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Mass spectrometry-based proteomic analysis to characterise barley breeding lines

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Abstract

Barley is a key ingredient in the malting and brewing industry, and it is the fourth most important crop being cultivated worldwide. The protein content of the barley grain is one of the main components determining the quality and nutritive value of the food and beverages prepared from barley. Mass spectrometry-based proteomic analysis is a valuable tool that can guide and inform plant breeding strategies and crop improvement programs. Understanding the proteome changes in barley grain under different growing locations, the impact of different environmental conditions and its relationship with malting characteristics have the potential to inform breeding programs to achieve high-quality malt. Moreover, hordeins, the major barley storage proteins, are among the known triggers of coeliac disease (CD). Therefore, investigating the changes in the overall grain proteome, especially hordeins provides valuable insight from a food safety perspective.

This thesis focuses on the proteomic investigation of barley grain to understand differences due to genetic and environmental factors and how these differences impact end use application after food processing steps such as malting. In Chapter 2 of this thesis, the proteome and malting characteristics of three different barley genotypes grown in three different locations in Western Australia were measured by applying a bottom-up proteomics workflow. First, using discovery proteomics, 1,571 proteins were detected and in the next step, by applying a global proteome quantitation workflow, 920 proteins were quantified in barley samples. Data analysis revealed that growing location outweighed the impact of genetic background, and samples were clustered into two major groupings of northern and southern growing locations. Also, a relationship between proteome measurements and malting characteristics using weighted gene co-expression network analysis (WGCNA) were investigated. The statistical analysis showed that both the genotypes and the growing locations strongly correlate with changes in the proteomes and desirable traits such as malt yield. Finally, linking meteorological data with proteomic measurements revealed how high-temperature stress in northern regions affects the seed temperature tolerance during malting, resulting in a higher malt yield.

In Chapter 3, a targeted proteomics approach was used to investigate the changes of hordein peptides after malting in grain samples of previously developed hordein-reduced barley lines, including a triple-hordein-reduced ultra-low gluten (ULG) barley line and their corresponding malt samples. Peptides representing hordein-like proteins, including B-, D- and γ-hordeins and avenin-like proteins (ALPs), were tracked using relative quantitation across single-, double-, and triple-hordein-reduced barley grain and malt samples. Further analysis showed that malting further reduced the quantity of

B-, D- and γ-hordeins and ALPs in the ULG malt sample compared to the unmalted grain. Moreover, the detection and quantitation of globulin proteins in the experimental samples indicated a compensation mechanism of storage proteins leading to the biosynthesis of seed storage globulins (vicilin-like globulins) in the ULG-line derived grain and malt sample compared to the wild type. Taken together, these results suggest that the compensation effect enables the hordein-reduced ULG line to maintain the balance of overall N-rich reservoir accumulation.

In Chapter 4, the impact of malting of barley grain was investigated by unbiased proteome comparison of the grain and malt. Using discovery proteomics, 2,688 proteins were detected in the barley grain and 3,034 proteins in the malt samples of which 807 proteins were unique to malt samples. Next, Gene Ontology (GO) enrichment analysis was performed on the unique proteins and revealed that "hydrolysing activity" was the most significant GO term enriched in malt over barley. By conducting quantitative proteomics using SWATH-MS, 2,654 proteins were quantified in the barley grain and malt samples. Based on their proteome level quantitation, the unsupervised clustering analysis showed two distinct clusters representing grain flour and malt samples. Moreover, a relationship between hordein-reduced backgrounds and proteome data was established. The results showed that the inclusion of C-hordein-reduced lines significantly impacted the proteome level changes in the grain and malt samples, more so than the inclusion of the B- and D-hordein-reduced lines. Furthermore, univariate analyses were performed to identify the differentially abundant proteins in each hordein-reduced background by comparing barley grain to malt samples. Finally, GO enrichment analysis was performed on the up- and down-regulated proteins detected from the pair-wise comparisons. GO enrichment analyses revealed that the up-regulated proteins in C-hordein-reduced lines were primarily involved in the small molecule metabolic process and provided more energy during malting to facilitate seed germination.

Advancements in mass spectrometry-based proteomics approaches and cutting-edge bioinformatics tools have revolutionised protein detection and quantitation from model and non-model species, enabling us to obtain unprecedented views on changes in the barley grain proteomes at the molecular level. The results generated from this PhD project have further illustrated the underlying complex regulatory mechanisms controlling storage protein accumulation upon malting in barley grains. The approaches used and the insights gleaned have the potential to accelerate the development of new varieties with desired traits of interest. Specifically, the foundational knowledge and workflow developed from this thesis can be applied in the selection of unique germplasm by barley breeders for barley food and beverage applications.

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Declaration

Declaration: I certify that this thesis does not, to the best of my knowledge and belief:

(i) incorporate without acknowledgement any material previously submitted for a degree or diploma in any institution of higher education.

(ii) contain any material previously published or written by another person except where due reference is made in the text; or

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Publications and conference presentations arising from this thesis

Published manuscripts

1. Bahmani M., Juhász A., Bose U., Broadbent J. A., Nye-Wood M., Edwards I. B., and Colgrave M. L. (2022). Proteome phenotypes discriminate growing location and malting traits in field-grown barley. *Journal of Agricultural and Food Chemistry, 70*(34), 10680-10691.

2. Bose U., Juhász A., Yu R., Bahmani M., Byrne K., Blundell M., Broadbent J. A., Howitt C. A., and Colgrave M. L. (2020). Proteome and nutritional shifts observed in coeliac-friendly hordein double-null barley lines. *Frontiers in Plant Science, 12*, 718504.

3. Bahmani M., O'Lone C. E., Juhász A., Nye-Wood M., Dunn H., Edwards I. B., and Colgrave M. L. (2021). Application of Mass Spectrometry-Based Proteomics to Barley Research. *Journal of Agricultural and Food Chemistry, 69*(31), 8591-8609.

4. Bahmani, M., Juhász, A., Bose, U., Nye-Wood, M. G., Blundell, M., Howitt, C. A., & Colgrave, M. L. (2024). From grain to malt: Tracking changes of ultra-low-gluten barley storage proteins after malting. *Food Chemistry, 432*, 137189.

5. Bahmani M., Juhász A., Bose U., Nye-Wood M., Blundell M. J., Howitt C. A., and Colgrave M. L. (2023). Proteome changes resulting from malting in hordein-reduced barley lines. *Journal of Agricultural and Food Chemistry, 71*(38), 14079–14091.

Conference presentations

1. Bahmani M., Juhász A., Bose U., Nye-Wood M., Blundell M. J., Howitt C. A., and Colgrave M. L. (2023, May 8-10). Protein quantitation from hordein-reduced barley and malt types. Joint 11th Asia Oceania Human Proteomics Oraganization (AOHUPO) and 7th Asia Oceania Agricultural Proteomics Organization (AOAPO) Congress, Singapore. (Oral presentation)

2. Bahmani M., Juhász A., Bose U., Nye-Wood M., Blundell M. J., Howitt C. A., and Colgrave M. L. (2023, March 21-23). *Protein quantitation from hordein-reduced barley and malt*. 37th Asia Pacific IBD Convention, Adelaide. (Poster presentation)

3. Bahmani M., Juhász A., Bose U., Nye-Wood M., Blundell M. J., Howitt C. A., and Colgrave M. L. (2022, November 24-25). *Protein quantitation from hordein-reduced barley and malt*. 1st Young

Food Proteomics Scientists Conference. Institute of Applied Biosciences, Department of Bioactive and Functional Food Chemistry Karlsruhe, Germany. (Oral presentation)

4. Bahmani M., Juhász A., Bose U., Nye-Wood M., Blundell M. J., Howitt C. A., and Colgrave M. L. (2022, August 22-25). *Protein identifications from hordein-reduced barley and malt*. 20th Australian Barley Technical Symposium. Gold Coast, Queensland, Australia. (Three minutes oral and poster presentation- award winning- Best three-minutes oral presentation)

5. Bahmani M., Juhász A., Broadbent A. J., Nye-Wood M., Dunn H., Edwards I. E., and Colgrave M. L. (2022, February 3-6). *Proteomics analysis of environment effect on malting barley near isogenic lines*. 27th Annual Lorne Proteomics Symposium, Lorne, Australia. (Oral presentation- award winning- APS Student oral presentation)

6. Bahmani M., Juhász A., Broadbent A. J., Nye-Wood M., Dunn H., Edwards I. E., and Colgrave M. L. (2021, November 21-25). *Investigating the effect of environment on barley near isogenic lines by mass spectrometry-based proteomics*. The Australian and New Zealand Society for Mass Spectrometry (ANZSMS). 28th biennial Conference, Canberra, Australia. (Poster presentation- award winning-Runner-up poster presentation)

7. Bahmani M., Juhász A., Nye-Wood M., Dunn H., Edwards I. E., and Colgrave M. L. (2021, February 4-5). *Effect of gas phase fractionation on protein identification rates in discovery proteomics of barley lines*. 26th Lorne Proteomics Symposium, Lorne, Australia. (Poster presentation)

8. Bahmani M., Juhász A., Nye-Wood M., Dunn H., Edwards I. E., and Colgrave M. L. (2020, October 30). *Effect of gas phase fractionation on discovery proteomics of barley lines*. Annual Post Graduate Symposium, School of Science, ECU, WA, Australia. (Oral presentation)

1. Chapter 1: Application of mass spectrometry-based proteomics to barley research

The first chapter of this thesis focuses on a thorough literature review to reveal the methods applied in proteomics research, specifically in barley studies The aim was to develop a detailed understanding of the research area and identify gaps in barley proteomic research.

The literature review yielded comprehensive insights in the latest advancements in the rapidly evolving proteomics field, and the gathered information was contributed as a review paper for the agricultural proteomics community in the *Journal of Food and Agriculture Chemistry* in 2021, including the following sections of Chapter 1, sections 1.1 to 1.8 of the thesis. This chapter serves as a general introduction to the application of MS-based proteomics in barley research.

1.1. Publication and Statement of Contributions

This chapter is drawn from the following publication:
Bahmani, M., O'Lone, C. E., Juhasz, A., Nye-Wood, M., Dunn, H., Edwards, I. B., & Colgrave, M. L. (2021). Application of mass spectrometry-based proteomics to barley research. *Journal of Agricultural and Food Chemistry*, *69*(31), 8591-8609.

Mahya Bahmani (candidate)
Reviewed the literature, processed gathered information, conducted visualisation, interpretation, and drafted the manuscript.

Signat 

Date: 29/03/2023

Clare O'Lone

Conducted literature search, drafted some sections of the manuscript and reviewed the manuscript.

Signa 

Date: 03/04/2023

Angéla Juhász

Provided guidance for manuscript structure and reviewed the manuscript.

Signatu 

Date: 30/03/2023

Mitchell G. Nye-Wood

Reviewed the manuscript.

Signatu 

Date: 03/04/2023

Hugh Dunn

Reviewed the manuscript.

Signature [redacted]

Date: 25/05/2023

Ian B. Edwards

Reviewed the manuscript.

Signature: Unfortunately, Dr. Edwards Passed away and therefore, we are unable to obtain his signature.

Michelle L. Colgrave

Conceived the outline of the study. Provided guidance and reviewed the manuscript.

Signature [redacted]

Date: 30/03/2023

1.2. Abstract:

Barley (Hordeum vulgare) is the fourth most cultivated crop in the world in terms of production volume and it is also the most important raw material of the malting and brewing industries. Barley belongs to the grass (Poaceae) family and plays an important role in food security and food safety for both humans and livestock. With the global population set to reach 9.7 billion by 2050, but with less available and/or suitable land for agriculture, the use of biotechnology tools in breeding programs are of considerable importance in the quest to meet the growing food gap. Proteomics as a member of "omics" technologies has become popular for the investigation of proteins in cereal crops and particularly barley and its related products such as malt and beer. This technology has been applied to study how proteins in barley respond to adverse environmental conditions including abiotic and/or biotic stresses, are impacted during food processing including malting and brewing and to study the presence of proteins implicated in CD. Moreover, proteomics can be used into the future to inform breeding programs that aim to enhance the nutritional value and broaden the application of this crop in new food and beverage products. Mass spectrometry analysis is a valuable tool that, along with genomics and transcriptomics can inform plant breeding strategies that aim to produce superior barley varieties. In this review recent studies employing both qualitative and quantitative mass spectrometry approaches with a focus on their application in cultivation, manufacturing, processing, quality, and safety of barley and its related products.

1.3. Introduction

Hordeum vulgare (barley) is among one of the first domesticated cereal crops derived from its wild relative *Hordeum spontaneum* with domestication occurring approximately 11,000 years ago in the Fertile Crescent (Lister et al., 2018). Barley has become the fourth most important member of the cereal grain family with a total production of 158 million metric tons worldwide in 2019 (FAO – FAOSTAT, 2022). Barley belongs to the grass (Poaceae) family, and it plays an important role in food security and food safety for both humans and livestock. It has been malted for beer and whisky and also has been used as a food product due to its health benefits, such as lowering blood cholesterol (Wang et al., 2017), improving regulation of blood sugar (AbuMweis et al., 2016), and modulation of gut microbiota (Ghaffarzadegan et al., 2018; Gong et al., 2018). Barley grain is a rich source of carbohydrate (including dietary fiber), protein, E group vitamins such as tocopherols and tocotrienols, and B group vitamins such as thiamine, riboflavin and niacin (Idehen et al., 2017; Kellner, 2000; Petrovska-Avramenko et al., 2017). The global barley market is expected to grow

from 19.98 billion USD in 2017 to 25.18 billion USD in 2022 (TechNavio, 2018). Its application can be categorized in two main sectors: (1) food and beverages; and (2) other applications such as animal feed, cosmetic products, pharmaceuticals, biofuels, and nutraceuticals (Eckert et al., 2018; Lee et al., 2013; Qureshi et al., 2010; Tricase et al., 2018). It has been used in diets of several countries worldwide such as the Middle East, Russia, Poland, Tibet, Japan, North African countries, and India. It can be used as a food in meals such as soups, stews, casseroles, different kinds of pastas, noodles, and bakeries to produce flat bread and pastries (Baik & Ullrich, 2008; Newman & Newman, 2006). According to Roman texts barley has been used as a staple for gladiators and interestingly they were known as *hordearii* which means "barley men". Hordearii followed a particular diet that consisted of barley and beans to gain weight and also provide subcutaneous fat with the aim of having more protection during battle (Lösch et al., 2014). Different methods of processing barley for food products include pearling, malting, grinding or roller milling, flaking, and extruding (Baik & Ullrich, 2008) and the main products of barley after this processing include de-hulled barley, malt, barley flour, pearl barley, and pot barley (Tricase et al., 2018). Malting is a food processing procedure and this intricate biochemical transformation involves the controlled germination of grains, primarily barley, followed by the arrest of germination through kilning. The malting process activates enzymes, such as amylase and protease, within the grains, leading to the breakdown of complex carbohydrates and proteins into simpler sugars and amino acids (Tricase et al., 2018). This enzymatic activity is pivotal for subsequent processes like brewing, where sugars are fermented by yeast to produce alcohol. Additionally, malting contributes to the development of desirable flavours, colours, and aromas in malted grains, influencing the final characteristics of the end product. The precise regulation of temperature, humidity, and germination time during malting is essential for achieving the desired enzymatic activity and flavour profile. Consequently, malting plays a fundamental role in the overall quality and sensory attributes of malt-derived products, making it a key focus of research and optimization within the brewing and distilling industries (Rani & Bhardwaj, 2021).

Concerns about nutrition (health facts) and safety (origin, allergy) of food are increasingly affecting consumers' choices. Specific medical conditions or dietary preferences have led to free-from diets such as gluten-free and dairy-free, or vegetarian/vegan and more recently plant-based diets. Consumers are also searching for foods that contain healthy ingredients, often fortified with minerals, vitamins, or bioactive molecules. Plant-based proteins have received considerable attention in recent years and owing to soluble fiber such as β-glucan and high protein content barley attracted significant attention. In addition, barley proteins glutelin demonstrated oil-binding

capacities and considerable emulsifying stability, while barley hordein showed good foaming properties which provide opportunities for value-added food applications for barley protein (Baik & Ullrich, 2008; Eckert et al., 2018; Lösch et al., 2014; Newman & Newman, 2006; Wang et al., 2010). The impact of these proteins on the final product will be discussed later in this paper. The use of barley as a high-quality protein ingredient in confectionary products showed the potential to improve product flavour, texture, and storage stability (Baik & Ullrich, 2008; Eckert et al., 2018; Fox & Watson-Fox, 2021; Lösch et al., 2014; Newman & Newman, 2006; C. Wang et al., 2010). There is a high demand for foods that contain high quality protein or that are free-from specific proteins. This demand coupled with constraints on both land and resources requires further optimization of food systems, for instance by meeting crop yield potentials. To achieve this goal, sustainable agriculture needs to adopt modern biotechnological tools of which proteomics is one of the key technologies that can support crop breeding programs.

The proteome refers to the whole set of expressed proteins in a cell, tissue or organism at a specific time and condition and the term proteomics describes the global identification and characterization of the protein complement of a biological sample (Wilkins et al., 1996). The results obtained from proteomics can be beneficial in many different research fields. For instance, to provide information about detection of diagnostic markers and alteration of expression patterns in response to different signals to understand pathogenicity mechanisms (Anderson et al., 2000; Domon & Gallien, 2015; Mora et al., 2018). In agriculture and food studies, proteomics is applied for the identification and characterization of proteins and to elucidate their function and interactions. Functional analysis of proteins is often achieved by qualitative and quantitative measurements of plant tissues at specific developmental or physiological stages (Mock et al., 2018; Ortea et al., 2016; Ramalingam et al., 2015).

Quantitative mass spectrometry (MS) based proteomics can be used to provide information on the grain proteome, that is, all proteins expressed within the edible part of cereal crops. Genomics and transcriptomics studies focus on genes present in a genome and their expression, whilst proteomics defines the qualitative and quantitative composition of expressed proteins as well as how the expressed levels change under different environmental conditions. In addition to protein abundance, the presence of protein modifications can be informative from both understanding biological processes (*i.e.* signaling) or chemical alterations (*i.e.* food processing) (Andjelković & Josić, 2018; Chawade et al., 2019).

There have been several valuable reviews covering different aspects of crops and particularly in barley which the reader can be referred to for further information (Diaz-Mendoza et al., 2019; Finnie et al., 2011; Mock et al., 2018; Willows et al., 2017). In recent years, advances in MS-based tools and methods have resulted in increasing application of proteomics to barley employing a range of different techniques. This review aims to give an overview of barley proteomics studies and to highlight the application of mass spectrometry to different parts of the value chain from cultivation to processing and final product quality and safety. These research studies range from qualitative to quantitative approaches as described in the subsequent sections.

1.3.1. Major proteins in barley

Barley produces structurally diverse proteins that play fundamental roles in plant development, cellular renewal, nutrient uptakes and transport, and biotic and abiotic stress responses (Steinwand & Ronald, 2020). In mature barley grain, 8-15% of the total dry weight consists of protein; however, the level varies depending on the genetic background, environmental conditions and nitrogen availability (Gubatz & Shewry, 2010).

Although the Osborne solubility-based classification of barley proteins has been an enormous contribution to cereal protein studies, modern proteomics enables and requires more systematic categorization (Osborne, 1895). Therefore, barley proteins can be categorized by more practically classification according to their molecular functions: (1) storage proteins; (2) metabolic and structural proteins; and (3) protective proteins (Figure 1.1) (Shewry & Halford, 2002).

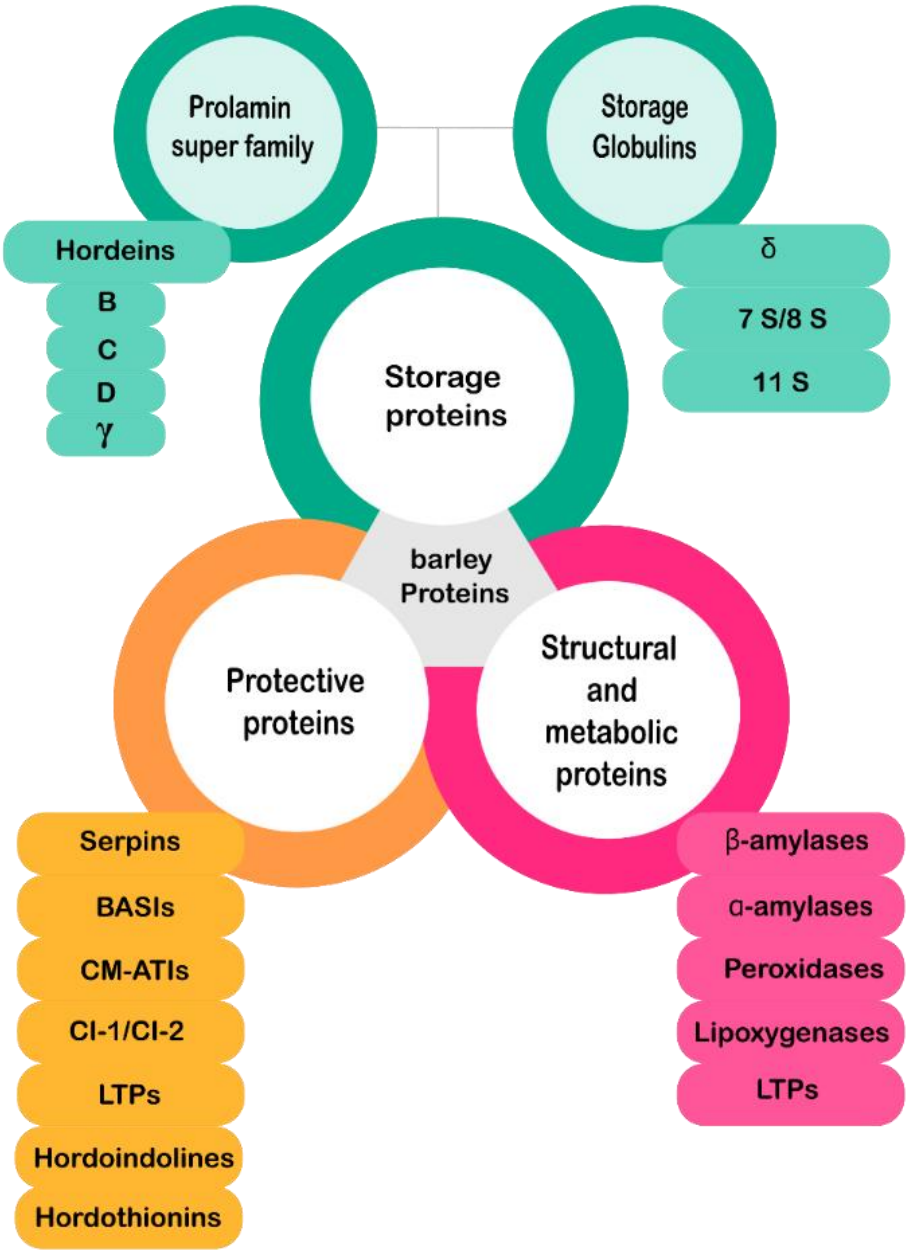


Figure 1.1. Barley protein classification (Gubatz & Shewry, 2010; Shewry & Halford, 2002). Abbreviations: BASI - Barley amyla/subtilisin inhibitor; CM-ATI - Chloroform methanol soluble alpha/amylase/trypsin inhibitors; CI - Chymotrypsin inhibitors; LTPs, Lipid transfer proteins; Serpins - serine protease inhibitors.

1.3.1.1. Storage proteins

Storage proteins provide energy and are considered as a source of nitrogen, sulfur, and carbon to fuel germination and the growth of the seedlings. Barley storage proteins include proteins both

from the prolamin superfamily and storage globulins. The grain prolamins are present in the tribe Triticeae including wheat (gliadins and glutenins), barley (hordein), rye (secalin), and other grains such as corn (zein), sorghum (kafirin), and oats (avenin). Prolamin proteins share a conserved pattern of cysteine residues, and they are classified into sulfur-rich, sulfur-poor, and high molecular weight prolamins (Juhász et al., 2018). Based on recent evidence, the avenin-like proteins in wheat, that are also present in barley and share amino acid sequence homology with hordeins, are also involved in stress responses including abiotic (drought) and biotic (Fusarium head blight) stresses (Zhang, 2018). The hordeins, are the dominant proteins in the endosperm and they comprise ~55% of total grain protein (Lexhaller et al., 2019; Schalk et al., 2017). They are rich in proline and glutamine residues, hence the term prolamin (Guerrieri & Cavaletto, 2018). Hordeins are classified into four subgroups according to their molecular weight, the D-hordeins with an approximate size of 105 kDa, the sulfur-poor C-hordeins of size 55-65 kDa, the B-hordeins of size ~50 kDa, and the sulfur-rich γ-hordeins of size 35-45 kDa (Baxter, 1981; Houde et al., 2018; Juhász et al., 2018; Tanner et al., 2019). The storage globulins of barley grain are present in the embryo, endosperm and aleurone layers. These proteins include 7-8S globulins which can be found both in aleurone layer and embryo and the 11-12S globulins which are found exclusively in the endosperm (Mahalingam, 2017). Seed storage proteins are produced at a specific stage of seed development in the endosperm (in cereals), they accumulate in organelles known as protein bodies, and fractions of storage proteins show polymorphism between genotypes (Izydorczyk & Edney, 2017; Shewry et al., 1995).

1.3.1.2. Metabolic and structural proteins

Metabolic and structural proteins are diverse and have different properties, and they may have other roles than metabolic or structural activities. In barley they include (1) enzymes such as β-amylases, α-amylases, peroxidases (Prx) and lipoxygenases (LOX); and (2) small sulfur-rich proteins such as non-specific lipid-transfer proteins (ns-LTPs) (Gubatz & Shewry, 2010). Amylases are hydrolytic enzymes that degrade starch, a major energy reserve of barley seed, during germination into sugars and oligosaccharides. Amylases are important to the malting and brewing process involved in fermentable sugar production during mashing (Henson et al., 2020). β-amylases are different in resting and germinated seeds. During grain development, β-amylases are synthesized, and a portion of the enzyme becomes insoluble during maturation and desiccation. The presence and abundance of β-amylases play a crucial role in the mashing process (Guerrieri & Cavaletto, 2018; Swanston & Molina-Cano, 2001).

Peroxidase enzymes of which barley seed-specific peroxidase 1 (BSSP1) and barley peroxidase 1 (BSP1) have been already identified (Rasmussen et al., 1997), oxidize a wide range of substrates in the barley during grain filling and germination. Lipoxygenases are involved in metabolic processes and catalyse the synthesis of xylipins, compounds derived from polyunsaturated fatty acids (Feussner & Wasternack, 2002). In barley they are present in three isoforms; (1) LOX-1 is present in quiescent grains; (2) LOX-2 is a germination-associated LOX isoform; and (3) LOX isoform expression has been detected only after germination and is similar to that of LOX-2, and it has several roles in brewing such as causing stale flavour in beer as a result of degradation and oxidation of polyunsaturated fatty acids during the malting and mashing processes (Holtman et al., 1996).

The barley seed proteome includes LTPs which have been implicated in several biological processes including developmental processes, metabolic and protective roles (Mikołajczak et al., 2016; Yeats & Rose, 2008).

1.3.1.3. Protective proteins

Seeds are a rich source of proteins and nutrients and as such are subject to different biotic stresses such as attack by pests and pathogens. Several grain proteins play protective roles, and their accumulation can be increased under these situations. In barley, the protective proteins include enzyme inhibitors like serine protease inhibitors (serpin), α-amylases, and trypsin inhibitors (ATI). ATIs are composed of three sub-groups of (1) chloroform-methanol soluble proteins (CM); (2) dimeric ATIs; and (3) monomeric ATIs. Additionally, barley amylase/subtilisin inhibitor (BASI), chymotrypsin inhibitors (CI) Cl-1/ Cl-2, LTPs, hordothionins, hordoindolines, and defensins also belong here (Gubatz & Shewry, 2010; Guerrieri & Cavaletto, 2018; Shewry & Tatham, 1990; Willows et al., 2017).

Like other cereals, barley contains protein inhibitors that can act against α-amylases and proteases from pathogens and pests. Serpins inhibit chymotrypsin-like enzymes from insects and pathogens, in developing barley grain two isoforms, BS24 and BS27 are expressed and suggested to be present in endosperm and aleurone (Jones, 2005; Shewry & Lucas, 1997). ATIs have roles in grain filling and maturation, and many can be selectively extracted by chloroform/methanol (CM) as such they are termed as CM-proteins (Barber et al., 1986; Bose, Juhász, et al., 2020). In barley CMa, CMb, CMd inhibit α-amylases, while CMc and CMe were observed to have inhibitor activity against trypsin (Barber et al., 1986; Bose, Juhász, et al., 2020). BASI is another protein that inhibits both

subtilisin and amylase-2 enzymes during premature sprouting, this inhibitor is a member of the Kunitz-type protein inhibitor family and is an abundant protein of the endosperm and the aleurone layers of the mature seed (Nielsen et al., 2004).

Chymotrypsin inhibitors of barley include CI-1 and CI-2, they lack Cys and hence disulphide bonds, and they belong to a family of proteins including the potato inhibitor I and the leech inhibitor elgin (Svendsen et al., 1982). Another abundant protein group in barley aleurone layers is the non-specific lipid transfer protein (ns-LTP) family. They are involved in the plant defence mechanisms to biotic and abiotic stresses and have protective roles in the assembly of extracellular hydrophobic polymers, these proteins survive during malting and brewing (Gubatz & Shewry, 2010). Hordoindolines are reported to be present in the mature barley endosperm as two isoforms of *a* and *b* proteins, with hordoindoline *b* being the major isoform in the mature barley endosperm which also survive in malting and mashing stages (Darlington et al., 2000). Hordothionins are cysteine-rich proteins and barley grain composed of two forms of thionins as α- and β-hordothionins, they inhibit the growth of pathogens such as fungi and bacteria and they survive during brewing process (Shewry & Lucas, 1997). Plant defensins are the final group of protective proteins, they appear to be among the most widespread antifungal peptides in plants, there are two types of defensins in barley termed γ- and ω-hordothionins which are sulphur-rich proteins, and they share homology to α- and β-hordothionins (Osborn & Broekaert, 1999).

1.3.2. Major proteins in malt

During the malting process, proteases play an essential role in the partial degradation of storage proteins to yield peptides and amino acids which are important contributors to wort and beer quality. Several studies have investigated the role of proteins and their modifications in the malting and brewing processes, and they indicated the role of proteins in the final beer foam, haze stability, and flavour (Colgrave et al., 2013; March et al., 2012a; Niu et al., 2018; Schulte et al., 2016; Schulz et al., 2018). During the malting process, enzymes such as α-amylases, β-amylases, limit dextrinases, α-glucosidases, β-glucanases and more than 40 proteases are present and carry out their functions (Fox et al., 2003; Gupta et al., 2010; Jones, 2005).

Upon commencement of germination, α-amylase expression is increased, and it acts to cleave α-(1-4)-glycosidic bonds in starch. This enzyme has three forms, α-amylase I, II, and III, and they appear at different stages of germination (Bamforth, 2009). The actions of β-amylase liberate maltose by cleaving non-reducing ends of amylose and amylopectin, which is the most abundant

sugar produced during mashing stage the resulting maltose serves as a source of energy for yeast during fermentation (Gous & Fox, 2017). This enzyme has three forms (Sd1, Sd2, and Sd3) and they vary in their thermostability, and as Swanston and Molina-Cano (2001) indicated this characteristic depends on the barley variety, noting that they also reported that for the studied genotypes, there was a small, but highly significant, effect of environment on the proportion of total beta-amylase that was water soluble (Swanston & Molina-Cano, 2001). Limit dextrinase (LD) is another enzyme that hydrolyses α-(1-6)-glycosidic bonds in amylopectin and branched dextrin, and it can be in three forms of active, inactive, or bound during germination (Gous & Fox, 2017). According to Huang et al. (2016), the activity of LD is increased during germination, and during mashing, it continues to convert dextrins into linear maltosachharides (Huang et al., 2016). Mashing temperature and pH are known to affect the activity of LD (Gous & Fox, 2017; Huang et al., 2016).

The enzyme, α-glucosidase is synthesized in the aleurone layer and embryo tissue during germination, and its activity increases in the presence of gibberellic acid (GA) as are other enzymes in barley grain. Alpha-glucosidase catalyses the α-glycosidic bonds in oligosaccharides and glucans to produce glucose (Sun et al., 2018). Its efficiency depends on critical parameters like the type of the substrate (oligosaccharides or starch polymers), temperature, and pH (Wenwen et al., 2019).

During germination, the enzyme β-glucanase catalyzes the hydrolysis of β-glucan. Studies have shown it has two isoenzymes EI and EII (Bamforth & Martin, 1983). The degradation of β-glucan, a major component of the barley cell wall, affects the malt quality. The enzyme β-glucanase and xylanase are considered important because high amounts of β-glucan and arabinoxylan in the final beer is considered a negative factor, impacting viscosity and the filtering process (Bamforth & Martin, 1983; Rimsten et al., 2002).

The last group of enzymes is the proteases, which break the proteins into peptides and amino acids to provide nitrogen source for the seedling. They are categorized into four classes according to their active-site residues in barley: cysteine-, serine-, aspartic-, and metalloproteases (Jones et al., 2000). The classes are differentiated by the catalytic mechanisms of the enzyme and the chemicals that inhibit their activity. Cysteine proteases contain the amino acid cysteine at their active centres, and they are the main proteases involved in the germination, serin proteases possess serin residue in their active site, aspartic proteases have two Asp-residues in the active centre and a conserved three-dimensional structure, metalloproteases use a metal ion in their catalytic reactions (Jones et al., 2000; Osman et al., 2002). Jones and Marinac (2000) demonstrated that by increasing the temperature during mashing proteases were inactivated, and most of the proteolytic activity

occurred during malting and mashing (Jones et al., 2000). Dormant barley grain possesses less proteolytic activity but during malting, this activity increases (Osman et al., 2002).

1.3.3. Major proteins in beer

Beer is one of the most consumed beverages around the world. Beer quality is affected by the proteins that remain intact after malting, mashing, and brewing. During malting, proteases partially hydrolyse storage proteins into free amino acids and soluble peptides and glucanases and xylanases hydrolyse the endosperm cell wall substrates. Poor hydrolysis of beta-glucans and arabinoxylans results in run-off and filtration and haze issues in the final beer. Hydrolysed proteins may play positive roles such as delivering body and mouthfeel or foam formation; or negative roles, such as haze formation.

Beer foam stability, flavour, mouthfeel, and haze formation are considered important characteristics in beer production (Bamforth & Martin, 1983; Rimsten et al., 2002; Rossi et al., 2014). Therefore, numerous studies identified and characterized proteins in beer that influence these traits. Among those proteins, ns-LTPs and the serpin protein Z have been shown to have major effects which will be explained in the following sections (Sørensen & Ottesen, 1978).

Non-specific LTPs are polypeptides characterized by an eight-cysteine motif, and as the name suggests, play a role in transferring lipids within plant membranes (Evans et al., 1999). Although their specific role is still unknown, they are known as pathogenesis-related (PR) proteins and play a role in defence mechanisms under biotic and abiotic stresses (Carvalho & Gomes, 2007). They are small cysteine-rich proteins, and they are classified into two types of nsLTP1 (9 kDa) and nsLTP2 (7 kDa) according to their molecular size. There is also a modern classification of LTPs including five major (LTP1, LTP2, LTPc, LTPd, and LTPg) and four minor types with fewer members (LTPe, LTPf, LTPh, LTPj, and LTPk), this classification is based on the position of the conserved intron, the identity of the amino acid sequence, and the spacing between the Cys residues (Salminen et al., 2016).

In barley seeds, the LTPs are deposited in the aleurone layer and persist in beer. LTP1 was found to be a surface-active protein that is modified and accumulates in beer foam during brewing (Stanislava, 2007). LTP1 is not only protease-resistant but also stable under high temperatures. During malting, barley grain is subjected to long-term high temperatures, during which conversion of starch to monosaccharides occurs, and proteins are glycated. The interaction of D-glucose with

free amine groups leads to a product called a Schiff base, and it is modified to form a more stable compound known as an Amadori product (Picariello et al., 2017). LTP1 glycation inhibits its unfolding and accumulation during the boiling step. In a study undertaken by Jegou et al. (2000), LTP1 in its unfolded state was shown to affect beer quality with unfolding observed to take place after wort boiling (Jegou et al., 2000). High temperature during boiling (103-110°C) increases protein precipitation and reduces the level of LTP1 in beer, therefore lower wort boiling temperatures near 96°C maintains LTP1 level in beer (Lindorff-Larsen & Winther, 2001; Perrocheau et al., 2006). In brewing, LTP1 stabilizes beer foam by binding foam stabilizing lipids, and a high amount of LTP1 can prevent the formation of stale flavour in beer because it binds to certain intermediate compounds, such as α-ketol 9-hydroxy-10-oxo-12 (z)-octadecenoic acid (Gordon et al., 2018).

Another protein that particularly affects beer flavour is lipoxygenase. This enzyme catalyses the oxygenation of polyunsaturated fatty acids (PUFAs) (Hirota et al., 2006). In barley seed, LOX-1 and is present in the germ and it carries out the oxidative degradation of PUFAs to produce compounds that influence beer flavour (Porta & Rocha-Sosa, 2002). Linoleic acid, one of the lipids in malt, undergoes oxidation by LOX-1 and as a result produces a compound called 2(E)-nonenal, which causes stale flavour in long storage beers (Schmitt & Van Mechelen, 1997). Therefore, malting cultivars that have low LOX-1 activity are desirable (Kuroda et al., 2002; Niu et al., 2018).

Serpins are another protein family that impact beer quality. They were first found to be active as serine-protease inhibitors, but they have other functions. All of the serpin types share three β-sheets, 8–9 α-helices, and a semi-conserved reactive centre loop domain in their secondary structures (Cohen et al., 2019). Their regulatory function relates to the control of cell death by inhibiting endogenous proteinases. In cereals, they have a defensive role by inhibiting the chymotrypsin-like enzymes of pests and pathogens (Gubatz & Shewry, 2010).

Protein Z, a member of serpin family, contains at least four antigenically identical molecular forms with different isoelectric points and molecular masses near to 40 kDa (Roberts et al., 2003). Protein Z comprises two cysteines and 20 lysine residues per monomer, and it is also rich in hydrophobic residues (Steiner et al., 2011). The glycation of protein Z is commenced from the early stages of malting (Bobálová et al., 2010). The quantity of protein Z is positively correlated with beer foam stability. The addition of purified protein Z from barley malt into the finished beer was shown to enhance the beer foam stability (Niu et al., 2018).

Two isoforms, protein Z4 and Z7, are present both in free and bound forms, and these two share an approximately 70% sequence homology (Evans et al., 1999). Protein Z4 has high elasticity and surface viscosity, and according to Evans et al. (1999) when malt is less modified the effect of protein Z in foam stability is lower (Carvalho & Gomes, 2007). When modification is increased the impact of the protein Z on the foam stability is noted to be enhanced. The amount of protein Z in the final beer has been observed to be dependent on nitrogen fertilization rates (Iimure & Sato, 2013).

As previously mentioned, hordeins are barley storage proteins that are grouped into four families: B-hordeins (30–45 kDa), C-hordeins (45–75 kDa), D-hordeins (105 kDa), and γ-hordeins (35-40 kDa). Hordeins like other barley proteins are subjected to chemical (Maillard reaction, hydrogen bond formation) and enzymatic (proteolysis) modifications that mainly occur during malting and mashing. These proline-rich proteins are considered as the main cause of haze formation in beer, that is related to protein-polyphenol interaction (Mastanjević et al., 2018; Siebert & Lynn, 2008). In this interaction, proline involves in the binding site of protein to polyphenols. Numerous studies demonstrated that hordeins are mainly responsible for chill haze formation (Asano et al., 1982; Iimure & Sato, 2013). It is also reported that polypeptides derived from hordein influence beer foam since they concentrated in beer foam fraction (Bamforth & Kanauchi, 2003). Aside from influencing the quality of the beer, hordeins are also known to trigger gluten sensitivities and autoimmune disorders termed CD. Among the barley hordeins, B- and C-hordeins contain higher numbers of immunogenic peptides which are implicated in CD. Therefore, much work has focused on those proteins using quantitative proteomic techniques to ensure the safety and quality of produced barley and its related products (Colgrave et al., 2012; Tanner et al., 2014; Tye-Din et al., 2010).

1.4. Proteomic methods used for barley analysis

Proteome characterization provides a path to understanding barley biochemistry and is of fundamental importance to improve productivity for sustainable agriculture, future food security and resource conservation, especially under changing climate conditions (Steinwand & Ronald, 2020). Proteomics is applied for the identification and characterization of proteins to elucidate their function and interactions. Additionally, understanding the modifications that proteins undergo and their interactions within the cell is also critical. Functional analysis of proteins is often achieved by qualitative and quantitative measurements of plant tissue at specific developmental or physiological

stages (Mock et al., 2018; Ortea et al., 2016). In an effort to achieve maximum coverage and resolution, proteomics studies benefit from the use of different but complementary technologies and approaches. There are crucial stages in proteomic research, including preparation, separation, identification, and quantitation of proteins in a sample. According to the aim of the study, the selection and application of approaches for each stage may be different (Andjelković & Josić, 2018).

1.4.1. Gel-based proteomics

Generally, gel electrophoresis techniques can be identified according to the dimension of the gel system and the labelling procedure for visualization of proteins. Two-dimensional gel electrophoresis (2-DE) is a form of gel electrophoresis technique which was introduced by O'Farrell and Klose in 1975 (Görg et al., 1988; Jorrin-Novo et al., 2019). In 2-DE proteins are separated according to their isoelectric points in the first dimension, and in the second dimension, they are separated according to their molecular mass through SDS-PAGE (Jorrin-Novo et al., 2019). In these methods the basis of gel staining is detection of proteins by visual inspection. Typically, a protein-specific dye-binding chemical reaction is conducted in proteins within the gel. A photograph of the stained gel is used for more investigation. By technology development UV light box or scanners have been used for documentation of the stained gel. There are numbers of staining methods that are used including Coomassie Brilliant Blue, silver, fluorescent dye, Zinc and functional group staining (Jorrin-Novo et al., 2019).

Difference Gel Electrophoresis (DIGE) is one of the powerful comparative techniques in proteomics in which samples are labelled with cyanine dyes prior to electrophoresis and the efficiency of this method is based on natural or modified differences in charge between individual polypeptide chains and dissimilarities of their molecular size under native or denatured conditions. There are some advantages of using this method including being applicable for large-scale proteomic studies, direct protein visualization, fluorescent labelling with highly sensitive dyes however there are some disadvantages which caused limitation in usage of this method such as restriction in separation of complex protein mixtures, cross-contamination of individual protein spots for highly abundant polypeptides, and under-representing some protein species (extremely low/high pI, highly hydrophobic proteins) in 2-DE gels (Görg et al., 1988; Jorrin-Novo et al., 2019).

Early research studies in barley proteomics implemented 2-DE technique to study the barley leaf proteome to discriminate and characterize cultivars based on the obtained spot patterns (Görg et al.,

1988). Although gel-based techniques were successful in identifying barley proteins, they do have some limitations such as incomplete separation of the entire proteome in a complex sample, wherein large abundant proteins mask the low abundant ones so they cannot be detected. Therefore, other analytical techniques such as different chromatography techniques have been applied for separation of proteins and subsequent MS analyses to identify proteins.

To identify and characterize proteins, there are two fundamental MS-based approaches which are termed “top-down” and “bottom-up” wherein most barley proteomics studies use a bottom-up proteomics workflow (Figure 1.2).

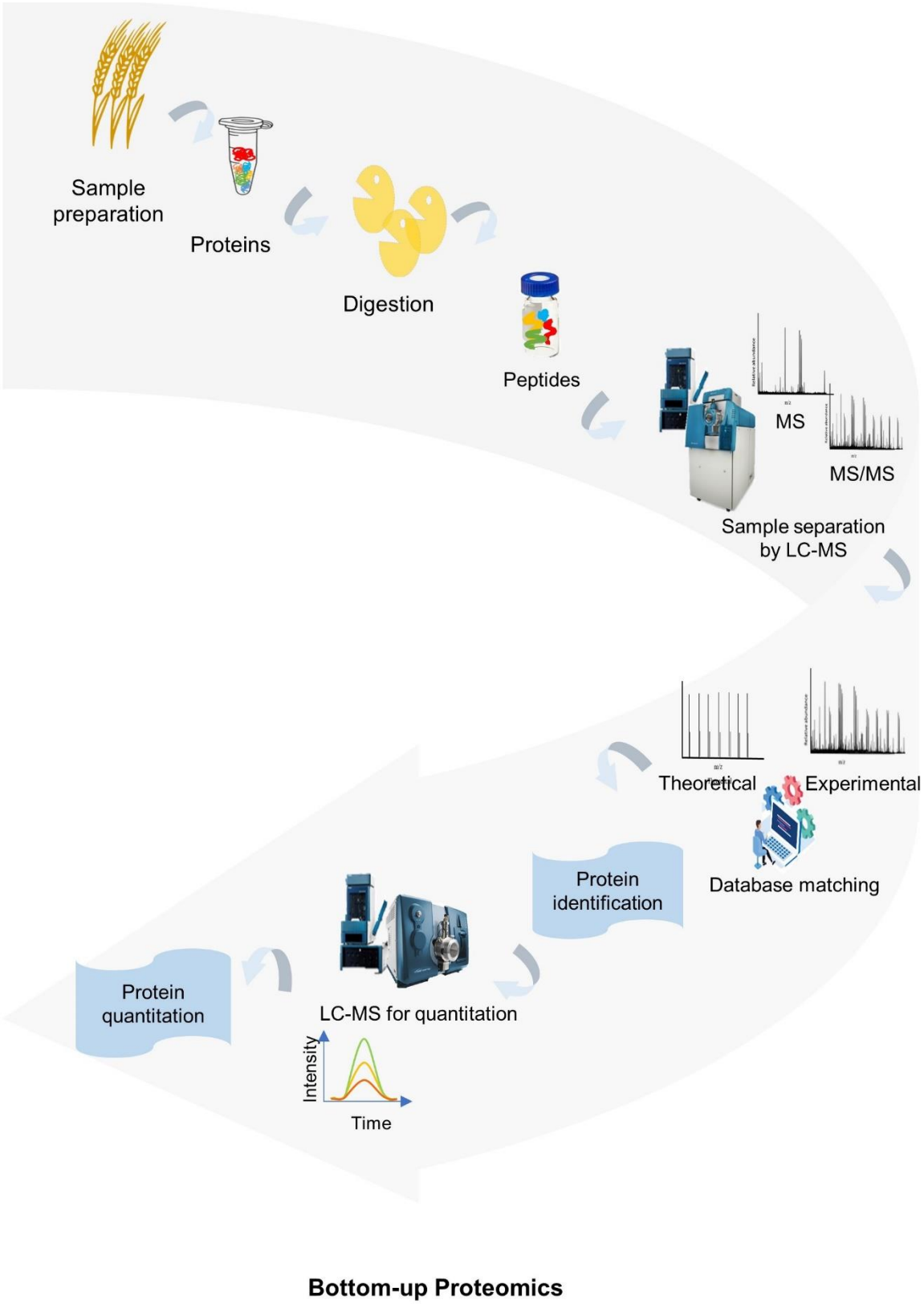


Figure 1.2. Bottom-up proteomic workflows in MS-based proteomics.

1.4.2. Bottom-up proteomics

Bottom-up proteomics or the peptide-centric approach is a common strategy that can be performed through different methods depending on the goal of the research. In this strategy, proteins are extracted and digested by a protease such as trypsin. This produces peptides that are subsequently separated before MS analysis. The peptides are then analysed and detected within the mass spectrometer to determine their mass and are commonly fragmented within the mass spectrometer to yield MS/MS spectra that reveal the mass of the product ions (or fragments) that are subsequently used to identify the peptides.

There are three different acquisition modes that are commonly used to acquire mass spectra in proteomics studies: (1) data-dependent acquisition (DDA); (2) quantitative acquisition; and (3) data-independent acquisition (DIA) (Table 1.1) (Ahsan et al., 2016; Manes & Nita-Lazar, 2018; Teo et al., 2015). These are discussed in the section below.

Table 1.1. Comparison of three different data acquisition methods employed in bottom-up proteomics.

Bottom-up proteomics acquisition methods		
Data-dependent acquisition (DDA)	Targeted MS	Data-independent acquisition (DIA)
Data analysis is easy	Number of peptides that can be quantified in each injection is limited (10s-100s)	Generates highly reproducible data
Number of most abundant ions for fragmentation should be defined	Data analysis is easier	Able to quantify 10000s of peptides
Lower reproducibility	Generates highly reproducible data	Information should be defined including mass range, precursor ion window width and number of MS/MS scans
Generates product ion spectra of peptides for either identification or as SWATH-MS ion library	Highly specific and sensitive	Requires creation of spectral library by DDA
Often used as a prerequisite for targeted MS method development	Requires optimization of method for target peptides	Data analysis and interpretation is more complex with specific software
Abbreviations: SWATH - sequential Window Acquisition of all Theoretical fragmentation spectra.		

Bottom-up acquisition methods has seen great application in the grain science area in recent years

due to technical innovation and optimization of techniques that have increased the depth of coverage provided more accurate information, in the following sections application of three advanced acquisition methods in barley proteomics will be explained (Table 1.2).

Table 1.2. Proteomic studies in barley.

Plant material	Technique	Number of identified spots/proteins	Quantitation	Reference
leaf	2-DE	29	-	(Görg et al., 1988)
seed and malt	2-DE and MS	27 for seeds, 3 for malt	-	(Østergaard et al., 2002)
seed	2-DE and MS	19	-	(Finnie et al., 2002)
seed and malt	2-DE and MS	62 in total	-	(Bak-Jensen et al., 2004)
seed	2-DE and MS	250	yes	(Finnie, Maeda, et al., 2004)
seed, malt, and beer	2-DE and LC-MS/MS	40 for seed, 41 for malt, 30 for beer	-	(Perrocheau et al., 2005)
seed	2-DE and MS	48	-	(Bønsager et al., 2007)
seed	2-DE and MS	48	-	(Finnie & Svensson, 2009)
malted beer	2-DE and LC-MS/MS	85	-	(Iimure et al., 2010)
wort	2-DE and MS	63	-	(Iimure et al., 2012)
seed, germinated grain, green malt and malt	2-DE, MALDI-TOF/TOF MS	6 (focus on hordeins)	yes, iTRAQ	(Flodrová et al., 2012)
beer	LC-MS/MS	33	yes, emPAI	(Picariello et al., 2012)
flour, wort and beer	LC-MS/MS	144 in flour, 27 in wort,79 in beer	yes, MRM	(Colgrave et al., 2012)
barley and malt	2-DE, MALDI-TOF/TOF MS	12 in barley, 9 in malt	-	(Flodrová et al., 2015)
seeds and breakfast products	LC-MS/MS	96	yes, MRM	(Colgrave, Byrne, Blundell, & Howitt, 2016)
seed	2-DE and MS	23	-	(Guo et al., 2016)
leaf	2-DE and LC-MS/MS	9,258	yes, intensity	(Mason et al., 2016)
grain	2-DE, LC-MS/MS	136 DE spots	-	(Schmidt et al., 2016)
grain	2-DE, LC-MS/MS	63 DE proteins	-	(Lee et al., 2016)
seeds	LC-MS/MS	1,168	yes, SPC	(Mahalingam, 2017)

seeds	LC-MS/MS	focus on prolamin oxidation	yes, MRM	(Huang et al., 2017)
malt	LC-MS/MS	1,418	yes, SPC	(Mahalingam, 2018)
wort and beer	LC-MS/MS	210	yes, SWATH	(Schulz et al., 2018)
seed	LC-MS/MS	220	yes, SWATH	(Kerr, Phung, et al., 2019)
spent grain	2-DE, LC-MS/MS	1,346	yes, intensity	(Bi et al., 2018)
Leaf	LC-MS/MS	1,800	yes, TMT	(Wang et al., 2018)
wort	LC-MS/MS	87	yes, SWATH	(Kerr, Caboche, et al., 2019)
grain	LC-MS/MS	1,483	-	(Bose, Broadbent, et al., 2019)
seeds	LC-MS/MS	6 (focus on ATIs)	yes, MRM	(Bose, Byrne, et al., 2019)
grain	2-DE, LC-MS/MS	hordein accumulation	-	(Tanner et al., 2019)
malt rootlet	LC-MS/MS	2,111	-	(Mahalingam, 2019)
wort	2-DE, LC-MS/MS	protein Z4 & Z7 analysis	-	(Luo et al., 2020)
leaf	LC-MS/MS	6,921	yes, DIA	(Wang et al., 2020)
grain	LC-MS/MS	1,907	yes, SWATH	(Bose, Broadbent, et al., 2020)

Abbreviations: ATIs - α-amylase trypsin inhibitirs, 2-DE - 2-dimensional electrophoresis, emPAI - exponentially modified protein abundance index; iTRAQ - isobaric tags for relative and absolute quantitation; LC - liquid chromatography; MALDI - matrix-assisted laser desorption ionization; MRM - multiple reaction monitoring; MS - mass spectrometry; SPC - spectral counting; SWATH - sequential Window Acquisition of all Theoretical fragmentation spectra; TMT - tandem mass tag, TOF - time of flight.

1.4.3. Data-dependent acquisition

In discovery proteomics by DDA methods, all ions which co-elute at a specific time in the chromatogram result in a mass spectrum. The instrument then switches to acquiring product ion mass spectra. The precursor ions are selected from the previous survey scan and are sequentially isolated and fragmented (Bateman et al., 2014). This approach is used to identify the maximum number of proteins in the sample; however, it often results in repeated identification of peptides

derived from high abundant proteins and is limited by the stochastic nature of ion sampling (Ting et al., 2015).

In an initial analysis of barley seed and malt, Østergaard et al. (2002) by using DDA acquisition method of MALDI-TOF MS demonstrated that in seed protein extracts, α-amylase/trypsin inhibitor was one of the proteins that caused variation between barley cultivars. Moreover, in malt extracts, multiple forms of the α-amylase isozyme 2 were identified according to varying spot patterns of the cultivars (Østergaard et al., 2002). Tissue-specific studies performed on barley grain by DDA technique revealed that although the starchy endosperm comprises nearly 85% of a seed's dry weight, it includes less than 50% of soluble proteins and interestingly, the aleurone layer and embryo showed significant contribution in the number of identified proteins using 2-DE (Finnie & Svensson, 2003).

Concerning analysis of proteins both in barley grain and the corresponding malt, Bak-Jensen et al. (Bak-Jensen et al., 2004) identified an increased number of proteins by implementing tandem MS wherein the proteins identified were involved in glycolysis, pathogen defence, nutrient storage, protein folding, detoxification, and nitrogen metabolism. Further research explored the environmental impact on grain filling, such as how nitrogen availability can influence seed proteome changes (Finnie, Steenholdt, et al., 2004).

Subsequently, discovery proteomics by DDA has been applied to study the early stages of barley grain development and protein changes in malt. For instance, Perrocheau et al. (2005), explored the barley proteome changes during the malting and brewing processes. In this study employing gel electrophoresis combined with MS, 40 proteins were identified from barley grain, 41 proteins from malt, and 30 proteins from beer. They reported that most of the heat-stable proteins during brewing are disulphide-rich proteins and these are involved in defence mechanisms and include protein Z, amylase-protease inhibitors, and LTPs (LTP1 and LTP2).

Another significant study focused on the proteome changes of aleurone, embryo, and endosperm across the timeframe of germination. Late embryogenesis abundant (LEA) proteins, ABA-induced proteins, a HSP70 fragment, and a β-type proteasome subunit showed alteration in abundance during the early stages of germination whilst the pattern of redox-related proteins altered at the end of germination (Bønsager et al., 2007).

A proteomic study of barley genetic diversity used a proteome map that was integrated for chromosome 1H, 2H, 3H, 5H, and 7H and the results indicated that more than 60 protein spots

showed variation between cultivars, including peroxidases, serpins, and proteins with unknown functions. MS data confirmed that single nucleotide polymorphisms (SNP) in the coding gene region can change the function of proteins and represent a connection between a cultivar's genome, proteome, and phenotype (Finnie et al., 2009). Further studies used MS-based proteomics to address barley quality improvement for beer production. This was accomplished by constructing a beer proteome map, which showed eight families of barley proteins that included protein Z (Z4, Z7), BDAI-1, CMb, LTP1, TAI, BTI-CMe, and subtilisin-chymotrypsin inhibitor CI-1B (Iimure et al., 2010). Progress and achievement in genome sequencing and annotation of the barley cultivar Morex had a huge impact on the implementation of modern MS methods to investigate the proteome of barley and its products (Mayer et al., 2012). Comparative proteome analysis is often applied to barley. For instance, using 2-DE and MS a feed barley cultivar and a malting barley cultivar were subjected to comparative study with the purpose of identifying protein markers, which have the potential to affect the grain protein composition and quality. The results identified 23 proteins, and malting quality suggested to be characterized by an accumulation of a serpin protein, α-amylase/trypsin inhibitor CMb, and α-amylase inhibitor BDAI-1(Guo et al., 2016). Another comparative analysis explored flag leaves of near-isogenic late- and early-senescing barley germplasm by applying both gel-based and gel-free quantitative techniques, wherein > 9,000 proteins were reported with pathogenesis-related proteins, membrane and intracellular receptors and coreceptors, involved enzymes in attacking pathogen cell walls and DNA repair enzymes up-regulated in early-senescing line. Additionally, a link between early-senescence and up-regulated defence functions was observed (Mason et al., 2016).

So-called 'shotgun' approaches have been more common in barley grain proteomic studies in recent years. A comparison of two-rowed and six-rowed cultivars was conducted to provide a comprehensive characterization of barley seed proteome by shot gun proteomics. This study could identify 1,168 proteins, and among these proteins, specifically hordoindolines were differentiated. It was reported that the type of hordoindoline proteins may cause seed hardness differences between two cultivars. Differences in protein profiles of cultivars were suggested to be utilized for investigation of important complex traits such as mating quality (Mahalingam, 2017). Later, using gel-free shotgun proteomics changes during different stages of malting was explored and results revealed more than 1400 identified proteins during different stages of malting. These proteins were associated with carbohydrate metabolism and enzyme regulation which offer potential targets for breeding with the aim of improving malting quality. Moreover, this research confirmed that most of the proteins necessary for seed germination are synthesized during later stages of seed maturation

(Mahalingam, 2018). In a recent study the same label-free shotgun approach was applied to investigate barley rootlets, a by-product of malting process, and as a result 800 proteins were identified. Gene ontology (GO) enrichment analysis of barley rootlets highlighted the enrichment of primary metabolism-related terms including glycolysis/gluconeogenesis, TCA cycle, and pentose phosphate shunt that involve in sugar production are enriched. Furthermore, GO term analysis for molecular function and cellular component identified the translation process as a key feature in barley rootlet proteome. This study also revealed that pathways that are involved in stress responses such as ascorbat-glutathione pathways were significantly enriched due to steeping regime that seeds undergo during malting process (Mahalingam, 2019).

A recent study compared different buffer compositions commonly used in cereal grain protein extraction, to assess the extraction efficiency experiments and was performed by means of shotgun proteomics. A total of 1,497 proteins were identified from two barley varieties (Hindmarsh and Commander) using an optimized extraction protocol and the results revealed 272 (18.2%) commonly extracted proteins by three experimented extraction methods including Tris-HCl, urea and isopropyl alcohol/dithiothreitol (IPA/DTT). As demonstrated in the results Tris-HCl and urea-based buffers extracted maximum number of proteins of all functional classes from barley compared to IPA/DTT (Bose, Broadbent, et al., 2019).

As it can be seen in the barley proteomics literature, results obtained through discovery proteomics laid the foundation for developing targeted proteomics methods and caused the discovery proteomics to improve to hypothesis-based approaches which is explained in the following.

1.4.4. Quantitative mass spectrometry

MS-based quantitation can be classified into two types according to the goal of the study: relative and absolute quantitation. Alternatively, they can be classified according to the technology used: label-based and label-free (Ankney et al., 2018).

Label-based quantitation method is based on the comparison between samples through labelling and detecting them according to specific changes in size, and this method includes three kinds of labelling: metabolic, chemical, and enzymatic labelling (Anand et al., 2017).

Isobaric Tags for Relative and Absolute Quantification (iTRAQ) is one of the stable isotope labelling approaches which have been implemented for quantitative proteomic analysis. In barley studies, this technique was implemented to investigate the effect of malting process on hordein

composition and results was shown that majority of the hordein components in barley grain are present in all stages of malting process and the amount of hordeins was reported to be decreased during malting, specifically C-hordein decreased by 65%. This technology has a high level of sensitivity and specificity, and it considered as a high throughput method which can be used for quantifying proteins across wide ranges of MW and pI; however, it is time consuming and expensive (Anand et al., 2017).

Label-free quantitation is an easy and cost-effective method for relative quantitation of proteins that does not require expensive reagents for labelling. In this method samples are injected independently in MS and quantitation can be done at the MS scan level by measuring the area under the curve (AUC) or signal intensity through extracted ion chromatograms (XICs). It can be performed at the MS/MS scan level by counting the number of peptide-to-spectrum matches (PSMs) for each protein, termed spectral counting. The advantage of this method is that an unlimited number of samples can be measured and compared, and it has relatively high quantitative proteome coverage. Yet the main disadvantage of this method is a higher variation that can result from individual preparation of each sample (Anand et al., 2017). There are some solutions to reduce the variation such as training of personnel, application of robotics and experimental design. Moreover, the application of internal standards can decrease variability that arises from instrument responses (Lyutvinskiy et al., 2013).

Targeted MS methods aim to detect and quantify protein targets of interest often in complex protein mixtures. Selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) is a targeted scan type commonly employed on triple quadrupole (QQQ) mass spectrometers. The development of targeted methods requires prior knowledge of the precursor/product ion pairs (Lesur & Domon, 2015).

In MRM, by filtering out all other ions, one can dramatically increase sensitivity to specific transitions. Quantitation by MRM can be achieved by relative and absolute approaches but also by label-based and label-free workflows (Picotti & Aebersold, 2012). Since 2012 studies have increasingly employed quantitative methods in addition to qualitative identification. For example, a shotgun proteome analysis of beer by means of HPLC-ESI-MS/MS demonstrated the presence of low-molecular-weight beer components (hordein-derived peptides), potentially harmful to people with CD (Picariello et al., 2012). Application of targeted MS methods such as MRM has facilitated proteomic studies that have increased sensitivity with greater accuracy (Picotti & Aebersold, 2012). This technique has now seen widespread application in plant proteomics studies, including barley.

For instance, targeted approaches were used to identify B-, C-, D-, and γ-hordeins in the wort and beer samples. In this study, researchers identified 144 proteins in flour, 27 proteins in wort, and 79 proteins in beer with the majority of them non-specific LTP1 and α-amylase trypsin inhibitors. They also identified degradation of C-hordein products in wort but these C-hordein fragments were not detected after the brewing process (Colgrave et al., 2012). Additionally, LC-MS/MS was used to develop a scheduled MRM method using nine barley-specific peptides that enabled the detection of barley contamination as a means to provide food safety assurance in gluten-free food production (Colgrave, Byrne, Blundell, & Howitt, 2016). Concerning immunogenic proteins of barley, it is known that degradation of cereal prolamin proteins and peptides can reduce their toxicities for coeliac disease (CD) patients, in a research MRM method was established to detect and quantify proline oxidation fragments in barley. Additionally, fragmentation, aggregation and side chain modifications were identified, free thiol loss, carbonyl formation, and dityrosine formation were among those modifications. The result of this study reported that the immunoreactivity of the oxidized hordein isolate was considerably decreased in all metal-catalysed oxidation systems (Huang et al., 2017). In an LC–MS discovery proteomics analysis of barley cultivars, extraction efficiency of three buffers were investigated (Bose, Broadbent, et al., 2019). and targeted MRM quantitation method was used with a focus on 6 α-amylase trypsin inhibitors across 12 barley cultivars. The research indicated that relative targeted quantitation approach by MRM can be used for identifying and quantifying ATIs involved in autoimmune responses, in order to develop barley lines with a low amount of immune responsive ATIs (Bose, Byrne, et al., 2019).

Quantitation of targeted peptides in label-free approach relies on the direct evaluation of mass spectrometry signal intensities of naturally occurring peptides contained in a sample.[160] Quantitation by MRM is ideally suited for projects that involve quantitation of low-abundant proteins and peptides with maximal accuracy (Schmidlin et al., 2016).

1.4.5. Data-independent acquisition

An alternative approach that does not rely on the pre-selection of target proteins and their peptide derivatives is called data-independent acquisition (DIA) MS. In DIA-MS, precursor ions are sampled across a defined mass range, and all those precursor ions are subjected to simultaneous fragmentation resulting in the generation of mosaic MS/MS spectra. DIA-MS can be implemented on a range of different mass spectrometers, but generally, these approaches help to increase the detection of low abundant and isobaric peptides, and as a result increased the identification of low

abundant proteins (Li et al., 2017). One variant of DIA is termed Sequential Window Acquisition of all THeoretical fragmentation spectra (SWATH) (Gillet et al., 2012).

The SWATH method mostly depends on the peptide spectral library, which is required to be established in advance by shotgun proteomics. It is worth mentioning that SWATH-MS can quantify an unlimited number of target peptides as long as they have been previously observed by shotgun MS (Huang et al., 2015). Therefore, a 'discovery' data-dependent experiment followed by data-independent quantitation by SWATH is a suitable choice when reproducible and accurate quantitation is among the main goals of the project (Mostek et al., 2015; Witzel et al., 2014).

Application of novel methods such as SWATH-MS has enabled investigation of the beer proteome. For instance, one study used a global untargeted SWATH analysis during beer production revealing protein modifications by protease digestion, glycation, or oxidation during the processing steps. They suggested that heat and high concentrations of protein catalyse glycation and oxidation modification, and the result is reduced sugars present in wort. These sugars are critical contributors to the colour and flavour of beer. The SWATH-MS results for sweet wort, hopped wort, beer was compared and demonstrated difference between boiling and fermentation stages, protein abundance of high molecular weight proteins were decreased during the boil, while hydrophobic proteins with high grand average hydrophobicity (GRAVY) scores were reduced in abundance during fermentation. This study showed the opportunities that modern MS-based techniques can offer for investigating and understanding the brewing process. The authors suggested that SWATH-MS methods can be used for exploring beer biochemistry to improve beer quality (Schulz et al., 2018). The implemented SWATH-MS workflow applied to investigate variability of barley seed to explore the difference of barley variety and burden of fungal disease at the proteome level. Obtained results of this study demonstrated that abundance of several proteins across the investigated diseases and locations were significantly affected by disease burden. Among those proteins, oxalate oxidase 2 (OXO2) abundance was significantly increased under pathogen infection. The importance of this protein is due to its role in plant stress responses and production of hydrogen peroxide in the apoplast. This study highlighted the differences between barley varieties with application in feed or malting. Those malting varieties showed higher levels of proteins involved in starch synthesis and beer quality than those varieties used for feed. The results of this study showed the potential of using SWATH-MS workflow for quality control purposes of barley products (Kerr, Phung, et al., 2019).

Later on, SWATH-MS approach was implemented to address the dynamics of protein abundance and modification during brewing process. Results of this research revealed that both wort and beer proteomes showed interplay of partial proteolysis and temperature-dependent protein unfolding as well as protein modification; different types of modifications were identified including oxidation, glycation, proteolytic cleavage in proteins of wort. Interestingly these modifications can alter the thermal stability of proteins and their ability to survive the boil intact. Additional results indicated an increase of most proteins during the maltose rest (63°C) and sugar rest 1 (73°C), and then substantial decrease of those proteins during sugar rest 2 (78°C) and into the boil (102°C) (Bi et al., 2018). Although this study helped to uncover post-translational modifications of wort and beer, it lacks the information regarding modifications of seed germination stage.

Quantitative proteomics by SWATH-MS have been used recently to investigate protein abundance changes in Tibetan barley responses to osmotic stress. The study provided a glimpse into application of multidimensional proteomic data obtained by SWATH-MS workflow with the aim of improvement of osmotic/drought stress tolerance in hull-less barley. Functional characterization analysis of differentially abundant proteins revealed that cytokinin synthesis degradation, UDP glucosyl and glucoronyl transferases, and wax-related proteins were likely to be an exclusive response in the drought-sensitive cultivar, while GDSL-motif lipase, DUF26 kinase, and plasma membrane intrinsic protein (PIP) were the three main functional terms in the drought-tolerant cultivar. In a closer examination it was found that MAPK and PR10 showed higher abundance in the treatment group over all time points of drought tolerant and drought sensitive cultivars, highlighting that these genes might play essential roles in plant defence responses to osmotic stress (Wang et al., 2020) .

In a recent study on hordeins, SWATH-MS acquisition was used to measure proteome-wide abundance differences between wild-type and single hordein-null (B-, C-, and D-null) barley lines to explore the effect of hordein deletion on proteome-level. Comparative analysis between single-null lines and wild type (WT) showed a significant difference between the C-null line and WT and B- and D-null lines. As a result, 1122 proteins were identified with significantly different protein abundance patterns in experimented samples. Additional GO enrichment analysis of these differentially expressed proteins showed that the top three biological processes associated with the differences were metabolic, single organism, and cellular processes. It was concluded that there is a linkage between downregulation of different storage protein families and upregulation of proteins related to primary metabolism, transcription, and enzymatic biosynthesis processes and enzyme

inhibitors with the absence of B-, C-, and/or D-hordeins. Furthermore, results revealed an increase in globulins, lipid transfer proteins, and proteins rich in essential amino acids in the null lines. This increase in non-gluten storage proteins occurred as the consequence of a specific reduction in hordein proteins. Implementing SWATH-MS, this study highlights one of the main applications of this advanced method in proteome-level alterations studies for investigating modified crops (Bose, Broadbent, et al., 2020).

The application of MS-based proteomic approaches for investigating cereals including barley in different steps of the food production chain have increased in the past decade. The following sections aim to shed light on the application of proteomics in the three critical sectors of barley studies including: 1) breeding; 2) manufacturing and processing; and 3) final product quality and food safety.

1.5. MS application in barley breeding

Barley represents a major cereal crop grown in temperate climate areas worldwide. The development of crop resilience to environmental changes is a crucial breeding goal of agricultural programs. Development of methods and techniques to address food security with an increasing population and global climate change is significant.

MS-based proteomics offers the potential to inform breeding programs and improve existing barley varieties or develop new ones that are high yielding and stress-resistant. Proteins play an important role in cellular mechanisms and are involved in various biological processes. In an organism, proteins are more directly related to the phenotypic changes when compared to gene expression profile changes and investigating the differential abundance patterns of protein profiles can complement information obtained from genomics and transcriptomics analyses (Kausar et al., 2013; Mora et al., 2018).

Different proteomics methods including discovery (shotgun) proteomics, absolute and relative quantitative approaches have been used to broaden the knowledge of barley proteome changes under specific abiotic and biotic stress conditions (Table 1.3) and to explore impact of post-translational modification (PTMs) and protein interactions (Anand et al., 2017; Finnie & Svensson, 2003; Ghaffari et al., 2019; Ribeiro et al., 2013; Willows et al., 2017; Yang, Jensen, Spliid, et al., 2010).

Table 1.3. Proteomic studies on abiotic and biotic stress response in barley (Kosová et al., 2014).

Plant material	Tissue	Methods	Quantitation	Reference
Drought				
barley cv. Basrah (T), Golden Promise (S)	leaf, root	2-DE-MALDI-TOF	yes	(Wendelboe-Nelson & Morris, 2012)
barley cv. 2 Egyptian accessions; 15141 (T), 15163 (S)	leaf	2-DE-MALDI-TOF	-	(Ashoub et al., 2013)
barley cv. Golden Promise	leaf	2-DE-MS	-	(Ghabooli et al., 2013)
spring malting barley cv. Amulet	leaf, crowns	2-DE, MALDI-TOF/TOF	yes	(Vítámvás et al., 2015)
spring barley cv. Maresi (T), Syrian breeding line – Cam/B1/CI (T)	leaf, root	2-DE, MALDI-TOF/TOF	yes	(Chmielewska et al., 2016)
spring barley cv. Maresi x Syrian breeding line – Cam/B1/CI (T), 100 RILs	leaf. root	MALDI-TOF/TOF	yes	(Rodziewicz et al., 2019)
Low temperature (cold/frost tolerance)				
winter barley cv. Luxor (T)	crown, leaf	2-DE-MALDI-TOF	yes	(Hlaváčková et al., 2013)
spring barley cv. Aths (S)	leaf, root	2-DE, LC-MS/MS	-	(Longo et al., 2017)
10 DH lines; DH534 (T), DH602 (T), DH561 (t), DH61 (t), DH435 (t), DH584 (t), DH363 (S), DH575 (S), DH158 (S), DH65 (S)	leaf	2-DE-MALDI-TOF/TOF	-	(Gołębiowska-Pikania et al., 2017)
Osmotic stress (Polyethylene glycol PEG-6000)				
Tibetan hull-less barley (*Hordeum vulgare* L. var. *nudum*) inbred lines, drought-resistant XL (T), drought-sensitive DQ (S)	leaf	LC-MS/MS	yes	(Wang et al., 2020)
Salinity				
barley cv. OUK305 (T), OUI743 (S)	root	2-DE, LC-MS/MS	yes	(Sugimoto & Takeda, 2009)
barley cv. Morex (T), Steptoe (S)	root	2-DE, LC-MS/MS	yes	(Witzel et al., 2009)
barley cv. Afzal (T), L-527 (S)	leaf	2-DE, MALDI-TOF/TOF	yes	(Rasoulnia et al., 2011)
barley cv. Afzal(T), L-527 (S)	leaf	2-DE MALDI-TOF/TOF	yes	(Fatehi et al., 2012)
Barley cv. Morex (T), Steptoe (S)	leaf, root	2-DE, MALDI-TOF; LC-	yes	(Witzel et al., 2014)

			qTOF MS/MS		
barley cv. DH187 (T), DH14 (S)		seed	2-DE, MALDI-TOF/TOF	yes	(Mostek et al., 2015)
barley DH population cross TX9425 and NasoNijo two pairs of NILs (N33 (S) and T46 (T), N53 (S) and T66 (T)		leaf, root	2-DE, MALDI TOF/TOF	yes	(Zhu et al., 2020)
Combined stress					
drought and osmotic stress	barley genotypes 004223 (T) 004186 (S)	leaf	2-DE MALDI-TOF; LC-MS/MS	yes	(Kausar et al., 2013)
drought and heat stress	Arta (T), Keel (T)	leaf	2-DE, MALDI-TOF/TOF	yes	(Rollins et al., 2013)
drought and *Piriformospora. indica*	barley cv. Golden Promise	leaf	2-DE, LC-MS/MS	yes	(Ghaffari et al., 2019)
Pathogenes					
fungal pathogen - Fusarium head blight (FHB)					
barley cv. Scarlett (S)		spikelet	2-DE, LC-MS/MS	yes	(Yang, Jensen, Spliid, et al., 2010)
barley cv. Scarlett (S)		seed	2-DE, MALDI-TOF, MS/MS	yes	(Yang, Jensen, Svensson, et al., 2010)
naked barley *(Hordeum vulgare ssp. nudum*)		seed	2-DE MALDI-TOF-MS, LC-MS/MS	yes	(Eggert & Pawelzik, 2011)
net blotch – *Pyrenophora teres*					
barley cv. La Trobe, Charger, Oxford, Commander, Fairview, Compass		seed	LC-MS/MS, SWATH-MS	yes	(Kerr, Caboche, et al., 2019)
barley cv. Oxford, Commander, Compass, Scope, Shepherd, Flagship		seed	LC-MS/MS, SWATH-MS	yes	(Kerr, Caboche, et al., 2019)
barley cv. Baudin		leaf	2-DE, LC-MS/MS, SWATH-MS	yes	(Hassett et al., 2020)
leaf rust – *Puccinia hordei*					

barley cv. LaTrobe, Commander, Compass, Scope, Shepherd, Fathom	seed	LC-MS/MS, SWATH-MS	yes	(Kerr, Caboche, et al., 2019)
Powdery mildew – *Blumeria graminis*				
barley cv. Golden Promise	leaf	LC-MS/MS	-	(Godfrey et al., 2009)
barley cv. Golden Promise	leaf	LC-MS/MS	yes	(Lambertucci et al., 2019)
Abbreviations: 2-DE - two-dimensional electrophoresis; DH - double haploid (line); LC - liquid chromatography; MALDI-TOF/TOF - matrix-assisted laser desorption ionization time-of-flight/time-of-flight (spectrometry); MS, mass spectrometry; qTOF - quadrupole time-of-flight; S - sensitive (genotype); SWATH - sequential window acquisition of all theoretical spectra; T - tolerant (genotype); t - genotype less tolerant than T.				

The agricultural production of barley is limited by a wide range of biotic and abiotic stress factors. Biotic stress factors including pathogens and insects, and abiotic stress factors including drought, salinity, cold, and frost severely limit plant growth and development as well as the final yield in barley crops. Recent reviews on plant protein reactions to pathogens (Gonzalez-Fernandez & Jorrin-Novo, 2012) and abiotic stresses (Marzano et al., 2020; Stelk et al., 2013) provide a critical overview of plant responses to stresses on a proteome level. These stresses pose a drastic threat to barley production and affect proteins and consequently plant phenotypes. Therefore, comprehensive knowledge about plant responses to different stresses is a prerequisite for the progress of breeding programs to deliver stress-tolerant crops. MS-based proteomics along with advances in sequencing technology and bioinformatics have greatly assisted in achieving this goal (Gupta et al., 2013). One valuable method that is used to link plant proteome changes to genetic variations, is proteomic quantitative trait locus (pQTL) analysis, which can be defined as a way to correlate protein abundance patterns with genetic polymorphism or QTL that controls variation in protein profiles (Colgrave et al., 2014; Ghaffari et al., 2019; Stelk et al., 2013). Using this approach, differences in protein abundance are considered as a molecular phenotype (Salekdeh & Komatsu, 2007).

Due to the essential roles of proteins in plant-stress interaction, they can be potential candidates for proteomics investigation by using pQTL approaches for producing resistant crops. This technique has been used for the identification of drought-responsive proteins in barley and valuable reviews published upon this subject (Ghaffari et al., 2019; Stelk et al., 2013). Large-scale barley leaf and root proteomic analysis performed by 2-DE and MS revealed 48 pQTLs for roots and 31 pQTLs

for leaves and a genetic linkage map was established for the studied recombinant inbred lines relative to the proteomic data (Rodziewicz et al., 2019).

Using the same method, a comprehensive study was undertaken on double-haploid introgression lines including wild-type line (Hs213, *Hordeum vulgare* subsp. *spontaneum*) within a modern cultivar background (*H. vulgare* cv. Brenda). As the primary stage of searching for QTLs, Li and his colleagues (2005) identified QTLs for malting quality and yield components and protein content was one of the measured traits in this study (Li et al., 2005). As a result of this research, three malting quality traits were evaluated in two years, two QTLs for increased protein content were detected which accounted for 11.8% and 14.6% of the phenotypic variance. Furthermore, three QTLs were identified for seed friability. One major limitation of the study was that it was based on the measured protein content as a phenotypic trait. In later years the difference in protein abundance of barley grain, considered as a molecular phenotypic trait, was used to map a QTL that regulates protein expression. Further QTL analysis was performed on the proteome level on the same population (Witzel et al., 2011). Grains from 45 barley lines were analysed and their 2-DE proteome patterns were used for QTL analysis. MS identification for 49 segregating spots was achieved, and functional protein annotation of proteins revealed that most were involved in defence mechanisms and metabolism processes. The DE spots include α-amylase inhibitor BDAI, protein disulphide isomerase, adenosine kinase, NADP malic enzyme, and peroxidase BP1 and also disease-related processes proteins. Proteins showing altered expression were mapped to the same chromosomal locations as the coding genes (Witzel et al., 2011).

With the aim of establishing a linkage between proteins and malting characteristics, the referred QTL approach was applied to a subset of near-isogenic wild barley introgression lines. In the mentioned study, 2-DE and MS applied to identify related proteins by using two QTLs specifically linked to malting quality, and the results identified 14 candidate proteins that affected this trait (March et al., 2012a).

The genotypic differences at a proteomic level can be exploited for the identification of candidate proteins that can be further analysed for improving stress-specific tolerance in barley and development of biomarkers (Kausar et al., 2013). The application of MS combined with the pQTL approach can be considered a practical tool to identify desirable genes for barley breeding programs that aim to deliver barley lines that are resistant to major stresses or containing desirable characteristics for the malting purposes. Further research aiming to investigate the relationship of the barley genome and proteome will allow the investigation of complex stress tolerance traits, the

analysis of the molecular basis of these traits and the development of the next generation of barley crops adapted to the changing climate that will be critically important for food security.

1.6. MS application in food manufacturing and processing in barley

MS proteomic applications in food sciences have increased dramatically in the past decade, largely due to significant advances in sample separation techniques and MS instrumentation. In the past food proteomic approaches relied heavily on electrophoretic methods. The application of MS technologies allows protein profiling, protein characterization, and analysis of proteins in complex matrices, such as food (Korte & Brockmeyer, 2017).

Food proteomics is complicated by several different factors that need consideration: (1) being complex matrices most foods have no comprehensive proteome data available; (2) complex modifications occur in food proteins during processing, for example, the Maillard reactions that is in general not measured in classical proteomics applications; (3) unknown dynamics in the proteome during storage and processing can cause high protein degradation or modification; and (4) interference or interactions of food proteins with carbohydrates or lipids can significantly impact the food matrices and complicate proteome analysis (Gupta et al., 2010).

Food losses contribute considerably to food security, food quality, and safety, both on an economic and environmental level. According to a study, one-third of food produced globally is wasted with developed country losses as high as in developing countries at 40% (Gustavsson et al., 2011). A reduction in post-harvest losses is considered to be the most efficient and cost-effective method for ensuring food security, as post-harvest savings not only increase food production, but the increases are sustainability achieved without additional land and water usage (Gustavsson et al., 2011). Post-harvest management systems and smart food processing technologies are equally necessary to guarantee the availability of safe, high-quality food in the coming years (Pedreschi et al., 2013).

After harvest, barley crops are exposed to a series of stresses. Exposure can be either abiotic stresses during, or before they reach the final consumer, for example: (1) physical stress due to handling; (2) variation in temperature and vibration caused by transportation; (3) alternation in atmospheric condition or temperature reduction in regards to storage, or (4) combination of biotic and abiotic stresses, such as, cereals containing fungal spores that will proliferate during storage (Pedreschi et al., 2013). During transportation and storage barley will undergo a series of changes trying to adjust to or acclimate to imposed stress that will change the composition of the proteome. Since proteins

are involved in stress responses, for example, (1) as enzymes catalysing changes in the regulation of protein levels, (2) as structural proteins of the cytoskeleton; or (3) as protein-bound receptors, proteomic studies designed to understand the physiological processes involved in stress tolerance of barley crops are of significant importance. These varying states can allow for comparative proteomic investigations related to changes in protein concentration and physiological parameters associated with stress during storage and shelf-life related to susceptibility (Kosová et al., 2011). For instance, the first requirement to reduce losses post-harvest temperature control combined with post-harvest technologies to delay product degradation (Mora et al., 2018).

While the majority of studies address the genomic and transcriptomic data, there is growing interest to understand proteomic involvement in physiological variations during post-harvest processing (Pedreschi et al., 2013). Applications of such knowledge would allow for biomarker identification of post-harvest disease and disorders. Additionally, this information would support selection and breeding programs, drive harvesting strategies, optimize processing, and storage conditions with the goal of reducing product losses (Mora et al., 2018).

The discovery of novel biomarkers is an important and exciting part of food safety, but to impact human health biomarkers must ensure efficacy and safety. Unfortunately, verification can be time-consuming, expensive, and can limit the number of biomarkers. A typical verification process involves either developing novel antibodies or validating existing antibodies against the new biomarkers so that these new biomarkers can be tested in a broader research program using an immunoassay (Sang, 2018). However, both the development and validation of antibodies take time and money, particularly when the discovery program has identified a panel of biomarkers to move into verification.

In comparison to ELISA methodology, LC-MS/MS methods for biomarker verification are more efficient because they don't rely on the development of new antibodies to measure biomarkers. In addition, LC-MS/MS can accurately and precisely measure multiple biomarkers in a single sample. The advantage of this technique is that it can regulate the bioanalysis of the instrument and can quantify various biomarkers in various states, such as seeds, flour, raw, and processed foods. The major disadvantage of this technique is the sensitivity of large molecules compared to ligand binding assay (Agrawal et al., 2013; Li et al., 2017).

1.7. MS application in final-product quality and food safety in barley

The global scale of food supply chains is a challenge for ensuring food safety. Most countries have implemented regulations and laws in an effort to guarantee food safety (Li et al., 2017). More importantly, increased consumers' attention to the biochemical composition, processing, and functional ingredients have led to making better-informed decisions (Stewart et al., 2018). Cost-effective, efficient, robust analytical methods with greater sensitivity are important to ensure the traceability, quality, and safety of foods. Over recent years, proteomic analysis has emerged as a tool for quality control assessment in food processing and production as well as in food safety Andjelković & Josić, 2018).

Barley proteins contribute significantly to quality parameters, such as flavour, texture, colour, with changes resulting from response to stress (Mora & Toldrá, 2021). Knowledge of the proteins present, their biological role, structures, and functions in raw food materials as well as in final food products is critical for process optimization. The protein content is an essential parameter for food barley and malt barley selection. The hordein content can also be considered, given that hordeins constitute the largest portion of the barley grain and impact the endosperm texture, but also play a role in influencing modification during malting and hordein peptides persist in beer (Fox, 2020). Hordeins are degraded by endoproteases into smaller peptides during malting, but some of the proteins survive the heat of kilning and mashing constituting approximately one-third of the proteins present in the final beer (Colgrave et al., 2012; Iimure & Sato, 2013; Stanislava, 2010). Flodrová et al. (Flodrová et al., 2012) compared two proteomic approaches (2-DE and LC) to monitor hordein profiles during malting. Their results suggested that LC was more efficient due to its capacity to detect more hordein proteins in a single experiment, facilitating the creation of a barley hordein map that can be applied to brewing optimisation (Colgrave et al., 2012; Tanner et al., 2014; Tye-Din et al., 2010). Measuring hordein content, as well as protein content, can be beneficial from a food safety perspective as food intolerance is linked to hordeins.

It has been reported that during processing, increased pressure or temperatures can modify gluten proteins, resulting in changes to the protein structure through alteration of the peptide sequence (Juhász et al., 2020; Popping, 2013). Therefore, the gluten content of products is difficult to calculate and can result in underestimation which poses a health risk for people with CD. The detection of allergenic ingredients is often accomplished using enzyme-linked immunosorbent assays (ELISA). Although this approach has some disadvantages, ELISAs are sensitive but can be challenged by limited reproducibility in food matrices, cross-reactivity, and it is not possible to

simultaneously detect multiple allergens (Stelk et al., 2013). Mass spectrometry-based proteomics approaches overcome some of these disadvantages, allowing high throughput multiplexed analyses (Marzano et al., 2020).

Tanner et al. (2015), compared the main characteristics between ELISA and MS-based proteomics and found that hordein (gluten) measurement in beer via ELISA analysis was problematic, where measured levels of hordein varied by four orders of magnitude (Tanner et al., 2013). It was concluded that ELISA detects only the antigenic peptides that may be absent in hydrolysed gluten present in fermented products (Colgrave et al., 2014). MS quantitation is carried out using peptides that are unique and specific, enabling the quantitation of individual hordein isoforms, therefore making it a more reliable approach for detection and speciation of gluten (Tanner et al., 2013). Furthermore, Lock (Lock, 2013) confirmed that in comparison to ELISA, LC-MS/MS, demonstrating that LC-MS/MS can be used to detect gluten in processed complex food matrices and food ingredients. Therefore, biomarkers can be developed for gluten and more specifically for barley hordeins.

A common proteomic workflow for the analysis of gluten in barley involves the identification of specific hordein peptide markers followed by quantitative analysis of the protein in food using multiple reaction monitoring (MRM) methods (Colgrave, Byrne, Blundell, Heidelberger, et al., 2016). This LC-MS/MS methodology has been broadly applied to gluten detection in barley, malt, and beer. However, further developmental work and standardization of methods are still required. This not only applies to the analytical approach but also food sample processing and database development (Fischer & Creydt, 2020). A further example of the application of proteomics in food science was the development of ultra-low gluten barley, for food and beverage production (Tanner et al., 2016). Starting with reduced hordein barley lines, Tanner et al. (2016) used a conventional breeding approach to produce an ultra-low gluten barley variety Kebari® (Tanner et al., 2016). Proteomics was used to confirm the reduced gluten content (~5 mg/kg) a ~19,000-fold reduction of hordein content when compared to conventional barley, below the level recommendation for classification as gluten-free by the World Health Organisation (WHO). Further proteomic analysis of this line uncovered that while the levels of α-amylase remained at levels similar to the control, the β-amylase levels were reduced remarkably by approximately 50-fold. Additionally, the overall protein content in this line was comparable to control barley, but there was a 10- to 15-fold increase in some free amino acid levels (Tanner et al., 2016).

Food and beverage proteomes are complex wherein the matrices can interfere with detection of proteins (qualitative and quantitative) (Samperi et al., 2015). LC-MS/MS-based proteomics methods are now available for the detection of various foodborne proteins and can be used as a complementary approach in food testing applications due to its accuracy, precision, sensitivity, and robust quantitative ability.

1.8. Conclusions

Advanced MS-based proteomics tools coupled with the progress in genomics and transcriptomics provide a platform to investigate barley, its expressed proteins and their biological roles. Gel-based techniques such as 2-DE while time and labour intensive have been effectively utilized for protein separation and visualization. More recently, liquid chromatography has become more common to improve the speed and precision of protein separation. The application of mass spectrometry in barley proteomics has provided a robust platform for the identification and quantitation of proteins. In parallel, developments in sample preparation, protein identification algorithms and the used background databases have enabled greater depth of coverage of the barley proteome as well as application to raw ingredients, i.e. barley, or its products: malt and beer. Proteomics can be used in molecular breeding to reveal more information about target proteins which are linked to desired malting characteristics, understanding and optimising health-related proteins, investigating modifications of those proteins during malting, mashing and fermentation as well as measuring the expression level of each protein. Furthermore, proteomics coupled with other omics technologies for providing a multidisciplinary system biology platform.

We are now able to not only explore the proteins present in barley, but we can do so from a truly quantitative standpoint. We can investigate the changes that occur because of environmental/experimental manipulations and that will lead to changes in barley cultivation practices, food processing and can be used for food safety assessment. Applying global quantitative proteomics to barley will change the way that barley is bred, grown, and processed and ensure food safety.

1.9. Aims and objectives

The literature review provided in sections 1.1-1.8 summarises past applications of proteomics to barley research. This provides a solid understanding of advancements in the field and also identified current research gaps. It was evident from the review that there were opportunities to develop

protein extraction protocols and to adopt cutting-edge analytical and bioinformatics strategies to propel and reinvigorate the barley proteome repertoire. Numerous studies have been published on barley proteomics; however, the impact of growing location and environment on grain proteome compositional changes has largely been overlooked. In addition to the genotype and environment-dependent global grain proteome changes, the literature review revealed that MS-based proteomics could offer an accurate and high-throughput platform for the monitoring of immunogenic proteins and peptides from barley. Proteomics offers the potential to be incorporated to guide breeding programs for developing low-immunogenic cultivars.

In this PhD project, two sets of barley samples were used to explore the changes in the barley proteome between grain and malt and provide insight into breeding programs by studying various developing barley lines. The first set of samples was from the breeding company Edstar Genetics, where three breeding lines that differed in their genetic backgrounds were grown in 2019 at three locations in Western Australia: Mingenew (-29.222513142453078, 115.44604997548826), Toodyay (-31.5511748, 116.4671695), and Munglinup (-33.7073279, 120.8652063). The results of this experiment have been published in the *Journal of Agriculture and Food Chemistry* (Bahmani et al., 2022) and are presented in Chapter 2.

The specific aims of this experiment include:

1) Investigation of the impact of different growing locations on the proteome of barley lines with different genetic backgrounds.
2) Establishing a relationship between proteomic measurements and malting specification data to understand grain proteins that define malting characteristics.

The second set of samples subjected to proteomic experiments included seven hordein-reduced barley lines that differed based on the presence or absence of a specific class of hordeins. The Commonwealth Scientific and Industrial Research Organisation (CSIRO) developed and provided these lines. Experiments using these samples and the findings of this study are presented in Chapters 3 and 4. The major aims of this part of the research include:

1) Understanding the impact of the genetic background of grain and malting as a food processing step on changes in the immune reactive protein classes of barley.
2) Identification and characterisation of the impact of grain proteome differences of the hordein-reduced lines on the malted grain samples by comparing proteomes of grain samples and their corresponding malt samples.

2. Chapter 2: Proteome phenotypes discriminate growing location and malting traits in field-grown barley

This chapter focuses on the first two aims mentioned in the Aims and Objectives (section 1.9), investigating the impact of different growing locations on the proteome of barley lines with different genetic backgrounds and to establish a relationship between proteomic measurements and malting specification data to obtain the concordance between proteome and malting data.

The current chapter is published in the *Journal of Food and Agriculture Chemistry* under the following citation:

Bahmani, M., Juhász, A., Broadbent, J., Bose, U., Nye-Wood, M. G., Edwards, I. B., & Colgrave, M. L. (2022). Proteome Phenotypes Discriminate the Growing Location and Malting Traits in Field-Grown Barley. Journal of Agricultural and Food Chemistry, 70(34), 10680–10691. https://doi.org/10.1021/ACS.JAFC.2C03816

2.1. Publication and Statement of Contributions

This chapter is drawn from the following publication:

Bahmani, M., Juhász, A., Broadbent, J., Bose, U., Nye-Wood, M. G., Edwards, I. B., & Colgrave, M. L. (2022). Proteome phenotypes discriminate the growing location and malting traits in field-grown barley. *Journal of Agricultural and Food Chemistry*, *70*(34), 10680-10691.

Mahya Bahmani (candidate)

Prepared the samples, developed processing pipeline for data processing and analysis, conducted data visualisation, interpretation, and drafted the manuscript.

Signat[redacted]

Date: 29/03/2023

Angéla Juhász

Conceived the design of the study. Provided guidance for data analysis, visualisation, and interpretation. Assisted with bioinformatics analysis and reviewed the manuscript.

Signatu[redacted]

Date: 30/03/2023

James A. Broadbent

Provided guidance for data analysis, visualisation, and interpretation. Assisted with development of mass spectrometry data acquisition strategies and reviewed the manuscript.

Signatur[redacted]

Date: 04/04/2023

Utpal Bose

Assisted with statistical analysis, data visualisation and reviewed the manuscript.

Signature 

Date: 31/03/2023

Mitchell G. Nye-Wood

Assisted with development of mass spectrometry methodologies, data acquisition and reviewed the manuscript.

Signatu 

Date: 03/04/2023

Ian B. Edwards

Provided barley samples and reviewed the manuscript.

Signature: Unfortunately, Dr. Edwards Passed away and therefore, we are unable to obtain his signature.

Michelle L. Colgrave

Provided guidance for mass spectrometry data acquisition, analysis, visualisation, and interpretation and reviewed the manuscript.

Date: 30/03/2023

2.2. Abstract:

Barley is one of the key cereal grains for the 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 applying liquid chromatography-mass spectrometry (LC-MS). Using data-dependent acquisition LC-MS, 1,571 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 the barley lines' genetics and their growing locations strongly correlated between proteins and desired traits such as malt yield. Linking meteorological data with proteomics measurements revealed how high-temperature stress in northern regions affects seed temperature tolerance during malting, resulting in 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.

2.3. Introduction

Barley (*Hordeum vulgare* L.) is a member of the Poaceae family and is ranked the fourth major cereal crop by yield globally (FAO - FAOSTAT (2022)).The importance of this crop stems from its wide application as human food and animal feed and essential to meet malting and brewing demand. Australia is the largest exporter of malting barley providing more than 30% of the world's supply (Luo et al., 2019).

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 (Gubatz & Shewry, 2010). 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 brewer's requirements or to decide on alternative use of grain. Malting barley varieties are bred and grown to select for optimal malt quality specifications like high enzyme activity, yield, and flavour characteristics (Herb et al., 2017). Therefore, it is essential to select and breed barley variety with desired malting specifications. In this regard, the total protein content of the barley seed is between 8-15 % — depending on the cultivar and growing environment — and this trait is central to grain quality due to its relationship with enzyme content and malt specifications (Gubatz & Shewry, 2010). There have been efforts to find candidate proteins associated with the malting specifications quantitative trait loci (QTL) (Daba et al., 2019; Li et al., 2005; Singh et al., 2017a; Witzel et al., 2011)and to map QTL associated with protein expression variation in barley; researchers reported that the detection of 14 proteins using mass spectrometry including heat shock proteins (HSP), Late Embryogenesis Abundant (LEA) proteins and enzyme inhibitors (March et al., 2012a).

The background genetics and growing conditions for barley lines have been shown to influence malt characteristics and quality (HONG & ZHANG, 2020). As a result, the combination of biotic and abiotic stresses that affect during the growth and development of the barley plant in field has been investigated in numerous studies (Dai et al., 2007; Laidig et al., 2017; Luo et al., 2019; Torp et al., 1981; Zhang et al., 2006). These stresses have been shown to cause changes at the molecular and physiological levels. For instance, the growing environment can significantly affect barley

phytic acid content, nutritional composition, and seed protein concentration (Dai et al., 2007). Likewise, growing barley in different environmental conditions can impact its amylopectin directly affecting a direct effect on germination and malt characteristics (Izydorczyk et al., 2001). Furthermore, environmental factors can affect malt specifications (Fox et al., 2003) and influence the subsequent malt and beer flavour (Herb et al., 2017).

Liquid chromatography (LC) coupled with mass spectrometry (MS) is a powerful tool to measure the barley proteome, protein quality and the changes occur during the germination events. Our recent review on the application of cutting-edge LC-MS-based proteomics approaches in barley protein research has demonstrated its potential to inform plant breeding (Bahmani, O'Lone, et al., 2021). Research to date has reported label-free quantitative MS-based proteomics to study: barley malting (Osama et al., 2021); quality and flavour (Herb et al., 2017); responses to infection (Kerr, Phung, et al., 2019); in-depth profiling of storage proteins (Bose, Broadbent, et al., 2019); and potential allergens and enzymes (Schalk et al., 2017); 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 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 having broad applicability to other malting cereals.

2.4. Materials and methods

2.4.1. Plant material

Three malting barley lines (006, 007 and 008) used in this study (Table 2.1). These lines were developed by Edstar Genetics Ltd Pty and each line was cultivated in two northern — Toodyay (T) (-31.5511748, 116.4671695) and Mingenew (Mi) (-29.222513142453078, 115.44604997548826) — and one southern region — Munglinup (Mun) (-33.7073279, 120.8652063) — across WA. Hereafter, three locations will be indicated as T, Mi and Mun throughout the manuscript.

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

These lines were sown in early May and harvested in late-November 2019. 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 (Colgrave et al., 2015). All three lines from each three locations were micro malted by the Australian Grain Export Innovation Centre (AEGIC) in Perth, WA, in 2019 and same malting process was used for all lines. Malting specification data is shown in Table 2.2 and 2.3. Average monthly temperature recordings from the three growing locations during 2019 was downloaded from the Australian Bureau of Meteorology (Australia's official weather forecasts & weather radar - Bureau of Meteorology). The average accumulated temperature has been calculated by adding all growing days for each region between May and November and divide the sum amount to the number of days.

Table 2.2. Malting specifications for barley lines (part 1).

line	location	test wt (kg/hl)	grain wt.	protein (% d.b)	moist. after 24 hrs germ. (%)	malt yield (%)	protein NIR (% d.b)	malt moisture (%)	malt NIR malt extract (%)	oven Moisture %	extract: fine grind EBC (%)	wort colour	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

Table 2.3. 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)	beta-glucan EBC (ppm)	malt alpha-amylase (U/g)	beta-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

2.4.2. Protein extraction and digestion

A total of 100 mg of flour was weighed for each of the four biological replicates into 1.5 mL micro-tubes 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 (Bose, Broadbent, et al., 2019). Samples were thoroughly mixed and sonicated (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. (Colgrave, Byrne, Blundell, Heidelberger, et al., 2016). 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) (Bose, Byrne, et al., 2019).

2.4.3. 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 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. (2017) (Colgrave et al., 2017). 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 585– 1250 *m/z*, both with 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 the 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 the ProGroup algorithm for conservative protein inference and grouping (Shilov et al., 2007). The DDA data were searched against a sequence database that included *Hordeum vulgare* proteins from the 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 Biognosys iRT pseudo-protein sequence.

2.4.4. Data-independent acquisition by SWATH MS

Samples were analysed in six batches. LC and MS source conditions for SWATH acquisition were identical to that described for DDA. 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 assigned considering each window centre 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 — and interspersed throughout — each batch.

2.4.5. 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® v2.2 software (SCIEX). Using the Microapp, six transitions per peptide and twenty-five 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: peptide confidence threshold - 91%; peak group FDR threshold - 1%; XIC width - 75 ppm; and, RT extraction window - 5 min. Peak groups were extracted and scored before exporting the peak group score report. Thereafter, the report used to filter the ion library wherein the original twenty-five peptides per protein was 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.

2.4.6. 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 after which the remaining missing values were imputed using the K-nearest neighbours (KNN) imputation algorithm (Troyanskaya et al., 2001). 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 (Ritchie et al., 2015) whereafter the Most Likely Ratio (MLR) method was applied for data normalization (Lambert et al., 2013). Peptide peak areas were summed to obtain a protein measurement data frame for further analysis.

2.4.7. 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 (Metsalu

& Vilo, 2015). Heatmap and HCA was performed in the Phantasus R package(Zenkova et al., 2018). 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 one between completely uncorrelated data. Pairwise comparisons were performed using 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, i.e., differences between groups are assumed not to be due to random chance alone at $p<0.05$.

Supervised oPLS-DA (Orthogonal Partial Least Square discriminant analysis) 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 (Zoppi et al., 2021). Briefly, a protein co-expression network was constructed, wherein scale-free topology was established using a softpower (β) of 10. Thereafter modules were established using the dynamic tree cut algorithm. 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 were assessed using Pearson correlation. Statistical significance for modules-trait associations is assumed not to be due to random chance alone at $p<0.05$. Modules with correlation to malting specifications were analysed further using each protein's variable importance in projection (VIP) scores from PLS regressions. The Phantasus R package (Zenkova et al., 2018) was used for matrix visualization and analysis. Gene ontology (GO) term and network enrichment analysis was conducted using ShinyGO v0.741 (Ge et al., 2020) using *H. vulgare* genome as a background; enrichment analysis was calculated based on hypergeometric distribution followed by 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).

2.5. Results

2.5.1. SWATH-MS spectral library generation

Three barley breeding lines were grown in three locations — Toodyay (T), Mingenew (Mi)and Munglinup (Mun), across Western Australia. Barley grain was commercially malted and subjected

to proteome measurement. In total, 1,517 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 2.1A). Samples from each barley line cluster together, indicating the effect of location outweighs the effect of line.

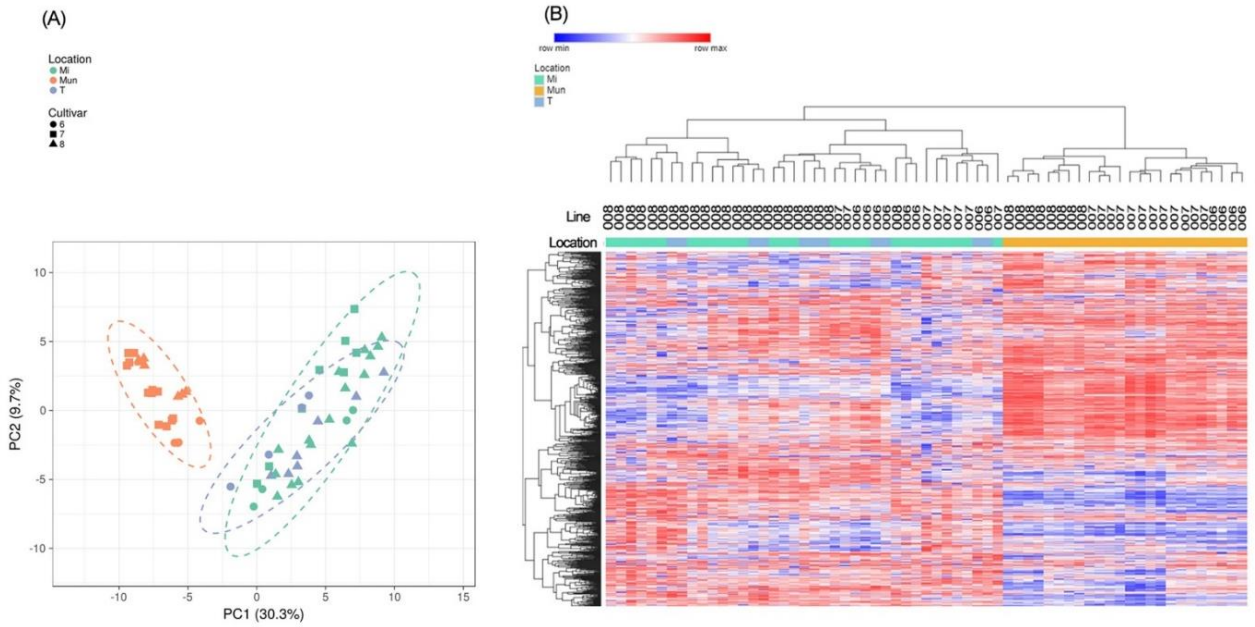


Figure 2. Overview of proteome composition between three barley lines harvested across three locations. (A) The PCA plot shows that the major variance in proteome composition is concordant with growing location (PC1), while the second-highest variance (PC2) is not explainable by barley lines or locations. Shapes represent breeding lines and colours refer to locations. (B) The heatmap depicts relative abundance levels (log10) of all proteins quantified from SWATH data; one minus Pearson correlation metric was used for HCA; colours represent differences in the abundance of proteins in rows; two major sample clusters (column) align with the northern and southern locations; genotypes show some propensity to cluster within these two major groupings.

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 2.1B). This further supports the effect of growing location on proteome composition across the three barley lines as well as highlighting 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 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) displaying the correlation of proteins versus separation between 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.

2.5.2. Relationship between malting specifications and proteome correlation network modules

WGCNA was performed to measure the relationships between the 27 malting specifications (Table 2.2 and 2.3) 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 2.2).

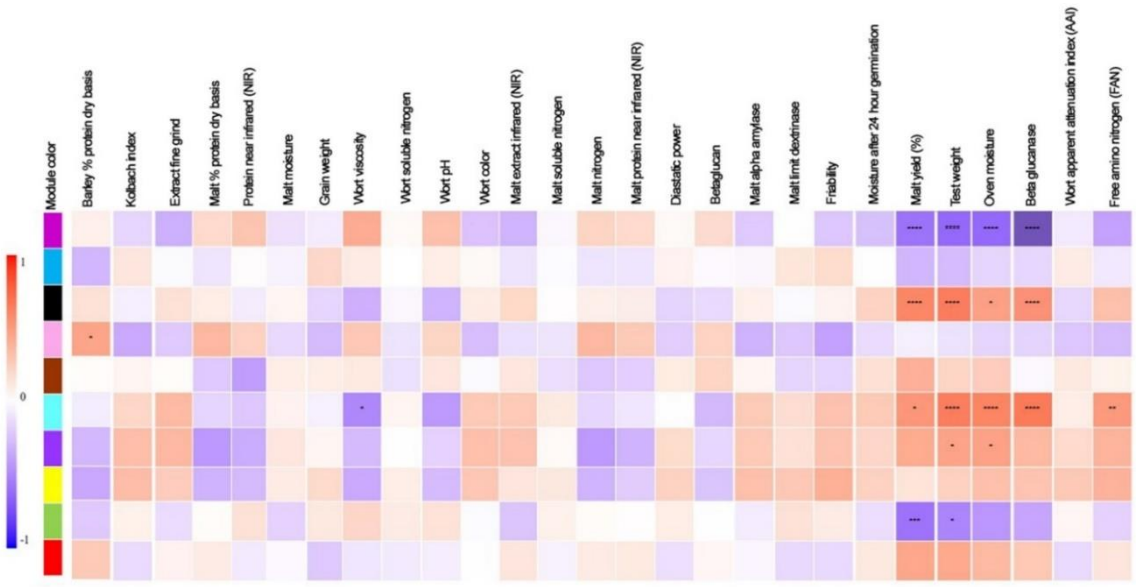


Figure 2.2. Module-trait relationship between malting specifications and barley proteome dataset. The left colour panel shows the 10 modules, and the orange-purple colour scale shows the module-malting specifications using Pearson correlation method to link modules to malting traits, correlation ranging from 1 to -1. Each row corresponds to a module eigengene and is named after a colour, while each column corresponds to a malting trait. The colour of each cell represents the

Pearson correlation coefficient between row and column reflected. P-values obtained from a univariate regression model between the module eigengene (PC1 of relevant protein measurements) and malting traits are shown by asterisk (∗∗∗∗) $p < 0.00001$, (∗∗∗) $p < 0.0001$, (∗∗) $p < 0.001$, and (∗) $p < 0.05$.

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 number of malt traits such as 'malt yield' and beta-glucanase, as well as others including test weight, oven moisture and free amino nitrogen (FAN) (Figure 2.2 and Table S3). Proteins categorized into these modules were more abundant in samples with higher malt yield. Similarly, proteins in the modules magenta and green were significantly negatively associated with these same traits, indicating these proteins are less abundant in samples with higher malt yield Malt yield is defined as the weight of the obtained final dehydrated malt divided by the weight of applied barley seed reported as percentage loss of grain mass during germination in malting procedure (Farzaneh et al., 2017). 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 < 0.001) and negatively correlated with magenta and green modules (p-value <0.00001) (Figure 2.2). Correlation of protein profiles in each module that positively and negatively influence the malt yield trait was undertaken. Overall, 203 proteins were associated with malt yield (Table S2); 82 positively associated and 121 proteins were negatively associated. PCA analysis of these 203 proteins (Figure 2.3A) shows the southern Mun location clustering separately to Mi and T and the three genotypes showing less clustering, similar to Figure 2.1A. In Figure 2.3A, PC1 explains 48% of the separation of samples and the same proteins tend to dominate PC1 and PC2 in Figure 2.1. The comparative analysis for malt yield between the two regions (Figure 2.3B) showed significant differences; with samples grown in the northern regions producing higher malt yield (p-value < 0.05) (Figure 2.3B).

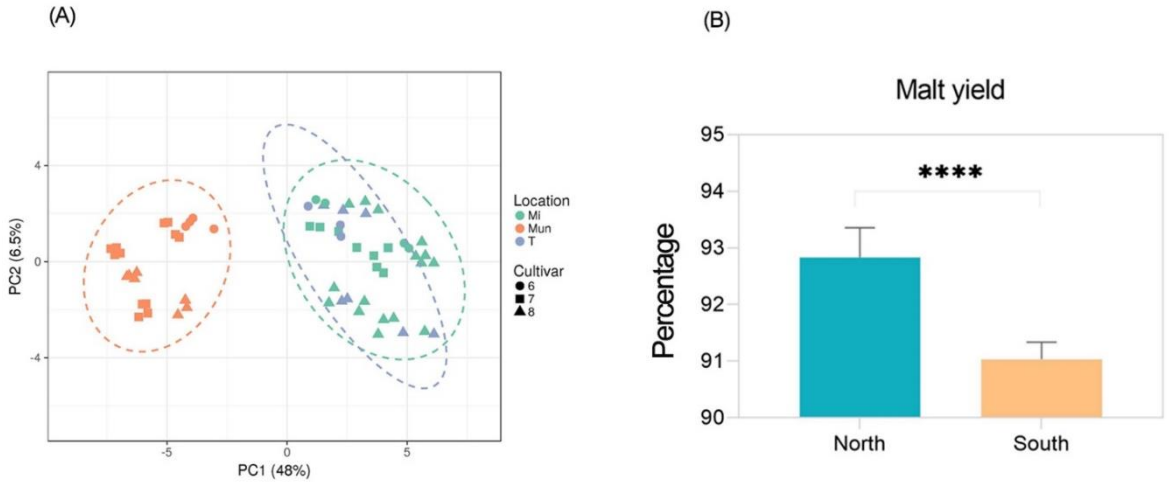


Figure 2.3. Malt yield related protein abundance stratify barley lines by growing location. (A) PCA plot shows the separation of samples according to growing locations using only proteins related to malt yield. Each shape represents one barley line and colours refer to locations. (B) Malt yield is different between the northern and the southern growing locations. (***) $p < 0.0001$ as analysed by unpaired t-test. Error bars show 95% confidence intervals.

Of the 203 proteins that are associated with malt yield, there are several protein groups, including protein inhibitors, enzymes — such as chitinases, beta-amylase, peroxidase, carboxylase, hydrolases, folding and unfolding related proteins. The most significant protein functions are shown in the GO analysis (Figure 2.4). Molecular function GO terms of proteins positively associated with malt yield was related to protein self-association, unfolded protein binding, endopeptidase and peptidase inhibitor and regulator activities, enzyme inhibitor activity and nutrient reservoir activity (Figure 2.4A). Analysis of GO terms (biological process) revealed the molecular processes related to response to hydrogen peroxide, negative regulation hydrolase activity, response to heat stress and response to reactive oxygen species (Figure 2.4B).

GO enrichment analysis of proteins that negatively impacted malt yield endowed with the molecular functions such as chitinase activity, threonine-type endopeptidase, and peptidase activities (Figure 2.4C) and biological processes including protein catabolic process, defence response to biotic stress (fungus) and chitin metabolic process (Figure 2.4D).

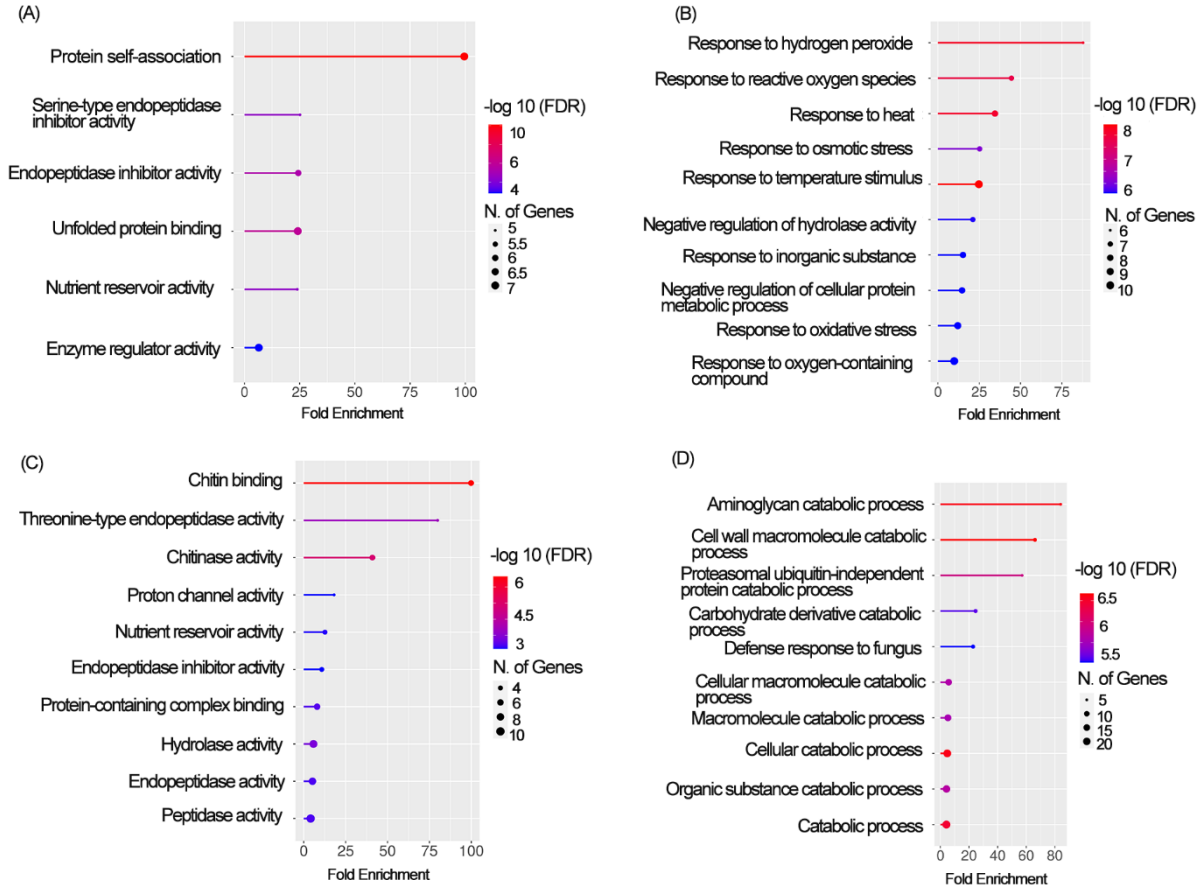


Figure 2.4. Gene Ontology enrichment analysis for genes of proteins that positively and negatively impact barley malt yield. (A) molecular functions of proteins positively related to malt yield. (B) biological process of proteins positively related to malt yield. (C) molecular functions negatively related to malt yield. (D) biological processes of proteins negatively related to malt yield. Colour scales indicate the FDR corrected p-value (<0.05) for each term and fold enrichments define as the percentage of genes related to proteins belonging to a term, divided by the corresponding percentage in the background genes (*H. vulgare* L).

To understand 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 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 location (north vs south) on these proteins were 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 malt yield. It can be concluded that growing

the same lines in different locations impacted protein expression with environmental differences contributing to protein changes (Figure 2.5A). Chitinase showed a significant difference between the two growing regions (higher in southern regions) (Figure 2.5B).

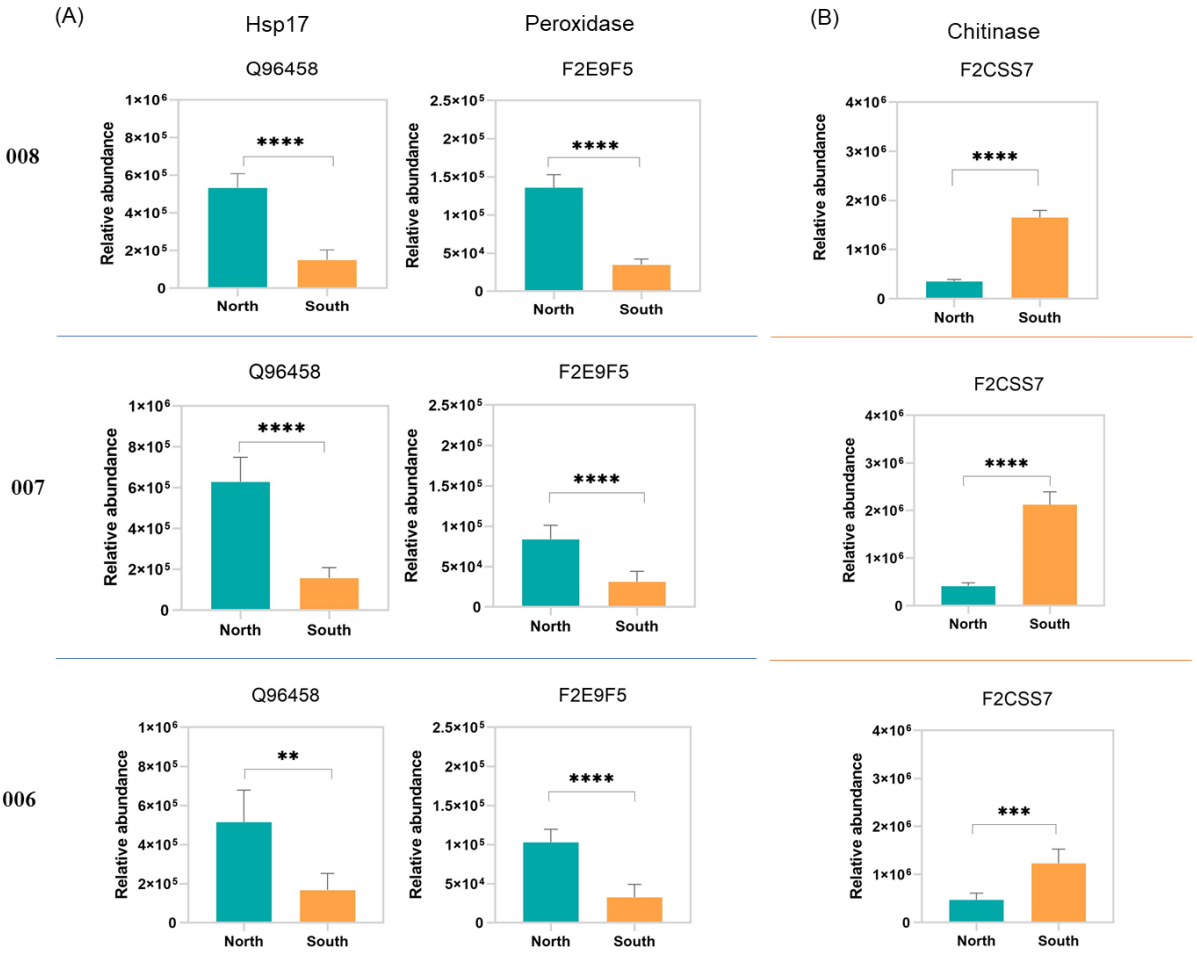


Figure 2.5. Proteins correlated with malt yield are perturbed between locations. (A) positively and (B) negatively correlated proteins are consistently perturbed across the three barley lines between northern and southern growth conditions. (***) p< 0.0001, (**) p< 0.001, and (*) p< 0.05 as analysed by unpaired t-test. Error bars show 95% confidence intervals.

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

To better understand the relationship between protein relative abundance, malt yield and temperature, a 3D scatter plot was created (Figure 2.6), which shows the relative abundance of the

top three proteins that were correlated with malt yield across the accumulated temperature during growing season in 2019. The higher temperature in the northern region averaged for both locations together resulted in higher malt yield and higher abundance of proteins that positively influence malt yield (Figure 2.6A and 2.6B). While for the top protein negatively associated with malt yield, there was a higher abundance of the protein in southern region where the temperature was lower (Figure 2.6C).

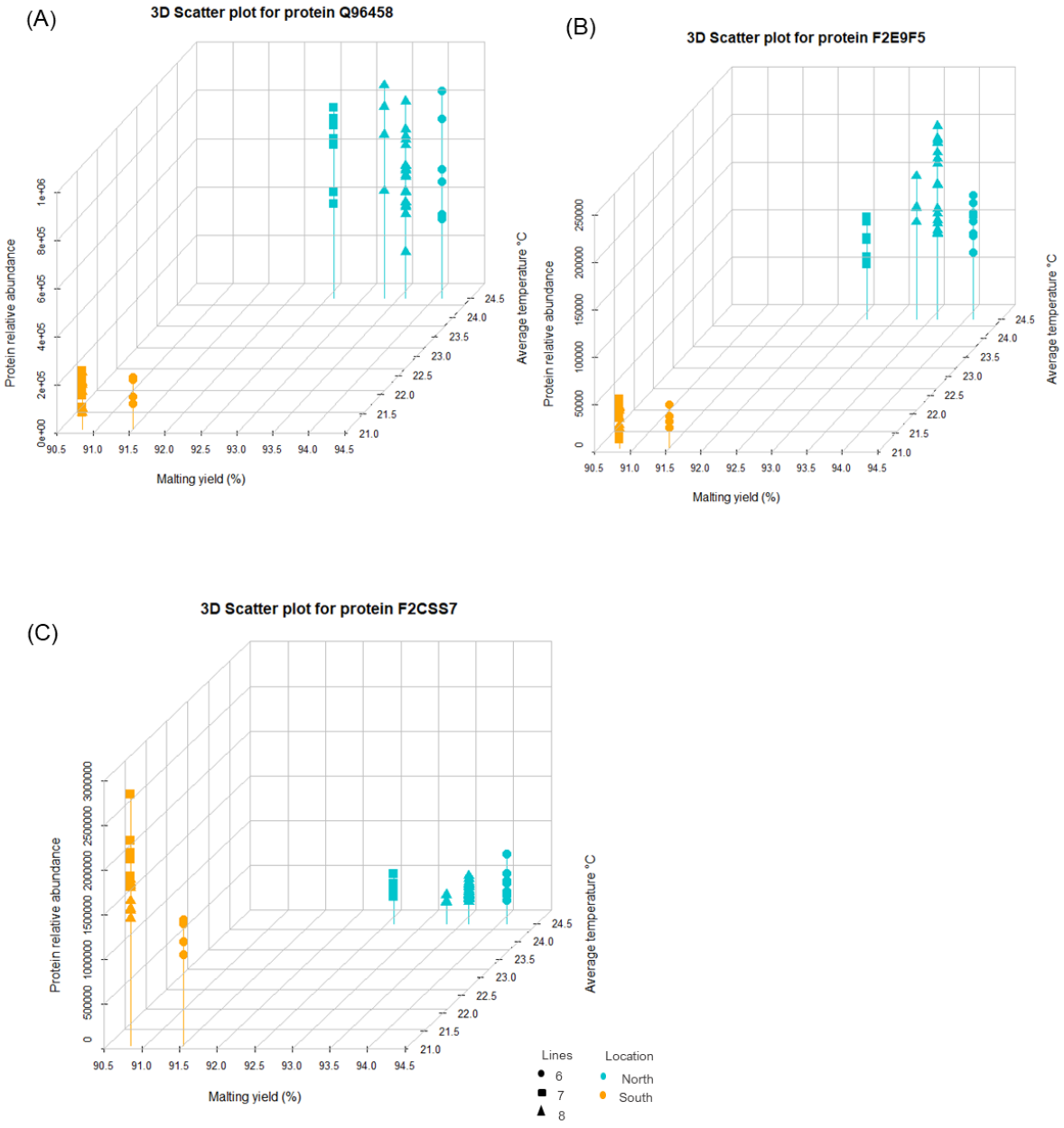


Figure 2.6. The relationship between protein relative abundance, malt yield and temperature. (A) and (B) Two top proteins that positively correlate with malt yield including (Q96458 and F2E9F5),

(C) top protein impacting malt yield negatively (F2CSS7). Shapes represent lines and colours show location.

2.6. 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 phenotype; identify sets of malt yield-related proteins and their functional themes; highlight individual proteins with a strong association to malt yield; and uncover an axis of proteome phenotype, malt yield and environment (Figure 2.6).

By analysing three different barley lines, proteome phenotypes were measured across environments and genotypes. Multivariate and HCA analyses showed that growing location is the stronger factor affecting proteome composition of three experimental barley genotypes. As noted, the samples are grouped according to their growing locations, i.e., northern, and southern regions (Figure 2.1). Our results were aligned with a previous study that investigated the effect of cultivar and environment on wheat proteins quality where environmental factors influenced wheat storage protein quality more than genetic background (Panozzo & Eagles, 2000). In addition, the influence of cultivar and environment on the quality of different Latin American wheat genotypes were studied. This study reported the important portion of variability observed within detected proteins related to wheat quality was influenced by the environment; however, the precise environment parameter that caused positive or negative impact on quality was not reported (Vázquez et al., 2012). In our study, the relationship between malting traits and proteomic data was established using weighted correlation network analysis (Figure 2.2), 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 malt yield, test weight, free amino nitrogen, and beta-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 malting process among all significant correlations. Two modules (black and turquoise labelled) were found to have a positive correlation with malt yield; two modules (magenta and green labelled) showed negative correlation with this trait. Furthermore, proteins in each module that positively and negatively correlated with malt yield were identified and stratification of proteins by growing location was observed (Figure 2.3). Although numerous studies have investigated the effect of environmental

factors such as fertilizer input (mainly nitrogen) or genetics on malt yield (Agu, 2003; Kassie & Tesfaye, 2019; Verma et al., 2003), no studies have linked proteome measurements with malting traits. Malt yield mainly is the result of endosperm starch mobilization to provide the mass of the growing embryo and biochemical energy (Lewis & Young, 2001). 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 malt yield (Verma et al., 2003).

In the present study, GO enrichment analysis revealed that proteins that positively correlated with malt yield trait have a molecular function including protein self-association, endopeptidase inhibitor activity, enzyme regulator activity, unfolded protein binding, and nutrient reservoir activity (Figure 2.4A). These proteins are involved in response to temperature stimulus, heat stress, hydrogen peroxide and reactive oxygen species (Figure 2.4B). A list of proteins that showed positive correlation with malting yield includes HSPs, peroxidases, serpin domain containing proteins, putative ripening proteins, starch synthase enzymes and beta-amylase (Table S2). Among the proteins that positively correlated with malt yield, the top three proteins were selected according to their stronger correlation with this trait. These were serpin domain-containing protein, HSP17 and peroxidase (Figure S2A). HSPs act as molecular chaperones to facilitate protein folding processes and protecting proteins that have been mis-folded or lost their conformation due to biotic or abiotic stresses (ul Haq et al., 2019). These proteins are also involved in protection of enzymes from degradation during malting, and associations with specific malting traits have been reported previously (Kochevenko et al., 2018; Potokina et al., 2004). The HSPs are induced in locations with higher temperature conditions, suggesting that their abundance might help protect plants from heat stress events (ul Haq et al., 2019). 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 (Chauhan et al., 2012). In addition to HSPs, we also identified peroxidases, an enzyme subclass that utilize hydrogen peroxide to oxidize compounds in all cells to avoid plant cell injury under environmental stress (Rasmussen et al., 1997). These proteins are correlated with higher malt yield which these proteins were up-regulated 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 (Laugesen et al., 2007). Peroxidases are vital to seed germination as they can neutralize reactive oxygen species (ROS) which have been induced by abiotic stresses and protecting seed from the subsequent peroxidation damage (Ahmad et al.,

2010). Serpin domain containing proteins possess a conserved reactive centre loop (RCL) domain that is the shared domain among all serpins. Abiotic stresses can cause cell death via vacuolar collapse by the involvement of serpin and protease interaction, for instance in Arabidopsis, overexpression of serpin1 caused lower sensitivity to water stress compared to the wild type (Koh et al., 2016). A recent study also showed that the serpin domain containing protein in hull-less barley seed has been expressed through different stages of development (Zhang et al., 2021).

Barley is an important cereal that is adapted to environments with optimum temperature of 15°C during grain filling; however, in Australian grain belt barley is exposed to high temperature (days above 30°C) (Wardlaw & Wrigley, 1994). The enrichment of high temperature-related proteins in the present study (Figure 2.4B) and consideration of temperature data (Figure 2.6) indicated that locations with higher temperature during grain seed filling increases the abundance of defence-related proteins peroxidase, HSP17, and serpin-domain containing proteins (Figure S2A).

A higher abundance of defence-related proteins suggests that these proteins may induce tolerance or resistance during the temperature-dependent malting process during the germination step when temperature reached up to 22 °C or above. Through the analysis of meteorological data and considering the accumulated temperature (Figure 2.6A and 2.6B), it was observed that the northern regions that revealed a higher abundance of defence 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 2.6B). This result suggests that a lesser degree of germination and consequently less production of root and shoot coupled with 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 2.6A and 2.6B) further strengthen our hypothesis that temperature stress occurrence in northern locations induced tolerance to temperature-dependent germination process during malting.

The top three proteins that negatively correlated with malt yield are related to pathogen defence mechanisms including chitinases and germin-like proteins. These proteins play roles in cell wall function and defence against invading pathogens (Dunwell et al., 2008). Chitinases belong to pathogenesis-related proteins and cleave the glycoside bond of chitin by 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 pathogens attack. It has been suggested that this difference might be related to the rain and

humidity of the growing environment (Jin et al., 2013). Plant endochitinases have antifungal properties and a potential inhibitory effect against fungal pathogens was previously reported in barley (Khan et al., 2019). In Arabidopsis, abiotic stresses, particularly heat stress brought about downregulation of most chitinase genes (Grover, 2012). 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 pathogen attack or hydrogen peroxide are strong signals for germin-like protein subfamilies (Zimmermann et al., 2006). Research on the tea plant (*Camellia sinensis*) also showed that germin-like proteins showed down-regulation in response to rising temperature (Fu et al., 2018). In the present research, it was observed that chitinases have higher abundance in samples that were grown in the southern location and can influence malt yield negatively (Figure 2.6C). 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 cause less 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 malt yield was significantly correlated by changes in the quantitative proteome composition and identified proteins with positive or negative associations to 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 which resulting in less germination thus resulted in higher malt yield. Although the limitation to access to more 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 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 malt yield that may

find utility for maltsters in meeting different brewing requirements. This investigation has delineated a protein – malting specifications – environment axis. The measurement of proteins related to malting quality can readily support breeding or grain testing programs in reaching more consistent seed and product quality.

3. Chapter 3: From grain to malt: tracking changes of ultra-low-gluten barley storage proteins after malting

In the previous chapter, the impact of growing locations on the proteome of different barley genotypes was explored. The research presented in Chapters 3 and 4 primarily focuses on the proteomic changes observed between grain and malted samples of hordein-reduced barley lines. Plant material that was used for the experiments in these next two chapters was previously developed by CSIRO. The research described in this chapter contributed to a better understanding of the grain protein composition of these hordein-reduced barley lines by investigation of the grain proteome and nutritional shifts observed in the experimented hordein-reduced barley lines contrasting malt to unmodified grain. The results of the aforementioned study (Bose *et al*, 2020) were published in the journal of *Frontiers in Plant Science*. My contribution to this research was annotation of the proteomics dataset and performing bioinformatics analysis.

Bose U., Juhász A., Yu R., Bahmani M., Byrne K., Blundell M., Broadbent J. A., Howitt C. A., and Colgrave M. L. (2020). Proteome and nutritional shifts observed in coeliac-friendly hordein double-null barley lines. Frontiers in Plant Science, 12, 718504.

In Chapter 3, the primary aim was to understand the quantitative changes of immunogenic gluten proteins in hordein-reduced barley lines to evaluate and measure the changes of these specific proteins in barley grains after malting by applying MS-proteomics techniques. Moreover, the influence of genetic modification of hordein-reduced barley lines on other storage proteins is explored.

The manuscript presented in this chapter has been published in *Food Chemistry in 2023* under following citation:

Bahmani, M., Juhász, A., Bose, U., Nye-Wood, M. G., Blundell, M., Howitt, C. A., & Colgrave, M. L. (2024). From grain to malt: Tracking changes of ultra-low-gluten barley storage proteins after malting. *Food Chemistry*, 432, 137189.

3.1. Publication and Statement of Contributions

This chapter is drawn from the manuscript that is submitted to Food Chemistry Journal:
Bahmani, M., Juhász, A., Bose, U., Nye-Wood, M. G., Blundell, M., Howitt, C. A., & Colgrave, M. L. (2024). From grain to malt: Tracking changes of ultra-low-gluten barley storage proteins after malting. *Food Chemistry*, *432*, 137189.
Title of paper: **From grain to malt: tracking changes of ultra-low-gluten barley storage proteins after malting**

Mahya Bahmani (candidate)
Prepared the samples, developed processing pipeline for data processing and analysis, conducted data visualisation, interpretation, and drafted the manuscript.

Signatur [redacted]

Date: 29/03/2023

Angéla Juhász
Provided guidance for data analysis, visualisation, and interpretation. Assisted with bioinformatics analysis and reviewed the manuscript.

Signat [redacted]

Date: 30/03/2023

Utpal Bose
Assisted with data visualisation, interpretation and reviewed the manuscript.

Signat [redacted]

Date: 3

Mitchell G. Nye-Wood

Assisted with development of mass spectrometry methodologies, data acquisition and reviewed the manuscript.

Signatu 

Date: 03/04/2023

Malcolm Blundell

Provided samples and reviewed the manuscript.

Signature

Date:16 May 2023

Crispin Howitt

Provided samples and reviewed the manuscript.

Signature

Date:

15/05/2023

Michelle L. Colgrave

Conceived the design of the study. Provided guidance for mass spectrometry data acquisition, analysis, visualisation, and interpretation and reviewed the manuscript.

Signature

Date: 30/03/2023

3.2. Abstract:

Barley (Hordeum vulgare L.) is a major cereal crop produced globally. Hordeins, the major storage proteins in barley, can trigger immune responses leading to celiac disease or symptoms associated with food allergy. Here, proteomics approaches were employed to investigate the proteome level changes of grain and malt from the malting barley cultivar, Sloop, and single-, double- and triple hordein-reduced lines. The triple hordein-reduced line is an ultra-low gluten barley cultivar, Kebari®. Using discovery proteomics, 2,688 and 3,034 proteins in the barley and malt samples were detected respectively. Through the application of targeted proteomics, a significant reduction in the quantity of B-, D-, and γ-hordeins, as well as avenin-like proteins, was observed in the ultra-low gluten malt sample. A compensation mechanism was observed evidenced by increased biosynthesis of seed storage globulins, specifically vicilin-like globulins. Overall, this study has provided insights into protein compositional changes after malting in celiac-friendly barley varieties.

3.3. Introduction

Barley (*Hordeum vulgare* L.) is one of the important crops of the Poaceae family and is used for animal feed and is a raw material for malting, brewing and distilling industries. About 8-10% of barley grain (by weight) is protein. Seed storage proteins in barley include albumins, globulins, prolamins and glutenins (Gubatz & Shewry, 2010). The barley prolamins are called hordeins and can be classified into four major groups according to their molecular weight: HMW glutenin homolog D-hordeins (105 kDa), sulfur-poor C-hordeins (55-65 kDa) and the sulfur-rich B- (50 kDa) and γ-hordeins (35-45 kDa) (Bahmani, O'Lone, et al., 2021). Globulins represent minor storage proteins in barley that can be classified according to their sedimentation coefficients into two main groups: vicilin-type 7S globulins and legumin-type 11S globulins. Both are deficient in methionine and cysteine (Shewry & Tatham, 1990). Avenin-like proteins (ALPs) are encoded by a multicopy gene family also belonging to the prolamin super-family that are considered atypical storage proteins in barley (Juhász et al., 2018).

Hordeins are recognized as triggers for celiac disease (CD), food allergy, and non-celiac cereal sensitivity. Immunological studies have unveiled potential peptide sequences in barley that can stimulate T cells associated with CD (Dale et al., 2019; Tye-Din et al., 2010; Walker et al., 2017). CD is an immune-mediated disease that triggers immune responses upon consumption of cereals with epitope-containing gluten-related sequences (Glissen Brown & Singh, 2018). To date, the gluten-free diet is the only treatment for people with CD to avoid gluten-related sensitivities. Conventional breeding was used to develop an ultra-low gluten barley, now commercially known as Kebari®, that meets the classification threshold (below 20 ppm) of gluten-free products according to the WHO (Tanner et al., 2016). Single hordein-reduced lines lacking B-, C-, and D hordeins were used to develop a suite of single-, double- and triple-hordein reduced lines. Previously, proteome compositional changes in the single and double hordein reduced lines have been investigated using barley grain (Bose, Broadbent, et al., 2020; Bose et al., 2021); however, how malting affects the grain compositional changes in the proteome level of the hordein-reduced lines remained unknown.

Malting is a biotechnological process that starts with seed germination under controlled conditions (Bahmani et al., 2022). Hydrolytic enzymes are expressed and processes in the seed during malting affect the composition of storage proteins and starch, in turn impacting the end product quality. Recently the application of mass spectrometry (MS)-based proteomics in barley research (Bahmani, O'Lone, et al., 2021), in particular label-free targeted quantification, has been used as a reliable

method to investigate proteome changes (Chawade et al., 2016; Domon & Gallien, 2015; Liebler & Zimmerman, 2013).

In the present study, discovery and targeted MS proteomic approaches were employed to explore the effect of malting, as a food processing step, on changes in immune reactive protein classes in barley. The grain proteomes of single-, double-, and triple-hordein-reduced barley lines were compared with the proteomes of the corresponding malt samples.

3.4. Materials and methods

3.4.1. Materials

Creation of hordein-reduced barley lines were previously described by Tanner et al. (Tanner et al., 2016). The single-, double-, and triple-hordein-reduced barley lines and wild-type barley (cv. Sloop) were provided by CSIRO and micro-malted following the same conditions for all lines by the Australian Grain Export Innovation Centre in Perth, WA, in 2019. In this study, barley grain and malt samples of single-, double- and triple-hordein-reduced lines were used for targeted quantitation by LC-MRM-MS experiments.

3.4.2. Protein extraction and digestion

Barley grain and malt sample preparation was performed according to the previously described protocol (Colgrave, Byrne, Blundell, & Howitt, 2016). In brief, after freeze drying, a total of 100 mg of flour was weighed for each of the four biological replicates into 1.5 mL micro-tubes and mixed with 1 mL of 8 M urea and 2% (w/v) dithiothreitol (DTT) in 100 mM Tris-HCl buffer (pH 8.5) to extract proteins. Samples were thoroughly vortexed and sonicated (Soniclean™ Ultrasonic Cleaner 250HD, 650 W, 43 kHz) for 5 min at room temperature. Protein reduction, cysteine alkylation and on-filter digestion steps using trypsin (Promega, NSW, Australia) were performed according to established methods (Colgrave, Byrne, Blundell, Heidelberger, et al., 2016). Digested samples 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, and the combined filtrates were evaporated to dryness in a Savant SpeedVac concentrator (Thermo Fisher Scientific™, MA, USA). Dried peptides were stored at -20°C until analysis, whereupon they were reconstituted in 100 µl of 1% formic acid and iRT reference peptide solution was added to the samples (1 pmol; Biognosys, Zurich, Switzerland).

3.4.3. Data-dependent acquisition (DDA) and protein identification

Digested proteins were analysed as previously described (Bahmani et al., 2022). Briefly the trypsin digests of four biological replicates were pooled and 1 μL was chromatographically separated using an Ekspert nanoLC415 chromatograph (Eksigent™, Dublin, CA, USA) with eluent directed to a TripleTOF 6600 MS (SCIEX™, Redwood City, CA, United States); the analysis method and LC-MS/MS parameters were the same as described previously (Colgrave et al., 2017). Gas phase fractionation (GPF) was used to maximize proteome coverage in DDA data collection (Kennedy & Yi, 2008), where two iterative injections recorded MS1 scans over the mass range 350 – 595 *m/z*, followed by one over 585– 1250 *m/z*. MS1 accumulation time was 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. Dynamic ion exclusion of precursors was set to 15 s after two acquisitions with the mass tolerance of 100 ppm.

ProteinPilot v5.0.3 software (SCIEX, Redwood City, CA, United States) encompassing the Paragon Algorithm was employed for protein identification for peptide spectrum matching and scoring and the ProGroup algorithm for conservative protein inference and grouping (Shilov et al., 2007). The DDA data were searched against a sequence database that included *Hordeum vulgare* proteins from the UniProt-KB [227,490 total entries accessed on 17/06/2022] supplemented with proteins listed on the Common Repository of Adventitious Proteins (thegpm.org/crap) as well as Biognosys iRT pseudo-protein sequence. Search parameters were included iodoacetamide modified with cysteine alkylation, modification set to “biological” as provided with the Protein Pilot software, results were produced at 1%FDR and zero missed cleavage was selected.

3.4.4. Targeted Proteomics

As a result of protein identification by GPF-DDA, 16 hordeins and 4 globulins were detected. Using CLC Main Workbench v20.0.4 (Qiagen, Denmark), hordein-type specific peptides were identified and unique peptides to each hordein-type were selected. Tryptic peptides were chromatographically separated on an Exion LC-40AD UHPLC system (SCIEX, Redwood City, CA, United States) and analysed on a 6500^{+} QTRAP mass spectrometer (SCIEX, Redwood City, CA, United States). To build the methods, a FASTA file containing all hordein and globulin proteins identified was imported into Skyline (MacLean et al., 2010) and tryptic peptides between 6-30 amino acids long were selected. The three most intense transitions at the expected retention

time without interference with a signal-to-noise (S/N) ratio >5 were used in the scheduled MRM method for quantitation. For hordein type-specific groups, 39 unique peptides and 117 transitions mapped to 16 protein accessions and were targeted in one method. Four globulin proteins were targeted in a separate method, including 21 peptides and 63 transitions.

Tryptic peptides were chromatographically separated on an Exion LC-40AD UHPLC system (SCIEX) and analysed on a 6500+ QTRAP mass spectrometer (SCIEX). A 30 s detection window was used for each MRM transition, the hordein method used a 0.4 s cycle time, and the globulin method used a 0.6 s cycle time. According to the results of the DDA, 4 globulin and 16 hordein proteins were identified; for quantitation of hordein peptides, a total of 16 proteins, including B-hordeins, γ-hordeins, C- and D-hordein and ALPs, with 39 peptides and 117 transitions overall were quantified. A total of 4 proteins with 21 peptides and 63 transitions were quantified for globulins quantification. Data files were collected for four technical replicates of each sample and the total peak areas were used to assess relative peptide abundance and graphed using R software (v. 2022.12.0) (Team, 2021).

3.4.5. Statistical analysis

Unsupervised Principal Component Analysis (PCA) was performed to detect outliers and evaluate relationships in the samples with R software (v. 2022.12.0) using the ggfortify package (Tang et al., 2016). Heatmap and hierarchical clustering analysis (HCA) was performed and visualized using Phantasus (Zenkova et al., 2018). Pairwise comparisons were performed in R (Team, 2021). A p-value of less than 0.05 was deemed as significant, i.e., differences between groups are assumed not to be due to random chance alone at $p<0.05$.

3.5. Results

3.5.1. Discovery proteomics using data-dependent acquisition

In the present study seven hordein-reduced barley lines and corresponding malt samples including single-, double- and triple-hordein-reduced lines as well as wild type (WT) malting barley line, Sloop, were subjected to discovery and targeted proteomic analysis. In discovery proteomics, we identified 2,688 proteins in the barley and 3,034 proteins in the malt samples at 1% false discovery rate (FDR). From these proteins, 16 were ALP-, C-, B-, D- and γ-hordeins (Table S4), and 4 were globulin proteins. As the C-hordeins are not digested efficiently by trypsin because of the low

number of Lys and Arg residues in their sequence, C-hordein peptides were excluded from the downstream analysis.

3.5.2. Targeted proteomics

Hordeins that were detected in at least one genotype of grain or malt were categorized into six type-specific groups: ALPs, B-hordeins, C-hordeins, D-hordeins, γ-hordeins, and globulins. The peptides quantified by sMRM were aligned to these proteins in CLC Main Workbench v20.0.4 (Qiagen, Denmark), and those that were specific to one group were used to assess the relative abundance of that protein group (details in Tables S4 and S5). Peptide abundance heatmaps and HCA revealed two major clusters of samples (Figure 3.1).

The barley lines that have the C-hordein reduced line in their pedigree were clustered together on the left side of the heatmap (Figure 3.1A) and showed different patterns of hordein and globulin content. The hordein abundance (B- and γ-) was generally lower in malt and grain from samples that crossed with C-hordein-reduced line, while globulins showed the opposite pattern and were generally higher. Unsupervised PCA showed that samples are stratified by type of hordein-reduced lines, wherein PC1 (hordein-reduced line component) and PC2 together explain 83% of the variation in the dataset (Figure 3.1B). There is clustering of genotypes, especially those that do not have C-hordein reduced background. The grains from CD-reduced lines are different from their malt sample. The CD- and BC-reduced malt are closely clustered together. Additionally, in the PC2 although the WT, B- and D-reduced grain and malt samples clustered in the same coordinate, there is a within-class variation. For example, the B- and D-reduced grain and malt shows similarity based on the measured protein abundance, whereas WT grain, malt and B-reduced malt clustered together.

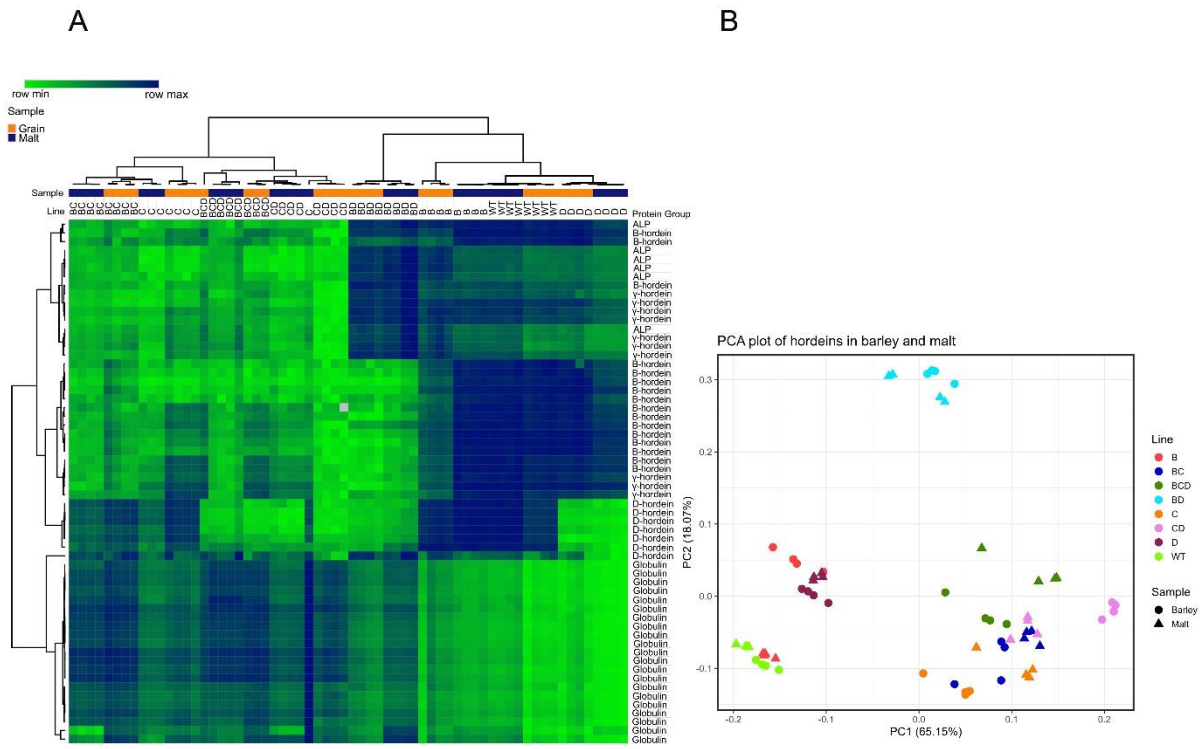


Figure 3.1. Hordein and globulin peptide abundance quantified across WT and hordein-reduced lines in barley grain and malt samples. A) The heatmap depicts relative abundance levels (log10) of all peptides quantified from sMRM data. One-minus Pearson correlation metric was used for HCA; colours represent differences in the abundance of peptides in rows. B) The PCA plot shows the quantified peptides of hordeins for barley and malt samples. It highlights differences between sample types (barley grain and malt) and clusters of hordein-reduced lines. Shapes represent samples and colours refer to lines.

3.5.3. Changes in hordein abundance after malting

For each line, the peak area of each quantified peptide in grain was subtracted from its peak area in the corresponding malt to calculate the change in abundance. The net change for the different hordein types was then calculated by finding the overall change for all peptides mapping to B-hordeins, γ-hordeins, D-hordeins, and ALPs, respectively. Changes in abundance of each protein group are presented in Figure 3.2. In WT, C-, D-, BC-, and BCD-reduced lines all hordein types were less abundant in malt than in barley. In the BD-reduced line the same trend was seen, though lesser in magnitude. However, in B- and CD- lines, and, to a lesser extent, the BD-reduced line,

there are some hordein type-specific patterns (Figure 3.2). In all three cases the B-hordein peptides showed a higher abundance in malt than barley.

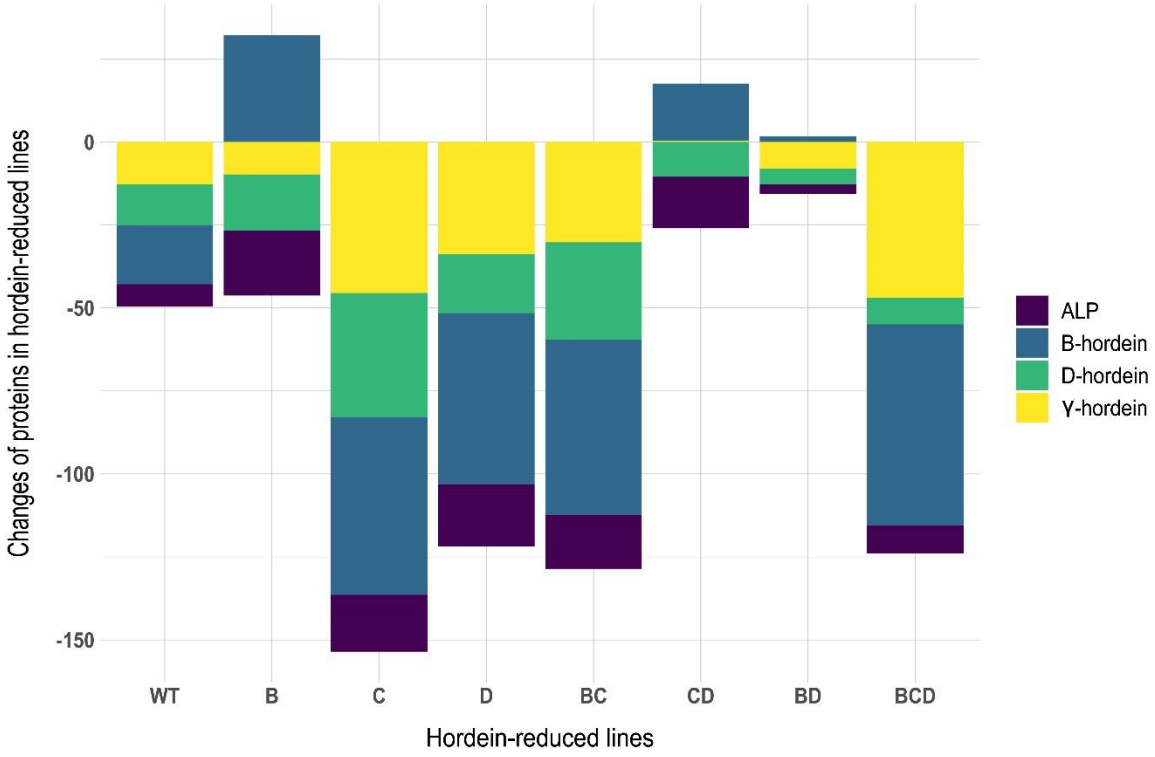


Figure 3.2. Relative changes in peptide abundance classified into protein groups (B-, D- and γ-hordeins in addition to ALP) across hordein-reduced barley lines and corresponding malt samples. Each colour represents an individual protein group. Negative changes demonstrate higher abundance of protein in grain samples compared to malt.

To further explore how malting affects each protein group in these different lines, grain samples were compared to their corresponding malt samples for each line (Figure. S5-S8). When this is done for B-hordein peptides, our results revealed that B-hordeins were further reduced significantly after malting in the C-, D-, BC-, CD-, and BCD-reduced lines (Figure. S5). The opposite trend was observed for B- and CD-reduced lines, wherein the abundance of B-hordeins was significantly higher after malting. The WT and BD-reduced lines did not show significant differences ($p<0.05$) between grain and malt samples for B-hordeins (Figure S1). Examining γ-hordein content before and after malting showed this protein group is significantly reduced in the WT, B-, C-, D-, BC-, and BCD-reduced lines after malting. No difference was observed between grain and malt samples in BD- and CD-reduced lines (Figure S7). In all lines except BD-reduced and WT, ALP abundance was significantly lower in malt than grain (Figure S8) and showed the same trend as B-hordein.

Monitoring D-hordein revealed a significant decrease from grain to malt samples in the WT, B-, C-, D-, BC-, CD- and BCD-reduced lines, though in the BD-reduced line it was not noted (Figure S8).

Previous research on the hordein-reduced barley lines suggested that, in barley, the reduction of hordeins can result in the increased accumulation of other storage proteins like globulins (Bose, Broadbent, et al., 2020). Four globulins were detected in this investigation, including A0A8I6X8S0 (vicilin-type), A0A8I7BA12 (vicilin-type), F2CYL7 (vicilin-type), and A0A8I6Y3C5 (vicilin-type). To test for a potential compensatory expression of globulins in the hordein-null barley and malt, these globulins were targeted for quantification. These four globulins were significantly more abundant in the BCD-, C-, BC-, and CD-reduced lines showed significantly higher abundance than WT, in both grain and malt (Figure 3.3).

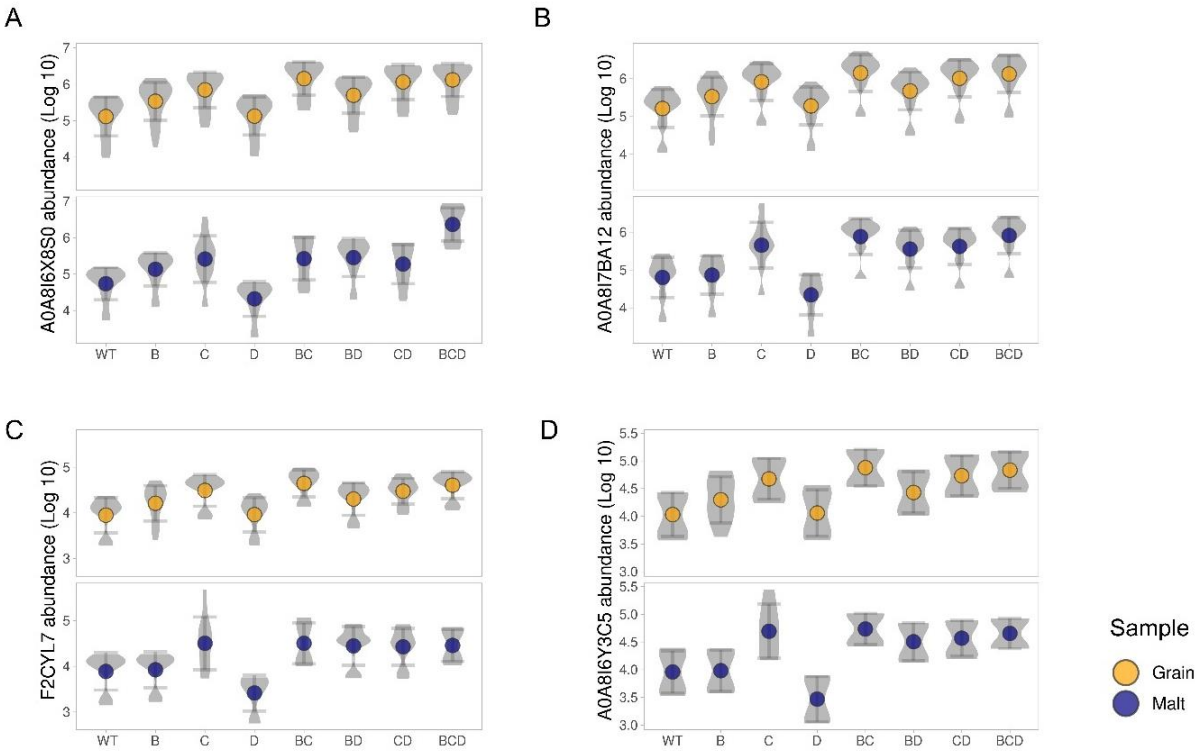


Figure 3.3. Relative abundance of the four detected globulin proteins in grain and malt for all lines assessed. Peak area of all quantified peptides mapping to A) A0A8I6X8S0, B) A0A8I7BA12, C) F2CYL7 D) A0A8I6Y3C5 were log10 transformed (n=4) and averaged to give a measure of relative protein abundance. Circles show mean values and colours refer to sample type (grain or malt).

To explore whether the compensation effect was evident in malt from Kebari grain, globulin protein abundance in the triple hordein-reduced BCD-reduced line was compared to the WT (Figure 3.4). The abundance of these globulins was significantly higher in the BCD-reduced line, which is the opposite trend to the hordein proteins in this line.

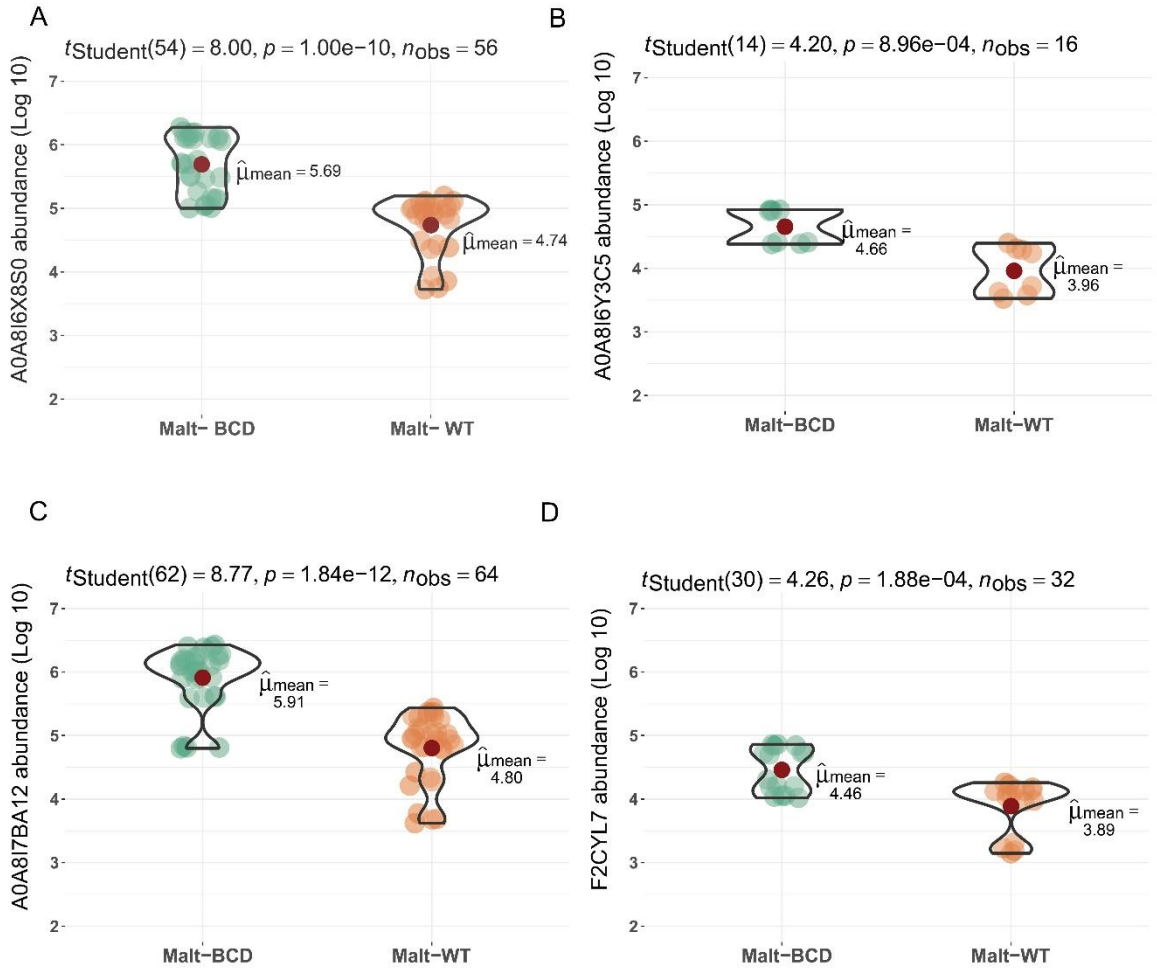


Figure 3.4. Relative quantitation of globulin proteins in malt from the triple hordein-reduced line (the BCD line) alongside wild type. A) A0A8I6X8S0, B) A0A8I7BA12, C) F2CYL7, D) A0A8I6Y3C5. Peak area of all peptides for each protein group are log10 transformed. Pairwise comparisons were performed using Student's t-test (n=4). Red dots show means.

3.6. Discussion

The present study investigated the impact of malting on key storage proteins in barley grain and malt, and examined a triple-hordein reduced line as well as its' double and single hordein reduced

ancestors and WT. Hierarchical clustering showed that grain and malt samples from the C-hordein reduced line clustered separately from samples lacking the C-hordein reduced line in their genetic background and showed similar hordein and globulin accumulation patterns in the experimented barley grain and malt samples (Figure 3.1A). This result corroborates a previous study that measured hordein content in barley samples from these hordein-reduced barley lines (Colgrave, Byrne, Blundell, Heidelberger, et al., 2016). It appears that the genetic background of the C-hordein-reduced lines is associated with a different composition of storage proteins. In the experimented lines, the C-hordein-reduced line (Risø 1508) is a result of a mutation in the transcription factor PBF (prolamin binding factor), which is expressed in the starchy endosperm, embryo, and aleurone during seed development and germination, and directly affects the expression of B- and C-hordeins (Moehs et al., 2019). This mutation of PBF caused the cysteine-rich hordeins to be down-regulated and consequently, lines including this mutation in their pedigree are more susceptible to digestive enzymes during malting compared to those lines that do not contain this mutation in their backgrounds. Due to the mutated TF, the genetic background of this C-hordein-reduced line differs from the WT, having reduced-amounts of two types of hordeins (B and C) and all the lines that clustered with it had this line in their pedigrees (Bose, Broadbent, et al., 2020).

In previous studies on single and double hordein-reduced lines, relative quantification of hordeins by SWATH-MS showed that the proteome profiles of WT and D-reduced lines were closely related and the reason for this similar pattern is that D-hordein-reduced line originated from an Ethiopian landrace (R118) and is a natural D-hordein null. A stop codon was identified at position 151 in the D-hordein mature sequence resulting in the low-level expression of a truncated D-hordein variant. The mutant containing line was backcrossed with WT (Colgrave, Byrne, Blundell, Heidelberger, et al., 2016). Interestingly, in the same study the C-hordein-reduced line showed an opposite pattern to the WT (Bose, Broadbent, et al., 2020; Bose et al., 2021). The D-hordein reduced line possesses the complete B-hordein locus and the active PBF (Tanner et al., 2016), and the B- and C-hordeins are expressed in a similar amount to the WT indicating fully functional transcriptional machinery, which is why proteome composition and changes of the D-hordein reduced line are more like WT than other single null lines. The B-hordein-reduced line possesses a chromosomal deletion in the B-hordein locus of chromosome 1H, which led to a decreased accumulation of B-hordeins.

One of the interesting results of the present study is the significant reduction of B-, γ- and D-hordeins in addition to ALPs in the BCD-reduced malt sample compared to the unmalted grain (Figure S5G, S6G, S7G, S8G). The ULG grain sample has reduced amounts of all gluten proteins,

known to be recalcitrant to digestion, which implies this line could be more susceptible to digestive enzymes during malting and hydrolytic enzymes further digest gluten proteins. Also, this observation could imply that hydrolytic enzymes make up a larger fraction of the proteome and result in more digestion of proteins on the ULG line which warrants further investigation. Similar effects of malting were seen on the remaining B-hordeins in the C-, D-, BC-reduced lines.

Among all hordein types, the D-hordeins are digested more extensively than B- and C-hordeins during malting (Flodrová et al., 2012) which explains the significant reduction of this hordein in most hordein-reduced malt samples including B-, C-, D-, BC-, CD- and WT (Figure S8). The significantly lower B- and γ-hordein content in malt samples compared to grain might be related to the structural homology of these two protein types and their location in the sub-aleurone layer of the outer endosperm (Chandra et al., 1999; Davies et al., 1993), which makes them more accessible for proteases. It has been reported that a well-modified malt contains less hordeins compared to the mature grain (Gupta et al., 2010), and according to our results, we observed a further reduction of all gluten proteins abundance after malting.

While B- and CD-hordein-reduced lines showed higher abundance of B-hordeins in malt than in grain (Figure 3.2), this could be related to the increased liberation of peptides of B-hordeins in these lines during digestion which needs further exploration. Both γ- and B-hordeins exhibit a significant sequence homology, and they both possess conserved cysteine residues within their sequences (Gubatz & Shewry, 2010). Similar to LMW-glutenins and gamma-gliadins in wheat (Juhász et al., 2018), they are regulated by the same transcriptional machinery (Moehs et al., 2019). Therefore, the expression patterns of these two hordein groups was expected to be similar due to their common origin and the conserved regulatory machinery, namely PBF. In previous research on these hordein-reduced barley lines, two ALPs (F2EGD5 and M0VEH1) were identified which share homology with γ-hordeins while being smaller (~17 kDa), and containing a higher glutamine content than other gluten proteins (Tanner et al., 2019). According to our results, malting impacted the abundance of quantified ALP proteins in most of hordein-reduced lines (Figure S7); ALPs play a role in malting process by serving as a source of nitrogen for the growing plant (Zhang et al., 2018). However, there have been reports of ALP detection in malt products including wort and beer (Colgrave et al., 2012; Iimure et al., 2015), which suggests that these proteins are resistant to digestion during malting. A longer germination time or an altered malting regime might lead to more digestion of these resistant proteins.

Storage proteins are major macronutrients of the barley seed and hordeins are not the only storage proteins; globulins represent storage proteins in barley and are mainly expressed in the endosperm embryo and outer aleurone layers (Shewry & Halford, 2002). In previous studies on the hordein-reduced barley lines, it has been suggested that their expression is increased to compensate for suppressed hordein expression (Bose, Broadbent, et al., 2020). The observations of the present study are consistent with this, as we observed a lower hordein content in the BCD-reduced line than WT, and globulin abundance was higher (Figure 3.4) (Bose et al., 2020). Compensation response in plants can be defined as changes in RNA or protein levels that can functionally compensate for the loss of function of another gene or protein (El-Brolosy & Stainier, 2017). This response has been reported in previous studies, for instance, an RNA interference approach was applied to silence α-gliadin genes in hexaploid wheat, and it was found that the lack of α-gliadins was compensated for by an increase in globulins (Becker et al., 2012). Furthermore, it has been reported that downregulation of α-gliadins was associated with the compensation mechanism and caused an increase in globulin abundance (Wieser et al., 2007). Plants express storage proteins in the developing seed according to their genetic background, and if some are absent then there may be compensatory expression of others. Here, our results suggest that the compensation effect occurred to maintain the balance of overall N-rich reservoir accumulation in lines with reduced hordeins. In the barley grain, the major storage proteins in the aleurone layer are represented by 7S globulins (Heck et al., 1993) and the function of globulins in cereals are associated with structural and metabolic activities. Our results of this study showed that the genetic background of the triple hordein-null lines is associated with a lower hordein content, higher globulin protein content, and a lower abundance observed after malting.

3.7. Conclusions

Our investigation shows the impact of malting on hordein-reduced barley lines and demonstrates the utility of targeted proteomics in exploring allergen content in food processing. By applying LC-MRM-MS, gluten and globulin peptides were quantified and changes in abundance of key storage proteins were measured in hordein-reduced barley lines. The reduced hordein content was associated with a higher globulin content, demonstrating that when hordeins, the major protein group in barley grain, are not expressed, the plants try to adapt to the new condition by reprogramming the storage protein synthesis. Moreover, after triple-null hordein barley lines are malted, the residual hordein content is further reduced compared to the initial barley grain. These investigations of hordeins and globulins in barley grain and malt provide insights into the altered

proteome composition arising from malting in hordein-reduced barley varieties. The findings of this study not only corroborate previous studies but demonstrate that malting further decreases the hordein content. Notwithstanding these results, further research is required to understand the immunogenic potential of these malted products in addition to the future products obtained from these modified malt grains for people with gluten-related disorders.

4. Chapter 4: Proteome changes resulting from malting in hordein-reduced barley lines

In the preceding chapter, the impact of malting on the abundance of specific storage proteins in hordein-reduced lines was explored. The current chapter focuses on the final aim of the thesis: to understand how efforts to breed a barley variety with lower hordein content impact on the broader proteome, both in barley and their corresponding malt. This builds upon the storage protein composition measurements in Chapter 3 and compares quantitative proteomic profiles from the various ancestral lines to the triple-hordein reduced line.

The manuscript presented in this chapter is published in the *Journal of Food and Agriculture Chemistry* in 2023.

4.1. Publication and Statement of Contributions

This chapter is drawn from the manuscript that is published in *Journal of Food and Agriculture Chemistry* under the following citation:

Bahmani, M., Juhász, A., Bose, U., Nye-Wood, M. G., Blundell, M., Howitt, C. A., & Colgrave, M. L. (2023). Proteome Changes Resulting from Malting in Hordein-Reduced Barley Lines. *Journal of Agricultural and Food Chemistry, 71* (38), 14079-14091.

Title of paper: **Proteome changes caused by genetic modification in hordein-reduced barley grain and malt.**

Mahya Bahmani (candidate)
Prepared the samples, developed processing pipeline for data processing and analysis, conducted data visualisation, interpretation, and drafted the manuscript.

Signatu[redacted]

Date: 29/03/2023

Angéla Juhász
Provided guidance for data analysis, visualisation, and interpretation. Assisted with bioinformatics analysis and reviewed the manuscript.

Signatur[redacted]

Date: 30/03/2023

Utpal Bose
Assisted with data visualisation, interpretation and reviewed the manuscript.

Signature: [redacted]

Date: 31/03/2023

Mitchell G. Nye-Wood

Assisted with development of mass spectrometry methodologies, data acquisition and reviewed the manuscript.

Signatu

Date: 03/04/2023

Malcolm Blundell

Provided samples and reviewed the manuscript.

Date: 16 May 2023

Crispin Howitt

Provided samples and reviewed the manuscript.

Signature

Date:

15/05/2023

Michelle L. Colgrave

Conceived the design of the study. Provided guidance for mass spectrometry data acquisition, analysis, visualisation, and interpretation and reviewed the manuscript.

Signature

Date: 30/03/2023

4.2. Abstract:

Hordeum vulgare L., commonly known as barley, is primarily used for animal feed and malting. The protein content and compositions in barley seeds can affect the grain and the malting quality. Major storage proteins in barley are hordeins, known triggers of 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, we detected 2,688 and 3,034 proteins were detected from the grain and malt samples, respectively. By utilizing label-free relative quantitation through SWATH-MS, a total of 2,654 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. Up-regulated 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 to understand the regulation of protein compositional changes during the malting process, we uncovered genetic background-related changes in the proteome after malting in hordein-reduced barley lines.

4.3. Introduction

Barley (*Hordeum vulgare* L.) is one of the oldest cereal crops from the ancient agricultural system (Badr et al., 2000). 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 (Bahmani et al., 2022). 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) (Tanner et al., 2019). The hordeins from barley can trigger adverse immune responses in people that suffer from CD. Upon ingestion of barley, the patients show various symptoms like gastrointestinal discomfort, respiratory problems, and skin rashes (Tanner et al., 2014; Wieser & Koehler, 2008). 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 (Tanner et al., 2016). 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 (Tanner et al., 2016). 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 composition.

Malting involves three main steps: steeping, germination, and kilning. During the steeping step, barley grains are soaked in water to start the germination processes. 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 (Gupta et al., 2010). Various techniques have been employed to study how malting affects grain protein composition and the different types of storage proteins (Dai et al., 2014; Flodrová et al., 2012; March et al., 2012a; Strouhalova et al., 2019). 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 (Bose, Broadbent, et al., 2020; Bose et al., 2021; Colgrave, Byrne, Blundell, Heidelberger, et al., 2016). 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 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.

4.4. Materials and methods

4.4.1. Plant material

Previously Tanner et al. (2016) described the process of creating barley lines with reduced levels of hordein (Tanner et al., 2016). 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 hours, followed by 8 hours of air rest, then 3 hours of wetting, 4 hours of air rest, and finally 1 hour of wetting. Germination was allowed for 96 hours, with 48 hours at 18°C and another 48 hours at 16°C, while maintaining the moisture level at 46% after 24 hours. The kilning process included 2 hours at 45°C, 3 hours at 50°C, 4 hours at 55°C, 3 hours at 60°C, 3 hours at 65°C, 3 hours at 70°C, 2 hours at 75°C, and 4 hours at 80°C. Lastly, any malt rootlets were eliminated using a specially designed deculming machine. Malting specification of experimented lines is demonstrated in Table 4.1 and 4.2.

Table 4.1. Malting specifications for barley lines (part 1).

Line	WT	D-B-reduced	D-reduced	B-reduced	B-C-D-reduced	C-reduced	D-C-reduced	B-C-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 hrs ex. Steep (%)	45.9	48.1	45.2	46.6	54.9	50.1	51.3	53.2
Moisture after 24 hrs germ. (%)	45.1	47.3	44.3	45.5	54.4	49	50.5	52.6
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

Table 4.2. Malting specifications for barley lines (part 2).

Line	WT	D-B-reduced	D-reduced	B-reduced	B-C-D-reduced	C-reduced	D-C-reduced	B-C-reduced
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

4.4.2. Protein extraction and digestion

Barley grain and rootlet-removed malt samples were prepared for proteomics analysis as described by Bahmani et al. (Bahmani et al., 2022). 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 maximal 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 minutes. 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) (Bose, Byrne, et al., 2019). 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 peptide solution was added to the samples (1 pmol/µL; Biognosys, Zurich, Switzerland) for further proteomic analysis.

4.4.3. 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), where previously-established analysis methods were used (Colgrave et al., 2017). Gas phase fractionation was employed for DDA data collection (Kennedy & Yi, 2008), 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 (Shilov et al., 2007). The DDA data was 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. Search parameters were included iodoacetamide modified with cysteine

alkylation, modification set to “biological” as provided with the Protein Pilot software, results were produced at 1%FDR and zero missed cleavage was selected.

4.4.4. Data-independent acquisition by SWATH-MS

Samples were divided into two batches and analysed 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 (PBQC) sample was prepared by combining the pooled replicate samples and was injected at the beginning and intermittently throughout each batch.

4.4.5. 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 (Demichev et al., 2018). 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 FDR threshold of 1%. Only fully tryptic peptides with a length of 7 to 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.

4.4.6. Data analysis

Raw peak area data points were imputed using the K-nearest neighbors (KNN) imputation algorithm employing MetImp 1.2 (Lazar, 2015). These measurements were used as input to remove batch effects using the Limma R package (Ritchie et al., 2015) whereafter the Most Likely Ratio

(MLR) method was applied for data normalization (Lambert et al., 2013). Peptide peak areas were summed to obtain a protein measurement data frame for further analysis.

4.4.7. Statistical analysis and data visualization

The results of identified proteins were visualized by upset plots using the UpSetR package (Conway et al., 2017). 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 (Team, 2021). Heatmap and HCA were performed in the Phantasus R package (Zenkova et al., 2018). 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, and to visualize the differentially expressed proteins, we utilized the DEBrowser tool and generated MA plots (Kucukural et al., 2019), 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 (Zoppi et al., 2021). In summary, to establish the relationship between protein expression, genetic background, and sample type (barley grain or malt), a protein co-expression 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 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 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 (Team, 2021; Zoppi et al., 2021). Violin-box plots of top positively and negatively correlated proteins were visualized using the ggplot2 R package (Wickham, 2011).

The process of gene ontology (GO) term and network enrichment analysis was carried out using agriGO (Tian et al., 2017) and ShinyGO v0.741 (Ge et al., 2020); 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.

4.5. Results

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

4.5.1. Discovery proteomics using DDA

Using discovery proteomics, we identified 2,688 proteins in barley grain and 3,034 proteins in barley malt samples (combined 3,495 proteins) at 1% FDR (Figure 4.1). The UpSet plots show that out of these proteins, 1,255 and 1,194 were common to all barley (Figure 4.1A) and malt (Figure 4.1B) samples, respectively. Further analysis revealed ~ 64% overlap (2,227 proteins) between the identified proteins of grain and malt. Additionally, there were 461 proteins unique to grain and 807 proteins unique to malt (Figure 4.1C).

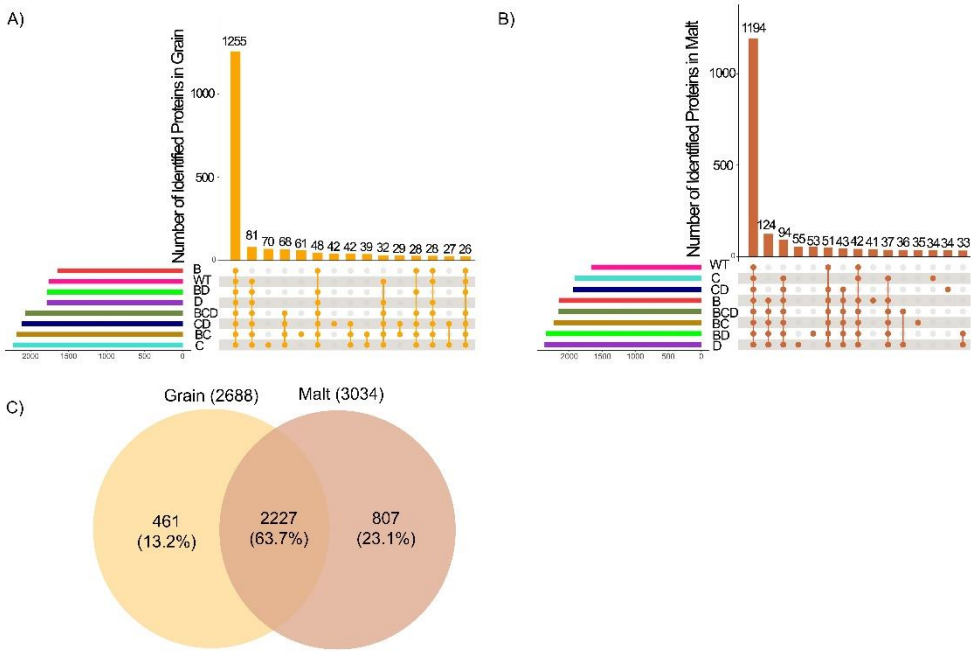


Figure 4.1. Analysis of identified proteins in barley grain and malt in hordein-reduced barley lines. A) Upset plot of all identified proteins for the top fifteen most frequent combination in the hordein-reduced lines for the grain samples. Rows represent each hordein-reduced line and columns depicts 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.

Upon analyzing the results depicted in Figure 4.1, 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 4.3. 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+) activity. 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.

Table 4.3. Most significant terms for identified unique proteins in grain and malt samples based on Figure 1.

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

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 4.2). The most significantly enriched terms resulting from the GO analysis for molecular function from the unique malt samples were hydrolase activity, peptidase, peroxidase, and oxidoreductase activities. Amylases and dextrinase were identified from barley grain and malt samples, a list of identified enzymes with their accession has been provided in Table S6.

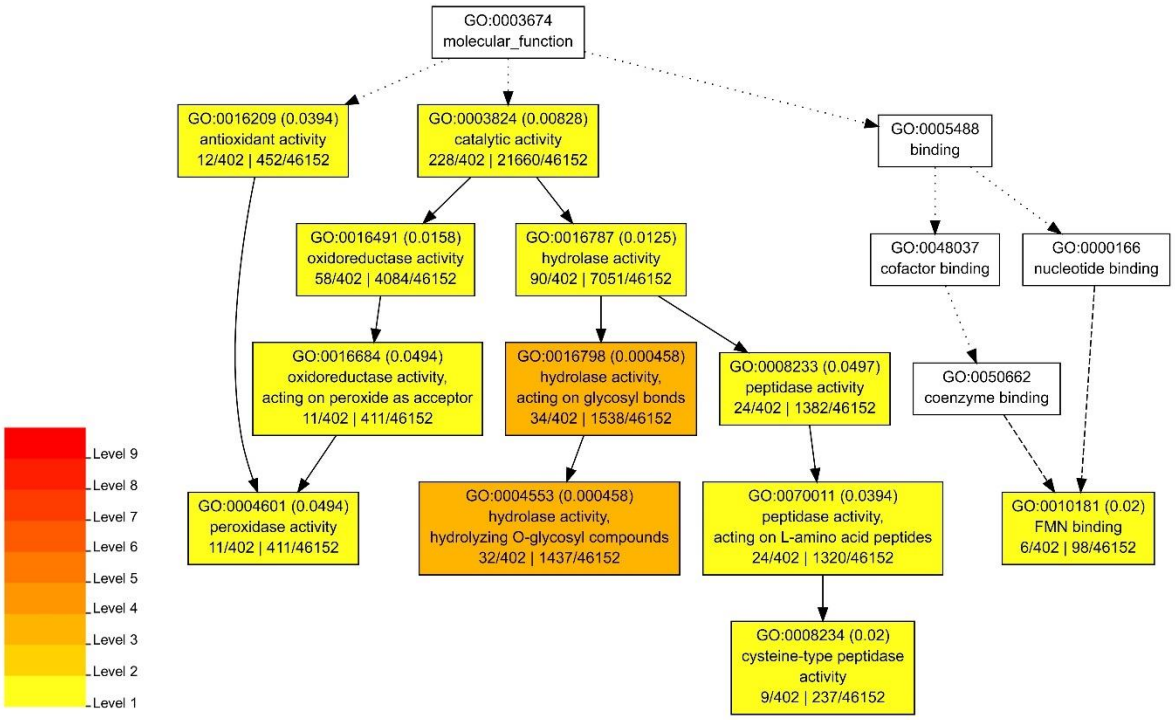


Figure 4.2. Hierarchical tree graph of GO terms in Molecular function of unique proteins identified in malt samples of hordein-reduced barley lines showing the molecular functions. Boxes display GO terms and corresponding ID, statistical information, and definitions. Significant terms are identified by an adjusted p-value of <0.05; colored according to significance, while the non-significant terms are white.

4.5.2. Relative quantitation of proteins by SWATH-MS

In the present study, a total of 2,654 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 4.3A). 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 abovementioned lines were clustered together. Grain samples of WT, B, D, and BD formed a cluster, and their malt sample excluding the BD-reduced line are grouped in a separate cluster.

Next, hierarchical clustering analysis (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 4.3B), which reveals the separation of sample types and impact of malting on changing the proteome. Furthermore, within the main clusters, there are sub-clusters of hordein-reduced lines dominated by their genetic background.

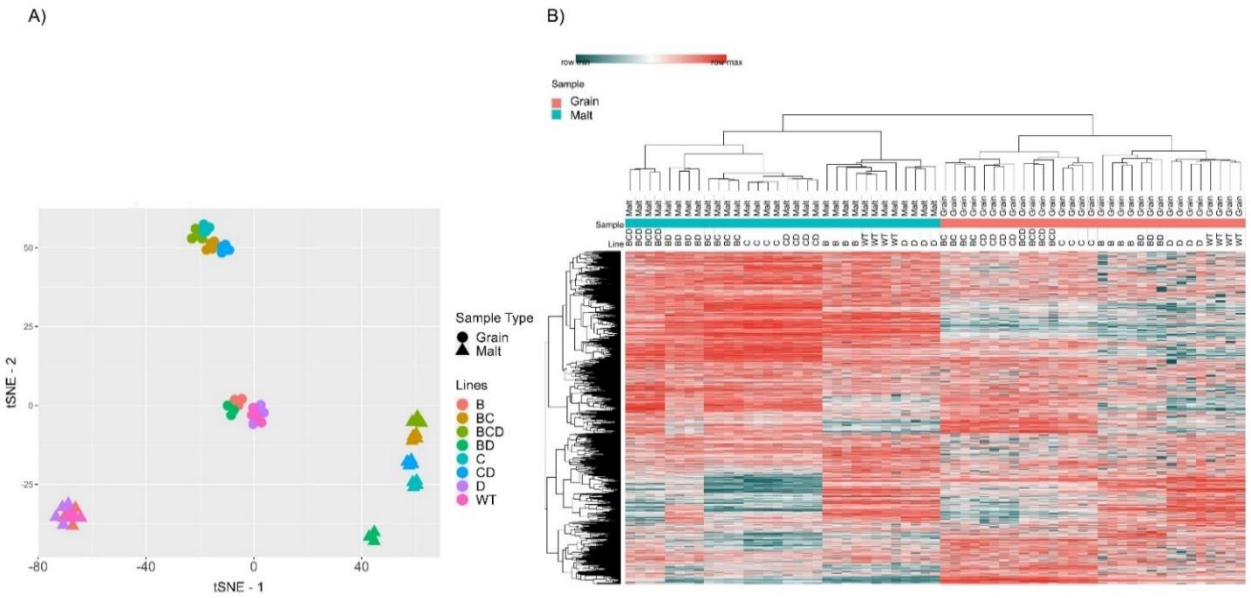


Figure 4.3. Overview of quantified proteins in hordein-reduced and WT barley grain and malt samples using SWATH-MS. A) t-SNE plot showing proteomic profiles in grain and malt of all hordein-reduced lines in addition to WT. Shapes (circle = grain, triangle = malt) represent sample type and colours differentiate genotypes. B) The heatmap represents relative abundance levels (log10) of all quantified proteins. HCA was performed using a one-minus Pearson correlation

metric. Rows in the heatmap are coloured 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.

4.5.3. 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, WGCNA analysis was performed (Figure S9). This analysis resulted in 16 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 S9). Each protein's variable importance in projection (VIP) scores from PLS regressions were used to identify proteins contributing significantly to the C-hordein-reduced background (Table S6). 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 4.4. These proteins were uncharacterized proteins, ripening-related 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 4.5 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.

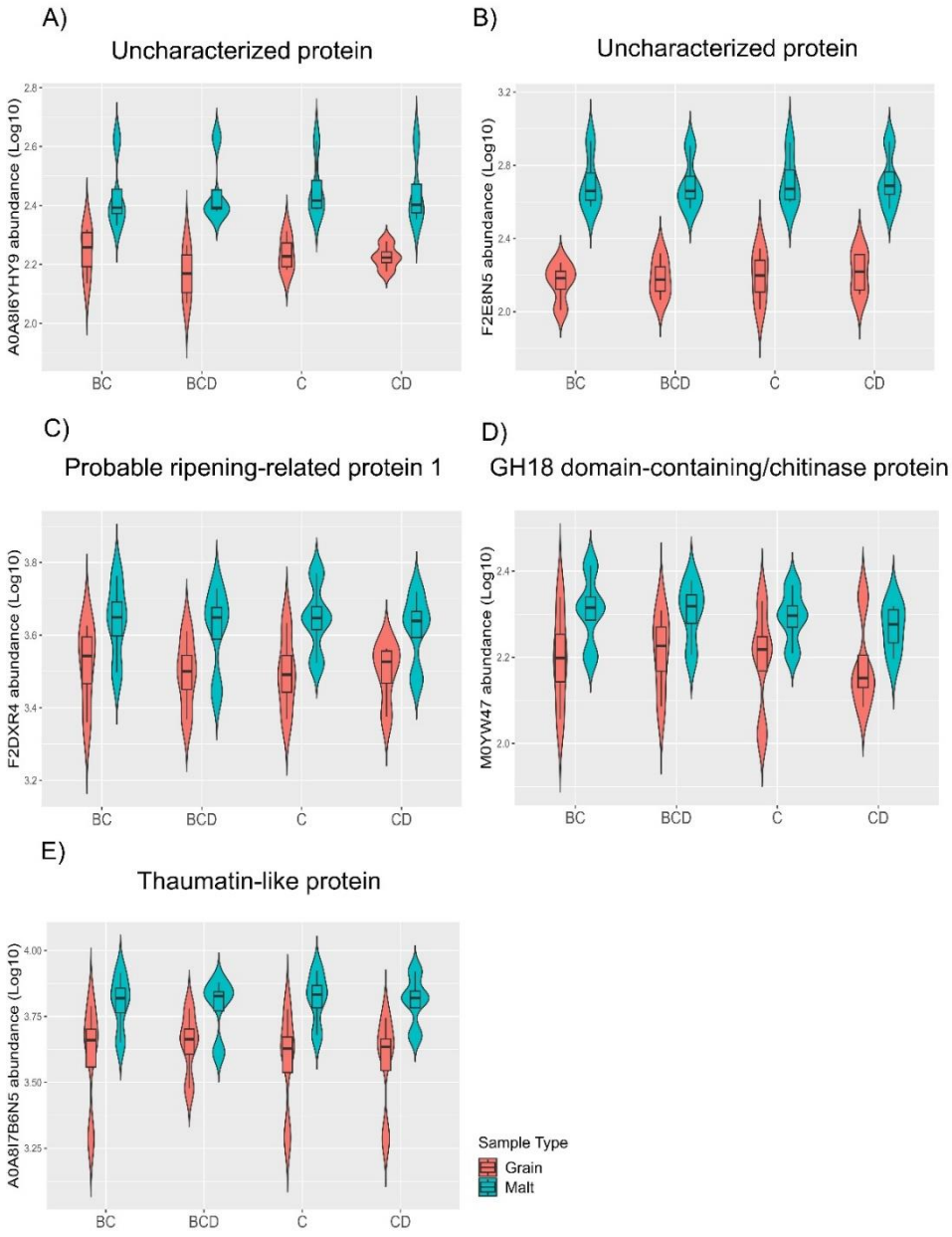


Figure 4.4. Relative abundance of top 5 proteins that positively correlated with C-hordein reduced background lines in barley grain and malt samples. A) Uncharacterized protein(A0A8I6YHY9), B) Uncharacterized protein (F2E8N5), C) Probable ripening-related protein (F2DXR4), D) GH18 domain-containing or chitinase (M0YW47), E) thaumatin-like proteins (A0A8I7B6N5). The y-axis shows the log10 transformed relative abundance of protein and x-axis represents lines that possess C-hordein-reduced line 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. Colour depicts different sample types (grain and malt).

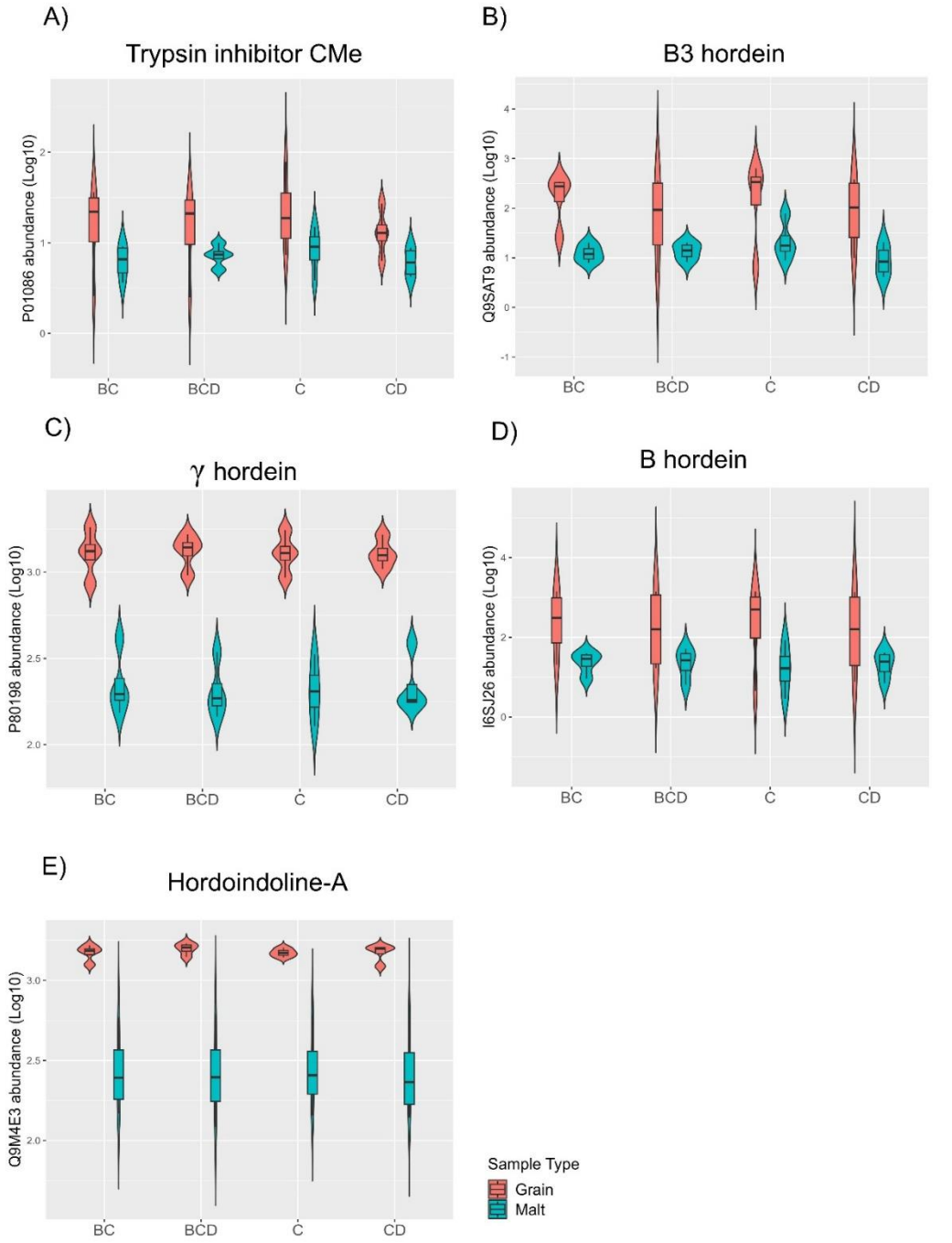


Figure 4.5. Relative abundance of top 5 proteins that negatively correlated with C-hordein reduced background lines in barley grain and malt samples. A) Trypsin inhibitor CMe (P01086), B) B3 hordein (Q9SAT9), C) γ-hordein (P80198), D) B-hordein (I6SJ26), E) hordoindoline (Q9M4E3). The y-axis shows the log10 transformed relative abundance of protein and 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 represents the interquartile range and the line inside each box represents the mean. Colour depicts different sample type (grain and malt).

4.5.4. 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 y-axis, versus the average abundance level (A) of two conditions on the x-axis. We used MA plot 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 S10A, B, C, D). GO enrichment analysis showed the top five significant (*p*-value < 0.05) enriched biological process terms for each genetic background and WT (Figure 4.6A and 4.6B). 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 4.6A). Significant biological process terms for down-regulated proteins were only found in C-background and these terms were "response to temperature stimulus" and "response to cold" (Figure 4.6B). 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).

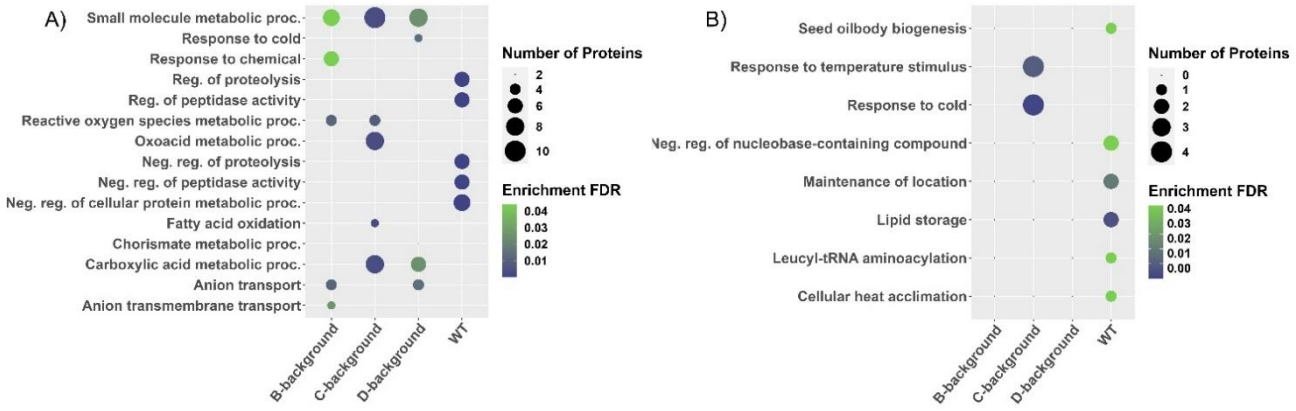


Figure 4.6. Gene Ontology enrichment analysis of the top 5 biological process terms for up- (A) and down-regulated (B) proteins. Size of dots show the number of proteins for each term and colour scale of dots represents the FDR-corrected p value (<0.05) for each term.

GO enrichment analysis for top five significant molecular function terms for up-regulated 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", "racemase and epimerase activity" (Figure S11A). In 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 down-regulated 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 S11B).

4.6. 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 4.3B) and t-SNE (Figure 4.3A) analyses demonstrated that the grain and malt samples were tightly grouped based on their proteome profile. During malting, hydrolytic 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 accord with previous proteomic studies (Strouhalova et al., 2019). Discovery proteomics results revealed a higher number of identified proteins in malt samples compared to grain (Figure 4.1) 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 (Mahalingam, 2018; Osman et al., 2002; Qin et al., 2021). However, there were unique proteins identified in individual hordein-reduced malt samples which revealed differences between the lines (Table 4.3). 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 (Han et al., 2018). They are involved in the biosynthesis and modification of secondary metabolites, including flavonoids, phenolic compounds, and glycosides (Han et al., 2018). 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 (Rani & Bhardwaj, 2021).

In our GO analysis, “hydrolase activity, hydrolyzing O-glycosyl compounds” was the most significant molecular function term (Figure 4.2) 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, arabinofuranosidase, sucrose galactosyl transferase, and as Table S7 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 (Power, 2003). 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 (Finnie et al., 2011), in particular the identification of enzymes like amylase and limit dextrinase (March et al., 2012b).

β-amylase enzyme, which accumulated during development of the grain and 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 disulphide reductase or by proteolytic enzymes (Sopanen & Laurière, 1989). Comparison of the abundance of this enzyme in barley grain and malt in all hordein reduced lines as shown in the Figure S12 indicates that the level of this enzyme was lower in those lines containing C-hordein reduced line in their genetic background. C-hordein reduced line used in the ULG line (Risø 1508) breeding program is different from other lines in the sense that it is a result of a mutation in the prolamin binding factor (PBF) (Tanner et al., 2016). 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 (Henson & Duke, 2016). 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 S12). Conversely, lines without reduced C-hordein backgrounds exhibited higher DP values.

4.6.1. 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 prolamin binding factor (PBF) (Moehs et al., 2019). 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 S9). 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. (Shewry & Halford, 2002).

Three proteins in our analysis (GH18 domain-containing, and thaumatin-like protein) belong to the pathogenesis related protein (PR) 10 and 5 families (Huang et al., 2012; Iqbal et al., 2020; Jenkins et al., 2005) with known function in defense and developmental processes in plants. As barley seeds are under temperature stresses during germination and kilning of malting process, these temperature fluctuations might trigger the higher abundance of these proteins in malt samples. Thaumatin-like proteins have been reported to play roles in barley seed germination and ripening (Singh et al., 2017b).

Proteins with significantly negative correlation with C-hordein reduced genetic background mostly belong to hordeins (B-, γ-hordeins and hordoindoline-A) and trypsin inhibitors (Figure 4.5) as they were reported previously (Tanner et al., 2016, 2019). 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 S13). While previous reports indicated an unclear connection between grain hardness and hordoindolines (Darlington et al., 2001), observations by Takahashi et al. revealed that hordoindolines might indeed hold a pivotal role in determining barley grain hardness (Takahashi et al., 2010). The abundance of this protein was significantly lower in lines with C-hordein-reduced line in their genetic background compared to the WT (13), 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 4.2), 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 (Bose, Broadbent, et al., 2020; Tanner et al., 2016).

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.

4.6.2. Investigating changes of proteins after malting

Early barley growth and development depends on glycolysis, which includes proteins with the biological process term of "small molecule metabolic process" and molecular function of "enzyme regulator activity" (James & Bunting, 1941), such as those involved in the tricarboxylic acid (TCA) cycle (Ma et al., 2016a), amino acid metabolism (Sreenivasulu et al., 2008), and lipid metabolism (MacLeod & White, 1961). In glycolysis, glucose is broken down to pyruvate and utilized in further metabolic processes to ultimately produce energy and is important during germination (James & Bunting, 1941). The TCA cycle is one of the crucial series of mechanisms happening in the mitochondria of barley cells for energy production through the oxidation of acetyl-CoA, which originated from amino acids, glucose, and fatty acids (Ma et al., 2016a). 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 (Sreenivasulu et al., 2008). 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 (Dong et al., 2015; Sreenivasulu et al., 2008; Yu et al., 2014). Enrichment of the "small molecule metabolic process" GO 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 highest number of proteins related with this term compared to other genetic backgrounds (Table S8). 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 (Ma et al., 2016b). 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 (Blackwood & Miflin, 1976). Additionally, the activation of the glyoxylate cycle during germination supports the β-oxidation of fatty acids (Holtman et al., 1994). This explains the observed upregulation of the glyoxysomal fatty acid beta-oxidation multifunctional protein.

The PBF transcription factor plays a crucial role in the expression regulation of prolamin-type storage protein coding and starch synthesis related genes in cereals (Mena et al., 1998), and exhibits a specific affinity towards 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 muted leading to the down-regulation 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 4.2), lines including C-hordein reduced in their genetic background showed significantly lower extract amount compared to the other lines (WT, B-, D-, BD-) which shows the relationship between starch and hordeins in the experimented lines. This result is aligned to the previous findings on these hordein-reduced lines (Bose, Broadbent, et al., 2020).

In the GO enrichment analysis of the down-regulated 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 down regulated in the malt samples of the C-hordein reduced background was ferritin which is a protein that involves in regulation and storage of iron (Borg et al., 2009). 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 (Distelfeld et al., 2007; Velu et al., 2017). 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 (Ciésla et al., 2016). 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 down-regulated proteins in C-hordein reduced background was "calcium ion binding activity". Upon closer look at the proteins that contributed to this molecular function term in the C-hordein reduced background, we found that these proteins were caleosin (Q6UFY6), calcium-dependent protein kinases (A8WEN6, A0A221C9D5), and NADH-ubiquinone reductase (F2CRG8). In grains, lipids are stored in spherical organelles named as oil bodies or oleosomes. Caleosin is a protein that is found in the surface of oil bodies and possesses a conserved calcium-binding domain (Liu et al., 2005). It has been reported that caleosins are in specific domains of the ER, implicating their involvement in lipid transfer (Næsted et al., 2000). Notably, caleosin gene expression aligns with the accumulation of storage products, such as protein bodies, during embryo development (Næsted et al., 2000). 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

which impacted some parts of B-hordein-loci yet some B-hordeins were left in the grain (Bose, Broadbent, et al., 2020). 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 (Bose, Broadbent, et al., 2020).

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 TCA cycle to provide more ATP during germination, while down-regulated proteins were related to the temperature stimulus processes GO term and included proteins like caleosin which includes a calcium-binding domain that are 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 on the impact of storage proteins abundance changes on the proteome in barley grain and malt samples.

5. Chapter 5: Summary of Conclusions and General Discussion

5.1. Summary of conclusions

The world population is projected to reach 9.8 billion by 2050, which will put additional pressure on the farming system to meet the increasing demands for food (Tilman et al., 2011). In addition, high global demand for food and beverages and declining arable land for agriculture will need further acceleration to increase and sustain food production. To achieve this goal, modern agriculture practices must adopt next-generation approaches in crop breeding research, wherein proteomics can play an important role. Cereals have been important crops for humankind and play a critical role in human and livestock diets. Barley, a member of true grasses (Poaceae), is the fourth most important crop worldwide. This crop has been subjected to breeding programs to incorporate desired traits for industry-driven biotechnology, including seed germination and maturation, malting and brewing. This project used bottom-up proteomics to explore barley grain and malt proteomes using a range of barley cultivars grown in different locations and a unique set of genotypes differing in storage protein compositions. Using two different barley sample sets, proteomics experiments were developed that use complementary analytical chemistry techniques and cutting-edge bioinformatics approaches were conducted to address the overall aims of the study, i.e., to understand the impact of growing location on the proteome and the proteome level changes across the hordein-reduced barley lines after malting. Analysing the results of these experiments holistically gives a deeper insight into the conclusions that when viewed alone.

In Chapter 2, three Australian barley lines with different genetic backgrounds were grown in three locations in WA and assessed using a bottom-up proteomics approach. This experiment was designed to understand how genetic backgrounds, growing location differences and the corresponding environmental parameters influence proteome level changes. After employing discovery proteomics, 1,517 proteins were identified at 1% FDR and 920 proteins were quantified using SWATH-MS. PCA revealed that samples were grouped into two major clusters based on location (Figure 2.1A). Furthermore, hierarchical clustering served to stratify samples according to their growing locations, wherein location as a variable had more impact on the proteome than genetic background (Figure 2.1B).

Chapter 2 describes the investigation of the relationship between malting traits and the proteome. WGCNA was used to identify proteins that correlated with desirable malting traits in each growing location, by assessing the relationship between 27 desirable malting characteristics (Table 2.2, and

2.3) and the measured grain protein abundances. This analysis revealed 19 significant correlations between modules (measured protein abundances) and malting specification measurements (traits) (Figure 2.2). Three significantly positive modules were related to desirable malt characteristics, such as malt yield. Malt yield is a key parameter indicative of barley seeds' malting performance, reflecting the percentage loss of grain mass during the germination process of the malting procedure (Farzaneh et al., 2017). By uncovering proteins related to those positively correlated modules, it was demonstrated that these proteins were more abundant in samples with higher malt yield. Subsequently, proteins significantly negatively correlated with malt yield were selected and interestingly, these proteins showed lower abundance with higher malt yield. Further analysis of proteins in positively and negatively correlated modules to malt yield showed a significant difference between the two regions: samples grown in northern locations produced higher malt yield than those grown in the southern location (Figure 2.3). Previously it has been reported that agronomic practices like nitrogen fertiliser amount or water availability can significantly affect malt yield (Verma et al., 2003). The GO analysis of proteins positively and negatively impacted malt yield demonstrated that proteins with the molecular function of protein self-association, endopeptidase inhibitor activity and enzyme regulator and nutrient reservoir activities were positively correlated with malt yield. These proteins are associated with response to temperature stimulus (Figure 2.4). Top proteins positively correlated with malt yield trait included HSPs, peroxidase and serpin-domain containing proteins. Most of these proteins have been reported that play roles in abiotic stresses (Potokina et al., 2004; ul Haq et al., 2019). Elevated temperatures may have triggered increased expression of defence-related proteins including peroxidases and HSPs during the grain-filling stage of barley seed. The higher abundance of these proteins suggests that they confer tolerance during steps of malting where there is a fluctuation in temperature.

Malting is an industrial, value-adding process wherein producing a high quality and uniform product as a raw material to brewers is a goal of maltsters. In this chapter, the obtained results suggested that growing location impacted the proteome profile and led to variation in malt yield, which is an undesirable outcome from a malting point of view if consistency and uniformity are desired. The impact of different growing environments on the malt yield is novel and warrants further investigation. Although several studies have investigated the impact of environmental parameters such as fertiliser and genetics, studies have yet to explore the link between malting characteristics and proteome to our knowledge. Therefore, the results from the Chapter 2 of this thesis have demonstrated the potential application of proteomics to provide an additional layer of information to the maltsters and breeders.

Chapter 3 focused on a unique collection of barley lines previously used for the selective breeding program to reduce the immunogenic storage proteins responsible for CD and NCGS. These lines were previously bred through a conventional breeding program to develop ULG barley line which is a commercial product under the name of Kebari® (Colgrave, Byrne, Blundell, Heidelberger, et al., 2016; Tanner et al., 2016). Proteomic analyses have been performed on these barley lines, including single- and double- hordein reduced lines (Bose, Broadbent, et al., 2020; Bose et al., 2021). In these prior studies, the primary focus was on grain flour samples to investigate compensation mechanisms and nutritional shifts. However, the malting quality assessment using these lines and proteome-level changes using the targeted analysis had not been conducted previously. Therefore, in Chapter 3, targeted proteomic analyses were performed on these hordein-reduced lines to understand how grain storage proteins, predominantly the hordeins, were affected by malting. In this chapter, the main storage proteins of barley were investigated in the grain and malt from hordein-reduced barley lines. The hordeins and ALP, proteins with known immunogenic potential in coeliac disease and allergy were targeted, and the abundance of the peptides derived from them, revealed two major clusters, separated according to the incorporation, or not, of the C-hordein reduced line in their pedigree (Figure 3.1). This is attributable to C-hordein null lines possessing a mutation in a transcription factor called prolamin binding factor (PBF) that controls prolamin-like storage protein accumulation. In contrast, the other mutant lines that clustered together are lines that include the B- and D-hordein reduced genotypes (Risø 56 and R118) in their pedigree and possess a normal functional PBF. Monitoring peptide abundance changes in barley and malt samples of each hordein class (B-, γ- and D-hordein) as well as ALPs in each hordein-reduced line revealed that independent of the hordein type, hordeins generally decreased after malting. All gluten-related proteins including ALPs, B-, D-, and γ-hordeins were significantly further reduced in the malt samples compared to barley grain in the ultra-low gluten barley line (BCD-reduced).

Previous research on hordein-reduced barley lines suggested that altered hordein accumulation (targeted decrease) impacts the abundance of other storage proteins such as seed storage globulins (Bose, Broadbent, et al., 2020). Therefore, globulins were additionally selected to develop a targeted quantitative method. In all lines, including C-hordein-reduced lines in their genetic background, the four seed storage globulins showed significantly higher abundance than in the WT cultivar, Sloop. When the malt samples of the ULG line were compared to WT malt, globulins were notably higher and reduction of hordeins in the ULG lines was evident (Figure 3.4). This is consistent with the compensation mechanism described by Bose *et al.* (2020), whereby a change in

RNA or protein expression occurs to compensate for loss of traditional seed storage proteins. Taken together, these results show that barley with lower hordein expression use this compensation mechanism to achieve adequate seed N storage and, importantly, this effect is stronger when multiple hordein classes are implicated, such as in the ULG barley line.

In Chapter 4, the impact of malting on the broader proteome of the same hordein-reduced barley lines was investigated using discovery proteomics and SWATH-MS. Overall, 2,688 and 3,034 proteins identified at 1% FDR in barley grain and malt samples, respectively (Figure 4.1) and GO enrichment analysis of 807 proteins unique to malt showed that the most significant terms for these proteins are related to hydrolysis activity. These findings aligned with the expectations characteristic of enzymatic modification during malting, wherein carbohydrates are converted to fermentable sugars during brewing (Gupta et al., 2010). Whilst in the previous chapter, targeted quantitation of storage proteins was investigated; herein, the SWATH-MS method was employed. The aim was to quantify the proteomes of hordein-reduced barley lines after malting to understand the interplay between genetic differences in conjunction with physical and biochemical modification that occurs during the malting process. It was noticed that grain and malts were grouped according to their proteome abundance profiles (Figure 4.2). As reported previously, a range of hydrolytic enzymes are expressed during malting and certain storage proteins are digested, especially amylase enzymes and isoforms of protein Z which leads to the separation of grain proteome from malt (Strouhalova et al., 2019).

To understand the impact of genetic background on the proteome of grain after malting, WGCNA was employed to investigate the proteome alterations in the B-, C- and D-reduced genetic backgrounds. WGCNA was initially developed for microarray datasets but is applicable to high-throughput proteomics and metabolomics datasets (Pei et al., 2017), as described in Chapter 2, where the results led us to identify a linkage between proteome abundance profiles and malting characteristics. Hence, we took the advantage of this analysis in Chapter 4 to connect genetic differences of hordein-reduced backgrounds with the quantified grain and malt proteomes. This analysis revealed that the C-hordein reduced genetic background had the highest number of significant modules related to the quantitative changes in the proteomic dataset (Figure S9). Proteins with significant correlations to the C-hordein mutant background were identified and the top proteins were selected to evaluate the distribution of protein abundances (Figure 4.4). Among those that showed higher abundance in C-hordein reduced malt compared to grain were primarily ripening- and pathogenesis-related proteins, like GH18 domain-containing and thaumatin-like

proteins. These proteins are thought to play roles in defence and plant development (Iqbal et al., 2020; Singh et al., 2017a); and results suggest that due to temperature fluctuation during germination and kilning stages of malting these proteins were triggered to be expressed in higher abundance.

The top proteins significantly negatively correlated with the C-hordein-reduced genetic background were primarily represented by B-hordeins, γ-hordeins and trypsin inhibitors (Figure 4.5). Interestingly, these proteins showed lower abundance in malt samples when compared to grain samples. Of note is the γ-hordein reduction in malt. In the original breeding program reduction of all hordeins reduction was intended, except for the γ-hordeins that were not intentionally altered. Despite this, reduced levels of γ-hordeins were observed in the ULG line (Tanner et al., 2016). The result that C-hordein reduced genetic background correlates with the reduction of γ-hordein is an interesting finding and the further reduction after malting is pertinent to the safety of this line and its products. MA analysis was conducted for each genetic background to determine the impact of malting on the proteome. In each comparison, the barley grain proteome was compared to the malt. Results demonstrated up- and down-regulated proteins of each genetic background and GO enrichment analysis was conducted (Figure 4.6). The top enriched biological process terms for up- and down-regulated proteins of each genetic background were identified. Most significant enriched terms belonged to C-hordein reduced genetic background and they were related to the small molecule metabolic process. These results refer to the processes that lead to the breakdown of carbohydrates, proteins, and fatty acids during germination in the malting procedure. In addition, within the down-regulated proteins of the C-hordein reduced lines one of the most significant terms that were enriched due to the malting was the response to temperature stimulus; proteins involved in these processes may have the role of saving energy for germination during malting.

5.2. General Discussion

The barley protein content is one of the critical determinants of end-product quality. Genetic background and environmental conditions are major protein content and composition determinants. In this study, proteins were identified and quantified using LC-MS in samples grown in three locations within Western Australia. The results highlighted the importance of growing locations on the grain proteome composition. Barley grown in northern locations of WA showed significantly different proteome changes compared to the same barley varieties grown in the southern location, and these differences were related to the meteorological differences such as monthly average

temperature and rainfall of these two regions in 2019. Differences caused by growing locations (temperature) impacted the grain proteome profile, which is important as consistency is of great focus in most applications of barley grain. Barley cultivars grown for malting purposes are expected to maintain a sustainable grain and malting quality, however experiments performed in this thesis indicate that barley grown in an environment with higher temperatures during grain filling significantly affects the grain composition with a clear impact on malting characteristics.

For malting purposes, maltsters prefer using a raw material with consistent malting characteristics to avoid variation in their final product. In this study, the proteome of barley lines grown in different locations was measured, these samples were malted and the relationship between the proteome and malting traits was established using WGCNA. A set of proteins significantly correlated with malt yield, test weight, free amino nitrogen and β-glucanase. Proteins with positive and negative correlations with malt-yield, a desirable trait that refers to the mobilisation of endosperm starch to provide energy for the mass of the growing embryo were identified. Those proteins positively correlated with malt yield are involved in responses to temperature stimuli like heat stress and response to reactive oxygen species. A higher abundance of these proteins in barley grown in the warmer northern locations due to a probable abiotic stress response in northern locations, conferring tolerance to the temperature fluctuations during malting; therefore, these samples may be less affected by the temperature changes in malting steps.

Hordeins are the major storage proteins in barley, a protein group known for triggering adverse immune reactions leading to CD, food allergy and NCGS. A targeted proteomics method was developed and applied to monitor immunogenic hordein peptides and measure their abundance in the hordein-reduced lines before and after malting. This was performed on single-, double-, and triple-hordein-reduced barley and their corresponding malt samples to investigate how differences in the grain hordein content and composition are further affected by malting. The malting process lowered the abundance of residual hordein proteins as evidenced by the decrease in peptides originating from hordeins and avenin-like proteins. In addition, it was concluded that suppression of hordein expression altered the protein allocation leading to compensatory abundance pattern changes in grains. As a result of hordein suppression, globulins and other storage proteins in barley were increased compared to the wild-type cultivar, Sloop. The results of this study provide insight into protein compositional changes in hordein-reduced barley lines and confirm that food processing steps such as malting can further reduce the immune reactive protein content in barley.

Malting is a process that changes the grain proteome, particularly during germination, when a cascade of molecular events occurs, which impact the structure, macronutrient, and protein composition of the grain. SWATH-MS, a quantitative proteomic approach was used to explore the changes in the broader barley proteome in genetically related ULG breeding lines before and after malting. Proteins in hordein-reduced barley grain and malt were identified, some of which were unique to malt and primarily represented proteins with hydrolysing activities. Interestingly, in exploring proteomic differences between seven genetic backgrounds, it has been found that C-hordein reduced background had the most significant impact on changes in the proteome. This is related to the presence of a mutated prolamin binding factor (PBF) transcription factor in the C-hordein reduced lines that were developed from the EMS mutated cultivar Risø 1508 (Moehs et al., 2019). The PBF transcription factor has a dual role in seed development and germination: during seed development it has a known regulatory effect on hordein and starch accumulation in the developing grain while regulating the expression of lysine-rich, energy providing proteins during germination (Bose et al., 2021; Moehs et al., 2019). The C-hordein reduced lines possess this mutation leading not only to a reduced B and C hordein accumulation but also changes in the accumulation of starch and fat biosynthesis related proteins and reverse material accumulation (Bose, Broadbent, et al., 2020; Bose et al., 2021; Tanner et al., 2016). These changes have a clear impact on the malted proteomes as highlighted in our results. The B- and D-hordein reduced lines, that represented backgrounds where the B hordein locus was partially absent (B-reduced line) (Tanner et al., 2016) and an inactive D-hordein null allele (D-hordein reduced line) is responsible for the decrease in B and D hordein abundance had less changes and differences compared to the WT (Colgrave, Byrne, Blundell, Heidelberger, et al., 2016). The results obtained in this thesis confirm the previous findings that in the B and D reduced lines the changes primarily affect the accumulation of the beforementioned hordein classes and have less impact on the overall storage material accumulation (Bose, Broadbent, et al., 2020; Bose et al., 2021). Similarly, we observed lower impact of the B and D-reduced background on the malted proteomes. Differentially abundant proteins in each genetic background were identified, and up-regulated proteins with C-genetic background in their pedigree were seen to be involved in energy metabolism-related small molecule metabolic processes including glycolysis, TCA cycle and fatty acid degradation. In contrast, down-regulated proteins were related to the temperature stimulus processes to facilitate seed germination. Overall, these results demonstrated the applicability of proteomics-based approaches not only to linking the proteome-level compositional changes with the functional level but also to offer a platform to monitor the processing effects such as malting.

5.3. Future research plans

Numerous research studies have been undertaken on the proteome of barley grain applying different techniques (Bahmani, O'Lone, et al., 2021; Finnie & Svensson, 2009; Mock et al., 2018; Willows et al., 2017). However, little research has been conducted to establish a connection between the impact of genetic background and/or environmental conditions on the malting process and the related malting traits. While the observations noted in Chapter 2 demonstrate a promising application, if more environmental variables were measured during the growing season, then WGCNA could be further used to elucidate specific relationships between protein expression and environment. Moreover, the application of malt in different food products providing flavour, antioxidants and nutrients has increased lately. Apart from use of malt in brewing and distilling industry, it also can be used to produce non-alcoholic malt beverages, malt vinegar, malt milkshakes, breakfast cereals, baked goods and confectionery. Another example of malt application is malt extract powders, dehydrated forms of malt extract. They are convenient to use and can be incorporated into a variety of applications, including nutritional supplements, energy bars, protein shakes, and meal replacements. Opening new horizons in malt applications also requires the precise understanding of beneficial and antinutritive components, such as bioactive peptides and allergens, where applications of mass spectrometry-based approaches are essential.

As shown in this thesis, malting affects the overall proteome in barley. Future research can focus on investigating the impact of the growing environment and additional agronomic practices, like fertiliser usage, soil profiles and weather meta-data on the proteome changes. Furthermore, proteome differences due to environmental changes in addition to monitoring the proteome in different time points and steps during malting will uncover additional changes in each time point and steps of malting. Also, metabolomics studies can be incorporated in future to identify pathways related to sensory profiles of beers produced from barley lines with proteome and environmental factors complementing WGCNA analyses to find desirable malting varieties for specific products. This information would assist breeders, maltsters and the food industry to better understand the raw material before processing to produce a malt with desired characteristics.

Abbreviations

AEGIC — Australian Export Grain Innovation Centre

CD — Coeliac disease

MS — mass spectrometry

DDA — data-dependent acquisition

DIA — data independent acquisition

DTT — dithiothreitol

PBF — protein binding factor

SWATH-MS — sequential window acquisition of all theoretical mass spectra

T — Toodyay

Mi — Mingenew

Mun — Munglinup

NCGS — Non-coeliac gluten sensitivity

TCA — tricarboxylic acid

HCA — hierarchical clustering analysis

WGNCA —weighted gene co-expression network analysis,

GO — gene ontology

FDR — false discovery rate

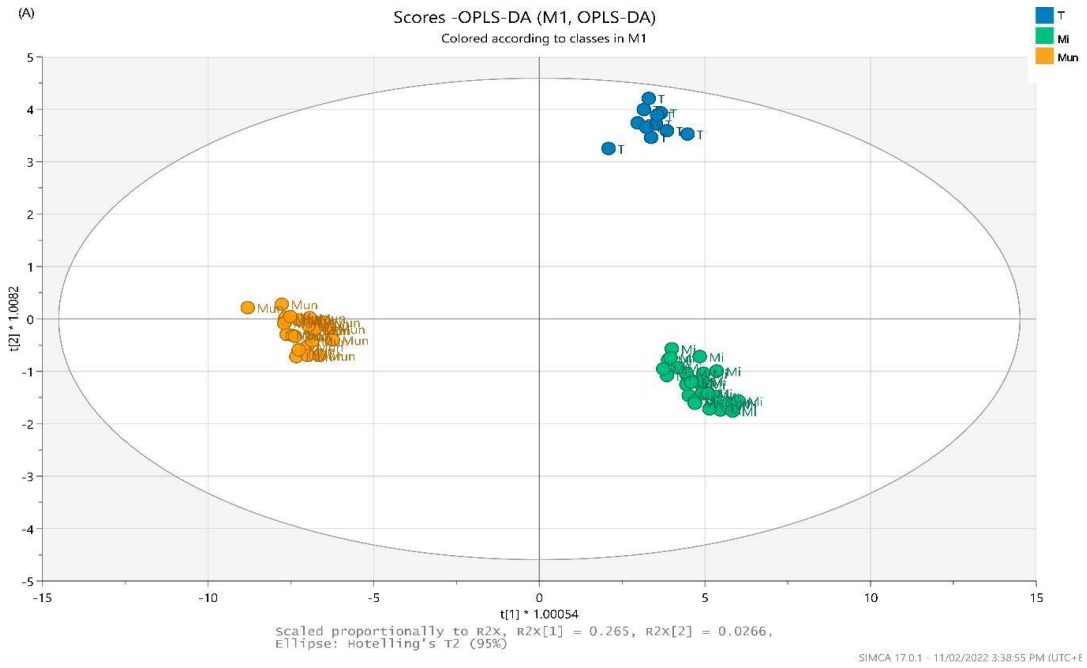
VIP — variable importance for the projection

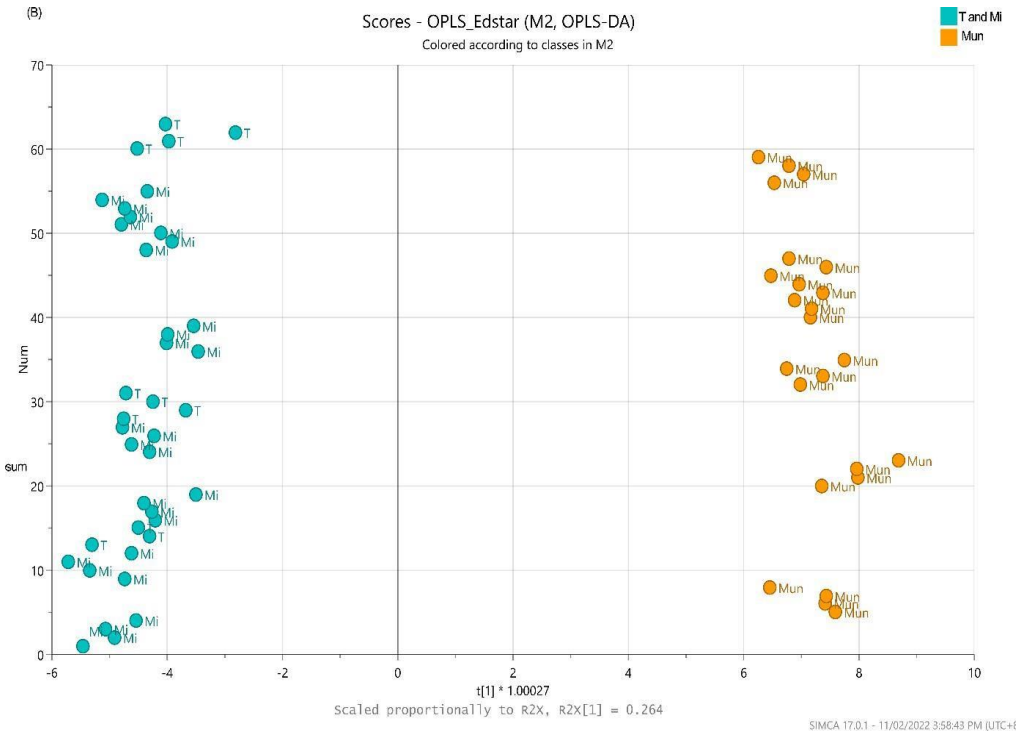
ULG — ultra-low gluten

WA —Western Australia

Appendices

Supplementary Figure 1. oPLS-DA score, OPLS and S-plots of proteins for three growing locations.





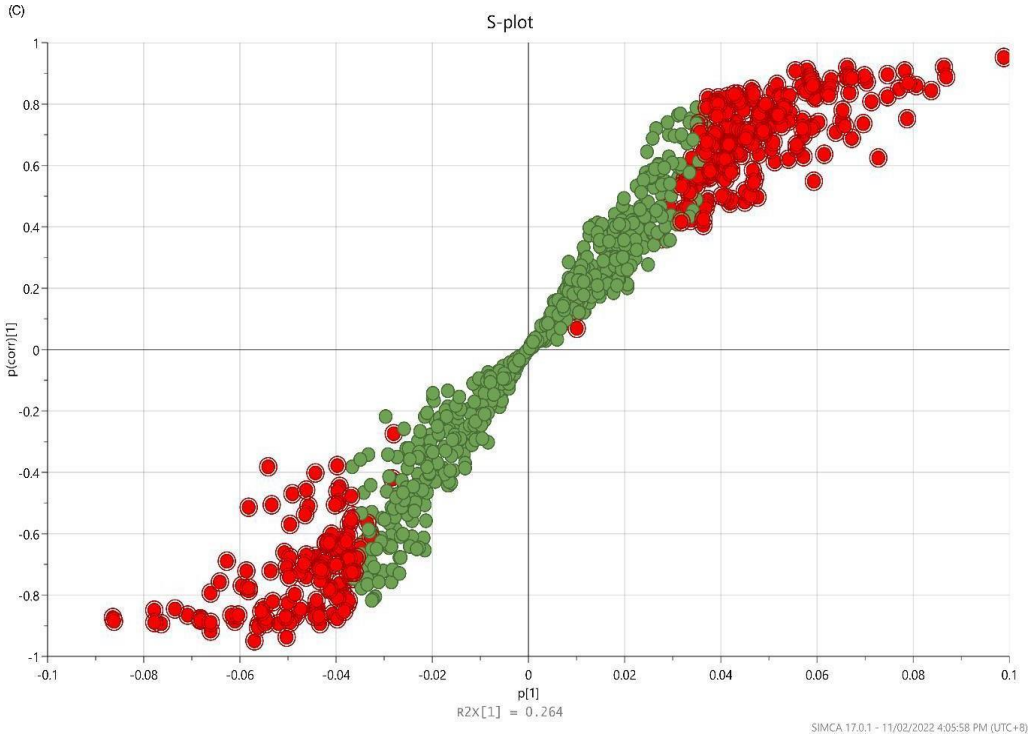
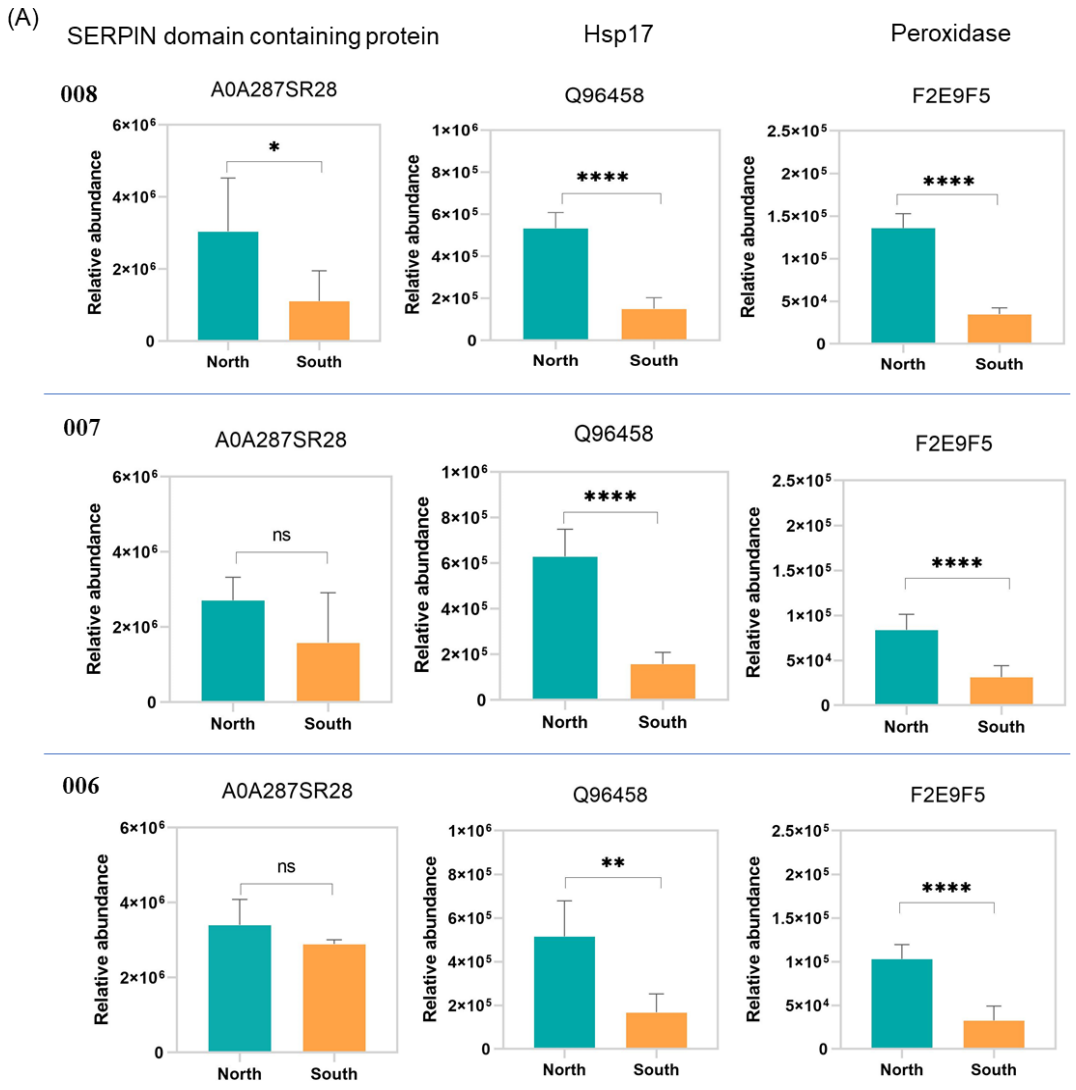


Figure S1. A) oPLS-DA score plot of proteins for three growing locations, each colour represents a growing location. B) OPLS shows the variation within two groups of northern and southern locations. C) displaying the correlation of proteins versus separation between two regions of north and south with the proteins with VIP>1 marked in red.

Supplementary Figure 2. Major proteins altered between location correlated malt yield.



(B)

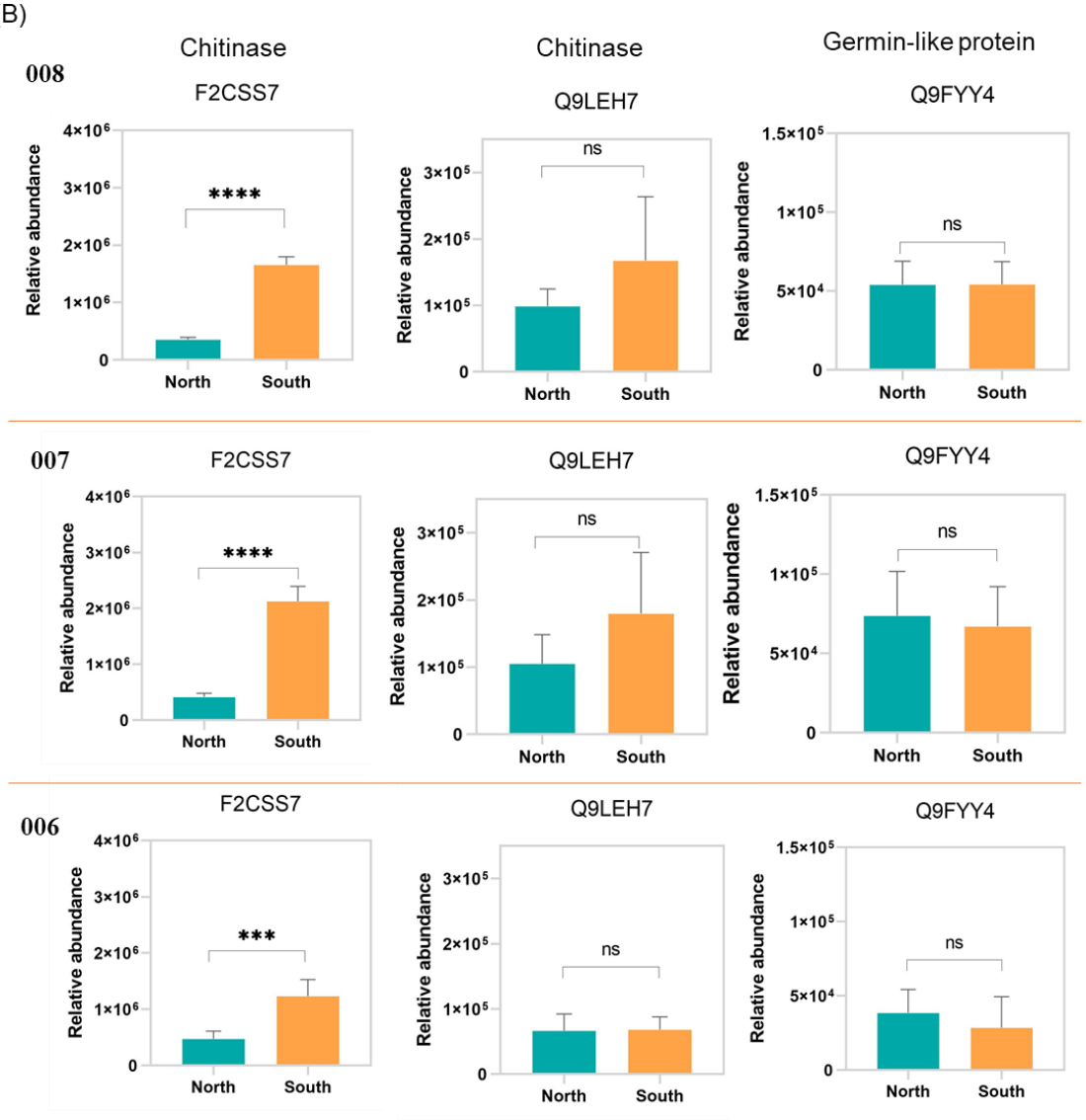


Figure S2. Major proteins altered between location correlated malt yield. (A) positively and (B) negatively and their relation to biotic and abiotic stresses. (∗∗∗) p< 0.0001, (∗∗) p< 0.001, and (∗) p< 0.05 as analysed by unpaired t-test. Error bars show 95% confidence intervals.

Supplementary Figure 3. Biplot demonstrates malting specification data.

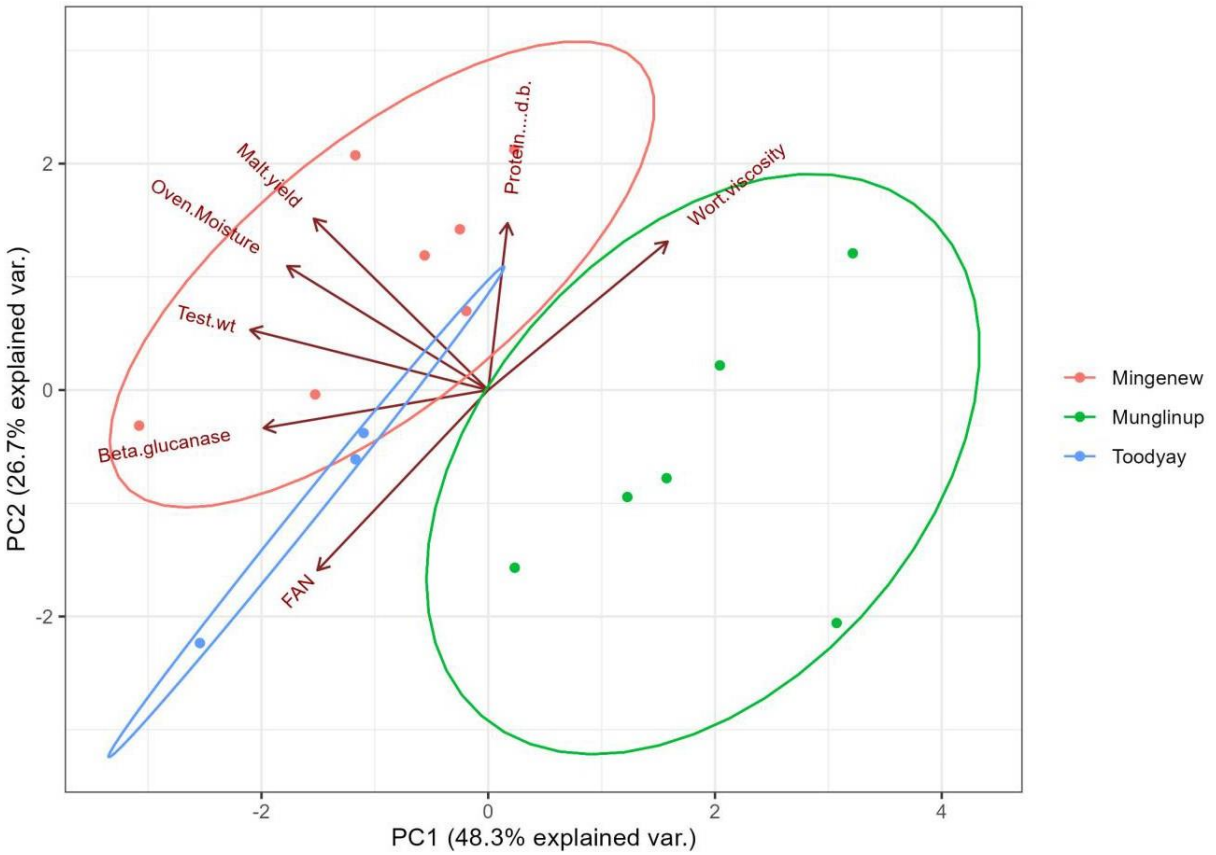


Figure S3. Biplot shows malt specification data that showed significant correlation between barley proteome and malting specification. The major variance in malt data is concordant with growing locations. Each colour represents one location.

Supplementary Figure 4. Average monthly temperature recordings from the three growing locations during 2019.

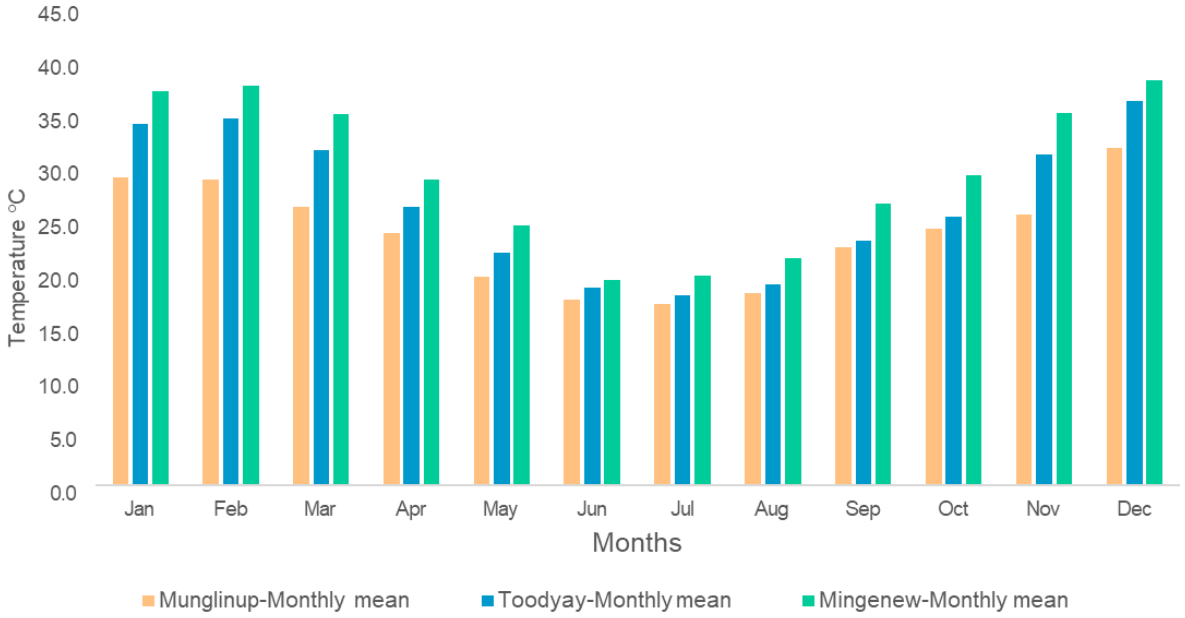


Figure S4. Average monthly temperature recordings from the three growing locations during 2019. Data was collected from the Australian Bureau of Meteorology for 2019.

Supplementary Table 1. List of proteins causing separation between two regions with the VIP> 1.

Protein ID	Protein names	M1.VIP[2+3+0]	M1.VIP[2]cvse * 2.44693
F2EI71	Defensin-like protein	2.29435	0.827392
A0A287Q3F4	Beta-amylase (EC 3.2.1.2)	2.28845	0.968119
F2CSS7	Chitinase (EC 3.2.1.14)	2.28043	0.186501
Q9LEH7	Chitinase (EC 3.2.1.14)	2.04016	0.220039
Q96458	class I small heat shock protein	2.03936	0.406942
F2E8F5	Galactose-binding domain-like protein [Dioscorea alata]	2.0206	0.239985
F2E9F5	Peroxidase (EC 1.11.1.7)	2.00813	0.443313
F2CR03	4-hydroxy-7-methoxy-3-oxo-3,4- dihydro-2H-1,4-benzoxazin-2-yl glucosidebeta-D-glucosidase (EC 3.2.1.182)	1.97701	0.449046
Q8S3U1	Beta-1,3-glucanase II	1.95737	0.185978
F2DZM1	Peroxidase (EC 1.11.1.7)	1.94448	0.389794
Q3YAF9	B hordein	1.9246	1.82586
A0A287T3E6	Putative ripening-related protein 6	1.90795	0.189046
A0A287K1P5	SHSP domain-containing protein	1.90528	0.12184
Q9FYY4	Germin-like protein	1.88076	0.94064
F2DXK0	Histone H1-like	1.87942	0.421368
F2DFY0	Putative Transcription factor SPEECHLESS [Cocos nucifera]	1.87057	0.33781
F2ELE1	Chitinase (EC 3.2.1.14)	1.86815	0.486731
F2CV55	Peroxidase (EC 1.11.1.7)	1.85869	0.291654
D2KZ45	Tonoplast intrinsic protein	1.84989	0.21303
F2CWX3	Allene oxide synthase 2-like	1.84506	0.217272
F2DGG6	Histone H2A	1.82544	0.729439
F2EHT9	Small heat shock protein, chloroplastic-like	1.81898	0.158404
A0A287IXZ5	Hsp70-Hsp90 organizing protein-like	1.81764	0.187189
F2DHQ4	Ribosomal protein L37	1.81562	0.865784
A0A287HUA7	60S ribosomal protein L38	1.78731	0.686756

Supplementary Table 2. Proteins that positively and negatively influence malt yield trait.

Protein ID	Protein name	Type of effect on malt yield
P04399	Protein synthesis inhibitor II (EC 3.2.2.22) (Ribosome-inactivating protein II) (rRNA N-glycosidase)	positive
P23951	26 kDa endochitinase 2 (EC 3.2.1.14) (CHI-26)	positive
A0A287EEX5	Gamma-gliadin-like	positive
A0A287EIP9	Gamma-gliadin-like isoform X1	positive
A0A287EZH9	Heat shock 70 kDa protein 15-like	positive
A0A287IXZ5	Hsp70-Hsp90 organizing protein- like	positive
A0A287K1P5	SHSP domain-containing protein	positive
A0A287K6X2	Translocon-associated protein subunit beta	positive
A0A287LZY0	VAMP-like protein YKT61	positive
A0A287Q3F4	Beta-amylase (EC 3.2.1.2)	positive
A0A287Q3Y5	Uncharacterized protein	positive
A0A287QYS2	Hypothetical protein CFC21_066433 [Triticum aestivum]	positive
A0A287RSX4	Uncharacterized protein	positive
A0A287SCT1	Usp domain-containing protein	positive
A0A287SR28	SERPIN domain-containing protein	positive
A0A287SR97	SERPIN domain-containing protein	positive
A0A287SUL6	AB hydrolase-1 domain-containing protein	positive
A0A287UK56	Usp domain-containing protein	positive
A0A287USY2	SHSP domain-containing protein	positive
A0A287VKA5	Phosphopyruvate hydratase (EC 4.2.1.11)	positive
A0A287W6A4	Pyrophosphate--fructose 6- phosphate 1-phosphotransferase subunit beta (PFP) (EC 2.7.1.90) (6- phosphofructokinase, pyrophosphate dependent) (PPi-PFK) (Pyrophosphate-dependent 6-phosphofructose-1-kinase)	positive
A0A287WCP3	Protein MOTHER of FT and TFL1 homolog 1-like	positive
A0A287Y1T9	Chitinase (EC 3.2.1.14)	positive

D6BU16	Beta-amylase (EC 3.2.1.2)	positive
F2CR89	Protein DJ-1 homolog D	positive
F2CTR2	Prolyl-tRNA synthetase (EC 6.1.1.15) (Fragment)	positive
F2CU34	Heat shock protein 81-1	positive
F2CWR0	Formate dehydrogenase, mitochondrial (FDH) (EC 1.17.1.9) (NAD-dependent formate dehydrogenase)	positive
F2CXF2	Protein BTR1	positive
F2CYX9	Phosphoenolpyruvate carboxylase (EC 4.1.1.31)	positive
F2CZV8	Malate synthase (EC 2.3.3.9)	positive
F2D009	DEAD-box ATP-dependent RNA helicase 12	positive
F2D2I7	Hypothetical protein TRIUR3_15226 [Triticum urartu]	positive
F2D351	Hypothetical protein TRIUR3_00701 [Triticum urartu]	positive
F2DAW3	Universal stress protein PHOS32- like	positive
F2DCE3	Pyruvate, phosphate dikinase (EC 2.7.9.1)	positive
F2DDV2	Probable pyridoxal 5'-phosphate synthase subunit PDX1.1	positive
F2DF14	Signal recognition particle subunit SRP72	positive
F2DIK1	Desiccation-related protein PCC13- 62-like	positive
F2DL78	Ubiquitin domain-containing protein DSK2a-like	positive
F2DNA9	Arginase 1, mitochondrial	positive
F2DVM8	Zinc finger CCCH domain- containing protein 12-like isoform X2	positive
F2DXR4	Putative ripening-related protein 2	positive
F2DY46	CBS domain-containing protein CBSX1, chloroplastic-like	positive
F2E3J0	Vesicle-associated protein 1-2-like	positive
F2E785	Enhancer of mRNA-decapping protein 4-like isoform X1	positive
F2E9B5	Hypothetical protein D1007_40451	positive
F2E9F5	Peroxidase (EC 1.11.1.7)	positive
F2E9N0	Glutelin type-B 1-like	positive
F2ED95	Non-specific lipid-transfer protein	positive
F2EDC3	21.9 kDa heat shock protein-like	positive
F2EDK4	Hypothetical protein D1007_31087	positive

F2EEA9	17.5 kDa class II heat shock protein- like	positive
F2EG33	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex (EC 2.3.1.-)	positive
F2EGM1	Antimicrobial peptides-like	positive
F2EHT9	Small heat shock protein, chloroplastic-like	positive
F2EI71	Defensin-like protein 1	positive
F2EJ52	Heat shock protein 17	positive
I6TEV2	Gamma 3 hordein	positive
I6TRT2	B1 hordein	positive
M0UGW6	23.2 kDa heat shock protein-like	positive
M0URB8	X8 domain-containing protein	positive
M0VDB7	SHSP domain-containing protein	positive
M0W9B7	Subtilisin-chymotrypsin inhibitor CI-1A	positive
M0X9E3	Hypothetical protein TRIUR3_00885 [Triticum urartu]	positive
M0XJ70	UBC core domain-containing protein	positive
M0XMW5	Stearoyl-[acyl-carrier-protein] 9-desaturase 1, chloroplastic-like	positive
M0YQS0	Expansin-B15-like	positive
M0YS80	Abscisic acid receptor PYL9-like	positive
O23983	L-ascorbate peroxidase (EC 1.11.1.11)	positive
O49862	BTI-CMe2.2 protein (Barley trypsin inhibitor CMe 2.2)	positive
Q1ENF0	Cystatin Hv-CPI8	positive
Q2V8X0	Limit dextrinase inhibitor	positive
Q40069	Peroxidase (EC 1.11.1.7)	positive
Q6UFY6	Caleosin 2	positive
Q760G1	Starch synthase, chloroplastic/amyloplastic (EC 2.4.1.-)	positive
Q7XZK6	1,4-alpha-glucan branching enzyme (EC 2.4.1.18) (Fragment)	positive
Q84LE9	D-Hordein	positive
Q84NG7	Globulin	positive
Q96458	17 kDa class I small heat shock protein	positive
Q9T2L5	26 kDa heat shock protein	positive
T2FH00	Alpha-1,4 glucan phosphorylase (EC 2.4.1.1) (Fragment)	positive

P11955	26 kDa endochitinase 1 (EC 3.2.1.14)	negative
P12940	Bowman-Birk type trypsin inhibitor	negative
P35793	Pathogenesis-related protein PRB1-3 (HV-8) (PR-1B)	negative
A0A287EFH8	AAI domain-containing protein	negative
A0A287EJ97	Histone H2A	negative
A0A287EWS1	Importin subunit alpha	negative
A0A287F0D6	Proteasome subunit beta	negative
A0A287F2Q7	ATP synthase subunit gamma	negative
A0A287FK93	Histone H4	negative
A0A287GCX8	Root-specific lectin	negative
A0A287HUA7	60S ribosomal protein L38	negative
A0A287IL79	PLAT domain-containing protein	negative
A0A287JZA2	BOWMAN_BIRK domain- containing protein	negative
A0A287K0Y8	Basic 7S globulin	negative
A0A287MGM7	Thioredox_DsbH domain-containing protein	negative
A0A287N132	Germin-like protein	negative
A0A287P0V3	EGF_CA domain-containing protein	negative
A0A287P6H5	Pyruvate decarboxylase (EC 4.1.1.1)	negative
A0A287P882	GST N-terminal domain-containing protein	negative
A0A287PP23	Uncharacterized protein	negative
A0A287Q8Y3	Cysteine synthase (EC 2.5.1.47)	negative
A0A287RUW1	Proteasome subunit beta	negative
A0A287RV85	60S ribosomal protein L7a	negative
A0A287RXT6	Proteasome subunit beta	negative
A0A287SLM8	Ribos_L4_asso_C domain-containing protein	negative
A0A287T069	PCI domain-containing protein	negative
A0A287T3E6	Putative ripening-related protein 6	negative
A0A287TMN7	CBS domain-containing protein	negative
A0A287X2E8	UBC core domain-containing protein	negative
A0A5B9D476	ATP synthase protein MI25	negative
A2SXS0	Uricase (EC 1.7.3.3) (Urate oxidase)	negative
A5CFY2	Tubulin alpha chain	negative

B5TWD0	HVA1 (Late embryogenesis abundant protein) (Predicted protein)	negative
D2KZ45	Probable aquaporin TIP3-1	negative
F2CPQ3	Hypothetical protein D1007_46620	negative
F2CQ03	YTH domain-containing family protein 2-like	negative
F2CQP9	ATP synthase subunit epsilon, mitochondrial	negative
F2CR03	4-hydroxy-7-methoxy-3-oxo-3,4-dihydro-2H-1,4-benzoxazin-2-yl glucosidebeta-D-glucosidase (EC 3.2.1.182)	negative
F2CR14	Antifreeze protein Maxi-like	negative
F2CRF1	14-3-3-like protein A [Aegilops tauschii subsp. strangulata]	negative
F2CSS7	Chitinase (EC 3.2.1.14)	negative
F2CSW4	Proteasome subunit alpha type	negative
F2CT17	Peroxidase (EC 1.11.1.7)	negative
F2CTK4	Glyoxysomal fatty acid beta-oxidation multifunctional protein MFP-a-like isoform X1	negative
F2CU93	Aspartyl protease family protein 2- like	negative
F2CUT7	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, mitochondrial	negative
F2CV55	Peroxidase (EC 1.11.1.7)	negative
F2CWX3	Allene oxide synthase 2-like	negative
F2CZ44	Inorganic diphosphatase (EC 3.6.1.1)	negative
F2CZ65	60S ribosomal protein L28-1 [Aegilops tauschii subsp. strangulata]	negative
F2CZA8	Phosphoribosylformylglycinamidine cyclo-ligase (EC 6.3.3.1)	negative
F2D3S4	low-temperature-induced 65 kDa protein-like isoform X1	negative
F2D4L0	Glutathione transferase (EC 2.5.1.18)	negative
F2D6F6	Hypothetical protein PVAP13_7NG118900 [Panicum virgatum]	negative
F2D7C5	Thionin BTH7	negative
F2D894	Proteasome subunit alpha type	negative
F2D9P7	Ras-related protein RABD1-like	negative
F2DC21	Stem-specific protein TSJT1	negative
F2DFY0	Hypothetical protein D1007_05479	negative

F2DGG6	Histone H2A	negative
F2DH85	60S acidic ribosomal protein P0	negative
F2DHE3	Reticulon-like protein	negative
F2DHQ4	Ribosomal protein L37	negative
F2DIC8	Ricin B-like lectin R40C1	negative
F2DKD2	Hypothetical protein D1007_47535	negative
F2DPX6	HMG-Y-related protein A-like	negative
F2DSU6	Guanine nucleotide-binding protein subunit beta-like protein A	negative
F2DTB2	Triosephosphate isomerase, chloroplastic	negative
F2DVV7	Probable 6- phosphogluconolactonase (EC 3.1.1.31)	negative
F2DXK0	Histone H1-like	negative
F2DYD4	GTP-binding protein SAR1A [Aegilops tauschii subsp. strangulata]	negative
F2DZ09	Peroxidase (EC 1.11.1.7)	negative
F2DZM1	Peroxidase (EC 1.11.1.7)	negative
F2E0C0	60S ribosomal protein L7-2-like	negative
F2E0Q7	Hypothetical protein CFC21_040712 [Triticum aestivum]	negative
F2E1T7	Protein disulfide-isomerase (EC 5.3.4.1)	negative
F2E1T8	Non-specific lipid-transfer protein	negative
F2E2K8	Aquaporin PIP2-1	negative
F2E4Y5	Nucleolin 1 isoform X2	negative
F2E546	Aminopeptidase P1 isoform X4	negative
F2E7G3	rRNA 2'-O-methyltransferase fibrillarin 1	negative
F2E8C1	Hypothetical protein HU200_036959 [Digitaria exilis]	negative
F2E8F5	Hypothetical protein D1007_59579	negative
F2E8X7	Cysteine proteinase inhibitor	negative
F2EEQ1	Hypothetical protein TRIUR3_19582 [Triticum urartu]	negative
F2EEX6	Glucan endo-1,3-beta-glucosidase GIV-like	negative
F2ELA8	Protein-synthesizing GTPase (EC 3.6.5.3)	negative
F2ELE1	Chitinase (EC 3.2.1.14)	negative
M0V0J0	Aspartate aminotransferase (EC 2.6.1.1)	negative
M0VDQ5	Peptidase A1 domain-containing protein	negative
M0VFE4	Reticulon-like protein	negative

M0WI75	WHy domain-containing protein	negative
M0WJV7	RRM domain-containing protein	negative
M0WRG0	Lipoxygenase (EC 1.13.11.-)	negative
M0WVV5	ATP synthase subunit O, mitochondrial	negative
M0X0Z2	CCT-eta	negative
M0X9E5	Keratin, type I cytoskeletal 10-like	negative
M0XM18	Galactokinase	negative
M0XNN4	Adenylyl cyclase-associated protein	negative
M0XP26	CN hydrolase domain-containing protein	negative
M0XTF9	CCT-alpha (T-complex protein 1 subunit alpha)	negative
M0XTM7	11-beta-hydroxysteroid dehydrogenase A-like	negative
M0XUU4	Cupincin-like	negative
M0Y075	Bowman-Birk type trypsin inhibitor	negative
M0Y8M7	PABS domain-containing protein	negative
M0YAZ9	Vacuolar proton pump subunit B (V- ATPase subunit B) (Vacuolar proton pump subunit B)	negative
M0YS04	Stearoyl-[acyl-carrier-protein] 9-desaturase 2, chloroplastic	negative
M0YW98	Chaperone protein ClpC1, chloroplastic	negative
O49861	BTI-CMe2.1 protein	negative
Q40009	18,9 kDa ABA-induced protein (Stress responsive protein)	negative
Q42839	Chitinase (EC 3.2.1.14)	negative
Q4LB19	Adenosylhomocysteinase (EC 3.3.1.1)	negative
Q5UNP2	Non-specific lipid-transfer protein	negative
Q6BCT3	5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase (EC 2.1.1.14)	negative
Q70XK1	ADP-ribosylation factor 1-like protein	negative
Q852R3	Glutathione peroxidase	negative
Q8S3U1	Beta-1,3-glucanase II	negative
Q8W011	Beta-D-xylosidase	negative
Q9FYY4	Germin-like protein	negative
Q9LEH7	Chitinase (EC 3.2.1.14)	negative

Q9SES7	Glutathione transferase (EC 2.5.1.18)	negative

Supplementary Table 3. Module – trait association table demonstrates correlation data.

Module	Trait	Correlation coefficient	Significance value
black	Test wt kg.hl.	-0.645195337	3.00E-06
blue	Test wt kg.hl.	-0.292721498	1
brown	Test wt kg.hl.	0.650176704	2.00E-06
green	Test wt kg.hl.	-0.127470057	1
magenta	Test wt kg.hl.	0.219324542	1
pink	Test wt kg.hl.	0.641513457	4.00E-06
purple	Test wt kg.hl.	0.474360915	0.02
red	Test wt kg.hl.	0.239586915	1
turquoise	Test wt kg.hl.	-0.526893883	0.002
yellow	Test wt kg.hl.	0.442547546	0.08
black	Grain wt.	-0.084829183	1
blue	Grain wt.	0.202281272	1
brown	Grain wt.	-0.17531204	1
green	Grain wt.	-0.288698296	1
magenta	Grain wt.	0.091733883	1
pink	Grain wt.	-0.069528173	1
purple	Grain wt.	0.053962523	1
red	Grain wt.	0.188664336	1
turquoise	Grain wt.	0.119039931	1
yellow	Grain wt.	-0.230020367	1
black	Protein d.b	0.084880075	1
blue	Protein d.b.	-0.317425912	1
brown	Protein d.b.	0.152007454	1
green	Protein d.b.	0.46251078	0.04
magenta	Protein d.b.	0.014327583	1
pink	Protein d.b.	-0.079790647	1
purple	Protein d.b.	-0.307343539	1
red	Protein d.b.	-0.373337254	0.7
turquoise	Protein d.b.	-0.226901638	1
yellow	Protein d.b.	0.271307497	1
black	Moist after 24 hrs germ		
		-0.266666203	1
blue	Moist after 24 hrs germ		
		-0.004827848	1
brown	Moist after 24 hrs germ		
		0.229757302	1
green	Moist after 24 hrs germ		
		-0.145825159	1
magenta	Moist after 24 hrs germ		
		0.16583659	1
pink	Moist after 24 hrs germ		
		0.278470292	1

purple	Moist after 24 hrs germ		
		0.205473225	1
red	Moist after 24 hrs germ		
		0.234102366	1
turquoise	Moist after 24 hrs germ		
		-0.142735336	1
yellow	Moist after 24 hrs germ		
		0.119827196	1
black	Malt yield	-0.614180547	2.00E-05
blue	Malt yield	-0.327556526	1
brown	Malt yield	0.601227833	5.00E-05
green	Malt yield	-0.063702267	1
magenta	Malt yield	0.403777338	0.3
pink	Malt yield	0.520134678	0.003
purple	Malt yield	0.429296837	0.1
red	Malt yield	0.149209475	1
turquoise	Malt yield	-0.605400681	4.00E-05
yellow	Malt yield	0.446663605	0.07
black	Protein NIR d.b.	0.29894658	1
blue	Protein NIR d.b.	0.009574027	1
brown	Protein NIR d.b.	-0.078526364	1
green	Protein NIR d.b.	0.233814184	1
magenta	Protein NIR d.b.	-0.422079208	0.2
pink	Protein NIR d.b.	-0.235434032	1
purple	Protein NIR d.b.	-0.356559981	1
red	Protein NIR d.b.	-0.293555146	1
turquoise	Protein NIR d.b.	0.15306355	1
yellow	Protein NIR d.b.	-0.087568501	1
black	Malt moisture	-0.134136686	1
blue	Malt moisture	-0.059630628	1
brown	Malt moisture	0.05011757	1
green	Malt moisture	-0.160639188	1
magenta	Malt moisture	0.087164355	1
pink	Malt moisture	0.057413492	1
purple	Malt moisture	0.125319706	1
red	Malt moisture	0.10020936	1
turquoise	Malt moisture	-0.189797095	1
yellow	Malt moisture	-0.032973443	1
black	Malt NIR malt extract	-0.323306742	1
blue	Malt NIR malt extract	-0.120210293	1
brown	Malt NIR malt extract	0.193119188	1
green	Malt NIR malt extract	-0.131395455	1
magenta	Malt NIR malt extract	0.123544099	1
pink	Malt NIR malt extract	0.27008663	1
purple	Malt NIR malt extract	0.311783368	1
red	Malt NIR malt extract	0.131757825	1

turquoise	Malt NIR malt extract	-0.246725828	1
yellow	Malt NIR malt extract	0.132289301	1
black	Oven Moisture	-0.639290038	5.00E-06
blue	Oven Moisture	-0.184957238	1
brown	Oven Moisture	0.483314487	0.02
green	Oven Moisture	-0.184477158	1
magenta	Oven Moisture	0.264397888	1
pink	Oven Moisture	0.617029474	2.00E-05
purple	Oven Moisture	0.484982135	0.02
red	Oven Moisture	0.324483723	1
turquoise	Oven Moisture	-0.442330793	0.08
yellow	Oven Moisture	0.355815277	1
black	Extract fine grind EBC		
		-0.337321575	1
blue	Extract fine grind EBC		
		-0.013245358	1
brown	Extract fine grind EBC		
		0.15350597	1
green	Extract fine grind EBC		
		-0.220167829	1
magenta	Extract fine grind EBC		
		0.021124897	1
pink	Extract fine grind EBC		
		0.358954049	1
purple	Extract fine grind EBC		
		0.367655379	0.8
red	Extract fine grind EBC		
		0.264617125	1
turquoise	Extract fine grind EBC		
		-0.145940647	1
yellow	Extract fine grind EBC		
		0.058129721	1
black	Wort colour	-0.259157295	1
blue	Wort colour	0.036193117	1
brown	Wort colour	0.103138239	1
green	Wort colour	-0.279512887	1
magenta	Wort colour	-0.022007359	1
pink	Wort colour	0.271326927	1
purple	Wort colour	0.326709684	1
red	Wort colour	0.290370822	1
turquoise	Wort colour	-0.012266795	1
yellow	Wort colour	-0.005149625	1
black	Wort pH	0.329384477	1
blue	Wort pH	0.089935484	1
brown	Wort pH	-0.320216766	1
green	Wort pH	0.21688046	1
magenta	Wort pH	0.122494161	1
pink	Wort pH	-0.437050652	0.09

purple	Wort pH	-0.189933679	1
red	Wort pH	-0.289883497	1
turquoise	Wort pH	0.14641729	1
yellow	Wort pH	-0.07718777	1
black	Wort soluble nitrogen dumas N.m.m.		
		0.026625584	1
blue	Wort soluble nitrogen dumas N.m.m.		
		0.001351798	1
brown	Wort soluble nitrogen dumas N.m.m.		
		-0.03152585	1
green	Wort soluble nitrogen dumas N.m.m.		
		-0.118596094	1
magenta	Wort soluble nitrogen dumas N.m.m.		
		-0.132669272	1
pink	Wort soluble nitrogen dumas N.m.m.		
		0.041616053	1
purple	Wort soluble nitrogen dumas N.m.m.		
		-0.005889975	1
red	Wort soluble nitrogen dumas N.m.m.		
		0.095543119	1
turquoise	Wort soluble nitrogen dumas N.m.m.		
		0.099381258	1
yellow	Wort soluble nitrogen dumas N.m.m.		
		-0.091101498	1
black	Wort viscosity EBC	0.426966731	0.1
blue	Wort viscosity EBC	0.10292125	1
brown	Wort viscosity EBC	-0.34119498	1
green	Wort viscosity EBC	0.28341548	1
magenta	Wort viscosity EBC	0.11570454	1
pink	Wort viscosity EBC	-0.515697801	0.004
purple	Wort viscosity EBC	-0.293287964	1
red	Wort viscosity EBC	-0.360236502	1
turquoise	Wort viscosity EBC	0.205114798	1
yellow	Wort viscosity EBC	-0.112260086	1
black	Wort aal.	-0.087783181	1
blue	Wort aal.	0.111360611	1
brown	Wort aal.	-0.166366588	1
green	Wort aal.	-0.243023341	1
magenta	Wort aal.	0.118441065	1
pink	Wort aal.	0.090311914	1
purple	Wort aal.	0.190738763	1
red	Wort aal.	0.280280016	1
turquoise	Wort aal.	0.04221781	1
yellow	Wort aal.	-0.15273551	1
black	Malt soluble nitrogen d.b.		
		-0.031530352	1
blue	Malt soluble nitrogen d.b.		
		-0.018083227	1

brown	Malt soluble nitrogen d.b.		
		0.009830479	1
green	Malt soluble nitrogen d.b.		
		-0.113742286	1
magenta	Malt soluble nitrogen d.b.		
		-0.139180168	1
pink	Malt soluble nitrogen d.b.		
		0.112552919	1
purple	Malt soluble nitrogen d.b.		
		0.03072935	1
red	Malt soluble nitrogen d.b.		
		0.119227936	1
turquoise	Malt soluble nitrogen d.b.		
		0.073934913	1
yellow	Malt soluble nitrogen d.b.		
		-0.053400134	1
black	Malt nitrogen	0.219855324	1
blue	Malt nitrogen	-0.116755243	1
brown	Malt nitrogen	0.080148814	1
green	Malt nitrogen	0.362668565	0.9
magenta	Malt nitrogen	-0.232691658	1
pink	Malt nitrogen	-0.172580048	1
purple	Malt nitrogen	-0.440818475	0.08
red	Malt nitrogen	-0.319872759	1
turquoise	Malt nitrogen	0.021458111	1
yellow	Malt nitrogen	0.105532482	1
black	NIR Malt protein d.b.	0.188166795	1
blue	NIR Malt protein d.b.	-0.109484133	1
brown	NIR Malt protein d.b.	0.101753568	1
green	NIR Malt protein d.b.	0.270932161	1
magenta	NIR Malt protein d.b.	-0.204521772	1
pink	NIR Malt protein d.b.	-0.110208874	1
purple	NIR Malt protein d.b.	-0.331641258	1
red	NIR Malt protein d.b.	-0.215247932	1
turquoise	NIR Malt protein d.b.	0.007899672	1
yellow	NIR Malt protein d.b.	0.11098193	1
black	Malt protein d.b.	0.225642413	1
blue	Malt protein d.b.	-0.118563205	1
brown	Malt protein d.b.	0.088197099	1
green	Malt protein d.b.	0.364659221	0.9
magenta	Malt protein d.b.	-0.221895003	1
pink	Malt protein d.b.	-0.181084504	1
purple	Malt protein d.b.	-0.442145982	0.08
red	Malt protein d.b.	-0.329046742	1
turquoise	Malt protein d.b.	0.016996316	1
yellow	Malt protein d.b.	0.10362192	1
black	Kolbach index	-0.175800431	1

blue	Kolbach index	0.130137045	1
brown	Kolbach index	-0.067280756	1
green	Kolbach index	-0.364321205	0.9
magenta	Kolbach index	0.051410385	1
pink	Kolbach index	0.203044569	1
purple	Kolbach index	0.333614442	1
red	Kolbach index	0.341886271	1
turquoise	Kolbach index	0.066727698	1
yellow	Kolbach index	-0.144468471	1
black	Diastatic power WK.d.b.		
		0.024406707	1
blue	Diastatic power WK.d.b.		
		0.062760105	1
brown	Diastatic power WK.d.b.		
		-0.186933737	1
green	Diastatic power WK.d.b.		
		-0.208118278	1
magenta	Diastatic power WK.d.b.		
		0.106571948	1
pink	Diastatic power WK.d.b.		
		0.00567486	1
purple	Diastatic power WK.d.b.		
		0.185779528	1
red	Diastatic power WK.d.b.		
		0.225022628	1
turquoise	Diastatic power WK.d.b.		
		0.089811874	1
yellow	Diastatic power WK.d.b.		
		-0.136148549	1
black	Free amino nitrogen EBC.ppm.		
		-0.39584734	0.4
blue	Free amino nitrogen EBC.ppm.		
		-0.1027032	1
brown	Free amino nitrogen EBC.ppm.		
		0.32691918	1
green	Free amino nitrogen EBC.ppm.		
		-0.29169353	1
magenta	Free amino nitrogen EBC.ppm.		
		0.063536726	1
pink	Free amino nitrogen EBC.ppm.		
		0.518738199	0.004
purple	Free amino nitrogen EBC.ppm.		
		0.388134929	0.5
red	Free amino nitrogen EBC.ppm.		
		0.395581395	0.4
turquoise	Free amino nitrogen EBC.ppm.		
		-0.191408013	1
yellow	Free amino nitrogen EBC.ppm.		
		0.132102233	1
black	Beta glucan EBC.ppm.		
		0.182048515	1

blue	Beta glucan EBC.ppm.		
		-0.024545867	1
brown	Beta glucan EBC.ppm.		
		-0.157863616	1
green	Beta glucan EBC.ppm.		
		0.228974437	1
magenta	Beta glucan EBC.ppm.		
		0.221297837	1
pink	Beta glucan EBC.ppm.		
		-0.296138668	1
purple	Beta glucan EBC.ppm.		
		-0.172735524	1
red	Beta glucan EBC.ppm.		
		-0.246324996	1
turquoise	Beta glucan EBC.ppm.		
		-0.003826268	1
yellow	Beta glucan EBC.ppm.		
		0.044086303	1
black	Malt alpha amylase U.g.		
		-0.225816147	1
blue	Malt alpha amylase U.g.		
		-0.037896523	1
brown	Malt alpha amylase U.g.		
		0.082910667	1
green	Malt alpha amylase U.g.		
		-0.329550665	1
magenta	Malt alpha amylase U.g.		
		0.046649273	1
pink	Malt alpha amylase U.g.		
		0.267487894	1
purple	Malt alpha amylase U.g.		
		0.271912362	1
red	Malt alpha amylase U.g.		
		0.330777012	1
turquoise	Malt alpha amylase U.g.		
		-0.083127081	1
yellow	Malt alpha amylase U.g.		
		-0.041936254	1
black	Beta glucanase U.kg.	-0.583493765	1.00E-04
blue	Beta glucanase U.kg.	-0.175382627	1
brown	Beta glucanase U.kg.	0.551945107	7.00E-04
green	Beta glucanase U.kg.	-0.181615352	1
magenta	Beta glucanase U.kg.	-0.033642277	1
pink	Beta glucanase U.kg.	0.655904073	1.00E-06
purple	Beta glucanase U.kg.	0.361353668	1
red	Beta glucanase U.kg.	0.305186948	1
turquoise	Beta glucanase U.kg.	-0.383757788	0.5
yellow	Beta glucanase U.kg.	0.287619808	1
black	Malt Limit Dextrinase U.kg.		
		0.003788849	1
blue	Malt Limit Dextrinase U.kg.		
		0.132995848	1

brown	Malt Limit Dextrinase U.kg.		
		-0.021381659	1
green	Malt Limit Dextrinase U.kg.		
		-0.239831744	1
magenta	Malt Limit Dextrinase U.kg.		
		-0.140621812	1
pink	Malt Limit Dextrinase U.kg.		
		0.171728989	1
purple	Malt Limit Dextrinase U.kg.		
		0.159541926	1
red	Malt Limit Dextrinase U.kg.		
		0.286528708	1
turquoise	Malt Limit Dextrinase U.kg.		
		0.152326219	1
yellow	Malt Limit Dextrinase U.kg.		
		-0.164030626	1
black	Friability	-0.242450599	1
blue	Friability	0.184623424	1
brown	Friability	0.05616672	1
green	Friability	-0.409843787	0.2
magenta	Friability	-0.179954897	1
pink	Friability	0.318518723	1
purple	Friability	0.308844764	1
red	Friability	0.406739457	0.3
turquoise	Friability	0.097774371	1
yellow	Friability	-0.159211232	1

Supplementary Table 4. Hordein-type and ALP specific peptides quantified by LC-sMRM-MS method.

Protein group	Accession	Peptide sequence
ALP	F2EGD5	QQCCQPLAQISEQAR
ALP	F2EGD5	QQQGQSFTQPQQQQSQSFGQPQQQVPVEVMR
ALP	F2EGD5	CQAVCSMAQVIMR
ALP	M0VEH1	QQCCQPLAQISEQNR
ALP	M0VEH1	MVLQTLPSMCR
ALP	M0VH55	QQCCQPLAHISEQAR
B hordein	I6SJ22	LQMLQLSSCHVLQQQCCQQLPQISEQFR
B hordein	I6SJ22	MCNVNVPLYDIMPPDFWH
B hordein	I6SJ22	MPQLIAR
B hordein	I6SJ22	VFLQQQCSPVR
B hordein	P06470	VFLQQQCSPVPVPQR
B hordein	I6SJ26	ILPFGIDTR
B hordein	I6SJ26	TLPMMCSVNVPFYR
B hordein	I6TRT5	IVPLAIDTR
B hordein	I6TRT5	SQMLQQSSCHVLQQQCCQQLPQIPEQLR
B hordein	I6TRT5	TLPTMCSVNVPLYR
B hordein	I6TRT5	VFLQQQCSPVAMSQR
B hordein	I6TRT5	AIVYSIVLR
B hordein	Q3YAF9	QLPQIPEQFR
B hordein	A0A8I6WDG9	VFLQQQCSPVPMPQR
B hordein	Q40022	SQMLQQSSCHVLQQQCCQQLPQIPEQFR
B hordein	I6TMV2	LQMLQQSSCHVLQQQCCQQLPQISEQFR
C hordein	Q40055	QLNPSHQELQSPQQPFLK
D hordein	Q84LE9	AQQLAAQLPAMCR
D hordein	Q84LE9	DVSPECRPVALSQVVR
D hordein	Q84LE9	ELQESSLEACR

D hordein	Q84LE9	GGSFYPGGTAPPLQQGGWWGTSVK
D hordein	Q84LE9	QYEQQTEVPSK
D hordein	Q84LE9	LEGGGGLLASQ
γ hordein	I6TMV6	APFVGVVTGVGGQ
γ hordein	I6TMV6	CTAIDSIVHAIFMQQGQR
γ hordein	I6TMV6	QGVQIVQQQPQPQQVGQCVLVQGQGVAQPQQLA QMEAIR
γ hordein	I6TMV6	VMQQQCCLQLAQIPEQYK
γ hordein	I6TMV6	ILQQSSCR
γ hordein	A0A8I6WNF0	CTTIDSIVHAIFMQQGQR
γ hordein	A0A8I6WNF0	QGVQIVQQQPQPQEVGQCVLVQGR
γ hordein	A0A8I6WNF0	APFFSLVNAGML
γ hordein	I6TEV2	EFLLQQCTLDEK
γ hordein	I6TEV2	QQCCQQLANINEQSR

Supplementary Table 5. Globulin peptides quantified by LC-sMRM-MS method.

Protein group	Accession	Peptide sequence
Globulin (vicilin-type)	A0A8I6X8S0	IIQSDHGFVR
Globulin (vicilin-type)	A0A8I6X8S0	ALRPFDQVSR
Globulin (vicilin-type)	A0A8I6X8S0	VAIMEVNPR
Globulin (vicilin-type)	A0A8I6X8S0	EGDVIVAPAGSIMHLANTDGR
Globulin (vicilin-type)	A0A8I6X8S0	SFHALANQDVR
Globulin (vicilin-type)	A0A8I6X8S0	LGSPAQELTFGRPAR
Globulin (vicilin-type)	A0A8I6X8S0	AQDQDEGFVAGPEQQSR
Globulin (vicilin-type)	A0A8I7BA12	VACLDAAPR
Globulin (vicilin-type)	A0A8I7BA12	AFLQPSHYDADEIAYVR
Globulin (vicilin-type)	A0A8I7BA12	EGEGVVVLLR
Globulin (vicilin-type)	A0A8I7BA12	EGDVFVIPAGSIVYSANTHR
Globulin (vicilin-type)	A0A8I7BA12	GEGEISEASEEQIR
Globulin (vicilin-type)	A0A8I7BA12	LHQITGDQCPHLR
Globulin (vicilin-type)	A0A8I7BA12	ALAFPQQVR
Globulin (vicilin-type)	A0A8I7BA12	AQTEAVFHDGPQQQR
Globulin (vicilin-type)	F2CYL7	LVESEGGSVHVVR
Globulin (vicilin-type)	F2CYL7	NKPQFLVGPTSVLR
Globulin (vicilin-type)	F2CYL7	VILGPELAAGLGVPLK
Globulin (vicilin-type)	F2CYL7	VAVIEPSLPDK
Globulin (vicilin-type)	A0A8I6Y3C5	VTYIQEGGSETSSLEVQR
Globulin (vicilin-type)	A0A8I6Y3C5	VYAIFTSNAINCDDPSHPK

Supplementary Figure 5. Comparison of relative quantitation of B-hordein peptides in barley grain (green) to malt (orange) samples.

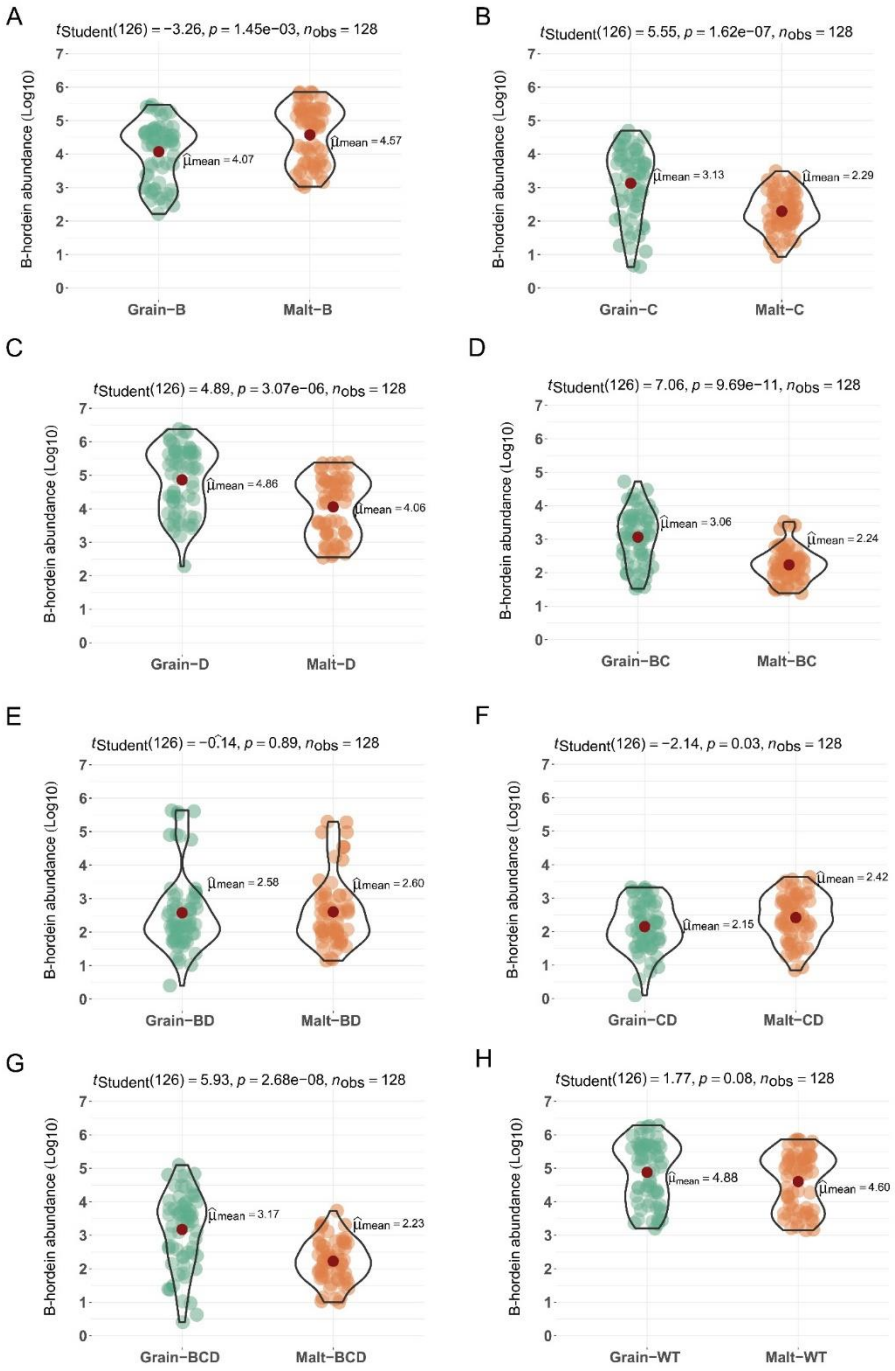


Figure S5. Comparison of relative quantitation of B-hordein peptides in barley grain (green) to malt (orange) samples in all single, double, and triple hordein-reduced lines individually including A) B line, B) C line, C) D line, D) BC line, E) BD line, F) CD line, G) BCD line, H) WT. Peak area of all peptides for each protein group are log10 transformed. Pairwise comparisons were performed using Student's t-test, red dots show means (n=4).

Supplementary Figure 6. Comparison of relative quantitation of γ-hordeins peptides in barley grain to malt samples.

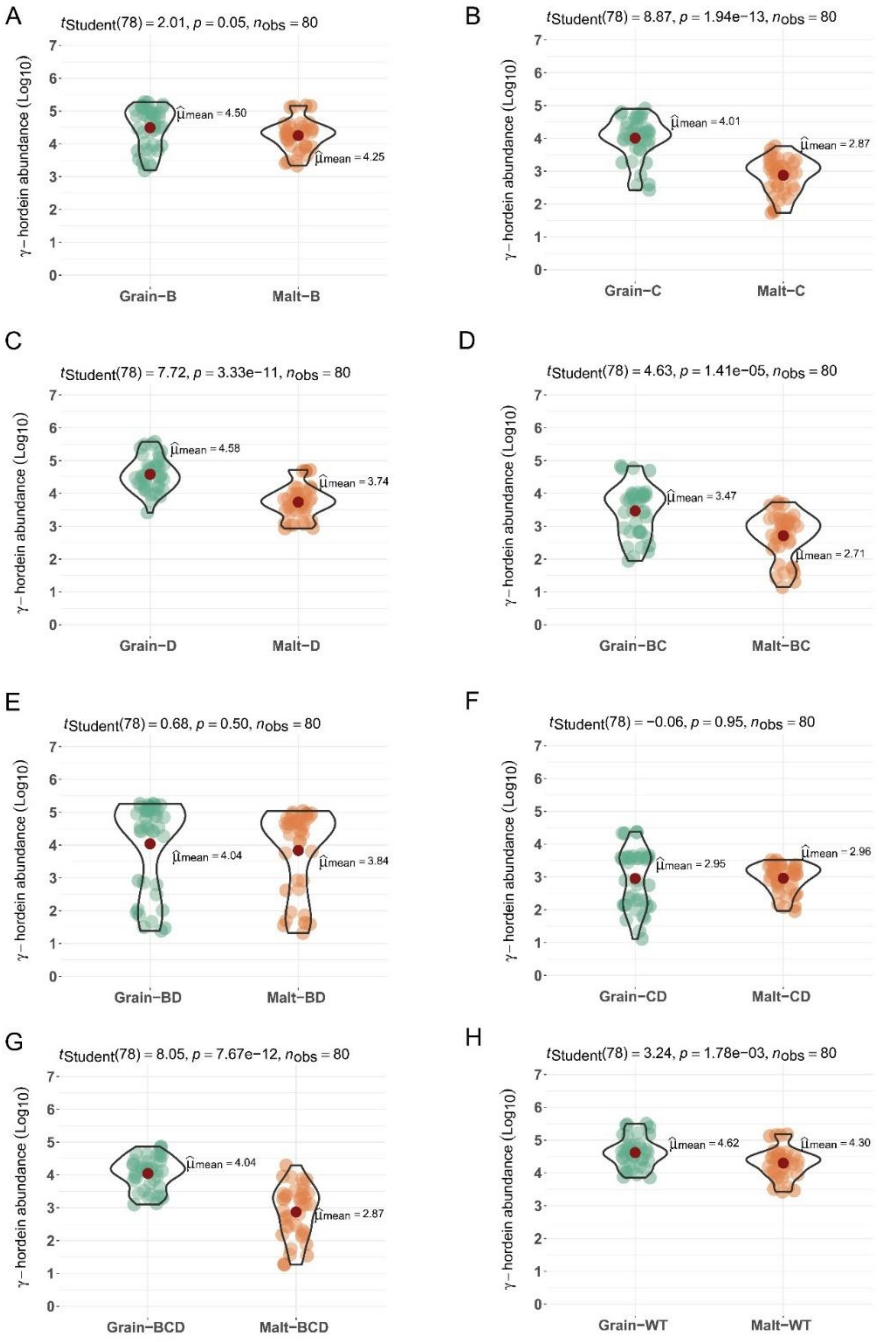


Figure S6. Comparison of relative quantitation of γ-hordeins peptides in barley grain to malt samples in all single, double, and triple hordein-reduced lines individually including A) B line, B) C line, C) D line, D) BC line, E) BD line, F) CD line, G) BCD line, H) WT. Peak area of all peptides for each protein group are $\log_{10}$ transformed. Pairwise comparisons were performed using Student's t-test, red dots show means (n=4).

Supplementary Figure 7. Comparison of relative quantitation of ALP peptides in barley grain to malt samples.

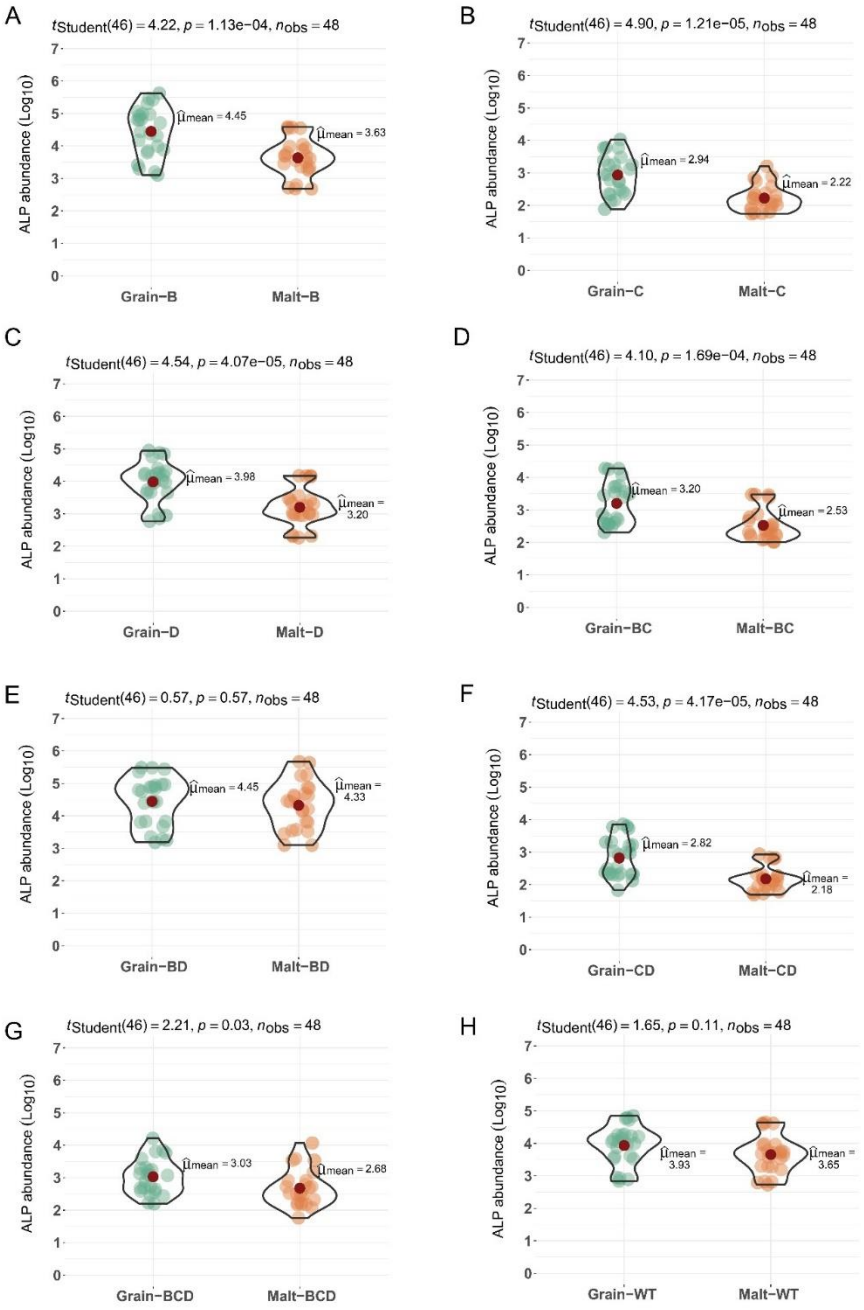


Figure S7. Comparison of relative quantitation of ALP peptides in barley grain to malt samples in all single, double, and triple hordein-reduced lines individually including A) B line, B) C line, C) D line, D) BC line, E) BD line, F) CD line, G) BCD line, H) WT. Peak area of all peptides for each protein group are $\log_{10}$ transformed. Pairwise comparisons were performed using Student's t-test, red dots show means (n=4).

Supplementary Figure 8. Comparison of relative quantitation of D-hordeins peptides in barley grain to malt samples.

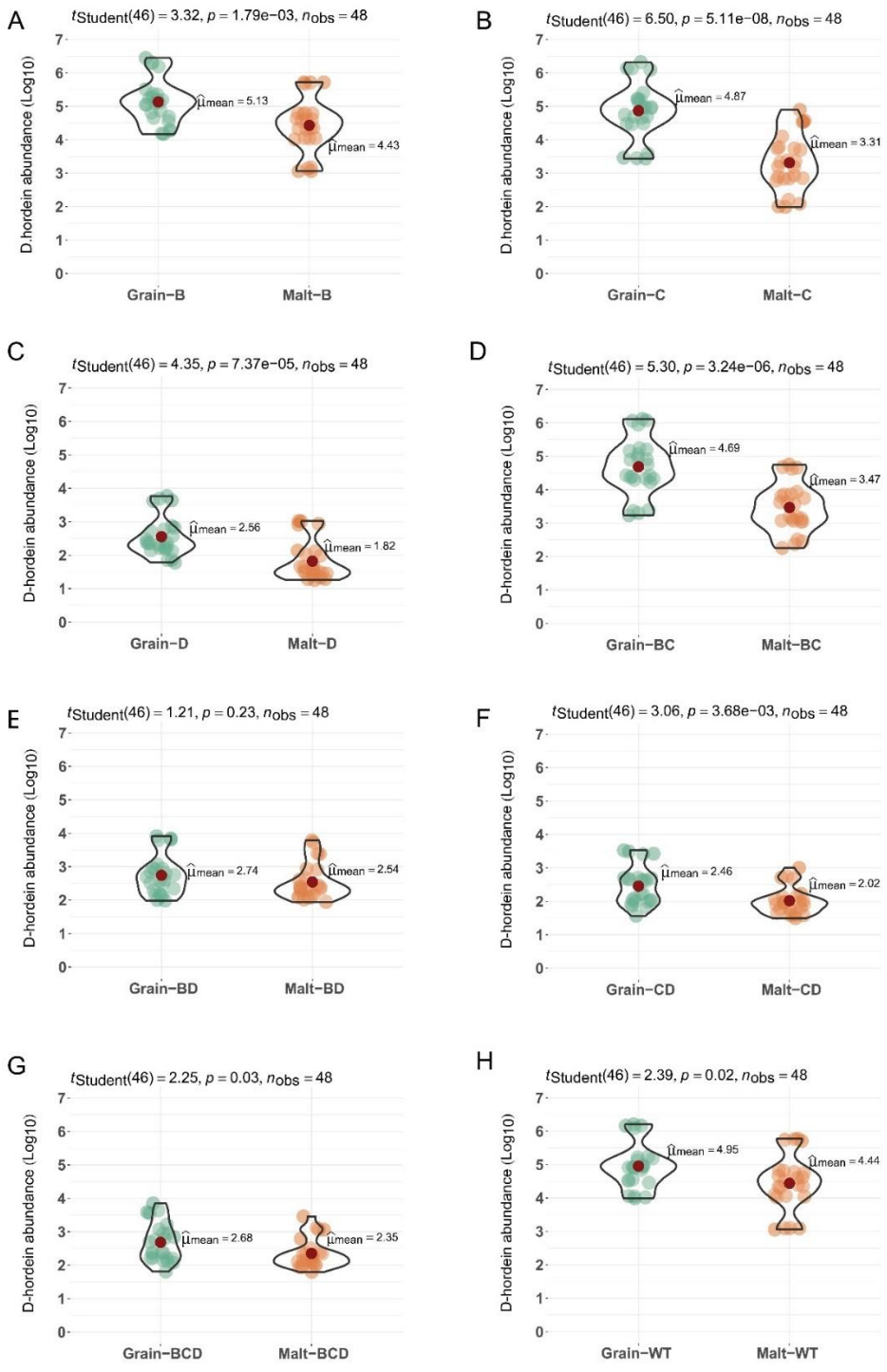


Figure S8. Comparison of relative quantitation of D-hordeins peptides in barley grain to malt samples in all single, double, and triple hordein-reduced lines individually including A) B line, B) C line, C) D line, D) BC line, E) BD line, F) CD line, G) BCD line, H) WT. Peak area of all peptides for each protein group are $\log_{10}$ transformed. Pairwise comparisons were performed using Student's t-test, red dots show means (n=4).

Supplementary Figure 9. Module- eigengene relationship between different hordein-reduced backgrounds including C-, B-, D-reduced in addition to WT and sample type (grain or malt).

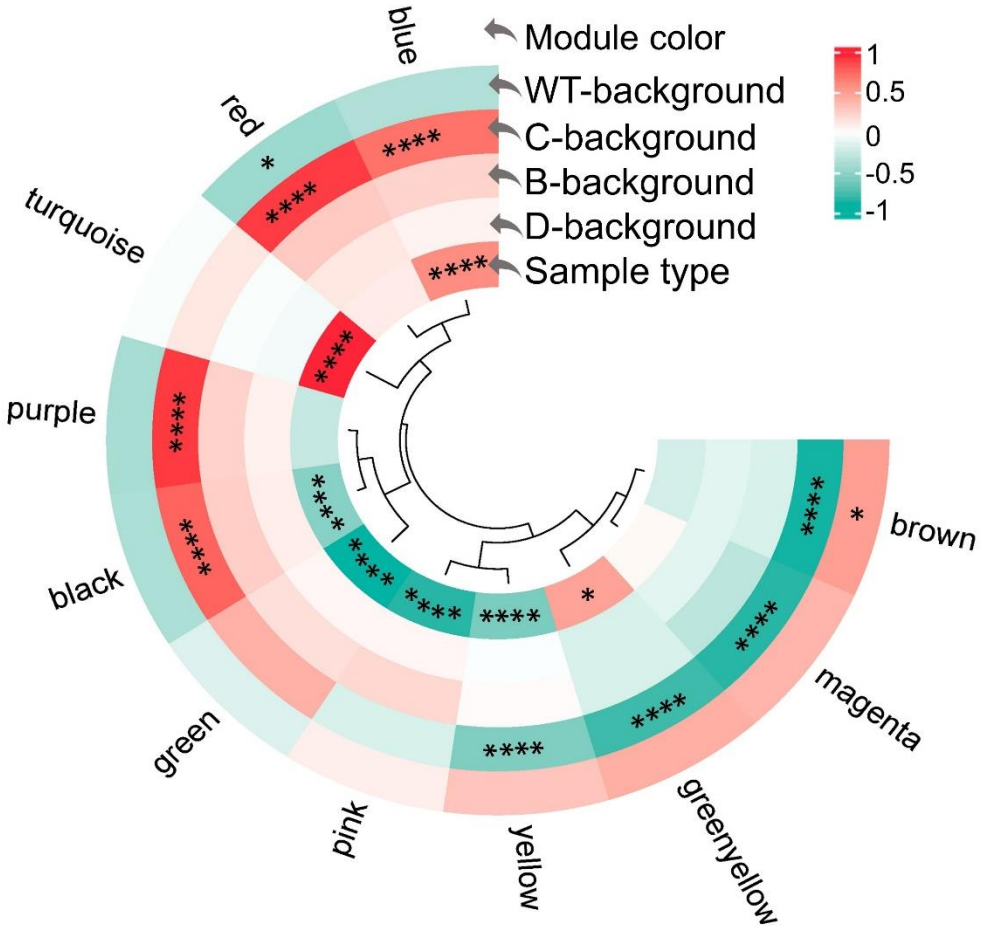


Figure S9. Module-trait relationship between different hordein-reduced backgrounds including C-, B-, D-reduced in addition to WT and sample type (grain or malt). First row from the top of the heatmap shows the 11 modules and green to red colour scale shows the correlation of genetic backgrounds and sample types with modules using the Pearson correlation method. Each module correlates to genetic background and sample type with a correlation ranging from -1 to 1.

Supplementary Figure 10. MA plots of B-, C-, and D-hordein reduced background lines in addition to WT.

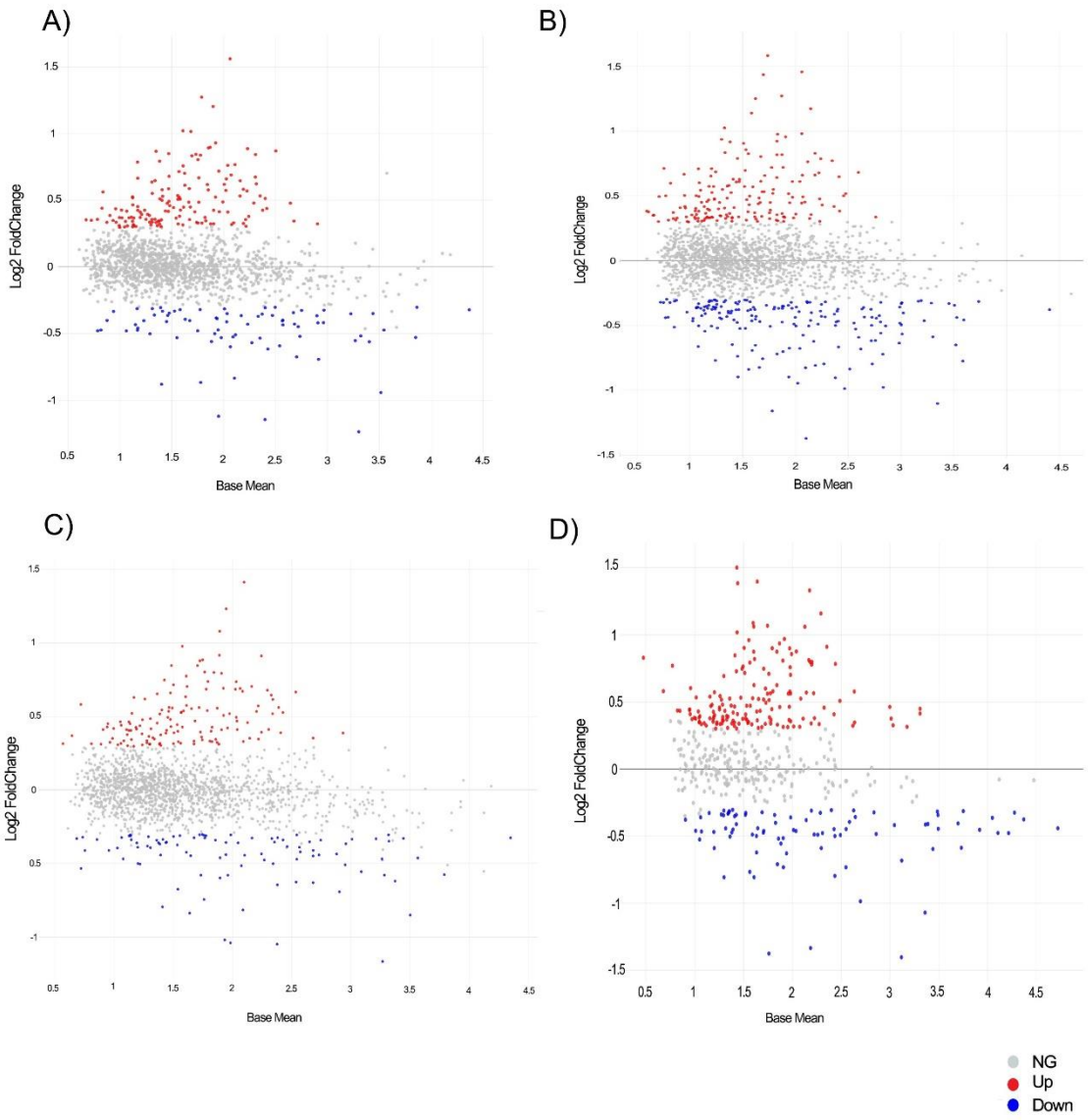


Figure S10. MA plots showing differential protein abundance between barley grain and malt in hordein-reduced barley lines of A) B-hordein reduced background, B) C-hordein reduced background, C) D-background) and D) WT. Each dot represents a protein, with the Y-axis referring to the log2 fold change (M) and x-axis showing the mean average of abundance level (A) of proteins in two comparing sample types (grain vs. malt). Proteins that are significantly (P-value <0.05, and fold change>2) upregulated are shown with red colour, proteins that are significantly down-regulated are depicted in blue and non-significant proteins are shown in grey.

Supplementary Figure 11. GO enrichment analysis of molecular function terms for up- and down-regulated proteins in lines including hordein-reduced genetic background and WT.

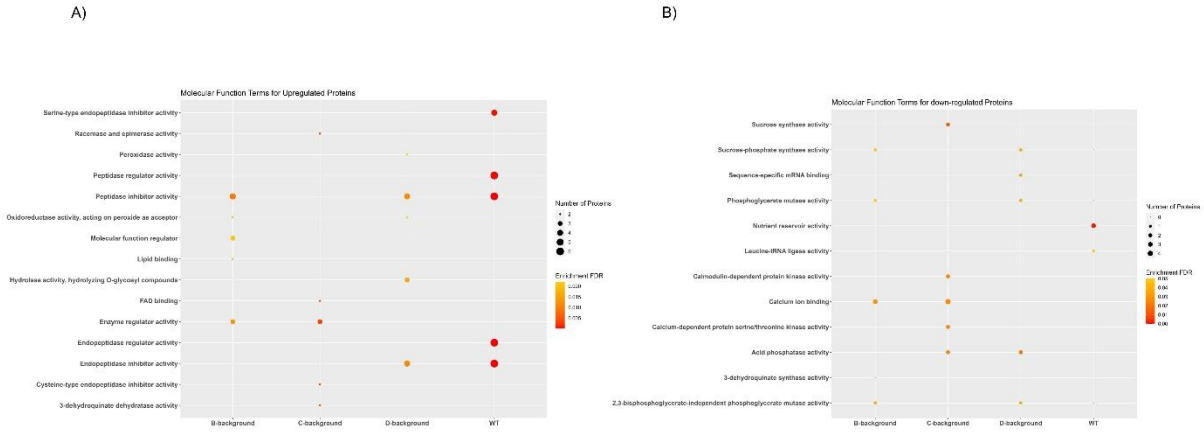


Figure S11. Gene Ontology enrichment analysis of top 5 molecular process terms for up- and down-regulated proteins. A) up-regulated and B) down-regulated in three hordein-reduced genetic background and WT. Y-axis shows the GO terms for biological process and x-axis represents hordein-reduced genetic background (B-, C-, and D-) lines and WT. Size of dots show the number of proteins for each term and color scale of dots represents the FDR-corrected p value (<0.05) for each term.

Supplementary Figure 12. Beta amylase (Q9AVJ8) abundance and Diastatic power measured in hordein reduced lines.

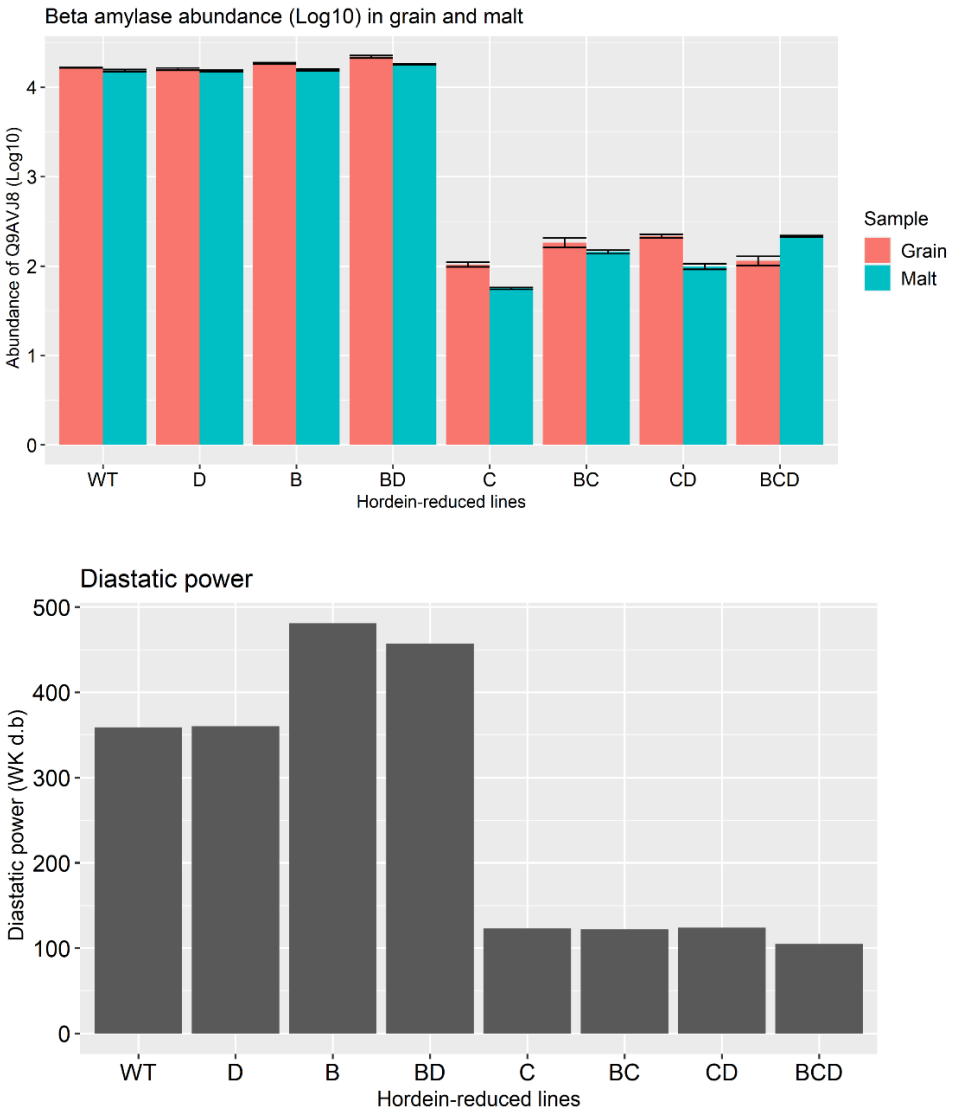


Figure 12. Beta amylase (Q9AVJ8) abundance and Diastatic power measured in hordein reduced lines.

Supplementary Figure 13. Relative abundance of papain-like cysteine proteases

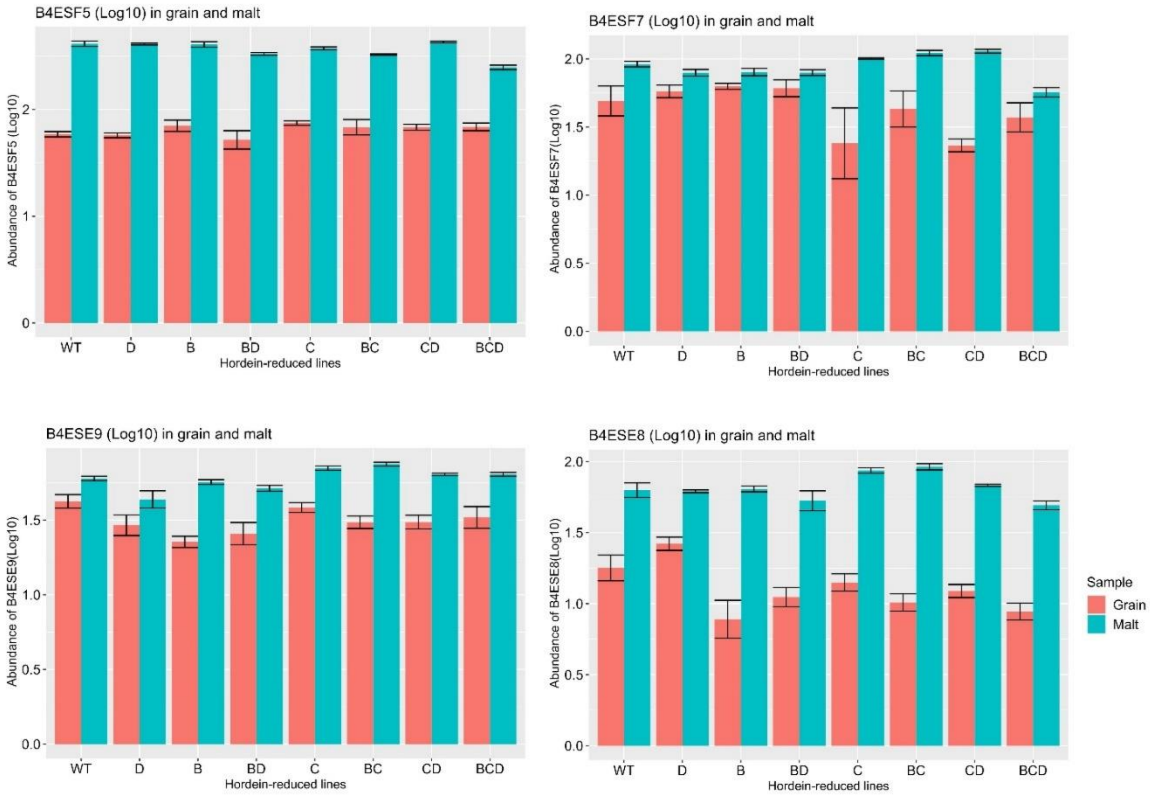
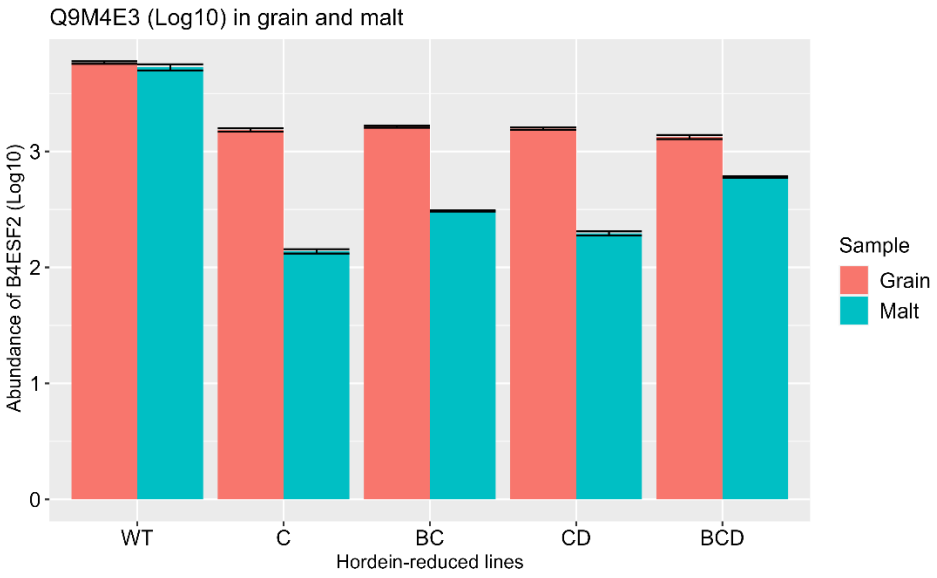


Figure 13. Relative abundance of papain-like cysteine proteases (B4ESF5, B4ESF7, B4ESE9, B4ESE8) in hordein-reduced barley grain and malt samples. Red color shows the grain and aqua refers to the malt samples.

Supplementary Figure 14. Abundance of hordoindoline protein.



Supplementary Figure 14. Abundance of hordoindoline protein in lines with C-hordein reduced background and WT

Supplementary Table 6. Proteins that positively and negatively correlated with C-hordein reduced line in their genetic background.

Protein	VIP score	Module colour	Correlation
A0A8I6YHY9	3.9	Blue	Positive
M0YW47	3.78	Purple	Positive
F2E8N5	3.7	Blue	Positive
A0A8I7B6N5	3.64	Blue	Positive
F2DXR4	3.6	Black	Positive
M0VNU5	3.37	Red	Positive
Q58IJ7	2.95	Blue	Positive
A0A8I6X2P8	2.95	Red	Positive
A0A8I6X7D5	2.85	Blue	Positive
D2KZ45	2.77	Blue	Positive
A0A8I6X3H5	2.65	Blue	Positive
A0A8I6XS01	2.46	Blue	Positive
F2CY72	2.44	Blue	Positive
A0A8I6XCS6	2.35	Black	Positive
F2DN23	2.34	Blue	Positive
A0A8I6YTI2	2.32	Blue	Positive
A0A8I6Y3F5	2.31	Blue	Positive
A0A8I6WUM9	2.3	Black	Positive
Q06572	2.3	Blue	Positive
A0A8I6YAS8	2.28	Blue	Positive
A0A8I6XB73	2.26	Red	Positive
Q40009	2.18	Blue	Positive
F2EGH1	2.13	Red	Positive
Q946Z0	2.12	Blue	Positive
A0A8I6YTT6	2.11	Red	Positive
P33044	2.08	Blue	Positive
Q6KE44	2.08	Blue	Positive
F2E9A5	2.05	Purple	Positive
A0A8I6WHB0	2.04	Red	Positive
Q8GT53	2.03	Red	Positive
F2EEH9	2.2	Red	Positive
A0A8I6YC55	1.98	Blue	Positive
A0A287T8X2	1.97	Blue	Positive
F2CRQ8	1.97	Red	Positive
A0A8I6XF91	1.96	Blue	Positive
F2EBZ7	1.93	Black	Positive
A0A8I6WIG4	1.88	Blue	Positive
K9J8S8	1.88	Blue	Positive
A0A8I7B2L4	1.86	Black	Positive

F2EFN1	1.85	Red	Positive
A7YA60	1.83	Blue	Positive
F2EFB5	1.82	Red	Positive
F2DLZ6	1.78	Blue	Positive
F2EBU5	1.78	Purple	Positive
A0A8I6XQT6	1.75	Blue	Positive
F2DEF4	1.74	Blue	Positive
Q5MBN3	1.73	Black	Positive
F2DI09	1.73	Blue	Positive
M0ZBM5	1.72	Blue	Positive
A0A8I6YVL6	1.68	Black	Positive
F2CR56	1.68	Blue	Positive
A0A8I6XPA9	1.68	Purple	Positive
A0A8I6XSQ9	1.66	Blue	Positive
A0A8I6YMG7	1.66	Blue	Positive
P55308	1.66	Blue	Positive
A0A8I6YTX6	1.65	Blue	Positive
B4ESD9	1.63	Black	Positive
M0WQ79	1.63	Blue	Positive
A0A8I6WVW5	1.62	Blue	Positive
F2CRR7	1.62	Blue	Positive
F2CWG0	1.62	Blue	Positive
F2DG65	1.6	Black	Positive
A0A8I6XAI5	1.54	Black	Positive
A0A8I7B654	1.54	Blue	Positive
A0A8I7BFW3	1.54	Blue	Positive
F2DTQ3	1.54	Red	Positive
F2E600	1.53	Black	Positive
F2DTB4	1.53	Blue	Positive
A0A8I6X7Y1	1.52	Blue	Positive
A0A8I6X8A8	1.48	Blue	Positive
F2DHE3	1.47	Black	Positive
A0A8I6WZ81	1.46	Black	Positive
F2D7S3	1.46	Black	Positive
F2D5Q2	1.46	Red	Positive
F2CS80	1.45	Blue	Positive
Q84L51	1.45	Red	Positive
A0A8I6Y358	1.44	Black	Positive
Q42841	1.44	Blue	Positive
A0A8I7BDH5	1.43	Red	Positive
C4PKL2	1.41	Blue	Positive
F2DSV2	1.41	Blue	Positive
Q43766	1.4	Blue	Positive
F2EDM7	1.4	Purple	Positive
A0A2Z6DS22	1.38	Black	Positive

A0A8I6XRD3	1.38	Blue	Positive
F2E3M1	1.38	Blue	Positive
A0A8I6X2S1	1.37	Blue	Positive
F2CV17	1.37	Blue	Positive
F2D3M4	1.37	Blue	Positive
P33045	1.36	Black	Positive
A0A8I6W4A1	1.36	Blue	Positive
A0A8I6YIG1	1.36	Blue	Positive
Q8SA48	1.36	Blue	Positive
A0A287QXJ4	1.35	Blue	Positive
A0A8I6WTE6	1.35	Blue	Positive
F2D3N8	1.35	Blue	Positive
F2DZ78	1.35	Blue	Positive
A0A8I7BBY6	1.34	Blue	Positive
F2DC79	1.34	Blue	Positive
F2D5F9	1.33	Blue	Positive
O22462	1.33	Red	Positive
F2DNL2	1.32	Blue	Positive
Q0GR09	1.32	Blue	Positive
P28814	1.32	Red	Positive
F2EGB7	1.31	Black	Positive
A0A8I7B5W4	1.31	Blue	Positive
A0A8I7B6H8	1.31	Blue	Positive
F2D6F2	1.31	Blue	Positive
F2CZM5	1.29	Blue	Positive
A0A8I7B211	1.29	Purple	Positive
A0A8I6YBK0	1.28	Blue	Positive
F2E5P4	1.27	Blue	Positive
P93180	1.27	Blue	Positive
F2D961	1.27	Red	Positive
A0A8I6XJW6	1.26	Blue	Positive
F2E2A1	1.26	Blue	Positive
F2EIJ2	1.26	Blue	Positive
A0A8I6YYQ5	1.24	Blue	Positive
A0A8I7B995	1.23	Black	Positive
Q40036	1.23	Blue	Positive
F2CTC0	1.22	Black	Positive
A0A8I6X1G8	1.22	Blue	Positive
A0A8I6Y7N8	1.22	Blue	Positive
A0A8I6XKT3	1.21	Black	Positive
A0A8I7B312	1.21	Black	Positive
F2E9D5	1.21	Black	Positive
A0A8I6XX62	1.21	Blue	Positive
A0A8I7B598	1.21	Blue	Positive
A0A8I7B799	1.21	Blue	Positive

F2DUK4	1.21	Blue	Positive
F2D3W3	1.21	Red	Positive
F2CSP8	1.2	Blue	Positive
F2DLX6	1.2	Blue	Positive
A0A8I6XYW9	1.19	Blue	Positive
A0A8I7B0V1	1.19	Blue	Positive
F2CW75	1.19	Blue	Positive
F2D6V0	1.19	Blue	Positive
B1NC18	1.19	Red	Positive
A0A8I7BDB9	1.18	Black	Positive
F2D043	1.18	Blue	Positive
F2DY62	1.18	Blue	Positive
F2E4G1	1.18	Blue	Positive
A0A8I6YHE1	1.17	Black	Positive
F2DMT1	1.17	Blue	Positive
F2DWC8	1.16	Black	Positive
F2E276	1.16	Black	Positive
P32024	1.16	Blue	Positive
P45851	1.16	Blue	Positive
F2E7F7	1.16	Purple	Positive
Q2KM86	1.16	Purple	Positive
F2CYL7	1.15	Black	Positive
A0A1C9ZVX7	1.15	Blue	Positive
A0A8I7BDQ9	1.15	Blue	Positive
F2DUF5	1.15	Blue	Positive
F2E0R2	1.15	Blue	Positive
F2ED32	1.15	Blue	Positive
A0A8I7B3K6	1.14	Blue	Positive
A0A8I6XX25	1.13	Black	Positive
A0A8I6YRZ3	1.13	Blue	Positive
F2DA79	1.13	Blue	Positive
F2E637	1.13	Blue	Positive
Q8H1V6	1.12	Black	Positive
A0A8I6XBR0	1.11	Blue	Positive
A0A8I6Y5W1	1.11	Blue	Positive
A0A8I6X3E8	1.11	Red	Positive
F2DGF0	1.1	Black	Positive
A0A1C9ZW58	1.1	Blue	Positive
F2DDV8	1.1	Blue	Positive
M0Z0W7	1.1	Blue	Positive
F2CW94	1.1	Red	Positive
F2D4C8	1.1	Red	Positive
A0A8I7B530	1.09	Black	Positive
A0A8I7B3A8	1.09	Blue	Positive
F2CRG8	1.09	Blue	Positive

F2DWV6	1.09	Blue	Positive
F2CRH6	1.08	Blue	Positive
A0A8I6X6P0	1.07	Black	Positive
F2CSR1	1.07	Black	Positive
F2DK64	1.07	Black	Positive
F2CZT5	1.07	Blue	Positive
F2EI80	1.07	Blue	Positive
F2DH57	1.07	Purple	Positive
F2D3Z1	1.07	Red	Positive
F2E8C1	1.07	Red	Positive
A0A8I6X850	1.06	Blue	Positive
A0A8I6XCW4	1.06	Blue	Positive
A0A8I6XQJ3	1.06	Blue	Positive
F2DW69	1.06	Blue	Positive
Q9M4Q0	1.06	Blue	Positive
A0A8I6WJ36	1.05	Black	Positive
A0A8I6YTU3	1.05	Blue	Positive
A0A8I7BH22	1.05	Blue	Positive
F2CYU9	1.05	Blue	Positive
F2DW39	1.05	Blue	Positive
F2CSZ1	1.05	Red	Positive
A0A8I6WMP9	1.04	Black	Positive
F2CUE5	1.04	Blue	Positive
F2CPQ3	1.04	Red	Positive
F2E858	1.04	Red	Positive
A0A191TDJ4	1.03	Blue	Positive
F2DSZ9	1.03	Blue	Positive
Q8LLB9	1.03	Blue	Positive
W8E7E3	1.03	Blue	Positive
A0A287SUL6	1.03	Red	Positive
Q42839	1.03	Red	Positive
A0A5B9D476	1.02	Blue	Positive
A0A8I6WND3	1.02	Blue	Positive
A0A8I6WZD3	1.02	Blue	Positive
F2DIK7	1.02	Blue	Positive
F2E6W5	1.02	Blue	Positive
Q0KKC3	1.02	Blue	Positive
A0A8I6WEA7	1.02	Purple	Positive
F2DIV0	1.02	Red	Positive
P51106	1.02	Red	Positive
A0A287MHI9	1.01	Blue	Positive
A0A8I6YGM2	1.01	Blue	Positive
F2DG78	1.01	Blue	Positive
M0UNE9	1.01	Blue	Positive
F2DHC9	1.01	Purple	Positive

A0A8I6WZY3	1	Black	Positive
F2DAA1	1	Black	Positive
F2DF28	1	Black	Positive
A0A8I6WBR4	1	Blue	Positive
A0A8I6YKR1	1	Blue	Positive
F2DXZ9	1	Blue	Positive
Q94L27	1	Purple	Positive
A0A8I7B149	1	Red	Positive
A0A8I6Y012	0.99	Blue	Positive
A0A8I6Y6L6	0.99	Blue	Positive
A0A8I7BHG9	0.99	Blue	Positive
F2DVQ3	0.99	Blue	Positive
D2KFI1	0.99	Red	Positive
F2DNH5	0.98	Blue	Positive
F2DU45	0.98	Blue	Positive
F2E0G2	0.98	Blue	Positive
F2CQI5	0.98	Purple	Positive
A0A8I6WT14	0.98	Red	Positive
F2CUN1	0.97	Black	Positive
F2DAL6	0.97	Blue	Positive
A0A8I6Y1C6	0.96	Blue	Positive
F2EJA0	0.96	Blue	Positive
A0A3S9KBF5	0.95	Blue	Positive
A0A8I6Y9U7	0.95	Blue	Positive
F2EKA8	0.95	Blue	Positive
A3RHH8	0.95	Purple	Positive
F2D218	0.94	Blue	Positive
A0A8I6WVQ0	0.93	Black	Positive
F2CS25	0.93	Blue	Positive
F2D449	0.93	Blue	Positive
F2DFZ2	0.93	Blue	Positive
F2DJ62	0.93	Blue	Positive
A0A8I6WYC1	0.93	Red	Positive
F2CYS2	0.92	Black	Positive
A0A8I6XYA1	0.92	Blue	Positive
F2D539	0.92	Blue	Positive
F2D663	0.92	Blue	Positive
F2DQP5	0.92	Purple	Positive
P29114	0.92	Purple	Positive
A0A8I6X2Q8	0.91	Blue	Positive
F2CTS4	0.91	Blue	Positive
Q6UFY6	0.91	Purple	Positive
F2EBG2	0.91	Red	Positive
A0A8I6YFE3	0.9	Blue	Positive
F2CSI6	0.9	Blue	Positive

F2D5L7	0.9	Blue	Positive
A0A8I7BFL1	0.89	Black	Positive
A0A8I6YGK1	0.89	Blue	Positive
F2CZC7	0.89	Blue	Positive
F2CZV2	0.89	Blue	Positive
Q94IM7	0.89	Blue	Positive
A0A8I6YMC7	0.89	Purple	Positive
F2CSS7	0.89	Purple	Positive
Q9M4C7	0.89	Red	Positive
A0A8I6Y933	0.88	Blue	Positive
A0A8I6Z8J3	0.88	Blue	Positive
F2CQ43	0.88	Blue	Positive
F2D318	0.88	Blue	Positive
F2DMV0	0.88	Blue	Positive
A0A8I6WYI0	0.88	Purple	Positive
F2ELV4	0.88	Purple	Positive
A0A8I6WUL6	0.87	Black	Positive
F2ED94	0.87	Blue	Positive
F2EFA8	0.87	Blue	Positive
M0X1N3	0.87	Blue	Positive
A0A8I6Y722	0.86	Blue	Positive
F2DA71	0.86	Blue	Positive
Q5D5T4	0.86	Blue	Positive
P05336	0.86	Purple	Positive
F2DJC5	0.85	Black	Positive
A0A8I6WI88	0.85	Blue	Positive
F2CZ44	0.85	Blue	Positive
A0A8I7BCF9	0.85	Red	Positive
F2CWX3	0.85	Red	Positive
A0A8I7BBP4	0.84	Blue	Positive
F2E4D2	0.84	Blue	Positive
M0Y818	0.84	Blue	Positive
F2E7E5	0.84	Purple	Positive
A0A8I6YNT4	0.84	Red	Positive
F2DF97	0.83	Black	Positive
A0A8I6WMV8	0.83	Blue	Positive
A0A8I6XD39	0.83	Blue	Positive
A0A8I7B1D8	0.83	Red	Positive
F2CQY9	0.82	Black	Positive
A0A8I6X3G0	0.82	Blue	Positive
F2D8B8	0.82	Blue	Positive
F2DHA3	0.82	Purple	Positive
F2DEK7	0.82	Red	Positive
A0A8I6Y560	0.81	Black	Positive
F2DCD7	0.81	Blue	Positive

F2DNM2	0.81	Blue	Positive
Q9SME8	0.81	Blue	Positive
A0A8I7B6Q0	0.81	Red	Positive
F2DPK1	0.81	Red	Positive
A0A8I6Y961	0.8	Blue	Positive
A0A8I7B9Q5	0.8	Blue	Positive
F2DR21	0.8	Blue	Positive
F2DY13	0.8	Blue	Positive
A0A8I6XNK1	0.8	Red	Positive
A0A8I6Z0L1	0.8	Red	Positive
F2EKQ3	0.79	Black	Positive
F2CXU9	0.79	Blue	Positive
D2XV82	0.79	Purple	Positive
A0A8I6Y4I6	0.79	Red	Positive
A0A8I6Z0V9	0.78	Black	Positive
F2CZV3	0.78	Blue	Positive
F2DY57	0.78	Blue	Positive
A0A8I6XI84	0.78	Purple	Positive
A0A8I6YR29	0.78	Purple	Positive
F2E1V1	0.78	Red	Positive
F2CS51	0.77	Black	Positive
A0A8I6YDP8	0.77	Blue	Positive
F2DJE0	0.77	Blue	Positive
F2DLJ7	0.77	Blue	Positive
F2EL27	0.77	Purple	Positive
A0A8I6Y5C8	0.77	Red	Positive
A0A8I6WIN4	0.76	Blue	Positive
A0A8I6XBR3	0.76	Blue	Positive
F2DI94	0.76	Blue	Positive
F2CYQ8	0.76	Red	Positive
F2D779	0.76	Red	Positive
Q5D5Y6	0.76	Red	Positive
F2D9S4	0.75	Black	Positive
F2DCC0	0.75	Blue	Positive
Q4QXS7	0.75	Blue	Positive
A0A8I7B9Z9	0.75	Red	Positive
Q58IJ6	0.75	Red	Positive
F2CS48	0.74	Blue	Positive
F2DFL5	0.73	Black	Positive
F2D642	0.73	Blue	Positive
O23979	0.73	Blue	Positive
A0A8I6XLF0	0.73	Purple	Positive
F2D698	0.73	Red	Positive
F2DXU2	0.73	Red	Positive
Q8VWW3	0.73	Red	Positive

A0A8I6YSR5	0.72	Blue	Positive
A0A8I7B2X4	0.72	Blue	Positive
F2CTJ9	0.72	Blue	Positive
F2DIN7	0.72	Blue	Positive
A7DX57	0.72	Red	Positive
A0A8I6YE09	0.71	Black	Positive
A0A8I6WQG0	0.71	Blue	Positive
A0A8I6Y6U9	0.71	Blue	Positive
A0A8I6Y7M3	0.71	Blue	Positive
F2DQW5	0.71	Blue	Positive
A0A287VK78	0.71	Red	Positive
Q9AYT7	0.71	Red	Positive
A0A8I6WWC5	0.7	Black	Positive
F2DQM8	0.7	Black	Positive
A0A287R7V7	0.7	Blue	Positive
A0A8I6XS59	0.7	Blue	Positive
A0A8I7B3U1	0.7	Blue	Positive
F2CQQ9	0.7	Blue	Positive
M0YWT6	0.7	Blue	Positive
A0A8I7B8E9	0.7	Purple	Positive
F2DI46	0.7	Purple	Positive
Q9T2L5	0.69	Black	Positive
F2CR14	0.69	Blue	Positive
F2CSE0	0.69	Blue	Positive
F2CSX0	0.69	Blue	Positive
M0X0A5	0.69	Purple	Positive
A0A8I6XDU2	0.68	Blue	Positive
A0A8I7B0Y3	0.68	Blue	Positive
A0A8I7BH75	0.68	Blue	Positive
F2E644	0.68	Purple	Positive
M0YTF7	0.68	Purple	Positive
F2E1A0	0.68	Red	Positive
A0A8I6XKI3	0.67	Black	Positive
A0A8I6XDB4	0.67	Blue	Positive
A0A8I6Y7Q2	0.67	Blue	Positive
F2D3D5	0.67	Blue	Positive
F2DV26	0.67	Blue	Positive
F2E8Y5	0.67	Blue	Positive
A0A8I6YE31	0.67	Purple	Positive
A0A8I6XH40	0.66	Blue	Positive
F2CQR0	0.66	Blue	Positive
F2D114	0.66	Blue	Positive
F2E0P3	0.66	Blue	Positive
O81135	0.66	Red	Positive
Q9ZTX1	0.66	Red	Positive

F2CY82	0.65	Black	Positive
F2DSB9	0.65	Black	Positive
A0A8I6XPK4	0.65	Blue	Positive
A0A8I6Y1D4	0.65	Blue	Positive
F2CSH7	0.65	Blue	Positive
F2CUU1	0.65	Blue	Positive
F2D3T5	0.65	Blue	Positive
F2DJ48	0.65	Blue	Positive
F2E2V8	0.65	Blue	Positive
Q711R0	0.65	Blue	Positive
F2CW31	0.65	Purple	Positive
Q8H1X2	0.65	Red	Positive
A0A8I6X2Y8	0.64	Black	Positive
A0A8I6X3P4	0.64	Blue	Positive
F2CY71	0.64	Blue	Positive
F2DWU3	0.64	Blue	Positive
P27337	0.64	Blue	Positive
A0A8I6Y288	0.64	Purple	Positive
A0A8I6YK17	0.64	Red	Positive
P23902	0.64	Red	Positive
A0A287GWL8	0.63	Blue	Positive
A0A8I6X1V3	0.63	Blue	Positive
A0A8I6XZ94	0.63	Blue	Positive
A0A8I6ZAA9	0.63	Blue	Positive
F2DFA7	0.63	Blue	Positive
F2DVI5	0.63	Blue	Positive
F2E7Q2	0.63	Blue	Positive
F2DVJ4	0.63	Red	Positive
A0A8I6Y9D8	0.62	Blue	Positive
A0A8I6YA60	0.62	Blue	Positive
F2DM56	0.62	Blue	Positive
F2E2J6	0.62	Blue	Positive
A0A8I7B1F6	0.62	Purple	Positive
F2EG62	0.62	Purple	Positive
F2CTT1	0.62	Red	Positive
A0A8I6X0G0	0.61	Black	Positive
A0A8I6Y879	0.61	Blue	Positive
C6H0M2	0.61	Blue	Positive
F2DYY1	0.61	Blue	Positive
P02902	0.61	Blue	Positive
A0A8I6XDC4	0.61	Red	Positive
F2CYX9	0.61	Red	Positive
Q6V759	0.61	Red	Positive
F2D4L0	0.6	Black	Positive
A0A8I6YGZ0	0.6	Blue	Positive

A0A8I7BAE5	0.6	Blue	Positive
F2CS67	0.6	Blue	Positive
F2CTP1	0.6	Red	Positive
A0A8I6XST8	0.59	Blue	Positive
A0A8I6XVT7	0.59	Blue	Positive
F2DC74	0.59	Blue	Positive
F2DKD7	0.59	Blue	Positive
Q9XGJ1	0.59	Blue	Positive
A0A8I6Y3C5	0.59	Purple	Positive
A0A8I6Y0G6	0.59	Red	Positive
A0A8I6YLV6	0.59	Red	Positive
F2CT03	0.59	Red	Positive
Q5MX86	0.59	Red	Positive
F2D8R0	0.58	Black	Positive
F2DNL6	0.58	Blue	Positive
O49866	0.58	Blue	Positive
D2KZ38	0.58	Red	Positive
F2CR13	0.58	Red	Positive
A0A8I6XIU5	0.57	Blue	Positive
A0A8I6YQG4	0.57	Blue	Positive
F2DGN6	0.57	Blue	Positive
F2E8K5	0.57	Blue	Positive
F2ELT5	0.57	Purple	Positive
A0A8I6WWD2	0.56	Blue	Positive
F2DHX6	0.56	Blue	Positive
M0YAR4	0.56	Blue	Positive
F2EIJ3	0.56	Red	Positive
F2E1T7	0.55	Black	Positive
A0A8I6WUM3	0.55	Blue	Positive
A0A8I6XBW8	0.55	Blue	Positive
F2DJW8	0.55	Blue	Positive
A1X810	0.55	Purple	Positive
A0A8I6XU09	0.55	Red	Positive
A0A8I6Y2X6	0.55	Red	Positive
F2D7Z6	0.55	Red	Positive
A0A8I6XFJ2	0.54	Blue	Positive
A0A8I6Z7P9	0.54	Blue	Positive
F2CRG7	0.54	Blue	Positive
F2DCR7	0.54	Blue	Positive
Q8LLS0	0.54	Blue	Positive
Q5EXM3	0.54	Purple	Positive
A0A8I7BG65	0.54	Red	Positive
F2DAH2	0.54	Red	Positive
A0A8I6XRS7	0.53	Black	Positive
F2D8X9	0.53	Black	Positive

F2E0T8	0.53	Black	Positive
F2E8J8	0.53	Black	Positive
A0A8I6XYU0	0.53	Blue	Positive
A0A8I7B6V2	0.53	Blue	Positive
F2CVZ3	0.53	Blue	Positive
F2CY28	0.53	Blue	Positive
F2D2J4	0.53	Blue	Positive
P36183	0.53	Blue	Positive
F2D712	0.53	Purple	Positive
A0A8I6WRJ2	0.52	Black	Positive
A0A8I7B8L0	0.52	Black	Positive
A0A8I7BDF4	0.52	Blue	Positive
F2CWH1	0.52	Blue	Positive
A0A287XD56	0.52	Red	Positive
F2CZA8	0.51	Black	Positive
F2CQQ1	0.51	Blue	Positive
F2CUF1	0.51	Blue	Positive
F2DIL7	0.51	Blue	Positive
F2CYR9	0.51	Red	Positive
F2D270	0.51	Red	Positive
A0A8I6W5E6	0.5	Black	Positive
A0A8I6XHD2	0.5	Blue	Positive
A0A8I6YJ60	0.5	Blue	Positive
F2DC26	0.5	Blue	Positive
P12948	0.5	Blue	Positive
F2E3V0	0.5	Purple	Positive
A0A8I6Z9Q5	0.5	Red	Positive
A0A8I7B8T8	0.49	Blue	Positive
A0A8I7B9K7	0.49	Blue	Positive
F2CWS1	0.49	Blue	Positive
F2CZD9	0.49	Blue	Positive
F2DMF8	0.49	Blue	Positive
P42210	0.49	Blue	Positive
A0A8I6YEU1	0.48	Black	Positive
A0A8I6Y124	0.48	Blue	Positive
F2D5X4	0.48	Blue	Positive
A0A8I6Y6I1	0.48	Red	Positive
A0A8I7BJH9	0.48	Red	Positive
F2DRG2	0.48	Red	Positive
D2XV68	0.47	Black	Positive
A0A8I6W6Q7	0.47	Blue	Positive
F2DLC2	0.47	Blue	Positive
F2DSU7	0.47	Blue	Positive
F2E311	0.47	Blue	Positive
Q84LA5	0.47	Purple	Positive

A0A8I6YSA7	0.46	Black	Positive
A0A8I6ZC09	0.46	Blue	Positive
A1X809	0.46	Blue	Positive
A0A8I6YE65	0.46	Red	Positive
A0A8I6WN64	0.45	Black	Positive
Q69EY5	0.45	Black	Positive
A0A8I6XXP7	0.45	Blue	Positive
A0A8I6XYU1	0.45	Blue	Positive
A0A8I6Y3B8	0.45	Blue	Positive
F2CPN8	0.45	Blue	Positive
F2D0V3	0.45	Blue	Positive
F2DPK4	0.45	Purple	Positive
F2E4H3	0.45	Purple	Positive
A0A8I6YHR5	0.45	Red	Positive
M0Z854	0.45	Red	Positive
F2CXE8	0.44	Black	Positive
F2E5L9	0.44	Black	Positive
A0A8I6YF41	0.44	Blue	Positive
A0A8I6YPX2	0.44	Blue	Positive
F2CQF7	0.44	Blue	Positive
F2DEZ2	0.44	Blue	Positive
F2DXB7	0.44	Blue	Positive
F2E6G5	0.44	Blue	Positive
A0A8I6XV73	0.44	Red	Positive
A0A8I6Y6H5	0.44	Red	Positive
A0A8I6Z4K8	0.44	Red	Positive
A0A8I7B9G4	0.44	Red	Positive
F2CX02	0.43	Black	Positive
F2DNM8	0.43	Black	Positive
F2EE28	0.43	Black	Positive
A0A8I6YEN8	0.43	Blue	Positive
F2DHH7	0.43	Blue	Positive
A0A8I6WNB3	0.43	Red	Positive
A0A8I6WSV9	0.43	Red	Positive
F2DQU4	0.43	Red	Positive
A0A8I6WKM0	0.42	Red	Positive
A0A8I6XY61	0.41	Black	Positive
A0A8I7BEF2	0.41	Black	Positive
F2E6V0	0.41	Black	Positive
A0A8I6XBW3	0.41	Blue	Positive
F2E0P9	0.41	Blue	Positive
A0A8I6WJQ4	0.41	Red	Positive
A0A8I6XNV5	0.41	Red	Positive
F2CU44	0.4	Black	Positive
A0A8I6XJ54	0.4	Blue	Positive

A0A8I7B532	0.4	Blue	Positive
F2DLQ7	0.4	Blue	Positive
F2DQZ9	0.4	Blue	Positive
F2DP00	0.39	Black	Positive
A0A8I6WD99	0.39	Blue	Positive
F2D6C1	0.39	Blue	Positive
Q9SME7	0.39	Blue	Positive
A0A8I6XPB3	0.39	Red	Positive
F2CXY0	0.39	Red	Positive
A0A8I6WWX5	0.38	Blue	Positive
A0A8I6YCQ7	0.38	Blue	Positive
A0A8I6YNZ7	0.38	Blue	Positive
A0A8I6Z004	0.38	Blue	Positive
F2D086	0.38	Blue	Positive
F2DNF4	0.38	Blue	Positive
A0A8I6YM43	0.38	Red	Positive
F2D342	0.38	Red	Positive
A0A8I6XXQ4	0.36	Black	Positive
F2DJ37	0.36	Black	Positive
A0A8I6X6D4	0.36	Blue	Positive
A0A8I6YE56	0.36	Blue	Positive
A0A8I7B4T0	0.36	Blue	Positive
B5U8Z1	0.36	Blue	Positive
F2D009	0.36	Blue	Positive
F2D668	0.36	Blue	Positive
M0WT98	0.36	Red	Positive
A0A8I6XAP9	0.35	Blue	Positive
A0A8I7BA39	0.35	Blue	Positive
A0A8I7BJ44	0.35	Blue	Positive
F2E732	0.35	Blue	Positive
A0A8I6X8T8	0.35	Red	Positive
Q8H1V3	0.35	Red	Positive
A0A8I7BEP7	0.34	Black	Positive
F2DWX8	0.34	Black	Positive
A0A8I6WF89	0.34	Blue	Positive
F2DEG1	0.34	Blue	Positive
F2E710	0.34	Blue	Positive
A0A8I6YR61	0.33	Black	Positive
A0A8I6WH86	0.33	Blue	Positive
A0A8I6XN54	0.33	Blue	Positive
A0A8I6XVL6	0.33	Blue	Positive
A0A8I6YWJ8	0.33	Red	Positive
F2E2M4	0.33	Red	Positive
A0A287U5M5	0.32	Blue	Positive
A0A8I6Y763	0.32	Blue	Positive

A0A8I6Z4A5	0.32	Blue	Positive
F2DVB6	0.32	Blue	Positive
F2EA86	0.32	Blue	Positive
Q1PBI1	0.32	Blue	Positive
F2CRI1	0.32	Purple	Positive
F2D3U6	0.31	Black	Positive
A0A7D5DB82	0.31	Blue	Positive
A0A8I6WUK1	0.31	Blue	Positive
A0A8I6XD03	0.31	Blue	Positive
F2EG33	0.31	Purple	Positive
A0A8I6XA13	0.3	Black	Positive
A4UUF3	0.3	Black	Positive
A0A8I6WGG3	0.3	Blue	Positive
A0A8I6Y3E0	0.3	Blue	Positive
F2DQ10	0.3	Blue	Positive
A0A8I7B2H8	0.29	Black	Positive
A0A8I7B6J3	0.29	Blue	Positive
F2CQD4	0.29	Blue	Positive
F2CSW4	0.29	Blue	Positive
F2CU86	0.29	Blue	Positive
F2CX32	0.29	Red	Positive
A0A287QE55	0.28	Black	Positive
A0A8I6Y9Z6	0.28	Blue	Positive
A0A8I6YRT4	0.28	Blue	Positive
F2D6B9	0.28	Blue	Positive
F2DAT1	0.28	Blue	Positive
F2DRC5	0.28	Blue	Positive
A0A8I6YPI7	0.28	Purple	Positive
F2DH88	0.27	Black	Positive
F2CUF4	0.27	Blue	Positive
F2D1J8	0.27	Blue	Positive
F2D643	0.27	Blue	Positive
A0A8I6XJD4	0.27	Purple	Positive
A0A8I6YWR4	0.27	Purple	Positive
A0A8I6WIX1	0.26	Black	Positive
F2DKY1	0.26	Blue	Positive
F2E3G7	0.26	Blue	Positive
F2DA22	0.26	Purple	Positive
A0A8I6YGY2	0.25	Blue	Positive
A0A8I7B2U1	0.25	Blue	Positive
A0A8I7B7C6	0.25	Blue	Positive
A0A8I7B8K2	0.25	Blue	Positive
A0A8I7BDD0	0.25	Blue	Positive
A0A8I7BFP1	0.25	Blue	Positive
M0WZB5	0.25	Blue	Positive

A0A8I6YZG5	0.24	Black	Positive
A0A8I6WRG9	0.24	Blue	Positive
A0A8I6X928	0.24	Blue	Positive
F2DYJ0	0.24	Blue	Positive
F2CQ08	0.24	Red	Positive
F2D1K5	0.24	Red	Positive
F2D6E2	0.23	Blue	Positive
F2E724	0.23	Blue	Positive
F2DZK6	0.23	Red	Positive
A0A8I6WK56	0.22	Blue	Positive
A0A8I6WQV8	0.22	Blue	Positive
A0A8I6YB06	0.22	Red	Positive
F2DUF1	0.21	Black	Positive
A0A8I7BIT9	0.21	Blue	Positive
F2E3M7	0.21	Blue	Positive
F2D4D8	0.2	Black	Positive
F2DIA6	0.2	Black	Positive
A0A8I6YGF9	0.2	Blue	Positive
A0A8I7BES8	0.2	Blue	Positive
F2CU19	0.2	Blue	Positive
F2CYT7	0.19	Blue	Positive
F2DCE8	0.19	Blue	Positive
F2CQP8	0.19	Purple	Positive
Q3S835	0.19	Red	Positive
A0A8I6XEC2	0.18	Black	Positive
A0A8I6W6F0	0.18	Blue	Positive
F2DDK5	0.18	Blue	Positive
A0A8I6WKG3	0.17	Black	Positive
A0A8I7BBS8	0.17	Blue	Positive
M0UVQ5	0.16	Black	Positive
F2DX84	0.16	Blue	Positive
A0A8I6Y5V6	0.14	Blue	Positive
A0A8I6YME5	0.14	Blue	Positive
A0A8I6YP10	0.14	Blue	Positive
F2CRE3	0.14	Blue	Positive
F2CTY5	0.14	Blue	Positive
A0A8I6XV06	0.13	Blue	Positive
A0A8I6YBK2	0.13	Blue	Positive
F2DB00	0.13	Blue	Positive
Q3V4F7	0.13	Blue	Positive
F2DB07	0.12	Blue	Positive
F2E4V3	0.12	Blue	Positive
F2DC78	0.12	Red	Positive
A0A8I6YZB3	0.11	Black	Positive
F2DEW2	0.11	Blue	Positive

F2DQB8	0.09	Blue	Positive
A0A8I7BAU1	0.08	Black	Positive
F2D294	0.07	Blue	Positive
O48604	0.07	Blue	Positive
A0A287EZQ9	0.06	Black	Positive
A0A8I6XZW6	0.06	Blue	Positive
A0A8I7B0N1	0.06	Blue	Positive
F2CW97	0.06	Blue	Positive
F2E8W3	0.06	Blue	Positive
A0A8I6Y2R3	0.05	Black	Positive
A0A8I6WM65	0.05	Blue	Positive
F2E3J0	0.05	Blue	Positive
Q40025	0.04	Blue	Positive
F2DFX0	0.03	Blue	Positive
F2DY46	0.03	Blue	Positive
A0A8I6YDQ5	0.02	Blue	Positive
F4ZC51	0.01	Blue	Positive
A0A8I6Y4S2	0	Blue	Positive
F2DRK6	0	Blue	Positive
P01086	5.36	Brown	Negative
P80198	4.9	Yellow	Negative
Q9M4E3	4.8	Yellow	Negative
Q9SAT9	4.53	Brown	Negative
I6SJ26	4.15	Brown	Negative
D6BU17	4	Magenta	Negative
A0A8I6WDG9	3.9	Brown	Negative
Q9AVJ8	4.1	Brown	Negative
Q40021	3.97	Brown	Negative
A0A8I6YCK0	3.84	Yellow	Negative
F2EGD5	3.58	Brown	Negative
Q43492	3.43	Brown	Negative
P17990	3.33	Brown	Negative
Q673G6	3.2	Yellow	Negative
Q70IB4	3.08	Brown	Negative
A0A8I7BCH5	2.97	Brown	Negative
A8V3Q5	2.91	Yellow	Negative
A0A8I6WNF0	2.76	Brown	Negative
F2DTH9	2.75	Brown	Negative
F2EFW5	2.75	Yellow	Negative
A0A8I6YBJ3	2.69	Brown	Negative
A9E4D5	2.54	Brown	Negative
A0A8I6YB73	2.5	Yellow	Negative
A0A8I6YMM5	2.39	Yellow	Negative
A0A8I6X3C2	2.34	Yellow	Negative
P16968	2.32	Brown	Negative

A0A8I6XS48	2.3	Brown	Negative
P40880	2.29	Yellow	Negative
F2E6J2	2.28	Yellow	Negative
P21742	2.18	Brown	Negative
A0A8I7B6U7	2.12	Brown	Negative
P06293	2	Brown	Negative
A0A8I6WSD4	1.99	Yellow	Negative
F2DTG3	1.97	Magenta	Negative
C3W8K9	1.97	Yellow	Negative
P01545	1.95	Brown	Negative
A0A8I6YQ86	1.94	Magenta	Negative
G1UH43	1.9	Brown	Negative
P31923	1.9	Yellow	Negative
F2DIF8	1.8	Yellow	Negative
F2EAZ9	1.79	Greenyellow	Negative
P80284	1.75	Yellow	Negative
F2ECB9	1.73	Yellow	Negative
F2CYT1	1.66	Greenyellow	Negative
P31922	1.65	Yellow	Negative
A0A8I6YAJ5	1.63	Greenyellow	Negative
A0A8I6YFB9	1.61	Yellow	Negative
A0A060ILL2	1.57	Brown	Negative
A0A8I7B9F0	1.56	Yellow	Negative
F2CPS8	1.53	Yellow	Negative
F2DZW3	1.53	Yellow	Negative
A0A8I6WGH5	1.51	Greenyellow	Negative
A0A8I6WNV8	1.49	Yellow	Negative
F2E2T2	1.48	Yellow	Negative
C3W8L2	1.47	Yellow	Negative
C3W8M2	1.47	Yellow	Negative
F2DCE3	1.46	Yellow	Negative
A0A8I6WIL2	1.45	Greenyellow	Negative
A0A8I6YA81	1.45	Greenyellow	Negative
A0A8I6X522	1.45	Yellow	Negative
F2DXJ9	1.44	Yellow	Negative
C3W8M1	1.43	Brown	Negative
A0A8I6Y5D8	1.42	Greenyellow	Negative
A0A8I6XKX5	1.42	Yellow	Negative
P11643	1.41	Brown	Negative
F2DMG8	1.39	Yellow	Negative
F2DMU4	1.39	Yellow	Negative
P30524	1.39	Yellow	Negative
A0A3G9EF67	1.38	Yellow	Negative
Q7XZK6	1.37	Brown	Negative
P13691	1.36	Brown	Negative

F2CT85	1.36	Magenta	Negative
F2EDB2	1.35	Greenyellow	Negative
Q8H1Y7	1.34	Greenyellow	Negative
F2CTL8	1.34	Yellow	Negative
F2DA67	1.34	Yellow	Negative
P28041	1.33	Brown	Negative
F2DFV4	1.33	Yellow	Negative
A0A8I7B7Q8	1.28	Brown	Negative
F2D1M6	1.28	Yellow	Negative
P32936	1.24	Brown	Negative
A0A8I6YA05	1.24	Yellow	Negative
F2D4M6	1.23	Yellow	Negative
F2EI64	1.22	Magenta	Negative
M0YQS0	1.19	Brown	Negative
A0A221C9D5	1.15	Yellow	Negative
A0A8I6X956	1.15	Yellow	Negative
F2E7L1	1.15	Yellow	Negative
P22244	1.14	Brown	Negative
W8VR77	1.13	Greenyellow	Negative
F2CTX3	1.11	Brown	Negative
A0A8I7B6P3	1.11	Magenta	Negative
C3W8L4	1.11	Magenta	Negative
F2DA55	1.1	Magenta	Negative
A0A8I6Y4H4	1.08	Brown	Negative
F2D350	1.07	Magenta	Negative
A0A8I7BAP0	1.07	Yellow	Negative
A0A8I7B0X5	1.06	Greenyellow	Negative
M0WE81	1.05	Yellow	Negative
F2DVE5	1.04	Greenyellow	Negative
F2D9P7	1.04	Yellow	Negative
A0A8I6YP98	1.03	Brown	Negative
A0A8I6WVY3	1.03	Yellow	Negative
F2EE76	1.02	Brown	Negative
F2DVU2	1.02	Greenyellow	Negative
F2CWQ4	1.02	Yellow	Negative
A0A8I6X9X3	1.01	Brown	Negative
A0A8I6YDN6	1.01	Greenyellow	Negative
A0A8I6XDV2	0.99	Brown	Negative
Q43772	0.99	Greenyellow	Negative
F2DSJ2	0.99	Yellow	Negative
E7BB45	0.98	Brown	Negative
F2D692	0.98	Magenta	Negative
P16062	0.98	Yellow	Negative
Q673G5	0.97	Brown	Negative
A0A8I7BA67	0.97	Greenyellow	Negative

F2E8P4	0.97	Magenta	Negative
A0A8I6WSY6	0.95	Brown	Negative
B5TWK6	0.95	Brown	Negative
P04399	0.95	Brown	Negative
A0A8I6X4E1	0.95	Greenyellow	Negative
A0ZV97	0.95	Magenta	Negative
A0A8I6XRH2	0.94	Greenyellow	Negative
F2D7E7	0.94	Yellow	Negative
A0A8I7BFU9	0.93	Brown	Negative
A0A8I6YIH2	0.93	Yellow	Negative
F2D903	0.92	Yellow	Negative
A0A8I6Z3M4	0.91	Brown	Negative
F2CRC2	0.9	Brown	Negative
A0A8I6WUQ1	0.9	Greenyellow	Negative
A0A287ME54	0.9	Yellow	Negative
Q40054	0.89	Brown	Negative
A0A287KP21	0.89	Yellow	Negative
A0A8I6X9X5	0.89	Yellow	Negative
F2CS69	0.89	Yellow	Negative
F2E3I5	0.89	Yellow	Negative
A0A8I6XVS0	0.88	Yellow	Negative
A0A8I7B6P8	0.88	Yellow	Negative
D6PY83	0.87	Brown	Negative
A9UKM4	0.87	Greenyellow	Negative
F2CYM3	0.87	Yellow	Negative
A0A8I6X7K0	0.86	Greenyellow	Negative
F2EKQ2	0.86	Greenyellow	Negative
A0A8I6XB71	0.86	Yellow	Negative
A0A8I6XCG6	0.86	Yellow	Negative
F2EJY5	0.86	Yellow	Negative
A0A8I7B5U1	0.85	Yellow	Negative
F2D4E7	0.84	Brown	Negative
Q40069	0.84	Brown	Negative
F2EFZ6	0.84	Yellow	Negative
A0A8I6XA39	0.82	Brown	Negative
A0A8I6XYT6	0.82	Brown	Negative
F2DWR2	0.82	Brown	Negative
A0A8I6Y697	0.82	Yellow	Negative
F2D2W7	0.82	Yellow	Negative
P35266	0.81	Greenyellow	Negative
A0A8I6WZY4	0.8	Yellow	Negative
A0A8I6WTT5	0.79	Brown	Negative
F2D510	0.79	Greenyellow	Negative
C3W8L3	0.79	Magenta	Negative
A0A287LWH7	0.78	Brown	Negative

A5CFY3	0.78	Yellow	Negative
F2DM06	0.77	Yellow	Negative
A0A8I6X1I0	0.76	Yellow	Negative
F2DCF7	0.76	Yellow	Negative
A0A8I6Y6V9	0.75	Magenta	Negative
F2EDK4	0.74	Brown	Negative
Q7XZK3	0.74	Greenyellow	Negative
A0A8I6YGT2	0.74	Yellow	Negative
A0A8I7BBJ7	0.74	Yellow	Negative
A0A8I6WGX2	0.73	Yellow	Negative
I3WA82	0.71	Brown	Negative
F2CQN1	0.71	Greenyellow	Negative
F2CSX2	0.71	Magenta	Negative
A0A8I7BH19	0.71	Yellow	Negative
F2DJH8	0.71	Yellow	Negative
T2FH00	0.71	Yellow	Negative
A0A8I6XBP3	0.7	Brown	Negative
A0A8I6WY90	0.69	Yellow	Negative
A0A8I6XGW9	0.68	Brown	Negative
A0A8I6YJY1	0.68	Brown	Negative
F2CR55	0.68	Brown	Negative
Q949H0	0.68	Greenyellow	Negative
A0A8I6WBS1	0.68	Magenta	Negative
A0A8I7BGJ1	0.68	Magenta	Negative
A0A8I6WPK0	0.67	Brown	Negative
A0A8I6WXW1	0.67	Brown	Negative
A0A8I6X660	0.67	Brown	Negative
F2DDB1	0.67	Greenyellow	Negative
A2T578	0.67	Yellow	Negative
F2E9B5	0.66	Brown	Negative
G1APU2	0.66	Brown	Negative
P09842	0.66	Brown	Negative
A5CFY4	0.66	Greenyellow	Negative
F2DEP3	0.66	Greenyellow	Negative
F2D1U7	0.66	Yellow	Negative
A0A8I6WH70	0.65	Brown	Negative
A0A8I7BCU3	0.64	Brown	Negative
F2DBW7	0.64	Greenyellow	Negative
A0A8I6XLD0	0.64	Magenta	Negative
F2CW55	0.64	Yellow	Negative
F2D351	0.64	Yellow	Negative
A0A8I6YIP0	0.63	Greenyellow	Negative
A0A8I7B4C5	0.63	Greenyellow	Negative
A0A8I7BI25	0.63	Yellow	Negative
F2DWT1	0.63	Yellow	Negative

A0A8I6Y7Y1	0.62	Brown	Negative
F2D101	0.62	Brown	Negative
F2CYV4	0.62	Yellow	Negative
A0A8I6WQM2	0.61	Brown	Negative
A0A8I6Z0S4	0.61	Brown	Negative
F2CYK2	0.61	Yellow	Negative
F2D868	0.61	Yellow	Negative
A0A8I6YZW8	0.6	Brown	Negative
A0A8I7BFH9	0.6	Brown	Negative
F2D4A4	0.6	Greenyellow	Negative
A0A8I6Y6E8	0.6	Yellow	Negative
F2CY57	0.6	Yellow	Negative
F2CWF9	0.59	Greenyellow	Negative
F2D3Q4	0.59	Greenyellow	Negative
F2DCK8	0.59	Magenta	Negative
A0A8I6XVE2	0.58	Brown	Negative
F2DMG9	0.58	Yellow	Negative
A0A8I6XIA0	0.57	Brown	Negative
A0A8I6YG19	0.56	Brown	Negative
A0A8I6WGR4	0.56	Magenta	Negative
F2CQC7	0.56	Magenta	Negative
F2EEU3	0.56	Magenta	Negative
A0A8I6WCK6	0.56	Yellow	Negative
F2EG52	0.56	Yellow	Negative
A0A8I6WH75	0.55	Brown	Negative
A0A8I6X3D1	0.55	Yellow	Negative
F2CR61	0.55	Yellow	Negative
F2DBD1	0.54	Magenta	Negative
A0A8I6XJN9	0.53	Brown	Negative
F2CXF2	0.53	Brown	Negative
F2CTM9	0.53	Magenta	Negative
F2D6F6	0.53	Magenta	Negative
F2E2F1	0.53	Magenta	Negative
F2E556	0.53	Magenta	Negative
F2EB41	0.52	Brown	Negative
F2CXV3	0.52	Magenta	Negative
A0A0U2GJL1	0.52	Yellow	Negative
A0A8I6WT86	0.52	Yellow	Negative
F2CXC1	0.52	Yellow	Negative
F2DFQ8	0.51	Magenta	Negative
A0A8I7BB75	0.51	Yellow	Negative
A0A8I6Y635	0.5	Magenta	Negative
A0A8I6WQI8	0.5	Yellow	Negative
F2DNB2	0.5	Yellow	Negative
A0A287WY76	0.49	Brown	Negative

A0A8I6YQ76	0.49	Brown	Negative
F2CRB3	0.49	Yellow	Negative
A0A8I7B7U0	0.48	Brown	Negative
Q9AXH9	0.48	Brown	Negative
F2DSW5	0.48	Magenta	Negative
F2DGG1	0.48	Yellow	Negative
Q84QC6	0.48	Yellow	Negative
Q850M2	0.48	Yellow	Negative
A0A8I7B981	0.47	Brown	Negative
A0A8I7BBR7	0.47	Brown	Negative
F2E1G7	0.47	Brown	Negative
P50888	0.47	Brown	Negative
F2DE13	0.47	Magenta	Negative
A0A287EBT3	0.46	Brown	Negative
Q673G1	0.46	Brown	Negative
A0A8I6XXA7	0.46	Greenyellow	Negative
A0A8I6WIM9	0.46	Yellow	Negative
A0A8I6XM59	0.45	Brown	Negative
C3W8L0	0.45	Brown	Negative
H1ACH0	0.45	Brown	Negative
A0A8I6XA06	0.45	Magenta	Negative
F2E1Y8	0.45	Magenta	Negative
A0A8I6WN81	0.45	Yellow	Negative
P0CG87	0.45	Yellow	Negative
F2CVW9	0.44	Brown	Negative
F2D963	0.44	Brown	Negative
F2EB17	0.44	Brown	Negative
F2E6K2	0.44	Magenta	Negative
A0A8I6WKV5	0.44	Yellow	Negative
A0A8I6Y3M0	0.43	Brown	Negative
F2E7H5	0.43	Brown	Negative
F2E8B4	0.43	Brown	Negative
F2DIP0	0.43	Yellow	Negative
Q9M3U8	0.43	Yellow	Negative
A0A8I6WK14	0.42	Brown	Negative
F2CSC5	0.42	Brown	Negative
F2D0T5	0.42	Brown	Negative
F2CXB8	0.42	Magenta	Negative
F2DAK3	0.42	Magenta	Negative
F2DUJ6	0.42	Yellow	Negative
A0A8I6XX74	0.41	Brown	Negative
A0A8I6Y1Z6	0.41	Brown	Negative
F2DUI0	0.41	Brown	Negative
A0A8I6YPQ8	0.41	Yellow	Negative
Q9AUH2	0.41	Yellow	Negative

F2E8J4	0.4	Brown	Negative
F2EGL3	0.4	Brown	Negative
P07596	0.4	Brown	Negative
F2D2Y2	0.4	Greenyellow	Negative
A0A8I6WZJ4	0.4	Magenta	Negative
F2DZN2	0.39	Brown	Negative
Q9ZTB6	0.39	Brown	Negative
F2DD34	0.39	Greenyellow	Negative
A0A8I6WUT0	0.39	Yellow	Negative
A0A8I6X9C3	0.39	Yellow	Negative
A0A8I6WGF6	0.38	Brown	Negative
A0A8I6XT50	0.38	Brown	Negative
A0A8I6YTH6	0.38	Brown	Negative
A0A8I6Z702	0.38	Brown	Negative
F2DS44	0.38	Brown	Negative
F2CT73	0.38	Magenta	Negative
F2D702	0.38	Magenta	Negative
A0A8I6XG35	0.38	Yellow	Negative
A0A8I6Y9P4	0.38	Yellow	Negative
A0A8I6Z375	0.37	Brown	Negative
F2E8H2	0.37	Brown	Negative
F2CV33	0.37	Magenta	Negative
F2D448	0.37	Magenta	Negative
F2EFR1	0.37	Magenta	Negative
A0A8I6WNW9	0.37	Yellow	Negative
A0A8I6XLS1	0.37	Yellow	Negative
A0A8I6Y4Z6	0.37	Yellow	Negative
F2CQ27	0.37	Yellow	Negative
A0A8I6WS33	0.36	Brown	Negative
F2DFA6	0.36	Greenyellow	Negative
F2DIR3	0.36	Magenta	Negative
A0A8I6WGF3	0.36	Yellow	Negative
A0A8I6YLD4	0.36	Yellow	Negative
F2DBM9	0.36	Yellow	Negative
F2EG53	0.36	Yellow	Negative
A0A8I6YNS2	0.35	Brown	Negative
A0A8I6ZBD0	0.35	Brown	Negative
F2EE62	0.35	Brown	Negative
F2EEX6	0.35	Brown	Negative
A0A8I6YCK7	0.35	Yellow	Negative
A0A8I6YC52	0.34	Brown	Negative
A0A8I6YJD5	0.34	Brown	Negative
A0A8I6YQ17	0.34	Brown	Negative
C9ELM8	0.34	Brown	Negative
F2CV88	0.34	Magenta	Negative

A0A287L8A3	0.34	Yellow	Negative
A0A8I6XCC9	0.34	Yellow	Negative
F2CZZ8	0.34	Yellow	Negative
A0A8I6XI22	0.33	Brown	Negative
A0A8I6XI44	0.33	Brown	Negative
A0A8I6Y3Y3	0.33	Brown	Negative
C3W8M5	0.33	Brown	Negative
Q8VWM4	0.33	Brown	Negative
F2EAU8	0.33	Magenta	Negative
D2XV76	0.33	Yellow	Negative
Q5TIW4	0.33	Yellow	Negative
P06353	0.32	Brown	Negative
A0A8I6XF13	0.32	Yellow	Negative
F2E3N4	0.32	Yellow	Negative
F2EKF1	0.32	Yellow	Negative
A0A8I6XWI9	0.31	Brown	Negative
A0A8I6YGD1	0.31	Brown	Negative
A0A8I6YIE8	0.31	Brown	Negative
A0A8I6YWE2	0.31	Brown	Negative
F2DLR6	0.31	Brown	Negative
F2DZH1	0.31	Brown	Negative
F2CT61	0.31	Magenta	Negative
C3W8L6	0.31	Yellow	Negative
F2E2W5	0.31	Yellow	Negative
F2E4A7	0.31	Yellow	Negative
A0A8I6X8N4	0.3	Brown	Negative
A0A8I6YJI3	0.3	Brown	Negative
A0A8I6Z3Z5	0.3	Brown	Negative
F2D861	0.3	Brown	Negative
F2DM10	0.3	Brown	Negative
A0A8I6XEY1	0.3	Yellow	Negative
F2EHD3	0.3	Yellow	Negative
A0A8I6YSZ9	0.29	Brown	Negative
A0A8I6YUM7	0.29	Brown	Negative
A0A8I7BEZ0	0.29	Brown	Negative
F2D017	0.29	Brown	Negative
F2EG92	0.29	Brown	Negative
Q8H1V4	0.29	Brown	Negative
A0A8I6W6I7	0.29	Yellow	Negative
A0A8I6XC32	0.28	Brown	Negative
F2CXK4	0.28	Brown	Negative
F2DRT8	0.28	Magenta	Negative
F2EAX5	0.28	Magenta	Negative
A0A8I6Y5X5	0.28	Yellow	Negative
F2CVC1	0.28	Yellow	Negative

F2DT12	0.28	Yellow	Negative
O65305	0.28	Yellow	Negative
A0A287XXH1	0.27	Brown	Negative
A0A8I6WF01	0.27	Brown	Negative
A0A8I6XND6	0.27	Brown	Negative
A0A8I6YXP5	0.27	Brown	Negative
F2CX95	0.27	Brown	Negative
F2D7A1	0.27	Brown	Negative
F2EFK9	0.27	Brown	Negative
J7GP58	0.27	Yellow	Negative
F2CTJ0	0.26	Brown	Negative
F2D587	0.26	Brown	Negative
F2DGV1	0.26	Brown	Negative
M0YZK1	0.26	Brown	Negative
P07597	0.26	Brown	Negative
A0A8I6XGX5	0.26	Yellow	Negative
F2DMD1	0.26	Yellow	Negative
F2D0Z7	0.25	Brown	Negative
F2DJY3	0.25	Brown	Negative
P20145	0.25	Brown	Negative
F2E598	0.25	Magenta	Negative
Q40034	0.25	Magenta	Negative
A0A8I7B8A2	0.25	Yellow	Negative
A0A8I6YT58	0.24	Brown	Negative
Q19D39	0.24	Brown	Negative
A0A287KZV7	0.24	Yellow	Negative
A0A8I7B5C7	0.24	Yellow	Negative
A0A8I6XG95	0.23	Brown	Negative
F2D0A7	0.23	Brown	Negative
F2D1G5	0.23	Brown	Negative
F2D5W2	0.23	Brown	Negative
F2DCD4	0.23	Brown	Negative
F2DGN1	0.23	Brown	Negative
Q9M4D6	0.23	Brown	Negative
F2D483	0.23	Magenta	Negative
A0A287Q7U2	0.23	Yellow	Negative
A0A8I6WYS3	0.23	Yellow	Negative
A0A8I6YFU0	0.23	Yellow	Negative
F2DQH1	0.23	Yellow	Negative
A0A8I6WL88	0.22	Brown	Negative
A0A8I6XB38	0.22	Brown	Negative
F2EDE8	0.22	Brown	Negative
F2DC41	0.22	Yellow	Negative
A0A8I6YB85	0.21	Brown	Negative
A0A8I7BD26	0.21	Brown	Negative

F2DY31	0.21	Brown	Negative
F2EAV7	0.21	Brown	Negative
Q84KB8	0.21	Brown	Negative
A0A8I6XBF6	0.21	Yellow	Negative
A0A8I6XM85	0.2	Brown	Negative
A0A8I6Y060	0.2	Brown	Negative
A0A8I6YEZ5	0.2	Brown	Negative
A0A8I6YPP1	0.2	Brown	Negative
F2E5L1	0.2	Brown	Negative
F2EID5	0.2	Brown	Negative
F2D1P1	0.2	Magenta	Negative
A0A287T3E6	0.2	Yellow	Negative
A0A8I6YCT4	0.2	Yellow	Negative
A0A8I6YLQ8	0.2	Yellow	Negative
F2D424	0.2	Yellow	Negative
A0A8I6WHT4	0.19	Brown	Negative
A0A8I6WIE7	0.19	Brown	Negative
F2CSR6	0.19	Brown	Negative
F2CT83	0.19	Brown	Negative
F2D6E9	0.19	Brown	Negative
F2DLB0	0.19	Brown	Negative
F2ECY1	0.19	Brown	Negative
F2EFA5	0.19	Brown	Negative
F2CW48	0.19	Magenta	Negative
A0A161G109	0.19	Yellow	Negative
A0A8I6X969	0.19	Yellow	Negative
A0A8I6YFW7	0.19	Yellow	Negative
Q43479	0.19	Yellow	Negative
A0A287XP46	0.18	Brown	Negative
A0A8I6WS69	0.18	Brown	Negative
A0A8I6XK58	0.18	Brown	Negative
F2CQY5	0.18	Brown	Negative
F2D179	0.18	Brown	Negative
F2D6X2	0.18	Brown	Negative
F2DER7	0.18	Brown	Negative
A0A8I7BC59	0.18	Yellow	Negative
A0A8I6XMQ4	0.17	Brown	Negative
A0A8I6XV79	0.17	Brown	Negative
A0A8I6YNV4	0.17	Brown	Negative
A0A8I7B6G8	0.17	Brown	Negative
F2DV95	0.17	Brown	Negative
F2E6W0	0.17	Brown	Negative
A0A8I6XTH8	0.17	Yellow	Negative
M0X794	0.17	Yellow	Negative
A0A8I6W4Q8	0.16	Brown	Negative

A0A8I6WL52	0.16	Brown	Negative
F2CWR5	0.16	Brown	Negative
F2CZ53	0.16	Brown	Negative
F2DIE3	0.16	Brown	Negative
F2DLQ1	0.16	Brown	Negative
F2DN53	0.16	Brown	Negative
M0Y7Q2	0.16	Brown	Negative
A0A8I6YUS4	0.16	Yellow	Negative
F2DBD4	0.16	Yellow	Negative
A0A8I6X5V6	0.15	Brown	Negative
A0A8I6XCS9	0.15	Brown	Negative
A0A8I6XFW5	0.15	Brown	Negative
A0A8I6XP22	0.15	Brown	Negative
A0A8I6XTS4	0.15	Brown	Negative
F2CQ57	0.15	Brown	Negative
F2D9Z5	0.15	Brown	Negative
F2DIT7	0.15	Brown	Negative
F2EDJ3	0.15	Brown	Negative
M0UJR6	0.15	Brown	Negative
A0A8I6XYC7	0.15	Yellow	Negative
F2DVV7	0.15	Yellow	Negative
F2EF67	0.15	Yellow	Negative
A0A8I6W6B5	0.14	Brown	Negative
A0A8I6WJW9	0.14	Brown	Negative
A0A8I6Y6W0	0.14	Brown	Negative
A0A8I6YCQ8	0.14	Brown	Negative
F2DED9	0.14	Brown	Negative
F2DUY9	0.14	Brown	Negative
F2EBT8	0.14	Brown	Negative
A0A8I6WC09	0.14	Yellow	Negative
C4PFJ4	0.14	Yellow	Negative
F2CX63	0.14	Yellow	Negative
Q70WD6	0.14	Yellow	Negative
A0A8I6X164	0.13	Brown	Negative
A0A8I6XQR4	0.13	Brown	Negative
A0A8I7B3C6	0.13	Brown	Negative
F2CQR1	0.13	Brown	Negative
F2CZQ3	0.13	Brown	Negative
F2D5V3	0.13	Brown	Negative
F2DP88	0.13	Brown	Negative
F2DSK9	0.13	Brown	Negative
Q40066	0.13	Brown	Negative
A0A8I6XXB5	0.13	Yellow	Negative
A0A8I7B965	0.13	Yellow	Negative
F2CVL1	0.13	Yellow	Negative

F2DXI0	0.13	Yellow	Negative
A0A8I6WI52	0.12	Brown	Negative
A0A8I7BBK0	0.12	Brown	Negative
F2CQH9	0.12	Brown	Negative
F2DF14	0.12	Brown	Negative
F2DJJ2	0.12	Brown	Negative
P52184	0.12	Brown	Negative
A0A8I6XZV8	0.12	Yellow	Negative
F2DPN4	0.12	Yellow	Negative
Q9LEH6	0.12	Yellow	Negative
A0A8I7BIP3	0.11	Brown	Negative
F2CR90	0.11	Brown	Negative
F2CWU8	0.11	Brown	Negative
F2D5K0	0.11	Brown	Negative
F2D5P0	0.11	Brown	Negative
F2D788	0.11	Brown	Negative
F2DSH0	0.11	Brown	Negative
F2EA93	0.11	Brown	Negative
A0A8I6WVY4	0.11	Yellow	Negative
A0A8I6YHZ7	0.11	Yellow	Negative
A0A8I7BEG8	0.11	Yellow	Negative
F2DTF1	0.11	Yellow	Negative
M0YUI3	0.11	Yellow	Negative
A0A8I6XI28	0.1	Brown	Negative
A0A8I6XUK6	0.1	Brown	Negative
Q6B6L8	0.1	Brown	Negative
F2D847	0.1	Yellow	Negative
M0XYS5	0.1	Yellow	Negative
A0A8I6X6U3	0.09	Brown	Negative
A0A8I6Y0T5	0.09	Brown	Negative
F2CS01	0.09	Brown	Negative
F2CYH7	0.09	Brown	Negative
F2D5G5	0.09	Brown	Negative
F2E474	0.09	Brown	Negative
A0A0U2GJM5	0.09	Yellow	Negative
A0A8I6Z015	0.09	Yellow	Negative
F2D9F0	0.09	Yellow	Negative
F2E1V6	0.09	Yellow	Negative
F2EGI1	0.09	Yellow	Negative
A0A8I6XUL9	0.08	Brown	Negative
A0A8I6Y6R7	0.08	Brown	Negative
A0A8I6YG23	0.08	Brown	Negative
A0A8I7B466	0.08	Brown	Negative
A0A8I7BAJ8	0.08	Brown	Negative
A0A8I7BCY8	0.08	Brown	Negative

A5CFY2	0.08	Brown	Negative
F2CXB2	0.08	Brown	Negative
F2DAY3	0.08	Brown	Negative
F2DI93	0.08	Brown	Negative
F2D1H7	0.08	Magenta	Negative
A0A287PU65	0.08	Yellow	Negative
A0A8I6X6I4	0.08	Yellow	Negative
F2DDF2	0.08	Yellow	Negative
F2CQY1	0.07	Brown	Negative
F2D6A6	0.07	Brown	Negative
F2EFD1	0.07	Brown	Negative
F2D293	0.07	Magenta	Negative
A0A8I6YJY4	0.07	Yellow	Negative
F2D2U4	0.07	Yellow	Negative
F2D3C1	0.07	Yellow	Negative
F2EGN2	0.07	Yellow	Negative
A0A8I6WX78	0.06	Brown	Negative
F2CRV8	0.06	Brown	Negative
F2DLJ6	0.06	Brown	Negative
F2DKY2	0.06	Yellow	Negative
F2EEQ1	0.06	Yellow	Negative
F2EL68	0.06	Yellow	Negative
M0UGD2	0.06	Yellow	Negative
A0A8I6WZE6	0.05	Brown	Negative
A0A8I6Y4X2	0.05	Brown	Negative
F2DVK7	0.05	Brown	Negative
A0A8I6WWH2	0.05	Yellow	Negative
F2DXD4	0.05	Yellow	Negative
A0A8I6X6F8	0.04	Brown	Negative
F2DM74	0.04	Brown	Negative
F2DN24	0.04	Brown	Negative
F2DW24	0.04	Brown	Negative
A0A8I6WP44	0.04	Yellow	Negative
F2D3R8	0.04	Yellow	Negative
F2D426	0.04	Yellow	Negative
F2CQ90	0.03	Brown	Negative
F2EFS3	0.03	Brown	Negative
F2CVF1	0.03	Magenta	Negative
A0A8I6WY36	0.03	Yellow	Negative
A0A8I6XCC4	0.03	Yellow	Negative
A0A8I6Y6Z6	0.03	Yellow	Negative
F2DSU6	0.03	Yellow	Negative
A0A8I6Y184	0.02	Brown	Negative
F2CU93	0.02	Brown	Negative
F2DHD8	0.02	Brown	Negative

A0A8I6WH53	0.02	Yellow	Negative
A0A8I6XRQ1	0.02	Yellow	Negative
A0A8I7B6R1	0.02	Yellow	Negative
F2DJ14	0.02	Yellow	Negative
Q0KKA5	0.02	Yellow	Negative
Q40040	0.02	Yellow	Negative
Q42840	0.02	Yellow	Negative
A0A8I6WUT1	0.01	Brown	Negative
A0A8I6X3A9	0.01	Yellow	Negative
A0A8I6X589	0.01	Yellow	Negative
A0A8I6Y2M3	0.01	Yellow	Negative
A0A8I6YXP8	0.01	Yellow	Negative
Q96468	0.01	Yellow	Negative
W8VR74	0.01	Yellow	Negative
A0A8I7BFC0	0	Yellow	Negative

Supplementary Table 7. Identified amylases in barley grain and malt samples.

Sample	**Accession**	**Protein**
Barley grain	Q9FUK6	Beta-amylase (EC 3.2.1.2)
	A0A482LNP1	Beta-amylase (EC 3.2.1.2)
	A0A8I6XP65	Alpha-amylase (EC 3.2.1.1) (1,4-alpha-D-glucan glucanohydrolase)
Barley malt	O48541	Limit dextrinase,
	D3XAZ8	Alpha-amylase (EC 3.2.1.1) (1,4-alpha-D-glucan glucanohydrolase)
	A0A8I6XP65	Alpha-amylase (EC 3.2.1.1) (1,4-alpha-D-glucan glucanohydrolase)
	C3W8N0	Alpha-amylase (EC 3.2.1.1) (1,4-alpha-D-glucan glucanohydrolase)
	D3XB18	Alpha-amylase (EC 3.2.1.1) (1,4-alpha-D-glucan glucanohydrolase)
	Q84T19	Beta-amylase (EC 3.2.1.2)
	D3XAZ4	Alpha-amylase (EC 3.2.1.1) (1,4-alpha-D-glucan glucanohydrolase)
	K4N2F5	alpha-amylase (EC 3.2.1.1) (1,4-alpha-D-glucan glucanohydrolase)
	Q40015	Alpha-amylase (EC 3.2.1.1) (1,4-alpha-D-glucan glucanohydrolase)
	K4MPS2	Alpha-amylase 2
	F2DM00	Beta-amylase (EC 3.2.1.2)
	Q9FUK6	Beta-amylase (EC 3.2.1.2)
	Q03651	Alpha-amylase (EC 3.2.1.1) (1,4-alpha-D-glucan glucanohydrolase)

Supplementary Table 8. Number of proteins for “small molecule metabolic process” GO term in three hordein-reduced genetic backgrounds.

line	enrichment FDR	number of proteins for GO term
C-hordein reduced	0.0032	10
D-hordein reduced	0.025	8
B-hordein reduced	0.044	6

Supplementary Table 9. Summary of pairwise comparisons results for proteases in barley grain and malt in hordein reduced lines.

B4ESF5 (malt samples) papain-like cysteine protease		
Comparison	**p-value (Bonferroni)**	**Significancy**
WT vs BC	0.0014302	** p<0.01
WT vs BCD	1.3024e-07	** p<0.01
WT vs BD	0.0026490	** p<0.01
WT vs C	0.4033440	insignificant
WT vs CD	3.1644204	insignificant
WT vs D	5.8196902	insignificant
WT vs B	5.3502830	insignificant
B4ESE8 (malt samples) papain-like cysteine protease		
Comparison	**p-value (Bonferroni)**	**Significancy**
WT vs BC	0.0011465	** p<0.01
WT vs BCD	0.2052177	insignificant
WT vs BD	1.0635469	insignificant
WT vs C	0.0098408	** p<0.01
WT vs CD	3.7617758	insignificant
WT vs D	4.9535754	insignificant
WT vs B	6.8135652	insignificant
B4ESE9 (malt samples) papain-like cysteine protease		
Comparison	**p-value (Bonferroni)**	**Significancy**
WT vs BC	0.0119436	* p<0.05
WT vs BCD	2.4843912	insignificant
WT vs BD	0.3999759	insignificant
WT vs C	0.1424359	insignificant
WT vs CD	2.6417986	insignificant
WT vs D	0.0083919	** p<0.01
WT vs B	3.1567251	insignificant
B4ESF7 (malt samples) papain-like cysteine protease		
Comparison	**p-value (Bonferroni)**	**Significancy**
WT vs BC	0.0224587	insignificant
WT vs BCD	2.6528e-05	** p<0.01
WT vs BD	0.3073868	insignificant
WT vs C	0.9040438	insignificant
WT vs CD	0.0055381	** p<0.01
WT vs D	0.3079612	insignificant
WT vs B	0.4295261	insignificant
B4ESF5 (grain samples)		
Comparison	**p-value (Bonferroni)**	**Significancy**
WT vs BC	1.3485569	insignificant
WT vs BCD	2.0171707	insignificant
WT vs BD	6.1508084	insignificant
WT vs C	1.7564679	insignificant
WT vs CD	4.3213540	insignificant
WT vs D	0.6141514	insignificant

WT vs B	1.1201441	insignificant
B4ESE8 (grain samples)		
Comparison	**p-value (Bonferroni)**	**Significancy**
WT vs BC	0.1183264	insignificant
WT vs BCD	0.4128596	insignificant
WT vs BD	0.2281864	insignificant
WT vs C	0.0445077	* p<0.05
WT vs CD	0.3057959	insignificant
WT vs D	1.2468886	insignificant
WT vs B	0.0393260	* p<0.05
B4ESE9 (grain samples)		
Comparison	**p-value (Bonferroni)**	**Significancy**
WT vs BC	0.2175874	insignificant
WT vs BCD	0.2361928	insignificant
WT vs BD	0.1591019	insignificant
WT vs C	0.7614274	insignificant
WT vs CD	0.0507096	insignificant
WT vs D	3.0196889	insignificant
WT vs B	0.0054461	** p<0.01
B4ESF7 (grain samples)		
Comparison	**p-value (Bonferroni)**	**Significancy**
WT vs BC	4.7029536	insignificant
WT vs BCD	0.1460891	insignificant
WT vs BD	4.5555298	insignificant
WT vs C	2.0299775	insignificant
WT vs CD	3.5049680	insignificant
WT vs D	0.9895492	insignificant
WT vs B	2.9127700	insignificant

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