Edith Cowan University

Research Online

2023

Proteome changes resulting from malting in hordein-reduced barley lines

Mahya Bahmani
Edith Cowan University

Angéla Juhász
Edith Cowan University

Utpal Bos
Edith Cowan University

Mitchell G. Nye-Wood
Edith Cowan University

Malcolm Blundell

See next page for additional authors

Authors

Mahya Bahmani, Angéla Juhász, Utpal Bos, Mitchell G. Nye-Wood, Malcolm Blundell, Crispin A. Howitt, and Michelle L. Colgrave

Article

Proteome Changes Resulting from Malting in Hordein-Reduced Barley Lines

Mahya Bahmani, Angéla Juhász, Utpal Bose, Mitchell G. Nye-Wood, Malcolm Blundell, Crispin A. Howitt, and Michelle L. Colgrave*

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: *Hordeum vulgare* L., commonly known as barley, is primarily used for animal feed and malting. The major storage proteins in barley are hordeins, known triggers of celiac disease (CD). Here, sequential window acquisition of all theoretical mass spectra (SWATH)-MS proteomics was employed to investigate the proteome profile of grain and malt samples from the malting barley cultivar Sloop and single-, double-, and triple hordein-reduced lines bred in a Sloop background. Using a discovery proteomics approach, 2688 and 3034 proteins were detected from the grain and malt samples, respectively. By utilizing label-free relative quantitation through SWATH-MS, a total of 2654 proteins have been quantified from grain and malt. The comparative analyses between the barley grain and malt samples revealed that the C-hordein-reduced lines have a more significant impact on proteome level changes due to malting than B- and D-hordein-reduced lines. Upregulated proteins in C-hordein-reduced lines were primarily involved in the tricarboxylic acid cycle and fatty acid peroxidation processes to provide more energy for seed germination during malting. By applying proteomics approaches after malting in hordein-reduced barley lines, we uncovered additional changes in the proteome driven by the genetic background that were not apparent in the sound grain. Our findings offer valuable insights for barley breeders and maltsters seeking to understand and optimize the performance of gluten-free grains in malt products.

KEYWORDS: *barley, malting, hordein, mass spectrometry, proteomics, SWATH-MS*

INTRODUCTION

Barley (*Hordeum vulgare* L.) is one of the oldest cereal crops from the ancient agricultural system.[1] Barley is used as animal feed, malt, or in various food preparations in modern days. The main storage proteins in barley known as hordeins, belong to the prolamin superfamily. The hordein sequences are primarily enriched in proline and glutamine amino acids.[2] Hordeins, homologous proteins of the wheat gluten proteins, gliadins, and glutenins, are classified based on their molecular weights to D- (∼105 kDa), C (55−65 kDa), B- (∼50 kDa), and γ-hordeins (∼35−45 kDa).[3] The hordeins from barley can trigger adverse immune responses in people who suffer from celiac disease (CD). Upon ingestion of barley, the patients show various symptoms like gastrointestinal discomfort, respiratory problems, and skin rashes.[4,5] To reduce the gluten-protein amount in barley, scientists were successful in developing an ultra-low gluten (ULG) barley variety, which is a commercial product under the name of Kebari for the gluten-intolerant population.[6] To develop this ULG line, first the single hordein mutants, i.e., B-, C-, D-hordein-reduced lines were selected and then cross-bred multiple times with a commercial malting variety Sloop to generate three double hordein-reduced lines: BC-, BD-, and CD-reduced lines.[6] Due to the presence of different quantities of storage proteins in these lines, these hordein-reduced samples presented an opportunity to study the effect of malting on lines with varied protein compositions.

Malting involves three main steps: steeping, germination, and kilning. During the steeping step, barley grains are soaked

in water to start the germination process. Gibberellic acid (GA) is naturally produced and can be added exogenously to activate enzymes needed for breaking down the grain macromolecules such as proteins, starch, and lipids. In the final step, germinated grains are dried with a gradual increase in temperature to halt the germination process.[7] Various techniques have been employed to study how malting affects grain protein composition and the different types of storage proteins.[8−12] With advances in proteomics, high-throughput quantitative approaches like MS-based proteomics by sequential window acquisition of all theoretical mass spectra (SWATH)-MS can shed light on the proteome level changes by quantifying the global proteome profile in the experimental samples.

Previous research investigated the impact of suppression of hordeins on the proteome and nutritional shifts in hordein-reduced barley grain samples, using proteomics approaches including SWATH-MS and targeted proteomics.[13−15] However, there is a lack of knowledge on the impact of malting on hordein-reduced lines and the mechanism of how the reduction of individual hordein subtype affects malting. In this study, we used the bottom-up proteomics method using

Table 1. Barley Grain Quality Traits[a]

line	WT	BD-reduced	D-reduced	B-reduced	BCD-reduced	C-reduced	CD-reduced	BC-reduced
protein NIR (% d.b)	13.2	13.5	12.1	12.2	12.7	10.7	11.6	12.5
NIR moisture (%)	11.6	11.1	11.3	11.4	12.6	10.5	11.8	11.1
NIR barley extract (%)	79.2	78.1	79.8	78.3	77.9	79.8	78.7	79.5
Minolta a^*: grains	5.7	5.5	5.8	5.5	4.8	5.4	5.3	5.1
Minolta b^*: grains	23.3	22.2	22.9	22.8	20.4	21.4	21.3	21.6
Minolta L^*: grains	64.9	64	65.8	65.7	60.3	62.7	63.8	62.6
protein (% d.b)	12.7	14.4	11.7	12.7	15.9	12.2	13.4	14.7
moisture 2 h ex. steep (%)	45.9	48.1	45.2	46.6	54.9	50.1	51.3	53.2
moisture after 24 h germ. (%)	45.1	47.3	44.3	45.5	54.4	49	50.5	52.6

[a]Abbreviations: NIR, near-infrared: a rapid and nondestructive analysis of various properties of barley grains; d.b, dry basis; Minolta, color evaluation instrument in which values of L^* vary from black (0) to white (100), values of a^* range from green (−60) to red (+60) and b^* values, from blue (−60) to yellow (+60); h, hours; germ, germination.

Table 2. Malt Quality Traits[a]

line	WT	BD-reduced	D-reduced	B-reduced	BCD-reduced	C-reduced	CD-reduced	BC-reduced
malt yield (%)	90	89.5	90.4	90	88.9	88.3	89.2	87
extract: fine grind EBC (%)	78.4	73.6	77.5	75.5	65.6	73.1	69.9	69.7
malt NIR malt extract (%)	77.8	75	78	76.1	73.9	77.3	75.5	75.2
malt moisture (%)	3.9	3.6	4.2	3.9	3.2	3.5	3.1	3.4
wort clarity	1	1	1	1	5	2	1	5
wort color	3.2	5.1	3.5	3.8	7.4	6	6.7	7.8
wort pH	6.07	5.89	6.07	5.94	5.93	5.89	5.93	5.86
wort viscosity EBC	1.52	1.44	1.51	1.43	1.56	1.58	1.45	1.69
malt protein (d.b)	12	14.2	11.6	12.1	15	12.7	13.8	14.6
wort soluble nitrogen dumas (%N m/m)	1034	1381	989	1069	1620	1530	1527	1801
Kolbach index	47.8	53.2	46.9	48.4	58.8	66	60.2	67.3
friability (%)	94.4	99.4	98.5	98	98.8	99.2	99.1	98.8
diastatic power (WK d.b)	359	457	360	481	105	123	124	122
free amino nitrogen EBC (ppm)	170	254	170	175	341	339	346	413
beta-glucan EBC (ppm)	67	61	71	66	62	48	50	46
malt limit dextrinase (U/kg)	715	1040	705	915	545	1137	1197	1169
malt alpha-amylase (U/g)	235	320	203	257	229	486	418	417
beta-glucanase (U/kg)	545	655	485	564	421	709	762	636
Wort - aal%	84.4	85.4	84.3	85.3	70.7	82.8	82	80.8

[a]Abbreviations: NIR, near-infrared; d.b, dry basis; EBC, European Brewers Convention; d.b, dry basis; WK d.b, Windisch−Kolbach dry basis; ppm, parts per million; kg, kilogram; g, gram; aal, apparent attenuation limit.

the SWATH-MS approach to identify and relatively quantify changes in proteins due to malting in hordein-reduced barley lines and their corresponding malt samples. By quantifying proteins and establishing a relationship between the proteome and genetic backgrounds, we determined the differentially abundant proteins due to malting in hordein-reduced lines. The results of this research will benefit breeding programs and provide insight into protein changes caused by malting in cereals.

■ MATERIALS AND METHODS

Plant Material. Previously, Tanner et al. described the process of creating barley lines with reduced levels of hordein.[6] Single- (B-, C-, D-), double- (BC-, BD-, CD-), and triple- (BCD-) hordein-reduced barley lines and wild-type barley (cv. Sloop) were grown together at CSIRO Ginninderra Experiment Station (GES), Canberra. All lines were micro-malted and tested for malt quality in 2019 by the Australian Export Grains Innovation Centre (AEGIC) in Perth, WA, Australia. The process of malting was carried out using an Automated Joe White Malting Systems Micro-malting Unit, following a standard program provided by Joe White Maltings. Before micro-malting, the barley samples were cleaned and passed through a 2.2-mm screen without any additional substances. A standard malting schedule that was followed involved steeping the barley at 19 °C for 7 h followed by

8 h of air rest, then 3 h of wetting, 4 h of air rest, and finally 1 h of wetting. Germination was allowed for 96 h, with 48 h at 18 °C and another 48 h at 16 °C, while maintaining the moisture level at 46% after 24 h. The kilning process included 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. Finally, any malt rootlets were removed using a specially designed deculming machine. Malting specification of experimented lines is demonstrated in Tables 1 and 2.

Protein Extraction and Digestion. Barley grain and rootlet-removed malt samples were prepared for proteomics analysis as described by Bahmani et al.[2] In brief, four replicates comprising 100 mg grain flour were weighed precisely, mixed with 10 mL/g of 8 M urea and 2% (w/v) dithiothreitol (DTT) in 100 mM Tris buffer, pH 8.4, and sonicated (Soniclean Ultrasonic Cleaner 250HD) for 5 min with the aim of extracting highest number of proteins. Samples went through protein reduction and cysteine alkylation on an Amicon 10 kDa molecular weight cutoff centrifuge filter (Merck, VIC, Australia). Proteins were digested on-filter by trypsin (Promega, NSW, Australia), and digested samples were collected in fresh collection tubes by centrifugation at 20,800 × g for 15 min. Filters were washed with 200 μL of 0.1 M ammonium bicarbonate, and the combined filtrates were evaporated to dryness in a Savant SpeedVac concentrator (Thermo Fisher Scientific, MA, USA).[16] Dried peptides were stored at −20 °C until proteomics analysis. Finally, the samples were reconstituted in 100 μL of 1% formic acid and iRT reference
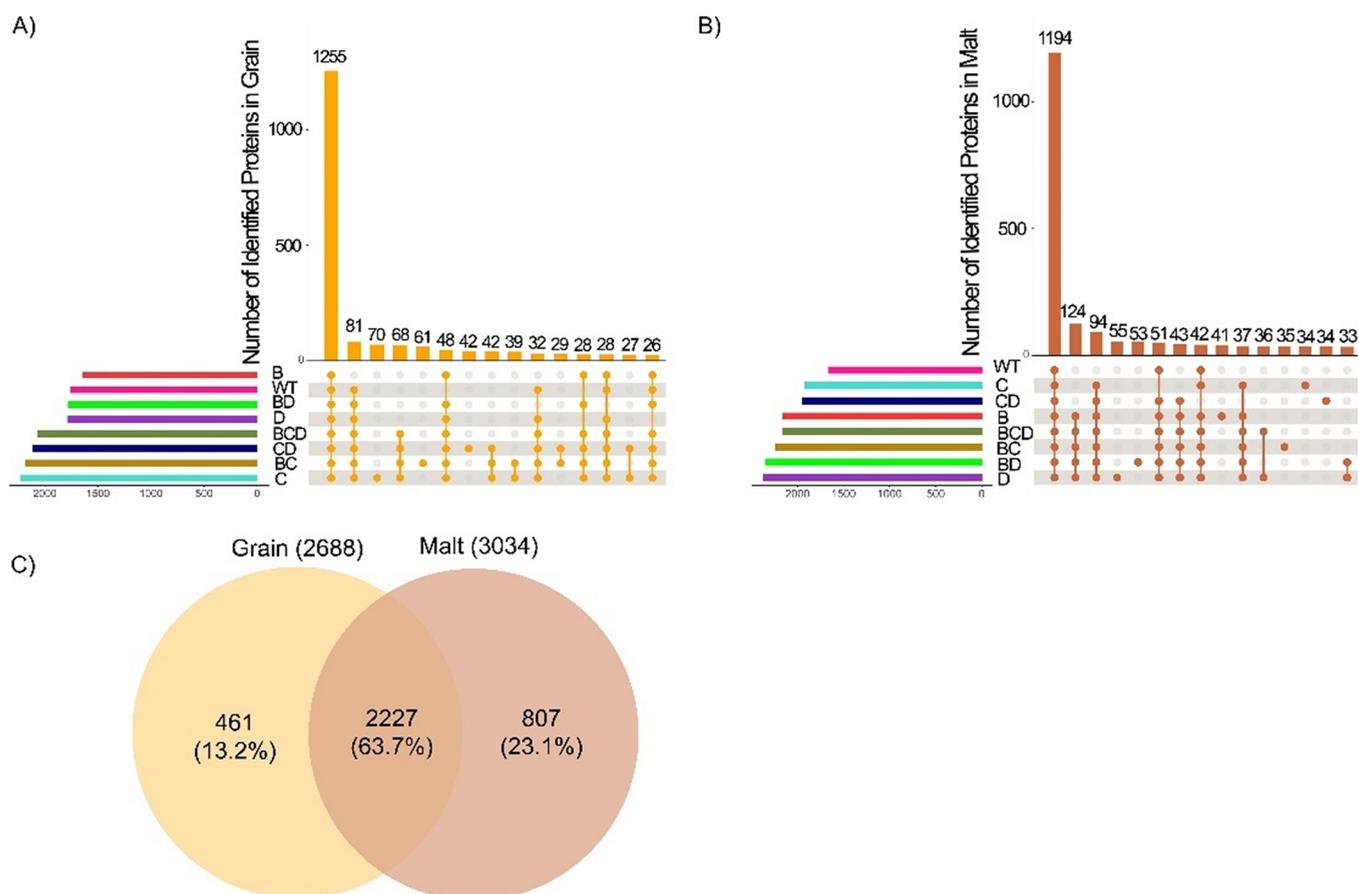
Figure 1. Analysis of identified proteins in barley grain and malt in hordein-reduced barley lines. (A) Upset plot of all identified proteins for the top 15 most frequent combination in the hordein-reduced lines for the grain samples. Rows represent each hordein-reduced line and columns depict the shared proteins between a series of lines. Numbers on the columns refer to the number of shared proteins among lines highlighted and connected by dots. (B) Upset plot of all identified proteins in malt samples of all hordein-reduced lines. (C) Venn diagram showing the overlap of identified proteins between grain and malt samples. A total of 2688 and 3034 proteins were identified in grain and malt, respectively, at 1% FDR.

peptide solution was added to the samples (1 pmol/μL; Biognosys, Zurich, Switzerland) for further proteomic analysis.

Data-Dependent Acquisition (DDA). For DDA analysis, pooled samples (1 μL) were chromatographically separated on an Ekspert nanoLC415 (Eksigent, Dublin, CA, USA) system and subjected to a TripleTOF 6600 MS (SCIEX, Redwood City, CA, United States) analysis, where previously established analysis methods were used.[17] Gas phase fractionation was employed for DDA data collection,[18] beginning with an MS1 scan of the mass range 350−595 m/z followed by an autonomous injection that targeted the mass range 585−1250 m/z. In both cases, the MS1 accumulation time was 0.25 s, and the top 30 precursor ions were selected for DDA-MS, with dynamic exclusion of peptides for 15 s after two acquisitions, with a mass tolerance of 100 ppm. MS2 spectra were acquired across mass ranges of 100−1800 m/z with an accumulation time of 0.05 s per spectrum.

The process of identifying proteins was carried out using ProteinPilot v5.0.3 software.[19] The DDA data were compared against a sequence database that included *Hordeum vulgare* proteins from UniProt-KB [55,750 total entries accessed on 06/2022] were also included from the common Repository of Adventitious Proteins (thegpm.org/crap) and Biognosys iRT pseudo-protein sequence.

Data-Independent Acquisition by SWATH-MS. Samples were divided into two batches and analyzed using SWATH-MS acquisition with LC and MS source conditions as described for DDA-MS. The SWATH variable window calculator v1.1 (SCIEX) was used to generate a 65-window acquisition scheme across a mass range of 350−1250 m/z within a 2.9 s total cycle time. Collision energy (CE) was determined for each window center by using the input m/z for SCIEX CE equations, and a 5 eV CE spread was used for m/z

variance over each SWATH window. To evaluate the instrument performance over the data acquisition period, iRT peptides in the samples were used. Additionally, a pooled biological quality control sample was prepared by combining the pooled replicate samples and was injected at the beginning and intermittently throughout each batch.

SWATH-MS Data Processing Using DIA-NN. DIA-NN, a software that corrects interference and uses a neural network for quantitative proteomics using data-independent acquisition (DIA) data, was used to analyze SWATH-MS data.[20] The spectral library was generated by DIA-NN using a FASTA file consisting of all protein sequences identified in the GPF-DDA analysis of cultivar pools at a global false discovery rate (FDR) threshold of 1%. Only fully tryptic peptides with a length of 7−30 amino acids and no missed cleavages were used. Carbamidomethylation of cysteine was selected as a fixed modification, and no variable modifications were included in the analysis. A precursor m/z range of 350−1250 and a fragment ion m/z range of 200−1800 were chosen, and 1% precursor FDR was applied for filtering. Mass accuracy, MS1 accuracy, and scan window were set to automatic mode, and the software removed interferences predicted by the neural network classifier in a single-pass mode. Quantification was performed using a high-accuracy strategy, and cross-run normalization was conducted in a retention time-dependent manner.

Data Analysis. Raw peak area data points were imputed using the K-nearest neighbors imputation algorithm employing MetImp 1.2.[21] These measurements were used as input to remove batch effects using the Limma R package[22] whereafter the Most Likely Ratio method was applied for data normalization.[23] Peptide peak areas were summed to obtain a protein measurement data frame for further analysis.

Table 3. Most Significant Terms for Identified Unique Proteins in Grain and Malt Samples Based on Figure 1[a]

| sample type	line	number of identified unique proteins	most significant term for molecular function of unique proteins	most significant term for biological process of unique proteins
grain	C	70	CTP synthase activity	regulation of alcohol biosynthetic process
	BC	61	maltose transmembrane transporter activity	maltose transport
	CD	42	isocitrate dehydrogenase (NADP+) activity	isocitrate metabolic process
malt	D	55	ER retention sequence binding	negative regulator of ethylene biosynthetic process
	BD	53	mannosyl-oligosaccharide 1,2-alpha-mannosidase activity	cellular heat acclimation
	B	41	UDP-glucose 4-epimerase activity	triterpenoid biosynthetic process
	BC	35	RNA cap binding	RNA splicing, via endonucleolytic cleavage and ligation
	C	34	UDP-glycosyltransferase activity	polysaccharide biosynthetic process
	CD	34	Hsp90 protein binding	immune effector process

[a]Abbreviations: CTP, cytidine triphosphate; ER, endoplasmic reticulum; NADPH, nicotinamide adenine dinucleotide phosphate; RNA, ribonucleic acid; UDP, uridine diphosphate, Hsp, heat shock protein.
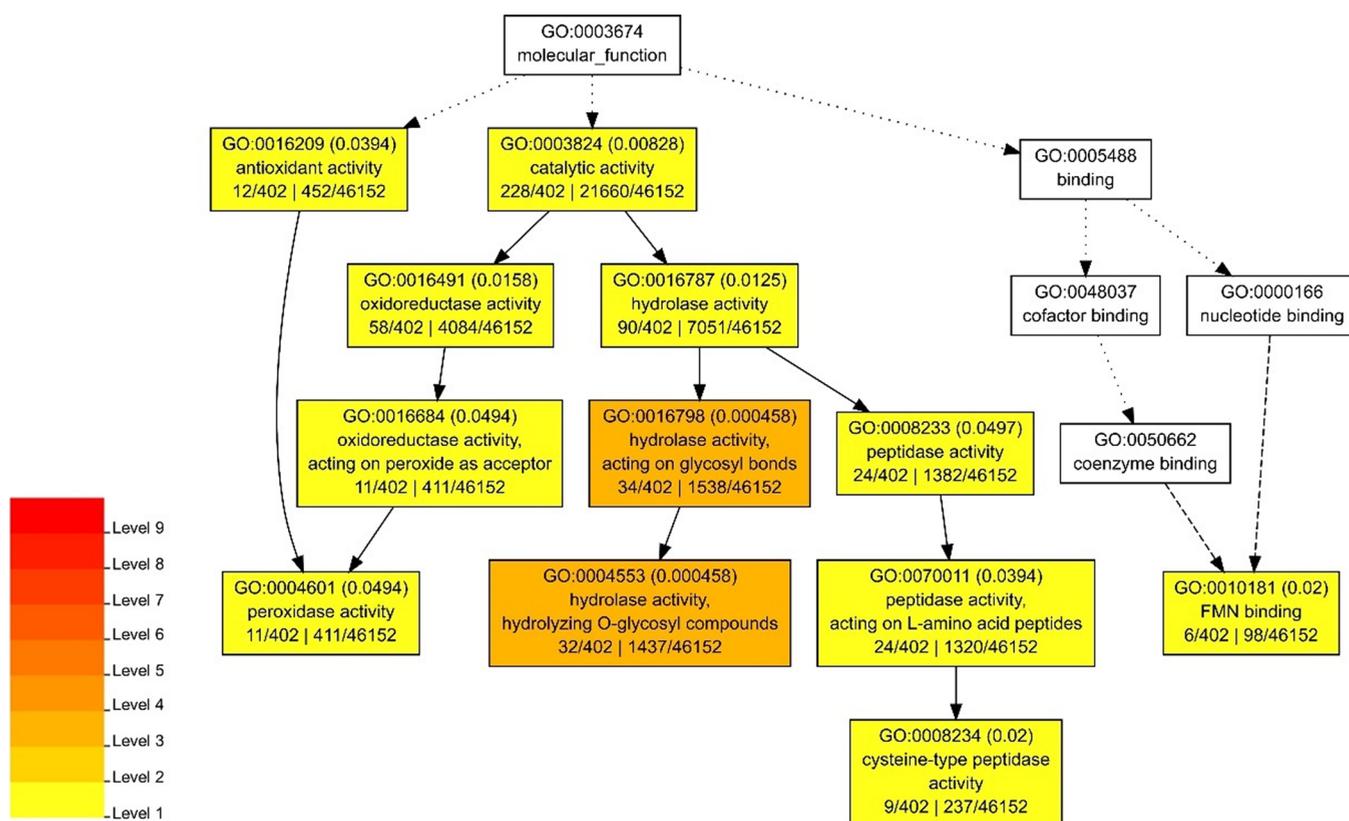
Figure 2. Hierarchical tree graph of GO terms in molecular function of unique proteins identified in malt samples of hordein-reduced barley lines showing the molecular functions. The graph shows boxes that display GO terms and their corresponding ID, statistical information, and definitions. Significant terms are identified by an adjusted p-value of <0.05, colored, while the nonsignificant terms are white.

Statistical Analysis and Data Visualization. The results of identified proteins were visualized by upset plots using the UpSetR package.[24] To visualize high-dimensional data in a 2D space and understand the structures in the data, t-SNE (t-Distributed Stochastic Neighbor Embedding) technique was used in R.[25] Heatmap and hierarchical clustering analysis (HCA) was performed in the Phantasus R package.[26] One-minus Pearson correlation coefficient was used to calculate distances for constructing a tree diagram with complete-linkage clustering method. To compare two conditions (malt vs grain), within each hordein-reduced background (B, C, D) as well as the wild type (WT), and to visualize the differentially expressed proteins, we utilized the DEBrowser tool and generated MA plots,[27] i.e., scatter plots with log2 fold changes on the y-axis and the mean of normalized abundance values on the x-axis.

The Mibiomics Shiny-R package was used for weighted gene correlation network analysis (WGCNA) to determine the correlation between genetic background, sample types, and proteome measurements.[28] In summary, to establish the relationship between protein expression, genetic background, and sample type (barley grain or malt), a protein coexpression network was created using a soft power of 10 to establish scale-free topology. This network was then divided into modules using the dynamic tree-cut algorithm, and the Pearson rank correlation method was used for network construction. Pearson correlation was used to determine the association between the first principal component of each module (eigengene values) and genetic background and sample type. Modules that showed significant correlations (positively or negatively) were further analyzed using each protein's variable importance in projection (VIP) scores from orthogonal partial least square regressions (oPLSR), where it provides
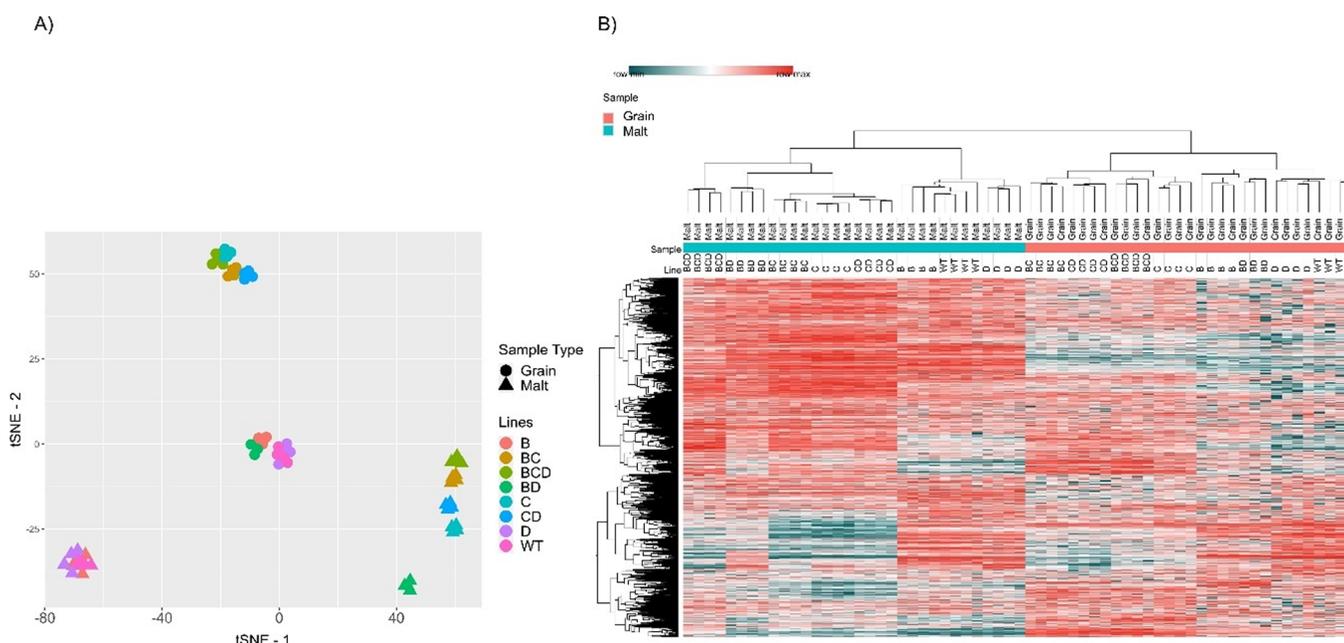
Figure 3. Overview of quantified proteins in hordein-reduced barley grain and malt samples using SWATH-MS. (A) t-SNE plot showing proteomic profiles in grain and malt of all hordein-reduced lines in addition to WT. Shapes (circle = grain, triangle = malt) represent sample type (grain or malt) and colors depict hordein-reduced lines. (B) Heatmap represents relative abundance levels ($\log_{10}$) of all quantified proteins. HCA was performed using a one-minus Pearson correlation metric. Rows in the heatmap are colored to represent relative variations in protein abundance. Two revealed clusters are aligned with sample types (grain or malt) and single, double, and triple hordein-reduced lines in addition to WT exhibit a proclivity to cluster within the two major groups.

a measure of the relative importance of each protein in explaining the variation in the response variable of interest. To perform the oPLSR analysis in Mibiomics, an R package was used[29] in which protein expression data was used as the predictor variable, and the response variable was the module groupings of different hordein-reduced genetic backgrounds.[28] Violin-box plots of top positively and negatively correlated proteins were visualized using the ggplot2 R package.[30]

The process of gene ontology (GO) term and network enrichment analysis was carried out using agriGO[31] and ShinyGO v0.741,[32] with the genome of *H. vulgare* as the background for the analysis. Enrichment analysis was performed using hypergeometric distribution followed by FDR correction with standard settings, 0.05 FDR *p*-value threshold.

RESULTS

This study used seven hordein-reduced barley lines, including single-, double-, and triple-reduced lines and a WT for the discovery of proteomics and relative protein quantitation with SWATH-MS of both raw grain and finished malt.

Discovery Proteomics Using DDA. Using discovery proteomics, we identified 2688 proteins in barley grain and 3034 proteins in barley malt samples (combined 3495 proteins) at 1% FDR (Figure 1). The UpSet plots show that out of these proteins, 1255 and 1194 were common to all barley (Figure 1A) and malt (Figure 1B) samples, respectively. Further analysis revealed ~64% overlap (2227 proteins) between the identified proteins of grain and malt. Additionally, there were 461 proteins unique to grain and 807 proteins unique to malt (Figure 1C).

Upon analyzing the results depicted in Figure 1, our focus was to identify the molecular functions and biological processes that exhibited the highest enrichment among the unique proteins discovered in hordein-reduced lines. A summary of these findings can be found in Table 3. Our

analysis revealed notable enrichments in specific enzymatic functions within the identified unique proteins from C-, BC-, and CD-hordein-reduced grain samples. These functions included cytidine triphosphate (CTP) synthase activity, maltose transmembrane transporter activity, and isocitrate dehydrogenase (NADP+) activities. Additionally, we observed distinct molecular functions associated with the most significantly enriched terms in D-, BD-, B-, BC-, C-, and CD-hordein-reduced malt samples. These functions comprised endoplasmic reticulum (ER) retention sequence binding, mannosyl-oligosaccharide 1,2-alpha-mannosidase activity, RNA cap binding, uridine diphosphate-glycosyltransferase activity, and heat shock protein (HSP) 90 protein binding activities.

We found 807 unique proteins from the malt samples. Next, we performed GO enrichment analysis of molecular function terms enriched in these proteins to further investigate the functional properties of these unique proteins (Figure 2). The most significantly enriched terms resulting from the GO analysis for molecular function unique to malt samples were hydrolase activity, peptidase, peroxidase, and oxidoreductase activities. Amylases and dextrinase were identified from barley grain and malt samples, and a list of identified enzymes with their accession has been provided in Table S2.

Relative Quantitation of Proteins by SWATH-MS. In the present study, a total of 2654 proteins were quantified from SWATH-MS acquisition in both barley grain and malt. The quantitative data matrix was initially assessed using a t-SNE plot to display the high-dimensional proteomic dataset of barley grain and malt for hordein-reduced lines and WT in a lower-dimensional space (Figure 3A). The t-SNE plot revealed a distinct cluster of malt samples with C-hordein-reduced lines in their background (C, BC, CD, and BCD lines) in the lower right quadrant of the plot where the grain samples of the
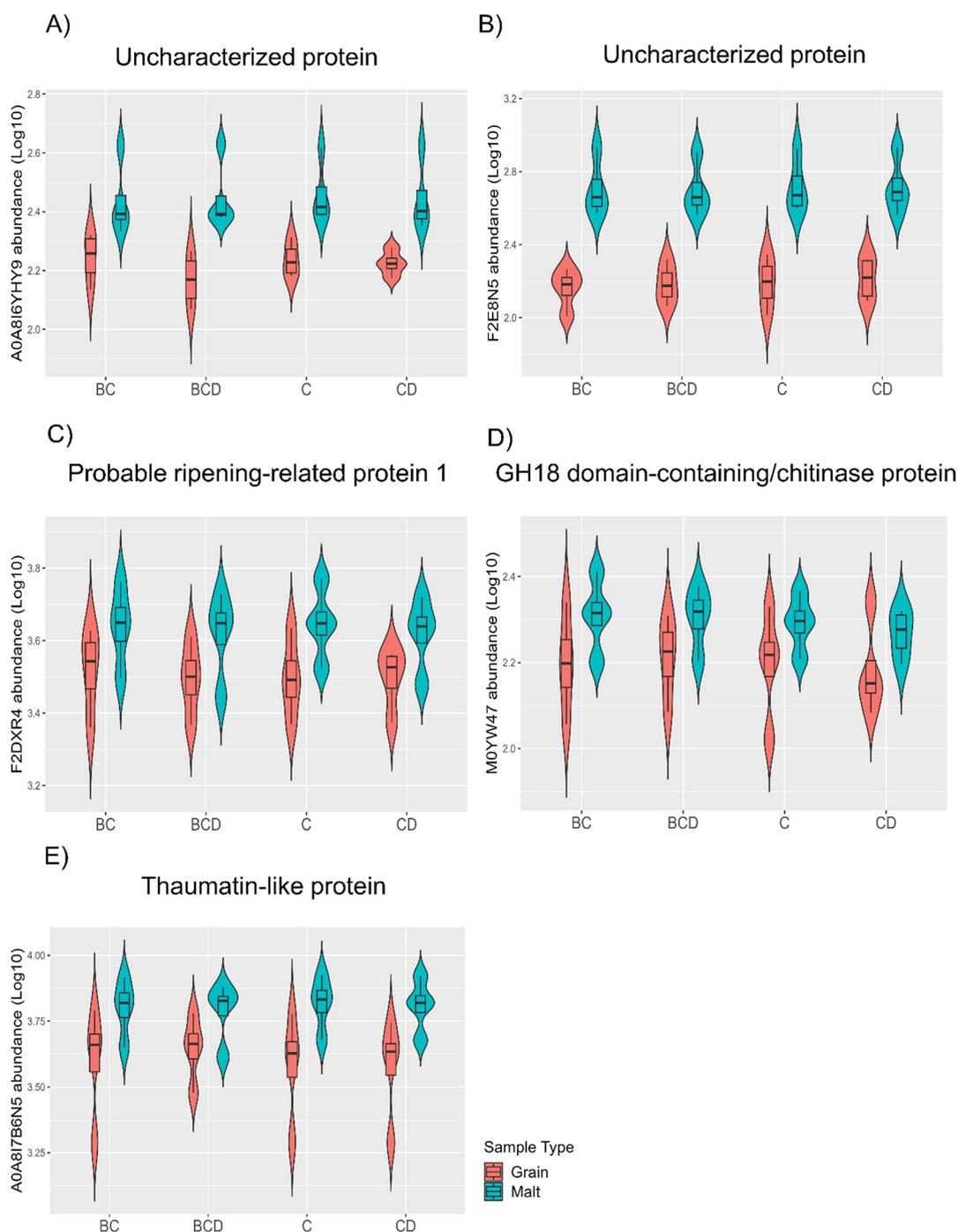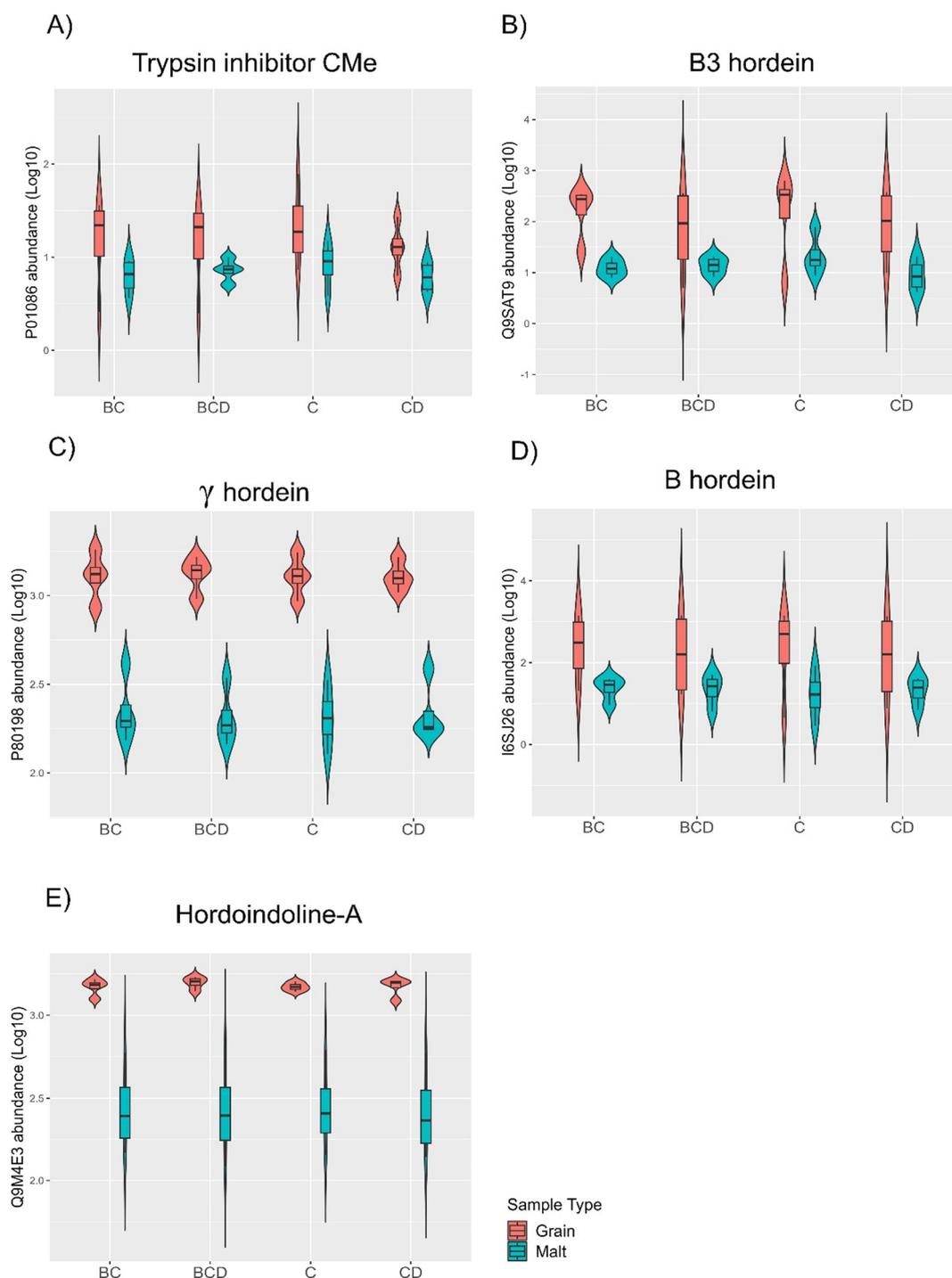
Figure 4. Relative abundance of top five proteins that positively correlated with C-hordein-reduced background lines in barley grain and malt samples. (A) Uncharacterized protein (A0A8I6YHY9), (B) uncharacterized protein (F2E8N5), (C) probable ripening-related protein (F2DXR4), (D) GH18 domain-containing or chitinase (M0YW47), and (E) thaumatin-like proteins (A0A8I7B6N5). The y-axis shows the log10 transformed relative abundance of protein and the x-axis represents lines that possess C-hordein-reduced lines in their genetic background. Violin plots show the abundance distribution in each line and box plots represent the interquartile range and the line inside each box represents the mean. Color depicts different sample types (grain and malt).

abovementioned lines were clustered together. Grain samples of WT, B, D, and BD formed a cluster, and their malt samples excluding the BD-reduced line are grouped in a separate cluster.

Next, HCA was performed to further delineate the patterns. Results showed that all experimental samples are grouped in two primary clusters of grain and malt (Figure 3B), which reveals the separation of sample types and the impact of

malting on changing the proteome. Furthermore, within the main clusters, there are subclusters of hordein-reduced lines dominated by their genetic background.

Relationship between Proteome Correlation Network Modules and Genetic Background. To identify and measure the relationship between the impact of the genetic background of B-, C-, and D-hordein-reduced lines and the proteome profiles of each line in barley grain and malt,

Figure 5. Relative abundance of top proteins—(A) trypsin inhibitor CMe (P01086), (B) B3 hordein (Q9SAT9), (C) γ-hordein (P80198), (D) B-hordein (I6SJ26), and (E) hordoindoline (Q9M4E3)—that negatively correlated with C-hordein-reduced background lines in barley grain and malt samples. The y-axis shows the log10 transformed relative abundance of protein and the x-axis represents lines that possess C-hordein-reduced line in its genetic background. Violin plots show the distribution of the abundance in each line and box plots represent the interquartile range and the line inside each box represents the mean. Color depicts different sample types (grain and malt).

WGCNA analysis was performed (Figure S1). This analysis resulted in 17 significant (p value of <0.05) correlations between module eigengenes, genetic background, and sample type. Several significant correlations were identified through the WGCNA analysis and establishing the relationship between protein modules and the genetic background of hordein-reduced lines. Proteins belonging to the black, blue, purple, and red modules were significantly positively correlated

(r value = 0.76, 0.7, 0.91, 0.9) with C-hordein-reduced genetic background (C-, BC-, CD-, and BCD) (Figure S1). Each protein's VIP scores from PLS regressions were used to identify proteins contributing significantly to the C-hordein-reduced background (Table S1). From all positively significant modules, we selected the top five proteins with VIP score > 3, and the distribution of the relative abundance of these proteins is shown in Figure 4. These proteins were ripening-related
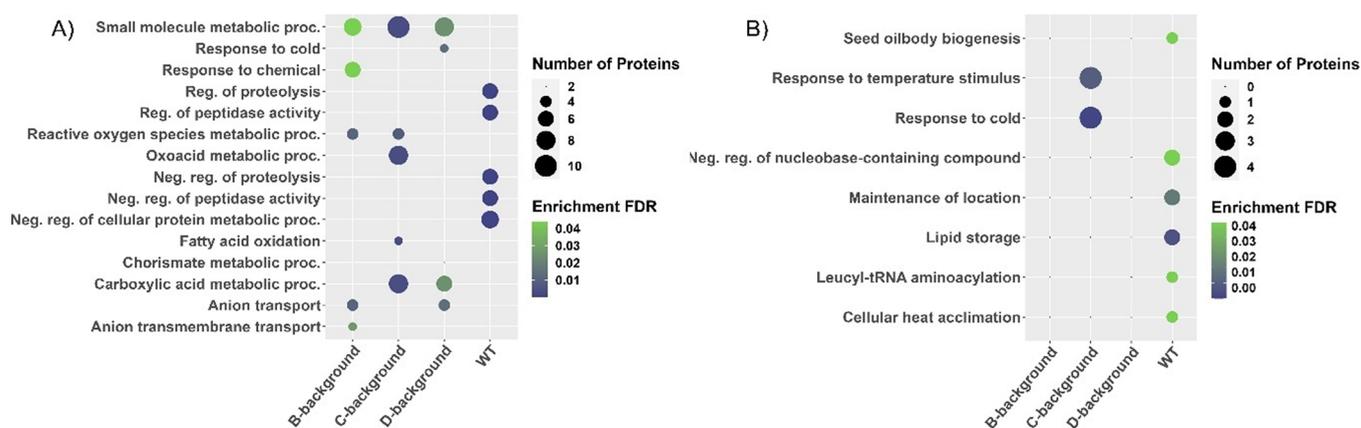
Figure 6. Gene ontology enrichment analysis of the top five biological process terms for upregulated (A) and downregulated (B) proteins. The size of dots shows the number of proteins for each term and the color scale of dots represents the FDR-corrected p value (<0.05) for each term.

protein 1, GH18 domain-containing protein, and thaumatin-like protein, all of which showed higher abundance in malt samples.

Likewise, proteins classified in brown, green-yellow, magenta, and yellow modules were significantly negatively correlated (r value = −0.88, −0.8, −0.85, −0.58) with the C-hordein-reduced genetic background. Proteins belonging to these modules were selected and plotted separately. Figure 5 shows the distribution and comparison of these top five proteins across lines that contain the C-hordein-reduced line in their background; these proteins were trypsin inhibitor CMe, two B-hordeins and one γ-hordein, and hordoindoline-A, all of which showed a decreased abundance in malt.

Investigating Changes of Proteins after Malting. We performed an analysis to compare protein abundances between barley grain and malt, where M stands for a scatterplot of the log-fold change between abundance levels of two compared conditions on the y-axis versus the average abundance level (A) of two conditions on the x-axis. We used MA analysis of the proteomic data to evaluate the impact of malting on the proteome and its relation to genetic background in hordein-reduced lines. The MA plot shows the $\log_2$ fold change versus the average abundance of proteins in barley grain and malt samples for each hordein-reduced genetic background and the WT line. According to our applied criteria of absolute value of 2-fold change and p-value of <0.05, we identified 226, 350, 235, and 249 differentially abundant proteins in B-, C-, and D-hordein-reduced genetic backgrounds and WT, respectively (Figure S2A−D). GO enrichment analysis showed the top five significant (p-value <0.05) enriched biological process terms for each genetic background and WT (Figure 6A,B). In the comparison of grain to malt, the C-background showed the highest enrichment of the biological processes in the upregulated proteins. These terms included small molecule metabolic process, oxoacid metabolic process, carboxylic acid metabolic process, reactive oxygen species metabolic process, and fatty acid oxidation. The most significant terms of hordein-reduced backgrounds were different from those in WT while there were shared terms between three main hordein-reduced background like small molecule metabolic process, reactive oxygen species metabolic process, and carboxylic acid metabolic process (Figure 6A). Significant biological process terms for downregulated proteins were only found in C-background and these terms were response to temperature stimulus and response to cold (Figure 6B). Upon closer

examination of the proteins in both genetic backgrounds associated with response to temperature stimulus, we identified four downregulated proteins in malt with this term in the C-hordein-reduced background: ferritin (B1NC18), oleosin (Q43769), calcium-dependent protein kinase (A0A221C9D5), and glycine-rich protein, RNA-binding protein (Q40052). Conversely, in the D-hordein-reduced background, the following proteins were upregulated in malt: RNA helicase (F2E4I7), glycine-rich protein, RNA-binding protein (Q40052), and 20 kDa chaperonin (F2DDU3).

GO enrichment analysis for top five significant molecular function terms for upregulated proteins belonged to WT and included serine-type endopeptidase inhibitor activity, peptidase regulator activity, and endopeptidase regulator and inhibitor activities, while in C-hordein-reduced background MF enriched terms included enzyme regulator activity, cysteine-type endopeptidase, and racemase and epimerase activity (Figure S3A). In the GO enrichment analysis of down-regulated proteins, samples originating from C-hordein-reduced background showed enrichment in proteins with sucrose synthase activity, calmodulin-dependent protein kinase, calcium ion binding, and acid-phosphatase activity. Terms enriched for downregulated proteins in the WT were different from enriched terms in all three hordein-reduced genetic backgrounds, while some terms were shared among those backgrounds like calcium ion binding and acid phosphatase activity (Figure S3B).

■ DISCUSSION

Herein, we applied discovery and quantitative proteome measurements and a suite of statistical analyses to uncover the proteome profiles of hordein-reduced lines and to explore the relationship between changes in barley grain proteome after malting and according to the genetic background. The relative quantitation by SWATH-MS across hordein-reduced barley grain and malt samples was carried out, and HCA (Figure 2) and t-SNE (Figure 3) analyses demonstrated that the grain and malt samples were tightly grouped based on their proteome profile. During malting, hydrolytic enzymes are implicated in numerous interconnected processes like germination, starch conversion, enzyme activation, and inactivation, resulting in a change in the overall proteome leading to the separation of barley grain and malt independent of genotype, in accordance with previous proteomic studies.[10] Discovery proteomics results revealed a higher number of identified

proteins in malt samples compared to grain (Figure 1) independent of the genetic background of the lines, indicating active gene expression and metabolic changes in the barley grain during the malting process. This result was aligned to previous findings on changes of barley proteome during malting.[12,33,34] However, there were unique proteins identified in individual hordein-reduced malt samples, which revealed differences between the lines (Table 3). Unique proteins in C-hordein-reduced line were involved in UDP-glycosyltransferase molecular function, which refers to a group of enzymes that catalyze the transfer of glycosyl groups from UDP donors to acceptor molecules, such as sugars, lipids, hormones, and secondary metabolites. In malt samples, UDP-glycosyltransferases play a crucial role in the modification and conjugation of various molecules with sugar moieties.[35] They are involved in the biosynthesis and modification of secondary metabolites, including flavonoids, phenolic compounds, and glycosides.[35] Interestingly, UDP-glycosyltransferases in C-hordein-reduced malt samples are involved in polysaccharide biosynthetic process. In malt barley, various polysaccharides contribute to the structural components of the grain cell walls, storage reserves, and other essential cellular functions. During the malting process, enzymes are activated to break down stored polysaccharides into smaller sugar units. Subsequently, during the kilning stage, enzymes are inactivated, and the sprouted grains are dried and processed to produce malt. Throughout these stages, there are active biosynthetic processes leading to the production of flavonoids, phenolic compounds, and glycosides, which lead to enrichment of this term in malt samples.[36]

In our GO analysis, hydrolase activity, hydrolyzing O-glycosyl compounds was the most significant molecular function term (Figure 2) of the identified unique protein from the malt samples. Proteins with this molecular function are involved in degrading O-glycosyl compounds in malt, and these proteins included alpha-galactosidase, chitinase, arabino-furanosidase, sucrose galactosyl transferase, and as Table S2 shows identified alpha-amylase and limit dextrinase. Proteins with the abovementioned GO term are involved in the hydrolysis of the bond between the O-glycosyl group and the aglycone molecule, releasing free sugars like glucose, maltose, and maltotriose.[37] These released sugars are crucial for embryo growth (during germination), or from a brewing perspective, provide a carbon source for yeast during fermentation. The differences observed between barley grain and malt generally align with previous observations that more proteins are identified after malting,[38] in particular the identification of enzymes like amylase and limit dextrinase.[9]

β-amylase is an enzyme that accumulates during the development of the grain and is not synthesized during germination. The β-amylase that we could quantify was from the endosperm-specific BMY1 gene (Q9AVJ8), which is a bound form. The bound β-amylase is released during germination either by disulfide reductase or by proteolytic enzymes.[39] Comparison of the abundance of this enzyme in barley grain and malt in all hordein-reduced lines as shown in Figure S4 indicates that the level of this enzyme was lower in those lines containing C-hordein-reduced line in their genetic background. The C-hordein-reduced line used (Risø 1508) in the ULG line breeding program is a result of a mutation in the prolamin binding factor (PBF).[6] β-amylases are also regulated by PBF; therefore, a nonfunctional PBF in the C-hordein reduced background will result not only in the decreased

storage prolamin accumulation but also decreased β-amylase accumulation. Diastatic power (DP) of barley malt indicates the effectiveness of multiple starch-degrading enzymes, with β-amylase playing a significant role as the main contributor to DP.[40] In our study, the values of DP showed lower amounts for lines including C-hordein reduced in their genetic background, indicating a potential reduction in β-amylase activity in these lines (Figure S4). Conversely, lines without reduced C-hordein backgrounds exhibited higher DP values.

Relationship between Proteome Correlation Network Modules and Genetic Background. The present study used hordein-reduced barley lines that were created through selective breeding. In developing the final ULG barley, three single-hordein-reduced lines were employed, including B-, C-, and D-reduced, and they were crossed with the WT line. The C-hordein-reduced line used in the ULG line (Risø 1508) breeding program is a result of a mutation in the PBF.[41] Unlike the C-hordein-reduced line, the B-hordein-reduced line was a result of chromosomal deletion, and the D-hordein reduced is an Ethiopian landrace (R118) and considered as a natural D hordein-null genotype. Aligned with the breeding strategy to create triple and double hordein-reduced lines and their presence or absence in the grain and malt samples, we performed WGCNA to discover the relationship between their proteome profile and genetic backgrounds (Figure S1). This analysis revealed that incorporation of the C-hordein-reduced genetic background showed the most significant effect. Of the proteins that are positively correlated with the C-hordein-reduced genetic background, two uncharacterized proteins (A0A8I6YHY9, F2E8N5), which contain the DUF 538 domain, as well as probable ripening-related protein (F2DXR4), and chitinase (M0YW47) showed increased abundance in malt. Some of these proteins also play a role in N storage and serve the aim of providing amino acids as a source of nitrogen for growth.[42]

Two proteins in our analysis (GH18 domain-containing and thaumatin-like protein) belong to the pathogenesis-related protein (PR) 10 and 5 families[43−45] with known functions in defense and developmental processes in plants. Two were uncharacterized proteins with the DUF 538 domain; however, there is less known about these proteins and it has been reported that they have functions in the plant defense response.[46,47] The application of thermal stresses, as experienced by barley seeds during the germination and kilning phases of malting, could potentially lead to an increased abundance of these proteins in malt samples. Thaumatin-like proteins have been reported to play roles in barley seed germination and ripening.[48]

Proteins with a significantly negative correlation with C-hordein-reduced genetic background included B-, γ-hordeins, hordoindoline-A, and trypsin inhibitors (Figure 5), as also reported previously.[3,6] The abundance of these proteins was higher in grain and reduced after malting. Reduction of hordeins across the breeding lines was expected as it was the intention of the breeding program. Hordoindoline was another protein that was noted to be higher in grain and was notably lower in the lines with C-hordein reduced in their genetic background reduced after malting, which could be related to the higher abundance of proteases in malt samples compared to grains (Figure S5). While previous reports indicated an unclear connection between grain hardness and hordoindo-lines,[49] observations by Takahashi et al. revealed that hordoindolines might indeed hold a pivotal role in determining

barley grain hardness.[50] The abundance of this protein was significantly lower in lines with C-hordein-reduced line in their genetic background compared to the WT (Figure S5), which suggests a probable concurrent increase in grain hardness. It is worth mentioning that kernel structure is defined by various parameters in the grain, like grain protein content, grain softness genes, ratio of starch to proteins, and hordoindolines are one of those factors. In malting, harder grains require extended steeping time, increasing the time needed for grain modification.

During malting, enzymes break down proteins and release amino acids and peptides, assessed as the concentration of free amino nitrogen (FAN). The lines with C-hordein-reduced line in their genetic background showed higher FAN levels (Table 2), which indicates that although hordeins were reduced in these lines, the total amount of protein was not reduced, suggesting a probable compensation mechanism has occurred in the grain and other proteins have been increased.[6,13]

In our WGCNA analysis, there were not any significant correlation between protein modules and B- or D-hordein reduced genetic backgrounds. This observation could be related to the normal function of PBF in mentioned lines; therefore, the effect of genetic background on storage protein accumulation is less than grain/malt sample type in these lines.

Investigating Changes of Proteins after Malting. Early barley growth and development depend on glycolysis, which includes proteins with the biological process term of small molecule metabolic process and molecular function of enzyme regulator activity,[51] such as those involved in the tricarboxylic acid (TCA) cycle,[52] amino acid metabolism,[53] and lipid metabolism.[54] In glycolysis, glucose is broken down to pyruvate and utilized in further metabolic processes to ultimately produce energy and is important during germination.[51] The TCA cycle is one of the crucial series of mechanisms that occurs in the mitochondria, leading to energy production through the oxidation of acetyl-CoA, which originated from amino acids, glucose, and fatty acids.[52] Amino acids are the building blocks of proteins, and in barley, they are metabolized through several pathways like transamination, deamination, and decarboxylation, which lead to producing energy for germination.[53] Lipids in barley are metabolized through lipid degradation, beta-oxidation, and fatty acid synthesis. Previous studies have also reported activation and an increase in metabolic pathways during cereal seed germination.[53,55,56] Enrichment of the "small molecule metabolic process" GO term was observed for all three hordein-reduced backgrounds, and it was the most significant in lines with C-hordein-reduced background. This genetic background showed the highest number of proteins related to this term compared to other genetic backgrounds (Table S4). Proteins contributing to this GO term included chorismite synthase, fructose-biphosphatase, malate dehydrogenase, cysteine-tRNA ligase, ATP synthase, glyoxysomal fatty acid beta-oxidation multifunctional protein, and fatty acid beta-oxidation multifunctional protein. Most of these proteins are involved in the TCA cycle; for instance, malate dehydrogenase is involved in TCA cycle during cellular respiration and facilitates the production of adenosine triphosphate (ATP), which roles as an energy currency for cells. Malate is found in abundance in the endosperm of barley seeds. An acidic environment (pH 4.5–5.0) is necessary for starch breakdown during malting, and this acidity is achieved through the production of organic acids during the TCA cycle, leading to endosperm acidification.[52]

The aforementioned proteins play a crucial role in converting malate to pyruvate during the germination stage of the malting process, providing additional energy for germination.[57] Additionally, the activation of the glyoxylate cycle during germination supports the β-oxidation of fatty acids.[58] This explains the observed upregulation of the glyoxysomal fatty acid beta-oxidation multifunctional protein.

The PBF transcription factor plays a crucial role in the transcriptional regulation of prolamin-type storage protein-coding and starch synthesis-related genes in cereals[59] and exhibits a specific affinity toward the promoter sequences of the genes that encode prolamin proteins. In normal developing seed, PBF is negatively regulated by GA hormone, which also upregulates the production of amylases and hydrolytic enzymes. In the C-hordein-reduced line, the PBF is mutated leading to the downregulation of cysteine-rich prolamins and starch synthases, resulting in significant changes in protein storage reservoirs of the grain. This change was reflected in the malt extract quantities (extract fine grind) (Table 2), lines including C-hordein reduced in their genetic background showed significantly lower extract amount compared to the other lines (WT, B-, D-, BD-) which show the relationship between starch and hordeins in the experimented lines. This result is aligned with the previous findings on these hordein-reduced lines.[13]

In the GO enrichment analysis of the downregulated proteins, among the three main genetic backgrounds, C-hordein-reduced lines showed a significant effect in the response to temperature stimulus GO term, although a low number of proteins are implicated. However, this term was enriched in the upregulated proteins of the D-hordein-reduced background. One of the proteins that was downregulated in the malt samples of the C-hordein-reduced background was ferritin which is a protein that is involved in regulation and storage of iron.[60] The C-hordein-reduced line possesses a mutation in the PBF transcription factor, which belongs to the NAC transcription factor family. This mutation led to starch and storage protein accumulation changes. It has been reported that NAC transcription factors affect the grain protein content as well as grain iron and zinc.[61,62] As in the C-hordein-reduced background, mutation of the NAC transcription factor that impacts hordeins could also affect ferritin and iron storage.

Another protein that was downregulated in the C-hordein-reduced genetic background was calcium-dependent protein kinase, which is an enzyme that regulates numerous cellular processes and is involved in signal transduction.[63] This downregulation observed in malt samples from the C-hordein-reduced background could imply reduced reliance on calcium-mediated signaling pathways. This could be attributed to the lower levels of hordein and starch accumulation, stemming from the PBF mutation in the C-hordein-reduced background, and this mutation has the potential to disrupt regulatory interactions and signaling pathways. Conversely, in the D-hordein-reduced background where PBF is functioning normally, we observed an upregulation of this protein. This points toward the influence of PBF mutation on the regulation and influence on signaling pathways, which necessitates further investigations.

One of the observed enriched terms for the molecular function of downregulated proteins in C-hordein-reduced background was calcium ion binding activity. Upon closer examination of the proteins contributing to this specific

molecular function in the C-hordein-reduced background, a notable set included caleosin (Q6UFY6), calcium-dependent protein kinases (A8WEN6, A0A221C9D5), and NADH—ubiquinone reductase (F2CRG8). These proteins have distinct roles within the grain. Caleosin, for instance, is located on the surface of oil bodies, spherical organelles that store lipids. It boasts a conserved calcium-binding domain.[64] It has been reported that caleosins are in specific domains of the ER, implicating their involvement in lipid transfer.[65] Notably, caleosin gene expression aligns with the accumulation of storage products, such as protein bodies, during embryo development.[65] Given that the C-hordein-reduced line involves a mutated PBF transcription factor and different mechanisms of prolamins and starch accumulation, this finding suggests potential shifts in signaling pathways. The downregulation of lipid transfer proteins in conjunction with the downregulation of prolamins in this context demands further detailed investigation.

In our study, we showed that SWATH-MS protein quantitation can be used to track proteome changes in hordein-reduced barley and malt samples and explore the overall perturbation of proteins caused by malting. Our findings demonstrated that barley grain and malt have different protein profiles also including unique proteins present in malt samples. Moreover, WGNCA analysis helped us to identify the correlation between each genetic background and proteome profiles of hordein-reduced lines. Interestingly, we could determine the most significant correlations and impactful genetic background in the experimented samples were primarily related to C-hordein-reduced background. The reason that C-hordein-reduced background showed the most significant correlation is related to the development of the original hordein-reduced lines that were used in this study. In barley, the short arm of barley chromosome 1H encodes the B-hordeins (Hor 2 locus), while the Hor 1 locus on the short arm of 1H encodes the C-hordeins. In the high-lysine mutant barley line Risø 56, there is a significant gamma-ray-induced genomic deletion of at least 85 kb genomic region which impacted some parts of B-hordein-locus, yet some B-hordeins were left in the grain.[13] On the other hand, Risø 1508 has a mutation induced by ethyl methanesulfonate in the lys3 locus on chromosome 5H, resulting in the inactivation of the PBF transcription factor and it acted both on the C-hordeins and B-hordeins.[13]

We used MA plot analysis to compare grain to malt samples in each genetic background. The GO enrichment analysis results suggested the underlying biological processes in the lines with the C-hordein-reduced genotype in their genetic pedigree. Our results indicate that upregulated proteins in the lines with mutation of the PBF transcription factor are more involved in small molecule metabolic processes such as fatty acid oxidation and the TCA cycle; the output of these cycles is ATP, potentially providing more energy during germination. Exploring ATP quantitation during germination could serve as an intriguing avenue for future research on these lines. Downregulated proteins were related to the temperature stimulus processes GO term and included proteins like caleosin, which includes a calcium-binding domain that is involved in signaling and lipid transfer. This downregulation of proteins involved in signaling pathways and lipid transfer could be a consequence of related gene expression regulation leading to the downregulation of storage proteins (hordeins). Findings of this study provide insights into the impact of storage proteins abundance changes on the proteome in barley grain and malt samples. Application of proteomics approaches revealed genetic background-related changes in the proteome after malting in hordein-reduced barley lines. Our results showed that the inclusion of the C-hordein-reduced line had the greatest impact on the malt proteome. Together, our results offer valuable insights for barley breeders and maltsters seeking to enhance gluten-free malt products.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.3c02292.

Module—eigengene relationship between different hordein-reduced backgrounds including C-, B-, D-reduced in addition to WT and sample type (grain or malt); MA plots of B-, C-, and D-hordein-reduced background lines in addition to WT; GO enrichment analysis of molecular function terms for upregulated and downregulated proteins in lines including hordein-reduced genetic background and WT; cysteine protease abundance in grain and malt samples of the hordein-reduced barley lines; abundance of hordoindoline protein in grain and malt of lines with C-hordein-reduced background; proteins that positively and negatively correlated with C-hordein-reduced line in their genetic background; identified amylases in barley grain and malt samples; number of proteins for the "small molecule metabolic process" GO term in three hordein-reduced genetic backgrounds; and summary of pairwise comparison results for proteases in barley grain and malt in hordein-reduced lines (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Michelle L. Colgrave − *Australian Research Council Centre of Excellence for Innovations in Peptide and Protein Science, School of Science, Edith Cowan University, Joondalup, WA 6027, Australia; CSIRO Agriculture and Food, St Lucia, QLD 4067, Australia;* orcid.org/0000-0001-8463-805X; Email: m.colgrave@ecu.edu.au

Authors

Mahya Bahmani − *Australian Research Council Centre of Excellence for Innovations in Peptide and Protein Science, School of Science, Edith Cowan University, Joondalup, WA 6027, Australia;* orcid.org/0000-0002-7337-4412

Angéla Juhász − *Australian Research Council Centre of Excellence for Innovations in Peptide and Protein Science, School of Science, Edith Cowan University, Joondalup, WA 6027, Australia;* orcid.org/0000-0002-4317-2027

Utpal Bose − *Australian Research Council Centre of Excellence for Innovations in Peptide and Protein Science, School of Science, Edith Cowan University, Joondalup, WA 6027, Australia; CSIRO Agriculture and Food, St Lucia, QLD 4067, Australia;* orcid.org/0000-0003-1156-6504

Mitchell G. Nye-Wood − *Australian Research Council Centre of Excellence for Innovations in Peptide and Protein Science, School of Science, Edith Cowan University, Joondalup, WA 6027, Australia;* orcid.org/0000-0001-9560-9916

Malcolm Blundell − *CSIRO Agriculture and Food, Black Mountain, ACT 2601, Australia;* ◉ orcid.org/0000-0002-9961-8243

Crispin A. Howitt − *CSIRO Agriculture and Food, Black Mountain, ACT 2601, Australia*

Complete contact information is available at:
https://pubs.acs.org/10.1021/acs.jafc.3c02292

■ ACKNOWLEDGMENTS

■ ABBREVIATIONS

AEGIC, Australian Export Grain Innovation Centre; MS, mass spectrometry; DDA, data-dependent acquisition; DIA, data independent acquisition; DTT, dithiothreitol; PBF, prolamin binding factor; SWATH-MS, sequential window acquisition of all theoretical mass spectra; TCA, tricarboxylic acid; HCA, hierarchical clustering analysis; WGNCA, weighted gene coexpression network analysis; GO, gene ontology; FDR, false discovery rate; VIP, variable importance for the projection

■ REFERENCES

(1) Badr, A.; Müller, K.; Schäfer-Pregl, R.; El Rabey, H.; Effgen, S.; Ibrahim, H. H.; Pozzi, C.; Rohde, W.; Salamini, F. On the Origin and Domestication History of Barley (*Hordeum vulgare*). *Mol. Biol. Evol.* **2000**, *17*, 499−510.

(2) Bahmani, M.; Juhász, A.; Broadbent, J.; Bose, U.; Nye-Wood, M. G.; Edwards, I. B.; Colgrave, M. L. Proteome Phenotypes Discriminate the Growing Location and Malting Traits in Field-Grown Barley. *J. Agric. Food Chem.* **2022**, *70*, 10680−10691.

(3) Tanner, G. J.; Colgrave, M. L.; Blundell, M. J.; Howitt, C. A.; Bacic, A. Hordein Accumulation in Developing Barley Grains. *Front. Plant Sci.* **2019**, *10*, 649.

(4) Tanner, G. J.; Colgrave, M. L.; Howitt, C. A. Gluten, Celiac Disease, and Gluten Intolerance and the Impact of Gluten Minimization Treatments with Prolylendopeptidase on the Measurement of Gluten in Beer. *J. Am. Soc. Brew. Chem.* **2014**, *72*, 36−50.

(5) Wieser, H.; Koehler, P. The Biochemical Basis of Celiac Disease. *Cereal Chem.* **2008**, *85*, 1−13.

(6) Tanner, G. J.; Blundell, M. J.; Colgrave, M. L.; Howitt, C. A. Creation of the First Ultra-Low Gluten Barley (*Hordeum vulgare* L.) for Coeliac and Gluten-Intolerant Populations. *Plant Biotechnol. J.* **2016**, *14*, 1139−1150.

(7) Gupta, M.; Abu-Ghannam, N.; Gallaghar, E. Barley for Brewing: Characteristic Changes during Malting, Brewing and Applications of Its by-Products. *Compr. Rev. Food Sci. Food Saf.* **2010**, *9*, 318−328.

(8) Flodrová, D.; Šalplachta, J.; Benkovská, D.; Bobálová, J. Application of Proteomics to Hordein Screening in the Malting Process. *Eur. J. Mass Spectrom.* **2012**, *18*, 323−332.

(9) March, T. J.; Richter, D.; Colby, T.; Harzen, A.; Schmidt, J.; Pillen, K. Identification of Proteins Associated with Malting Quality in a Subset of Wild Barley Introgression Lines. *Proteomics* **2012**, *12*, 2843−2851.

(10) Strouhalova, D.; Benkovska, D.; Bobalova, J. ITRAQ-Based Quantitative Proteomic Analysis of Key Barley Proteins Reveals Changes after Malting. *J. Liq. Chromatogr. Relat. Technol.* **2019**, *41*, 1−6.

(11) Dai, H.; Zhang, X. Q.; Harasymow, S.; Roumeliotis, S.; Broughton, S.; Eglinton, J.; Wu, F.; Li, C. MALDI-TOF Mass Spectrometry Provides an Efficient Approach to Monitoring Protein Modification in the Malting Process. *Int. J. Mass Spectrom.* **2014**, *371*, 8−16.

(12) Mahalingam, R. Temporal Analyses of Barley Malting Stages Using Shotgun Proteomics. *Proteomics* **2018**, *18*, No. 1800025.

(13) Bose, U.; Broadbent, J. A.; Byrne, K.; Blundell, M. J.; Howitt, C. A.; Colgrave, M. L. Proteome Analysis of Hordein-Null Barley Lines Reveals Storage Protein Synthesis and Compensation Mechanisms. *J. Agric. Food Chem.* **2020**, *68*, 5763−5775.

(14) Bose, U.; Juhász, A.; Yu, R.; Bahmani, M.; Byrne, K.; Blundell, M.; Broadbent, J. A.; Howitt, C. A.; Colgrave, M. L. Proteome and Nutritional Shifts Observed in Hordein Double-Mutant Barley Lines. *Front. Plant Sci.* **2021**, *12*, No. 718504.

(15) Colgrave, M. L.; Byrne, K.; Blundell, M.; Heidelberger, S.; Lane, C. S.; Tanner, G. J.; Howitt, C. A. Comparing Multiple Reaction Monitoring and Sequential Window Acquisition of All Theoretical Mass Spectra for the Relative Quantification of Barley Gluten in Selectively Bred Barley Lines. *Anal. Chem.* **2016**, *88*, 9127−9135.

(16) Bose, U.; Byrne, K.; Howitt, C. A.; Colgrave, M. L. Targeted Proteomics to Monitor the Extraction Efficiency and Levels of Barley α-Amylase Trypsin Inhibitors That Are Implicated in Non-Coeliac Gluten Sensitivity. *J. Chromatogr. A* **2019**, *1600*, 55−64.

(17) Colgrave, M. L.; Byrne, K.; Howitt, C. A. Liquid Chromatography-Mass Spectrometry Analysis Reveals Hydrolyzed Gluten in Beers Crafted to Remove Gluten. *J. Agric. Food Chem.* **2017**, *65*, 9715−9725.

(18) Kennedy, J.; Yi, E. C. Use of Gas-Phase Fractionation to Increase Protein Identifications. *Methods Mol. Biol.* **2008**, 217−228.

(19) Shilov, I. V.; Seymourt, S. L.; Patel, A. A.; Loboda, A.; Tang, W. H.; Keating, S. P.; Hunter, C. L.; Nuwaysir, L. M.; Schaeffer, D. A. The Paragon Algorithm, a Next Generation Search Engine That Uses Sequence Temperature Values and Feature Probabilities to Identify Peptides from Tandem Mass Spectra. *Mol. Cell. Proteomics* **2007**, *6*, 1638−1655.

(20) Demichev, V.; Messner, C. B.; Vernardis, S. I.; Lilley, K. S.; Ralser, M. DIA-NN: Neural Networks and Interference Correction Enable Deep Coverage in High-Throughput Proteomics. *bioRxiv* **2020**, No. 282699.

(21) Lazar, C. ImputeLCMD: A Collection of Methods for Left-Censored Missing Data Imputation. *R package, version*, 2015, vol 2.

(22) Ritchie, M. E.; Phipson, B.; Wu, D.; Hu, Y.; Law, C. W.; Shi, W.; Smyth, G. K. Limma Powers Differential Expression Analyses for RNA-Sequencing and Microarray Studies. *Nucleic Acids Res.* **2015**, *43*, e47−e47.

(23) Lambert, J. P.; Ivosev, G.; Couzens, A. L.; Larsen, B.; Taipale, M.; Lin, Z. Y.; Zhong, Q.; Lindquist, S.; Vidal, M.; Aebersold, R.; Pawson, T.; Bonner, R.; Tate, S.; Gingras, A. C. Mapping Differential Interactomes by Affinity Purification Coupled with Data Independent Mass Spectrometry Acquisition. *Nat. Methods* **2013**, *10*, 1239.

(24) Conway, J. R.; Lex, A.; Gehlenborg, N. UpSetR: An R Package for the Visualization of Intersecting Sets and Their Properties. *Bioinformatics* **2017**, *33*, 2938−2940.

(25) Team, R. C. *R: A Language and Environment for Statistical Computing*, 2021. Published Online 2020. http://www.R-project.org/.

(26) Zenkova, D.; Kamenev, V.; Sablina, R.; Artymov, M.; Sergushichev, A. *Phantasus: Visual and 715 Interactive Gene Expression Analysis*, 2018.

(27) Kucukural, A.; Yukselen, O.; Ozata, D. M.; Moore, M. J.; Garber, M. DEBrowser: Interactive Differential Expression Analysis and Visualization Tool for Count Data 06 Biological Sciences 0604

Genetics 08 Information and Computing Sciences 0806 Information Systems. *BMC Genomics* **2019**, *20*, 6.

(28) Zoppi, J.; Guillaume, J.-F.; Neunlist, M.; Chaffron, S. MiBiOmics: An Interactive Web Application for Multi-Omics Data Exploration and Integration. *BMC Bioinf.* **2021**, *22*, 1−14.

(29) Mevik, B.-H.; Wehrens, R. *The Pls Package: Principal Component and Partial Least Squares Regression in R*, 2007.

(30) Wickham, H. Ggplot2. *Wiley Interdiscip.: Rev. Comput. Stat.* **2011**, *3*, 180−185.

(31) Tian, T.; Liu, Y.; Yan, H.; You, Q.; Yi, X.; Du, Z.; Xu, W.; Su, Z. AgriGO v2.0: A GO Analysis Toolkit for the Agricultural Community, 2017 Update. *Nucleic Acids Res.* **2017**, *45*, W122−W129.

(32) Ge, S. X.; Jung, D.; Jung, D.; Yao, R. ShinyGO: A Graphical Gene-Set Enrichment Tool for Animals and Plants. *Bioinformatics* **2020**, *36*, 2628−2629.

(33) Osama, S. K.; Kerr, E. D.; Yousif, A. M.; Phung, T. K.; Kelly, A. M.; Fox, G. P.; Schulz, B. L. Proteomics Reveals Commitment to Germination in Barley Seeds Is Marked by Loss of Stress Response Proteins and Mobilisation of Nutrient Reservoirs. *J. Proteomics* **2021**, *242*, No. 104221.

(34) Qin, Q.; Liu, J.; Hu, S.; Dong, J.; Yu, J.; Fang, L.; Huang, S.; Wang, L. Comparative Proteomic Analysis of Different Barley Cultivars during Seed Germination. *J. Cereal Sci.* **2021**, *102*, No. 103357.

(35) Han, Z.; Zhang, J.; Cai, S.; Chen, X.; Quan, X.; Zhang, G. Association Mapping for Total Polyphenol Content, Total Flavonoid Content and Antioxidant Activity in Barley. *BMC Genomics* **2018**, *19*, 81.

(36) Rani, H.; Bhardwaj, R. D. Quality Attributes for Barley Malt: "The Backbone of Beer.". *J. Food Sci.* **2021**, *86*, 3322−3340.

(37) Power, R. F. Enzymatic Conversion of Starch to Fermentable Sugars. In *The Alcohol Textbook*; Notthingham University Press: Notthingham, 2003; pp 23−32.

(38) Finnie, C.; Andersen, B.; Shahpiri, A.; Svensson, B. Proteomes of the Barley Aleurone Layer: A Model System for Plant Signalling and Protein Secretion. *Proteomics* **2011**, *11*, 1595−1605.

(39) Sopanen, T.; Laurière, C. Release and Activity of Bound β-Amylase in a Germinating Barley Grain. *Plant Physiol.* **1989**, *89*, 244−249.

(40) Henson, C. A.; Duke, S. H. Maltose Effects on Barley Malt Diastatic Power Enzyme Activity and Thermostability at High Isothermal Mashing Temperatures: I.β-Amylase. *J. Am. Soc. Brew. Chem.* **2016**, *74*, 100−112.

(41) Moehs, C. P.; Austill, W. J.; Holm, A.; Large, T. A. G.; Loeffler, D.; Mullenberg, J.; Schnable, P. S.; Skinner, W.; van Boxtel, J.; Wu, L.; McGuire, C. Development of Decreased-Gluten Wheat Enabled by Determination of the Genetic Basis of Lys3a Barley. *Plant Physiol.* **2019**, *179*, 1692−1703.

(42) Shewry, P. R.; Halford, N. G. Cereal Seed Storage Proteins: Structures, Properties and Role in Grain Utilization. *J. Exp. Bot.* **2002**, *53*, 947−958.

(43) Jenkins, J. A.; Griffiths-Jones, S.; Shewry, P. R.; Breiteneder, H.; Mills, E. N. C. Structural Relatedness of Plant Food Allergens with Specific Reference to Cross-Reactive Allergens: An in Silico Analysis. *J. Allergy Clin. Immunol.* **2005**, *115*, 163−170.

(44) Iqbal, I.; Tripathi, R. K.; Wilkins, O.; Singh, J. Thaumatin-Like Protein (TLP) Gene Family in Barley: Genome-Wide Exploration and Expression Analysis during Germination. *Genes* **2020**, *11*, 1080.

(45) Huang, Q. S.; Xie, X. L.; Liang, G.; Gong, F.; Wang, Y.; Wei, X. Q.; Wang, Q.; Ji, Z. L.; Chen, Q. X. The GH18 Family of Chitinases: Their Domain Architectures, Functions and Evolutions. *Glycobiology* **2012**, *22*, 23−34.

(46) Araujo, A. C. G.; Guimaraes, P. M.; Mota, A. P. Z.; Guimaraes, L. A.; Pereira, B. M.; Vinson, C. C.; Lacerda, A. L.; Martins, A. C. Q.; Brasileiro, A. C. M. Overexpression of DUF538 from Wild Arachis Enhances Plant Resistance to Meloidogyne Spp. *Agronomy* **2021**, *11*, 559.

(47) Gholizadeh, A. Pectin Methylesterase Activity of Plant DUF538 Protein Superfamily. *Physiol. Mol. Biol. Plants* **2020**, *26*, 829.

(48) Singh, S.; Tripathi, R. K.; Lemaux, P. G.; Buchanan, B. B.; Singh, J. Redox-Dependent Interaction between Thaumatin-like Protein and β-Glucan Influences Malting Quality of Barley. *Proc. Natl. Acad. Sci. U. S. A.* **2017**, *114*, 7725−7730.

(49) Darlington, H. F.; Rouster, J.; Hoffmann, L.; Halford, N. G.; Shewry, P. R.; Simpson, D. J. Identification and Molecular Characterisation of Hordoindolines from Barley Grain. *Plant Mol. Biol.* **2001**, *47*, 785−794.

(50) Takahashi, A.; Ikeda, T. M.; Takayama, T.; Yanagisawa, T. A Barley Hordoindoline Mutation Resulted in an Increase in Grain Hardness. *Theor. Appl. Genet.* **2010**, *120*, 519−526.

(51) James, W. O.; Bunting, A. H. On the Mechanism of Glycolysis in Barley. *New Phytol.* **1941**, *40*, 268−275.

(52) Ma, Z.; Marsolais, F.; Bernards, M. A.; Sumarah, M. W.; Bykova, N. V.; Igamberdiev, A. U. Glyoxylate Cycle and Metabolism of Organic Acids in the Scutellum of Barley Seeds during Germination. *Plant Sci.* **2016**, *248*, 37−44.

(53) Sreenivasulu, N.; Usadel, B.; Winter, A.; Radchuk, V.; Scholz, U.; Stein, N.; Weschke, W.; Strickert, M.; Close, T. J.; Stitt, M.; Graner, A.; Wobus, U. Barley Grain Maturation and Germination: Metabolic Pathway and Regulatory Network Commonalities and Differences Highlighted by New MapMan/PageMan Profiling Tools. *Plant Physiol.* **2008**, *146*, 1738−1758.

(54) MacLeod, A. M.; White, H. B. Lipid metabolism in germinating Barley. *J. Inst. Brew.* **1961**, *67*, 182−190.

(55) Yu, Y.; Guo, G.; Lv, D.; Hu, Y.; Li, J.; Li, X.; Yan, Y. Transcriptome Analysis during Seed Germination of Elite Chinese Bread Wheat Cultivar Jimai 20. *BMC Plant Biol.* **2014**, *14*, 1−19.

(56) Dong, K.; Zhen, S.; Cheng, Z.; Cao, H.; Ge, P.; Yan, Y. Proteomic Analysis Reveals Key Proteins and Phosphoproteins upon Seed Germination of Wheat (Triticum Aestivum L.). *Front. Plant Sci.* **2015**, *6*, 1017.

(57) Blackwood, G. C.; Miflin, B. J. The Effects of Salts on NADH Malate Dehydrogenase Activity in Maize and Barley. *Plant Sci. Lett.* **1976**, *7*, 435−446.

(58) Holtman, W. L.; Heistek, J. C.; Mattern, K. A.; Bakhuizen, R.; Douma, A. C. β-Oxidation of Fatty Acids Is Linked to the Glyoxylate Cycle in the Aleurone but Not in the Embryo of Germinating Barley. *Plant Sci.* **1994**, *99*, 43−53.

(59) Mena, M.; Vicente-Carbajosa, J.; Schmidt, R. J.; Carbonero, P. An Endosperm-Specific DOF Protein from Barley, Highly Conserved in Wheat, Binds to and Activates Transcription from the Prolamin-Box of a Native B-Hordein Promoter in Barley Endosperm. *Plant J.* **1998**, *16*, 53−62.

(60) Borg, S.; Brinch-Pedersen, H.; Tauris, B.; Holm, P. B. Iron Transport, Deposition and Bioavailability in the Wheat and Barley Grain. *Plant Soil* **2009**, *325*, 15−24.

(61) Distelfeld, A.; Cakmak, I.; Peleg, Z.; Ozturk, L.; Yazici, A. M.; Budak, H.; Saranga, Y.; Fahima, T. Multiple QTL-Effects of Wheat Gpc-B1 Locus on Grain Protein and Micronutrient Concentrations. *Physiol. Plant.* **2007**, *129*, 635−643.

(62) Velu, G.; Singh, R. P.; Cardenas, M. E.; Wu, B.; Guzman, C.; Ortiz-Monasterio, I. Characterization of Grain Protein Content Gene (GPC-B1) Introgression Lines and Its Potential Use in Breeding for Enhanced Grain Zinc and Iron Concentration in Spring Wheat. *Acta Physiol. Plant.* **2017**, *39*, 1−9.

(63) Ciésla, A.; Mituła, F.; Misztal, L.; Fedorowicz-Strónska, O.; Janicka, S.; Tajdel-Zielińska, M.; Marczak, M.; Janicki, M.; Ludwików, A.; Sadowski, J. A Role for Barley Calcium-Dependent Protein Kinase CPK2a in the Response to Drought. *Front. Plant Sci.* **2016**, *7*, 222815.

(64) Liu, H.; Hedley, P.; Cardle, L.; Wright, K. M.; Hein, I.; Marshall, D.; Waugh, R. Characterisation and Functional Analysis of Two Barley Caleosins Expressed during Barley Caryopsis Development. *Planta* **2005**, *221*, 513−522.

(65) Næsted, H.; Frandsen, G. I.; Jauh, G. Y.; Hernandez-Pinzon, I.; Nielsen, H. B.; Murphy, D. J.; Rogers, J. C.; Mundy, J. Caleosins: Ca2+-Binding Proteins Associated with Lipid Bodies. *Plant Mol. Biol.* **2000**, *44*, 463−476.