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Bread wheat milling behavior: effects of genetic and environmental factors, and modeling using grain mechanical resistance traits

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Abstract

Near-isogenic lines of bread wheat that differ in hardness, due to distinct puroindoline-b alleles (the wild-type, *Pinb-D1a*, or the mutated forms, *Pinb-D1b* or *Pinb-D1d*), were grown in different environments and under two nitrogen fertilization levels, to study genetic and environmental effects on milling behavior. Milling tests used a prototype mill, equipped with two break steps, one sizing step and two reduction steps, this enabled 21 individual or aggregated milling fractions to be collected. Four current grain characters, thousand grain weight, test weight, grain diameter and protein content, were measured, and three characters known to influence grain mechanical resistance, NIRS hardness, SKCS hardness index and grain vitreousness (a character affecting the grain mechanical behavior but generally not studied). As expected, the wild-type or mutated forms of *Pinb-D1* alleles led to contrasted milling behavior: soft genotypes produced high quantities of break flour and low quantities of reduction flour, whereas reverse quantities were observed for hard genotypes. This different milling behavior had only a moderate influence on total flour production. NIRS-hardness and vitreousness were respectively the most important and the second most important grain characters to explain milling behavior. However, contrary to NIRS hardness, vitreousness was only involved in endosperm reduction and not in the separation between the starchy endosperm and the outer layers. The highest flour yields were obtained for SKCS values comprised between 30 and 50, which corresponded either to soft vitreous or hard mealy grains. Prediction equations were defined and showed a good accuracy estimating break and reduction flours portions, but should be used more cautiously for total flour.

Keywords: Bread wheat - puroindoline - hardness - vitreousness - SKCS - milling behavior - flour yield.

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), milling tests, data analysis, research conception.

CM: production of UFS NILs, field experiments, grain characteristics (SKCS).

AD: prototype mill conception and building.

OG, EH and BR: field experiments.

MR: production of INRA NILs, research conception and organization.

CBLH: milling tests, flour ash content, research conception and organization.

JA: research conception and organization.

VLP: grain characteristics (vitreousness), milling tests, data analysis, drafting, research conception and organization.

Key message

Genetic (*Pinb-D1* alleles) and environment (through vitreousness) have important effects on bread wheat milling behavior. SKCS optimal values corresponding to soft vitreous or hard mealy grains were defined to obtain the highest total flour yield.

Introduction

The aim of wheat grain milling process is to recover the starchy endosperm (the main inner grain tissue) from the other parts of the grain, through successive steps of grinding and sieving (Posner 2009). The first steps, corresponding to grain breaking, use one or more pairs of corrugated break rolls with their associated sieves. These break rolls lead to the grain opening and the production of large particles corresponding to relatively intact bran, while the endosperm shatters in smaller particles. In that way, the break steps contribute to the separation of endosperm from bran by sieving, and to the production of flour particles at each step. The endosperm particles that do not fall through the sieve are too large to be considered as flours and are sent to the reduction steps, constituted by one or more pairs of smooth reduction rolls and their accompanying sieves. The reduction steps enable a progressive size reduction of the endosperm particles, and again the production of flour at each stage.

Wheat grain mechanical resistance is a key determinant of the milling behavior as it has an impact on the required energy for grinding and on the size of generated particles (Haddad et al. 1999, 2001; Campbell et al. 2007; Greffeuille et al. 2007). Wheat grain mechanical resistance was found to depend on genetic factors and to be linked with 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 (PIN-A) and Puroindoline-B (PIN-B). When both wild-type alleles of these two genes (*Pina-D1a* and *Pinb-D1a*) are present, the wheat grain displays a soft texture, whereas deletion or mutations in one of them result in a hard texture which corresponds to higher grain mechanical resistance to grinding (Giroux and Morris 1998; Beecher et al. 2002; Morris 2002). Indeed, the PINs, when both present as wild forms, were hypothesized to play a role of anti-adhesion at the interface between the starch granules and the protein matrix (Feiz et al. 2009; Pauly et al. 2013; Chichti et al. 2015), decreasing the cohesion in the starchy endosperm. Therefore, when mutated PINs are present, a higher level of starch damage can be observed at grinding due to higher adhesion between the starch granules and the protein matrix. This starch damage is known to impact the flour water absorption properties (Morrison and Tester, 1994; Li et al. 2014).

Wheat grain mechanical resistance was also found to depend on environmental factors, which affect a grain optical property corresponding to the degree of grain translucence or vitreousness (Haddad et al. 1999, 2001; Greffeuille et al. 2006, 2007; Lasme et al. 2012). Moreover,

vitreousness appeared to be partly related to grain protein content (GPC), as an increase in GPC is generally associated to an increase in vitreousness (Oury et al. 2015).

Grain mechanical resistance is commonly measured using the Particle Size Index (PSI), based on the particle size distribution obtained after grinding (Williams and Sobering 1986; Turnbull and Rahman 2002; Pearson et al. 2007), or with the Single Kernel Characterization System (SKCS) developed by Martin et al. (1993), which measures the force required to crush individual grains (Gaines et al. 1996) and is referred to as the hardness index (HI). Near-infrared reflectance spectroscopy (NIRS) is also often used instead of PSI, as it allows a good prediction of PSI values (Saurer 1978). The two classes defined by the puroindoline alleles (soft and hard), and the two classes defined by NIRS and SKCS measurements are roughly concordant. However, in the following, to avoid any confusion, the “soft” and “hard” terms will be devoted to the two classes defined by the alleles at gene *Pinb-D1* (wild-type allele conferring a soft texture, versus the mutated alleles conferring a hard texture). Indeed, only the genetic hardness (a discrete variable) is unambiguous. This is not the case for NIRS or SKCS measurements, which correspond to continuous variables with possible overlap between the highest values obtained by soft genotypes and the lowest values obtained by hard genotypes. For example, depending on the environmental conditions, mealy or vitreous grains can be found in both soft and hard types. Consequently, there is a range of SKCS-HI values (approximately from 25 to 45) which can be obtained either by soft wheat samples displaying vitreous grains, or by hard wheat samples displaying mealy grains (Oury et al. 2015).

The aim of wheat milling is to achieve a high flour yield with minimal bran contamination. However, improvement of these two components of the milling value remains difficult. Indeed, there is no indirect test to predict milling quality, and the direct evaluation methods are destructive and require large amounts of grains. Consequently, the assessment of wheat milling value occurs only belatedly in the breeding process, inducing low selection efficiency. Moreover, previous studies revealed a negative relationship between grain mechanical resistance and break flour yield (Martin et al. 2001; Hogg et al. 2005; Martin et al. 2007; Carter et al. 2012), and on the contrary a positive relationship between hardness and reduction flour yield (Garland-Campbell et al. 2001; Ma et al. 2009; Choy et al. 2015), which make more difficult the improvement of the total extraction rate.

In a previous article (Oury et al. 2015), near-isogenic lines (NILs) carrying a distinct allele at gene *Pinb-D1* (with soft/hard NILs displaying either the wild-type allele *Pinb-D1a* or the mutated allele *Pinb-D1b*; and hard/hard NILs displaying either the mutated alleles *Pinb-D1b* or *Pinb-D1d*), were used to study the respective roles of *Pinb-D1* alleles and environmental

conditions in the variation of the grain characteristics related to wheat grain mechanical resistance (NIRS hardness, SKCS-HI and vitreousness), and to examine relationships between these parameters. In the present article, the same NILs were used to establish the respective importance of genetic and environmental factors on the milling behavior. The obtained data were also used to construct prediction models of bread wheat milling value, using the different measured grain characteristics, and especially those involved in the mechanical properties, as explicative variables. To enlarge the conclusions drawn from the NILs study, and to validate the prediction equations, we used an independent dataset constituted by a panel of elite lines carrying either the wild-type allele (*Pinb-D1a*), or the most predominant alleles (*Pinb-D1b*, *Pinb-D1c* and *Pinb-D1d*) present in European hard wheats (Huang and Röder, 2005). To gather a sufficiently robust dataset, NILs and elite lines were grown for two consecutive years in a multisite experimental design, with two levels of nitrogen fertilization.

Materials and Methods

Plant material

A first set of material comprised five pairs of NILs differing by the allelic form at gene *Pinb-D1*. Two pairs (1010a/1010b and 1259a/1259b) were produced by Institut National de la Recherche Agronomique (INRA) and displayed either the wild-type *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 three other pairs of NILs (VM1b/VM1d, VM2b/VM2d and VM3b/VM3d) were produced by Union Française des Semenciers (UFS) and displayed 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 (NILs VM2b/d and VM3b/d originated from the same cross) after selection of the two allelic types at the F6:F7 step. All the INRA and UFS NILs expressed the wild-type allele *Pina-D1a* encoding the wild form of PIN-A.

Due to their genetic similarity, differences observed between the two lines constituting a pair of NILs can mainly be attributed to an effect of the *Pinb-D1* allele present at the *Ha* locus. This genetic similarity was verified using Diversity Array Technology (DArT) markers (Akbari et al. 2006) generated by Triticarte Pty.Ltd (www.triticarte.com.au).

The French wheat cultivar Soissons (*Pinb-D1d*) was used as a control, leading to a maximum of 11 genotypes (10 NILs and one control) in the experimentations. This first set of material (regrouping INRA NILs, UFS NILs and the control Soissons) will be referred as the NILs dataset.

The second set of material comprised 32 elite lines provided by INRA and UFS breeders. All these 32 lines carried the wild-type allele of *Pina-D1* (*Pina-D1a*) and were chosen according to their *Pinb-D1* allele: seven lines displayed the *Pinb-D1a* allele (soft class), 10 lines the *Pinb-D1b* allele, four lines the *Pinb-D1c* allele and 11 lines the *Pinb-D1d* allele (these last 25 lines belong to the hard class).

Field experimentations

Rainfed trials were conducted for the two sets of plant material. All the trials corresponded to randomized complete block designs with two replicates. Typical plot sizes were between 7 and 10 m², with a plant density around 250 plants/m². 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 (N) fertilization were used: the first one (N1 treatment) was adjusted to high yield objectives (around 9 t/ha, depending on the location); for the second one (N2 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 N1 trial were near the two replicates of the N2 trial (no difference in soil fertility for the two treatments).

For INRA NILs, trials were conducted in 2007 and 2008, with the three same sites each year: Clermont-Ferrand (CF: 45°46'N/3°04'E), Estrées-Mons (EM: 49°52'N/3°00'E) and Rennes (RE: 48°06'N/1°40'W). UFS NILs were cultivated only in 2008, in four sites: Cappel-en-Pévèle (CA: 50°30'N/3°10'E), CF, Maule (MA: 48°55'N/1°51'E) and Orgeval (OR: 48°55'N/1°58'E).

For the panel of elite lines, trials occurred in 2007 and 2008. The sites of the first year of experimentation were CF, Chartainvilliers (CH: 48°32'N/1°33'E) and Louville-la-Chenard (LO: 48°19'N/1°47'E). The sites for 2008 were MA and OR.

Grain characteristics

For each trial, the two harvest sacks corresponding to the two replicates were bulked into a single sample. Then 1kg of grains was picked up and used to evaluate all the characters listed below (one measurement for each “genotype*N*site*year” combination). A consequence of the loss of the replicate information was that the “genotype*N*site*year” interaction could not be statistically tested.

Test weight (TW) was measured according to the AFNOR method NF-EN-ISO7971 (www.boutique.afnor.org), and expressed in kg/hl.

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

GPC 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 sections (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 grain diameter and a mean SKCS-HI according to AACC method 55-31 (AACC 2000).

Milling behavior

Five hundred grams of cleaned grains were conditioned to 16% moisture and milled after 24h at room temperature using a patented prototype developed in order to evaluate the wheat milling value (French Patent Chopin, INRA, ARVALIS, N°0905572; Abecassis et al. 2009) and now commercialized as Labmill by Chopin Technologies. This mill was equipped with two pairs of break rolls, one pair of sizing rolls and one pair of reduction rolls (detailed in supplementary Fig. 1).

Sieves of 1000µm, 500µm and 200µm mesh were used to isolate the different fractions at break1 (B1) and break2 (B2) stages, while sieves of 500µm and 160µm mesh were used for the sizing (S) stage, and a 160µm mesh was used to isolate the flour at the two reduction stages (R1 and R2). Thus the milling process allowed the production of eight final fractions: two break flours (B1 and B2 flours), one sizing flour and two reduction flours (R1 and R2 flours), as well as coarse bran, fine bran and a shorts fraction. Each collected fraction was weighed at each step, including seven intermediate fractions: two overtails fractions obtained at the B1 and R1 stages, two coarse middlings (obtained at the B1 and B2 stages) which were reduced at the S stage, and

three fine middlings (obtained at the B1, B2 and S stages), which were reduced at the R1 stage. Six aggregated fractions were also calculated: coarse middlings (regrouping of the B1 and B2 coarse middlings), fine middlings (regrouping of the B1, B2 and S fine middlings), total bran (regrouping of the coarse and fine bran fractions), break flour (regrouping of the B1 and B2 flours), reduction flour (regrouping of the sizing, R1 and R2 flours), and total flour (regrouping of the break and reduction flours). Finally, the milling behavior of the different genotypes was characterized through the weights of the 21 fractions obtained (supplementary Fig. 1). However, for reasons of simplification, the results presented will mainly concern nine fractions: the shorts, coarse bran and fine bran final fractions, and the coarse middlings, fine middlings, total bran, break flour, reduction flour and total flour aggregated fractions.

After milling, total flour ash content was measured according to the AFNOR method NF-EN-ISO2171 (www.boutique.afnor.org).

It must be noted that all the measurements were not available for the different datasets:

- for the NILs dataset (see also supplementary Table 1), the different grain and milling characters were available for all the “genotype*N*site*year” combinations ($4*2*3*2 = 48$ for INRA NILs; $6*2*4*1 = 48$ for UFS NILs). However, for the control Soissons, measurements were only available for 11 out of the $2*9 = 18$ possible “N*(site*year)” combinations. Flour ash content was also not measured on this dataset.
- for the elite lines panel (see also supplementary Table 2), as milling process was a time consuming activity, the prototype mill was used only for a subset of the samples. Finally, the different grain and milling characters were available for 163 out of the $32*2*5 = 320$ existing “genotype*N*(site*year)” combinations, except for vitreousness which was not measured on this dataset, and flour ash content which was only measured on 145 out of these 163 combinations.

Statistical analyses

Analyses of variance (ANOVA) were used to assess the effects of environmental and genetic factors, and their interactions, on the variation of the different characters studied. They were performed using the following model:

$$\text{model 1: } Y_{ijk} = \mu + N_i + \text{Env}_j + \text{Pinb}_k + N_i * \text{Env}_j + N_i * \text{Pinb}_k + \text{Env}_j * \text{Pinb}_k + \varepsilon_{ijk}$$

where Y is the response variable (the different characters measured), μ the overall mean, N_i the effect of N treatment i , Env_j the effect of environment j (each environment being one of the different “site*year” combinations), $Pinb_k$ the effect of *Pinb-DI* allele k , and ε an error term.

Two other models were also used to underline (through their r^2) the contribution of the environmental factors (model 2), or the genotype factor (model 3):

$$\text{model 2: } Y_{ij} = \mu + N_i + Env_j + N_i * Env_j + \varepsilon_{ij}$$

$$\text{model 3: } Y_{ijkl} = \mu + N_i + Env_j + Pinb_k + Pinb_k(Geno_l) + N_i * Env_j + N_i * Pinb_k + Env_j * Pinb_k + \varepsilon_{ijkl}$$

where $Geno_l$ is the effect of genotype l (the *Geno* factor being nested in the *Pinb* factor).

Multivariate analyses were performed with the different milling characters as response variables and the seven grain characteristics as explanatory variables. Two methods were used: - tree-based modeling (Chambers and Hastie, 1992), which is an exploratory technique to uncover structure in a dataset. A segmentation tree is constructed by binary recursive partitioning, whereby a dataset is successively split into increasingly homogenous subsets which are the nodes of the tree. The top node is called the root and the terminal nodes are called the leaves. The deviance function provides the basis for choosing partitions. The deviance of a node is defined as:

$$D_{\text{node}} = \sum (y_i - \mu)^2 \quad \text{where } y_i \text{ is the response value for each individual } i \text{ present in the node and } \mu \text{ the node average.}$$

At a given node, the dichotomy chosen is the one that maximizes the change in deviance:

$$\Delta D = D_{\text{node}} - D_{\text{children.nodes}}$$

Goodness-of-fit can be estimated by the reduction in deviance enabled by the model and given by:

$$1 - (D_{\text{leaves}}/D_{\text{root}})$$

- Partial Least Squares (PLS) regressions, which enable 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).

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

Results

Effects of environmental and genetic factors on the grain characteristics and milling behavior

As expected, N2 treatment enabled an increase in protein content: for the NILs dataset, over the whole environments, average GPC were respectively 11.8% and 12.5% for N1 and N2 treatments; and these average values were 11.9% and 12.5% in the case of the elite lines dataset. As DArT markers indicated that the differences inside each pair of NILs never exceeded 1.6% of the total number of markers, far below the theoretical level (3.12%) of residual heterozygosity at this selfing step (for more details, see Lasme et al. 2012; Oury et al. 2015), the differences observed for a character inside a pair of NILs could reasonably be attributed to an effect of the *Pinb-D1* allele present at the *Ha* locus.

Analyses of variance were applied to the two balanced datasets corresponding to INRA NILs and UFS NILs, to estimate the effects of the *Pinb* factor in a context of genetic similarity. Detailed results obtained with ANOVA model 1 were reported in Table 1. Comparison between r^2 of model 1 and r^2 obtained with ANOVA model 2 (taking into account only the environmental factors) highlighted the contribution of the *Pinb-D1* alleles to the variation of the different characters, and the comparison between r^2 of model 1 and r^2 of ANOVA model 3 (including the genotype factor) pointed out the contribution of the genetic background.

Grain characteristics

The present NILs dataset (n=107) was a subset of a larger one (n=173), for which grain characteristics have been exhaustively studied (Oury et al., 2015). We summarized here the main results concerning the environmental and genetic effects observed on the present subset, which were similar to those previously reported.

Table 1

Comparison between lines carrying *Pinb-D1a* or *Pinb-D1b* (INRA NILs) revealed that environmental effects were strong for TGW, TW, GPC and vitreousness (r^2 of model 2 > 0.6), but weak for grain diameter, NIRS hardness and SKCS-HI (r^2 < 0.3). They mainly corresponded to effects of the Env factor (i.e. the different “site*year” combinations), except for GPC and vitreousness which were also influenced by the N factor (Table 1). The N*Env interactions

were always non-significant. Effects of *Pinb-D1* alleles were non-significant for TGW, TW, grain diameter and GPC, with no interaction with the two environmental factors, therefore r^2 of model 1 remained similar to those of model 2. On the contrary, *Pinb-D1* alleles had highly significant effects for NIRS hardness, vitreousness and SKCS-HI, with significant Env*Pinb interactions for vitreousness and SKCS-HI. For these three characters related to grain mechanical resistance, r^2 of model 1 were higher than 0.9, which corresponded to a considerable increase when compared to r^2 of model 2, especially for NIRS hardness and SKCS-HI. For all the characters, r^2 obtained with model 3 taking into account the Geno factor were very high (at least 0.81). However the increase, when compared to r^2 of model 1, was only important for TGW and grain diameter.

In the case of comparison between lines carrying *Pinb-D1b* or *Pinb-D1d* (UFS NILs), environmental effects were due principally to effects of the Env factor, with some effects of the N factor for GPC and NIRS hardness (Table 1). However, r^2 of model 2 ranged from 0.27 for grain diameter to 0.76 for vitreousness. As all these lines belong to the hard class, effects of the Pinb factor, as well as the N*Pinb and Env*Pinb interactions, were non-significant for most of the characters. Consequently r^2 of model 1 had generally the same magnitude than r^2 of model 2. However, for NIRS hardness, r^2 increased from model 2 to model 1, revealing a highly significant effect of the Pinb factor, due to a relatively strong difference between the mean values of lines carrying the *Pinb-D1b* (82.1) and lines carrying the *Pinb-D1d* allele (74.5), as already demonstrated in Lasme et al. (2012). Moreover, for all of the characters except NIRS hardness, the Geno factor present in model 3 enabled an important increase of r^2 value, when compared to model 2.

Milling behavior

For the comparison between lines carrying *Pinb-D1a* or *Pinb-D1b* (INRA NILs), the effects of environmental factors only appeared strong for the total bran and total flour fractions (r^2 of model 2 > 0.5). For the other milling fractions, r^2 of model 2 were generally lower than 0.3 (Table 1). These environmental effects corresponded mainly to highly significant effects of the Env factor, with sometimes a supplementary effect of the N factor. The N*Env interactions were never significant. *Pinb-D1* alleles had highly significant effects for all of the milling fractions, except for the shorts fraction. The Pinb*N interactions were always non-significant, as well as the Pinb*Env interactions. For most of the milling fractions, r^2 of model 1 were higher than 0.8. They were lower than 0.55 only for the shorts fraction obtained at the end of the

milling process. There were considerable increases in r^2 from model 2 to model 1, except for the shorts fraction, which was the only one not significantly influenced by the environmental or Pinb factors. The Geno factor introduced with model 3 appeared nearly of no use to further improve the r^2 . With this model, the r^2 obtained for the shorts fractions remained relatively low (< 0.55).

For the comparison between lines carrying *Pinb-D1b* or *Pinb-D1d* (UFS NILs), the environmental effects explained an important part of the variability as r^2 of model 2 were often higher than 0.6, and were lower than 0.3 only for the coarse bran and total bran fractions (Table 1). These environmental effects corresponded principally to highly significant effects of the Env factor, with a supplementary significant effect of the N factor for some fractions. In this context of hard genotypes, effect of the Pinb factor was only significant for the fine bran fractions. Moreover, the N*Pinb and Env*Pinb interactions were always non-significant. Consequently, there was only a slight increase of r^2 value from model 2 to model 1. After all, model 3 taking into account the Geno factor enabled important improvements in r^2 values (most of the values being higher than 0.8).

Elaboration of flour yield according to the different Pinb-D1 alleles

Mean weights of the different milling fractions (the nine previously defined in Table 1 + B1 fine middlings) were reported in Table 2 for the three datasets (flour ash content was added in the case of the elite lines panel). Coefficients of variation, which enabled to compare the variability level of the different characters, were also given.

Table 2

For INRA NILs, in accordance with the significant effects of the *Pinb-D1* alleles demonstrated in Table 1, milling behaviors appeared highly contrasted between hard and soft genotypes, except for the B1 fine middlings and shorts fractions which presented no differences between the two classes. Lines carrying the *Pinb-D1a* allele produced higher amounts of coarse bran, total bran and break flour, whereas lines carrying the *Pinb-D1b* allele produced more coarse middlings, fine middlings, fine bran and reduction flour. The opposite effects of *Pinb-D1* alleles on coarse and fine bran fractions reduced the variability observed for total bran in comparison to that of its two components: on the whole dataset coefficients of variation were 0.09 for total

bran, and respectively 0.17 and 0.16 for coarse bran and fine bran (Table 2). On the same manner, the opposite effects of *Pinb-D1* alleles on break flour and reduction flour fractions led to a coefficient of variation considerably lower for the total flour fraction (0.03) than for its two components (0.2 for break flour and 0.19 for reduction flour). However, a difference appeared between hard and soft NILs for the total flour production: on average hard genotypes obtained a higher total flour yield (75%) than soft genotypes (73.5%).

For UFS NILs, lines carrying the *Pinb-D1b* allele displayed higher values for the three characters (NIRS hardness, vitreousness and SKCS-HI) related to grain mechanical resistance than the lines carrying the *Pinb-D1d* allele (data not shown). However, the difference was only significant in the case of NIRS hardness (Table 1). For the milling fractions, the differences between *Pinb-D1b* and *Pinb-D1d* genotypes were generally in the same direction than the differences observed for the hard/soft comparison in the case of INRA NILs (Table 2). Therefore, an increase in grain mechanical resistance had similar consequences on the milling fractions in a context of hard genotypes, even though these effects had a smaller magnitude and were generally non-significant (Table 1). Total flour yields were equivalent for *Pinb-D1b* and *Pinb-D1d* genotypes, and reached 78%, a value higher than that obtained by the hard genotypes in the case of INRA NILs.

For the panel of elite lines, the differences between the soft and hard classes were similar to that observed for INRA NILs (Table 2), except for the shorts fraction. Moreover, the findings made for the comparison *Pinb-D1a/Pinb-D1b* in the case of INRA NILs can be generalized to other mutated alleles, as the results were similar for the comparisons *Pinb-D1a/Pinb-D1c* and *Pinb-D1a/Pinb-D1d*. On average, total flour yield was 75% for soft genotypes and reached 78% for hard genotypes.

Figure 1

Some effects of the *Pinb-D1* alleles on the most important milling fractions were illustrated in Fig. 1. A clear distinction between hard and soft genotypes for break and reduction flours was observed as they produced opposite quantities of these flours. However, the differences were less clear for total flour, as some hard genotypes (Quebon, Orvantis, Oakley, Carenius and Timber) appeared mixed with the soft genotypes on the graphic. The milling behavior of variety Innov appeared peculiar, as its production of total flour was very low, due to a low production of reduction flour not completely counter-balanced with a high production of break flour. Discarding Innov, the range observed for total flour (369.1g - 400g) was approximately three

times lower than the range observed for break flour (117g - 201g) or for reduction flour (168.5g - 270.2g). Fig. 1 also indicated that there was no hierarchy between the hard genotypes carrying either of the *Pinb-D1b*, *Pinb-D1c* or *Pinb-D1d* allele, as they appeared mixed on the graphic whatever the flour type. For flour ash content, there was no apparent effect of *Pinb-D1*: the four alleles were distributed all over the graphic (Fig.1). This result was confirmed in Table 2, where the average values appeared very similar for the different *Pinb-D1* alleles.

Figure 2

The opposite effects between soft and hard *Pinb-D1* alleles during the break and reduction stages, led to a very strong negative linear relationship between break and reduction flours, which was illustrated in Fig. 2 for the NILs and elite lines datasets. This relation appeared very stable, as the linear regression was nearly the same (similar slope and intercept) for the two independent datasets. It must be noted that there was no co-linearity between the regression line and the line of perfect compensation between the two flour fractions (i.e. the line corresponding to a constant production of total flour, here equivalent to the mean total flour value obtained on the considered dataset). Most of the soft genotypes were below this line of perfect compensation, and on the contrary most of the hard genotypes were above, which reflected the fact that on average hard genotypes led to higher total flour yield than soft genotypes (Table 2). However, the angle between the regression and the perfect compensation lines appeared very acute (Fig. 2), indicating that differences between the two classes for the total flour production were not very large.

Globally (Table 2 and Fig. 2), break and reduction flours each contributed approximately to 50% of the total flour in the case of soft genotypes. These contributions were approximately 35% and 65% respectively, in the case of hard genotypes.

Relationships between grain mechanical resistance and milling fractions

The relationships between SKCS-HI and different milling fractions were presented in Fig. 3, in the case of the elite lines dataset. Results were similar for the NILs dataset (graphics not shown).

Figure 3

Three types of relationships could be distinguished:

- a linear trend with a positive slope, which was observed for the coarse middlings, fine middlings and fine bran fractions. For the fine bran fraction, there was a higher dispersion of the points around the smoothing line (Fig. 3).
- a linear trend with a negative slope in the case of break flour.
- a two-parts curve, with a more or less pronounced change in the slope. In all cases, the abscissa of the inflexion point corresponded approximately to the separation between soft and hard genotypes for SKCS-HI values. For the coarse bran fraction (Fig. 3), the inflexion point corresponded to a minimum, and it was the same for the total bran and shorts fractions (not shown). For B1 fine middlings, sizing flour and total flour, the inflexion point corresponded to a maximum. In the case of reduction flour the curve at first increased markedly, and then more slightly.

Multivariate analyses to explain the milling behavior

Segmentation trees

Two tree-based models were used to estimate the effects of the seven grain characters on the different milling fractions and the flour ash content (considered as response variables). Model 1 used NIRS hardness, vitreousness, TGW, TW, grain diameter and GPC as explanatory variables and was only applied to the NILs dataset, as vitreousness was not measured on elite lines panel. Model 2 used SKCS-HI, TGW, TW, grain diameter and GPC as explanatory variables, and was applied both to the NILs and elite lines datasets.

Figure 4

As an example, Fig. 4 presents the segmentation tree obtained with model 1 for the B1 fine middlings, in the case of NILs dataset. The sum of the deviances of the leaves (830) represented 31% of the total deviance at the root of the tree (2676), so segmentation model 1 enabled a deviance reduction of 69%. Five explanatory variables contributed to this deviance reduction (only grain diameter was never retained for the binary partitioning), but NIRS hardness (34%) and vitreousness (24.2%) were the most important. The highest values of B1 fine middlings were obtained for the values of the explanatory variable superior to the threshold defined by the algorithm in the case of the binary partitioning involving NIRS hardness (values ≥ 23.5), TGW (values ≥ 40.0) and GPC (values ≥ 13.9). On the contrary, the highest values of B1 fine middlings were obtained for the values of the explanatory variable inferior to the threshold

defined by the algorithm for the binary partitioning involving vitreousness (values < 56.7 or < 64.3) and TW (values < 79.2).

Results from the different segmentation trees obtained with the two models, for the overall milling characters and the two datasets, were summarized in Table 3 (for a good understanding, the results presented in Fig. 4 can be related to the deviance reductions reported in the second line of Table 3).

Table 3

In the case of NILs dataset, segmentation models 1 and 2 enabled an important deviance reduction (>78%) for coarse and fine middlings, coarse bran, total bran, break flour and reduction flour. Deviance reductions were lower (between 63 and 74%) for B1 fine middlings, fine bran and total flour, and were particularly weak (<38%) for the shorts fraction.

For most of the milling fractions, the main explanatory variables were the three characters related to grain mechanical resistance (NIRS hardness and vitreousness for model 1; SKCS-HI for model 2). However, TGW also contributed to the deviance reduction for the three bran fractions (coarse, fine and total bran), and for B1 fine middlings and total flour, especially in the case of model 2. Moreover, for total bran and total flour, the usual substitution between “NIRS hardness + vitreousness” and SKCS-HI when using model 2 instead of model 1, did not occur: indeed, NIRS hardness was the most important explanatory variable in the case of model 1, but it was TGW that contributed the most to the deviance reduction in the case of model 2. This substitution between NIRS hardness and TGW was due to a relatively strong relationship between the two characters ($r^2=0.38$ for NILs carrying *Pinb-D1a*; $r^2=0.39$ for NILs carrying *Pinb-D1b* or *Pinb-D1d*). It must be noted that this relationship was not recovered for the elite lines dataset. For the fine bran and shorts fractions, TW and grain diameter also contributed to the deviance reduction.

Whatever the segmentation model, four milling fractions (coarse and fine middlings, break and reduction flours) presented a high deviance reduction mainly due to the grain mechanical resistance traits. Globally, for these four milling fractions, in the case of model 1, the average deviance reductions due to NIRS hardness and vitreousness were respectively of 72.8% and 17.1%; and the average deviance reduction due to SKCS-HI was 80% in the case of model 2.

In the case of the elite lines panel, the results obtained with model 2 were not very different from those obtained for the NILs dataset. Again, the lowest deviance reduction was observed

for the shorts fraction (<43%), and high deviance reductions (>78%) were obtained for coarse and fine middlings, break and reduction flours. However, there was a decrease in deviance reduction for coarse and total bran, which were found in the same group than B1 fine middlings, fine bran and total flour (deviance reductions between 57 and 67%). Globally, for the four fractions with a high deviance reduction, the average deviance reduction due to SKCS-HI was 76.2%, a value similar to that obtained for model 2 with the NILs dataset. For flour ash content, the deviance reduction enabled by model 2 was the lowest of all the milling characters (42.6%), and the main explanatory variables were TGW and GPC.

With the two segmentation models and for most of the milling fraction, the three characters related to mechanical resistance acted in the same direction: highest values of coarse bran, total bran and break flour were always obtained for values of SKCS-HI, NIRS hardness and vitreousness inferior to the thresholds defined by the segmentation algorithm (in Table 3, deviance reductions were in the column “<” and there were 0 in the column “≥”); and highest values of coarse and fine middlings, fine bran and reduction flour were always obtained for values of SKCS-HI, NIRS hardness and vitreousness superior to the thresholds defined by the algorithm (deviance reductions in the column “≥” and 0 in the column “<”). All these results were coherent with the opposite effect of genetic hardness on the break and reduction stages. However, for B1 fine middlings, the three characters related to mechanical resistance did not act in the same direction. In the case of model 1, the highest productions of B1 fine middlings were obtained for NIRS hardness values superior to the threshold defined by the algorithm, and for vitreousness values inferior to the thresholds (see also Fig.4). For segmentation model 2, increases in B1 fine middlings were obtained with values of SKCS-HI inferior to the threshold in some parts of the segmentation tree, and with values superior to the threshold in other parts (deviance reductions both in columns “<” and “≥” in Table 3). It was the same for the shorts and total flour fractions (and also for total bran in the case of elite lines panel).

PLS regressions

On a preliminary stage, different combinations of explanatory variables were tested to determine the most relevant PLS regression models. The models which enabled the highest values of r^2 (for the relation between observed and fitted values) and the lowest values of Root Mean Square Error of Calibration (RMSEC), with as few explanatory variables as possible, were chosen. The model including only GPC, TGW, TW and grain diameter as explanatory variables appeared clearly insufficient, and quality of the model increased strongly if a character

related to grain mechanical resistance was included (results not shown). The presence/absence of allele *Pinb-D1a* appeared as a useful explanatory variable, especially if SKCS-HI was the only model variable related to grain mechanical resistance. On the contrary, grain diameter and the three alleles of *Pinb-D1* corresponding to hard genotypes were of no use to improve the quality of the prediction. Finally, three models including GPC, TGW, TW and the presence/absence of the *Pinb-D1a* allele as explanatory variables were retained. These models were either completed with NIRS hardness and vitreousness (model PLS1), or SKCS-HI (model PLS2), or these three characteristics together (model PLS3). Results obtained with these PLS models were given in supplementary Table 3. As vitreousness measurements were not available for the elite lines panel, only model PLS2 could be applied to this dataset.

In the case of NILs dataset, models PLS1, PLS2 and PLS3 gave very similar results in terms of r^2 and RMSEC for all of the milling fractions. With r^2 higher than 0.8, the level of explanation enabled by the three models appeared particularly high for coarse and fine middlings, coarse bran, total bran, break and reduction flours. For fine bran and total flour, the r^2 were lower (between 0.55 and 0.71), and the shorts fraction was poorly explained ($r^2 < 0.2$).

In the case of the elite lines panel, the PLS2 model gave similar determination coefficients than those found for NILs dataset (supplementary Table 3). However, a decrease in r^2 was observed for coarse bran (and consequently for total bran) and for total flour. With a r^2 lower than 0.3, flour ash content also appeared as a character that weakly depended on the explanatory variables of model PLS2.

Prediction of the final milling fractions

Different PLS models were used for the prediction of the final milling fractions (shorts; coarse, fine and total bran; break flour, reduction flour and total flour – see also supplementary Fig.1). Model PLS2 defined in the former paragraph, which could be applied to the two datasets, was retained, and two other models (PLS4 and PLS5) were used to test whether adding parts of the milling process could improve the prediction. The explanatory variables of model PLS4 included the five variables of model PLS2 (GPC, TGW, TW, presence/absence of the *Pinb-D1a* allele and SKCS-HI) and the four milling fractions obtained at break B1. Model PLS5 included the explanatory variables of model PLS4 and the four milling fractions produced at break B2. For a given dataset, the level of explanation enabled by a given PLS model corresponds to the r^2 of the relation between observed and fitted values, these fitted values being calculated with the PLS equation obtained on the considered dataset. On the other hand, quality

of the prediction corresponds to the r^2 of the relation between observed and predicted values, these predicted values being calculated with the PLS equation obtained with the other dataset. In that way, the prediction equations were validated with an independent dataset.

Results obtained with these three models were summarized in Table 4. Taking into account the whole datasets, it can be noted that whatever the PLS models, r^2 between observed and predicted values were not far below the r^2 between observed and fitted values, except for the shorts fraction.

Table 4

None of the three models enabled a good fit (and consequently a good prediction) of the shorts fraction.

For the bran fractions, r^2 values obtained with model PLS2 were never sufficient for fine bran, and were high (>0.75) for coarse bran and total bran only in the case of NILs dataset (Table 4). Goodness of fit was improved using PLS4 model, but the quality of the prediction remained insufficient. Model PLS5 used coarse bran as an explanatory variable, therefore this fraction needed no more prediction. With this last model, fine bran and therefore total bran displayed a good fit as well as a good prediction.

Figure 5

Among the three aggregated flour fractions, only break and reduction flours presented high determination coefficients ($r^2 > 0.75$) for both relationships (fitted/observed and predicted/observed) using model PLS2 (Table 4). However, detailed analyses of the relationships between predicted and observed values presented in Fig. 5, confirmed the efficiency of PLS2 model only in the case of break flour (illustrated for the NILs dataset, but the graphic was similar for the elite lines panel). For reduction flour, despite global r^2 higher than 0.75, the prediction using model PLS2 appeared acceptable for hard genotypes but insufficient for soft genotypes (Fig. 5). When the fractions produced at B1 were taken into account (model PLS4), predictions were greatly improved, especially for soft genotypes (Table 4 and Fig. 5). As model PLS5 did not appear better, model PLS4 was retained as the most relevant for the prediction of reduction flour. In the case of total flour, model PLS4 also enabled some improvement in the case of soft genotypes, but not in the case of hard genotypes. Model PLS5 permitted further improvement for hard genotypes (for the two datasets), and also for soft

genotypes but only in the case of the elite lines panel (Table 4). Finally, model PLS5 appeared as the most relevant for the prediction of total flour, even though the quality of the prediction was not as high as that obtained for break and reduction flours (Fig. 5). Prediction of total flour appeared usable in the case of the NILs dataset ($r^2=0.74$), but was still insufficient in the case of the elite lines panel ($r^2=0.62$).

For flour ash content, the quality of model PLS2 was very low (supplementary Table 3), and an attempt was made to explain this character with a PLS model involving five grain characteristics (TGW, TW, GPC, grain diameter and SKCS-HI) and the 15 individual milling fractions as explanatory variables. It appeared that the information brought by the whole milling process was of no use to improve the explanation of flour ash content: the determination coefficient was only slightly increased ($r^2 = 0.36$) and there was no improvement of RMSEC (0.05).

As the validation process of the PLS models using independent datasets appeared successful (the quality of the prediction was good for break and reduction flours, and acceptable for total flour), it was possible to define prediction equations for the three aggregated flour fractions. These equations were obtained with model PLS1, using the NILs dataset ($n=107$), and with models PLS2, PLS4 and PLS5, using the regrouping of the NILs and elite lines datasets ($n=270$) to increase the robustness of the equation. The main features of these models and their corresponding equations were given in supplementary Table 4.

Discussion

“The purpose of wheat milling is to separate bran and germ from endosperm so as to extract as much flour as possible, at minimum operating cost, while maintaining high flour quality” (Campbell et al, 2007). Therefore, as milling is an obligatory step for most of the bread wheat end-uses (especially in the case of human nutrition), milling value has a great economical importance. However, paradoxically, milling value is not taken into account for the registration of wheat lines to the French National List of varieties and, consequently, is rarely considered in French breeding programs due to difficulties in its measurement using low grain quantities. The prototype mill used in this study allows to only mill 500g of grains, and therefore appears an interesting tool for this purpose. This prototype has been built to well reflect the milling

values and to well classify wheat samples on this criteria (Abecassis et al., 2009), even if a single moisture conditioning is applied. With this mill, the total flour yield ranged from 73% (NIL 1259a) to 80% (control Soissons) in the case of NILs dataset, and from 68% (for the very peculiar variety Innov, the second lowest yield being 74% for variety Astuce) to 80% (Soissons) for the elite lines dataset (Fig. 1). These results are consistent with those obtained with commercial mill, for which the extraction rates are generally in the range 70-80% (Campbell et al., 2007). The values obtained by Soissons, which is considered by millers as a variety with a very high milling value, were also consistent, as Soissons obtained the best total flour yield for the two independent datasets, and the second lowest value for ash content (Fig. 1). Moreover Crousty, which is recognized by millers as a good variety for the soft class, also presented expected high total flour yield (77 %).

Effects of *Pinb-D1* alleles on milling behavior

The NILs used in this study demonstrated a strong effect of *Pinb-D1* alleles on the characters related to grain mechanical resistance (especially NIRS hardness and SKCS-HI), and consequently on the milling fractions (Table 1). Moreover, for the NILs and the elite lines datasets, the wild-type or mutated forms of *Pinb-D1* alleles led to opposite milling behavior: on average, soft genotypes produced higher quantities of break flour and lower quantities of reduction flour than hard genotypes (Table 2 and Fig. 1). Several authors have already mentioned a strong negative relationship between genetic hardness and break flour, and a positive relationship between genetic hardness and total flour (Hogg et al. 2005; Ma et al. 2009; Choy et al. 2015), but the underlying compensation between break flour and reduction flour has rarely been studied. Fig. 2 highlighted a very strong negative linear relationship between break and reduction flours ($r^2=0.91$ for the two datasets). It appeared that the higher production of break flour from soft genotypes was generally insufficient to completely compensate the lowest production of reduction flour, since part of the regression line corresponding to the soft genotypes was slightly under the perfect compensation line, whereas part corresponding to the hard genotypes was slightly above. However, in the case of INRA NILs, for which the differences between the two types could be attributed with some certainty to the *Pinb-D1* effect, total flour yield was only 1.5% higher for hard genotypes than for soft ones. Therefore, the opposite effect of wild-type and mutated *Pinb-D1* alleles on break and reduction flours seemed to have only a moderate incidence on the final total flour production.

The slight superiority of hard genotypes for total flour yield could be due to a globally more efficient endosperm reduction, or to a better separation between the endosperm and the outer layers of the grain, leading to a smaller loss of endosperm particles dragged in bran fraction. This second point was corroborated by the fact that hard genotypes produced smaller quantities of total bran (Table 2). More precisely, even if there was a compensatory effect between coarse and fine bran, the highest quantities of coarse bran obtained from soft genotypes were not completely compensated by lowest quantities of fine bran.

However, with so contrasted milling behavior, it is doubtful that the same moisture conditioning and a unique mill setting could be optimal for hard and soft genotypes. Moreover, these water tempering conditions and mill adjustments have been defined to be suitable to a majority of wheat samples, and therefore are favorable to hard genotypes which represent a high proportion of the cultivated varieties in France. An adaptation of the water conditioning and mill settings to soft genotypes could possibly lead to a regression line between break and reduction flours still closer to the perfect compensation line, and consequently a lower discrepancy between the two wheat classes for the total flour production.

Effects of grain characteristics on milling behavior

Relationships between grain mechanical resistance and the milling fractions

To go further than the average effect of *Pinb-D1* alleles, the influence of grain hardness on the milling process was studied. SKCS-HI was the character retained to measure the grain mechanical resistance, as it directly measures the force required for crushing the grains and was found to both reflects the genetic hardness (effects of *Pinb-D1* alleles) and the vitreousness (depending on environmental conditions). This was demonstrated when studying the relationships between NIRS-hardness, SKCS-HI and vitreousness (Oury et al., 2015): a strong positive linear relationship appeared between SKCS-HI and vitreousness, but with two distinct and parallel regression lines for hard and soft genotypes. Our present results indicated that the total deviance reductions obtained with segmentation models 1 and 2 were equivalent (Table 3), and that the r^2 and RMSEC of models PLS1, PLS2 and PLS3 were very similar (supplementary Table 1), which confirmed that SKCS-HI contained approximately the same information related to milling extraction as NIRS hardness and vitreousness combined.

Plotting milling fractions versus SKCS-HI (Fig. 3), the opposite effect of hard and soft *Pinb-D1* alleles on the two components of total flour was recovered through a negative linear trend for break flour, and an increasing curve with an inflexion point roughly corresponding to the

separation between soft and hard genotypes for the reduction flour. Consequently, the relationship between total flour and SKCS-HI was a curve with an optimum (Fig. 3): the lowest flour yields were obtained for the lowest or the highest values of grain mechanical resistance, and the highest flour yields were obtained for moderate SKCS-HI values (between 30 and 50). Previous results (Oury et al. 2015), indicated that SKCS-HI values between 25 and 45 could correspond either to soft genotypes with vitreous grains or to hard genotypes with mealy grains. Therefore, the two-parts curve obtained for total flour can be interpreted in the following manner: the increasing part corresponds to a raise in total flour production from soft genotypes with mealy grains to soft genotypes with vitreous grains; and the decreasing part to a total flour production lowering from hard genotypes with mealy grains to hard genotypes with vitreous grains. It must be noted that two other fractions issued from different steps of the milling process exhibited the same curve shape than the total flour fraction. The first one was the B1 fine middlings fraction, followed by the sizing flour fraction (Fig. 3). Consequently, the increasing-decreasing curve observed for total flour was the resultant of events occurring at different stages of the milling process, and for which the optimal grain texture corresponded to soft vitreous or hard mealy grains.

Multivariate analyses

Segmentation trees or PLS models enabled a good explanation for several important milling fractions, and the percentages of total deviance reduction or the r^2 , obtained respectively with these two types of multivariate analyses, were generally of the same magnitude for the different milling fractions (Table 3 and supplementary Table 3). Unfortunately, due to the compensation between break flour and reduction flour, the level of explanation obtained for total flour was not as high as those observed for its two components. Moreover, flour ash content, which is another important parameter of the wheat milling value, was only poorly explained by the different grain characters.

Whatever the models, the major explanatory variables were the characters related to grain mechanical resistance. That was expected, as numerous authors already pointed out that grain hardness was involved in the grinding properties (Dobraszczyk et al. 2002; Campbell et al. 2007; Martin et al. 2007; Osborne et al. 2007; Edwards et al. 2008; Lasme et al. 2012; Choy et al., 2015). However, our results highlighted the importance of vitreousness, a parameter generally not measured, in milling behavior. Indeed, for segmentation and PLS models, high levels of explanation were reached if vitreousness was taken into account, either directly or indirectly with SKCS-HI (Tables 3 and 4). Interestingly, segmentation models indicated that

vitreousness was implicated in endosperm reduction and not in the separation between the endosperm and the outer layers. Indeed, over the milling fractions related to endosperm reduction (coarse and fine middlings, break flour, reduction flour and shorts) and with segmentation model 1 (Table 3), vitreousness enabled on average a deviance reduction of 15.9%, when it was 58.8% for NIRS hardness, and 1.4%, 2.7%, 0.2% and 1.8% respectively for TGW, TW, GPC and grain diameter. The average contributions were very different for the two fractions (coarse bran and fine bran) related to the dissociation of endosperm and outer layers, as vitreousness contributed to only 0.9%, whereas it was 58.8% for NIRS hardness, and 6.6%, 7.4%, 0.6% and 0.6% respectively for TGW, TW, GPC and grain diameter.

Breeding for milling value

Our results indicated that most of the milling fractions were subjected to a strong genetic determinism, moderate environmental effects and nearly no interactions between these factors (Table 1). Therefore, one can expect some success in breeding for total flour yield. However, this must be tempered, as the strong genetic determinism principally corresponds to an effect of the *Pinb-D1* alleles. This *Pinb-D1* effect masked the genotypic effect when soft and hard genotypes were considered together (case of INRA NILs in Table 1), and a genotypic effect was only recovered when genotypes belonging to the same hardness category were studied (case of UFS NILs in Table 1). Moreover, due to the negative linear relationship between break and reduction flours, and as the regression line was not far from the line of perfect compensation between the two components (Fig. 2), most of the variability (whatever its genetic or environmental origin) observed for break and reduction flours was nearly of no use for breeding purposes. In fact, the variability of interest to improve total flour yield corresponds to the deviations from the regression line, and as the negative relationship was very strong these deviations appeared limited. That could be related to the narrower range observed for total flour, when compared to the ranges observed for break flour and reduction flour (Fig. 1), which was also a direct consequence of the opposite effect of wild-type and mutated *Pinb-D1* alleles. This rather restricted genetic variability could increase the difficulty to select for total flour yield. Besides, as vitreousness appears as an important character for milling behavior, breeding for flour yield proves to be more complex than simply dealing with the effects of genetic hardness. Indeed, vitreousness is submitted to environmental effects (Table 1), and the optimal grain texture to obtain high total flour yield (soft vitreous grain or mealy hard grains), could be a difficult breeding objective to reach.

Another difficulty encountered in breeding for milling value relates to the fact that milling tests are destructive, require large amounts of grains and are time consuming. Hence, they can be applied only on advanced lines (F7 generation or later), which induces a low selection efficiency. Therefore, a prediction of milling quality through grain characteristics easy to obtain and available at an early stage, would be of great interest. Some attempts have already been made to predict milling yield, especially in the case of durum wheat where models have been developed to predict semolina yield from grain characteristics such as SKCS measurements (Sissons et al. 2000; Haraszi et al. 2016), whole grain image analyses (Novaro et al. 2001), or NIR spectroscopy (Sissons et al. 2006). In the case of bread wheat, Choy et al. (2015) used the Brabender hardness tester to predict with some success the production of break flour and reduction flour, but the prediction of total flour was not mentioned. In our study, PLS1 and PLS2 models used grain characteristics as explanatory variables. Their associated PLS equations presented generally a good accuracy (high r^2 values and low RMSEC – supplementary Table 3), and the loss of accuracy was very moderate when they were validated on independent datasets (Table 4). Consequently, models PLS1 and PLS2 enabled high quality predictions for break and reduction flours. However, due to the compensation between these two fractions, and the associated narrower range of variation for total flour, the accuracy was lower for total flour yield, which is the most important parameter of milling value. Therefore, the prediction equations given in supplementary Table 4 for models PLS1 and PLS2, can be used without any doubt for the prediction of break and reduction flours, but should be restricted to the assignment of the genotypes to two or three contrasted classes (for example “low yield”, “medium yield” and “high yield”) in the case of total flour. To obtain predictions of higher quality for total flour yield, it appears necessary to introduce the information given by the break milling stages. The increase of accuracy from model PLS2 to models PLS4 and PLS5 (Table 4 and supplementary Table 2) indicated that a milling process reduced to one or two break stages could be sufficient. This could be really interesting, as the time saved when omitting the reduction stages is significant, and as the amount of grains needed is necessarily smaller than for the whole milling process.

Concerning the flour ash content, which is another important parameter of milling value, our results indicated that it depended very weakly on the grain characters measured, and that it was also independent from the milling behavior. Other studies are needed, but weighting the flour ash content by the total grain ash content could be helpful to progress in the total flour ash prediction.

Conclusions

The wild-type or mutated forms of *Pinb-D1* alleles led to contrasted milling behaviors for soft and hard genotypes. However, the opposite effect of genetic hardness on break and reduction flours had only a moderate incidence on the total flour production, because the regression line corresponding to the strong negative relationship between the two components of total flour was found close to the line of perfect compensation between these two fractions.

NIRS hardness values were confirmed to be related to *Pinb-D1* alleles, and were found to influence both the endosperm reduction (higher for mutated alleles) and its separation from the outer layers. Vitreousness appeared influenced by environmental effects, and was only involved in the endosperm reduction. A strong relationship was found between total flour production and SKCS-HI values which reflect both the presence of wild-type or mutated *Pinb-D1* alleles and vitreousness level. Moderate SKCS values between 30 and 50, corresponding either to soft vitreous or hard mealy grains, appeared optimal for high total flour production.

PLS prediction equations requiring at least presence/absence of the *Pinb-D1a* allele, TGW, TW, GPC and SKCS-HI as explanatory variables were determined and validated, and should be useful to select the lines with a better total flour yield potential.

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Ethical standards

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

Conflict of interest

The authors declare that they have no conflict of interest.

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	terms of model 1							r ² model 1	r ² model 2	r ² model 3	
	N	Env	N*Env	Pinb	N*Pinb	Env*Pinb	residuals				
df for INRA NILs	1	5	5	1	1	5	29				
grain characters for INRA NILs	TGW	5.81 ns	684 ***	38.4 ns	15.3 ns	1.51 ns	22.2 ns	364	0.68	0.64	0.87
	TW	0.02 ns	549 ***	10.7 ns	0.52 ns	0.19 ns	14.8 ns	40.9	0.93	0.91	0.93
	grain diameter	0.01 ns	0.12 ns	0.02 ns	0 ns	0.01 ns	0.01 ns	0.54	0.25	0.22	0.81
	GPC	6.09 ***	18.5 ***	2.19 ns	0.01 ns	0.18 ns	0.51 ns	6.15	0.82	0.8	0.89
	NIRS-hardness	18.7 ns	3146 ***	212 ns	18723 ***	1.33 ns	199 ns	1105	0.95	0.14	0.97
	Vitreousness	308 **	11865 ***	197 ns	4290 ***	21.9 ns	829 ***	782	0.96	0.68	0.96
	SKCS-HI	22.7 ns	5148 ***	123 ns	12642 ***	11 ns	523 **	674	0.96	0.28	0.97
milling fractions for INRA NILs	coarse middlings	305 *	5073 ***	86.3 ns	22342 ***	47.1 ns	219 ns	1628	0.95	0.18	0.98
	fine middlings	420 *	13211 ***	161 ns	34170 ***	13.1 ns	323 ns	1689	0.97	0.26	0.98
	shorts	58.1 ns	274 ns	256 ns	125 ns	1.76 ns	180 ns	876	0.5	0.32	0.51
	coarse bran	21.4 ns	1192 ***	37.7 ns	4003 ***	15.2 ns	26.3 ns	526	0.91	0.21	0.95
	fine bran	2.66 ns	126 ***	4.3 ns	714 ***	0.02 ns	9.6 ns	110	0.89	0.14	0.95
	total bran	13.2 ns	1885 ***	55.3 ns	1314 ***	7.86 ns	36.9 ns	336	0.91	0.53	0.92
	break flour	440 *	10822 ***	118 ns	37061 ***	12.4 ns	1165 *	1918	0.96	0.22	0.98
	reduction flour	356 ns	21206 ***	332 ns	46897 ***	6.18 ns	1360 ns	3856	0.95	0.28	0.96
	total flour	15.5 ns	3116 ***	198 ns	604 ***	1.78 ns	174 ns	1121	0.79	0.64	0.79
df for UFS NILs	1	3	3	1	1	3	35				
grain characters for UFS NILs	TGW	1.17 ns	196 ***	9.74 ns	2.95 ns	0.09 ns	12.2 ns	288	0.44	0.41	0.82
	TW	1.33 ns	27.2 ***	0.96 ns	1.02 ns	0.02 ns	0.6 ns	18.3	0.63	0.6	0.74
	grain diameter	0 ns	0.11 *	0.01 ns	0 ns	0 ns	0 ns	0.32	0.29	0.27	0.74
	GPC	8.33 **	31.2 ***	1.96 ns	0.24 ns	0 ns	0.37 ns	33.6	0.56	0.55	0.88
	NIRS-hardness	275 **	885 ***	1003 ***	698 ***	13 ns	35.9 ns	1268	0.7	0.52	0.73
	Vitreousness	360 ns	11457 ***	129 ns	232 ns	2.21 ns	108 ns	3463	0.78	0.76	0.94
	SKCS-HI	72.5 ns	3921 ***	74.9 ns	93.5 ns	3.52 ns	47.9 ns	1866	0.69	0.67	0.92
milling fractions for UFS NILs	coarse middlings	1091 ***	4125 ***	117 ns	106 ns	14.5 ns	6.52 ns	2025	0.73	0.71	0.8
	fine middlings	862 *	6786 ***	3175 ns	224 ns	26.7 ns	11.9 ns	4803	0.63	0.61	0.88
	Shorts	20.1 ns	315 ***	15.9 ns	39.9 ns	2.15 ns	0.8 ns	363	0.52	0.46	0.84
	coarse bran	111 *	171 ns	5.35 ns	42 ns	0.64 ns	8.05 ns	777	0.3	0.26	0.75
	fine bran	9.04 *	106 ***	3.8 ns	25.8 ***	1.42 ns	0.21 ns	69.7	0.68	0.55	0.82
	total bran	56.7 ns	33 ns	5.26 ns	1.95 ns	0.16 ns	6.24 ns	693	0.13	0.12	0.74
	break flour	860 **	5800 ***	185 ns	108 ns	33.8 ns	21.7 ns	3089	0.69	0.68	0.84
	reduction flour	1055 ***	3991 ***	101 ns	30.6 ns	15.1 ns	16.2 ns	2472	0.68	0.67	0.79
	total flour	9.97 ns	232 **	16.6 ns	23.9 ns	3.71 ns	6.96 ns	525	0.36	0.32	0.83

Table 1: Analyses of variance (Sums of Squares and F Probability) for model 1 with two environmental factors (nitrogen fertilization and site*year combination), and a genetic factor (allele at gene *Pinb-D1*), for the grain characteristics and milling fractions. ANOVAs were made separately for INRA NILs (*Pinb-D1a* or *Pinb-D1b* alleles; 48 observations) and for UFS NILs (*Pinb-D1b* or *Pinb-D1d*; 48 observations). Determination coefficients (r²) were given for model 1, and also for model 2 (including only the environmental factors) and model 3 (model 1 with a supplementary genotype factor).

N = nitrogen treatment; Env = environment = site*year combination; Pinb = allele at gene *Pinb-D1*.

TGW = thousand grain weight; TW = test weight; GPC = grain protein content; SKCS-HI = SKCS hardness index.

df = degree of freedom; *** = p-value < 0.001; ** = p-value < 0.01; * = p-value < 0.05; ns = non significant.

	INRA NILs			UFS NILs			elite lines panel					
	<i>Pinb-D1a</i> (n=24)	<i>Pinb-D1b</i> (n=24)	whole dataset (n=48)	<i>Pinb-D1b</i> (n=24)	<i>Pinb-D1d</i> (n=24)	whole dataset (n=48)	<i>Pinb-D1a</i> (n=32)	<i>Pinb-D1b</i> (n=49)	<i>Pinb-D1c</i> (n=24)	<i>Pinb-D1d</i> (n=58)	hard genotypes (n=131)	whole dataset (n=163)
coarse middlings (g)	97.6 (0.14)	140.7 (0.08)	119.2 (0.21)	151.3 (0.08)	148.4 (0.09)	149.9 (0.08)	103 (0.14)	142.3 (0.1)	145.6 (0.08)	137.6 (0.09)	140.8 (0.09)	133.4 (0.15)
B1 fine middlings (g)	81.7 (0.08)	82.5 (0.05)	82.1 (0.06)	85.2 (0.04)	86.2 (0.04)	85.7 (0.04)	89.1 (0.06)	89.1 (0.04)	88.4 (0.04)	91.1 (0.06)	89.9 (0.05)	89.7 (0.05)
fine middlings (g)	172.6 (0.12)	225.5 (0.08)	199.6 (0.16)	238.4 (0.07)	234.1 (0.07)	236.2 (0.07)	177.2 (0.08)	228.7 (0.07)	233.9 (0.06)	222.6 (0.06)	227 (0.06)	217.2 (0.11)
shorts (g)	32.6 (0.16)	35.6 (0.18)	34.1 (0.18)	31.4 (0.13)	29.6 (0.13)	30.5 (0.13)	32.2 (0.3)	27.9 (0.1)	27.8 (0.14)	26.1 (0.12)	27 (0.12)	28.1 (0.2)
coarse bran (g)	74.3 (0.09)	56 (0.11)	65.1 (0.17)	48.7 (0.08)	50.6 (0.11)	49.7 (0.1)	69.4 (0.12)	51.3 (0.1)	54.8 (0.11)	53.9 (0.12)	53.1 (0.11)	56.3 (0.16)
fine bran (g)	25.4 (0.1)	33.2 (0.07)	29.4 (0.16)	31.1 (0.06)	29.6 (0.07)	30.4 (0.07)	22.7 (0.1)	28.8 (0.08)	28.5 (0.1)	27 (0.09)	27.9 (0.09)	26.9 (0.12)
total bran (g)	99.7 (0.07)	89.2 (0.08)	94.4 (0.09)	79.8 (0.05)	80.2 (0.06)	80 (0.05)	92.1 (0.08)	80.1 (0.06)	83.3 (0.08)	80.9 (0.08)	81.1 (0.07)	83.2 (0.09)
break flour (g)	189.2 (0.11)	133.6 (0.1)	161.4 (0.2)	129.8 (0.11)	132.8 (0.12)	131.3 (0.11)	186 (0.09)	136.5 (0.12)	130.5 (0.11)	141.3 (0.09)	137.5 (0.11)	147 (0.17)
reduction flour (g)	179.2 (0.16)	241.5 (0.08)	211.1 (0.19)	258.9 (0.04)	257.3 (0.05)	258.1 (0.05)	189.8 (0.15)	255.5 (0.07)	258.4 (0.04)	251.8 (0.05)	254.4 (0.06)	241.7 (0.13)
total flour (g)	367.8 (0.03)	375.1 (0.03)	371.5 (0.03)	388.7 (0.01)	390.1 (0.01)	389.4 (0.01)	375.7 (0.04)	392 (0.02)	388.8 (0.02)	393.1 (0.02)	391.9 (0.02)	388.7 (0.03)
flour ash content (%dm)	-	-	-	-	-	-	0.582 (0.12) n=26	0.592 (0.09) n=45	0.58 (0.08) n=19	0.595 (0.11) n=55	0.591 (0.1) n=119	0.590 (0.1) n=145

Table 2: Elementary statistics (mean and coefficient of variation) obtained in the case of INRA NILs, UFS NILs and the elite lines panel, for 10 milling fractions. For each dataset, results were given for the subsets corresponding to the different alleles at gene *Pinb-D1* and for the whole dataset.

B1 = break 1.

		Contribution of the different explanatory variables to the deviance reduction (%)														total
		SKCS-HI		NIRS hardness		vitreousness		TGW		TW		grain diameter		GPC		
		≥	<	≥	<	≥	<	≥	<	≥	<	≥	<	≥	<	
segmentation model 1 for the NILs Dataset	coarse middlings			71.5	0	17.9	0	1.5	0	0	0	0	0	1.2	0	92.2
	B1 fine middlings			34	0	0	24.2	4.8	0	0	2	0	0	4	0	69
	fine middlings			71.3	0	18.1	0	2.1	0	0	0	0	0	0	0	91.5
	Shorts			1.3	1.3	10.2	1.1	1.6	0	0	13.3	0	8.9	0	0	37.7
	coarse bran			0	80	0	0	0	3	0	1.3	0	0	0	0	84.3
	fine bran			37.6	0	1.8	0	0	10.2	9.6	4	0	1.2	0	1.2	65.6
	total bran			0	72.7	0	0	0	6.5	0	0	0	0	0	0	79.2
	break flour			0	67.8	0	25	0	0	0	0	0	0	0	0	92.8
	reduction flour			80.6	0	7.3	0	1.9	0	0	0	0	0	0	0	89.8
	total flour			55.9	0	0	3.4	9.3	0	5.5	0	0	0	0	0	74.2
segmentation model 2 for the NILs Dataset	coarse middlings	78.6	0					4	0	1.2	0	0	0	0	0	83.8
	B1 fine middlings	11.1	21.6					27.9	1.4	0	0	0	0	1.3	0	63.4
	fine middlings	82.1	0					3.5	0	0	0	0	0	0	0	85.6
	Shorts	8.7	1.5					6.6	6.5	0	13.3	0	0	0	0	36.7
	coarse bran	0	59.8					0	18.5	0	0	0	0	0	0	78.3
	fine bran	44.1	0					0	6.1	6	5.5	0	0	0	3.2	64.9
	total bran	0	25.1					1.1	50.3	1.7	1.1	0	0	0	0	79.2
	break flour	0	86.8					0	1	0	0	0	0	0	0	87.8
	reduction flour	72.4	0					6.9	0	0	0	0	0	0	0	79.3
	total flour	15.4	6.1					45.5	0	0	0	2.2	0	0	1.3	70.5
segmentation model 2 for the elite lines dataset	coarse middlings	68.3	0					2.2	0	0	0	0	0	12.2	0	82.7
	B1 fine middlings	6.6	21.8					20.4	5.9	2.9	0	0	0	0	0	57.7
	fine middlings	82.9	0					1.6	0	0	0	0	0	3.4	0	87.9
	Shorts	8.8	20.4					0	0	0	0	12.7	0	1	0	42.9
	coarse bran	0	46.5					0	4.6	0	3.8	0	1.8	0	4.4	61.1
	fine bran	58.9	0					0	0	0	4.2	2.6	0	1.1	0	66.8
	total bran	10.1	30.7					0	1.9	0	17.5	0	0	0	1.8	62
	break flour	0	83.8					0	0	0	0	2.1	0	0	0	85.8
	reduction flour	69.8	0					0	0	0	0	0	2.3	6.4	0	78.5
	total flour	34.4	12.4					1.6	0	6.7	0	0	4.2	0	0	59.3
flour ash content	6.5	0					0	10.6	0	3.9	4.3	0	0	17.3	42.6	

Table 3: Results of segmentation model 1 (6 explanatory variables: NIRS hardness, vitreousness, TGW, TW, grain diameter and GPC) and segmentation model 2 (5 explanatory variables: SKCS-HI, TGW, TW, grain diameter and GPC), for 10 milling fractions considered as response variables. Segmentations were applied to the NILs dataset regrouping INRA NILs, UFS NILs and the control Soissons (models 1 and 2), and to the elite lines dataset (only model 2, with flour ash content as a supplementary response variable). Given values are the contributions of the different explanatory variables to the deviance reduction in %. For each explanatory variable, the column “≥” corresponds to binary partitioning for which the highest values of the response variable are obtained for values of the explanatory variable superior to the threshold defined by the algorithm. On the contrary, the column “<” corresponds to binary partitioning for which the highest values of the response variable are obtained for values of the explanatory variable inferior to the threshold defined by the algorithm.
SKCS- HI = SKCS hardness index; TGW = thousand grain weight; TW = test weight; GPC = grain protein content; B1 = break 1.

		model PLS2				model PLS4				model PLS5			
		NILs dataset		elite lines dataset		NILs dataset		elite lines dataset		NILs dataset		elite lines dataset	
		fitted / observed	predicted / observed	fitted / observed	predicted / observed	fitted / observed	predicted / observed	fitted / observed	predicted / observed	fitted / observed	predicted / observed	fitted / observed	predicted / observed
all genotypes	Shorts	0.17	0.03	0.18	0.02	0.31	0.03	0.32	0.01	0.37	0.03	0.34	0
	coarse bran	0.87	0.86	0.59	0.58	0.91	0.85	0.78	0.71	1	1	1	1
	fine bran	0.55	0.52	0.64	0.61	0.78	0.71	0.76	0.72	0.89	0.8	0.83	0.76
	total bran	0.8	0.77	0.53	0.5	0.85	0.75	0.73	0.61	0.99	0.98	0.97	0.96
	break flour	0.91	0.89	0.89	0.87	0.99	0.99	0.99	0.99	1	1	1	1
	reduction flour	0.89	0.87	0.8	0.78	0.97	0.94	0.96	0.95	0.98	0.97	0.98	0.96
	total flour	0.68	0.6	0.49	0.43	0.77	0.58	0.69	0.53	0.87	0.74	0.82	0.62
soft genotypes	break flour	0.87	0.75	0.49	0.4	1	0.99	0.99	0.98	1	1	1	1
	reduction flour	0.78	0.5	0.29	0.16	0.95	0.89	0.92	0.87	0.98	0.91	0.95	0.84
	total flour	0.41	0.01	0.07	0	0.6	0.37	0.68	0.48	0.85	0.39	0.78	0.55
hard genotypes	break flour	0.83	0.81	0.81	0.78	0.98	0.98	0.98	0.98	1	1	1	1
	reduction flour	0.72	0.64	0.61	0.54	0.86	0.78	0.9	0.81	0.96	0.9	0.96	0.82
	total flour	0.58	0.53	0.51	0.48	0.73	0.52	0.61	0.3	0.9	0.74	0.83	0.44

Table 4: Determination coefficients (r^2) obtained with three PLS regression models used for the explanation or the prediction of the final milling fractions. The explanatory variables were GPC, TGW, TW, SKCS-HI and the presence/absence of *Pinb-D1a* for model PLS2. PLS4 include the same explanatory variables than PLS2 + the four fractions obtained at B1 stage (B1 overtails, B1 coarse and fine middlings and B1 flour). PLS5 include the same explanatory variables than PLS4 + the four fractions obtained at B2 stage (coarse bran, B2 coarse and fine middlings and B2 flour). For a given dataset, the level of explanation enabled by a given PLS model corresponded to the r^2 of the relation between observed and fitted values, these fitted values being calculated with the PLS equation obtained on this dataset. The quality of the prediction corresponded to the r^2 of the relation between observed and predicted values, these predicted values being calculated with the PLS equation obtained with the other dataset (validation on an independent dataset).

B1 = break 1; B2 = break 2.

Figure captions

Fig. 1: Weight values obtained with the 32 elite lines on the entire experimental design, for break flour, reduction flour and total flour (n=163), and for flour ash content (n=145). Genotypes are ordered according to their mean value given on the right side of the graphic and represented by a vertical bar on the plot. Legends appear in the first figure panel: contrasting colors and distinct symbols mark the different *Pinb-D1* alleles and the different “site*year*N treatment” combinations respectively.

Fig. 2: Linear regression between break and reduction flours, for the NILs dataset (regrouping INRA NILs, UFS NILs and the control Soissons) and the elite lines panel. Hard genotypes were plotted in red and soft genotypes were plotted in blue. The black line passes by the mean point with a -1 slope, and then represents the perfect compensation between break and reduction flour to obtain a constant total flour production (equivalent to the mean total flour value observed on the considered dataset).

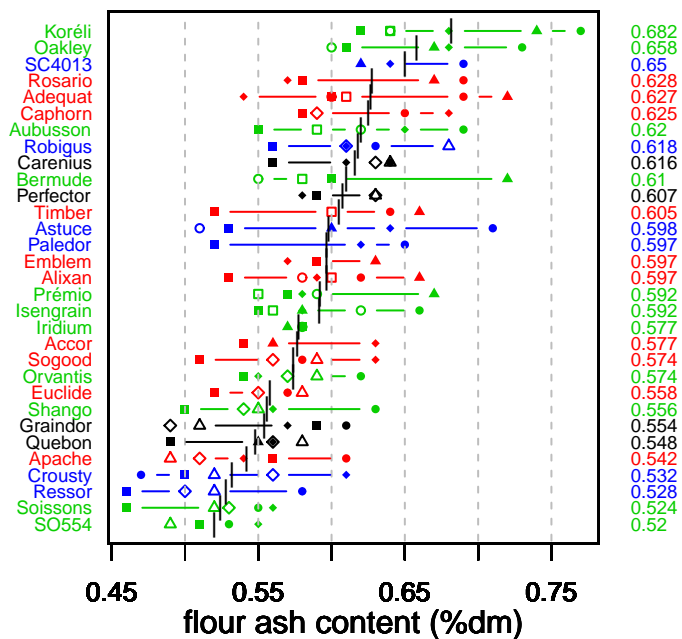
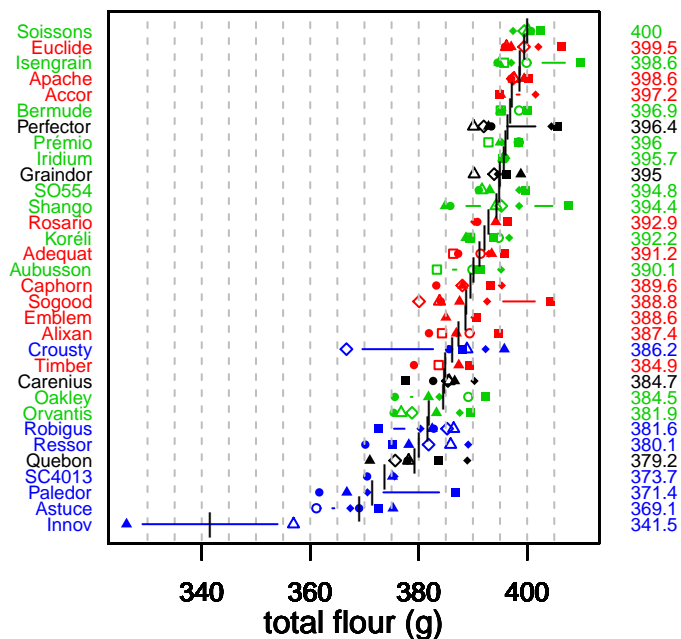
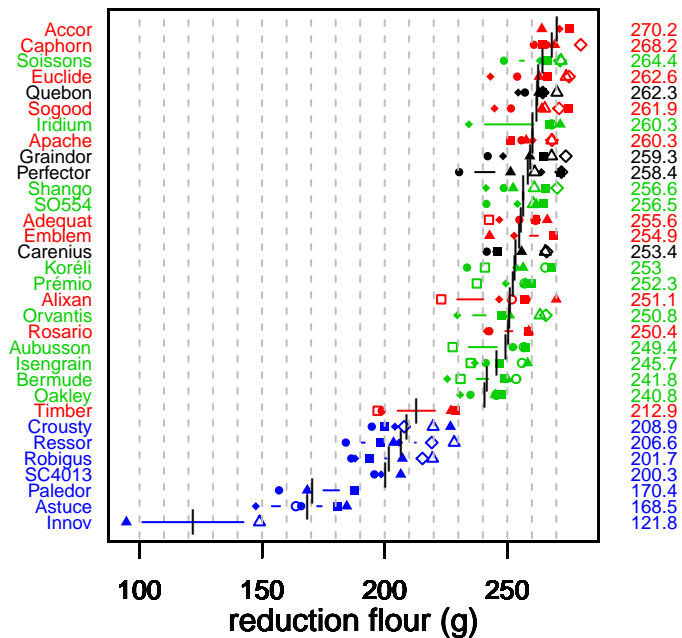
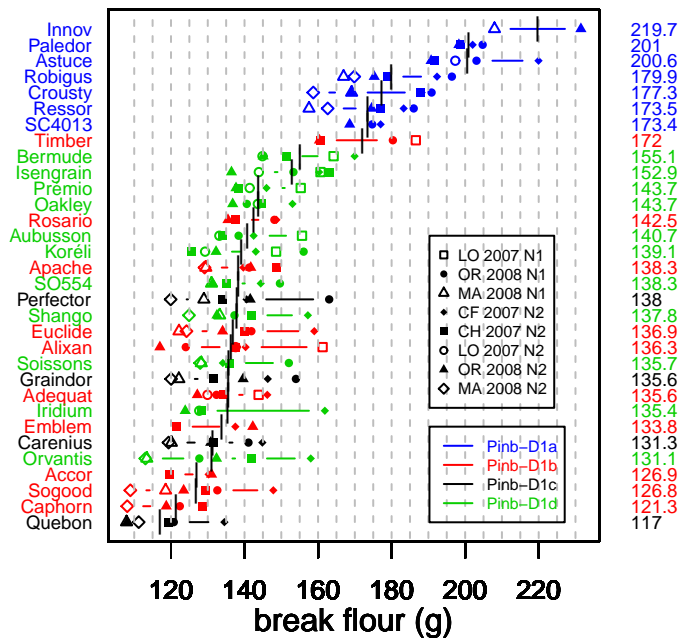
Fig. 3: Relationships between SKCS hardness index and some individual or aggregated fractions from the milling process, in the case of the elite lines dataset. Soft genotypes (*Pinb-D1a*) are plotted in blue, and hard genotypes (*Pinb-D1b* or *Pinb-D1c* or *Pinb-D1d*) in red. The black line corresponds to a smooth of the data, giving the shape of the relation.

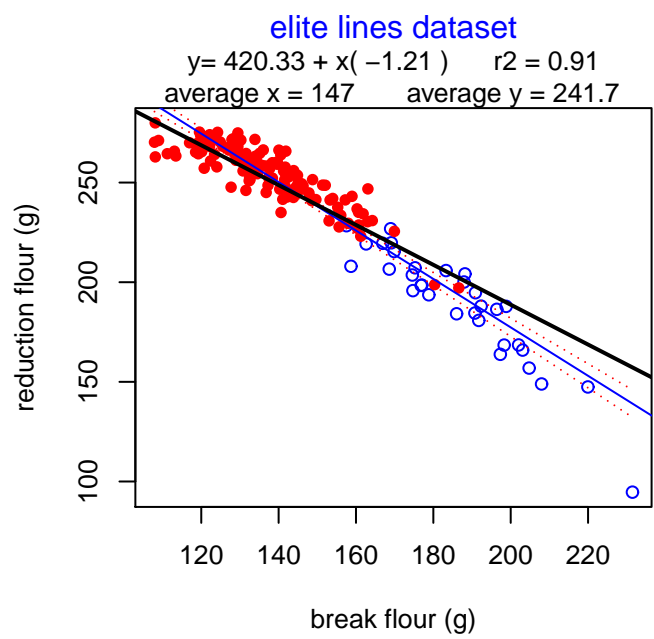
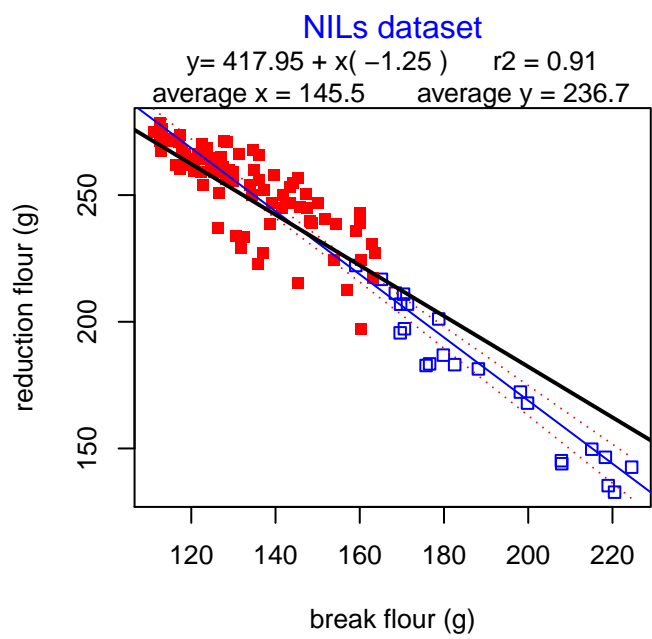
B1 = break 1.

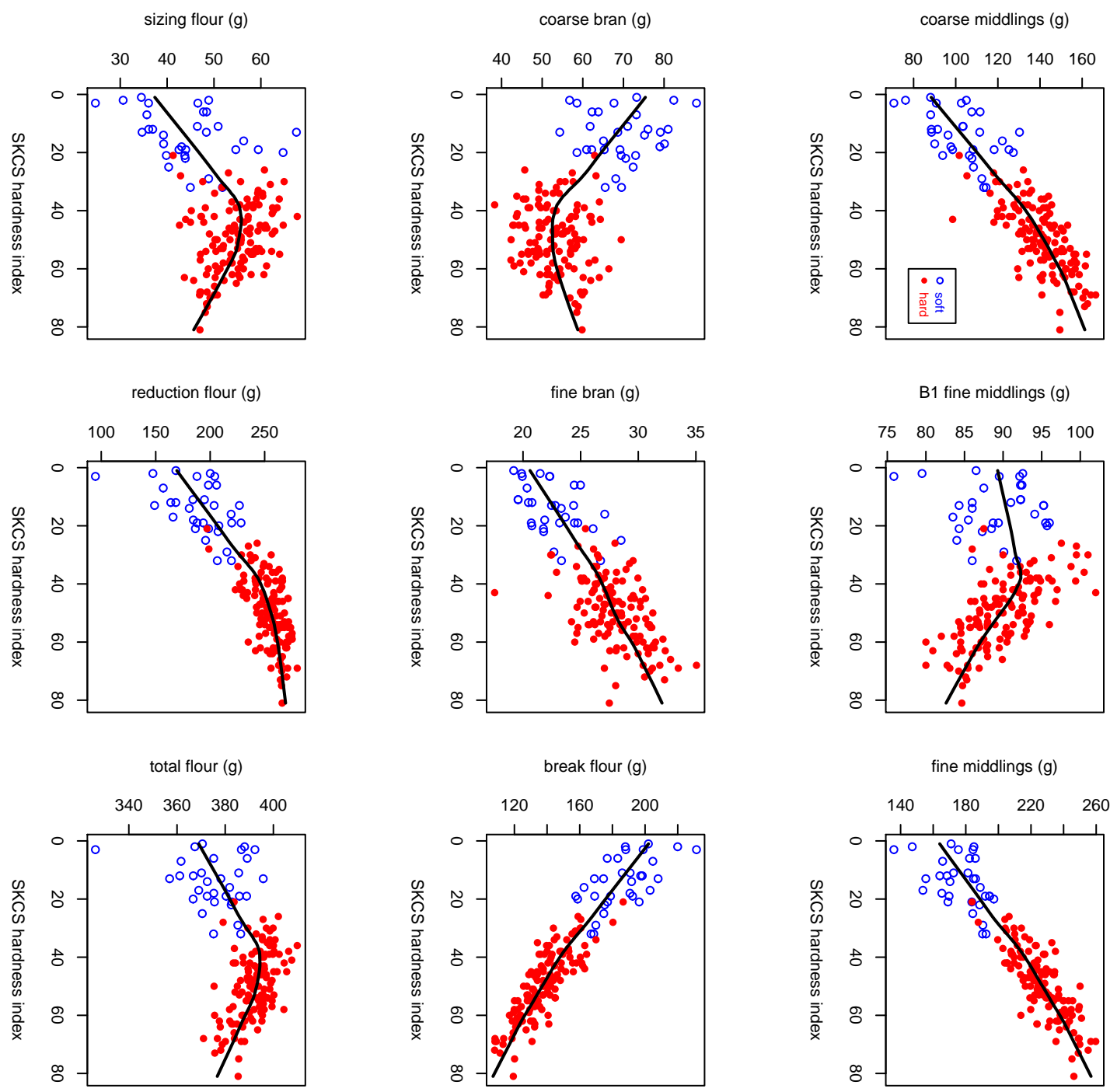
Fig. 4: Segmentation tree for the response variable corresponding to B1 fine middlings (fMidB1), using segmentation model 1 with six explanatory variables (NIRS hardness, vitreousness, TGW, TW, grain diameter and GPC), in the case of the NILs dataset. Nodes are surrounded by circles and leaves are surrounded by rectangles. For each node or leaf are given the mean value of the response variable for the n genotypes present, as well as the deviance value. The explanatory variables used for the binary partitioning (with the thresholds defined by the algorithm) are given for the different branches of the tree. Nodes and leaves with mean values lower than the first quartile of the distribution of the response variable are in green, and in red for mean values higher than the third quartile.

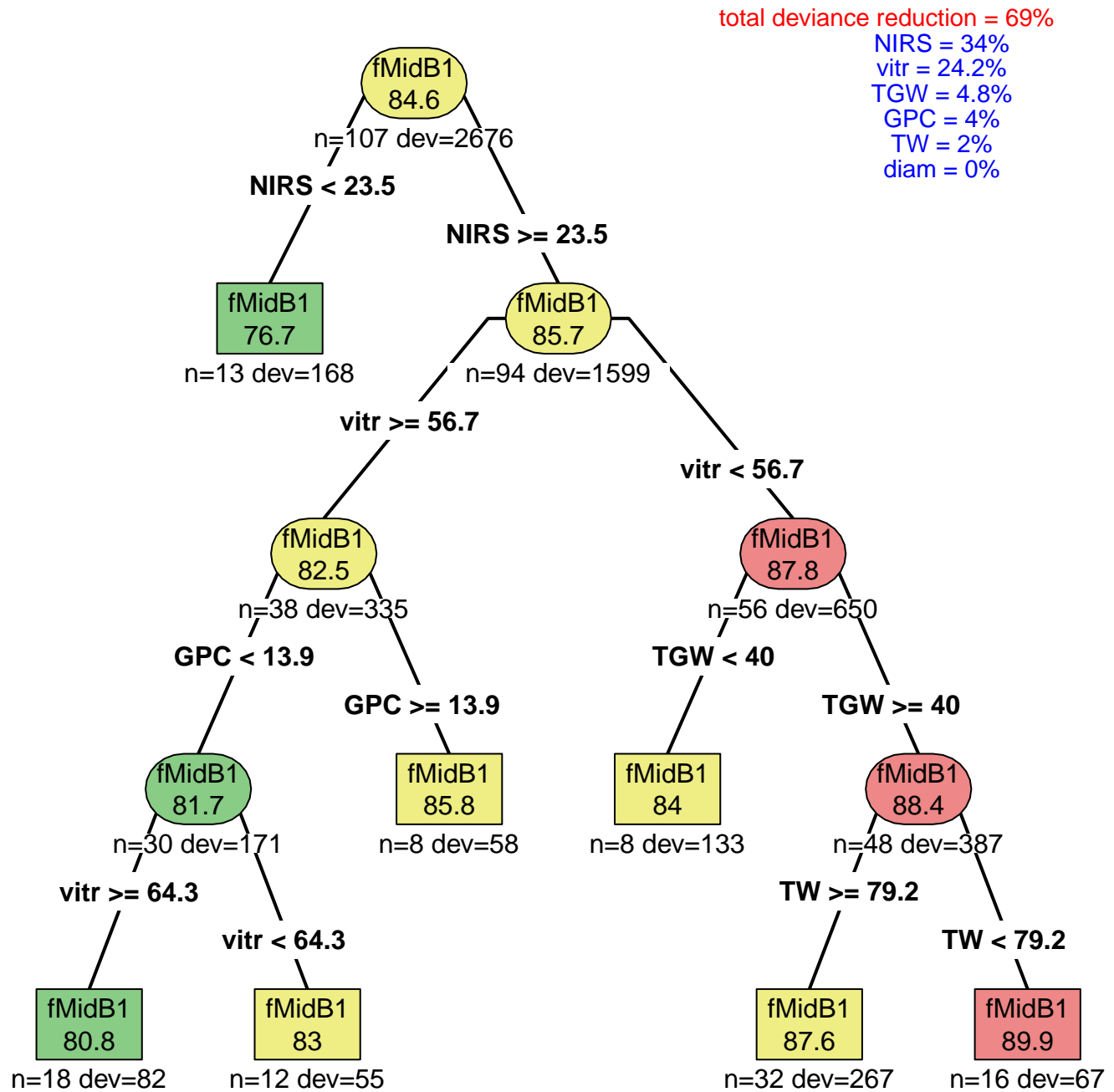
dev = deviance; NIRS = NIRS hardness; vitr = vitreousness; GPC = grain protein content; diam = grain diameter; TW = test weight; TGW = thousand grain weight.

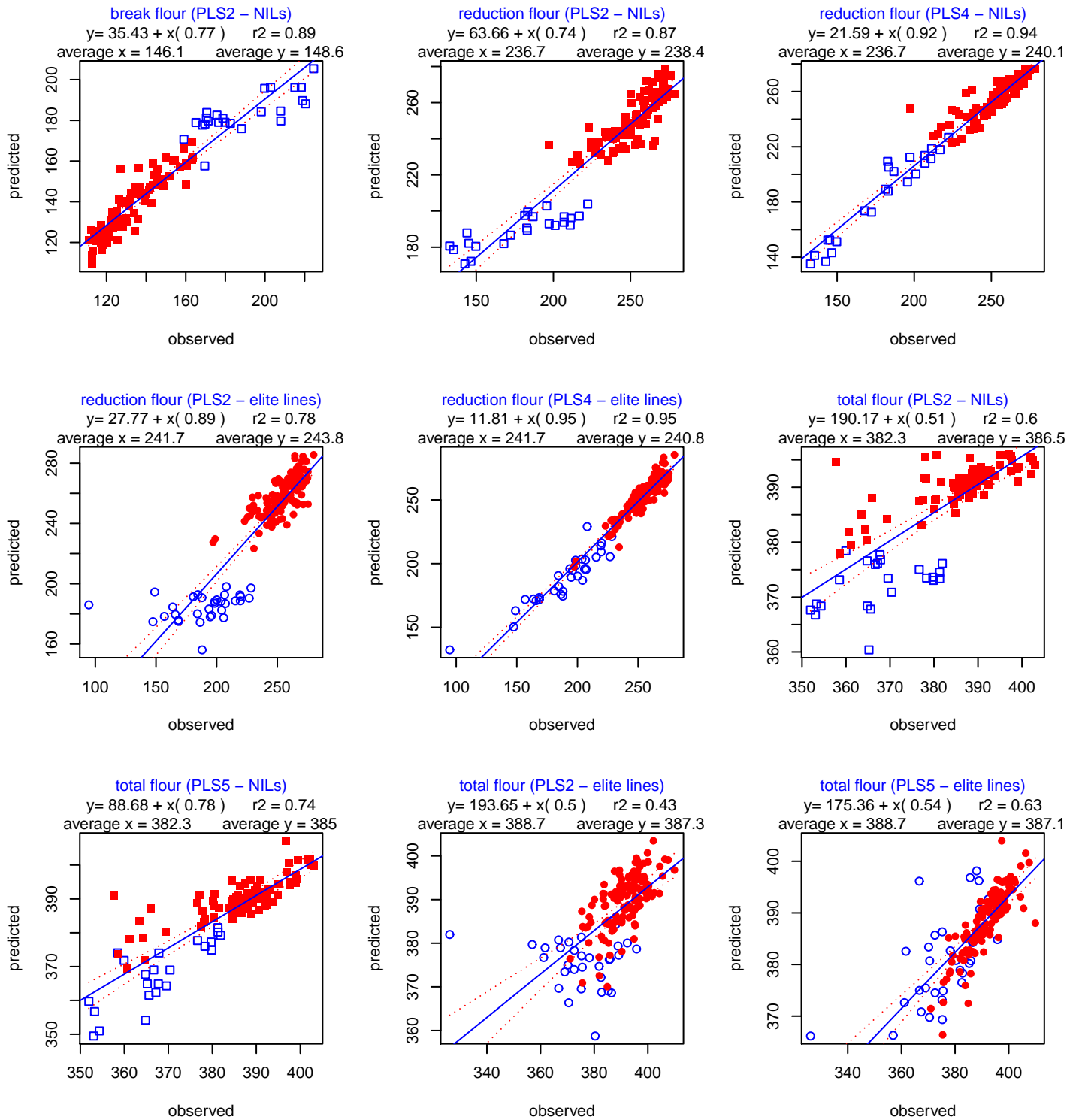
Fig. 5: Relationships between observed and predicted values for the three aggregated flour fractions, and for the NILs (square symbols) or the elite lines (circle symbols) datasets. Results obtained with model PLS2 (GPC, TGW, TW, SKCS-HI and the presence/absence of *Pinb-D1a* as explanatory variables) are all presented, except for break flour in the case of the elite lines dataset. Results obtained with model PLS4 (same explanatory variables than model PLS2 + the four fractions obtained at break 1 stage) are presented for reduction flour. Results obtained with model PLS5 (same explanatory variables than model PLS4 + the four fractions obtained at break 2 stage) are presented for total flour. For a given dataset, the predicted values were calculated with the PLS equations obtained on the other dataset (validation on an independent dataset). Soft genotypes (*Pinb-D1a*) are plotted in blue, and hard genotypes (*Pinb-D1b* or *Pinb-D1c* or *Pinb-D1d*) are plotted in red.











		2007						2008											
		CF		EM		RE		CA		CF		EM		OR		MA		RE	
		N1	N2	N1	N2	N1	N2	N1	N2	N1	N2	N1	N2	N1	N2	N1	N2	N1	N2
INRA NILs	1010a	•	•	•	•	•	•			•	•	•	•					•	•
	1010b	•	•	•	•	•	•			•	•	•	•					•	•
	1259a	•	•	•	•	•	•			•	•	•	•					•	•
	1259b	•	•	•	•	•	•			•	•	•	•					•	•
UFS NILs	VM1b							•	•	•	•			•	•	•	•		
	VM1d							•	•	•	•			•	•	•	•		
	VM2b							•	•	•	•			•	•	•	•		
	VM2d							•	•	•	•			•	•	•	•		
	VM3b							•	•	•	•			•	•	•	•		
	VM3d							•	•	•	•			•	•	•	•		
Soissons			•		•		•	•	•	•	•			•	•	•	•		

Supplementary Table 1: Data available for INRA NILs, UFS NILs and the control Soissons, within an experimental design including two years, six sites and two nitrogen treatments for each site.

Empty space = missing value. • = measurements available for the seven grain characters (thousand grain weight, test weight, grain diameter, grain protein content, NIRS hardness, SKCS hardness index and vitreousness) and the 21milling fractions (flour ash content was not measured on the NILs).

CA = Cappelle-en-Pévèle; CF = Clermont-Ferrand; EM = Estrées-Mons; MA = Maule; OR = Orgeval; RE = Rennes.

N1 = standard nitrogen fertilization; N2 = additional supply of 50kg/ha at flowering.

	<i>Pinb-DI</i> allele	2007						2008			
		CF		CH		LO		MA		OR	
		N1	N2	N1	N2	N1	N2	N1	N2	N1	N2
Accor	b		•		•						•
Adequat	b		•		•	•	•			•	•
Alixan	b		•		•	•	•			•	•
Apache	b		•		•			•	•	•	○
Astuce	a		•		•		•			•	•
Aubusson	d		•		•	•	•			•	
Bermude	d		•		•	•	•				•
Caphorn	b		•		•				•	•	○
Carenius	c		•		•			•	•	•	○
Crousty	a		•		•			•	•	•	○
Emblem	b		•		•						•
Euclide	b		•		•			•	•	•	○
Graindor	c		•		•			•	•	•	○
Innov	a							○			○
Iridium	d		•		•		•				•
Isengrain	d		•		•	•	•			•	•
Koréli	d		•		•	•	•			•	•
Oakley	d		•		•		•			•	•
Orvantis	d		•		•			•	•	•	○
Paledor	a		•		•					•	○
Perfector	c		•		•			•	•	○	○
Premio	d		•		•	•	•				•
Quebon	c		•		•			•	•	○	•
Ressor	a		•		•			•	•	•	○
Robigus	a		•		•			•	•	•	○
Rosario	b		•		•					•	•
SC4013	a		•							•	•
Shango	d		•		•			•	•	•	○
SO554	d		•		•			•		•	○
Sogood	b		•		•			•	•	•	○
Soissons	d		•		•			•	•	•	
Timber	b				•	•				•	•
sample size		0	30	0	30	8	10	15	14	26	30

Supplementary Table 2: Data available for the panel of elite lines, within an experimental design including two years, five sites, and two nitrogen treatments.

Empty space = missing value. • = measurements available for six grain characters (thousand grain weight, test weight, grain diameter, grain protein content, NIRS hardness and SKCS hardness index), the 21 milling fractions and flour ash content. ○ = no measurement for flour ash content.

CF = Clermont-Ferrand; CH = Chartainvilliers; LO = Louville-la-Chenard; MA = Maule; OR = Orgeval.
N1 = standard nitrogen fertilization; N2 = additional supply of 50kg/ha at flowering.

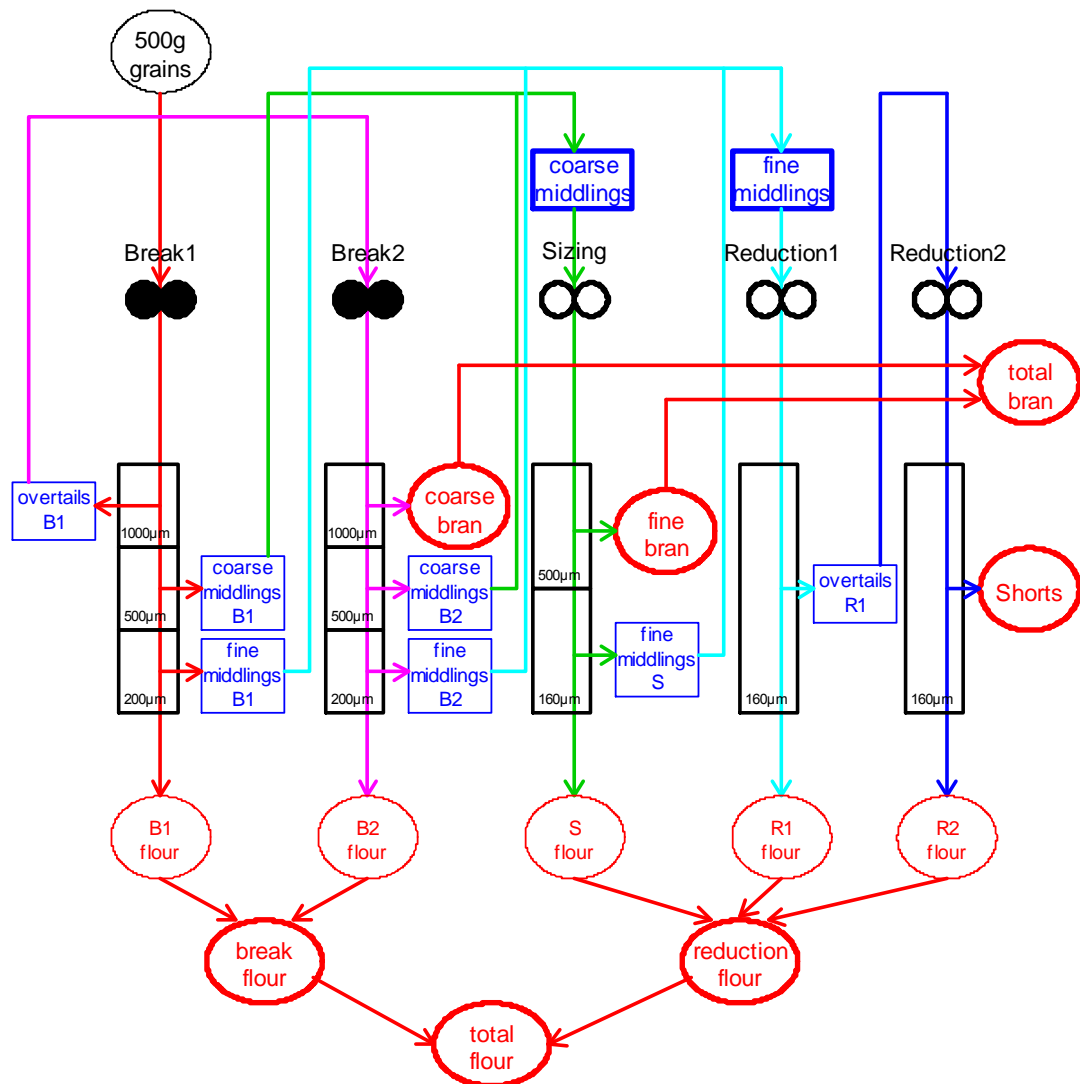
	NILs dataset			elite lines dataset
	model PLS1	model PLS2	model PLS3	model PLS2
coarse middlings r^2 (RMSEC)	0.93 (6.98)	0.92 (7.26)	0.93 (7.16)	0.83 (8.34)
fine middlings	0.92 (8.86)	0.93 (8.39)	0.94 (8.05)	0.89 (8.32)
Shorts	0.18 (5.5)	0.17 (5.32)	0.19 (5.4)	0.18 (5.17)
coarse bran	0.88 (4.27)	0.87 (4.36)	0.88 (4.25)	0.59 (6.19)
fine bran	0.56 (2.49)	0.55 (2.5)	0.58 (2.5)	0.64 (2.04)
total bran	0.81 (4.72)	0.8 (4.9)	0.81 (4.84)	0.53 (5.47)
break flour	0.91 (9.36)	0.91 (8.86)	0.92 (8.49)	0.89 (8.53)
reduction flour	0.9 (12.22)	0.89 (12.35)	0.9 (12.35)	0.8 (14.95)
total flour	0.7 (7.44)	0.68 (7.45)	0.71 (7.43)	0.49 (8.09)
flour ash content	-	-	-	0.28 (0.05)

Supplementary Table 3: Results obtained for three PLS regression models used to relate the variation of the milling characteristics (response variables) to the variation of grain characteristics. The three models included GPC, TGW, TW and the presence/absence of *Pinb-D1a* as explanatory variables. NIRS hardness and vitreousness were added in the case of model PLS1, SKCS-HI in the case of model PLS2, and the three characters related to grain mechanical resistance together for model PLS3. In the absence of vitreousness measurements for the elite lines dataset, models PLS1 and PLS3 could only be applied to the NILs dataset. r^2 = determination coefficient for the relation between observed and fitted values; RMSEC = root mean square error of calibration.

response variable		NILs dataset n = 107		total dataset (NILs + elite lines) n = 270					
		break flour	reduction flour	break flour		reduction flour		total flour	
PLS model		PLS1	PLS1	PLS2	PLS4	PLS2	PLS4	PLS4	PLS5
number of explanatory variables		6	6	5	8	5	8	8	12
r^2 (observed/fitted)		0.91	0.90	0.89	0.99	0.84	0.96	0.70	0.82
RMSEC		9.36	12.22	8.98	2.51	13.98	7.04	6.97	5.65
coefficients of the PLS equation	intercept	282.740	-75.8279	272.103	41.1305	-29.907	275.293	318.538	-1630.38
	TGW	0.14621	0.41319	-0.75982	-0.23306	1.28432	0.28126	0.04311	-0.15138
	TW	-1.04379	2.73535	-0.03509	0.42725	1.64837	0.59569	1.01518	0.2921
	GPC	-2.15938	3.83549	-4.02515	0.10988	4.59533	-1.59252	-1.48268	-0.73335
	NIRS hardness	-0.30446	0.47254	-	-	-	-	-	-
	vitreousness	-0.50551	0.26093	-	-	-	-	-	-
	SKCS-HI	-	-	-0.97029	-0.21053	0.73032	-0.01767	-0.22341	0.06051
	<i>Pmb-D1a</i>	31.99189	-42.5654	18.08735	-2.21671	-41.00309	-9.7757	-11.94388	-1.85295
	B1 overtails	-	-	-	0.18754	-	-0.33906	-0.1574	-0.74616
	B1 coarse middlings	-	-	-	-0.35656	-	0.58911	0.22957	4.05618
	B1 fine middlings	-	-	-	0.00787	-	0.43554	0.44719	4.45231
	B1 flour	-	-	-	0.94322	-	-1.06076	-0.11987	4.27112
	coarse bran	-	-	-	-	-	-	-	3.76619
	B2 coarse middlings	-	-	-	-	-	-	-	3.90676
	B2 fine middlings	-	-	-	-	-	-	-	5.14473
	B2 flour	-	-	-	-	-	-	-	4.64952

Supplementary Table 4: Characteristics of four PLS regression models which can be used for the prediction of break flour, reduction flour and total flour, and coefficients of the corresponding equations.

r^2 = determination coefficient; RMSEC = root mean square error of calibration; TGW = thousand grain weight; TW = test weight; GPC = grain protein content; SKCS-HI = SKCS hardness index; B1 = break 1; B2 = break 2.



Supplementary Fig. 1: Flow diagram for the patented LabMill prototype provided by CHOPIN Technologies including two break stages, a sizing stage and two reduction stages and their associated sifters (sieve cloth openings are given in Microns). Terminal fractions in red are surrounded by ellipses, and intermediate fractions in blue are surrounded by rectangles. The intermediate and final fractions which have been particularly studied are surrounded in bold. Filled cylinders represent corrugated rolls whereas empty cylinders represent smooth rolls.

B1 = break 1; B2 = break 2; S = sizing; R1 = reduction 1; R2 = reduction 2.