Ultra-low gluten barley

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Abstract
This study describes the creation of an ultra-low gluten (ULG) barley for food and brewing applications for coeliacs and gluten intolerants. Two barley lines, Risø 56 which does not accumulate B-hordeins and Risø 1508 which does not accumulate C-hordeins, were combined to remove the dominant hordein (gluten) protein families. The best hordein double-null line, ULG 2.0, had hordein levels reduced to approximately 1,600 ppm, about 3% of wild-type. A line that did not accumulate D-hordeins was crossed to ULG 2.0 to produce a hordein tri-null line (ULG 3.0) with a reduced hordein level of approximately 3-5 mg/kg (ppm). Both ULG 2.0 (33.4 mg/seed; with high screenings: 53% grain <2.8 mm) and 3.0 (41.8 mg/seed; 54% screenings) suffer from a shrunken seed phenotype compared to wild-type cv Sloop (53.0 mg/seed; 4% screenings). ULG 3.0 was crossed to large-seeded lines cv Sloop, Yagan and Baudin, and the resulting hordein tri-null F2 lines selfed and intercrossed to produce 12,000 hordein tri-null lines. The best tri-null lines (ULG 3.2) had improved seed weight (44-48 mg) and screenings (<3%) similar to commercial malting barley, while the hordein level was <5 ppm. The best ULG hordein tri-null line, ULG 3.2, may be useful for the preparation of malt, food and beverages for the estimated 5% of the population (approximately 344 million people) in the world who suffer from coeliac disease, gluten allergy and gluten intolerance.

Introduction
Coeliac disease (CD) is a condition that is estimated to affect about 1% of most populations worldwide [1] and at present the only treatment is a lifelong gluten-free diet with the exclusion of gluten-like proteins in wheat (gliadin and glutenins), barley (hordeins), rye (secalins) and in some coeliacs, oats (avenins) [2]. Untreated coeliacs face adverse health outcomes including low bone mineral density and increased intestinal malignancy [3]. Gluten-free diets are traditionally more expensive, low in fibre, high in fat and which in turn can lead adverse health outcomes [4-6].
In addition to CD a second condition known as non-coeliac gluten sensitivity (NCGS) is thought to affect up to 10% of the population in some countries [7, 8]. Little is known about NCGS, but it has been shown that for those who suffer NCGS a gluten-free diet is beneficial, see [9] and reference therein.
Barley contains four classes of hordeins, the B-, C-, D- and γ-hordeins. The B and C hordeins are the major classes representing over 90% of the hordeins [10, 11]. The B- hordeins and C- hordeins are encoded by the *Hor 2* and *Hor* 1 loci respectively, with both loci located on the short arm of Chromosome 1. In the 1970s the search for high lysine barleys at the Carlsberg institute identified two mutants, Risø 56 and Risø 1508, which had reduced hordein content. Risø 56 does not accumulate B-hordeins due to the deletion of the *Hor 2* locus [10]. While Risø 1508 does not accumulate C-hordeins and this is the result of a mutation in the *lys3* locus on chromosome 5H [12]. The D-hordeins are encoded by the *Hor3* locus, which encodes a single 105 kDa protein [13], located about 9 cM from the centromere on the long arm of chromosome 1H [14]. An Ethiopian derived landrace, R118 [15], 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) [16, 17]. The γ-hordein loci are very tightly linked to the B-hordein locus at 0.2 cM [18, 19].
We report the isolation of the first ultra-low gluten barley with hordein levels 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. 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.

Materials and Methods
Barley lines cv Sloop and Bomi (wild-types) were obtained from the Australian Winter Cereals Collection (Tamworth, Australia). The single hordein-null lines, Risø 56 and Risø 1508 were obtained

from the Nordic Germplasm Bank (Alnarp, Sweden). A line which was null for D-hordein, Ethiopian R118 [15], was obtained from The John Innes Centre Public Collections, Norwich. The Ethiopian R118 line was a landrace observed to segregate for 2-row and 6-row phenotypes and for black pigmented and green seeds. A two-rowed, green seeded line was selected and backcrossed to Sloop to produce a BC2 D-hordein null line with 87.5% Sloop background.

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 [20]. Starch, β-glucan, α- and β-amylase were determined by the methods of McCleary [21-24]. Free sugar composition was by the anthrone method [25]. Fatty acids were extracted and analysed according to the method of Zhou [26]. Free amino acids were determined by the Australian Proteome Analysis Facility (Macquarie University, Sydney) according to the method of Cohen [27] with norvaline (Sigma) as an internal standard [28].

Protein Analysis

Protein extraction, one and two dimensional SDS PAGE and ELISAs were carried out as described previously [29]. Mass spectrometry was conducted as described previously [30-32] .

Results and Discussion

Creation and analysis of ULG 3.0

Risø 56 and Risø 1508 were intercrossed to produce a line that lacked B- and C- hordeins, a *Hor2, Lys3a* double mutant and was called ULG 2.0. The hordein concentration was lowered further by crossing ULG 2.0 with the Ethiopian R118 line in a Sloop BC2 background to produce ULG 3.0, which lacked B- C- and D- hordeins (*Hor2, Hor3, Lys3a* triple mutant). Analysis of the hordein content in these lines by ELISA (Table 1) revealed that it had been reduced to approximately 4 ppm in ULG 3.0. 2D-PAGE, in-gel digestion and MS/MS analysis of ULG 3.0 confirmed that the only detectable hordein in an alcohol soluble protein fraction from ULG 3.0 was γ-3-hordein (Uniprot: P80198) (data not shown).

Line	**Hordein content (ppm)**
Sloop	56,600 ± 3,300
R56	33,300 ± 1,100
R1508	4,900 ± 260
ULG 2.0	1,670 ± 70
ULG 3.0	3.9 ± 0.7

Table 1: Hordein content of ULG lines as measured by ELISA, values are mean ± S.E, n=3.

Seeds of both ULG 2.0 and 3.0 were shrunken, similar to those of Risø 1508, most likely due to a pleiotropic effect of the *Lys3a* gene derived from Risø 1508 [33], and had high levels of screenings. When grown in the field at Ginninderra Experiment Station, ACT, seed weight of ULG 2.0 was 33.5 ± 0.40 mg/ kernel, while ULG 3.0 had slightly larger seeds 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).

Improving seed size, development of ULG 3.2

ULG 2.0 was crossed to cv Sloop, Yagan and Baudin. F2 hordein double-null lines were identified in the three backgrounds 2S (Sloop), 2Y (Yagan) and 2B (Baudin) respectively, each containing 50% of the respective parental germplasm. These hordein double-null lines were crossed with a ULG 3.0 line named T2 and F2 hordein tri-nulls identified in the three backgrounds T2S, T2Y and T2B respectively. The hordein tri-null lines were intercrossed in pairs in both directions e.g. T2S x T2Y and T2Y x T2S, to form F1 lines, SY and YS respectively to create biparental lines, all with hordein tri-null phenotypes. The F1 lines were either selfed to create F2 bi-parental families or intercrossed and selfed to create segregating quad-parental families all with a hordein tri-null phenotype.

Through a field selection process over two generations the best 20 lines were identified. Progeny of these lines were carried through a further three generations of field selection to identify agronomically acceptable lines that produced seeds that were larger, with short, well-filled heads. The best two lines,

43.2 and 124.1, were selected and fixed by three rounds of single-seed descent. These lines had the lowest hordein levels, and improved seed weight (>47.2 mg), and improved screenings near that expected for commercial malting barley (3-14%).

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 whether removal of seed hordeins, a significant seed sink, impacted other components of the grain (Table 2).

The starch content of ULG 3.0 was lower than the controls Sloop and Baudin, while the two ULG 3.2 lines 043.1 and 124.1 had starch contents similar to the controls. There was no consistent trend in monosaccharide content between the lines with 043.2 and 124.1 having similar levels to that seen in Baudin. The protein content of ULG 3.0 was higher than that seen in the control lines, while the 043.2 and 124.1 had a lower protein content than the controls. Interestingly the β-glucan content of the ULG 3.0 lines was significantly lower than that the controls, with 043.2 and 124.1 having a lower β-glucan content than ULG 3.0. The cause of this is unknown at present. The total free amino acid content in the ULG 3.0 lines was up to 15-fold higher than the levels in the controls and is likely to be a direct result of the absence of the hordeins and the inability of the lines to incorporate proline and glutamine, which are present in high concentrations in the hordein proteins, into other proteins in the absence of the hordeins.

Table 2: Proximal analysis of grain components from the ULG lines

Line	Starch (% flour)	Mono-saccharides (% flour)	Protein (% flour)	β-glucan (% flour)	Free amino acids (mg/g)
Sloop	69.5± 0.5	2.70 ± 0.19	12.7 ± 2.7	2.43 ± 0.02	3.63 ± 0.01
Baudin	67.8 ± 1.1	3.58 ± 0.16	11.6 ± 0.3	2.85 ± 0.15	1.60 ± 0.02
Hindmarsh	59.9 ± 0.6	2.69 ± 0.06	12.3 ± 0.5	4.24 ± 0.07	1.70 ± 0.03
Risø 56	75.5 ± 1.3	4.19 ± 0.19	10.0 ± 0.7	2.17 ± 0.01	5.67 ± 0.08
Risø 1508	65.9 ± 1.5	4.34 ± 0.54	11.5 ± 1.2	2.29 ± 0.03	5.22 ± 0.09
ULG 3.0	64.1 ± 0.6	2.65 ± 0.01	14.1 ± 0.2	1.10 ± 0.04	18.10 ± 0.63
043.2	71.1 ± 1.9	3.38 ± 0.10	10.9 ± 0.8	0.51 ± 0.03	18.56 ± 0.08
124.1	68.4 ± 1.5	3.60 ± 0.25	11.0 ± 0.7	0.76 ± 0.02	19.82 ± 0.25

Values are means plus or minus standard deviation, n = 3.

Amylase activity

The level of α-amylase of the ULG 3.2 lines was not significantly different from the malting lines cv Sloop, Baudin, Commander or Hindmarsh. Conversely, the level of β-amylase in the ULG 3.2 lines was reduced by approximately 10-fold compared to the malting lines above (data not shown).

Conclusion

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.

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