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Article

Proteome Phenotypes Discriminate the Growing Location and Malting Traits in Field-Grown Barley

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ABSTRACT: Barley is one of the key cereal grains for malting and brewing industries. However, climate variability and unprecedented weather events can impact barley yield and end-product quality. The genetic background and environmental conditions are key factors in defining the barley proteome content and malting characteristics. Here, we measure the barley proteome and malting characteristics of three barley lines grown in Western Australia, differing in genetic background and growing location, by applying liquid chromatography−mass spectrometry (LC−MS). Using data-dependent acquisition LC−MS, 1571 proteins were detected with high confidence. Quantitative data acquired using sequential window acquisition of all theoretical (SWATH) MS on barley samples resulted in quantitation of 920 proteins. Multivariate analyses revealed that the barley lines' genetics and their growing locations are strongly correlated between proteins and desired traits such as the malt yield. Linking meteorological data with proteomic measurements revealed how high-temperature stress in northern regions affects seed temperature tolerance during malting, resulting in a higher malt yield. Our results show the impact of environmental conditions on the barley proteome and malt characteristics; these findings have the potential to expedite breeding programs and malt quality prediction.

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

INTRODUCTION

Barley (*Hordeum vulgare* L.) is a member of the Poaceae family and is ranked as the fourth major cereal crop by yield globally.[1] The importance of this crop stems from its wide application as human food and animal feed and essentiality to meet malting and brewing demand. Australia is the largest exporter of malting barley, providing more than 30% of the world's supply.[2]

Malting is a value-adding process that prepares barley for brewing or food production. It is a three-step biotechnological process including steeping, germination, and kilning of the barley grain under controlled temperature and moisture conditions. The primary purpose of malting is to initiate controlled germination of the seed where hydrolytic enzymes digest the endosperm cell walls and the proteins surrounding starch granules to produce enzymes, simple sugars, and amino acids. Kilning then halts the process in preparation for further food processing. Malt modification refers to the level of endosperm hydrolysis within the malting process.[3] To obtain desired malting characteristics of barley breeding lines, small-scale malting studies can assist in understanding the malting quality of the barley grain to meet the brewer's requirements or to decide on an alternative use of grain. Malting barley varieties are bred and grown to select for optimal malt quality specifications such as high enzyme activity, yield, and flavor characteristics.[4] Therefore, it is essential to select and breed the barley variety with desired malting specifications. In this regard, the total protein content of the barley seed is between 8 and 15% depending on the cultivar and growing environment, and this trait is central to the grain quality due to its relationship with enzyme content and malt specifications.[3] There have been efforts to find candidate proteins associated with the malting specification's quantitative trait loci (QTL)[5−8] and to map QTL associated with the protein expression variation in barley; researchers reported the detection of 14 proteins using mass spectrometry including heat shock proteins (HSP), late embryogenesis abundant (LEA) proteins, and enzyme inhibitors.[9]

The background genetics and growing conditions for barley lines have been shown to influence the malt characteristics and quality.[10] As a result, the combination of biotic and abiotic stresses that have an effect during the growth and development of the barley plant in fields has been investigated in numerous studies.[2,11−14] These stresses have been shown to cause changes at the molecular and physiological levels. For instance, the growing environment can significantly affect the barley phytic acid content, nutritional composition, and seed protein concentration.[13] Likewise, growing barley in different environmental conditions can impact its amylopectin, directly affecting germination and malt characteristics.[15] Furthermore, environ-

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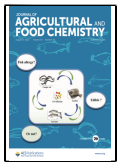

mental factors can affect malt specifications[16] and influence the subsequent malt and beer flavor.[4]

Liquid chromatography (LC) coupled with mass spectrometry (MS) is a powerful tool to measure the barley proteome, the protein quality, and the changes that occur during the germination events. Our recent review on the application of cutting-edge LC−MS-based proteomic approaches in barley protein research has demonstrated its potential to inform on plant breeding.[17] Research to date has reported label-free quantitative MS-based proteomics to study barley malting,[18] quality and flavor,[4] responses to infection,[19] in-depth profiles of storage proteins,[20] and potential allergens and enzymes;[21] however, investigation of the growing environment and its influence on the barley seed proteome remains lacking.

In the present study, a bottom-up MS-based proteomic approach was employed to explore the effect of variable growing locations across Western Australia (WA) on three field-grown barley lines that differ in their genetic backgrounds. The relationship between proteomic measurements and malting specification data was established to understand the concordance between the growing location and malting traits. The result of this study provides information that can support the breeding of barley lines for malting purposes while also providing broad applicability to other malting cereals.

MATERIALS AND METHODS

Plant Material. Three malting barley lines (006, 007, and 008) were used in this study (Table 1). These lines were developed by

Table 1. Barley Lines' Information

barley line	pedigree	growing locations
006	Wimmera/barley yellow dwarf virus-18	Toodyay, Mingenew, Munglinup
007	Yangsimi3/Hindmarsh × 90/La Trobe	Toodyay, Mingenew, Munglinup
008	Yangsimi3/Hindmarsh × 225/La Trobe	Toodyay, Mingenew, Munglinup

Edstar Genetics Pty Ltd., Australia, and each line was cultivated in two northern regions, namely, Toodyay (T) (−31.5511748, 116.4671695) and Mingenew (Mi) (−29.222513142453078, 115.44604997548826), and one southern region, namely, Munglinup (Mun) (−33.7073279, 120.8652063), across WA, Australia. Hereafter, three locations will be indicated as T, Mi, and Mun throughout the manuscript.

These lines were sown in early May and harvested in late November 2019. The barley seeds were transported to the laboratory and milled using a mixer mill (model MM400 Retsch, Germany) and sifted. Fine flour was obtained using a 300 μm sieve (Endecotts Pty Ltd., Sieves, London, England) as previously described.[22] All three lines from each three locations were micro-malted by the Australian Grain Export Innovation Centre (AEGIC) in Perth, WA, in 2019, and the same malting process was used for all lines. Malting specification data is shown in Tables 2 and 3. The average monthly temperature recordings from the three growing locations during 2019 were downloaded from the Australian Bureau of Meteorology (Australia's Official Weather Forecasts and Weather Radar—Bureau of Meteorology).[23] The average accumulated temperature has been calculated by adding all the growing days for each region between May and November and dividing the sum by the number of days.

Protein Extraction and Digestion. A total of 100 mg of flour was weighed for each of the four biological replicates into 1.5 mL microtubes and mixed with 1 mL of 8 M urea and 2% (w/v) dithiothreitol (DTT) in 100 mM Tris buffer (pH 8.5) to extract maximal proteins.[20] Samples were thoroughly mixed and sonicated

Table 2. Malting Specifications for Barley Lines (Part 1)

line	location	test wt (kg/hl)	grain wt (mg)	protein (% d.b.)	moist. after 24 h germ. (%)	malt yield (%)	protein NIR (% d.b.)	malt moisture (%)	malt NIR malt extract (%)	oven moisture (%)	extract: fine-grind EBC (%)	wort color	wort pH	wort soluble nitrogen dumas (%N m/m)	wort viscosity EBC	wort (aal %)
006	Mingenew	67.2	42.9	12.1	44.5	94.1	11.5	4.1	78.6	4.3	77.9	3.1	6.12	716	1.48	85.5
008	Mingenew	68.0	37.8	12.3	44.4	93.6	12.6	4.1	77.5	4.1	77.5	3.3	6.06	767	1.42	84.7
007	Mingenew	66.6	37.6	12.6	44.4	93.5	12.2	4.3	77.5	4.1	77.3	3.2	6.13	711	1.49	85.6
008	Mingenew	67.2	34.6	13.0	43.5	93.3	13.2	4.0	77.6	3.9	76.9	3.1	6.12	770	1.48	80.9
007	Mingenew	66.5	40.6	12.4	45.9	92.6	12.1	3.9	76.5	4.1	75.3	3.2	6.10	774	1.47	84.4
008	Mingenew	67.0	38.0	13.0	45.4	92.5	12.1	4.0	76.3	4.1	75.6	2.8	6.08	748	1.47	84.4
008	Mingenew	65.6	32.8	13.1	45.2	92.4	12.5	4.0	77.5	4.1	77.6	2.9	6.04	798	1.44	85.8
006	Toodyay	67.8	40.5	11.5	43.8	92.3	11.0	4.0	79.6	4.1	79.2	3.6	6.11	749	1.45	86.1
008	Toodyay	67.9	38.7	12.0	45.0	92.1	12.6	4.0	78.4	4.0	77.5	3.7	6.06	801	1.44	83.7
008	Toodyay	68.4	39.2	11.1	42.7	91.9	11.4	4.0	79.8	4.1	79.2	4.2	6.06	824	1.43	84.2
006	Munglinup	63.0	36.6	11.6	45.3	91.5	12.5	4.0	77.0	3.8	77.0	2.9	6.08	783	1.51	85.1
007	Munglinup	63.3	42.4	12.5	44.2	91.2	12.4	3.9	77.3	3.8	76.5	3.1	6.10	774	1.52	84.1
007	Munglinup	65.2	41.0	11.6	43.5	91.1	12.3	4.1	77.4	3.9	76.9	3.4	6.11	759	1.45	84.6
008	Munglinup	64.6	40.5	12.1	42.9	90.8	12.3	4.0	78.4	3.9	78.3	3.3	6.09	801	1.45	85.7
008	Munglinup	63.4	34.2	12.3	44.1	90.8	13.7	3.9	77.0	3.9	76.9	3.1	6.14	752	1.50	83.9
007	Munglinup	61.6	39.3	12.2	44.4	90.8	12.3	4.2	77.2	3.3	75.6	2.7	6.07	801	1.45	85.7

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Table 3. Malting Specifications for Barley Lines (Part 2)

lines	location	malt soluble nitrogen (% d.b.)	malt nitrogen (%)	NIR malt protein (% d.b.)	malt protein (d.b.)	Kolbach index	diastatic power (WK d.b.)	free amino nitrogen EBC (ppm)	β-glucan EBC (ppm)	malt α-amylase (U/g)	β-glucanase (U/kg)	malt limit dextrinase (U/kg)	friability (%)
006	Mingenew	0.64	1.75	11.3	10.9	36.5	429	151	148	256	820	1053	81.2
008	Mingenew	0.68	1.9	12.1	11.9	35.8	378	172	76	298	913	1090	87.0
007	Mingenew	0.63	1.83	11.9	11.4	34.6	456	151	181	278	754	1059	76.2
008	Mingenew	0.68	1.98	12.7	12.4	34.5	401	160	114	261	797	1066	74.0
007	Mingenew	0.69	1.96	12.3	12.2	35.0	428	162	159	279	764	1062	74.2
008	Mingenew	0.66	2.01	12.8	12.5	33.1	394	160	136	244	813	1077	77.3
008	Mingenew	0.71	1.83	11.3	11.4	38.8	444	172	101	282	877	1088	85.3
006	Toodyay	0.67	1.64	11.0	10.3	40.6	460	166	90	274	803	1114	89.1
008	Toodyay	0.71	1.90	12.7	11.9	37.5	473	161	84	268	833	1076	88.8
008	Toodyay	0.73	1.72	11.5	10.7	42.6	445	181	56	322	870	1077	91.8
006	Munglinup	0.69	1.82	11.5	11.4	38.0	412	164	101	281	805	1114	86.7
007	Munglinup	0.69	1.95	11.8	12.2	35.1	390	143	119	228	723	1056	79.6
007	Munglinup	0.67	1.86	12.1	11.6	36.1	465	157	111	275	717	1117	85.4
008	Munglinup	0.71	1.88	12.2	11.8	37.7	490	170	79	290	815	1113	86.7
008	Munglinup	0.67	1.99	12.8	12.4	33.5	380	151	116	236	790	1068	78.5
007	Munglinup	0.70	1.84	11.9	11.5	38.2	459	162	108	309	723	1077	82.6

(Soniclean Ultrasonic Cleaner 250HD, 650 W, 43 kHz) for 5 min at room temperature. Protein reduction, cysteine alkylation, and digestion steps were performed following the previously described method by Colgrave et al.[24] Proteins were digested by trypsin (Sigma-Aldrich Inc., St. Louis, MO, USA), and digested samples in the filters were transferred to fresh collection tubes and centrifuged at 20,800 × *g* for 15 min and washed with 200 μL of 0.1 M ammonium bicarbonate; the combined filtrates were evaporated to dryness in a Savant SpeedVac concentrator (Thermo Fisher Scientific, MA, USA).[25]

Data-Dependent Acquisition (DDA). Digested proteins were reconstituted in 100 μL of 0.1% formic acid (FA), and iRT reference peptide solution was added to the samples (1 pmol; Biognosys, Zurich, Switzerland). Pooled samples of biological replicates were used for DDA analysis. The peptides (1 μL) were chromatographically separated using an Ekspert nanoLC415 chromatograph (Eksigent, Dublin, CA, USA) with the eluent directed to a TripleTOF 6600 MS (SCIEX, Redwood City, CA, United States); the analysis method and LC−MS/MS parameters were precisely described in Colgrave et al.'s work (2017).[26] Gas phase fractionation was employed for DDA data collection where a top 30 mode MS1 scan of mass range 350−595 *m*/*z* was performed first followed by an independent injection targeting the mass range of 585−1250 *m*/*z*, both with the accumulation time set to 0.25 s. MS2 spectra were acquired across mass ranges of 100−1800 *m*/*z* with an accumulation time of 0.05 s per spectrum and dynamic exclusion of peptides for a 15 s interval after two acquisitions with a mass tolerance of 100 ppm.

Protein identification was conducted using ProteinPilot v5.0.3 software encompassing the Paragon Algorithm for peptide spectrum matching and scoring (SCIEX) and ProGroup algorithm for conservative protein inference and grouping.[27] The DDA data were searched against a sequence database that included *Hordeum vulgare* proteins from UniProt-KB [139,559 total entries accessed on 08/2020] supplemented with proteins listed on the common Repository of Adventitious Proteins (thegpm.org/crap) as well as the Biognosys iRT pseudo-protein sequence.

Data-Independent Acquisition by SWATH-MS. Samples were analyzed in six batches. LC and MS source conditions for SWATH acquisition were identical to those described for DDA. The SWATH variable window calculator v 1.1 (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. The collision energy (CE) was assigned considering each window center as the input *m*/*z* for SCIEX CE equations, and a 5 eV CE spread was used for *m*/*z* variance over each SWATH window. The iRT peptides in the samples were used to evaluate the instrument performance over the data acquisition period; moreover, a pooled biological quality control (PBQC) sample was prepared by combining the pooled replicate samples and was injected at the beginning, which interspersed throughout each batch.

Spectral Library Processing. DDA data acquired from the PBQC gas phase fractions were searched and used as input for the ion library within the SWATH Acquisition MicroApp plugin for Peakview v 2.2 software (SCIEX). Using the MicroApp, 6 transitions per peptide and 25 peptides per protein were selected. The library was exported and filtered to remove modified peptides. Shared peptides were retained in their first instance only (i.e., attributed to the top-ranked protein according to the ProteinPilot search result). This initial ion library was imported into the SWATH MicroApp, and RT calibration was performed by manually selecting the iRT peptides. Extraction settings were the following: peptide confidence threshold of 91%, peak group FDR threshold of 1%, XIC width of 75 ppm, and RT extraction window of 5 min. Peak groups were extracted and scored before exporting the peak group score report. Thereafter, the report was used to filter the ion library wherein the original 25 peptides per protein were reduced to the six best peptides per protein, according to the mean peak group score. This ion library was then imported back into PeakView for extracting the final peak area data using the same settings as described above.

Data Analysis. A custom R script was used for the curation of the raw peak area data. In summary, fragment ions with more than 20% missing values across the samples were removed, and after which, the remaining missing values were imputed using the K-nearest neighbors (KNN) imputation algorithm.[28] Fragment ions were then summed to obtain peptide-level measurements. These measurements were used as input to remove batch effects using the Limma R package[29] whereafter the most likely ratio (MLR) method was applied for data normalization.[30] Peptide peak areas were summed to obtain a protein measurement data frame for further analysis.

Statistical Analysis. Unsupervised principal component analysis (PCA) was performed with SIMCA software version 17.0.1.26957 (SIMCA Software, Umetrics, Sweden) to detect outliers and evaluate relationships in the samples. PCA plots were visualized using the ClustVis open web tool.[31] Heat mapping and HCA were performed in the Phantasus R package.[32] The one-minus Pearson correlation coefficient was used to calculated distances for the construction of a tree diagram. This measure was used so that perfectly correlated data would correspond to no distance between samples, increasing to a maximum distance of 1 between completely uncorrelated data. Pairwise comparisons were performed using a two-tailed T-test with Welch's correction, and data analysis was performed with GraphPad Prism version 9.3.1 (GraphPad Software, San Diego, California, USA). A *p* value of less than 0.05 was deemed as significant, that is,

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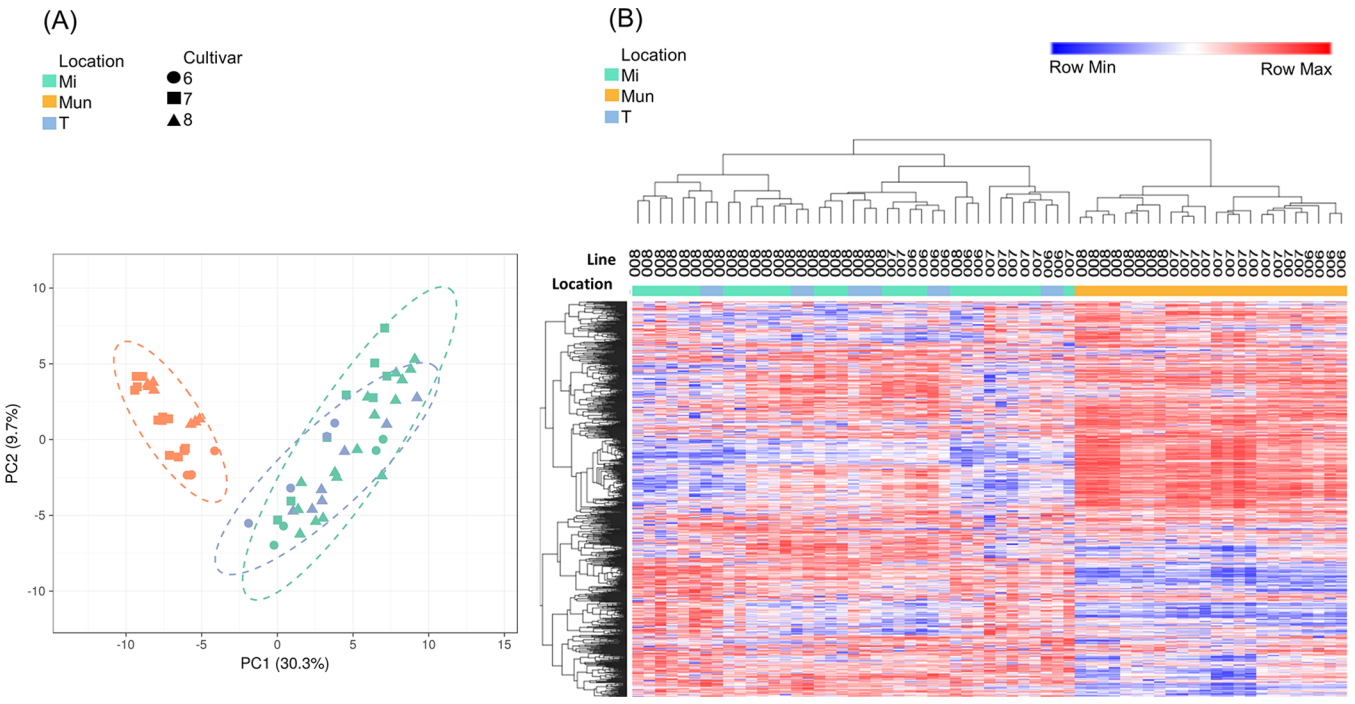

Figure 1. Overview of the proteome composition between three barley lines harvested across three locations. (A) The PCA plot shows that the major variance in the proteome composition is concordant with the growing location (PC1), while the second-highest variance (PC2) is not explainable by barley lines or locations. Shapes represent the breeding lines, and colors refer to locations. (B) The heatmap depicts relative abundance levels (log 10) of all proteins quantified from SWATH data; a one-minus Pearson correlation metric was used for HCA; colors represent differences in the abundance of proteins in rows; two major sample clusters (column) align with the northern and southern locations; and genotypes show some propensity to cluster within these two major groupings.

differences between groups are assumed not to be due to random chance alone at $p < 0.05$.

Supervised orthogonal partial least-square discriminant analysis (oPLS-DA) was performed in SIMCA software version 17.0.1.26957 (SIMCA Software, Umetrics, Sweden) to stratify locations and identify the proteins responsible for this stratification. The relation between malting specifications received from AEGIC and proteome measurements was established using a weighted gene correlation network analysis (WGCNA) in the Mibiomics Shiny-R package.[33] Briefly, a protein co-expression network was constructed, wherein a scale-free topology was established using a softpower (β) of 10. Thereafter, modules were established using the dynamic tree cut algorithm. A Spearman rank correlation was selected as the correlation method for network construction. The association between protein modules' eigengene values (the first principal component of the module) and malting specifications was assessed using the Spearman correlation. Statistical significance for module−trait associations is assumed not to be due to random chance alone at $p < 0.05$. Modules with correlation to malting specifications were analyzed further using each protein's variable importance in projection (VIP) scores from PLS regressions. The Phantasus R package[32] was used for matrix visualization and analysis. Gene ontology (GO) term and network enrichment analysis was conducted using ShinyGO v 0.741[34] using an *H. vulgare* genome as a background; enrichment analysis was calculated based on a hypergeometric distribution followed by an FDR correction with standard settings (0.05 FDR *p*-value threshold). Statistical analysis was performed with GraphPad Prism software version 9.3.1 for Windows (GraphPad Software, San Diego, California, USA).

RESULTS

SWATH-MS Spectral Library Generation. Three barley breeding lines were grown in three locations, namely, Toodyay (T), Mingenew (Mi), and Munglinup (Mun), across Western Australia. Barley grain was commercially malted and subjected to proteome measurements. In total, 1517 proteins were identified at 1% FDR using DDA and 920 proteins were quantified from SWATH-MS acquisition. An initial assessment of the SWATH-MS data was performed using unsupervised PCA, revealing that samples are stratified by location, wherein PC1 (location component) and PC2 explain 40% of the variation in the dataset (Figure 1A). Samples from each barley line cluster together, indicating that the effect of the location outweighs the effect of line.

Hierarchical clustering analysis (HCA) showed that samples are clustered according to their growing location into two major groups of northern regions including T and Min and the southern Mun region (Figure 1B). This further supports the effect of the growing location on the proteome composition across the three barley lines as well as highlights the substantial shift in the proteins' abundances between the locations. It also shows a strong secondary clustering of samples by genotype within the locations.

Supervised multivariate analysis was performed to identify the proteins responsible for the stratification seen in the PCA and HCA. oPLS-DA confirmed the results of PCA and HCA in that the two northern locations appear closer compared to the southern location. The S-plot derived from the oPLS-DA model (Figure S1) displayed the correlation of proteins versus separation between the two regions of north and south with the proteins with VIP > 1 marked in red. A list of proteins with a VIP score of >1 was extracted and deemed to be the major cause of the separation of the northern locations (Mi and T) from the southern location (Mun) (Table S1). In total, 357 proteins were perturbed and influenced the separation between the two northern locations.

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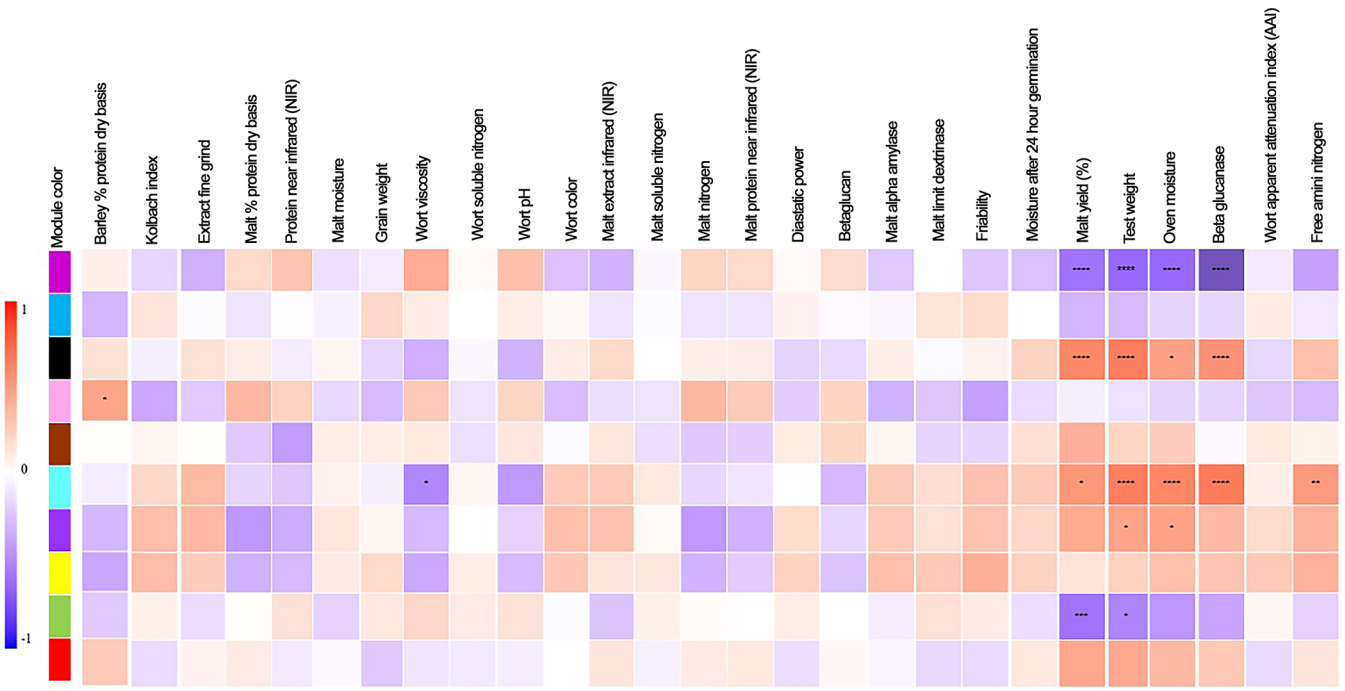

Figure 2. Module−trait relationship between malting specifications and barley proteome dataset. The left color panel shows the 10 modules, and the orange−purple color scale shows the module-malting specifications using the Pearson correlation method to link modules to malting traits with the correlation ranging from 1 to −1. Each row corresponds to a module eigengene and is named after a color, while each column corresponds to a malting trait. The color of each cell represents the Pearson correlation coefficient between rows and columns reflected. *P* values obtained from a univariate regression model between the module eigengene (PC1 of relevant protein measurements) and malting traits are shown by asterisks ****$p < 0.00001$, ***$p < 0.0001$, **$p < 0.001$, and *$p < 0.05$.

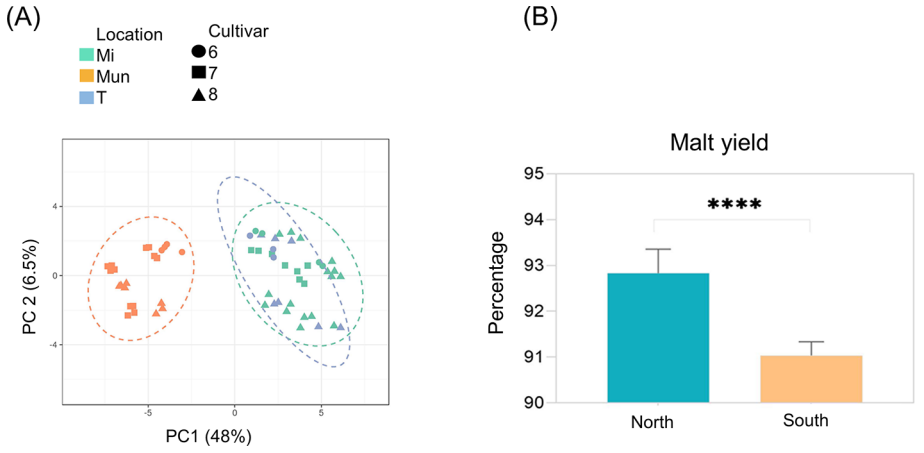

Figure 3. Malt yield-related protein abundance stratifying barley lines by the growing location. (A) PCA plot shows the separation of samples according to growing locations using only proteins related to the malt yield. Each shape represents one barley line, and colors refer to locations. (B) The malt yield is different between the northern and the southern growing locations. ***$p < 0.0001$ as analyzed by unpaired t-test. Error bars show 95% confidence intervals.

Relationship between Malting Specifications and Proteome Correlation Network Modules. WGCNA was performed to measure the relationships between the 27 malting specifications (Tables 2 and 3) and the modular structure within the proteome correlation network. The WGCNA analysis revealed the presence of 19 significant correlations between module eigengenes and malting specification measurements (Figure 2).

Analysis of the module−trait relationship reveals the presence of several significant associations: the proteins categorized into the modules black, turquoise, and purple were significantly positively associated with the number of malt traits such as the malt yield and *β*-glucanase as well as others including the test weight, oven moisture, and free amino nitrogen (FAN) (Figure 2 and Table S3). Proteins categorized into these modules were more abundant in samples with a higher malt yield. Similarly, proteins in the modules magenta and green were significantly negatively associated with these same traits, indicating that these proteins are less abundant in samples with a higher malt yield. The malt yield is defined as the weight of the obtained final dehydrated malt divided by the weight of applied barley seed reported as the percentage loss of

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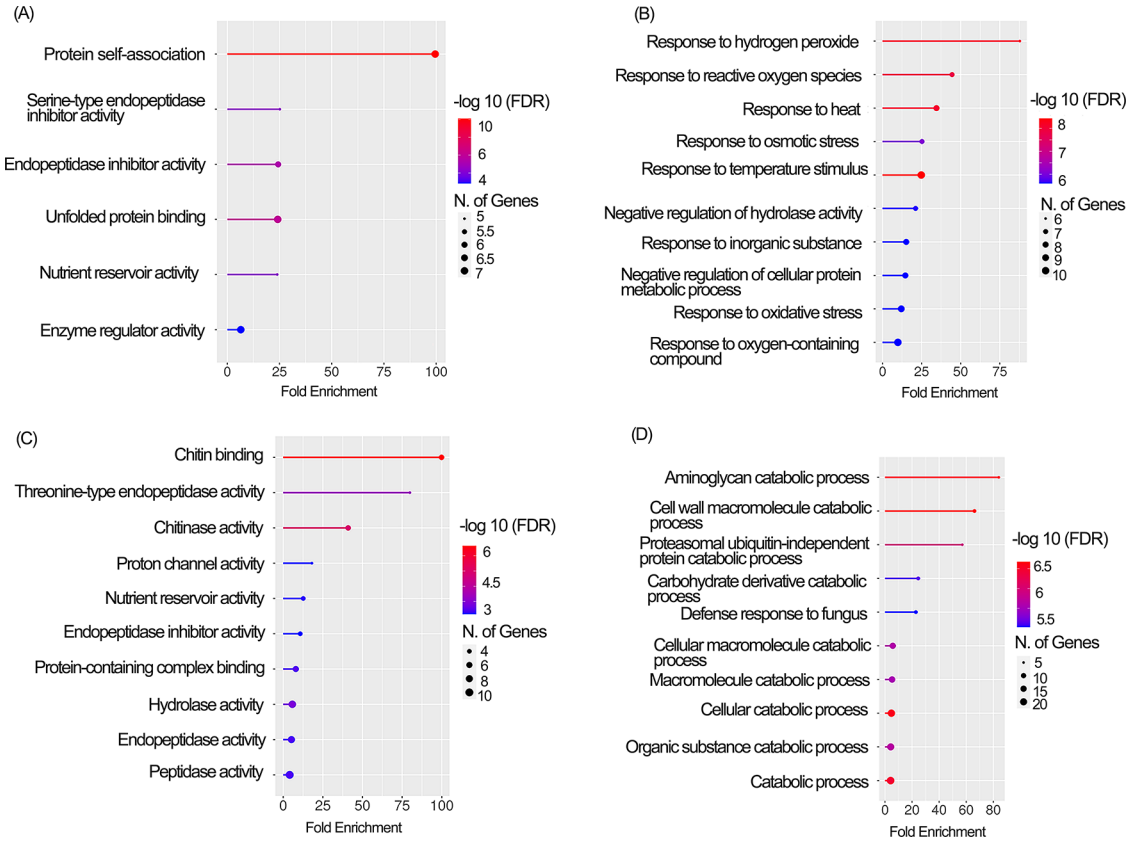

Figure 4. Gene ontology enrichment analysis for genes of proteins that positively and negatively impact the barley malt yield. (A) Molecular functions of proteins positively related to the malt yield. (B) Biological process of proteins positively related to the malt yield. (C) Molecular functions negatively related to the malt yield. (D) Biological processes of proteins negatively related to the malt yield. Color scales indicate the FDR-corrected p value (<0.05) for each term, and fold enrichments are defined as the percentage of genes related to proteins belonging to a term divided by the corresponding percentage in the background genes (*H. vulgare* L.).

grain mass during germination in the malting procedure.[35] The malt yield was one of the traits that was strongly associated and showed similar directions of trend. As such, this trait is relevant to the malting performance of barley seed. This trait was positively correlated with black and turquoise modules (p-value of <0.001) and negatively correlated with magenta and green modules (p value of <0.00001) (Figure 2). The correlation of protein profiles in each module that positively and negatively influence the malt yield trait was undertaken. Overall, 203 proteins were associated with the malt yield (Table S2); 82 were positively associated, and 121 proteins were negatively associated. PCA analysis of these 203 proteins (Figure 3A) shows the southern Mun location clustering separately to Mi and T and the three genotypes showing less clustering, similar to Figure 1A. In Figure 3A, PC1 explains 48% of the separation of samples, and the same proteins tend to dominate PC1 and PC2 in Figure 1. The comparative analysis for malt yields between the two regions (Figure 3B) showed significant differences with samples grown in the northern regions producing a higher malt yield (p value of <0.05) (Figure 3B).

Of the 203 proteins that are associated with the malt yield, there are several protein groups, including protein inhibitors, enzymes such as chitinases, β-amylase, peroxidase, carboxylase, and hydrolases, and folding and unfolding-related proteins. The most significant protein functions are shown in the GO analysis (Figure 4). The molecular function GO terms of proteins positively associated with the malt yield were related to protein self-association, unfolded protein binding, endopeptidase and peptidase inhibitor and regulator activities, enzyme inhibitor activity, and nutrient reservoir activity (Figure 4A). Analysis of GO terms (biological process) revealed the molecular processes related to the response to hydrogen peroxide, negative regulation hydrolase activity, response to heat stress, and response to reactive oxygen species (Figure 4B).

GO enrichment analysis of proteins that negatively impacted the malt yield showed that they were endowed with molecular functions such as chitinase, threonine-type endopeptidase, and peptidase activities (Figure 4C) and biological processes including a protein catabolic process, defense response to biotic stress (fungus) and chitin metabolic process (Figure 4D).

To understand the individual protein abundance perturbation related to growing locations, the top three proteins of each positive and negative protein group were selected according to their VIP score (Figure S2). Of note, the two proteins that are positively associated with the malt yield were related to heat and oxidative stress; as shown by GO analysis, these proteins were serpin (serine protein inhibitor) domain-containing proteins: HSP (heat shock protein) 17 and peroxidase. The effect of the location (north vs south) on these proteins was assessed using a Student's t-test. A significant difference in protein abundance between the two locations was noted with northern regions expressing a higher abundance of proteins influencing the malt yield. It can be concluded that growing the

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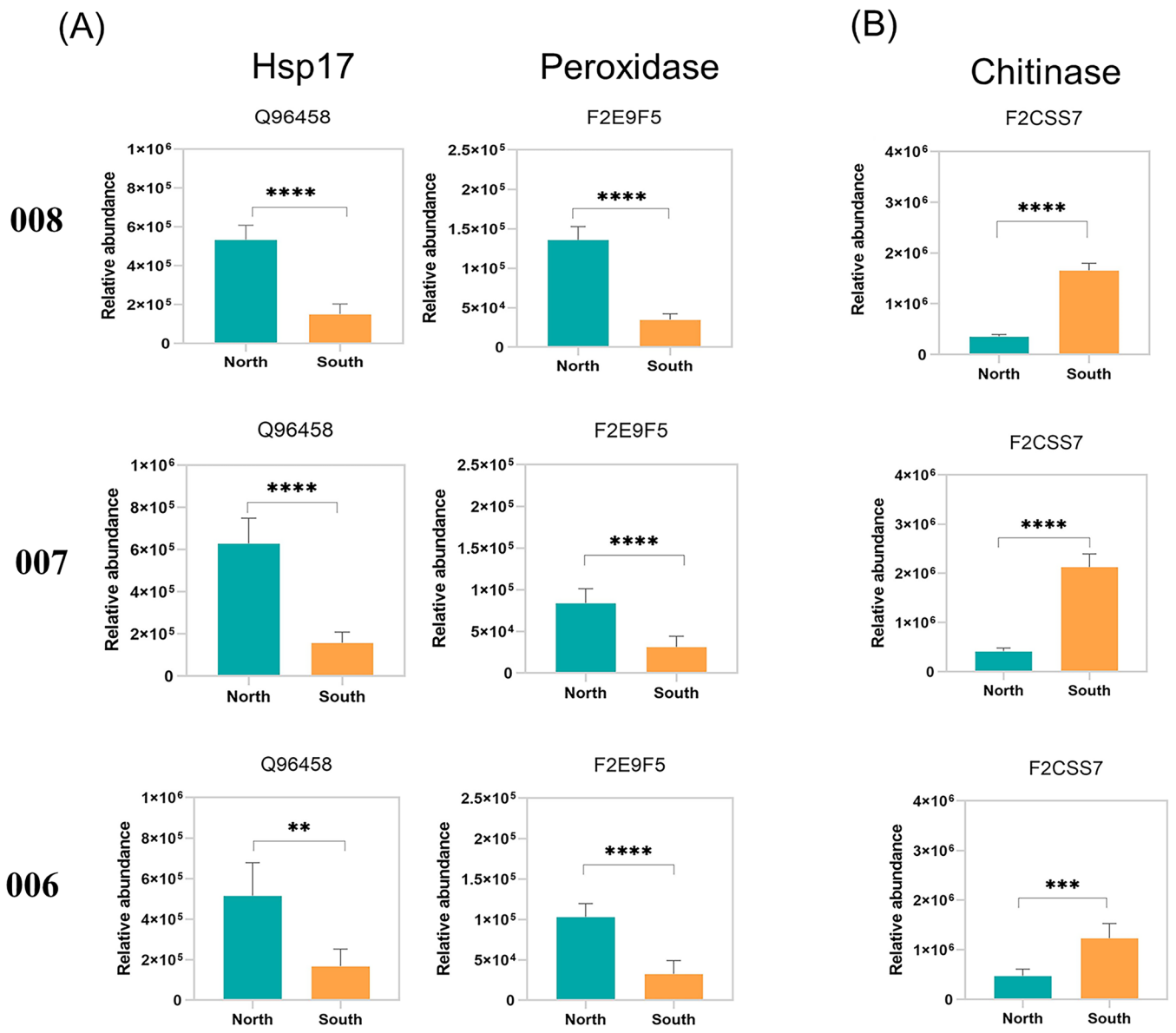

Figure 5. Proteins correlated with the malt yield are perturbed between locations. (A) Positively and (B) negatively correlated proteins are consistently perturbed across the three barley lines between northern and southern growth conditions. ***$p < 0.0001$, **$p < 0.001$, and *$p < 0.05$ as analyzed by an unpaired t-test. Error bars show 95% confidence intervals.

same lines in different locations impacted protein expression with environmental differences contributing to protein changes (Figure 5A). Chitinase showed a significant difference between the two growing regions (higher in southern regions) (Figure 5B).

To assess the impact of temperature fluctuations during the growing season, the average monthly temperature was plotted. The weather pattern shows higher temperatures for the northern region compared to the southern region during the growing season between May and November in 2019 (Figure S4).

To better understand the relationship between the protein relative abundance, malt yield, and temperature, a 3D scatter plot was created (Figure 6), which shows the relative abundance of the top three proteins that were correlated with the malt yield across the accumulated temperature during the growing season in 2019. The higher temperature in the northern region averaged for both locations together resulted in a higher malt yield and higher abundance of proteins that positively influence the malt yield (Figure 6A,B). While for the top protein negatively associated with the malt yield, there was a higher abundance of the protein in the southern region where the temperature was lower (Figure 6C).

DISCUSSION

This study explores the proteome phenotypes of three barley lines grown across different environments to delineate and discover proteome-malting specifications relationships. Herein, we assess the genetic and environmental influence on proteome phenotypes, identify sets of malt yield-related proteins and their functional themes, highlight individual proteins with a strong association to the malt yield, and uncover an axis of the proteome phenotype, malt yield, and environment (Figure 6).

By analyzing three different barley lines, proteome phenotypes were measured across environments and geno-

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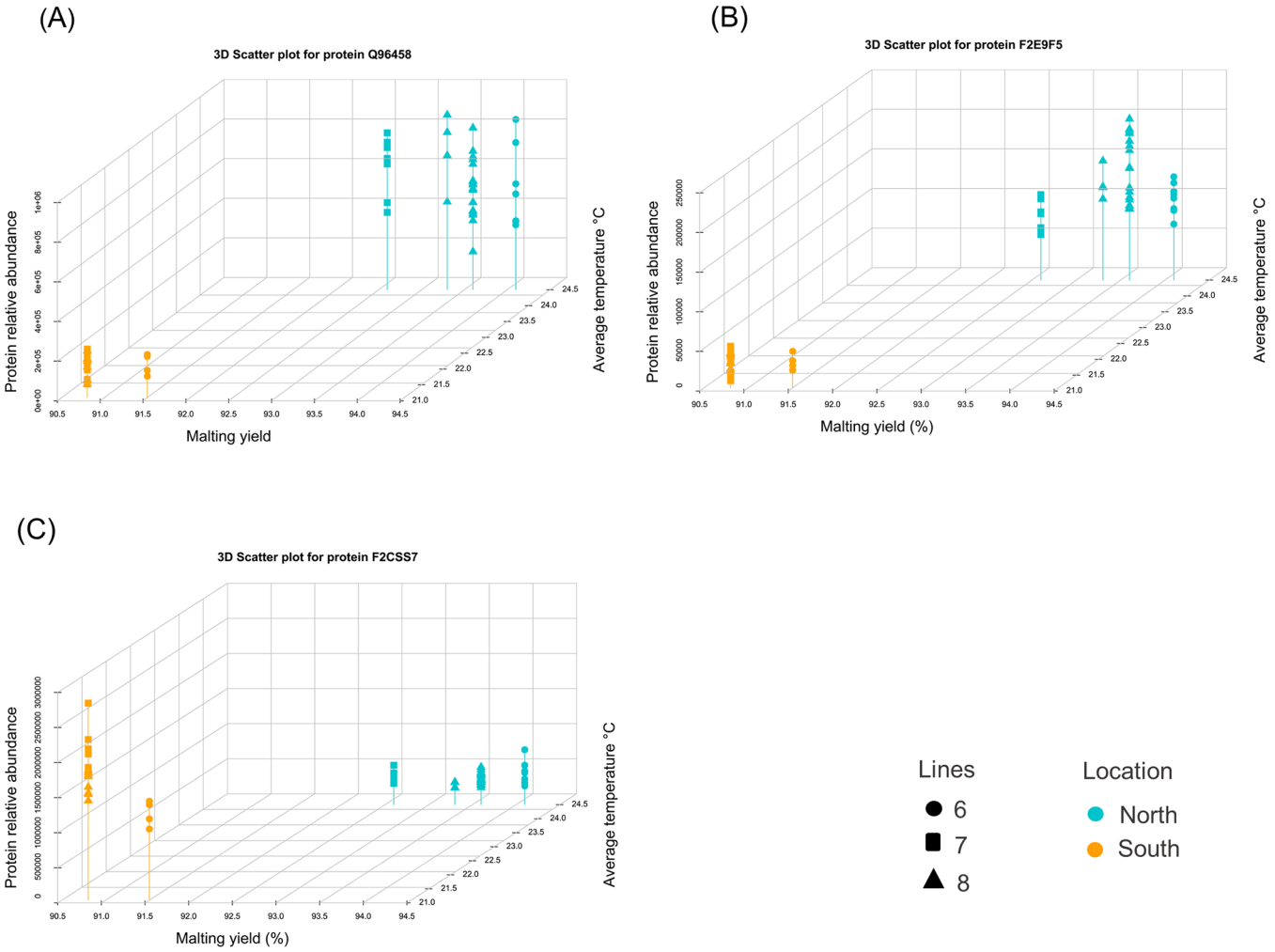

Figure 6. Relationship between the protein relative abundance, malt yield, and temperature. (A, B) Top two proteins that positively correlate with the malt yield (Q96458 and F2E9F5) and (C) top protein impacting the malt yield negatively (F2CSS7). Shapes represent lines, and colors show the location.

types. Multivariate and HCA analyses showed that the growing location is the stronger factor affecting the proteome composition of three experimental barley genotypes. As noted, the samples are grouped according to their growing locations, that is, northern and southern regions (Figure 1). Our results were aligned with a previous study that investigated the effect of cultivar and the environment on wheat proteins' quality where environmental factors influenced the wheat storage protein quality more than the genetic background.[36] In addition, the influence of cultivar and the environment on the quality of different Latin American wheat genotypes was studied. This study reported that the important portion of variability observed within detected proteins related to the wheat quality was influenced by the environment; however, the precise environment parameter that caused a positive or negative impact on the quality was not reported.[37] In our study, the relationship between malting traits and proteomic data was established using weighted correlation network analysis (Figure 2), and this investigation found a network structure comprising 10 modules of correlating proteins. Upon assessment of module−trait relationships, 19 significant correlations were identified.

Significant correlations were found for the malt yield, test weight, free amino nitrogen, and β-glucanase with a set of shared proteins correlating with these malting traits. Here, we focused on the malt yield trait as it is the most relevant trait to barley germination and the malting process among all significant correlations. Two modules (black and turquoise labeled) were found to have a positive correlation with the malt yield; two modules (magenta and green labeled) showed a negative correlation with this trait. Furthermore, proteins in each module that were positively and negatively correlated with the malt yield were identified and stratification of proteins by growing location was observed (Figure 3). Although numerous studies have investigated the effect of environmental factors such as fertilizer input (mainly nitrogen) or genetics on the malt yield,[38−40] no studies have linked proteome measurements with the malting traits. The malt yield mainly is the result of endosperm starch mobilization to provide the mass of the growing embryo and biochemical energy.[41] Additionally, it has been shown that environmental variables including the level of nitrogen fertilizer input, water availability, and the cultivar-specific genetic background all significantly impact the malt yield.[38]

In the present study, GO enrichment analysis revealed that proteins that are positively correlated with the malt yield trait have a molecular function including protein self-association, endopeptidase inhibitor activity, enzyme regulator activity,

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J. Agric. Food Chem. 2022, 70, 10680−10691

unfolded protein binding, and nutrient reservoir activity (Figure 4A). These proteins are involved in responses to a temperature stimulus, heat stress, hydrogen peroxide, and reactive oxygen species (Figure 4B). A list of proteins that showed a positive correlation with the malting yield includes HSPs, peroxidases, serpin domain-containing proteins, putative ripening proteins, starch synthase enzymes, and β-amylase (Table S2). Among the proteins that are positively correlated with the malt yield, the top three proteins were selected according to their stronger correlation with this trait. These were serpin domain-containing proteins HSP17 and peroxidase (Figure S2A). HSPs act as molecular chaperones to facilitate protein folding processes and protecting proteins that have been misfolded or lost their conformation due to biotic or abiotic stresses.[42] These proteins are also involved in protection of enzymes from degradation during malting, and associations with specific malting traits have been reported previously.[43,44] The HSPs are induced in locations with higher temperature conditions, suggesting that their abundance might help protect plants from heat stress events.[42] The study of the impacts of high-temperature stress on wheat and *Arabidopsis* has revealed that heat stress during early stages of seed development led to the expression of HSPs before constitutive accumulation at advanced stages of seed maturation when it undergoes the desiccation phase.[45] In addition to HSPs, we also identified peroxidases, an enzyme subclass that utilizes hydrogen peroxide to oxidize compounds in all cells to avoid plant cell injury under environmental stress.[46] These proteins are correlated with a higher malt yield in which these proteins were upregulated in samples grown in northern locations (T and Mi). A proteomics-based study has revealed that these enzymes are involved in barley germination, and results showed that different isozymes of peroxidase appeared in different stages of the barley seed germination.[47] Peroxidases are vital to seed germination as they can neutralize reactive oxygen species (ROS), which have been induced by abiotic stresses, and protect seeds from the subsequent peroxidation damage.[48] Serpin domain-containing proteins possess a conserved reactive center loop (RCL) domain that is the shared domain among all serpins. Abiotic stresses can cause cell death via vacuolar collapse by the involvement of a serpin and protease interaction, for instance, in Arabidopsis, overexpression of serpin1 caused lower sensitivity to water stress compared to the wild type.[49] A recent study also showed that the serpin domain-containing protein in hull-less barley seed has been expressed through different stages of development.[50]

Barley is an important cereal that is adapted to environments with an optimum temperature of 15 °C during grain filling; however, in the Australian grain belt, barley is exposed to high temperature (days above 30 °C).[51] The enrichment of high temperature-related proteins in the present study (Figure 4B) and consideration of temperature data (Figure 6) indicated that locations with higher temperatures during grain seed filling increases the abundance of defense-related proteins peroxidase, HSP17, and serpin domain-containing proteins (Figure S2A).

A higher abundance of defense-related proteins suggests that these proteins may induce tolerance or resistance during the temperature-dependent malting process during the germination step when the temperature reaches up to 22 °C or above. Through the analysis of meteorological data and considering the accumulated temperature (Figure 6A,B), it was observed that the northern region samples that revealed a higher abundance of defense proteins were, in fact, less impacted by the temperature changes during malting and less (or slower) germination occurred compared to the samples from the southern region (Figure 6B). This result suggests that a lesser degree of germination and consequently less production of root and shoot coupled with a lower weight loss due to germination resulted in a higher final malt weight. Revealing the higher abundance of the three aforementioned proteins in grain grown in the higher-temperature environments (Figure S4 and Figure 6A,B) further strengthens our hypothesis in that temperature stress occurrence in northern locations induced tolerance to the temperature-dependent germination process during malting.

The top three proteins that are negatively correlated with the malt yield are related to pathogen defense mechanisms including chitinases and germin-like proteins. These proteins play roles in cell wall function and defense against invading pathogens.[52] Chitinases belong to pathogenesis-related proteins and cleave the glycoside bond of chitin by a hydrolytic cleavage. Pathogenesis-related proteins such as chitinase were previously found as differentially expressed proteins in different growing locations of malt barley lines to protect grains during germination against pathogen attacks. It has been suggested that this difference might be related to the rain and humidity of the growing environment.[53] Plant endochitinases have antifungal properties, and a potential inhibitory effect against fungal pathogens was previously reported in barley.[54] In Arabidopsis, abiotic stresses, particularly heat stress, brought about downregulation of most chitinase genes.[55] Germin-like proteins are also involved in responses to pathogen and abiotic stresses in plants; in a study on the multigene family-encoding germin-like proteins of barley, it has been found that a pathogen attack or hydrogen peroxide are strong signals for germin-like protein subfamilies.[56] Research on the tea plant (*Camellia sinensis*) also showed that germin-like proteins showed downregulation in response to rising temperature.[57] In the present research, it was observed that chitinases have a higher abundance in samples that were grown in the southern location and can influence the malt yield negatively (Figure 6C). In accordance with the previous findings, the results from the present study indicate that the upregulation of mentioned proteins (chitinases and germin-like proteins) in the southern region may not be related to temperature stress. Further investigation would be helpful to understand the impact of environmental changes on barley grain that causes a lower malt yield during the malting procedure.

Our study demonstrated that SWATH-MS can be a powerful tool for exploring the impact of the environment on the proteome of malting barley. Our results indicate that location represented a major factor impacting proteome compositional changes of each barley line. Using WGCNA analysis, we established a relationship between malting traits and proteomic data, and we observed that the malt yield was significantly correlated by changes in the quantitative proteome composition and identified proteins with positive or negative associations to the malt yield.

GO enrichment analysis suggested that the occurrence of probable abiotic stress such as high-temperature stress influenced samples that were grown in locations with a higher average temperature. These samples were found to be more tolerant to temperature changes during the malting procedure, resulting in less germination, which thus resulted in a higher malt yield. Although the limitation to access to more

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physiological and phenotype data represents a challenge to interpreting obtained results, the integration of meteorological datasets and physiological observations coupled with obtained proteomic results could be informative to understand the impact of changes on the barley yield and malt specifications. Results of this study indicate that the applied proteomics pipeline can be used for future crop improvement studies especially in barley malt research as uniformity of barley seed malting traits can be very beneficial from a malting perspective. Moreover, we identified candidate proteins as potential markers of the malt yield that may find utility in maltsters in meeting different brewing requirements. This investigation has delineated a protein−malting specification−environment axis. The measurement of proteins related to the malting quality can readily support breeding or grain testing programs in reaching a more consistent seed and product quality.

■ ASSOCIATED CONTENT

Data Availability Statement

The datasets presented in this study can be found at: https://massive.ucsd.edu/ as dataset MSV000090084 [https://doi.org/doi:10.25345/C5M03Z216] [dataset license: CC0 1.0 Universal (CC0 1.0)].

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.2c03816.

oPLS-DA score, OPLS, and S-plots of proteins for three growing locations, major proteins altered between location-correlated malt yields, biplot demonstrating malting specification data, average monthly temperature recordings from the three growing locations in 2019, list of proteins causing separation between two regions with the VIP > 1, proteins that positively and negatively influence the malt yield trait, and a module−trait association table demonstrating correlation data (PDF)

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■ ABBREVIATIONS

AEGIC, Australian export grain innovation Centre; MS, mass spectrometry; DDA, data-dependent acquisition; DIA, data independent acquisition; T, Toodyay; Mi, Mingenew; Mun, Munglinup; DTT, dithiothreitol; SWATH-MS, sequential window acquisition of all theoretical mass spectra; PCA, principal component analysis; HCA, hierarchical cluster analysis; XIC, extracted ion chromatogram; WGNCA, weighted gene co-expression network analysis; GO, gene ontology; FDR, false discovery rate; oPLS-DA, orthogonal projections to latent-structure discriminant analysis; VIP, variable importance for the projection; HSP, heat shock protein

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