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ORIGINAL PAPER

Grain characterization and milling behaviour of near-isogenic lines differing by hardness

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Abstract Wheat grain hardness is a major factor affecting the milling behaviour and end-product quality although its exact structural and biochemical basis is still not understood. This study describes the development of new near-isogenic lines selected on hardness. Hard and soft sister lines were characterised by near infrared reflectance (NIR) and particle size index (PSI) hardness index, grain protein content, thousand kernel weight and vitreousness. The milling behaviour of these wheat lines was evaluated on an instrumented micromill which also measures the grinding energy and flour particle size distribution was investigated by laser diffraction. Endosperm mechanical properties were measured using compression tests. Results pointed out the respective effect of hardness and vitreousness on those characteristics. Hardness was shown to influence both the mode of fracture and the mechanical properties of the whole grain and endosperm. Thus, this parameter also acts on milling behaviour. On the other hand, vitreousness was found to mainly play a role on the energy required to break the grain. This study allows us to distinguish between consequences of hardness and vitreousness. Hardness is suggested to influence the adhesion forces between starch granules and protein matrix whereas vitreousness would rather be related to the endosperm microstructure.

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Introduction

Hardness is one of the important factors used to characterize the wheat grain quality and cultivars of common wheats are usually classified into two major classes: hard and soft. Hardness is a whole grain characteristic defined as the grain resistance to applied deformation (Greenaway 1969) and is largely determined by the endosperm properties considering its proportion in grain. It affects tempering conditions, milling behaviour and performance and finally the end-use quality of wheats (Pomeranz and Williams 1990). Starch damage and size of the flour particles were found to generally increase with wheat hardness. Therefore, hard wheat flours are usually used for bread making while soft wheat flours are more suitable for biscuits, cookies and cake production.

Hardness was found to be mainly controlled by one major genetic factor, the *Hardness* (*Ha*) locus, on the short arm of chromosome 5D (Mattern et al. 1973). This is a complex locus that encodes for the puroindolines a (PINa) and b (PINb) (Sourdille et al. 1996) which are basic cystein-rich proteins with a molecular weight around 15 kDa (Blochet et al. 1993). These proteins were found in the starchy endosperm (PINa and PINb) and the aleurone layer (PINb) by Dubreil et al. (1998). Soft texture results from the expression of *Pina-D1* and *Pinb-D1* genes in their wild-type allelic state while hard texture could either be due to a null allele in *Pina-D1* (*Pina-D1b*) or a mutation in *Pinb-D1* (Giroux and Morris 1997, 1998). Several mutations were already reported in *Pinb-D1* locus (Lillemo and Morris 2000). The majority of the historically important hard red winter wheat cultivars of North-America carries the *Pinb-D1b* allele hardness mutation (Morris et al. 2001). The precise biological function of puroindolines is still unknown, and is the subject of continuing research. However, it can be pointed out that, with the exception of the tryptophan-rich region, puroindolines display strong sequence identity with lipid transfer proteins, including similar pattern of disulphide bonds (Le Bihan et al. 1996; Douliez et al. 2000). Hogg et al. (2005), studying the technological value of transgenic lines over-expressing PINa and/or PINb and thus exhibiting increased puroindolines content and decreased grain hardness, described that softer grains gave a lower flour yield but a higher break flour yield as well as a lower protein and ash content in total flour. Furthermore, comparison of *Pinb-D1b* wheats with *Pina-D1b* wheats showed a distinct impact of the allelic variation on softness. Indeed, *Pinb-D1b* wheats exhibited softer grains and better milling value than *Pina-D1b* (Martin et al. 2001). While important progress has been made in the understanding of the genetic origin of hardness, the exact structural and biochemical foundation of hardness is still not clearly understood and hypotheses only rely on protein matrix structure or binding forces between starch and proteins (Simmonds et al. 1973; Stenvert and Kingswood 1977; Darlington et al. 2000).

Milling behaviour could also be influenced by endosperm vitreousness, a characteristic often confused with hardness. Vitreousness is an optical parameter which is mainly influenced by growing conditions rather than genetic factors. Vitreous endosperm has glassy appearance which may, as suggested by Dobraszczyk (2002), be due to a greater protein matrix density than those found in mealy grain.

Most of the methods to assess grain hardness are empirical and based on the overall properties of whole grains. Particle size index (PSI) and Near Infrared Reflectance Spectroscopy (NIRS) methods rely on differences in particle size distribution of ground whole grain meal but the first is based on sieving behaviour whereas the second relies on spectral characteristics. NIRS method was already shown to provide a better discrimination between wheats of different hardness classes (Morris et al. 1999) and displayed values comprised between 0 and 100 (soft wheat score being under 45) whereas PSI scale is narrower and inverted with values between 20 to more than 35 for soft wheats and 1–20 for hard ones. Another method relies on the measurement of the endosperm mechanical properties and was already shown to be in good agreement with wheat milling behaviour (Haddad et al. 1998). Beside these indirect methods, another way to assess hardness could be the use of biochemical markers such as the PINa/PINb ratio that already showed good correlation with PSI score but was not validated for all types of puroindoline mutations (Day et al. 1999, 2001).

Wheat breeding programs in France are aimed at selecting new highly productive cultivars with very diversified grain quality characteristics. Therefore, the lines used as genitors in INRA wheat breeding programs are widely variable for their grain composition and hardness. Thus, near-isogenic-lines (NILs) that differ in their allelic state (*Ha*, soft vs. *ha*, hard) enable to investigate the hardness characteristics against a comparatively uniform genetic background. In this study, the development of two sets of NILs differing by hardness and exhibiting a high agronomic value is described and their grain characteristics, endosperm properties and milling behaviour are investigated.

Materials and methods

Plant material

Among INRA breeding material grown in Clermont-Ferrand (France) during year 2003, F_5 progenies from two crosses, CF9825/DI9812 and VM9203/Ornicar, were investigated. Each F_5 progeny corresponded to 30–40 plants derived from a single F_4 plant. The original parental lines DI9812 (hard type), CF9825 (soft type) and VM9203 (hard type) are inbred lines with a high degree of homozygosity selected in the INRA breeding programs and 'Ornicar' is a soft cultivar registered on the official list in 1997 (CC Benoist company, Orgerus, France). F_5 progenies were plant per plant analysed in order to identify advanced progenies segregating for hardness, i.e. with residual heterozygosity in the region of the *Ha* cluster. Two cases were revealed (Fig. 1):

1. A very small "between-plants within-progeny" phenotypic variation for hardness was observed and thus considered as non-genetic variation for hardness (for example, progenies 1007 and 1257 or

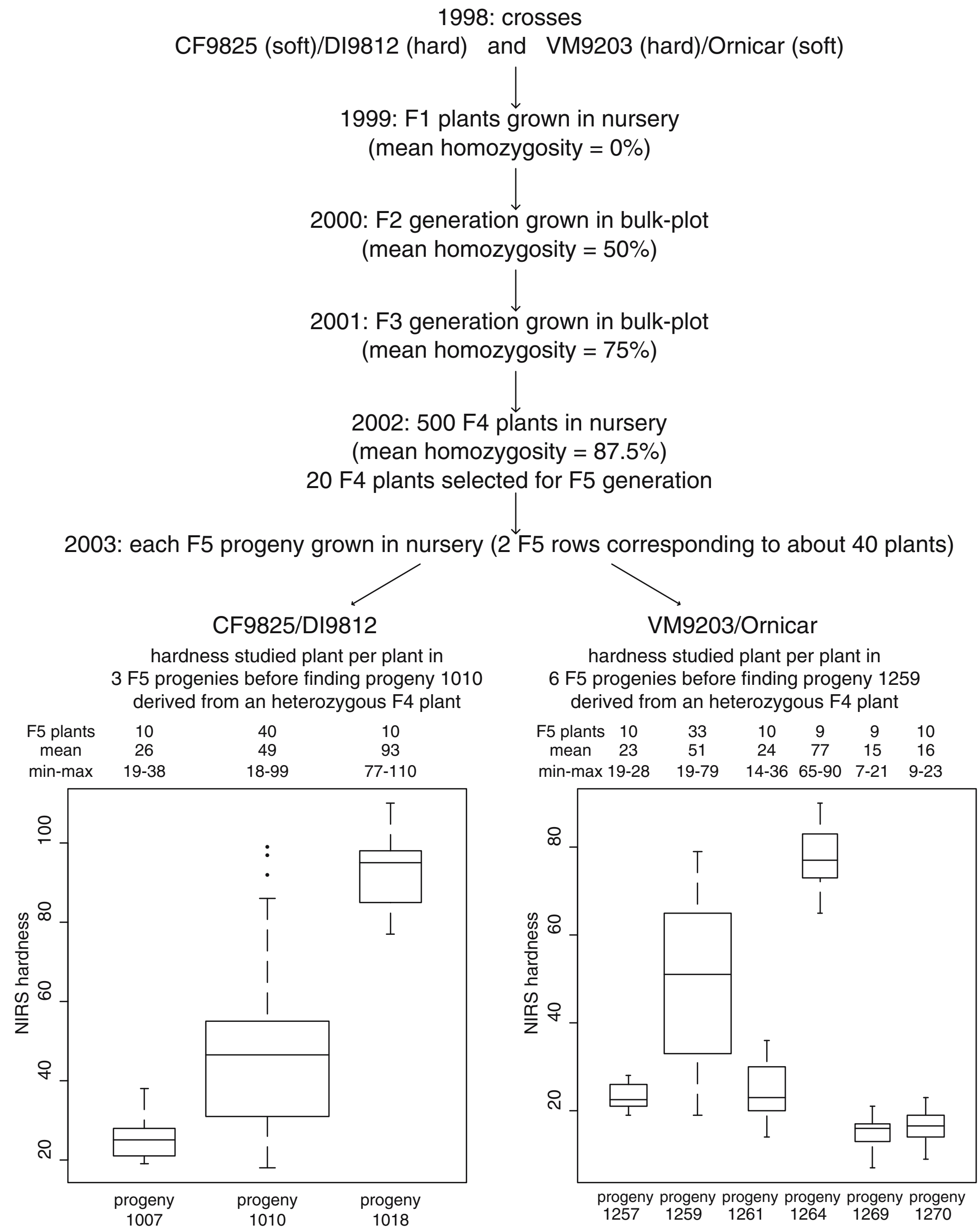

Fig. 1 Scheme of development of near-isogenic-lines (*NILs*) for hardness within breeding populations. Each F_5 progeny was identified by the name of the F_4 plant from which it was derived. Minima, maxima and mean hardness values and number of plants are noted for each progeny

progenies 1018 and 1264 which appeared respectively fixed for the *Ha* or the *ha* alleles). In this case only ten plants per progeny were analysed among all the harvested plants,

2. A very large "between-plants within-progeny" phenotypic variation for hardness was observed in the first ten plants studied and was considered as a clear genetic variation for hardness due to segregation of hardness genes. It was the case for progenies 1010 and 1259 (Fig. 1), and then all the harvested plants (30–40 plants) were analysed with the aim to cover the total "within progeny" genetic variation.

During year 2004, selfed F_6 derived from F_5 progenies 1010 and 1259 were grown in Clermont-Ferrand (one F_6 row for each F_5 plant studied in 2003), with two aims:

- F_6 progenies derived from F_5 plants classified in the extreme zones of the distribution (Fig. 2) were

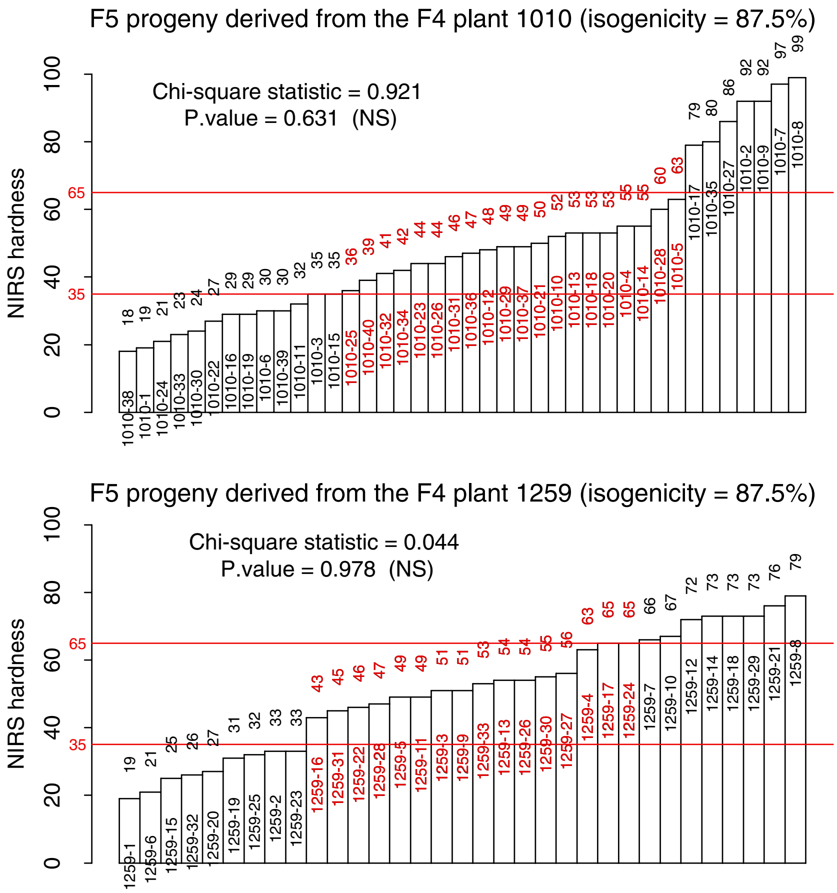

Fig. 2 Grain hardness distribution in F_5 progenies derived from heterozygous *Ha/ha* F_4 plants from the two crosses DI9812/CF9825 and VM9203/Ornicar and results of the Chi-square test comparing the empirical proportions of *Ha/Ha*, *Ha/ha* and *ha/ha* types with the 25–50–25% theoretical proportions. Names of each plant and hardness scores are specified in and up the barplot. Plants below 35 were homozygous *Pinb-D1a/Pinb-D1a*, plants above 65 were homozygous *Pinb-D1b/Pinb-D1b* and plants between 35 and 65 were heterozygous *Pinb-D1a/Pinb-D1b*

considered as homozygous *Ha/Ha* or *ha/ha*, and constituted the near-isogenic-lines. As these NILs were derived from the same F_4 plant, the theoretical isogenicity of this material was 87.5%. Grains from these F_6 rows were harvested and used for seed multiplication and hardness study.

- F_6 progenies derived from F_5 plants classified in the intermediary region of the distribution (Fig. 2) were supposed heterozygous *Ha/ha*, and then were used to increase isogenicity through more recombinational events.

During year 2005, this scheme was continued. Selfed F_7 derived from the different batches of F_6 NILs were multiplied in nursery rows and microplots, the typical size of these plots being 7 m^2.

In this study, among a number of couples of NILs available (Fig. 2), we focused on the couple 1010-33 (soft)/1010-2 (hard) for progeny 1010 (NILs-A), and the couple 1259-15 (soft)/1259-18 (hard) for progeny 1259 (NILs-B). These NILs were all of the generation with a theoretical 87.5% isogenicity. These analysed samples were chosen based on their hardness differences in 2003 and their characterisation with molecular markers. All the investigations presented were made on grains from NILs-A and NILs-B grown in Clermont-Ferrand and harvested in 2004 (selfed F_6) or in 2005 (selfed F_7).

Each year, head-rows of each progeny were compared for their plant phenotype all along their growth, to judge the similarities between hard and soft sister lines. Characters like heading stage and height were measured. Agronomic value of the NILs was also evaluated with the micro-plots in 2005.

Analytical methods

Molecular markers

To characterize the plant material for the hardness locus, we used the molecular markers specific to the *Pinb-D1a* versus *Pinb-D1b* allele alternatives. The *Pinb-D1b* mutation is known to modify PINb with a change from Gly-46 (soft type) to Ser-46 (hard type) (Giroux and Morris 1997). These co-dominant markers allowed us to clearly distinguish between the two homozygous *Pinb-D1a/Pinb-D1a* (wild-type) and *Pinb-D1b/Pinb-D1b* (mutation) and the heterozygous type *Pinb-D1a/Pinb-D1b*.

Thousand kernel weight and vitreousness

Wheat grains were all characterized for their thousand kernel weight (TKW) (AFNOR NF V03-702, Dec. 1981). Grain vitreousness was assessed by analysis of kernel cross-sections (obtained with a Pohl kernel cutter, Versuchs und Lehranstalt für Brauerei, Berlin, Germany) and expressed by the percentage of vitreous versus mealy grains.

Grain moisture, proteins and ash contents

Moisture, grain protein content (GPC) and ash content of the grains were determined according respectively to approved method 44-19 (AACC 2000), NF V18-120 (1997) and 08-12 (AACC, 2000), respectively.

NIRS and PSI hardness measurement

Grain hardness was evaluated with a Percon NIRS apparatus (approved method 39-70A, AACC 1995). As it is a destructive method a small sample of grains was milled for a plant to plant approach and a few non-milled grains were saved to preserve the progeny.

PSI was determined according to the adapted AACC method 55-30 (Le Brun et al. 1988). Twenty grams of each wheat sample were milled on a KT30 grinder and the product obtained was sieved for 10 min using 75 μm sieve (LS-PRO, Hosokawa Alpina). PSI corresponds to the percentage mass of particles able to pass through the sieve.

Tempering and micromilling

One hundred grams of cleaned wheat grains from each sample were hydrated to 15.0% (w/w) moisture content and tempered for 24 h (including 1 h of agitation) before milling. Grains were milled according to the process already described by Bar L'Helgouac'h et al. (2004). Milling diagram included two breaking stages, one sizing and one reduction stages and gave four flour fractions, coarse and fine bran fractions as well as two shorts fractions. Furthermore the micromill was equipped with on-line torque transducers to measure mechanical energy consumption at each stage. Energy needed to produce 1 kg of break flour can be calculated and is represented by the K' index (kJ/kg of flour) (Pujol et al. 2000).

Particle size distribution of flour

Particle size distribution of the flours were obtained from a laser beam particle size analyser (Coulter Co., Miami, FL). Distribution is expressed as the proportion in volume for the different classes of particle size (from 0 to 1,000 μm).

Mechanical properties measurements

Preparation of endosperm test samples

Mechanical tests were conducted only on the NIL-A hard and soft wheats harvested in both 2004 and 2005. Parallelepipedal test samples were prepared by abrasion method as already described by Haddad et al. (1998). Samples were equilibrated at 17% moisture content by leaving them in balanced conditions with saturated saline [NaCl] solutions at 25°C for 3 days (i.e. 75% relative humidity) as already described in Haddad et al. (2001). Grains harvested in 2004 were all selected for their mealy appearance whereas grains harvested in 2005 were all selected for their vitreous appearance in order to retain samples which are the most representative of the batches characteristics. At least ten samples were tested for each of the four batches of NILs-A grains.

Mechanical tests

Compression tests were performed using a traction-compression static-machine (Swick 1, Zwick/Roell, Villepinte, France) at strain rate of 0.1 mm/s. Test samples were placed directly beneath the rheometer and data capture rate was 200 points per second. The stress (σ, MPa) according to the strain (ε, %) was plotted as a curve in each test. Young's modulus (E, GPa) was determined as the slope of the stress/strain curve and rupture energy (W, MJ/m^3) was the area of the curve up to the peak force. The coefficient of variation for these three characteristics was around 15%.

Results

Construction and characterization of the wheat near-isogenic lines

Crosses between parental lines exhibiting opposite values for hardness lead to heterozygous *Ha/ha* F_1 plants and the probability to get an heterozygous *Ha/ha* F_4 plant after three generations of selfing without selection is 1/8. This probability is not too low, and effectively we found segregant F_5 progenies derived from such heterozygote F_4 plant in both crosses (Fig. 1).

Hardness distributions for segregating progenies from the two different crosses are presented in Fig. 2.

The NIRS grain hardness of individual plants varied from 18 to 99 and from 19 to 79 for progenies 1010 (NIL-A) and 1259 (NIL-B), respectively. For these progenies, plants with a hardness value lower than 35 were supposed to be homozygous *Ha/Ha*, and plants with a hardness value higher than 65 were supposed homozygous *ha/ha*. This analysis was confirmed using the *Pinb-D1* molecular marker, as plants below 35 were found homozygous *Pinb-D1a/Pinb-D1a* whereas plants above 65 were homozygous *Pinb-D1b/Pinb-D1b*. The selfed progeny of an heterozygous *Ha/ha* F_4 plant is theoretically composed of 25% hard type F_5 plants (*ha/ha*), 25% soft type F_5 plants (*Ha/Ha*) and 50% heterozygous F_5 plants (*Ha/ha*). Chi-square statistics indicated that the null hypothesis of equality of the empirical and theoretical distributions could not be rejected for both crosses (Fig. 2).

In each NILs-A and NILs-B progeny, no clear morphological differences were observed between the hard and soft sister-lines followed year-to-year (2004, 2005). All lines studied showed the same date of heading, i.e. 141 days after January the first whatever the year considered. Furthermore, the plant heights were similar for the two NILs in a same year (90 and 80 cm for 2004 and 2005, respectively). Yields were comprised between 7.9 and 8.27 tons/ha, i.e. between 100.2 and 104.9% of the mean yield obtained with Apache, Caphorn, Charger and Orvantis, the four most cultivated cultivars in France in year 2005, in the same location. Even if this agronomic characterization of the NILs is quite rough (only one site and 1 year for yield evaluation), results indicate that these NILS correspond to bread wheat well-adapted to North-European agriculture.

Hypothetical phenotypic differences between NILs belonging to the same progeny could be due to genetic differences for characters other than hardness, as residual heterozygosity is still of 12.5% in this material. These divergences could be localized either in the region of the *Ha* gene or in a completely different unlinked region. Another hypothesis to explain phenotypic differences could be a pleiotropic effect of the *Ha* gene itself on characters other than grain texture.

Grain characterisation from wheat near-isogenic lines

NILs showed TKW comprised between 33.4 and 38.0 g of dry matter (Table 1). NILs-B displayed slightly smaller grains than NILs-A. However, hard and soft types for NILs-A and NILs-B did not differ significantly in TKW as already shown by Turnbull et al. (2002). Thus, hardness does not influence grain morphology or density.

NIRS scores of grains from hard and soft NILS-A and NILs-B cultivated in 2004 or 2005 were compared in Table 1. NIRS hardness score from hard NILs-A and NILs-B were shown to decrease by 10 or 13 points, respectively, in 2005 compared with 2004 but was not detected by PSI score that did not markedly change. By contrast, soft NILs-A and NILs-B showed increased hardness in 2005 compared to 2004 as assessed by both NIRS and PSI methods. It could be noted that the PSI score obtained for soft NIL-A in 2005 even corresponds to a medium hard class according to Le Brun and Mahaut (1988) and AACC method 55-30. However, since the scale measurement is narrower in PSI method, classification of intermediate hardness wheats could be misleading.

NILs-A vitreousness was shown to be equivalent for hard and soft types but differ largely according to the crop year. For example, in 2005, NILs-A vitreousness was increased by 60% for soft and hard types. However, part of the grains designed as vitreous presented also semi-vitreous endosperms with both vitreous and mealy parts. In both NILs-A and NILs-B, a strong effect of the growing year was observed. If soft NIL-B showed very low vitreousness, NIL-B hard exhibited 30–55% vitreous grains depending on the crop year. Grain protein contents decreased in 2005 compared to 2004 except for soft NIL-B. Furthermore, if NILs-A hard and soft types displayed similar protein contents, those of NILs-B differed both according to hardness type and according to the year.

Table 1 Wheat grain characteristics measured on NILs-A and NILs-B for harvests 2004 and 2005

	NIL-A hard		NIL-A soft		NIL-B hard		NIL-B soft	
	2004	2005	2004	2005	2004	2005	2004	2005
Thousand kernel weight (g d.m.)[a]	38.0	36.9	36.4	36.4	35.0	35.1	33.4	36.5
NIRS hardness [b]	70	60	15	26	67	54	13	16
PSI hardness [c]	14	13	22	19	14	14	24	20
Vitreousness (%)[d]	10	70	0	60	30	55	1	7
Protein content (% d.m.)[e]	10.9	10.1	11.1	10.3	10.8	9.8	9.5	10.2

Coefficients of variations were: [a] <2%, [b] <5%, [c] <10%, [d] <10%, [e] <2%

Milling behaviour

Isogenic wheats were milled with an instrumented micromill which gives a good description of the milling behaviour (Bar L'Helgouac'h et al. 2004).

The instrumented micromill was primarily designed to measure mechanical energy during grain milling (Pujol et al. 2000). K' index corresponds to the energy necessary to produce 1 kg of flour and enables discrimination between the hardness classes. The K' measurement at the first break is the most pertinent factor for all wheat types (Bar l'Helgouac'h et al. 2004). Results shown in Table 2 indicate that, for each analysed crop year, hard type wheats required more energy than soft type wheats to produce the same amount of flour.

Results obtained with the micromill also showed that soft NILs produced more total break flour (mean = 34.3%) than hard NILs (mean = 29.4%) and this is mainly due to the second break stage where soft NILs flour production was greater than those from hard NILs.

Soft NILs also led to greater amounts of total and coarse bran compared to hard NILs but lower amounts of fine bran. Thus, soft wheats not only produced more bran but also bran of larger size than those from hard wheats. Concurrently, hard NILs produced more farina and thus more sizing and reduction flours than soft ones. For hard NILs, the greatest flour yield is obtained at the reduction stage whereas soft NILs could produce as much flour in the second break stage than in the reduction stage. Additionally, hard NILs produced more shorts than soft ones. Therefore, hard and soft isogenic lines showed, respectively, a hard and soft wheat typical milling behaviour (Willm 1995).

The effect of the year of harvest on milling was investigated for NILs-A. As already pointed out grains from NILs-A collected in 2004 or 2005 differed mainly by their vitreousness and to a lesser extent by their protein content. Comparing data from hard and soft NIL-A samples showed an increase of the K' value when grains grown in 2005 were milled compared to grains grown in 2004. These results indicate that an increase in vitreousness results in a higher energy requirement to break wheat grains independently to their hardness. However, the K' value at first break of hard NIL-A appeared to be more influenced by the vitreousness increase than those of soft NIL-A. Indeed, even if hard and soft NIL-A displayed a similar vitreousness increase, the K' value increase was higher for hard NIL-A. In 2005 soft NIL-A displayed even higher K' than found for hard NIL-B in the first year of culture.

In 2005, soft NIL-A produced less first break flour but equivalent amounts of second break flour resulting in slightly lower amount of total break flour. Concomitantly soft NIL-A produced more farina and fine bran but lower amounts of coarse and total bran than in 2004. Hard NIL-A also led to lower coarse and total bran production and higher fine bran production than observed in 2004. A decrease in first break flour production was also observed from hard NIL-A, in 2005, and was even more pronounced than for soft NIL-A. However, concomitantly a greater amount of second break flour is obtained, leading to approximately identical amounts of total break flour than in 2004. Contrary to what was observed for soft NIL-A, hard NIL-A did not show a noteworthy increase of the farina production in 2005.

To summarize, the vitreousness increase seems to have resulted in lower first break flour production, lower coarse and total bran production and higher K' at first break whatever the hardness. Moreover, vitreousness appeared to slightly increase the second break flour production in hard NIL-A but not in soft ones and acted on the farina production in soft wheats but not in hard ones.

The increase in farina production from soft NIL-A was also found to lead to higher amount of sizing and

Table 2 Flours, shorts, bran and farina fractions yield value (% whole grain, w/w) and K' values (kJ/kg flour) for NILs-A (harvests 2004 and 2005) and NILs-B (harvest 2004)

	Hard NIL-A		Soft NIL-A		Hard NIL-B	Soft NIL-B
	2004	2005	2004	2005	2004	2004
K' break 1	108.8	149.1	81.7	106.7	82.9	61.0
Break 1 flour	12.7	9.5	13.0	10.5	11.5	13.0
Break 2 flour	17.0	20.0	21.8	22.0	17.6	22.7
Total break flour	*29.7*	*29.5*	*34.8*	*32.5*	*29.1*	*35.7*
Coarse bran	10.6	7.5	19.1	13.3	9.1	15.8
Fine bran	7.6	8.8	5.5	8.0	7.2	5.5
Total bran	*18.2*	*16.3*	*24.6*	*21.3*	*16.3*	*21.3*
Farina	28.7	29.2	20.2	24.2	29.4	21.4
Sizing flour	14.2	13.9	10.3	11.6	16.9	10.4
Reduction flour	28.7	28.2	23.0	25.2	29.9	24.0
Shorts	9.1	12.2	7.4	9.4	7.7	7.2
Total flour	72.7	71.5	68.1	69.3	75.9	71.0

reduction flour in 2005. This behaviour was not verified for hard NIL-A which did not produce more farina and even showed a slight decrease of sizing and reduction flour production. Furthermore, increased vitreousness gave rise to higher shorts production in 2005 whatever the hardness class.

Total flour yield obtained with the micromill is slightly higher for hard wheats than generally observed (Bass 1988; Greffeuille et al. 2005). However the bulk yield is not always representative of the yield at a given ash content and hardness usually shows little influence on the yield at a given purity (Fowler and De La Roche 1975). Indeed, analysis of the ash content of break and total flours from the two NIL samples show a lower level in soft total flours for a similar ash total amount in grains (Table 3). This suggests that flour yield at a given purity could even be better for soft wheats.

Table 3 Ash content (% d.m.) of grains, first break flour and total flour obtained after micro-milling

	NIL-A hard		NIL-A soft		NIL-B hard	NIL-B soft
	2004	2005	2004	2005	2004	2004
Grain	1.76	1.92	1.73	1.87	1.73	1.77
Break flour	0.41	0.72	0.30	0.53	0.38	0.32
Total flour	0.48	0.68	0.47	0.59	0.57	0.40

Coefficients of variations for Ash content ≤2%

Flour particle size distribution

Figure 3 shows the particle size distribution of flours from NILs B obtained with micromill but similar profiles were observed for NILs A. The particle size distribution of the isogenic wheats corresponds to typical hard and soft wheat flour particle size profile. As usually observed, soft wheat flour mean particle size is lower than those from hard wheats. Furthermore, the size distribution of flour particles from soft wheats is bimodal showing a first population around 25 μm diameter and a second population around 150 μm size.

According to Devaux et al. (1998) the mode at 20–25 μm is representative of the amount of isolated starch granules in the flour. On the contrary, hard wheat flour particle size distribution shows only one peak around 150 μm diameter. Moreover, influence of vitreousness on flour particle size distribution at first break was also examined and reveals a slight effect on flour from soft wheat grains which shows a higher mean particle size (Fig. 4).

Mechanical properties of the starchy endosperm

Mechanical characteristics of the wheat kernels were determined by examination of the stress/strain curves obtained from compression tests of endosperm samples. Only NILs A were analysed as both hardness and

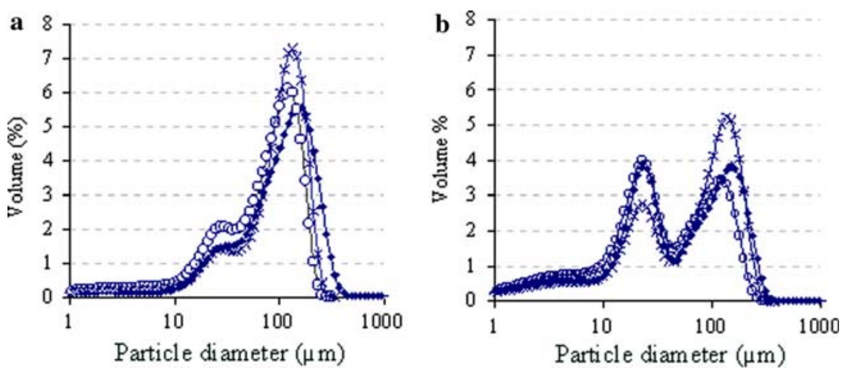

Fig. 3 Particle size distribution of flours from hard (**a**) and soft (**b**) NIL-B obtained at the first break (*filled circle*), sizing (*open circle*) and reduction (*multi symbol*) stages after treatment of the grains with an instrumented micromill already described by Pujol et al. (2000)

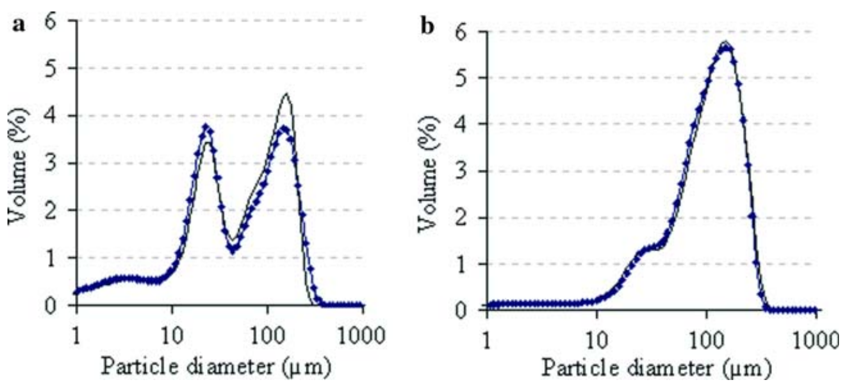

Fig. 4 Particle size distribution of the first break flour from soft (**a**) and hard (**b**) NIL-A harvested in 2004 (*filled circle*) and 2005 (*dashed lines*)

vitreousness effects on endosperm mechanical properties could be studied with these plants. The starchy endosperm exhibited typical elastic mechanical behaviour with fragile type of rupture as already shown for mealy grains (Haddad et al. 1998, 2001). This behaviour characterizes heterogeneous material that breaks because of cracks in the links between components. Neither soft nor hard NILs-A exhibited plastic stage. Endosperms from hard NILs-A exhibited greater E, σ_{max}, ε_{max} and W than soft ones suggesting that greater energy would be necessary to break hard wheat grains (Table 4). Vitreousness influenced mechanical properties leading to higher values of E, σ_{max} and W values whatever the hardness. Additionally vitreousness also led to greater extensibility of endosperm samples from hard type wheats. As a consequence, in 2005, W values were multiplied by 1.9 for endosperm from soft wheats and by 3.4 from hard wheats.

Discussion

Grain characteristics

The overall results show that hardness is a stable genetic factor that could be moderately contrasted by growing conditions. No clear morphological differences appeared between hard and soft type either for NILs-A or NILs-B. Turnbull et al. (2002) already showed that near isogenic grains did not differ in kernel size and weight. There was no pleiotropic effect of the *Ha/ha* alleles on these grain characters. However, as already shown (Pomeranz et al. 1985), the growing conditions could affect the thousand-kernel weight.

The differences in PSI and NIRS scores observed between years for soft NIL-A and NIL-B may result from influences of different factors other than hardness. Among factors that potentially influence hardness score, protein content, growing conditions and vitreousness have already been monitored by several investigations (Miller et al. 1984; Pomeranz et al. 1985; Le Brun and Mahaut 1988; Willm 1995). It was difficult to conclude on the potential effect of the first two factors in this study, and indeed the third factor, vitreousness, is often linked to the two others. Hardness and vitreousness are two factors often mixed up because they both refer to kernel texture. However vitreousness is an optical parameter which is mainly influenced by the environmental conditions especially during the grain filling period. Furthermore, hard grains are generally recognized to be more vitreous than soft ones (Anjum and Walker 1991). All NILs presented markedly show increased vitreousness in 2005 compared to 2004. Thus, the slightly lower PSI scores of soft wheats in 2005 could result from increased vitreousness. However, differences in hardness appeared to be minor compared to the large vitreousness increase so that differences observed on milling behaviour and physical properties of the endosperm between 2004 and 2005 must be attributed to vitreousness.

It has to be pointed out that the vitreousness increase was not accompanied by an increase in the protein content unlike what is usually observed (Matveef 1963; Dexter et al. 1989). However environmental conditions, and more precisely temperature and light intensity, during grain filling and the rate of drying at maturity were also shown to be determinant factors affecting grain vitreousness (Parish and Halse 1968). Thus, the growing conditions, and particularly the high temperature and sunny weather during 2005 harvest may have contributed to high vitreousness level even with no change in the grain protein content.

Milling behaviour, flour particle size distribution and endosperm texture

Measurement of K' allows to obtain indications on the grinding resistance (or mechanical behaviour under grinding process) of kernel whereas the particle size distribution reveals the ability of a wheat to produce fine particles and thus its mode of fracture. In addition, endosperm texture was characterised by compression tests allowing description of its rheological properties. K' at first break indicates that hard NILs wheats require more energy than soft ones to produce the same amount of flour and confirm previous data obtained on cultivated common wheats (Pujol et al. 2000; Kilborn et al. 1982). These results obtained on whole grain well corroborates with the mechanical characteristics measured on the

Table 4 Average values (n = 9–11) and standard deviations (SD) of the NILs-A endosperm mechanical characteristics: Young's modulus (E), maximum stress to rupture (σ_{max}), maximum strain to rupture (ε_{max}) and rupture energy per unit area (W)

	E (GPa)		σ_{max} (MPa)		ε_{max} (%)		W (MJ/m³)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Soft								
2004 mealy	0.39	0.05	11.7	1.4	3.9	0.5	0.28	0.06
2005 vitreous	0.68	0.09	21.7	3.2	3.8	0.5	0.52	0.11
Hard								
2004 mealy	0.52	0.1	23.8	3.5	6,5	0.7	0.96	0.02
2005 vitreous	0.74	0.3	44.1	5.1	11.0	1.1	3.3	0.06

endosperm. Furthermore, K' index and rheological properties of the endosperm were found to be influenced by both hardness and vitreousness whereas particle size distribution was only found slightly affected by vitreousness in soft NIL-A.

Analysis of milling behaviour at the breaking stage and flour particle size distribution showed that harder wheats tend to break in coarser particles referred to as farina whereas soft varieties led directly to fine particles. When kernels from soft wheat are crushed, fracture tends to pass trough the cell and starch–protein interface so that many individual starch granules are released. On the contrary, hard wheats are harder to break and produce larger regular shaped particles, mainly composed of whole endosperm cells (Pomeranz et al. 1990; Campbell et al. 2001). This results in higher break flour yield from soft wheats while hard wheats produce more farina at the breaking stage.

Particle size distribution from hard NIL-A was unchanged in 2005 whereas vitreousness increase led to higher K' values and endosperm mechanical resistance. Thus vitreousness may not affect the particle size distribution of ground hard wheat (=the way the grain fracture), whereas it influences the energy necessary to break the hard and soft grains as already suggested by Pujol et al. (2000). In soft NIL-A, higher vitreousness also acts on K' values and endosperm mechanical properties and to a lesser extent on particle size distribution of first break flour. Thus, soft vitreous grains still contain weakness zones allowing release of individual starch granules but also show reinforced microstructure resulting in higher K' and rheological values except the extensibility parameter.

These overall results highlight the respective effect of hardness and vitreousness on kernel texture and allows to distinguish between the two. Hardness was shown to be a major determinant of the way endosperm breaks and more precisely appears to determine the weakness zones in the endosperm. Thus hardness influences the adhesion forces between starch granules and the surrounding matrix as suggested by Barlow et al. (1973). The greater starch–protein matrix adhesion in hard wheats compared to soft ones would explain both the greater energy necessary to break the grain (illustrated by K' and mechanical parameters measured on the endosperm) and the differences observed in flour particle size distribution. On the other hand, results show that vitreousness affects mainly K' and flour production at first break as well as the rheological parameters especially in hard NIL. Consequently, lack of vitreousness, i.e. mealy state, could be considered as microstructural weakness due to a higher number of fragility points in the endosperm that could explain the decrease in energy necessary to break the grain and higher amount of flour production at first break. As it was furthermore found to be unrelated to the grain protein content, this endosperm weakness could be related to the overall porosity of the endosperm structure as already suggested by other authors (Dobraszczyk et al. 2002; Sadowska et al. 1999; Dexter et al. 1989).

The results obtained also point out the impact of hardness and vitreousness on the other products obtained by the milling process. As expected, hard NILs produce bran in lower amount and with smaller size than bran obtained from soft wheats (Moss et al. 1980; Greffeuille et al. 2005) but production of finer bran particles increases with the grain vitreousness. These differences in bran size distribution could be assigned to the mechanical properties of either starchy endosperm or/and peripheral tissues. Indeed the additional pressure exerted during milling on outer layers by harder starchy endosperm could result in bran size reduction. The K' index at first break showed that hard wheats need more energy to break the grains so that outer layers could be more damaged. On the other hand, differences in bran size could be due to intrinsic properties of outer layers, those from hard wheats being potentially less resistant than those from soft wheats.

Furthermore, results show that hard NILs produced more shorts than soft ones indicating that hard wheats were more difficult to reduce and would need additional reduction stages to complete flour extraction. Vitreousness appeared to emphasize this hardness effect as illustrated by the greater amount of shorts obtained in 2005.

Total flour yield corresponds to the amount of flour produced by milling for a given amount of grains. It is representative of the milling value of wheat even if it does not include purity assessment. Data seem to indicate that hardness positively influences the amount of flour collected after milling independently of the other grain characteristics. However, ash content in total flours showed that soft wheat flours obtained with micromill was generally of greater purity. Then, results suggested that hard wheats present the advantage of giving greater amount of flour while soft wheats lead to flour of greater purity. Similar milling product characteristics were observed with transgenic lines (overexpression of *pina-D1* and/or *pinb-D1*) exhibiting increased puroindolines content and decreased grain hardness that were assessed for their technological values (Hogg et al. 2005).

Conclusions

Near isogenic lines were shown to constitute an interesting material to determine the impact of hardness on grain characteristics and milling behaviour. Clear differences in milling behaviour between hard and soft grains from two different NILs were highlighted and showed that hardness impacts on the way grains break and on the energy necessary to break the grain. Hard NILs need more energy to break the grain as measured by the K' index at first break and produce larger endosperm particles than soft NILs. Soft NILs break more easily and thus produce more flour in the first stages than hard ones. This study also indicates that vitreousness could influence differently the milling value of wheats depending on their hardness. Soft wheats milling behaviour appeared to be improved by increasing vitreousness since this leads to greater flour amount. On the contrary in hard wheat sample used in this study, higher vitreousness appeared to be disadvantageous mainly because farina reduction was more difficult.

Analysis of the overall results allowed us to precise hypothesis on the structural origin of hardness and vitreousness. Hardness appeared to be related to adhesion forces between starch granules and protein matrix and found to influence both the way endosperm breaks and the energy necessary to crush the grain. Vitreousness was suggested to act more on the overall microstructure, a decrease in vitreousness increasing the number of defects and weakness points in the endosperm leading to milling behaviour differences at first break and lower endosperm resistance to rupture. Furthermore, vitreousness seems to affect hard wheat milling behaviour with a greater extent than soft wheat.

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