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ORIGINAL PAPER

# Relationships between wheat grain physical characteristics studied through near-isogenic lines with distinct puroindoline-b allele

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**Abstract**

***Key message*** **Genetic (different forms of puroindoline-b) and environment (through variations in vitreousness), have important effects on wheat grain mechanical properties. The two methods of hardness measurements (NIRS, SKCS) do not give the same information.**

*Abstract* Bread wheat near-isogenic lines differing in hardness, due to distinct puroindoline-b alleles (the wild type, *Pinb-D1a*, or the mutated forms, *Pinb-D1b* or *Pinb-D1d*), were grown for three years in seven sites and under two nitrogen fertilization levels, to study genetic and environmental effects on grain mechanical properties. Two methods, Near-Infrared Reflectance Spectroscopy (NIRS) and Single Kernel Characterization System (SKCS), currently used for grain hardness characterization, were carried out. Grain vitreousness, which is known to affect the grain mechanical behavior but is generally not studied, was also measured, as well as three other characters (Thousand Grain Weight, Test Weight and protein content). The relationships between the different characters were studied. Results revealed a clear effect of the different *Pinb-D1* alleles on NIRS hardness, and a marked impact of the environmental conditions on vitreousness. SKCS hardness was influenced by both *Pinb-D1* alleles and environmental conditions. The relationship between SKCS and NIRS hardness was strong when considering together soft and hard genotypes, but moderate within a class of genetical hardness. Vitreousness had only a weak effect on NIRS hardness, whereas vitreousness and SKCS values were strongly correlated, with two distinct regressions for soft and hard genotypes. Vitreousness was positively related to protein content, especially in the case of hard genotypes, which were able to reach high vitreousness values never observed for soft genotypes.

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## Introduction

The mechanical properties of bread wheat grains are recognized to affect the grinding resistance, the milling behavior, the flour particle size distribution, and the degree of starch damage which impacts the water absorption capacity of flours (Pomeranz and Williams 1990; Haddad et al. 1999, 2001; Greffeuille et al. 2006, 2007).

Two classical methods are generally undertaken to classify wheat grain samples depending on their mechanical properties (Turnbull and Rahman 2002; Pearson et al. 2007). The Particle Size Index (PSI) is based on the particle size distribution obtained after grinding. PSI corresponds to the percentage of particles below 75 µm which have been generated by grain grinding (Williams and Sobering 1986). For convenience sake, near-infrared reflectance spectroscopy (NIRS) is often used instead of PSI (Saurer 1978), as specific wavelengths allow a good prediction equation calibrated on PSI values. NIRS hardness is however expressed

on a larger and inverted scale in comparison with PSI. The Single Kernel Characterization System (SKCS) developed by Martin et al. (1993), represents the second current method. It measures the force required to crush individual grains (Gaines et al. 1996). A hardness index (HI) is then calculated to express the average crushing resistance of the overall grain sample. All these methods allow the identification of two grain hardness classes (the soft and the hard type) with contrasted mechanical behaviors.

Part of the grain mechanical properties are genetically controlled by the Hardness (*Ha*) locus located on the short arm of Chromosome 5D (Chantret et al. 2005). At this locus, two genes (*Pina-D1* and *Pinb-D1*) encode for specific proteins called puroindoline-a and -b, which are suggested to play a role at the interface between starch granules and the protein matrix (Feiz et al. 2009; Pauly et al. 2013). The wild alleles (*Pina-D1a* and *Pinb-D1a*) of these two genes lead to a soft mechanical behavior of the endosperm, whereas deletion or mutations in one of these genes result in grains displaying a hard phenotype (Giroux and Morris 1998; Lillemo and Morris 2000; Beecher et al. 2002; Morris and Massa 2003; Day et al. 2006; Wanjugi et al. 2007). Morris and Bhave (2008) recently reviewed the current mutations found in the puroindoline genes. In Europe, the most frequent mutation leading to a hard wheat grain phenotype concerns the *Pinb-D1* gene (allele *Pinb-D1b*) and corresponds to a point mutation resulting in a Gly to Ser substitution in position 46 (Lillemo and Morris 2000). Two other relatively frequent mutations are represented by the alleles *Pinb-D1c* and *Pinb-D1d*, which correspond, respectively, to a Leu to Pro change in position 60, or to a Trp to Arg change in position 44 (Huang and Röder 2005). In the following, to avoid any confusion, the terms "hard" and "soft" will be reserved to the two classes defined by the alleles at gene *Pinb-D1* (wild allele versus mutant alleles). Indeed, even if there is a good concordance between the two classes defined by NIRS, PSI or SKCS measurements, and the two classes defined by the puroindoline alleles, only the genetical hardness (a discrete variable) is unambiguous. This is not the case for grain mechanical hardness, which corresponds to continuous variables with possible difficulties to define the upper bound of soft type or the lower bound of hard type.

Grain mechanical properties are also known to be influenced by the degree of grain vitreousness (Greffeuille et al. 2006), which can be modulated by environmental factors. Vitreousness is an optical property generally measured by the degree of grain translucence. It has been clearly related to different agro-climatic factors like temperature and light intensity during grain filling, rate of grain drying, drought and level of nitrogen fertilization (Parish and Halse 1968; Sharma et al. 1983; Weightman et al. 2008). It must be noted that there is some confusion, in the literature, between the terms hardness and vitreousness, which are sometimes considered as synonymous.

Even though the literature has clearly demonstrated that alleles at puroindoline genes and vitreousness level are involved in the grain mechanical properties, their relationships with classical mechanical measurement methods used to characterize wheat grain hardness have seldom been explored. It is especially the case for vitreousness, a character rarely measured.

The aim of this study was to establish the respective roles of *Pinb-D1* alleles and environmental conditions in the variation of vitreousness, and in the variation of wheat grain mechanical properties as measured by NIRS or SKCS hardness. It was also to examine the relationships between these three measurements, and the possible influence of some other characteristics as Thousand Grain Weight (TGW), Test Weight (TW) or the protein content on them. To gather a significant dataset, near-isogenic lines (NILs) carrying a distinct allele at gene *Pinb-D1* (with hard/soft NILs displaying either the wild allele *Pinb-D1a* or the mutant allele *Pinb-D1b*; and hard/hard NILs displaying either the mutant allele *Pinb-D1b* or the mutant allele *Pinb-D1d*), were grown for three consecutive years in seven distinct sites, with two levels of nitrogen fertilization.

## Materials and methods

### Plant material

Four pairs of NILs differing by the allelic forms at *Pinb-D1* gene were used. Two pairs (1010a/1010b and 1259a/1259b) were produced by Institut National de la Recherche Agronomique (INRA) and displayed the puroindoline-b isoform encoded by either the wild *Pinb-D1a* or the mutated *Pinb-D1b* allele, which, respectively, conferred to grains the soft or the hard phenotype. These two pairs were derived from two different crosses after selection of the two allelic forms at the F6:F7 selfing generation (F7 siblings issued from the same F6 parent plant). For more details on the NILs creation, see Greffeuille et al. (2006). The two other pairs of NILs (VM1b/VM1d and VM2b/VM2d) were produced by Union Française des Semenciers (UFS) and displayed the puroindoline-b isoform encoded by either the mutant allele *Pinb-D1b* or the mutant allele *Pinb-D1d*, and thus were all hard grain phenotypes. UFS NILs were also derived from two different crosses after selection of the two allelic types at the F6:F7 step. All the INRA and UFS NILs expressed the wild allele *Pina-D1a* for puroindoline-a.

Genetic similarity within NILs was confirmed using diversity array technology (DArT) markers (Akbari et al. 2006) generated by Triticarte Pty.Ltd (www.triticarte.com.au). For INRA NILs, it was done with the set of

DArT markers available in 2007 (around 1100 markers), and for UFS NILs with the set available in 2009 (around 2150 markers). DArT markers were also used to assess the genetic variability between the different pairs of NILs.

The French wheat cultivar Soissons (*Pinb-D1d*) was used as a control, leading to a maximum of nine genotypes (eight NILs and one control) in the experimentations.

### Field experimentations

Rainfed trials were conducted for three consecutive years, with seven sites each year, to get a great variability of environmental conditions, maximizing the probability to obtain a large range of grain mechanical properties. Four sites, Clermont-Ferrand (45°46′N/3°04′E), Estrées-Mons (49°52′N/3°00′E), Orgeval (48°55′N/1°58′E) and Rennes (48°06′N/1°40′W), were common to the 3 years. The three other locations were Chartainvilliers (48°32′N/1°33′E), Louville-la-Chenard (48°19′N/1°47′E) and Allonnes (48°19′N/1°39′E) in 2007; Cappelle-en-Pévèle (50°30′N/3°10′E), Estrées-Saint-Denis (49°25′N/2°38′E) and Maule (48°55′N/1°51′E) in 2008; and Auchy-lez-Orchies (50°28′N/3°12′E), Froissy (49°34′N/2°13′E) and Milly-la-Forêt (48°24′N/2°28′E) in 2009.

All the trials corresponded to randomized complete block designs with two replicates. Typical plot sizes were between 7 and 10 $m^2$, with a plant density around 300 plants/$m^2$. The sowing dates reflected common agricultural practices (from early October to late October, according to the location). Crop management methods corresponded to intensive farming, with full insecticide and fungicide protection. Two types of nitrogen fertilization were used: the first one (N treatment) was adjusted to high yield objectives (around 9 t/ha depending on the location); for the second one (N+ treatment), an additional supply of 50 kg/ha was made at flowering, to potentially obtain some variation in vitreousness through an increase in protein content. For a given location, the two replicates of the N trial were near the two replicates of the N+ trial (no difference in soil fertility for the two managements).

Finally, the experimental design was constituted of 42 different environments (each corresponding to one "site × nitrogen fertilization × year" combination).

### Physical characteristics of the grain

For each trial, the two harvest sacks corresponding to the two replicates were bulked into a single sample. Then 500 g of grains were taken, which were used to evaluate all the characters listed below (one measure for each "genotype × site × nitrogen fertilization × year" combination). A consequence of the loss of the replicate information was that the "genotype × N management" interaction could not be statistically tested.

TW was measured according to the AACC Method 55-10 (AACC 2000), and expressed in kg/hl.

TGW was measured according to the AFNOR method NF-EN-ISO520 (www.boutique.afnor.org), and expressed in g.

Grain protein content and NIRS hardness were evaluated using a Percon NIRS apparatus (Inframatic 8620), according to AACC methods 39-35 and 39-70A (AACC 2000), respectively.

Grain vitreousness was assessed by visual analysis of grains cross Sects. (500 grains studied per sample), according to Lasme et al. (2012), and using a Pohl grain cutter (Versucht and Lehranstalt, Brauerei, Berlin, Germany).

A Perten Single Kernel Characterization System (SKCS) 4100 (Perten Instruments North America INC, Springfield, IL) was used to collect data on 100 grains per sample and calculate a mean HI according to AACC method 55-31 (AACC 2000).

As reported in Table 1, it must be noted that all the measurements were not possible on the complete design. First, UFS NILs were not available in 2007, and N+ treatment was absent in site Auchy-lez-Orchies in year 2009. Nine samples were also missing for the control Soissons in year 2007. Moreover, SKCS measurements were only made on a sub-sample of the whole "genotype × site × nitrogen fertilization × year" combinations. Finally, on a theoretical maximum number of 378, our dataset contained 304 measurements for TGW, TW, protein content, NIRS hardness and vitreousness, and 173 measurements for SKCS HI.

### Statistical analyses

All statistical analyses were made using R (R Development Core Team 2009).

Analyses of variance (ANOVA) were run with type II sum of squares required in the case of unbalanced datasets. Tukey's test was used for comparisons of means.

Pair-wise comparison of two linear regressions was done using a Fisher-test (Tomassone et al. 1983).

Multivariate analyses were performed for the two response variables SKCS HI and vitreousness, using Partial Least Squares (PLS) regressions. PLS regression enables to avoid the problems encounter in multiple linear regression when the predictors are correlated or when the number of predictors is high (Tenenhaus et al. 1995; Tenenhaus 1998).

## Results

### Genetic diversity among the studied genotypes

Using DArT markers covering the whole genome, it appeared that differences between the two lines in each of

**Table 1** Data available for the different characters, within the total experimental design including three years, seven sites each year, and two nitrogen treatments for each site

| Genotypes | 2007 | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CF | | EM | | OR | | RE | | AL | | CH | | LO | |
| | N | N+ | N | N+ | N | N+ | N | N+ | N | N+ | N | N+ | N | N+ |
| Soissons | | ● | | ● | | | | ● | | | | ● | | ● |
| 1010a | ● | ● | ● | ● | ○ | ○ | ● | ● | ○ | ○ | ● | ● | ● | ● |
| 1010b | ● | ● | ● | ● | ○ | ○ | ● | ● | ○ | ○ | ● | ● | ● | ● |
| 1259a | ● | ● | ● | ● | ○ | ○ | ● | ● | ○ | ○ | ○ | ○ | ○ | ○ |
| 1259b | ● | ● | ● | ● | ○ | ○ | ● | ● | ○ | ○ | ○ | ○ | ○ | ○ |
| Total/site | 9 (9) | | 9 (9) | | 8 (0) | | 9 (9) | | 8 (0) | | 9 (5) | | 9 (5) | |
| Total 2007 61 (37) | | | | | | | | | | | | | | |
| | 2008 | | | | | | | | | | | | | |
| | CF | | EM | | OR | | RE | | CA | | ED | | MA | |
| | N | N+ | N | N+ | N | N+ | N | N+ | N | N+ | N | N+ | N | N+ |
| Soissons | ● | ● | ○ | ○ | ● | ● | ○ | ○ | ● | ● | ○ | ○ | ● | ● |
| 1010a | ● | ● | ● | ● | ○ | ○ | ● | ● | ○ | ○ | ○ | ○ | ○ | ○ |
| 1010b | ● | ● | ● | ● | ○ | ○ | ● | ● | ○ | ○ | ○ | ○ | ○ | ○ |
| 1259a | ● | ● | ● | ● | ○ | ○ | ● | ● | ○ | ○ | ○ | ○ | ○ | ○ |
| 1259b | ● | ● | ● | ● | ○ | ○ | ● | ● | ○ | ○ | ○ | ○ | ○ | ○ |
| VM1b | ● | ● | ○ | ○ | ● | ● | ○ | ○ | ● | ● | ○ | ○ | ● | ● |
| VM1d | ● | ● | ○ | ○ | ● | ● | ○ | ○ | ● | ● | ○ | ○ | ● | ● |
| VM2b | ● | ● | ○ | ○ | ● | ● | ○ | ○ | ● | ● | ○ | ○ | ● | ● |
| VM2d | ● | ● | ○ | ○ | ● | ● | ○ | ○ | ● | ● | ○ | ○ | ● | ● |
| Total/site | 18 (18) | | 18 (8) | | 18 (10) | | 18 (8) | | 18 (10) | | 18 (0) | | 18 (10) | |
| Total 2008 126 (64) | | | | | | | | | | | | | | |
| | 2009 | | | | | | | | | | | | | |
| | CF | | EM | | OR | | RE | | AU | | FR | | MI | |
| | N | N+ | N | N+ | N | N+ | N | N+ | N | N+ | N | N+ | N | N+ |
| Soissons | ● | ● | ● | ● | ● | ● | ● | ● | ○ | | ○ | ○ | ○ | ○ |
| 1010a | ● | ● | ● | ● | ● | ● | ● | ● | ○ | | ○ | ○ | ○ | ○ |
| 1010b | ● | ● | ● | ● | ● | ● | ● | ● | ○ | | ○ | ○ | ○ | ○ |
| 1259a | ● | ● | ● | ● | ● | ● | ● | ● | ○ | | ○ | ○ | ○ | ○ |
| 1259b | ● | ● | ● | ● | ● | ● | ● | ● | ○ | | ○ | ○ | ○ | ○ |
| VM1b | ● | ● | ● | ● | ● | ● | ● | ● | ○ | | ○ | ○ | ○ | ○ |
| VM1d | ● | ● | ● | ● | ● | ● | ● | ● | ○ | | ○ | ○ | ○ | ○ |
| VM2b | ● | ● | ● | ● | ● | ● | ● | ● | ○ | | ○ | ○ | ○ | ○ |
| VM2d | ● | ● | ● | ● | ● | ● | ● | ● | ○ | | ○ | ○ | ○ | ○ |
| Total/site | 18 (18) | | 18 (18) | | 18 (18) | | 18 (18) | | 9 (0) | | 18 (0) | | 18 (0) | |
| Total 2009 117 (72) Total over years 304 (173) | | | | | | | | | | | | | | |

Empty space: missing value, ○ measurements available for five characters (thousand grain weight, test weight, protein content, NIRS hardness and vitreousness), ● measurements available for six characters (the five precedent ones + SKCS hardness index)

Numbers in parentheses corresponded to the sums for SKCS hardness index

*AL* Allones, *AU* auchy-lez-Orchies, *CA* cappelle-en-Pévèle, *CF* clermont-Ferrand, *CH* chartainvilliers, *ED* Estrées-Saint-Denis, *EM* Estrées-Mons, *FR* froissy, *LO* Louville-la-Chenard, *MA* maule, *MI* Milly-la-Forêt, *OR* orgeval, *RE* rennes, *N* standard nitrogen fertilization, *N+* additional supply of 50 kg/ha at flowering

**Table 2** Genetic similarity assessed with DArT markers for different pairs of genotypes, including the comparisons within and between the different pairs of NILs

| Pairs of genotypes | Number of available DArT markers ($n1$) | Number of differences ($n2$) | Genetic similarity $(1-n2/n1) \times 100$ (%) |
|---|---|---|---|
| 1010a–1010b | 1108 | 15 | 98.6 |
| 1259a–1259b | 1068 | 17 | 98.4 |
| 1010a/b–1259a/b | 1082 | 401 | 62.9 |
| Valoris–Isengrain | 1063 | 286 | 73.1 |
| Renan–Récital | 1030 | 487 | 52.7 |
| VM1b–VM1d | 2113 | 30 | 98.6 |
| VM2b–VM2d | 2167 | 20 | 99.1 |
| VM1b/d–VM2b/d | 2181 | 569 | 73.9 |
| 1010a/b–VM1b/d | 274 | 106 | 61.3 |
| 1010a/b–VM2b/d | 274 | 105 | 61.7 |
| 1259a/b–VM1b/d | 275 | 99 | 64 |
| 1259a/b–VM2b/d | 275 | 97 | 64.7 |

The DArT markers used were not the same for INRA NILs (set of markers available in 2007) and for UFS NILs (set available in 2009), which explained the differences in the total number of DArT markers

the four pairs of NILs never exceeded 1.6 % of the total number of markers (Table 2), far below the theoretical level (3.12 %) of residual heterozygosity at this selfing step. This high genetic similarity enabled us, when differences were observed between the two lines constituting a pair of NILs, to mainly attribute them to an effect of the *Pinb-D1* allele present at the *Ha* locus.

Moreover, the genetic variability between NILs with different origins was consistent with the values commonly observed when comparing cultivars adapted to North-West European agriculture (Table 2). Observed differences between INRA and UFS NILs concerned around 37 % of the markers (35.3 to 38.7 %). In accordance to their diverse genetic origin, differences between NILs 1259a/b and NILs 1010a/b hit 37.1 % of the markers. It was lower in the case of NILs VM1b/d and VM2b/d, which differed for only 26.1 % of the markers. For comparison, differences between cultivars Valoris and Isengrain concerned 26.9 % of the DArT markers, when two cultivars with distant origins, Renan and Récital, differed for 47.3 % of the markers.

## Effects of environmental conditions on the different characters studied

To evaluate the effects of environmental conditions on individual characters, all the available data were considered (304 observations for TGW, TW, protein content, NIRS hardness and vitreousness, and 173 observations for SKCS HI), to take advantage of the largest possible number of environments despite an unbalanced dataset (see Table 1). Results of the analyses of variance with three factors (nitrogen fertilization, year and location nested in year), and their interactions, were reported in Table 3.

For TGW and TW, ANOVA revealed highly significant year and location effects, but no clear effect of nitrogen fertilization. However, the three environmental factors tested appeared much more relevant to explain the variability of TW (adjusted $r^2 = 0.82$, against 0.41 for TGW). For protein content, the three factors had highly significant effects, and the adjusted $r^2$ of the model was quite high (0.65). As expected, N+ treatment appeared efficient to enhance the protein content (on average, the protein content was 11.33 % for N treatment and reached 12.31 % for N+ treatment).

For NIRS hardness, only the year effect appeared significant, and the environmental factors tested were clearly not relevant to explain the variability of the character (adjusted $r^2 = 0.06$). In contrast, ANOVA revealed highly significant effects of year, location and nitrogen fertilization in the case of vitreousness, and the adjusted $r^2$ of the model appeared quite high (0.67). As an illustration (data not shown), vitreousness values were higher in 2008 than in 2007 and 2009, and there were also important differences between sites within each year (for example, low vitreousness values were obtained in 2007, except at Estrées-Mons, and it was the contrary in 2008, with low vitreousness values only obtained at Cappelle). The case of SKCS HI appeared intermediate, with a highly significant year effect, but nitrogen fertilization and location effects only significant at the 5 % level, and an adjusted $r^2$ (0.18) only slightly higher than for NIRS hardness.

## Effects of Pinb-D1 alleles on the different characters studied

To study the effects of *Pinb-D1* alleles, calculations were made independently on the two subsets corresponding, respectively, to INRA NILs (contrast *Pinb-D1a*/*Pinb-D1b*; 164 observations for TGW, TW, protein content, NIRS hardness and vitreousness, and 108 observations for SKCS HI), and UFS NILs (contrast *Pinb-D1b*/*Pinb-D1d*; 86 observations for the first five characters and 64

**Table 3** Analyses of variance with three environmental factors (nitrogen fertilization, year and location nested in year), carried out on the whole dataset for the six characters studied (304 observations for the first five characters and 173 observations for SKCS hardness index). Sample size and elementary statistics (average, range and standard deviation) were also given for all characters

| | *df* | TGW | | TW | | Protein content | | NIRS hardness | | Vitreousness | | *df* | SKCS HI | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | SumSq | Pr (F) | SumSq | Pr (F) | SumSq | Pr (F) | SumSq | Pr (F) | SumSq | Pr (F) | | SumSq | Pr (F) |
| *N* | 1 | 21.84 | NS | 4.52 | (*) | 69.41 | *** | 944.42 | NS | 4214.94 | *** | 1 | 997.97 | * |
| Year | 2 | 874.25 | *** | 651.09 | *** | 23.19 | *** | 19587.14 | *** | 32374.38 | *** | 2 | 9062.69 | *** |
| year (location) | 18 | 1475.97 | *** | 846.79 | *** | 62.87 | *** | 7906.32 | NS | 32,964.76 | *** | 12 | 5618.32 | * |
| *N**year | 2 | 1.48 | NS | 3.40 | NS | 1.42 | (*) | 253.72 | NS | 1399.86 | ** | 2 | 279.26 | ns |
| *N* *year(location) | 17 | 157.75 | NS | 148.30 | *** | 17.34 | *** | 1353.32 | NS | 1619.05 | ns | 12 | 511.07 | ns |
| Residuals | 263 | 2650.57 | | 310.27 | | 78.78 | | 130,739.08 | | 28,942.52 | | 143 | 35,223.18 | |
| $r^2$ | | 0.49 | | 0.84 | | 0.7 | | 0.18 | | 0.71 | | | 0.32 | |
| Adjusted $r^2$ | | 0.41 | | 0.82 | | 0.65 | | 0.06 | | 0.67 | | | 0.18 | |
| Sample size | | 304 | | 304 | | 304 | | 304 | | 304 | | | 173 | |
| Average (range) | | 43.6 (32.1–52.9) | | 79.2 (71–84) | | 11.8 (9.5–14.8) | | 53.6 (2–91) | | 37.6 (3.9–80) | | | 41.4 (2–73) | |
| Standard deviation | | 4.13 | | 2.54 | | 0.92 | | 23 | | 18.28 | | | 17.31 | |

*TGW* thousand grain weight, *TW* test weight, *SKCS HI* SKCS hardness index, *N* nitrogen treatment, *NS* non significant

*** *p* value <0.001; ** *p* value <0.01; * *p* value <0.05, (*) *p* value < 0.1

observations for SKCS HI). Doing so, the two designs were only slightly unbalanced (see Table 1), with less sites considered for SKCS HI (12 among 21 for INRA NILs, and eight among 14 for UFS NILs).

Results of the analyses of variance with three environmental factors (nitrogen fertilization, year and location nested in year), a genetic factor (allele at gene *Pinb-D1*), and their interactions, were reported in Table 4. As they were obtained on a large set of environments, the differences between the average values calculated for the different *Pinb-D1* alleles could be considered as quite good estimates of the additive effect of these alleles, for each character considered.

For INRA NILs and UFS NILs, ANOVA indicated no effect of *Pinb-D1* alleles on TGW, TW or protein content. Consequently, the adjusted $r^2$ obtained with this additional genetic factor were quite similar to those given in Table 3. Turnbull et al. (2002), using another pair of NILs (Heron hard/soft) where the *Pina-D1* gene was deleted or not, also pointed out the absence of relationships between genetical hardness and TGW.

As expected, for INRA NILs, ANOVA revealed a highly significant effect of *Pinb-D1* allele on NIRS hardness and SKCS HI, corresponding to the good concordance between genetical hardness and the two hardness classes classically defined by these two tests. The additional genetic factor induced a great increase in model accuracy, with adjusted $r^2$ higher than 0.92. More interestingly, a highly significant effect of *Pinb-D1* alleles was also found for vitreousness, even though *Pinb-D1b* values for this character were on average only 1.5 times higher than *Pinb-D1a* values (3 times higher for NIRS hardness and SKCS HI).

For UFS NILs, the effect of *Pinb-D1* alleles appeared highly significant for NIRS hardness, and only slightly significant for SKCS HI. In the two cases average values were higher for *Pinb-D1b* than for *Pinb-D1d*, and the increase in adjusted $r^2$ was lower than for INRA NILS. There was no effect of *Pinb-D1* allele on vitreousness, with no change in adjusted $r^2$ when compared to Table 3.

Due to the absence of *Pinb-D1* effect on TGW, TW and protein content, the effects of *Pinb-D1* alleles pointed out above for NIRS hardness, SKCS HI and vitreousness, could be considered as free of any bias due to differences in TGW, TW or protein content.

## Genotype effects on the different characters studied

To avoid any bias due to missing values, genotype effects were studied on a subset of 10 "site × nitrogen fertilization × year" combinations for which all the measurements were available. This subset concerned the sites Clermont-Ferrand (2008, 2009), Estrées-Mons (2009), Orgeval (2009) and Rennes (2009), with the two nitrogen

**Table 4** Analyses of variance with three environmental factors (nitrogen fertilization, year and location nested in year) and a genetic factor (allele at gene *Pinb-D1*), for the six characters studied. Calculations were made separately for INRA NILs (contrast *Pinb-D1a*/*Pinb-D1b*; 164 observations for the first five characters and 86 observations for SKCS hardness index) and for UFS NILs (contrast *Pinb-D1b*/*Pinb-D1d*; 108 observations for the first five characters and 64 observations for SKCS hardness index). Average values obtained by the two lines carrying the same *Pinb-D1* allele were also given

| | *df* | TGW | | TW | | Protein content | | NIRS hardness | | Vitreousness | | *df* | SKCS HI | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | SumSq | Pr (F) | SumSq | Pr (F) | SumSq | Pr (F) | SumSq | Pr (F) | SumSq | Pr (F) | | SumSq | Pr (F) |
| INRA NILs | | | | | | | | | | | | | | |
| *N* | 1 | 3.66 | NS | 0.81 | NS | 40.60 | *** | 124.26 | (*) | 1953.57 | *** | 1 | 209.14 | ** |
| Pinb | 1 | 23.10 | NS | 0.09 | NS | 0.22 | NS | 70280.64 | *** | 6243.92 | *** | 1 | 23466.86 | *** |
| Year | 2 | 295.42 | *** | 444.68 | *** | 15.49 | *** | 2494.58 | *** | 12335.77 | *** | 2 | 5015.94 | *** |
| Year (location) | 18 | 963.49 | *** | 480.28 | *** | 32.06 | *** | 5241.96 | *** | 20750.23 | *** | 9 | 3179.60 | *** |
| *N**Pinb | 1 | 0.25 | NS | 0.73 | NS | 0.53 | NS | 0.31 | NS | 84.59 | NS | 1 | 0.43 | NS |
| *N**year | 2 | 6.32 | NS | 2.97 | NS | 0.48 | NS | 185.56 | NS | 511.05 | *** | 2 | 136.34 | * |
| Pinb*year | 2 | 9.16 | NS | 5.28 | NS | 0.78 | NS | 821.68 | *** | 914.01 | *** | 2 | 504.21 | *** |
| *N**year(location) | 17 | 141.19 | NS | 86.57 | *** | 9.31 | *** | 435.56 | NS | 584.96 | NS | 9 | 72.92 | NS |
| Pinb*year(location) | 18 | 43.18 | NS | 20.72 | NS | 2.17 | NS | 644.70 | NS | 1837.79 | *** | 8 | 217.30 | NS |
| Residuals | 101 | 1356.06 | | 166.37 | | 19.92 | | 4335.57 | | 3339.18 | | 50 | 952.95 | |
| $r^2$ | | 0.52 | | 0.86 | | 0.84 | | 0.95 | | 0.93 | | | 0.97 | |
| Adjusted $r^2$ | | 0.23 | | 0.78 | | 0.74 | | 0.92 | | 0.89 | | | 0.95 | |
| Average *Pinb-D1a* | | 42.3 | | 78.7 | | 11.9 | | 20.2 | | 29.2 | | | 18.9 | |
| Average *Pinb-D1b* | | 41.5 | | 78.8 | | 11.8 | | 61.6 | | 41.5 | | | 52.3 | |
| UFS NILs | | | | | | | | | | | | | | |
| *N* | 1 | 6.88 | NS | 3.88 | * | 26.20 | *** | 440.35 | ** | 2831.54 | *** | 1 | 540.56 | * |
| Pinb | 1 | 0.53 | NS | 0.48 | NS | 1.02 | NS | 1045.33 | *** | 212.91 | NS | 1 | 289.00 | (*) |
| Year | 1 | 55.04 | *** | 95.50 | *** | 13.38 | *** | 5382.99 | *** | 14521.90 | *** | 1 | 885.06 | ** |
| Year (location) | 12 | 409.79 | *** | 290.50 | *** | 25.61 | *** | 2707.52 | *** | 11361.70 | *** | 6 | 2599.44 | ** |
| *N**Pinb | 1 | 0.53 | NS | 0.12 | NS | 0.08 | NS | 20.35 | NS | 5.05 | NS | 1 | 0.56 | NS |
| *N**year | 1 | 0.02 | NS | 0.57 | NS | 0.97 | NS | 0.06 | NS | 356.43 | (*) | 1 | 121.00 | NS |
| Pinb*year | 1 | 5.91 | NS | 0.41 | NS | 0.02 | NS | 88.34 | Ns | 125.20 | NS | 1 | 0.06 | NS |
| *N**year (location) | 11 | 38.52 | NS | 52.44 | *** | 6.41 | NS | 1086.10 | * | 960.98 | NS | 6 | 132.94 | NS |
| Pinb*year (location) | 12 | 13.88 | NS | 3.23 | NS | 1.10 | NS | 164.17 | NS | 321.32 | NS | 6 | 26.94 | NS |
| Residuals | 66 | 289.39 | | 64.32 | | 35.79 | | 2791.15 | | 7961.42 | | 39 | 3960.44 | |
| $r^2$ | | 0.65 | | 0.87 | | 0.68 | | 0.8 | | 0.8 | | | 0.54 | |
| Adjusted $r^2$ | | 0.43 | | 0.8 | | 0.48 | | 0.67 | | 0.67 | | | 0.25 | |
| Average *Pinb-D1b* | | 46.2 | | 79.9 | | 11.8 | | 73.3 | | 44.9 | | | 51.1 | |
| Average *Pinb-D1d* | | 46.3 | | 79.7 | | 11.6 | | 67.1 | | 42.1 | | | 46.9 | |

*TGW* thousand grain weight, *TW* test weight, *SKCS HI* SKCS hardness index, *N* nitrogen treatment, *Pinb* allele at gene *Pinb-D1, NS* non significant

*** $p$ value < 0.001, ** $p$ value < 0.01, * $p$ value < 0.05; (*) $p$ value < 0.1

treatments in each site (see Table 1). Values obtained by the nine genotypes were presented in Fig. 1 for the six characters studied. Tukey's test for comparisons of means was used to underline the significant differences.

With no significant difference within each pair of NILs for TGW, TW and protein content, Fig. 1 confirmed that *Pinb-D1* alleles had no effect on these three characters. It also confirmed indirectly the high isogenicity of the plant material, as already demonstrated in Table 2.

Moreover, Tukey's test also indicated that:

- NILs VM2b/d obtained significantly higher values than other genotypes for TGW, when Soissons and NILs 1259a/b obtained significantly lower values. With average values ranging from 41.1 to 47.7, the genetic variability for TGW appeared quite large among the nine genotypes.
- For TW, values obtained by NILs 1010a/b were slightly above that obtained by the other genotypes. However,

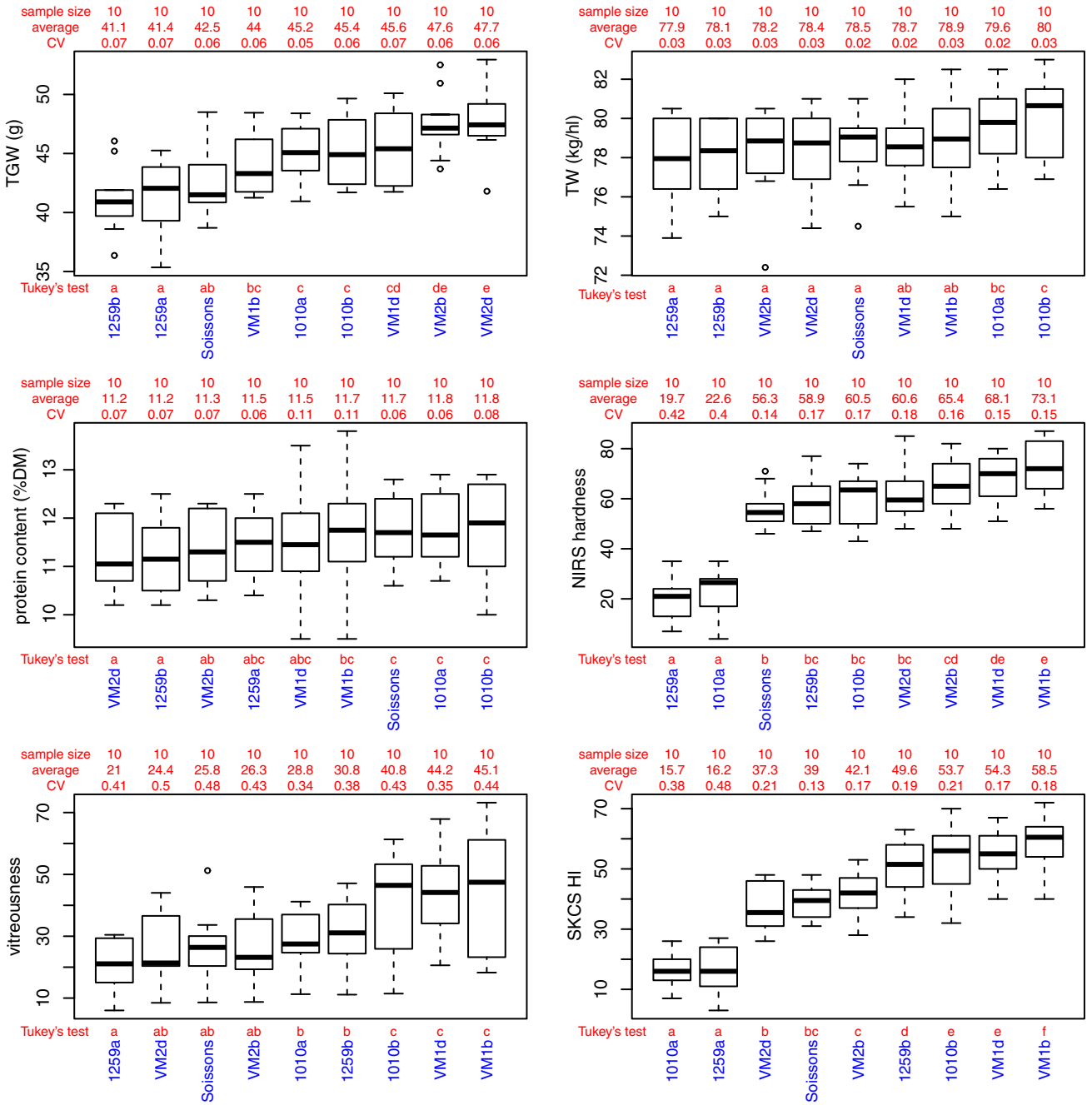


**Fig. 1** Distributions of TGW, TW, protein content, NIRS hardness, vitreousness and SKCS HI for the nine genotypes under study. These distributions concerned a subset of the whole dataset (10 "site × nitrogen fertilization × year" combinations, for which all the measurements were available), and for each character the boxplots were ordered according to increasing average values. Sample size, average value and coefficient of variation (CV) were specified over each *boxplot*

with average values ranging from 77.9 to 80 kg/hl, the variability for TW appeared narrow, all the genotypes presenting rather high TW values.

– With average values ranging from 11.2 to 11.8 %, and a wide overlap between the groups defined by Tukey's test, there were only slight differences between the nine genotypes for protein content.

As expected, a highly significant difference appeared for NIRS hardness and SKCS HI between the two soft lines (1010a and 1259a) and the lines carrying the different mutated *Pinb-D1* alleles (Fig. 1).

For INRA NILs, in the case of vitreousness, the significant effect of *Pinb-D1* allele demonstrated in Table 4, was recovered at the genotype level: values obtained by

lines 1259a and 1010a were significantly lower than values obtained, respectively, by lines 1259b and 1010b. However, vitreousness values obtained by the three hard genotypes Soissons and NILs VM2b/d were not significantly different from that of the two soft lines.

For UFS NILs, the significant effect of *Pinb-D1* allele reported in Table 4 for NIRS hardness and SKCS HI, appeared on Fig. 1 but with reverse intensity: average values obtained by lines VM1b and VM2b were higher than average values obtained, respectively, by lines VM1d and VM2d, but the differences were no more significant in the case of NIRS hardness, and on the contrary became highly significant in the case of SKCS HI. For the two characters, NILs VM1b/d obtained higher values than NILs VM2b/d, but the difference appeared significant only in the case of SKCS HI, which could explain the reverse intensity of the effect of *Pinb-D1* alleles, when observed at the aggregate level (Table 4) and at the genotype level (Fig. 1).

Within the lines carrying allele *Pinb-D1b*, Tukey's test indicated that VM1b obtained significantly higher values for NIRS hardness than VM2b, 1010b and 1259b. Rankings were not the same in the case of vitreousness, for which VM1b and 1010b presented values significantly higher than 1259b and VM2b. For SKCS HI, rankings appeared similar to those observed for vitreousness, but the four lines were considered significantly different from each other according to Tukey's test. Within the lines carrying allele *Pinb-D1d*, VM1d displayed significantly higher values than VM2d and Soissons for NIRS hardness, vitreousness and SKCS HI.

For NIRS hardness, Fig. 1 also indicated that environmental effects could not be completely neglected: for each genotype, there was a quite large dispersion of NIRS hardness values among the 10 environments considered (corresponding CVs were, respectively, around 0.41 and 0.16 for soft and hard lines). Environmental effects appeared to have the same magnitude in the case of SKCS HI: CVs were, respectively, around 0.43 and 0.18 for soft and hard genotypes. For vitreousness, CVs were similar (around 0.4) in the case of soft lines, but higher (around 0.43) for hard lines, which could be related to the fact that, on this subset of 10 environments, lines carrying the *Pinb-D1a* allele displayed maximum vitreousness values around 40, whereas lines carrying the *Pinb-D1b* or *Pinb-D1d* allele had maximum vitreousness values around 70. On the whole, environmental effects appeared greater for vitreousness than for NIRS hardness and SKCS HI, as already seen in Table 3.

## Interactions between genotype and environment for the different characters studied

Analyses of variance with three environmental factors (nitrogen fertilization, year and location nested in year), the genotype factor, and their interactions, were carried out on the whole dataset (304 observations for TGW, TW, protein content, NIRS hardness and vitreousness; 173 observations for SKCS HI). They indicated highly significant ($p$ value <0.001) genotype*year interactions for the six characters studied, and also highly significant genotype*year (location) interactions, except for NIRS hardness (data not shown).

To go further, correlations between the values obtained by the different genotypes for a given character were calculated for all available pairs of environments. Indeed, correlations allow the separation of crossover and non-crossover interactions: high correlations mean that rankings of the genotypes are conserved from one environment to another, indicating low Genotype × Environment (G × E) crossover interactions (independently from the significance of non-crossover interactions). Inversely, provided that the variability present in the observations is sufficient, low correlations indicate high G × E crossover interactions. In our case, this approach was possible for all the characters, except TW, for which the variability was too low (the nine genotypes under study displayed high TW values).

Histograms of the Pearson's correlation coefficients for protein content, NIRS hardness, SKCS HI and vitreousness were presented in Fig. 2. For protein content, the average value of the Pearson's correlations was 0.31, with values ranging from −1 to +1 and a high proportion of negative values, which was consistent with the well known non-stability of this character. For NIRS hardness, the average value was very high (0.96), as well as for SKCS HI (value only slightly lower: 0.9), indicating very low G × E crossover interactions for these two characters.

For vitreousness, there was a very high proportion of positive correlations and the average value reached 0.66, indicating that rankings of the genotypes were relatively stable from one environment to another. In that way, vitreousness appeared quite similar to TGW: for this last character, the histogram looked like the one obtained for vitreousness, with an average value of 0.67 (data not shown).

## Relationships between NIRS hardness, SKCS HI and vitreousness

To avoid sampling effects, and to take into account a maximum of environmental conditions, the relationships between the different characters were studied on the subset of 173 "genotype × site × nitrogen fertilization × year" combinations for which data for SKCS and thus all the six characters was available. Linear regressions between NIRS hardness, SKCS HI and vitreousness were illustrated in Fig. 3. They were calculated on the whole sub-sample, and also for the soft and hard types separately, with a pair-wise comparison of the two linear regressions.

For NIRS hardness and SKCS HI, the determination coefficient appeared quite high ($r^2 = 0.61$), but only for

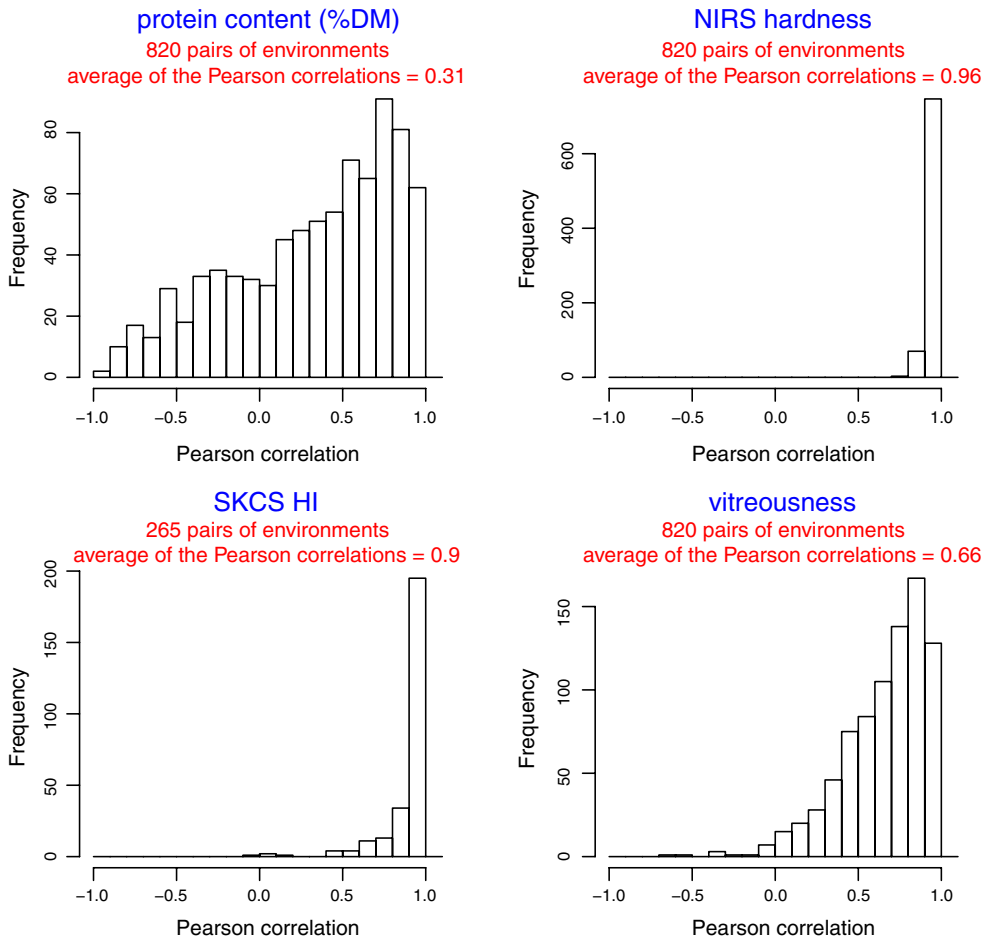


**Fig. 2** Histograms of the Pearson correlations obtained for all of the possible pairs of environments, for protein content, NIRS hardness, SKCS HI and vitreousness (only the correlations calculated with more than two degrees of freedom were retained)

the whole sub-sample. When soft and hard types were distinguished, the correlations became moderate, with $r^2$ values lower than 0.25. It must be noted that NIRS hardness permitted a perfect discrimination between *Pinb-D1a* and *Pinb-D1b* or *Pinb-D1d* genotypes, which was not the case for SKCS HI (indeed, SKCS HI values between 25 and 45 could be obtained whatever the allele at gene *Pinb-D1*).

With weak correlations between the two characters, an increase in vitreousness only led to a slight increase in NIRS hardness values: $r^2$ calculated on the whole sub-sample or for the hard genotypes were lower than 0.2, and for soft genotypes the relationship was only slightly stronger ($r^2 = 0.3$). Vitreousness was not at all efficient for the discrimination between soft and hard types: values ranging from 0 to 60 could be obtained whatever the allele at *Pinb-D1*. However, interestingly in Fig. 3, vitreousness values higher than 60 were only obtained by hard genotypes.

On the contrary, vitreousness had a strong impact on SKCS HI: on the whole sub-sample, the two characters appeared quite highly correlated ($r^2 = 0.54$). Moreover, the correlations increased strongly ($r^2$ around 0.75) when considering separately soft and hard genotypes. The graphic clearly indicated that the soft and hard genotypes which obtained SKCS HI values in the range of 25–45 corresponded more precisely to soft genotypes with quite vitreous grains or to hard genotypes with mealy grains.

It must be noted that whatever the pair of characters considered, the regression calculated for soft genotypes was always highly significantly different from the regression calculated for hard genotypes ($p$ value $\approx 0.01$ for NIRS hardness versus SKCS HI; $p$ value <0.001 for vitreousness versus NIRs hardness or SKCS HI).

## Influence of protein content on the 3 characters related to grain mechanical behavior

The relationships between protein content and NIRS hardness, SKCS HI or vitreousness were presented in Fig. 4. These relationships were illustrated on the same sub-sample ($n = 173$) used for Fig. 3.

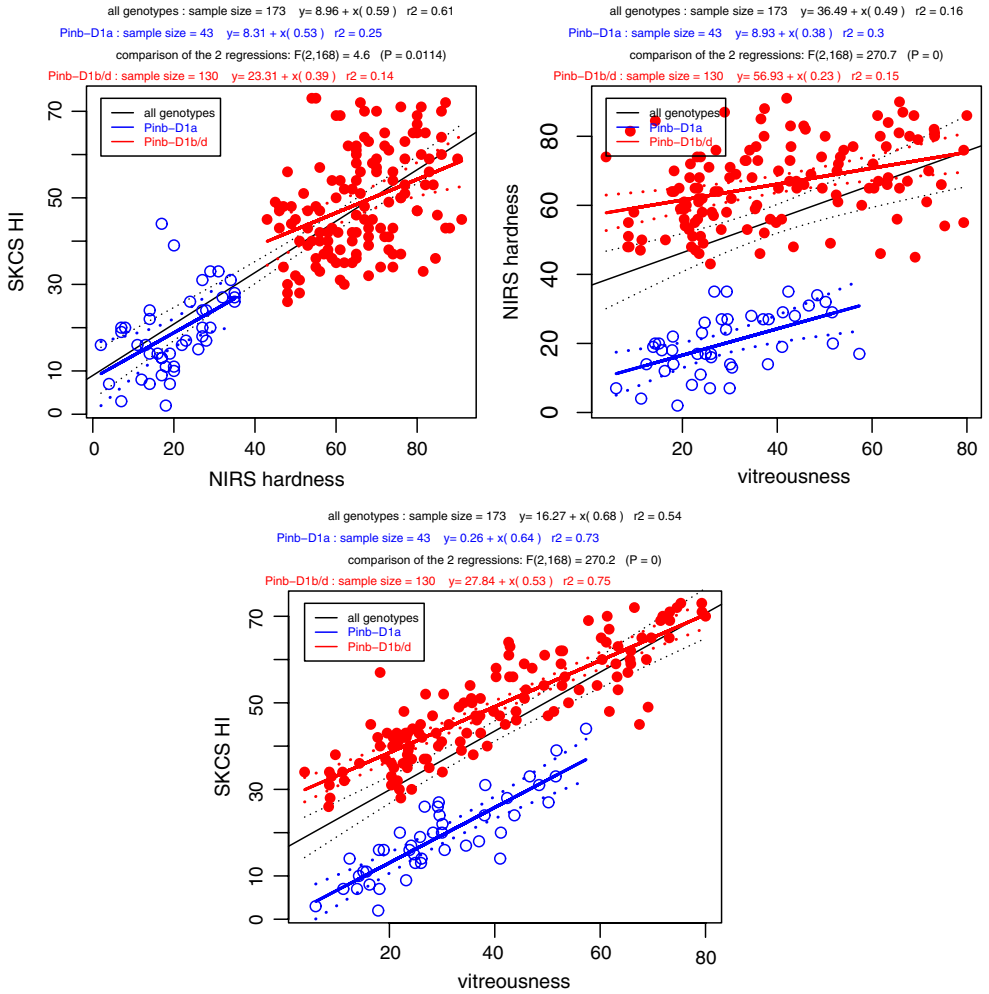


**Fig. 3** Relationships between NIRS hardness, SKCS HI and vitreousness. Equations of the linear regressions were given for the whole sample (in *black*), for soft genotypes (*Pinb-D1a*, plotted in *blue*) and hard genotypes (*Pinb-D1b* or *Pinb-D1d*, plotted in *red*). Results of the Fisher test for the pair-wise comparison of the two regressions corresponding to soft and hard type were also given (color figure online)

A very weak influence of protein content on NIRS hardness was observed. The determination coefficients were near zero when considering the whole sub-sample or only the soft genotypes, and only a low correlation ($r^2 = 0.18$) could be pointed out for hard genotypes, with a slight increase in NIRS hardness for higher values of protein content.

On the contrary, vitreousness appeared quite strongly influenced by the protein content ($r^2 = 0.35$ on the whole sub-sample), but this effect could be split into a weak correlation ($r^2 = 0.15$) for soft genotypes, and a stronger one ($r^2 = 0.42$) for hard genotypes, with a high increase in vitreousness for higher values of protein content. Once more, the particular role of hard genotypes with high values of vitreousness (>60) was highlighted, as these genotypes were also characterized by high protein content values (in most cases >12.5 %), and thus were responsible for the relatively strong correlation.

Relationships between SKCS HI and protein content were slightly higher than in the case of NIRS hardness. Very low correlations were observed for the whole sub-sample ($r^2 = 0.08$) and the soft genotypes ($r^2 = 0.06$), but a less weak correlation ($r^2 = 0.22$) appeared for hard genotypes.

## Multivariate analyses to explain SKCS HI and vitreousness

The strong relationships between vitreousness and SKCS HI (especially when the *Pinb-D1* allele was taken into account), and also between vitreousness and protein content (Figs. 3, 4), incited us to test PLS regressions with different combinations of the grain characteristics as explanatory variables, and SKCS HI or vitreousness as response variables. Some features of these PLS models were given in Table 5.

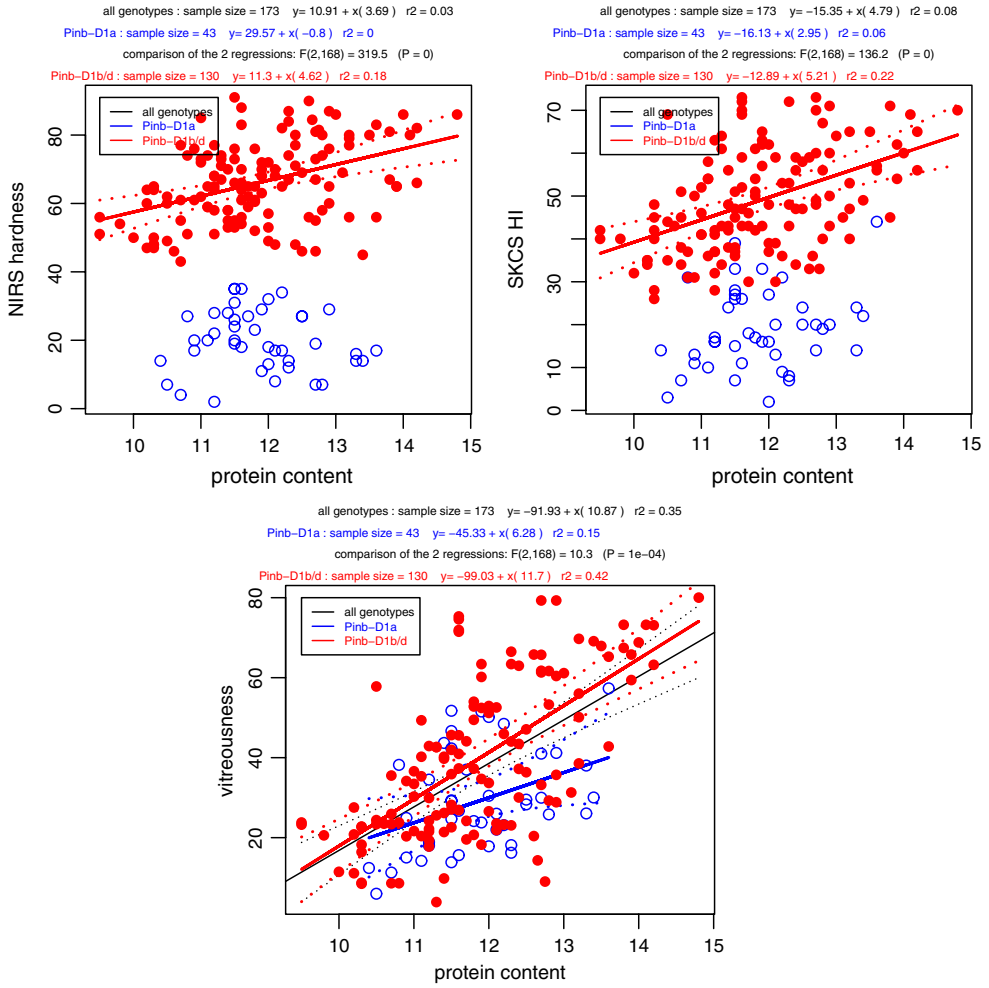


**Fig. 4** Relationships between protein content and NIRS hardness, SKCS HI or vitreousness. Equations of the linear regressions were given for the whole sample (in *black*), for soft genotypes (*Pinb-D1a*, plotted in blue) and hard genotypes (*Pinb-D1b* or *Pinb-D1d*, plotted in *red*). Results of the Fisher test for the pair-wise comparison of the two regressions corresponding to soft and hard type were also given (color figure online)

NIRS hardness and vitreousness appeared as determining predictors for SKCS HI: with these two explanatory variables alone, root mean square error of prediction (RMSEP) was lower than 7.5 and the $r^2$ of the model higher than 0.80. TW was not efficient as an additional predictor, but protein content, TGW and presence/absence of *Pinb-D1a* allele enabled some improvement of the model, leading to a fit with an $r^2$ value of 0.9 and a RMSEP value of 5.51.

For vitreousness, PLS models became acceptable (RMSEP lower than 10 and $r^2$ higher than 0.75) as soon as NIRS hardness, SKCS HI and protein content were associated as explanatory variables. TGW did not improve the model, but TW and the presence/absence of *Pinb-D1a* allele appeared as interesting additional predictors and enabled to reach an $r^2$ value of 0.83 and a RMSEP value of 7.98.

## Discussion

As the production of NILs is not an easy task, our results were obtained on a quite restricted genetic variability (nine genotypes). However, DArT markers indicated (Table 2) that the genetic diversity present in the four pairs of NILs (plus the additional diversity brought by Soissons), was sufficient to enable a pertinent study of the effects of genotype and environmental conditions on wheat grain characteristics.

NIRS hardness was markedly related to the presence of a wild or a mutated form of the puroindoline-b whatever the genetic background (Fig. 1; Table 4). Significant differences in NIRS hardness were also observed between isogenic lines carrying either *Pinb-D1b* or *Pinb-D1d* allele. That confirmed and reinforced the previous results reported in Lasme et al. (2012) obtained on a sub-sample (4 sites in

**Table 5** Characteristics of seven PLS regression models tested to relate SKCS HI or vitreousness to different combinations of explanatory variables

| | PLS regression models (response = SKCS HI) | | | | | | |
|---|---|---|---|---|---|---|---|
| | PLS1 | PLS2 | PLS3 | PLS4 | PLS5 | PLS6 | PLS7 |
| Number of explanatory variables | 2 | 2 | 2 | 3 | 4 | 4 | 5 |
| $r^2$ | 0.64 | 0.58 | 0.82 | 0.84 | 0.87 | 0.85 | 0.90 |
| RMSEP | 10.53 | 11.51 | 7.35 | 6.99 | 6.48 | 6.78 | 5.51 |
| Coefficients of the PLS regression | | | | | | | |
| Intercept | −21.617 | 59.167 | 0.153 | 31.813 | 59.924 | 92.398 | 56.096 |
| Protein content | 2.670 | −4.025 | – | −2.932 | −2.916 | −3.225 | −1.955 |
| NIRS hardness | 0.575 | – | 0.442 | 0.430 | 0.494 | 0.440 | 0.157 |
| Vitreousness | – | 0.811 | 0.464 | 0.564 | 0.500 | 0.605 | 0.550 |
| TGW | – | – | – | – | −0.677 | – | −0.368 |
| TW | – | – | – | – | – | -0.751 | – |
| *Pinb-D1a* | – | – | – | – | – | – | −17.780 |
| | PLS regression models (response = vitreousness) | | | | | | |
| | PLS1 | PLS2 | PLS3 | PLS4 | PLS5 | PLS6 | PLS7 |
| Number of explanatory variables | 2 | 2 | 2 | 3 | 4 | 4 | 5 |
| $r^2$ | 0.45 | 0.62 | 0.70 | 0.76 | 0.76 | 0.80 | 0.83 |
| RMSEP | 14.14 | 11.77 | 10.37 | 9.31 | 9.35 | 8.67 | 7.98 |
| Coefficients of the PLS regression | | | | | | | |
| Intercept | −94.724 | 8.458 | −81.714 | −72.958 | −80.492 | −183.708 | 128.840 |
| Protein content | 9.932 | – | 7.686 | 7.243 | 7.311 | 7.089 | 5.429 |
| NIRS hardness | 0.256 | −0.370 | – | −0.323 | −0.351 | −0.346 | −0.051 |
| SKCS HI | – | 1.176 | 0.666 | 1.007 | 1.037 | 0.981 | 1.128 |
| TGW | – | – | – | – | 0.162 | – | – |
| TW | – | – | – | – | – | 1.459 | 0.664 |
| *Pinb-D1a* | – | – | – | – | – | – | 21.059 |

$r^2$ determination coefficient, *RMSEP* root mean square error of prediction, *TGW* thousand grains weight, *TW* test weight

year 2008) of the presently studied dataset. Moreover, the significant differences observed between genotypes carrying the same allelic form at gene *Pinb-D1* (Fig. 1), indicated that NIRS hardness could also be influenced by some minor genes not located at the *Ha* locus, as already suggested by different QTL analyses (Sourdille et al. 1996; Campbell et al. 1999; Breseghello et al. 2005) which revealed some genomic regions influencing NIRS hardness and not located on chromosome 5DS.

Vitreousness appeared much more dependent on environment than NIRS hardness (Table 3). Within INRA NILs, the lines carrying *Pinb-D1a* allele obtained lower average values for vitreousness than those carrying the mutant alleles *Pinb-D1b* or *Pinb-D1d* (Fig. 1; Table 4). However, differences between the hard and soft lines were considerably lower than for NIRS hardness, and were mainly observed for the sites leading to high vitreousness values (for example, sites from year 2008 except Cappelle–data not shown-). Moreover, some hard genotypes (Soissons and NILs VM2b/d) obtained vitreousness values equivalent to those of soft genotypes (Fig. 1). Thus, *Pinb-D1* could not be considered as a major gene for vitreousness. As it was the case for NIRS hardness, some significant differences appeared between genotypes carrying the same *Pinb-D1* allele (Fig. 1), indicating that genes not situated at the *Ha* locus could have an effect on vitreousness. These genes could be different from the minor genes influencing NIRS hardness, as rankings of the genotypes were not the same for vitreousness and for NIRS hardness. Together with the differences in the effect of gene *Pinb-D1* (major for NIRS hardness; less important for vitreousness), it could suggest quite different genetic determinisms for these two characters. It must be noted that the genes implicated in the low vitreousness values of the hard genotype Soissons should be of some importance, as Soissons is considered as a genotype with a very high milling value.

Like NIRS hardness, SKCS HI appeared strongly influenced by *Pinb-D1* allele (Fig. 1; Table 4), but at the same time was more dependent on the environmental conditions than NIRS hardness (Table 3).

G*E interactions appeared very low for NIRS hardness and SKCS HI. These results confirmed those reported by Gazza et al. (2008) with a set of genotypes including soft and hard types, and also by Hazen and Ward (1997)

with only soft genotypes. Concerning vitreousness, G × E interactions were higher, but rankings of the genotypes appeared quite stable from one environment to another (Fig. 2). Thus, the high environmental effects demonstrated for vitreousness in Fig. 1 and Table 3 (especially for hard genotypes), affected principally the dispersion of the values, leading essentially to non-crossover G × E interactions with only moderate crossover interactions. In that way, vitreousness can be compared to TGW, a quite stable character for which the distinction between large-grain genotypes and small-grain genotypes is usually easy, even though high environmental effects are possible.

A low degree of correlation was pointed out between protein content and NIRS hardness (Fig. 4). The relationship between protein content and SKCS HI was also weak, as already reported by Gazza et al. (2008), with only a slightly stronger regression for genotypes carrying the mutated alleles of *Pinb-D1*. On the contrary, vitreousness appeared quite strongly influenced by protein content, especially in the case of hard genotypes which were the only ones that could obtain vitreousness values higher than 60 (Figs. 1, 3, 4). Most of these hard genotypes displaying high values of vitreousness were also found to display a high protein content (in most cases >2.5 %), as similarly reported in durum wheat by Dexter et al. (1989) and Samson et al. (2005). However, protein content was not sufficient to explain grain vitreousness, and soft genotypes having high protein content (around 13 %) never reached vitreousness values as high as hard genotypes with comparable protein content (Fig. 4). This difference in the vitreousness level reached when protein content increased, could thus reflect a distinct physical organization of the endosperm constituents for soft and hard genotypes, leading to more or less porosity. Interestingly, modelling of the endosperm rupture (Topin et al. 2009) also revealed a different impact of the protein content depending on the adhesion force between starch granules and the protein network. Indeed, for a higher level of adhesion (suggested to be linked to the presence of a mutated puroindoline), the protein content was found to play a crucial role on the proportion of broken bonds in the material and thus on the potential proportion of starch damage, whereas for a low adhesion level a low proportion of broken bonds was observed whatever the protein content.

The relationship between NIRS hardness and SKCS HI appeared clearly weaker within a hardness class, than when soft and hard genotypes were considered together (Fig. 3). This was also pointed out by Morris and Massa (2003). Moreover, only NIRS hardness permitted a perfect discrimination between soft and hard genotypes. These results clearly illustrated the statement from Dobraszczyk et al. (2002) who pointed out that "there appears to be some confusion over what is meant by the term hardness: it has come to have several different meanings depending on the type of test used to measure it".

Actually, our study highlighted an important difference between NIRS hardness and SKCS HI, which concerned their relation with vitreousness. A weak correlation was observed between NIRS hardness and vitreousness (Fig. 3), in agreement with the work of Weightman et al. (2008) who also observed identical ranges of vitreousness values for hard and soft genotypes. On the contrary, a relatively strong relationship was found between vitreousness and SKCS HI. That confirmed previous observations in bread wheat (Orucevic et al. 2007) or in durum wheat (Sissons et al. 1999), but for the first time, our results clearly demonstrated an increase of the positive correlation between vitreousness and SKCS HI, when taking into account *Pinb-D1* allele. Indeed, two highly significantly different relationships appeared for hard and soft genotypes, and a quite constant deviation was observed between the two linear regressions, indicating that for a same level of vitreousness a hard genotype should obtain approximately a SKCS HI value 25 points higher than a soft genotype. Consequently, PLS regression models including NIRS hardness and vitreousness as explanatory variables, enabled a good prediction of SKCS HI (Table 5).

At this stage, we can suggest some explanations to the differences between NIRS hardness and SKCS HI, taking into account both the distinct principles of these two methods used for grain characterization, and the respective influence of genetic and environmental factors on them:

- NIRS hardness is related to the distribution of particle sizes after grinding, independently of the energy required for this grinding, and appears to strongly depend on the nature of the *Pinb-D1* allele (considering the "vitreousness-NIRS hardness" relationship given in Fig. 3, there was a constant deviation of approximately 50 points of NIRS hardness between the regression lines calculated for soft and hard genotypes), with only minor influence of environmental factors. This can be related to the results of Greffeuille et al. (2006), where a change in vitreousness only induced a slight shift in the distribution of flour particle sizes, for the bimodal distribution typical of soft genotypes, as well as for the unimodal distribution characteristic of hard genotypes. Thus, NIRS hardness does not reflect the effects of the environmental conditions which, through the variations in vitreousness, play a role in the grain milling behavior. NIRS hardness can be used to easily distinguish between the two classes of genetical hardness, but is not sufficient to appreciate the grain mechanical properties.
- SKCS HI corresponds to the force required for crushing the grains, and in that way gives a global insight on grain mechanical resistance. This force can be influ-

enced by gene *Pinb-D1*, as the form of puroindoline-b (wild or mutated) is suggested to be involved in the adhesion between starch granules and the endosperm protein network. Figure 3 indicates that this effect of gene *Pinb-D1* corresponds approximately to 25 points in SKCS unit. But this force can also be influenced by vitreousness, which is suggested to be related to the overall porosity of the endosperm structure (Dobraszczyk et al. 2002). For example a mealy state, which is associated to a high porosity, can lead to some endosperm weakness and consequently to a decrease in the necessary force to break the grain. Similar effects of both the nature of *Pinb-D1* allele and environmental conditions (through changes in vitreousness), have already been reported in Greffeuille et al. (2006), with a measurement of the energy during grinding and an assessment of the endosperm rupture curve.

As vitreousness (and consequently SKCS HI) is influenced by the environmental conditions, experimentations restricted to a limited number of environments could lead to results biased by sampling effects (i.e., results reflecting only some peculiarity of the concerned environments). For example, in our study, high vitreousness values appeared specific of hard genotypes (Figs. 1, 3, 4). Thus, environments leading to high levels of vitreousness could induce a clear separation between soft and hard genotypes for vitreousness values. It could be the case for the experiments of Morris and Beecher (2012), where the NILs cultivated in a glasshouse obtained high levels of protein content (around 16 %), probably inducing high levels of vitreousness, and where a quasi complete association was found between the hardness class and vitreousness. It made these authors conclude that the locus *Ha* play a major role in vitreousness, which was not confirmed by our results obtained on a large range of environments.

More generally, as SKCS HI is influenced by both genetic and environment, these two factors must be studied to correctly interpret SKCS values. Otherwise, it could lead to a wrong attribution of the observed effects to the puroindoline form rather than to variations in vitreousness. Genetical hardness is easily obtained through NIRS hardness, or through the determination of the puroindoline form using genomic tools. On the contrary, vitreousness is tedious to measure, and is consequently rarely available, although it could be a character particularly important to consider. Our study indicated that PLS regression models could enable a quite good prediction of vitreousness (Table 5), using different associations of five explanatory variables (protein content, NIRS hardness, SKCS HI, TW and presence/absence of the *Pinb-D1a* allele). Estimated values could therefore potentially replace the time-consuming vitreousness measurements, which generally dissuade millers to characterize this important grain parameter.

## Conclusions

Our study demonstrated that NIRS hardness and SKCS HI gave non-redundant information on wheat grain mechanical properties. NIRS hardness appeared mainly determined by the allelic composition at the *Ha* locus. It permitted a perfect discrimination between grains expressing wild or mutated puroindoline-b, and our results also revealed that *Pinb-D1b* and *Pinb-D1d* alleles induced significant differences in NIRS hardness values. In contrast, SKCS HI was found to not only depend on *Pinb-D1* alleles, but also on environmental conditions. It only allowed a rough discrimination between grains carrying wild or mutated *Pinb-D1* alleles, as a zone of uncertainty existed for values between 25 and 45 which could rather correspond to vitreous grains from soft genotype or mealy grains from hard genotypes. A great difference between these two methods used for grain characterization was found in their relation to vitreousness, which was weak in the case of NIRS hardness and strong in the case of SKCS HI. Vitreousness appeared as a character greatly influenced by environmental conditions, which did not permit to distinguish the two classes of genetical hardness. It could be quite correctly predicted from other grain characteristics through PLS regressions, to avoid time-consuming measurements. Vitreousness was also found to be significantly and positively related to protein content, and differences between soft and hard genotypes were observed for the effects of protein accumulation on the level of vitreousness. This probably suggested differences in the physical organization of the endosperm constituents leading to more or less porosity, which could be interesting to study.

**Author contribution statement** FXO: production of INRA NILs, field experiments, grain characteristics (PMG, PS, NIRS hardness, protein content), data analysis, drafting, research conception. PL: grain characteristics (vitreousness), data analysis, research conception. CM: production of UFS NILs, field experiments, grain characteristics (SKCS). MR: production of INRA NILs, research conception and organization. JA: research conception and organization. VLP: grain characteristics (vitreousness), data analysis, drafting, research conception and organization.

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**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical standards** The authors declare that the experiments described in this manuscript comply with the current laws in France.

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