Hordoindolines are associated with a major endosperm-texture QTL in Barley (Hordeum vulgare)


Abstract: Endosperm texture has a tremendous impact on the end-use quality of wheat (Triticum aestivum L.). Cultivars of barley (Hordeum vulgare L.), a close relative of wheat, also vary measurably in grain hardness. However, in contrast to wheat, little is known about the genetic control of barley grain hardness. Puroindolines are endosperm-specific proteins found in wheat and its relatives. In wheat, puroindoline sequence variation controls the majority of wheat grain texture variation. Hordoindolines, the puroindoline homologs of barley, have been identified and mapped. Recently, substantial allelic variation was found for hordoindolines among commercial barley cultivars. Our objective was to determine the influence of hordoindoline allelic variation upon grain hardness and dry matter digestibility in the ‘Steptoe’ × ‘Morex’ mapping population. This population is segregating for hordoindoline allele type, which was measured by a HinA/HinB/Gsp composite marker. One-hundred and fifty lines of the ‘Steptoe’ × ‘Morex’ population were grown in a replicated field trial. Grain hardness was estimated by near-infrared reflectance (NIR) and measured using the single kernel characterization system (SKCS). Variation attributable to the HinA/HinB/Gsp locus averaged 5.7 SKCS hardness units (SKCS U). QTL analysis revealed the presence of several areas of the genome associated with grain hardness. The largest QTL mapped to the HinA/HinB/Gsp region on the short arm of chromosome 7 (5H). This QTL explains 22% of the SKCS hardness difference observed in this study. The results indicate that the Hardness locus is present in barley and implicates the hordoindolines in endosperm texture control.

Key words: puroindolines, grain hardness, digestibility.

Résumé : La texture de l’albumen a un impact considérable sur la qualité du blé selon les différentes utilisations projetées. Les cultivars de l’orge (Hordeum vulgare L.), un proche parent du blé (Triticum aestivum L.), montrent aussi une grande variation pour ce qui est de la dureté du grain. Cependant, contrairement à la situation chez le blé, bien peu de choses sont connues quant au contrôle génétique de la dureté du grain chez l’orge. Les puroindolines sont des protéines spécifiques de l’albumen qu’on retrouve chez le blé et les espèces voisines. Chez le blé, la variation de la séquence de puroindolines détermine largement la variation quant à la texture des grains. Les hordoindolines, les homologues des puroindolines chez l’orge, ont été identifiées et cartographiées. Récemment, une importante variation allélique au niveau des hordoindolines a été observée chez des cultivars commerciaux de l’orge. L’objectif des auteurs était de déterminer l’impact de la variation allélique au niveau des hordoindolines sur la dureté des grains et la digestibilité de la matière sèche au sein de la population de cartographie dérivée du croisement ‘Steptoe’ × ‘Morex’. Cette population est en ségrégation pour le type d’hordoindoline, lequel a été déterminé à l’aide du marqueur composite HinA/HinB/Gsp. Cent cinquante lignées de la population ‘Steptoe’ × ‘Morex’ ont été cultivées en parcelles d’essai. La dureté des grains a été mesurée par réflectivité en proche infrarouge (NIR) à l’aide d’un seul grain (SKCS : « single kernel characterization system »). La variation attributable au locus HinA/HinB/Gsp était en moyenne de 5.7 unités de dureté SKCS. Une analyse QTL a révélé l’association de plusieurs régions génomiques avec la dureté des grains. Le QTL le plus important était localisé dans la même région que le locus HinA/HinB/Gsp sur le bras court du chromosome 7 (5H). Ce QTL permet d’expliquer 22 % des différences de dureté des grains observées lors de cette étude. Ces résultats indiquent que
le locus "Hardness" est présent chez l‘orge et que les hordoindolines seraient impliquées dans le contrôle de la texture de l‘albumen.

Mots clés : puroindolines, dureté des grains, digestibilité.

[Traduit par la Rédaction]

Introduction

Amélioration de la qualité de l‘élevage est une partie importante de l‘amélioration des cultures. Des ressources considérables ont été consacrées à la mappage et au caractérisation des gènes responsables pour la variation dans le seigle (Hordeum vulgare L.) grain qualité. Le mappage de la nature de chromosomes associés à des caractéristiques quantitatives tels que la mélange ou la qualité du grain (Hayes et al. 1993; Rouvès et al. 1996). Le contrôle génétique de la texture du grain dans le seigle a reçu peu d‘attention, bien que des preuves suggèrent que c‘est important. La mélange, une mesure de la texture du grain, apparaît à corrélérer négativement avec la qualité du mélange (Allison 1986; Brennan et al. 1996). En outre, il a été montré que l‘endosperme particules taille affecte la digestion de la nourriture (Bowman et al. 2001). Des avancées en sélection pour le mélange et la qualité de la nourriture peuvent donc dépendre de la variation des caractéristiques de la texture du grain en endosperm. Par conséquent, il est important de mappage et de caractérisation des gènes responsables pour ce phénomène.

Dans les autres membres des Triticaceae, ainsi qu‘un seigle (Triticum aestivum L.), grain texturale différences traditionnellement été une grande partie en sélection de nouvelles variétés owing to their large impacts on end-product quality. Because endosperm texture is one of the primary determinants of end-product quality, hardness is a major market-class distinction for wheat (i.e.wheat is marketed as either hard or soft.)

Endosperm hardness can be measured in many ways. Traditionnellement, le grain durcissement a été mesuré par le particule index (PSI) ou infrarouge réflexion (NIR), mais la mesure de la durcissement de grain a été largement limité à la mélange (Symes 1965; Norris et al. 1989). Le seigle grain caractérisation système (SKCS), qui mesure a fonction du harnais requiert pour le mélange du grain entier, peut être utilisé pour les deux espèces et est devenue un moyen populaire de mesurer ce trait (Gaines et al. 1996; Psotha 1996).

Wheat-grain hardness is simply inherited and controlled primarily by a single locus, termed "Hardness" (Ha) (Symes 1965; Baker 1977) that resides on the short arm of chromosome 5D (Mattern et al. 1973; Law et al. 1978). Soft wheats (Ha) are physically softer in endosperm texture and produce flours of smaller size and size distribution than hard wheats (ha). Three genes closely linked to Ha have been identified (Rahman et al. 1994; Sourdille et al. 1996; Giroux and Norris 1998). These are the structurally related genes puroindoline a (pinA), puroindoline b (pinB), and Gsp-1a. All hard wheats characterized to date have a sequence alteration in either pinA or pinB (Giroux and Morris 1998). The most common mutation is a glycine-serine alteration of the pinB, which is present in the majority of American hard wheats surveyed (Morris et al. 2001). Expression of wild type pinB in a hard wheat containing this mutant pinB sequence fully restored the soft phenotype (B. Becker, A. Bettge, and M.J. Giroux, unpublished data). PinA and PinB expression has also been shown to confer a softer endosperm texture in transgenic rice plants (Krishnamurthy and Giroux 2001). Thus, the evidence is strong that puroindolines are responsible for the function of Ha in wheat.

The puroindoline homologs of barley, the hordoindolines, have been identified (Gautier et al. 2000). They map to the short arm of chromosome 7 (SH), the same chromosomal location of puroindolines in wheat (Rouvès et al. 1996; Beecher et al. 2001). A recent study suggests that substantial allelic variation exists in barley for both hordoindoline a (hina) and hordoindoline b (hinB) sequences (Beecher et al. 2001). The chromosomal region in which the hordoindolines are found appears to be involved in grain texture dependent traits such as milling energy and level of fine grind extract, as well as malt extract yield (Thomas et al. 1996; Mather et al. 1997; Beecher et al. 2001). Our objective was to identify regions of the genome that affect barley-grain hardness in a QTL analysis. We were also particularly interested in determining whether hordoindoline allelic variation influenced endosperm texture and related traits in a doubled haploid population segregating at this locus.

Materials and methods

Plant material

The barley mapping population consisted of 150 doubled-haploid lines (DHLs) generated from a ‘Steptoe’ (C115229) × ‘Morex’ (C115773) cross (Kleinhofs et al. 1993). The 150 lines and parents were grown in randomized trials in Bozeman, Mont., in 1992 and 2000. Seed was bulked from replications grown in the field.

Seed texture measurement

Barley seed texture was analyzed by both the SKCS 4100 (Perten Instruments, Springfield, Ill.) and NIR InfraAlyzer 400 (Technicon Corp., Tarrytown, N.Y.) NIR analysis (method 39-70A) was performed on replicates of approximately 10 g wheat seeds ground into a wholewheat flour on a UDY mill (UDY Co., Fort Collins, Colo.) fitted with a 0.5-mm screen. The SKCS machine was used to estimate hardness, kernel weight (wt.), and kernel diameter. SKCS analysis was performed on samples of 100 seeds.

Dry-matter digestibility

Many of the doubled haploid lines in the ‘Steptoe’ × ‘Morex’ population have poor agronomic characteristics. Dry-matter digestibility (DMD) was performed on 115 lines from the ‘Steptoe’ × ‘Morex’ mapping population that produced sufficient grain for all required analyses. Grain samples were cracked using a Buhler mill (Buehler-Miag, Braunschweig, Germany) to simulate the dry-rolling processing done before feeding barley. To measure ruminal DMD (Vanzant et al. 1998), four 5-g samples of each line were weighed and placed into 10 × 20 cm, 50-µm pore poly-
Fig. 1. QTL Analysis of the ‘Steptoe’ × ‘Morex’ mapping population. QTL scans of barley grain protein percentage (blue trace), grain diastatic power (red trace), heading date (green trace), and grain hardness as measured by the SKCS (black trace). LOD scores reported at left. Dotted line indicates LOD = 2.0. The heading date gene (most likely Ppd1) on chromosome 2 exerts so great an effect on phenotype that the QTL scales could not easily be justified for heading date and grain protein percentage. The LOD score scale for heading date is shown in green, whereas that for the other three traits is shown in black. The position of the HinA/HinB/Gsp markers on chromosome 7 is noted.

Ester bags (Ankom Technology, Fairport, N.Y.). Two of the four polyester bags for each line were placed in the rumen of each of two ruminally cannulated steers and incubated for 3 h. All animals were cared for under guidelines equivalent to those laid down by the Canadian Council on Animal Care. Two additional empty blank bags were included with each incubation to correct for DM content owing to microbial contamination. After removal from the rumen, the bags were rinsed under cold water until the wash water ran clear. The bags were dried at 60°C for 48 h, and then weighed. Dry-matter (DM) content of the cracked barley samples was measured (AOAC 1997). Ruminal DMD (measured in grams per kilogram) was calculated according to the following equation:

\[ \text{Dry matter digestibility} = \frac{\text{(sample wt. in × sample DM content) – (sample wt. out – blank)×1000}}{(\text{sample wt. in} × \text{sample DM content})} \]

**Grain and agronomic quality**

Grain protein and heading date were measured in both 1992 and 2000 as described by See et al. (2002). Diastatic power was measured in 1992 only and the data set is available from http://wheat.pw.usda.gov/ggpages/.

**QTL analysis**

Phenotype measurements were obtained for both 1992 and 2000 and entry means were merged with the ‘Steptoe’ × ‘Morex’ DHL marker dataset available from http://wheat.pw.usda.gov/ggpages/. Linkage maps were reconstructed using Mapmaker 3.0 (Lander et al. 1987) with linkage indicated by a minimum LOD of 4.0 and the maximum distance between linked genes set at 35 cM. In this dataset, no recombination has been observed among the markers HinA, HinB, and Gsp. Therefore, our analysis relied upon a HinA/HinB/Gsp composite hardness (Ha) marker. The Ha-s locus is defined as the HinA/HinB/Gsp alleles carried by ‘Steptoe’ and the Ha-m locus is defined as the HinA/HinB/Gsp alleles carried by ‘Morex’. The LOD score and percentage of phenotypic variance accounted for by the Ha locus was taken directly from the Mapmaker QTL output. We considered the LOD scores greater than 3.0 as likely indicators of QTL.

**Statistical analysis**

Analyses of variance were computed for each trait using the environments as replications. The entry’s source of variation was partitioned by including a fixed effect for hordoidinolene class and a random effect for entries within classes using PROC GLM in SAS (SAS Institute Inc. 1988).

**Results**

A major QTL for barley grain hardness is located on the short arm of chromosome 7 (5H)

Quantitative trait loci (QTLs) impacting grain hardness could be found on barley chromosomes 1, 4, 5, and 7 (Fig. 1). The chromosome-1 gene region impacting hardness impacted no other measured trait. The Hardness gene region on chromosome 4, near ABG319A, reflected the impact of one or more genes modifying grain protein content. The effect of the Hardness gene region on chromosome 5 was nearly concordant with variation in diastatic power. The two gene regions on chromosome 7, centered around HinA/HinB/Gsp and CDO504, showed no significant impact on grain protein content, diastatic power, or heading date. From this point forward, merely for readability, we will refer to these chromosomal regions as QTL.

The grain hardness QTL cosegregating with the HinA–HinB–Gsp gene family accounts for approximately 22% of the total phenotypic variation in grain hardness, whereas the other four QTL indicated account for between 9 and 13%. Two of these QTL appear to impact hardness in a pleiotropic manner, one through gross-grain composition (grain protein percentage) and the other perhaps by regulating the amount of β-amylase, a large contributor to overall endosperm protein composition in barley and the primary determinant of diastatic power. The three genes that have no obvious pleiotropic correlants and may be provisionally considered to impact grain hardness in a direct fashion account for approximately 45% of the phenotypic variation for this trait, with the effect centered around the HinA/HinB/Gsp complex responsible for about half of this overall impact. For simplicity, this hordoidinolene complex will hereafter be referred to as Hardness (Ha).

Hordoidinolene allelic state has a significant impact upon both kernel hardness and DMD

The 150 lines of the ‘Steptoe’ × ‘Morex’ mapping population segregated 77 Ha-m (Hardness allele from the ‘Morex’ parent) : 73 Ha-s (Hardness allele from the ‘Steptoe’ parent), or approximately 1:1 (χ² < 0.2). Hardness, kernel weight, kernel diameter, and dry-matter digestibility traits were measured for all lines. Dry-matter digestibility was measured on a subset of 115 lines where sufficient grain was available. SKCS hardness values ranged from 37.2 to 76.7 units, whereas NIR-estimated hardness values ranged from –33.6 to –6.3 units. The correlation between NIR-estimated hardness and hardness directly measured by SKCS was significant, but weak (0.24, Table 1). Kernel weight ranged from 30.1 to 48.6 mg and kernel diameter ranged
Correlations among measured traits

Correlation analysis was performed for all trait combinations (Table 1). The SKCS and NIR methods of measuring grain hardness exhibited a positive, but poor, correlation \( r = 0.24 \). This likely reflects the fact that grain hardness in barley has not been well studied and that the calibration for NIR hardness used was based on a set of wheat genotypes varying in grain hardness. SKCS hardness showed a bimodal distribution with the harder Ha-m class having a greater range and completely overlapping the softer Ha-s class (Fig. 2). Distribution for dry-matter digestibility was also bimodal, but the range within the hordoindoline classes was similar (Fig. 3).

**Discussion**

QTL analysis was performed on the 'Steptoe' × 'Morex' mapping population to determine which regions of the genome contribute to endosperm texture. The most significant QTL for hardness in this study was located on the distal end of the short arm of chromosome 7 (5H), coincident with the location of the HinA–HinB–Gsp markers (Kleinholfs et al. 1993; Rouvès et al. 1996; Beecher et al. 2001). This chromosomal region has previously been implicated in explaining a portion of the level of fine-grind extract and extract viscosity differences (Mathe et al. 1997). This location coincides with the Hardness locus of wheat that resides on the short arm of chromosome 5D (Mattern et al. 1973; Law et al. 1978). Grain texture has been well studied in wheat, barley's close relative. In wheat, the puroindoline genes are associated with the majority of grain-hardness variation (Giroux and Morris 1998). Recent evidence indicates that the puroindolines directly control rice and wheat grain texture (Krishnamurthy and Giroux 2001; B. Beecher, A. Bettge, and M.J. Giroux, unpublished data). They appear to accomplish this by physically interacting with the surface of the starch granule (Greenwell and Schofield 1986). Like puroindolines, hordoindolines have also been found on the surface of barley starch granules (Greenwell and Schofield 1986; Darlington et al. 2000). It seems likely that these homologous sequences control endosperm texture to a large degree in both wheat and barley.

Barley cultivars differ in traits related to grain texture. A survey of barley cultivars found that milling energy, another measure of grain hardness, correlates negatively with malting quality (Allison 1986; Brennan et al. 1996). This study indicates that SKCS hardness is negatively correlated with percentage dry-matter digestibility by ruminants. However, larger particles and slower dry-matter digestibility is associated with higher feed quality (Bowman et al. 2001). Therefore, the development of softer cultivars may benefit malting-quality traits and the development of harder barleys may benefit feed-quality traits. However, until now, the genetic control of barley grain hardness has not received much attention. Perhaps this is because barley has been thought to exhibit relatively little texture variation. In fact, barleys appear to behave in a similar fashion to hard hexaploid wheat. That is, the barley lines in this study averaged 60.3 SKCS U, placing them within the hard range of wheat varieties. The average SKCS hardness difference between lines varying in hordoindoline allele types was 5.7, similar to the average difference of 6 SKCS hardness units reported in a similar study involving various Hardness alleles found in hard wheats (Giroux et al. 2000). The average hardness difference, which is conferred by hordoindoline allele type, is similar to that reported among the different alleles of Hardness in hard wheat (Martin et al. 2001; Lillemo and Morris 2000).

The differences between the SKCS hardness assay and NIR estimates of hardness appear largely because of the wheat-based hardness calibration used for the NIR estimates.
If market-class segregation (malting vs. feeding) is to be done for barley at the elevator, a better NIR calibration for barley-kernel hardness should be developed. Thus, it appears that barley cultivars may be analogous to hard wheat cultivars. That is, barley cultivars exhibit small but significant amounts of variation in grain hardness that affect grain quality, which is controlled to some extent by the Hardness locus. The small differences between the hordoindoline sequences of barley and their soft-wheat homologs may be important in this context (Beecher et al. 2001). Some of these amino-acid sequence changes might influence the degree to which hordoindolines function in grain texture. A difference of as little as one amino acid has been shown to greatly decrease the function of puroindolines in reducing grain hardness (Giroux and Morris 1997; Beecher, Bettge, and Giroux, unpublished data). The changes in the hordoindoline sequences of barley relative to those in soft wheats may be fixed in the species, and therefore the natural potential for softness may be less than for wheat. The diploid progenitors of hexaploid wheat are soft textured (Williams 1986). However, that does not appear to be the case for barley. A survey of several populations of wild barley (Hordeum spontaneum) found significant variability in grain hardness as measured by milling energy (Ellis et al. 1993). Interestingly, the observed variability in the wild material ranged towards the hard end of the observed variation for domestic cultivars. Other research has included a wider range of cultivated germplasm in barley grain-hardness studies. Thus far, no barley varieties have been reported as having endosperm texture typical of soft

Table 2. Hordoindoline allele class means, range, and parent means for grain hardness, kernel morphology traits, and dry matter digestibility of the ‘Steptoe’ × ‘Morex’ doubled-haploid population.

<table>
<thead>
<tr>
<th>Grain hardness</th>
<th>SKCS</th>
<th>NIRS</th>
<th>Kernel wt. (mg)</th>
<th>Kernel diameter (mm)</th>
<th>Dry-matter digestibility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entry</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ha-m</td>
<td>63.0</td>
<td>–20.4</td>
<td>37.6</td>
<td>2.26</td>
<td>39.2</td>
</tr>
<tr>
<td>Ha-s</td>
<td>57.3**</td>
<td>–23.3**</td>
<td>38.9**</td>
<td>2.32**</td>
<td>41.1*</td>
</tr>
<tr>
<td>Range</td>
<td>37.2–76.7</td>
<td>–33.6–(6.3)</td>
<td>30.1–48.6</td>
<td>1.88–2.64</td>
<td>27.1–52.3</td>
</tr>
<tr>
<td>‘Morex’</td>
<td>53.6</td>
<td>–24.1</td>
<td>38.7</td>
<td>2.41</td>
<td>32.7</td>
</tr>
<tr>
<td>‘Steptoe’</td>
<td>56.1</td>
<td>–25.6</td>
<td>42.0</td>
<td>2.39</td>
<td>36.5</td>
</tr>
<tr>
<td>CV%</td>
<td>6.2</td>
<td>23</td>
<td>6.3</td>
<td>5.0</td>
<td>19</td>
</tr>
</tbody>
</table>

Note: Date is based on the mean from two years.* difference between hordoindoline class means is significant at the 0.05 probability level; **, difference between hordoindoline class means is significant at the 0.01 probability level.

1 Single-kernel characterization system.

2 Near-infrared reflectance.

Fig. 2. Distribution of 77 lines within the Ha-m class and 73 lines within the Ha-s class for SKCS hardness from the ‘Steptoe’ (Ha-s allele) × ‘Morex’ (Ha-m allele) doubled haploid mapping population. SKCS average hardness values shown on the x axis. The y axis denotes number of individuals per hardness class. White bars represent individuals that contain the Ha-m allele. Black bars represent individuals carrying the Ha-s allele. Two-year means reported.

Fig. 3. Distribution of 77 lines within the Ha-m class and 73 lines within the Ha-s class for dry matter digestibility (DMD) percentage from the ‘Steptoe’ (Ha-s allele) × ‘Morex’ (Ha-m allele) doubled-haploid mapping population. Dry matter digestibility values shown on the x axis. The y axis denotes number of individuals per digestibility class. White bars represent individuals that contain the Ha-m allele. Black bars represent individuals carrying the Ha-s allele. Two-year means are reported.
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