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MALDI-TOF mass spectrometry provides an efficient approach to monitoring protein modification in the malting process

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ABSTRACT

Proteins in barley grains are determinants of beer quality, which are modified during malting to provide nutrition for yeast during brewing. Different malting barley varieties behave differently during malting. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to characterize storage proteins including albumin, globulin, hordein and glutelin in grains and malt of parental varieties and a doubled haploid (DH) population. Each parental variety produces malt with particular quality profiles. The protein profiles of analyzed extracts in seed and malt were compared to find the most influenced proteins during the malting process. Our results revealed that malting procedure dramatically affected the composition and amounts of albumin, then hordein, glutelin and globulin in barley. Moreover, hordein and glutelin profiles in the DH population exhibited high levels of polymorphism. The genetic information represented by individual hordein and glutelin profiles was successfully mapped as molecular markers in a genetic linkage map. Twelve and 8 (hordein in seed and malt, respectively) or 10 and 8 (glutelin in seed and malt, respectively) segregating peaks were scored as polymorphic across the population, and all peaks were located to the chromosome 1H. The MALDI-TOF MS provides a method with high resolution and throughput to characterize mass patterns of extracted storage proteins in barley and malt.

1. Introduction

Barley (*Hordeum vulgare* L.), the fourth largest cereal worldwide after wheat, rice and maize, can be used as livestock fodder and more popularly for malt production in the brewing industry [7]. Barley grain contains 8–13% (w/w) protein depending on the variety [41]. Based on the Osborne's method, barley storage protein could be classified on the basis of solubility in water (albumin), salt (globulin), alcohol solutions (prolamin) and alkali (glutelin). Albumin and globulin proteins (constituting about 15–30%) are enriched in the bran and germ [10]. Hordein (prolamin) and glutelin are the two major storage proteins accounting for 35–55% and 35–40% total protein, respectively, and are mainly contained in barley endosperm. Based on their electrophoresis mobilities and sulphur content, hordeins are classified into three broad groups named sulphur-rich (S-rich), sulphur-poor (S-poor) and high molecular weight (HMW) prolamins, with several subgroups within the S-rich group [35]. The B (S-rich, 28–45 kDa) and C (S-poor, 49–72 kDa) hordeins represent the two main fractions accounting for 70–90% and 10–20% of the total hordeins, respectively, while the D (around 100 kDa) and γ fractions (molecular mass less than 20 kDa) are the minor components (less than 5% of the total hordein fraction) [34]. Glutelin is generally polymerized and defined as an alkali/acidic-soluble protein after hordein extraction. However, it is impossible to prepare a glutelin fraction totally free from hordein contamination

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[4]. Moreover, each of the original isolated storage proteins corresponding to Osborne's fractionation is now known to be a complex mixture of different polypeptides, and the most varied fractions or profiles during malting process need to be answered in the beer industry.

The malting process – barley seed germination under controlled conditions – is important to produce diastatic enzymes, digest internal cell walls, starch and proteins of the endosperm [1]. Some of the proteins survive through multiple steps of malting and brewing processes, which are crucial to malting and beer quality [19]. Water-soluble barley proteins such as lipid transfer protein 1 (LTP1) and protein Z derived from barley malt are regarded as the proteins involved in the formation of head foams [8]. Moreover, the proline-rich hordeins from malt play a major role in haze formation [17]. Therefore, it is important to investigate the changing patterns of storage proteins including albumin and hordein during the malting process.

When considering the separation/discrimination of storage proteins in barley grains or malt, the choice of analytical techniques includes sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), high-performance capillary electrophoresis (HPCE), or MALDI-TOF MS [4,23,29,42]. SDS-PAGE is a manual, slow, labor-intensive procedure and the results are difficult to quantify, while CE results are reported to be easily influenced by experimental conditions such as pH value, buffer composition, temperature, and types of capillary tubes [7]. MALDI-TOF MS has demonstrated many technical advantages for identifying unknown compounds, including high throughput, high resolution and accuracy, and is thus ideal for dealing with a large number of samples in a short time [21,31]. MS analysis can also be used in comparative experiments due to its simplicity and significant rapidity in obtaining results [21]. Thus, MS may provide an efficient approach to understand protein modification during the malting process.

The objectives of the present research were: (1) to compare albumin, globulin, hordein and glutelin compositions in barley grain and malt in order to uncover the change of protein profile related to the malting process; (2) to construct a genetic linkage map by exploring the MALDI-TOF MS individual hordein and glutelin peaks as markers in a double diploid (DH) barley population.

2. Material and methods

2.1. Barley materials

Baudin is a malting barley variety developed by the Department of Agriculture and Food Western Australia. It has high diastatic power and fermentability, thus it is considered ideal malting barley for starch adjunct brewing. In contrast, Dhow has low diastase power and fermentability making it suitable for pure malt brewing or sugar adjunct brewing. A doubled haploid (DH) population was developed from the F1 of Baudin/Dhow by anther culture. Barley grain samples and malt of 90 DH lines, together with their parents, were used in this study.

2.2. Micromalting

Barley samples were cleaned and sieved over a 2.2 mm screen prior to micro-malting in a Joe White Systems micro-malting unit without the use of additives. A standard malting schedule was used: steeping at 19 °C for 7 h wet, 8 h air rest, 3 h wet, 4 h air rest, 1 h wet. Germination was for 96 h (48 h at 18 °C followed by 48 h at 16 °C), and moisture adjusted to 46% at 24 h. Kilning was 2 h at 45 °C, 3 h at 50 °C, 4 h at 55 °C, 3 h at 60 °C, 3 h at 65 °C, 3 h at 70 °C, 2 h at 75 °C and 4 h at 80 °C.

2.3. Construction of molecular linkage map

Ninety DH lines and their parents were genotyped by Diversity Arrays Technology Pty Ltd. (http://www.diversityarrays.com) using the barley version 2.0 array. More than 900 DArT markers were scored in the population. In addition, 446 commonly-used SSR markers were synthesized using information from previous publications and used for screening polymorphic markers. In total, 629 DArT and SSR markers were mapped in this population. Software package Joinmap 4.0 [38] was used to determine initial linkage groups using the Kosambi map function [20]. RECORD [39] was used to optimize the marker order of linkage groups and the final map was then defined by Map Manager QTX.

2.4. Storage protein extraction

The extraction protocol (seed and malt) is based on the differences in solubility of grain proteins. Albumin and globulin were extracted according to Chmelik et al. (2002) method, while hordein and glutelin were extracted according to the methods of Wang et al. [40] with minor modification. Briefly, barley seed and malt (100 mg) were crushed with TissueLyser at speed 25 Hz for 10 min in a tube, and then extracted with Milli-Q water at room temperature (RT). The supernatant contained water-soluble albumins. The residues were mixed with 5% (w/v) NaCl solution and the extraction procedure was repeated; the supernatant contained salt-soluble globulins. After that, hordein and glutelin fractions were obtained respectively with 60% (v/v) ethanol added 2% dithiothreitol (DTT) and NaOH (pH 11.5) extracting solution. All extractions were performed with 0.4 mL of specific extraction agent and repeated three times to remove the corresponding proteins. The first supernatant of one sample was used for subsequent mass spectrometric analysis. Each extraction step started by vortex mixing for 5 min and then shaking for 30 min at room temperature, except for hordein extracted in 60 °C water bath for 1 h to extract the hordein completely [35]. The extracts were centrifuged at 12,000 g for 10 min. In summary, the protein mixture were isolated by sequential extraction with various solvents from barley grain and malt, and then mixed with matrix solution for the preparation of loading sample into equipment.

2.5. Sample preparation and instrument settings for MALDI-TOF MS

The matrix solution was prepared by dissolving sinapinic acid (SA) in 60% acetonitrile (ACN) and 35% methanol (v/v) at a concentration of 40 mg/mL. The supernatant (1.5 μL) was transferred into a new tube and mixed to 10 μL of SA solution. After a short vortexing, the sample/matrix solution (1.5 μL) was spotted onto the sample plate and dried at room temperature. The spotting procedure was repeated twice. MALDI-TOF MS was carried out on a Voyager DE-PRO TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a 337 nm UV nitrogen laser and delayed extraction. Analyses were carried out on a positive linear ion mode at a mass range of 3000–21,000 D (albumin and globulin) or 30,000–65,000 D (hordein and glutelin) with an accelerating voltage of 25 kV and a delay time of 600 ns or 850 ns. The other parameters of the instrument were: grid voltage 92%, guide wire 0.2%, bin size 4 ns, and input bandwidth at 20 MHz. The final spectrum was generated from the average of 400 laser shots for each sample. Data generated from MALDI-TOF MS instrument was further processed using Data Explorer 4.0 software (Applied Biosystems, Framingham, MA, USA).

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2.6. Marker allocation

Hordein peaks of the two parents were compared and the polymorphic peaks were scored across all progeny lines. The polymorphic peak segregation pattern was recorded in an Excel file and used to determine these peak chromosome locations via mapping.

3. Results and discussion

Malting is a biological process that turns barley into malt for the brewing and food industries. It is a three-stage process consists of steeping, germination and kilning. In barley grain, protein content is one of the major factors which impacts the malting and brewing processes and the quality of the end product – beer [3]. Generally, barley protein ranging from 10–12.5% suits maltsters to meet many brewers' needs. In our study, we used MALDI-TOF MS to determine the changing pattern of storage proteins in two elite malting genotypes – Baudin and Dhow – and their DH population. Storage protein in barley was affected in the malting process.

Monitoring storage protein digestion during malting was performed by comparing protein patterns of the samples (grain and malt). Overlaid traces of protein profiles of the parents Baudin and Dhow were also carried out to determine the different proteins between them. Compared to seed albumin, many proteins were remarkably degraded or even disappeared in malt, especially the major groups of profiles (e.g., 7112, 7958, 8611, 8792, 8998, 9685 and 9983 Da) with molecular weights (MW) ranging from 6500 to 11,000 Da (including LTP1b) in addition to the peak with MW around 16,994 Da (Fig. 1A and B). Simultaneously, three mainly-new protein profiles with MWs of 4038, 6803 and 12,166 Da were generated in malt. However, most of the proteins with MWs from 12,500 to 16,500 Da were less broken down by the malting process, which indicates enzymatic digestions lead to part-modification of the proteins during malting. Interestingly, seed albumin in these two parents exhibited similar protein patterns (Fig. 1C). In contrast, significant differences between the two malts were evident: a special protein with MW of 9226 Da showed in the malt of Baudin but not in Dhow (Fig. 1D). To date, only three protein peaks have been previously identified in malting process, which were protein Z, LTP1 and LTP1b with molecular masses of 4033 Da, 9689 Da and 9983 Da, respectively [21,15,16]. LTP1 (9685 Da), a protease resistant and heat stable albumin, is abundant in the aleurone layers of barley endosperm [27]. LTP1b is another form of LTP1 bound to an aspartate residue [16]. Apparently, the trace of peaks belonging to glycated LTP1b multiplied after malting (Fig 1 A and B), which can be used as a marker for characterization of glycation during malting [2]. Protein Z (4038 Da)—one of the major lysine-

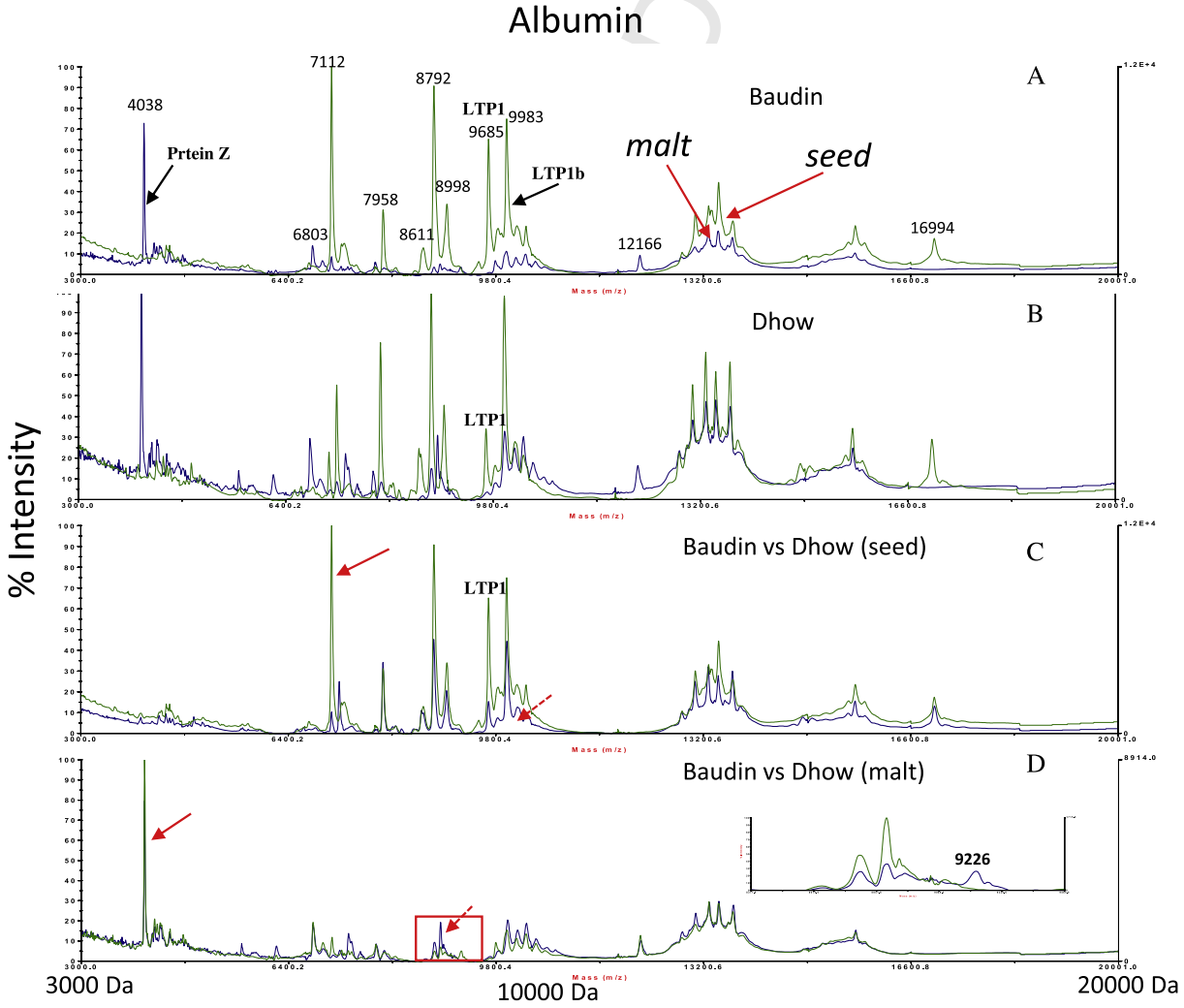

Fig. 1. Overlaid traces of MALDI-TOF-based barley albumin profiles of seed (green line) and malt (blue line) in Baudin (A) and Dhow (B), respectively, and overlaid traces of seed (C) or malt (D) of Baudin (solid arrow) and Dhow (dotted arrow). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.) Q6

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rich endosperm albumins, resistant to denaturation and proteolytic modification during malting [13]—is also represented by a peptide fragment in the mass spectra. While different to LTP1, protein Z is obviously detectable in the mass spectra of extracts from the barley malt not in grain [21]. In our study, no constitutively polymorphic albumin profiles were observed in the DH population (data not shown), which might be attributed to lack resolution of the isotopic patterns in the presented mass spectra. Thus, the peaks might represent the average of the respective amino acid in albumin. MALDI-TOF MS analysis of albumin revealed that the proteins were sharply modified during the malting process, demonstrating a large amount of albumin proteins were used to trigger hydrolysis of storage compounds at seed germination. Therefore, two-dimensional [2-D] gel electrophoresis and mass spectrometry were combined to identify the proteome changes during barley seed development and germination [9]. However, except for identifying some of the proteins in albumin or hordeins [28,11,37], the concrete composition of the other albumin or hordeins protein peaks has not been identified during the malting process, not to mention the globulin or glutelin. The role of these differently-expressed proteins has yet been determined in the final product. Therefore, MALDI-TOF MS provides an effective approach in distinguishing and identifying specific polypeptides, but further study is needed such as combining with a MS/MS analysis.

Similar to albumin, the amount of globulin in grain was dramatically reduced during the malting process (Fig. 2A and B). However, the composition of globulin in barley grain was less altered during malting comparing to albumin. In grain, a small protein peak (MW 7268 Da) was found in Dhow, but not in Baudin (Fig. 2C). With respect to the difference in malt, two small protein peaks were expressed in Baudin (MW 7141 Da, 7311 Da) at a low level, and one in Dhow (MW 7425 Da) (Fig. 2D). In barley, the major protein reserve in the aleurone layer belongs to the 7S globulin, which is controlled by a major gene *Begl* and a minor gene *Beg2* [12]. According to the biological role in cereals, globulin together with albumin were classified into structural and metabolic proteins [33]. However, there is only limited research which has mainly focused on the relationship between albumin or hordein content and malt quality [14,21]. The relationship between the globulin fraction composition and malt quality is still not clear. In the present study, the globulin in barley seed and malt was first monitored and compared using MALDI-TOF MS technique, visually demonstrating the change pattern of its complex composition. Moreover, globulin was relatively resistant to proteolysis compared with the other three storage proteins, which means that

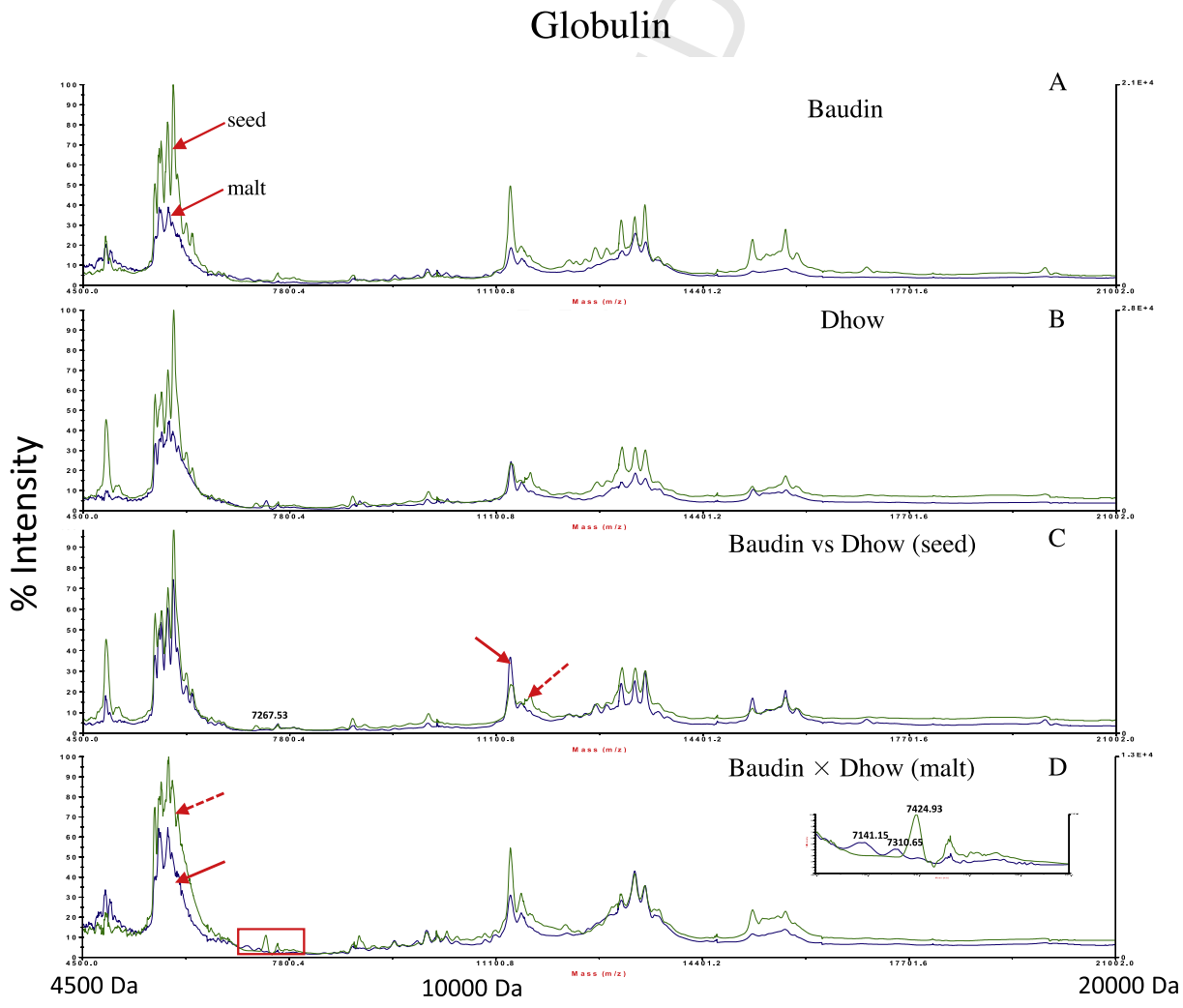

Fig. 2. Overlaid traces of MALDI-TOF-based barley globulin profiles of seed (green line) and malt (blue line) in Baudin (A) and Dhow (B), respectively, and overlaid traces of seed (C) or malt (D) of Baudin (solid arrow) and Dhow (dotted arrow). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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most of the globulin would exist in the next process in beer production such as mashing and brewing. Therefore, the three different profiles observed in globulin in view of seed and malt indicates its potentially important role in malting quality and warrants further investigation.

The composition of hordein was also modified in the malting process. Both qualitative and quantitative differences among these two cultivars were evident in both B and C hordein subclass ranges. Seed protein peaks at MWs of 40,427, 42,428, 54,278 and 57,680 Da in Baudin and 42,341 Da in Dhow were significantly degraded compared to malt (Fig. 3A and B), demonstrating hordeins B and C were markedly influenced during malting. Moreover, no obvious C hordeins profiles were found in Dhow. Previous studies showed that a high total content of C hordein was consistently associated with greater water absorption during malting and better malting quality [25]. Recently, Holopainen et al. [14] proved that C hordeins could affect water uptake in the steeping phase of malting. Thus, the presence or absence of C hordein might explain the differences in malting quality between Baudin and Dhow. Moreover, MALDI-TOF MS-based hordein peaks of seed and malt were constitutively expressed and showed high polymorphism for different genotypes. For the analysis of the genetic population, 12 polymorphic peaks (a–i in Baudin, j–l in Dhow) were identified in grains differentiating the two parental lines and scored across the whole seed DH population, while 8 (a–e in Baudin, f–h in Dhow) were identified in the malted samples (Fig. 3C, D and E). The analysis of recombinant protein peaks in the population revealed four and two segregating loci in seed and malt, respectively. The polymorphic peaks were mapped onto chromosome 1H with LOD scores ranging from 6.85–27.1 (data not shown) (Fig. 4). Our results confirm that hordein production is controlled by multigenes. In previous studies, B hordeins were encoded by two segregating loci Hor2 (major) and Hor4 (minor) [36], and C hordeins were controlled by Hor1 [22]. Since the hordein B and C profiles exhibited differently

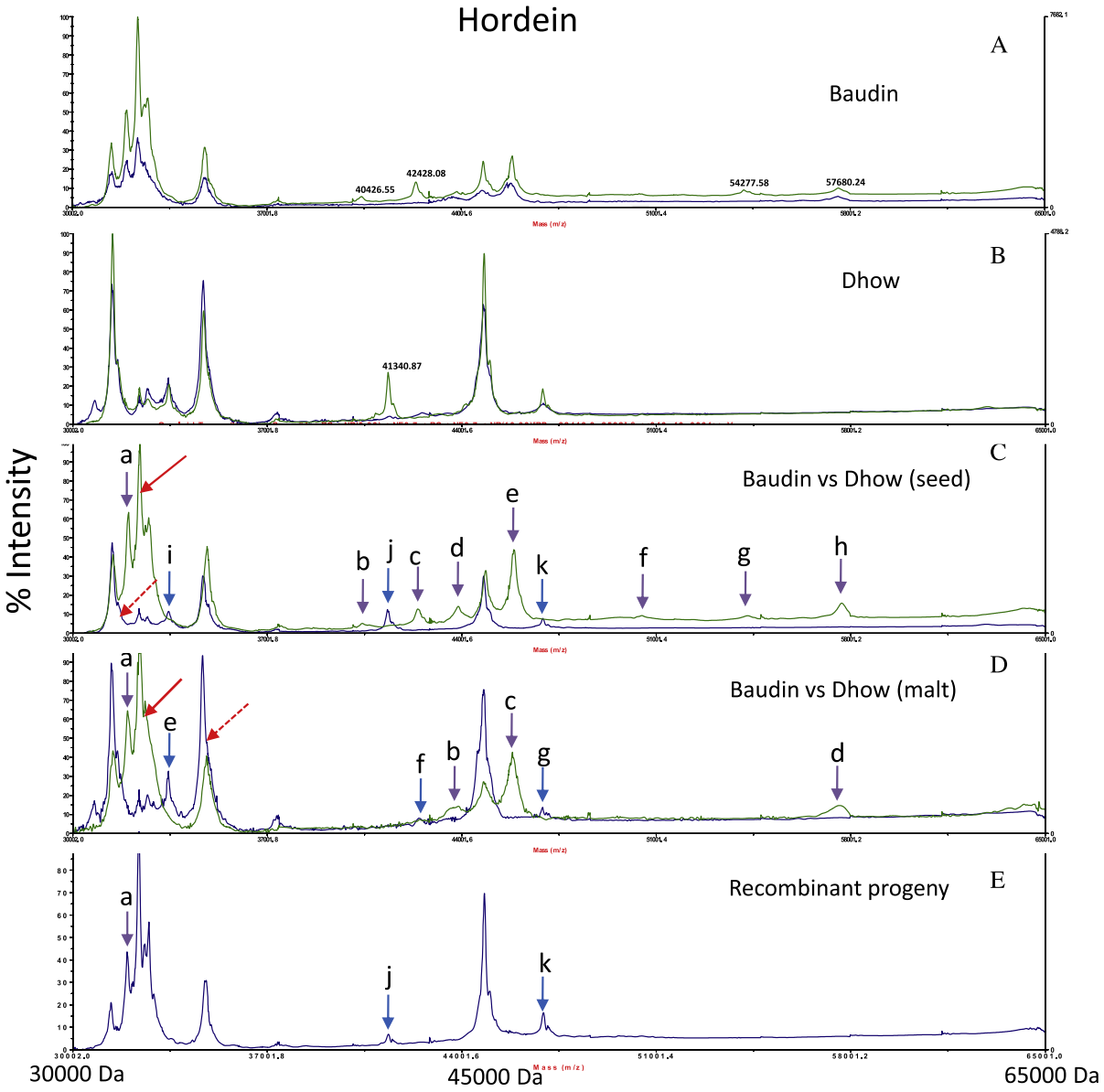

Fig. 3. Overlaid traces of MALDI-TOF-based barley hordein profiles. (A) Baudin (seed vs malt); (B) Dhow (seed vs malt); (C) seed Baudin vs seed Dhow; (D) malt Baudin vs malt Dhow; (E) recombinant progeny of seed. Peaks a–k are dominant markers, i.e., either present or absent. Only those with clear segregation patterns across progeny lines are scored as polymorphisms.

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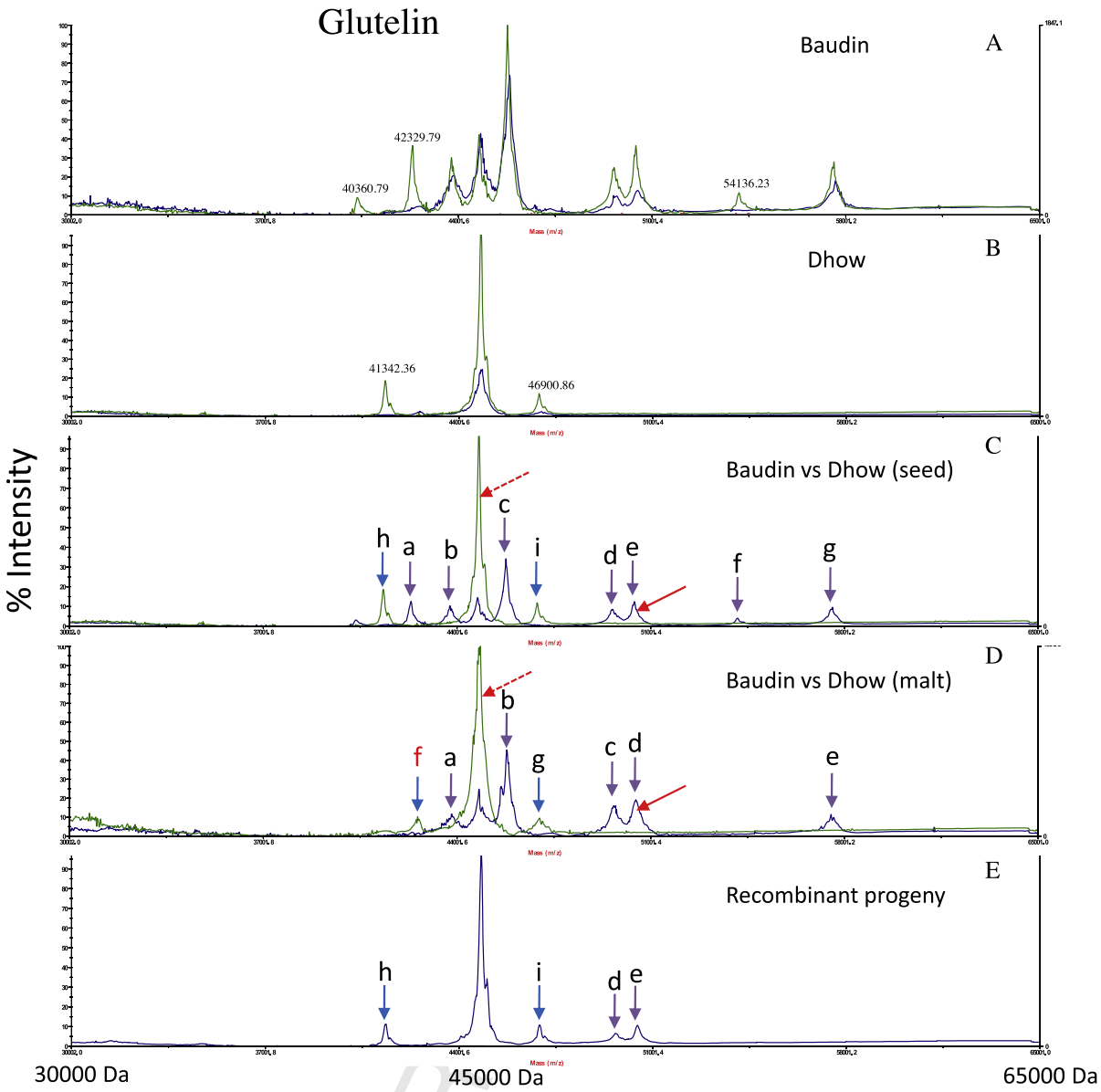

Fig. 4. Overlaid traces of MALDI-TOF-based barley glutelin profiles. (A) Baudin (seed vs malt); (B) Dhow (seed vs malt); (C) seed Baudin vs seed Dhow; (D) malt Baudin vs malt Dhow; (E) recombinant progeny of seed. Peaks a–i are dominant markers, i.e., either present or absent. Only those with clear segregation patterns across progeny lines are scored as polymorphisms.

between grain and malt, the expression of Hor1, Hor2, Hor4 in Baudin, and Hor2, Hor4 in Dhow seem to be related to malting quality.

Dithiothreitol (DTT), a strong reducing agent, is frequently used to reduce disulfide bonds both within and between polypeptide chains of proteins, and thus could improve the extraction efficiency of prolamins [31]. However, the addition of DTT is not an indispensable reagent for identifying storage protein hordeins in barley [17,42]. Chen et al. [5] also used DTT to extract gliadin (prolamin) in wheat grain, and demonstrated the gliadin profiles in a DH population could be used as molecular marker mapped to the expected chromosomes. In the present study, we also determined the hordein patterns in a DH population without DTT. Apparently, the number of hordein profiles was less without DTT when compared with DTT addition condition, which indicated the disulfide bonds existing in the hordein proteins. Ten sharp hordein peaks were characterized by MALDI-TOF MS in Baudin, while there were six peaks in Dhow. Interestingly, a recombinant line with eight peaks was identified in the DH population (Fig. S1). Eight peaks (a–h) inherited from Baudin (a–k) and Dhow (g and h) showed hordein polymorphism, thus could be used as molecular markers to score the population. Their coding genes also are located on chromosome 1H (Fig. S2). With regard to variety identification and hordein peaks used as molecular marker in mapping, the hordeins can be extracted in aqueous solution of ethanol with or without DTT.

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The changing patterns of glutelin after malting are similar to hordein. In particular, three proteins peaks in Baudin grain (MW 40,361, 42,330 and 54,136 Da) almost disappeared in malt. While in Dhow, the glutelin protein peaks similar to hordein (MW 41,342 and 46,901 Da) were significantly degraded during the malting process (Fig. 5A–B). Ten glutelin dominant profiles (a–h in Baudin, i and j in Dhow) in the population were identified as polymorphic markers in grain, while 8 (a–f in Baudin, g and h in Dhow) showed

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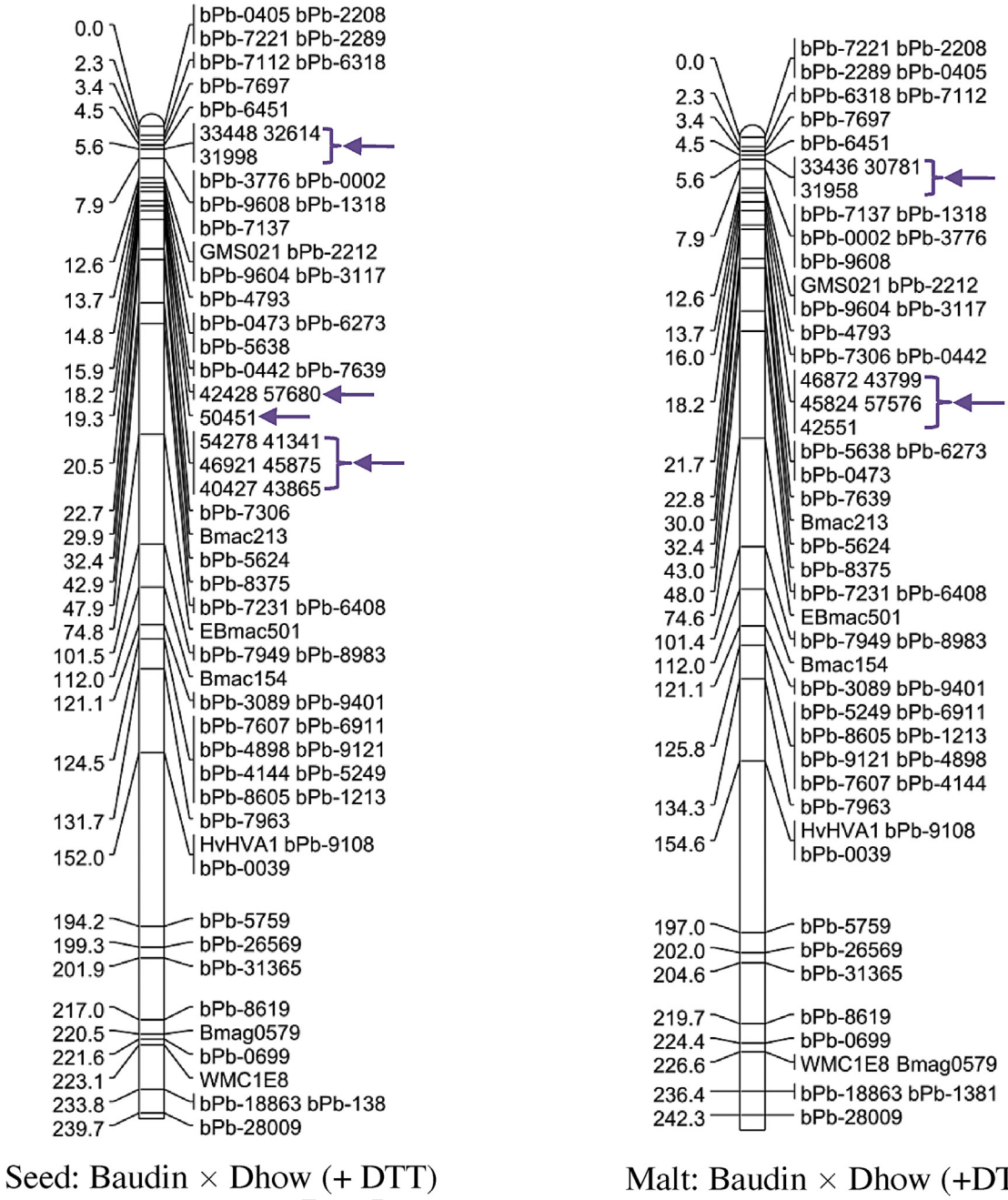

Fig. 5. Map locations of segregating hordein peaks in chromosome 1H. Arrows indicate mapped hordein locations; markers correspond to those from Fig. 3; the parent names are listed under each linkage group.

in malt (Fig. 4C and D). Combined with the genetic linkage map constructed by the molecular makers, these segregating peaks were scored as polymorphic across the population and mapped on chromosome 1H (Fig. 6A and B). Although MALDI-TOF MS could provide the information of the molecular weight of proteins profile, a second method such as the typical digest of SDS separated protein extracts is needed to valid the annotation of specific proteins. In our study, we noticed that some of the glutelin peaks also presented in hordein profiles, which demonstrated the difficulty of dividing them in barley grain [4]. By using a total of 1618 DArT markers to evaluate a subset of 61 barley genotypes, Jin et al. [18] found that 44 DArT markers were consistently associated with globulin, glutelin and hordein. In our study, we also noticed that some of the glutelin peaks presented in hordein profiles, which demonstrated the difficulty of dividing them in barley grain [4]. This result visually confirmed that hordein and glutelin could be controlled by the same or tightly linked genes. Unlike previously extensive studies on hordein, most work on barley glutelin has focused on its functional properties in food chemistry [40,43]. To the best of the authors' knowledge, this is the first report using MALDI-TOF MS monitoring for barley glutelin composition, whose polymorphic peaks as molecular markers are mapped on chromosome 1H.

Barley grain and malt provide a unique set of protein peaks detected by MALDI-TOF MS. In contrast to albumin and globulin, hordein and glutelin peaks exhibited high levels of polymorphism in the DH population (individual protein profiles Figs. S2–S7). Previous study has shown that malting quality is a complex trait controlled by numerous biochemical pathways [32]. At the genetic level, QTL clusters harboring effects on eight traits including

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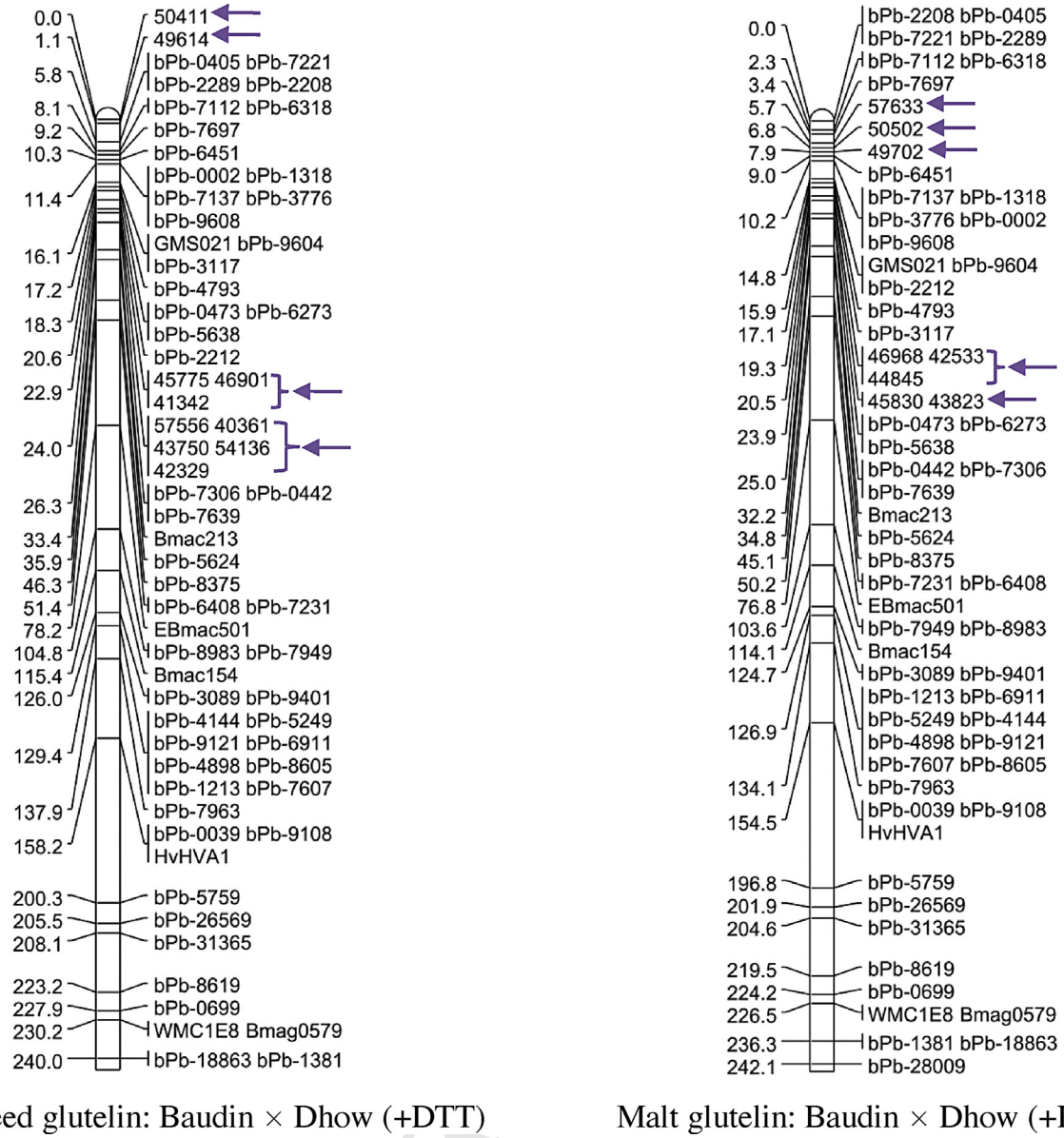

Fig 6. Map locations of segregating glutelin peaks in chromosome 1H. Arrows indicate mapped glutelin locations; markers correspond to those from Fig. 4; the parent names are listed under each linkage group.

α-amylase activity, fine-grind malt extract, friability, grain protein content, grain sieving fraction, Kolbach index (soluble protein/total protein ratio), viscosity of wort and Hartong 45 °C (the ratio of the extraction at 45 °C) were mapped to the region of chromosome 1H [32]. Recently, March et al. [24], using a subset of barley introgression lines containing wild barley alleles at chromosome 1H of approximately 15 centimorgans (cM), validated the association of this region with malting quality. In our study, the segregating peaks of hordein and glutelin in Baudin × Dhow DH population were easily scored as polymorphic markers using MALDI-TOF MS, and mapped on chromosome 1H. The results indicated that hordein and glutelin might be associated with these malt parameters such as fine-grind extract, Kolbach index and friability, grain protein content and wort viscosity. The polymorphisms observed here are related to the presence or absence of particular peaks in the mass spectrum, which may be due to single amino acid substitutions. Technically, it is more challenge to align such polymorphisms into the barley physical map (http://mips.helmholtz-muenchen.de/plant/barley/index.jsp) or select the polymorphisms using marker assisted selection. On the other hand, single nucleotide polymorphisms (SNPs) are developed as a type of DNA molecular marker and now predominate the applications in modern genetic analysis such as genome mapping, quantitative trait locus (QTL) analysis, and genome association analysis [6,26,30]. Future research should focus on conversion of the polymorphisms of protein peaks into gene-based molecular markers (SNPs) and align the polymorphisms into the barley physical map for better understanding the relationship of the protein polymorphisms and the QTLs for malting quality.

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4. Conclusion

Our results proved that the MALDI-TOF MS approach exhibits good sensitivity and provides high resolution and high throughput characteristic mass patterns of extracted barley storage proteins. By comparing protein profiles of grain and malt, we found that the

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malting process significantly affected the composition and amounts of albumin, followed by hordein, glutelin and globulin in barley. Furthermore, hordein and glutelin profiles in the DH population showed high levels of polymorphism, which could be explored as molecular markers. We also verified that the addition of DTT in ethanol has no effect on the extraction and identification of hordein peaks. The candidate proteins formed during malting remain to be identified through a MS/MS analysis of peptide sequences, particularly those with significant differences between genotypes.

Acknowledgements

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