Puroindolines: the molecular genetic basis of wheat grain hardness

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Abstract

The variation in grain hardness is the single most important trait that determines end-use quality of wheat. Grain texture classification is based primarily on either the resistance of kernels to crushing or the particle size distribution of ground grain or flour. Recently, the molecular genetic basis of grain hardness has become known, and it is the focus of this review. The puroindoline proteins a and b form the molecular basis of wheat grain hardness or *texture*. When both puroindolines are in their 'functional' wild state, grain texture is soft. When either one of the puroindolines is absent or altered by mutation, then the result is hard texture. In the case of durum wheat which lacks puroindolines, the texture is very hard. Puroindolines represent the molecular-genetic basis of the *Hardness* locus on chromosome 5DS and the soft (*Ha*) and hard (*ha*) alleles present in hexaploid bread wheat varieties. To date, seven discrete hardness alleles have been described for wheat. All involve puroindoline a or b and have been designated *Pina-D1b* and *Pinb-D1b* through *Pinb-D1g*. A direct role of a related protein, grain softness protein (as currently defined), in wheat grain texture has yet to be demonstrated.

Abbreviations: BAC, bacterial artificial chromosome; ELISA, enzyme-linked immunosorbent assay; mmt, million metric tons; NIL, near-isogenic line; NIR, near-infrared spectroscopy; PCR, polymerase chain reaction; PSI, particle size index; RILs, recombinant inbred lines; RSLs, recombinant substitution lines; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SKCS, Single-Kernel Characterization System; TCA, trichloroacetic acid; TX114, Triton X-114

Introduction

Among all crop plants, bread wheat (*Triticum aestivum* L.) and durum or pasta wheat (*T. turgidum* L. var. *durum*) have the unique ability to produce a broad range of nutritious, appealing foods. Bread in its various forms results primarily from its unique complement of seed storage proteins which form gluten upon hydration and mixing, and allow it to retain gases produced during fermentation. Although this aspect of wheat grain quality is well-known, it is the variation in grain hardness or *texture* that is the single most im-

portant trait that determines end-use and technological utilization. The world produces about 600 million metric tons (mmt) of wheat annually, of which about 100 mmt is traded internationally. Grain hardness forms the fundamental basis of differentiating world trade of wheat grain.

In the vernacular, 'grain hardness' has both a qualitative and a quantitative meaning. It is common to differentiate 'soft' and 'hard' hexaploid wheats, and 'very hard' durum wheat as three distinct qualitative classes. Grain hardness also refers to the quantitative variation within and across these qualitative classes. Here, the term 'texture' will be used in preference to 'hardness'. In the past 15 years, the underlying molecular genetic basis for this key trait of wheat has been resolved and shown to result from the puroindoline

proteins. In this review, I shall briefly summarize the means of measuring wheat grain texture, the classical genetic studies on wheat grain texture, the discovery of friabilin, the discovery and eventual reconciliation of puroindoline a and puroindoline b as the main components of friabilin, a clarification of the role of grain soft protein (GSP), the characterization of mutations in the puroindoline genes that result in hard kernel texture, and lastly, the occurrence of puroindolines in other species. Prior reviews on the general subject of wheat grain texture are by MacRitchie (1980), Hoseney (1987), Pomeranz and Williams (1990), Anjum and Walker (1991), Autran *et al.* (1997), and Douliez *et al.* (2000).

Measuring wheat grain texture

Generally, methods that measure the texture of wheat grain quantify the texture phenotype of bulk grain lots or individual kernels. As such, most methods provide a discrete, numerical separation of the qualitative classes of soft and hard. Although different methods provide slightly different phenotypes (cf. Morris *et al.*, 1999a), the underlying utility of these methods is that they capture the physical manifestations of the *Hardness* gene. As discussed below, the *Hardness* gene has two primary phenotypes (alleles), those being soft (*Ha*) and hard (*ha*). Soft wheats are more friable, require less energy to mill and produce flours and meals with reduced particle-size distribution, including many free starch granules (Cutler and Brinson, 1935; Devaux *et al.*, 1998). Hard wheat meals are coarser, have more broken and damaged starch granules, but flow and bolt more easily. When the gene is absent, as is the case of durum, the third, very hard phenotype is observed.

To facilitate information exchange, some standardization of texture methods has occurred (Williams and Sobering, 1986; Norris *et al.*, 1989; Gaines *et al.*, 1996; AACC, 2000). The three most commonly used texture methods are the particle size index (PSI), near-infrared reflectance (NIR) and the Single-Kernel Characterization System (SKCS) (see below). As MacRitchie (1980) noted, methods of texture measurement may be grouped according to whether they grind, crush, abrade or indent the sample, but, 'none of the methods, however, measures a fundamental material property. One of the obstacles to accomplishing this is the difficulty which the sample geometry of the grain presents.' Glenn *et al.* (1991) addressed this

issue by devising a means of producing cylinders of uniform dimension from the center of the endosperm. However, this method is very labor-intensive and has not been widely adopted (Jolly *et al.*, 1996; Delwiche, 2000; Osborne *et al.*, 2001). Of the three common methods mentioned above, the two most wide-spread approaches to texture measurement rely on differences in granularity (particle size distribution) of meals or flours after grinding or milling, respectively. The first, PSI, quantifies granularity by sifting the ground or milled material and expressing the proportion of material that passes through a sieve of defined aperture (Worzella and Cutler, 1939). Consequently, higher numbers indicate softer texture (due to the lower particle size distribution of soft wheat meals). The second, NIR, provides an indirect assessment of particle size through the optical reflectance of ground samples. More recently, a desire in the U.S. system to identify mixtures of soft and hard wheats led to the development of a single-kernel crushing device (Martin *et al.*, 1993) which is now commercially available as the Single-Kernel Characterization System (model 4100) (Perten Instruments, Springfield, IL). Of historical interest, apparently the first mechanical means of measuring wheat grain texture was developed around 1908 (Roberts, 1910) and determined the force required to crush individual kernels.

Before proceeding further, it is important to reiterate the difference between texture, as defined in the previous section, versus vitreosity. Although the literature is replete with instances where the two terms are used interchangeably (and often incorrectly), they refer to different phenomena. Variation in wheat grain texture is primarily influenced by the *Hardness* gene, but is also influenced by grain protein and other secondary factors. Vitreosity is primarily a function of grain protein content, with more vitreous kernels having a higher protein content and more continuous protein matrix. However, by manipulating N fertility and other environmental factors, vitreous and non-vitreous (mealy) kernels may be produced from soft, hard and durum wheats.

Classical genetics of wheat grain texture

Although the physical difference among grain lots known as texture has been long recognized (Cobb, 1896; Biffen, 1908) and attributed as a 'varietal' (i.e. genetic) factor (Cutler and Brinson, 1935; Worzella, 1942; Berg, 1947; Greer, 1949; Greer and Hinton,

1950; Symes, 1961), it was only in the second half of the 20th century that the means of measuring grain texture (see above) and the commercial interest in segregating and breeding soft and hard wheats combined to prompt careful genetic studies of the mode of texture inheritance. Early reports of Symes (1965, 1969), Mattern *et al.* (1973), Baker and Dyck (1975), Law *et al.* (1978) all identified the major effect of a single gene on grain texture. Mattern *et al.* (1973) and Doekes and Belderok (1976) localized the gene on 5D. Law *et al.* (1978) localized the locus on the short arm of chromosome 5D (5DS) and designated the gene *Hardness*, with the soft allele, *Ha*, and the hard allele, *ha*.

The discovery of friabilin

In the now landmark paper of Philip Greenwell and J. David Schofield, published in 1986, a M_r 15 000 protein was extracted from water-washed wheat starch and separated by gradient SDS-PAGE. In more than 150 different wheats including seven durum varieties, an unbroken relationship was established: abundant 15 kDa protein associated with soft wheat starch, small amounts associated with hard wheat starch, and none with durum starch. Further, they associated the occurrence of the protein with chromosome 5D using whole-chromosome substitution lines between 'Cappelle-Desprez' (soft, recipient line) and 'Bezostaya-1' (hard, donor line) (data supporting this observation were later published in Wrigley and Bietz, 1988, and MacRitchie *et al.*, 1990, and reviewed in Greenwell, 1987, and Schofield and Greenwell, 1987). A prior report[1] was given on October 17, 1985, and appeared in the Bulletin of the FMBRA (Greenwell, 1986). This 15 kDa protein was eventually named 'friabilin' to highlight the fact that soft wheats are more friable than hard ones (Greenwell and Schofield, 1989).

In subsequent papers (Greenwell, 1987; Schofield and Greenwell, 1987; Greenwell and Schofield, 1989) the survey of the grain softness-starch friabilin observation was extended to ca. 300 wheats. Further, based on extraction characteristics with SDS, friabilin was

[1] The Bulletin of the Flour Milling and Baking Research Association (FMBRA) is protected by copyright and could not be previously cited; recent permission from the Association has been obtained to cite the work here. In 1995 the FMBRA merged with Campden Food and Drink Association into what is now Campden & Chorleywood Food Research Association Group, Chipping Campden, UK.

shown to be associated with the surface of the starch granule ('surface' vs. 'integral' starch granule proteins). They also examined a set of 56 recombinant substitution lines between cv. Chinese Spring (CS) and CS with a pair of 5D chromosomes substituted from the hard wheat cv. Hope. For all but three lines, the association between a high level of friabilin and softness (as judged by PSI of Bühler milled flours) or, conversely, low level of friabilin and hardness was observed (Greenwell, 1987; Schofield and Greenwell, 1987). The three exceptional lines exhibited intermediate levels of friabilin and hard grain texture. The friabilin-soft wheat association held among two pairs each of hard and soft NILs produced from cvs. Falcon and Heron (Greenwell, 1987). Friabilin levels quantified from SDS-PAGE were about 10-fold higher in the starch derived from the soft lines compared to the hard ones. A re-examination of the earlier work with the CS × 'CS(Hope 5D)' RILs (Greenwell, 1987; Schofield and Greenwell, 1987) resulted in only one line deviating from the expected pattern. This line, it was suggested, may have been monosomic for 5D. Antibodies purportedly localized friabilin to the surface of the starch granule. Analysis of F_1 hybrid grain derived from eight different hard by soft crosses indicated that the PSI of Quadrumat-milled flours were intermediate to the hard and soft parents and were proportional to the *Hardness* gene dosage in the triploid endosperm. A preliminary report from Greenwell, Schofield and co-workers examined 'soft' durum wheats which were found to have PSI values between 'normal' durum and hard hexaploid wheats and, like normal durum wheats, lacked starch-associated friabilin (Schofield *et al.*, 1991a).

The report of Greenwell and Schofield (1986) prompted research in several laboratories. Bakhella *et al.* (1990), Glenn and Saunders (1990), Oda *et al.* (1992) and Rogers *et al.* (1993) extended the observation of friabilin and kernel softness. These studies added strong support to a general model implicating some unknown role of friabilin in wheat grain softness.

Rahman and co-workers (Jolly *et al.*, 1990, 1993, 1996; Jolly, 1991; Rahman *et al.*, 1991, 1994) conducted extensive studies on friabilin and grain softness. They isolated friabilin, which they termed 'grain softness protein' or GSP, from the soft cultivar Rosella and used it to raise polyclonal antibodies (GSP antiserum, asGSP). Using this asGSP in western analysis they showed that GSP was present in whole-meal extracts of hard as well as soft wheats. Examination

of soft and hard BC_7 near-isogenic lines (NILs) of Symes (1969) derived from cvs. Heron (soft) and Falcon (hard) showed about four-fold more GSP in the whole meal of the soft NILs and Heron compared to hard NILs and Falcon. When the analysis of GSP was extended to other soft and hard wheats, the level was found to be more variable among the hard wheats than among the soft ones. The level of GSP of some cultivars was equivalent to that found in soft wheats (0.8–1.3 μg GSP per mg whole meal) whereas others were similar to Falcon or even lower (0.4 μg/mg). However, with water-washed starch the original soft wheat-abundant friabilin observation of Greenwell and Schofield (1986) held. By using as-GSP they found that there was about 20-fold more GSP in whole meal compared to that associated with the surface of water-washed soft wheat starch. Linkage analysis was conducted using SDS-PAGE and Coomassie Blue staining of proteins extracted from individual F_2 and F_3 half kernels derived from crossing the 'hard Falcon' NIL and the 'soft Falcon' NIL. Texture phenotype was determined on geometrically uniform endosperm cylinders (Glenn et al., 1991). Among 44 F_2 plants and selected $F_{2:3}$ progeny, the association between GSP occurrence and soft texture held true without exception.

The most significant result of this work was that friabilin was present in whole meal of hard as well as soft wheats. Although some hard wheat cultivars exhibited a reduced amount of friabilin, these data necessitated a re-evaluation of the then existing model which suggested that a qualitative difference in friabilin level (the 'non-stick' protein as it was called) produced the difference between soft and hard wheats. With the results of Rahman and co-workers, the phenomenon of friabilin occurrence on water-washed starch had to be viewed as a 'partitioning phenomenon' and, in a sense, a result of the starch isolation procedure itself. Using asGSP they showed that among fractionated flours (starch, gluten and water solubles) friabilin partitioned to the gluten and starch fractions but not the water solubles. Greenwell (1992b) also had discovered that friabilin was present in similar amounts in endosperm of both hard and soft wheats based on western analysis. Unfortunately, this report was not available outside the FMBRA.

Morris and co-workers conducted studies on the effects of the hardness gene on end-use quality (Morris, 1992b; Bettge and Morris, 1995; Miller et al., 1997) and gained new information regarding the friabilin-soft wheat phenomenon (Bettge et al., 1992, 1995;

Morris, 1992a; Morris et al., 1992, 1994a, b, c; Greenblatt et al., 1992, 1995; DeMacon et al., 1993, 1994). During attempts to reproduce the SDS-PAGE results of Greenwell and Schofield (1986), it was found that friabilin was soluble in the common gel fixative, methanol/water/acetic acid, and could be selectively leached out of gels. TCA, on the other hand, proved an effective fixative. At this time the attention of several research groups was turning to the issue of friabilin extraction and characterization. The solubility of friabilin in methanol/water/acetic acid prompted us to conduct a survey of solvents and extraction protocols for friabilin. We eventually settled on 50 mM NaCl in 50% v/v propan-2-ol. This solvent allowed extraction of friabilin without the use of ionic detergents (i.e. SDS). Earlier, Greenwell (1987) showed that when using 1% SDS, reducing the temperature from 50 °C to 20 °C reduced starch granule swelling and therefore eliminated the confounding extraction of integral proteins. To reduce the interference of other kernel proteins to a minimum, purification of starch was essential. Generally this was accomplished by the common 'dough-ball' method of Wolf (1964). However, B granules were discarded in this procedure. These limitations were overcome by the starch isolation procedure of Morrison and co-workers (South and Morrison, 1990; Sulaiman and Morrison, 1990). A kernel macerate was centrifuged through 80% w/v CsCl, and a nearly protein-free total starch fraction was recovered. Friabilin association with the surface of the soft wheat starch granule was variably affected (cf. Greenblatt et al., 1992). We also found that stepwise acetone precipitation of friabilin after extraction from granules effected further purification. Our work and that of Rahman and co-workers indicated that some portion of 'friabilin', that is, the protein(s) of ca. 15 kDa, extracted from the surface of water-washed soft wheat starch, was composed of protein(s) that differed in solubility (Morris et al., 1992, 1994c) and was antigenically related to the α-amylase inhibitor family (Jolly, 1991; Rahman et al., 1991; Jolly et al., 1993). Oda and Schofield (1997) provided direct amino acid sequence confirmation of the identity of α-amylase inhibitors. Western analysis showed that this 'contaminant' was present in variable amounts, was unrelated to kernel texture and could be removed selectively with 0.1 M NaCl. By replacing Coomassie Blue with silver stain in PAGE, a significant increase in sensitivity and detection of friabilin was gained. These and other improvements in technique allowed the characterization of friabilin from as little as 2 mg equivalents

of starch, and the analysis of friabilin occurrence among F_1 seeds of reciprocal soft by hard crosses (Bettge *et al.*, 1992, 1995). That study showed that expression of the *Hardness* gene was additive and proportional to the gene dosage in the triploid endosperm. Among the four *Hardness* allelic combinations, friabilin occurrence followed a linear relationship with texture phenotype and gene dosage. All of these improvements in technique, though often appearing trivial, are still useful today in studying the occurrence of friabilin and characterization of unknown genotypes.

The unique extraction and solubility characteristics of friabilin prompted further studies regarding the interaction of friabilin with the starch granule surface. Greenblatt *et al.* (1992, 1995) showed that the interaction exhibited both ionic and hydrophobic characteristics. The combination of propan-2-ol and salt were required to remove friabilin from the granule surface. Pre-extraction with propanol/water followed by Tris-salt was effective, whereas pre-extraction with hexane was not. Tris-salt and propanol individually were largely ineffective. Rahman *et al.* (1994) and Schofield and co-workers (in Oda and Schofield, 1997) also found that organic solvents and salt interacted in the extraction of friabilin. Greenblatt *et al.* (1992, 1995) and Morris (1995) demonstrated that the occurrence of bound polar lipids follows the same pattern as friabilin. Glycolipids and phospholipids were abundant on the surface of water-washed soft wheat starch but scarce on hard wheat starch. Yet, similar levels were present in soft and hard wheat flours. Greenwell (1992a) found that water-washed starch from soft wheat had ca. 7.5-fold more $CHCl_3$-extractable surface lipid than hard wheat starch.

As studies continued and greater resolution of friabilin was accomplished, it became clear that friabilin was likely composed of multiple proteins. Although Rahman and co-workers reported preliminary results that western blots of 2D-IEF-SDS-PAGE gels identified only a single protein (Jolly *et al.*, 1990), their later paper stated that no polypeptide of the expected molecular mass could be detected (Rahman *et al.*, 1994). RP-HPLC had suggested two poorly resolved GSP proteins (Rahman *et al.*, 1991). Using 2D-NEPHGE and SDS-PAGE, one elongated GSP band from the hard cv. Cook and soft cv. Rosella with a pI greater than 10 was resolved (Rahman *et al.*, 1994). Greenblatt *et al.* (1992) reported preliminary results that indicated that friabilin was a single protein in IEF with a pI of about 7.5 to 8.0. Oda (1994) found four and Oda and Schofield (1997) two major spots

on 2D-NEPHGE-SDS-PAGE that corresponded to friabilins. Sulaiman *et al.* (1993) reported that western blot identified mono-, di-, tri-, and tetrameric forms of unreduced friabilin.

Greenwell (1992a, b, c) and Greenwell and Brock (1993)[2] reported that friabilin is a mixture of 'several' proteins. A major component of friabilin was isolated from starch using 1 M NaCl and purified using Sephadex G50 and sulfopropyl-Sephadex (S-Sephadex) chromatography. This component was termed 'friabilin(basic)' because of its apparent pI of greater than 10 (Greenwell, 1992b). Greenwell (1992c) elaborated on this preliminary report and showed how S-Sephadex chromatography separated three highly basic (pI 11–12) and one neutral (pI 6.5–7.6) proteins from starch of the soft wheat cv. Galahad. NEPHGE indicated that the neutral fraction comprised about 12 bands; HPLC also indicated 12 components. The three basic components were resolved by NEPHGE into 'friabilin(basic-1)', 'friabilin(basic-2)', and 'friabilin(basic-3)'. In terms of pI, they ranged in order from most basic: friabilin (basic-2), -1, and -3. 'Friabiline' and 'puroindoline' were obtained from D. Marion and coincided with friabilin(basic-1) and -3, respectively, on NEPHGE.

Morris *et al.* (1994c) conducted SDS-PAGE with unreduced friabilin and clearly resolved two proteins that differed by only about 0.7 kDa. Jolly *et al.* (1993) had noted a reduction in molecular mass of unreduced GSP on SDS-PAGE indicting the probability of intramolecular disulfide bonds. Proprietary reports by Greenwell (1992b, c) indicated that unreduced friabilin migrated at a molecular mass of 10 kDa.

Amino acid sequence data from several sources shed new light on the friabilin phenomena and supported the fact that friabilin exists in at least two major forms. Direct amino acid analysis was provided by Jolly (1991), Rahman *et al.* (1991), Greenwell (1992b), Oda *et al.* (1992), Jolly *et al.* (1993), Morris *et al.* (1994c), and Oda and Schofield (1997) (Figure 1). What soon came to the light was the fact that the N-terminal sequence data showed either perfect correspondence or high homology with two Triton X-114-soluble proteins, 'peak 5' and 'peak 7', of Blochet *et al.* (1991). The uniqueness of these sequences provided a strong argument that friabilin and the Triton-soluble proteins were one and the same. The

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[2]Information supplied by Christopher Brock in a typed transcript of a presentation made at the annual meeting of the AACC, Miami Beach, FL (Greenwell and Brock, 1993).
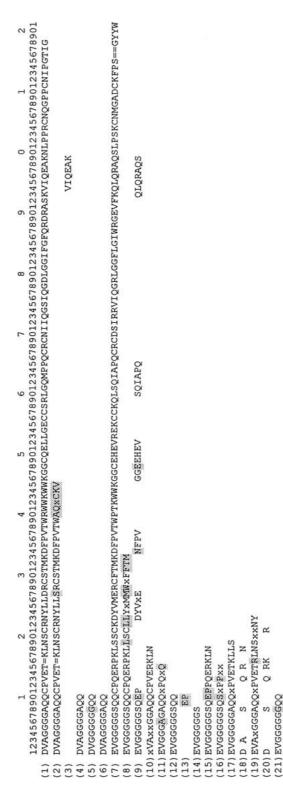
```
                1                   2                   3                   4                   5                   6                   7                   8                   9                   0                   1                   2
      1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
 (1)  DVAGGGGAQQCPVET=KLNSCRNYLLDRCSTMKDFPVTWRWKWWKGGCQELLGECCSRLGQMPPQCRCNIIQGSIQGDLGGIFGFQRDRASKVIQEAKNLPPRCNQGPPCNIPGTIG
 (2)  DVAGGGGAQQCPVET=KLNSCRNYLLSRCSTMKDFPVTWAQxCKV
 (3)                                                                                                                        VIQEAK
 (4)  DVAGGGGAQQ
 (5)  DVGGGGGQQ
 (6)  DVAGGGGAQQ
 (7)  EVGGGGGSQQCPQERPKLSSCKDYVMERCFTMKDFPVTWPTKWWKGGCEHEVREKCCKQLSQIAPQCRCDSIRRVIQGRLGGFLGIWRGEVFKQLQRAQSLPSKCNMGADCKFPS==GYW
 (8)  EVGGGGGSQQCPQERPKLSSCLIYxxMWWxFFTM
 (9)  EVGGGGSQEP            DYVxE          NFPV          GGEEHEV            SQIAPQ                              QLQRAQS
(10)  xVAxxGGAQQCPVERKLN
(11)  EVGGGAGAQQxPQxQ
(12)  EVGGGGGSQQ
(13)              EP
(14)  EVGGGGGS
(15)  EVGGGGGSQEPPQERKLN
(16)  EVGGGGGSQSxPPxx
(17)  EVGGGGGAQQxPVETKLLS
(18) DA       S     QRN
(19)  EVAxGGGAQQxPVETRLNSxxNY
(20) D       S     QRK     R
(21)  EVGGGGGQQ
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Figure 1. Puroindoline amino acid gene sequences. (1) puroindoline a cDNA, Gautier *et al.*, 1994; (2) peak 5, Blochet *et al.*, 1991; (3) peptide 1, Jolly, 1991, Jolly *et al.*, 1993; (4) albumin, Kasarda *et al.*, personal communication; (5) friabilin, Oda, 1992; (6) peak 3, Oda and Schofield, 1997; (7) puroindoline b cDNA, Gautier *et al.*, 1994; (8) peak 7, Blochet *et al.*, 1991; (9) peptides 9, 6, 7, 2, 10, and 4, Jolly, 1991, Jolly *et al.*, 1993; (10) GSP, Jolly, 1991; (11) GSP, Kortt and Rahman (Jolly, 1991); (12) peptide, Kortt and Rahman (Jolly, 1991); (13) ditto, alternate residues; (14) Rahman *et al.*, 1991; (15) GSP Jolly *et al.*, 1993; (16) friabilin(basic), Greenwell, 1992b; (17) friabilin, Morris *et al.*, 1994; (18) ditto, alternate residues; (19) friabilin, Morris *et al.*, 1994; (20) ditto, alternate residues; (21) peak 4, Oda and Schofield, 1997. 'x' denotes unknown residue; '=' introduced gap to improve alignment. Residues not accounted for by the cDNA deduced amino acid sequence of either puroindoline a or b are highlighted.

stage was now set for the next series of advances in our understanding of wheat grain texture.

The discovery of puroindolines

Blochet *et al.* (1991) reported results that would prove to be the crucial link between the friabilin proteins and wheat grain texture. Interestingly, these researchers were studying lipid-binding proteins and the efficacy of different non-ionic and zwitterionic detergents to extract flour lipids, and membrane and/or lipid binding proteins. Triton X-114 (TX114) was chosen as the most desirable detergent due to its unique ability to form phase separations when warmed (Bordier, 1981), and its efficacy at solubilizing polar lipids and proteins. Separation of a <20 kDa TX114-soluble protein fraction from flour of cv. Capitole on RP-HPLC produced six major peaks. Amino acid composition of the first three identified them as the purothionins. The three remaining proteins were subjected to N-terminus amino acid sequencing. Based on the sequence (Figure 1), all were previously uncharacterized. Two, 'peak 5' and 'peak 7', showed high homology. The more abundant of the two species, 'peak 5', was later sequenced in its entirety (Blochet *et al.*, 1993). Due to the presence of a 'tryptophan domain' where five of seven residues (positions 39–45) were tryptophan, it was named 'puroindoline'. Subsequent work by Marion and co-workers (Gautier *et al.*, 1994) isolated two families of cDNA clones using a synthetic oligonucleotide based on puroindoline sequence as a probe. One clone from each family was sequenced in its entirety. One corresponded perfectly to the direct amino acid sequence of Blochet *et al.* (1993) (Figure 1) and was named puroindoline a. The second corresponded to 'peak 7' of Blochet *et al.* (1991) and was named puroindoline b.

Comparison of N-terminus and peptide sequence obtained on friabilin (or GSP) provided convincing evidence that the puroindoline proteins were indeed the primary components of friabilin (Jolly, 1991; Rahman *et al.*, 1991; Greenwell, 1992b; Oda *et al.*, 1992; Jolly *et al.*, 1993; Morris *et al.*, 1994c; Oda and Schofield, 1997). As seen in Figure 1, most sequences were limited to between 10 and 20 residues. Clearly, each can be categorized as representing puroindoline a, puroindoline b, or a mixture of the two proteins. Yet, nearly all show some non-correspondence with either puroindoline a or puroindoline b. Generally these mismatched residues occur near the end of the reported

sequence and may simply reflect the uncertainty associated with a reduction in chromatogram signal as the sequencing reactions proceed.

Some variation in total length of the mature proteins seems to be a common feature of the puroindolines due to post-translational processing (Blochet *et al.*, 1993; Gautier *et al.*, 1994). In addition to two possible cleavage sites for 'pre-protein' processing and possible targeting, variation in both N- and C-terminus of 1–5 amino acids were present in 'mature' proteins. Whether such variation could explain poor resolution in one-dimensional SDS-PAGE and the sometimes large number of spots identified in 2D-NEPHGE is at present unknown. Certainly the number of structural genes of the puroindolines, and possibly closely related proteins, is of great interest. Gautier *et al.* (1994) sequenced two clones for puroindoline a and although both coded for the same polypeptide, one had an additional 51 nucleotides at the 3′ end. Whether this suggests that duplicate genes for puroindoline a exist or simply variable post-translational processing is unknown. However, it is interesting to speculate that gene duplication for puroindoline a might explain the generally greater abundance of this protein relative to puroindoline b. Direct chromatographic data for puroindoline a vs. b were provided by Blochet *et al.* (1991, 1993), Greenwell (1992c), Oda and Schofield (1997), and Day *et al.* (1999).

Grain softness protein (GSP)

For this review it is important to discuss some of the results concerning GSP. As described above, the earlier studies of Rahman and co-workers (Jolly *et al.*, 1990, 1993, 1996; Jolly, 1991; Rahman *et al.*, 1991, 1994) on the protein, which they referred to as 'grain softness protein', can be considered to be directly applicable to friabilin (and, hence, puroindolines). However, during the transition from these direct GSP protein studies to the isolation of GSP DNA clones, there was a departure from what we now define as puroindoline a and b.

Jolly *et al.* (1990) described the isolation of the cDNA clone, pSR3.1, from a λgt11 expression library using antiserum raised against GSP (asGSP). The sequence of this clone reportedly did not match the N-terminus amino acid sequence obtained directly from purified GSP. They stated that this clone detected GSP gene(s) on all three group 5 chromosomes in Southern analysis. A subsequent report (Rahman *et al.*, 1991)

described the isolation of additional cDNA clones and a genomic clone from libraries prepared from the soft wheat cv. Rosella (the original clone was isolated from a library prepared from the hard wheat cv. Timgalen). These later clones were all similarly highly homologous to one another and to pSR3.1, but did not code for the N-terminus obtained on the original GSP.

A more complete description of these studies appeared in a follow-up report (Rahman *et al.*, 1994). Originally, three cDNA clones were isolated from the Timgalen expression library, one of which was designated 'TG15.5'. A portion of this clone was used to re-screen the same library propagated in λgt10, and a number of clones were isolated including SR3.1 (pSR3.1). SR3.1 was used to screen an additional cDNA library prepared from a 'soft Falcon' NIL. Again, several clones were isolated and sequenced. However, none corresponded to the GSP amino acid sequence obtained previously except for a peptide, ARTVQTA, which is not present in either of the puroindolines (Figure 1).

All of the isolated clones were categorized into three 'GSP-1 sub-families' (GSP-1a–1c) (Rahman *et al.*, 1994). The three sub-families varied about 10% from each other at the amino acid level. Translation of these GSP-1 clones indicated about 50% homology with an oat avenin (Fabjinanski *et al.*, 1998) and about 42% homology with puroindoline a (Blochet *et al.*, 1993). The deduced amino acid sequence of GSP-1a and -1b show high homology with the N-terminus of 'peak 6' reported by Blochet *et al.* (1991). Clearly, 'peak 6', which eluted between the two puroindolines, may indeed be a GSP-1.

These and other studies (Turner *et al.*, 1993, 1996, 1999; Gill *et al.*, 1996; Jolly *et al.*, 1996; Giroux and Morris, 1997a; Boyko *et al.*, 1999; Tranquilli *et al.*, 1999; Turnbull *et al.*, 1999) have indicated that three orthologous loci for GSP-1 exist in hexaploid wheat. These genes are located on the distal end of the homoeologous group 5 chromosomes. The GSP-1 locus on 5DS is closely linked to the puroindoline genes and thus is linked to grain texture. Clearly, GSP-1 is closely related to the puroindolines and is a member of the same protein 'superfamily' that includes α-amylase/trypsin inhibitors, the 'CM' proteins, and non-specific lipid transfer proteins (Gautier *et al.*, 1994). Our current model of *Ha*-locus-controlled grain texture involves only puroindoline a and b. No direct or indirect relationship of GSP-1 with grain texture has been demonstrated. In summary, it appears that when considering the studies of GSP (the protein), GSP is equivalent to friabilin. However, when GSP refers to nucleic acid data (i.e. '*GSP-1*'), GSP and puroindoline a and b are not equivalent.

Mutations in puroindolines result in hard kernel texture

Early investigations in my laboratory have been described above. We were struggling (as were others) with the enigma of marked differences in friabilin occurrence on water-washed wheat starch between soft, hard and durum wheats, but similar levels of friabilin in the native endosperm of soft and hard wheats. Further, the friabilin levels on isolated starch were small relative to those found in the endosperm. With the discovery of the puroindolines, we gained new approaches to the problem of wheat grain texture.

Preliminary work in this area focused on the concurrence of friabilin and the TX114-soluble puroindolines during grain development (Giroux *et al.*, 1996). A paper which influenced our research was published in the fall of 1996: Bechtel *et al.* (1996) showed how 'Karl' hard red winter wheat could be made 'soft' simply by freeze-drying kernels from 15–28 days after flowering (up to the point of natural seed desiccation). Other drying treatments resulted in hard texture. Clearly, the way that cellular constituents coalesced during seed desiccation and maturity played a key role in grain texture. From previous work, we considered the amyloplast membrane as the place likely involved in these phenomena and questioned how the puroindolines (i.e. friabilin) might effect these differences in grain texture (Giroux *et al.*, 1996; Giroux and Morris, 1997b; Bettge and Morris, 1997, 2000).

Kota and Dvorak provided us with a set of 83 chromosome 5D recombinant substitution lines (RSLs) constructed using CS and CS with the 5D chromosomes substituted with those of 'Cheyenne' (substitution line designated 'CS(CNN5D)'). CS is a soft wheat and Cheyenne is a hard wheat. In theory, all progeny possess the CS genome except chromosome 5D, which is a random recombination of the CS and Cheyenne chromosomes. Among these lines, the majority of phenotypic variation in grain texture was clearly assignable to the *Hardness* alleles (DeMacon *et al.*, 1993, 1994; Giroux and Morris, 1997a; Morris *et al.*, 1999a). During this time, attempts at differential display with these same soft and hard RSLs proved to be moderately successful (Giroux and Morris, data not shown). Northern analysis using two hard and two

soft RSLs, the soft cultivar Hill 81 and the hard cultivar Wanser demonstrated that puroindoline a and b transcript levels were similar regardless of grain texture (Giroux and Morris, 1997a). These results were consistent with prior protein data that indicated similar levels of friabilin in native endosperm.

We sequenced puroindoline a and b transcripts from Hill 81, Wanser, CS and CS(CNN5D). Although the puroindoline a sequence from all the lines matched that of Gautier *et al.* (1994), a single-nucleotide change was discovered in puroindoline b from the hard cultivar Wanser and the hard CS(CNN5D). The puroindoline b sequence of the soft cultivars Hill 81 and CS matched that reported by Gautier *et al.* (1994). The nucleotide change present in the hard wheats converted a glycine codon at position 46 to a serine. This single-base change was exploited to design discriminating PCR primers for the two types of sequence. The 83 RSLs described above were assayed for the presence of the Gly-46 or Ser-46 puroindoline b sequence using the discriminating PCR technique. Without exception, all hard RSLs possessed the Gly-to-Ser change, whereas all of the soft RSLs possessed the Gly sequence. Based on these results, we designated the soft puroindoline allele sequences as *Pina-D1a* and *Pinb-D1a* in keeping with the revised 'Guidelines for Nomenclature of Biochemical/Molecular Loci in Wheat and Related Species' (McIntosh *et al.*, 1995). The Ser-46 mutation was designated *Pinb-D1b* (Table 1). In that same study (Giroux and Morris, 1997a), the relationship between starch-associated friabilin, grain softness, and puroindoline gene sequence held true among the 83 RSLs without exception. Since no recombination was observed, the maximum linkage distance was 4.28 cM at a 95% confidence level. We considered the locus to be one and the same.

As we extended and tested the hypothesis that wheat grain hardness resulted from the Gly-46 to Ser-46 codon change (which we considered to be a loss-of-function mutation) in puroindoline b, we encountered hard wheats that lacked this sequence change (Giroux and Morris, 1998). Although seven of 11 hard wheats examined did possess the Ser-46 mutation, four did not. Like soft wheats, these four genotypes possessed the Gly-46 puroindoline b sequence. Even though puroindoline a could be generated using PCR from genomic DNA, none of these four genotypes exhibited puroindoline a transcripts in northern analysis. Similarly, close examination of SDS-PAGE of TX114 proteins indicated that these four genotypes lacked puroindoline a protein. Among these genotypes was

Falcon; it proved to lack puroindoline a transcript and protein. This 'puroindoline a null' genotype was examined among the set of Symes' (Symes, 1969) hard and soft NILs in the Heron and Falcon backgrounds. Among all 44 NILs, all but two showed discrete classification of soft texture and presence of puroindoline a protein, and hard texture and absence of puroindoline a. The two exceptional lines proved to be heterogenous for hardness and the components of each followed the same rule (Morris *et al.*, 1998, 20001a; Morris and Allan, 2001). This research identified the second hardness allele in wheat and was designated *Pina-D1b* (Table 1).

To test and validate our texture model we conducted additional surveys of wheat genotypes. A survey of US and Canadian spring wheats found that of 15 hard spring wheats, eight were *Pina-D1b* and seven were *Pinb-D1b* (Morris *et al.*, 1998). More extensive surveys of 343 wheats of northern European origin (Lillemo and Morris, 2000) and 152 wheats of North American origin (Giroux *et al.*, 1998; Morris *et al.*, 1999b, 2000b, 2001b) identified five new hardness mutations (Table 1). In the study of Lillemo and Morris (2000), 34 lines were soft, 18 were *Pina-D1b* and 191 were *Pinb-D1b*. Of the remaining 100 hard wheat lines, 97 possessed a single-nucleotide change in the codon of Leu-60, converting it to Pro. The remaining three lines possessed a single-nucleotide change in the codon of Trp-44, converting it to Arg. These mutations were designated *Pinb-D1c* and *Pinb-D1d*, respectively (Table 1). In the survey of historically significant North American wheats (Morris *et al.*, 2001), 71 hard spring wheats were found to comprise18 *Pina-D1b*, 47 *Pinb-D1b*, and 4 *Pinb-D1c*. The two remaining hard wheats possessed a single-nucleotide mutation in the codon of Trp-39 causing it to be a 'stop' signal (*Pinb-D1e*, Table 1). One spring wheat, 'Utac', which was mixed for grain texture, had the hard component isolated, sequenced and shown to possess a single-nucleotide mutation in the codon of Trp-44, again causing a 'stop' signal (*Pinb-D1f*, Table 1). Of the winter wheat cultivars, 52 of 54 hard wheats all shared the same *Pinb-D1b* mutation present in 'Turkey Red' and 'Kharkof'. Of the two exceptional wheats, one possessed the *Pinb-D1e* allele discovered in the spring lines, the other once again possessed a single nucleotide 'stop' mutation, in this instance in the codon for Cys-56 (*Pinb-D1f*, Table 1). In all these surveys, all soft wheats possessed the 'soft', wild-type sequences of puroindoline a and b. Similarly, all hard wheats exhibited a change in one or the other puroin-

Table 1. Puroindoline a and b grain hardness (*Hardness*) (kernel texture) alleles, kernel phenotype, and the molecular changes in the puroindoline DNA and protein sequence.

Puroincoline locus		Phenotype, molecular change
Pina-D1	*Pinb-D1*	
Pina-D1a	*Pinb-D1a*	soft, wild-type
Pina-D1b	*Pinb-D1a*	hard, puroindoline a null
Pina-D1a	*Pinb-D1b*	hard, puroindoline b, GGC→AGC, Gly-46 to Ser-46
Pina-D1a	*Pinb-D1c*	hard, puroindoline b, CTG→CCG, Leu-60 to Pro-60
Pina-D1a	*Pinb-D1d*	hard, puroindoline b, TGG→AGG, Trp-44 to Arg-44
Pina-D1a	*Pinb-D1e*	hard, puroindoline b null, TGG→TGA, Trp-39 to stop codon
Pina-D1a	*Pinb-D1f*	hard, puroindoline b null, TGG→TGA, Trp-44 to stop codon
Pina-D1a	*Pinb-D1g*	hard, puroindoline b null, TGC→TGA, Cys-56 to stop codon

doline. All of the mutations in puroindoline b involved single-nucleotide base changes. The specific cause of the *Pina-D1b* 'null' mutation is still under investigation (M. Lillemo, personal communication; cf. Digeon *et al.*, 1999).

Dubreil *et al.* (1994, 1998) identified four wheat cultivars that lacked puroindoline a (by ELISA) and cv. Lobo which lacked puroindoline b (by RP-HPLC). These authors stated that 'the lack of puroindoline-b is often obtained for spring cultivars...' (Dubreil *et al.*, 1994). The relationship of these cultivars to the mutations described above are at present only partly known. In Dubreil *et al.* (1998) two of the four cultivars lacking puroindoline a were the Canadian hard red spring wheats 'Prinqual' and 'Glenlea' which were shown by Morris *et al.* (1998) to have the *Pina-D1b/Pinb-D1a* genotype. Day *et al.* (1999) examined the puroindoline proteins by capillary electrophoresis. Of the 11 hard wheat cultivars in their study, five were shown to lack puroindoline a. The genotype(s) of the other hard wheats is unknown. Igrejas *et al.* (2001) reported levels of puroindoline a and b among 40 wheat cultivars. Two cultivars had significantly lower puroindoline contents (ca. 0.04 g/kg) as determined by a puroindoline-specific monoclonal antibody test in ELISA. Linkage studies with puroindoline a detected RFLPs and hardness (Sourdille *et al.*, 1996), and the Gly-46 vs. Ser-46 puroindoline b sequence (Giroux and Morris, 1997) and hardness (Campbell *et al.*, 1999, 2001) have been reported.

Turnbull *et al.* (2000) examined 15 Australian cultivars, of which eight were soft and seven hard. Among the seven hard cultivars, two ('Janz' and 'Halberd') possessed the *Pinb-D1b* mutation. Consistent with our extensive surveys, these cultivars also possessed the 'soft' *Pina-D1a* allele. The other five hard wheats possessed the 'soft' *Pinb-D1a* allele. ELISA showed that of these, the 'Falcon hard' NIL, 'Eagle', and 'Hartog' lacked puroindoline a and would therefore possess the *Pina-D1b* allele (consistent with Giroux and Morris, 1988). The two remaining hard wheats, 'Diaz' and 'Cook', both contain the 'soft' Gly-46 sequence and normal levels of puroindoline a. It will be interesting to see if they possess new or existing hardness mutations. Turnbull *et al.* (2000) stated that these two cultivars are closely related.

Whether or not different hardness mutations provide differences in grain hardness and technological utilization of wheat grain and flour (end-use quality) is of particular interest. Because the different genetic background of different cultivars can greatly influence end-use quality, more precise approaches are desirable (cf. Dubreil *et al.*, 1998; Igrejas *et al.*, 2001). One such approach is to use NILs. Even though NILs exist for *Hardness*, I am not aware of any for individual *ha* alleles. A second approach is to use large populations, such as RILs, RSLs, and doubled haploids. A somewhat less precise approach due to the limited amount of inbreeding was that of Giroux *et al.* (2000), where three breeding populations derived from crossing *Pina-D1b* × *Pinb-D1b* (i.e. hard × hard) wheat genotypes were studied. Whole-grain NIR hardness across the three populations was higher for the *Pina-D1b* genotype. A follow-up study (Martin *et al.*, 2000, 2001) which used 139 *Pina-D1b* × *Pinb-D1b* RILs and grown in replicated field plots in two environments confirmed that those lines lacking puroindoline a (*Pina-D1b*) had harder kernels by SKCS and NIR, lower break flour yields, lower flour yields, lower milling score, lower bread loaf volume, but slightly

lower flour ash. Although the differences were modest compared to the total variation contributed by the two parents and the environment, the differences were nonetheless important. The conclusion to be drawn is that a normal (i.e. wild-type) puroindoline a and mutant puroindoline b are together slightly more effective in softening grain texture than is a normal puroindoline b in the absence of puroindoline a.

Puroindolines in other species

Since the discovery of friabilin, researchers have explored the occurrence of friabilin (and puroindolines) in other species. As noted earlier, puroindolines are members of a large family of related proteins which includes the CM proteins, α-amylase/trypsin inhibitors, non-specific lipid transfer proteins and GSP-1. The delineation of whether or not puroindolines occur in other species becomes an exercise in assessing varying degrees of sequence homology. Just as puroindoline a and b share sequence similarities, so do many of the proteins listed above. The classification of such proteins and genes continues to be debated. Clearly, puroindoline-like proteins in rye (*Secale cereale* L.) are found on water-washed starch granules and likely effect kernel softness in this species in a similar fashion as wheat. Because of the contribution of this rye locus (which we might designate *Pina-R1/Pinb-R1*), triticales (× *Triticosecale* Wittmack) are soft, too. Similarly, puroindoline-like sequences are found in most all of the diploid members of the Triticeae, including *Aegilops tauschii*, the donor of the D-genome in the evolution of hexaploid wheat (Gautier *et al.*, 2000; Lillemo *et al.*, 2000; Morris *et al.*, 2000a).

In addition to puroindolines, GSP-1 is also found near *Ha* on 5DS, as noted above. Studies on *A. tauschii* show that GSP-1 and puroindoline a and b all reside within ca. 100 kb of DNA with puroindoline a between puroindoline b and GSP-1, and closer to GSP-1 (Turnbull *et al.*, 1999). Similar studies using BAC clones from *T. monococcum* showed that in this species the three genes also resided within ca. 100 kb of sequence with puroindoline a and b occurring within a span of 36 kb (Tranquilli *et al.*, 1999). This work also ordered the three genes along the chromosome in the same way.

As noted above, GSP-1 loci are also found in a similar position on the distal end of 5AS and 5BS, yet, no puroindoline genes are present in AB tetraploids or on the A and B genomes of hexaploid wheat (see Giroux

and Morris, 1997; Gautier *et al.*, 2000). Several extensive surveys of the Triticeae have been conducted (Jolly, 1991; Morrison *et al.*, 1992; Gautier *et al.*, 2000; Morris *et al.*, 2000a).

Of specific interest is the recent work on barley (*Hordeum vulgare* L.; Jagtap *et al.*, 1993; Rouvès *et al.*, 1996; Darlington *et al.*, 2000; Gautier *et al.*, 2000), oat (*Avena sativa* L.; Tanchak *et al.*, 1998; Gautier *et al.*, 2000), and AA, BB, SS, and DD diploid taxa (Gautier *et al.*, 2000; Lillemo *et al.*, 2000; Morris *et al.*, 2000a). Gautier *et al.* (2000) make a compelling case for both 'puroindolines' as well as GSP-1-like proteins in oat.

Perhaps the key delineation for comparing these various puroindoline-like sequences will be to assess the extent to which they influence grain texture. In this regard the mutations characterized in the puroindoline b sequence of hexaploid wheat may assist in revealing which portions of the protein are critical for conferring softness. Clearly, the conclusion must be drawn that both puroindoline a and b must be 'functional' to create soft texture. If either is missing or altered, then hard texture results. Yet, we may also consider that these hard hexaploid wheat genotypes have puroindolines that are 'partially functional' since the hardness of these wheats is less than that of durum wheats where puroindolines are completely absent.

One means of gaining greater insight into the role of puroindolines in grain texture is through the analysis of transgenics. Krishnamurthy and Giroux (2001) transformed rice (*Oryza sativa* L.) with puroindolines a and b. Expression of the transgene(s) reduced grain hardness as evidenced by reduced force to crush kernels, reduced starch damage after grinding, and increased proportion of small (<75 μm) particles. The softest transformed rice line was that which expressed both puroindoline a and b. Another means of assessing the relative effects of the puroindolines in grain texture is through the use of NILs. Morris and co-workers (Morris and Allan, 2001; Morris and Konzak, 2001; Morris *et al.*, 2001a) developed additional hard and soft NIL sets, and are in the process of back-crossing all known puroindoline hardness mutations into the soft white spring wheat cultivar 'Alpowa.'

Closing

Considering the thousands of years that man has utilized wheat grain for sustenance and enjoyment, it has been only during the past century that the recognition

and importance of kernel texture (hard versus soft) has been established. With the discovery of puroindolines, breeders, cereal chemists, millers and food processors may take the 'hard-soft' differentiation to a new level: specific puroindoline hardness alleles may better suit certain end-use requirements. And research aimed at characterizing all grain texture variation will no doubt continue for years to come.

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