Review

The Effect of Friabilin on Wheat Grain Hardness

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Abstract: A wheat marketing system established the primary classification of hexaploid wheat based on the endosperm texture, i.e. hardness or softness of the grain. Hardness affects a range of characters including the milling (tempering, milling yield, flour particle size, shape and density of flour particles), baking and end-use properties. Wheat grain hardness is largely controlled by genetic factors but it can also be affected by the environmental and other factors. The endosperm texture is primarily associated with the *Hardness* (*Ha*) locus on the short arm of chromosome 5D. It is regulated by friabilin. This 15 kDa starch surface protein complex is present in larger amounts in soft wheats compared to hard ones and consists of three major polypeptides: puroindoline a (*Pina*), puroindoline b (*Pinb*) and grain softness protein 1 (*Gsp-1*). The soft grain texture in wheat is a result of both puroindoline genes being in the wild type active form and bound to starch. When one of the puroindolines is either absent or altered by mutation, then the result is a hard texture. Gene sequence variation and mutation of both puroindoline genes account for the majority of variation in the wheat grain texture. The latter may serve as the potential for improvement of milling and baking wheat quality. However, many wheat varieties have the intermediately (mixed) hard endosperm and there is a wide variation between soft and hard grain texture. Grain hardness is affected by a number of factors beyond genetics including N management, tillage system, pest infestations, environment (location of growth, temperature and rainfall during the growing season) and their interactions, and factors such as moisture, gliadin composition, and content of lipids, starch and pentosans.

Keywords: friabilin; grain softness protein (*Gsp-1)*; puroindoline a (*Pina)*; puroindoline b (*Pinb)*

Wheat grain hardness

The variation in grain hardness (hard or soft grain texture) is one of the most important traits that determine the utilization and marketing of wheat. Wheat grain texture is the degree of hardness or softness of the grain. Hardness is defined as "difficult to penetrate or reduce to smaller fragments". This is one of the most important characteristics that affect the functionality of wheat. It affects a range of characters including the milling (tempering, milling yield, flour particle size, shape and density of flour particles), baking and end-use properties (Giroux & Morris 1998; Morris 2002). An important functional difference between hard and soft wheats is in their water absorption. Hard wheat varieties are typically higher in protein content (12–15%) and stronger gluten-forming proteins than soft wheat (5–10%). Grain hardness was negatively correlated with break flour yield, flour yield, and mixing score and positively correlated with flour ash (Martin *et al.* 2001). Grain hardness was not correlated with loaf volume or crumb grain score (Hogg *et al.* 2005).

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The hardness of the grain appears to be determined by the degree of adhesion between the starch granules and the protein matrix (Csóti *et al*. 2005) but possibly also between the matrix proteins and the cell walls. Due to strong adhesion, starch granules fragmentize during the milling of hard wheat. This fragmentation is called starch damage. It is the most important factor in determining the water absorption of flour. It also determines the amount of carbohydrates available to yeasts for fermentative activity. Therefore, it positively affects gas production, loaf volume and, as a result, baking quality.

Softness is the opposite of hardness. An important functional difference between hard and soft wheats is in their water absorption. Flours milled from hard wheat have a higher baking absorption, giving higher quality and increased profit. Soft wheats have the softer endosperm texture (require less energy to mill) and yield smaller particles with less starch damage upon milling than do hard wheats (Hogg *et al*. 2005). Soft wheat flour is typically used for biscuits, cakes, cookies, crackers, pastries, and noodles and hard wheat flour for bread, buns and other yeast-raised products (Tipples *et al.* 1994).

Many wheat varieties have the intermediately (mixed) hard endosperm and there is a wide variation between soft and hard grain texture (semi-hard and medium-soft). The grain hardness is a result of many factors: genetical, biological, biochemical, biophysical and environmental ones.

Friabilin

Wheat hardness (the degree of adhesion between the starch granules and the protein matrix) is regulated by the protein called friabilin. The discovery of this 15 kDa protein on the surface of water-washed starch granules provided a biochemical way to distinguish between hard and soft wheats (Greenwell & Schofield 1986). This surface protein complex is present in larger amounts in soft wheats compared to hard ones and consists of three major polypeptides (Gautier *et al.* 1994; Rahman *et al.* 1994; Giroux & Morris 1997): puroindoline a (*Pina*), puroindoline b (*Pinb*) and grain softness protein 1 (*Gsp-1*). Friabilin levels quantified by SDS-PAGE were about 10-fold higher in starch derived from the soft lines compared to the hard ones. The results of Greenblatt *et al*. (1995) showed an interaction of friabilin with the starch granule surface. This interaction exhibited both ionic and hydrophobic characteristics.

Puroindolines (*Pina* and *Pinb*)

The term "puroindoline" is a derived term from Greek puro = wheat and indoline for the indole ring of tryptophan. Puroindolines are the main components of friabilin. In wheat, it is located in the starchy endosperm and in the aleurone layer (Dubreil *et al.* 1998) and it is also known because of foaming properties and antimicrobial activity which it has. The puroindolines may be a membrano-toxin that might be important in the defence mechanism of plants against microbial pathogens (Blochet *et al*. 1993). Jing *et al.* (2003) found that a 13-residue fragment of puroindoline a (*Pina*) exhibits the activity around both gram-positive and gram-negative bacteria. The microbial effect of *Pina* may be due to interactions with bacterial membranes (Charnet *et al.* 2003). These endosperm-specific low molecular weight cathionic proteins bind lipids and are found in abundance on the surface of soft wheat starch relative to their small amount present on hard wheat starch (Csóti *et al.* 2005). Presence of both puroindolines a and b *(Pina* and *Pinb)* is necessary for the soft phenotype (Morris 2002). Previous studies suggested that grain hardness is correlated with soft type *Pina* and *Pinb*, not total puroindoline (Swan *et al.* 2006). Evaluating a multilocal collection of European cultivars and advanced lines, Igrejas *et al.* (2001) found *Pinb* content to be more closely correlated with grain hardness than *Pina* content.

Both puroindolines are cysteine-rich proteins (ten cysteine residues forming five disulphide bridges) having a unique tryptophan-rich domain and a molecular mass of approximately 13 kDa (Douliez *et al*. 2000; Day *et al.* 2006). They are strongly basic proteins with isoelectric points of pI 10.5 for *Pina* and pI 10.7 for *Pinb* (Gautier *et al*. 1994). Whereas *Pina* is capable of binding tightly to both wheat phospholipids and glycolipids, *Pinb* interacts tightly only with negatively charged phospholipids and forms loose lipoprotein complexes with glycolipids. Ionic, hydrogen, and hydrophobic bonds contribute to the stability of puroindoline-polar lipid complexes, and the integrity of the tryptophan-rich domain is essential for the interaction with neutral polar lipids (Dubreil *et al.* 1997, 2003). Until now there has existed no information about the way the puroindolines prevent adhesion between starch granules and matrix protein (namely gliadin, glutenin...). It is not clear either why puroindolines are necessary for the grain softness.

Grain softness protein 1 (*Gsp-1*)

Grain softness proteins (*GSPs*) are closely related to puroindolines and all belong to the same group of proteins that includes the chloroform–methanol-soluble proteins and the non-specific lipid transfer proteins (Gautier *et al.* 1994). The grain softness protein family *GSP-1* includes *GSP-1a*, *GSP-1b* and *GSP-1c* (Rahman *et al.* 1994). The Grain softness protein-1 gene (*Gsp-D1*) is closely linked to puroindoline genes.

The wheat starch 15-kDa protein *GSP* consists of a major polypeptide and several minor polypeptides (Rahman *et al.* 1994). An antiserum raised against *GSP* was used to screen the wheat cDNA library. The cDNA family encoding approximately 15-kDa proteins that included a heptapeptide sequence previously isolated from protease digests of *GSP* was identified. Partial cDNA was used in a prokaryotic expression system to produce a fusion protein which reacted strongly against the original anti-*GSP* serum. A new antiserum raised against the fusion protein produced a weak reaction against a 15-kDa polypeptide extracted from wheat seeds. The results suggest that the proteins encoded by the cDNA family form a minor component of the mixture of 15-kDa polypeptides defined as *GSP*. RNA complementary to the cDNAs could be extracted from both soft and hard wheat grains from about half-way through grain filling. The encoded proteins are novel members of the 2S superfamily of seed proteins, a diverse family of proteins which maintain the characteristic framework of cysteine residues.

No clear evidence on the role of *Gsp-1* genes in relation to the grain texture has been obtained so far. Some authors are convinced that the lipid-binding properties of *GSP* polypeptides may influence grain softness. On the other hand, Tranquilli *et al.* (2002) recognized that deletions or allelic variants of *Gsp-A1* and *Gsp-B1* did not produce any significant effects on the grain texture, suggesting that these genes do not have a critical role in relation to the grain hardness. Giroux and Morris (1998) also believed that the grain softness protein was not associated with grain hardness.

Genetic aspects of wheat hardness

A single locus *Hardness* (*Ha*) was identified on the short arm of chromosome 5D (5DS) for the grain endosperm texture (Law *et al.* 1978). They designated the gene *Hardness*, with the soft allele *Ha* and the hard allele *ha*. Softness is in fact a dominant trait (Symes 1965, 1969). It is a simply inherited character.

Chantret *et al.* (2004) reported the complete sequence of a 101-kb BAC clone from *Triticum monococcum* (A^m genome) which includes three genes: *Puroindoline-a* (*Pina-A^m1*), *Puroindoline-b* (*Pinb-A^m1*) and *Grain Softness Protein* (*Gsp-A^m1*). The genes *Gsp-A^m1*, *Pina-A^m1* and *Pinb-A^m1* are separated by 37 kb and 32 kb, respectively, and are organized in the same transcriptional orientation. Four additional genes, including a pair of duplicated genes, were identified upstream of *Gsp-A^m1* within a high-density gene island. These additional genes were found in the same order and orientation, and the same relative distances apart as similar genes previously annotated on rice chromosome 12. An interesting discovery was a small not annotated putative rice gene that was similar to the *Gsp-A^m1* gene of *T. monococcum* and that was disposed in the same orientation, and located in the same position relative to the other orthologous genes. The high gene density observed in this BAC (1 gene per 14 kb) was expected for a distal chromosome region, but the level of microcollinearity with rice was higher than that reported in similar distal regions of other wheat chromosomes. Most of the BAC sequence was represented by repetitive elements, mainly concentrated in regions adjacent to the genes *Pina-A^m1* and *Pinb-A^m1*. Rearrangements among these repetitive elements might provide an explanation for the frequent deletions observed at this locus in the genomes of polyploid wheat species.

The soft grain texture in wheat is a result of both puroindoline genes being in the wild type active form and bound to starch (Morris 2002). When one of the puroindolines is either absent or altered by the mutation, then the result is a hard texture (Morris & Allan 2001; Morris & Konzak 2001; Morris *et al.* 2001a,b; Morris 2002). In the case of durum wheat which lacks puroindolines, the grain texture is very hard.

The presence of a single major gene is contrary to the allohexaploid nature of wheat (*T. aestivum* L.) ($2n = 6x = 42$ chromosomes; genomes AABBDD) because most genes exist in triplicated homoeologous sets, one from each genome. Alleles of the hardness gene are present on the 5A and 5B chromosomes of hexaploid wheat but are not expressed. For this reason, durum wheats

(*T. turgidum* L. var. *durum*) (2*n* = 4*x* = 28 chromosomes; genomes AABB), which lack the D genome, are generally harder textured than hard hexaploid wheat (Dexter *et al.* 1988).

Giroux and Morris (1997) reported a single nucleotide change in the *Pinb* gene that may change the secondary or tertiary structure of *Pinb* preventing the binding to starch granules, resulting in a hard grain texture. The variations of both genes mainly involve absence or single base mutation in the coding region (Giroux & Morris 1997; Lillemo & Morris 2000).

Seven hard alleles of puroindoline b and one hard allele in puroindoline a have been identified (Morris 2002). Data are shown in Table 1. Three alleles result in amino acid substitution (*Pinb-D1b*, *Pinb-D1c*, *Pinb-D1d*) and three result in "stop" codon TGA (*Pinb-D1e*, *Pinb-D1f*, *Pinb-D1g*). The exact mutation that nullifies the action of the *Pina* gene, resulting in the complete absence of the puroindoline a protein, has been unexplained so far.

Pina-D1a (wild type) is present in all soft hexaploid wheats and possibly all hard hexaploid wheats carrying a hardness mutation in puroindoline b.

The null *Pina-D1b* was found in some hard hexaploid wheats, allowing Hard Red Calcuta, Marroqui, Red Egyptian (Giroux & Moris 1997). However, it is often in Chinese landraces and historical cultivars from China (Chen *et al.* 2006). The *Pina-D1b* (a-null) is associated with harder texture than *Pinb-D1b* mutation (Morris & Massa 2003; Gedye *et al.* 2005).

Pina-D1c is the first null allele due to a point mutation that has been identified at the *Pina-D1* locus (Gazza *et al.* 2005). It was achieved in Fortuna and Glenman cultivars and was shown to have a cytosine deletion at position 267 in the coding region of the *Pina-D1a* gene, which resulted in a TGA stop codon.

Pinb-D1a (wild type) occurs in all soft hexaploid wheats and possibly in all hard hexaploid wheats carrying the *Pina-D1b* mutation.

Pinb-D1b is a "loss of function" mutation resulting from the replacement of glycine by serine at position 46. The mutation is prevalent among a wide set of both recent and historical wheat varieties (Giroux & Morris 1997). This amino acid change resides in a region thought to be important for the lipid-binding properties of puroindolines. Lines possessing the *pinb-D1b* mutation had higher break flour yields, higher flour yields, lower flour ash, improved crumb grain scores, and larger loaf volumes compared with lines carrying the *pina-D1b* mutation (Martin *et al.* 2001; Hogg *et al.* 2005). Other authors (Cane *et al.* 2004) supported these findings by reporting that Australian hard cultivars possessing the *pinb-D1b* mutation had higher flour yields, lower water absorption, and a smaller particle size than those hard cultivars possessing the *pina-D1b* mutation.

Pinb-D1c is characterized as involving a leucine to proline change at position 60. The mutation is frequently present in hard wheats from Northern Europe (Lillemo & Morris 2000). On the other

Table 1. Scheme of seven grain hardness alleles in wheat

Puroindoline locus		Phenotype	Puroindoline	Change in DNA	Change in protein sequence
Pina-D1	*Pinb-D1*				
Pina-D1a	*Pinb-D1a*	soft	wild type		
Pina-D1b	*Pinb-D1a*	hard	*Pina* null		
Pina-D1a	*Pinb-D1b*	hard	*Pinb*	GGC → AGC	Gly-46 to Ser-46
Pina-D1a	*Pinb-D1c*	hard	*Pinb*	CTG → CCG	Leu-60 to Pro-60
Pina-D1a	*Pinb-D1d*	hard	*Pinb*	TGG → AGG	Trp-44 to Arg-44
Pina-D1a	*Pinb-D1e*	hard	*Pinb* null	TGG → TGA	Trp-39 to stop codon
Pina-D1a	*Pinb-D1f*	hard	*Pinb* null	TGG → TGA	Trp-44 to stop codon
Pina-D1a	*Pinb-D1g*	hard	*Pinb* null	TGC → TGA	Cys-56 to stop codon

A – adenosine, C – cytidine, G – guanosine, T – tymidine, Arg – arginine, Cys – cysteine, Gly – glycine, Leu – leucine, Pro – proline, Ser – serine, Trp – tryprophan

hand, in North American wheats it occurs relatively scarcely. Out of 90 spring wheats, the allele was found only in four varieties.

Pinb-D1d possesses an amino acid substitution in the codon of tryptophan-44, converting it to arginine. It was located in three winter wheats from Sweden and Netherlands (Lillemo & Morris 2000).

The null allele *Pinb-D1e* includes a single nucleotide stop mutation in this instance in the codon for tryptophan-39. Chiefkan winter wheat carried the same *Pinb-D1e* allele as Canadian red and Gehun spring wheats (Morris *et al*. 2001b).

Pinb-D1f involved a single nucleotide change so that tryptophan-44 became a stop codon. This null allele is the least frequent.

Similarly, the allele *Pinb-D1g* is rare. Allowing, it occurred in Andrews hard red winter wheat (Morris *et al*. 2001b). The null allele *Pinb-D1g* involves a single nucleotide change of cysteine-56 to stop codon.

Besides, recently Chen *et al*. (2005, 2006) discovered a number of novel types of *Pina* (*Pina-D1g*, *Pina-D1l*, *Pina-D1m*, *Pina-D1n* and *Pina-D1p*) and types of *Pinb* (*Pinb-D1p*, *Pinb-D1u*, *Pinb-D1v* and *Pinb-D1w*) carrying a single base mutation. They were found in some common wheat as well as in spelt cultivars (Chang *et al*. 2006).

An allele designed as *Pina-D1l* was detected in five Chinese landraces with a cytosine deletion at position 265 in *Pina* locus.

Pina-D1m was detected in the landrace Hongheshang, from Jiangsu province, with the characterization of a proline to serine substitution at position 35 in the coding region of *Pina* gene (Chen *et al.* 2006).

Another novel allele *Pina-D1n* was identified in six Chinese landraces, with the characterization of an amino acid change from tryprophan-43 to a stop codon in the coding region of *Pina* gene. In this territory the *Pinb-D1p* allele was also found sporadically (Chen *et al.* 2006).

A new *Pinb-D1t* allele was identified in two landraces, Guangtouxianmai and Hongmai from the Guizhou province, with the characterization of a glycine to arginine substitution at position 47 in the coding region of *Pinb* gene (Chen *et al*. 2006).

Results of many authors (Giroux *et al.* 2000; Campbell *et al.* 2001; Lillemo & Ringlund 2002; See *et al*. 2004; Breseghello *et al.* 2005; Gedye *et al.* 2005) conclusively indicated that hybridizations between hard and soft wheat types could be a source of novel variations for wheat quality improvement. The identification of the *Pin*a and *Pinb* alleles may serve for breeders and researchers to understand the molecular mechanism of wheat grain texture.

A segregating population of 115 recombinant inbred lines originating from a cross between the hexaploid Synthetic wheat (*Triticum durum* × *Aegilops tauschii*) and the cultivar Opata was studied in two different experimental years to detect Quantitative Trait Loci (QTL) for three traits: grain hardness, *Pina* and *Pinb* contents (Igrejas *et al*. 2002). Negative correlation coefficients (–0.86 and –0.80) were identified between grain hardness and puroindoline content (*Pina* and *Pinb*, respectively) on data obtained in 1996. Results obtained in 1999 confirmed the negative correlation between hardness and *Pina* (–0.73), however a positive correlation coefficient was found with *Pinb* content (0.41). For each hardness, *Pina* and *Pinb* traits one major QTL was detected on the short arm of chromosome 5D, located close to the *mta9* allele (*Pina*). For the first year (1996) the QTL in this region explained around 63% of the phenotypic variability in grain hardness, 77% in *Pina* and 45% in *Pinb* contents. These values were confirmed in trials carried out in 1999 with the R^2 value of 0.71, 0.72 and 0.25 for hardness, *Pina* and *Pinb*, respectively. In 1996 and 1999 a second major QTL was detected for grain hardness on the long arm of the same chromosome. Present results indicate that it cannot be definitely concluded that puroindoline content represents a linear explanation for variations in grain hardness.

However, four additional regions located on chromosomes 2A, 2D, 5B and 6D were shown to have single-factor effects on wheat hardness, while three others situated on chromosomes 5A, 6D and 7A had interaction effects (Sourdille *et al*. 1996). The QTL analysis of other authors has identified specific regions of the wheat genome that are not linked to the *Ha* locus and are associated with the endosperm texture. These QTLs have been identified on chromosomes 2A, 2DL and 6B (Campbell *et al*. 1999) and 1A and 6D (Perretant *et al.* 2000).

In order to study the role of puroindoline content in the texture variation, the quantity of *Pina* and *Pinb* was determined (Igrejas *et al*. 2001). Eleven bread-making parameters were obtained from 40 bread wheat cultivars grown in four experimen-

tal locations. All bread-making parameters were significantly influenced by the genotype whereas the location did not significantly affect *Pina* and *Pinb* and loaf volume. The results strengthened the significant role of *Pinb* in bread-making (loaf volume), and indicated that biochemical factors other than puroindolines are involved in the grain hardness variation.

Other factors affecting wheat grain hardness

Three basic mechanisms of grain hardness have been postulated (Anjum & Walker 2006): (1) chemically induced adhesion between protein and starch granule, (2) continuity of the protein matrix and (3) net charge on the protein. While a significant proportion of variation in the endosperm texture between hard and soft wheats is due to the *Ha* gene on chromosome 5D, up to 40% of the variation in hardness is due to other unknown factors, with evidence for a strong genotype × environment (G × E) interaction. The environmental component of the variation is known to show a strong correlation with the visual endosperm characteristic of vitreosity, indicative of an effect on the physical structure of the endosperm (Stenvert & Kingswood 2006).

Grain hardness is affected by a number of factors beyond genetics including N management, tillage system, pest infestations, environment, and their interactions and factors such as moisture, gliadin composition, lipid and pentosan content (Huebner & Gaines 1992; Peterson *et al*. 1992; Turnbull & Rahman 2002).

Nitrogen fertilizer is known to increase grain protein levels in wheat. The use of adequate levels of N fertilizer might help to ensure that grains from hard wheat cultivars are classified as hard wheat, although the amount of fertilizer required will probably vary with timing and amount of growing season precipitation, cultivar, and pest infestations (Lyon & Shelton 1999). In addition to fertilizer management, fallow management may also influence the wheat grain hardness (Lyon & Shelton 1999).

Simple linear correlation coefficients between the wheat endosperm hardness and its lipid composition indicated that hardness was positively correlated with the content of free glycolipids and negatively correlated with the content of surface lipids of starch, especially with their non-polar fraction. The typical feature of harder wheat varieties was a substantially higher content of oleic acid in lipids of the starch surface (Konopka *et at*. 2005).

Softer textured wheats had higher lipid-complex amylose and starch phosphorus contents and lower total starch content. Softer textured wheats had larger starch granules and harder textured wheats had smaller starch granules. Smaller granules have a larger surface area available for non-covalent bonding with the endosperm protein matrix and they may also pack more efficiently, producing the harder endosperm (Gaines *et al.* 2000).

Pentosans (primarily arabinoxylans and arabinogalactans) were found to have an effect on the endosperm texture, especially in soft wheat. Among the hard wheat samples, pentosans had a minimal role in modifying the grain hardness. However, among the soft wheat samples, pentosans appeared to have significant hardness-modifying effects that carried over into end-use quality (Bettge & Morris 2000)

A multidisciplinary approach combining genetical, fine structural, biochemical, molecular and biophysical inputs would provide a detailed understanding of the molecular interactions on the starch granule surface that determine the grain hardness – this in turn would facilitate the application of genetic engineering to produce new types of wheat in which the hardness was optimized for specific end-uses for the benefit of manufacturer and consumer.

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