University of São Paulo
"Luiz de Queiroz" College of Agriculture

Multi-method research strategy for understanding changes in barley grain protein composition and its relation to improved nutritional quality

Daiana Schmidt

Thesis presented to obtain the degree of Doctor in Science. Area: Genetics and Plant Breeding

Piracicaba
2015

Daiana Schmidt
Agronomist

Multi-method research strategy for understanding changes in barley grain protein composition and its relation to improved nutritional quality

versão revisada de acordo com a resolução CoPGr 6018 de 2011

Advisor:
Prof. Dr. **RICARDO ANTUNES DE AZEVEDO**

Thesis presented to obtain the degree of Doctor in Science. Area: Genetics and Plant Breeding

Piracicaba
2015

Dados Internacionais de Catalogação na Publicação
DIVISÃO DE BIBLIOTECA - DIBD/ESALQ/USP

Schmidt, Daiana
Multi-method research strategy for understanding changes in barley grain protein composition and its relation to improved nutritional quality / Daiana Schmidt. - - versão revisada de acordo com a resolução CoPGr 6018 de 2011. - - Piracicaba, 2015.
136 p. : il.

Tese (Doutorado) - - Escola Superior de Agricultura “Luiz de Queiroz”.

1. Proteínas de grãos 2. Hordeínas 3. Proteínas ricas em lisina 4. Aminoácidos 5. Espectrometria de massa 6. qRT-PCR I. Título

CDD 633.16
S349m

To my parents, Ivanildo and Erotides,

To my Love, Marcelo

To my "Prince" who is coming,

With love I dedicate this work.

ACKNOWLEDGMENTS

To Fundação de Amparo à Pesquisa no Estado de São Paulo (FAPESP), for the financial support to the project entitled “Entendendo o metabolismo de lisina em milho de alta qualidade proteica (QPM) e em cevada geneticamente modificada” (Grant number 2011/21123-8).

To Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the scholarship granted to carry out the sandwich program (PDSE) (Grant number BEX 5361/13-3) from 08/01/2013 to 04/30/2014 at Aarhus University (Slagelse, Denmark).

To Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the scholarship granted from 11/01/2011 to 03/31/2011 (Grant number 161100/2011-2).

To Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the scholarship granted from 08/01/2011 to 10/31/2011.

To “Escola Superior de Agricultura Luiz de Queiroz” for the opportunity to carry out my Doctor of Science studies.

To Centro Nacional de Pesquisa em Energia e Materiais (CNPEM) for the opportunity to perform the mass spectrometry analysis in the Brazilian Biosciences National Laboratory (LNBio), and particularly to Romenia Ramos Domingues and Bianca Alves Pauletti for the technical support.

To my advisor Prof. Dr. Ricardo Antunes de Azevedo, for giving me the chance to be involved in this very interesting research and be a part of an excellent team. I am very grateful for his continue support during my Doctorate, for his wisdom, commitment and patience.

To Dr. Salete A. Gaziola, I would like to thank our friendship during all these years. I really appreciate all her advices, technical and scientific guidance.

To Dr. Eva Vincze, of Aarhus University, Denmark, for guidance during my period in Denmark. I really appreciate her attention and inspiring discussions regarding science and everyday life.

To Dr. Giuseppe Dionisio, of Aarhus University, Denmark, for the technical support during my period in Denmark, for his patient and friendship.

To Dr. Agnieszka Kaczmarczyk, for technical support on the qRT-PCR experiments.

To the teaching, technical and administrative staff of the Genetics Department of ESALQ-USP, which in some way contribute to my Doctorate.

To all my friends at the laboratory of plant biochemical genetics, I thank everyone who contributed to my Doctorate and for providing a more enjoyable path during this period. Without citing names, for the fear of forgetting someone, I thank greatly you all for the companionship, friendship, exchange of experiences and contributions!!

To my parents, Ivanildo and Erotides, my life examples, my strength and my balance. I would like to express my profound gratitude to them, who gave me the possibility to complete this thesis.

To my beloved husband Marcelo, who was next to me in the most difficult moments…and always believed in me!

To all that contributed scientifically and emotionally to the development of this work.

I sincerely thank you all!

Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning.

(Albert Einstein)

CONTENTS

RESUMO 11
ABSTRACT 13
1 INTRODUCTION 15
References 20
2 MULTI-PROTEIN-STUDY-METHOD FOR UNRAVELING THE COMPLEX MIXTURE OF PROTEIN FROM MATURE BARLEY GRAINS 25
Abstract 25
2.1 Introduction 25
2.2 Development 28
2.2.1 Material and methods 28
2.2.1.1 Plant material 28
2.2.1.2 Protein extraction 29
2.2.1.3 Protein quantification 30
2.2.1.4 Amino acid composition 30
2.2.1.5 Sample preparation for shotgun proteomics (nano LC-MS/MS) on the Osborne fractions of whole mature barley grains 31
2.2.1.6 SDS-PAGE of protein fractions on whole mature barley grains 31
2.2.1.7 In-gel digestion of proteins separated by SDS-PAGE and protein identification 32
2.2.2 Results 33
2.2.2.1 Protein quantification 33
2.2.2.2 Amino acid composition 34
2.2.2.3 Proteins identified by shotgun proteomics (LC-MS/MS) on protein fractions of whole mature barley grain 35
2.2.2.4 SDS-PAGE and protein identification of excised bands from the gel 45
2.2.3 Discussion 63
2.3 Conclusions 69
References 69
3 PROTEOME ANALYSIS OF ANTISENSE C-HORDEIN MATURE BARLEY GRAINS 75
Abstract 75
3. 1 Introduction 75
3.2 Development 78
3.2.1 Material and methods 78
3.2.1.1 Biological material, growth conditions and experiment design 78
3.2.1.2 Mature barley grains protein extraction 78

3.2.1.3 Two-dimensional electrophoresis 79
3.2.1.4 Software-based evaluation of differentially abundant proteins 79
3.2.1.5 In-gel digestion for mass spectrometry 80
3.2.1.6 Protein identification 81
3.2.2 Results 81
3.2.3 Discussion 95
3.3 Conclusions 102
References 103
4 EFFECTS OF NITROGEN FERTILIZATION ON THE TRANSCRIPT LEVEL OF DIFFERENT MEMBERS OF HORDEIN GENES AND ON MATURE GRAIN PROTEIN COMPOSITION IN BARLEY 111
Abstract 111
4.1 Introduction 111
4.2 Development 113
4.2.1 Material and methods 113
4.2.1.1 Plant material 113
4.2.1.2 Transcriptome analyses 114
4.2.1.3 Protein analyses 115
4.2.2 Results 116
4.2.2.1 Response of hordein genes at transcriptional level to N supply at 20 DAP 116
4.2.2.2 Response of subfamily of c-hordein multigene family at transcriptional level to N supply at 20 DAP 120
4.2.2.3 Response of total protein content to N supply in the mature grain 121
4.2.2.4 Response of hordeins to N supply in the mature grain 122
4.2.3 Discussion 126
4.3 Conclusion 130
References 130
5 FINAL CONSIDERATIONS 135

RESUMO

Estratégia de pesquisa multimétodo para compreender a composição de proteínas de grãos de cevada e sua relação com a melhoria da qualidade nutricional

A cevada (*Hordeum vulgare* L.) é o quarto cereal mais produzido no mundo. Cerca de dois terços desta produção é utilizada na alimentação animal. A principal desvantagem dos grãos de cevada na alimentação de animais monogástricos é a deficiência de aminoácidos essenciais, principalmente lisina, treonina e metionina. Esta composição desfavorável ocorre devido à principal proteína de reserva dos grãos, as hordeínas, que representam cerca de 50% do teor total de proteína no grão. O nitrogênio promove a expressão e o acúmulo de C-hordeínas, o subgrupo com o menor teor de aminoácidos essenciais e o maior teor de aminoácidos não essenciais. Devido à importância do teor e composição de proteínas nos grãos na determinação de sua qualidade no uso final, o principal objetivo do presente trabalho foi obter uma visão detalhada sobre a síntese e acúmulo de proteínas de grãos de cevada e sua relação com a melhoria da qualidade nutricional. Análises proteômicas e transcriptômicas integradas foram realizadas em um conjunto de linhagens transgênicas de cevada com o perfil de proteínas de reserva alterados em comparação à linhagem não transgênica cv. Golden Promise. Os resultados foram apresentados na forma de três manuscritos (capítulos 2, 3 e 4). O primeiro (capítulo 2) descreveu um novo método de extração de proteínas dos grãos em combinação com métodos de estudo de proteínas diversos, incluindo a quantificação bioquímica, composição de aminoácidos, eletroforese em gel de poliacrilamida-dodecil sulfato de sódio (SDS-PAGE) seguido de identificação por espectrometria de massas (MS) e estratégia *shotgun* para identificação e quantificação relativa das proteínas. Os resultados mostram a mutabilidade das proteínas entre os diferentes grupos e a importância da escolha de um método adequado para a sua identificação de acordo com a complexidade das misturas proteicas. No segundo manuscrito (capítulo 3) o perfil proteico diferencial da linhagem não transgênica e transgênica foi obtido por eletroforese bidimensional (2-DE) para proteínas solúveis, e aquelas expressas diferencialmente foram identificadas por MS. Os resultados demonstram que a supressão das C-hordeínas não afeta exclusivamente a síntese e o acúmulo de hordeínas, e que a composição de aminoácidos mais equilibrada destas linhagens pode ser uma consequência de fontes de proteína distintas entre os diferentes eventos de transgenia, embora a regulação positiva de proteínas ricas em lisina foi estável. No terceiro manuscrito (capítulo 4) foram avaliados os efeitos da adubação nitrogenada sobre a família das hordeínas. Os resultados mostraram que as respostas foram diferentes entre as linhagens não transgênica e transgênica. Um efeito específico de supressão e respostas particulares foi verificado entre os subgrupos da família multigênica das C-hordeínas na linhagem transgênica. Em resumo, a estratégia de pesquisa multimétodo foi aplicada com sucesso na obtenção de informações abrangentes sobre a síntese e o acúmulo de proteínas nos grãos de cevada, e pelo menos em parte, explicou sua relação com a melhoria da qualidade nutricional. Esses resultados podem ser úteis em programas de melhoramento de cevada que visam alterações seletivas de alelos/homólogos específicos para alterar a composição de aminoácidos, através de mudanças nas proporções relativas das proteínas dos grãos.

Palavras-chave: Proteínas de grãos; Hordeínas; Proteínas ricas em lisina; Aminoácidos; Espectrometria de massa; qRT-PCR

ABSTRACT

Multi-method research strategy for understanding changes in barley grain protein composition and its relation to improved nutritional quality

Barley (*Hordeum vulgare* L.) is the fourth largest produced cereal worldwide. About two thirds of barley production is used to animal feed. When used to feed monogastric animals, the main shortcoming of barley grains is the deficiency of essential amino acids, especially lysine, threonine and methionine. The unbalanced amino acid composition is due to the main storage protein, the hordeins, which account for about 50% of total grain protein content. The nitrogen fertilization promotes C-hordein expression and accumulation, the hordein subgroup with the lowest content of essential amino acids, and the highest content of non-essential amino acids. Due to the importance of grain protein content and composition in the end use grain quality the key objective of the present study was to obtain a detailed insight into synthesis and accumulation of barley grain proteins and their relation to improved nutritional quality. An integrated proteomic and transcriptomic analysis have been undertaken in a set of transgenic antisense barley lines with the grain protein profile altered in comparison to the non-transgenic line cv. Golden Promise. The results were presented in three manuscripts in the thesis (chapters 2, 3 and 4). The first manuscript (chapter 2) reported a new grain protein extraction method combined with multi-method protein evaluation, including biochemical quantification, amino acid composition, sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) couple with mass spectrometry (MS) identification and a gel free shotgun MS identification and relative quantification. The results showed the changeability of proteins between protein groups and the importance of choosing an adequate proteomic-based method for protein identification according to the complexity of protein mixtures. In the second manuscript (chapter 3) a differential protein profile of non-transgenic barley cv. Golden Promise and the transgenic antisense C-hordein barley lines was achieved by two-dimensional gel electrophoresis (2-DE) for salt soluble proteins and the differentially expressed proteins were identified by MS. The key results showed that the suppression of C-hordeins, the poor nutritional hordein subgroup, does not exclusively affects hordein synthesis and accumulation, and that the more balanced amino acid composition of these lines may be a consequence of distinct protein sources among different transgenic events, though a stable lysine-rich proteins upregulation occurs in all lines. In the third manuscript (chapter 4) the effects of nitrogen fertilization on hordein family at transcriptional and proteome level were assessed. The main results showed differential responses to N nutrition between non-transgenic and transgenic lines. In relation to C-hordein, specific C-hordein downregulation effect and in particular different responses to N were verified among subgroups of C-hordein multigene family in the transgenic line at transcriptional and proteomic level. In summary, the multi-method strategy used in the present work was successfully applied to obtain comprehensive information about barley grain proteins synthesis and accumulation and explain, at least in part, their relation to improved nutritional quality. These results can be useful in barley breeding programs aiming selective alterations of specific alleles/homologues to change amino acid composition by changing the relative proportions of the grain proteins in order to improve the barley grains nutritional quality.

Keywords: Grain proteins; Hordeins; Lysine-rich proteins; Amino acids; Mass spectrometry; qRT-PCR

1 INTRODUCTION

The modern barley (*Hordeum vulgare* subsp. *vulgare*) derived from its wild progenitor *Hordeum vulgare* subsp. *spontaneum* belongs to one of the most economically important plant groups worldwide, the Triticeae tribe of the Poaceae (true grasses) family (VON BOTHMER; KOMATSUDA, 2010). One of the oldest cultivated crops in the world, barley was first domesticated in the 'Fertile crescent' in the Near East, with its first remains discovered in archeologic sites dating around 8,000 B.C, and subsequently spread to Africa and Europe (NEWMAN; NEWMAN, 2006).

Barley's distribution is worldwide and undoubtedly the most widely adapted cereal grain species, which has been cultivated either in high-input agricultural systems or in marginal and subsistence low-input environments, especially due to its high drought, cold and salt tolerance (NEWTON et al., 2011).

In 2013, barley ranked fourth in cereal production worldwide, after maize, rice and wheat, with an annual production which exceeded 143 million tons (Mt) (FAO, 2015). Continentally, barley production was highest in Europe (59.7%), followed by Asia (15.2%), Americas (14.7%), Oceania (5.5%) and Africa (5%). Russian Federation, Germany, France and Canada were the largest producing countries in 2013 (FAO, 2015). In this scenario, Brazil ranked 38^{o} in barley world production, with 330,682.00 tons harvested in an area of 88,321.00 hectares, traditionally growing in the south region (IBGE, 2015).

Barley grains were presumably first used as human food, however, in recent times, the largest use of barley is for animal feed, accounting 55%-60% of global barley production, whilst around 30%-40% of barley is malted, 2%-3% is used for human food and about 5% for seed (ULLRICH, 2011).

Barley is utilized to feed a variety of animals, including ruminant and non-ruminant livestock, poultry and fish, normally growing in temperate regions of the world (FOX et al., 2003; BLAKE et al., 2010). The desirable characteristics of barley grains for feed vary greatly among the livestock species and their uses (BLAKE et al., 2010), thus to understand and adjust true feed quality is not an easy task. Thereby, animal performance was traditionally used and measured by average daily weight gain to determine barley feed quality (GOUS et al., 2012).

Most of the efforts to improve barley nutritional feed quality have been developed for non-ruminant animals (pigs and poultry) because of their simple stomach and limited abilities to utilize the grain components (KACZMARCZYK, 2012). Among the set of traits normally

considered to contribute improving animal performance (acid detergent fiber, starch, protein and dry matter digestibility) (SURBER et al., 2000), the protein composition is the major determinant of feed quality when used to feed non-ruminant animals, once they are not capable to synthesize nine of the twenty amino acids normally incorporated into proteins, thus their requirements must be meet through diet (AZEVEDO; LANCIEN; LEA, 2006; AZEVEDO; ARRUDA, 2010).

Despite of this, the majority of barley breeding programs is based on grain yield, which probably is the main reason why most of the barley used for feeding is malting barley that failed to meet malting standards (ULLRICH, 2002; JACOB; PESCATORE, 2012).

Grain yield, as the majority of agronomically and economically important traits, is a quantitative characteristic controlled by many genes, wherein the phenotype is a result of a series of components including spike number, grain number, grain weight and thousand-grain weight (WANG et al., 2014). Although substantial increases in grain yield of plant crops have been achieved in genetic breeding programs through the development of superior varieties, environment management is largely employed in agricultural system to delivery high yield crops (EVENSON; GOLLIN, 2003) once polygenic traits are known to be extensively affected by environmental factors.

One of the most employed environment management strategy to maximize crop yields is the use of large amounts of nitrogen fertilizer. Over the past four decades a seven-fold increase in the use of nitrogen fertilizer was reported against a two-fold increase of food production worldwide (HIREL et al., 2007). However, the excessive use of this mineral has had negative impacts on the environment, threatens the quality of air, soil and water (SUTTON et al., 2011). As a consequence, in recent times there is an increasing pressure to reduce nitrogen usage. Therefore, the challenge of genetic breeding is to develop new crop varieties with improved yield in low nitrogen environments (RENGEL; MARSCHNER, 2005) delivering desirable characteristics to end use.

Nitrogen is the most important nutrient for plant growth and development (XU et al., 2014) and has effect on protein level and composition of cereal crops (CHOPE et al., 2014), which in turn have significant impacts on grain end use proprieties. The effects include the ratio of storage to non-storage proteins, the proportions of individual storage proteins, and the ratios of storage protein polymers to monomers (SHEWRY, 2011).

Similarly to other cereals, the major storage protein in barley are prolamins, accounting to at least 50% of total grain nitrogen and due to its high proportion in total protein, they determine the nutritional quality of barley grains (HOLOPAINEN et al., 2012).

Hordeins, as they are named in barley, are classified in relation to their structural and evolutionary relationship into three subgroups, sulfur-rich (S-rich; B- and γ-hordeins), sulfur-poor (S-poor; C-hordeins) and high molecular weight (HMW) glutelins homologues (D-hordeins), distinguishable by their mobility in SDS-PAGE and amino acid composition (TATHAM; SHEWRY, 2012).

C-hordeins is the second most abundant hordein subgroup, accounting for about 10-20% of total hordein, and the subgroup which contain the highest proportion of the dispensable amino acids glutamine and proline and the lowest content of indispensable amino acids lysine, threonine and methionine (SHEWRY; HALFORD, 2002). Due to these features, C-hordeins are considered to be responsible to low nutritional quality of barley for feeding.

All the three gene families encoding for S-rich, S-poor and HMW prolamins are subjected to strict tissue-specific (starchy endosperm) and temporal regulation with coordinated expression (SHEWRY; TATHAM; HALFORD, 2001). The primary regulation of prolamin genes occurs at transcriptional level, with the accumulation of all three hordein subgroups being directly related to their respective genes transcription rates (RAHMAN et al., 1983). However, a fine tuning at translational level occurs when the sulfur availability is limited, leading to an increased efficiency of C-hordein mRNA translation and a reduced rate of B-hordeins synthesis. Besides that, C-hordein promoter responded positively to nitrogen fertilisation during grain development (SHEWRY, 2007) due to two specific GCN4 motif (ATGA(C/G)TCAt) and an endosperm specific promoter TGTAAAGT (MÜLLER; KNUDSEN, 1993). Low nitrogen acts on the GCN4 box by repressing its gene expression while the endosperm box has the task of fine tuning the optimal nitrogen response to further upregulate or silence the effect of the CGN4 box (MÜLLER; KNUDSEN, 1993). Despite the barley genome is sequenced (CONSORTIUM, 2012), all the members of the C-hordein alleles have not been cloned and promoter differences cannot be inferred, as well as more detailed C-hordeins gene regulation.

Different strategies have been exploited to improve nutritional quality of barley grains, which includes the use of natural mutants (e.g. high-lysine barley) in traditional breeding programs, mutagenesis and genetic engineering altering amino acid metabolism and/or the grain protein pattern (SUN; LIU, 2004; GALILI; AMIR, 2013). At present, one of the most promising strategies seems to be by changing the grain protein pattern, specifically altering the relative proportions of the major storage protein families by antisense or RNAi technologies (HANSEN et al., 2007).

Thus, in order to silence the poorest hordein subgroup (C-hordein) and improve the nutritional quality of barley grains Lange et al. (2007) employed an antisense strategy, wherein a 480 bp fragment of the 3′ region of a C-hordein encoding gene (Gene Bank accession number S66938) was amplified from barley genomic DNA and cloned in the antisense orientation. According to the authors, the antisense construct was assembled by the insertion of the antisense C-hordein fragment between the ubiquitin promoter and the *nos* terminator (Ubi-AsHorC-nosT construct). An *Agrobacterium tumefaciens* mediated transformation of immature embryos at approximately 14 days after pollination of barley cv. Golden Promise was performed using hygromycin as selectable marker. A total of 48 barley lines were regenerated and the transformation confirmed by Southern blot analysis using a T-DNA probe of 1.4 kb fragment of the ubiquitin promoter. Hybridizing bands were found in 35 lines of the primary transformants. Five of 35 lines were chosen for further investigation based on RT-PCR for expression of the Ubi-AsHorC-nosT construct with RNA isolated from leaves and due to its greater suppression of C-hordein synthesis revealed by SDS-PAGE of mature barley grains (LANGE et al., 2007).

The Southern blot analysis of the screened set of antisense C-hordein T2 barley lines (L1-5) revealed two lines with a single locus integration sites (L1 and L4), one line with two integration sites (L2), and four integration sites were identified in two lines, (L3 and L5) (LANGE et al., 2007). Moreover, the authors reported rearrangements and/or deletions in L1, L2, L3 and L5.

From the nutritional point of view, this set of antisense barley lines are characterized by a more balance amino acid composition, due to the increased level of essential amino acids including lysine (up to 16%), threonine (up to 13%), methionine (up to 11%), isoleucine (up to 6.6%), leucine (up to 8.1%) and valine (up to 17.1%); and reduced level of non-essential amino acids, such as proline (up to.3%) and glutamine (up to 9.8%) (LANGE et al., 2007).

Apart from the improved nutritional quality of the barley grains obtained, this set of antisense C-hordein barley lines, which contain an altered protein profile has become an important tool of genetic, physiology and metabolic studies of grain proteins, including protein expression, synthesis, accumulation and their relation with amino acid composition.

Therefore, the general objective of the present work was to obtain a detailed insight into barley grain proteins to understand the molecular and biochemical mechanisms underlining the storage and non-storage protein synthesis in antisense C-hordein barley lines. To reach the objective, the work was divided into three steps, which are present here as three separated chapters.

The objective of the first step (described in chapter 2) was to perform a broad characterization of a modified grain protein extraction method through multi-protein study methods in the whole mature barley grain of cv. Golden Promise. The novelty of this chapter was the identification of the proteins solubilized by each protein extractor (salt, water, alcohol and alkali solution) through nano ultra-performance liquid chromatography with tandem mass spectrometry (nano UPLC-MS/MS). Although the barley grain protein fractionation is widely spread worldwide and extensive efforts have been developed to improve protein extraction methodology as well as protein separation by SDS-PAGE, until now, at least in the light of our knowledge, there is no report in the literature on the individual protein identification in each fraction. Besides that, it was determined to each fraction the protein quantity through the two most widely protein quantification methodologies (Bradford and Lowry), the protein profile through SDS-PAGE and the amino acid composition through high-performance liquid chromatography.

In the second step (described in chapter 3) the main goal was to address questions on the association of non-storage proteins (globulins and albumins) and storage proteins (prolamins) profile in relation to a grain protein balance perturbation, in this case the suppression of C-hordeins. In the original paper describing the generation of the antisense C-hordein barley lines Lange et al. (2007) reported the altered storage protein profile, wherein the relative contribution of each hordein subgroups (B-, C-, D- and γ-hordeins) on total hordein changed in comparison to the parental line cv. Golden Promise. Moreover, the relative contribution of non-storage protein on total grain protein also changed. Thus, based on a two-dimensional gel electrophoresis (2-DE)-based approach in combination with mass spectrometry, the originality of this chapter was the identification of individual non-storage proteins differentially expressed in the antisense C-hordein barley lines, which could be also strongly related with the more balanced amino acid composition of these transgenic lines, especially when the lysine content is concerned.

The objective of the last step (described in chapter 4) was to assess the effect of nitrogen fertilization (three dosages) on hordein synthesis and composition in an antisense C-hordein line (L5) and obtain comprehensive information about the impact of nitrogen fertilization on the different C-hordein isoforms through RT-qPCR and SDS-PAGE tools. Although the effects of nitrogen fertilization on hordeins accumulation in barley grains are widely studied, the responsiveness of this phenomenon is more complex, involving metabolic

feedback control and regulation of hordein genes expression, which required detailed studies for increased understanding.

References

AZEVEDO, R.; ARRUDA, P. High-lysine maize: the key discoveries that have made it possible. **Amino Acids**, Wien, v. 39, n. 4, p. 979-989, 2010.

AZEVEDO, R.A.; LANCIEN, M.; LEA, P.J. The aspartic acid metabolic pathway, an exciting and essential pathway in plants. **Amino Acids**, Wien, v. 30, n. 2, p. 143-162, 2006.

BLAKE, T.; BLAKE, V.C.; BOWMAN, J. G. P.; ABDEL-HALEEM, H. Barley feed uses and quality improvement. In: ULLRICH, S.E. (Ed.). **Barley:** production, improvement, and uses. Oxford, UK: Wiley-Blackwell, 2010. chap. 16, p. 522-531.

CHOPE, G.A.; WAN, Y.; PENSON, S.P.; BHANDARI, D.G.; POWERS, S.J.; SHEWRY, P.R.; HAWKESFORD, M.J. Effects of genotype, season, and nitrogen nutrition on gene expression and protein accumulation in wheat grain. **Journal of Agricultural and Food Chemistry**, Easton, v. 62, n. 19, p. 4399-4407, 2014.

CONSORTIUM, T. I. B. G. S. A physical, genetic and functional sequence assembly of the barley genome. **Nature**, London, v. 491, n. 7426, p. 711-716, 2012.

EVENSON, R.E.; GOLLIN, D. Assessing the impact of the green revolution, 1960 to 2000. **Science**, Washington, v. 300, n. 5620, p. 758-762, 2003.

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS (FAO). **FAOSTAT crop production and trade**. Disponível em:<http://faostat3.fao.org/browse/Q/QC/E>. Acesso em: 01 Jun. 2015.

FOX, G.P.; PANOZZO, J. F.; LI, C.; LANCE, R.; INKERMAN, P.A.; HENRY, R.J. Molecular basis of barley quality. **Crop and Pasture Science**, Victoria, v. 54, n. 12, p. 1081-1101, 2003.

GALILI, G.; AMIR, R. Fortifying plants with the essential amino acids lysine and methionine to improve nutritional quality. **Plant Biotechnology Journal**, Oxford, v. 11, n. 2, p. 211-222, 2013.

GOUS, P.; MARTIN, A.; LAWSON, W.; KELLY, A.; FOX, G.; SUTHERLAND, M. QTL associated with barley (*Hordeum vulgare*) feed quality traits measured through in situ digestion. **Euphytica**, Wageningen, v. 185, n. 1, p. 37-45, 2012.

HANSEN, M.; LANGE, M.; FRIIS, C.; DIONISIO, G.; HOLM, P. B.; VINCZE, E. Antisense-mediated suppression of C-hordein biosynthesis in the barley grain results in correlated changes in the transcriptome, protein profile, and amino acid composition. **Journal of Experimental Botany**, Oxford, v. 58, n. 14, p. 3987-3995, 2007.

HIREL, B.; LE GOUIS, J.; NEY, B.; GALLAIS, A. The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches. **Journal of Experimental Botany**, Oxford, v. 58, n. 9, p. 2369-2387, 2007.

HOLOPAINEN, U.R.M.; WILHELMSON, A.; HOME, S.; POUTANEN, K.; SHEWRY, P. R. Day-length effects on protein localisation affect water absorption in barley (*Hordeum vulgare*) grains. **Journal of the Science of Food and Agriculture**, London, v. 92, n. 15, p. 2944-2951, 2012.

INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA (IBGE). **Produção Agrícola Municipal**. Disponível em:<http://www.sidra.ibge.gov.br/bda/tabela/protabl.asp?c=1612&z=t&o=11&i=P>. Acesso em: 01 Jun. 2015.

JACOB, J.P.; PESCATORE, A.J. Using barley in poultry diets - a review. **The Journal of Applied Poultry Research**, Athens, v. 21, n. 4, p. 915-940, 2012.

KACZMARCZYK, A. **Multi-method research strategy for understanding changes in storage protein composition in developing barley grain to improve nutritional profile**. 2012. 176p. PhD thesis (Molecular Biology and Genetics) - Faculty of Science and Technology, Aarhus University, Slagelse, 2012.

LANGE, M.; VINCZE, E.; WIESER, H.; SCHJOERRING, J.K.; HOLM, P.B. Suppression of C-hordein synthesis in barley by antisense constructs results in a more balanced amino acid composition. **Journal of Agricultural and Food Chemistry**, Easton, v. 55, n. 15, p. 6074-6081, 2007.

MÜLLER, M.; KNUDSEN, S. The nitrogen response of a barley C-hordein promoter is controlled by positive and negative regulation of the GCN4 and endosperm box. **The Plant Journal**, Washington, v. 4, n. 2, p. 343-355, 1993.

NEWMAN, C.; NEWMAN, R. A brief history of barley foods. **Cereal Foods World**, Minneapolis, v. 51, n. 1, p. 4-7, 2006.

NEWTON, A.; FLAVELL, A.; GEORGE, T.; LEAT, P.; MULLHOLLAND, B.; RAMSAY, L.; REVOREDO-GIHA, C.; RUSSELL, J.; STEFFENSON, B.; SWANSTON, J.S.; THOMAS, W. B.; WAUGH, R.; WHITE, P.; BINGHAM, I. Crops that feed the world 4. Barley: a resilient crop? Strengths and weaknesses in the context of food security. **Food Security**, Amsterdam, v. 3, n. 2, p. 141-178, 2011.

RAHMAN, S.; SHEWRY, P. R.; FORDE, B. G.; KREIS, M.; MIFLIN, B. J. Nutritional control of storage-protein synthesis in developing grain of barley (*Hordeum vulgare* L.). **Planta**, Berlin, v. 159, n. 4, p. 366-372, 1983.

RENGEL, Z.; MARSCHNER, P. Nutrient availability and management in the rhizosphere: exploiting genotypic differences. **New Phytologist**, Cambridge, v. 168, n. 2, p. 305-312, 2005.

SHEWRY, P.R. Improving the protein content and composition of cereal grain. **Journal of Cereal Science**, London, v. 46, n. 3, p. 239-250, 2007.

SHEWRY, P.R. Effects of nitrogen and sulfur nutrition on grain composition and properties of wheat and related cereals. In: HAWKESFORD, M.J.; BARRACLOUGH, P. (Ed.). **The Molecular and Physiological Basis of Nutrient Use Efficiency in Crops**. Chichester: Wiley-Blackwell, 2011. chap. 6, p. 103-120.

SHEWRY, P.R.; HALFORD, N.G. Cereal seed storage proteins: structures, properties and role in grain utilization. **Journal of Experimental Botany**, Oxford, v. 53, n. 370, p. 947-958, 2002.

SHEWRY, P.; TATHAM, A.; HALFORD, N. Nutritional control of storage protein synthesis in developing grain of wheat and barley. **Plant Growth Regulation**, New York, v. 34, n. 1, p. 105-111, 2001.

SUN, S.; LIU, Q. Transgenic approaches to improve the nutritional quality of plant proteins. **In Vitro Cellular and Developmental Biology - Plant**, Columbia, v. 40, n. 2, p. 155-162, 2004.

SURBER, L.; BOWMAN, J.; BLAKE, T.; HINMAN, D.; BOSS, D.; BLACKHURST, T. Prediction of barley feed quality for beef cattle from laboratory analyses. **Proceedings, Western Section American Society of Animal Science**, Davis, v. 51, p. 454-457, 2000.

SUTTON, M.A.; OENEMA, O.; ERISMAN, J.W.; LEIP, A.; VAN GRINSVEN, H.; WINIWARTER, W. Too much of a good thing. **Nature**, London, v. 472, n. 7342, p. 159-161, 2011.

TATHAM, A.S.; SHEWRY, P.R. The S-poor prolamins of wheat, barley and rye: revisited. **Journal of Cereal Science**, London, v. 55, n. 2, p. 79-99, 2012.

ULLRICH, S. Genetics and breeding of barley feed quality attributes. In: SLAFER, G. A.; MOLINA-CANO, J.L.; SAVIN, R.; ARAUS, J.L.; ROMAGOSA, I. (Ed.). **Barley Science:** Recent Advances from Molecular Biology to Agronomy of Yield and Quality. New York: Food Products Press, 2002. chap. 6, p. 115-142.

ULLRICH, S.E. Significance, adaptation, production, and trade of barley. In: ULLRICH, S. E. (Ed.). **Barley:** production, improvement, and uses. Oxford, UK: Wiley-Blackwell, 2011. chap. 1, p. 3-13.

VON BOTHMER, R.; KOMATSUDA, T. Barley origin and related species. In: ULLRICH, S. E. (Ed.). **Barley:** production, improvement, and uses. Oxford, UK: Wiley-Blackwell, 2010. chap. 2, p. 14-62.

WANG, J.; YANG, J.; JIA, Q.; ZHU, J.; SHANG, Y.; HUA, W.; ZHOU, M. A new QTL for plant height in barley (*Hordeum vulgare* L.) showing no negative effects on grain yield. **PLoS One**, San Francisco, v. 9, n. 2, p. e90144, 2014.

XU, Y.; WANG, R.; TONG, Y.; ZHAO, H.; XIE, Q.; LIU, D.; ZHANG, A.; LI, B.; XU, H.; AN, D. Mapping QTLs for yield and nitrogen-related traits in wheat: influence of nitrogen and phosphorus fertilization on QTL expression. **Theoretical and Applied Genetics**, Berlin, v. 127, n. 1, p. 59-72, 2014.

2 MULTI-PROTEIN-STUDY-METHOD FOR UNRAVELING THE COMPLEX MIXTURE OF PROTEIN FROM MATURE BARLEY GRAINS

Abstract

Barley grain proteins extraction and identification/quantification are still a major challenge in plant proteome studies. Proteomic approaches have been used extensively using two-dimensional gel electrophoresis (2-DE) and collecting spots for protein identification. Gel excision from one dimensional (1D) SDS-PAGE gel has also been an alternative in plant proteomics, although each band containing several proteins at once. Recently, the high throughput proteomics based on LC-MS/MS has enabled the high load of nano ultrahigh liquid performance chromatography (nanoULPC) runs coupled to mass spectrometry (MS) identification. In the present study, a modification of the Osborne protein extraction technique has been used to separate barley grain proteins and shotgun MS strategy was employed to unraveling the proteins qualitative identification and relative quantification, which compose each one of the five protein extracts obtained in the new procedure. Results have generated lists of abundant proteins per extract and these proteins hits have been compared to 1D SDS-PAGE gel excised bands of the same extract. Therefore, it is possible to achieve a higher throughput with the gel free shotgun MS method. As a control experiment such dataset of shotgun proteins identified that refers to untransformed barley cv. Golden Promise has been checked against the differential MS identification using 1D SDS-PAGE protein bands of antisense C-hordeins barley lines (L1-5). The most prominent protein of each protein extract with altered abundancy profile were excised from the gel and the protein identified by MS. As expected, from the comparison to the parental line cv. Golden Promise, some C-hordeins isoforms were downregulated. Regardless the particular shortcoming of C-hordeins analysis due to the high repetitive motifs in their sequences and lack of C-hordeins sequences deposited on databases with high number of redundancy, we suggest that 1D SDS-PAGE excision technique was a reasonable tool for storage protein profile (prolamin) comparisons. On the other hand, due to the high number of proteins identified per each band excised from salt soluble proteins, to obtain accurate information from 1D SDS-PAGE excision technique for other Osborne fraction, it should be recommended the employment of 2-DE or the MS shotgun strategy.

Keywords: Plant proteomics; SDS-PAGE; Mass spectrometry; LC-MS/MS; Amino acids

2.1 Introduction

Barley is the fourth most important cereal crop worldwide, after maize, rice and wheat, with a production of 143 million tons (Mt) in 2013, which represented 5.2% of total cereal production (FAO, 2015). Barley grains are mainly use for livestock feed, industry processing (malting, brewing, distilling) and human food (GUBATZ; SHEWRY, 2010).

Barley grains, as well other cereal grains, are excellent source of metabolizable energy, available as starch and dietary fiber, however, when it comes to the end use for non-ruminant animal feed and human food as protein source some limitations exist, the amount and the quality of it proteins. The protein content of whole barley grain on the basis of dry

weigh varies from 8% to 15% according to environment factors, especially nitrogen supply and genetic background (SHEWRY, 2007). Although the amount of protein is low, the main shortcoming is the low nutritional quality of proteins. The nutritive value of a protein is accessed through the amino acid composition and the bioavailability of individual amino acids as well the protein digestibility (BOYE; WIJESINHA-BETTONI; BURLINGAME, 2012).

Due to the importance of barley grain protein content and composition for the end use properties, they have been studied over 200 years (GUBATZ; SHEWRY, 2010), and since the beginning the researchers have classified the grain proteins into categories according to an arbitrary criterion. In the past few years grain proteins have been classified on the basis of their functions into metabolic and protective, storage and structural proteins (SHEWRY; HALFORD, 2002), yet, the remarkable studied developed by T. Osborne in 1900's is still widely used. The Osborne study established a protein classification based on their solubility in a series of solvents. Proteins soluble in dilute salt solutions, water, aqueous alcohols and dilute acid or alkali are termed, globulins, albumins, prolamins and glutelins, respectively (OSBORNE, 1909).

In barley, globulins constitutes about 15% of total grain protein, whereas albumins, prolamins, which in barley are called hordeins, and glutelins account to the remaining 15%, 37% and 30%, respectively (STEINER; GASTL; BECKER, 2011). However, it is known that these fractions are complex mixtures of proteins and there is an overlap between the classes (shared solubility). Therefore, in order to improve the distinction among the protein classes, extensive modifications have been done to the original "Osborne fractionation", either methodologically and terminologically.

In barley, the main concern about the "Osborne fractionation" is related to the separation of the major constituents of the barley grain, the prolamins, better termed, hordeins. In the original study proposed by Osborne, the term prolamins is limited to the fraction which is soluble in alcohol-water mixtures without any component added to the extraction buffer. However, in that condition only half of the storage proteins are extractable being the remained proteins, soluble in dilute acid or alkali, detergents or aqueous alcohol mixtures containing a reducing agent, are termed glutelins (TATHAM; SHEWRY, 2012). However, the later fraction forms a complex mixture of proteins in which it is difficult to determine accurately the true glutelins, once many components are structurally related to hordeins, wherein individual reduced subunits are alcohol-soluble and are proline and glutamine-rich proteins, similarly to hordeins (NEWMAN; NEWMAN, 1992).

Therefore, due to the lack of complete separation of hordeins and glutelins, currently, the most widely used solvent to extract hordeins comprises an aqueous-alcohol solution (usually 55% of 2-propanol) containing a reducing agent (usually β-mercaptoethanol), being the remained proteins soluble in dilute acid or alkali termed glutelins (SHEWRY; HALFORD, 2002).

Moreover, it is known that prolamins are categorized into three major groups based on their structure and evolutionary relationships. They are sulfur-rich (S-rich), sulfur-poor (S-poor) and high molecular weight proteins (HMW), which in barley is further subdivided into four subgroups, B and γ-hordeins (S-rich), C-hordeins (S-poor) and D-hordeins (HMW) (SILVA et al., 2008). Due to its abundance and economic importance, extensive efforts has been given to extent the knowledge about hordeins, such as those related to high resolution separation, structure, chemical and physical properties, and post translational modifications.

In the past two decades we have witnessed an astonishing advance towards methods for studying proteins, and since the term proteomics was used for the first time in the mid of 1990's (WILKINS et al., 1996) a huge amount of data have been generated through the employment of different technologies, from diverse protein extraction methods of different plant species (VILHENA et al., in press) to protein separation using for example 2-DE (ARRUDA et al., 2011) and protein separation, identification and quantification by diverse MS-based high-throughput techniques (ZHANG et al., 2014). The primary concept of proteomics is the study of the complete set of proteins expressed by an organism, tissue or cell (WILKINS et al., 1996), however alternative concepts have been emerged encompassing all aspects of proteins, which means, to define and characterize the identities, quantities, structures and functions of proteins in different cellular context (PHIZICKY et al., 2003). Moreover, post-translation modifications as well as protein/protein interactions analyses are also included in proteomics (BAGINSKY, 2009).

Initially, the studies were basically descriptive using two-dimensional polyacrylamide gel electrophoresis (2-DE) technique to access the protein profile of a given tissue or organelle (VANDERSCHUREN et al., 2013). Nevertheless, with the development of mass spectrometry and high-throughput analysis, the studies using proteomics approaches became rather than descriptive, and now it is possible to access the relative or absolute quantification of identified proteins (SCHULZE; USADEL, 2010). To get detailed information on proteomes, coupling more than one technique such as 2-DE or liquid chromatography followed by mass spectrometry, has been largely used in more recent years.

Nowadays, the advances in proteomics tools are providing new opportunities to access the complex mixture of proteins obtained from the Osborne fractionation. Therefore, the aim of this study was to identify and quantify the barley protein composition of the major grain protein fractions (salt soluble, prolamin and glutelin) for future proteomics based diagnostic studies based on differential proteomics of barley antisense C-hordein barley lines (LANGE et al., 2007) and its parental line cv. Golden Promise. Thus, based on the original sequential barley protein extraction described by Shewry et al. (1983), we employed experimental modifications suggested by Landry; Delhaye; Damerval (2000) using lower amount of grain flour and performing an additional step of extraction after prolamin solubilization, wherein proteins associate to prolamins in maize were reported.

Therefore, we identify and estimate the relative abundance of five protein extracts from the whole mature barley grains of cv. Golden Promise by a shotgun proteomics strategy and also by protein identification of excised protein bands from SDS-PAGE by nano ultra-high performance liquid chromatography (nanoUPLC) with tandem mass spectrometric (MS/MS). Besides that, we also investigated the amino acid composition of each protein extract and determined the protein quantity through traditional methods.

2.2 Development

2.2.1 Material and methods

2.2.1.1 Plant material

Mature barley grains (*Hordeum vulgare* L. cv. Golden Promise) were used for protein extraction, shotgun proteomics (nano UPLC-MS/MS) on the Osborne fractions, protein identification of excised protein bands from SDS-PAGE by nano UPLC-MS/MS, protein quantification and amino acid composition. An additional five antisense C-hordein barley lines (L1-5) (LANGE et al., 2007), generated at University of Aarhus, Denmark, and kindly provided by M. Lange, were used for protein extraction of the Osborne fractions and further separated by SDS-PAGE as a preliminary proteome differential analysis of this set of lines and its parental line cv. Golden Promise.

The plants were grown in a glasshouse in Piracicaba, São Paulo, Brazil (22^{o} 42’ 30’’ S, 47^{o} 38’ 00’’ W), under natural daylight conditions during the autumn/winter season of 2010. Twenty-liter-pots containing soil and 1 g of NPK (10-10-10) were used to grow three plants. The soil chemical is shown in Table 2.1. Nitrogen fertilization with ammonium sulfate was applied at 40 and 55 days after sowing, to a total amount of 2 g per 20 liter-pot.

Table 2.1 – Soil chemical characteristics used in the experiment. P, S, B, Cu, Fe, Mn and Zn expressed as $mg.dm^{-3}$. K, Ca, Mg, Al, H+Al, SB and CTC expressed as $mmol.dm^{-3}$. V expressed as percentage (%)

pH	P	S	K	Ca	Mg	Al	H+Al	SB[a]	CTC[b]	V[c]	B	Cu	Fe	Mn	Zn
6.1	585	39	3.9	165	30	0	13	198.7	212	94	0.82	1.7	75	2.5	14.1

[a] Total of exchangeable bases
[b] Cation-exchange capacity
[c] Base saturation

Mature barley grains from three different pots and from three plants per pot were harvest randomly and bulk for all analysis.

2.2.1.2 Protein extraction

The protein extraction procedure was based on the sequential extraction described by Shewry et al. (1983) with modifications reported by Landry et al. (2000) using 100 mg of flour and 1 ml of extraction buffer. Whole grains from three different plants were ground in a sample mill and passed through a screen in order to obtain homogenized flour.

Prior to protein extraction, the samples were treated with 1 ml of hexane during 15 min at room temperature and centrifuged at 10 000*g* for 5 min to extract lipids. The supernatant was discarded and the flour dried overnight. Salt soluble proteins (E_1) were extracted in 500 mM NaCl for 30 min at 4 ^{o}C. After centrifugation at 10 000*g* for 5 min the supernatant was stored and the pellet used for a second round of extraction in deionized water for 30 min at 4 ^{o}C to solubilize the remained salt soluble proteins in the pellet (E_2). For both extractions an additional 10 µl of protease inhibitor cocktail (Sigma-Aldrich) were added for each 1 ml of extraction buffer. Prolamins were extracted from the previous pellet in a solution of 55% 2-propanol containing 0.6% 2-mercaptoethanol (2ME) for 30 min at room temperature followed by centrifugation at 10 000*g* for 5 min at room temperature and was named prolamins I (E_3). The pellet was afterwards used to extract the fraction called prolamins II (E_4) in 500 mM NaCl, pH 10 (0.025M sodium borate buffer) containing 0.6% 2ME. After centrifugation, a 0.5% SDS solution in 0.025M sodium borate buffer pH 10 containing 0.6% 2ME was added to the pellet and the glutelins (E_5) were extracted for 30 min at room temperature.

The procedures described for E_1 and E_4 were repeated twice and the supernatants of each fraction were combined. E_2 extraction was also repeated twice, but the second supernatant was discarded. E_3 and E_5 were extracted three times and the supernatants of each fraction combined. The samples were stored at -80 ^{o}C until further analysis.

In order to get detailed information about protein quantity and distribution in the modified extraction protein method presented here, in all forward analysis the extracts were analyzed separately (E_1, E_2, E_3, E_4 and E_5).

2.2.1.3 Protein quantification

Two of the most employed methods to quantify proteins, Bradford (BRADFORD, 1976) and Lowry (GERHARDT, 1994) were used to determine the protein concentration.

Firstly, in order to avoid chemical interferences in both quantification methods all protein extracts were precipitate with two volumes of acetone and the pellet reconstituted in 300 µl of 2M urea. An aliquot of the reconstituted sample was used to determine the protein concentration according to Bradford (1976) using bovine serum albumin (BSA) as a standard.

The Lowry method was employed as described by Gerhardt et al. (1994) with some modifications. The samples were diluted in 2M urea to a final volume of 500 µl, to which 700 µl of fresh Lowry solution (Solution A =0.143 N NaOH and 0.135 N Na_2CO_3 + Solution B =0.057 M $CuSO_4$ and 1% w/v Na_2 tartrate + Solution C = 0.124 M Na_2 tartrate, with a ratio of 100:1:1) was added. The samples were incubated for 20 min at room temperature in the dark following an addition of 100 µl of diluted Folin–Ciocalteu's phenol reagent (1N). After 30 min of incubation at room temperature in the dark, the samples were vortex and the absorbance determined at 750 nm. A standard curve based on known amount of BSA was used to determine the protein concentration.

2.2.1.4 Amino acid composition

The amino acid composition was determined from protein hydrolysates of each extract independently (E_1, E_2, E_3, E_4 and E_5). The hydrolysis of 100 µg of protein in 40 µl of 6 N HCl solution was performed at 110 ^{o}C during 22 h (FOUNTOULAKIS; LAHM, 1998). The samples were lyophilized and reconstituted in ultra-pure water. The samples were analyzed as *o*-phthaldialdeyde (OPA) derivates by reverse-phase high performance liquid chromatography (HPLC) on a Spherisorb ODS-2 (C18) column through a linear elution gradient of methanol from 20 to 100% (AZEVEDO et al., 2003). The derivates were detected trough fluorescent signals using a fluorometer (Shimadzu RF350) adjusted to the excitation wavelength of 250 nm and an emission wavelength of 480 nm. The amino acids standard solution AAS18 (Sigma-Aldrich) was used.

2.2.1.5 Sample preparation for shotgun proteomics (nano LC-MS/MS) on the Osborne fractions of whole mature barley grains

Protein identification of fraction has been performed in duplicate by shotgun proteomics using nano UPLC-MS/MS identification according to CHRISTENSEN et al. (2014). A volume of 50 µl of soluble proteins for each protein extract were denatured and reduced mixing it with 150 µl of 8 M urea, 5 µl of 1 M DTT, 2 µl of 0.5 M EDTA and 25 µl of 100 mM ammonium bicarbonate (ABC) buffer pH 8.0 and incubating the mixture at 37 ^{o}C for 30 min. After this period, the reduced cysteines alkylation was performed by adding 50 µl of 0.5 M iodoacetamide (IAA) and incubation of the samples at room temperature in the dark for 45 min. The alkylated proteins were desalted using a Zip Tip® pipette tips C18 (Millipore) and subsequently dried down. The pellet were reconstitute with 100 µl of 100 mM ABC buffer pH 8.0 containing 0.1% *Rapi*Gest™ SF Surfactant (Waters). The protein digestion was performed by adding 4 µl of proteomic grade trypsin endoproteinase (250 ng.µl^{-1}) (TPCK treated, MS grade, Thermo Scientific) for E_1 and E_2, and chymotrypsin endoproteinase (250 ng.µl^{-1}) (TLCK treated, MS grade, Thermo Scientific) for E_3, E_4 and E_5. The samples were incubated at 37 ^{o}C overnight and the protein digestion stopped by adding 5 µl of 5% formic acid (FA). The peptides were desalted using a Zip Tip® pipette tips C18 (Millipore). The peptides were reconstituted in 0.1% of FA, 3% acetonitrile (ACN) and subsequent analyzed by nano UPLC-MS/MS. Per each sample, 2 µg of desalted peptides were analyzed in triplicate in a Xbridge BEH130 C18 5 µm desalting/trap column coupled with a BEH300 C18 1.7 µm nanoUPLC analytical capillary column (100µm x 250 mm) on an Acquity nano UPLC-LC system interfaced with a nano-Source to a Q-TOF Premiere MS (Waters, Milford, MA, USA). The entire length of the LC run was 120 min starting with a gradient from 0 to 40% of ACN in 0.1 mM FA from 0 to 100 min followed by 95% ACN wash.

Data acquisition was performed in V positive mode. MS and MS/MS data was recorded in DDA mode (MS scan every 1 sec, MS/MS every 1 sec with selection of 6 ions and real time mass exclusion). DDA runs were analyzed by PEAKS Studio v. 6.0 (Bioinformatics Solutions Inc., Canada).

2.2.1.6 SDS-PAGE of protein fractions on whole mature barley grains

Electrophoretic analysis of each protein fraction were carried out under denaturing condition in a NuPAGE® 4-12% Bis-Tris gel with a NuPAGE® MOPS SDS running buffer

(Novex® by Life technologies™) according to the manufacturer's instructions. The the HiMark™ Pre-stained protein standard (Novex® by Life technologies™) was used as protein standard. The gels were run at 150 volts for approximately 1.5 h and stained with Coomassie blue solution (SCHÄGGER; VON JAGOW, 1987).

2.2.1.7 In-gel digestion of proteins separated by SDS-PAGE and protein identification

Bands of interest were cut out from the gels and submitted to in gel trypsin or chymotrypsin digestion. The excised bands were further cut into small pieces (1x1 mm) and completely destained in 50% ACN containing 25 mM ABC buffer pH 8.0. The gels pieces were submitted to a100% ACN and dried down in a vacuum concentrator for 30 min at room temperature. After that, the gel pieces were soaked in 100 µl of 100 mM ABC buffer pH 8.0 containing 25 mM dithiothreitol (DTT) at 56 ^{o}C for 60 min for proteins reduction. After cool the samples down, the proteins alkylation was performed soaking the gel pieces in 100 µl of 100 mM ABC buffer pH 8.0 containing 55 mM IAA at room temperature in the dark for 45 min. The gel pieces were washed with 100 µl of 100 mM ABC buffer pH 8.0 for 10 min and dehydrated in 250 µl of ACN 100%. The liquid phase was removed and the gel pieces dried down in a vacuum concentrator. Gel pieces were covered with 100 µl of 100 mM ABC buffer pH 8.0 containing 0.1% *Rapi*Gest™ SF Surfactant (Waters) and 4 µl of trypsin endoproteinase (TPCK treated, MS grade, Thermo Scientific) or chymotrypsin endoproteinase (TLCK treated, MS grade, Thermo Scientific) (250 ng.µl^{-1}) and rehydrated at 4 ^{o}C for 45 min. Subsequently the samples were incubated at 37 ^{o}C overnight and centrifuged at 4 000*g* for 1 min at room temperature. The supernatant containing the peptides was transferred to a new tube and the gel pieces submitted to further peptides extraction by adding 50 µl of 20 mM ABC buffer pH 8.0. After centrifugation at 4 000*g* for 1 min the supernatant was combined with the first one. The gel pieces were subjected to another peptide extractions using 50 µl of 5% FA in 50% ACN for 20 min and repeated three times and the peptides were then pooled. The samples were desalted and purified using a Zip Tip® pipette tips C18 (Millipore) and subsequently dried down. The peptides were reconstituted in 25 µl of 5% FA in 50% ACN and subsequently analyzed by nano LC-MS/MS as described above.

Data acquisition was performed in V positive mode. MSe/DDA data acquisition was performed by Masslynx version 4.1 (Waters). MSe runs were analyzed by the Protein Lynx Global Server 2.5 software using Leu-Enk (leucine-enkephaline) lock mass standard with 556.2771 m/z for, 30 ppm error tolerance for MS data and 30 ppm error for MSe data. Peptide search was performed against a barley (*Hordeum vulgare*, chose as organism, Os – organism

specie) protein databases downloaded from Uniprot (www.uniprot.org, combined Swissprot=”sp” and TrEMBL=”tr” accessions). Search parameters used in PEAKS Studio v. 6.0 (Bioinformatics solutions, Canada) and Protein Lynx Global (PLGS) server v. 2.4 (Waters, Milford, USA), included semi-trypsin/chymotrypsin as standard protease. The fixed modification was the carbamidomethyl cysteine (ΔMass + 57.02), and many other variable modifications as follows: deamination NQ (+.98), oxidation M,H,W (15.99), carbamilation N-term (+43.01), dehydration DSTY,C-term (-18.01), hydroxylation D,K,N,P,R,Y (+15.99), pyrrolidinone P (-30.01), pyroglutamic P (13.98), pyro-glu from Q (-17.03), and pyro-glu from E (-18.01).

2.2.2 Results

2.2.2.1 Protein quantification

Two different protein quantification methods (Bradford and Lowry) were used to estimate the contribution of each protein fraction on total extractable proteins (Table 2.2). A total of 16.27 mg of protein per 100 mg of the whole mature barley grain flour was extracted according to the Lowry quantification method, while 21.03 mg of protein was quantified by the Bradford method (Table 2.2). In both quantification methods, the relative contribution of each protein fraction on total extracted protein followed the same decreasing order, in which the highest contribution belonged to E_5 (glutelins), followed by E_1+E_2 (salt soluble proteins), E_3 (prolamin I) and E_4 (prolamin II) (Table 2.2). The E_2 was also quantified in order to check how efficient was this step of complementary solubilization of salt soluble proteins. The Lowry quantification method resulted in lower absolute value of extracted protein in all protein fractions than the Bradford method (Table 2.2).

Table 2.2 - Mature barley grain protein fractions quantification of cv. Golden Promise by Lowry and Bradford methodologies. Data are expressed as mg.100mg^{-1} of flour ± standard deviation and as percentage. The values are mean of three replicates

Protein fraction	**Lowry**		**Bradford**	
	mg/100mg	%	mg/100mg	%
E_1	4.16 ± 0.23	25.56	4.29 ± 0.26	20.39
E_2	0.16 ± 0.02	0.97	0.07 ± 0.01	0.31
E_3	2.87 ± 0.66	17.62	3.91 ± 1.46	18.60
E_4	1.08 ± 0.03	6.63	1.55 ± 0.05	7.39
E_5	8.01 ± 0.16	49.23	11.21 ± 0.54	53.30
Total (%)	16.27		21.03	

2.2.2.2 Amino acid composition

The amino acid composition of each extract was determined following the hydrolysis of total protein of the respective extract. The glutamine and asparagine contents were accordingly included in the glutamate and aspartate fractions, respectively. A number of differences in the amino acid content among E_1, E_2, E_3, E_4 and E_5 were revealed (Table 2.3).

Table 2.3 - Amino acid composition of E_1, E_2, E_3, E_4 and E_5 of mature barley grains cv. Golden Promise. Data are expressed as $mg.g^{-1}$ of protein ± standard deviation. Values are mean of two replicates

Amino acids	Protein fractions					
	E_1	E_2	E_1+E_2	E_3	E_4	E_5
asp	8.53 ± 1.47	14.42 ± 0.21	22.947	3.75 ± 1.77	7.79 ± 0.02	28.71 ± 0.02
glu	11.75 ± 1.06	25.27 ± 1.74	37.024	13.20 ± 5.06	14.06 ± 0.15	41.90 ± 0.78
ser	3.06 ± 1.27	7.01 ± 1.23	10.070	6.23 ± 2.77	7.34 ± 0.37	25.21 ± 0.24
his	1.80 ± 0.57	4.11 ± 0.44	5.912	3.47 ± 0.81	4.26 ± 0.37	12.18 ± 2.17
gly	4.82 ± 1.03	6.94[a] ± 0.81	11.760[b]	3.63 ± 0.22	4.44 ± 0.07	21.13 ± 0.84
thr	4.01 ± 0.85		10.946[b]	4.96 ± 1.77	7.05 ± 0.10	17.27 ± 0.68
arg	7.41 ± 2.34	8.89 ± 1.41	16.298	7.30 ± 1.44	11.31 ± 0.61	26.32 ± 2.62
ala	6.69 ± 0.51	8.57 ± 0.39	15.261	5.72 ± 0.13	6.21 ± 0.33	23.10 ± 0.07
tyr	2.61 ± 0.66	6.09 ± 0.49	8.706	8.20 ± 0.65	8.67 ± 0.58	21.63 ± 2.17
met	0.63 ± 0.11	1.22 ± 0.10	1.855	1.90 ± 0.04	3.30 ± 0.09	5.10 ± 0.09
val	4.09 ± 1.11	4.09 ± 0.95	8.181	6.22 ± 0.81	4.78 ± 0.35	18.63 ± 1.01
phe	4.95 ± 1.20	6.00 ± 0.34	10.947	10.78 ± 0.51	6.32 ± 0.35	26.81 ± 0.48
ile	2.45 ± 0.86	2.46 ± 0.73	4.914	8.15 ± 1.11	2.46 ± 0.05	13.95 ± 3.28
leu	6.49 ± 1.39	9.81 ± 0.22	16.300	9.84 ± 0.69	8.57 ± 0.21	27.63 ± 1.38
lys	4.23 ± 0.58	4.79 ± 0.23	9.018	2.15 ± 0.05	4.51 ± 0.17	21.67 ± 2.14

[a] Glycine plus threonine as an unique peak in the chromatogram
[b] Sum considering glycine plus threonine of E_2

Glutamine/glutamate was the most abundant amino acid in all protein extracts, however the abundance in E_5 was around 4-fold higher than E_1, which showed the lowest content of this amino acid (Table 2.3). Asparagine/aspartate was the second most abundant amino acid in E1, E_2 and E_5, while in E_3 asparagine/aspartate abundance was less than half of the content observed in E_4, in which this amino acid was the fifth in quantity (Table 2.3).

In E_1, E_2 and E_4 extracts the lysine contents were similar, whilst around a 50% lower content was observed in E_3, whereas in E_5 a five-fold higher content was observed (Table 2.3). When the amino acid methionine is concerned, the lowest content was found in E_1, followed by E_2, E_3, E_4, and the highest content by up to eight-fold was observed in E_5 (Table 2.3). The isoleucine content was similar in E_1, E_2 and E_4, however it was over three-fold higher in E_3 and six-fold higher in E_5 (Table 2.3).

While the content of serine, tyrosine and histidine were similar in E_2, E_3 and E_4, around half of its content was observed in E_1, and again a higher content was observed in E_5 (Table 2.3). For other amino acids such as arginine, alanine, leucine, phenylalanine, threonine and valine, the contents were very similar in E_1, E_2, E_3 and E_4, but again higher in E_5 (Table 2.3).

2.2.2.3 Proteins identified by shotgun proteomics (LC-MS/MS) on protein fractions of whole mature barley grain

E_1 exhibited the highest number of protein groups and protein isoforms identified among all protein fractions. Among the 514 peptide sequences identified with 3.3% false discovery rate (FDR), 285 possible protein isoforms could be detected into 114 protein groups. The identified protein list of E_1 and their characteristic are shown in Table 2.4.

In E_2 only 28 protein groups have been identified. A total of 76 possible protein isoforms from 77 peptide sequences with 3.9% FDR were identified belonging to the 28 protein groups. Table 2.5 presents the identified proteins and their most important characteristics.

Similarly, a low number of protein groups was identified in E_3 and E_4, totalizing 16 and 11 groups, respectively. Among 51 peptide sequences with 13.7% of FDR, a total of 42 possible protein isoforms could be detected in E_3. The protein list of E_3 is shown in Table 2.6. A similar number of identified proteins, to a total of 46 proteins could be detected from 53 peptides sequences of E_4 (Table 2.7).

Finally, in E_5 31 protein groups have been found related to 81 possible isoforms combinations from 112 peptide sequences (20.5% of FDR). The protein list of E_5 is shown in Table 2.8.

The relative abundance of the MS identified proteins of each extract was also determined by their spectral counting visualized by the -10lgP parameter value, which is shown in Tables 2.4-2.8. Such parameter given by the software PEAKS Studio v. 6.0 (Bioinformatics solutions, Canada) quantify the number of peptides identified for the protein in question, hence the higher is the -10lgP parameter the higher is the abundance of the chosen protein in analysis.

Among the 285 identified proteins belonging to E_1, the most abundant proteins were β-amylase, α-amylase/trypsin inhibitors (barley dimeric α-amylase inhibitor (BDAI-1), α-amylase-trypsin inhibitor (CMd, CMb), barley α-amylase inhibitor (BMAI-1)), protein

synthesis inhibitor I and II, storage globulins (globulin-1S, embryo globulin 7S) and metabolic enzymes (Table 2.4).

As expected, similarly to E_1, β-amylase, protein synthesis inhibitor, α-amylase/trypsin inhibitors (barley dimeric α-amylase inhibitor (BDAI-1), barley α-amylase inhibitor (BMAI-1), α-amylase-trypsin inhibitor (CMc, CMb) were the most abundant identified proteins in E_2 (Table 2.5).

As expected the majority of the 42 identified proteins in E_3 belonged to the hordein family (Table 2.6). In decreasing order of protein abundance, B-hordeins (B3-, B2- and B1-, respectively), C-hordeins, β-amylase, γ-hordeins (γ2- and γ-1, respectively) and D-hordeins were identified in this extract (Table 2.6).

On the other hand, among the hordein families only D-hordein was identified in E_4, dominating this fraction in relative abundance, followed by 7S globulin, 11S globulin and β-amylase, typically salt soluble proteins (Table 2.7).

In E_5, 7S globulin, D-hordein and 11S globulin were the most abundant proteins, and unexpectedly B-hordeins (B3- and B1), were identified in high abundance (Table 2.8).

Table 2.4 - Proteins identified by shotgun proteomics on the E_1 of the modified grain protein method extraction on the barley mature grains. Proteins were digested by trypsin and searched by PEAKS v. 6.0 search engine against barley Uniprot protein database. The -10lgP parameter indicate their relative abundance since correspond to the spectral counting of the total of the peptides detected. Post translational modification (PTM) presence was indicated by Y=yes else by N=no

(continues)

Description	Accession code	Mw (Da)	-10lgP	Peptides	Unique	Coverage (%)	PTM
Beta-amylase	Q9FUK7_HORVU	59387	163.23	21	2	36	Y
Barley dimeric alpha-amylase inhibitor (BDAI-1)	Q546U1_HORVU	16429	161.94	18	18	61	Y
Beta-amylase	Q84T19_HORVD	59639	161.13	20	0	36	Y
Beta-amylase	C1IIM6_HORVU	59744	158.82	19	1	36	Y
Beta-amylase	AMYB_HORVS	59639	156.38	19	1	31	Y
Alpha-amylase-trypsin inhibitor CMd	M0Y227_HORVD	18526	143.37	10	10	77	Y
Protein synthesis inhibitor I	RIP1_HORVU	29973	137.07	13	0	46	Y
Protein synthesis inhibitor II	RIP2_HORVU	29863	136.71	14	2	50	Y
rRNA N-glycosidase	B5TWK6_HORVU	29551	135.65	12	1	48	Y
Globulin-1S (S7 globulin)	M0XUU4_HORVD	47981	132.89	14	13	23	Y
Embryo globulin (7S globulin)	Q03678_HORVU	72253	132.46	16	15	25	Y
Alpha-amylase-trypsin inhibitor CMb	M0ULY1_HORVD	15817	124.98	13	13	73	Y
Beta-glucosidase	Q40025_HORVU	57445	121.54	11	11	20	Y
Alpha-amylase inhibitor BMAI-1	IAA1_HORVU	15816	120.41	6	6	32	Y
Haem peroxidase	F2EEV5_HORVD	38693	119.13	11	10	36	Y
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	F2D6I8_HORVD	36612	118	11	4	35	Y
Alpha-amylase/trypsin inhibitor CMa	M0YS73_HORVD	15470	116.01	12	1	63	Y
Beta-hordothionin	F2EE63_HORVD	14573	115.98	10	5	53	Y
1-Cys peroxiredoxin PER1 (Thioredoxin peroxidase)	M0Y4T3_HORVD	23963	115.19	7	7	43	Y
Alpha-amylase/trypsin inhibitor CMc	M0VFV9_HORVD	15735	112.23	8	8	53	Y
Alpha-amylase/trypsin inhibitor CMa	IAAA_HORVU	15500	112.14	12	1	56	Y
Alpha-amylase/trypsin inhibitor BTI-CMe3.1	IAAE_HORVU	16136	109.21	7	7	39	Y
Non-specific lipid-transfer protein 1	NLTP1_HORVU	12301	108.31	8	8	60	Y
Late embryogenesis abundant protein (PHV A1)	LEA1_HORVU	21749	107.91	8	8	29	Y
Protein disulphide isomerase (PDI)	PDI_HORVU	56429	107.8	8	8	18	Y
Triosephosphate isomerase, cytosolic	TPIS_HORVU	26737	107.4	5	5	26	Y
Glucose-ribitol dehydrogenase	F2CSK4_HORVD	37516	107.04	9	7	29	Y
Serpin-Z7 (chymotrypsin inhibitor)	BSZ7_HORVU	42821	106.72	8	2	25	Y
Alpha-hordothionin	THNA_HORVU	13597	103.97	7	2	43	Y
Late embryogenesis abundant protein B19.1A (LEA)	LE19A_HORVU	9962	103.94	5	2	53	Y
Glyoxalase_1	F2CQP8_HORVD	32546	103.7	9	9	33	Y
Serpin	M0UEE6_HORVD	43220	102.71	10	8	28	N

Table 2.4 - Proteins identified by shotgun proteomics on the E_1 of the modified grain protein method extraction on the barley mature grains. Proteins were digested by trypsin and searched by PEAKS v. 6.0 search engine against barley Uniprot protein database. The -10lgP parameter indicate their relative abundance since correspond to the spectral counting of the total of the peptides detected. Post translational modification (PTM) presence was indicated by Y=yes else by N=no

(continued)

Description	Accession code	Mw (Da)	-10lgP	Peptides	Unique	Coverage (%)	PTM
Fructose-bisphosphate aldolase	F2CXT7_HORVD	38868	101.05	6	6	25	Y
Glyceraldehyde-3-phosphate dehydrogenase 1, cytosolic	G3PC1_HORVU	36514	100.94	8	0	23	Y
D-hordein	Q84LE9_HORVU	80410	94.12	7	7	11	Y
Late embryogenesis abundant (LEA) protein	F2EKY2_HORVD	70560	90.13	4	1	9	N
Hordoindoline b-2	Q5IU17_HORVD	16066	88.98	5	0	39	Y
Late embryogenesis abundant protein B19.3 (LEA5)	F2EG29_HORVD	14605	88.64	4	1	27	N
Serpin	M0Z9Q3_HORVD	28814	88.3	6	1	22	Y
Hordoindoline b	Q56GB1_HORVD	16127	86.61	4	1	39	Y
Enolase	F2CR08_HORVD	48059	85.81	6	6	17	Y
Phosphoglycerate kinase	F2CZV0_HORVD	40189	84.49	5	5	17	N
Late embryogenesis abundant protein D-34-like	M0ZDL8_HORVD	27503	82.11	4	1	24	N
Heat shock protein 70 (HSP70)	F2E4C2_HORVD	71905	80.11	7	2	15	N
Hordoindoline b-2	G1UH44_HORVS	16034	79.14	4	1	39	Y
Subtilisin-chymotrypsin inhibitor CI-1C	ICIC_HORVU	8258	78.29	3	1	36	N
Grain softness protein	Q5ITF2_HORVD	18177	78.27	3	3	25	Y
Grain softness protein	Q5ITH3_HORVD	18177	78.27	3	3	25	Y
Grain softness protein	D7F5D2_HORVS	18338	78.27	3	3	25	Y
Grain softness protein	Q5ITG0_HORVD	18398	78.27	3	3	25	Y
Grain softness protein	Q0GIL0_HORVU	18368	78.27	3	3	25	Y
Aldo-keto NADPH-dependent reductase	F2DXQ5_HORVD	35807	78.06	4	4	13	Y
Malate dehydrogenase	M0Z0D3_HORVD	33175	76.9	3	3	12	Y
Hordoindoline-b2	Q5IUG4_HORVD	13631	76.87	5	0	46	Y
Hordoindoline-b2	Q5IU86_HORVD	16052	76.87	5	0	39	Y
Beta-amylase	M0WHZ5_HORVD	49562	76.53	5	2	8	Y
Beta-amylase	Q4VM11_HORVD	57114	76.53	5	2	7	Y
Subtilisin-chymotrypsin inhibitor CI-1A	ICIA_HORVU	8882	73.86	3	1	34	N
Kunitz-type Alpha-amylase/subtilisin inhibitor, Isa	IAAS_HORVU	20742	73.7	4	4	26	Y
Subtilisin-chymotrypsin inhibitor 2B (bci-7)	Q9M4C9_HORVU	7918	72.19	2	2	46	Y
Subtilisin-chymotrypsin inhibitor 2A	A8V3Q5_HORVS	9381	70.3	4	2	54	Y
Chymotrypsin inhibitor-2	ICI2_HORVU	9413	70.3	4	2	54	Y
Haem_peroxidase	F2E8A9_HORVD	40952	69.58	3	2	14	Y
Late embryogenesis abundant protein D-34-Like	F2EL01_HORVD	22520	68.81	4	3	22	Y

Table 2.4 - Proteins identified by shotgun proteomics on the E_1 of the modified grain protein method extraction on the barley mature grains. Proteins were digested by trypsin and searched by PEAKS v. 6.0 search engine against barley Uniprot protein database. The -10lgP parameter indicate their relative abundance since correspond to the spectral counting of the total of the peptides detected. Post translational modification (PTM) presence was indicated by Y=yes else by N=no

(continued)

Description	Accession code	Mw (Da)	-10lgP	Peptides	Unique	Coverage (%)	PTM
Bowman-Birk type trypsin inhibitor	M0Y075_HORVD	19190	68.56	2	2	9	Y
Aldose 1-epimerase	M0WGK7_HORVD	39038	66.74	2	2	7	N
60S ribosomal protein L12	M0YPS4_HORVD	17648	66.07	3	3	18	Y
Cold-regulated protein (Raf kinase inhibitor-like protein, YbhB/YbcL family protein)	Q9FSI8_HORVU	17613	64.1	2	2	18	N
Malate dehydrogenase	F2CQR0_HORVD	35867	63.88	3	3	13	N
Hordoindoline-b2	Q5IUF4_HORVD	16023	62.15	4	1	39	Y
Heat shock protein 70 (HSP70)	M0X093_HORVD	73146	61.93	2	1	5	N
Glyoxalase	F2EJ79_HORVD	15293	60.64	2	2	17	N
Chymotrypsin inhibitor-2	A8V3Q4_HORVS	9327	59.54	3	1	48	Y
Elongation factor 1-alpha	F2DCL0_HORVD	50208	57.44	4	4	7	Y
Dehydroascorbate reductase	F2D6B1_HORVD	23337	56.22	3	3	19	N
Trypsin/alpha amylase inhibitor	F2EEH7_HORVD	11760	54.42	2	2	21	Y
Translationally-controlled tumor protein homolog	TCTP_HORVU	18884	50.05	2	2	14	N
Thaumatin (xylanase inhibitor)	F2DNP3_HORVD	24307	48.97	2	2	10	Y
7S Globulin storage protein (cupin, vicilin-like)	F2CYL7_HORVD	55883	48.94	1	1	3	N
Barwin-like_endoglucanase	BARW_HORVU	13737	48.89	1	1	10	N
Pathogenesis-related protein 4	P93180_HORVU	15694	48.89	1	1	9	N
40S ribosomal protein S5	M0ZD23_HORVD	12172	48.7	1	1	14	Y
Peptidyl-prolyl cis-trans isomerase	M0WCQ8_HORVD	17366	47.66	2	2	13	N
ABC transporter substrate-binding protein	M0VJA1_HORVD	10380	46.64	1	1	14	N
Globulin 2, cupin, vicilin-like (7S globulin)	F2EBM4_HORVD	76924	45.9	2	2	4	N
Thaumatin (xylanase inhibitor, basic pathogenesis-related protein PR5)	Q946Z0_HORVU	23726	43	2	2	13	Y
Glucose-1-phosphate adenylyltransferase	F2D9F4_HORVD	57261	42.29	1	1	3	N
40S ribosomal protein S20	M0WQH6_HORVD	7183	42.15	1	1	19	N
Epoxide hydrolase-like protein	F2EIH2_HORVD	38700	41.92	1	1	3	N
Embryo-specific protein(DUF1264)	F2DJC5_HORVD	26155	41.38	2	2	11	N
UTP--glucose-1-phosphate uridylyltransferase	UGPA_HORVU	51644	41.28	2	2	5	N
Late embryogenesis abundant protein D-34-like	F2EC88_HORVD	30458	38.34	1	1	4	N
Late embryogenesis abundant protein (LEA 2)	M0WI75_HORVD	16264	32.04	1	1	5	N
Thioredoxin H-type	Q7XZK3_HORVD	12754	31.85	1	1	9	N
Scarecrow	M0VAL0_HORVD	72034	30.03	2	0	2	Y

Table 2.4 - Proteins identified by shotgun proteomics on the E_1 of the modified grain protein method extraction on the barley mature grains. Proteins were digested by trypsin and searched by PEAKS v. 6.0 search engine against barley Uniprot protein database. The -10lgP parameter indicate their relative abundance since correspond to the spectral counting of the total of the peptides detected. Post translational modification (PTM) presence was indicated by Y=yes else by N=no

(conclusion)

Description	Accession code	Mw (Da)	-10lgP	Peptides	Unique	Coverage (%)	PTM
40S ribosomal protein S28-like	F2CWF9_HORVD	7464	29.9	1	1	18	N
Acetyl-carboxylase	F2EIZ4_HORVD	247960	29.52	2	0	0	Y
RNA polymerase-associated RTF1-like protein	M0YGN1_HORVD	91995	29.37	2	0	1	Y
Eukaryotic initiation factor 4A-1	M0X5Z0_HORVD	41850	29.16	1	1	3	N
Eukaryotic initiation factor 4A-1	M0YHR5_HORVD	54268	29.16	1	1	2	N
Late embryogenesis abundant protein	F2ECH4_HORVD	38674	29.09	1	1	5	N
Proline-rich protein PRCC	M0Z8N5_HORVD	36973	28.34	2	0	3	Y
40S ribosomal protein S13	F2CVF1_HORVD	17126	27.84	1	1	8	N
Transcription initiation factor TFIID subunit 15b	F2D9E7_HORVD	46074	26.78	2	1	3	Y
Histone H2A	F2EBJ8_HORVD	16392	26.57	1	1	6	N
Histone H2A	M0X5A2_HORVD	14322	26.57	1	1	7	N
Histone H2A	F2DKC2_HORVD	14401	26.57	1	1	6	N
Histone H2A	F2DR58_HORVD	15653	26.57	1	1	6	N
Histone H2A	F2EF71_HORVD	16434	26.57	1	1	6	N
60S ribosomal protein L6	F2DAK3_HORVD	24372	25.99	1	1	4	N
Late embryogenesis abundant protein (LEA3)	M0YHZ3_HORVD	20130	25.64	1	1	9	N
Pentatricopeptide repeat-containing protein (PPR motif), chloroplastic	F2D374_HORVD	93306	25.42	2	1	1	Y
Cell division cycle protein 48-like protein	F2E3Y2_HORVD	65469	24.91	2	2	3	Y
B3-hordein	HOR3_HORVU	30195	24.25	1	1	3	N
B3 hordein	I6TEV5_HORVU	35463	24.25	1	1	3	N
Glutaredoxin	M0X7U5_HORVD	11640	24.15	1	1	12	N
CAP-Gly domain-containing linker protein	M0VFT1_HORVD	73637	23.46	1	1	1	Y
Haem_peroxidase	F2E8K5_HORVD	32178	21.78	1	1	3	N
Pyruvate kinase	F2CX32_HORVD	57436	21.32	1	1	5	N
Malic enzyme	M0XZU6_HORVD	34958	21.12	1	1	5	N
Acetyl-CoA carboxylase	M0WX43_HORVD	228745	21.03	1	0	0	Y
Late Embryogenesis Abundant protein (LEA3)	F2DW12_HORVD	19962	20.99	1	1	11	Y
ABC transporter G family member 28-like	M0X5C9_HORVD	118127	20.63	1	1	1	Y

Table 2.5 - Proteins identified by shotgun proteomics on the E_2 of the modified grain protein method extraction on the barley mature grains. Proteins were digested by trypsin and searched by PEAKS v. 6.0 search engine against barley Uniprot protein database. The -10lgP parameter indicate their relative abundance since correspond to the spectral counting of the total of the peptides detected. Post translational modification (PTM) presence was indicated by Y=yes else by N=no

Description	**Accession code**	**Mw (Da)**	**-10lgP**	**Peptides**	**Unique**	**Coverage (%)**	**PTM**
Beta-amylase	AMYB_HORVU	59387	117.33	10	0	22	Y
Beta-amylase	AMYB_HORVS	59639	116.49	10	1	22	Y
Protein synthesis inhibitor (rRNA N-glycosidase)	RIP1_HORVU	29863	80.15	4	4	19	N
Dimeric alpha-amylase inhibitor (BDAI-1)	IAA2_HORVU	16429	79.39	5	5	43	Y
Alpha-amylase inhibitor BMAI-1	IAA1_HORVU	15816	76.51	4	4	24	Y
Late embryogenesis abundant protein B19.1A	LE19A_HORVU	9962	67.14	3	3	37	N
Peroxidase BP1	F2EEV5_HORVD	38693	57.56	4	4	11	N
Alpha-amylase/trypsin inhibitor CMc	M0VFV9_HORVD	15735	53.13	3	3	23	Y
Beta-glucosidase	Q40025_HORVU	57445	51.58	2	2	5	N
Alpha-amylase-trypsin inhibitor CMb	IAAB_HORVU	15817	48.94	3	3	22	Y
Protein disulphide isomerase (PDI)	F2D284_HORVD	56429	48.61	4	4	10	N
Protein disulfide isomerase	PDI_HORVU	56463	48.61	4	4	10	N
Non-specific lipid-transfer protein 1	NLTP1_HORVU	12301	46.65	1	1	15	Y
Serpin-Z7	BSZ7_HORVU	42821	46.15	2	1	5	N
Alpha-amylase/trypsin inhibitor CMa	IAAA_HORVU	15500	44.46	3	3	27	Y
Subtilisin-chymotrypsin inhibitor-2A	M0VN21_HORVD	7966	40.17	1	1	18	N
Alpha-amylase/trypsin inhibitor CMe	IAAE_HORVU	16136	39.57	1	1	9	Y
alpha amylase/trypsin inhibitor BTI-CMe2.2 protein	O49867_HORVU	16171	39.57	1	1	9	Y
Serpin	M0UEE6_HORVD	43220	38.37	2	1	6	N
Kunitz-type alpha-amylase/subtilisin inhibitor, Isa	IAAS_HORVU	20742	35.02	1	1	6	N
Triosephosphate isomerase, cytosolic	TPIS_HORVU	26737	34.36	1	1	5	N
B3-hordein	HOR3_HORVU	30195	28.86	1	1	5	Y
B3 hordein	I6TRT5_HORVU	31819	28.86	1	1	5	Y
B hordein	C7FB17_HORVD	30146	28.86	1	1	5	Y
Subtilisin-chymotrypsin inhibitor CI-1A	ICIA_HORVU	8882	28.13	1	1	11	N
Glucose-ribitol dehydrogenase	F2CSK4_HORVD	37516	25.23	1	1	4	N
Glyoxalase	F2CQP8_HORVD	32546	24.89	1	1	4	N
Cysteine-rich receptor-like protein kinase 25 (stress/antifungal response)	M0XDY0_HORVD	51080	23.33	1	1	3	Y
Alpha-hordothionin	THNA_HORVU	13597	22.88	1	1	11	N
Haem_peroxidase	F2E8K5_HORVD	32178	21.73	1	1	3	N

Table 2.6 - Proteins identified by shotgun proteomics on the E_3 of the modified grain protein method extraction on the barley mature grains. Proteins were digested by chymotrypsin and searched by PEAKS v. 6.0 search engine against barley Uniprot protein database. The -10lgP parameter indicate their relative abundance since correspond to the spectral counting of the total of the peptides detected. Post translational modification (PTM) presence was indicated by Y=yes else by N=no

Description	**Accession code**	**Mw (Da)**	**-10lgP**	**Peptides**	**Unique**	**Coverage (%)**	**PTM**
B3-hordein	I6TEV5_HORVU	35463	70.17	10	3	31	Y
C-hordein	Q41210_HORVU	36508	61.52	8	3	39	Y
B3-hordein	I6SJ26_HORVU	35426	57.5	6	1	15	Y
B2-hordein	C7FB14_HORVD	30291	57.5	6	1	18	Y
B2-hordein	C7FB16_HORVD	30214	57.5	6	1	18	Y
B1-hordein	HOR1_HORVU	33422	57.31	7	0	23	Y
B1-hordein	Q0PIV6_HORVD	33030	54.52	7	0	22	Y
C-hordein (Hor1-17)	Q40053_HORVU	30397	54	6	1	23	Y
B2-hordein	I6R4A7_HORVD	30904	51.2	5	0	19	Y
Beta-amylase	F2EFV1_HORVD	42880	50.67	4	4	7	Y
Beta-amylase	AMYB_HORVS	59312	50.67	4	4	5	Y
Beta-amylase	AMYB_HORVU	59647	50.67	4	4	5	Y
Beta-amylase	Q84T20_HORVD	59572	50.67	4	4	5	Y
B1-hordein	I6TRT2_HORVU	32153	49.1	5	0	17	Y
B1-hordein	I6SJ13_HORVD	33799	49.1	5	0	16	Y
B2-hordein	I6TMW0_HORVU	29127	48.92	5	0	19	Y
B3-hordein	Q3YAF9_HORVD	34465	46.81	4	0	10	Y
Gamma 2-hordein	Q70IB4_HORVU	29033	44.9	2	2	14	Y
Gamma 1-hordein	I6TMV6_HORVU	34663	44.9	2	2	12	Y
Gamma 1-hordein	HOG1_HORVU	34737	44.9	2	2	12	Y
D-Hordein	Q40054_HORVU	75108	37.81	2	2	2	N
D-Hordein	I6TRS8_HORVU	77221	37.81	2	2	2	N
D-Hordein	I6SW23_HORVU	79349	37.81	2	2	2	N
D-Hordein	I6SW34_HORVD	79351	37.81	2	2	2	N
D-Hordein	F2EA67_HORVD	79321	37.81	2	2	2	N
D-Hordein	Q84LE9_HORVU	80410	37.81	2	2	2	N
Guanylate-binding protein 4	M0YH39_HORVD	55860	21.18	1	1	2	Y
Haem_peroxidase	F2DA71_HORVD	34589	20.49	1	1	2	Y

Table 2.7 - Proteins identified by shotgun proteomics on the E_4 of the modified grain protein method extraction on the barley mature grains. Proteins were digested by chymotrypsin and searched by PEAKS v. 6.0 search engine against barley Uniprot protein database. The -10lgP parameter indicate their relative abundance since correspond to the spectral counting of the total of the peptides detected. Post translational modification (PTM) presence was indicated by Y=yes else by N=no

Description	**Accession code**	**Mw (Da)**	**-10lgP**	**Peptides**	**Unique**	**Coverage (%)**	**PTM**
D-hordein	Q40054_HORVU	75108	99.04	12	12	25	Y
D-Hordein	F2EA67_HORVD	79321	99.04	12	12	24	Y
Globulin 1-S (7S globulin)	M0XH58_HORVD	46366	97.33	7	7	16	Y
Embryo globulin (7S globulin)	Q03678_HORVU	72253	97.33	7	7	11	Y
11S Seed storage globulin (cupin)	F2E9N0_HORVD	63902	60.33	5	5	10	Y
11S Seed storage globulin (cupin)	M0Z4S0_HORVD	63547	60.33	5	5	10	Y
Beta-amylase	Q6SNP7_HORVU	57635	53.45	4	4	6	Y
Beta-amylase	AMYB_HORVU	59387	53.45	4	4	6	Y
Beta-amylase	AMYB_HORVS	59639	53.45	4	4	6	Y
Aldose 1-epimerase	M0WGK6_HORVD	19653	44.72	2	2	13	N
Globulin 2, cupin, vicilin-like (7S globulin)	M0V1Y3_HORVD	58915	36.1	1	1	4	N
Globulin-1S (S7 globulin)	M0XUU4_HORVD	47981	35.07	3	3	6	N
Kunitz-type alpha-amylase/subtilisin inhibitor	IAAS_HORVU	22164	31.55	2	2	6	N
Late embryogenesis abundant protein	M0Z6A4_HORVD	17718	25.53	1	1	8	N
Late embryogenesis abundant protein (LEA3)	M0YHZ3_HORVD	20130	25.53	1	1	7	N
Late embryogenesis abundant protein (PHV A1)	LEA1_HORVU	21820	25.53	1	1	6	N
poly(rC)RNA-binding protein	M0XYZ0_HORVD	55407	21.01	1	1	1	Y
Beta-hordothionin	THNB_HORVU	14603	20.06	1	1	8	Y

Table 2.8 - Proteins identified by shotgun proteomics on the E_5 of the modified grain protein method extraction on the barley mature grains. Proteins were digested by chymotrypsin and searched by PEAKS v. 6.0 search engine against barley Uniprot protein database. The -10lgP parameter indicate their relative abundance since correspond to the spectral counting of the total of the peptides detected. Post translational modification (PTM) presence was indicated by Y=yes else by N=no

(continues)

Description	Accession code	Mw (Da)	-10lgP	#Peptides	#Unique	Coverage (%)	PTM
Embryo globulin (7S globulin)	Q03678_HORVU	72253	112.4	12	12	19	Y
D-hordein	Q40054_HORVU	75108	110.32	7	7	15	Y
D-hordein	I6SW23_HORVU	79349	110.32	7	7	14	Y
11S seed storage globulin (cupin)	M0Z4S0_HORVD	63547	84.2	7	7	12	Y
11S seed storage globulin (cupin)	F2E9N0_HORVD	63902	84.2	7	7	12	Y
B3-hordein	I6TEV5_HORVU	35463	84.05	11	1	26	Y
Serpin-Z7	BSZ7_HORVU	42821	77.38	4	2	11	Y
B1-hordein	HOR1_HORVU	33422	72.55	8	2	26	Y
B1-hordein	Q0PIV6_HORVD	33030	71.18	6	0	15	Y
B3-hordein	HOR3_HORVU	30195	70.98	7	1	19	Y
B3-hordein	M0WA02_HORVD	15500	64.14	4	0	22	Y
Fructose-bisphosphate aldolase	C1J960_HORVU	38689	62.91	2	2	10	N
Alpha-amylase/trypsin inhibitor CMe	IAAE_HORVU	16136	61.22	3	3	24	Y
Alpha-amylase/trypsin inhibitor BTI-CMe3.1	M0UY52_HORVD	16290	61.22	3	3	23	Y
Gamma 2-hordein	Q70IB4_HORVU	29033	57.06	2	2	7	N
Gamma 1-hordein	I6TMV6_HORVU	34663	57.06	2	2	6	N
Haem peroxidase	F2EEV5_HORVD	38693	53.07	2	1	6	Y
Enolase	F2CR08_HORVD	48059	51.66	1	1	3	N
Beta-amylase	Q84T19_HORVD	59639	49.89	4	3	7	Y
Beta-amylase	AMYB_HORVU	59647	49.89	4	3	7	Y
Glyceraldehyde-3-phosphate dehydrogenase 1, cytosolic	M0YRV8_HORVD	36605	47.92	2	2	4	N
Serpin	M0Z714_HORVD	28803	44.65	3	1	11	N
Sucrose synthase 2	SUS2_HORVU	92575	38.03	4	4	5	Y
Aminotransferase (Transaminase)	M0USC9_HORVD	49988	37.86	1	1	4	N
Hordoindoline-a	D5KQP2_HORVS	16501	36.93	2	1	8	N
Elongation factor 1-alpha	Q6LAA4_HORVU	49169	34.69	3	3	7	N
Elongation factor 1-alpha	M0Y9X9_HORVD	32095	34.69	3	3	10	N
Elongation factor 1-alpha	EF1A1_HORVU	49142	34.69	3	3	7	N
Serpin-Z4	SPZ4_HORVU	43276	33.56	3	3	12	Y
Oleosin	Q43769_HORVU	18494	33.47	2	2	12	N
Protein disulphide isomerase (PDI)	F2D284_HORVD	56429	31.01	2	2	4	N
Alpha-amylase inhibitor BDAI-1	IAA2_HORVU	16429	30.92	2	2	30	Y
Malate dehydrogenase	F2D4W6_HORVD	35601	26.58	2	2	12	Y
Aldo-keto reductase family	F2DTH9_HORVD	37202	24.43	1	1	4	N

Table 2.8 - Proteins identified by shotgun proteomics on the E_5 of the modified grain protein method extraction on the barley mature grains. Proteins were digested by chymotrypsin and searched by PEAKS v. 6.0 search engine against barley Uniprot protein database. The -10lgP parameter indicate their relative abundance since correspond to the spectral counting of the total of the peptides detected. Post translational modification (PTM) presence was indicated by Y=yes else by N=no

(conclusion)

Description	**Accession code**	**Mw (Da)**	**-10lgP**	**#Peptides**	**#Unique**	**Coverage (%)**	**PTM**
Cold-regulated protein	Q9FSI8_HORVU	17613	22.92	1	1	9	N
Phosphoglycerate kinase	M0Y9H9_HORVD	42161	21.93	1	1	3	N
Barwin-like_endoglucanase	M0YQS0_HORVD	27822	20.85	1	1	6	N
Rho guanyl-nucleotide exchange factor	F2EIU8_HORVD	61198	20.47	1	1	3	Y

2.2.2.4 SDS-PAGE and protein identification of excised bands from the gel

The whole mature barley grain protein profile of each protein extract (E_1, E_2, E_3, E_4 and E_5) was assessed for a set of antisense C-hordein barley lines (L1-5) and its parental line cv. Golden Promise (Figure 2.1).

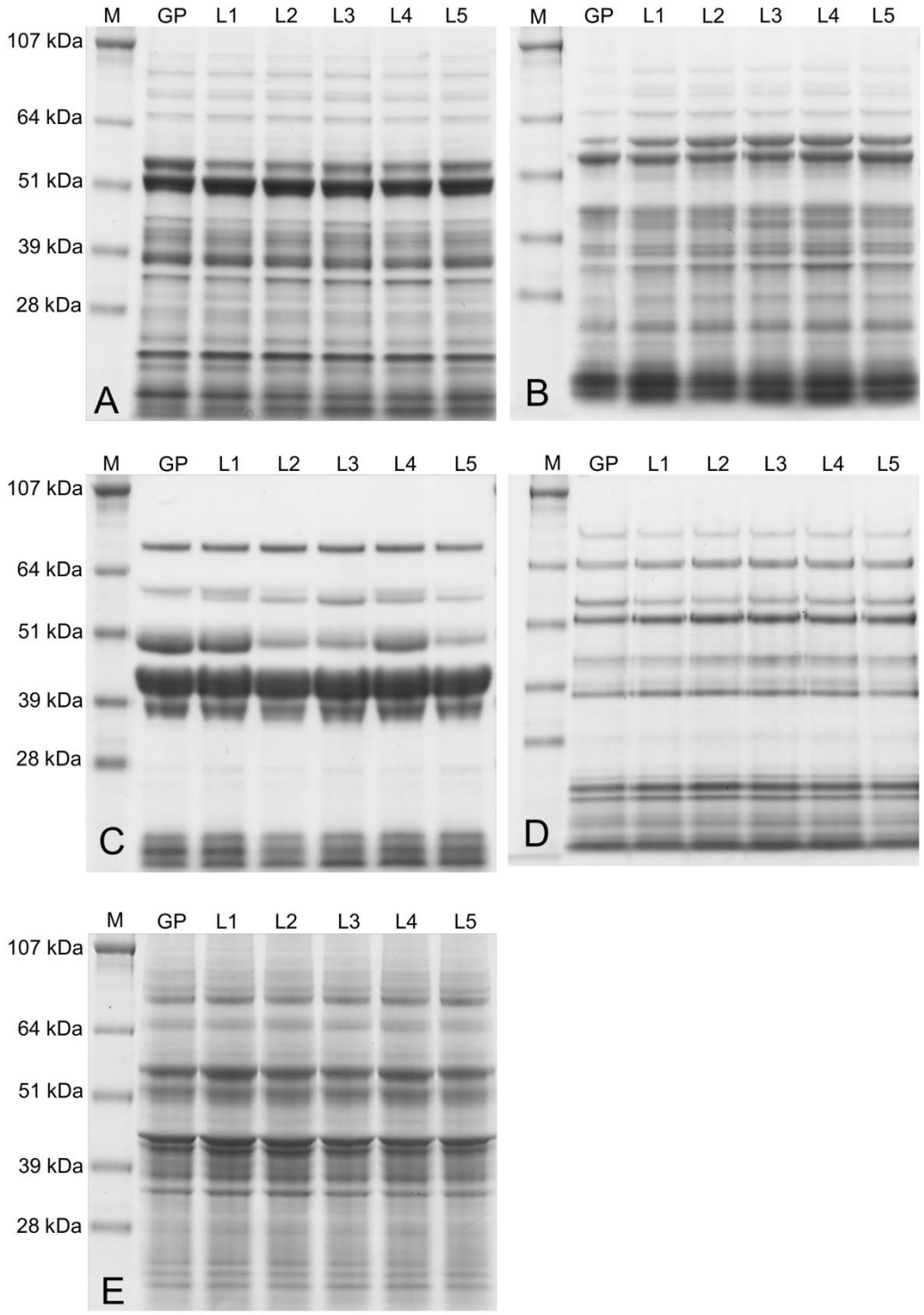

Figure 2.1 - SDS-PAGE of the extracted Osborne fractions (A) E1, (B) E2, (C) E3, (D) E4 and (E) E5 from the whole mature barley grains of *Hordeum vulgare* cv. Golden Promise (GP) and antisense C-hordein lines (L1-5). The molecular weights of the marker (M) were given in kilodalton (kDa)

In order to associate the one dimensional SDS-PAGE gel band to which proteins such band contained per each extract, the gel excision technique coupled to trypsin/chymotrypsin MS identification have been applied. The most abundant proteins in a SDS-PAGE band was determined by protein coverage and peptide mapping shown also by the number of peptides

counted as given by the software Protein Lynx Global (PLGS) server v. 2.4 (Waters, Milford, USA).

E_1, E_2 and E_3 were selected for band protein identification because great differences in the SDS-PAGE profiles were observed comparing the set of antisense C-hordein barley lines and its parental line cv. Golden Promise (Figure 2.2, 2.3 and 2.4, respectively). Thus, the efforts of this step were dispended towards getting more detailed information that could be useful for future studies with these transgenic lines.

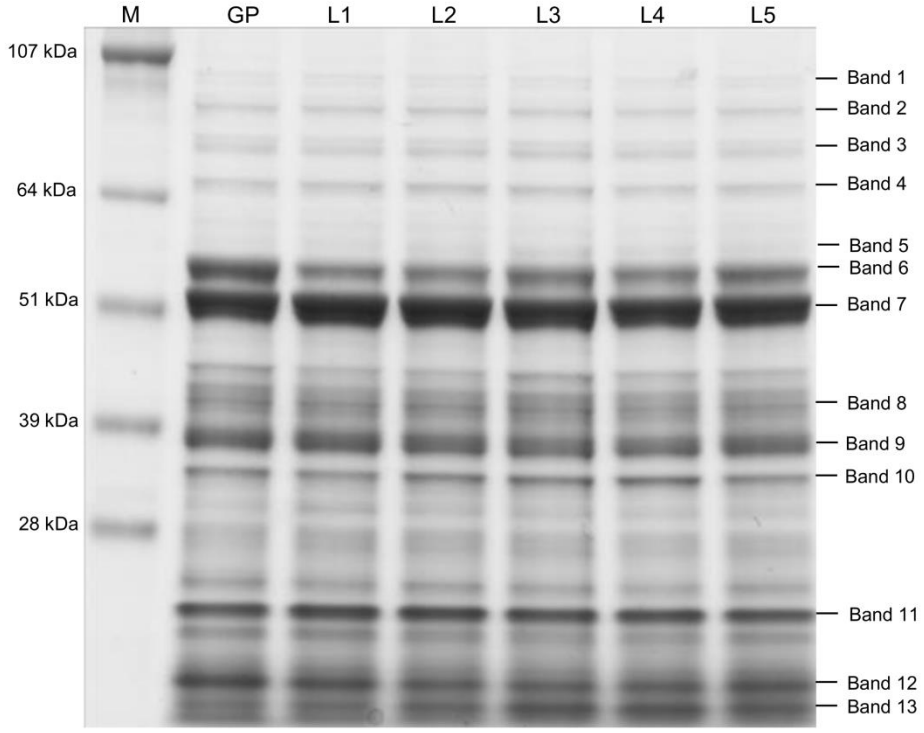

Figure 2.2 - SDS-PAGE of E_1 from the whole mature barley grains of *Hordeum vulgare* cv. Golden Promise (GP) and antisense C-hordein lines (L1-5) indicating the excised bands for protein identification. The molecular weights of the marker (M) were given in kilodalton (kDa)

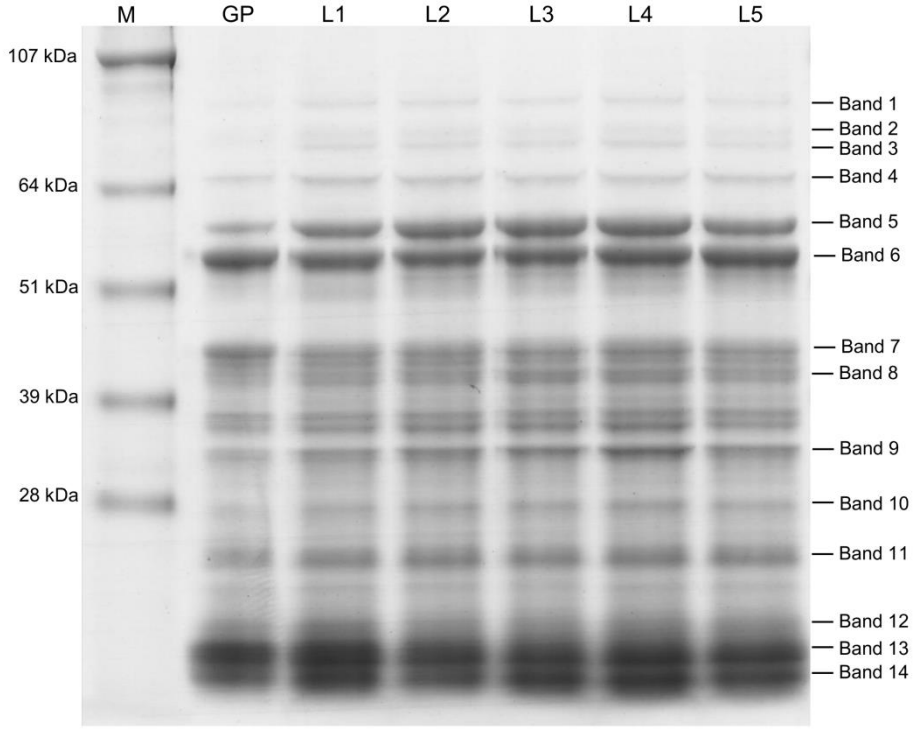

Figure 2.3 - SDS-PAGE of E_2 from the whole mature barley grains of *Hordeum vulgare* cv. Golden Promise (GP) and antisense C-hordein lines (L1-5) indicating the excised bands for protein identification. The molecular weights of the marker (M) were given in kilodalton (kDa)

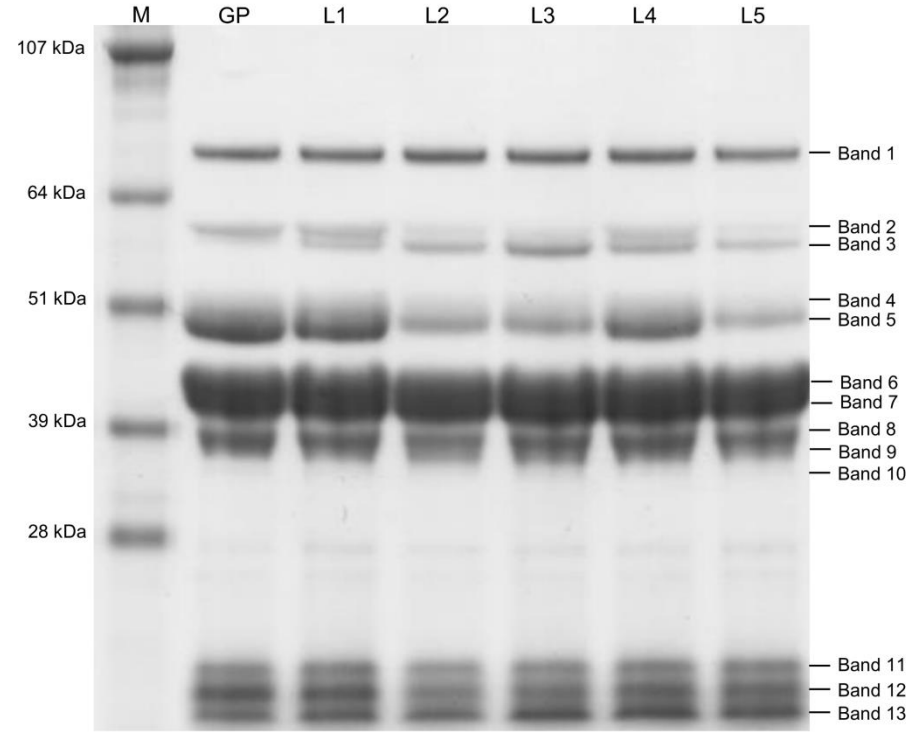

Figure 2.4 - SDS-PAGE of E_3 from the whole mature barley grains of *Hordeum vulgare* cv. Golden Promise (GP) and antisense C-hordein lines (L1-5) indicating the excised bands for protein identification. The molecular weights of the marker (M) were given in kilodalton (kDa)

A total of 12 SDS-PAGE bands of E_1 were excised from the gel for protein identification according to Figure 2.2. The detailed overview of top protein hits for each band excised of E_1 is presented in Table 2.9. A large number of protein hits were found to each band, with the majority being related to metabolic and protective functions (Table 2.9). The most abundant proteins identified were timeless circadian protein (band number 1); lipoxygenase (band number 2); heat shock protein 90, methionine synthase and cytosolic heat shock protein 90 (band number 3); heat Shock Protein 70 HSP70 (band number 4); malic enzyme and protein disulphide isomerase (band number 5); beta glucosidase and beta amylase (band number 6); globulin 1S and beta-amylase (band number 7); serpin Z4, globulin 1S, serpin Z7, phosphoglycerate kinase, fructose bisphosphate aldolase and glyceraldehyde 3 phosphate dehydrogenase (band number 8); glyoxalase 1, globulin 1S, haem peroxidase and aldo keto NADPH dependent reductase (band number 9); protein synthesis inhibitor I and II (band number 10); 60S ribosomal protein, 17 kDa class I small heat shock protein and phosphatidylethanolamine binding protein (band number 11); histone H4, globulin 1S and α-amylase inhibitor (band umber 12) (Table 2.9).

A total of 14 bands were cut out from the SDS-PAGE of E_2 according to Figure 2.3. The detailed information of the identified proteins per band is presented in Table 2.10. Similarly to E_1, a large number of protein hits was found for each band, with exception of band number 2, in which only the enzyme used for protein digestion was identified. As expected the majority protein identified are related to metabolic and protective functions. The most abundant proteins identified in each excised band were lipoxygenase (band number 1); heat shock HSP70 (band number 4); malic enzyme, protein disulphide isomerase and beta-amylase (band number 5); beta-amylase and beta glucosidase (band number 6); serpin Z4 (band number 7); rRNA N glycosidase, protein synthesis inhibitor I and II (band number 8); serpin Z7 (band number 9) and protein synthesis inhibitor II (band number 10) (Table 2.10).

A total of 13 bands were excised from E_3 gel as shown Figure 2.4. The detailed overview of top protein hits for each band is presented in Table 2.11. As expected the majority of identified proteins belonged to the hordein superfamily. D-hordein, C-hordein and C-hordein were uniquely identified with high abundance in band number 1, 2 and 3, respectively (Table 2.11). C-hordein and Hor1 17 C-hordein were identified in both bands number 4 and 5, but C-hordein was more abundant in band number 4, and Hor1 17 C-hordein in band number 5 (Table 2.11). Proteins belonging to B-hordein family were identified as the most abundant proteins in bands number 6, 7 and 8, whilst γ-hordeins were identified in bands

number 9 and 10 (Table 2.11). The most abundant protein identified in the low molecular weight bands (bands number 11, 12 and 13) was α-amylase/trypsin inhibitor BDAI-1 (monomeric) (Table 2.11).

Table 2.9 - Protein identification of excised bands from SDS-PAGE of E_1. Protein bands were digested by trypsin and searched by the software Protein Lynx Global (PLGS) server v. 2.4. The protein abundance was determined by protein coverage and peptide mapping shown also by the number of peptides counted as given by the software PLGS. Proteins with high abundance (+++), moderate abundance (++) and low abundance (+) are presented

(continues)

Band	Description	Accession code	Mw (Da)	pI (pH)	PLGS Score	Peptides	Coverage (%)
1	Globulin 1S (++)	M0XUU4_HORVD	47951	7.93	11.20	2	6.22
	Timeless circadian protein (+++)	M0WDA4_HORVD	95806	5.16	10.42	1	2.09
	Beta amylase (++)	Q6SNP7_HORVU	57598	5.58	9.06	3	9.09
2	Globulin 1S (++)	M0XUU4_HORVD	47951	7.93	11.20	2	8.53
	Uncharacterized protein (+)	M0VT04_HORVD	10004	11.84	10.84	1	21.74
	Translation elongation factor EFG (++)	F2CWX1_HORVD	85087	5.72	10.60	3	5.37
	Lipoxygenase (++)	F2D961_HORVD	96460	6.34	10.51	1	1.27
	Lipoxygenase (+++)	M0WRG0_HORVD	96333	5.68	10.51	12	16.13
	Beta amylase (+)	Q6SNP7_HORVU	57598	5.58	8.76	2	6.77
3	Globulin 1S (++)	M0XUU4_HORVD	47951	7.93	11.20	1	3.92
	Heat shock protein 90 (+++)	F2CU34_HORVD	80356	4.78	11.19	7	11.57
	Late embryogenesis abundant protein (+)	M0ZDL8_HORVD	27486	5.56	11.18	1	5.07
	Heat shock protein 90 (+++)	F2CRR4_HORVD	81740	4.74	10.51	9	14.25
	Phosphatidylinositol 4 phosphate 5 kinase (+)	M0VLX9_HORVD	86861	8.15	10.46	1	1.68
	Methionine synthase (+++)	M0Z2S8_HORVD	84511	5.65	9.73	8	15.56
	Cytosolic heat shock protein 90 (+++)	F2DX25_HORVD	80382	4.78	3.09	6	10.86
4	Heat shock HSP70 protein (+++)	F2DYT5_HORVD	70972	4.95	11.20	13	23.77
	Heat shock protein 70 HSP70 (+++)	F2E4C2_HORVD	71860	4.94	11.20	15	23.90
	Heat shock HSP70 protein (+++)	M0YSB2_HORVD	71102	4.90	11.20	11	19.79
	Uncharacterized protein (+)	M0WFD1_HORVD	12040	6.72	11.20	1	9.73
	Heat Shock Protein 70 HSP70 (++)	M0UW40_HORVD	34063	5.44	11.15	1	5.06
	Heat Shock Protein 70 HSP70 (+++)	Q40058_HORVU	66974	5.63	10.75	6	11.51
	Uncharacterized protein (+)	M0X9N6_HORVD	114667	6.25	8.19	1	1.24
5	Malic enzyme (+++)	F2ELT5_HORVD	68256	6.47	11.20	4	7.87
	Protein Disulphide Isomerase PDI (+++)	F2D284_HORVD	56393	4.80	10.51	7	21.83
	Uncharacterized protein (+)	M0VZ60_HORVD	52290	6.11	10.36	1	2.03
	Beta amylase (++)	F2EFV1_HORVD	42852	5.51	9.69	2	6.49
6	Beta-amylase (+)	M0W919_HORVD	6117	4.88	11.20	1	15.38
	Globulin 1S (++)	M0XUU4_HORVD	47951	7.93	11.20	3	10.83
	Beta glucosidase (+++)	Q40025_HORVU	57408	7.34	11.18	15	26.92
	Beta amylase (+++)	D6BU16_HORVS	59573	5.50	11.13	25	45.23
	Pyruvate kinase (++)	M0WHN6_HORVD	39460	5.06	11.00	3	13.22

Table 2.9 - Protein identification of excised bands from SDS-PAGE of E_1. Protein bands were digested by trypsin and searched by the software Protein Lynx Global (PLGS) server v. 2.4. The protein abundance was determined by protein coverage and peptide mapping shown also by the number of peptides counted as given by the software PLGS. Proteins with high abundance (+++), moderate abundance (++) and low abundance (+) are presented

(continued)

Band	Description	Accession code	Mw (Da)	pI (pH)	PLGS Score	Peptides	Coverage (%)
6	Ketol acid reductoisomerase chloroplastic like (+)	F2D434_HORVD	62179	6.03	10.51	1	1.91
	12 oxophytodienoic acid reductase 2 (+)	F2DMD8_HORVD	40496	6.22	10.51	1	2.46
	R Zinc sensing zinc finger B box TF (+)	K7YDM1_HORVD	21487	4.36	10.51	1	9.71
	Beta amylase (+++)	Q9FUK7_HORVU	59349	5.59	10.39	28	50.09
	Beta glucosidase (+)	M0VPC0_HORVD	32707	6.34	10.36	1	2.38
7	Enolase (++)	F2CR08_HORVD	48028	5.24	11.20	2	7.62
	Predicted protein (+)	F2CSR6_HORVD	21800	8.62	11.20	1	5.97
	Enolase (++)	F2D4W3_HORVD	48201	5.24	11.20	3	10.91
	Enolase (+)	F2E895_HORVD	39327	6.01	11.20	1	4.16
	Globulin 1S (+++)	M0XUU4_HORVD	47951	7.93	11.20	16	17.05
	Trypsin proteomics grade (+)	TRYP_PIG	24393	6.91	11.20	3	12.12
	Allene oxide synthase Cyt P450 family (++)	M0VBP5_HORVD	28796	9.17	11.15	3	13.46
	Embryo globulin (+)	Q03678_HORVU	72209	6.83	11.08	10	14.91
	Galactinol sucrose galactosyltransferase 6 like isoform (+)	F2DAE8_HORVD	59371	5.56	10.82	1	1.26
	56kDa Selenium binding protein SBP56 (+)	G8CN39_HORVS	33870	5.99	10.51	1	3.61
	Elongation factor 1 alpha (++)	F2CRH2_HORVD	49137	9.48	10.50	6	19.46
	Deoxymugineic acid synthase 2 (+)	M0XLU9_HORVD	22037	4.82	10.28	1	7.28
	Beta amylase (+++)	Q9FUK7_HORVU	59349	5.59	10.17	9	22.14
	Xylose isomerase (+)	F2DNL2_HORVD	53396	5.11	10.10	1	2.51
8	Histone H1/H5 (+)	F2DXK0_HORVD	27360	11.23	11.20	1	7.43
	Serpin Z4 (+++)	M0UEE6_HORVD	43193	5.53	11.20	6	20.75
	Aspartic Proteinase Nepenthesin 1 (++)	M0W9B2_HORVD	48915	8.22	11.20	2	5.96
	basic-leucine zipper (bZIP) transcription factor (+)	M0XRT1_HORVD	21435	9.51	11.20	1	4.59
	Globulin 1S (+++)	M0XUU4_HORVD	47951	7.93	11.20	5	14.06
	Serpin I4 (+++)	M0Z714_HORVD	28784	5.46	11.20	12	38.08
	Serpin Z7 (+++)	BSZ7_HORVU	42794	5.33	11.20	13	29.97
	Hordein B3 (fragment) (++)	Q9SAT9_HORVU	3366	9.86	11.20	1	29.03
	Aldose 1 epimerase (++)	M0WGK6_HORVD	19641	10.73	11.18	3	21.74
	Chaperone DnaJ (HPS40) (++)	M0VDD8_HORVD	16679	9.63	11.17	2	26.00
	Phosphoglycerate kinase (+++)	Q850M2_HORVD	31301	4.86	11.15	5	27.85
	UDP glycosyltransferase (UGT) (+)	M0VZ81_HORVD	14302	6.13	11.12	1	12.40

Table 2.9 - Protein identification of excised bands from SDS-PAGE of E_1. Protein bands were digested by trypsin and searched by the software Protein Lynx Global (PLGS) server v. 2.4. The protein abundance was determined by protein coverage and peptide mapping shown also by the number of peptides counted as given by the software PLGS. Proteins with high abundance (+++), moderate abundance (++) and low abundance (+) are presented

(continued)

Band	Description	Accession code	Mw (Da)	pI (pH)	PLGS Score	Peptides	Coverage (%)
8	UDP D glucuronate decarboxylase (+)	M0Y7M9_HORVD	14571	8.78	10.95	1	8.46
	Glyceraldehyde 3 phosphate dehydrogenase 2 cytosolic (++)	G3PC2_HORVU	33214	6.19	10.91	3	16.07
	Aminotransferase transaminase (+)	F2DS59_HORVD	35843	9.66	10.78	1	3.02
	Globulin 2 Cupin Vicilin like 7S (++)	F2EG51_HORVD	40893	5.03	10.66	1	2.79
	Serpin ZX (++)	SPZX_HORVU	42920	6.93	10.59	3	7.79
	Late embryogenesis abundant protein, LEA-14 (++)	F2DED9_HORVD	36224	4.76	10.53	1	3.67
	Fructose bisphosphate aldolase (+++)	C1J960_HORVU	38664	6.02	10.51	12	49.44
	Predicted protein (+)	F2DWM8_HORVD	115738	4.58	10.51	1	1.93
	Aldose 1 epimerase (+)	F2E008_HORVD	39719	7.48	10.51	1	6.72
	Glyceraldehyde 3 phosphate dehydrogenase (+++)	F2CUE9_HORVD	36490	6.78	10.49	10	46.59
	Elongation factor 1 alpha (++)	M0Y9X9_HORVD	32074	9.71	10.01	2	11.95
	Alcohol dehydrogenase 1 (++)	O65114_HORVS	28241	7.61	9.11	3	8.40
9	Glyoxalase 1 (+++)	F2CQP8_HORVD	32525	5.16	11.20	16	45.70
	Malate dehydrogenase (++)	F2CQR0_HORVD	35844	8.11	11.20	4	24.06
	Predicted protein (+)	F2CYK7_HORVD	103213	6.65	11.20	1	0.96
	DEAD-like helicase (+)	F2D7P3_HORVD	14335	11.26	11.20	1	6.45
	Predicted protein (+)	F2DV26_HORVD	34070	9.60	11.20	1	4.35
	Predicted protein (+)	F2E904_HORVD	113145	5.91	11.20	1	1.57
	Serpin Z4 (++)	M0UEE6_HORVD	43193	5.53	11.20	3	12.50
	Tryptophan synthase beta subunit-like (++)	M0VBS3_HORVD	24927	6.40	11.20	1	5.06
	Globulin 1S Cupin 12 (+++)	M0XH59_HORVD	34764	10.58	11.20	20	45.28
	Globulin 1S (++)	M0XUU4_HORVD	47951	7.93	11.20	6	16.13
	Malate dehydrogenase (++)	F2D4W6_HORVD	35578	5.50	11.20	5	14.41
	Haem peroxidase (+++)	F2E8A9_HORVD	40926	8.75	11.20	11	32.11
	Malate dehydrogenase (++)	M0XD85_HORVD	22832	9.78	11.17	4	37.79
	Late embryogenesis abundant protein (++)	M0Z8T0_HORVD	10727	4.02	11.16	1	27.72
	Globulin 11 S seed storage protein cupin (++)	M0Y822_HORVD	18295	6.35	11.15	2	14.29
	Haem peroxidase (+++)	F2EEV5_HORVD	38668	6.12	11.10	12	40.39
	Serpin Z7 (++)	M0Z714_HORVD	28784	5.46	11.03	2	11.92
	Concanavalin A-like lectin/glucanase (+)	F2DFV1_HORVD	57717	7.02	10.68	1	1.73
	Caleosin (++)	M0WIB6_HORVD	18231	5.79	10.54	1	8.93

Table 2.9 - Protein identification of excised bands from SDS-PAGE of E_1. Protein bands were digested by trypsin and searched by the software Protein Lynx Global (PLGS) server v. 2.4. The protein abundance was determined by protein coverage and peptide mapping shown also by the number of peptides counted as given by the software PLGS. Proteins with high abundance (+++), moderate abundance (++) and low abundance (+) are presented

(continued)

Band	Description	Accession code	Mw (Da)	pI (pH)	PLGS Score	Peptides	Coverage (%)
9	Ribosomal protein L18 (+)	M0XVN5_HORVD	137482	5.63	10.53	1	0.83
	Predicted protein (+)	F2D1P1_HORVD	34301	9.60	10.51	1	2.99
	Annexin (+)	F2DAW2_HORVD	35282	6.35	10.51	1	3.82
	60S acidic ribosomal protein P0 (++)	F2DBD4_HORVD	34528	5.21	10.51	3	18.44
	Epoxide hydrolase like (+)	F2EIH2_HORVD	38674	6.07	10.51	2	5.68
	Uncharacterized protein (+)	M0YFZ5_HORVD	20059	4.42	10.26	1	4.00
	Spermidine/spermine_ synthase (++)	M0Y8M6_HORVD	28695	4.76	10.25	1	4.94
	Cellulose synthase like CslF10 (+)	M0URI5_HORVD	89483	6.47	10.23	1	3.31
	Alcohol dehydrogenase1 (+)	M0VG07_HORVD	12834	8.45	10.12	1	8.00
	aldo keto NADPH dependent reductase (+++)	F2DXQ5_HORVD	35784	6.54	10.10	10	29.69
	Translation elongation factor EF1A/initiation factor IF2gamma (++)	M0Y9X9_HORVD	32074	9.71	10.07	2	10.92
	Glyceraldehyde 3 phosphate dehydrogenase (++)	A3RHT3_HORVD	25005	5.69	9.24	1	6.01
10	Glyoxalase 1 (+)	F2CQP8_HORVD	32525	5.16	11.20	1	3.78
	Secreted Lipoprotein DUF1264 (++)	F2EBF9_HORVD	27654	8.03	11.20	3	15.32
	Glucose and ribitol dehydrogenase like protein (+)	M0V5B1_HORVD	27321	5.82	11.20	15	40.39
	Globulin 1S (++)	M0XUU4_HORVD	47951	7.93	11.20	3	10.83
	Protein synthesis inhibitor II (+++)	RIP2_HORVU	29844	10.00	11.20	15	38.57
	Peroxidase (++)	Q42852_HORVU	19729	7.09	11.19	3	25.56
	Protein synthesis inhibitor I (+++)	RIP1_HORVU	29953	10.30	11.14	16	38.43
	Late embryogenesis abundant protein (++)	M0Z5Y1_HORVD	23197	5.22	11.01	2	22.55
	Globulin 1S Cupin 12 (+)	M0XH59_HORVD	34764	10.58	10.62	1	3.58
	Initiation factor eIF-4 gamma (++)	M0YZB7_HORVD	70227	5.18	10.61	2	2.34
	Ferritin 2 like protein desiccation related protein (+)	M0VN23_HORVD	33649	4.71	10.51	1	5.05
	GrpE nucleotide exchange factor (+)	M0YSD3_HORVD	18063	5.00	10.43	1	6.71
	Glucan endo 1 3 beta glucosidase GII (++)	E13B_HORVU	35171	9.22	10.12	2	9.88
	LEA3 late embryogenesis abundant protein (+)	F2CUJ9_HORVD	33857	8.64	10.10	1	6.56
	Protease inhibitor I4 serpin (++)	F2DTC0_HORVD	28881	6.44	9.90	1	3.86
	Peroxidase (+)	F2D359_HORVD	27567	5.40	8.83	1	6.49
11	40S ribosomal protein S13 like (+)	F2CVF1_HORVD	17115	10.96	11.20	1	7.95

Table 2.9 - Protein identification of excised bands from SDS-PAGE of E_1. Protein bands were digested by trypsin and searched by the software Protein Lynx Global (PLGS) server v. 2.4. The protein abundance was determined by protein coverage and peptide mapping shown also by the number of peptides counted as given by the software PLGS. Proteins with high abundance (+++), moderate abundance (++) and low abundance (+) are presented

(continued)

Band	Description	Accession code	Mw (Da)	pI (pH)	PLGS Score	Peptides	Coverage (%)
11	Redoxin family protein (++)	F2DDK1_HORVD	24089	9.31	11.20	1	7.23
	60S ribosomal protein L12 1 (+++)	F2DEP3_HORVD	17727	9.41	11.20	3	22.89
	Ribosomal S19e protein (++)	F2E598_HORVD	17073	10.25	11.20	2	12.26
	Peptidyl prolyl cis trans isomerase (+)	M0WCQ8_HORVD	17354	8.71	11.20	2	13.04
	17 kDa class I small heat shock protein (+++)	Q96458_HORVU	16831	5.73	11.20	3	22.00
	50S ribosomal protein L35 (+)	M0VT04_HORVD	10004	11.84	10.84	1	21.74
	Protein synthesis inhibitor II (+)	M0Z8M4_HORVD	15195	10.03	10.77	1	10.42
	HistoneH2a variant 1 like (+)	F2CXB8_HORVD	14634	10.78	10.51	1	6.47
	Translationally controlled tumor protein homolog TCTP (++)	F2DWT1_HORVD	18872	4.33	10.51	2	13.69
	Phosphatidylethanolamine binding protein PEBP (+++)	F2DAA1_HORVD	17601	4.75	10.51	3	26.22
	Histone H2A 6 (++)	M0YQR0_HORVD	5026	12.30	9.94	1	19.15
	Histone H2B (++)	F2E2R1_HORVD	14376	10.52	9.18	2	18.18
	Predicted protein (+)	F2DSM8_HORVD	17923	12.47	-2.67	1	4.27
12	Histone H4 (+++)	F2DY33_HORVD	11727	11.77	11.20	3	17.59
	Alpha amylase trypsin inhibitor CMc (+)	M0VFV9_HORVD	15724	4.94	11.20	1	7.53
	LEA Protein (+)	M0VJA1_HORVD	10374	4.92	11.20	1	14.43
	Barwin-like_endoglucanase (++)	M0VKD9_HORVD	18200	8.13	11.20	1	8.82
	Trypsin proteomics grade (+)	TRYP_PIG	24393	6.91	11.20	10	20.78
	Globulin 1 S vicilin 7S like (+++)	M0XH58_HORVD	46337	6.60	11.13	2	6.30
	Barwin (++)	BARW_HORVU	13728	7.63	10.94	2	26.40
	50S ribosomal protein L35 (+)	M0VT04_HORVD	10004	11.84	10.84	1	21.74
	Alpha amylase inhibitor BMAI 1 (++)	IAA1_HORVU	15805	5.15	10.79	3	14.38
	LRR protein (+)	M0WUL7_HORVD	109847	6.52	10.66	1	0.59
	Alpha amylase trypsin inhibitor CMb (++)	M0ULY1_HORVD	15806	5.13	10.62	2	13.29
	Alpha hordothionin (++)	THNA_HORVU	13587	6.17	10.55	1	11.02
	Histone H4 (+++)	F2CUP3_HORVD	11402	11.88	10.51	4	26.21
	Alpha amylase trypsin inhibitor CMa (+)	M0YS73_HORVD	15459	5.84	10.51	1	3.45
	Thioredoxin (++)	M0YSP2_HORVD	6651	8.89	10.35	1	18.33
	Hordoindoline A 1 (+)	Q8S409_HORVD	15648	7.95	10.28	1	7.04
	Alpha amylase inhibitor BDAI 1 (+++)	IAA2_HORVU	16417	5.19	10.15	5	32.89

Table 2.9 - Protein identification of excised bands from SDS-PAGE of E_1. Protein bands were digested by trypsin and searched by the software Protein Lynx Global (PLGS) server v. 2.4. The protein abundance was determined by protein coverage and peptide mapping shown also by the number of peptides counted as given by the software PLGS. Proteins with high abundance (+++), moderate abundance (++) and low abundance (+) are presented

(conclusion)

Band	Description	Accession code	Mw (Da)	pI (pH)	PLGS Score	Peptides	Coverage (%)
12	Alpha amylase trypsin inhibitor BTI CMe2 1 protein (+)	O49861_HORVS	16212	6.89	10.12	1	12.84
	Hordoindoline b2 (++)	Q5IUG4_HORVD	13621	8.50	9.83	2	18.25
	Non-specific lipid transfer protein (+)	A8YPK3_HORVU	12293	8.31	9.81	2	13.68
	LEA 5 Protein (+)	F2EHN7_HORVD	9970	5.39	9.81	1	12.90
	Hordoindoline b2 (+)	G1UH43_HORVD	16110	8.30	9.59	2	15.65
	Glyceraldehyde 3 phosphate dehydrogenase (+)	A3RHT3_HORVD	25005	5.69	9.49	1	6.01
	Hordoindoline b1 (+)	G1UH41_HORVS	16110	8.30	9.12	2	15.65
	ER membrane carboxypeptidase M14 (+)	F2CSR6_HORVD	21800	8.62	7.53	1	5.97
	Uncharacterized protein (+)	M0VWK8_HORVD	65995	8.45	7.53	1	0.83

Table 2.10 - Protein identification of excised bands from SDS-PAGE of E_2. Protein bands were digested by trypsin and searched by the software Protein Lynx Global (PLGS) server v. 2.4. The protein abundance was determined by protein coverage and peptide mapping shown also by the number of peptides counted as given by the software PLGS. Proteins with high abundance (+++), moderate abundance (++) and low abundance (+) are presented

(continues)

Band	Description	Accession code	Mw (Da)	pI (pH)	PLGS Score	Peptides	Coverage (%)
1	Uncharacterized protein (+)	M0X1E5_HORVD	43206	9.98	10.88	1	2.58
	Chitinase class II group glycoside hydrolase family 18 (+)	F2D094_HORVD	33071	8.06	10.51	1	4.04
	Lipoxygenase (+++)	M0WRG0_HORVD	96333	5.68	10.51	11	16.13
	Uncharacterized protein (+)	M0VIB5_HORVD	28392	8.51	10.24	1	3.11
2	Trypsin proteomics grade (+)	TRYP_PIG	24393	6.91	11.20	5	20.78
3	Late embryogenesis abundant protein (+)	M0ZDL8_HORVD	27486	5.56	11.06	1	5.07
	Heat shock protein 90 (++)	F2CU34_HORVD	80356	4.78	9.61	1	1.71
	Cobalamin-independent methionine synthase MetE (+)	M0WNE8_HORVD	42817	5.08	9.23	1	2.53
4	Heat shock HSP70 protein (+++)	F2DYT5_HORVD	70972	4.95	11.20	13	23.30
	Heat shock HSP70 protein (++)	M0YSB2_HORVD	71102	4.90	11.20	9	15.80
	Heat shock HSP70 protein (++)	M0Z8C6_HORVD	59737	4.80	11.20	7	16.61
	Heat shock HSP70 protein (++)	M0UW40_HORVD	34063	5.44	11.20	1	5.06
	Heat shock protein 70 HSP70 (+++)	F2E4C2_HORVD	71860	4.94	11.20	16	30.75
	Heat shock HSP70 protein (++)	M0WFD3_HORVD	59915	4.82	11.14	5	13.19
	Heat Shock Protein 70 HSP70 (+)	Q40058_HORVU	66974	5.63	10.86	6	11.51
	Heat shock HSP70 protein (++)	M0XNU3_HORVD	72297	5.23	10.55	4	8.90
5	Malic enzyme (+++)	F2ELT5_HORVD	68256	6.47	11.20	6	13.96
	Uncharacterized protein (+)	M0W2S1_HORVD	15144	10.08	11.20	1	15.11
	Protein Disulphide Isomerase PDI (+++)	F2D284_HORVD	56393	4.80	10.50	12	31.97
	Uncharacterized protein (+)	M0ZFG2_HORVD	32888	7.31	10.48	1	6.97
	Uncharacterized protein (+)	M0VZ60_HORVD	52290	6.11	10.36	1	2.03
	Beta amylase (+++)	Q9FUK7_HORVU	59349	5.59	10.19	10	27.02
	Predicted protein (+)	F2DLW4_HORVD	42164	6.14	10.14	1	5.08
	Predicted protein (+)	F2D207_HORVD	103604	5.74	-35.86	1	4.09
6	Beta amylase (+++)	D6BU16_HORVS	59573	5.50	11.20	45	59.63
	Predicted protein (+)	F2DDE5_HORVD	48613	4.70	11.20	1	1.11
	Predicted protein (+)	F2DS64_HORVD	47938	11.23	11.20	1	4.85
	Predicted protein (+)	F2EAQ7_HORVD	112147	5.44	11.20	1	0.78
	Beta amylase (+++)	F2EFV1_HORVD	42852	5.51	11.20	33	60.52
	Uncharacterized protein (+)	M0W919_HORVD	6117	4.88	11.20	1	15.38
	Beta glucosidase Fragments (+)	Q9S9H4_HORVU	14314	9.63	11.20	4	29.63

Table 2.10 - Protein identification of excised bands from SDS-PAGE of E_2. Protein bands were digested by trypsin and searched by the software Protein Lynx Global (PLGS) server v. 2.4. The protein abundance was determined by protein coverage and peptide mapping shown also by the number of peptides counted as given by the software PLGS. Proteins with high abundance (+++), moderate abundance (++) and low abundance (+) are presented

(continued)

Band	Description	Accession code	Mw (Da)	pI (pH)	PLGS Score	Peptides	Coverage (%)
	Uncharacterized protein (+)	M0VKF4_HORVD	24682	7.77	11.17	2	9.77
	Beta glucosidase (+++)	Q40025_HORVU	57408	7.34	10.72	21	35.17
	Beta glucosidase (+)	M0VPC0_HORVD	32707	6.34	10.71	1	2.38
	Uncharacterized protein (+)	M0Y1B0_HORVD	149274	5.04	10.69	2	0.60
6	Beta amylase (+++)	A8CFR3_HORVU	59601	5.50	10.50	46	59.63
	Uncharacterized protein (+)	M0Y6M8_HORVD	31569	5.43	10.44	1	4.96
	Uncharacterized protein (+)	M0W3A2_HORVD	40559	5.25	10.44	1	3.00
	Uncharacterized protein (+)	M0WHZ8_HORVD	15241	6.32	8.64	7	24.44
	Beta amylase (+++)	Q9FUK7_HORVU	59349	5.59	0.78	47	59.85
	Late embryogenesis abundant protein (++)	F2ECH4_HORVD	38651	6.53	11.20	2	9.12
	B3 hordein (++)	I6SJ26_HORVU	35402	8.09	11.20	2	9.35
	Serpin Z4 (+++)	M0UEE6_HORVD	43193	5.53	11.20	12	37.00
	Serpin Z7 (++)	M0Z714_HORVD	28784	5.46	11.20	3	16.15
	Uncharacterized protein (+)	M0YUA8_HORVD	34958	9.28	10.90	1	5.31
	B3 hordein (++)	I6TRT5_HORVU	31798	7.85	10.73	6	26.88
7	ThiJ PfpI family protein (+)	F2EDR4_HORVD	42134	8.69	10.58	1	3.04
	B hordein (++)	C7FB13_HORVD	30164	7.86	10.51	5	24.91
	B hordein (++)	C7FB14_HORVD	30271	8.10	10.51	2	16.23
	Phosphoglycerate kinase (+)	Q850M2_HORVD	31301	4.86	10.43	1	5.70
	Uncharacterized protein (+)	M0V861_HORVD	41108	4.86	10.41	1	3.43
	Uncharacterized protein (+)	M0YJX2_HORVD	33153	5.11	10.38	1	6.00
	Beta amylase (++)	Q6SNP7_HORVU	57598	5.58	9.23	3	9.09
	rRNA N glycosidase (+++)	B5TWK6_HORVU	29532	10.10	11.20	9	31.77
	Glyoxalase 1 (+)	F2CQP8_HORVD	32525	5.16	11.20	1	3.78
	Haem peroxidase (++)	F2E8K5_HORVD	32157	8.02	11.20	4	14.38
	Secreted Lipoprotein DUF1264 (+)	F2EBF9_HORVD	27654	8.03	11.20	1	4.03
	Beta glucosidase glycoside hydrolase family 17 (+)	F2EEX6_HORVD	35935	7.26	11.20	1	5.83
8	rRNA N glycosidase (+++)	M0WCD7_HORVD	18803	10.16	11.20	11	44.44
	Protein synthesis inhibitor II (+++)	RIP2_HORVU	29844	10.00	11.20	14	33.21
	Protein synthesis inhibitor I (+++)	RIP1_HORVU	29953	10.30	11.20	14	33.10
	Glucose and ribitol dehydrogenase like protein (++)	M0V5B1_HORVD	27321	5.82	11.19	6	22.75
	Endochitinase (+)	M0YCY4_HORVD	19099	8.26	11.14	2	12.43

Table 2.10 - Protein identification of excised bands from SDS-PAGE of E_2. Protein bands were digested by trypsin and searched by the software Protein Lynx Global (PLGS) server v. 2.4. The protein abundance was determined by protein coverage and peptide mapping shown also by the number of peptides counted as given by the software PLGS. Proteins with high abundance (+++), moderate abundance (++) and low abundance (+) are presented

(continued)

Band	Description	Accession code	Mw (Da)	pI (pH)	PLGS Score	Peptides	Coverage (%)
8	Serpin Z7 (++)	M0Z714_HORVD	28784	5.46	11.03	2	11.92
	Ferritin 2 like protein desiccation related protein (++)	M0VN22_HORVD	12444	4.90	10.95	1	24.17
	Uncharacterized protein (+)	M0VT04_HORVD	10004	11.84	10.84	1	21.74
	Glucan endo 1 3 beta glucosidase GII (++)	E13B_HORVU	35171	9.22	10.13	2	9.88
9	Serpin Z4 (++)	M0UEE6_HORVD	43193	5.53	11.20	5	16.75
	Serpin ZY (++)	M0Z714_HORVD	28784	5.46	11.20	10	37.31
	Serpin Z7 (+++)	BSZ7_HORVU	42794	5.33	11.20	10	24.43
	B3 fragment (+)	Q9SAT9_HORVU	3366	9.86	11.20	2	74.19
	B3 hordein (++)	Q3YAF9_HORVD	34442	8.68	10.57	2	7.67
	Phosphoglycerate kinase (+)	Q850M2_HORVD	31301	4.86	10.43	1	5.70
	Fructose bisphosphate aldolase (++)	M0YV25_HORVD	35526	8.47	9.99	3	12.20
	Glyceraldehyde 3 phosphate dehydrogenase (++)	A3RHT3_HORVD	25005	5.69	9.77	2	16.74
	Uncharacterized protein (+)	M0VTB3_HORVD	141167	6.00	4.17	1	1.02
	Uncharacterized protein (+)	M0V4S7_HORVD	53948	4.83	1.01	1	2.71
10	Tetratricopeptide repeat-containing protein (++)	M0V771_HORVD	33648	7.67	11.20	1	3.02
	Protein synthesis inhibitor II (+++)	M0Z8M4_HORVD	15195	10.03	11.20	3	20.83
	Triosephosphate isomerase (++)	F2EHF8_HORVD	26761	5.22	10.51	2	9.09
	Late embryogenesis abundant protein (++)	B5TWD1_HORVD	21735	9.53	9.83	1	9.43
11	Protein synthesis inhibitor II (+++)	M0Z8M4_HORVD	15195	10.03	10.77	1	10.42
	PR17c pathogenesis related PR basic secretory proteins BSPs (+)	A7YA60_HORVD	24373	9.65	10.51	5	17.33
	Kunitz type soybean trypsin inhibitor STI Isa (+++)	J9V7J5_HORVD	20729	6.85	10.36	3	20.00
	Thaumatin (+++)	F2DNP3_HORVD	24290	7.48	10.25	3	20.60
	Alpha amylase inhibitor BDAI 1 (++)	IAA2_HORVU	16417	5.19	10.12	1	14.47
	Late embryogenesis abundant protein (++)	B5TWD1_HORVD	21735	9.53	9.82	1	9.43
	Barperm1 thaumatin (++)	O22462_HORVU	21642	7.69	9.75	2	17.56
12	Avenin Like Protein (+++)	M0VEH1_HORVD	18063	7.56	11.20	5	28.66
	Uncharacterized protein (+)	M0XFE6_HORVD	80563	8.52	11.20	1	2.88
	Superoxide dismutase Cu Zn (++)	M0VCA1_HORVD	10365	5.37	10.82	1	22.33
	Alpha amylase trypsin inhibitor CMb (++)	M0ULY1_HORVD	15806	5.13	10.55	1	7.69
	UDP glycosyltransferase (UGT) (++)	F2CUV1_HORVD	50038	5.16	10.51	1	6.57

Table 2.10 - Protein identification of excised bands from SDS-PAGE of E_2. Protein bands were digested by trypsin and searched by the software Protein Lynx Global (PLGS) server v. 2.4. The protein abundance was determined by protein coverage and peptide mapping shown also by the number of peptides counted as given by the software PLGS. Proteins with high abundance (+++), moderate abundance (++) and low abundance (+) are presented

(continued)

Band	Description	Accession code	Mw (Da)	pI (pH)	PLGS Score	Peptides	Coverage (%)
12	Alpha amylase trypsin inhibitor CMd (+++)	M0Y227_HORVD	18513	6.07	10.51	12	58.48
13	Alpha amylase trypsin inhibitor CMc (++)	M0VFV9_HORVD	15724	4.94	11.20	3	19.86
	Barwin (+++)	BARW_HORVU	13728	7.63	11.05	8	36.80
	Alpha amylase inhibitor BMAI 1 (+)	IAA1_HORVU	15805	5.15	10.93	2	14.38
	Enoyl-CoA hydratase/isomerase (++)	M0UR68_HORVD	16366	4.77	10.76	1	13.92
	Alpha amylase trypsin inhibitor CMb (+++)	M0ULY1_HORVD	15806	5.13	10.75	4	19.58
	Alpha amylase trypsin inhibitor CMe (++)	IAAE_HORVU	16124	7.42	10.59	4	35.14
	Uncharacterized protein (+)	M0WUL7_HORVD	109847	6.52	10.58	1	0.59
	Alpha amylase trypsin inhibitor CMa (+)	M0YS73_HORVD	15459	5.84	10.51	1	16.55
	Uncharacterized protein (+)	M0YSP2_HORVD	6651	8.89	10.35	1	18.33
	Uncharacterized protein (+)	M0XNS8_HORVD	34187	7.30	10.34	1	6.69
	Uncharacterized protein (+)	M0W866_HORVD	42564	7.75	10.32	2	5.33
	Alpha amylase inhibitor BDAI 1 (+++)	IAA2_HORVU	16417	5.19	10.20	6	32.89
	Hordoindoline b 2 (++)	G1UH43_HORVD	16110	8.30	9.59	3	20.41
	Hordoindoline b 1 (++)	G1UH41_HORVS	16110	8.30	9.12	3	20.41
	Hordoindoline b 2 (++)	G1UH45_HORVS	16011	8.30	9.00	3	20.41
14	Alpha amylase trypsin inhibitor CMc (+)	M0VFV9_HORVD	15724	4.94	11.20	1	7.53
	Subtilisin chymotrypsin inhibitor CI 1C (+++)	ICIC_HORVU	8252	7.69	11.20	3	36.36
	Barwin (+++)	BARW_HORVU	13728	7.63	11.13	7	56.00
	Alpha amylase inhibitor BMAI 1 (+)	IAA1_HORVU	15805	5.15	10.98	3	14.38
	Translation elongation factor EF1A/initiation factor IF2gamma (++)	M0VS47_HORVD	12299	10.41	10.94	2	17.70
	Thioredoxin (+)	Q7XZK2_HORVD	13156	4.94	10.68	1	9.02
	Alpha amylase trypsin inhibitor CMb (+)	M0ULY1_HORVD	15806	5.13	10.59	1	7.69
	Uncharacterized protein (+)	M0X1R0_HORVD	92697	5.59	10.51	1	2.10
	Uncharacterized protein (+)	M0W4X5_HORVD	9414	7.73	10.51	1	11.24
	Subtilisin chymotrypsin inhibitor CI 1A (+++)	M0W9B7_HORVD	8876	5.07	10.51	6	73.49
	Subtilisin chymotrypsin inhibitor CI 1B (+++)	M0XBS5_HORVD	8957	5.14	10.51	6	72.29
	Barley trypsin inhibitor CMc (++)	E7BB45_HORVD	11241	7.44	10.49	3	45.19
	Alpha amylase inhibitor BDAI 1 (++)	IAA2_HORVU	16417	5.19	10.39	17	59.87
	Uncharacterized protein (+)	M0WP44_HORVD	29568	4.11	10.37	1	5.68
	Uncharacterized protein (+)	M0YSP2_HORVD	6651	8.89	10.35	1	18.33

Table 2.10 - Protein identification of excised bands from SDS-PAGE of E_2. Protein bands were digested by trypsin and searched by the software Protein Lynx Global (PLGS) server v. 2.4. The protein abundance was determined by protein coverage and peptide mapping shown also by the number of peptides counted as given by the software PLGS. Proteins with high abundance (+++), moderate abundance (++) and low abundance (+) are presented

(conclusion)

Band	Description	Accession code	Mw (Da)	pI (pH)	PLGS Score	Peptides	Coverage (%)
14	Subtilisin chymotrypsin inhibitor CI 1A (+++)	M0W9B7_HORVD	8876	5.07	10.51	6	73.49
	Subtilisin chymotrypsin inhibitor CI 1B (+++)	M0XBS5_HORVD	8957	5.14	10.51	6	72.29
	Barley trypsin inhibitor CMc (++)	E7BB45_HORVD	11241	7.44	10.49	3	45.19
	Alpha amylase inhibitor BDAI 1 (++)	IAA2_HORVU	16417	5.19	10.39	17	59.87
	Uncharacterized protein (+)	M0WP44_HORVD	29568	4.11	10.37	1	5.68
	Uncharacterized protein (+)	M0YSP2_HORVD	6651	8.89	10.35	1	18.33
	Alpha amylase trypsin inhibitor BTI CMe2 1 protein (+)	O49861_HORVS	16212	6.89	10.11	1	12.84
	Uncharacterized protein (+)	M0Z4D5_HORVD	37545	5.82	10.07	1	2.65
	D hordein fragment (+)	Q40045_HORVU	50754	7.59	9.95	1	3.37
	Hordoindoline b2 (++)	Q5IUG4_HORVD	13621	8.50	9.83	2	18.25
	Non-specific lipid transfer protein (+++)	A8YPK3_HORVU	12293	8.31	9.81	5	23.93
	Alpha amylase trypsin inhibitor CMd (+)	M0Y227_HORVD	18513	6.07	9.81	1	9.94
	Hordoindoline b2 (++)	G1UH43_HORVD	16110	8.30	9.59	2	15.65
	Hordoindoline b1 (++)	G1UH41_HORVS	16110	8.30	9.12	2	15.65
	Chymotrypsin inhibitor 2 (++)	A8V3P3_HORVD	9375	7.66	8.49	1	16.67
	Grain softness protein (+)	Q0GIK9_HORVU	18164	4.76	7.82	1	14.11
	Uncharacterized protein (+)	M0YET1_HORVD	78689	7.92	-16.48	1	2.50

Table 2.11 - Protein identification of excised bands from SDS-PAGE of E_3. Protein bands were digested by chymotrypsin and searched by the software Protein Lynx Global (PLGS) server v. 2.4. The protein abundance was determined by protein coverage and peptide mapping shown also by the number of peptides counted as given by the software PLGS. Proteins with high abundance (+++), moderate abundance (++) and low abundance (+) are presented

Band	Description	Accession code	Mw (Da)	pI (pH)	PLGS Score	Peptides	Coverage (%)
1	D hordein (+++)	I6SW34_HORVD	79303	7.96	4516.8	2	5.09
2	C hordein (+++)	I6TEV8_HORVD	35291	9.50	54033.6	16	28.48
3	C hordein (+++)	I6TEV8_HORVD	35291	9.50	11301.4	3	11.59
4	C hordein (+++)	Q41210_HORVU	36485	7.71	85778.4	9	25.16
	Hor1 17 C hordein (++)	Q40053_HORVU	30377	7.65	43459.6	6	8.46
5	C hordein (++)	Q41210_HORVU	36485	7.71	172565.0	34	28.06
	Hor1 17 C hordein (+++)	Q40053_HORVU	30377	7.65	106090.3	43	25.77
6	B hordein (+++)	Q3YAF9_HORVD	34465	8.67	2341109.0	4	14.00
	B3-hordein (+)	HOR3_HORVU	30195	7.60	2712945.0	2	11.36
7	B3 hordein (+++)	I6TEV5_HORVU	35440	7.51	54185.2	10	15.81
	B3 hordein (+++)	I6SJ26_HORVU	35402	8.09	21483.3	11	18.39
	Gamma 1 hordein (+)	I6SJ17_HORVD	34714	7.95	13871.1	1	3.61
8	B1 hordein (+++)	I6SJ22_HORVU	32776	8.30	211676.0	35	18.53
	Gamma 1 hordein (+++)	I6TMV6_HORVU	34640	7.95	129089.8	38	39.02
	Gamma 1 hordein	I6SJ17_HORVD	34714	7.95	14056.0	23	17.70
	B hordein (++)	Q3YAF9_HORVD	34442	8.68	70171.2	15	7.67
	B hordein (++)	I6QP72_HORVD	33481	7.06	64906.5	24	7.93
	B hordein (+)	Q2XQF1_HORVD	30136	7.86	22717.5	6	14.34
9	B1 hordein (++)	I6SJ22_HORVU	32776	8.30	32382.0	6	16.78
	Gamma 1 hordein (+++)	I6TMV6_HORVU	34640	7.95	30361.3	22	30.49
	Gamma 3 hordein (++)	I6TEV2_HORVU	33763	6.70	7668.3	6	9.15
10	rRNA N glycosidase (++)	B5TWK6_HORVU	29532	10.10	64122.8	5	13.00
	Gamma 1 hordein (++)	I6SJ17_HORVD	34714	7.95	2615.2	2	4.26
11	Oleosin (++)	F2CWL7_HORVD	18496	10.02	36352.8	4	20.11
	α-amylase/trypsin inhibitor BDAI-1 (monomeric) (+++)	F2EI31_HORVD	16605	5.19	22841.8	9	42.21
	Oleosin (+)	F2CRF2_HORVD	16091	9.59	18523.2	2	7.55
12	Alpha-amylase/trypsin inhibitor BDAI-1 (monomeric) (+++)	F2EI31_HORVD	16605	5.19	255814.9	41	56.49
	Barley trypsin inhibitor CMc Fragment (++)	E7BB45_HORVD	11241	7.44	30296.0	16	75.96
	Hordoindoline b1 (++)	Q5IUH9_HORVD	16110	8.30	23826.0	11	14.29
13	Alpha-amylase/trypsin inhibitor BDAI-1 (monomeric) (+++)	F2EI31_HORVD	16605	5.19	284504.3	43	52.60
	Non-specific lipid transfer protein (++)	F2ED95_HORVD	12323	8.31	131753.9	20	45.30
	Barley trypsin inhibitor CMc Fragment (++)	E7BB45_HORVD	11241	7.44	31333.1	10	75.96

2.2.3 Discussion

Barley grain protein separation by biochemical techniques is based on the solubility of the different protein classes, which is also related to their hydrophilicity/hydrophobicity index. Osborne (1909) has introduced a cereal grain protein separation method based on their sequential solubility in hydrophilic and hydrophic extraction solutions. Modifications to this method exist and the protein extractability/distribution in the related fractions is different according to the Osborne modification technique (FIDO et al., 2004). In the present study we have applied some modifications of the classical sequential barley grain protein extractions described by Landry; Delhaye; Damerval (2000). Thus, five protein extracts of barley grains were obtained and characterized. The salt soluble proteins are presented here as E_1 and E_2, prolamin as E_3, an additional extract associate to prolamin as E_4 and finally glutelin as E_5. It is known that relatively slight changes of experimental conditions results in marked changes in protein distribution (LANDRY; DELHAYE; DAMERVAL, 2000), therefore sophisticated techniques were employed here to unraveling the complex mixtures of proteins of each protein extract obtained with the modified method.

Although the modifications used in the present study was first reported to improve the maize grain proteins separation, this methodology had already been employed to characterize hull-less barley grain proteins, however, only SDS-PAGE-based characterization was reported (HELM et al., 2005).

Firstly, the single protein extract contribution were determined by two classical biochemical protein quantification methods, Lowry (GERHARDT, 1994) and Bradford (BRADFORD, 1976) (Table 2.2). Despite the discrepancy of the two methods and their limits of protein detection, the results showed that the major grain protein fraction was E_5 (glutelins) accounting for about 50% of the total extracted protein, followed by 30-25% and 20-25% of E_1+E_2, respectively (salt soluble proteins), 17-19% of E_3 (prolamins) and 6-8% of E_4 (Table 2.2).

The most widely employed barley grain protein fractionation developed by Shewry et al. (1983) revealed a large variation in protein fractions distribution. Changes in protein distribution are primarily depended upon environment fluctuations and to a lesser extent on genotypic differences. Remarkable changes in the contribution of salt soluble proteins and prolamins were reported, and at a lesser extent on glutelin. While salt soluble proteins represent 38.7% and 42.3% of total N per g of dry weight under low sulfur nutrition (S-low), 15.1% and 16.6% were obtained under high sulfur nutrition (S-high) for cv. Athos and

Sundance, respectively. Similarly, the prolamin contribution varied greatly, from 28.1% and 28.2% under S-low to 51.5% and 48.7% under S-high for cv. Athos and Sundance, respectively. On the other hand, less variation was observed on glutelin contribution, from 33.2% and 29.4% under S-low to 33.4% and 34.7% under S-high for cv. Athos and Sundance, respectively (SHEWRY et al., 1983). Similar grain protein distribution was reported by Kirkman; Shewry; Miflin (1982) for barley cv. Bomi growing under medium nitrogen (100 $Kg.ha^{-1}$), in which the salt soluble proteins represented 18.59%, prolamins accounted for 50.34% and glutelin for 31.05% of total N per g of dry weight. These authors also reported large variations according to nitrogen fertilization. Even using a different protein extraction method and high performance liquid chromatography-based protein quantification, Lange et al. (2007) reported similar results of protein distribution for cv. Golden Promise. Wang et al. (2007) reported divergent results from those reported in the literature, but similar to those reported in the present study. The salt soluble proteins represented 21.43% and 21.64% of total extracted protein per g of dry weight; prolamins corresponded to 14.50% and 20.02% and glutelin accounting for the 64.06% and 58.33% on Xiumai 3 and 92-11 barley cultivars, respectively.

Nonetheless, comparing data obtained from these reports is a great challenge in view of the multiplicity of factors affecting the protein composition in barley grains, including genotype, environmental fluctuations (especially nitrogen and sulfur nutrition) (SHEWRY, 2007) and the extraction/quantification protein methods used. Therefore, we suggest that the results of protein fractions distribution alone should be analyzed carefully, and that further analysis must follow these results, and be taken into consideration for conclusions. Moreover, once hordeins is the major determinant of barley grain quality due to their essential amino acid deficiencies (especially lysine, methionine and threonine) (SHEWRY, 2011), we suggest the employment of direct methods of protein extraction for hordeins, which has been proved to solubilize up to 90% of the total hordeins, when focusing on hordein characterization.

The analysis of amino acid composition of protein fractions can be a powerful tool on protein characterization and complement protein distribution data. It is known that considerable difference in amino acid composition of different protein fractions exist (IVANKO, 1971) depending directly of which protein composes the respective fraction. For example, prolamin amino acid composition is characterized by high glutamine and proline contents and very low contents of essential amino acids, especially lysine, methionine and threonine, once the polypeptides of all hordein (prolamin) consist primarily of these two amino acids and few residues of essential amino acids (SHEWRY et al., 1986). Besides that,

although all hordein subgroups (B-, C-, D- and γ-hordeins) are rich in glutamine and proline, differences on the proportion of these two amino acids and other exist among them (SHEWRY; HALFORD, 2002; GUBATZ; SHEWRY, 2010). On the other hand, salt soluble proteins and glutelins contain similar amino acid composition characterized to be more balanced from the nutritional point of view.

In the present study, the amino acid composition of each protein extract was accessed and the results are presented in Table 2.3. In agreement with literature data, the E_3 protein extract, which is supposed to comprise mainly hordeins exhibited the lowest content of lysine and other essential amino acids. However, divergent results were obtained for E_1+E_2 (salt soluble proteins) and E_5 (glutelins), wherein E_5 contained the highest amount of all amino acids compared to E_1+ E_2 (Table 2.3).

The high proportion of E_5 (glutelin) on total grain protein distribution (Table 2.2) and the highest amino acid content compared to other fractions (Table 2.3) suggest that not only true glutelins were solubilized in the E_5, but that the glutelin fraction is composed by a complex mixture of proteins, whose many components are structurally related to hordeins (NEWMAN; NEWMAN, 1992). The last methodology employed to separate prolamin and glutelin comprised an aqueous-alcohol solution (usually 55% of 2-propanol) containing a reducing agent (usually β-mercaptoethanol), being the remained proteins soluble in dilute acid or alkali termed glutelins (SHEWRY; HALFORD, 2002). However, according to our shotgun results, these extraction buffers do not properly separate these fractions.

Therefore, the label free spectral counting shotgun technique have been applied in the present study to MS identify and relatively quantify the protein abundance in the modified barley grain protein fractions (E_1, E_2, E_3, E_4 and E_5) (Table 2.4-2.8). Although the technique employed in this study was able to produce a list of identified proteins and their relative abundancy, the real picture of their absolute amount remains uninvestigated since it is required a more sophisticated quantification MS method. Recently the introduction of a spiked amount of a known protein standard in the trypsin/chymotrypsin digest has been used for absolute quantification in the shotgun proteomics identification techniques (CHRISTENSEN et al., 2014). Therefore, we suggest that in future implementation of this modified Osborne fractionation, the absolute quantification could solve the problem of identification and quantification of the proteins in the different solubilized barley grain fractions. Apart from the absolute amount of each protein, the results unraveled the

composition of each protein fraction, which complement and might explain the results obtained in Tables 2.2 and 2.3.

Although the Osborne protein classification proved to be remarkable useful and still widely used, it is more usually today classified cereal grain proteins into three major groups according to their functions into storage proteins, metabolic and protective proteins, and structural proteins (SHEWRY; HALFORD, 2002). Thus, the Osborne salt soluble fraction (albumin and globulin) includes storage globulins and metabolic and protective proteins, whereas the prolamin family consists of storage hordeins and the glutelins is mainly related to structural proteins. Gubatz and Shewry (2010) listed the most abundant proteins in barley grains, which are storage hordeins, storage globulins, enzymes (β-amylase, peroxidase), enzyme inhibitors (CM proteins, chymotrypsin inhibitors, serpins, barley amylase/subtilisin inhibitor) and other small S-rich proteins (nonspecific lipid transfer proteins, hordoindolines, hordothionins), which were identified in the present study among all extracts analyzed (Tables 2.4-2.8).

As expected, the majority and the most relative abundant proteins identified in E_1 (salt soluble proteins), belong to the metabolic and protective protein groups and storage globulins (Table 2.4). However, even relatively less abundant, storage hordein (D- and B-hordein) and structural proteins (histone, ribosomal proteins) were also found.

Hordeins are readily soluble in aqueous alcohol solution (CELUS; BRIJS; DELCOUR, 2006), however Weiss, Postel and Görg (1992) showed by a 2-DE coupled with MS identification-approach that high-salt buffer extracts mainly albumin, globulin and also part of hordeins. One alternative suggested by Østergaard et al. (2002) to suppress the release of hordeins in the salt soluble fraction was the use of a low-salt solution. It has been demonstrated that the majority of hordeins is not readily soluble in this type of extraction buffer (FINNIE et al., 2002; ØSTERGAARD et al., 2002; FINNIE et al., 2004; ØSTERGAARD et al., 2004; FINNIE et al., 2006), however, Bak-Jensen et al. (2004) and Jin et al. (2012) identified B-hordein among salt soluble proteins, in agreement with the present study (Tables 2.4 and 2.5). On the other hand, at least in the light of our knowledge, there is no report in the literature reporting the presence of structural proteins, such as histone and ribosomal, in salt soluble fraction. Indeed, little information is found in the literature about these proteins in barley grains.

As a control experiment, a 1D SDS-PAGE gel excised band MS identification was performed with the same extract. In general, the band excision technique followed by MS identification is qualitative more than quantitative and the results overlap with the shotgun

technique, less laborious and faster. Figure 2.1A shows the protein profile of E_1 whereas Figure 2.2 is showing which bands were cut out from the gel and MS identified. The protein hits found per each band of E_1 is shown in Table 2.9. The majority of the band proteins identified was present in the shotgun protein list of E_1. The exceptions are mainly protein hits with low abundance (Table 2.9), confirming the shotgun experiment.

In E_2, which consists of the remaining proteins that belong to the salt soluble fraction, exhibited a similar list of proteins that was generated with less number of hits (Table 2.5), which was expected. Although structural proteins were not found in this extract, again storage hordeins were identified (Table 2.5). The lower number of identified proteins in E_2 is in agreement with the very low E_2 contribution on total protein content of the grains (Table 2.2). The similarity of amino acid composition of both extracts (Table 2.3) is due to the similarity of proteins composing these fractions. Although an extensive list of protein hits was generated by the excised band of the SDS-PAGE of E_2 (Table 2.10), the majority of the most abundant protein of each band was compatible to the shotgun list (Table 2.5).

The list generated by shotgun MS identification of E_3 is presented in Table 2.6. As expected the majority and the most abundant protein identified belonged to the hordein superfamily. Metabolic proteins related to salt soluble fraction were also found (β-amylase, guanylate-binding protein 4 and haem-peroxidase) (Table 2.6). Nowadays, the hordein superfamily is divided into four subgroups distinguishable by their electrophoretic mobility and amino acid composition, such as B-hordeins which account for 70-80%; C-hordeins 10-20%; D-hordeins 2-4% and γ-hordeins <5% of the total hordein fraction (KACZMARCZYK et al., 2012). However, some decades ago an extra hordein subgroup (A-hordeins) was also included. This subgroup was supposed to consist of low-molecular weight proteins, probably alcohol-soluble albumins or globulins or breakdown products of larger hordeins (BAXTER, 1981). Indeed, the hordein electrophoretic pattern is characterized by the presence of the low-molecular weight proteins (CELUS; BRIJS; DELCOUR, 2006), although the A-hordein nomenclature has not been used anymore. The identity of these proteins was revealed by SDS-PAGE band excision and MS identification (Figure 2.4 and Table 2.11). As expected, all proteins identified are low-molecular weight proteins and are related to salt soluble protein fraction, but no hordein fragment was detected. The most abundant protein in all three bands was α-amylase/trypsin inhibitor BDAI-1 (Table 2.11). These results were not in agreement with shotgun experiment (Table 2.6) due to the absence of the low-molecular weight proteins in the shotgun list and the absence of protein hits with β-amylase of the excised bands.

Apart from the low molecular weight proteins, the list of proteins identified by SDS-PAGE excise band technique is in agreement with the electrophoretic pattern of hordeins, distinguished by their molecular weight (SHEWRY; TATHAM; HALFORD, 2001). Besides that, in Figures 2.1C and 2.4, prolamins (E_3) SDS-PAGE of cv. Golden Promise versus antisense C-hordein barley lines (L1-5) shows the downregulation of C-hordeins as described by Lange et al. (2007) and Hansen et al. (2007).

Furthermore, our results provided the identification of individual isoforms of each hordein subgroup by SDS-PAGE, which can support more detailed hordein SDS-PAGE-based comparative studies. However, the handicaps of this technique must be taken in consideration, especially when C-hordein isoforms are concerned. It is thought that 20-30 genes per haploid genomes encoding C-hordeins (SHEWRY et al., 1985) exist, however, only a few full length sequences are available for C-hordeins in databanks, and there is a discrepancy in the molecular weights determined MS and those based on the sequences, indicating that the major expressed C-hordeins may not yet have been cloned and sequenced (TATHAM; SHEWRY, 2012). Therefore, we suggest that this information must be taken into consideration when analyzing the results presented in Table 2.6 and 2.11.

Interestingly, the most abundant proteins identified in E_4 are proteins which play storage functions (storage hordein and storage globulin) (Table 2.7). Metabolic and protective proteins were also found, but they were less abundant. Although, this fraction was related to prolamins in maize (LANDRY; DELHAYE; DAMERVAL, 2000), in barley only one subgroup of hordeins was found (D-hordein), suggesting that it is more related with salt soluble protein in barley, for two reasons: the list of proteins comprises a large number of soluble proteins (Table 2.7) and the amino acid composition (Table 2.3) showed high amounts of essential amino acids in comparison to E_3. It seems that the non-solubilized salt soluble proteins and hordeins in the previous steps of the sequential fractionation were able to solubilize in the presence of a reducing agent.

Finally, the list of proteins generated in E_5 is presented in Table 2.8. Proteins belonging to all Osborne fractions were found. With the exception of C-hordeins, all hordein subgroups were solubilized in E_5. Indeed, the majority of these proteins appeared in high abundance in this fraction, and can explain the low contribution of E_3 to total protein content, once they were able to solubilize only in the last step of the sequential extraction procedure. The mixture of proteins in E_5 was not a totally unexpected result, once glutelin is poorly understood. The poor solubility of glutelin components require extreme extraction conditions and powerful solvents, which often cause denaturation and even degradation of the proteins,

making this fraction hardly understood. Besides that, glutelin is the last fraction to be extracted, thus it is frequently affected by previous treatments and contaminated with residual proteins from other fractions, mainly hordeins (STEINER; GASTL; BECKER, 2011).

2.3 Conclusions

Although this modified method seemed to introduce a new redistribution of the barley grain proteins, their precise composition has been unraveled in this work by MS identification and their relative abundancy given for each fraction. Taken together the total protein determination of each fraction and the composition of the most abundant protein in each fraction we can conclude that this method can be applied to future differential proteomics applications of genetic modified barley plants in order to find out changes in their protein distribution and abundancy. Moreover, the SDS-PAGE excised band and MS identified prolamins and at a lesser extent of salt soluble proteins, can support SDS-PAGE-based comparative analysis for the most abundant proteins in each fraction.

References

ARRUDA, S.C.C.; BARBOSA, H.D.; AZEVEDO, R.A.; ARRUDA, M.A.Z. Two-dimensional difference gel electrophoresis applied for analytical proteomics: fundamentals and applications to the study of plant proteomics. **Analyst**, London, v. 136, n. 20, p. 4119-4126, 2011.

AZEVEDO, R.A.; DAMERVAL, C.; LANDRY, J.; LEA, P.J.; BELLATO, C.M.; MEINHARDT, L.W.; LE GUILLOUX, M.; DELHAYE, S.; TORO, A.A.; GAZIOLA, S. A.; BERDEJO, B.D.A. Regulation of maize lysine metabolism and endosperm protein synthesis by opaque and floury mutations. **European Journal of Biochemistry**, Berlin, v. 270, n. 24, p. 4898-4908, 2003.

BAGINSKY, S. Plant proteomics: Concepts, applications, and novel strategies for data interpretation. **Mass Spectrometry Reviews**, New York, v. 28, n. 1, p. 93-120, 2009.

BAK-JENSEN, K.S.; LAUGESEN, S.; ROEPSTORFF, P.; SVENSSON, B. Two-dimensional gel electrophoresis pattern (pH 6–11) and identification of water-soluble barley seed and malt proteins by mass spectrometry. **Proteomics**, Weinheim, v. 4, n. 3, p. 728-742, 2004.

BAXTER, E.D. Hordein in barley and mal - a review **Journal of the Institute of Brewing**, London, v. 87, n. 3, p. 173-176, 1981.

BOYE, J.; WIJESINHA-BETTONI, R.; BURLINGAME, B. Protein quality evaluation twenty years after the introduction of the protein digestibility corrected amino acid score method. **British Journal of Nutrition**, Cambridge, v. 108, n. Supplement S2, p. S183-S211, 2012.

BRADFORD, M.M. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. **Analytical Biochemistry**, New York, v. 72, n. 1/2, p. 248-254, 1976.

CELUS, I.; BRIJS, K.; DELCOUR, J.A. The effects of malting and mashing on barley protein extractability. **Journal of Cereal Science**, London, v. 44, n. 2, p. 203-211, 2006.

CHRISTENSEN, J.B.; DIONISIO, G.; POULSEN, H.D.; BRINCH-PEDERSEN, H. Effect of pH and recombinant barley (*Hordeum vulgare* L.) endoprotease B2 on degradation of proteins in soaked barley. **Journal of Agricultural and Food Chemistry**, Easton, v. 62, n. 34, p. 8562-8570, 2014.

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS (FAO). **FAOSTAT crop production and trade**. Disponível em:<http://faostat3.fao.org/browse/Q/QC/E>. Acesso em: 01 Jun. 2015.

FIDO, R.; MILLS, E.N.C.; RIGBY, N.; SHEWRY, P. Protein extraction from plant tissues. In: CUTLER, P. (Ed.). **Protein Purification Protocols**: Humana Press, 2004. chap. 3, p. 21-27.

FINNIE, C.; BAK-JENSEN, K.S.; LAUGESEN, S.; ROEPSTORFF, P.; SVENSSON, B. Differential appearance of isoforms and cultivar variation in protein temporal profiles revealed in the maturing barley grain proteome. **Plant Science**, Limerick, v. 170, n. 4, p. 808-821, 2006.

FINNIE, C.; MELCHIOR, S.; ROEPSTORFF, P.; SVENSSON, B. Proteome analysis of grain filling and seed maturation in barley. **Plant Physiology**, Washington, v. 129, n. 3, p. 1308-1319, 2002.

FINNIE, C.; STEENHOLDT, T.; RODA NOGUERA, O.; KNUDSEN, S.; LARSEN, J.; BRINCH-PEDERSEN, H.; BACH HOLM, P.; OLSEN, O.; SVENSSON, B. Environmental and transgene expression effects on the barley seed proteome. **Phytochemistry**, New York, v. 65, n. 11, p. 1619-1627, 2004.

FOUNTOULAKIS, M.; LAHM, H. W. Hydrolysis and amino acid composition analysis of proteins. **Journal of Chromatography A**, Amsterdam, v. 826, n. 2, p. 109-134, 1998.

GERHARDT, P. **Methods for general and molecular bacteriology**. 2nd . ed.: American Society for Microbiology, 1994. 791p.

GUBATZ, S.; SHEWRY, P.R. The development, structure, and composition of the barley grain. In: ULLRICH, S.E. (Ed.). **Barley:** production, improvement, and uses. Oxford: Wiley-Blackwell, 2010. chap. 13, p. 391-448.

HANSEN, M.; LANGE, M.; FRIIS, C.; DIONISIO, G.; HOLM, P.B.; VINCZE, E. Antisense-mediated suppression of C-hordein biosynthesis in the barley grain results in correlated changes in the transcriptome, protein profile, and amino acid composition. **Journal of Experimental Botany**, Oxford, v. 58, n. 14, p. 3987-3995, 2007.

HELM, C.V.; DE FRANCISCO, A.; GAZIOLA, S.A.; FORNAZIER, R.F.; POMPEU, G. B.; AZEVEDO, R.A. Hull-less barley varieties: storage proteins and amino acid distribution in relation to nutritional quality. **Food Biotechnology**, New York, v. 18, n. 3, p. 327-341, 2005.

IVANKO, S. Changeability of protein fractions and their amino acid composition during maturation of barley grain. **Biologia Plantarum**, Praha, v. 13, n. 3, p. 155-164, 1971.

JIN, Z.; MU, Y.-W.; SUN, J.-Y.; LI, X.-M.; GAO, X.-L.; LU, J. Proteome analysis of metabolic proteins (pI 4–7) in barley (*Hordeum vulgare)* malts and initial application in malt quality discrimination. **Journal of Agricultural and Food Chemistry**, Easton, v. 61, n. 2, p. 402-409, 2012.

KACZMARCZYK, A.; BOWRA, S.; ELEK, Z.; VINCZE, E. Quantitative RT-PCR based platform for rapid quantification of the transcripts of highly homologous multigene families and their members during grain development. **BMC Plant Biology**, London, v. 12, n. 1, p. 184, 2012.

KIRKMAN, M.A.; SHEWRY, P.R.; MIFLIN, B.J. The effect of nitrogen nutrition on the lysine content and protein composition of barley seeds. **Journal of the Science of Food and Agriculture**, London, v. 33, n. 2, p. 115-127, 1982.

LANDRY, J.; DELHAYE, S.; DAMERVAL, C. Improved method for isolating and quantitating alpha-amino nitrogen as nonprotein, true protein, salt-soluble proteins, zeins, and true glutelins in maize endosperm. **Cereal Chemistry**, Saint Paul, v. 77, n. 5, p. 620-626, 2000.

LANGE, M.; VINCZE, E.; WIESER, H.; SCHJOERRING, J.K.; HOLM, P.B. Suppression of C-hordein synthesis in barley by antisense constructs results in a more balanced amino acid composition. **Journal of Agricultural and Food Chemistry**, Easton, v. 55, n. 15, p. 6074-6081, 2007.

NEWMAN, C.W.; NEWMAN, R.K. Nutritional aspects of barley seed structure and composition. In: SHEWRY, P. R. (Ed.). **Barley**: genetics, biochemistry, molecular biology and biotechnology Wallingford: CBA International, 1992. chap. 17, p. 351-368.

OSBORNE, T.B. **The vegetable proteins**. London: Longmans, Green , 1909. 152 p. (Monographs on Biochemistry).

ØSTERGAARD, O.; FINNIE, C.; LAUGESEN, S.; ROEPSTORFF, P.; SVENNSON, B. Proteome analysis of barley seeds: identification of major proteins from two-dimensional gels (pI 4–7). **Proteomics**, Weinheim, v. 4, n. 8, p. 2437-2447, 2004.

ØSTERGAARD, O.; MELCHIOR, S.; ROEPSTORFF, P.; SVENSSON, B. Initial proteome analysis of mature barley seeds and malt. **Proteomics**, Weinheim, v. 2, n. 6, p. 733-739, 2002.

PHIZICKY, E.; BASTIAENS, P.I.H.; ZHU, H.; SNYDER, M.; FIELDS, S. Protein analysis on a proteomic scale. **Nature**, London, v. 422, n. 6928, p. 208-215, 2003.

SCHÄGGER, H.; VON JAGOW, G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. **Analytical Biochemistry**, New York, v. 166, n. 2, p. 368-379, 1987.

SCHULZE, W.X.; USADEL, B. Quantitation in mass-spectrometry-based proteomics. **Annual Review of Plant Biology**, Palo Alto, v. 61, p. 491-516, 2010.

SHEWRY, P.; TATHAM, A.; HALFORD, N. Nutritional control of storage protein synthesis in developing grain of wheat and barley. **Plant Growth Regulation**, New York, v. 34, n. 1, p. 105-111, 2001.

SHEWRY, P.R. Improving the protein content and composition of cereal grain. **Journal of Cereal Science**, London, v. 46, n. 3, p. 239-250, 2007.

SHEWRY, P.R. Effects of nitrogen and sulfur nutrition on grain composition and properties of wheat and related cereals. In: HAWKESFORD, M. J.; BARRACLOUGH, P. (Ed.). **The Molecular and Physiological Basis of Nutrient Use Efficiency in Crops**. Chichester: Wiley-Blackwell, 2011. chap. 6, p. 103-120.

SHEWRY, P.R.; BUNCE, N.A.C.; KREIS, M.; FORDE, B.G. Polymorphism at the *Hor1* locus of barley (*Hordeum vulgare* L.). **Biochemical Genetics**, New York, v. 23, n. 5/6, p. 391-405, 1985.

SHEWRY, P.R.; FRANKLIN, J.; PARMAR, S.; SMITH, S. J.; MIFLIN, B.J. The effects of sulphur starvation on the amino acid and protein compositions of barley grain. **Journal of Cereal Science**, London, v. 1, n. 1, p. 21-31, 1983.

SHEWRY, P.R.; HALFORD, N.G. Cereal seed storage proteins: structures, properties and role in grain utilization. **Journal of Experimental Botany**, Oxford, v. 53, n. 370, p. 947-958, 2002.

SHEWRY, P.R.; SMITH, S.J.; LEW, E. J.-L.; KASARDA, D.D. Characterization of prolamins from meadow grasses: homology with those of wheat, barley and rye. **Journal of Experimental Botany**, Oxford, v. 37, n. 5, p. 633-639, 1986.

SILVA, F.; NOGUEIRA, L.C.; GONÇALVES, C.; FERREIRA, A.A.; FERREIRA, I.M.P. L.V.O.; TEIXEIRA, N. Electrophoretic and HPLC methods for comparative study of the protein fractions of malts, worts and beers produced from Scarlett and Prestige barley (Hordeum vulgare L.) varieties. **Food Chemistry**, London, v. 106, n. 2, p. 820-829, 2008.

STEINER, E.; GASTL, M.; BECKER, T. Protein changes during malting and brewing with focus on haze and foam formation: a review. **European Food Research and Technology**, Berlin, v. 232, n. 2, p. 191-204, 2011.

TATHAM, A.S.; SHEWRY, P.R. The S-poor prolamins of wheat, barley and rye: revisited. **Journal of Cereal Science**, London, v. 55, n. 2, p. 79-99, 2012.

VANDERSCHUREN, H.; LENTZ, E.; ZAINUDDIN, I.; GRUISSEM, W. Proteomics of model and crop plant species: Status, current limitations and strategic advances for crop improvement. **Journal of Proteomics**, Amsterdam, v. 93, n. 0, p. 5-19, 2013.

VILHENA, M.B.; FRANCO, M.R.; SCHMIDT, D.; CARVALHO, G.; AZEVEDO, R.A. Evaluation of protein extraction methods for enhanced proteomic analysis of tomato leaves and roots. **Anais da Academia Brasileira de Ciencias**, Rio de Janeiro, in press.

WANG, J.; CHEN, J.; DAI, F.; WU, F.; YANG, J.; ZHANG, G. Protein fractions in barley grains as affected by some agronomic factors and their relationships to malt quality. **Cereal Research Communications**, Szeged, v. 35, n. 1, p. 129-140, 2007.

WEISS, W.; POSTEL, W.; GÖRG, A. Application of sequential extraction procedures and glycoprotein blotting for the characterization of the 2-D polypeptide patterns of barley seed proteins. **Electrophoresis**, Weinheim, v. 13, n. 1, p. 770-773, 1992.

WILKINS, M.R.; PASQUALI, C.; APPEL, R.D.; OU, K.; GOLAZ, O.; SANCHEZ, J.-C.; YAN, J.X.; GOOLEY, A.A.; HUGHES, G.; HUMPHERY-SMITH, I.; WILLIAMS, K.L.; HOCHSTRASSER, D.F. From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and amino acid analysis. **Nature Biotechnology**, London, v. 14, n. 1, p. 61-65, 1996.

ZHANG, Z.; WU, S.; STENOIEN, D. L.; PAŠA-TOLIĆ, L. High-throughput proteomics. **Annual Review of Analytical Chemistry**, Washington, v. 7, n. 1, p. 427-454, 2014.

3 PROTEOME ANALYSIS OF ANTISENSE C-HORDEIN MATURE BARLEY GRAINS

Abstract

Hordeins are the major storage proteins in barley grains and responsible for their low nutritional quality. Previously, generated antisense C-hordeins barley lines were shown to contain a more balanced amino acid composition and an altered storage protein profile. In the present study a two-dimensional gel electrophoresis (2-DE) based proteomic approach in combination with mass spectrometry was used to identify changes in the protein profile of non-storage proteins (salt soluble fraction) in the antisense C-hordein barley lines (L1, L2 and L3) and map differentially expressed proteins compared with the non-transgenic control (*Hordeum vulgare* cv. Golden Promise). Besides that, we also wanted to correlate protein changes with the more balanced amino acid composition of these lines, especially with respect to lysine content. The results revealed that the suppression of C-hordeins does not exclusively affect hordein synthesis and accumulation. Therefore, the more balanced amino acid composition of the transgenic lines L1, L2 and L3 is an indirect result of profound alterations also in the pattern of non-storage proteins. The changes observed included the upregulation of proteins involved in stress and detoxification (L1) and defence system (L2 and L3), as well as storage globulins (L1, L2 and L3). At a lesser extent, proteins involved in grain metabolism were also changed. Thus, the increased essential amino acids content may be a consequence of distinct protein sources among the three antisense C-hordeins lines analyzed, although the upregulation of lysine-rich proteins is consistently observed in all lines.

Keywords: Two-dimensional electrophoresis; Mass spectrometry; Non-storage proteins; Lysine rich-proteins

3. 1 Introduction

Barley is the fourth most important cereal crop worldwide, used mainly for animal feed (ca. two-thirds of harvested grains), for malting in the alcoholic beverage industry (ca. one-third) and directly for human food (only 2%) (DAY, 2013). The traits that determine barley grain quality vary according to the end use purposes. Physical aspects (e.g. color, weight, size, hardness, hull content and so forth) and chemical composition (e.g. carbohydrates, non-starch polysaccharides, amino acids, fiber, protein, fat, minerals and vitamins) are the major features evaluated in barley grains used for feed (BLEIDERE; GAILE, 2012). Despite the fact that barley is an excellent source of energy, the grains also constitute an important protein source (HELM et al., 2005; SCHMIDT et al., 2015). Therefore, protein quality, which is determined basically by its amino acid composition, is one of the most concerned aspects of barley grains for feed, once monogastric animals are not capable to synthesize essential amino acids, which must be acquired from diet (AZEVEDO; LANCIEN; LEA, 2006; WU et al., 2013).

Such as for other cereals, barley grain proteins are classified according to their solubility in water (albumin), salt (globulin), aqueous alcohol (prolamins) and dilute acid or alkali (glutelins) (SHEWRY; HALFORD, 2002). Alternatively, grain proteins have been classified according to their function into metabolic and protective, storage and structural proteins (GUBATZ; SHEWRY, 2010). The prolamins, named hordeins in barley, which exert storage functions, account for ca. 50% of the total grain protein and are responsible for the low nutritional quality of the barley grain for feeding purposes, once they are characterized by excess proline and glutamine and only traces of essential amino acids in their sequences, especially lysine and the sulfur-amino acids methionine and cysteine (TATHAM; SHEWRY, 2012). Although all hordeins share some of these features, they are further classified into four subgroups accordingly to their molecular mass and amino acids composition into: sulfur-rich (B-,γ-hordeins), sulfur-poor (C-hordeins) and high molecular mass proteins (D-hordeins).

In a successful attempt to improve nutritional quality of barley grains for feed, Lange et al. (2007) produced a more balanced amino acid composition barley grains through the expression of a C-hordein gene in the antisense orientation in order to suppress the poorest storage protein subgroup from the nutritional point of view. Besides the impact on barley grain quality for end-use, these antisense C-hordein barley lines are valuable tools for genetic and biochemistry studies, especially grain protein accumulation (HANSEN et al., 2007) and amino acid metabolism (SCHMIDT et al., 2015). The antisense C-hordein barley lines exhibited an altered quantitative protein profile in comparison to the non-transgenic control cv. Golden Promise, but such differences were not limited to the storage proteins, but also affected others with protective and metabolic (albumins and globulins) as well as structural (glutelins) functions (LANGE et al., 2007).

Apart from the original studies with natural and induced mutants for a number of cereal crops (BRIGHT; MIFLIN; ROGNES, 1982; FALCO et al., 1995; BRENNECKE et al., 1996; GAZIOLA et al., 1999; AZEVEDO, 2002; AZEVEDO et al., 2003; AZEVEDO et al., 2004a; AZEVEDO et al., 2004b), the proteome rebalancing induced by mutations or the introduction of transgenes that suppress or knockdown one or more storage proteins in grains have been exploited to produce heterologous proteins in plants (STREATFIELD, 2007). However, the expression of a foreign protein or a suppression of intrinsic proteins can cause changes in grain metabolism and in the grain proteome due to changes of source-sink relation and also because of the insertion site into the genome (FINNIE et al., 2004a). Therefore, to understand the proteome rebalancing in transgenic grains it is important to drive genetic manipulation avoiding unpredictable outcome (SCHMIDT; HERMAN, 2008).

The field of proteomics has been generating a large amount of valuable information in general (BARBOSA et al., 2012; ARRUDA et al., 2013; VIDAL et al., 2015), including on cereal grain metabolism, remarkably in barley. A number of studies including protein accumulation during grain filling and maturation in the whole grain and in dissected tissues (aleurone layer, starch endosperm and embryo) (FINNIE et al., 2002; FINNIE et al., 2004b), responses to environmental and genetic fluctuations (FINNIE et al., 2004a; YANG et al., 2010; EGGERT; PAWELZIK, 2011) as well as to elucidate the relation between protein and grain quality for different end-use purposes (ØSTERGAARD et al., 2002; BAK-JENSEN et al., 2004; ØSTERGAARD et al., 2004; FINNIE et al., 2006; JIN et al., 2012) are just some examples.

Two-dimensional gel electrophoresis (2-DE) was the first tool employed in proteomics to visualize and compare complex mixtures of proteins (VILHENA et al., in press). Since the first remarkable paper using 2-DE was published (O'FARRELL, 1975), a wide range of procedure improvements have been done. Although some drawbacks still remains unsolved, to cite, the low efficiency to analyze hydrophobic proteins and the high sensitivity to great range of protein amount distribution, i.e. low abundance proteins are masked spatially in the gel for high abundance proteins (RABILLOUD et al., 2010), its advantages prevails. The robustness, parallelism and its unique capability to analyze intact proteins at high resolution make this technique widely spread (RABILLOUD et al., 2010). Hence, when coupling with mass spectrometry it becomes a powerful tool to resolve an enormous variety of biological questions (SCHUCHARDT; SICKMANN, 2007; MATAVELI; ARRUDA, 2014).

In the present study a 2-DE-based proteomic approach in combination with mass spectrometry was used to address questions on the association of non-storage proteins (albumin and globulin) and storage proteins profile front of a grain protein balance perturbation, in this case the suppression of C-hordeins in barley. The main objective was to visualize the changes in the protein spot pattern of non-storage proteins in the antisense C-hordein barley lines, to identify differentially abundant proteins in comparison to the non-transgenic control, and to relate them to the more balanced amino acid composition of these transgenic lines, especially the lysine content. In addition, we also aimed to point out candidates, e.g. upregulated lysine-rich proteins, which could be used for further improvement of nutritional quality of barley grains. In order to achieve these goals, non-storage proteins extractable in salt solution buffer (albumins and globulins) were extracted from the whole mature barley grains of three selected antisense C-hordein lines (L1, L2 and L3) due to its

representative genetic characteristics among all five lines, i.e, L1 contains a single locus integration site, L2, two integration sites and L3, four integration sites; and compared to the non-transgenic control proteins (*Hordeum vulgare* cv. Golden Promise).

3.2 Development

3.2.1 Material and Methods

3.2.1.1 Biological material, growth conditions and experiment design

Mature barley (*Hordeum vulgare*) grains of cv. Golden Promise (non-transgenic control) and three antisense C-hordein lines named AsHorC L1-3 (LANGE et al., 2007), were kindly provided by M. Lange (Center for Biological Sequence Analysis, BioCentrum, Technical University of Denmark). Plants were growth in 20-liters-pots in triplicate (three plants per pot) containing soil and 1 g of chemical fertilizer (NPK, 10-10-10), under natural daylight conditions during the autumn/winter season of 2010 in a glasshouse in Piracicaba, São Paulo, Brazil. After sowing, a total of 2 g ammonium sulfate was applied in two periods (40 and 55 days after sowing). Grains from three plants per pots were harvested at maturity and mixed.

3.2.1.2 Mature barley grains protein extraction

The protein extraction for 2-DE was carried out following the procedure described by Eggert and Pawelzik (2011) with some modifications. The whole mature barley grains were ground in a sample mill until a fine and homogeneous flour was obtained. Flour samples (100 mg) were treated with 1 ml of diethyl ether for 15 min to solubilized lipids, which were discarded after centrifugation at 14,000*g* for 10 min. The samples were dried out in vacuum to remove any residual ether. Ninety mg of delipidate flour was weighted and the protein extraction was procedure by the addition of 4.5 ml of 50 mM Tris-HCl pH 7.4 extraction buffer containing 50 mM NaCl, 1% (v/v) tween-80 and 200 mM β-mercaptoethanol. The proteins were solubilized by 30 min of continuous agitation at 37 ^{o}C and the supernatant recovered by centrifugation at 14,000*g* for 20 min at room temperature. In a new container the proteins were precipitated by the addition of 18 ml of ice cold-ethanol followed by 2 h of incubation at -20 ^{o}C. The precipitated protein was recovery by centrifugation at 10,000*g* for 20 min at 4 ^{o}C. After two days in vacuum, the dried pellet was re-dissolved in 500 µl of

solubilization buffer containing 7 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.5% (v/v) Triton X-100 and 50 mM dithiothreitol (DTT). The samples were placed in an ultrasonic bath for 10 min to improve the protein solubilization.

The protein concentration was determined by Bradford method (BRADFORD, 1976), using a bovine serum albumin (BSA) standard curve.

3.2.1.3 Two-dimensional electrophoresis

The Ettan IPGphor 3 IEF system (GE Healthcare) was employed for isoelectric focusing (IEF) with immobilized pH gradient (IPG) strips (Immobiline DryStrip gels, GE Healthcare) (pH 3.0-10.0, non-linear gradient, 18 cm). A final volume of 375 µl of solubilization buffer containing 0.8% (v/v) of IPG buffer and 0.002% (w/v) bromophenol blue (BPB) and 800 µg of protein was applied onto the IPG strip.

The strips were submitted to a rehydration step for 4 h at 20 ^{o}C before the IEF, which was carried out following the voltage settings: 30 V for 12 h, 100 V for 1 h, 200 V for 1 h, 400 V for 1 h, 700 V for 1 h, 1.000 V for 1 h, 5.000 V for 10 h, 8.000 V for 4 h and 100 V for 3 h.

After IEF, the proteins in the strips were reduced with 6 ml of equilibration buffer (75 mM Tris-HCl pH 8.8, 8 M urea, 30% (v/v) glycerol, 2% (w/v) sodium dodecyl sulphate (SDS) and 0.002% (w/v) BPB) containing 1% (w/v) DTT for 15 min at room temperature, and then incubated with the same equilibration buffer containing 2.5% (w/v) iodoacetamide (IAA) in the same conditions above, for protein alkylation.

The denatured proteins in the strip were separated in the second dimension by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12.5% polyacrylamide gel, at 15 mA per gel for 1 h and then 25 mA per gel for around 5 h in a 25 mM Tris-HCl pH 8.8 running buffer containing 192 mM glycine and 0.1% (w/v) SDS.

After separation, the gels were stained with colloidal coomassie brilliant blue (cCBB) G-250 according to manufacturer´s recommendations, and scanned using an image scanner with the LabScan™ V. 5.0 software (Amersham Biosciences).

3.2.1.4 Software-based evaluation of differentially abundant proteins

The gel images were imported as MEL file to ImageMaster 2D Platinum 7.0 software for spot detection, background subtraction, spot matching and spot normalization (protein

quantification). The parameters contrast, smooth, saliency and minimum area were subjected to an adjustment for automatic spot detection, matching and normalization. Artifacts, e.g. false and faint spots as well as mismatching among replicates, were checked manually for higher accuracy of the results. Spot quantification was determined automatically by the ImageMaster algorithm using the parameters intensity, area and volume. The protein amount was expressed as %Vol, which is a normalized value considering the total volume over all the spots in the image (individual spot volume/sum of all spot volumes), which avoid protein loading and staining variations.

The analyses were performed through a set of pairs comprising the comparison between the non-transgenic control and each transgenic line (L1, L2 and L3) independently.

One-way analysis of variance (ANOVA) was used to analyze differences in protein abundance between samples of gels, and to draw conclusions about the significance of the protein abundance changes, i.e., the software calculated the probability of observing a certain difference between sample means be real or a coincidence of random sampling. Thus, the one-way ANOVA tested the null hypothesis that all populations have identical means and generated a P value based on the same assumptions as the t-test. Therefore, it was considered differentially abundant spots those with P value < 0.05 and a minimum change of 50% compared with the non-transgenic control.

3.2.1.5 In-gel digestion for mass spectrometry

In this step all solutions employed were freshly prepared with mass spectrometry grade chemicals and sterilized purified water. Glassware and containers were brand new, rinsed with methanol and exclusively used for mass spectrometry analyses.

The protocol for in-gel digestion described by Shevchenko et al. (2006) was adopted with some modifications. Differentially abundant spots were excided from the gels and destained with 500 µl of a destain solution containing 50% (w/v) methanol and 2.5% (v/v) acetic acid for 2 h at room temperature. After incubation the destain solution was removed and the procedure repeated. The destained spots were dehydrated twice with 200 µl of 100% acetonitrile (ACN) for 5 min at room temperature. Dehydrated spots were reduced with 10 mM DTT followed by an alkylation with 50 mM IAA for 30 min at room temperature for each step. The spots were washed with 100 mM ammonium bicarbonate followed by a dehydration step with 200 µl of 100% ACN. This procedure was repeated twice. The spots were dried in a vacuum concentrator (Thermo Scientific™ SpeedVac™) for 5 min to ensure complete removal of ACN. The spots were then treated with 30 µl (20 $ng.\mu l^{-1}$) of sequencing

grade modified trypsin (Promega) and rehydrated in an ice bath for 30 min. The digestion was performed overnight at 37 ^{o}C in a dry bath. Peptide extraction was performed with 10 µl of 5% (v/v) formic acid (FA) in purified water for 10 min at room temperature. After centrifugation, the supernatant was transferred to a new tube and another step of peptides extraction was performed with 12 µl of 5% (v/v) FA in 50% (v/v) ACN. The peptides from both extractions were mixed and concentrate in a vacuum concentrator for approximately 45 min and re-dissolved in 0.01% (v/v) FA for identification.

3.2.1.6 Protein identification

The samples were subjected to an ultra-performance liquid chromatography (NanoACQUITY UPLC®, Waters) coupled with nanoelectrospray source (ESI) on a hybrid quadrupole/time-of-flight (Quad-TOF) mass analyzer (Premier spectrometer, Waters).

The data were acquired using MassLynx™ software (version 4.1; Waters) in the data dependent acquisition (DDA) mode, wherein the three most intense peptides of each spectrum (MS) were selected for fragmentation (MS/MS). The raw data file acquired were converted to a peak list format (Mascot Generic File- mgf) using the Mascot Distiller software (version 2.3.2.0; Matrix Science). Protein identification was performed by searching the spectra (mgf) against the National Center for Biotechnology Information non-redundant sequence database (NCBInr_062013; 25877237 sequences; 8945087761 residues) with a specific taxonomy for green plants (*Viridiplantae*) (762307 sequences) using the Mascot software (version 2.3; Matrix Science). The MS/MS ion search was employed with the following parameters: monoisotopic mass value, unrestricted protein mass, peptide mass tolerance of ± 0.1 Da, fragment mass tolerance of ± 0.1 Da, maximum missed cleavage of 1, fixed protein modification included carbamidomethyl of cysteine and variable modification included oxidation of methionine.

3.2.2 Results

The 2-DE using a broad range pH 3.0-10.0 resolved a total of 294 spots in the reference gel of non-transgenic control with 93% of shared spots among the three replicates (Table 3.1). A similar number of spots, with averages of 266, 257 and 267 were obtained in the reference gel of L1, L2 and L3 transgenic lines, respectively (Table 3.1).

Table 3.1 - Comparative software-based analysis of two-dimensional gel electrophoresis (2-DE) (non-linear gradient pH 3.0 -10.0) between non-transgenic control cv. Golden Promise and antisense C-hordein barley lines (L1, L2 and L3). The results were statistically evaluated by analysis of variance (ANOVA). Differentially abundant spots were those with P value < 0.05 and a minimum change of 50% of protein quantity of its respective spot in the control line

	Total number of spots	Number of spots with P value < 0.05	Matches (%)	**Differentially abundant spots**			
				Upregulated	Downregulated	Exclusive	Total
L1	266	83	93	35	04	02	41
L2	257	110	95	37	13	05	55
L3	267	107	90	27	16	02	45

In the comparative software-based image analysis, the spots present in the antisense C-hordein lines that exhibited change of 50% higher (upregulated) or lower (downregulated) in protein quantity (%Vol) than the non-transgenic control were considered differentially abundant and cut out from the gel for protein identification by mass spectrometry. The one-way ANOVA between 2-DE gels of each antisense C-hordein line and the non-transgenic control revealed a total of 41, 55 and 45 differentially abundant spots (P value < 0.05 and a minimum change of 50% of protein quantity of its respective spot in the control line) in L1, L2 and L3, respectively (Table 3.1).

The reference gel of non-transgenic control and each antisense C-hordein line (L1, L2 and L3) is shown in Figure 3.1, indicating all spots cut out from the gels for protein identification. The differentially abundant proteins identified belonged to different proteins groups, which were classified into 6 categories according to their function: 1. storage, protein synthesis and amino acids metabolism; 2. glycolysis, starch metabolism and citric acid cycle; 3. defence proteins; 4. stress and detoxification; 5. chaperones or folding proteins and 6. other or undefined.

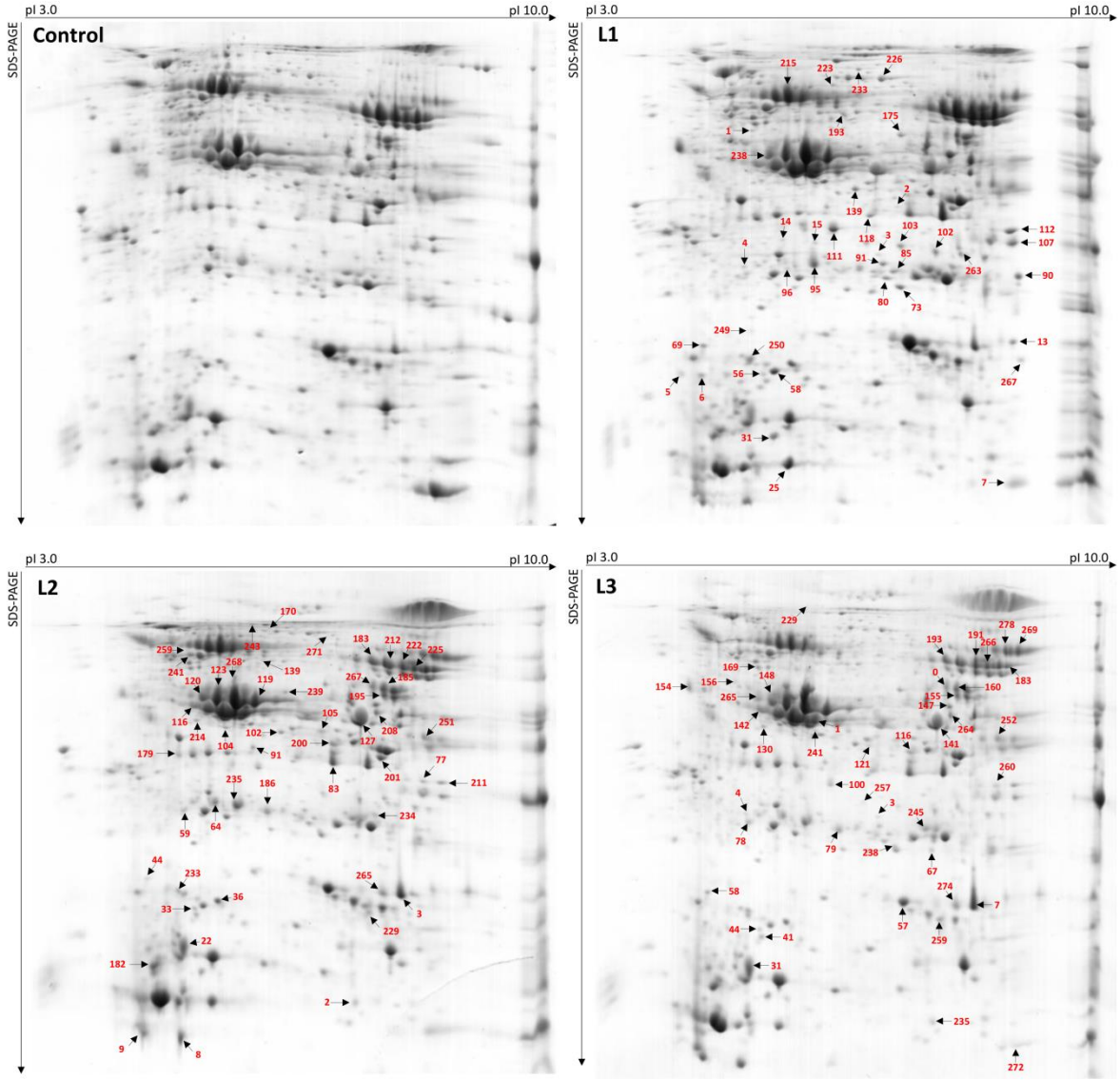

Figure 3.1 - Master gel representing the non-transgenic control and the spots differentially abundant and cut out from the gel for protein identification of antisense C-hordein line L1; L2 and L3. Each arrow indicates one spot labeled with its respective identification number (spot id) in the tail of the arrow. The spot id was independently numbered in each antisense C-hordein line through an independent comparative analysis with non-transgenic control cv. Golden Promise

The relative distribution of differently abundant proteins among the functional categories of each antisense C-hordein line is shown in Figure 3.2. The majority of the proteins of L1 (25.6%) belonged to the stress and detoxification protein category. For a large number of identified proteins in this line (23.1%) the functional annotation was not available or not defined. The proteins which play a role in carbohydrate metabolism represented 20.5% of the identified proteins in L1, while those involved in protein or amino acids metabolism, as well as storage proteins accounted for 17.9%. A minor percentage of the proteins belonged to defence proteins (7.7%) and chaperones or folding proteins (5.1%) (Figure 3.2).

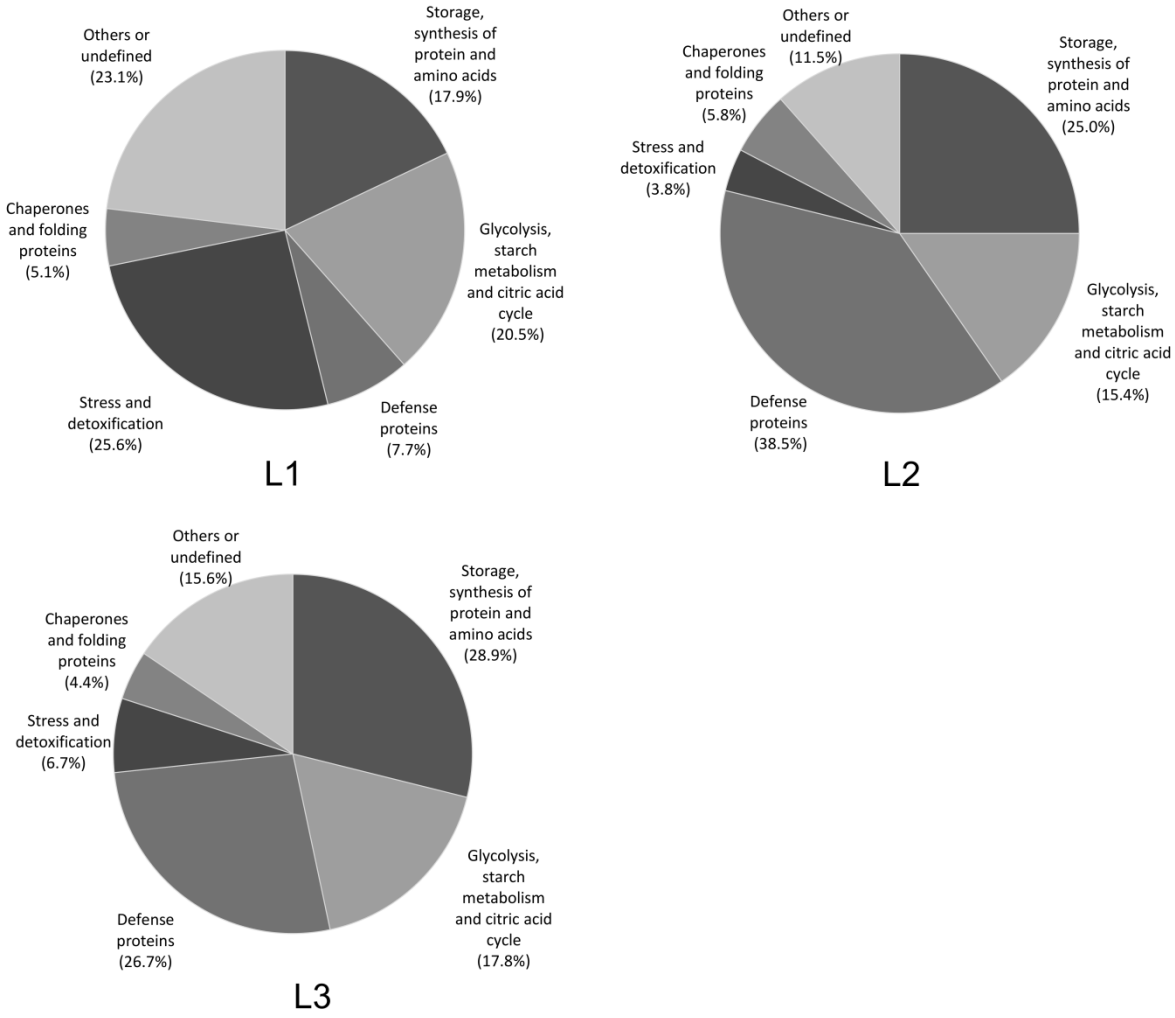

Figure 3.2 - Functional distribution of differently abundant proteins identified by mass spectrometry in antisense C-hordeins lines (L1, L2 and L3)

The relative distribution of identified proteins in L2 and L3 was very similar as can be observed in Figure 3.2. For both lines, the majority of the differently abundant proteins belonged to defence proteins group, including α-amylase, trypsin and chymotrypsin inhibitor and serpins (38.5% and 26.7% in L2 and L3, respectively) and those related to storage, protein and amino acids metabolism accounted to 25.0% and 28.9% in L2 and L3, respectively. Proteins involved in carbohydrate metabolism represented 15.4% (L2) and 17.8% (L3) of total identified proteins. The category which includes other or undefined function proteins corresponded for 11.5% (L2) and 15.6% (L3), and the group with minor representative proteins was stress and detoxification (3.8% and 6.7% in L2 and L3, respectively) and chaperones or folding proteins (5.8% and 4.4% in L2 and L3, respectively) (Figure 3.2).

The differently abundant protein identified by mass spectrometry in each antisense C-hordein lines and the most important characteristics of each protein are presented in Tables 3.2, 3.3 and 3.4 (L1, L2 and L3, respectively).

Table 3.2 - Proteins differentially abundant in antisense C-hordein line 1 (L1) compared with non-transgenic control cv. Golden Promise

(continue)

Spot Id	Ratio	P value	Identification	Organism	Accession n[o]	UniProtKB/ Swiss-Prot/ TrEMBL	pI[a]	Mw[a] (Kda)	Score	MP[b]
1	1.60	0.016	Os02g0196800 protein	*Oriza sativa* subsp. *japonica*	gi|115444831	Q6H7M1	5.62	47.10	159	7
2	2.00	0.009	Glucose and ribitol dehydrogenase homolog	*H. vulgare*	gi|7431022	T06212[c]	6.54	31.64	74	4
3	1.86	0.012	Proteasome subunit alpha type-4-2 (Os06g0167600)	*Oriza sativa* subsp. *japonica*	gi|115466646	Q5VRG3	6.44	27.02	325	11
4	2.18	0.003	Triosephosphate isomerase, cytosolic	*H. vulgare*	gi|2507469	P34937.3	5.39	26.73	66	1
5	2.07	0.001	Dehydrin	*Triticum durum*	gi|61657604	Q5CAQ2	4.78	16.25	114	3
6	1.84	0.047	Embryo globulin	*H. vulgare*	gi|167004	Q03678	6.80	72.25	154	3
13	2.71	<0.001	Dehydrin 7	*H. vulgare*	gi|296198	Q40043	8.00	14.11	117	2
14	1.89	0.004	Soluble inorganic pyrophosphatase	*H. vulgare*	gi|4033417	O23979.1	5.85	24.04	389	14
15	2.24	0.027	Gamma hydroxybutyrate dehydrogenase-like protein (Os02g0562700)	*O. sativa* subsp. *japonica*	gi|115446749	Q84VC8	6.18	30.49	189	3
25	1.57	0.004	Late embryogenesis abundant protein B19.1A	*H. vulgare*	gi|547817	Q05190.1	6.33	9.96	319	6
31	1.56	0.006	Glyceraldehyde-3-phosphate dehydrogenase 1, cytosolic	*H. vulgare*	gi|120680	P26517.1	6.67	36.51	140	2
56	1.78	0.002	Embryo globulin	*H. vulgare*	gi|167004	Q03678	6.80	72.25	167	6
58	1.60	0.001	Late embryogenesis abundant protein B19.4	*H. vulgare*	gi|547819	Q05191.1	5.58	16.89	189	4
69	1.98	<0.001	Hypothetical protein	*O. sativa* subsp. *japonica*	gi|22535646	Q8LHX6	6.75	15.84	86	6
73	1.94	0.003	manganese superoxide dismutase	*T. aestivum*	gi|1654387	P93606	7.89	25.29	648	20
80	1.69	0.029	Glutathione-S-transferase	*H. vulgare*	gi|6683765	Q9SES7	6.34	25.03	647	38
85	2.05	0.006	Glutathione-S-transferase, I subunit	*H. vulgare*	gi|21212950	Q8LPD5	5.86	23.48	484	42
90	2.43	0.001	Cyclophilin A-2	*T. aestivum*	gi|13925734	Q93XQ6	8.52	18.37	55	2
91	1.59	0.046	Glutathione transferase	*H. vulgare*	gi|18479038	Q8VWW3	5.82	24.90	476	29
102	1.73	0.035	Hypothetical protein SORBIDRAFT_01g005440	*Sorghum bicolor*	gi|242037831	C5WY16	6.31	51.49	96	6
103	1.70	0.001	Protein z-type serpin	*H. vulgare*	gi|1310677	P06293	5.61	43.22	99	4

Table 3.2 - Proteins differentially abundant in antisense C-hordein line 1 (L1) compared with non-transgenic control cv. Golden Promise

(continued)

Spot Id	Ratio	P value	Identification	Organism	Accession n[o]	UniProtKB/ Swiss-Prot/ TrEMBL	pI[a]	Mw[a] (Kda)	Score	MP[b]
107	1.87	0.002	No hits							
111	1.66	0.003	Lipoprotein-like (Os01g0749000)	*O. sativa* subsp. *japonica*	gi|115439929	Q94J20	7.79	28.05	62	1
112	1.74	0.015	Lipoprotein-like (Os01g0749000)	*O. sativa* subsp. *japonica*	gi|115439929	Q94J20	7.79	28.05	196	30
118	1.72	0.031	Glucose and ribitol dehydrogenase homolog	*H. vulgare*	gi|7431022	T06212[c]	6.54	31.64	158	5
139	1.55	0.020	Guanine nucleotide-binding protein subunit beta-like protein A (Os01g0686800)	*O. sativa* subsp. *japonica*	gi|115439261	P49027	5.97	36.23	155	6
175	1.77	0.005	Elongation factor 1 gamma-like protein	*O. sativa* subsp. *japonica*	gi|29367381	Q5Z627	6.11	47.07	287	10
193	1.66	0.019	Succinate semialdehyde dehydrogenase	*Z. mays*	gi|226529619	B6TPI6	7.56	56.17	357	13
223	1.80	0.012	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial (Os07g0134800)	*O. sativa* subsp. *japonica*	gi|115470493	Q6ZDY8	6.61	68.85	333	9
226	1.50	0.038	Malic enzyme Os01g0743500	*O. sativa* subsp. *japonica*	gi|115439879	Q5JKW5	6.50	64.26	351	12
233	2.05	0.012	OSJNBa0072D08.7 protein (Os04g0266900)	*O. sativa* subsp. *japonica*	gi|115457470	Q7XWP9	6.76	77.64	97	7
238	1.53	0.028	Protein z-type serpin	*H. vulgare*	gi|1310677	P06293	5.61	43.22	928	33
249	1.74	0.008	No hits							
250	2.49	0.001	Embryo globulin	*H. vulgare*	gi|167004	Q03678	6.80	72.25	54	4
263	1.68	0.011	Methionine synthase	*H. vulgare*	gi|50897038	Q6BCT3	5.67	84.50	125	2
7	-5.80	0.015	Chain A, non-specific lipid transfer protein 1 from barley in complex with L-alfa-lysophosphatidylcholine, laudoyl	*H. vulgare*	gi|47168353	1MID_A[d]	8.19	9.69	252	6
95	-2.19	0.020	Glutathione transferase	*H. vulgare*	gi|18479038	Q8VWW3	5.82	24.90	92	2
96	-2.76	0.003	Triosephosphate isomerase, cytosolic	*H. vulgare*	gi|2507469	P34937.3	5.39	26.73	136	5
215	-2.03	0.014	Beta-amylase	*H. vulgare*	gi|113786	P16098.1	5.58	59.64	396	19

Table 3.2 - Proteins differentially abundant in antisense C-hordein line 1 (L1) compared with non-transgenic control cv. Golden Promise

(conclusion)

Spot Id	Ratio	P value	Identification	Organism	Accession n[o]	UniProtKB/ Swiss-Prot/ TrEMBL	pI[a]	Mw[a] (Kda)	Score	MP[b]
267	-	<0.001	Predicted protein	*Micromonas pusilla* CCMP1545	gi|303280645	C1MV69	6.12	42.06	40	1
268	-	0.001	Aldose reductase	*H. vulgare*	gi|113595	P23901.1	6.51	35.80	373	8

[a] Theoretical pI and Mw calculated using the software compute pI/Mw available at http://www.expasy.org/
[b] Matched peptides
[c] Accession code for PIR databank. Protein not found at UniProtKB/Swiss-Prot/TrEMBL
[d] Accession code for PDB databank. Protein not found at UniProtKB/Swiss-Prot/TrEMBL

Table 3.3 - Proteins differentially abundant in antisense C-hordein line 2 (L2) compared with non-transgenic control cv. Golden Promise

(continue)

Spot Id	Ratio	P value	Identification	Organism	Accession n°	UniProtKB/ Swiss-Prot/ TrEMBL	pI[a]	Mw[a] (Kda)	Score	MP[b]
2	2.77	0.002	Trypsin inhibitor CMc	*H. vulgare*	gi|161784337	P34951.2	6.68	15.17	82	4
3	1.51	0.016	Bifunctional α-amylase/subtilisin inhibitor (BASI)	*H. vulgare*	gi|18916	P07596	7.77	22.24	237	14
8	2.54	<0.001	Subtilisin-chymotrypsin inhibitor CI-1B	*H. vulgare*	gi|124127	P16063.1	5.33	8.96	244	11
9	1.69	0.013	Subtilisin-chymotrypsin inhibitor CI-1B	*H. vulgare*	gi|124127	P16063.1	5.33	8.96	349	18
22	1.94	0.001	α-amylase inhibitor BMAI-1	*H. vulgare*	gi|2506771	P16968.2	5.36	15.81	225	19
33	2.22	0.001	Embryo globulin	*H. vulgare*	gi|167004	Q03678	6.80	72.25	241	9
36	1.65	0.003	Embryo globulin	*H. vulgare*	gi|167004	Q03678	6.80	72.25	398	44
44	1.52	0.004	Cold-regulated protein	*H. vulgare*	gi|10799810	Q9FSI8	4.93	17.61	61	2
59	2.04	0.004	Triosephosphate isomerase, cytosolic	*H. vulgare*	gi|2507469	P34937.3	5.39	26.73	784	22
64	2.77	0.002	27K protein	*T. aestivum*	gi|283480515	D2TE73	6.06	22.71	48	1
77	1.92	0.006	Lipoprotein-like (Os01g0749000 protein)	*O. sativa* subsp. *japonica*	gi|115439929	Q94J20	7.79	28.05	200	4
83	1.64	0.008	Glucose and ribitol dehydrogenase homolog	*H. vulgare*	gi|7431022	T06212[c]	6.54	31.64	302	21
91	1.56	0.039	Serpin-Z7	*H. vulgare*	gi|75282567	Q43492.2	5.45	42.82	409	16
102	1.64	0.033	Guanine nucleotide-binding protein subunit beta-like protein A (Os01g0686800)	*O. sativa* subsp. *japonica*	gi|115439261	P49027	5.97	36.23	237	6
104	2.49	0.001	Putative NAD-malate dehydrogenase	*O. sativa* subsp. *japonica*	gi|42407501	Q6YWL3	7.02	41.53	178	3
105	1.54	0.013	Putative ornithine carbamoyltransferase	*O. sativa* subsp. *japonica*	gi|41053142	Q6YVI0	6.37	39.66	64	2
116	2.14	0.017	Serpin-Z7	*H. vulgare*	gi|75282567	Q43492.2	5.45	42.82	425	12
119	1.87	0.006	Protein z-type serpin	*H. vulgare*	gi|1310677	P06293	5.61	43.22	421	19
120	2.21	0.001	Protein z-type serpin	*H. vulgare*	gi|1310677	P06293	5.61	43.22	748	23
123	1.56	0.032	Protein z-type serpin	*H. vulgare*	gi|1310677	P06293	5.61	43.22	540	32
139	2.10	0.033	Alanine aminotransferase 2	*H. vulgare*	gi|1703227	P52894.1	5.93	52.87	495	13

Table 3.3 - Proteins differentially abundant in antisense C-hordein line 2 (L2) compared with non-transgenic control cv. Golden Promise

(continued)

Spot Id	Ratio	P value	Identification	Organism	Accession n[o]	UniProtKB/ Swiss-Prot/ TrEMBL	pI[a]	Mw[a] (Kda)	Score	MP[b]
170	2.03	0.012	OSJNBa0072D08.7 protein (Os04g0266900)	*O. sativa* subsp. *japonica*	gi|115457470	Q7XWP9	6.76	77.64	228	7
179	1.70	0.022	hypothetical protein SORBIDRAFT_04g004700	*S. bicolor*	gi|242060654	C5XW38	6.11	38.97	61	2
182	1.64	0.002	trypsin/amylase inhibitor pUP13	*H. vulgare*	gi|225102	225102[e]	5.35	14.74	181	7
185	2.70	<0.001	Triticin	*T. aestivum*	gi|171027813	B2CGM5	6.37	64.88	153	7
186	2.15	0.002	Proteasome subunit beta type (Os08g0529100)	*O. sativa* subsp. *japonica*	gi|115477445	Q6ZIB4	5.81	23.81	104	2
195	1.92	0.004	HSP70	*H. vulgare*	gi|476003	Q40058	5.76	67.01	422	9
200	2.42	0.001	Chain A, Crystal Structure Of Barley Grain Peroxidase 1	*H. vulgare*	gi|157830301	1BGP_A[d]	6.51	33.82	1050	32
201	1.58	0.030	Peroxidase BP 1	*H. vulgare*	gi|167081	Q40069	7.57	38.82	354	10
208	2.35	0.003	Glyceraldehyde-3-phosphate dehydrogenase 1, cytosolic	*H. vulgare*	gi|120680	P26517.1	6.67	36.51	1100	45
214	2.12	0.003	Serpin-Z7	*H. vulgare*	gi|75282567	Q43492.2	5.45	42.82	473	14
217	1.76	0.047	Protein z-type serpin	*H. vulgare*	gi|1310677	P06293	5.61	43.22	165	20
233	1.84	0.032	Embryo globulin	*H. vulgare*	gi|167004	Q03678	6.80	72.25	237	4
235	1.87	0.005	Triosephosphate isomerase, cytosolic	*H. vulgare*	gi|2507469	P34937.3	5.39	26.73	80	1
239	2.03	0.039	Protein z-type serpin	*H. vulgare*	gi|1310677	P06293	5.61	43.22	420	10
241	1.67	0.026	Enolase 2, putative, expressed (Os03g0248600 protein)	*O. sativa* subsp. *japonica*	gi|115451911	Q10P35	5.32	47.97	982	22
243	2.18	0.002	Methionine synthase	*H. vulgare*	gi|50897038	Q6BCT3	5.67	84.50	1080	32
127	-2.40	0.016	Protein z-type serpin	*H. vulgare*	gi|1310677	P06293	5.61	43.22	100	3
183	-2.19	<0.001	Serpin-Z4	*H. vulgare*	gi|131091	P06293.2	5.72	43.27	173	30
211	-2.06	0.051	Lipoprotein-like (Os01g0749000)	*O. sativa* subsp. *japonica*	gi|115439929	Q94J20	7.79	28.05	63	1
212	-2.15	0.004	Hypothetical protein SORBIDRAFT_01g005440	*S. bicolor*	gi|242037831	C5WY16	6.31	51.49	100	4

Table 3.3 - Proteins differentially abundant in antisense C-hordein line 2 (L2) compared with non-transgenic control cv. Golden Promise

(conclusion)

Spot Id	Ratio	P value	Identification	Organism	Accession n°	UniProtKB/ Swiss-Prot/ TrEMBL	pI[a]	Mw[a] (Kda)	Score	MP[b]
222	-3.01	0.039	Hypothetical protein SORBIDRAFT_01g005440	*S. bicolor*	gi|242037831	C5WY16	6.31	51.49	92	2
225	-4.47	0.012	Hypothetical protein SORBIDRAFT_01g005440	*S. bicolor*	gi|242037831	C5WY16	6.31	51.49	100	1
229	-2.57	0.001	No hits							
234	-2.26	0.014	No hits							
251	-2.23	0.018	Hypothetical protein SORBIDRAFT_01g005440	*S. bicolor*	gi|242037831	C5WY16	6.31	51.49	74	1
252	-2.06	0.038	Cytosolic 3-phosphoglycerate kinase	*H. vulgare*	gi|28172913	Q850M2	5.05	31.32	137	6
255	-3.22	<0.001	Protein z-type serpin	*H. vulgare*	gi|1310677	P06293	5.61	43.22	233	7
256	-3.87	0.013	Protein disulfide-isomerase	*H. vulgare*	gi|1709617	P80284.2	5.02	56.46	105	4
259	-3.10	0.010	Xylose isomerase	*H. vulgare*	gi|1296807	Q40082	5.34	53.58	224	6
265		<0.001	Bifunctional α-amylase/subtilisin inhibitor	*H. vulgare*	gi|18916	P07596	7.77	22.24	258	10
267		<0.001	Triticin	*T. aestivum*	gi|171027813	B2CGM5	6.37	64.88	151	7
268		0.009	Protein z-type serpin	*H. vulgare*	gi|1310677	P06293	5.61	43.22	1270	55
271		0.002	No hits							
273		0.003	Elongation factor 2 (Os02g0519900)	*O. sativa* subsp. *japonica*	gi|115446385	Q6H4L2	5.85	94.02	323	13

[a] Theoretical pI and Mw calculated using the software compute pI/Mw available at http://www.expasy.org/
[b] Matched peptides
[c] Accession code for PIR databank. Protein not found at UniProtKB/Swiss-Prot/TrEMBL
[d] Accession code for PDB databank. Protein not found at UniProtKB/Swiss-Prot/TrEMBL
[e] Accession code for PRF databank. Protein not found at UniProtKB/Swiss-Prot/TrEMBL

Table 3.4 - Proteins differentially abundant in antisense C-hordein line 3 (L3) compared with non-transgenic control cv. Golden Promise

(continue)

Spot Id	Ratio	P value	Identification	Organism	Accession n[o]	UniProtKB/ Swiss-Prot/ TrEMBL	pI[a]	Mw[a] (Kda)	Score	MP[b]
0	2.32	0.002	Triticin	*T. aestivum*	gi|171027813	B2CGM5	6.37	64.88	222	10
1	1.58	0.037	Serpin-Z7	*H. vulgare*	gi|75282567	Q43492.2	5.45	42.82	286	9
3	2.06	0.007	Proteasome subunit alpha type-4-2 (Os06g0167600)	*O. sativa* subsp. *japonica*	gi|115466646	Q5VRG3	6.44	27.02	353	13
4	2.40	0.010	Triosephosphate isomerase, cytosolic	*H. vulgare*	gi|2507469	P34937.3	5.39	26.73	91	3
7	2.16	0.037	Bifunctional α-amylase/subtilisin inhibitor (BASI)	*H. vulgare*	gi|18916	P07596	7.77	22.24	233	8
31	1.75	0.002	α-amylase inhibitor BMAI-1	*H. vulgare*	gi|2506771	P16968.2	5.36	15.81	199	21
41	1.54	0.006	40S ribosomal protein S12 (Os07g0229900)	*O. sativa* subsp. *japonica*	gi|115471261	Q8H2J8	5.33	14.77	200	7
44	1.91	0.010	Embryo globulin	*H. vulgare*	gi|167004	Q03678	6.80	72.25	81	5
58	1.77	0.007	Uncharacterized protein LOC100278663	*Z. mays*	gi|226503157	B6UBZ3	6.75	15.88	100	3
67	2.10	0.021	Late embryogenesis abundant protei	*H. vulgare*	gi|199582497	B5TWC9	8.83	21.93	94	3
78	1.63	0.006	Triosephosphate isomerase, cytosolic	*H. vulgare*	gi|2507469	P34937.3	5.39	26.73	850	23
79	2.11	0.011	Glyceraldehyde-3-phosphate dehydrogenase 1, cytosolic	*H. vulgare*	gi|120680	P26517.1	6.67	36.51	200	4
116	2.12	0.004	Chain A, Crystal Structure Of Barley Grain Peroxidase 1	*H. vulgare*	gi|157830301	1BGP_A[d]	6.51	33.82	945	35
121	2.10	0.034	Guanine nucleotide-binding protein beta subunit-like protein	*Z. mays*	gi|219363167	B4FKM1	6.13	36.23	100	5
130	1.59	0.004	Serpin-Z7	*H. vulgare*	gi|75282567	Q43492.2	5.45	42.82	415	12
141	1.61	0.008	Protein z-type serpin	*H. vulgare*	gi|1310677	P06293	5.61	43.22	111	4
142	1.80	0.014	Serpin-Z7	*H. vulgare*	gi|75282567	Q43492.2	5.45	42.82	342	17
148	1.81	0.004	Protein z-type serpin	*H. vulgare*	gi|1310677		5.61	43.22	529	18
155	1.82	0.002	HSP70	*H. vulgare*	gi|476003	Q40058	5.76	67.01	223	5
160	2.28	0.001	Triticin	*T. aestivum*	gi|171027813	B2CGM5	6.37	64.88	72	2

Table 3.4 - Proteins differentially abundant in antisense C-hordein line 3 (L3) compared with non-transgenic control cv. Golden Promise

(continued)

Spot Id	Ratio	P value	Identification	Organism	Accession n[o]	UniProtKB/ Swiss-Prot/ TrEMBL	pI[a]	Mw[a] (Kda)	Score	MP[b]
235	3.11	0.001	Trypsin inhibitor CMc	*H. vulgare*	gi|161784337	P34951.2	6.68	15.17	163	8
238	2.68	0.022	Manganese superoxide dismutase	*T. aestivum*	gi|1654387	P93606	7.89	25.29	202	6
241	2.32	0.004	2-alkenal reductase	*H. vulgare*	gi|62765876	Q2KM86	5.87	38.25	468	19
260	2.46	0.015	Lipoprotein-like (Os01g0749000 protein)	*O. sativa* subsp. *japonica*	gi|115439929	Q94J20	7.79	28.05	171	5
264	3.47	0.001	Glyceraldehyde-3-phosphate dehydrogenase 1, cytosolic	*H. vulgare*	gi|120680	P26517.1	6.67	36.51	1019	40
265	2.98	0.004	Protein z-type serpin	*H. vulgare*	gi|1310677	P06293	6.61	43.22	1268	49
269	3.02	0.006	Beta-glucosidase	*H. vulgare*	gi|804656	Q40025	7.18	57.44	430	21
57	-2.28	0.001	Embryo globulin	*H. vulgare*	gi|167004	Q03678	6.80	72.25	236	21
100	-3.16	0.000	Lipoprotein-like (Os01g0749000)	*O. sativa* subsp. *japonica*	gi|115439929	Q94J20	7.79	28.05	62	1
147	-2.06	0.007	Glyceraldehyde-3-phosphate dehydrogenase 1, cytosolic	*H. vulgare*	gi|120680	P26517.1	6.67	36.51	321	6
154	-2.03	0.002	Hypothetical protein SORBIDRAFT_01g043980	*S. bicolor*	gi|242041881	C5WUN6	6.24	75.12	76	2
156	-2.12	0.035	Protein z-type serpin	*H. vulgare*	gi|1310677	P06293	5.61	43.22	174	8
169	-2.13	0.008	Eukaryotic initiation factor 4A	*T. aestivum*	gi|1170509	P41378.1	5.31	46.92	1048	44
183	-2.41	0.025	Hypothetical protein SORBIDRAFT_01g005440	*S. bicolor*	gi|242037831	C5WY16	6.31	51.49	92	4
191	-2.70	0.003	Hypothetical protein SORBIDRAFT_01g005440	*S. bicolor*	gi|242037831	C5WY16	6.31	51.49	96	2
193	-2.24	0.002	Hypothetical protein SORBIDRAFT_01g005440	*S. bicolor*	gi|242037831	C5WY16	6.31	51.49	74	1
229	-2.28	0.034	Phospoenolpyruvate carboxylase	*T. aestivum*	gi|3341490	O82072	5.61	110.15	194	9
245	-2.81	0.017	Hypothetical protein SORBIDRAFT_01g005440	*S. bicolor*	gi|242037831	C5WY16	6.31	51.49	100	8
252	-2.43	0.003	Hypothetical protein SORBIDRAFT_01g005440	*S. bicolor*	gi|242037831	C5WY16	6.31	51.49	85	3
257	-2.36	0.022	Hypothetical protein SORBIDRAFT_01g005440	*S. bicolor*	gi|242037831	C5WY16	6.31	51.49	47	2
259	-3.05	0.004	Hordothionin beta	*H. vulgare*	gi|225008	225008[e]	5.60	13.74	77	2

Table 3.4 - Proteins differentially abundant in antisense C-hordein line 3 (L3) compared with non-transgenic control cv. Golden Promise

(conclusion)

Spot Id	Ratio	P value	Identification	Organism	Accession n[o]	UniProtKB/ Swiss-Prot/ TrEMBL	pI[a]	Mw[a] (Kda)	Score	MP[b]
266	-2.35	0.009	Hypothetical protein SORBIDRAFT_01g00544	*S. bicolor*	gi|242037831	C5WY16	6.31	51.49	100	2
272	-4.12	0.018	Chain A, non-specific lipid transfer protein 1 from barley in complex with L-alfa-lysophosphatidylcholine, laudoyl	*H. vulgare*	gi|47168353	1MID_A[d]	8.19	9.69	180	10
274		<0.001	Bifunctional α-amylase/subtilisin inhibitor (BASI)	*H. vulgare*	gi|18916	P07596	7.77	22.24	360	9
278		<0.001	Beta-glucosidase	*H. vulgare*	gi|804656	Q40025	7.18	57.44	401	15

[a] Theoretical pI and Mw calculated using the software compute pI/Mw available at http://www.expasy.org/
[b] Matched peptides
[c] Accession code for PIR databank. Protein not found at UniProtKB/Swiss-Prot/TrEMBL
[d] Accession code for PDB databank. Protein not found at UniProtKB/Swiss-Prot/TrEMBL
[e] Accession code for PRF databank. Protein not found at UniProtKB/Swiss-Prot/TrEMBL

3.2.3 Discussion

In mature barley grains the non-storage proteins albumins and globulins are concentrated mainly in the embryo and aleurone layer (FINNIE; SVENSSON, 2009). These fractions are composed predominantly by a mixture of enzymes and their inhibitors, as well as storage globulins in less extent (WEISS; POSTEL; GÖRG, 1992). In the present study, all 136 differentially abundant spots in the antisense C-hordein lines belonged to these types of proteins (Tables 3.2, 3.3 and 3.4). Østergaard et al. (2002) had previously reported the appearance of these proteins in the salt soluble protein profile of whole mature barley grains, as well as in malt level of four barley cultivars. Besides the whole mature barley grains, the salt soluble protein profile were also reported during grain filling (ØSTERGAARD et al., 2004), grain maturation (FINNIE et al., 2002), grain germination (FINNIE et al., 2004b; FINNIE et al., 2006), in response to environmental fluctuation (FINNIE et al., 2004), malt extracts (JIN et al., 2012) and in the whole mature grains infected with the fungi *Fusarium graminearum* and *F. culmorum* (EGGERT; PAWELZIK, 2011). All these studies reported the appearance of the same protein categories cited above, even though some variation in the methodology procedure was observed among them. In a similar manner to these studies, the identified polypeptides in this work were categorized according to their functions into six classes. The distribution of differentially abundant proteins among these classes is shown in Figure 3.2. The proteins listed were mostly shown to be involved in protein and amino acids metabolism as well as storage globulins, glycolysis, starch metabolism and citric acid cycle, defence system, stress and detoxification, and protein folding (Figure 3.2).

Interestingly, L2 and L3 exhibited a very similar protein group distribution, whilst L1 diverged greatly. This result may be associated with intrinsic aspects, i.e. genetic reasons, such as the number of copies of the transgene, or the site of integration into the genome, once all lines were submitted to the same growing and experimental conditions, suggesting that such variation is not likely to be due to external aspects. Moreover, in a previous report Schmidt et al. (2015) when investigating lysine metabolism and the amino acid composition of five grain protein fractions also observed that L1 exhibited the most divergent profile when compared to the non-transgenic control.

Regardless the functional grouping of the proteins, the majority of the differentially abundant spots in the antisense C-hordeins barley lines was upregulated (Table 3.1). This result is in agreement with Lange et al. (2007) who reported increased relative proportion of albumins and globulins in the antisense C-hordein lines (12.3%, 10.9% and 11.2% in L1, L2 and L3, respectively) than in the non-transgenic control (9.2%). In addition, L1 once again

exhibited the greatest differences when compared with the non-transgenic control, wherein only 4 of a total 41 spots were downregulated (Tables 3.1 and 3.2). The increase or reduction of a specific albumin and/or globulin in the antisense C-hordein lines would be expected as a consequence of proteome rebalancing triggered by transgene expression and its effect on the grain protein profile.

The majority of the differentially abundant spots in L1 were shown to be related to stress and detoxification process (Figure 3.2), including polypeptides of dehydrin, late embryogenesis abundant protein (LEA), manganese superoxide dismutase, glutathione transferases and aldose reductase (Table 3.2). The LEA family is composed of small polypeptides (10-30 kDa) synthesized and highly accumulated during the late period of grain development and maturation, playing a protective role in desiccation tolerance (HONG-BO; ZONG-SUO; MING-AN, 2005). In addition, these proteins can play a role of atypical storage functions, once they disappear quickly during grain germination, and their amino acids utilized while the storage proteins are at an early stage of degradation (SHIH; HOEKSTRA; HSING, 2008). Nevertheless, LEA proteins gene expression have no grain-specificity, occurring also in vegetative tissues, such stems, leaves and roots, and in an abscisic acid-dependent pathway when exposed to water deficit associated with drought, salt or cold stress (GRELET et al., 2005).

Using a 2-DE based proteomic approach Finnie et al. (2006) monitored the protein profile during barley grain filling and maturation over a 5 week period experiment. A protein belonging to LEA family was one of 23 spots (19 proteins) that appeared only later than the onset of grain dissection or only in mature grain pattern. In addition, the same authors also reported difference in the time point appearance between cultivars, with LEA proteins appearing earlier in cv. Morex than cv. Barke. In the present study once neither non-transgenic control nor the antisense C-hordein lines were subjected to any type of induced stress, the upregulation of LEA proteins (spots #25 and #58) may be related to their atypical storage function rather than a response to environmental stimuli.

Dehydrins were other stress-related proteins to be upregulated in L1 (spots #5 and #13) (Table 3.2). These proteins are members of the LEA protein family (Group 2) (BIES-ETHÈVE et al., 2008). They are characterized by a highly conserved lysine-rich motif of 15 amino acids sequence (EKKGIMDKIKEKLPG), known as K-segment, which can be found from one to 11 copies within a single dehydrin polypeptide (WANG et al., 2014). The dehydrin defining feature (lysine-rich proteins) strongly supports our hypothesis that upregulated LEA protein, especially dehydrin, in L1, are one if not the most important protein

group contributing for the higher total lysine content of this line in comparison to the non-transgenic control.

Interestingly, some oxidative-stress related proteins were upregulated in L1, e.g. manganese superoxide dismutase (spot #73), glutathione S-transferase (spots #80 and #85) and glutathione transferase (spots #91 and #95) (Table 3.2). Molecular oxygen (O_2) is the biological electron acceptor that plays vital roles in cellular functions. However, aerobic cells are subjected to the formation of reactive oxygen species (ROS) such as superoxide (O2 •-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH•) (GRATÃO et al., 2005). Stress-induced ROS overproduction can cause negative effects on cellular structures and metabolism, causing damage in proteins, nucleic acids and lipids, and in extreme cases, cell death (SCANDALIOS, 2005). In counterpart, aerobic organisms evolved non-enzymatic and enzymatic antioxidant defence system to prevent the negative effects of ROS. Among the enzymatic antioxidant defence system in plants, enzymes such as catalases, peroxidases, superoxide dismutases (SOD) and glutathione S-transferases (GST) play key roles in the dismutation of ROS (GHELFI et al., 2011; CIA et al., 2012; DOURADO et al., 2013; BOARETTO et al., 2014). A 2-DE based proteomic studies of barley under abiotic (SUGIMOTO; TAKEDA, 2009; WITZEL et al., 2009; RASOULNIA et al., 2011; WENDELBOE-NELSON; MORRIS, 2012; GHABOOLI et al., 2013) and biotic stress (YANG et al., 2010) showed that some enzymes of the antioxidant defence system were de-regulated in vegetative tissues, while scarce studies are available for barley grains. One example is the work by Yang et al. (2011) who demonstrated that GST (1.5-fold spot intensity increase) and manganese superoxide dismutase (Mn-SOD, 1.7-fold spot intensity increase) were upregulated in germinating barley grains after 3 days of inoculation with *Fusarium graminearum*, but as far as we are aware, there is no information on the association of ROS production and/or regulation of the antioxidant defence system with genetic modifications in barley grains. In transgenic soybean seeds although only one protein (glycine-rich RNA-binding protein) related to ROS production was shown by 2-DE to be differently abundant in comparison to non-transgenic seeds, differences of enzymatic activity of antioxidant system enzymes (ascorbate peroxidase, catalase, and glutathione reductase and superoxide dismutase) were observed (BARBOSA et al., 2012). The authors suggested that the transgenic soybean seeds exhibited a level of stress higher than non-transgenic seeds. In the present study, although enzymatic assays were not performed, based on the increased spot intensity of Mn-SOD (spot #73), GST (spots #80, #85, #91 and #95) detected by 2-DE, it is possible to assume that the steady-state of antioxidant defence system in L1 may be altered due to the

genetic modification rather than environmental fluctuations, however, through distinct mechanisms than those reported for the in transgenic soybean (BARBOSA et al., 2012).

The genetic modification in antisense C-hordein lines resulted in a reduced storage protein content, which, at least in L1, might have induced a mechanism to stimulate the antioxidant system via overexpression of proteins involved in these processes in order to ensure grain germination, seedling establishment and production of offspring.

Differently of L1, the majority of the de-regulated proteins in L2 and L3 belonged to the defence system category (Figure 3.2). Similar result was observed in a comparative analysis of the whole mature barley grain proteome of doubled-haploid introgression lines representing a wild genome (*H. spontaneum* Hs213) within a modern cultivar background (*H. vulgare*. cv. Brenda), in which from a total of 49 identified segregating spots, the majority (30%) was related to disease/defence system processes, including proteases inhibitors (WITZEL et al., 2011). Protease inhibitors, one of the 17 pathogenesis-related protein family of plant defence systems, are intrinsically abundant in cereal grains (up to 10% of total protein content) (GORJANOVIĆ, 2009). In barley grains, the main protease inhibitors are the bifunctional barley α-amylase/subtilisin inhibitor (BASI), chymotrypsin/subtilisin inhibitors 1 and 2 (CI-1 and CI-2), a barley Bowman-Birk type trypsin inhibitor (BBBI), a large group of inhibitors representing bifunctional α-amylase/trypsin inhibitors (CM proteins) and serpins (MUTHUKRISHNAN et al., 2001). In the present study, serpins was the most affected protease inhibitor family of all antisense C-hordein lines (Tables 3.2, 3.3 and 3.4). Serpins, termed serine proteinase inhibitors, were categorized as defence proteins in the majority barley grain proteome studies, including the present one, however the physiological functions of plant serpins are still unclear (ROBERTS; HEJGAARD, 2008). In barley grains, the serpin protein family comprises polypeptides of around 43 kDa (BRANDT; SVENDSEN; HEJGAARD, 1990), localized in the central and peripheral starchy endosperm, subaleurone and at to a lesser extent in the aleurone layer (ROBERTS et al., 2003), accounting for ca. 5% of albumin fraction (HEJGAARD, 1982). In the present study, similarly to other barley grain proteome studies (FINNIE et al., 2002; FINNIE et al., 2004a; FINNIE et al., 2004b; ØSTERGAARD et al., 2004; FINNIE et al., 2006; JIN et al., 2012), serpins were identified in multiple spots in the antisense C-hordein lines (Tables 3.2, 3.3 and 3.4). However, to date, only three serpin subfamilies are recognized in barley (BSZx, BSZ4 and BSZ7), together known as protein Z (ROBERTS; HEJGAARD, 2008). This feature on 2-DE of serpins appearance (the same protein appear in different spots) are due to post-translational

modifications (FINNIE; SVENSSON, 2009), once this technique is able to detected such modifications, and/or protein degradation (JIN et al., 2012).

Interestingly, the majority of the differentially abundant serpins were upregulated in the antisense C-hordein lines, with 2 spots in L1 (spots #103 and #238), 9 spots in L2 (spots #91, #116, #119, #120, #123, #214, #217, #239 and #268) (the last one was exclusive in the transgenic line) and 6 spots in L3 (spots #1, #130, #141, #142, #148 and #265) (Tables 3.2, 3,3 and 3.4). None of other protein families exhibited such a high number of increased appearance among all de-regulated spots in the antisense C-hordein lines when compared with the non-transgenic control. Based on the previous results obtained from studies developed to elucidate the physiological role of serpins in plants, we suggest two hypotheses to explain these outcomes. Firstly, the proteins composing the serpin family could be replacing C-hordeins in the grains, as a result of the proteome rebalance, likewise Lange et al. (2007) observed among hordeins subgroups, in which B/γ- and D-hordeins relative content increased in detrimental of C-hordeins. Our hypothesis is strongly supported by the fact that serpins share at least three conserved characteristic with hordeins, such as the protein synthesis pattern during grain filling, the responsiveness to nitrogen nutrition and effects on mutations, suggesting storage functions to serpins in barley grains (ROBERTS; HEJGAARD, 2008). In addition, the regulation of gene expression of one serpin subfamily (BSZ4) is associated with two high-lysine alleles in barley, *lys1* enhances while *lys3a* represses the expression of *Paz1*, which encodes a member of BSZ4 (HEJGAARD; BOISEN, 1980; SØRENSEN; CAMERON-MILLS; BRANDT, 1989). The second hypothesis, based on the possible biological function of serpins in the plant defence system, is that the reduced storage protein content in the antisense C-hordein lines requires enhanced serpins synthesis to prevent degradation of hordeins (present in the grains, but in lower amounts) from insect proteinases, similarly to the functional role of wheat serpins (ØSTERGAARD et al., 2000). As a consequence of higher accumulation of serpins in detrimental of C-hordeins in the antisense C-hordein barley lines, we further suggest that this could explain partially, at least for L2 and L3, the increased lysine content of these lines, once it is known that in a normal barley grain phenotype serpins contribute to 5% of total lysine content of the grain, but 7% in high-lysine phenotypes (HEJGAARD; BOISEN, 1980). This suggestion is strongly supported by a direct comparison of these antisense C-hordein lines with the spontaneous high-lysine barley mutant Hiproly, which contains the single recessive allele *lys1* and the hordein percentage was shown to be slighted reduced (MUNCK et al., 1970). Some authors attributed the high-lysine content of this mutant and its derivatives to an increase of lysine-rich proteins composing the salt

soluble protein fraction, such as protein Z (whose expression is enhanced by *lys1*), β-amylase and chymotrypsin inhibitors (CI-1) and CI-2 (BRANDT, 1976; HEJGAARD; BOISEN, 1980; WILLIAMSON; FORDE; KREIS, 1988).

Besides serpins, other defence proteins were de-regulated in the antisense C-hordein lines, such as proteins belonging to α-amylase/trypsin inhibitor and Kunitz-type trypsin inhibitor family. All these spots were upregulated in the antisense C-hordein lines, together accounting for 3 spots in L2 (spots #2, #22 and #182) and 2 spots in L3 (spots # 31 and #235) (Tables 3.3 and 3.4).

The α-amylase/trypsin inhibitor family is composed by small polypeptides of 12-16 kDa, apparently restricted to the starchy endosperm. They belong to the salt soluble protein fraction, however because some polypeptides of this family are extractable in chloroform and methanol they are also known as CM proteins (MORALEJO et al., 1993). These proteins act against heterologous amylases from insects, mite, mammalian and bacteria, as well as trypsin like proteins, however endogenous hydrolytic enzymes are not affected (SALCEDO et al., 2004). Besides this role in the plant defence system, these proteins might be related to their nutritive value (PAZ-ARES et al., 1986). Therefore, it appears that the upregulation of these proteins observed in this study are related to the defence system, similarly to the serpins, to protect the storage protein from degradation by pest proteinases, and consequently altering the amino acid composition of the antisense C-hordein barley lines.

Other upregulated spots in L2 (spots #3 and #265) and L3 (spots #7 and #274) belonging to the defence system category was identified as the bifunctional amylase/subtilisin inhibitor (BASI) (Tables 3.3 and 3.4). This protein is a member of Kunitz-type trypsin inhibitor family comprising polypeptides of around 20 kDa (GUBATZ; SHEWRY, 2010). These proteins are synthesized during grain filling and abundantly stored in the endosperm and aleurone layers of the mature barley grain. Likewise other inhibitors, their precise biological functions are still unclear. However, it is known that they act against endogenous α-amylase isoenzyme 2 (AMY2), probably preventing precocious germination, and inhibiting subtilisin-type serine proteases of pathogens and pests (NIELSEN et al., 2004). The BASI gene expression is likely associated with high-lysine alleles, however, conversely to serpins gene regulation, the *lys1* allele exerts a negative effect on the expression of the gene encoding BASI, whilst *lys3a* exerts a positive effect (LEAH; MUNDY, 1989).

The chemically induced high-lysine mutant Risø 1508 (obtained from a Danish two-rowed spring barley cv. Bomi treated with ethyleneimine) containing the single recessive allele *lys3a*, confers a reduction of 80% and 93% of C- and B-hordeins, respectively, and an

increase of 36% in lysine content (INGVERSEN; KØIE; DOLL, 1973; EGGUM; BRUNSGAARD; JENSEN, 1995). In this high-lysine mutant, BASI transcript level was 2-4-fold higher with extended accumulation during grain filling in comparison to the normal line cv. Bomi. However, BASI proteins are not lysine-rich and its high accumulation could not be related the high-lysine phenotype in barley (LEAH; MUNDY, 1989). Therefore, it is possible to suggest that the upregulated BASI proteins is possibly a collateral effect of proteome rebalancing acting against insects or pest proteinases in order to maintain the storage proteins safe for grain germination in the appropriate time. However, unlike serpins and α-amylase/trypsin inhibitors, minor effects on amino acids composition, especially on lysine content, would be expected due to upregulated BASI. In addition, the number of differently abundant spots corresponding to BASI was lower than for other inhibitors (Tables 3.3 and 3.4).

In barley seeds, two immunochemically distinct (CI-1 and CI-2), but with 46% of similarity, inhibitors of bacterial and fungal alkaline proteases and chymotrypsin are found. In the present study, two spots identified as subtilisin-chymotrypsin inhibitor (CI-1B) (spots #8 and #9) were upregulated in L2 (Table 3.3). These proteins are lysine-rich (8 residues per molecule) and cysteine lacking small proteins with molecular masses around 8 kDa synthesized in the aleurone layer and starchy endosperm (GORJANOVIĆ, 2009). In barley, CI-1 and CI-2 are known to be a potential source of essential amino acids (SHEWRY et al., 1994). Nonetheless, some examples of successful modification, mainly regarded to multiple lysine substitution of CI-2 in order to improve nutritional quality of barley grains can be found in the literature (ROESLER; RAO, 1999; FORSYTH et al., 2005). In addition, as mentioned above, higher expression of CI-1 and CI-2 is associated with the high-lysine phenotype in barley (WILLIAMSON et al., 1987; WILLIAMSON; FORDE; KREIS, 1988).

Although pronounced differences were observed between L1 and the other lines (L2 and L3) when the majority of differentially abundant proteins (stress and detoxification category versus defence proteins) are concerned, smaller variation of protein grouping distribution was observed for other categories among all three antisense C-hordein lines (Figure 3.2).

Interestingly, multiple spots in L1 (spots #6, #56 and #250), L2 (spots #33, #36 and #236) and L3 (spot #44) were identified as embryo globulin, whereas other spots in L2 (spots #185 and #267) and L3 (spots #160 and #0) were identified as homologous to triticins (Tables 3.2, 3.3 and 3.4). Cereal embryo globulins may be homologues to the 7S vicilins of legumes and dicotyledonous plants, while triticins are related to legumins with a sedimentation

coefficient of 11-12S (SHEWRY; HALFORD, 2002), which belong to storage globulins, readily extractable in salt solution. Embryo globulins are found in the mature embryo and outer aleurone layer of the endosperm in cereals, whilst triticins, a minor storage protein group of wheat endosperm, accounting for only 5% of the total seed protein, are located in the starchy endosperm and deposited in protein bodies of wheat (SHEWRY; HALFORD, 2002).

Storage globulins apparently only play a role as storage, and although their amino acid composition contain relatively low sulfur-containing amino acids and tryptophan, the amounts of other essential amino acids, especially lysine, are much greater than in cereal grains (DURANTI, 2006). Therefore, it is possible that the increased storage globulins may be contributing to the more balanced amino acid composition encountered in the antisense C-hordein lines when lysine and sulfur amino acid contents are concerned, since the major cereal storage protein and storage globulin are nutritionally complementary. Moreover, a high content of storage globulins in cereals are directly related to high nutritional value, as notice for example in oat grains in which 50–80% of the grain protein are storage globulins (ANDERSON, 2014).

Although other spots were differentially abundant in the antisense C-hordein lines (Tables 3.2, 3.3 and 3.4), their de-regulated appearance was less impressive when compared to all other altered proteins already discussed.

The data obtained in this work suggest that the suppression of C-hordein, at least in the lines used here, do not exclusively affect hordein synthesis and accumulation. Therefore, the more balanced amino acid composition of these lines is the indirect result of profound alterations also in the pattern of non-storage protein. These changes include proteins involved in stress and detoxification (L1) and defence system (L2 and L3), as well as storage globulins (L1, L2 and L3). At lesser extent, proteins involved in grain metabolism, such as carbohydrate metabolism (e.g. glyceraldehyde-3-phosphate dehydrogenase 1, spots #31 (L1); #208 (L2); #79, #264, #147 (L3)), protein folding (e.g. heat shock protein HsP70, spots #195 (L2); #155 (L3) and amino acid metabolism (e.g. methionine synthase, spots #263 (L1), #243 (L2)), among others.

3.3 Conclusions

Although we demonstrated that the increased lysine content or other essential amino acids may be the consequence of distinct protein sources among the three antisense C-hordeins lines, the upregulation of lysine-rich proteins was consistently observed in all lines and this

information must be taken in combination to the data previously obtained for the same lines when the regulation of lysine metabolism was shown to be altered, and different patterns and trends reported (SCHMIDT et al., 2015), suggesting that the high-lysine phenotype of the antisense C-hordein transgenic barley lines cannot be explained by one mechanism, but by a combination of many which includes altered expression of proteins and altered lysine metabolism.

References

ANDERSON, O.D. The spectrum of major seed storage genes and proteins in oats (*Avena sativa*). **PLoS One**, San Francisco, v. 9, n. 7, p. e83569, 2014.

ARRUDA, S.C.C.; BARBOSA, H.S.; AZEVEDO, R.A.; ARRUDA, M.A.Z. Comparative studies focusing on transgenic through cp4EPSPS gene and non-transgenic soybean plants: an analysis of protein species and enzymes. **Journal of Proteomics**, Amsterdam, v. 93, n. 0, p. 107-116, 2013.

AZEVEDO, R.A. Analysis of the aspartic acid metabolic pathway using mutant genes. **Amino Acids**, Wien, v. 22, n. 3, p. 217-230, 2002.

AZEVEDO, R.A.; DAMERVAL, C.; LANDRY, J.; LEA, P.J.; BELLATO, C.M.; MEINHARDT, L.W.; LE GUILLOUX, M.; DELHAYE, S.; TORO, A.A.; GAZIOLA, S. A.; BERDEJO, B.D.A. Regulation of maize lysine metabolism and endosperm protein synthesis by opaque and floury mutations. **European Journal of Biochemistry**, Berlin, v. 270, n. 24, p. 4898-4908, 2003.

AZEVEDO, R.A.; DAMERVAL, C.; LEA, P.J.; LANDRY, J.; BELLATO, C.M.; MEINHARDT, L. W.; LE GUILLOUX, M.; DELHAYE, S.; TORO, A.A.; GAZIOLA, S. A.; VARISI, V.A.; GRATAO, P. L. Genetic control of lysine metabolism in maize endosperm mutants. **Functional Plant Biology**, Victoria, v. 31, n. 4, p. 339-348, 2004a.

AZEVEDO, R.A.; LANCIEN, M.; LEA, P.J. The aspartic acid metabolic pathway, an exciting and essential pathway in plants. **Amino Acids**, Wien, v. 30, n. 2, p. 143-162, 2006.

AZEVEDO, R.A.; LEA, P.J.; DAMERVAL, C.; LANDRY, J.; BELLATO, C. M.; MEINHARDT, L. W.; LE GUILLOUX, M.; DELHAYE, S.; VARISI, V. A.; GAZIOLA, S. A.; GRATÃO, P. L.; TORO, A. A. Regulation of lysine metabolism and endosperm protein synthesis by the *opaque-5* and *opaque-7* maize mutations. **Journal of Agricultural and Food Chemistry**, Easton, v. 52, n. 15, p. 4865-4871, 2004b.

BAK-JENSEN, K.S.; LAUGESEN, S.; ROEPSTORFF, P.; SVENSSON, B. Two-dimensional gel electrophoresis pattern (pH 6–11) and identification of water-soluble barley seed and malt proteins by mass spectrometry. **Proteomics**, Weinheim, v. 4, n. 3, p. 728-742, 2004.

BARBOSA, H.; ARRUDA, S.C.; AZEVEDO, R.; ARRUDA, M.Z. New insights on proteomics of transgenic soybean seeds: evaluation of differential expressions of enzymes and proteins. **Analytical and Bioanalytical Chemistry**, Heidelberg, v. 402, n. 1, p. 299-314, 2012.

BIES-ETHÈVE, N.; GAUBIER-COMELLA, P.; DEBURES, A.; LASSERRE, E.; JOBET, E.; RAYNAL, M.; COOKE, R.; DELSENY, M. Inventory, evolution and expression profiling diversity of the LEA (late embryogenesis abundant) protein gene family in *Arabidopsis thaliana*. **Plant Molecular Biology**, Dordrecht, v. 67, n. 1/2, p. 107-124, 2008.

BLEIDERE, M.; GAILE, Z. Grain quality traits important in feed barley. In: Proceedings of the Latvian Academy of Sciences, Section B: Natural, Exact and Applied Sciences. 2012. p.1-9.

BOARETTO, L. F.; CARVALHO, G.; BORGO, L.; CRESTE, S.; LANDELL, M. G. A.; MAZZAFERA, P.; AZEVEDO, R. A. Water stress reveals differential antioxidant responses of tolerant and non-tolerant sugarcane genotypes. **Plant Physiology and Biochemistry**, Paris, v. 74, n. 0, p. 165-175, 2014.

BRADFORD, M.M. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. **Analytical Biochemistry**, New York, v. 72, n. 1-2, p. 248-254, 1976.

BRANDT, A. Endosperm protein formation during kernel development of wild-type and a high-lysine barley mutant. **Cereal Chemistry**, Saint Paul, v. 53, n. 6, p. 890-901, 1976.

BRANDT, A.; SVENDSEN, I.; HEJGAARD, J. A plant serpin gene. **European Journal of Biochemistry**, Berlin, v. 194, n. 2, p. 499-505, 1990.

BRENNECKE, K.; NETO, A.J.S.; LUGLI, J.; LEA, P.J.; AZEVEDO, R. A. Aspartate kinase in the maize mutants *ASK1-LT19* and *opaque-2*. **Phytochemistry**, New York, v. 41, n. 3, p. 707-712, 1996.

BRIGHT, S.W.J.; MIFLIN, B.J.; ROGNES, S.E. Threonine accumulation in the seeds of a barley mutant with an altered aspartate kinase. **Biochemical Genetics**, New York, v. 20, n. 3-4, p. 229-243, 1982.

CIA, M.C.; GUIMARÃES, A.C.R.; MEDICI, L.O.; CHABREGAS, S.M.; AZEVEDO, R. A. Antioxidant responses to water deficit by drought-tolerant and -sensitive sugarcane varieties. **Annals of Applied Biology**, Warwick, v. 161, n. 3, p. 313-324, 2012.

DAY, L. Proteins from land plants - potential resources for human nutrition and food security. **Trends in Food Science and Technology**, Cambridge, v. 32, n. 1, p. 25-42, 2013.

DOURADO, M.N.; MARTINS, P.F.; QUECINE, M.C.; PIOTTO, F.A.; SOUZA, L.A.; FRANCO, M.R.; TEZOTTO, T.; AZEVEDO, R.A. Burkholderia sp. SCMS54 reduces cadmium toxicity and promotes growth in tomato. **Annals of Applied Biology**, Warwick, v. 163, n. 3, p. 494-507, 2013.

DURANTI, M. Grain legume proteins and nutraceutical properties. **Fitoterapia**, Milano, v. 77, n. 2, p. 67-82, 2006.

EGGERT, K.; PAWELZIK, E. Proteome analysis of Fusarium head blight in grains of naked barley (*Hordeum vulgare* subsp. *nudum*). **PROTEOMICS**, Weinheim, v. 11, n. 5, p. 972-985, 2011.

EGGUM, B.O.; BRUNSGAARD, G.; JENSEN, J. The nutritive value of new high-lysine barley mutants. **Journal of Cereal Science**, London, v. 22, n. 2, p. 171-176, 1995.

FALCO, S.C.; GUIDA, T.; LOCKE, M.; MAUVAIS, J.; SANDERS, C.; WARD, R. T.; WEBBER, P. Transgenic canola and soybean seeds with increased lysine. **Bio/Technology**, New York, v. 13, n. 6, p. 577-582, 1995.

FINNIE, C.; BAK-JENSEN, K. S.; LAUGESEN, S.; ROEPSTORFF, P.; SVENSSON, B. Differential appearance of isoforms and cultivar variation in protein temporal profiles revealed in the maturing barley grain proteome. **Plant Science**, Limerick, v. 170, n. 4, p. 808-821, 2006.

FINNIE, C.; MAEDA, K.; OSTERGAARD, O.; BAK-JENSEN, K.; LARSEN, J.; SVENSSON, B. Aspects of the barley seed proteome during development and germination. **Biochemical Society Transactions**, London, v. 32, n. 3, p. 517-519, 2004b.

FINNIE, C.; MELCHIOR, S.; ROEPSTORFF, P.; SVENSSON, B. Proteome analysis of grain filling and seed maturation in barley. **Plant Physiology**, Washington, v. 129, n. 3, p. 1308-1319, 2002.

FINNIE, C.; STEENHOLDT, T.; RODA NOGUERA, O.; KNUDSEN, S.; LARSEN, J.; BRINCH-PEDERSEN, H.; BACH HOLM, P.; OLSEN, O.; SVENSSON, B. Environmental and transgene expression effects on the barley seed proteome. **Phytochemistry**, New York, v. 65, n. 11, p. 1619-1627, 2004a.

FINNIE, C.; SVENSSON, B. Barley seed proteomics from spots to structures. **Journal of Proteomics**, Amsterdam, v. 72, n. 3, p. 315-324, 2009.

FORSYTH, J.L.; BEAUDOIN, F.; HALFORD, N.G.; SESSIONS, R.B.; CLARKE, A.R.; SHEWRY, P.R. Design, expression and characterisation of lysine-rich forms of the barley seed protein CI-2. **Biochimica et Biophysica Acta, Proteins and Proteomics**, Amsterdam, v. 1747, n. 2, p. 221-227, 2005.

GAZIOLA, S.A.; ALESSI, E.S.; GUIMARAES, P.E.O.; DAMERVAL, C.; AZEVEDO, R. A. Quality protein maize: a biochemical study of enzymes involved in lysine metabolism. **Journal of Agricultural and Food Chemistry**, Easton, v. 47, n. 3, p. 1268-1275, 1999.

GHABOOLI, M.; KHATABI, B.; AHMADI, F. S.; SEPEHRI, M.; MIRZAEI, M.; AMIRKHANI, A.; JORRÍN-NOVO, J. V.; SALEKDEH, G. H. Proteomics study reveals the molecular mechanisms underlying water stress tolerance induced by *Piriformospora indica* in barley. **Journal of Proteomics**, Amsterdam, v. 94, n. 0, p. 289-301, 2013.

GHELFI, A.; GAZIOLA, S.A.; CIA, M.C.; CHABREGAS, S.M.; FALCO, M.C.; KUSER-FALCÃO, P.R.; AZEVEDO, R.A. Cloning, expression, molecular modelling and docking analysis of glutathione transferase from *Saccharum officinarum*. **Annals of Applied Biology**, Warwick, v. 159, n. 2, p. 267-280, 2011.

GORJANOVIĆ, S. A review: biological and technological functions of barley seed pathogenesis-related proteins (PRs). **Journal of the Institute of Brewing**, London, v. 115, n. 4, p. 334-360, 2009.

GRATÃO, P.L.; POLLE, A.; LEA, P.J.; AZEVEDO, R.A. Making the life of heavy metal-stressed plants a little easier. **Functional Plant Biology**, Victoria, v. 32, n. 6, p. 481-494, 2005.

GRELET, J.; BENAMAR, A.; TEYSSIER, E.; AVELANGE-MACHEREL, M.-H.; GRUNWALD, D.; MACHEREL, D. Identification in pea seed mitochondria of a late-embryogenesis abundant protein able to protect enzymes from drying. **Plant Physiology**, Washington, v. 137, n. 1, p. 157-167, 2005.

GUBATZ, S.; SHEWRY, P.R. The development, structure, and composition of the barley grain. In: ULLRICH, S. E. (Ed.). **Barley:** production, improvement, and uses. Oxford: Wiley-Blackwell, 2010. chap. 13, p. 391-448.

HANSEN, M.; LANGE, M.; FRIIS, C.; DIONISIO, G.; HOLM, P. B.; VINCZE, E. Antisense-mediated suppression of C-hordein biosynthesis in the barley grain results in correlated changes in the transcriptome, protein profile, and amino acid composition. **Journal of Experimental Botany**, Oxford, v. 58, n. 14, p. 3987-3995, 2007.

HEJGAARD, J. Purification and properties of protein Z - a major albumin of barley endosperm. **Physiologia Plantarum**, Copenhagen, v. 54, n. 2, p. 174-182, 1982.

HEJGAARD, J.; BOISEN, S. High-lysine proteins in Hiproly barley breeding: identification, nutritional significance and new screening methods. **Hereditas**, Lund, v. 93, n. 2, p. 311-320, 1980.

HELM, C.V.; DE FRANCISCO, A.; GAZIOLA, S.A.; FORNAZIER, R.F.; POMPEU, G. B.; AZEVEDO, R.A. Hull-less barley varieties: storage proteins and amino acid distribution in relation to nutritional quality. **Food Biotechnology**, New York, v. 18, n. 3, p. 327-341, 2005.

HONG-BO, S.; ZONG-SUO, L.; MING-AN, S. LEA proteins in higher plants: structure, function, gene expression and regulation. **Colloids and Surfaces B: Biointerfaces**, Amsterdam, v. 45, n. 3/4, p. 131-135, 2005.

INGVERSEN, J.; KØIE, B.; DOLL, H. Induced seed protein mutant of barley. **Cellular and Molecular Life Sciences**, Basel, v. 29, n. 9, p. 1151-1152, 1973.

JIN, Z.; MU, Y.-W.; SUN, J.-Y.; LI, X.-M.; GAO, X.-L.; LU, J. Proteome analysis of metabolic proteins (pI 4–7) in barley (*Hordeum vulgare)* malts and initial application in malt quality discrimination. **Journal of Agricultural and Food Chemistry**, Easton, v. 61, n. 2, p. 402-409, 2012.

LANGE, M.; VINCZE, E.; WIESER, H.; SCHJOERRING, J. K.; HOLM, P. B. Suppression of C-hordein synthesis in barley by antisense constructs results in a more balanced amino acid composition. **Journal of Agricultural and Food Chemistry**, Easton, v. 55, n. 15, p. 6074-6081, 2007.

LEAH, R.; MUNDY, J. The bifunctional α-amylase/subtilisin inhibitor of barley: nucleotide sequence and patterns of seed-specific expression. **Plant Molecular Biology**, Dordrecht, v. 12, n. 6, p. 673-682, 1989.

MATAVELI, L. R. V.; ARRUDA, M.A.Z. Expanding resolution of metalloprotein separations from soybean seeds using 2D-HPLC-ICP-MS and SDS-PAGE as a third dimension. **Journal of Proteomics**, Amsterdam, v. 104, n. 0, p. 94-103, 2014.

MORALEJO, M.A.; GARCÍA-CASADO, G.; SÁNCHEZ-MONGE, R.; LOPEZ-OTÍN, C.; ROMAGOSA, I.; MOLINA-CANO, J. L.; SALCEDO, G. Genetic variants of the trypsin inhibitor from barley endosperm show different inhibitory activities. **Plant Science**, Limerick, v. 89, n. 1, p. 23-29, 1993.

MUNCK, L.; KARLSSON, K. E.; HAGBERG, A.; EGGUM, B. O. Gene for improved nutritional value in barley seed protein. **Science**, Washington, v. 168, n. 3934, p. 985-987, 1970.

MUTHUKRISHNAN, S.; LIANG, G.; TRICK, H.; GILL, B. Pathogenesis-related proteins and their genes in cereals. **Plant Cell, Tissue and Organ Culture**, Dordrecht, v. 64, n. 2/3, p. 93-114, 2001.

NIELSEN, P.K.; BØNSAGER, B.C.; FUKUDA, K.; SVENSSON, B. Barley α-amylase/subtilisin inhibitor: structure, biophysics and protein engineering. **Biochimica et Biophysica Acta, Proteins and Proteomics**, Amsterdam, v. 1696, n. 2, p. 157-164, 2004.

O'FARRELL, P.H. High resolution two-dimensional electrophoresis of proteins. **Journal of Biological Chemistry**, Bethesda, v. 250, n. 10, p. 4007-4021, 1975.

ØSTERGAARD, H.; RASMUSSEN, S. K.; ROBERTS, T.H.; HEJGAARD, J. Inhibitory serpins from wheat grain with reactive centers resembling glutamine-rich repeats of prolamin storage proteins: cloning and characterization of five major molecular forms. **Journal of Biological Chemistry**, Bethesda, v. 275, n. 43, p. 33272-33279, 2000.

ØSTERGAARD, O.; FINNIE, C.; LAUGESEN, S.; ROEPSTORFF, P.; SVENNSON, B. Proteome analysis of barley seeds: identification of major proteins from two-dimensional gels (pI 4–7). **Proteomics**, Weinheim, v. 4, n. 8, p. 2437-2447, 2004.

ØSTERGAARD, O.; MELCHIOR, S.; ROEPSTORFF, P.; SVENSSON, B. Initial proteome analysis of mature barley seeds and malt. **Proteomics**, Weinheim, v. 2, n. 6, p. 733-739, 2002.

PAZ-ARES, J.; PONZ, F.; RODRÍGUEZ-PALENZUELA, P.; LÁZARO, A.; HERNÁNDEZ-LUCAS, C.; GARCÍA-OLMEDO, F.; CARBONERO, P. Characterization of cDNA clones of the family of trypsin/α-amylase inhibitors (CM-proteins) in barley (*Hordeum vulgare* L.). **Theoretical and Applied Genetics**, Berlin, v. 71, n. 6, p. 842-846, 1986.

RABILLOUD, T.; CHEVALLET, M.; LUCHE, S.; LELONG, C. Two-dimensional gel electrophoresis in proteomics: past, present and future. **Journal of Proteomics**, Amsterdam, v. 73, n. 11, p. 2064-2077, 2010.

RASOULNIA, A.; BIHAMTA, M.; PEYGHAMBARI, S.; ALIZADEH, H.; RAHNAMA, A. Proteomic response of barley leaves to salinity. **Molecular Biology Reports**, Dordrecht, v. 38, n. 8, p. 5055-5063, 2011.

ROBERTS, T.; HEJGAARD, J. Serpins in plants and green algae. **Functional & Integrative Genomics**, Berlin, v. 8, n. 1, p. 1-27, 2008.

ROBERTS, T.H.; MARTTILA, S.; RASMUSSEN, S. K.; HEJGAARD, J. Differential gene expression for suicide-substrate serine proteinase inhibitors (serpins) in vegetative and grain tissues of barley. **Journal of Experimental Botany**, Oxford, v. 54, n. 391, p. 2251-2263, 2003.

ROESLER, K.R.; RAO, A.G. Conformation and stability of barley chymotrypsin inhibitor-2 (CI-2) mutants containing multiple lysine substitutions. **Protein Engineering**, Oxford, v. 12, n. 11, p. 967-973, 1999.

SALCEDO, G.; SANCHEZ-MONGE, R.; GARCIA-CASADO, G.; ARMENTIA, A.; GOMEZ, L.; BARBER, D. The cereal α-amylase/trypsin inhibitor family associated with bakers' asthma and food allergy. In: MILLS, E. N. C.; SHEWRY, P. R. (Ed.). **Plant food allergens**. Oxford: Wiley-Blackwell, 2004. chap. 5, p. 70-84.

SCANDALIOS, J. G. Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses. **Brazilian Journal of Medical and Biological Research**, Ribeirão Preto, v. 38, p. 995-1014, 2005.

SCHMIDT, D.; RIZZI, V.; GAZIOLA, S. A.; MEDICI, L. O.; VINCZE, E.; KOZAK, M.; LEA, P. J.; AZEVEDO, R. A. Lysine metabolism in antisense C-hordein barley grains. **Plant Physiology and Biochemistry**, Paris, v. 87, n. 0, p. 73-83, 2015.

SCHMIDT, M.A.; HERMAN, E.M. Proteome rebalancing in soybean seeds can be exploited to enhance foreign protein accumulation. **Plant Biotechnology Journal**, Oxford, v. 6, n. 8, p. 832-842, 2008.

SCHUCHARDT, S.; SICKMANN, A. Protein identification using mass spectrometry: a method overview. In: BAGINSKY, S.; FERNIE, A. R. (Ed.). **Plant systems biology**. Verlag: Birkhäuser, 2007. chap. 7, p. 141-170.

SHEVCHENKO, A.; TOMAS, H.; HAVLIS, J.; OLSEN, J.V.; MANN, M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. **Nature Protocols**, London, v. 1, n. 6, p. 2856-2860, 2006.

SHEWRY, P.; TATHAM, A.; HALFORD, N.; BARKER, J. A.; HANNAPPEL, U.; GALLOIS, P.; THOMAS, M.; KREIS, M. Opportunities for manipulating the seed protein composition of wheat and barley in order to improve quality. **Transgenic Research**, London, v. 3, n. 1, p. 3-12, 1994.

SHEWRY, P R.; HALFORD, N.G. Cereal seed storage proteins: structures, properties and role in grain utilization. **Journal of Experimental Botany**, Oxford, v. 53, n. 370, p. 947-958, 2002.

SHIH, M.-D.; HOEKSTRA, F.A.; HSING, Y.-I. C. Late embryogenesis abundant proteins. In: JEAN-CLAUDE, K.; MICHEL, D. (Ed.). **Advances in botanical research**. New York: Academic Press, 2008. chap., p. 211-255.

SØRENSEN, M.; CAMERON-MILLS, V.; BRANDT, A. Transcriptional and post-transcriptional regulation of gene expression in developing barley endosperm. **Molecular and General Genetics**, New York, v. 217, n. 2-3, p. 195-201, 1989.

STREATFIELD, S.J. Approaches to achieve high-level heterologous protein production in plants. **Plant Biotechnology Journal**, Oxford, v. 5, n. 1, p. 2-15, 2007.

SUGIMOTO, M.; TAKEDA, K. Proteomic analysis of specific proteins in the root of salt-tolerant barley. **Bioscience, Biotechnology and Biochemistry**, Tokyo, v. 73, n. 12, p. 2762-2765, 2009.

TATHAM, A.S.; SHEWRY, P.R. The S-poor prolamins of wheat, barley and rye: revisited. **Journal of Cereal Science**, London, v. 55, n. 2, p. 79-99, 2012.

VIDAL, N.; BARBOSA, H.; JACOB, S.; ARRUDA, M. Comparative study of transgenic and non-transgenic maize (*Zea mays*) flours commercialized in Brazil, focussing on proteomic analyses. **Food Chemistry**, London, v. 180, n. 0, p. 288-294, 2015.

VILHENA, M.B.; FRANCO, M.R.; SCHMIDT, D.; CARVALHO, G.; AZEVEDO, R.A. Evaluation of protein extraction methods for enhanced proteomic analysis of tomato leaves and roots. **Anais da Academia Brasileira de Ciencias**, Rio de Janeiro, in press.

WANG, Y.; XU, H.; ZHU, H.; TAO, Y.; ZHANG, G.; ZHANG, L.; ZHANG, C.; ZHANG, Z.; MA, Z. Classification and expression diversification of wheat dehydrin genes. **Plant Science**, Limerick, v. 214, n. 0, p. 113-120, 2014.

WEISS, W.; POSTEL, W.; GÖRG, A. Application of sequential extraction procedures and glycoprotein blotting for the characterization of the 2-D polypeptide patterns of barley seed proteins. **Electrophoresis**, Weinheim, v. 13, n. 1, p. 770-773, 1992.

WENDELBOE-NELSON, C.; MORRIS, P.C. Proteins linked to drought tolerance revealed by DIGE analysis of drought resistant and susceptible barley varieties. **Proteomics**, Weinheim, v. 12, n. 22, p. 3374-3385, 2012.

WILLIAMSON, M.S.; FORDE, J.; BUXTON, B.; KREIS, M. Nucleotide sequence of barley chymotrypsin inhibitor-2 (CI-2) and its expression in normal and high-lysine barley. **European Journal of Biochemistry**, Berlin, v. 165, n. 1, p. 99-106, 1987.

WILLIAMSON, M. S.; FORDE, J.; KREIS, M. Molecular cloning of two isoinhibitor forms of chymotrypsin inhibitor 1 (CI-1) from barley endosperm and their expression in normal and mutant barleys. **Plant Molecular Biology**, Dordrecht, v. 10, n. 6, p. 521-535, 1988.

WITZEL, K.; PIETSCH, C.; STRICKERT, M.; MATROS, A.; RÖDER, M.; WESCHKE, W.; WOBUS, U.; MOCK, H.-P. Mapping of quantitative trait loci associated with protein expression variation in barley grains. **Molecular Breeding**, Dordrecht, v. 27, n. 3, p. 301-314, 2011.

WITZEL, K.; WEIDNER, A.; SURABHI, G.-K.; BÖRNER, A.; MOCK, H.-P. Salt stress-induced alterations in the root proteome of barley genotypes with contrasting response towards salinity. **Journal of Experimental Botany**, Oxford, v. 60, n. 12, p. 3545-3557, 2009.

WU, G.Y.; WU, Z.L.; DAI, Z.L.; YANG, Y.; WANG, W.W.; LIU, C.; WANG, B.; WANG, J.J.; YIN, Y L. Dietary requirements of "nutritionally non-essential amino acids" by animals and humans. **Amino Acids**, Wien, v. 44, n. 4, p. 1107-1113, 2013.

YANG, F.; JENSEN, J.D.; SPLIID, N.H.; SVENSSON, B.; JACOBSEN, S.; JØRGENSEN, L. N.; JØRGENSEN, H. J. L.; COLLINGE, D. B.; FINNIE, C. Investigation of the effect of nitrogen on severity of Fusarium head blight in barley. **Journal of Proteomics**, Amsterdam, v. 73, n. 4, p. 743-752, 2010.

YANG, F.; SVENSSON, B.; FINNIE, C. Response of germinating barley seeds to Fusarium graminearum: the first molecular insight into Fusarium seedling blight. **Plant Physiology and Biochemistry**, Paris, v. 49, n. 11, p. 1362-1368, 2011.

4 EFFECTS OF NITROGEN FERTILIZATION ON THE TRANSCRIPT LEVEL OF DIFFERENT MEMBERS OF HORDEIN GENES AND ON MATURE GRAIN PROTEIN COMPOSITION IN BARLEY

Abstract

Barley prolamins called hordeins represent approximately 55% of total proteins in barley grains. The different hordein groups are coded by large multigene families. The responses of the different hordein groups to the nitrogen (N) fertilization are extensively studied and among hordeins the C-hordeins claimed to be the most upregulated by N fertilization. We investigated what happens under different N regimes in the antisense C-hordein barley line L5 where the N responsive C-hordein group is downregulated. Our study revealed that the hordein transcripts distributions at 20 days after pollination (DAP) were not in direct agreement with the protein distribution detected in the mature grain by SDS-PAGE. In the parental control, the hordein transcript levels were highest at the medium N regime and decreasing under high N treatment at 20 DAP, while in the transgenic line the hordein transcript increased with increasing N dosage. The antisense C-hordein line exhibited a specific C-hordein downregulation effect and in particular the different subgroup of C-hordein multigene family responded differently to the N treatment compared to the control. In comparison, in mature grain, all hordeins families were upregulated positively correlating with increasing N treatments and the ratio of B-hordein/C-hordein decreased upon higher N fertilizations. Both in control and the transgenic line all B-, C-, D-, γ-1 and γ-3 hordeins showed increased protein level although the relative C-hordein level was lower in the transgenic line due to the antisense effect. Interestingly, in response to N treatment, the distribution of C-hordein isoforms differed considerably in the transgenic line versus the control.

Keywords: Storage proteins; Multigene families; C-hordein; qRT-PCR; Protein separation by SDS-PAGE

4.1 Introduction

The storage compounds (lipids, starch and proteins) which compose the plant seeds have become an indispensable source of food, feed and raw material for human purposes (SHEWRY, 2011). In cereals, the major storage constituent in grains is starch, which makes this group of plants the main source of metabolised energy in diets. However, the cereal grains have become also an important source of proteins (TATHAM; SHEWRY, 2012). With a grain protein content varying from 8-15% and the annual global production exceeding 143 million tons (Mt) in 2012 (FAO, 2015) , it is estimated that over 200 million tons of protein are harvested from cereal grains (SHEWRY, 2007).

Prolamins are the major storage protein in cereals, accounting at least 50% of total grain N. Due to its high proportion of the total protein, this group of proteins determine the nutritional quality of cereal grains. The balance of indispensable and dispensable amino acids, individual amino acid bioavailability and protein digestibility are the factors influencing the

nutritional value of a protein (BOYE; WIJESINHA-BETTONI; BURLINGAME, 2012). All prolamins are composed of proteins with extensive glutamine and proline rich repeated motifs, among other amino acids lysine and threonine are present as lowest indispensable amino acids. This unbalance amino acid composition of prolamins is responsible for the low nutritional quality of cereal grains proteins (SHEWRY; HALFORD, 2002).

Barley (*Hordeum vulgare* L.) is the fourth most important cereal cultivated, and two thirds of barley harvested has been used for animal feed (DAY, 2013). In barley the prolamins are called hordeins, are homologues to other prolamins of the species belonging to the Triticeae tribe. The hordeins are classified in relation to their structural and evolutionary relationship into three subgroups, sulfur-rich (S-rich; B- and γ-hordeins), sulfur-poor (S-poor; C-hordeins) and high molecular weight (HMW) glutelins homologues (D-hordeins) (TATHAM; SHEWRY, 2012). The hordein subgroups are distinguishable by their mobility in SDS-PAGE and amino acid composition. Besides that, the contribution of each subgroup on total hordein content is variable. The most abundant subgroup is B-hordein (apparent molecular mass of 35-46 kDa) accounting generally from 70-80% of total hordeins. Second in abundance are the C-hordeins (apparent molecular mass of 55-75 kDa) which account for about 10-20% of the total. The γ-hordeins (apparent molecular mass of 35-46 kDa) that contributes to about 5% of the total and as minor constituent, the D-hordeins (apparent molecular mass of 100 kDa) representing 2-4% of the total hordeins (HOLOPAINEN et al., 2012).

Among the hordein subgroups, C-hordeins contain the highest proportion of glutamine and proline (40-50 mol% glutamine and 20-30 mol% of proline) and the lowest content of indispensable amino acids lysine, threonine and methionine (AZEVEDO et al., 1997; SHEWRY; HALFORD, 2002). Therefore the suitability of barley for feed is largely determined by the proportion of this hordein subgroup in the grains. Similarly to other cereals, the protein content and composition of barley grains are strongly affected by environmental oscillations, most notably by N supply (SHEWRY, 2007). The impacts of N fertilizer on hordeins have been assessed in a range of barley cultivars, wherein consistently positive correlation between total hordein content and N availability was reported. The effect of increasing N supply on total hordeins is accompanied by a decrease of S-rich (B-hordeins) and S-poor (C-hordein) ratio (KIRKMAN; SHEWRY; MIFLIN, 1982; GRIFFITHS, 1987; MOLINA-CANO et al., 2001; QI; ZHANG; ZHOU, 2006; SAVIN; PRYSTUPA; ARAUS, 2006) and in total lysine content (KIRKMAN; SHEWRY; MIFLIN, 1982).

The hordeins are encoded by a highly homologues multigene families, with sequences comprising highly conserved tandem repeats (SHEWRY; HALFORD, 2002), making the molecular studies of these family genes a complicated task. Recently, Kaczmarczyk et al. (2012) developed a qRT-PCR methodology to determine the transcript amount of the highly homologues multigene families of B-, C-, D- and γ-hordeins and their individual members during barley grain development.

In order to improve the nutritional quality of barley grains for food and feed Lange et al. (2007) generated a set of antisense C-hordein barley with more balance amino acid composition, wherein up to 16%, 13% and 11% increased lysine, threonine and methionine content, respectively. Since this set of transgenic barley lines contain an altered storage protein profile, with reduced C-hordein abundance, and altered amino acids metabolism (SCHMIDT et al., 2015), they become an important tool to unravelling the molecular basis of the impacts of N on storage protein in barley, especially on C-hordeins.

Although the effects to N fertilization on hordeins accumulation in barley grains are widely studied, the responsiveness of this phenomenon is more complex, involving metabolic feedback control and regulation of hordein genes expression. Ultimately, the regulation affects the rates of hordein proteins synthesis which reflects into their protein accumulation and also at post-translation regulating the formation and deposition of protein bodies. Therefore, the aim of the present study was to assess the effect of N fertilization on hordein synthesis and composition in the antisense C-hordein line L5 (LANGE et al., 2007) and in its parental control cv. Golden Promise Secondarily, we aim to obtain comprehensive information about the impact of N fertilization on the different C-hordein isoforms and indicate specific alleles/homologues that can be altered to confer improved nutritional quality of barley grains. The L5 was chosen due to its superior nutritional quality (LANGE et al., 2007) and because it is the well characterized line (HANSEN et al., 2007).

4.2 Development

4.2.1 Material and Methods

4.2.1.1 Plant material

Three biological replicates of each N dosage were planted for both parental barley cultivar (*Hordeum vulgare* cv. Golden Promise) (in the text parental line) and antisense C-hordein line (L5) (in the text transgenic line) (LANGE et al., 2007). The pots were prepared by planting five grains in 10l pots containing peat with fertilizer. The fertilizer was mixed

with peat by adding three different N dosages (10 g/10 l peat (N1); 20 g/10 l peat (N2) and 40 g/10 l peat (N3) representing 50 $Kg.ha^{-1}$, 100 $Kg.ha^{-1}$ and 150 $Kg.ha^{-1}$, respectively. The used slow realize fertilizer were Basacote® Plus 16-08-12 (Compo GwbH Germany) containing 16% of N (7.4% as NO_3 and 8.6% as NH_4). Semifield experiments were conducted in a locked outdoor cage at Research Centre Flakkebjerg, Slagelse, Denmark (55° 24' 17.9" N, 11° 22' 25.8" E) under natural daylight conditions during the spring season of 2013. After sowing, the pots were on automatic watering system. Once germination was completed, plants numbers were reduced to three plants per pot. Individual spikes were tagged and harvested in the morning (09.00–10.00) at 20 days after pollination (DAP). The collected spikes were immediately frozen in liquid N and stored at -80 °C until further analysis. The remaining spikes were harvested at maturity.

4.2.1.2 Transcriptome Analyses

RNA isolation: RNA was isolated from three biological replicates. In each one immature barley grain (20 DAP) from the mid-section of the spike was ground in liquid N in a mortar with a pestle and homogenate in one ml of TRI Reagent® (Sigma-Aldrich). The homogenate was centrifuged at 21 000*g* during 2 min at 4 °C to remove cellular debris. The supernatant was transferred to a new nucleic acid free tube and one volume of 96% ethanol was added. The sample was mixed thoroughly in vortex. One aliquot of 800 ul of this mixture was used to procedure the RNA purification using the Direct-zol™ RNA Mini Prep (Zymo Research) following the manufacturer's instructions. The total RNA were treated with the TURBO DNA-*free*™ Kit TURBO™ DNase Treatment and Removal Reagents (ambion® by Life technologies™) according to manufacturer's protocol to ensure non-contamination with genomic DNA. The RNA quality was assessed an Agilent 2001 Bioanalyzer (Agilent Technologies, Inc.), and sample with RIN number above 8.0 were used for the cDNA synthesis.

cDNA synthesis: First strand cDNA was synthetize in a reaction volume of 40 µl containing 5 µl of 100 pmol. μl^{-1} of Oligo $(dT)_{18}$ primer (Invitrogen by Life technologies™), 4 µl of dNTP mix 10 mM each (Thermo Scientific), 7 µl of free nucleic-acid-water, 8 µl 5X first-strand buffer (Invitrogen by Life technologies™), 4 µl 0.1 M DTT (Invitrogen by Life technologies™), 2 µl of Recombinant RNasin® Ribonuclease Inhibitor (40 $U.\mu l^{-1}$) (Promega), 2 µl of SuperScript™ II Reverse Transcriptase (200 units) (Invitrogen by Life technologies™) and 8 µl of total RNA. The reactions were performed by heating the samples

at 25 ^{o}C for 3 min, 42 ^{o}C for 45 min, 48 ^{o}C for 10 min and 50 ^{o}C for 12 min. The obtained single strand cDNA were stored at -20 ^{o}C.

qPCR conditions: The qPCR was performed in triplicate from each biological samples in the Applied Biosystem® ViiA™ 7 Real-Time PCR System with 384-well block. The reactions were loaded onto a 384-well plate in a final volume of 10 µl, comprising 5 µl of SYBR® Select Master Mix (Applied Biosystems by Life technologies), 0.6 µl of forward and reverse primers (5 µM) (Invitrogen) (list of primers in Table 4.1), 1 µl of diluted cDNA template (1:10, 1:100, 1:1000) and MilliQ water. No-template controls (NTC) were also performed. The thermal profile set-up was: one cycle at 50 ^{o}C for 2 min; one cycle at 95 ^{o}C for 10 min; 40 cycles at 95 ^{o}C for 15 sec and 60 ^{o}C for 1 min. The dissociation curve was also performed. All samples were carried out in three technical and three biological repetitions. The transcripts level were determined as described by Kaczmarczyk et al. (2012) using a DNA standard curve from the cloned actin gene (HVSMEi0002G07f) in a serial dilution from 10^{-1} to 10^{-8} and expressed as amol of gene of interested per amol of actin. Our study conforms to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) (BUSTIN et al., 2009). Statistical distributions and interpreting P values were done using online GraphPad software (http://www.graphpad.com/ quickcalcs/distMenu/).

4.2.1.3 Protein analyses

Hordein extraction and SDS-Polyacrylamide gel electrophoresis: The milled harvested matured barley grains were used for hordein determination. Barley alcohol-soluble proteins (hordeins) were extracted from 50 mg of flour and the isolated proteins were separated on SDS-PAGE according to Uddin et al. (2014). A HiMark™ Pre-stained protein standard (Novex® by Life technologies™) was used as MW control.

Coomassie staining and calculating the percentage of the band volume: After the SDS-PAGE gel was stained with Coomassie blue (SCHÄGGER; VON JAGOW, 1987) and image was taken using BioRad GelDoc. ImageLab 4.01 was used for densitometric image analysis and calculating percentage of band volume in each lane. Different hordein bands were assigned according to their approximate molecular weight. Since B- and γ-hordein have similar molecular weight and with 1-D gel, it was not possible to distinguish them, in calculation they were assigned together. The ratio among different hordeins (from the band intensity) was calculated manually in MS excel 2007.

4.2.2 Results

4.2.2.1 Response of hordein genes at transcriptional level to N supply at 20 DAP

To assess the response to N fertilization of hordein genes (B-, C-, D- and γ-hordeins), a set of primers previously designed by Kaczmarczyk et al. (2012) were employed. The so called common primer sets were used catching the whole gene family of B- (common B), C- (Common C), D- (Common D), and γ-hordeins, this latter one was further divided into two gene subfamilies (Common γ-1 and Common γ-3) (Table 4.1).

Table 4.1 - The set of primers for hordein families, C-hordein subfamilies and reference genes

Name	Primer sequence forward	Primer sequence reverse
[1]Common B	TTTCCAACAACCTCAACCACA	GTAGGGTACGCAGCGCAAT
Common C	TCCTCACCTTTGTCCTCCTT	TTTAGTTGCCTAGCGGTAGTG
[1]Common D	CACCGTGTCTCTGCACCATG	TGCCGTAGTACAACTCGTTGG
[1]Common γ-1	CAACCGCAACAACTAGCTCA	CACCAACAAATGGTGCTTTG
[1]Common γ-3	GGTTGGGTCATTGGTGATTC	AGCAATAAGGTGGGACATGC
C1 (X60037)	TCAACCAGTCCCCCAGCA	CTTGTTGGGGTTGCGGTT
X60037_S66938	CCGCTAGGCAACTAAACCCTAGCAG	TGGATATGGCTACTGCAGATATGA
JQ867090	CAACCTCAACCAGTCCCCCAGCAG	TCGGAATTAGTTGTTCTGATCCC
M36941	CCAACGTCAGGAACCTTATG	ATTAGCCCAAGAGGGCTATATC
[1]Actin	CCTCAGTTGAGAAGAGCTACG	TCTGCGCCAATCGTGATC
Tubulin	GATTGCTGTTATGCGTGTTTCC	CCACCCTAACAACATCTTCAGTA

[1] Primer sequence taken from Kaczmarczyk et al. (2012)

Among the four hordein gene families, B-hordein was the most abundant transcript in both, parental and the transgenic lines, regardless the N dosage, representing up to 84.85% and 85.42% of total hordein transcripts in parental line and transgenic line, respectively (Table 4.2). In parental line, the B-hordein transcript amount was decreasing upon increasing N dosage (Figure 4.1A). Conversely, in the transgenic line the amount of B-hordein transcript was increased upon increasing N dosage, reaching the highest level upon N3, wherein the accumulation of B-hordein transcripts was nearly twice higher than parental line (Figure 4.1B).

Table 4.2 - Relative contribution (%) of transcript of hordein gene families upon three different nitrogen regimes (N1 (10 g/10l soil); N2 (20 g/10l soil) and N3 (40 g/10l soil), in the parental line cv. Golden Promise (control) and antisense C-hordein line (L5). The transcript amount was measured in grains at 20 days after pollination and expressed as amol of each set of primers per amol of actin

Hordein gene family	**Control**			**L5**		
	N1	N2	N3	N1	N2	N3
Common B	84.85	78.14	79.73	85.42	79.99	81.59
Common C	6.46	11.59	9.91	3.92	8.15	6.94
Common D	0.99	1.33	1.02	1.18	1.19	1.24
Common γ-1	2.48	3.05	3.30	3.39	3.44	2.47
Common γ-3	5.21	5.89	6.05	6.09	7.23	7.76

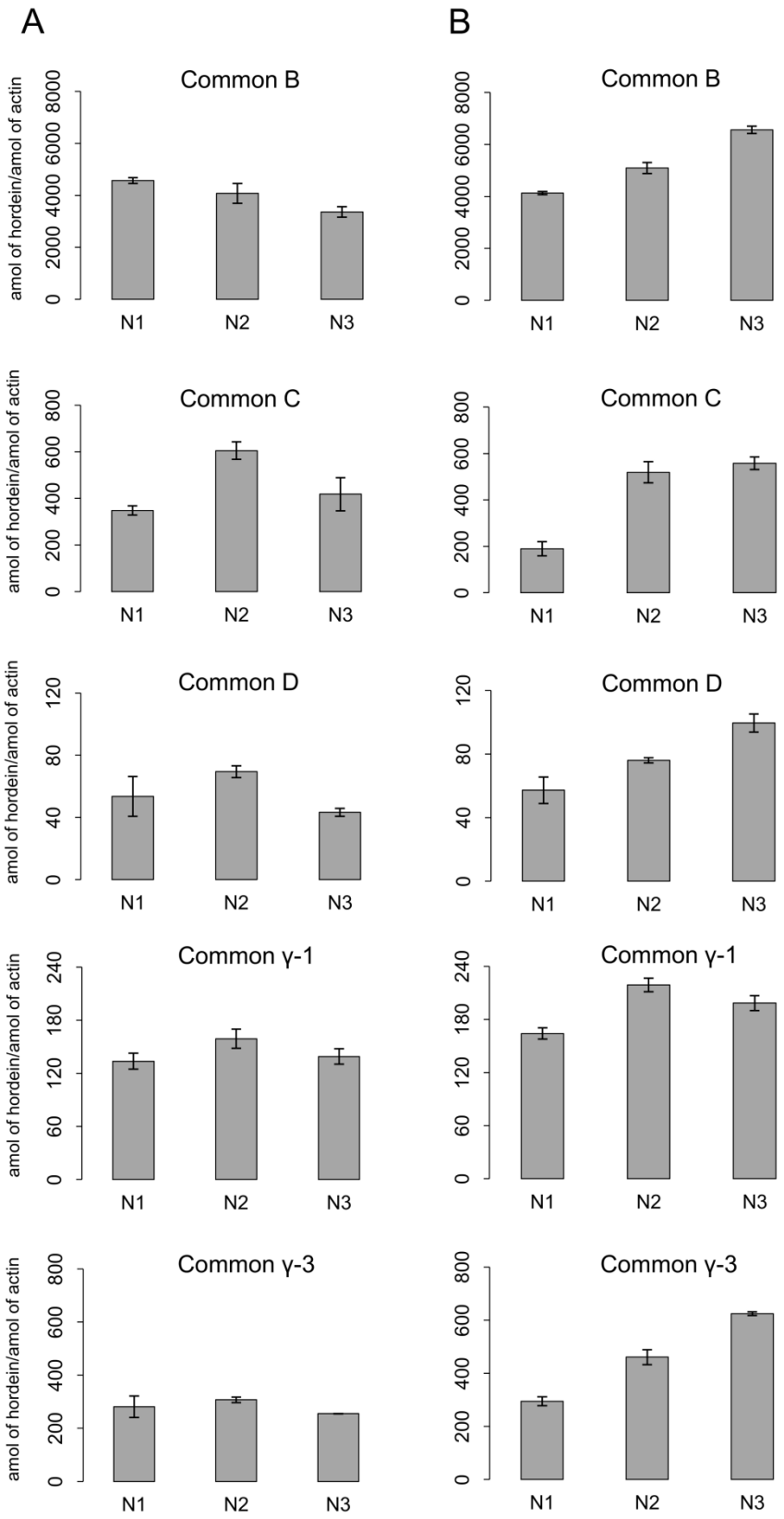

Figure 4.1 - The effects of nitrogen fertilization on the expression of B-, C-, D- and γ-hordeins transcripts in the (A) parental line cv. Golden Promise (control) and (B) antisense C-hordein line (L5). N1 (10 g/10l soil); N2 (20 g/10l soil) and N3 (40 g/10l soil). The expression was measured in grains at 20 days after pollination and expressed as amol of each set of primers per amol of actin. The transcript levels represent a mean of three technical and three biological replications ± standard error

As it was expected, regardless the N dosage, the relative contribution of C-hordein transcripts on total hordein transcripts was higher in parental line than in the transgenic line. In parental line, the total C-hordein genes expression level was represented from 6.46% to 11.59% of total hordeins, while in the transgenic line this percentage was lower, as expected, and varied from 3.92% to 8.15% (Table 4.2).

In the parental line, the C-hordein transcript amount was increasing from N1 to N2, however a reduction was observed from N2 to N3 (Figure 4.1A), while in the transgenic line the amount of C-hordein transcripts was increased upon increasing N dosage (Figure 4.1B). As we expected, at N1 and N2 the amounts of C-hordein transcripts at 20 DAP was lower in the transgenic line than in the parental line, however, at N3 the amount of C-hordein transcripts was higher in the transgenic line (Figure 4.1A and 4.1B). In both, parental and the transgenic lines the ratio of B-hordein/C-hordein transcripts decreased greatly from N1 to N2, however from medium to high N dosage, a slight increase was observed in both lines (Figure 4.2). As expected, regardless N dosage, the B ratio of B-hordein/C-hordein transcripts was higher in the transgenic line (Figure 4.2).

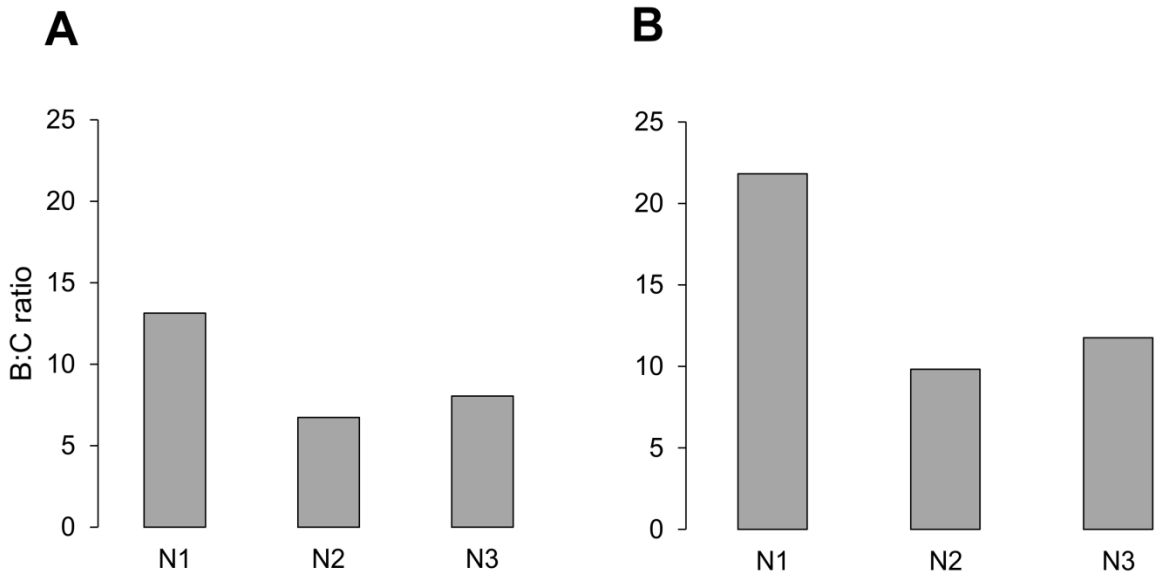

Figure 4.2 - The effects of nitrogen fertilization on the B:C hordein ratio in the hordein transcript of the (A) parental line cv. Golden Promise (control) and (B) antisense C-hordein line (L5). N1 (10 g/10l soil); N2 (20 g/10l soil) and N3 (40 g/10l soil). The B- and C-hordeins were determined by qRT-PCR

The lowest abundant hordein transcripts were D-hordeins, with a relative contribution on total hordeins up to 1.33% and 1.24% in parental and the transgenic lines, respectively (Table 4.2). The absolute amount of D-hordein transcripts was similar between the parental

and transgenic lines upon N1 and N2, however at N3 level there was a reduction in the parental line, whilst in the transgenic line the increase of D-hordein transcripts from N2 to N3 showed more than twice transcripts accumulated than in the parental line (Figure 4.2).

The γ-hordein gene family was divided into two gene subfamilies (γ-1 and γ-3). While the relative contribution of γ-3 genes on total hordeins represents up to 6.05% and 7.76% in the parental line and the transgenic line, respectively, the γ-1 genes represent approximately only half of this contribution, reaching a maximum of 3.30% and 3.44% (Table 4.2). In the parental line, for both γ-hordein genes (γ-1 and γ-3), a slight variation was observed among the three N dosages, with the highest transcript accumulation observed upon N2 fertilization regime (Figure 4.1A). In contrast, in the transgenic line, γ-3 subfamily was strongly responsive to increasing N fertilization, reaching the highest transcript amount upon N3 regime, which was more than twice than on N1 (Figure 4.1B). In the transgenic line, the γ-1 subfamily exhibited an increasing transcript changes upon increasing N fertilization, reaching the highest transcript amount upon N2 (Figure 4.1B).

4.2.2.2 Response of subfamily of C-hordein multigene family at transcriptional level to N supply at 20 DAP

The effects of N fertilization on the expression of transcripts of C-hordein gene family were determined by qRT-PCR using allele specific primers (Table 4.1). The expression of three known subfamilies from the C-hordeins (S66938, X60037 and JQ867090) was determined by qRT-PCR (Figure 4.3). While the expression of X60037 (C1) and JQ867090 subfamilies were determined through a specific set of primer recognizing them individually, the expression of S66938 subfamily were determined through a set of primer catching both X60037 and S66938 (X60037_S66938). Among the C-hordein subgroups, C1 presented the lowest transcript expression in both parental (Figure 4.3A) and transgenic lines (Figure 4.3B), and no conclusions can be drawn its overall contribution to the N treatments. On the other hand, both JQ867090 and S66938 subfamilies were responsive to the N fertilizations in both lines (Figure 4.3). While greater increase can be observed for subfamily JQ867090 and S66938 in parental line (Figure 4.3A), the most remarkable decrease was observed for JQ867090 subfamily when the parental line was compared to the transgenic line (Figure 4.3).

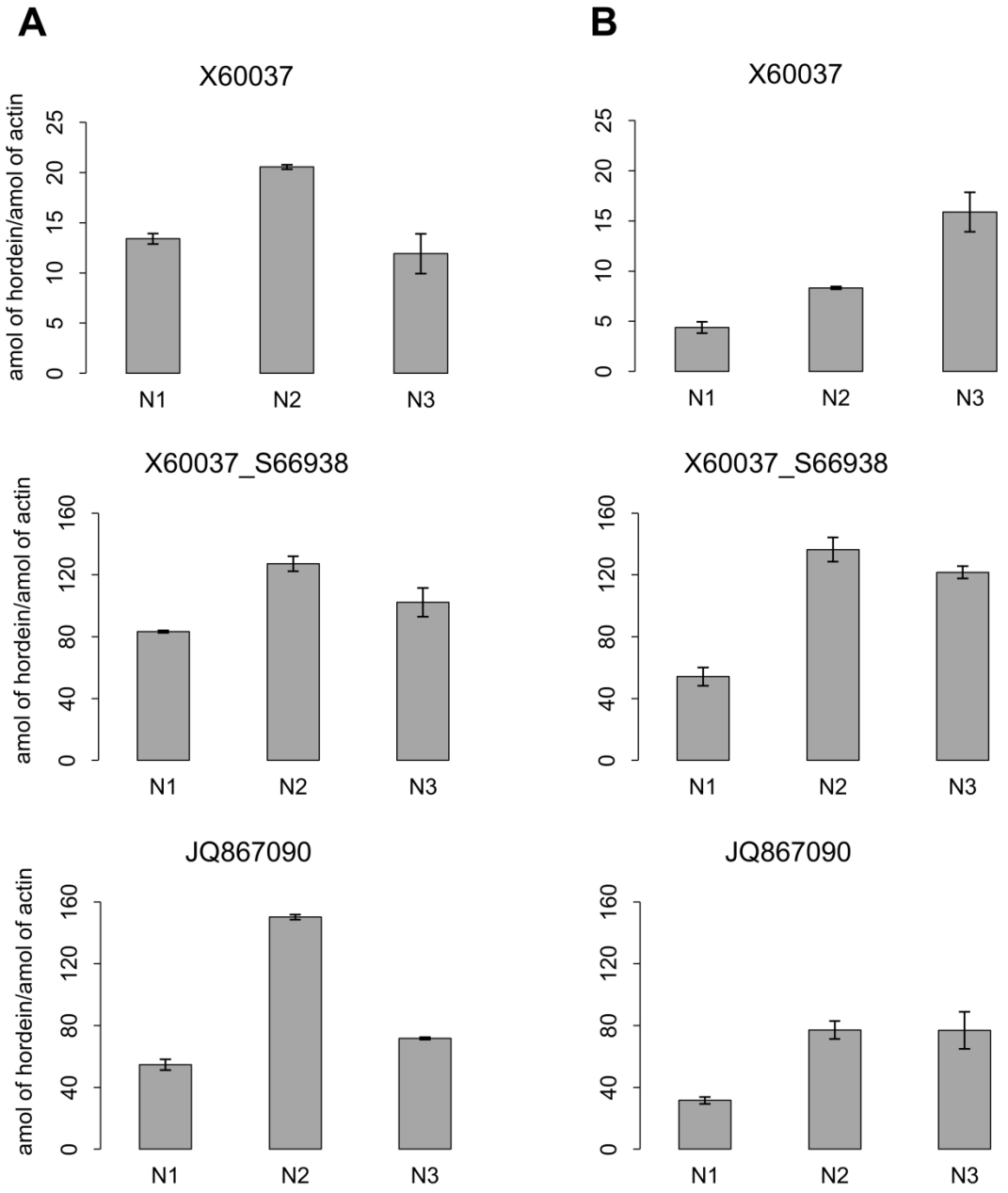

Figure 4.3 - The effects of nitrogen fertilization on the expression of subfamilies of C-hordeins transcripts in the (A) parental line cv. Golden Promise (control) and (B) antisense C-hordein line (L5). N1 (10 g/10l soil); N2 (20 g/10l soil) and N3 (40 g/10l soil). The expression was measured in grains at 20 days after pollination and expressed as amol of each set of primers per amol of actin. The identified C-hordein subfamilies had the following accession numbers: X60037; X60037_S66938 and JQ867090. The transcript levels represent a mean of three technical and three biological replications ± standard error

4.2.2.3 Response of total protein content to N supply in the mature grain

The protein content increased with increasing N dosages in both lines, however, while in the parental line the content varied from 11.11% to 13.52% in N1 and N3, respectively, in L5 greater increase was observed, from 10.88% to 19.30% in N1 and N3, respectively (Figure 4.4).

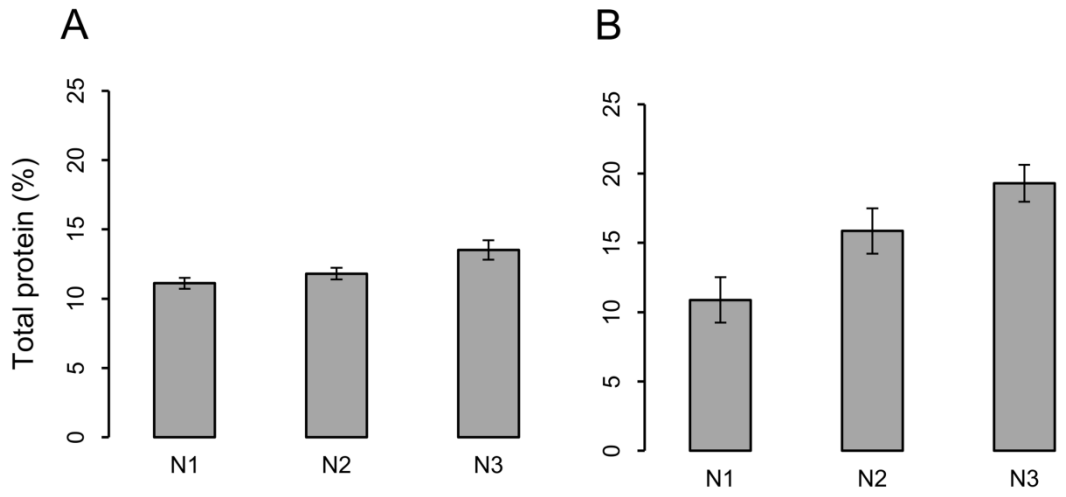

Figure 4.4 - The effects of nitrogen fertilization on the total protein content (%) in the (A) parental line cv. Golden Promise (control) and (B) antisense C-hordein line (L5). N1 (10 g/10l soil); N2 (20 g/10l soil) and N3 (40 g/10l soil). The protein content represents a mean of three technical and three biological replications ± standard error

4.2.2.4 Response of hordeins to N supply in the mature grain

The total hordeins fraction was isolated from the mature barley grains and separated by protein gel electrophoresis (SDS-PAGE). The increased N dosage, showed increased hordein levels in both parental and transgenic lines (Figure 4.5). The densitometric analysis of the bands in the SDS-PAGE gel revealed the overall increase upon increasing N dosage for both the parental and transgenic lines, besides that, through densitometric analysis of SDS-PAGE (Figure 4.5) the relative contribution of each hordein family in total hordein content was determined as shown in Table 4.3.

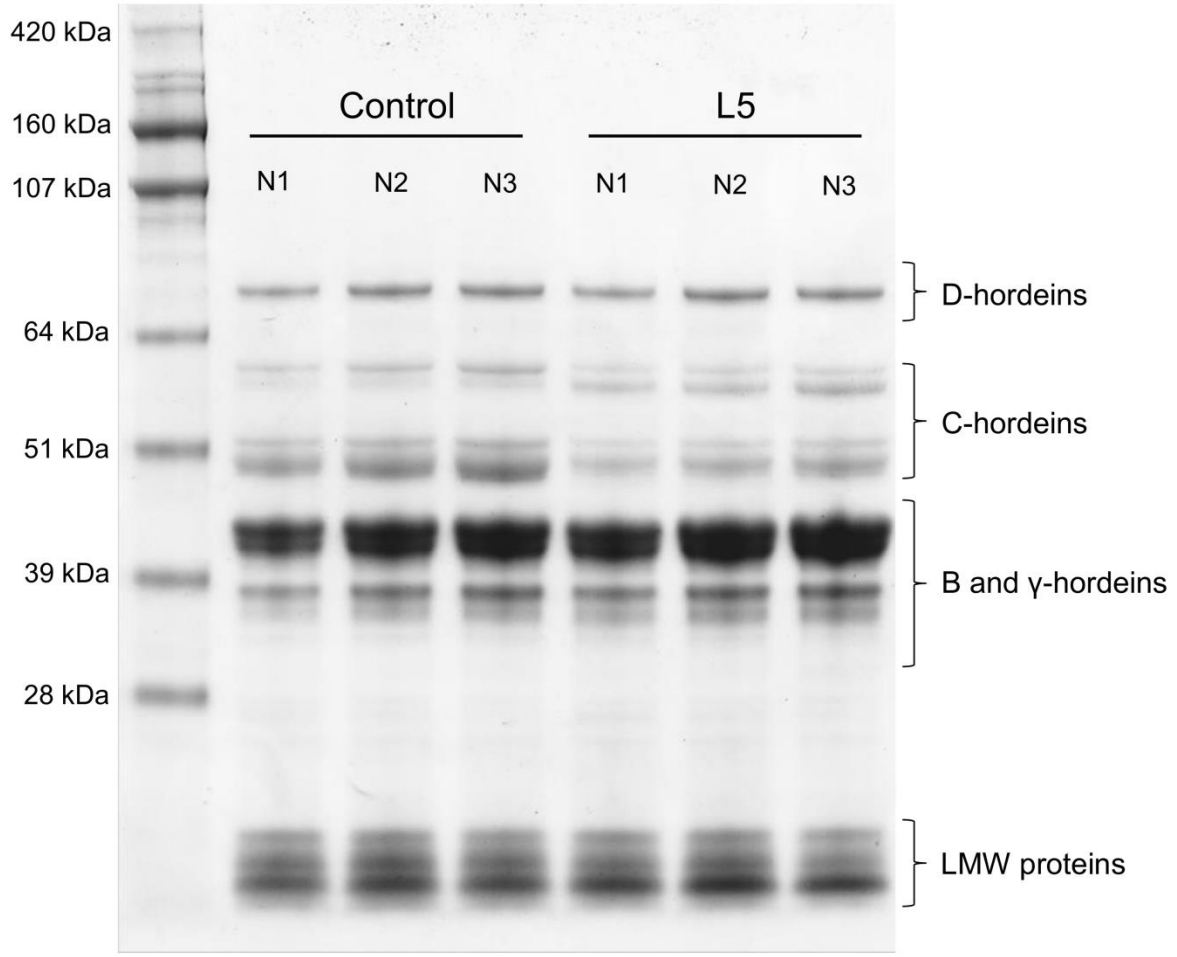

Figure 4.5 - SDS-PAGE analysis of reduced hordeins from the (A) parental line cv. Golden Promise (control) and the antisense C-hordein line (L5) under three different nitrogen regimes. N1 (10 g/10l soil); N2 (20 g/10l soil) and N3 (40 g/10l soil). Three hordeins families (B/γ-, C- and D-hordein) and low molecular weight proteins (LMW) were indicated. The molecular weights of the marker were given in kiloDalton (kDa)

In both, parental and transgenic lines, an increase of B/γ-hordeins abundance on the SDS-PAGE (Figure 4.5) was observed. The major increment occurred from N1 to N2 in both lines. Regardless of N dosage, the transgenic line exhibited higher B/γ-hordeins than the parental line, as expected (Figure 4.5). However, when the relative contribution of each hordein family was determined, in both, parental and transgenic lines, the relative proportion of B/γ-hordeins was decreased upon increasing N dosage (Table 4.3). Nevertheless, in the parental line this contribution on total hordeins decreased from 71.65% to 64.75%, whereas in the transgenic line this reduction was less expressive, which varied from 77.26% to 72.20% along N1 to N3 treatments, respectively (Table 4.3).

Table 4.3 - Relative contribution (%) of each hordein family on total hordein upon three different nitrogen regimes (N1 (10 g/10l soil); N2 (20 g/10l soil) and N3 (40 g/10l soil) in the parental line cv. Golden Promise (control) and antisense C-hordein line (L5). The relative proportions were determined based on densitometry from the SDS-PAGE

Hordein family	**Control**			**L5**		
	N1	N2	N3	N1	N2	N3
B/γ	71.65	68.03	64.75	77.26	73.92	72.20
C	19.17	21.37	25.45	13.02	14.79	18.93
D	9.18	10.59	9.80	9.72	11.29	8.87

Regardless to N dosage applied, as expected, the majority of C-hordeins bands were downregulated in the transgenic line compared to the parental line (Figure 4.5). Nevertheless, the C-hordeins were clearly positively correlated to the increased N dosage, in both parental and transgenic lines (Figure 4.5). The relative proportion of C-hordein was still lower in transgenic line than the parental line even in the highest N dosage (Table 4.3). In the parental line the relative proportion of C-hordein increased from 19.2% to 25.5% in N1 and N3, respectively, whereas in the transgenic line this percentage increased from 13.0% to 18.9% in the same given N conditions (Table 4.3). Besides that, the B:C ratio in the transgenic line was higher than in the parental line even in the highest N dosage (Figure 4.6).

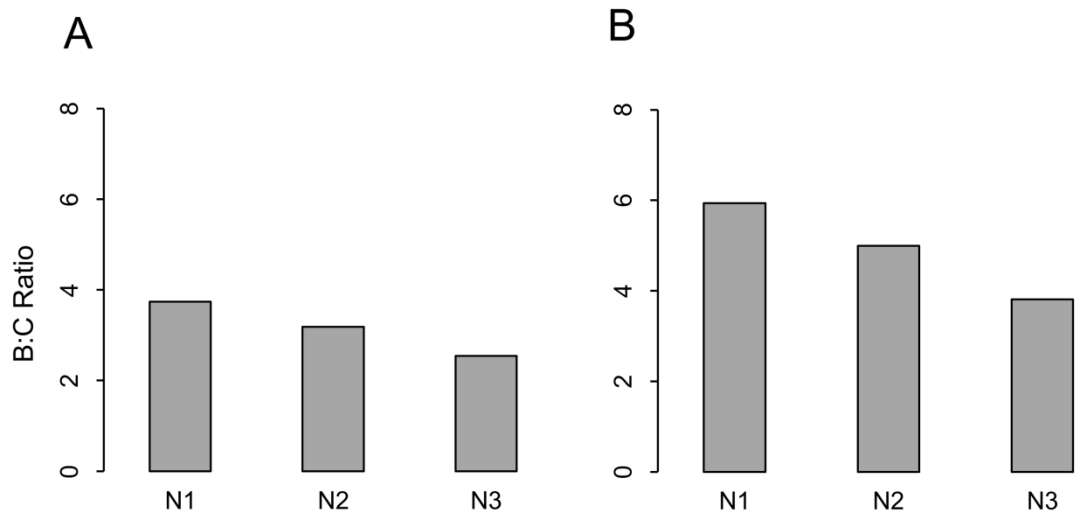

Figure 4.6 - The effects of nitrogen fertilization on the B:C hordein ratio in the (A) parental line cv. Golden Promise (control) and (B) antisense C-hordein line (L5). N1 (10 g/10l soil); N2 (20 g/10l soil) and N3 (40 g/10l soil). The B/γ-, C- and D-hordeins were determined by densitometry from the SDS-PAGE

It was observed in the SDS-PAGE that D-hordeins was also responsive to increasing N dosage for both, parental and transgenic lines (Figure 4.5). Nevertheless, when the relative contribution was determined, 9.18% to 10.59% and 9.72% to 11.29% of D-hordein was observed at N1 and N2 for the parental line and transgenic line, respectively (Table 4.3),

while a reduced contribution (9.80% and 8.87%) detected at N3 for the parental line and transgenic line, respectively (Table 4.3).

Besides each hordein family relative contribution on total hordein, the relative contribution of each C-hordein isoform on total C-hordein pool and their respective response to N fertilization were determined by densitometric analysis (Figure 4.7 and Table 4.5). Band number 3 was barely responsive to N increasing dosage in the parental line (Figure 4.7A), whereas the same band was upregulated 1.7 times in the transgenic line upon increasing N level (Figure 4.7B). On the other hand, bands number 2, 4 and 5 were moderately upregulated in both lines (Figure 4.7). Although, band number 5 seemed to comprise two polypeptides, which were considered together (Figure 4.7A), it is possible that similar response to the increasing N dosage was observed for the upper band in both lines, which was slightly more abundant (Figure 4.7A). This differential response of individual C-hordein isoforms altered the relative contribution of the observed polypeptides on the total hordeins along the three N dosages, as it can be better observed by their relative contribution in percentage of the total hordein content (Table 4.4). Band number 5 represents the majority of C-hordeins in both lines with 1.4 times upregulation in both from N1 to N3 (Table 4.4). At the same time the abundancy of band number 2 showed factor 1.4 fold versus 1.1 and band number 4 a factor 1.2 versus 1.7 as upregulation in the parental versus transgenic lines from N1 to N3 (Table 4.4).

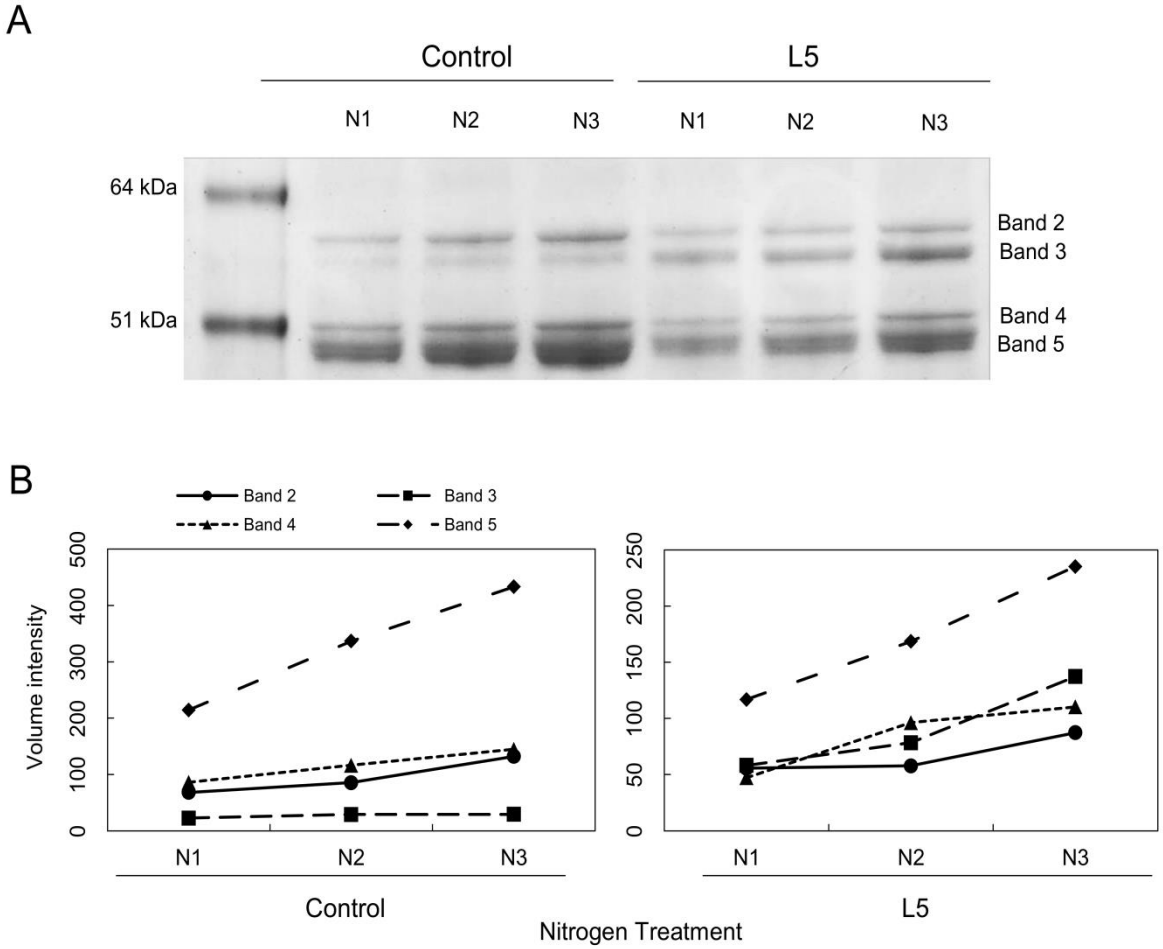

Figure 4.7 - The effects of nitrogen fertilization on different C-hordein isoforms in the (A) parental line cv. Golden Promise (control) and (B) antisense C-hordein line (L5). N1 (10 g/10l soil); N2 (20 g/10l soil) and N3 (40 g/10l soil). Band numbers (3-5) represented the separated C-hordeins isoforms. The volume intensity of each C-hordein isoform was determined by densitometry from the SDS-PAGE

Table 4.4 - Relative contribution (%) of individual C-hordein polypeptide on total hordein upon three different nitrogen regimes (N1 (10 g/10l soil); N2 (20 g/10l soil) and N3 (40 g/10l soil)), in the parental line cv. Golden Promise (control) and antisense C-hordein (L5). The relative proportions of individual C-hordeins were determined by densitometry from the SDS-PAGE

C-hordein	**Control**			**L5**		
	N1	N2	N3	N1	N2	N3
Band 2	3.33	3.22	4.55	2.61	2.13	2.90
Band 3	1.11	1.09	1.00	2.73	2.89	4.56
Band 4	4.22	4.38	4.98	2.21	3.55	3.66
Band 5	10.51	12.68	14.92	5.47	6.22	7.81
Total	19.17	21.37	25.45	13.02	14.79	18.93

4.2.3 Discussion

C-hordein is claimed to be the most N responsive hordein family. The increasing N supply is causing an increase in total hordein level but a decrease in B-hordeins and C-

hordeins ratio (KIRKMAN; SHEWRY; MIFLIN, 1982; GRIFFITHS, 1987; MOLINA-CANO et al., 2001; QI; ZHANG; ZHOU, 2006; SAVIN; PRYSTUPA; ARAUS, 2006). Studying the N responses of C-hordein downregulated transgenic line can give us better understanding about the storage protein n accumulation under different N regimes. From the five antisense C-hordeins lines (L1-L5) reported by Lange et al. (2007), we selected transgenic line L5 due to its superior nutritional quality for feeding. In addition, L5 was further investigated through a grain-specific array by Hansen et al. (2007), providing more information, which could compared to our findings.

In order to obtain more comprehensive information about the response of hordeins synthesis at transcript level and hordein composition in the mature grain we have used a combination of approaches: 1) at transcription level determining the amount of transcripts of each hordein gene family (B-, C-, D- and γ-hordeins) and of individual C-hordein alleles (qRT-PCR); 2) at protein level the hordein families and in particular the C-hordein isoforms (SDS-PAGE). The study the N responses of the characterized transgenic barley line (L5) (LANGE et al., 2007) provided us an unique opportunity to evaluate how the plant alter its hordein composition when one of the most N responsive protein, the C-hordein level is downregulated.

Rahman et al. (1983) demonstrated that the primary regulation of prolamin genes occurs at transcriptional level, with the accumulation of all three hordein subgroups being directly related to their respective genes transcription rates. However, a fine tuning at translational level occurs when the sulfur availability is limited, leading to an increased efficiency of C-hordein mRNA translation and a reduced rate of B-hordeins synthesis.

The data obtained in this research partially agreed with the above mentioned findings. The parental line cv. Golden Promise exhibited increased total B-, C-, D-, γ-1- and γ-3-hordeins transcript abundance at 20 DAP from low to medium N regimes, but under high N regime a decrease was observed for all hordein groups (Figure 4.1A). Similar results were obtained in another barley cultivar (cv. Barke) (KACZMARCZYK, 2012). In contrast, the antisense C-hordein line exhibited increased accumulation of total B-, C-, D- and γ-3-hordein transcripts abundance under increasing N fertilization including the high N dosage (Figure 4.1B). The observed increases of the total B-, C-, D-, γ-1- and γ-3-hordeins were relatively higher in the transgenic line in response to N, which could be explained by the increased relative proportion of D- and B/γ-hordeins to compensate the detrimental antisense effect of the C-hordein transcript (Figure 4.1). Although the C-hordein transcript level also increased, due to the antisense effect it reached lower levels than in the parental line at N1 and N2

(Figure 4.1). A possible explanation is that the higher level of C-hordein transcript in N3 treatment could be the higher general upregulation of the hordein transcript levels including C-hordeins. While the total B-, D-, γ-1- and γ-3-hordein transcript levels were higher in the transgenic line (N3) than in obtained highest N2 levels, C-hordeins at N3 exhibited lower levels than the parental line at N2. The detailed transcriptomic studies on the known C-hordein subfamilies revealed differences how the antisense line responded to the increased N (Figure 4.3). While all C-hordein bands (number 2 to 5) increased in both lines, bands 3 and 4 were more responsive in the transgenic line (Figure 4.7, Table 4.4). Whatever is the explanation for the obtained transcript abundance, the final results (the mature grain protein composition) showed that the translation of the C-hordein to protein was not satisfactory as the relative contribution of C-hordein in the transgenic line was lower than in the parental line in every studied N levels (Table 4.4).

It is known that under high N dosages there is a delay of the onset of N remobilization in comparison with low N fertilizer regimes (TA; WEILAND, 1992), postponing the senescence process of source organs, such as flag leaf, which has a special role in N availability for grain filling in barley, likewise in other cereals (MARTIN et al., 2005). Moreover, the storage proteins genes are under temporal regulation during grain development, as reported by Hansen et al. (2009) through a grain specific microarray experiment with field grown barley grains, under normal N dosage, that C-hordein genes showed late expression profile.

Even though the plants were grown and the developing grains harvested of both parental and transgenic lines were in the same conditions, reduction of total C-hordein transcript abundance from normal N dosage (N2) to high N dosage (N3) was not observed in the transgenic line (Figure 4.1B), so other molecular and biochemical aspects could be taken in consideration. According to Masclaux-Daubresse et al. (2008) grain sink strength, transfer processes in the source organs and the phloem pathway efficiency are the main factors regulating the N loading of grains. We speculate that once C-hordeins were suppressed through an antisense construct in the 3’ end region of a C-hordein gene (LANGE et al., 2007), it reduced grain sink strength for N. This is supported by the fact C-hordeins contain the highest content of glutamine (41.2 mol%) among all hordeins subgroups, which is the preferred N-compound exported in cereals. Moreover, the N recycling and re-assimilation in source organs is dependent of three major enzymes, the cytosolic glutamine synthetase (GS1), glutamate dehydrogenase (GDH) and asparagine synthetase (AS) (LEA; AZEVEDO, 2006; 2007). Interestingly, Hansen et al. (2007) reported that GDH2, GS1 and AS were

downregulated in the transgenic line (L5) at 20 DAP. In addition, these authors also reported that the ribulose bisphosphate carboxylase large chain precursor gene was also downregulated in this line. In C3 plants, such as barley, around 50% of total soluble protein content in the leaves is Rubisco (Ribulose bisphosphate carboxylase/oxygenase) (ZHU; PORTIS; LONG, 2004), and also the major source for N remobilization during chloroplast breakdown. These findings reported by Hansen et al. (2007) support our suggestion that the sink strength during grain filling in the antisense C-hordein line was reduced.

It is known that based on their mobility on SDS-PAGE the barley hordeins are divided into three subgroups: B/γ-, C- and D-hordeins (SHEWRY; TATHAM; HALFORD, 2001). As reported by many other studies, total hordein content increased in response to increasing N dosage in both the parental and transgenic lines (Figure 4.5). In addition, the relative contribution of each hordein subgroup (B/γ-, C-, D-hordein) was also altered at protein level in response to N and the most responsive hordein subgroup to N fertilization was C-hordein (Table 4.3). In agreement with several reports comparing different cultivars and/or years and locations (KIRKMAN; SHEWRY; MIFLIN, 1982; GRIFFITHS, 1987; MOLINA-CANO et al., 2001; QI; ZHANG; ZHOU, 2006; SAVIN; PRYSTUPA; ARAUS, 2006), the disproportional increase of C-hordeins in response to N fertilization leads to a decreased B:C ratio with increasing N dosage in both parental and transgenic lines (Figure 4.6). Although both, B- and C-hordeins are characterized by high amounts of glutamine and proline, these two hordein subgroups differ in their amino acid composition (SHEWRY, 1993). The major difference is related to sulfur-amino acids, methionine and cysteine. While the sulfur-rich B-hordein contains 2.5 mol% and 0.6 mol% of cysteine and methionine, respectively, no cysteine residues and only 0.2 mol% of methionine are found in C-hordeins. Therefore, some authors suggested that disproportional increase of C-hordein accumulation in relation to B-hordein may be a response to limitation of sulfur relative to N (KIRKMAN; SHEWRY; MIFLIN, 1982; SHEWRY, 2011). In our study, irrespective to the N dosage, the B:C ratio was higher in the transgenic line than the parental line (Figure 4.6), which can be explained due the fact that the sulfur-poor protein group (C-hordein) was suppressed in the studied transgenic line (LANGE et al., 2007).

Indeed, the impact of N on hordeins is more complex than those reported above due to the differential effects on individual gene/protein components. We therefore determined the effects of N fertilization on each C-hordein isoforms by quantitative scanning of SDS-PAGE. Such analysis revealed clear variable responses among different C-hordein isoforms within the same line and also between the parental line and the transgenic line (Figure 4.7), which in

turn affected the relative contribution of each C-hordein isoform on total hordein (Table 4.4). To our knowledge, differential responses to N fertilization have not been reported for C-hordeins isoforms. In a field-growth experiment with barley cv. Julia, with four N dosages, increasing from 50 to 200 $kg.ha^{-1}$ Kirkman et al. (1982) reported no apparent changes in the relative amounts of individual polypeptides within C-hordein subgroups, neither for B-hordein subgroups. Likewise, there was no effect on the relative contribution of individual polypeptides in both subgroups when the barley cv. Bomi were grown in a glasshouse with three increasing N dosage from 250 to 1000 mg of N (KIRKMAN; SHEWRY; MIFLIN, 1982).

4.3 Conclusion

Our research strategy for understanding changes in storage protein composition in response to different N regimes was based on studying all the gene families coding hordeins at transcriptome level at 20 DAP (qRT-PCR), followed by the protein studies on the mature grain (SDS-PAGE). The study included the evaluation and comparison of C-hordein alleles (mRNA) and isoforms (protein) in the parental and transgenic lines. We obtained major differences both in expression level and in the C-hordein isoform pattern. Further experiment needed to validate the mechanism of protein compensation triggered in C-hordein antisense line by N treatments in order to deviate the N flux into proteins qualitatively and improve the nutritional value by altered amino acids composition.

References

AZEVEDO, R.A.; ARRUDA, P.; TURNER, W.L.; LEA, P.J. The biosynthesis and metabolism of the aspartate derived amino acids in higher plants. **Phytochemistry**, New York, v. 46, n. 3, p. 395-419, 1997.

BOYE, J.; WIJESINHA-BETTONI, R.; BURLINGAME, B. Protein quality evaluation twenty years after the introduction of the protein digestibility corrected amino acid score method. **British Journal of Nutrition**, Cambridge, v. 108, n. Supplement S2, p. S183-S211, 2012.

BUSTIN, S.A.; BENES, V.; GARSON, J.A.; HELLEMANS, J.; HUGGETT, J.; KUBISTA, M.; MUELLER, R.; NOLAN, T.; PFAFFL, M.W.; SHIPLEY, G.L.; VANDESOMPELE, J.; WITTWER, C.T. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. **Clinical Chemistry**, Baltimore, v. 55, n. 4, p. 611-622, 2009.

DAY, L. Proteins from land plants - potential resources for human nutrition and food security. **Trends in Food Science and Technology**, Cambridge, v. 32, n. 1, p. 25-42, 2013.

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS (FAO). **FAOSTAT crop production and trade**. Disponível em:<http://faostat3.fao.org/browse/Q/QC/E>. Acesso em: 01 Jun. 2015.

GRIFFITHS, D.W. The ratio of B to C hordeins in barley as estimated by high performance liquid chromatography. **Journal of the Science of Food and Agriculture**, London, v. 38, n. 3, p. 229-235, 1987.

HANSEN, M.; FRIIS, C.; BOWRA, S.; HOLM, P.B.; VINCZE, E. A pathway-specific microarray analysis highlights the complex and co-ordinated transcriptional networks of the developing grain of field-grown barley. **Journal of Experimental Botany**, Oxford, v. 60, n. 1, p. 153-167, 2009.

HANSEN, M.; LANGE, M.; FRIIS, C.; DIONISIO, G.; HOLM, P.B.; VINCZE, E. Antisense-mediated suppression of C-hordein biosynthesis in the barley grain results in correlated changes in the transcriptome, protein profile, and amino acid composition. **Journal of Experimental Botany**, Oxford, v. 58, n. 14, p. 3987-3995, 2007.

HOLOPAINEN, U.R.M.; WILHELMSON, A.; HOME, S.; POUTANEN, K.; SHEWRY, P. R. Day-length effects on protein localisation affect water absorption in barley (*Hordeum vulgare*) grains. **Journal of the Science of Food and Agriculture**, London, v. 92, n. 15, p. 2944-2951, 2012.

KACZMARCZYK, A. **Multi-method research strategy for understanding changes in storage protein composition in developing barley grain to improve nutritional profile**. 2012. 176p. PhD thesis (Molecular Biology and Genetics) - Faculty of Science and Technology, Aarhus University, Slagelse, 2012.

KACZMARCZYK, A.; BOWRA, S.; ELEK, Z.; VINCZE, E. Quantitative RT-PCR based platform for rapid quantification of the transcripts of highly homologous multigene families and their members during grain development. **BMC Plant Biology**, London, v. 12, n. 1, p. 184, 2012.

KIRKMAN, M.A.; SHEWRY, P.R.; MIFLIN, B.J. The effect of nitrogen nutrition on the lysine content and protein composition of barley seeds. **Journal of the Science of Food and Agriculture**, London, v. 33, n. 2, p. 115-127, 1982.

LANGE, M.; VINCZE, E.; WIESER, H.; SCHJOERRING, J. K.; HOLM, P.B. Suppression of C-hordein synthesis in barley by antisense constructs results in a more balanced amino acid composition. **Journal of Agricultural and Food Chemistry**, v. 55, n. 15, p. 6074-6081, 2007.

LEA, P.J.; AZEVEDO, R A. Nitrogen use efficiency. 1. Uptake of nitrogen from the soil. **Annals of Applied Biology**, Warwick, v. 149, n. 3, p. 243-247, 2006.

LEA, P.J.; AZEVEDO, R.A. Nitrogen use efficiency. 2. Amino acid metabolism. **Annals of Applied Biology**, Warwick, v. 151, n. 3, p. 269-275, 2007.

MARTIN, A.; BELASTEGUI-MACADAM, X.; QUILLERÉ, I.; FLORIOT, M.; VALADIER, M.-H.; POMMEL, B.; ANDRIEU, B.; DONNISON, I.; HIREL, B. Nitrogen management and senescence in two maize hybrids differing in the persistence of leaf greenness: agronomic, physiological and molecular aspects. **New Phytologist**, Cambridge, v. 167, n. 2, p. 483-492, 2005.

MASCLAUX-DAUBRESSE, C.; REISDORF-CREN, M.; ORSEL, M. Leaf nitrogen remobilisation for plant development and grain filling. **Plant Biology**, Stuttgart, v. 10, n. Supplement S1, p. 23-36, 2008.

MOLINA-CANO, J.L.; POLO, J.P.; ROMERA, E.; ARAUS, J.L.; ZARCO, J.; SWANSTON, J.S. Relationships between barley hordeins and malting quality in a mutant of cv. Triumph I. Genotype by environment interaction of hordein content. **Journal of Cereal Science**, London, v. 34, n. 3, p. 285-294, 2001.

QI, J.C.; ZHANG, G.P.; ZHOU, M.X. Protein and hordein content in barley seeds as affected by nitrogen level and their relationship to beta-amylase activity. **Journal of Cereal Science**, London, v. 43, n. 1, p. 102-107, 2006.

RAHMAN, S.; SHEWRY, P.R.; FORDE, B.G.; KREIS, M.; MIFLIN, B.J. Nutritional control of storage-protein synthesis in developing grain of barley (*Hordeum vulgare* L.). **Planta**, Berlin, v. 159, n. 4, p. 366-372, 1983.

SAVIN, R.; PRYSTUPA, P.; ARAUS, J.L. Hordein composition as affected by post-anthesis source–sink ratio under different nitrogen availabilities. **Journal of Cereal Science**, London, v. 44, n. 1, p. 113-116, 2006.

SCHÄGGER, H.; VON JAGOW, G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. **Analytical Biochemistry**, New York, v. 166, n. 2, p. 368-379, 1987.

SCHMIDT, D.; RIZZI, V.; GAZIOLA, S.A.; MEDICI, L.O.; VINCZE, E.; KOZAK, M.; LEA, P.J.; AZEVEDO, R.A. Lysine metabolism in antisense C-hordein barley grains. **Plant Physiology and Biochemistry**, Paris, v. 87, n. 0, p. 73-83, 2015.

SHEWRY, P.; TATHAM, A.; HALFORD, N. Nutritional control of storage protein synthesis in developing grain of wheat and barley. **Plant Growth Regulation**, New York, v. 34, n. 1, p. 105-111, 2001.

SHEWRY, P.R. Barley seeds proteins. In: MACGREGOR, A.W.; BHATTY, R.S. (Ed.). **Barley: Chemistry and Technology**. Saint Paul: American association of cereal chemists, 1993. chap. 3, p. 131-197.

SHEWRY, P R. Improving the protein content and composition of cereal grain. **Journal of Cereal Science**, London, v. 46, n. 3, p. 239-250, 2007.

SHEWRY, P.R. Effects of nitrogen and sulfur nutrition on grain composition and properties of wheat and related cereals. In: HAWKESFORD, M.J.; BARRACLOUGH, P. (Ed.). **The Molecular and Physiological Basis of Nutrient Use Efficiency in Crops**. Chichester: Wiley-Blackwell, 2011. chap. 6, p. 103-120.

SHEWRY, P. R.; HALFORD, N. G. Cereal seed storage proteins: structures, properties and role in grain utilization. **Journal of Experimental Botany**, Oxford, v. 53, n. 370, p. 947-958, 2002.

TA, C.T.; WEILAND, R.T. Nitrogen partitioning in maize during ear development. **Crop Science**, Madison, v. 32, n. 2, p. 443-451, 1992.

TATHAM, A.S.; SHEWRY, P.R. The S-poor prolamins of wheat, barley and rye: revisited. **Journal of Cereal Science**, London, v. 55, n. 2, p. 79-99, 2012.

UDDIN, M.N.; NIELSEN, A L.-L.; VINCZE, E. Zinc blotting assay for detection of zinc-binding prolamin in barley (*Hordeum vulgare*) grain. **Cereal Chemistry**, Saint Paul, v. 91 , n. 3, p. 228-232, 2014.

ZHU, X.G.; PORTIS, A.R.; LONG, S P. Would transformation of C3 crop plants with foreign Rubisco increase productivity? A computational analysis extrapolating from kinetic properties to canopy photosynthesis. **Plant, Cell and Environment**, Oxford, v. 27, n. 2, p. 155-165, 2004.

5 FINAL CONSIDERATIONS

In the present study a multi-method research based on combination of different proteomic and transcriptomic methods has been undertaken to understand synthesis and accumulation of barley grain proteins and their relation to improved nutritional quality of non-transgenic and transgenic antisense C-hordein barley lines with the protein profile altered.

Chapter 2 presents a new procedure of barley grain protein extraction method and a multilevel characterization of each protein extracts. The gel free shotgun MS method is an important tool to unreveal complex mixture of proteins, including the generation of lists of proteins present in a protein mixture and also giving quantitative estimation of the relative abundance of each protein. Simpler alternative methodologies exist, such as 1D-SDS-PAGE excision band technique coupled to MS identification and showed to be promising tool for differential SDS-PAGE experiment of normal and genetic modified barley. The use of one or another depends on the complexity of the protein mixtures, thus we concluded that gel free shotgun MS method and 2-DE should be used for more complex samples, such salt soluble proteins and glutelins, while 1D-SDS-PAGE can be successfully employed for prolamins.

Chapter 3 reports the salt soluble profile of non-transgenic barley cv. Golden Promise and transgenic antisense C-hordein barley lines that were determined by 2-DE and the differentially expressed proteins identified by MS. The suppression of C-hordeins, the poor nutritional hordein subgroup, not exclusively affects hordein synthesis and accumulation. Upregulation of proteins involved in stress and detoxification, and defence system, as well as storage globulins occurs. The more balanced amino acid composition of the transgenic lines is an indirect result of profound alterations also in the pattern of non-storage proteins, however it may be a consequence of distinct protein sources among the three antisense C-hordeins lines analyzed. But, the upregulation of lysine-rich proteins is consistently observed in all lines.

Chapter 4 described the differential effects of three dosages nitrogen fertilization on the hordein families' synthesis and accumulation of non-transgenic barley cv. Golden Promise and in the transgenic antisense barley line 5. The response to nitrogen differs between transgenic and non-transgenic lines. At transcriptomic level in immature grains, the transgenic line exhibited a specific C-hordein downregulation effect and in particular different responses to N was verified among subgroups of C-hordein multigene family in the transgenic line. At proteomic level in mature grain, all hordeins families were upregulated positively correlating with increasing nitrogen treatments. The distribution of C-hordein isoforms differed considerably in the transgenic line versus the control in response to nitrogen treatment.

The multi method strategy employed in the present research was able to revealed important aspects in relation to synthesis and accumulation of barley grain proteins. The choice of each method to use depends on the biological questions which intent to be answered and the complexity of variables which must be investigated. However, method handicaps exist and the right choice can reduce them.