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Expression of wild-type *pinB* sequence in transgenic wheat complements a hard phenotype

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Abstract Wheat grain hardness is a major factor in the wheat end-product quality. Grain hardness in wheat affects such parameters as milling yield, starch damage and baking properties. A single locus determines whether wheat is hard or soft textured. This locus, termed *Hardness* (*Ha*), resides on the short arm of chromosome 5D. Sequence alterations in the tryptophan-rich proteins puroindoline a and b (PINA and PINB) are inseparably linked to hard textured grain, but their role in endosperm texture has been controversial. Here, we show that the *pinB-D1b* alteration, common in hard textured wheats, can be complemented by the expression of wild-type *pinB-D1a* in transformed plants. Transgenic wheat seeds expressing wild-type *pinB* were soft in phenotype, having greatly increased friabilin levels, and greatly decreased kernel hardness and damaged starch. These results indicate that the *pinB-D1b* alteration is most likely the causative *Ha* mutation in the majority of hard wheats.

Keywords Wheat · Endosperm texture · Grain hardness · *Puroindolines*

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Introduction

Among members of the Triticeae, endosperm texture is a grain trait that varies considerably among individual cultivars. The importance of endosperm texture differences is well established in wheat (*Triticum aestivum L.*), where grain hardness is one of the primary determinants of end product quality. Soft wheats have softer endosperm texture, require less energy to mill, and yield smaller particles with less starch damage upon milling than do hard wheats (Symes 1965; MacRitchie 1980). The flours of the two types behave differently as well; soft wheats make superior cakes, while hard wheats make superior breads (reviewed in Morris and Rose 1996).

The genetic basis of endosperm texture in wheat has been described (Symes 1965; Baker 1977). One major locus called *Hardness* (*Ha*) controls most of the variability of kernel hardness in this species. Wheat grain hardness is simply inherited, with the soft phenotype (*Ha*) dominant to the hard phenotype (*ha*). The *Ha* locus resides on the short arm of chromosome 5D in wheat (Mattern et al. 1973; Law et al. 1978). Three structurally related genes have been identified that are closely linked to *Ha* (Rahman et al. 1994; Sourdille et al. 1996; Giroux and Morris 1998). They are *puroindoline a* (*pinA*), *puroindoline b* (*pinB*) and *Gsp-1a*. Puroindolines contain a tryptophan-rich region that appears to be involved in lipid binding (Kooijman et al. 1997). The first evidence that puroindolines may be involved in grain hardness came in a publication by Greenwell and Schofield (1986). They reported a protein, termed friabilin, which is found in larger amounts on soft wheat starch than that of hard wheat, and is absent in durum wheat. Friabilin is controlled by chromosome 5D (Jolly et al. 1993), suggesting a direct relationship between the component(s) of the marker protein friabilin and the *Ha* locus. Subsequent N-terminal sequencing of this marker protein indicated the presence of two proteins (Oda and Schofield 1997). Comparison of the N-terminal sequences with that of the lipid binding proteins PINA and PINB made it

clear that friabilin N-terminal sequences (Jolly et al. 1993; Morris et al. 1994) consist primarily of a 1:1 molar ratio of PINA and PINB. All hard wheats characterized to-date have a sequence alteration in either *pinA* or *pinB* relative to soft wheats (Giroux and Morris 1998). The most common mutations observed are either a null mutation for *pinA*, or point mutations in the coding sequence of *pinB* (Giroux and Morris 1998; Lillemo and Morris 2000). All soft wheats characterized to-date have identical *pinA* and *pinB* sequences (Giroux and Morris 1997, 1998; Lillemo and Morris 2000). The cultivar 'Hi-Line' used here contains the glycine to serine change in *pinB* that is present in the majority of US hard wheat varieties (Morris et al. 2001). This glycine to serine change occurs in a conserved region in close proximity to the tryptophan-rich region, which could potentially affect its lipid-binding characteristics (Giroux and Morris 1997).

It has been suggested that puroindolines are responsible for wheat grain softness (Giroux and Morris 1998). However this hypothesis is in dispute, and other *Ha*-linked genes such as *Gsp-1a* have been put forward as controlling endosperm texture (Jolly et al. 1993; Rahman et al. 1994; Turnbull et al. 2000). Some of the confusion perhaps results from the fact that friabilin itself is composed of other components in addition to the puroindolines, such as GSP-1 and an alpha amylase inhibitor (Jolly et al. 1993; Morris et al. 1994). In short, while the alterations in PINA and PINB are intriguing, a direct cause and effect relationship has not been demonstrated for *puroindolines* and grain hardness. The puroindoline genes may simply be linked to the true *Ha*-locus functional genes. Further, the common glycine to serine *pinB* sequence alteration found in most hard textured wheats (Morris et al. 2001) may simply reflect a tight genetic linkage between *pinB* and *hardness*. We have carried out a complementation test of this putative *pinB Ha* locus mutation to address this question. The experiments involved transforming a hard wheat variety, which has the glycine to serine *pinB* sequence alteration, with the *pinB* sequence found in soft wheats. Successful complementation and restoration of grain softness demonstrated that *pinB* is a functional part of the *Ha* locus.

Materials and methods

Plant material and growth conditions

The wheat cultivars 'Hi-Line' and 'Chinese Spring' were used in this study. Hi-Line (Lanning et al. 1992) is a hard wheat variety containing the glycine to serine *pinB* variant sequence *pinB-D1b* and the "soft type" *pinA* sequence *pinA-D1a* (Giroux et al. 2000). Chinese Spring is a soft wheat which contains the *pinB-D1a* sequence found in all soft wheats so far examined and *pinA-D1a* (Giroux and Morris 1997). Plants were grown in 8-inch diameter pots in a greenhouse in the Montana State University-Bozeman Plant Growth Center. Two plants were grown per pot, and replicates consisted of two pots (four plants). Greenhouse conditions consisted of target temperatures of 22 °C and 14 °C for day and night respectively, and with supplemental lighting providing 400 μE m^{-2} s^{-1} consisting of 1,000 W metal halide lamps on from 5 a.m. until 9 a.m. and from 4 p.m. until 9 p.m. Plants were watered as needed with 0.25 g of Peters 20–20–20 General Purpose NPK plant food per liter of water.

Plasmid constructs for wheat transformation

The PINB expression construct, pGB4.20, was created in our laboratory using untranslated glutenin gene flanking sequences from pGlu10H5 (Blechl and Anderson 1996). The primers BBH5 (5′CGGGATCCCCATGAAGACCTTATTCCTCCTAGC3′) and BXP3 (5′ AACTGCAGTCTAGATCATCACCAGTAATAGCCACT3′) were used to amplify the *pinB-D1a* sequence from a 'Chinese Spring' genomic DNA preparation using *Taq* DNA polymerase (Promega) (Riede and Anderson 1996). The temperature regimen consisted of a 3-min initial denaturation step at 94 °C, followed by 40 cycles of 94 °C for 45 s, 55 °C for 30 s, 72 °C for 90 s, followed by a 5-min final extension at 72 °C. The amplified product was digested with *Pst*I and ligated to the Dy10 promoter and the Dx5 3′ downstream sequences of pGlu10H5 (Blechl and Anderson 1996). The result was the complete replacement of the glutenin coding sequence with that of *pinB*, along with the addition of the *Bam*HI and *Pst*I sequences. The entire upstream and downstream glutenin sequences were preserved in this expression sequence, which was placed in a modified pET28a (Invitrogen) backbone. Construct pRQ101A was used for the selection of transformed plant lines. It contains the *bar* gene (De Block et al. 1987) under the control of the cauliflower mosaic virus 35S (CaMV 35S) promoter and the nopaline synthase (NOS) terminator. The *bar* gene confers resistance to the herbicides bialaphos (Meiji Seika Kaisha Ltd, Japan) and glufosinate (AgrEvo USA Company, Wilmington, Del.).

Wheat transformation and regeneration

The approach used for the production of fertile transgenic wheat plants was adapted from that described by Weeks et al. (1993) and by Altpeter et al. (1996). Immature embryos approximately 0.5–1.5 mm in length were isolated from greenhouse-grown Hi-Line seeds and placed on S1 callus induction medium [4.32 g of MS Basal Medium (Sigma Chemical, St. Louis, USA), 150 mg of L-asparagine, 40 mg of thiamine, 20 g of maltose, 2 mg of 2,4-D, and 2.5 g of phytagel per liter, pH 5.7–5.8] for 5–8 days in the dark at 25 °C. The resulting calli were then moved to S1 medium supplemented with 0.4-M sorbitol 4 h before bombardment. Constructs pGB4.20 and pRQ101A were precipitated on 1-μm-diameter gold particles in a 5:1 molar ratio using a standard protocol (Bio-Rad). Calli were bombarded twice in a Biolistic PDS-1,000/He Particle Delivery System (Bio-Rad, Hercules, Calif.) using 1,550 psi rupture disks and a 6 cm target distance. After approximately 17 h, the calli were moved to a selection medium consisting of S1 medium supplemented with an additional 20 g/l of maltose and 5 mg/l of bialaphos and maintained for 3 weeks in the dark at 25 °C. Calli were then placed on regeneration medium. Regeneration medium was identical to the selection medium except for the replacement of the 2,4-D with 1 mg/l of kinetin and 0.5 mg/l of IAA. Regenerated plantlets were moved to rooting medium (2.16 g of MS Basal Medium, 75 mg of L-asparagine, 20 mg of thiamine, 10 g of maltose, 2.5 g of phytagel, 5 mg of bialaphos and 0.01 mg/l of NAA per liter, pH 5.7–5.8) in the light at 25 °C. Rooted plants were transferred to potting soil and allowed to grow to approximately 10 cm in height. They were then sprayed with 0.1% glufosinate to identify transgenics.

PCR and herbicide analysis of transgenic lines

PCR analysis was performed to identify transgenic plants containing the pGB4.20 sequence. Genomic DNA was prepared from young leaves (Riede and Anderson 1996). PCR reactions were performed on the genomic DNA using primer GP3S (5′ CAC AAT TTC ATC ATC ACC CACAACACCGAG 3′), which hybridizes

to the 3′ end of the glutenin promoter and BXP3 (described above). The PCR cycling regimen was as previously described. The presence of the pGB4.20 plasmid was indicated by the production of a 541-bp product. *Bar* expression was tested by the leaf-paint method. Briefly, individual leaves were marked and painted with 0.1% glufosinate. Resistance was measured 5 days post-application. Susceptible leaves are substantially yellow in color, while resistant ones maintain normal green coloration. Transgenic lines homozygous for both *bar* expression and pGB4.20 were chosen for further analysis.

Southern analysis

Southern-blot analysis was performed by standard methods described previously (Giroux and Morris 1997). Briefly, genomic DNA was prepared (Riede and Anderson 1996) from young greenhouse-grown leaf tissue and digested with *Bam*HI for 3 h. Fifteen micrograms of the digested DNA was fractionated on a 0.7% agarose gel for 20 h at 15 V, and blotted to a nylon membrane (Osmonics, Inc). Blots were hybridized to ^{32}P-labeled probes prepared by a random primer method (Gibco BRL). Following hybridization, the membranes were washed three times at low stringency (2 × SSPE, 0.1% SDS) and then two times at high-stringency (0.2 × SSPE, 0.1% SDS). All washes were 15 min at 65 °C. Washed membranes were exposed to Kodak Biomax MS film at –80 °C using an intensifying screen. Probes were made from the coding sequence of wheat *pinA* or *pinB*, amplified as previously described (Gautier et al. 1994).

Northern analysis

Northern-blot analysis was performed by standard methods as described previously (Giroux and Morris 1997). Wheat plants were grown in the greenhouse at Bozeman, Montana, in the spring of 2001. RNA was prepared from 20 days post-anthesis developing wheat seeds by a LiCl method (McCarthy 1986). Five micrograms of RNA were separated on a formaldehyde agarose gel and blotted to a nylon membrane. Blots were hybridized to ^{32}P-labeled probes prepared by the previously described random primer method. Following hybridization, the membranes were washed and exposed to X-ray film as described above. Probes were made from the coding sequence of wheat *pinA* or *pinB*, amplified as previously described (Gautier et al. 1994).

Starch damage determination

Whole meal wheat flour was prepared with a UDY mill (UDY Co., Fort Collins, Colo.) fitted with a 0.5 mm screen. The percentage of damaged starch was determined using the Megazyme starch damage assay kit [AACC (2000) Method 76-31, Megazyme International, Bray, Ireland]. Three independent replicates were performed per line tested.

Seed texture measurement

Wheat seed texture was analyzed by both the Single Kernel Characterization System, SKCS 4100 (Perten Instruments, Springfield, Ill.) and NIR InfraAlyzer 400 (Technicon Corp., Tarrytown, N.Y.). SKCS analysis was performed on three replicates of 100 seeds each. NIR analysis was performed on three replicates of approximately 10 g of whole wheat flour ground on a UDY mill as described above.

Protein extraction and analysis

Wheat friabilin was isolated from 60 mg of whole wheat flour as previously described (Bettge et al. 1995). Whole proteins were prepared by extracting 20 mg of whole wheat flour in 400 µl of standard SDS PAGE Laemmli loading buffer at 70 °C for 10 min. After dilution to the optimal concentration for visualization, 10-µl aliquots were separated in 10–20% Tris-Glycine gradient gels at 130 V and stained with Coomassie blue.

Starch granule visualization by SEM

UDY ground (0.5 mm mesh) wheat meal (100 mg) was placed on top of 1 ml of chloroform in a 1.7-ml microfuge tube at 22 °C. After standing for 1 h with occasional light stirring of the meal with a weighing spatula, the supernatant and remaining suspended wheat meal were aspirated off. The remaining starch granules were removed from the bottom of the tube and dried. After drying, the starch granules were attached to an aluminum electron microscope stub with double-sided tape and sputter coated to 100 angstrom thickness with gold. The granules were imaged on an Hitachi SEM-600 at 1 k magnification (20 kV).

Results

Preliminary analysis of transgenic plants

Biolistic transformation and tissue culture were used to generate T0 lines of the cultivar Hi-Line which were resistant to the herbicide glufosinate. Herbicide-resistant T0 lines were tested for the presence of the pGB4.20 expression construct (Fig. 1) by PCR using a construct-specific primer pair. T1 plants were analyzed for glufosinate resistance. Ratios of resistant to sensitive plants were approximately 3:1 (Table 1), consistent with a single-locus integration event for the glufosinate resistance construct. PCR screening was used to confirm the presence of pGB4.20 in at least 12 herbicide-resistant T1 seedlings. Of the 10 T1 lines analyzed, one failed to co-segregate (herbicide resistance and pGB4.20), and was dropped from further study. It is likely that both the glufosinate resistance construct and pGB4.20 reside at a single locus in co-segregating plants. Transgenic lines appeared largely phenotypically identical with untransformed controls. Six homozygous T2 lines were chosen for further analysis.

Fig. 1 *PinB* expression construct. The pGB4.20 wheat endosperm expression construct is shown schematically above. The complete *pinB* coding sequence amplified from ‘Chinese Spring’ genomic DNA was used to replace the glutenin coding region in the pGlu10(5) construct of Blechl and Anderson (1996). The resulting construct contains the wild-type *pinB* coding sequence under control of the Dy10 glutenin regulatory 5′ sequences and the Dx5 glutenin 3′ sequence

Table 1 pB4.20 PCR data and glufosinate resistance segregation for selected wheat lines

Line[a]	PCR[b] (pGB4.20)	Segregation[c] (resistant/susceptible)	Chi-square[d] (3:1)
Hi-Line	–	0/24	
161	–	24/7	0.09
GB-1	+	17/4	0.40
GB-5	+	17/7	0.22
GB-10	+	21/3	2.0
GB-12	+	20/2	3.0
GB-16	+	20/6	0.051
GB-18	+	16/9	1.6

[a] All plants are derivatives of the wheat cultivar 'Hi-Line'
[b] PCR screening was performed on samples of genomic DNA from T0 plants using a pGB4.20-specific primer pair
[c] T_0 progeny segregation data represent glufosinate selection for resistant plants. T1 seedlings were leaf-painted with 0.1% glufosinate. Seedlings which showed minimal adverse effects were scored as resistant
[d] Chi-square values test the fit of the resistant/susceptible progeny of the T0 plants to a 3:1 ratio

Molecular characterization of transgenic lines

Southern-blot analysis was used to confirm that each line studied resulted from an independent transformation event. Total genomic DNA was isolated from seedlings and restriction digested with *Bam*HI. *Bam*HI cleaves pGB4.20 at a single site at the 5′ end of the *pinB* coding region, so a unique pattern can be expected from each random integration event. Digested genomic DNA isolated from Hi-Line and transgenic line 161, showed one major strongly hybridizing band of approximately 5.0 kb, which most likely corresponds to the native mutant *pinB* sequence, along with two minor bands of 11 and 16 kb in size (Fig. 2). In contrast, the transgenics show the presence of additional multiple strongly hybridizing bands, which indicates that the *pinB* transgene is present in multiple copies in each line (Fig. 2). The banding pattern is unique for each transgenic line, indicating that each arose from an independent integration event (Fig. 2).

Northern-blot analysis of purolindoline transcript levels

Total RNA from developing wheat kernels was analyzed (Fig. 3). Control lines Hi-Line and 161, which do not contain pGB4.20, accumulated a measurable level of native mutant *pinB-D1b* transcript. However, all transformed lines showed increased levels of *pinB* transcript accumulation relative to the control lines corresponding to the *pinB-D1a* transgene (Fig. 3). The level of *pinA* transcript expressed from the endogenous *pinA-D1a* sequence did not vary substantially between lines. Transcript levels of *pinA* and *pinB* in Hi-Line and 161 are roughly equal on blots exposed for comparable lengths of time.

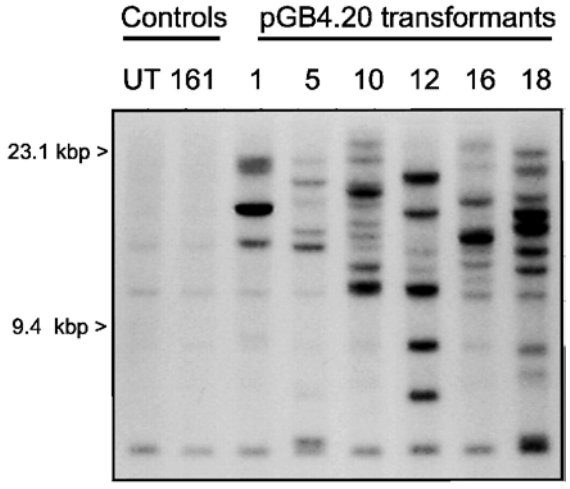

Fig. 2 Southern-blot analysis. DNA-blot analysis of restriction-digested wheat genomic DNA. Blots were hybridized to the entire 451-bp *pinB* coding region. Three invariant bands were observed in all genotypes. These three bands are present in the control lines UT (untransformed Hi-Line) and 161 (Hi-Line transformed with the *bar* marker construct only). Lines successfully transformed with pGB4.20 show additional bands, each with a unique pattern

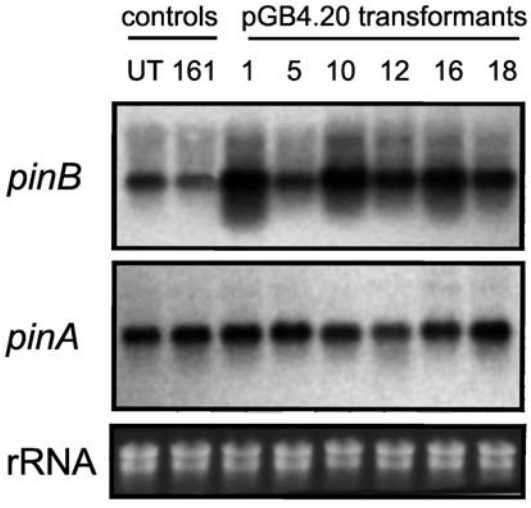

Fig. 3 Northern-blot analysis. RNA gel-blot analysis of *pinA* and *pinB* transcript accumulations in developing wheat kernels. The *pinA* sequence serves as a loading control, and is invariant among the lines. The *pinB* transcript accumulates to much greater levels in pGB4.20- transformed lines than in the untransformed Hi-Line (UT) and the transgenic control line 161. A duplicate ethidium bromide-stained agarose gel shows discrete bands of rRNA fractionated, indicating a similar loading from lane to lane and a lack of RNA degradation

Friabilin protein levels are increased in transgenic seeds expressing PINB-D1a

The effect of wild-type *pinB-D1a* expression upon the friabilin level, the classic marker for kernel softness that consists of PINA and PINB, was determined. Starch from mature seeds was washed with water, and the ad-

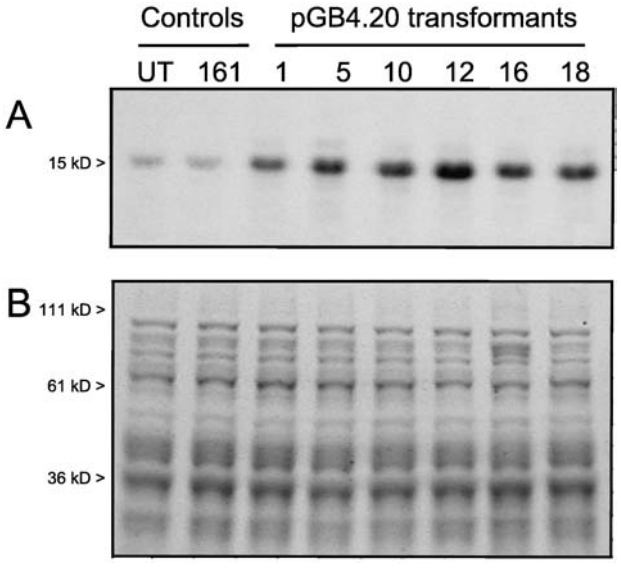

Fig. 4 Endosperm protein analysis. **A** SDS-PAGE gel of starch surface proteins isolated from water-washed starch. The friabilin levels of wild-type *pinB* expressors appear to be much greater (4 to 10 fold) than that of control lines HL and 161. This level in friabilin difference is comparable to that distinguishing hard and soft wheats. **B** SDS-PAGE gel of total wheat kernel proteins. Proteins soluble in SDS-PAGE buffer were separated and stained with Coomassie Blue. The protein banding pattern is unaltered by expression of *pinB* in transformed plants versus controls

hering proteins were extracted, separated on a gradient gel, and stained with Coomassie Blue. Control plants Hi-Line and 161 showed only trace amounts of friabilin (Fig. 4A). In contrast, starch extracted from *pinB-D1a*-expressing transgenics showed much higher levels. The increase in friabilin for the transgenics ranged from approximately four-fold for line 1 to approximately ten-fold for line 12. The overall pattern of storage protein accumulation did not vary among lines, as shown by the total protein control gel (Fig. 4B).

Table 2 Hardness (measured by both NIR and SKCS), particle size, and starch damage means for *pinB*-expressing lines and non-expressing controls

Line[a]	Grain hardness		% Starch damage[d]
	SKCS[b]	NIR[c]	
Hi-Line	70 +/– 2.8	60 +/– 6.8	3.36 +/– 0.50
161	71 +/– 2.6	65 +/– 3.3	3.71 +/– 0.18
GB-1	29 +/– 2.4	18 +/– 4.0	2.05 +/– 0.10
GB-5	22 +/– 2.9	6.4 +/– 5.3	1.33 +/– 0.10
GB-10	25 +/– 5.5	12 +/– 8.4	1.69 +/– 0.30
GB-12	9.8 +/– 1.7	5.8 +/– 3.2	0.99 +/– 0.05
GB-16	26 +/– 6.8	9.9 +/– 5.7	1.40 +/– 0.30
GB-18	37 +/– 7.2	18 +/– 7.2	2.56 +/– 0.30

[a] T^3 homozygous wheat seeds
[b] Single kernel hardness values are an average of three independent groups of seeds
[c] NIR hardness was measured on samples of wheat seeds ground as described in Materials and methods
[d] Starch damage was measured on whole meal flour using an enzymatic assay as described in Materials and methods

Seed hardness and starch damage are reduced by PINB-D1a expression

Data for seed hardness and starch damage are presented in Table 2. Hardness was measured by the Single Kernel Characterization System (SKCS) and by Near-Infrared Reflectance (NIR). Soft wheats typically have hardness

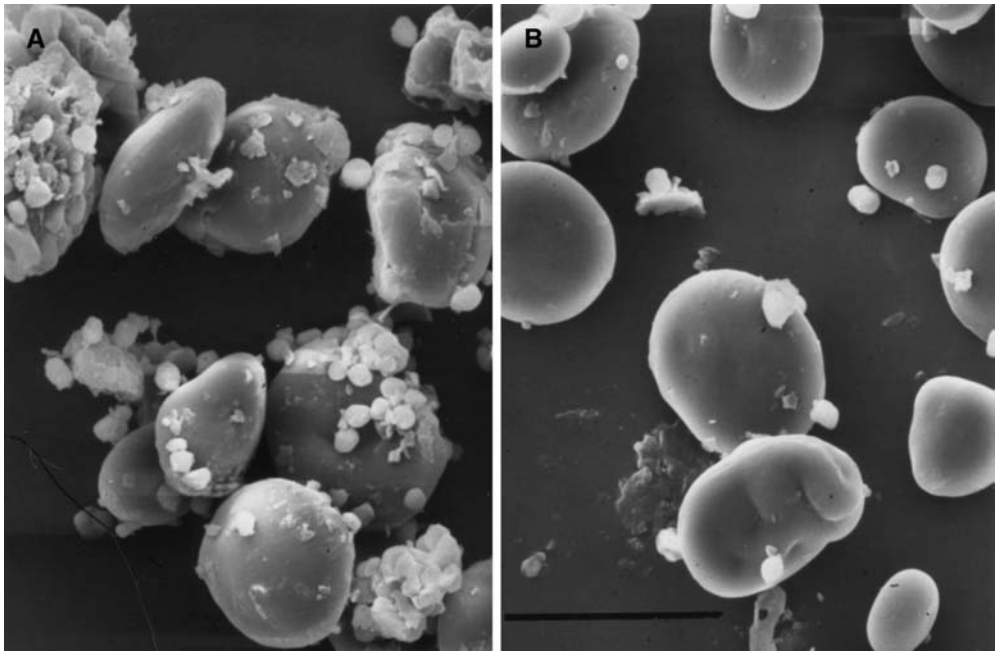

Fig. 5A, B Scanning electron microscopy (SEM) analysis. SEM analysis of purified starch from untransformed Hi-Line control and *pinB*-expressing transformant. **A** Scanning electron microscope (SEM) image of untransformed Hi-Line starch granules. The granules are rough in texture and appear to have material adhering to their surface. A (large, oblong) and B (smaller, round) granules occur as clumps with associated matrix material. This appearance is consistent with that previously observed for hard wheat starch (Barlow et al. 1973). **B** SEM image of starch granules from transgenic line-12 endosperm. These granules have a smooth surface, with little clumping or adhering material. The appearance is typical of soft wheat starch (Barlow et al. 1973). Size bar is 30 μm

values of approximately 15 to 40, while hard wheats range from about 45 to about 90 (Gaines et al. 1996; Norris et al. 1989; Perten Instruments 1999). Three replicates of mature wheat kernels were fractured by the SKCS instrument, and the means reported. Each replicate consisted of a pool of seed from four randomly selected greenhouse-grown plants. Hardness values of Hi-Line and 161 were 70 and 71, respectively, and fall in the range expected of a hard wheat. In contrast, hardness values of the *pinB-D1a* transgenics ranged from 37 for line 18 to 9.8 for line 12. These values are typical for soft wheat varieties. Whole wheat flour was used to measure hardness by the NIR method, again with three replications, each consisting of a sample of four greenhouse-grown plants. Average hardness values of the control lines were 60 and 65 for Hi-Line and 161, respectively. Hardness values for the transgenics ranged from 18 for line 18 to 5.8 for line 12, again typical of soft wheats.

An enzymatic starch-damage assay was performed on whole wheat flour. Three replicates were done, each consisting of a pool of seed from four greenhouse-grown plants. Since soft wheat flours have a lower degree of starch damage than do hard wheat flours, a decrease in the amount of starch damage measured would be consistent with a change to a softer phenotype. Average starch-damage percentages for Hi-Line and 161 were 3.36 and 3.71%, respectively. Levels of damage were substantially lower for the soft type *pinB*-expressing transgenic lines. Values ranged from 2.56% for line 18 to 0.99% for line 12.

SEM analysis of starch granules

Barlow et al. (1973) first reported that starch granules from soft and hard wheat varieties differed by the amount of material adhering to the surface. To examine whether this holds true for the complementation experiment in which the lines differed only by the presence of the *pinB* construct, starch granules were prepared non-aqueously from both transformed line 12 and untransformed Hi-Line, and analyzed by scanning electron microscopy (SEM). Both samples contained large and small (A and B type) granules. However, the appearance of the starch granule surface differed dramatically between the two lines (Fig. 5). Hi-Line starch granules (Fig. 5A) are rough in appearance, with the protein matrix clearly adhering to the majority. These granules were often present in aggregated clumps composed of multiple granules and the protein matrix. This is what Barlow et al. (1973) observed for hard wheat. In contrast, granules of pGB4.20-transformed line 12 are very smooth in appearance, and are often present as single discrete granules (Fig. 5B). This smooth appearance was described for soft wheat granules (Barlow et al. 1973).

Discussion

The *pinB-D1a* gene sequence isolated from a soft wheat was expressed in the endosperm of the hard wheat cultivar 'Hi-Line' which has the *pinB-D1b* gene. The resulting kernels were softer, contained higher levels of friabilin, and incurred less starch damage upon milling than those from control plants. The hardness and starch-associated friabilin levels observed in the transformants are typical of soft wheats. It can therefore be concluded that the glycine to serine *pinB-D1b* sequence in Hi-Line was genetically complemented in this study. The importance of this lies in the fact that Hi-Line contains the glycine-46 to serine-46 *pinB-D1b* sequence alteration (Giroux et al. 2000), which is by far the most common mutation observed in North American hard wheats (Morris et al. 2001). Genetic studies have previously implicated this sequence in the hard phenotype (Giroux and Morris 1998). However, since this *pinB* sequence change is minimal and the altered protein is present in amounts comparable to that in soft wheat, a direct role for *pinB* in the control of grain hardness has been controversial. An additional confounding factor is the fact that the *Ha* locus contains several tightly linked genes such as *Gsp-1a* (Jolly et al. 1996; Turnbull et al. 2000). In fact, in a wheat diploid relative, *pinA*, *pinB* and *Gsp-1a* are contained within a single 105-kb BAC clone (Tranquilli et al. 1999). The transgenic expression system employed here allowed the creation of true isogenic lines, which vary only in the expression of wild-type *pinB-D1a*. No other genetic system allows for the complete separation of the *puroindolines* from other linked genes at or near the *Hardness* locus. In this study, expression of the glycine-containing *pinB-D1a* sequence complemented the serine-containing sequence. This confirms that the glycine to serine change in *pinB-D1b* is responsible for the hard phenotype in Hi-Line, and likely in all hard cultivars containing this sequence. This mutation is the most prevalent in U.S. wheat cultivars (Giroux and Morris 1998). In fact, the *pinB-D1b* alteration is found in 52 of 54 (96%) U.S. hard winter wheats surveyed and 47 of 71 (66%) spring wheats surveyed (Morris et al. 2001). The next most common alteration consists of a *pinA* null allele (Giroux and Morris 1998). The *pinA-D1b* allele is found only in hard textured wheats (Giroux and Morris 1998; Morris et al. 2001). Additional sequence alterations have been found in *pinB* among hard wheats, and all soft wheats characterized have the *pinA-D1a* and *pinB-D1a* sequences (Morris et al. 2001). To-date, all wheats characterized have contained an alteration in *pinA* or *pinB* relative to the soft type sequences *pinA-D1a* and *pinB-D1a* (Morris et al. 2001).

These results are also consistent with the hypothesis that the puroindolines, as components of friabilin, are directly involved in maintaining a soft phenotype by reducing adhesion of starch granules to the protein matrix. Friabilin occurs in roughly equal amounts in hard and soft wheat flours; however, its presence at the surface of water-washed starch granules is much greater in soft vs hard wheat starch (Greenblatt et al. 1995). It has been suggested that the ability of the puroindolines to bind to the surface of the starch granule is controlled by their tryphophan-rich region (Gautier et al. 1994). Tryptophan

residues are believed to facilitate interactions between proteins and membrane phospholipids (Marion et al. 1994). The serine substitution in the PINB-D1b allele occurs in this conserved area. The probable effect of this sequence change would be a decrease in the membrane affinity of the tryptophan-rich region. In comparison to the zero hydrophobicity of a glycine residue (Thorgeirsson et al. 1996), the change to a serine would result in a negative hydrophobicity (–0.27). Confirmation that the glycine to serine change severely decreases the ability of PINB to bind to starch granules re-emphasizes the importance of this region of the molecule. The hydrophobic tryptophan-rich domains may mediate the site-specific localization of puroindolines to the starch granule surface. If so, the glycine-to-serine alteration in this region could be expected to reduce the association of PINB-D1b with the membrane lipids at the starch granule surface. A role for starch granule membrane lipids in endosperm texture was suggested by Barlow et al. (1973). They reported that the granule surface was the likely site of the functional difference between soft and hard wheats. However, because that study involved unrelated cultivars, it could not be definitively determined which of the many genetic differences between the two cultivars was responsible for the physical characteristics. This study, using truly isogenic material, determined that PINB alone is able to cause the change in starch granule appearance associated with soft and hard wheat. The starch granules of unmodified Hi-Line have little associated friablin and the rough appearance caused by adhering remnants of the proteinaceous material of the endosperm cell. In contrast, Hi-Line that expresses the wild-type PINB-D1a has high levels of starch-associated friabilin and smooth starch granules. This correlation of the wild-type PINB's ability to interact with the surface of the starch granules as friabilin, and cause smooth granule appearance and kernel softness, presents a clear picture. All of these results are consistent with the hypothesis that the puroindolines directly control grain softness by reducing the interaction between starch granules and their surrounding protein matrix.

In addition to effects on grain texture, previous reports demonstrated that puroindolines contribute to the formation and stability of dough foams, which in turn influence the breadmaking qualities of the flour (Dubreil et al. 1998). Even relatively minor differences in puroindoline composition and quality can have substantial impacts upon the milling and breadmaking qualities of wheats. A recent study of 139 recombinant inbred lines showed that one puroindoline allele was significantly superior to the other in milling yield, crumb grain and loaf volume (Martin et al. 2001). Therefore, in addition to dramatic changes in wheat grain texture, it is anticipated that transgene mediated alterations in puroindoline content will dramatically alter cereal end-use quality.

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References

AACC (2000) Approved methods of the American association of cereal chemists, 10th edn. American Association of Cereal Chemists, St. Paul, Minnesota

Altpeter F, Vasil V, Srivastava V, Stöger, E, Vasil IK (1996) Accelerated production of transgenic wheat (*Triticum aestivum* L.) plants. Plant Cell Rep 16:12–17

Baker RJ (1977) Inheritance of kernel hardness in spring wheat. Crop Sci 17:960–962

Barlow KK, Buttrose MM, Simmonds DH, Vesk M (1973) The nature of the starch-protein interface in wheat endosperm. Cereal Chem 50:443–454

Bettge AD, Morris CF, Greenblatt GA (1995) Assessing genotypic softness in single wheat kernels using starch granule-associated friabilin as a biochemical marker. Euphytica 86:65–72

Bio-Rad Laboratories, Hercules, California. Biolistic PDA-1000/He Particle Delivery System instruction manual

Blechl AE, Anderson OD (1996) Expression of a novel high-molecular-weight glutenin subunit gene in transgenic wheat. Nature Biotechnol 14:875–879

De Block M, Botterman J, Vandewiele M, Dockx J, Thoen C, Gosselé V, Rao Movva N, Thompson C, Van Montagu M, Leemans J (1987) Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO J 6:2513–2518

Dubreil L, Gaborit T, Bouchet B, Gallant DJ, Broekaert WF, Quillien L, Marion D (1998) Spatial and temporal distribution of the major isoforms of puroindolines (puroindoline-a and puroindoline-b) and non specific lipid transfer protein (ns-LTPe1) of *Triticum aestivum* seeds. Relationships with their in vitro antifungal properties. Plant Sci 138:121–135

Gaines CS, Finney PF, Fleege LM, Andrews LC (1996) Predicting a hardness measurement using the single-kernel characterization system. Cereal Chem 73:278–283

Gautier MF, Aleman ME, Guirao A, Marioen D, Joudier P (1994) *Triticum aestivum* puroindolines, two basic cystine-rich seed proteins: cDNA analysis and developmental gene expression. Plant Mol Biol 25:43–57

Giroux MJ, Morris CF (1997) A glycine to serine change in *puroindoline* b is associated with wheat grain hardness and low levels of starch-surface friabilin. Theor Appl Genet 95:857–864

Giroux MJ, Morris CF (1998) Wheat grain hardness results from highly conserved mutations in the friabilin components, *puroindoline* a and b. Proc Natl Acad Sci USA 95:6262–6266

Giroux MJ, Talbert L, Habernicht DK, Lanning S, Hemphill A, Martin JM (2000) Association of puroindoline sequence type and grain hardness in hard red spring wheat. Crop Sci 40:370–374

Greenblatt GA, Bettge AD, Morris CF (1995) The relationship among endosperm texture, friabilin occurrence, and bound polar lipids on wheat starch. Cereal Chem 72:172–176

Greenwell P, Schofield JD (1986) A starch granule protein associated with endosperm softness in wheat. Cereal Chem 63:379–380

Jolly CJ, Rahman S, Kortt AA, Higgins TJV (1993) Characterization of the wheat Mr 15000 'grain softness protein' and analyis of the relationship between its accumulation in the whole seed and grain softness. Theor Appl Genet 86:589–597

Jolly CJ, Glenn GM, Rahman S (1996) *Gsp-1* genes are linked to the grain hardness locus *(Ha)*. on wheat chromosome 5D. Proc Natl Acad Sci USA 93:2408–2413

Kooijman M, Orsel R, Hessing M, Hamer RJ, Bekkers ACAPA (1997) Spectroscopic characterization of the lipid-binding properties of wheat puroindolines. J Cereal Sci 26:145–159

Lanning SP, Talbert LE, McNeal FH, Alexander WL, McGuire CF, Bowman H, Carlson G, Jackson G, Eckhoff J, Kushnak G, Stewart V, Stallknecht G (1992) Registration of Hi-Line wheat. Crop Sci 32:283–284

Law CN, Young CF, Brown JWS, Snape JW, Worland JW (1978) The study of grain protein control in wheat using whole chromosome substitution lines. In: Seed protein improvement by nuclear techniques. International Atomic Energy Agency, Vienna, Austria, pp 483–502

Lillemo M, Morris CF (2000) A leucine to proline mutation in puroindoline b is frequently present in hard wheats from Northern Europe. Theor Appl Genet 100:1100–1107

MacRitchie F (1980) Physicochemical aspects of some problems in wheat research. In: Pomeranz Y (ed) Advances in cereal science and technology, vol. III, American Association of Cereal Chemists, St. Paul, Minnesota, pp 271–326

Marion D, Gautier M-F, Joudrier P, Ptak M, Pézolet, M, Forest E, Clark DC, Broekaert W (1994) In: Wheat kernel proteins-molecular and functional aspects. Universita Degli Studi Della Tuscia. S. Martino al Cimino, Viterbo (Italy). September 28–30, pp 175–180

Martin JM, Frohberg RC, Morris CF, Talbert LE, Giroux MJ (2001) Milling and bread baking traits associated with puroindoline sequence type in hard red spring wheat. Crop Sci 41:228–234

Mattern PJ, Morris R, Schmidt JW, Johnson VA (1973) In: Proceedings of the 4th International Wheat Genetics Symposium, University of Missouri, Columbia, Missouri, pp 703–707

McCarthy DR (1986) A simple method for extraction of RNA from maize tissues. Maize Genet Coop Newslett 60:61

Morris CF, Rose SP (1996) Wheat. In "Cereal Grain Quality". Chapman and Hall, New York, pp 3–54

Morris CF, Greenblatt GA, Bettge AD, Malkawi HI (1994) Isolation and characterization of multiple forms of friabilin. J Cereal Sci 21:167–174

Morris CF, Lillemo M, Simeon GM, Giroux MJ, Babb S, Kidwell K (2001) Prevalence of puroindoline grain hardness genotypes among historically significant North American spring and winter wheats. Crop Sci 41:218–228

Norris KH, Hruschka WR, Bean MM, Slaughter DC (1989) A definition of wheat hardness using near infrared spectroscopy. Cereal Foods World 34:696–705

Oda S, Schofield JD (1997) Characterisation of friabilin polypeptides. J Cereal Sci 26:29–36

Perten Instruments (1999) SKCS 4100 operation manual. Perten Instruments Inc. USA, Springfield, Illinois

Rahman S, Jolly CJ, Skerritt JH, Wallosheck A (1994) Cloning of a wheat 15-lDa grain softness protein (GSP): Gsp is a mixture of puroindoline-like polypeptides. Eur J Biochem 223:917–925

Riede CR, Anderson JA (1996) Linkage of RFLP markers to an aluminum tolerance gene in wheat. Crop Sci 36:905–909

Sourdille P, Perretant MR, Charmet G, Leroy P, Gautier MF, Joudrier P, Nelson JC, Sorrells ME, Bernard M (1996) Linkage between RFLP markers and genes affecting kernel hardness in wheat. Theor Appl Genet 93:580–586

Symes KJ (1965) The inheritance of grain hardness in wheat as measured by the particle size index. Aust J Agric Res 16:113–123

Thorgeirsson TE, Russel CJ, King DS, Shin YK (1996) Direct determination of the membrane affinities of individual amino acids. Biochem 35:1803–1809

Tranquilli G, Lijavetzky D, Muzzi G, Dubcovsky J (1999) Genetic and physical characterization of grain texture-related loci in diploid wheat. Mol Gen Genet 262:846–850

Turnbull, K-M, Gaborit T, Marion D, Rahman S (2000) Variation in puroindoline polypeptides in Australian wheat cultivars in relation to grain hardness. Aust J Plant Physiol 27:53–158

Weeks JT, Anderson OD, Blechl AE (1993) Rapid production of multiple independent lines of fertile transgenic wheat (*Triticum aestivum*). Plant Physiol 102:1077–1084