Creation of the first ultra-low gluten barley (*Hordeum vulgare* L.) for coeliac and gluten-intolerant populations

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Summary

Coeliac disease is a well-defined condition that is estimated to affect approximately 1% of the population worldwide. Noncoeliac gluten sensitivity is a condition that is less well defined, but is estimated to affect up to 10% of the population, and is often self-diagnosed. At present, the only remedy for both conditions is a lifelong gluten-free diet. A gluten-free diet is often expensive, high in fat and low in fibre, which in themselves can lead to adverse health outcomes. Thus, there is an opportunity to use novel plant breeding strategies to develop alternative gluten-free grains. In this work, we describe the breeding and characterization of a novel ultra-low gluten (ULG) barley variety in which the hordein (gluten) content was reduced to below 5 ppm. This was achieved using traditional breeding strategies to combine three recessive alleles, which act independently of each other to lower the hordein content in the parental varieties. The grain of the initial variety was shrunken compared to wild-type barleys. We implemented a breeding strategy to improve the grain size to near wild-type levels and demonstrated that the grains can be malted and brewed successfully. The ULG barley has the potential to provide novel healthy foods and beverages for those who require a gluten-free diet.

Introduction

Immunological markers for coeliac disease (CD) revealed by retrospective analysis of stored serum samples suggest a prevalence of about 1% of most populations worldwide (Fasano et al., 2003). Noncoeliac gluten sensitivity is thought to affect up to 10% of the population in some countries (Aziz et al., 2014; Golley et al., 2015). Both conditions require lifelong dietary exclusion of gluten-like proteins in wheat (gliadins and glutenins), barley (hordeins), rye (secalins) and in some 8% of coeliacs, oats (avenins) (Hardy et al., 2014). Gluten-free diets are traditionally low in fibre, high in fat and economically more costly (Lee et al., 2007; Ohlund et al., 2010; Wild et al., 2010). In addition up to 90% of coeliacs remain currently undiagnosed, a phenomenon termed the ‘coeliac iceberg’ (Catassi et al., 1996; Ravikumara et al., 2007). Untreated coeliacs face adverse health outcomes including low bone mineral density and increased intestinal malignancy (Green et al., 2003).

Bread wheat is hexaploid and contains three related genomes, containing 20–30 glutenin genes and in the order of one hundred gliadin genes, across nine loci, which together make up the gluten component of the grain (Shewry et al., 2009). This complicates selection for the many null alleles required for development of a gluten-free phenotype. However, in barley, a diploid, there are only four hordein protein families, the B-, C-, D- and γ-hordeins. The dominant hordeins comprise two multi-gene families consisting of at least 13 B-hordein genes (Kreis et al., 1983) and 20–30 C-hordein genes (Shewry et al., 1985a). The B-hordeins are encoded by the Hor 2 locus on the short arm of barley chromosome 1H. The high-lysine barley line Riso 56 contains a large gamma-ray-induced genomic deletion of at least 85 kb of DNA containing the entire B-hordein loci (Kreis et al., 1983) and does not accumulate B-hordeins. The C-hordeins are encoded by the Hor 1 locus on the short arm of 1H. Riso 1508 contains an ethyl methanesulfonate (EMS)-induced mutation in the lys3 locus on chromosome 5H (Karlsson, 1977), and this has been suggested to encode a demethylase gene, required for expression of C-hordeins in the endosperm (Wen et al., 2012; von Wettstein, 2009). Riso 1508 does not accumulate C-hordeins and has decreased B- and D-hordein levels (Doll, 1973, 1983), and transcription of the B- and C-hordein genes is drastically reduced (Sorensen, 1992). The B- and C-hordein loci are tightly linked and separated by approximately 12.6 cM representing 10 Mb of DNA (Pedersen and Linde-Laursen, 1995; Shewry and Parmar, 1987). The D-hordeins are encoded by the Hor3 locus, which encodes a single 105-kDa protein (Gu et al., 2003). An Ethiopian-derived landrace, R118 (Brennan et al., 1998), contains a single spontaneous mutation which prevents accumulation of the D-hordeins. The γ-hordein family comprises two genes, γ-1 and γ-3 hordein at the Hor5 locus on the short arm of 1H which encode three γ-proteins (1, 2 and 3) of approximate size 45, 40 and 38 kDa, respectively (Cameron-Mills and Brandt, 1988; Shewry et al., 1985b). The γ-2 protein appears to be derived from the γ-1 gene via post-translational modification which results in the deletion of 30 amino acids. The γ-hordein loci are very tightly linked to the B-hordein locus at 0.2 cM (Shewry and Parmar, 1987; Shewry et al., 1988).

We report the isolation of the first ultra-low gluten barley with hordein levels well below the Codex limit of 20 ppm for gluten in gluten-free food. In addition, the grain shape and size have been improved and are similar to current malting lines. Barley is not a large component of the diet; however, products from barley including malt and beer are common. The problem for coeliacs and gluten intolerants is these products are often included as ‘hidden ingredients’ and although they should be completely eliminated from the coeliac diet, in practice this is often difficult.
These new grains have utility in the preparation of malt, food and beverages for those who suffer from coeliac disease, gluten allergy and gluten intolerance.

Results and discussion

Barley hordein genes occur on chromosome 1H (Figure S1) comprising four gene families, the B-, C-, D- or \( \gamma \)-hordeins at the Hor 2, 1, 3 and 5 loci, respectively. In wild-type barley, the B-hordeins represent the dominant protein family. In chromatographs and SDS-PAGE analysis, the B-hordeins comigrate with and obscure the \( \gamma \)-hordeins.

Creation and properties of hordein double-null line ULG 2.0

The hordein level in barley was first reduced by combining the recessive mutations present in Riso 56 (B-hordein null) and Riso 1508 (C-hordein null). Sixteen putative hordein double-null lines were identified, by SDS-PAGE (Figure S2). The mean seed weight, tiller number, harvest index and seeds per head from F3 families of these 16 lines were determined and the best hordein double-null line, G1, was selected. G1 had the highest number of tillers per F3 plant (Figure S3) with a harvest index of 0.5, 15 seeds per head, and a kernel weight of 38 mg and was generally superior to the other hordein double-null lines. Unexpectedly, the F3 families, derived from several F2 hordein double nulls, segregated approximately 1:3 for B-hordeins by SDS-PAGE (Figure S4) and PCR (Figure S5). We concentrated on refining the G1 line. One seed (G1*) was selected which was null for B- and C-hordeins, and three rounds of single-seed descent were carried out to produce near homozygous, hordein double-null F6 seeds and the pooled seed from 12 of these individual plants became the basis of ultra-low gluten barley with two recessive alleles (called ULG 2.0). Seed was increased in the field for malting and brewing trials. ULG 2.0 revealed a hordein level reduced to ~3% of wild-type and coeliac immunoreactivity reduced 20-fold relative to wild type (Tanner et al., 2010).

In-gel digestion of the dominant protein bands in extracts of F3-F6 generations of ULG 2.0 showed the presence of D-hordein at ~100 kDa and \( \beta \)-glucosidase at 60 kDa and \( \gamma \)-3-hordein at 35 kDa. As expected, there was no detection of the B- and C-hordeins. Surprisingly, \( \gamma \)-1 and \( \gamma \)-2-hordeins were not detected by protein gel analysis. This was unexpected as there was no attempt to remove these proteins by introducing null genes; however, subsequent analysis has shown these are also low in Riso 1508. These hordein null characteristics were stably inherited through the F4 and later generations. The lack of the dominant hordein families did not hinder the germination of ULG 2.0.

Creation of hordein tri-null line ULG 3.0

ULG 2.0 was crossed to a line derived from Ethiopian R118 that had been refined by back-crossing thrice to the standard malting grain cv Sloop, to backcross level 2 (BC2) and that did not accumulate D-hordein, to create hordein tri-null germplasm (Figure 1). Due to time constraints, backcrossing of the D-null stopped at the BC2 level, that is, plants were on average 87.5% Sloop germplasm, but homozygous for the hordein null gene. The plants were agronomically acceptable at this level.

barley with three recessive alleles in a genetically unimproved background (ULG 3.0).

Grain properties of ULG 2.0 & 3.0

In crops grown together at CSIRO Ginninderra Experiment Station (GES), Canberra, ULG 2.0 and 3.0 both displayed a shrunken seed phenotype due to the pleiotropic effect of the \( l s 3 a \) gene derived from Riso 1508 (Gabert et al., 1995). There was an increase of kernel weight from ULG 2.0 (33.5 ± 0.40 mg/kernel) to ULG 3.0 (39.2 ± 0.31) probably due to the contribution of cv Sloop germplasm. The screenings also decreased dramatically from ULG 2.0 (96.2 ± 1.2% of grains <2.8 mm) to ULG 3.0 (41.3 ± 3.7% <2.8 mm). The grain characteristics of cv Sloop exemplify the commercially acceptable target of large plump seeds (53.6 ± 0.93 mg/kernel) and screenings at a minimum of 93% (6.1 ± 1.1% seeds <2.8 mm). The kernel weight of ULG 3.0 was higher (45.0 ± 0.7 mg) when the plants were grown under a mild climate in the glasshouse under 25 °C days with ambient sunlight and 15 °C nights. The effect of environment on kernel weight screenings was confirmed in field trials across three environments, with seed weight increasing and screenings decreasing in the mildest climate (Figure S6).

Hordein levels in ULG 3.0 grain

The grain from the line ULG 3.0 had no detectable hordein by Western blot of 2 \( \mu \)g of alcohol soluble protein visualized with three antigluten (hordein) antibodies (Tanner et al., 2013a). 1D-SDS-PAGE and in-gel digestion of the dominant, alcohol soluble protein bands in ULG 3.0, showed the presence of \( \beta \)-glucosidase, serpin Z4 (protein Z), endochitinase precursor, subtilisin \( \alpha \)-amylase inhibitor, \( \alpha \)-amylase inhibitor, lipid transfer protein-1 and \( \gamma \)-3-hordein (Figure S7). The hordein level in flour...
samples determined by ELISA fell from 4900 mg/kg flour (equivalent to ppm) in Riso 1508 to 1670 ppm (ULG 2.0) and 3.9 ppm (ULG 3.0). This represented 0.007% of that present in cv Sloop (Table 1).

Analysis by 2D-PAGE and in-gel digestion of Riso 56 identified at least six proteins that can be assigned to the C-hordein families and up to five protein spots that could be assigned to the D-hordein family (not shown). 2D-PAGE of ULG 3.0 (Figure 2), in-gel digestion and MS/MS analysis confirmed that the only detectable hordein in alcohol soluble protein from ULG 3.0 was γ-3-hordein (Uniprot: P80198). The γ-3-hordein level was estimated by three independent methods: (i) as a percentage of all spot volumes arising from analysis of 50 µg of protein: ULG 3.0 = 13.5 ± 1.6 ppm, (ii) relative to the spot intensity of BSA (1 µg loaded): ULG 3.0 = 10.9 ± 1.3 ppm or (iii) relative to the spot volume (intensity × area) of BSA (1 µg loaded): ULG 3.0 = 3.4 ± 0.41 ppm. These levels were in close agreement with the ELISA determination (Table 1).

Mass spectrometry (MS) of ULG 3.0 flour

Prototypic peptides specific to the major hordein protein isoforms were identified using a bottom-up proteomics approach (Colgrave et al., 2012, 2013; Tanner et al., 2013a). Peptides representative of each hordein family were subsequently monitored by multiple reaction monitoring (MRM) MS and the peak areas were integrated and compared to give the relative percentage compared to wild-type (cv Sloop) barley flour (Figure 3).

| Line     | Hordein mg/g flour | % of Sloop |
|----------|--------------------|------------|
| Sloop    | 56.6 ± 3.3         | 100.0      |
| Riso 56  | 33.3 ± 1.1         | 58.8       |
| Riso 1508| 4.90 ± 0.26        | 8.7        |
| ULG 2.0  | 1.67 ± 0.07        | 2.9        |
| ULG 3.0  | 0.0039 ± 0.0017    | 0.007      |

Table 1 Hordein content of hordein single-, double- and tri-null lines

Values are mean ± S.E, n = 3. Hordein was measured by ELISA, calibrated against an appropriate standard.

The graphs show the results of four replicate extracts of flour from control barley (wild type, cv Sloop); hordein single-null lines (Riso 56, Riso 1508 and D-null); the hordein double-null line ULG 2.0; and the single-seed descent, tri-null lines T2-4-8 (ULG 3.0) and T2-6-A5, all following trypsin digestion (Figure 3). In the case of the C-hordein monitored, the prototypic peptide was generated following chymotrypsin digestion (as C-hordeins have a low number of trypsin cleavage sites: Lys/Arg). In most cases, two prototypic peptides were monitored for each hordein family and yielded similar results. For clarity of presentation, the results for a single prototypic peptide are shown.

Each of the parental lines was shown to lack the expected hordein: for example, Riso 56 and ULG 2.0 lacked B-hordeins (Figure 3b), Riso 1508 and ULG 2.0 lacked C-hordeins (Figure 3c) and γ-1-hordeins (Figure 3 G1). The D-null plant had wild-type levels of B- and C-hordein (Figure 3b and c, respectively), but did not accumulate D-hordein (Figure 3d). Wild-type barley (cv Sloop), Riso 56 and the D-null line, accumulated high levels of avenin-like proteins, which were present at significantly reduced levels in ULG 2.0 (Figure 3a). The hordein tri-null lines, T2-4-8 and T2-6-A5, and Riso 1508 accumulated only trace levels of the avenin-like proteins (Figure 3a). It should be noted that the avenin-like proteins identified and monitored by MS (e.g. Uniprot: F2EGD5) have not been formally classified as hordein (and/or gluten). However, they contain regions that show significant homology with the γ-3-hordeins and as such have been included in the analysis.

The hordein tri-null lines, T2-4-8 and T2-6-A5, did not have any significant level of B-, C-, D- or γ-1-hordein, but there was a low level of γ-3-hordein. Serendipitously, the level of γ-3-hordein in T2-4-8 and T2-6-A5 was reduced to ~25% of that seen in cv Sloop and ULG 2.0 (Figure 3 G3). The reason for this was unknown. The γ-3 hordein is a minor hordein component, estimated to be ~1.6% of the total hordein content of cv Sloop.

Brewing properties of ULG 2.0 and ULG 3.0

With the confirmed reduction in hordein content of these lines, studies were undertaken to understand the impact on malting and brewing characteristics. Most properties of ULG 2.0 malt were not significantly different to ULG 3.0 malt. The high levels of grain protein measured by NIR in ULG 2.0 and ULG 3.0 led to an undesirably high Kolbach Index (the ratio of soluble protein to total malt protein) as well as total protein and

Figure 2 The proteomic profiles of extracts of barley cv Riso 56 or T2-4-8 (ULG 3.0) were investigated using 2-DE. Fifty micrograms of alcohol soluble protein, spiked with 1 µg each of landmark standards: BSA (1); soy trypsin inhibitor (2); and horse myoglobin (3), was loaded. The relative MW of the landmark proteins was 67, 21 and 17 kDa as deduced by comparison with a protein ladder. The identity of all protein spots was determined by in-gel digestion and MS analysis. Comparison of the alcohol soluble proteome of Riso 56 with that of ULG 3.0 showed the only hordein was γ-3-hordein. The position of C-hordeins (C-hor), γ-1-hordein (gamma-1), γ-2-hordein (gamma-2), γ-3-hordein (gamma-3) in cv Riso 56 is shown.

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soluble protein that were above commercial benchmarks (Table 2); however, this elevation may be an artefact of the NIR method (see Protein compensation, below). Elevation of these parameters is undesirable, because it may lead to impaired filtration during brewing and haze formation during storage. Small-scale brewing trials indicated that wort filtration was slower than normal as a result of the smaller size of the spent grains, which did not form an efficient filtration bed. The efficiency of digestion measured as percentage extract (65.2 ± 1.4%) or apparent attenuation limit (AAL; 74.8 ± 0.9%) was also similar and significantly worse (by ~10%) than cv Sloop. This means an additional 10% load of malt would be required to produce commercial strength wort from these grains. Wort β-glucan levels, which can reduce filtration at high levels, were 29.3 ± 0.3 mg/L but reduced compared to cv Sloop (Table 2). The diastatic power, which is a general measure of starch digestive power, was significantly lower in both ULG 2.0 and ULG 3.0 compared to cv Sloop. However, the α-amylase activity was significantly higher in the hordein double- and tri-null malts than wild-type cv Sloop. Despite the lower diastatic power, analysis of wort sugars by capillary electrophoresis showed that starch was completely converted to glucose, maltose and maltotriose in the wort of ULG 2.0, confirming there was no impediment to starch conversion in the hordein double-null malt. The lack of iodine staining shown by ULG 2.0 wort also confirmed that all starch was converted during the ‘amylase’ rest during mashing. These grains did not support fungal or microbial growth and no mycotoxins were detected in malt samples of ULG 2.0 or ULG 3.0. Despite some apparent drawbacks, small-scale brewing and taste testing showed it was possible to make an acceptable beer from both ULG 2.0 and ULG 3.0 grains.

**Improving the grain properties beyond ULG 3.0**

Processing of ULG 2.0 and ULG 3.0 worts was hindered by the shrunken grains, which slowed the final lautering (filtration), as the smaller spent grains did not form a suitable filtration bed. The seed size and weight were improved to values near wild-type through a biparental and triparental intercrossing programme (Figure 4). From an initial 12 000 hordein tri-null lines, the best 10 lines were selected based upon seed weight, screenings and agronomic characteristics (tiller number, head length and harvest index). These lines showed improved seed weight and screenings (Tables 3 and 4, Figure 5) near those of commercial malting barley varieties (Table S1). Of the ten best lines in the final selection, five lines came from only two individual plants in the first round selection (Table 3). Plant 8 from plot 8 gave rise to three elite lines, 124.1, 125.1 and 126.1; plant 38 from plot 12 gave rise to two elite lines, 148.1 and 148.2. This indicates that the selection scheme was capable of identifying rare beneficial gene combinations.

The best two lines, 43.2 and 124.1, had the lowest hordein levels, approximately 5 ppm, as determined by MRM-MS, improved seed weight (>47.2 mg) and improved screenings near that expected for commercial malting barley (3-14%). The screenings compared well to that of the control grains (Table S1). The progress in improving the overall seed shape and overcoming the shrunken seed phenotype, most likely associated with the lys 3a gene contributed by the Risø 1508 parent, can be seen in Figure 5 and Table 4.

**Figure 3** Relative quantification of prototypic peptides selected to represent major hordein families. Graphs show percentage (based on MRM peak area) relative to cv Sloop (wild type) for Risø 56 (B-null), Risø 1508 (C-null), ULG2.0 (B-, C-hordein double-null), T2-4-8 and T2-6-A5 (B-, C-, D-hordein tri-nulls). The prototypic peptides monitored were (a), avenin-like protein (CQAVCSMAQVIMR); (b), B-hordein (VFLQQQCSPVAMSQR); (c), C-hordein (IIPQQPQQPLPLQPHQPY); (d), D-hordein (DVSPECRPVALSQVVR), G1, γ-1-hordein (APFVGVVTGVGGQ); G3, γ-3-hordein (QQCCQQLANINEQSR). The hordein tri-null line T2-4-8 was the basis of the cultivar ULG 3.0.
Table 2  Malting properties of ULG 2.0 and ULG 3.0 compared to wild type (cv Sloop)

| Sample        | Extract (%) | Wort pH | Colour of wort | FANS (mg/L) | Diastatic power (WK) | Total protein (%) | Soluble protein (%) | Kolbach index (%) | AAL (%)       | Viscosity (mPa/s) |
|---------------|-------------|---------|----------------|-------------|----------------------|-------------------|--------------------|-------------------|---------------|------------------|
| Sloop         | 74.1±0.67   | 5.85±0.01 | 2.90±0.05      | 190±10      | 5.69±0.03            | 6.53±0.02        | 5.88±0.02          | 2.3±0.10         | 6.3±0.01     | 65.0±1.37       |
| ULG 2.0       | 65.2±1.47   | 6.22±0.08 | 3.26±0.14      | 189±10      | 5.67±0.03            | 6.53±0.02        | 5.88±0.02          | 2.3±0.10         | 6.3±0.01     | 65.0±1.37       |
| ULG 3.0       | 66.5±1.37   | 6.22±0.08 | 3.26±0.14      | 189±10      | 5.67±0.03            | 6.53±0.02        | 5.88±0.02          | 2.3±0.10         | 6.3±0.01     | 65.0±1.37       |
| ULG 3.2       | 65.0±1.37   | 6.22±0.08 | 3.26±0.14      | 189±10      | 5.67±0.03            | 6.53±0.02        | 5.88±0.02          | 2.3±0.10         | 6.3±0.01     | 65.0±1.37       |
| ULG 3.2       | 65.0±1.37   | 6.22±0.08 | 3.26±0.14      | 189±10      | 5.67±0.03            | 6.53±0.02        | 5.88±0.02          | 2.3±0.10         | 6.3±0.01     | 65.0±1.37       |

 failure to detect hordeins in ULG 3.2 lines by ELISA

Urea or alcohol soluble extracts of barley flour are normally diluted by a factor of 1000–5000 x (representing about 2 μg of flour in 100 μl) and added to ELISA wells to achieve a level of colour formation in the middle of the Sloop standard curve (Tanner et al., 2013). Extracts of ULG 3.0 (grown at Koroit, Ballarat or Yanco) and ULG 3.2 lines were diluted 1/50 and 1/20 and added directly to an ELISA Systems sandwich assay. There was no significant colour formation compared to the zero (no hordein added) control. There was a slight, but significant colour formation when 1/10 dilutions of ULG 3.0, but not ULG 3.2, lines were added to ELISA wells as above. The protein load was so extreme (equivalent to 2 mg of fresh weight of flour per well) that the slight colour formation in the ELISA reaction was suspected to be due to other proteins such as LTP1, serpin Z4 and β-glucanase in the extract, which have weak homology to the hordeins and can bind weakly to the antibodies in Western blots.

Hordein level

Analysis of the hordein content in ULG 3.2 lines by MS, as in Figure 3, revealed only γ-3-hordein at levels similar to that found in ULG 3.0 (data not shown). Several ULG 3.2 lines including 49.1, 55.2, 72.2, 124.1 and 148.2 had near-zero levels of the γ-3-hordein peptide.

Proximal analysis of ULG 2.0, 3.0 and 3.2

Starch, monosaccharides, β-glucan, free amino acids and fatty acid content were also determined to establish that removal of seed hordeins, a significant seed sink, did not result in reduced malting quality (as judged by decreased starch or increased β-glucan). Furthermore, the accumulation of potentially toxic compounds was assessed, such as increased concentrations of free asparagine which could generate acrylamide during malting. In addition, there was no accumulation of toxic fatty acids such as erucic acid. For simplicity, the results for the best two ULG 3.2 lines (124.1 and 43.2, grown in the field at GES in the summer of 2013) are shown along with ULG 2.0 (glasshouse grown), ULG 3.0 (grown under three environments in the summer of 2012; GES (ULG3G), Horsham (ULG 3H) and Yanco (ULG3Y), the single-null parents (Risø 56, Risø 1508, D-null) and commercial control lines (Sloop, Hindmarsh and Baudin, all grown at GES in the summer of 2012).

Starch

There was a continuous variation in the starch content of the ULG 3.2 lines from 61.5% to 71.1% (w/w) (Table S2), within the variation seen for the controls, which also ranged from 60 to 70% (w/w) (Figure 6a). The best two ULG 3.2 lines, 124.1 and 43.2, were not significantly different from the controls Sloop and Baudin. The starch content of the hordein single-null parents, the hordein double-null line (ULG 2.0) and the hordein tri-null line (ULG 3.0) was not significantly different from each other. The starch content in ULG 3.0 showed no variation when grown in three environments.

Monosaccharides

There were three ULG 3.2 lines: 72.2, 88.2 and 100.2 (Table S2) which yielded an average monosaccharide content of 5.85%, which was higher than the average for the three controls at 3.1%; however, the best two ULG 3.2 lines, 124.1 and 43.2,
were not significantly different from the controls (Figure 6b). The monosaccharide level in ULG 3.0 showed little variation when grown in two environments; Yanco and Horsham, but was significantly lower when grown at GES. HPLC analysis revealed a monosaccharide composition of glucose, fructose and sucrose.

\( \beta \)-Glucan

The average \( \beta \)-glucan content of controls was 3.2% (w/v) (Figure 6c). The average \( \beta \)-glucan content of the ULG 3.2 lines was 0.5% (w/v) (Table S2), significantly lower than controls \((P < 0.001)\). The \( \beta \)-glucan content of ULG 3.0 lines was also significantly lower than ULG 2.0 \((P < 0.001)\), but showed little variation across the three environments. Low \( \beta \)-glucan content is beneficial for brewing as it reduces wort viscosity during filtration. The reduced \( \beta \)-glucan content may also affect the cell wall structure and impact on starch extraction during mashing.

Examination of the cell wall structure of ULG lines is the subject of current investigations.

Dumas protein

There was a continuous variation in protein content for ULG 3.2 lines from 10.7% to 13.6% (w/v) (Table S2), which was within the variation seen for the controls (11.6–14.2%) (Figure 6d). The line with the lowest protein, 124.1 with 11.0%, was significantly lower than the line with the highest protein content, 72.2 with 13.6% protein, but not significantly lower than the standard Baudin (11.6%). The best ULG 3.2 lines, 124.1 and 43.2, were significantly lower than cv Sloop, but similar to cv Baudin and Hindmarsh. The protein level in 2nd intercross F1's:

\[ \text{T}2S \ (49.4\% \ S) \]
\[ \text{T}2B \ (25\% \ B) \]
\[ \text{T}2Y \ (25\% \ Y) \]

1st intercross F2's:

\[ \text{SB} \]
\[ \text{YB} \]
\[ \text{YS} \]

Bi-parental lines

2nd intercross F1's:

\[ \text{SBYB} \]
\[ \text{YBYS} \]
\[ \text{YSSB} \]

Self

Table 3 Third round of selection for improved grain characteristics in ULG 3.2 lines

| 1st round identity (plot, plant) | 3rd round identity (pot number) | Mean tiller size (cm) | Mean head size (cm) | Harvest Index (HI, %) | No. of tillers | Kernel weight (KW, mg) | Screenings (% <2.8 mm) | Pedigree |
|---------------------------------|---------------------------------|-----------------------|---------------------|-----------------------|----------------|------------------------|------------------------|----------|
| 5.11                            | 43.2                            | 86.7                  | 9.0                 | 29.5                  | 12            | 44.7                   | 14.6                   | YB       |
| 5.8                             | 48.1                            | 75.0                  | 7.0                 | 39.4                  | 8             | 47.2                   | 13.3                   | YB       |
| 4.8                             | 72.2                            | 84.7                  | 8.3                 | 39.0                  | 9             | 48.4                   | 11.7                   | SBYB     |
| 1.49                            | 88.2                            | 72.7                  | 6.3                 | 42.6                  | 8             | 45.4                   | 9.3                    | SB       |
| 1.24                            | 100.2                           | 79.3                  | 6.3                 | 46.6                  | 15            | 43.2                   | 11.7                   | SB       |
| 8.8                             | 124.1                           | 79.3                  | 7.3                 | 46.5                  | 8             | 43.7                   | 3.2                    | SY short |
| 8.8                             | 125.1                           | 78.0                  | 7.7                 | 42.0                  | 10            | 46.2                   | 4.7                    | SY short |
| 8.8                             | 126.1                           | 73.0                  | 6.0                 | 37.0                  | 10            | 43.1                   | 2.0                    | SY short |
| 12.38                           | 148.1                           | 93.3                  | 7.3                 | 35.0                  | 14            | 44.2                   | 10.0                   | SB short |
| 12.38                           | 148.2                           | 90.3                  | 6.3                 | 33.3                  | 7             | 42.5                   | 5.1                    | SB short |

The SE in determination of kernel weight (KW) was <0.5 mg, the SE in screenings was <1%. The various grain and plant properties were measured on field grown plants at GES planted in March 2011.

Table 4 Breeding for improved grain characteristics

| Line               | Kernel weight (KW, mg) | Screenings (% <2.8 mm) |
|--------------------|------------------------|------------------------|
| Sloop              | 53.6 ± 0.9             | 6.1 ± 1.1              |
| ULG 2.0            | 33.5 ± 0.4             | 96.2 ± 1.2             |
| ULG 3.0            | 39.2 ± 0.3             | 41.3 ± 3.7             |
| ULG 3.2            | 47.2 ± 0.5             | 13.3 ± 1.0             |

Values are mean ± S.E, n = 3.
ULG 3.0 was similar when grown in two environments; GES and Horsham, but was significantly lower when grown in the harsher climate of Yanco.

Protein compensation

The protein measured by NIR was elevated in ULG 2.0 and ULG 3.0 lines compared to the control cv Sloop (Figure 5), confirming that some compensation was occurring in the levels of several individual alcohol soluble proteins, but not in the total protein level.

Total free amino acids

There were several significance groups in the level of free amino acids (Figure 6e). The wild-type grains and hordein D-null in Sloop background had the lowest free amino acids, mean was 2.15 ± 0.25 mg/g (range 1.60–3.64 mg/g). The free amino acids in the other single-null mutants, Risø 1508 and Risø 56, were significantly higher: mean 5.45 ± 0.11 mg/g (range 5.14–5.64; P < 0.05). Total free amino acids in the hordein tri-null ULG 3.0Y were almost double (10.76 ± 0.06 mg/g) that seen with ULG 2.0 (6.85 ± 0.03 mg/g, P < 0.005). The tri-null ULG 3.0 was also affected by environment having the highest FAA when grown at GES (18.10 ± 0.37 mg/g). The ULG 3.2 lines were divided into two broad significance groups: (i) the FAA in the low group including the best lines, 124.1, 125.1, 43.2 and 48.1, was similar to the ULG 3.0 mean at 18.60 ± 0.26 mg/g (range 17.3–20.1 mg/g); (ii) the higher group (72.2, 88.2, 100.2, 126.1, 148.1 and 148.2) showed the highest levels of FAA and mean 25.94 ± 0.45 mg/g (range 23.3–28.7 mg/g) (Table S2) and was significantly higher than other barley lines (P < 0.05).

Dominant free amino acids

There were five dominant amino acids (Figure 7): Asp, Glu, Arg, Ala and Pro which accounted for 49% of the total amino acids in the wild-type and single-null controls, and 60% of the total amino acids in the ULG 3.2 lines. As expected for prolamin-rich tissues, two amino acids, Gln and Pro, were the most abundant accounting for an average of 15% of the total amino acids in the wild-type and single-null controls, and an average of 37% of the total amino acids in the ULG 3.2 lines.

Free asparagine and its significance to acrylamide formation

Asparagine was one of the five most abundant free amino acids in barley flour (Figure 7), with the mean free asparagine content for the ULG lines varying from 0.66 ± 0.00 mg/g (ULK 2.0) to 2.63 ± 0.04 mg/g (ULK 3.0G). The free asparagine content was lowest in the wild-type, D-null, ULG 2.0 and Risø 56 lines and was higher in the ULG 3.0 lines. ULG 3.0 had the highest free asparagine levels, which varied considerably with the environment. ULG 3.2 lines showed a continuous variation from a high of 1.88 ± 0.01 mg/g (148.1) down to a low of 1.34 ± 0.01 mg/g (72.2) (Table S2). The mean asparagine level in the commercial controls Baudin, Hindmarsh and Sloop was 2.5- to 4.2-fold lower than that observed for the best ULG 3.2 lines, 124.1 and 43.2.

The Swedish National Food Authority announced in April 2002, the finding of low levels of the neurotoxin/carcinogen acrylamide, in ppm levels, predominantly in carbohydrate-rich foods, which had been heated (www.slv.se, 2002). Zyzac et al. (Zyzak et al., 2003) subsequently showed that asparagine heated in a moist environment in the presence of starch or reducing sugar, such as encountered during barley malting was the main source of acrylamide in food (Taeymans et al., 2005; Zyzak et al., 2003). The tolerable daily intake (TDI) for neurotoxicity or cancer from acrylamide was estimated to be 40 and 2.6 μg/kg/day.
respectively (Tardiff et al., 2010). A 70 kg person could consume 182 μg acrylamide per day and not exceed the TDI for cancer. Assuming a similar rate of conversion of asparagine to acrylamide as seen by Zyzak et al. (2003), a 70 kg adult could consume 1.8 kg of ULG 3.2 barley and not exceed the more stringent TDI for cancer.

Amylase activity

Amylase activity was measured in the samples used for proximal analysis, along with an additional commercial control line, Commander, which was also grown at GES in the summer of 2012. The level of α-amylase of the ULG 3.2 lines was not
Figure 8. Amylase activity for the best two ULG 3.2 lines (124.1 and 43.2), ULG 3.0, ULG 2.0 and commercial Australian lines cv Sloop, Baudin, Commander and Hindmarsh: (a) α-amylase activity and (b) β-amylase activity. For each amylase, one-way ANOVA was carried out using Prism v6.02. The LSD’s are shown; means which differ by more than the LSD were significantly different.

Concluding Remarks

We report the selection and breeding of the first ultra-low gluten barley. The hordein level was extremely low, to the point where it was difficult to measure even with sensitive mass spectrometry or ELISA: The grain had a normal appearance and malting and brewing properties sufficient to make a useful malting grain. This grain has application in the preparation of food and beverages for coeliacs and gluten intolerants.

Experimental procedures

Plant material

Barley lines cv Sloop, Bomi, Baudin, Commander and Hindmarsh (wild types) were obtained from the Australian Winter Cereals Collection (Tamworth, Australia). The single hordein null lines, Risø 56 lacking B-hordeins and Risø 1508 lacking C-hordeins, were obtained from the Nordic Germplasm Bank (Alnarp, Sweden) and intercrossed to produce ULG 2.0.

D-null hordein line

A line which did not express the D-hordein gene, Ethiopian R118 (Brennan et al., 1998), was obtained from The John Innes Centre Public Collections, Norwich. The mutation was tracked by SDS-PAGE analysis and with a CAPS marker developed (Figures S8 and S9). Ethiopian R118 segregated for 2-row and 6-row phenotypes (Figures S8 and S9). The best two lines, 43.2 and 124.1, with acceptable seed size, were determined by the method of Dumas (Shea and Watts, 1939). The best 20 lines were replanted. About 1000 F4 seeds per pedigree were planted in rows in the field, and F5 plants again selected which were relatively shorter and produced seeds that were larger, with short, well-filled heads. Both early- and late-maturing plants were selected. In the following generation grown in the field, F6 lines were again selected which additionally exhibited relatively high harvest index and head length, optimal height (semi-dwarf), lack of lodging and disease resistance such as powdery mildew in the field.

The best two lines, 43.2 and 124.1, with acceptable seed properties and the low hordein levels, with null alleles at the three hordein loci, hor2-lys3a-hor3 were fixed by three rounds of single-seed descent.

Analytical methods

Plant analysis

Kernel weight, dimensions and screenings, a measure of seed size, were determined using a SeedCount™ SC4 (Seed Count Australasia, Condell Park, Australia). Total flour nitrogen was determined by the method of Dumas (Shea and Watts, 1939). Starch, β-glucan, α- and β-amylase were determined by the methods of McCleary (McCleary and Codd, 1989, 1991; McCleary et al., 1997, 2002). Free sugar composition was by the anthrone method (Yemm and Willis, 1954). Fatty acids were extracted from fine whole-meal flour, methylated in sodium methoxide, extracted into hexane and analysed according to the method of Zhou (Zhou et al., 2011). Free amino acids were determined by the Australian Proteome Analysis Facility (Macquarie University, Sydney). Analysis of barley grain for mycotoxins was carried out by Agrifood Technology (Werribee, Melbourne, Vic., Australia).

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Grain was malted and brewed according to the methods of the European Brewing Convention (EBC, 2014) by Barrett Burston Co. (Melbourne, Vic., Australia).

**Protein extraction and SDS-PAGE**

Alcohol soluble proteins in the endosperm half of seeds were extracted in a Savant Bead beater in 0.5 mL of 50% IPA/DDT at speed 4 for 30 s with 160-μg glass beads (0.15 mm dia) and a 6.3-mm ceramic sphere and clarified by centrifugation, 5000 g. Total protein was extracted in a Savant Bead beater as above, using the protocol and SDS-PAGE analysis as described previously (Tanner et al., 2013a).

**Two-dimensional polyacrylamide gel electrophoresis (2D PAGE)**

Alcohol soluble prolams were extracted from 20 mg whole flour as above and precipitated from the supernatant with two volumes of IPA at −20 °C. This precipitated all hordeins. The pellet was dissolved in Zoom 2, protein solubilising solution (Promega Sydney, NSW, Australia), containing additionally 3 mM Tris base, 1 μg/mL AEBSF, 0.5 μg/mL E64, 0.5% (w/v) pH 3-10 ampholytes, 0.005% (w/v) Bromophenol blue and 20 mM DTT (Zoom 2*).

Total protein was extracted in 500 μL Zoom 2* (as above) and centrifuged at 18 000 g for 10 min. The protein content was measured with a 2D Quant kit (Amersham, UK).

Two-dimensional protein gel electrophoresis was carried out with 200 μg of total protein or 100 μg of prolamin diluted to 170 μL with Zoom 2*, and 1 μg each of BSA, trypsin inhibitor and myoglobin added as internal standards. Zoom2 IPG strips (pH 3-10; Invitrogen Melbourne, Victoria, Australia) were passively rehydrated with sample solutions overnight at room temperature. The first dimension was focussed as follows: a linear gradient from 0 V to 175 V over 1 min, maintained at 175 V for 15 min, a linear gradient from 175 V to 2000 V over 45 min and maintained 2000 V for 30 min. The first dimension strips were reduced in NuPAGE LDS buffer (Invitrogen) containing 50 mM iodoacetamide and alkylated in NuPAGE LDS buffer containing 260 mM acrylamide. The first dimension strips were laid on top of a precast 1 mm NUPAGE 12% Bis–Tris Zoom Gel (Invitrogen), calibrated with a 10 kDa molecular weight marker (Invitrogen), and the second dimension electromorphosed at 200 V for 1 h in MTS buffer, stained in NuSep NuBlu Express Stain and destained in water overnight. Protein spots were cut from 1D- and 2D-PAGE gels, subject to in-gel digestion and the proteins identified by MS as described below.

**Enzyme-linked immunosorbent analysis (ELISA)**

Twenty milligrams of wholemeal flour or the endosperm half of seeds was crushed and washed thrice in 0.5 mL of MilliQ water in shaking in a 96-well Vibration Mill (Retsch GmbH, Rheinsche) and sedimented, 5000 g for 5 min, before extraction into 0.5 mL of 50% IPA/DDTT. Protein concentration was determined according to Bradford (Bradford, 1976). Forty nanograms of alcohol soluble protein from the control lines: Sloop, Risø 56, Risø 1508 and ULG 2.0; or 1900 ng alcohol soluble protein from ULG 3.0 was diluted with a solution containing ELISA Systems sample diluent containing 0.2 mM excess H₂O₂ added to quench any DTT remaining from the initial extract, and added to ELISA wells (ELISA Systems, Brisbane, Qld, Australia). ELISA plates were processed according to manufacturers’ instructions and calibrated against a 0.2 mM excess H₂O₂ quenched standard curve of the appropriate total hordein: Sloop total hordein for Sloop flour, Risø 56 total hordein for Risø 56 flour, Risø 1508 total hordein for Risø 1508 flour or ULG 2.0 total hordein for determination of hordein in ULG 2.0 flour. Hordein standards were purified by alcohol precipitation and FPLC (Tanner et al., 2010).

**Global proteomic profiling**

Protein bands for identification were excised and processed as described previously (Byrne et al., 2012) using both trypsin or chymotrypsin as the digestion enzymes. Proteolytic peptides (15 μL) were analysed as described previously (Colgrave et al., 2014) with chromatographic separation using a nano HPLC system (Shimadzu Scientific, Rydalmere, NSW, Australia) directly coupled to a 5600 TripleTOF MS (SCIEX, Foster City, CA). ProteinPilot™ 4.0 software (SCIEX) with the Paragon Algorithm (Shilov et al., 2007) was used for protein identification. Tandem mass spectrometry data were searched against in silico tryptic digests of Poaceae proteins of the Uniprot database (version 2014/07; 665 783 sequences). The search parameters were defined as iodoacetamide modified for cysteine alkylation and trypsin (or chymotrypsin) as the digestion enzyme. Modifications and cleavages were defined previously (Colgrave et al., 2014). The database search results were manually curated to yield the protein identifications using a 1% global false discovery rate (FDR) determined by the in-built FDR tool within ProteinPilot software (Tang et al., 2008).

**Targeted proteomic analysis employing multiple reaction monitoring (MRM) MS**

Each half-seed was milled to a fine flour, and the alcohol soluble proteins in 20 mg flour were extracted in 200 μL of a solution containing 55% IPA (v/v) and 2% DTT (v/v). An aliquot of the extract, equivalent to 5 mg flour, was subjected to buffer exchange into 200 μL of 8 M urea in 0.1% Tris-HCl, pH 8.5, by centrifuging twice, on a 10-kDa MW cut-off filter (Millipore Sydney, NSW, Australia). The cysteines were alkylated by addition of 100 μL of 50 mM iodoacetamide for 20 min at room temperature. The buffer was exchanged as above, to 200 μL of 50 mM ammonium bicarbonate, pH 8.5. Proteins were digested with 100 μL of 0.25 mg/mL trypsin (equivalent to 25 μg) with incubation at 37 °C for 18 h. Peptides were collected by filtration through a 10-kDa filter, dried and reconstituted in 30 μL of 1% formic acid (v/v) for LC-MRM-MS analysis. Peptides (10 μL) were separated by liquid chromatography on a Shimadzu Nexera HPLC with Phenomenex column (Kinetex, 1.7 μm, C18, 100 × 2.1 mm) with a gradient from 5 to 45% B over 10 min at a flow rate of 0.4 mL/min. Solvent A was 0.1% aqueous formic acid (v/v) and solvent B was 90% aqueous acetonitrile (v/v) containing 0.1% formic acid (v/v). The HPLC eluate was directly coupled to the mass spectrometer and MRM analysis was performed on 4000 QTRAP mass spectrometer (SCIEX) targeting hordein-derived tryptic (or chymotryptic) peptides. Data were acquired using Analyst v1.5 software and peak area integration was accomplished using MultiQuant v2.0.2 software.

**Statistical analysis**

One- or two-way analysis of variance (ANOVA) was carried out as indicated, using Prism v6.02. The significance of differences of means was determined either by Tukey’s test or by calculation of Fischer’s least significant difference (LSD).
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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Location of the hordein genes on chromosome 1H of barley.

Figure S2 The identity of proteins present in alcoholic extracts from hordein double nulls and controls.

Figure S3 Selection of the hordein double-null line G1 as basis of ULG 2.0.

Figure S4 Segregation of the B-hordeins in F3 lines of ULG 2.0.

Figure S5 Molecular marker for the presence or absence of the B-hordein loci.

Figure S6 The effect of the environment on ULG 3.0 seed width.

Figure S7 Alcohol soluble proteins present in flour from ULG 3.0.

Figure S8 DNA and protein sequences of the Hor3 alleles.

Figure S9 CAPS marker for the Hor3 null allele derived from Ethiopian R118.

Table S1 Kernel Weight and screenings of select malting barley varieties.

Table S2 Proximal analysis of grain components from the ULG 3.2 lines that were eliminated in the final round of selection.
