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ORIGINAL PAPER

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Puroindoline A-gene expression is involved in association of puroindolines to starch

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Abstract Puroindolines largely influence cereal grain hardness. In order to understand how they exert this influence, we carried out a molecular analysis of the *pina* and *pinb* genes of many Italian wheat cultivars. On the basis of their *pin* genotypes they could be divided into three groups: *Pina-D1a/Pinb-D1a*; *Pina-D1a/Pinb-D1b*; and *Pina-D1b/Pinb-D1a*. Five cultivars from each group were chosen to be studied to examine the quantity of puroindolines associated with starch (friabilin) and the amount not associated with starch. In addition, the level of *pina* expression was measured using RT-PCR. Soft cultivars (*Pina-D1a/Pinb-D1a*) exhibited the highest level of expression of *pina*; among the hard cultivars, those with the *Pina-D1a/Pinb-D1b* genotype showed a lower level of expression, while those with the *Pina-D1b/Pinb-D1a* genotype did not express *pina*. Total puroindoline and friabilin content was then measured by flow cytometry. Soft *Pina-D1a/Pinb-D1a* cultivars displayed high puroindoline content that was primarily starch associated. Hard *Pina-D1b/Pinb-D1a* cultivars had very low puroindoline content with no puroindoline bound to starch. Hard *Pina-D1a/Pinb-D1b* cultivars were highly heterogeneous with respect to both the content of puroindolines and the level of association with starch. The accurate quantification of puroindolines in starch-bound and not starch-bound forms in association with molecular analysis, indicates that *pina* expression and presence controls the abundance of total puroindoline and its association with starch.

Keywords Starch · Friabilin · *pina* · *pinb* · Flow cytometry · Kernel hardness

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Introduction

Wheat (*Triticum aestivum* L.) endosperm-texture influences the technological characteristics of wheat grain, particularly milling properties (Morris and Rose 1996). This trait is controlled by a single locus, called *Hardness* (*Ha*), located on the short arm of chromosome 5D (Symes 1965; Baker 1977). On the basis of endosperm texture, wheat cultivars are classified as hard or soft: the former are mainly used to make bread; the latter cookies, cakes and pastries (reviewed in Morris and Rose 1996).

The first indication of the difference between hard and soft wheats came with the discovery of friabilin. Friabilin, a starch granule protein associated with wheat endosperm softness, was reported first by Greenwell and Schofield (1986). Friabilin was defined as a 13,000 kDa protein(s) (Blochet et al. 1993; Gautier et al. 1994) present in much greater abundance on the surface of water-washed starch of soft-wheats than hards. Friabilin was thought to simply be a marker of grain hardness (Morris et al 1994; Bettge et al. 1995). N-terminal amino-acid sequencing demonstrated that friabilin is a mixture of two proteins (Greenwell 1992; Jolly et al. 1993; Gautier et al. 1994; Morris et al. 1994; Oda and Schofield 1997; Morris 2002). The two proteins are named puroindoline a (PINA) and puroindoline b (PINB) in view of the presence of a hydrophobic tryptophan-rich domain with apparent affinity for lipids (Gautier et al. 1994). Indeed, Greenblatt et al. (1995) have demonstrated that the interaction of PINA and PINB with starch is mediated by the residual polar lipids present at the surface of purified starch granules.

According to Oda and Schofield (1997), soft and hard wheats contain approximately the same quantity of total friabilin, but differ in the amount of friabilin associated with starch, which is high in soft wheats and low in hard wheats. On the basis of these results, they propose that kernel texture is determined exclusively by the fraction of friabilin present on starch granules.

The *pina* and *pinb* genes are located on chromosome 5D and the occurrence of friabilin and grain hardness are linked (Sourdille et al 1996; Giroux and Morris 1997). Recently, experiments with transgenic rice (Krishnamurthy and Giroux 2001) have directly demonstrated that puroindolines influence cereal grain texture. How this influence is exerted remains still poorly understood.

Giroux and Morris (1997) found that American cultivars of hard wheat commonly contain a glycine-to-serine change in the tryptophan-rich domain of PINB (*pinb-D1b* allele). The same mutation was absent in the soft cultivars examined which had the *pinb-D1a* allele. According to the authors, the decrease in hydrophobicity subsequent to the glycine-serine change lessens the strength of lipid binding. In a following study (Giroux and Morris 1998), the same authors found that American hard cultivars exhibit either the pinB-D1b allele or the absence of the *pina* protein (*pina-D1b*). On the basis of these studies, the authors concluded that grain hardness is the result of an alteration of either the *pina* or *pinb* gene.

In this study, we measured the quantity of puroindolines present in Italian soft- and hard-wheat cultivars and determined the amount of puroindolines starch, associated (friabilin) and not associated. To measure puroindoline content, we exploited flow cytometry with a specific monoclonal antibody able to recognize both PINA and PINB. The data presented here indicates that this technique can measure friabilin much more accurately than the standard methods of Western blotting and SDS-electrophoresis used in previous studies (Jolly et al. 1993; Bettge et al. 1995). Flow cytometry in its ordinary version cannot be applied to the dosage of soluble molecules. To overcome this limitation, in the present study puroindolines were extracted from meal, adsorbed on latex particles, and finally measured using specific antibodies and flow cytometry.

Materials and methods

Biological materials

The cultivars of *T. aestivum* L. (Golia; Manital; Sibilla; Barra; Guadalupe; Francia; Colfiorito; Pandas; Serio; Mieti; Bilancia; Centauro; Stroika and Lampo; Ariete) and *Triticum durum* Desf. (Ciccio) included in this study, were kindly provided by the Consorzio Nazionale Sementi, (Ravenna, Italy) and the Institute of Agronomy (University of Naples, Italy), respectively.

DNA was extracted from fresh leaves and RNA from developing seeds collected 13 days after flowering. Ears were immediately frozen in liquid nitrogen and stored at −70°C until used.

Total kernel protein-extraction

Kernels were ground and proteins extracted from 100 mg of meal using the Rhône Diagnostics Duro Test Kit (Glasgow, UK). The extract was diluted 10^{-1} and tested.

Friabilin purification

Friabilin was purified using a solid immuno-adsorbent prepared by covalently coupling the friabilin specific monoclonal antibody (MnαF) (Rhône diagnostics, Glasgow, UK) to amino-polystyrene particles (Polyscience, Warrington, Pa., USA) as described by the manufacturer.

The amino beads with the covalently bounded antibody were mixed end to end overnight at room temperature with 1 ml of meal extract, and then washed with PBS to remove excess proteins. To elute the protein, the microspheres were incubated three times with 0.1 M glycine at pH 3 for five min and the supernatant was recovered after centrifugation. The supernatants, containing friabilin, were then pooled and used for protein quantification (Biorad assay).

Extraction of proteins associated and non-associated with starch

Single kernels were crushed lightly and incubated for 30 min in 0.5 ml of 0.1 M NaCl, with occasional stirring (Bettge et al. 1995). The kernels were then lightly ground for a few min with a pestle forming a small dough-ball of gluten. Bran, germ and formed gluten were pushed to the bottom of the tube whil the top layer, containing starch, was quickly pipetted into a fresh tube. The extraction of starch with 0.1 M NaCl was repeated 3-times for each kernel to recover as much starch as possible.

All starch obtained was then centrifuged and the supernatant and pellet were treated separately. The former, containing all the proteins not associated with starch was ready to be tested by flow cytometry. The latter was treated as described by Bettge et al. (1995) in order to extract all the proteins associated with the starch granules. The dried starch surface proteins were then solubilized in 100 μl of PBS.

Bran, germ and gluten previously obtained were treated with 400 μl of extraction buffer (Rhône diagnostics, Glasgow, UK) and incubated for 30 min under end-to-end mixing. This incubation step was followed by centrifugation and the resulting supernatant was then analyzed by flow cytometry (to examine residual friabilin in bran, germ and gluten).

Cytometric assay

Latex particles of various diameter (Polyscience, Warrington, Pa., USA) were incubated (3 h at room temperature) under agitation with 1 ml of the sample previously diluted 10^{-1} in 0.1 M borate buffer, using tubes pre-treated with 2% (v/v) milk diluent/blocking solution and 20 × concentrate (Kirkegaard and Perry laboratories, Gaithersburg, Md., USA). The mixture was centrifuged and the pellet incubated for 30 min with 2% (v/v) milk. After washing with PBS, latex particles were incubated, in the following order, with 100 μl of ten-fold dilutions of Mouse Monocolonal antibody anti-friabilin (MnαF) and 100 μl of ten-fold dilutions of goat anti-mouse antiserum labelled with fluorescein (GαM^{FITC}) (Sigma, St. Louis, Mo., USA). Particles were washed twice and analysed with the flow cytometer (FacScan Becton Dickinson Immunocytometry Systems, San Josè, Calif., USA). The data of 3,000 events were collected for each sample and the results were presented as the mean channel (MC) of fluorescence of the treated sample subtracted by the mean channel of the control (sample incubated with PBS instead of MnαF).

DNA isolation

DNA was isolated from fresh tissue (700 mg of leaves) as described (Doyle and Doyle 1990), with minor modifications [the isolation buffer contained 1% polyvinylpyrrolidone; an additional extraction step with phenol-chloroform-isoamyl alcohol (25:24:1) preceded ethanol and ammonium-acetate precipitation]. To avoid extraction buffer interference, DNA concentration was measured running scalar dilutions of DNA on 0.7% (w/v) agarose gel stained with ethidium bromide. The least visible band was assumed to contain 2 ng of DNA (Sambrook et al. 1989).

Allele-specific PCR amplification

Pinb DNA sequences were amplified with the Gene Amp 5700 sequence-detection system (Applied Biosystems, Foster City, Calif., USA). The sequence of the sense-strand primer was: *5'ATGAAGACCTTATTCCTCCTA3'* (Gautier et al. 1994); and of the antisense-strand primer specific for the Gly-46: *5'CCTCAT-GCTCACAGCCGCC3'*; of the antisense-strand primer specific for the Ser-46: *5'CCTCATGCTCACAGCCGCT3'* . The reaction was carried out in a 25-μl volume containing 50 ng of genomic DNA, 1 × *Taq* DNA amplification buffer (Promega, Madison, Wis., USA), 5 pmol of each primer, 2.5 mM of $MgCl_2$, 10 pmol of each dNTP and 1 unit of *Taq* Polymerase (Promega). The thermal profile included a first step of denaturation at 94°C for 3 min, followed by 25 cycles, each one lasting 5 s at 94°C and 30 s at 60.5°C (Blaiotta, personal communication). The PCR products were then analysed on 1.5% (w/v) agarose gel stained with ethidium bromide.

RNA isolation

Developing seeds (700 mg) were ground in liquid nitrogen and the RNA was isolated as described (Chomczynski and Sacchi 1987).

RT-PCR analysis

Pina was amplified by reverse-transcription PCR from 1 μg of RNA using specific primers. The *pina* 5' primer was: *5'CAACAT-GAAGGCCCTCTTCCT3'*; the 3' primer was the reverse complement of: *5'CAATATAGCGAAGTTGTTGGCAGT3'* (Gautier et al. 1994). The cDNA synthesis was carried out using the RT-PCR kit (Promega), leaving out the annealing step and shortening the extension step to 30 min at 44°C. The cDNA (1 μl) was amplified (30 cycles; annealing temperature: 54°C) and analysed on a 2% (w/v) agarose gel stained with ethidium bromide.

Level of *pina* transcript expression

The cDNA (1 μl) of the cultivars was amplified (annealing temperature: 54°C) in five tubes and the reaction stopped after 15, 17, 20, 23 or 25 cycles. The presence of the transcript was detected on 2% (w/v) agarose gel stained with ethidium bromide.

Results

Molecular analysis

Allele-specific PCR showed that soft cultivars all have the *Pinb-D1a* allele, while the hard cultivars have either the *Pinb-D1a* or the *Pinb-D1b* allele (Fig. 1).

RT-PCR established that all soft cultivars express the *pina* transcript; the hard cultivars instead express the *pina* transcript only in association with the *Pinb-D1b* allele

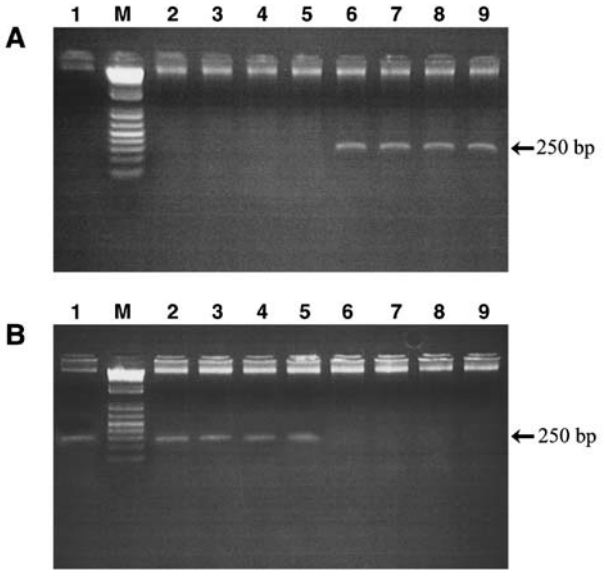

Fig. 1A, B Allele specific PCR of nine hard wheat cultivars amplified in the presence of the Gly-46 (**A**) or the Ser-46 (**B**) specific primer. M = 1-kb Plus DNA ladder

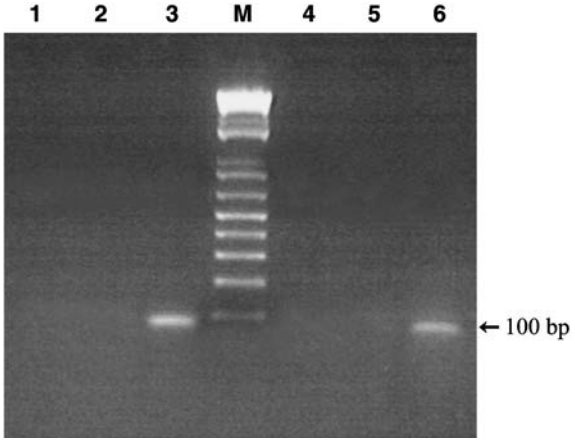

Fig. 2 Presence of the *pinA* transcript detected by RT-PCR in six hard wheat cultivars. Cultivars 1, 2, 4 and 5 have the *Pinb-D1a* allele; cultivars 3 and 6 the *Pinb-D1b* allele. M = 1-kb Plus DNA ladder

(Fig. 2). The analysis identified three groups of wheat: the soft cultivars, uniformly *Pina–D1a/Pinb-D1a*; the hard ones, either *Pina-D1a/Pinb-D1b* or *Pina-D1b/Pinb-D1a* (Table 1). Thus, the Italian cultivars examined express the *Pina-D1a* allele in association with the *Pinb-D1a* allele if soft and with the *Pinb-D1b* allele if hard, as is known to occur in the North American cultivars (Giroux and Morris 1998).

Table 1 Correlation between the percentage of starch-associated friabilin and the level of expression of the *PinA* transcript

Class	Cultivar	% Starch friabilin	Threshold cycle
Pina-D1b	1 Golia	nd[a]	–
	2 Manital	nd	–
Pinb-D1a	3 Sibilla	nd	–
	4 Barra	nd	–
	5 Guadalupe	nd	–
Pina-D1a	6 Francia	68	20
	7 Colfiorito	11	25
Pinb-D1b	8 Pandas	35	23
	9 Serio	60	20
	10 Mieti	55	20
Pina-D1a	11 Bilancia	87.5	17
	12 Centauro	89	17
Pinb-D1a	13 Stroika	90.5	17
	14 Lampo	90.6	17
	15 Ariete	92	17

[a] Not detectable

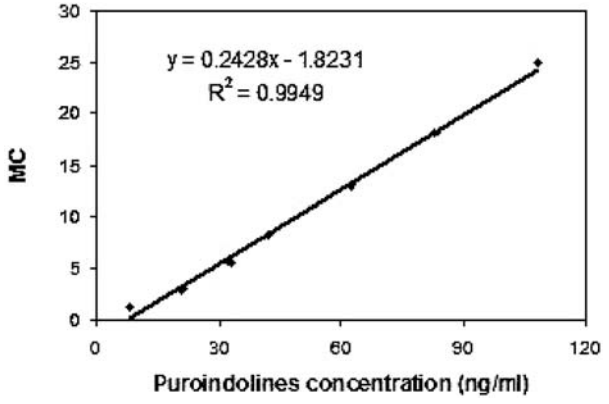

Fig. 3 Dosage of puroindolines by flow cytometry. Detection limit of the assay: 8 ng (MC of the 8-ng standard = 1.2. MC of the control = 0.21). Each point of the curve represents the average of three replicas. The intra-assay and inter-assay coefficients of variation were 0.2 and 0.4, respectively

Flow cytometric studies

The technique chosen to measure both total puroindolines and friabilin (PINA and PINB starch-bounded) was flow-cytometry.

Preliminary experiments were carried out to establish the optimal conditions for detection puroindolines. These conditions were: 4×10^4 latex particles with a diameter of 10 microns per tube incubated sequentially with 100 μl of meal extract diluted 10^{-1}, 100 μl of MnαF diluted 10^{-2}, and 100 μl of GαM^{FITC} diluted 2×10^{-3}.

Under these conditions, the sensitivity of the assay was 8 ng ml^{-1} (minimum amount of detectable puroindolines). This detection limit [mean channel (MC) = 1.2; n=10] represents approximately six-fold the value of the control tube (MC 0.21; n=10) (Fig. 3). The inter- and intra-assay coefficient of variation was respectively 0.2 and 0.4. Five samples of *T. durum* (which lacks the 5D chromosome)

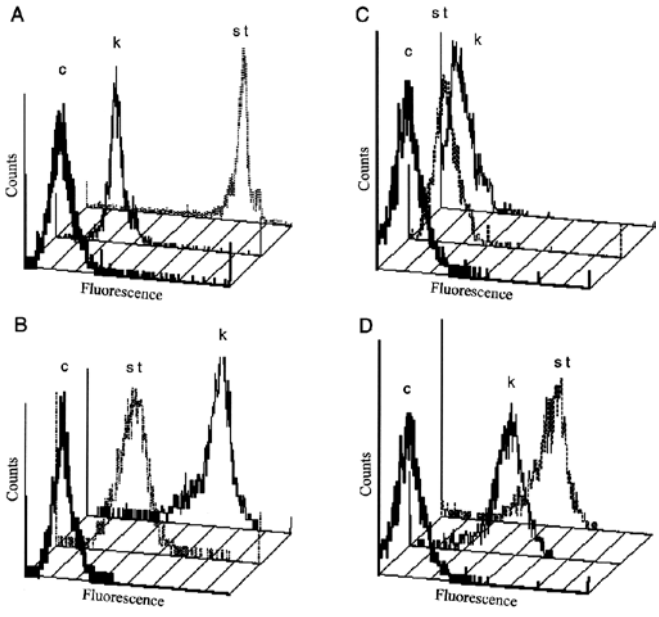

Fig. 4A–D Flow cytometric profile of four wheat cultivars. **A:** *Pina-D1a/ Pinb-D1a*. **B:** *Pina-D1a/Pinb-D1b*. **C:** *Pina-D1b/Pinb-D1a*. **D:** *Pina-D1a/Pinb-D1b*. Note the heterogeneity displayed by the *Pina-D1a/Pinb-D1b* cultivars. *c* control; *k* kernel; *st* starch

were tested under the conditions described above and their mean channels were the same as that of the control. Immunoblotting analysis performed on a puroindoline-a null wheat and a puroindoline-b null wheat showed that the monoclonal antibody used for the citofluorimetric assay was able to recognize both the puroindolines equally (data not shown). Both these results demonstrate that the assay is specific for puroindolines. The assay was used to measure, on single kernels, the total puroindolines and the fractions of puroindolines associated with starch granules (friabilin) and not associated with starch granules. The samples containing the proteins coming from bran and gluten extraction were tested, but only traces of friabilin were found in them (data not shown). These measurements were carried out on five cultivars of each of the three classes of grains established by molecular analysis. The soft cultivars displayed high puroindoline content, mainly associated with starch granules (Fig. 4A and Fig. 5). The *Pina-D1a/Pinb-D1b* hard cultivars were highly heterogeneous with respect to both puroindoline content and its association with starch. In particular, the cultivars Colfiorito and Pandas displayed high puroindoline content which was primarily not starch associated. The cultivars Francia and Serio instead showed a lower level of puroindolines, mainly associated with starch. The cultivar Mieti was characterized by an intermediate content of puroindolines, half of which associated with starch (Fig. 4B–D and Fig. 5). The hard cultivars *Pina-D1b/Pinb-D1a* exhibited a very low total puroindoline content, and their peaks almost overlapped with that of the negative control (Fig. 5).

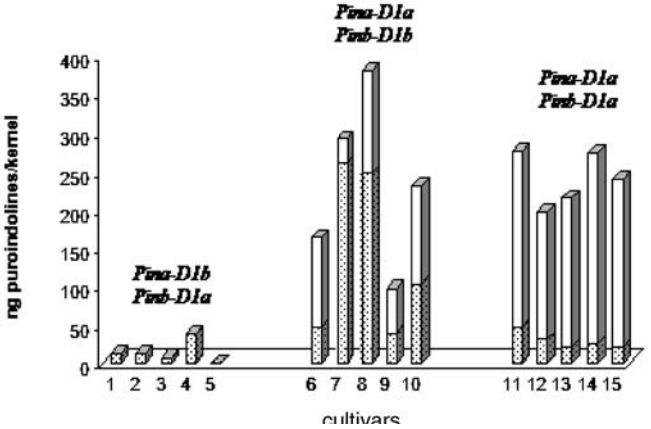

Fig. 5 Starch-association of puroindolines in kernel of 15 different wheat cultivars: (❑) starch associated and (■) not starch associated. (*1*=Golia; *2*=Manital; *3*=Sibilla; *4*=Barra; *5*=Guadalupe; *6*=Francia; *7*=Colfiorito; *8*=Pandas; *9*=Serio; *10*=Mieti; *11*=Bilancia; *12*=Centauro; *13*=Stroika; *14*=Lampo; *15*=Ariete)

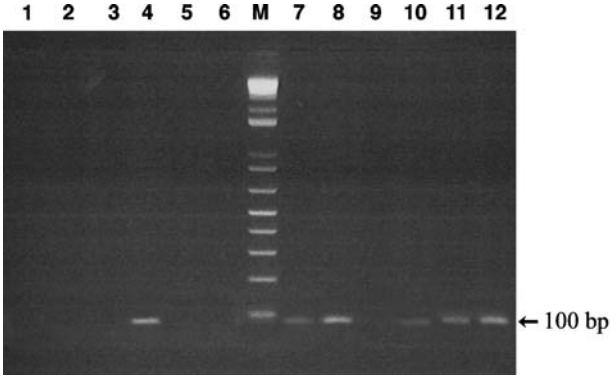

Fig. 6 Different levels of expression of the *pinA* gene in hard wheats. *Lanes* 1–4: *pinA* transcript expressed after 25 amplification cycles. *Lanes* 5–8: *pinA* transcript expressed after 23 amplification cycles. *Lanes* 9–12: *pinA* transcript expressed after 20 amplification cycles. M = 1-kb Plus DNA ladder

Level of expression of the *pina* gene

In order to investigate the role of *pina* in determining the hardness of the kernels, we compared the *pina* gene-expression level of hard (both *Pina-D1b/Pinb-D1a* and *Pina-D1a/Pinb-D1b*) and soft (*Pina-D1a/Pinb-D1a*) cultivars. For this purpose, in the course of RT-PCR, the cDNA amplification step was interrupted after 15, 17, 20, 23 or 25 cycles (Fig. 6).

In the soft cultivars, *pina* transcript was detectable after 17 cycles. In the *Pina-D1a/Pinb-D1b* hard cultivars 20 cycles or more were required until the *pina* transcript was detectable, while in the *Pina-D1b/Pinb-D1a* hard wheats, as expected, no *pina* transcript could be detected even after 25 cycles (Table 1 and Fig. 6).

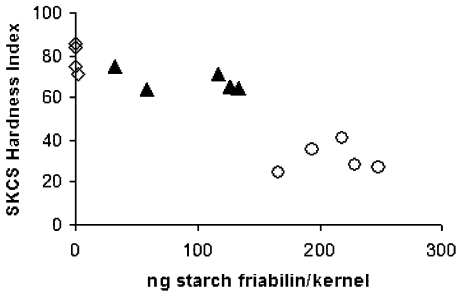

Fig. 7 The quantity of friabilin associated with starch can predict kernel hardness similar to the SKCS method. In addition, it can distinguish between *Pina-D1b/Pinb-D1a* (◇) and *Pina-D1a/Pinb-D1b* (▲) hard wheats. ◯ = soft *Pina-D1a/Pinb-D1a*

The dosage of starch-surface friabilin can measure kernel hardness

We compared the starch friabilin content of the samples included in this study with their hardness values. These values, measured by the single-kernel characterization system (SKCS), were taken from the literature (Ferraresi et al. 1997; Gazza et al. 2001). The results are shown in Fig. 7. They indicate that starch friabilin content is a valid parameter of the hardness.

Discussion

According to several studies (Jolly et al. 1993; Bettge et al. 1995) the puroindoline content of soft- and hard-wheat cultivars is approximately the same. However, in the course of this study we found that the majority of soft wheats contain much more total friabilin than the hard ones. Also, while the total puroindoline content of soft wheats does not vary very much, that of the hard wheats can vary from almost zero to a level sometimes higher than that observed in the soft wheats (Fig. 5). The contradiction in the results may reflect a characteristic of the Italian samples or the small number of cultivars examined in previous stuies (Jolly et al. 1993; Bettge et al. 1995). Another possible explanation for the discrepancy may reside in the technique adopted in this study. Our preliminary experiments in fact displayed that flow cytometry can measure friabilin more accurately than Western blotting and SDS-PAGE, the techniques used by Jolly et al. (1993) and Bettge et al. (1995), respectively.

It has been proposed (Gautier et al. 1994; Oda and Schofield 1997) that the association of friabilin with starch is an artefact caused by the breakdown of the cellular structures of the seed during the milling process. We have detected friabilin associated with the starch, even after gentle crushing of single kernels, a procedure unable to destroy seed structure as the milling process does. Therefore, we believe that the association of friabilin with starch is a real phenomenon, rather than an artefact, primarily due to ionic and hydrophobic interactions with the starch granule surface as suggested

by Greenblatt et al. (1995). Giroux and Morris (1998) have suggested that starch friabilin is the factor determining the hardness of the kernel; more exactly, that the higher is the quantity of starch friabilin, the lower is the hardness of the sample. To address this question directly, we plotted the starch friabilin content of wheat samples against their hardness values. The results (Fig. 7) demonstrate that starch friabilin content, as measured by flow cytometry, is a valid parameter of kernel hardness; indeed it is more discriminating than the standard method since it allows the ability to distinguish the two classes of hard wheats identified by molecular techniques.

In the course of this study we found also that the association of friabilin with starch is regulated by the level of expression of the *pina* transcript. There is a positive correlation between the number of cycles needed to detect the transcript (i.e. the abundance of the *pina* transcript) and the quantity of friabilin associated with starch. The evidence that in the absence of PINA (Fig. 5), all the puroindolines is not starch-associated, which supports this role for *pina*. It would be of utmost interest to investigate also the influence of *pinb* presence – absence and the level of expression on friabilin association with starch, but unfortunately no Italian PINB null cultivar was available for this purpose.

In conclusion, this study confirms the importance of friabilin associated with starch in determining the hardness of wheat kernels. The data also indicates that *pina* influences the abundance of friabilin since much-less than half of the amount of friabilin was found in cultivars lacking PINA versus that present in soft wheats. It seems possible that both puroindolines are required for full friabilin abundance and grain softness, and that fully functional friabilin requires both PINA and PINB and not simply total puroindoline content. Previous studies examining grain hardness and puroindoline have described mechanisms based on nucleotide alterations (Gly-46 versus Ser-46 mutation of PINB) or presence versus absence of a gene transcript (*pina*). The present paper displays a new means of control, where the transcript is present, but expressed at different levels. This new way to modulate gene expression whereby *pina* is reduced in expression in *pinB-D1b* varieties has been identified following an accurate analysis of the quantity of puroindolines associated and non-associated with the starch, made possible by flow cytometry.

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