrated by their presence in the aneuploid line CS Nulli5D/Tetra5B (Fig. 10, lane 6).

Molecular characterization of Pinb-D1 in bread wheat cultivars lacking Pin-a

All null Pin-a cultivars analyzed here gave PCR products of the expected size when amplified with the Pin-b specific primers developed by Gautier et al. (1994) (data not shown). Moreover, PCR amplification of cultivars Fortuna and Glenman with the sense strand primer 5′-CACATGATTCTAAATAC-3′ and the antisense strand primer 5′-TCACCAGTAATAGCCACTAGGGAA-3′ specific for the promoter region of the wild-type *Pinb-D1a* allele produced a size-specific fragment. Finally, the Pin-b gene in cultivars Fortuna and Glenman showed a wild-type sequence after sequencing of the amplification products obtained with primers specific for the coding region of *Pinb-D1a*.

Discussion

Extraction of puroindolines with different solvents

Greenwell and Schofield (1986) were the first authors to observe that soft wheat cultivars possess a prominent 15 kDa band in their SDS-PAGE patterns of proteins extracted from starch granules with 1% SDS, this band being faint or very faint in hard bread wheat cultivars, and absent in durum wheat. The same unbroken positive association between endosperm softness and the intensity of Pin-b in the A-PAGE pattern of starch granule proteins isolated by propan-2-ol/NaCl was observed

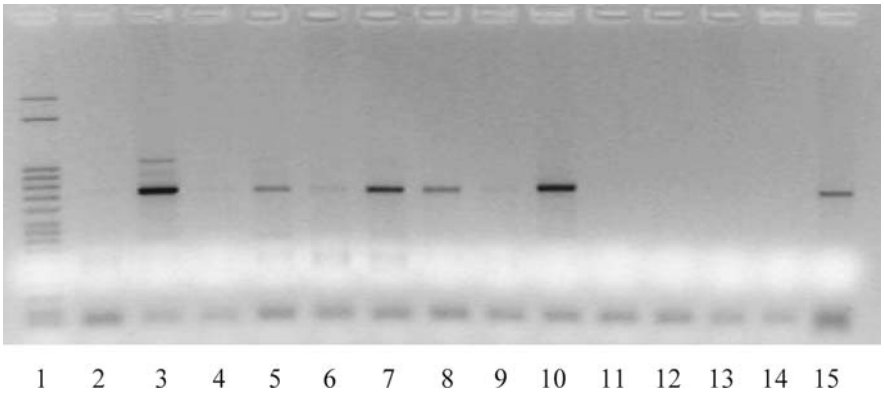

Fig. 8 PCR amplification products obtained with the sense strand primer prom-A1 specific to the promoter region of *Pina-D1a*. *Lane 1* DNA size marker (50 bp ladder), *lane 2* durum wheat cv. Langdon, *lane 3* cv. Chinese Spring, *lane 4* cv. Barra, *lane 5* cv. Marberg, *lane 6* cv. Newana, *lane 7* cv.Glenman, *lane 8* cv. Fortuna, *lane 9* cv. Mieti, *lane 10* Langdon 5D(5B), *lane 11* cv. Amidon, *lane 12* cv. Sibilla, *lane 13* cv. Eridano, *lane 14* cv. Golia, *lane 15* cv. Francia

A

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Fortuna & Glenman   ATGAAGGCCCTCTTCCTCATAGGACTGCTTGCTCTGGTAGCGAGCACCGCCTTTGCGCAA 60
Pina-D1a            ATGAAGGCCCTCTTCCTCATAGGACTGCTTGCTCTGGTAGCGAGCACCGCCTTTGCGCAA 60
                    ************************************************************

Fortuna & Glenman   TATAGCGAAGTTGTTGGCAGTTACGATGTTGCTGGCGGGGGTGGTGCTCAACAATGCCCT 120
Pina-D1a            TATAGCGAAGTTGTTGGCAGTTACGATGTTGCTGGCGGGGGTGGTGCTCAACAATGCCCT 120
                    ************************************************************

Fortuna & Glenman   GTAGAGACAAAGCTAAATTCATGCAGGAATTACCTGCTAGATCGATGCTCAACGATGAAG 180
Pina-D1a            GTAGAGACAAAGCTAAATTCATGCAGGAATTACCTGCTAGATCGATGCTCAACGATGAAG 180
                    ************************************************************

Fortuna & Glenman   GATTTCCCGGTCACCTGGCGTTGGTGGAAATGGTGGAAGGGAGGTTGTCAAGAGCTCCTT 240
Pina-D1a            GATTTCCCGGTCACCTGGCGTTGGTGGAAATGGTGGAAGGGAGGTTGTCAAGAGCTCCTT 240
                    ************************************************************

Fortuna & Glenman   GGGGAGTGTTGCAGTCGGCTCGGC-AAATGCCACCGCAATGCCGCTGCAACATCATCCAG 299
Pina-D1a            GGGGAGTGTTGCAGTCGGCTCGGCCAAATGCCACCGCAATGCCGCTGCAACATCATCCAG 300
                    ************************ ***********************************

Fortuna & Glenman   GGGTCAATCCAAGGCGATCTCGGTGGCATCTTCGGATTTCAGCGTGATCGGGCAAGCAAA 359
Pina-D1a            GGGTCAATCCAAGGCGATCTCGGTGGCATCTTCGGATTTCAGCGTGATCGGGCAAGCAAA 360
                    ************************************************************

Fortuna & Glenman   GTGATACAAGAAGCCAAGAACCTGCCGCCCAGGTGCAACCAGGGCCCTCCCTGCAACATC 419
Pina-D1a            GTGATACAAGAAGCCAAGAACCTGCCGCCCAGGTGCAACCAGGGCCCTCCCTGCAACATC 420
                    ************************************************************

Fortuna & Glenman   CCCGGCACTATTGGCTATTACTGGTGA 446
Pina-D1a            CCCGGCACTATTGGCTATTACTGGTGA 447
                    ***************************
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B

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Fortuna & Glenman   MKALFLIGLLALVASTAFAQYSEVVGSYDVAGGGGAQQCPVETKLNSCRNYLLDRCSTMK 60
Pin-a               MKALFLIGLLALVASTAFAQYSEVVGSYDVAGGGGAQQCPVETKLNSCRNYLLDRCSTMK 60

Fortuna & Glenman   DFPVTWRWWKWWKGGCQELLGECCSRLGK----CHRNAAATSSRGQSKAISVASSDFS-- 114
Pin-a               DFPVTWRWWKWWKGGCQELLGECCSRLGQMPPQCRCNIIQGSIQGDLGGIFGFQRDRASK 120

Fortuna & Glenman   VIGQAK---------------------- 120
Pin-a               VIQEAKNLPPRCNQGPPCNIPGTIGYYW 148
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Fig. 9 **a** Nucleotide sequence of the coding region of wild-type allele *Pina-D1a* as compared with that of novel *Pina-D1* allele present in cultivars Fortuna and Glenman. The cytosine lacking in cultivars Fortuna and Glenman is *arrowed*, whereas the new TGA stop codon is *underlined*. **b** Amino acid sequence of wild-type Pin-a compared with that of the protein encoded by the novel *Pina-D1* allele in cultivars Fortuna and Glenman

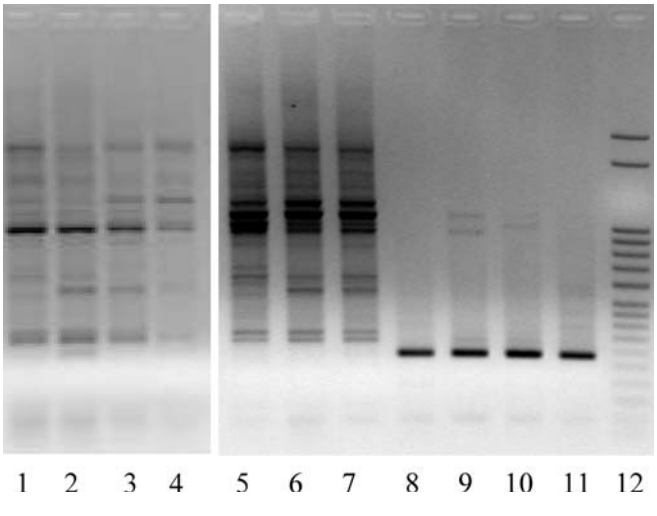

Fig. 10 PCR amplification products obtained with the antisense strand primer specific for the novel *Pina-D1* allele of cultivars Glenman and Fortuna. *Lane 1* cv. Eridano, *lane 2* cv. Falcon, *lane 3* cv. Golia, *lane 4* cv. Guadalupe, *lane 5* cv. Amidon, *lane 6* CS Nulli5D/Tetra5B, *lane 7* cv. Chinese Spring, *lanes 8 and 9* two single seeds of cv. Glenman, *lanes 10 and 11* two single seeds of cv.Fortuna, *lane 12* DNA size markers (50 bp ladder)

previously (Corona et al. 2001a, b; Pogna et al. 2002) and is confirmed here (Fig. 1). The finding that 1% SDS preferentially extracts Pin-b from starch granules as compared with Pin-a (Fig. 2, lane 2) suggests that the prominent 15 kDa band associated with grain texture described by Greenwell and Schofield (1986) corresponds to Pin-b.

On the contrary, sequential extraction of cracked kernels or starch granules with a Tris buffer containing 1% Triton X-114 (Giroux and Morris 1998) gave relatively high amounts of Pin-a with respect to Pin-b (Fig. 3, lane 2). In this context, it is noteworthy that the amount of Pin-a extracted with Triton X-114 from soft wheat cultivars by Turnbull et al. (2000, 2003) was at least twice the amount of Pin-b. Triton X-114 phase extraction has been widely used in biochemical and genetic studies of puroindolines, as well as in correlation studies between puroindoline composition and grain hardness or dough quality (Blochet et al. 1993; Dubreil et al. 1997, 1998; Giroux and Morris 1998; Giroux et al. 2000; Igrejas et al. 2001, 2002; Krishnamurthy and Giroux 2001; Lillemo et al. 2002; Branlard et al. 2003).

Characterization of bread wheat cultivars lacking Pin-a

With the exception of two genotypes, PCR studies have confirmed that hard wheat cultivars lacking Pin-a give no product when amplified with the terminal primers specific to the coding sequence of *Pina-D1a*. No amplification product was obtained with primers prom-A1 or prom-A2, which are specific to the promoter region of *Pina-D1a*, suggesting that major changes have occurred at the *Pina-D1* locus in these cultivars. In addition, the antiserum to Pin-a was not able to recognize any component in the 10–15 kDa region of the SDS-PAGE pattern of proteins extracted with Triton X-114. In contrast, the cultivars Fortuna and Glenman were rather peculiar in having a single-base deletion at position 267, downstream from the region coding for the tryptophan-rich domain of *Pina-D1a*. The occurrence of the same mutated allele, provisionally named *Pina-D1c*, in two genotypes can be accounted for by the fact that these cultivars are closely related genetically, the latter having cultivar Fortuna in its pedigree. The point mutation, unequivocally confirmed by PCR amplification with specific primers, should have a remarkable effect on the primary structure of the corresponding protein and on its interaction with starch granules. However, no protein with the expected mobility was observed in the A-PAGE or SDS-PAGE patterns of proteins extracted with either Triton X-114 or propan-2-ol/NaCl from either crushed kernels or starch granules. Furthermore, the antiserum to Pin-a was unable to recognize any component in the 10 kDa region of the SDS-PAGE patterns of cultivars Fortuna and Glenman. This was not unexpected since the anti-Pin-a antiserum was obtained by immunization of rabbits against a synthetic peptide located at positions 116–131 in the C-terminal region of Pin-a (Krishnamurthy and Giroux 2001). Furthermore, proteins extracted from cracked grains of cultivars Fortuna and Glenman showed very weak spot-blotting immunoreactions against the Durotest S antiserum. Therefore, no evidence has been obtained for the presence of a protein encoded by the novel *Pina-D1* allele in the endosperm of Fortuna and Glenman. *Pina-D1c* is the first null allele associated with a point mutation revealed in the Pin-a gene, the null alleles described so far being likely due to major alterations in both the promoter and the coding regions of *Pina-D1*.

In a previous study (Corona et al. 2001a), cultivars Fortuna and Glenman were assumed to possess allele *Pina-D1b* because of the absence of DNA fragments in their agarose gels after PCR amplification with the terminal primers specific to *Pina-D1a*. In the present study, about one-third of the 20 DNA samples extracted from single seeds of both cultivars did not give any size-specific product with those terminal primers (data not shown). However, all negative DNA samples exhibited the expected 1,303 bp fragments when amplified with primer prom-A2 specific to the promoter region of *Pina-D1a*. Moreover, nested PCR amplification of these fragments with the terminal primers specific to the coding region of *Pina-D1a* (Gautier et al. 1994) gave the expected size-specific products.

In common with all cultivars lacking Pin-a, cultivars Fortuna and Glenman showed very low amounts of Pin-b on the surface of starch granules, as demonstrated by A-PAGE fractionations (Fig. 1, lanes 3 and 4) and Western blot patterns with the anti-Pin-b antiserum (Fig. 6, lane 1). This result is consistent with a recent study showing reduced amounts of Pin-b in starch granules of developing kernels in a wheat cultivar lacking Pin-a as compared with its soft near-isogenic counterpart (Turnbull et al. 2003).

Using the anti-Pin-b antiserum, Krishnamurthy and Giroux (2001) did not obtain clear results in Western blots of SDS-PAGE fractionations of puroindolines isolated by Triton X-114 phase partitioning. However, this antiserum proved to be specific to Pin-b in ELISA experiments. The high amounts of Pin-b extracted by propan-2-ol/NaCl as compared to Triton X-114 could explain the positive results obtained here in Western blots with the anti-Pin-b antiserum.

The present results indicate that in the absence of Pin-a there are low levels of Pin-b on the starch granules of wheat endosperm, with significant effects on grain texture. This is consistent with previous results obtained by Corona et al. (2001a) and Capparelli et al. (2003). Adhesion of Pin-b to the surface of the starch granules is likely to be mediated by Pin-a, which seems to be tightly bound to membrane lipids, as demonstrated by its reduced extraction with 1% SDS and easy isolation with Triton X-114. Recently Hogg et al. (2004) provided evidence of interaction of puroindolines to form starch surface friabilin in transgenic wheat isolines. The presence of wild-type allele *Pinb-D1a* in null Pin-a cultivars raises the question of whether they synthesize Pin-b in amounts similar to those observed in soft wheat cultivars, and preferably allocate it in the endosperm protein matrix. This aspect is currently being investigated using the anti-Pin-b antiserum in near-isogenic lines with contrasting grain texture characteristics grown in replicated plots.

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