Composition and structure of barley (*Hordeum vulgare L.*) grain in relation to end uses

Ulla Holopainen-Mantila

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Academic dissertation

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List of original publications

This thesis is based on the following original publications, referred to in the text by their Roman numerals:

I **Holopainen URM, Wilhelmson A, Home S, Poutanen K, Shewry PR**. 2012. Day-length effects on protein localisation affect water absorption in barley (*Hordeum vulgare*) grains. *Journal of the Science of Food and Agriculture* **92**: 2944–2951.

II **Holopainen URM, Rajala A, Jauhiainen L, Wilhelmson A, Home S, Kauppila R, Peltonen-Sainio P.** Influence of sulphur application on hordein composition and malting quality of barley (*Hordeum vulgare* L.) in Northern European growing conditions. *Journal of Cereal Science, in press.*

III **Holopainen URM, Pihlava J-M, Serenius M, Hietaniemi V, Wilhelmson A, Poutanen K, Lehtinen P.** 2014 Milling, water uptake and modification properties of different barley (*Hordeum vulgare* L.) lots in relation to grain composition and structure. *Journal of Agricultural and Food Chemistry* 62: 8875–8882.

IV **Holopainen URM, Wilhelmson A, Salmenkallio-Marttila M, Peltonen-Sainio P, Rajala A, Reinikainen P, Kotaviita E, Simolin H, Home S.** 2005. Endosperm structure affects the malting quality of barley (*Hordeum vulgare* L.). *Journal of Agricultural and Food Chemistry* 53: 7279–7287.

The publications are reproduced with kind permission from the publishers. Additionally, unpublished results related to **Publication I** are also presented in the summary.

Author's contributions

I   Ulla Holopainen participated in experimental design and was responsible for the experimental work. She had the main responsibility for interpretation of the results and writing the publication.

II   Ulla Holopainen was responsible for the analysis of hordeins. The interpretation of the results was performed together with Lauri Jauhiainen, Annika Wilhelmson, Ari Rajala and Pirjo Peltonen-Sainio. Ulla Holopainen had the main responsibility for preparing and writing the publication.

III   Ulla Holopainen was responsible for planning of the research and the experimental work. She had the main responsibility for interpretation of the results and writing the publication.

IV   Ulla Holopainen was responsible for hordein extraction and analysis of endopeptidase activities. She had the main responsibility for interpretation of the results, preparing and writing the publication except for the RP-HPLC results, which were interpreted together with Helena Simolin.

Contents

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Abstract

Tiivistelmä

List of abbreviations

ANOVA   analysis of variance

AOAC   Association of Official Analytical Chemists

AU   arbitrary units

β-glucan   mixed-linkage (1→3,1→4)-β-D-glucan

CE   controlled-environment

dap   days after pollination

DTT   dithiothreitol

EBC   European Brewery Convention

ER   endoplasmic reticulum

FAN   free amino nitrogen

HMW   high molecular weight

ICP-AES   inductively-coupled plasma atomic emission spectroscopy

LMW   low molecular weight

LTm   light transmission meter

NIR   near-infrared

PUG   partially unmodified grains

QTL   quantitative trait locus

REML   restricted maximum likelihood

RP-HPLC   reverse-phase high-performance liquid chromatography

SDS-PAGE   sodium dodecyl sulphate polyacrylamide gel electrophoresis

Tukey's HSD post-hoc test   Tukey's honestly significant difference post-hoc test

1. Introduction

Barley, *Hordeum vulgare* L. (Poaceae), is an annual monocotyledonous herb. Belonging to tribe Triticeae, barley is evolutionarily closely related to two other small-grain cereal species, wheat and rye, although the genus *Hordeum* is known to have diverged c. 12 million years ago (von Bothmer and Komatsuda 2011).

The first signs of the pre-agricultural gathering of wild barley are found in the region of Fertile Crescent in south-western Asia c. 22 000 years ago, and domestication of barley has occurred independently also in Central Asia (Piperno et al. 2004; Morrell and Clegg 2007). The early selection by environmental factors and man and continued with modern breeding has resulted in hundreds of landraces and cultivars, which are grown from semi-arid subtropical to temperate climates, from equatorial to nearly circumpolar latitudes, and from sea-level to high altitudes. Characteristically for a grain crop, barley cultivated today has long heads and large grains in comparison to its wild ancestors. These features support high grain yield as well as quality.

Today, barley is a significant crop plant globally, and it is mainly exploited as feed or as a raw material for malt production. In Finland, feed and industrial uses of barley cover 59 and 14% of total barley usage, respectively (Tike 2013). Only a small proportion of barley (0.7% in Finland) is consumed as food. This is in contrast to regions such as Northern Africa and mountainous areas of Asia where it is a staple food. Only recently the high content of soluble dietary fibre present in barley and its proven health effects have boosted the status of barley as a food ingredient (Baik and Ullrich 2008).

The cultivation area of barley has diminished during the last two decades (FAO 2014). In Northern America, decline is related to competition with more profitable crops (Hertsgaard 2012), and the same might apply to European countries as well. Nevertheless, in Finland barley has maintained its position as an important crop plant due to its fast growth cycle, which is required to reach harvest-ripeness during the short growing season dominated by long day-length.

The current study was conducted to reveal how growing conditions, in particular those prevailing in Northern Europe, affect barley grain composition and structure. Furthermore, it was studied how the grain's compositional and structural properties are associated with end-use quality in milling as well as hydration and hydrolysis of endosperm occurring during malting. The study was focussed particularly

on the major storage proteins of barley, namely hordeins, which are centrally located in the grain.

1.1 Barley grain architecture

1.1.1 Structure and composition of barley grain

In botanical terms, the barley grain represents an indehiscent fruit type called a caryopsis. Caryopses develop from spikelets, which are attached to the rachis of the spike by short structures called rachillas. The barley grain has an elongated shape and is divided longitudinally in half by a crease extending over the whole length of the grain (**Figure 1A**). The crease marks the ventral side of the grain, and the opposite side is called the dorsal side. The end of the grain where the embryo is located is attached to the rachis (Evers and Millar 2002).

The major parts of the barley grain are the endosperm, the embryo and their covering layers of maternal origin (**Figure 1B**). The endosperm consists of the starchy endosperm and a surrounding aleurone layer (**Figure 2**). The starchy endosperm forms the largest morphological part of the barley grain comprising c. 75% of its weight (Evers and Millar 2002). The function of the starchy endosperm is to serve as a nutrient storage for the growing embryo during germination. It is comprised of dead cells lacking nuclei and containing starch granules embedded in a matrix of storage proteins. The surrounding cell walls consist of mixed-linkage $(1\rightarrow3,1\rightarrow4)$-β-D-glucan (β-glucan) and arabinoxylan in proportions of 75% and 20%, respectively (Fincher and Stone 1986). The cell shape varies in the different parts of the starchy endosperm, being irregular in the flanks, prismatic between the crease and the dorsal side of the grain and smaller and regular in size in the subaleurone, which is the outermost layer of the starchy endosperm (Bosnes et al. 1992). Subaleurone cells contain more storage protein than other starchy endosperm cells (Palmer 1993; Olsen 2001).

The aleurone layer in barley grain is comprised of 2–4 rows of cells with thick, two-layered cell walls consisting mainly of arabinoxylan, while β-glucan is a minor component (Fincher and Stone 1986). Aleurone cells contain protein, lipids, vitamins and minerals (Pomeranz 1973; Fincher 1976; Evers and Millar 2002). Aleurone cells are isodiametric in comparison with the cells of the starchy endosperm. Besides the embryo, the aleurone layer is the only part of the grain containing living cells.

The embryo consists of an acrospire (including coleoptile, leaf primordia, and apical meristem), a nodal region between the root and the shoot, and a primary root covered by coleorhiza. The embryo is separated from the endosperm upon germination by scutellum, which is a modified cotyledon. The outermost layer of the scutellum, the scutellar epithelium, faces towards the outermost layer of endosperm, which in this part of the grain is the layer of crushed cells formed of compressed cell wall material (Palmer 1998).
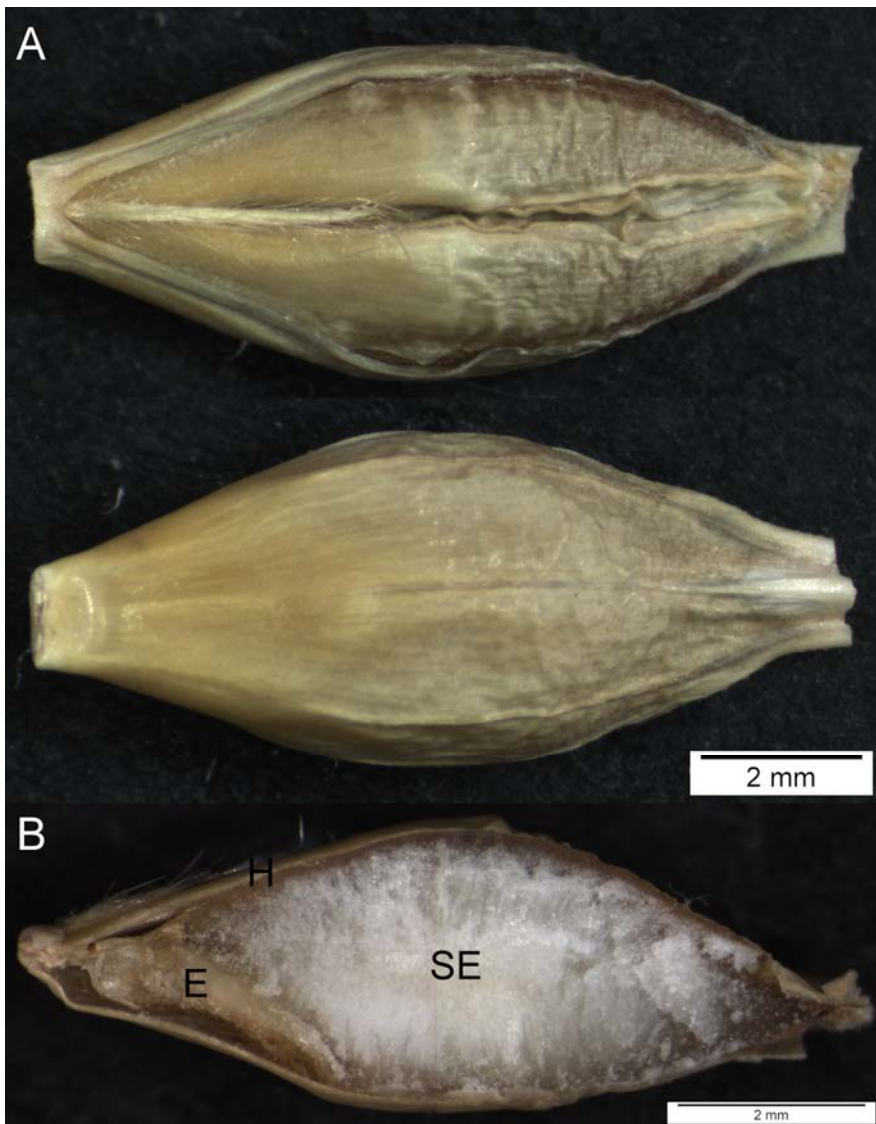
Figure 1. Appearance of barley grain from ventral (A, upper image) and dorsal (A, lower image) side and longitudinal-cut surface (B) showing husk, H, embryo, E, and starchy endosperm, SE.
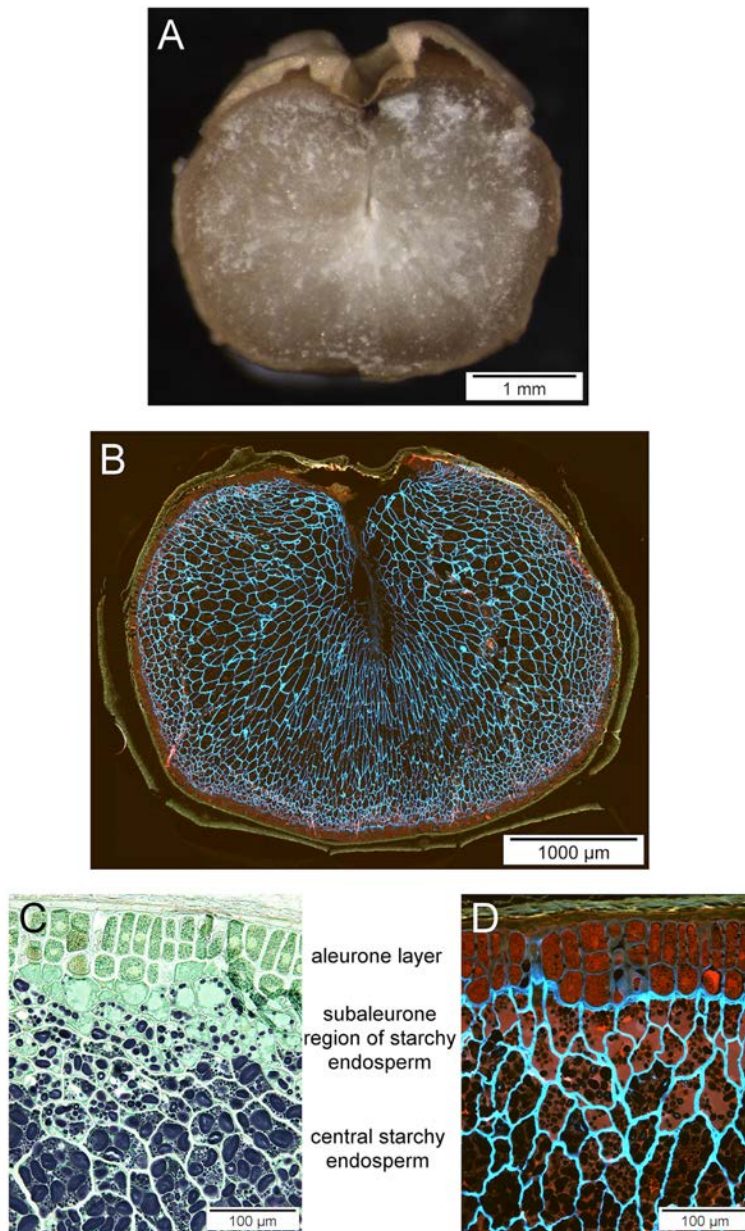
Figure 2. Structure of barley endosperm illustrated by a cross-cut surface (A), whole cross section (B) and close-ups (C and D) representing the middle third of the grain. In B and D, protein (red) and β-glucan in cell walls (blue) are visualized by Acid Fuchsin and Calcofluor, respectively. In C, protein (green) is stained with Light Green and starch (dark blue) with Lugol's iodine.

The innermost layer enclosing both the endosperm and embryo is the nucellar epidermis. It is a residue of a maternal tissue, which serves nutrients to the embryo and endosperm during the early stages of grain development. The nucellar epidermis consists of hyaline and no pigments are present (Duffus and Cochrane 1992). The testa (seed coat) is a thin layer surrounding the nucellar epidermis. It is composed of two cell layers with differing cell angle. Cells in the inner layer of the testa are parallel to the crease, while in the outer layer, the long axis of the cells is in right angle to the crease (Evers and Millar 2002). The pericarp (fruit coat) enclosing the testa also consists of several cell layers including hypodermis or layers of crushed cells, cross cell layers and a tube cell layer. The majority of the cells in the pericarp of mature barley kernels are dry and empty. Typically large intercellular spaces occur between shrunken cells in the pericarp (Freeman and Palmer 1984; Evers and Millar 2002).

The outermost layer of the grain is the husk. It forms 10–13% of the grain weight being thus the second largest part of the grain after the starchy endosperm (Evers and Millar 2002). The husk consists of two distinct overlapping structures called lemma and palea covering the ventral and dorsal side of the grain, respectively. Characteristic for barley, the husk is tightly attached to the pericarp layer by a cementing layer (Olkku et al. 2005). The grain outer layers are separated from each other by cutin layers. The cutin layer between testa and pericarp is the thickest of all three cutin layers present, and it is believed to be the main structure influencing water impermeability of the grain (Evers and Millar 2002; Olkku et al. 2005).

The genetic diversity of barley is shown by the variation present among cultivars. Barley cultivars may differ structurally, e.g. by the presence or absence of the hull (hulled and hull-less cultivars) or in the spike structure (2- and 6-rowed cultivars). Variation occurs also in the growth habit such as between spring- and winter-type barley cultivars. Barley cultivars vary compositionally having, e.g. a high content of β-glucan or lysine or containing no proanthocyanidins (Baik and Ullrich 2008). The amylose content of barley starch shows large variation, being 0% in zero-amylose barley, c. 5% in waxy barley, 20–30% in barley with normal starch composition, and up to 45% in high-amylose barley (Goering et al. 1973; Morrison et al. 1986; Henry 1988; Bhatty and Rossnagel 1997).

Typically, a hulled barley grain consists of about 56–67% starch, and the range is somewhat lower for waxy barley (54–60%) (Åman et al. 1985; Oscarsson et al. 1996, 1997; Holtekjølen et al. 2006). In the data of Holtekjølen et al. (2006) covering 29 hulled barley cultivars, protein contents varied from 8.2 to 14.5%, and the 10 hull-less cultivars showed variation between 12.6–18.5%. β-Glucan content is typically 2.5–5.5%, but contents of 6% or higher are typical to waxy cultivars and even a cultivar having a β-glucan content as high as of c. 15% exists (Lehtonen and Aikasalo 1987; Pérez-Vendrell et al. 1996; Andersson et al. 1999; Zhang et al. 2001; Holtekjølen et al. 2006). Contents of fat and minerals or ash are low being 3–4% for lipids, and 2–3% for ash (Åman et al. 1985; Andersson et al. 2008).

1.1.2 Storage proteins in barley grain

The main function of storage proteins in cereal grain is to act as a reserve of nitrogen. During germination, these proteins are mobilized by hydrolytic enzymes and the resulting peptides and amino acids are utilized for the growth of the developing seedling. The storage proteins in barley grains belong to two solubility classes, namely globulins (a fraction soluble in dilute salt solutions) and prolamins (a fraction soluble in aqueous alcohols) (Gubatz and Shewry 2011). These fractions are obtained using a sequential extraction procedure established by Osborne (Osborne 1895). Due to the salts present in the mature barley grain, water-soluble albumins not counted as storage proteins are extracted with globulins.

Globulins cover 10–20% of the total protein content of barley grains (Lásztity 1984). Not all globulins serve as storage proteins, some have metabolic and protective functions (Gubatz and Shewry 2011). β-Amylase is an exception, acting both as nitrogen storage during grain development and starch-hydrolysing enzyme during germination (Giese and Hejgaard 1984). Globulins having a storage protein role are mainly located in the aleurone cells, but also in the embryo (Yupsanis et al. 1990). Barley contains γ- and δ-globulins, which have sedimentation coefficients ($S_{20.w}$) of 7–8 S and 12 S, respectively. Barley γ-globulin is comprised of four subunits with relative molecular masses of 50, 40, 25 and 20 kDa (Burgess and Shewry 1986; Yupsanis et al. 1990). Based on partial amino acid sequencing, it is homologous with vicilin-like globulins present in cotton and legumes (Yupsanis et al. 1990). δ-Globulin has a molecular weight of 300 kDa, its structure and composition are not yet known (Shewry 1993). It is assumed to be related with legumin-like triticins in wheat endosperm (Shewry 1993; Gubatz and Shewry 2011).

The prolamins present in barley grains are called hordeins, and are located only in the cells of the starchy endosperm (Yupsanis et al. 1990; Shewry 1993). Hordeins can be considered major storage proteins, forming 35–55% of the protein content of barley grain, depending on nitrogen application rate (Kirkman et al. 1982). Prolamins of cereal species belonging to tribe Triticeae share many structural features and are part of the large prolamin superfamily, which covers a diverse group of plant proteins with a characteristic conserved cysteine skeleton (Shewry 1995; Kan et al. 2006).

Hordeins are classically differentiated by their mobility in sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE). D hordeins have the highest molecular weight (105 kDa) followed by C (55-75 kDa), B and γ (32–46 kDa), and low-molecular-weight (LMW) or avenin-like hordeins (22 and 16.5 kDa) (Faulks et al. 1981; Shewry and Miflin 1982; Salcedo et al. 1982; Festenstein et al. 1987; Rechinger et al. 1993). The status of LMW hordeins as a part of the hordein fraction was confirmed rather recently (Kan et al. 2006; Gubatz and Shewry 2011). B and C hordeins are the major hordein fractions accounting for 70–80% and 10–20%, respectively, of the grain hordein content while proportions of D and γ hordeins are lower than 5% (Kirkman et al. 1982; Shewry et al. 1983; Shewry 1992). The polypeptide composition of each hordein group varies with cultivar (Kreis et al.

1983). This high polymorphism of hordein groups among barley genotypes, resulting in unique hordein banding in SDS-PAGE, can be used as a tool in varietal identification (Marchylo and Kruger 1985).

High contents of glutamine and proline are characteristic of the hordeins (**Table 1**). Hordeins are also typically poor in lysine, which reduces their nutritional value (Kreis et al. 1984). Each hordein group has a characteristic amino acid composition, which is strongly influenced by the repetitive amino acid sequences covering 30-100% of these polypeptides (Shewry 1993; **Figure 3**). Typically, C hordein contains more phenylalanine than other hordeins. D and LMW hordeins contain less proline in comparison to other hordein groups. As compensation, these hordeins are rich in glycine, serine, and threonine.

Table 1. Amino acid composition of total hordeins and its fractions (mol%). Data is based on full length sequences except for LMW hordein.

Amino acid	Hordeins				
	B1	C	D	γ_1	LMW [a]
Alanine (A)	2.5	0.7	3.2	2.1	7.2
Arginine (R)	2.6	1.0	1.6	1.4	2.5
Asparagine (N)	0.7	1.0	0.9	1.7	} 2.2
Aspartic acid (D)	0.0	0.0	0.6	0.7	
Cysteine (C)	2.9	0.0	1.5	3.5	7.1
Glutamine (Q)	30.3	37.2	25.8	28.0	} 26.2
Glutamic acid (E)	1.8	1.4	2.2	2.1	
Glycine (G)	2.9	0.3	15.7	3.1	6.0
Histidine (H)	1.4	1.0	3.1	1.4	0.2
Isoleucine (I)	4.4	3.4	0.7	3.8	3.3
Leucine (L)	8.0	4.1	4.1	7.0	4.0
Lysine (L)	0.7	0.0	1.2	1.7	0.1
Methionine (M)	1.1	0.3	0.4	2.1	3.6
Phenylalanine (F)	4.7	7.9	1.3	5.6	2.1
Proline (P)	19.3	30.0	10.5	16.8	7.0
Serine (S)	4.7	4.1	10.5	5.6	12.2
Threonine (T)	2.2	1.4	7.3	3.1	9.6
Tryptophan (W)	0.7	1.4	1.2	0.7	-
Tyrosine (Y)	2.5	3.1	4.2	2.1	2.3
Valine (V)	6.2	1.4	4.1	7.3	4.4
Ref.	1, 2	2, 3	2	2, 4	5

References: 1) Forde et al. 1985 (cv. Sundance), 2) Shewry et al. 1999, 3) Sayanova et al. 1993, 4) Cameron-Mills and Brandt 1988, 5) Salcedo et al. 1982 (cv. Zephyr)
[a] LMW subunit of 16.5 kDa

The amino acid composition of hordeins defines to a large extent their functional properties. The high contents of glutamine and proline contribute to the characteristic solubility of prolamins in aqueous alcohols (Shewry and Miflin 1985; Kreis and Shewry 1989). Cysteine residues, in turn, play an important role in the intra- and inter-chain aggregation of hordeins, which happens via sulphur bridges. C hordein, which lacks cysteine, is present in monomeric form or as single polypeptide subunits. Part of the B hordein and part of the γ hordein are also in monomeric form having intramolecular disulphide bonds between conserved cysteine residues. The remaining B and γ hordeins and all D hordein are present in the mature grain in polymeric form as aggregates of polypeptide subunits linked by inter-chain disulphide bonds (Shewry 1992; Rechinger et al. 1993; Shewry et al. 1999). In B and γ hordeins, inter-chain sulphur bridges are probably formed between cysteine residues marked as "unpaired" in **Figure 3**. Due to disulphide bonds, polymeric hordeins are extractable only in the presence of a reducing agent. B and D hordein may form a mixed polymer, which has been recognized in the gel protein fraction (van den Berg et al. 1981; Smith and Lister 1983). It has been proposed that γ hordeins may also occur in mixed hordein aggregates (Gubatz and Shewry 2011). In addition to the linkages via sulphur bridges, repetitive domains of hordeins have been suggested to form regular spiral supersecondary structures relying on the β-reverse turns and poly-L-proline II structures (Tatham and Shewry 2012).

Hordeins are encoded by separate loci Hor1–Hor5 located on barley chromosome 1H(5) (reviewed by Halford and Shewry 2007). Hordein synthesis is regulated mainly on a transcriptional level with fine-tuning on a translational level (Shewry et al. 2001). The transcription of B, C and γ hordein genes is regulated by the "prolamin box", which is a c. 30 basepair-long sequence located c. 300 basepairs upstream of the translation initiation site (Forde et al. 1985). The prolamin box contains two conserved regions called the endosperm (E) and nitrogen (N) motifs. The regulation of transcription occurs through interaction of these two motifs and transcription factors. D hordein synthesis is controlled by an upstream "HMW prolamin enhancer" region, which is extremely tightly conserved among HMW prolamins of cereal species and shows only limited similarity with the prolamin box (reviewed by Halford and Shewry 2007; Shewry 2011).
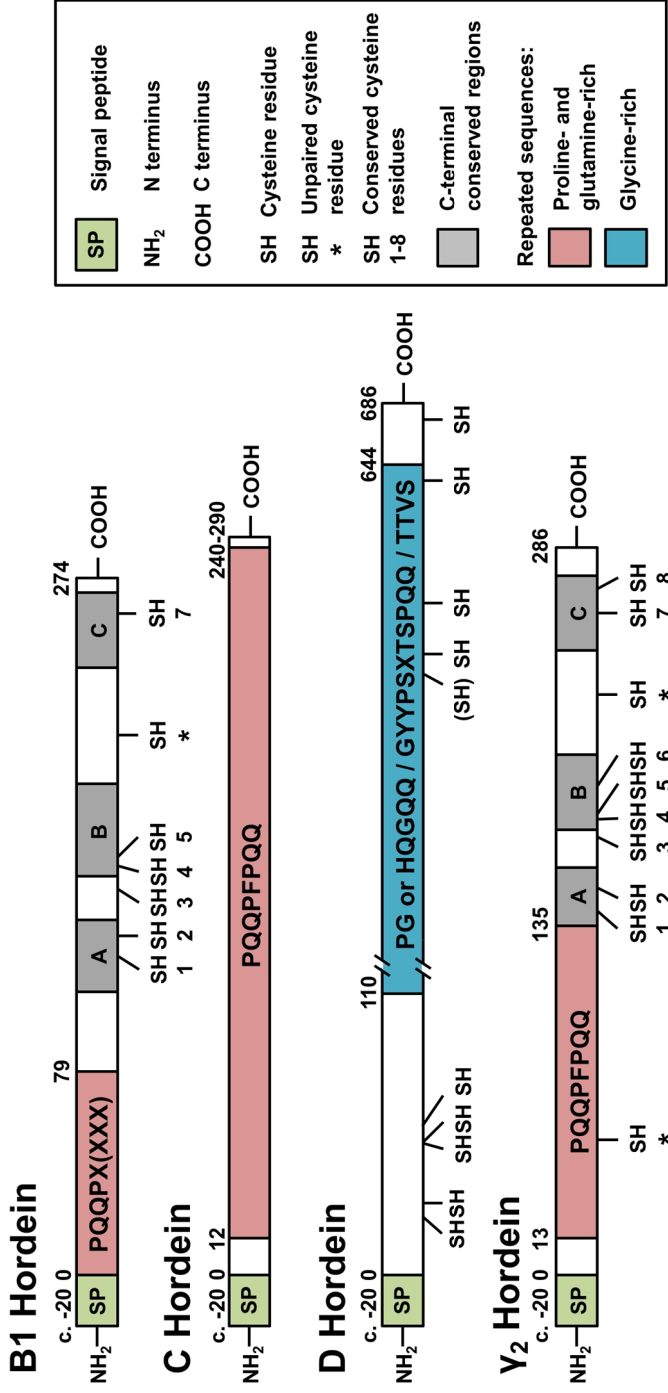
Figure 3. Schematic structures of barley hordeins.
The one-letter abbreviations of amino acids are given in **Table 1**.
(Based on data reviewed in Shewry et al. 1995, 1999; Gubatz and Shewry 2011).

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1.1.3 Packing of the starchy endosperm

In mature barley grains, the cells of the starchy endosperm are filled with starch granules embedded in a protein matrix (**Figure 4**). The subcellular structure of the starchy endosperm is formed during grain filling, which is characterized by the expansion of starchy endosperm cells due to intracellular accumulation of storage compounds (Bosnes et al. 1992). Starch packed in granules serves as the major storage of photoassimilates, forming the bulk of the mature starchy endosperm. Hordein storage proteins act as the reserve of nitrogen and sulphur, and they are the second major constituent of the mature starchy endosperm. In addition, for example phosphorus (in nucleic acids) and calcium are stored inside cells during grain filling (Ritchie et al. 2000).
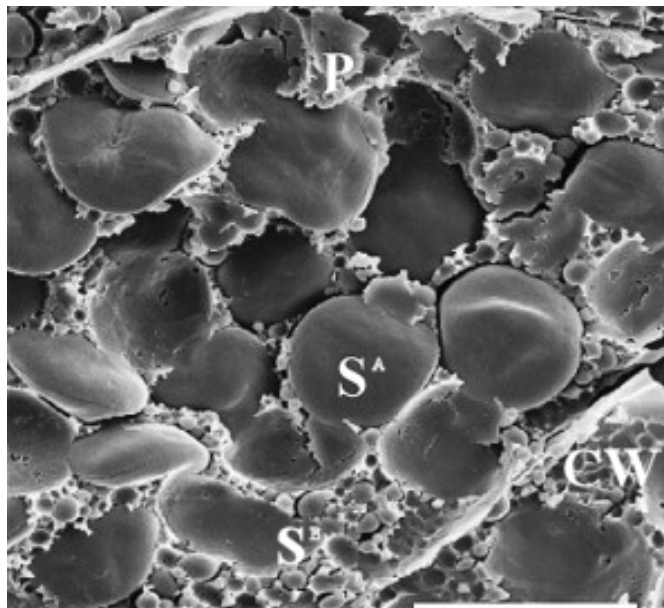
Figure 4. Location of protein (P) as a matrix which surrounds the large A-type starch granules (S^A) and between which small B-type starch granules (S^B) are embedded in mature starchy endosperm of barley grain (cell wall, CW). Scale bar corresponds to 30 μm. Reproduced from Nair, Knoblauch, et al. 2011 with permission from the publisher.

Grain filling starts already before the cells of the developing endosperm are fully differentiated and continues during the grain maturation stage until the starchy endosperm undergoes programmed cell death, and dehydrates (Young and Gallie

2000). Grain filling is preceded by the early phases of endosperm development including the nuclear divisions in the triploid endosperm and cellularization of this syncytial tissue (Bosnes et al. 1992). After the cellularization stage, at c. 8 days after pollination (dap), the differentiation or fate determination of cells to starchy endosperm and aleurone layer begins (Bosnes et al. 1992). At this stage, cell divisions start in the middle part of the developing grain proceeding towards the outer layers ceasing by c. 14 dap in the starchy endosperm (Bosnes et al. 1992; Brown et al. 1994; Olsen 2001). The differentiation stage is over at c. 21 dap when subaleurone cells, derived from the innermost cells of aleurone, have differentiated as the last tissue (Bosnes et al. 1992). During the cellularization and differentiation stages, the final cellular structure of the starchy endosperm is established by the development of anticlinal walls, succeeded by the formation of periclinal cross-walls and mitotic divisions of the cells formed (Bosnes et al. 1992). The fully differentiated starchy endosperm is characterized by the presence of three cell types (prismatic, irregular and isodiametric subaleurone cells) at the beginning of the maturation stage (Bosnes et al. 1992).

Grain filling begins with the appearance of the first starch granules at c. 10 dap or soon after cellularization is finished and the first cell walls have been formed (Bosnes et al. 1992; Duffus and Cochrane 1993). Starch is synthesized from glucose-1-phosphate by the action of four enzymes situated in specific membrane structures called amyloplasts (Duffus and Cochrane 1992; Hannah 2007; Hannah and James 2008). The first starch granules appear in the central stroma of each amyloplast, where one single starch granule is initiated and grows steadily in size (Duffus and Cochrane 1992; Wei et al. 2010). In mature endosperm, these first starch granules form a population of large, so called A-type starch granules, having a lenticular shape and diameter of 10–50 µm (Goering et al. 1973). The synthesis of lenticular starch granules is followed by the initiation of spherical B-type starch granules approximately at 14 dap (Duffus and Cochrane 1992). These round starch granules appear first in the oldest endosperm cells in the middle of the developing grain and are formed in the stroma-filled protrusions of the amyloplasts (Parker 1985; Langeveld et al. 2000; Wei et al. 2008). In mature endosperm, B-type starch granules are 1–10 µm in diameter (Goering et al. 1973). In some studies, the smallest B-type granules are classified a separate group of C-type starch granules having an average diameter of c. 2 µm (Takeda et al. 1999). In wheat, the timing of starch granule formation has been associated with their size, the smallest C-type granules being synthesized after the formation of B-type granules has ceased (Wei et al. 2010).

The accumulation of endosperm storage proteins commences several days after the formation of the first starch granules in the endosperm amyloplasts (Cameron-Mills and Wettstein 1980; Duffus and Cochrane 1993). The first hordeins in developing endosperm have been detected as early as 10-14 dap by SDS-PAGE (Shewry et al. 1979; Giese et al. 1983) and immunocytochemically as early as 5-6 dap (Davies et al. 1993; Pulido et al. 2006). The slight discrepancies between the reported first appearance of starch granules and storage protein bodies are probably due to differences in evaluation of the timing of pollination or in the

plant developmental schedule under different growing conditions. Hordein synthesis does not begin simultaneously in the different parts of the starchy endosperm. Accumulation of hordein has been shown to follow the pattern of transcriptional activity, which proceeds in a wave-like action from both grain ends through the dorsal, outer starchy endosperm continuing towards the central parts (Shewry et al. 1993; Davies et al. 1993).

On a subcellular level, hordeins are synthesized in the ribosomes on the surface of the rough endoplasmic reticulum (ER) in the cytosol (Brandt and Ingversen 1976; Matthews and Miflin 1980). Prolamins contain a signal peptide typical to secretory proteins, which control their transport into the lumen of the ER (Shewry et al. 1995). In the ER lumen, the signal peptide is cleaved and polypeptides take their conformation by folding and assembly, e.g. via disulphide bonds. Within the ER, prolamins are assumed to aggregate as protein bodies, although it is not known how these protein bodies are initially formed (Tosi 2012). Nevertheless, it is widely accepted that prolamins are deposited in protein storage organelles via two transport routes, one dependent and the other independent of the Golgi apparatus (Galili et al. 1993; Herman and Larkins 1999; Shewry and Halford 2002).

The first indications of the hordein routing to the storage vacuoles either through the Golgi apparatus or bypassing it in the developing starchy endosperm were presented by Cameron-Mills and Wettstein (1980) using transmission electron microscopy and by Miflin et al. (1981) using biochemical analysis of protein bodies. The current understanding is that part of the prolamins is transported via the Golgi to the protein storage vacuole and that this is mediated by *de novo* synthesis of vacuoles. The other part is kept within the ER from which protein bodies then are derived and adsorbed by storage protein vacuoles in a process similar to autophagy (Rubin et al. 1992; Levanony et al. 1992; Shewry et al. 1995). Prolamins do not contain known signal peptides for retention in the ER or targeting to storage vacuoles (Shewry and Halford 2002; Tosi 2012). It is not known therefore how the transport route for these proteins is determined. However, it has been shown that in older cells of the starchy endosperm in the more central part of the tissue, hordeins are deposited within the ER, whereas hordeins in the younger subaleurone cells are transported to the storage vacuoles via the Golgi apparatus (Okita and Rogers 1996). It has also been proposed that accumulation of starch could disrupt the Golgi-dependent pathway at the later stages of grain filling making accumulation of hordeins within the ER more preferable (Shewry 1993). Accordingly, it has been shown that in the developing wheat grain, the same prolamins can be transported by either route, probably depending on the developmental stage (Tosi et al. 2009).

Protein bodies of hordeins from both trafficking routes are ultimately located within large protein storage vacuoles (Cameron-Mills and Wettstein 1980). These vacuoles are formed by the fusion of smaller vacuoles and subsequent aggregation of protein bodies (Tosi 2012). The organization of hordeins within the protein bodies is not known. In wheat and maize, a segregation of different prolamins might occur both between and within protein bodies (Lending and Larkins 1989; Tosi et al. 2009, 2011).

Hordein accumulation during grain filling has been reported to last until c. 39–45 dap with the greatest accumulation rate from 23 to 29 dap (Rahman et al. 1982; Møgelsvang and Simpson 1998). C hordein is synthesized at the early stage of grain filling. Then higher accumulation of B hordein decreases the proportion of C hordein during the later stages (Rahman et al. 1982). D hordein is probably synthesized during the later stage of grain filling, as it has been detected only in mature grains (Schmitt et al. 1989).

Grain filling ceases after the mid-stage of grain maturation. At this point, the metabolic activities in the starchy endosperm are reduced, partly due to the induction of desiccation, but also as coordinated by ethylene-mediated programmed cell death (Young and Gallie 2000; Sreenivasulu et al. 2006). Unlike the programmed cell death occurring in the aleurone layer during germination, the programmed cell death of the starchy endosperm before full grain maturity does not involve degradation of cell contents and cell walls (Sabelli 2012). During these later stages of grain maturation, the integrity of the membranes of protein storage vacuoles as well as that of amyloplasts envelopes is lost (Miflin and Burgess 1982; Wei et al. 2008). As a result, starch granules are localized in a protein matrix in the mature starchy endosperm. In particular, small starch granules and hordein are similarly distributed in the mature starchy endosperm, both being more abundant in the outer than in the inner part (Palmer 1989, 1993; Davies et al. 1993). B and C hordeins are concentrated mainly in the subaleurone cells and D hordein in the central starchy endosperm cells (Shewry et al. 1996; Tesci et al. 2000). Despite the differences in the accumulation of these proteins, the developmental basis for the differential distribution of B, C and D hordeins is not known (Shewry and Halford 2002).

1.1.4 Texture of barley endosperm

The structure of the grain formed during grain development, and especially the accumulation of storage reserves, greatly affect not only chemical, but also physical properties of the barley grain. In the context of the texture of the barley grain, the terms hardness and steeliness (also known as vitreousness) are often used as synonyms, although they refer to separate characteristics of the grain. Grain hardness can be defined as the resistance of the kernel to deformation or fracture (Turnbull and Rahman 2002). More precisely, hardness is a property of endosperm tissue, as it is independent of the presence or absence of grain outer layers (Galassi et al. 2012). Steeliness is also clearly a property of the endosperm, describing the degree of packing of the endosperm cell contents. The assessment of steeliness is based either on the effect of air spaces on the light absorbance properties or on the appearance of the endosperm fracture plane. A loosely packed or mealy endosperm, with air spaces between protein and starch granules, absorbs light, while a densely packed, steely endosperm structure enables the passage of light (Chandra et al. 1997, 1999). Cross-cut surfaces of mealy grains appear

opaque or floury, while those of steely grains are translucent or glassy (Chandra et al. 1999; Mayolle et al. 2012). The difference in the appearance of the cross-cut surface of the grain is explained by the fact that in a steely grain, the fracture plane traverses the interface of starch granule and protein matrix. A mealy endosperm, in turn, shows the fracture plane around A-type starch granules, and B-type starch granules are well embedded in a protein matrix (Brennan et al. 1996). Despite the different basis of hardness and steeliness, these properties are linearly correlated (Nair et al. 2010; Mayolle et al. 2012). Accordingly, there is a positive correlation between the parameters, with grain density obviously related to endosperm packing and endosperm hardness (Walker and Panozzo 2011).

Mature endosperm in the wheat grain has been described as a cohesive granular material by Topin et al. 2008. Their model of mature wheat endosperm suggests that the texture of endosperm is dominated by the adherence between starch granules and protein matrix, and the protein content, which affects the degree of packing. In barley, protein also seems to play an important role in grain hardness. Grain protein content correlates positively with hardness and also with steeliness, but the correlation between hardness has not been found in all studies (**Table 2**). The protein matrix is more continuous in hard and steely barley grains than in soft and mealy ones (Nair, Knoblauch, et al. 2011). The continuity of the protein matrix is likely to be higher in a grain with higher protein content. The difference in the fracture planes mentioned above is related to the stronger adhesion of protein to starch granules in steely or hard endosperm (Brennan et al. 1996; Nair, Knoblauch, et al. 2011).

The adherence of storage proteins to starch granules is regulated by specific proteins, hordoindolines, which are homologous with puroindolines found in wheat (Gautier et al. 2000; Beecher et al. 2001; Darlington et al. 2001). In soft barley endosperms, hordoindolines are bound to the surface of starch granules and act on grain hardness by reducing the adhesion of the protein matrix to starch granules. In harder barley endosperms, hordoindolines are associated with the protein matrix and cannot thus impede efficiently the adhesion of protein on starch granules (Darlington et al. 2000). Accordingly, these proteins have been reported to increase the aggregation of storage proteins in hard wheat endosperm (Darlington et al. 2000; Lesage et al. 2011). The composition of hordoindolines may explain the absence of barley cultivars with endosperm texture as soft as in soft wheat cultivars (Galassi et al. 2012).

Table 2. Associations between barley grain texture and composition reported in literature indicated with + (positive association), 0 (no association) and – (negative association).

| | Grain texture | | | | | |
| | Hardness [a] | | | Steeliness [b] | | |
Grain composition	+	0	–	+	0	–
Starch content			Henry and Cowe 1990; Psota et al. 2007			
Protein content	Allison et al. 1979; Glennie Holmes 1990; Washington et al. 2001; Walker and Panozzo 2011	Henry and Cowe 1990; Psota et al. 2007; Gamlath et al. 2008; Nair et al. 2010		Agu and Palmer 1998; Chandra et al. 1999; Broadbent and Palmer 2001; Agu 2007		
β-Glucan content	Henry and Cowe 1990; Izydorczyk et al. 2003; Vejražka et al. 2008; Gamlath et al. 2008	Allison et al. 1979; Washington et al. 2001; Nair et al. 2010				Agu 2007

[a] includes results of different grain hardness analyses including milling energy, particle size distribution after grinding, and measurements by single kernel characterization system (SKCS)

[b] includes results of LTm analysis and visual inspection of vitreousness

Despite the similar function of hordoindolines and puroindolines, grain hardness in barley is less strongly linked to this protein class than in wheat (Igrejas et al. 2002; Galassi et al. 2012). In barley starchy endosperm, the cell walls are thicker and may thus affect endosperm texture more than in wheat grain (Dornez et al. 2011; Jääskeläinen et al. 2013). In fact, both the β-glucan and arabinoxylan contents of the barley endosperm correlate linearly with grain hardness and together explain c. 60% of grain hardness (Gamlath et al. 2008). Besides, many positive correlations between grain hardness and β-glucan content have been reported (**Table 2**). Hard barley grains also have thicker cell walls in starchy endosperm compared to soft grains (Nair, Knoblauch, et al. 2011). The role of cell walls in the formation of barley grain texture could explain why the correlation between protein content and hardness is not always found. Another explanation could lie in the composition or localization of hordeins, which are not routinely analysed. For example, more C hordein has been detected in steely grains compared to softer ones (Ferrari et al. 2010).The current study was focussed on hordeins in order to demonstrate their role in grain texture and further in grain processing.

Various analytical methods are available for testing grain hardness. One of these is the measurement of the milling energy needed for the grinding of barley grains (Allison, Cowe, et al. 1979)**.** Another method utilizes a single kernel characterization system (SKCS), in which individual kernels are crushed. By SKCS, endosperm hardness is calculated based on the crush force profiles corrected by the effects of kernel moisture and size (Martin et al. 1993). In addition, hardness measurements developed for wheat, based on the particle size distribution after grinding, have been applied for barley (Psota et al. 2007). Traditionally, the steeliness of barley has been examined visually on the basis of the endosperm appearance. For this, grains have been cut transversely or longitudinally in halves by a grain splitter or farinator (Briggs 1998). Today, steeliness is often examined as the passage of light through the grain either on a surface illuminated from below or using a light transmission meter (LTm). In LTm, endosperm steeliness is measured quantitatively from single kernels as the transmission of laser light through grain (Chandra et al. 2001). The use of laser light enables the assessment of barley grains without dehulling.

1.2 Growing conditions affecting barley endosperm protein and its composition

The wide range of geographical locations and climatic conditions in which barley is cultivated indicates that the genetic background of barley provides versatile capabilities for adapting the plant's growth habit to various growing conditions. In relation to this, total protein content of barley grain as well as its hordein content and composition are influenced by environmental factors.

The growing conditions in the field are a combination of biotic and abiotic environmental variables. However, there is only scarce information available on the

effect of biotic environmental factors such as pathogen infection on barley protein content or hordein composition. For example, no significant changes have been observed in total protein or hordein content in barley grains infected by *Fusarium* fungi (Eggert et al. 2010), although in wheat grains a moderate infection of *F. graminearum* was reported to cause an increase in total protein content by 6% and a major decrease in the amount of albumins and glutenins (Boyacıoğlu and Hettiarachchy 1995).

The abiotic environment encompasses aspects of light, temperature and availability of water and nutrients as well as edaphic factors. Some of these are dependent on latitudinal location (e.g. length of light period), whereas some of them can be controlled by farming practices (e.g. supply of nutrients). The effect of these environmental factors on yield formation of cereal plants including barley is well known. The effect of sulphur and nitrogen availability on barley grain protein has also been thoroughly investigated, but the impact of light, temperature and availability of water on total grain protein, its components or their localization has not been as intensively studied.

The influence of N application on yield and grain N content has been well established for wheat in comprehensive long-term studies reported, e.g. by Benzian and Lane (1981). In the case of barley, very similar results showing an increase in yield and grain N content with increasing N application rate have been reported e.g. by Oscarsson et al. (1998). N accumulation is sink-limited at the beginning of grain filling, but source-limited after the mid-point of the grain-filling period (Dreccer et al. 1997). In wheat, this has been linked to the timing of the accumulation of different protein fractions; structural and metabolic proteins are synthesized during the first half of grain filling period and most storage proteins after this (Martre et al. 2003). This is in line with observations on the positive correlation of N application and hordein amount (Kirkman et al. 1982; Giese et al. 1983; Giese and Hopp 1984; Brennan et al. 1998; Qi et al. 2006; Buiatti et al. 2009). Protein content is affected by the separate action of cultivar and nitrogen application rate, while hordein content is affected also by the interaction of cultivar and nitrogen rate (Qi et al. 2006; Wang et al. 2007).

The increase in total hordein content occurs as the result of increased amounts of B, C and D hordeins (Qi et al. 2006). However, the amount of C hordein has been noticed to increase more than that of B hordein causing a decrease in the B:C hordein ratio (Qi et al. 2006; Savin et al. 2006). Both limiting sulphur availability and the role of C hordein as the major nitrogen sink at high nitrogen rates have been suggested as reasons for this (Kirkman et al. 1982; Giese et al. 1983; Giese and Hopp 1984; Savin et al. 2006). C and D hordeins are affected more by nitrogen availability and B hordein more by cultivar (Qi et al. 2006).

Grain storage proteins do not serve only as the main storage for nitrogen but also for sulphur. In developing cereal endosperm, sulphur from glutathione or sulphate is incorporated mainly into two S-containing amino acids, cysteine and methionine, which are further deposited as units of polypeptide chains of storage proteins (Anderson and Fitzgerald 2001). Based on their cysteine and methionine contents, hordeins are classified as S-rich (B and D hordeins) or S-poor (C hordein).

The study of Shewry et al. (1983) on the grains of S-deficient and S-sufficient barley plants of two cultivars demonstrated that the availability of sulphur influenced the hordein fraction of grain protein, while the salt-soluble proteins and glutelins were not notably affected. The decrease in the total protein content of grain related to sulphur depletion was explained solely by the lower total hordein content in the grains of S-deficient plants. As a consequence of S deficiency, the proportion of hordeins decreased from 47–51% to 27% of grain nitrogen content. At the same time, the composition of hordeins was changed through a substantial increase in the proportion of S-poor C hordein and a decrease in the proportion of S-rich B and D hordein (Shewry et al. 1983). The absolute contents of B and D hordein also decreased, but the content of C hordein did not change. Additionally it was shown that asparagine acted as a non-protein nitrogen storage compound in the grains of S-deficient plants. This was indicated by the increased amount of aspartic acid, which is a hydrolysis product of asparagine. Asparagine thus assisted in maintaining the nitrogen content independently of the sulphur availability (Shewry et al. 1983).

A similar change in hordein composition due to sulphur deficiency was reported by Rahman et al. (1983). Low sulphur availability has been shown to decrease the B hordein accumulation rate already at the early stages of hordein synthesis. Thus the low content of B hordein in mature grains is not a consequence of suspension of protein synthesis at a later stage of grain maturation due to a diminishing amount of available sulphur (Rahman et al. 1983).

Potentially, sulphur application could increase the grain nitrogen content, because it has been shown to increase the nitrogen use efficiency in wheat (Salvagiotti et al. 2009). However, either no effect or a decrease in nitrogen content due to higher sulphur availability has been observed in barley grains grown in pot-scale or field conditions (Mortensen et al. 1992; Eriksen and Mortensen 2002; Zhao et al. 2006). The decrease in grain nitrogen content could be explained by the dilution effect caused by the increased yield level with higher sulphur availability (Zhao et al. 2006). Thus, the effect of sulphur may be related to nitrogen availability and in particular which one of these nutrients is primarily limiting. In wheat, the timing of sulphur application did not influence grain protein content (Steinfurth et al. 2012).

Hordein synthesis seems to be under strict nutritional regulation of gene transcription. It has been shown that in the expression of C hordein, the N motif of the prolamin box acts as a negative regulator at low nitrogen application rates. When nitrogen is sufficiently high, the N motif interacts with the E motif which results in high transcript levels (Muller and Knudsen 1993). The transcription factors binding to these elements are known, but the mechanisms by which the transcription factors sense the nutritional status are not (Halford and Shewry 2007; Shewry 2011). Likewise, the exact mechanism by which sulphur controls the hordein gene expression is not known (Shewry et al. 2001).

Light potentially influences grain protein content via photosynthetically active radiation, as protein content is dependent on the supply and transport of assimilation products into the grain (Boonchoo et al. 1998). When a high photosynthetic

rate is maintained by high radiation during grain filling, the grain dry matter content increases, mainly by accumulation of starch, which is synthesized from sucrose supplied from photosynthetic tissues. If the radiation level is decreased, e.g. due to a high density of plant stands causing shading, the grain protein content increases due to the diminished supply of assimilates, although protein synthesis is not affected (Grashoff and d'Antuono 1997). Barley grain nitrogen content can be nearly doubled due to shading (Angelino et al. 1997). In wheat, shading has been shown to influence the prolamin contents and composition (Cai et al. 2013).

Of all environmental variables, day-length is the only constant, latitude-dependent factor that shows the same alternation during every growing season. Nevertheless, some variation in the timing of growth stages with respect to day-length is caused by the fluctuation in sowing time. Day-length is known to affect the growth habit of cereal species. Under long-day conditions at high latitudes, tillers are dominated by the main shoot. Tiller yield potential is clearly not realized even under conditions favouring growth. This leads to lower production of biomass than with shorter day-length (Fairey et al. 1975; Peltonen-Sainio, Jauhiainen, et al. 2009). Northern European growing conditions are characterized by the short growing season with long day-length leading to intensive growth (Peltonen-Sainio, Rajala, et al. 2009). Due to the limited length of the growing season, the end-use quality of barley is easily affected by suboptimal weather conditions. For example, unrealized yield potential due to drought or disease epidemics may lead to the high accumulation of protein in grains due to an excessive amount of available nitrogen (Bertholdsson 1999; Rajala et al. 2007). In wheat grain, an increase in protein content due to a longer photoperiod has been reported for photoperiods of 13 h and 11 h (Metho et al. 1999). In lighting conditions of 10 h of light and 14 h of either darkness or low light intensity, the longer day-length condition also resulted in higher grain protein content (Kolderup 1975). Long days induced also an increase in the amount of gliadin and glutelin, which was confirmed by a simultaneous increase in glutamine and proline contents (Kolderup 1975).

Similarly to shading, high temperature during grain filling increases grain protein content indirectly by decreasing the accumulation of photoassimilates (Macleod and Duffus 1988; Wallwork et al. 1998; Passarella et al. 2002). The effect of temperature on barley protein composition has not been studied. In wheat, high temperature (+37°C at daytime) during grain filling increases protein content, especially the proportion of S-poor proteins and leads to a proportionally greater amount of protein matrix in starchy endosperm cells (DuPont et al. 2006; Hurkman and Wood 2011). The effects of high temperature and nitrogen application on accumulation of gluten proteins have been reported to be very similar in wheat (Hurkman et al. 2013).

Water availability also has an influence on grain protein content. During grain filling, the accumulation of starch is more sensitive to drought than protein. Under water deficit this leads to the concentration of protein in grains without any increase in nitrogen accumulation (Brooks et al. 1982; Jenner et al. 1991). Especially in the early phase of grain filling, drought combined with high nitrogen availability increases grain protein content (Coles et al. 1991; Fathi et al. 1997). Water

deficiency may affect the translocation of nitrogen to grains and emphasize the role of nitrogen accumulation during the vegetative growth phase (Jenner et al. 1991). It has been reported that in wheat, gluten proteins accumulated faster under water deficit (Giuliani et al. 2014).

Overall, the grain protein content and composition is influenced by numerous environmental variables. For the current study, the environmental factors were selected on the basis of the significance for grain protein (nitrogen application), as well as relevance to the Northern European growing conditions (day-length). In addition, the effect of sulphur application on barley protein content and composition has not been studied before in the Scandinavian growing conditions.

1.3 Genotype-dependency of barley grain protein content and endosperm texture

The inheritance of quantitative traits in barley has been comprehensively studied through investigation of quantitative trait loci (QTL). QTL analysis is a statistical method, which combines the phenotypic data (trait measurements) and genotypic data (e.g. molecular markers) in order to explain the genetic basis of variation in complex traits (Kearsey 1998). By QTL analysis, it is possible to link certain complex phenotypes to specific regions of chromosomes, and it aims to identify the action, interaction, number, and precise location of these regions. For conventional QTL mapping, a mapping population is developed from a bi-parental cross.

A QTL analysis of nine mapping populations has shown that there are regions linked to grain protein content in all seven chromosomes of barley (Zale et al. 2000). This indicates that grain protein content is a heritable trait and that the determination of grain protein level is complex (Baik et al. 2011). QTLs of grain protein content show also high interaction with the growth environment (Emebiri et al. 2005). Three major QTLs located on barley chromosomes 6H and 2H have been estimated to explain 56% of the total heritable variance of grain protein content (See et al. 2002). Two of these regions encode transcription factors, which are homologous with regions increasing grain protein content in wheat. They regulate senescence and nutrient remobilization from leaves to developing grains (Uauy et al. 2006). Recently, Cai et al. (2013) confirmed the association between barley protein content and these transcription factors by a genome wide association study combining high mapping resolution and wide genetic variation.

Grain hardness is a heritable trait with a heritability of >85%, and is affected also by the growing environment probably via grain protein or β-glucan content (Fox et al. 2007). QTLs associated with grain hardness measured by SKCS have been identified in all chromosomes of barley (Fox et al. 2007). The most significant QTL for grain hardness has been located in the distal end of the short arm of chromosome 5H, where also hordoindoline genes have been mapped (Gautier et al. 2000; Beecher et al. 2002). This "hardness" (Ha) locus explains 22% of the variation in barley grain hardness (Beecher et al. 2002).

QTLs of grain density linked with grain steeliness have been located in chromosomes 2H and 6H (Walker et al. 2013). Based on the effect of growing environment on the grain protein content and hardness, it is not surprising that also steeliness is influenced by environmental conditions during grain development (Vejražka et al. 2008). In wheat, steeliness is affected by several factors of growing environment such as water and nutrient availability, temperature, light intensity during grain filling and the rate of desiccation at grain maturity (Parish and Halse 1968; Turnbull and Rahman 2002).

1.4 Barley processing and quality requirements

Barley grains are used as raw material in feed and malt production, but also in food processing. These diverse end uses set a range of requirements related to the composition and structure of barley grain. In the following sections, the quality requirements for malting, feed and food barley are described.

1.4.1 Malting

Malting is a controlled germination process consisting of steeping or hydration of grains, a germination phase in moist conditions and finally the termination of the grain's physiological activities by heating during a phase called kilning. Fundamentally, the aim of malting is to unmask starch granules from the surrounding cell walls and protein matrix so that fermentable sugars can be optimally released from starch during the brewing process (Swanston et al. 2014).

In malt of good quality, cell walls, a part of the small starch granules and the surrounding protein matrix are broken down uniformly throughout the endosperm (Palmer 1993). This requires rapid distribution of water in the endosperm during hydration as well as fast and homogeneous endosperm modification (Davies 1989; Brennan et al. 1997). Modification or degradation of endosperm reserves for the needs of the growing seedling involves the coincident action of the enzymes hydrolysing protein, starch, and cell wall structures. Enzymes are synthesized or activated in the aleurone and scutellar cells by the action of embryonic gibberellin-activated signal transduction pathways and are secreted into the starchy endosperm (Cohen and Paleg 1967; Jones and Jacobsen 1991). Besides enzyme activities, degradation of endosperm reserves, and also hydration, are controlled by the structural pattern at the tissue and subcellular level (Brennan et al. 1996; Chandra et al. 1999). Thus both an endosperm structure that is easily hydrated and modified and a good enzyme-synthesizing capacity for ensuring fast modification are favourable features of a malting barley cultivar. However, modification should not be too extensive, because this causes too brittle malt which cannot be optimally milled for mashing. Over-modification leads also to the breakdown of starch granules and losses in fermentable yield. Overall, the nature of malting quality can be considered complex, as it is dependent not only on the grain struc-

ture and composition, but also on its germination physiology. Therefore, the evaluation of malting quality includes the assessment of characteristics of both barley and the malt produced from it.

Specifications of barley accepted for malting usually include requirements for parameters such as kernel size, moisture and nitrogen or protein content as well as proportions of damaged, contaminated or preharvest-sprouted kernels, (Swanston et al. 2014). The selection of barley lots with high germination capacity and low amount of poorly filled, dormant or water-sensitive grains or grains with low viability is related to the assurance of quick and even germination during malting. The protein content is routinely assessed, because high protein content in barley generally leads to a low malt extract yield (Bishop 1930, 1948; Glennie Holmes 1990; Agu and Palmer 1998, 2001; Agu 2003; Fox et al. 2003). The loss of extract yield is caused mainly by the inverse correlation of protein and starch contents in barley grain (Bishop 1930; Holtekjølen et al. 2006). In addition, grains with high protein content often have steelier structure limiting the modification of the endosperm (Glennie Holmes 1990; Agu and Palmer 1998). Barley with a nitrogen content of 1.5–1.7% (9.4–10.6% as converted to protein) is typically accepted for malting in Europe (Swanston et al. 2014).

The malting behaviour of barley and the resulting malt can be characterized by several analytical methods. The same methods are used in the evaluation of malting quality of barley lots for industrial maltings, for optimization of process conditions and in the selection of breeding lines for malting purposes. The parameters measured from malt reflect different aspects, e.g. rate, uniformity and extent of hydration and modification occurring during malting. The water content of barley and the amount of germinated grains are routinely assessed during malting in order to monitor the rate and evenness of the hydration and germination, respectively.

From the brewer's point of view, the most essential quality parameter of malt is extract yield. For its measurement, malt is ground and mashed or extracted, usually in a temperature-profiled mashing procedure ending at +70°C (Schwartz and Li 2011). Determined by the specific gravity of the wort produced in mashing, the extract yield reflects the extent of enzymatic degradation and the solubility of grain components after malting and mashing (Swanston et al. 2014). However, because only fermentable sugars are converted to alcohol by yeast in fermentation, the fermentability of the wort is often measured as attenuation limit indicating the proportion of original extract that can be utilized during fermentation (Schwartz and Li 2011).

Another way of testing the extent of malt modification is to analyse the friability or tendency of the endosperm to break into flour in a specified milling process (Chapon et al. 1979). This measurement is based on the more brittle structure of enzymatically hydrolysed endosperm compared to native barley endosperm. An increase in friability reflects thus a more extensive modification of the endosperm during malting, mostly with respect to the degradation of the protein matrix and cell walls (Chapon et al. 1979; Darlington and Palmer 1996). The friability measurement enables also the assessment of the homogeneity of modification as the proportion of undermodified endosperm material remaining unmilled.

While extract yield and friability measure the effect of modification in general, several analysis methods exist for measuring either the hydrolysis of cell walls or protein. Cell wall degradation in the starchy endosperm can be assessed by visualizing the main cell wall component, β-glucan, with fluorescent Calcofluor dye (Aastrup et al. 1981). The same dye can also be utilized in the analysis of β-glucan content of wort (Jorgensen et al. 1985). Measuring the extent of proteolysis during malting is essential, because formation of protein degradation products indicates not only the release of starch granules but also the generation of amino acids needed for yeast nutrition during fermentation and peptides involved, e.g. in turbidity of beer (Asano et al. 1982; Gibson et al. 2009). Proteolysis is measured as the amount of soluble nitrogen present in malt or as the proportion of soluble nitrogen to total malt nitrogen also called Kolbach index. The determination of free amino nitrogen (FAN) in wort reflects even more accurately the amount of amino acids available to yeast by yeast (Schwartz and Li 2011).

In addition, activities of the main starch-hydrolysing enzymes generated during malting are measured from malt. The activity of α-amylase describes the dextrinizing capacity of malt. The role of α-amylase in starch degradation is to reduce the size of starch and oligosaccharides by attacking α-(1→4)-glucosidic bonds in amylose and amylopectin (Swanston et al. 2014). The total capacity of the malt to convert starch to fermentable sugars is called diastatic power. This parameter is determined as the production of reducing sugars by the enzymes extracted from malt in the presence of excess starch (Schwartz and Li 2011).

1.4.2 Feed use

One reason for the high proportion of barley crop being used as feed is its nutritional suitability for a wide range of animals. Barley can be included in the diets of ruminants, monogastric livestock, poultry and even fish (Blake et al. 2011). However, certain aspects of animal nutrition set limits on the use of barley. The quality requirements of feed barley are related to grain composition and structure through supply of essential amino acids, phosphorus and minerals, energy content and digestibility.

Many requirements for feed barley are related to polysaccharides present in the barley grain. Starch serves as the primary source of digestible energy for livestock. A high starch content is thus preferred in barley used as feed (Fairbairn et al. 1999; Solà-Oriol et al. 2009). The high starch content is also supported by the requirement of high grain weight for feed barley (Baik 2014). In ruminal feed, a high starch content and low content of insoluble or acid detergent fibre fraction are favoured, because such a composition combined with high particle size after dry rolling has been associated with higher weight gain and a slower digestion rate without undesirable symptoms (Surber et al. 2000; Blake et al. 2011). A low content of cell wall β-glucan is required in barley used as feed for poultry due to its

tendency to reduce weight gain by increasing intestinal viscosity (Almirall et al. 1995; von Wettstein 2007).

Although cereals serve also as a protein source for livestock, the deficiency of essential amino acids, especially lysine, limits the use of barley as a feed for monogastric livestock. This imbalance in amino acid composition is to a large extent due to hordeins with low contents of lysine, threonine, tryptophan, cysteine and methionine. In barley grains with high protein content meaning usually an increased proportion of hordeins, the low content of the essential amino acids is even more pronounced (Shewry 1993). In addition, a high protein content has been associated with lower starch digestibility in poultry (Almirall et al. 1995).

With respect to nutrition of monogastric livestock, a low content of phytic acid in barley is a preferable characteristic for two reasons. First, this storage compound of phosphorus is poorly digested in monogastric animals leading to a requirement for phosphorus supplements, high content of phosphorus in faeces, waste runoff and pollution of soil and water (Cromwell et al. 1993; Correll 1998; Htoo et al. 2007). Secondly, phytic acid located in aleurone cells has an ability to chelate nutritionally important minerals and also protein and starch thus reducing their bioavailability (reviewed in Oatway et al. 2001).

Grain hardness also has an impact on processing of barley for feed purposes, as steam or dry flaking or rolling is a common processing technique used in feed production (Fox et al. 2009). Both grain hardness and particle size after dry rolling have been shown to correlate positively with the desired slower digestion rate in ruminants (Surber et al. 2000; Beecher et al. 2002).

1.4.3 Food use

The interest in the use of barley in other industrial food applications besides malting has recently grown as barley has the potential to be used as an alternative to the more commonly used cereals. Due to the low use rate of barley in the food industry, there are no generally accepted requirements for food barley, except for the limits of the prevalence of fungal toxins and other antinutritive compounds (EU 2006). Nevertheless, both physical and compositional properties of barley grain are important with respect to food use.

The food processing of hulled barley starts nearly invariably with the removal of the tightly adhered, inedible hull by techniques based on pearling or abrasion. The grains of hull-less barley cultivars or the grains already dehulled may also be pearled further, if the removal of bran layers is desired (Baik and Ullrich 2008). For the maximization of the hull removal and the minimization of the pearling loss, grains with uniform size and shape, shallow crease and thin hull (if not hull-less) are favoured for pearling (Pomeranz and Shands 1974; Jadhav et al. 1998). Pearled barley is utilized as such or halved as a substitute for rice, or it may be processed further by flaking, dry roasting, puffing or milling (Baik 2014).

In milling, the use of barley poses certain problems compared to wheat. The bran of the barley grain is easily shattered during roller milling, which causes darker colour and a higher ash content in barley flour in comparison to typical wheat flour (Bhatty 1987; Jadhav et al. 1998). Another typical processing feature of barley is flake formation during roller milling, which further challenges the separation of bran and reduces the flour yield (Jadhav et al. 1998). Barley with increased bran resilience would probably help in reducing both the shattering and flaking during milling (Baik and Ullrich 2008).

With respect to grain hardness, pearling and milling set different requirements for barley. In pearling, the hardness of the barley grain correlates linearly with the pearling time (Bhatty and Rossnagel 1998). A hulled, waxy-type barley cultivar with a hard-textured endosperm has been shown to produce less broken kernels during pearling compared to barley with regular starch composition (Edney et al. 2002). In milling, more mechanical energy and possibly changes in the milling process are required to produce flour of similar coarseness from harder barley grains (Nair, Ullrich, et al. 2011).

The main components of the barley grain, starch, protein and β-glucan, but also some minor components significantly affect the food-use quality of barley. Different barley cultivars display a large variation in starch amylose content. The amylose content affects the physicochemical properties of starch and leads to different processing and end-use properties. For example, pearled barley with waxy-type starch has been shown to have faster hydration and cooking time, but results in lower expansion and higher density in extrusion in comparison to barley with normal starch composition (Baik et al. 2004; Baik and Ullrich 2008).

The nutritional quality of barley protein has been evaluated as moderate. This is mainly due to the high proportion of hordeins (Baik and Ullrich 2008). Extensive studies and breeding of barley cultivars with high lysine content were carried out in 1980s, but the released cultivars have reached only limited cultivation areas (Eggum et al. 1996; Jacobsen et al. 2005). Recently, a patent in United States was applied for barley with a low content of hordein for production of gluten-free foods or beverages (Tanner and Howitt 2014).

Barley β-glucan has been shown to lower blood cholesterol, improve lipid metabolism and reduce post-prandial glycemic response (Li et al. 2003; Delaney et al. 2003; Behall et al. 2004a; b, 2006; Keenan et al. 2007). Thus, a high concentration of β-glucan would be favourable to produce food products with higher nutritional value. Due to a linkage between grain hardness and β-glucan found in some studies (summarized in **Table 2**), high β-glucan content probably sets similar requirements to milling and pearling as described above for grain hardness. As a hygroscopic compound, barley β-glucan has been shown to increase the water absorption of dough thus decreasing dough extension strength, limiting the hydration of starch and reducing bread volume (Izydorczyk et al. 2005, 2008; Symons and Brennan 2006; Sudha et al. 2007; Brennan and Cleary 2007; Jacobs et al. 2008). In contrast to these detrimental effects on bread quality, barley β-glucan improved the quality of noodles by increasing firmness and decreasing cooking loss (Marconi et al. 2000). In addition to β-glucan, barley is a good source of in-

soluble dietary fibre. In hull-less or dehulled grains, insoluble fibre is known to increase satiety and reduce post-prandial glycemic response (Samra and Anderson 2007; Freeland et al. 2009). Pearling should be restricted to the removal of husk, in order not to lose excessively this insoluble fibre fraction.

A major challenge in incorporating barley into various wheat-based products is its impact on the product colour. The addition of barley has been shown to increase the development of grey or darker colours, e.g. in dough, noodles, pasta and flatbread (Başman and Köksel 1999; Marconi et al. 2000; Quinde et al. 2004; Izydorczyk et al. 2005). The discolouration occurs as an action of polyphenol oxidase (Quinde-Axtell et al. 2006). This undesired effect is lower in proanthocyanidine-free barley cultivars (Quinde-Axtell et al. 2005). In order to avoid discolouration, barley cultivars with low content of especially dimeric proanthocyanidins and low activity of polyphenol oxidase should be chosen for food use.

Are different quality requirements contradictory?

Based on the requirement of lower protein content in malting barley in comparison with feed barley, it is often assumed that the majority of the quality requirements of malting and feed barley are competing. Nevertheless, ruminal digestibility of feed barley as well as its metabolizable energy in poultry correlate positively with extract yield in mashing indicating that the breeding targets of malting and feed quality are not entirely exclusive (Molina-Cano et al. 1997; Fox et al. 2009). Malting barley cultivars can represent even better feed quality than feed cultivars with respect to ruminal digestibility and daily weight gain (Fox et al. 2009). In the case of grain hardness, there might be a contradiction in the requirements between malting and feed barleys, as soft or mealier grains are usually of better malting quality and at least for ruminants harder barley grains represent better feed quality (Chandra et al. 1999; Surber et al. 2000; Beecher et al. 2002). The requirement for low β-glucan content in malting barley as well as in feed barley for monogastric livestock are clearly contradictory to the aims of high β-glucan content of food barley for higher soluble fibre content.

All in all, there seem not to be very remarkable differences between quality requirements for malting, feed and food barley. However, in practice, other factors lead to a preference for certain cultivars for specific purposes. For example the capability of six-rowed barley cultivars to achieve high grain protein content in a shorter time compared to two-rowed cultivars clearly leads to their preference for feed use at least in northern growing conditions (Peltonen-Sainio, Rajala, et al. 2009; Peltonen-Sainio et al. 2012).

2. Aims of the study

The structure of barley grain is formed through the accumulation and synthesis of various components during grain filling. Both genotype and growth environment are known to influence the final structure of the mature grain, which in turn affects grain processing properties. The role of protein composition in determining the structural properties of barley is not thoroughly understood. Hordeins, the major storage proteins in barley grain, form a matrix surrounding starch granules, and hence they are considered to play an important role in the structural properties of barley.

The main aim of this study was to demonstrate the role of hordeins in barley grain structure, both with respect to dependence of the grain structure on the growth environment, and its effects on end use properties.

The more specific aims of this study were

- to show the influence of day-length and nitrogen and sulphur availability typical to Northern European field conditions on grain structure and composition, particularly in relation to hordeins (**I** and **II**)
- to characterize the influence of grain composition and structure (determined as steeliness and localization of grain components) on the behaviour of barley grain in milling, hydration and endosperm modification during malting (**I–IV**)

3.   Materials and methods

The barley material, experimental procedures and analytical methods used in this study are described in detail in the original Publications **I–IV**, and only a brief summary is presented in this section.

3.1 Barley materials

Barley (*Hordeum vulgare* L.) material examined in Publications **I–IV** was cultivated at pot-scale in controlled environment (CE) cabinets in the UK and in a greenhouse as well as in field conditions in Finland and Scandinavia (**Table 3**). All the cultivars studied were spring-type barley cultivars, and most of them were 2-row malting barley cultivars. In Publication **III**, also one waxy type cultivar and 6-row, feed-type cultivars were included. Barke, Prestige and Scarlett represent cultivars which are grown for malting purposes across Europe, i.e. from Northern to Southern Europe.

  In addition to mature grains, developing main shoot head caryopses were collected for localization of hordeins (**I**) and for detection of malate:sulphate ratio during vegetative growth and total above-ground phytomass (**II**). The growing experiments are described in detail in each publication.

Table 3. Barley materials used in the current study.

Publication	Barley material	Growing site(s)	Samples collected	Barley cultivars studied
I	Barley grown at pot-scale under long-day (18/6 h) and short-day (14/10 h) conditions	CE cabinets at Rothamsted Research, Harpenden (UK)	Developing caryopses from the main shoot heads and mature grains	Barke (*2-rowed malting barley cultivar*)
II	Barley cultivated in pots with 0, 5, 10, 20, 40 and 80 mg S / kg soil	Greenhouse at MTT Agrifood Research, Jokioinen (Finland)	Leaf and phytomass samples during vegetative growth and mature plant stands with grains	Scarlett (*2-rowed malting barley cultivar*)
	Barley cultivated in the field with 60 or 90 kg N / ha and 0 or 6% S of total amount of fertilizer	Vihti, Inkoo and Jokioinen (Southern Finland)	Leaf samples at the beginning of tillering phase and mature grains	Barke, Prestige, Saana, Scarlett (*2-rowed malting barley cultivars*)
III	Barley cultivars grown in the same field in two consecutive years	Hauho (Southern Finland)	Mature grains	Pilvi, Vilde, Voitto (*6-rowed feed barley cultivars*) Saana, Scarlett, SW Makof (*2-rowed malting barley cultivars*) Magdalena (*2-rowed waxy barley cultivar*)
IV	Field-grown barley representing two crop years	Different growing sites in Scandinavia	Mature grains	Barke, Luberon, Scarlett, Wikingett (*2-rowed malting barley cultivars*)

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3.2 Analysis of growth and yield formation of barley

3.2.1 Assessment of phytomass production (II)

The amount of above-ground phytomass per plant was measured in order to eval-
uate the influence of sulphur supply on the growth of barley in the greenhouse
experiment described in Publication **II**. For this, plant stands from the replicate
pots were collected by cutting the plants at the soil surface at tillering, at the be-
ginning of stem elongation, at the flag leaf stage, and when mature. After drying
and weighing, the above-ground phytomass was calculated as g / plant.

3.2.2 Analysis of malate:sulphate ratio during vegetative growth (II)

For the evaluation of the degree of sulphur deprivation, malate:sulphate ratio was
analysed from the plant samples taken before ear emergence (**II**). In the green-
house experiment, plant samples were collected at tillering, at the beginning of
stem elongation, and at the flag leaf stage. In the field trials, plant samples for the
malate-sulphate test were taken at the beginning of the tillering phase. Malate and
sulphate contents of the samples were analysed at Hill Court Farm Research (UK)
using ion chromatography and the increase of malate:sulphate ratio was utilized to
evaluate sulphur deficiency during vegetative growth (Blake-Kalff et al. 2000).

3.2.3 Determination of pollination and sampling of developing caryopses (I)

The main shoot heads/ears of plants growing in CE cabinets under long and short
photoperiods were examined to determine the timing of pollination (**I**). The date of
pollination was determined by the maturity of anthers in florets of main shoot
heads. Developing caryopses were collected from the middle third of these main
shoot heads for immunolabelling. For this, the main shoots were sampled 14, 21,
28 and 35 days after pollination (dap).

3.2.4 Yield parameters (I, II, IV)

In order to characterize the yield formation in the greenhouse experiment, grain
yield (g / plant), ear number per plant, and grain number per ear were determined
from the pot-grown barley plants (**II**). Vegetative mass (g / plant) was calculated as
the difference between plant above-ground phytomass and grain yield. Yield in
field experiments was expressed as kg per hectare.

   In Publications **I, II** and **IV,** yield formation and grain filling was evaluated also
by measuring kernel size distribution and thousand-grain weight using the proto-

cols recommended by the European Brewing Convention (Analytica EBC 1998 and 2007: 3.11.1 and 3.4). In addition, numbers of pre-germinated (3.8.1) and damaged barley kernels (3.11.2) were analysed and reported in Publication **II**.

3.3 Analysis of composition and structure of barley grains

3.3.1 Barley composition (I-IV)

Standard methods used in the analysis of barley grain composition in Publications **I-IV** are presented in **Table 4**. In publication **II**, sulphur content of field-grown barley grains was analysed at Soil Analyses Service Ltd. (Mikkeli, Finland) using a routine method consisting of wet combustion in HNO_3, pressure cooking at 140°C and analysis using inductively-coupled plasma atomic emission spectroscopy (ICP-AES). Concentrations of amino acids were analysed with a Biochrom 20 amino acid analyser (Biochrom Ltd., Cambridge, England) using sodium citrate buffer (EC 1998; **II**).

Table 4. Standard methods used in barley grain composition analysis.

Analysis	Method	Publication	Ref.
Barley composition			
Moisture content	EBC 3.2 and/or 3.13[a]	I, II, IV	1
	AOAC 14.004 and 7.007	III	2
Protein content	EBC 3.13[a]	IV,II	
	EBC 3.3.1[b]	I	1
	AOAC 14.068[b]	II,III	2
Starch content	NIR[a] analyser	IV,II	
	AOAC 996.11	I	3
		III	4
β-Glucan content	EBC 3.10.2	I,III	1
Lipid content	AOAC 922.06	III	3
Ash content		III	5

References: 1) Analytica EBC 1998 and 2007, 2) AOAC Official methods 1980, 3) AOAC International Official methods 2005, 4) Bach Knudsen et al. 1987, 5) Nordlund et al. 2013
[a] method based on near-infrared (NIR) spectroscopy
[b] Kjeldahl method

3.3.2 Extraction and quantification of hordeins (I–IV)

To study hordein composition, proteins of barley (**I–IV**) and malt (**IV**) samples were extracted sequentially by their solubility using a method modified from the classical

Osborne fractionation procedure (Osborne 1895; Marchylo et al. 1986). In this modified protein extraction method, water- and salt-soluble proteins were first extracted with dilute sodium chloride solution and then hordeins were extracted in 50% (v/v) 1-propanol without and with reducing agent dithiothreitol (DTT) as described in Publications **I–IV**. The extractions were carried out at +60°C, except in experiments described in Publications **II** and **III;** salt-soluble and hordeins without reduction were extracted at room temperature. The residue after these extractions was finally extracted with urea solution (**IV**).

Hordeins in the propanol- and propanol+DTT–fractions were separated and quantified, either with reverse-phase high-performance liquid chromatography (RP-HPLC) (**IV**) or by SDS-PAGE and densitometric analysis of separated proteins (Publications **I–III**).

In RP-HPLC, described in detail in Publication **IV**, hordeins were separated and detected with an HPLC chromatograph with photodiode array detector using water and acetonitrile as running solvents. Elution was carried out by using a gradient extending from 31.2 to 54.0% acetonitrile and detection was at 210 nm. The amounts of B, C and D hordeins were calculated as chromatogram areas in arbitrary units (AU).

Prior to SDS-PAGE, the propanol- and propanol+DTT-soluble fractions were either dried (**I or II**) or precipitated (**III**) and resuspended in sample buffer. Hordeins in the propanol- and propanol+DTT-soluble fractions were separated by SDS-PAGE, either on 12% Tris-borate gels (**I** and **III**) or on 12% Tris-HCl gels (**II**). After SDS-PAGE, to achieve quantitative binding of the protein stain, gels were fixed and stained with Coomassie Brilliant Blue G-250 according to Neuhoff et al. 1988 (**I**) or with slight modifications mentioned in Publications **II** and **III**. After scanning the gels with a GS-710 calibrated imaging densitometer (Bio-Rad), hordeins were quantified with QuantityOne software (Bio-Rad) based on the intensity and area of the bands (**I**) or based on the band intensity and protein content of the hordein extracts (**II** and **III**).

Protein contents of the two propanol extracts containing hordeins (**II** and **III**) and NaCl extracts containing albumins and globulins (**III**) were determined with a 2D Quant protein kit (Amersham Biosciences). The total hordein content per dry matter of barley was defined as the sum of the two hordein extracts (**II** and **III**).

3.3.3 Localization of hordeins in developing barley caryopses (I)

An immunolabelling method described in detail in Publication **I** was developed to study accumulation patterns of hordeins during grain filling. For this, the developing caryopses from the main shoot heads of barley plants grown under long- or short-day conditions were collected 14, 21, 28 and 35 dap. Transverse sections of freshly sampled caryopses were fixed with paraformaldehyde in cacodylate buffer, dehydrated in a series of increasing concentrations of ethanol and embedded into diethylaminoethyl methacrylate (LR White resin, London Resin Company).

Hordeins in 1-µm thick grain sections, cut using an ultramicrotome (Ultracut E, Reichert-Jung, Vienna, Austria), were labelled with monoclonal antibodies BII (provided by A. Brandt, Carlsberg Laboratory, Denmark) and IFRN0614 (Brett et al. 1990). The specificities of the antibodies BII and IFRN0614 towards B hordeins and C hordein, respectively, were confirmed by western blotting against total hordeins as described in detail in Publication **I**. These primary antibodies were further labelled with secondary antibodies conjugated with alkaline phosphatase. The binding of antibodies was detected by adding SIGMA FAST™ FastRedTR/Naphthol AS-MX substrate, which forms an intense red colour in a reaction with alkaline phosphatase.

The labelled sections were examined in brightfield with an Olympus BX-50 microscope (Olympus Corp., Tokyo, Japan) and imaged using a PCO SensiCam CCD camera (PCO AG, Kelheim, Germany). The areas labelled with antibody in the endosperm, to a depth of 1330 µm from the surface of the grain, were measured by using the Cell^P image analysis software (Olympus) and calculated as a proportion of the total endosperm area excluding the aleurone.

3.3.4 Localization of hordeins and other selected grain components in mature barley grains (I, III)

Localization of grain components in mature grains was carried out using an abrasion method described in Publications **I** and **III**. In this method, intact barley grains are pearled progressively and the removal of the outer layers or the degree of pearling is measured as weight loss. The localization of grain components was detected by analysing the composition of the remaining pearled kernels and interpreting the enrichment or loss of components occurring along with the pearling as it proceeded towards the central part of the grain. In Publications **I** and **III**, this method was utilized for localization of total protein and hordein fractions. In publication **III**, also location of β-glucan and water- and salt-soluble proteins was studied using this method. The contents of these components in abraded kernels were analysed as described in sections 3.3.1 and 3.3.2 and in the original publications.

3.3.5 Analysis of barley grain microstructure by light microscopy (IV)

The microstructure of 20 grains per barley sample was examined by light microscopy. The transverse sections cut from the middle of each grain were fixed with glutaraldehyde in phosphate buffer, dehydrated in a graded ethanol series, and embedded into hydroxyethyl methylacrylate resin (Historesin, Leica). Semi-thin (2 µm) sections were cut with microtome and stained in order to visualize starch, protein and cell walls.

Protein and starch were stained with Light Green and diluted Lugol's iodine solution and imaged in brightfield. Light Green stains protein green and iodine stains the amylose component of starch blue and amylopectin brown (Parkkonen et al. 1994). Protein and cell walls were stained with Acid Fuchsin and Calcofluor White

(Fulcher and Wong 1980; Wood et al. 1983). In exciting light (excitation, 400–410 nm; fluorescence, >455 nm) intact cell walls containing β-glucan and stained with Calcofluor appear blue and proteins stained with Acid Fuchsin appear red. Starch is unstained and appears black. The samples were imaged as mentioned in section 3.3.3.

3.3.6 Analysis of grain steeliness by light transmission (IV)

The grain steeliness was studied as transmission of a laser light through barley grains (Chandra et al. 2001). In total, the steeliness of 97 grains per sample was analysed by measuring quantitatively the amount of light passing through grains using the light transmission meter (LTm). Steeliness of the grains was expressed as the mean of LTm values recorded, and the proportions of mealy and steely grains in each sample were also calculated.

3.4 Processing performance of barley grains

3.4.1 Assessment of milling behaviour of barley (III)

Whole-grain barley samples were milled with a fine impact mill (100 UPZ-Ib, Hosokawa Alpine, Augsburg, Germany) with stainless steel pin disc grinders using maximum speed (17700 rpm; tip speed 180 m/s) for the pin disc. Particle size distributions of pin-milled and two commercial barley flours were determined in triplicate using a set of 11 sieves with apertures from 1250 µm to 75 µm.

3.4.2 Malting and characterization of malt quality (I–IV)

Micromaltings were carried out as presented in **Table 5** (**I–IV**). Steeping parameters depended on the crop year and, in some cases, cultivar. Moisture content after steeping was analysed by weighing.

Table 5. Descriptions of process parameters in micromalting trials in Publications **I–IV**.

	Publication					
	I	**II**			**III**	**IV**
Batch size (g)	60	1000			60	1000
Process step						
Steeping, in total (h)	53	30^{c}	32^{d}	50^{e}	48	48
– of which wet steeps in total (h)	21	14	16	18	16	14^{a} 17^{b}
Temperature (°C)	14	13-15		14	14	13-15
Target moisture after steeping (%)	45	46-47		46	45	46
Germination, in total (d)	5	5		5^{f} / 6^{g}	5	5
Temperature (°C)	15	14		16	15	14
Kilning, in total (h)	21	22			19	22
Temperature (°C)	83	82-83			80	82

[a] crop 2001, [b] crop 2002, [c] crop 2004 (Vihti), [d] crop 2005 (Inkoo), [e] crop 2005 (Jokioinen), [f] cv. Saana, [g] cv. Barke

Viability and possible pre-germination of barley were analysed as germinative capacity (Analytica EBC 1998 and 2007: Method 3.5.1; **II and IV**). In Publications **I** and **III**, the proportions of germinated grains were visually inspected during malting.

The malting process was monitored by determining the grain moisture after steeping and malting losses (total, loss in rootless removal and respiration loss) based on weight loss at the end of malting (**II and IV**). The methods used in the analysis of final malts produced in Publications **I–IV** are shown in **Table 6**.

Table 6. Experimental procedures used in analysing final malts.

Analysis	Method	Publication	Ref.
Malt analyses			
Moisture content	EBC 4.2	I-IV	1
Protein content	EBC 4.3.1	IV	1
Friability	EBC 4.15	II, IV	1
Relative friability [a]		I, III	
Modification and homogeneity	EBC 4.14	IV	1
Congress mashing			
Extract content (fine)	EBC 4.5.1	II	1
Extract difference (fine/coarse)	EBC 4.5.2	IV	1
Soluble nitrogen, Kolbach index	EBC 4.9.1	II, IV	1
Free amino nitrogen (FAN)	EBC 4.10	II, IV	1
Wort β-glucan content	EBC 4.16.2	II, IV	1
Wort colour	EBC 4.7.2	II, IV	1
Wort viscosity	EBC 4.8	II, IV	1
Wort pH		II	
Enzyme activities in malt			
α-Amylase	Megazyme K-CERA [b]	IV	2
Free β-amylase	Megazyme K-BETA3	IV	3, 4
Total and free limit dextrinase	Megazyme T-LDZ [c]	IV	5
β-Glucanase	Megazyme K-MBGL	IV	6, 7
Endo-β-xylanase		IV	8
Endopeptidase	[d]	IV	9,10

References: 1) Analytica EBC 1998 and 2007, 2) McCleary and Sheehan 1987, 3)
Mathewson and Seabourn 1983, 4) McCleary and Codd 1989, 5) McCleary 1992, 6)
McCleary and Shameer 1987, 7) McCleary 1986, 8) Autio et al. 2001, 9) Zhang and Jones
1995, 10) Kihara et al. 2002
[a] Friability method recommended by EBC (EBC 4.15) was optimized for smaller sample size
as described in Publications **I** and **III**.
[b] Extraction time was changed to 30 min.
[c] Concentration of DTT in extraction buffer were changed to 62 mM.
[d] The crude endopeptidase extraction procedure was modified from the method of Zhang
and Jones 1995 and endopeptidase activity inhibited by E-64 was defined as cysteine pro-
teinase activity (Kihara et al. 2002) as described in Publication IV. The endopeptidase activi-
ty was measured spectrophotometrically using azo-casein as a substrate.

3.4.3 Assessment of water uptake during germination (I, III, IV)

Distribution of water in the endosperm was measured based on the methods of
Chapon (1960) and Kelly and Briggs (1992) with some modifications. For this,
grains were sampled after 48, 72, 96 and 144h (**I**) or after 48 h (**III**) of malting,
immersed in boiling water, air-dried and abraded longitudinally into halves. The
hydration of the endosperm was determined based on starch gelatinization in
wetted endosperm regions by heating. Non-hydrated endosperm regions ap-

peared opaque and those containing gelatinized starch appeared transparent. The extent of endosperm hydration was assessed visually by classifying 100 grains / sample into four (**III**) or six (**I**) groups based on the amount of hydrated endosperm in the longitudinal section.

In addition, water uptake during malting was measured by weighing grain lots after steeping (**II**), after 48 h (**IV**) or after 24, 48 and 72 h (**III**) of malting.

3.5 Statistical analysis (I-IV)

The results were calculated as averages of the analytical or biological replicates. The variation of the data was expressed as standard errors (**II**), standard deviations (**III, IV**) or 95% confidence intervals indicating the range of values within which true value is included 95% of the time, if the measurement is repeated (**I**). Data were statistically analysed using the two-tailed Student's t-test (**I, IV**) and one-way analysis of variance (ANOVA) with Tukey´s honestly significant difference (HDS) post-hoc test (IBM SPSS Statistics software for Windows v20.0 or earlier; IBM Corporation, Somers, NY, USA) (**I–III**). Correlation analysis in Publication **III** was performed by calculating the Spearman correlation coefficients.

In publication **II**, data on the field experiments was statistically analysed using a linear mixed model based on a split-split-plot experimental design in which the cultivar was the main plot, nitrogen application rate was sub-plot and sulphur application rate was sub-sub-plot factor. The model was modified by removing interactions or defining them as random effects when it was used for smaller sub-sample sets (n<384). In addition, all trials were analysed separately by the traditional mixed model for a split-split-plot design. Methods used included an assumption of the normal distribution of the data, and normality was checked by graphical methods. Generally, proportional data needed arc-sine transformation before normality. All mixed models were fit using SAS/MIXED software (SAS v9.3; SAS Institute Inc., Cary, NC, USA) with restricted maximum likelihood (REML) estimation method.

Within all statistical methods used, the differences were considered to be significant when $p < 0.05$.

4. Results

4.1 Day-length affects the localization of hordeins (I)

In the experiments carried out in CE cabinets, the period from sowing to pollination was shorter for the barley plants (cv. Barke) grown under long-day conditions compared to those grown under short-day conditions (**Table 7**). Based on the proportions of grains > 2.5 mm, grain filling was successful under both day-lengths (**I**, Table 1). Size distribution, weight or contents of starch, protein and β-glucan in mature grains were not affected by the length of the photoperiod.

Table 7. Duration of growth phases of barley plants (cv. Barke) grown under long and short photoperiods (n= 96 and n=79 for long- and short-day experiments, respectively; **I**, partly unpublished results).

Growth phase	Duration (days) under conditions of	
	long photoperiod (18/6h)	short photoperiod (14/10h)
From sowing to pollination	52 ± 0.4 [a]	74 ± 0.8
From pollination to full ripeness	51	57
In total	103	131

[a] average ± 95% confidence interval

Regardless of day-length, both B and C hordeins were initially observed in the subaleurone region at 14 dap in the developing caryopses (**Figure 5 A** and **E**). These hordeins remained concentrated in the subaleurone throughout development, although they also accumulated progressively in the inner endosperm of the developing caryopses (**Figure 5 B–D** and **F–H**). Immunolocalization showed that the B and C hordeins had different accumulation patterns in the later stages of grain filling, with B hordein ceasing to accumulate after 28 dap. Moreover, the immunolabelling of hordeins revealed that C hordein was significantly more concentrated in the outer layers of the endosperm and also extended deeper into the endosperm at 35 dap in developing caryopses grown under long-day conditions

(**Figure 5 D** and **H**; **I**, Figure 3). No differences related to day-length were observed in localization of the B hordein using immunolabelling.

The deeper location of C hordein within the endosperm was confirmed by the abrasion of mature grains (**I**, Table 2). Grains grown under long-day conditions contained proportionally more 1-propanol+DTT-soluble or entrapped C hordein and had a lower ratio of B:C hordeins in the 1-propanol+DTT-soluble hordein fraction derived from the more central part of the grain. The distribution of total protein in mature grains was not associated with day-length.

Thus, the results show that C hordein was located more deeply within endosperm of both developing grains at 35 dap and in mature grains under long-day conditions. Based on the analysis of mature grains, the C hordein fraction entrapped by aggregated hordeins accounted for the localization of C hordein in the more central endosperm.
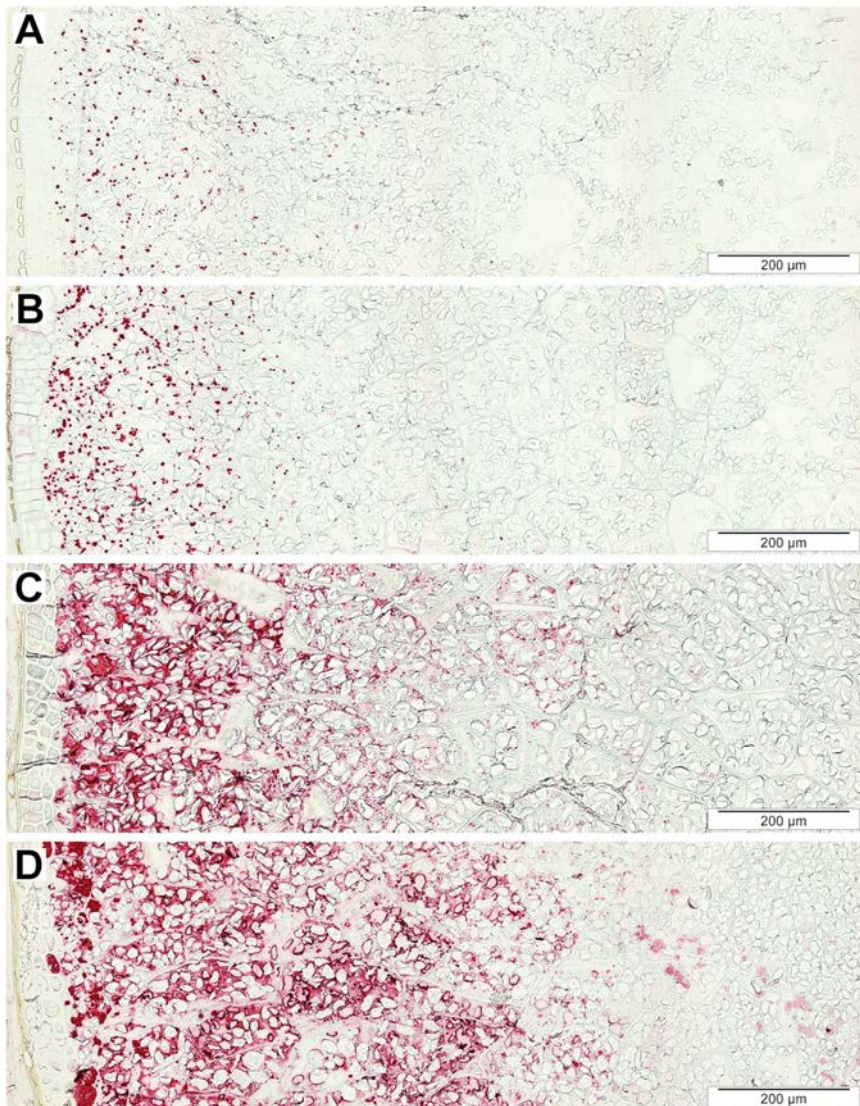
Figure 5. C hordeins in the developing main head caryopses of barley (cv. Barke) grown under long-day (A–D) and short-day (E–F) conditions labelled with antibody IFRN0614. Each picture is a compilation of four micrographs taken successively from the aleurone (left in the pictures) towards the centre of the grain (right in the pictures). Images A and E represent the caryopses collected at 14 dap, B and F 21 dap, C and G 28 dap, and D and H 35 dap. Reproduced with permission from the publisher.
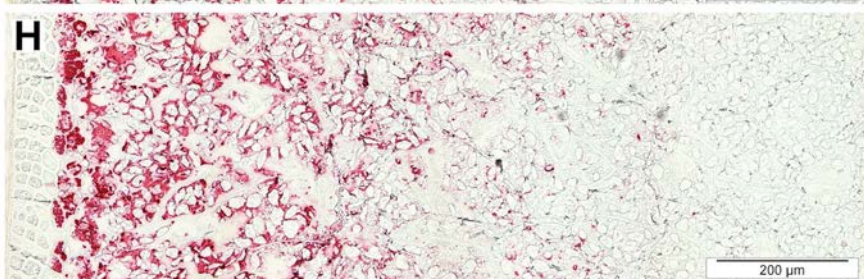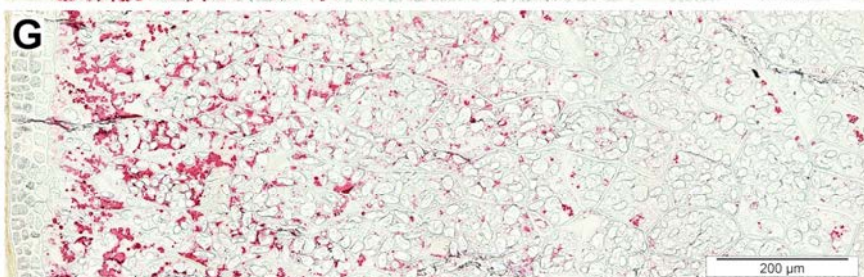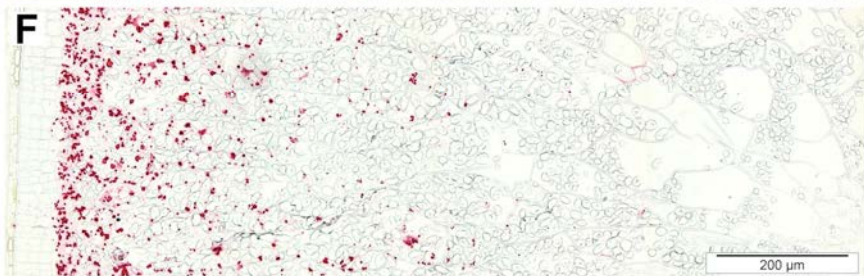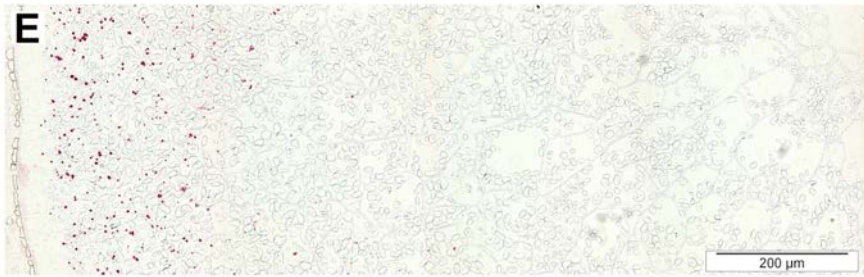
4.2 Hordeins are influenced by nitrogen and sulphur application (II)

The effect of sulphur application on growth, yield formation and grain composition was first demonstrated in the malting barley cultivar Scarlett in greenhouse conditions (**II**). Application rates of 0, 5, 10, 20, 40 and 80 mg of S / kg soil resulted in changes in several growth, yield and composition parameters in this pot-scale experiment as summarized in **Figure 6**.

Phytomass of barley plants at the tillering stage increased significantly when application rate changed from 0 to 5 mg S / kg soil, and at flag-leaf stage also from 5 to 10 mg S / kg soil. The final decrease in malate:sulphate ratio before levelling off, due to sufficient supply of sulphur, did not coincide with an increase in phytomass. The change in malate:sulphate ratio occurred at the change of application rate from 5 to 10 or from 10 to 20 mg S / kg soil at tillering or flag-leaf stage, respectively.

Plants grown without added sulphur were not capable of producing ears. No significant effect on the final vegetative mass was detected at an application rate of 5 mg S / kg soil or higher. Plants grown with application rates of 10 mg S / kg soil or higher, had greater grain yield per plant and grain weight was higher in comparison with plants supplied with 5 mg S / kg soil. The application rate did not affect significantly the nitrogen content of barley grains.

The amounts of S-containing amino acids in barley grains reflected the availability of sulphur. Content of cystine, which is formed of two cysteine residues, increased significantly with sulphur availability in the grains grown with 5, 10 or 20 mg S / kg soil. The amount of another S-containing amino acid, methionine, increased more gradually compared to that of cystine. The amount of aspartic acid in grains decreased when the amount of sulphur increased from 5 to 10 mg S / kg soil. The total content of amino acids increased significantly only at the highest sulphur application rate.

Changes in hordein composition of barley grains due to higher sulphur availability were observed. Total hordein content as well as that of B hordein increased gradually from the lowest to the highest sulphur application rate. The amount of C hordein was significantly lower in the grains grown with 20 mg S / kg soil or more than in grains grown with lower sulphur application rates. On the other hand, the D hordein content in grains was higher when 10 mg S / kg soil or more was added. When examining the hordein composition of barley grains, the major changes in proportions were the increase and decrease of the proportions of B and C hordein, respectively, occurring between the application rates of 5, 10 and 20 mg S / kg soil.
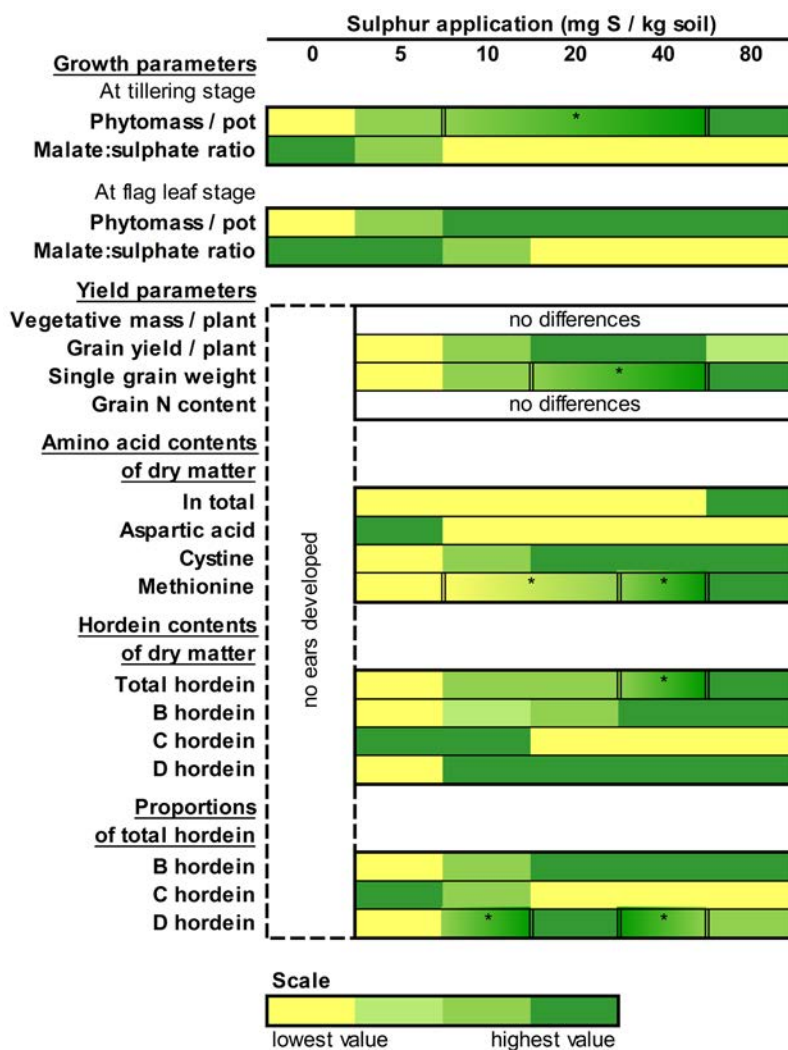
Figure 6. The effect of sulphur application rate on growth, yield and composition parameters of barley in a greenhouse experiment (**II**). Different colours indicate significant differences (p<0.05) between sulphur application rates based on Tukey's HSD post-hoc test (except for malate:sulphate ratio). Sulphur application rates indicated by asterisk were not significantly different from the ones on either side delimited by a double line.

In field conditions, the effect of sulphur application rate (0 or 6% S of total amount of fertilizer) was demonstrated in combination with two nitrogen application rates (60 or 90 kg N / ha). Higher sulphur availability was associated with lower malate:sulphate ratio in leaves and also a lower ratio of N:S in grain, although an increase in grain sulphur content was observed only in one location. Nitrogen application was associated with higher yield, protein and sulphur content in grains in all 384 samples studied.

Because no sulphur-related effect was found from three locations over two crop years, the response of hordeins to sulphur and nitrogen application was examined in detail in the data from the site where response to sulphur application in grain sulphur content was detected (Vihti in 2004). Total hordein content was increased by the main effect of sulphur application, while the effect of nitrogen application varied in the four cultivars studied. The amount of propanol-soluble B hordein increased in most of the cultivars as an effect of cultivar and sulphur application interaction, while the effect of nitrogen application was dependent on cultivar. The proportions of B (both total and propanol-soluble) and total C hordein increased and decreased, respectively, due to the sulphur application. The propanol+DTT-soluble fraction of C hordeins was affected by the interaction of sulphur and nitrogen application.

4.3 Milling performance of barley is dependent on grain composition (III)

The association between grain composition and milling properties was studied using barley material representing feed-type, malting and waxy barley cultivars from two crop years. On the basis of correlation analysis, the particle size distribution of the flours analysed by sieving was linked to grain composition. The proportion particles from 132 µm up to 180 µm in barley flours correlated negatively with protein and β-glucan contents and positively with starch content (**III**, Table 2). Similarly, negative correlations with β-glucan and positive correlations with starch content of grains were observed in the proportion of the finest flour particles (smaller than 132 µm), and opposite correlations were observed between β-glucan and starch contents and the proportion of the coarsest flour particles greater than 355 µm. Thus, barley grains with lower β-glucan and protein content and higher starch content produced finer flour upon milling. In line with this, starch content correlated negatively with both protein and β-glucan content in grains.

Grain milling properties were linked with grain composition and also crop year. Based on the cumulative proportions of flour particles smaller than 132 µm, most of the barley samples from the 2007 crop produced finer flour compared to those from the 2006 crop (**III**, Table 3). In line with this, β-glucan contents in each cultivar were substantially higher in 2006 than in 2007.

Localization of grain components in relation to milling performance was examined in the samples of malting barley cultivar Scarlett and feed-type cultivar Vilde

from 2006 and 2007 using an abrasion method. Grains of Scarlett had consistently higher contents of protein and β-glucan in the endosperm at all pearling rates compared to samples of Vilde, which was reflected in the coarser flour produced from Scarlett samples. In the grains from the 2006 crop, aggregated B hordein was concentrated in the peripheral endosperm. Deeper within the endosperm, there were lower proportions of aggregated B and D hordeins, and C hordein entrapped among them, and a higher proportion of non-trapped C hordein in comparison with grains from the 2007 crop (**III**, Table 5). Both the localization of hordeins and the higher total β-glucan content were associated with production of coarser flour upon milling of the 2006 grains. Contents of albumins, globulins or total hordein, the distribution of these protein fractions, or that of total protein within the grain, were not associated with milling behaviour. Correlations between localization of β-glucan within the grain and flour particle size distribution were not observed either.

The associations found between barley grain composition and milling properties are summarized in **Table 8**.

4.4 Localization of hordeins (and total β-glucan content) play a role in grain hydration during malting (I, III)

Grains of cultivar Barke grown under long-day conditions were shown to hydrate faster during malting in comparison with Barke grains grown with short days (**I**, Figure 5). This difference between grains grown under long- and short-day conditions, assessed as the proportion of hydrated endosperm, was observed at the sampling points of 48, 72 and 96 h from the beginning of malting. After 144 h, the samples were equally hydrated. The faster hydration of grains grown under long-day conditions correlated positively with the deeper location of C hordein within the endosperm. The localization of C hordein was analysed by immunolabelling and the abrasion method and the results are described in detail above in section 4.1. The C hordein found in the more central part of the endosperm was entrapped by aggregated B and D hordeins. The total contents of protein, starch, β-glucan or hordein fractions, and distribution of total protein in grain did not correlate with grain hydration (**I**, Table 1).

In the investigation of malting, feed-type and waxy barley cultivars, grains with lower β-glucan content hydrated faster during steeping; the water content of the grains at the time points of 24 and 48 h from the beginning of malting correlated negatively with β-glucan content (**III**, Tables 2 and 4). Accordingly, lower β-glucan contents in the samples of each cultivar grown in 2007 were associated with faster water uptake in comparison with the samples from the 2006 crop. This crop-year dependency was observed in the water content of grains from all cultivars after 24 and 48 h of malting. The exception was the malting barley cultivar Scarlett, in which there was no difference between the crop years at the 48 h time point. However, in the samples of Scarlett, as well as in some other samples from crop

2007, hydration had proceeded further by the 48 h time point compared to those from the 2006 crop as measured by the proportion of hydrated endosperm (**III**, Figure 1).

The effect of localization of grain components on hydration properties was studied in the samples of cultivars Scarlett and Vilde from 2006 and 2007. The grains from the 2007 crop contained less β-glucan, and aggregated B and D hordeins and entrapped C hordein were located within the more central endosperm while non-trapped C hordein was located less deeply. Grains with this composition and hordein localization had higher water content after 24 h of malting and a higher proportion of hydrated endosperm after 48 h (**III**, Tables 5). However, the effect of hordein localization could not be distinguished from that of the β-glucan, as they both were dependent on crop year. In contrast to total β-glucan content, the distribution of β-glucan within the kernel was not associated with water uptake in the samples of Scarlett and Vilde. Neither the contents of total protein, albumins and globulins or total hordein nor their distribution within the grain were linked to hydration characteristics of barley grain.

These results on the association of grain composition and structure on hydration are summarized in **Table 8.**

4.5 Grain structure and composition affect malting quality of barley (I, III and IV)

In Publication **IV**, the association of barley grain steeliness with their composition and malting quality was studied. Data from 27 barley samples representing two crop years showed that steelier grains contained more protein and were smaller than the mealier grains (**IV**, Table 1). Steelier grains also resulted in less friable malt which had higher activities of α-amylase and limit dextrinase and produced darker wort with a higher content of soluble nitrogen and free amino nitrogen compared to mealier grains.

From this barley material, three sample pairs, representing the same protein level but different steeliness, were selected for more detailed examination. For a certain protein level, steelier samples contained more starch than the mealier samples (**IV**, Table 2). However, no differences in the packing of endosperm cells or in the cell wall thickness associated with steeliness were found using light microscopy (**IV**, Figure 5). During malting, steelier barley produced less root mass, but had higher respiration loss (**IV**, Table 4). Steelier barley resulted in malt with less friable structure, more urea-soluble D hordein, FAN and soluble nitrogen in comparison with mealier samples (**IV**, Figure 3 and Table 5). Malts of steelier barley samples also showed higher activities of xylanase, total limit dextrinase, and both total and cysteine endopeptidase compared with the malts of mealy samples (**IV**, Figures 1–2).

Within all fourteen barley lots including malting, feed-type and waxy barley cultivars studied in Publication **III**, only barley starch content correlated positively with

friability and no significant correlations between protein or β-glucan content and friability were found (**III**, Table 2). The localization of grain components was studied in the Scarlett and Vilde samples, from crops of 2006 and 2007. In these samples, higher β-glucan content, concentration of aggregated B hordein in the peripheral endosperm, lower proportions of B and D hordeins and entrapped C hordein, and higher proportions of non-trapped C hordein deeper within the endosperm correlated with less-extensive endosperm modification during malting as measured by friability (**III**, Table 5). Due to its dependency on the crop year, the effect of hordein localization could not be differentiated from that of β-glucan content. Clear correlations between the total contents or distribution of albumins, globulins, total hordein or β-glucan in barley grains and friability were not observed.

The localization of hordeins was associated with malting quality also in Publication **I**. Analysis of friability revealed that malts produced from grains grown under long-day conditions contained significantly fewer partially unmodified grains (PUG) compared to the malts of grains grown under short-day conditions (**I**, Figure 5). The lower proportion of less-modified grains indicated that endosperm degradation had been more uniform and extensive in the grains grown under long-day conditions. The grains grown under long-day conditions contained C hordein deeper within the endosperm as described in detail in the results on the localization of hordeins in long- and short-day grown grains in section 4.1. The C hordein with more central localization in the endosperm represented the fraction entrapped by aggregated B and D hordeins. No associations between total contents of protein, starch, β-glucan or hordein fractions or distribution of total protein in grain with degradation of endosperm during malting was found.

Hordein composition was linked to malting quality parameters in Publication **II**. A higher content of total hordein was associated with an increase in soluble nitrogen of malt and a decrease in friability, extract yield and Kolbach index. The changes in FAN and wort colour were cultivar-dependent. However, the increase in the proportion of B hordein and the decrease in the proportion of C hordein were linked to an increase in FAN and colour of Congress wort in Barke and Prestige. In Scarlett the same change in hordein composition was related to a decrease in FAN and wort colour while no changes in these parameters was observed in Saana.

The summary of the influences of grain composition and structure is presented in **Table 8**.

Table 8. Summary of the associations of composition and structure of barley grains with their processing properties found in this study indicated with + (positive association) and – (negative association) (Publications **I, III and IV**).

Compositional or structural characteristics of barley grain	Association with		
	endosperm dissociation into flour particles upon milling	hydration during malting	modification during malting
Grain composition			
Starch content	**+** (III)	*no effect*	**+** (III), **–** (IV)[a]
Protein content	**–** (III)	*no effect*	**–** (IV)
β-Glucan content	**–** (III)	**–** (III)	**–** (III)[b]
Localization in subaleurone and central endosperm			
Protein content	**–** (III)	*no effect*	*no effect*
β-Glucan content	**–** (III)	*no effect*	*no effect*
Localization in subaleurone			
Proportion of aggregated B hordein	**–** (III)[c]	**–** (III)[c]	**–** (III)[c]
Localization in central endosperm			
Proportions of aggregated B and D hordeins	**+** (III)[c]	**+** (III)[c]	**+** (III)[c]
Proportion of non-trapped C hordein	**–** (III)[c]	**–** (III)[c]	**–** (III)[c]
Proportion of C hordein entrapped by aggregated B and D hordeins	**+** (III)[c]	**+** (I, III)[c]	**+** (I, III)[c]
Packing of endosperm			
Steeliness	*not studied*	*not studied*	**–** (IV)

[a] Observed in the sub-sample set of 6 barley lots with same protein content
[b] Observed in the sub-sample set of Scarlett and Vilde from 2006 and 2007, in which the effect could not be separated from that of hordein localization
[c] The effect could not be separated from that of β-glucan content

5.   Discussion

5.1 Effects of growing conditions on barley grain structure and composition

The length of photoperiod was found to have presumed impacts on the growth rhythm of barley plants in the current study and, in addition, novel findings concerning the influence of day-length on hordein localization were made. A longer day-length shortened the pre-anthesis phase of the life cycle of barley plants in comparison to shorter day-length. This is characteristic of the Northern European growing conditions, under which the growth rhythm of plants is known to be accelerated due to the long photoperiods occurring during the short growing season (Peltonen-Sainio, Rajala, et al. 2009). In general, day-length is important for the plant life-cycle, as the timing of many developmental phases are set as a photoperiodic response by plants (Jackson 2009). Barley is a long-day plant meaning that its flowering is induced by long photoperiods (Kirby and Appleyard 1980; Greenup et al. 2009), which is in accordance with the results obtained. The times between sowing and pollination under long and short photoperiod conditions found in the current study were similar to those reported earlier for barley grown in the field in Southern Finland and Central Europe at latitudes of c. 60° N and 48-52°, respectively (Lang 1966; Peltonen and Nissilä 1996). The photoperiods chosen therefore provided a suitable model for the illumination conditions in Southern Finland and in Central Europe during the growing season. Furthermore, the selection of cultivar Barke for the day-length trials supports the relevance of the results obtained as this cultivar is grown both in Northern and Central Europe for malting purposes and thus can be considered as an appropriate model cultivar for this kind of experimental setup.

As the main result of the day-length experiments, the localization of hordeins in particular in the subaleurone region of the grain was shown, for the first time, to be influenced by the growing conditions. In the current study, C hordein was found more deeply within the endosperm of developing grains at 35 dap under long-day conditions using immunolabelling. The pearling of the mature grains revealed that the fraction entrapped by or co-accumulated with aggregated B and D hordeins

accounted for the localization of C hordein in the more central endosperm. Distribution of B hordein and total protein as well as grain size, weight and composition of mature grains were independent of photoperiod. This is in contrast to earlier studies, in which the length of photoperiod have been shown to affect several developmental processes and ultimately the yield components of barley (Kirby 1969; Kirby and Appleyard 1980; Laurie et al. 1994). The reason for the lack of responses in yield components in the current study could be explained by the focus on main head grains and not on yield formation as such.

In the developing barley endosperm, synthesis of hordeins begins when the cellularization phase is over and the accumulation of starch has commenced (Cameron-Mills and Wettstein 1980). In accordance with our results, hordein accumulation in developing barley grain has been reported to begin at 5–14 dap, lasting until c. 39–45 dap, and showing the highest accumulation rate between 23 and 29 dap (Shewry et al. 1979; Rahman et al. 1982; Davies et al. 1993; Møgelsvang and Simpson 1998). In addition, our study showed that synthesis of B hordein begins in the subaleurone cells beneath the aleurone layer and extends towards more central endosperm. A similar hordein accumulation in developing grains has been reported earlier (Shewry et al. 1993; Davies et al. 1993), and it is logical that B hordein, which comprises the major proportion of total hordein follows this pattern. A similar pattern of accumulation was observed in the current study also for C hordein during grain filling, which has not been reported earlier. Our results on the localization of hordeins in mature barley grains with B and C hordein concentrated in the subaleurone layer and D hordein in the more central cells of the starchy endosperm were consistent with previous studies (Shewry et al. 1996; Tesci et al. 2000).

The deeper localization of C hordein within the endosperm in barley grown under long-day conditions is an interesting result which has not been reported earlier. It can be hypothesized that an accelerated growth rhythm may have affected nitrogen accumulation during the vegetative growth phase (Peltonen and Nissilä 1996; Peltonen-Sainio et al. 2011). The supply of nitrogen from vegetative organs could then have influenced hordein accumulation after the first half of the grain-filling period, when nitrogen accumulation, at least in wheat, is known to be source-limited (Dreccer et al. 1997; Martre et al. 2003). This is the time when most hordeins are synthesized (Møgelsvang and Simpson 1998). However, the impact of sink-source relationships or nitrogen supply from vegetative organs on prolamin localization is not known, although hordein genes are regulated by nitrogen availability (Muller and Knudsen 1993; Halford and Shewry 2007). Furthermore, this mechanism would not explain why localization of C hordein alone was affected.

Another possible mechanism for the photoperiod-dependent localization of C hordein is differential development of endosperm cell structure during early grain development. It is known that long days accelerate the cell-division rate in barley endosperm (Rajala et al. 2004). It is also known that subaleurone cells and cells below differ in their hordein accumulation pattern. This results in higher content of B and C hordein in the subaleurone, while D hordein is more abundant in the cells deeper within the starchy endosperm (Shewry et al. 1996; Tesci et al. 2000). More

intensive cell division could affect the determination of cell fate during the differentiation stage of grain development, so that the cells below the subaleurone get a more subaleurone-type identity. This could further lead to higher accumulation of hordeins than usual deeper in endosperm. However, the reasons why C hordein, but not B hordein, was affected by the longer photoperiod are unknown.

Subaleurone and central starchy endosperm cells are reported to differ in the hordein deposition also at subcellular level. In the younger subaleurone cells, hordeins are transported to storage vacuoles via the Golgi apparatus, while in the central endosperm cells the pathway to vacuoles goes through deposition within the ER (Okita and Rogers 1996). The hordein deposition route has been proposed to be dependent also on starch accumulation (Shewry 1993). A higher starch accumulation rate induced by the longer photoperiod could disrupt the Golgi-dependent pathway of hordein deposition. Additionally, based on recent studies on storage-protein deposition in developing wheat grains, different prolamins seem to be segregated to different deposition pathways. Segregation between different storage protein fractions within protein bodies also seems to occur (Tosi et al. 2009, 2011). All this could lead to differences in localization of hordein fractions in different parts of the mature endosperm, and explain the fact that the C hordein was found deeper within the mature endosperm and entrapped by aggregated B and D hordeins. However, many details of the transport of prolamins to protein bodies are still not understood (Tosi 2012).

All in all, the mechanism behind the greater accumulation of C hordein deeper in the endosperm under long-day conditions typical to Northern European growing conditions remains unclear. It also has to be kept in mind that this result was obtained in the current study with one genotype only. Interestingly, however, as shown in Publication **III**, a similar type of localization of entrapped C hordein, as well as differences in location of other hordein fractions, in the field-grown grains of two other barley cultivars were associated with crop year, indicating a dependence on environmental conditions. Together these results on hordein localization suggest that hordein deposition both at tissue and subcellular level during grain filling are affected by the growing environment.

Besides light influencing the growth rhythm of barley plants, grain composition is naturally affected by the availability of nutrients utilized in the formation of grain structure and their storage in subcellular storage organelles. Sulphur plays an essential role in plant metabolism, growth and development (Randall and Wrigley 1986). In the current study, greenhouse-grown plants without added sulphur were not capable of developing reproductive organs.

The application rates of 10 and 20 mg S / kg soil were proven to be critical for sulphur sufficiency by several parameters in the pot-scale experiment. The malate:sulphate ratio indicated sufficient sulphur availability for plants grown with 10 or 20 mg S / kg soil or more and, as a confirmation of this, grain yield per plant increased at these sulphur application rates.

Changes in grain metabolism due to increased sulphur availability led both to elevated accumulation of S-rich compounds and to decrease in the content of compounds that are accumulated when sulphur is not sufficiently available. The

application rates of 10 and 20 mg S / kg soil resulted in a significant increase in the amount of cystine in comparison to lower sulphur application rates. According to Shewry et al. (1983) this is an indication of improved sulphur availability. Logically, the content of S-rich B hordein containing several cysteine residues increased in a similar pattern to that of cysteine.

The content of aspartic acid decreased when the sulphur application increased from 5 to 10 mg S / kg soil. During hydrolysis of proteins and peptides for assessing amino acid composition, asparagine is deaminated resulting in formation of aspartic acid. Asparagine is known to act as a storage pool of non-protein nitrogen when protein synthesis is limited in S-deficient barley (Shewry et al. 1983; Mortensen et al. 1992). Therefore, the decrease in the content of aspartic acid marked the point when protein synthesis was not anymore limited by sulphur availability, and when nitrogen storage in proteins was preferred over non-protein storage. The nature of asparagine as a non-protein nitrogen pool explains also why no significant differences were observed in grain nitrogen content due to sulphur application rate.

Similarly to asparagine, C hordein is known to serve as a nitrogen sink in grains of barley plants deficient in sulphur (Rahman et al. 1983). The C hordein content and its proportion of total hordein did not level off simultaneously with the other nitrogen storage compounds, but only with addition of 20 mg S / kg soil or more. Based on the pattern of the changes in C hordein, S application of 20 mg / kg soil or more seemed to be sufficient, although the amount of aspartic acid indicated an increased level of protein synthesis even with lower sulphur application rates.

It is noteworthy that the contents of S-containing amino acids cystine and methionine responded differently to sulphur availability. The increase in methionine was more gradual in comparison to that of cystine. A similar difference due to sulphur availability has been shown by Mortensen et al. (1992). One reason for this difference may lie in the fact that the sulphur atom of methionine originates from cysteine via a metabolic pathway active in cereal endosperm during grain development (Wirtz and Droux 2005; Dupont 2008). The lower amount of methionine in hordeins in comparison to that of cystine could be a second reason why the amounts of these S-containing amino acids did not increase linearly with S application rate (Kreis et al. 1984).

It is possible that the hordein composition did not change further at higher sulphur application rates due to the depletion of nitrogen in the pot-scale experiment. On the other hand, the increase in the total hordein and B hordein contents continued also with the highest sulphur application rates showing that nitrogen was not totally depleted by the barley plants.

In field conditions, neither grain sulphur content nor grain N:S ratio indicated sulphur deficiency according to the definitions given by Randall and Wrigley (1986) and Withers et al. (1995). In addition, the increases in malate:sulphate ratio were substantially smaller in comparison to the changes observed at pot-scale between S additions of 10 and 20 mg/kg soil. Contrary to earlier findings (Reisenauer and Dickson 1961; Zhao et al. 2006), yield was not affected by sul-

phur application in the current study. The only observation referring to slight S deficiency was the decrease of grain size in four trial sites due to higher N application, although grain size is affected by several other variables as well (Reisenauer and Dickson, 1961). Therefore, it can be concluded that no apparent S-deficiency occurred in the field trial sites.

An interesting result of the current study is that, despite the fact that sulphur was sufficiently available, grain composition was affected by the sulphur application rate. A decrease in grain N:S ratio was observed in three trials. Such a decrease has been linked earlier to the dilution effect caused by the yield-increasing effect of S application, when N is deficient (Zhao et al. 2006). However, no yield increase due to S application was found in the current study. Yield, and both nitrogen and sulphur contents, of the grains increased with the higher nitrogen application rate, indicating that sulphur was not limiting. Nitrogen application rate did not change the grain N:S ratio.

In accordance with previous studies, sulphur and nitrogen application were associated with increased total hordein content (Kirkman et al. 1982; Giese et al. 1983; Shewry et al. 1983; Buiatti et al. 2009) and the proportions of B and C hordeins increased and decreased, respectively, with a higher S application rate (Shewry et al. 1983). The effect of nitrogen application on hordein composition was more cultivar-dependent than that of sulphur application. This cultivar-dependency may reflect the genotype-dependency of protein content.

To our knowledge, the effect of sulphur application on barley hordein composition in northern growing conditions has been reported for the first time in this study. The sulphur responses of hordein composition observed both in greenhouse and field conditions were in accordance with those reported earlier. This indicates that, e.g. the more intensive growth rhythm induced in Northern European growing conditions does not alter greatly the effect of S on grain composition.

5.2 Association of barley grain composition and structure with processing properties

Milling and malting represent different processing types, the former relying on the use of mechanical force and the latter being the controlled physiological process of germination consisting basically of hydration and subsequent enzymatic hydrolysis of grain structures. However, both these processes are dependent on the properties of the starchy endosperm, which was also proved in the current study.

5.2.1 Milling

Upon milling, the endosperm tissue is broken mechanically, leading to tissue degradation to smaller fragments and disintegration of cell walls and subcellular structures. In the current study, higher starch content and lower protein and β-glucan contents in barley grains were associated with production of flour with finer parti-

cles upon pin-milling (**III**). The higher fragility of an endosperm with a certain composition is probably associated with grain hardness, as the milling of softer grains is known to result in finer flour (Nair, Ullrich, et al. 2011). Hence, the results suggest that an endosperm containing more starch and less protein and β-glucan is softer than one having the opposite composition. The results are in accordance with earlier studies (summarized in **Table 2**). In most cases, starch content correlates negatively and β-glucan positively with grain hardness, whereas protein content correlates negatively with both grain hardness and steeliness. Nevertheless, these associations have not been found in all studies. It is thus probable that grain hardness or endosperm fragility upon milling cannot be explained by the amount of a single component but is rather a result of interaction of starch, protein, β-glucan and the packing of the endosperm. For example in the current study (**III**), crop year-related differences, probably in the endosperm structure, were more important for endosperm fragility than β-glucan content only.

The interdependency of starch, protein and β-glucan explains their close association with grain fragility. Here, starch content showed a negative correlation with both protein and β-glucan contents, and similar correlations have been found in a study covering a wide range of barley cultivars (Holtekjølen et al. 2006). Thus, the influence of starch content on grain fragility is probably related to either protein or β-glucan or both.

As a cell wall component, β-glucan could contribute to grain hardness via cell wall thickness, as thicker cell walls have been associated with hard-structured, steely barley grains (Nair, Knoblauch, et al. 2011). β-Glucan could act on grain hardness also through endosperm cell size, because higher β-glucan content in harder grains could mean smaller cells and thus a higher amount of cell walls. This would be in line with the negative correlation observed between starch and β-glucan contents. In fact, one explanation for the inverse relationship between starch and β-glucan contents could lie in the allocation of carbon between these polysaccharides during grain development.

Logically, regarding the localization of protein surrounding starch granules in the starchy endosperm, how protein influences grain hardness and fragility in milling is probably related to starch in one way or another. First, the continuity and compactness of the protein matrix in the endosperm has been linked to grain hardness and flour coarseness (Darlington and Palmer 1996; Chandra et al. 1999; Nair, Knoblauch, et al. 2011). Consistent with the negative correlation observed between protein and starch, the less continuous and compact protein matrix in softer grains could be a consequence of a higher volume of starch granules dispersing and compressing the protein matrix. Secondly, the lower adhesion of protein to starch granules has been related to a mealy endosperm and dissociation of endosperm into finer flour particles (Brennan et al. 1996; Nair, Knoblauch, et al. 2011).

In addition to the grain total protein content, the localization of hordeins also affected the dissociation of endosperm on milling, although the data did not support the separation of the effect of hordeins from that of β-glucan. The relevance of hordein distribution for milling properties could be questioned, because of the

small amount of hordein in relation to the volume of starch granules present in starchy endosperm cells. Nevertheless, steeliness has been shown previously to be associated with high C hordein content (Ferrari et al. 2010). Therefore, the lower proportion of non-trapped C hordein found in the current study within the central starchy endosperm of grains resulting in finer flour, could be expected to facilitate dissociation of the protein matrix and starch.

5.2.2 Hydration

Quick and even hydration has been considered a fundamental prerequisite for enzymatic modification occurring during germination, as it enables the action of hydrolysing enzymes (Amor et al. 1996). During hydration of the barley grain, water enters the starchy endosperm from the embryo through the scutellum, and at lower rates also from the distal end of the grain and through the aleurone layer (Axcell et al. 1983; Davies 1991; McEntyre et al. 1998). In the current study, lower grain β-glucan content (**III**) and a higher proportion of C hordein entrapped by aggregated hordeins and localized deeper within the endosperm (**I** and **III**), were associated with faster hydration in terms of distribution of water and the amount of water absorbed. In addition, the localization of aggregated B and D hordeins was found to be related to hydration (**III, Table 8**). Although the data does not distinguish between the effect of localization of aggregated hordeins on water uptake and the effect β-glucan content, the results are in line with an earlier study stating that the major grain components affecting water uptake are β-glucan and hordeins (Molina-Cano et al. 1995).

β-Glucan in the endosperm cell walls of barley grain consists of cellotriosyl, cellotetraosyl, and longer cello-oligomers connected via β-(1→3)-linkages. Due to this chain conformation, β-glucan is a hygroscopic compound capable of binding large hydrodynamic volumes (Izydorczyk and Dexter 2008). Accordingly, in the hydrated barley grains, high β-glucan content has been related to higher water content and water mobility (Fast Seefeldt et al. 2007). However in order to facilitate the action of hydrolysing enzymes, water uptake at the beginning of malting should result in hydration of both cell walls and protein and starch inside the cells of barley starchy endosperm. Thus the high water-retention properties of β-glucan may slow down or prevent the desired even distribution of water in the starchy endosperm. A relationship between high β-glucan and slower hydration has been shown (Gamlath et al. 2008), but there are also studies in which this relationship has not been detected (Molina-Cano et al. 2002; Mayolle et al. 2012).

There are several potential reasons for the lack of consistent evidence on the correlation between β-glucan content and water uptake within the barley grain. One explanation is that water uptake is not actually related to the total β-glucan content, but rather to its water-insoluble fraction as shown by Molina-Cano et al. (1995). The distribution of β-glucan within the grain has also been shown to be associated with hydration. Higher concentrations of β-glucan especially in the proximal end of the grain have been linked to slower hydration of the endosperm

(Chandra 1995). In the current study, no such correlation between hydration and distribution of β-glucan was found, because localization was assessed by pearling the grain outer layers gradually, not allowing examination of differences between grain ends. In addition to the properties and distribution of β-glucan, water uptake has been shown to correlate negatively with grain hardness or steeliness (Chandra et al. 1999; Gamlath et al. 2008). Hard and steely grains have also been shown to have the lowest porosity and water diffusivity, impeding hydration (Mayolle et al. 2012). Thus, the inconsistency in the reported correlations between grain β-glucan content and hardness or steeliness may explain why the correlation between hydration and β-glucan is not always found.

As discussed above, grain hardness or steeliness is a complex characteristic and probably not resulting from the content of a single compound in the grain. In fact, the same probably applies also to the association between grain composition and hydration. In the current study (III), the grains of each cultivar had higher β-glucan content and slower hydration in 2006 compared to the same cultivar in 2007. Despite the similar behaviour in water uptake, the cultivars differed in their β-glucan levels, indicating that water uptake was not regulated by β-glucan content only, but also by other properties related to crop year, and thus induced by similar environmental conditions. These non-compositional grain properties may be linked to packing or distribution of components in the starchy endosperm (Brennan et al. 1997; Chandra et al. 1999).

Another grain component, with which water uptake of barley grain is usually associated, is protein. Usually low protein content is associated with improved water uptake due to lower grain hardness or vitreousness (Chandra et al. 1999; Molina-Cano et al. 2002). Especially the compaction of starch granules and protein matrix has been supposed to limit endosperm hydration (Darlington and Palmer 1996). However, a correlation between protein content and water uptake is not always found (Leach et al. 2002; Swanston et al. 2006; Gamlath et al. 2008), and Mayolle et al. (2012) have even reported that the barley sample with the lowest protein content had the lowest maximal water uptake. In the current study, no relationship was found between the content or distribution of total protein or total hordein and water uptake. But interestingly, the distribution of hordein fractions was shown to affect the hydration properties of the barley grain. The most convincing evidence was found on the effect of entrapped C hordein on water uptake during the steeping phase of malting, indicating that the deeper localization of this hordein fraction facilitates the distribution of water in the starchy endosperm (I). This effect was related to the C hordein fraction entrapped by aggregated hordeins. A similar distribution of entrapped C hordein was found in Publication III, in which also the lower proportion of non-trapped C hordein was associated with quicker hydration. The actual mechanism by which C hordein affects hydration is not known, but it can be assumed that the phenomenon is real based on the known structure of C hordein protein and the effects related to C hordein in previous studies.

C hordeins are hydrophobic proteins based on their amino acid composition (Herman and Larkins 1999). C hordeins have high hydrogen bonding capacity due to their high content of glutamine residues, which suggests the potential of these

proteins to have strong interactions with each other (Tatham and Shewry 2012). In Publications **I** and **III**, it was shown that most C hordein polypeptide was soluble in alcohol without a reducing agent, and was thus not trapped among aggregated B and D hordeins. This C hordein fraction could account for the properties limiting hydration related to C hordein (Bénétrix et al. 1994). In the current study, a lower proportion of this fraction deeper within the starchy endosperm was indeed related to higher water uptake. It is not clear if the reported association of C hordein with steeliness (Ferrari et al. 2010) is somehow related to the hydrophobicity of C hordein. In some studies, conversely, a high total content of C hordein has been associated with greater water absorption during malting (Molina-Cano et al. 1995, 2002). In the current study, the greater water uptake was related to the minor proportion of C hordein, which is entrapped within aggregated hordeins. This C hordein fraction could facilitate hydration by restricting the aggregation of B hordein, or by changing the number and orientation of disulphide bonds between aggregated hordeins (Skerritt and Janes 1992; Molina-Cano et al. 1995). Entrapment of C hordein may be facilitated by non-covalent bonding to B hordeins (Shewry and Miflin 1985).

Grains with faster hydration have been shown to have a lower ratio of B and C hordeins (Molina-Cano et al. 1995). In line with the current study, the concentration of aggregated B hordein in the peripheral endosperm or in subaleurone has been associated with lower water uptake (Molina-Cano et al. 2002) and with impaired malting quality, probably due to uneven hydration (Millet et al. 1991).

5.2.3 Endosperm modification during malting

In the current study, both protein and starch content of barley were associated with the degree of malt modification in terms of friability. The results on the effect of barley starch content were not congruent, and both higher and lower starch content were related to better friability. The negative correlation between starch content and friability was observed in the sub-sample set of 6 barley lots with the same protein content and could be at least partly explained by the smaller grain size related to the higher starch content (Edney and Mather 2004). The positive correlation of starch with modification could be explained by the inverse relation of starch and protein contents of the barley grains observed also in the current study. A higher amount of starch with lower protein content could mean a more easily degraded, less dense or compact protein matrix surrounding starch granules resulting in higher modification of the endosperm (Chandra et al. 1999).

A negative association between protein content and friability is usually observed (Brennan et al. 1997; Agu and Palmer 2001; Fox et al. 2009). High protein may cause compaction of starch and protein in endosperm cells, which can, even if localized within a limited area, result in poor modification and malting quality in terms of low friability and insufficient β-glucan and protein degradation (Darlington and Palmer 1996; Koliatsou and Palmer 2004). In the current study (**II**), an in-

crease in the total hordein content was associated with a decrease in friability. As hordein content reflects the total protein content of the grain and is the grain component responsible for the increase in the total protein content, the effect of total hordein on malting quality is most probably very similar to that described above for total protein content (Wang et al. 2007; Buiatti et al. 2009). The other changes in malting quality associated with the increase in hordein content were also linked to the aspects of a lower modification rate. The extract yield may have been reduced as a consequence of a less modified endosperm caused by higher hordein content (Slack et al. 1979; Howard et al. 1996; Buiatti et al. 2009). The Kolbach index also decreased, indicating that less protein was hydrolysed during malting and more protein was left in malt due to a lower degree of modification. The role of hordein localization in modification is probably related to the action and role of hordeins in grain hydration as discussed above. Especially the correlation of deeper location of C hordein with more uniform modification (**I, III**) can be taken as an indication of this.

Barley β-glucan content and friability correlated negatively in the current study, having a limited sample set, although the effect of β-glucan content was not fully distinguishable from that of localization of different hordein fractions. Potentially, β-glucan content could be linked to modification and friability through its effect on grain hardness (Henry and Cowe 1990; Psota et al. 2007). However, due to the extensive degradation of β-glucan during malting, friability correlates more clearly with the amount of β-glucan left in the malt than the β-glucan content of unmalted barley (Henry 1988). A correlation between barley β-glucan content and modification is not common (Agu and Palmer 2001).

Endosperm hardness and structure is known to affect modification during germination. A mealy or soft endosperm has been reported to facilitate hydration and thus enzyme movements within the endosperm, leading to higher degradation of cell walls during malting (Chandra et al. 1999; Swanston et al. 2006; Gamlath et al. 2008). Accordingly, a negative correlation between grain hardness or steeliness and friability has been found in several studies (Henry and Cowe 1990; Psota et al. 2007; Vejražka et al. 2008) including the current study. Steelier grains resulted in less friable malt even in the sample set with the same grain protein content indicating independency of this association on protein content. Harder or steelier grains have also been related to lower extract yield (Psota et al. 2007; Vejražka et al. 2008; Nagamine et al. 2009), but no such correlation was observed in the current study. However, the malts of steelier barley resulted in darker wort, which can be interpreted as a sign of uneven modification (Palmer 1992).

Malts of steelier grains contained more soluble nitrogen and FAN than malts produced from mealier grains. This difference was observed even between samples of the same protein level, indicating that higher content of proteolytic products was not explained by the higher protein content. As a confirmation of the higher proteolytic activity, the malts of steelier samples were also found to contain higher activities of total and cysteine endopeptidases than the malts of mealier grains. However, it could be questioned whether the differences in the enzyme activities were large enough to cause such differences in the amounts of protein degrada-

tion products. Thus the explanation for differential proteolysis may lie in spatial patterns of endoproteolytic activity development or substrate localization. Differential activity of carboxypeptidases, needed for further degradation of peptides, could also be a reason for the difference in proteolysis between steely and mealy grains (Enari, 1986; Simpson 2001). In addition, rates of hordein degradation may be more closely associated with hordein structures than with the activity of endopeptidases (Palmer 1995).

Higher steeliness was also associated with higher activities of xylanase, α-amylase and limit dextrinase. Xylanases, which hydrolyse cell wall arabinoxylans, are released from the aleurone cells during the final stage of endosperm mobilization and are linked to the gibberellin-controlled programmed cell death occurring in aleurone cells during germination (Banik et al. 1997; Caspers et al. 2001; Simpson et al. 2003). Higher activities of xylanases may be an indication of a more advanced programmed cell death and thus of a higher metabolic rate of steely grains. The higher metabolic activity is supported by the higher respiration losses of the steely grains. The higher activities of starch-hydrolysing enzymes could also be related to the higher respiration rate. Similarly to results of the current study, high activities of α-amylase and also β-glucanase have been found in steely grains (Mayolle et al. 2012).

Likewise with the results on milling and hydration behaviour of barley grains, no individual grain component or structural parameter alone was found to explain the processability during malting in the current study. Starch and protein as the main components of barley grain as well as the grain hardness or steeliness seemed to be the factors affecting the malting performance. However, the significance of hordeins, and their localization in subaleurone in particular, was emphasized by the results obtained.

5.3 Methodological considerations

The grain material examined in the current study represented several barley cultivars and was produced both in field conditions and in more controlled conditions either in greenhouse or CE cabinets. Because the grain material was partly provided by larger research projects, it was not possible to have only certain barley cultivars. Despite the heterogeneity of the material, certain phenomena, such as the effect of deeper localization of a C hordein fraction on water uptake, were observed independently of the grain material. Therefore, generalization is, in many cases, justified. Naturally, having a higher number of samples and even higher variation in the grain composition would have facilitated the interpretation of the results.

As a research subject, hordeins set certain limitations in comparison to other plant proteins. The main issue is their solubility only in aqueous alcohols. The polymorphism of these proteins, resulting in cultivar-dependent patterns in SDS-PAGE separation, also makes hordein analysis challenging. In order to study

these proteins and their different fractions, these need to be extracted, separated and quantified. The methods used in each of these steps in the current study are discussed below.

In the current study, hordeins were extracted first with 50% (v/v) 1-propanol only and then with a reducing agent (DTT). Hordein extraction procedures vary in the choice of alcohol used (1- or 2-propanol, ethanol), extraction temperature (room temperature or +40–60°C), extraction volume and ratio of solid to liquid (e.g. Shewry et al. 1980; Kanerva et al. 2008). In the current study, these variables were not individually optimized, but the extraction conditions were adopted from those reported by Marchylo et al. (1986) with down-scaling of the extraction volume in order to ensure the effectiveness of the extraction.

Proportions of total hordein content extracted from total grain protein content were somewhat lower in the current study (c. 20%) compared to 35–55% reported in the literature (Kirkman et al. 1982). This discrepancy is likely explained by the differences in protein quantification methods used either for hordein extracts or determination of grain total protein content. Ideally, the same method would be used for both. In the current study, the total protein contents were measured based on NIR or calculated from the total nitrogen content determined by Kjeldahl method. The quantification of extracted hordeins was performed after acetone precipitation and dissolution in rehydration solution without any dye (**II** and **III**). This allowed the use of the 2-D Quant protein quantification method which is compatible with 7 M urea and 2 M thiourea present in the rehydration solution. It is also independent of amino acid composition unlike the widely-used protein quantification methods of Lowry and Bradford, which rely on the reactions with aromatic amino acid residues such as tyrosine and tryptophan or basic amino acids (primarily arginine, lysine and histidine), respectively (Lowry et al. 1951; Bradford 1976). Regarding the different amino acid composition of hordeins, the independence of the protein quantification method on the amino acid composition can be taken as an advantage.

Hordein groups were separated and quantified using two techniques: RP-HPLC (**IV**) and SDS-PAGE integrated with densitometric quantification (**I–III**). In RP-HPLC, the separation of molecules is based on the surface hydrophobicity, while in SDS-PAGE proteins are separated by their molecular size. This difference may affect the comparability of the results obtained using these two methods. Quantification of hordein groups after RP-HPLC is performed by the integration of chromatogram peaks and for this literature information on the elution order of different hordein groups is needed if fractionation and protein sequencing is not applied for reducing the risk of misinterpretation. Hordein groups were originally identified by SDS-PAGE separation, which clearly makes the identification of hordein groups easier with this method in comparison to RP-HPLC. In addition, the identification of hordein bands separated by SDS-PAGE can be confirmed by Western blotting using specific antibodies.

Sulphur deficiency in the barley plants was evaluated in the current study by analysing the ratio of malate and sulphate in leaves sampled during vegetative growth (**II**). This analysis is based on the inverse relationship between malate and

sulphate concentrations in the leaves due to the role of malate in maintaining the cytoplasmic pH in response to the accumulation of ions (Blake-Kalff et al. 2000). The malate:sulphate ratio has been used in predicting sulphur deficiency of winter and spring barley, although the method was originally developed for wheat and oilseed rape (Blake-Kalff et al. 2000; Carver 2005; Zhao et al. 2005). However, based on the results of Zhao et al. (2005) and Carver (2005), the critical value for sulphur deficiency may not be unequivocally determined; the malate:sulphate ratio in barley seems to be affected by growing conditions and varietal differences. In the current study, clear changes in the malate:sulphate ratio rather than exact values were used in the evaluation of the changes in sulphur availability experienced by the plant.

Milling behaviour of barley was examined in the current study by pin-milling (**III**). The barley endosperm may have been differently dissociated in another type of grinding device, e.g. in hammer or roller mill. However, no standard milling procedure for barley exists and thus pin-milling can be considered as an appropriate treatment for studying the milling properties of barley.

The evaluation of malting quality of malts produced in batches of 60 g was performed by down-scaled friability measurement (**I, III**). The friability values obtained by this modified procedure are not directly comparable with the values obtained with the standardized EBC method, but they allow comparison within the analysed sample set.

6. Conclusions

The current study provides novel information on the formation of barley grain structure and composition during endosperm development, and on the relationship of grain composition, structure and texture with the grain's end use quality.

First, it was shown with the cultivar Barke that growing conditions may influence grain filling by altering the hordein localization within the starchy endosperm. A longer photoperiod typical of Northern European latitudes induced, in particular, the fraction of C hordein entrapped by aggregated B and D hordeins to be more deeply located in the grain. Thus the impact of the growing environment on hordein deposition during grain filling was observed both at tissue and subcellular level. However, the mechanism behind the differential accumulation of C hordein under long-day conditions remains unclear. In addition to cultivar Barke, the deeper localization of entrapped C hordein was demonstrated to improve grain water uptake in malting also in two other barley cultivars. Hence the results suggest that the influence of growing environment on the hordein localization further affects the end-use properties of barley. In more detail, it can be concluded that day-length characteristic of Southern Finland during growing the season had a positive effect on barley processing performance. Moreover, the role of the subaleurone region in barley grain was found to be significant with respect to end use quality.

Secondly, the effects of sulphur availability on grain amino acid and hordein content were investigated for the first time in Northern European growing conditions. Asparagine and C hordein served as nitrogen storage pools when the sulphur application rate was lower than 20 mg S / kg soil, whereas total hordein and B hordein contents increased with higher sulphur application rates. In addition, the increase in sulphur availability was reflected as the decrease of the malate:sulphate ratio determined in leaves during vegetative growth. The current study also showed that even when sulphur is sufficiently available in field conditions, the hordein composition may still react to sulphur application. This result indicates the sensitivity of the developing grain to respond to nutrient availability. It also underlines the complexity of factors affecting grain filling in field conditions and the difficulty of their control. The observed sulphur responses were in accordance with those reported earlier on hordein composition, which indicates that, e.g. a more intensive growth rhythm induced in northern growing conditions does not alter greatly the effect of sulphur on grain composition.

Thirdly, the current study verified that the main grain components, starch, protein and β-glucan and also hordein localization influence grain processing properties including milling, hydration and endosperm modification. However, their influence on endosperm texture (hardness or steeliness), which also affects processing behaviour, cannot be directly derived or estimated on the basis of grain composition. Assessment of barley β-glucan content and grain hardness or steeliness along with the analysis of starch and protein content would give a good estimate of the grain processing behaviour in the milling and steeping phase of malting. Nevertheless, the significant role of hordeins in hydration found in the current study raises the question of whether hordeins should be also taken into account in evaluation of the processability of barley lots.

Acknowledgements

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Day-length effects on protein localisation affect water absorption in barley (*Hordeum vulgare*) grains

Research Article

Day-length effects on protein localisation affect water absorption in barley (*Hordeum vulgare*) grains

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Abstract

BACKGROUND: Hordeins are major storage proteins of barley (*Hordeum vulgare* L.) grains and are considered to influence malting and brewing by forming a matrix surrounding the starch granules which affects the release of fermentable sugars. However, the extent to which environmental factors affect hordein location, and the impact of this on malting performance, have not so far been studied. Therefore the relationship of hordein location to water uptake and malting quality were studied by growing barley cv. Barke under different daylengths (14 h and 18 h of light) in controlled environment conditions.

RESULTS: Differences in the locations of hordein storage proteins were observed, with C hordein being located more deeply within the endosperm of both developing grains at 35 days after anthesis and in mature grains under long-day conditions. This deeper location of C hordein was correlated positively with water uptake during the steeping phase of malting.

CONCLUSION: An effect of environment (daylength) on the localisation of C hordein was demonstrated. This difference in hordein localisation was also associated with differences in malting quality with water uptake in the steeping phase being associated positively with the deeper location of C hordein. These results indicate that environmental effects on protein location may affect malting performance of barley grains.
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Keywords: barley (*Hordeum vulgare*); daylength; hordein; malting quality

INTRODUCTION

In barley endosperm the starch granules are embedded in a protein matrix consisting of alcohol-soluble proteins, hordeins, which act as the major nitrogen store of the grain.[1,2] High contents of protein in malting barley are related to a tightly packed endosperm structure resulting in slow imbibition and uneven endosperm modification leading to low extract yield and to problems with filtration, flavour and haze formation during brewing.[3,4] However, not only the total protein content but also hordein composition has been related to malting quality.[5,6]

Hordeins belong to the prolamin superfamily of cereal storage proteins and are classified based on their molecular size and amino acid composition: B and C hordeins account for 70–80% and 10–20%, respectively, of grain hordein content while D and γ hordeins are minor components.[7–9] Growth conditions may affect the content and/or composition of hordeins, and several studies have compared samples grown at different latitudes. Barleys grown in Scandinavia have been reported to have higher proportions of B hordein leading to decreased malt extract yield in comparison to barleys grown in Spain and Portugal.[10] Barley grown in Spain had high levels of protein due to greater hordein accumulation, particularly of C hordein, in the later stages of grain maturation compared to barley grown in Scotland. Despite this, Spanish-grown barley had better water uptake in malting and higher extract yields in comparison to barley grown in

Scotland.[5,11] Similarly, Canadian barley with lower contents of protein and hordein, had lower malting quality compared to the Spanish-grown barley.[12] The growth environment, and its latitude, therefore appears to affect hordein composition and malting quality.

In addition to amount and composition, the distribution of proteins within the grain may also affect malting performance. B and C hordeins are concentrated mainly in the sub-aleurone cells and D hordein in the central starchy endosperm cells.[13,14] This peripheral localisation of B and C hordeins is consistent with their impact on water uptake in malting and on subsequent endosperm modification. Poor malting quality has been related to a high content of aggregated B hordein in the peripheral grain layers,[15] while a low B : C hordein ratio has been related to better hydration.[5,16] Nevertheless, C hordeins may also have negative effects on malting performance.[17] However, the extent to which environmental factors affect hordein location, and the impact of this on malting performance, have not so far been studied. We

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therefore show here that differences in hordein location can result from environmental impacts, and that these can in turn affect water uptake and hence malting quality.

MATERIALS AND METHODS

Plant material

Two-rowed spring-type barley (*Hordeum vulgare*) cultivar Barke was grown under long-day (18/6 h, LD) and short-day (14/10 h, SD) conditions in two replicate experiments under identical conditions. Seed was sown into pots of 12 cm diameter filled with a peat–loam–grit–vermiculite compost (75 : 12 : 10 : 3; Petersfield Products, Cosby, UK) fertilised with a slow-release fertiliser (3.5 kg m^{-3} OsmocotePlus, Scotts Professional, Ipswich, UK; NPK 15 : 11 : 13 and micronutrients) and a readily available fertiliser (0.5 kg m^{-3} PG mix, Hydro Agri (UK) Ltd, Belfast, UK; NPK 14 : 16 : 18 and micronutrients). Each replicate experiment comprised 96 plants (two plants per pot) and was carried out in controlled-environment cabinets equipped with an upward airflow distribution system (Sanyo SGC228.CFX.J; Sanyo, Osaka, Japan) at Rothamsted Research, Harpenden, UK. Lighting for 48 pots in each cabinet was provided by fluorescent lamps (Philips Master TL5 HO 49 w/830; Philips Lighting UK, Guildford, UK). The photosynthetically active radiation was 400 µmol m^{-2} s^{-1} (at 400–700 nm, at pot height) during light periods. The temperature regime was 12 h at 20 °C ± 0.5 °C and 12 h at 15 °C ± 0.5 °C for both daylengths, with the warmer period in the middle of the light period. The relative humidity was maintained in light at 70% ± 5% and in dark at 80% ± 5%. Plants were hand-watered daily and nutrients were supplied after 6 or 7 weeks after sowing using nutrient solution (2 g L^{-1} Phostrogen Balanced Plant Food; Phostrogen, Corwen, UK; NPK 14 : 10 : 27, MgO 2.5, SO$_3$ 15 and micronutrients).

Grains from all experiments were analysed for grain composition and hordein localisation as well as for water uptake in micromalting and friability. Additionally, the main shoot heads were tagged in the trials LDII and SDII and the date of pollination was determined by examining the anther maturity of their florets. Developing caryopses were collected 14, 21, 28 and 35 days after pollination (dap) from the middle third of these main shoot heads for immunolabelling. The average harvesting times were 52 dap and 74 dap for the plants grown under the long and short daylength conditions, respectively.

Analysis of barley grain

Grain size distribution, thousand grain weight, protein and β-glucan contents of mature grains >2.5 mm were determined according to the European Brewing Convention.[18] Starch concentrations were measured using the Megazyme total starch assay kit (Megazyme International, Ltd, Brey, Ireland). Analyses were performed in triplicate per experiment and averages were calculated for LD and SD conditions.

Western blotting for confirmation of antibody specificities

The specificities of the monoclonal antibodies BII (provided by A. Brandt, Carlsberg Laboratory, Denmark) and IFRN0614[19] raised against hordein fractions were confirmed by western blotting against total hordeins from grains of cultivars Barke and Risø 56 [a mutant line in which the absence of B hordeins allows clear resolution of the γ_1, γ_2 and γ_3 hordeins (41, 36 and 32 kDa, respectively)].

Grains of barley cultivars Barke and Risø 56 were milled using a Fritsch Pulverisette sample mill with a 0.5 mm sieve (Fritsch GmbH, Idar-Oberstein, Germany). Fifty milligrams of barley meal was extracted with 250 µL of 500 mL L^{-1} 1-propanol containing 10 g L^{-1} dithiothreitol (DTT) for 1 h at 60 °C. The lyophilised supernatants were resuspended into 200 µL of sample buffer (50 mmol L^{-1} Tris-HCl buffer (pH 6.8), 100 mL L^{-1} glycerol, 20 mg L^{-1} sodium dodecyl sulfate (SDS), 1 mg L^{-1} bromophenol blue and 200 mmol L^{-1} DTT), boiled for 3 min, centrifuged (20 800 × *g*, 10 min, 4 °C) and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 12% Tris-borate gels (pH 8.9) using 0.125 mol L^{-1} Tris-borate with 1 g L^{-1} SDS (pH 8.9) as running buffer.[20] After separation by using SDS-PAGE, hordeins were wet blotted onto nitrocellulose membranes (Protran BA83, pore size 0.2 µm; Schleicher & Schuell, Dassel, Germany) with MiniTrans-Blot® Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA, USA) using buffer containing 0.192 mol L^{-1} glycine, 25 mmol L^{-1} Tris, 200 mL L^{-1} ethanol and 1 g L^{-1} SDS. Membranes were blocked overnight at 4 °C in 20 mmol L^{-1} Tris-buffered saline (TBS) containing 5 mL L^{-1} Tween-20 and 50 g L^{-1} dried milk, incubated for 1 h at room temperature (RT) in TBS containing 5 mL L^{-1} Tween-20 and 20 g L^{-1} dried milk with monoclonal antibodies BII or IFRN0614 at 1 : 3000 dilution, rinsed and incubated similarly with Goat Anti-Mouse IgG-AP Conjugate (Bio-Rad) as secondary antibody at a 1 : 3000 dilution.

Antibody binding on membranes was detected using nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) (Roche Diagnostics, Mannheim, Germany) as an alkaline phosphatase substrate. Proteins that were not labelled with antibody were visualised with 2 g L^{-1} Ponceau solution containing 3 g L^{-1} trichloroacetic acid. Gels and membranes were scanned with a GS-710 calibrated imaging densitometer (Bio-Rad).

Immunolocalisation of hordeins in developing caryopses

Transverse sections of 400 µm from the middle third of the freshly sampled developing caryopses (14, 21, 28 and 35 dap) taken from the middle third of main shoot heads from trials LDII and SDII were cut submerged in mQ-H$_2$O with a Leica VT1000S vibrating blade microtome (Leica Microsystems GmbH, Nussloch, Germany).

Sections were fixed with 40 g L^{-1} paraformaldehyde in 0.05 mol L^{-1} sodium cacodylate buffer (pH 7.4), washed with 0.05 mol L^{-1} sodium cacodylate buffer (pH 7.4), dehydrated in a series of increasing concentrations of ethanol and infiltrated progressively with LR White resin (LRw; London Resin Company, Reading, UK) before embedding in LRw. Semi-thin sections (1 µm) were cut with a glass knife using an ultra-microtome (Ultracut E; Reichert-Jung, Vienna, Austria) and mounted on poly-l-lysine-coated microscopic slides.

Slides were preincubated for 30 min at RT in phosphate-buffered saline (PBS; Oxoid, Basingstoke, UK) with 10 g L^{-1} BSA and 1 mL L^{-1} Tween-20 (PBST-BSA). Sections were then incubated overnight at 4 °C with monoclonal antibodies diluted in PBST-BSA (BII 1 : 250, IFRN0614 1 : 100), washed and incubated for 30 min at RT using PBST-BSA. After washing, the sections were incubated (2 h, RT) with goat anti-mouse IgG-AP antibody (A3688; Sigma, St. Louis, MO) diluted 1 : 50 in PBST-BSA. Slides were rinsed with PBST-BSA and PBS. Binding of the antibodies was detected using SIGMA FAST™ FastRedTR/Naphthol AS-MX substrate (F4523; Sigma). Controls were the detection system only and labelling with the secondary labelling and the detection.

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The labelled sections were examined with an Olympus BX-50 microscope (Olympus Corp., Tokyo, Japan), imaged using a PCO SensiCam CCD camera (PCO AG, Kelheim, Germany) and analysed with the Cell P imaging software (Olympus). Nine series (three from both cheeks and three from the dorsal side of the grain) of four pictures were taken from three sampled grain per sampling point to show the protein labelling from the outer parts towards the centre of the grain. Images represented areas 0–310 µm, 310–650 µm, 650–990 µm and 990–1330 µm deep from the surface of the grain. In total, the area of proteins detected was determined in 36 images per grain (864 images per antibody) and calculated as a proportion of the endosperm area excluding the aleurone and the outer grain layers. Cheek parts of grains analysed were treated as individual samples and averages were calculated of cheeks ($n = 6$) and dorsal side ($n = 3$) per sampling point.

Hordein localisation by abrasion of mature grains

Samples of 100 g of grains (>2.5 mm) from each experiment were abraded using a barley dehuller–pearler fabricated at VTT Technical Research Centre of Finland. Abraded grains were sampled after every 60 s until 180 s of abrasion was reached. The protein content of abraded grains was determined using the Kjeldahl method ($6.25 \times$ N).

Protein fractions were extracted in triplicate using a modified Osborne fractionation method.[21] Twenty-five-microlitre batches of the 1-propanol- and 1-propanol+DTT-soluble hordein fractions were dried in a Speedvac centrifuge vacuum evaporator (Savant Instruments Inc., Holbrook, NY, USA), and resuspended into 40 µL of sample buffer (62.5 mmol L^{-1} Tris-HCl buffer (pH 6.8), 200 mL L^{-1} glycerol, 20 g L^{-1} SDS, 0.5 g L^{-1} bromophenol blue and 50 mL L^{-1} mercaptoethanol). After SDS-PAGE (described in the section 'Western blotting for confirmation of antibody specificities'), gels were fixed and stained with Coomassie brilliant blue G-250 according to Neuhoff *et al.*[22] After scanning the gels with a GS-710 calibrated imaging densitometer (Bio-Rad), hordeins were quantified with QuantityOne software (Bio-Rad) by the intensity and area of the bands.

Micromalting and assays for water uptake and friability

Sixty-gram samples of barley grains (>2.5 mm) of each experiment were malted in duplicate using a Joe White micromalting unit (Joe White Maltings Pty. Ltd., Adelaide, Australia). Malting consisted of steeping phase lasting 53 h in total [8 h wet, 16 h dry, 8 h (w), 16 h (d), 5 h (w)] at 14 °C and of germination lasting 5 days at 15 °C. Grains were kilned for 21 h to a final temperature of 83 °C.

Water uptake was measured based on the methods of Chapon[23] and Kelly and Briggs[24] with some modifications. Twelve grams of grains was sampled after 48, 72, 96 and 144 h of malting and immersed in boiling water for 30 s. Air-dried grains were pressed into wax blocks and abraded longitudinally into halves. The hydration of the endosperm was determined based on starch gelatinisation in wetted endosperm regions caused by heating. Non-hydrated endosperm regions appeared opaque and those containing gelatinised starch appeared transparent. The extent of endosperm hydration was assessed visually by classifying 100 grains per sample into six groups based on the amount of hydrated endosperm in the longitudinal section.

The EBC method[18] for determination of friability was optimised for smaller sample size using a malt of known friability as standard. Ten grams of kilned malt was processed in the friabilimeter for

3.25 min and the friability was calculated as the proportion of malt material passed through the mesh.

Statistical analysis

The data of two replicate experiments were combined and averages for both growth conditions were calculated. The results were statistically analysed using Student's *t* test and Tukey's HDS test (SPSS Statistics v17.0; SPSS Inc., Chicago, IL, USA).

RESULTS

### Hordein localisation in developing caryopses

Immunoblotting using total hordein extracts showed that the monoclonal antibody IFRN0614 recognised only bands corresponding in mobility to C hordeins while monoclonal antibody BII recognised the majority of B hordein bands but showed no cross-reactivity with γ hordeins (Fig. 1). Because the antibodies differed in their affinities for their respective antigens, differences in the relative intensity of labelling should not be taken to indicate differences in the absolute amounts of B and C hordeins. Nevertheless, they do show differences in localisation and changes in abundance of the antigens during grain development.

Irrespective of daylength, both B and C hordeins were initially observed in the sub-aleurone region at 14 dap, and remained concentrated in this layer throughout development, although they also accumulated progressively in the inner endosperm of the developing caryopses (Fig. 2).

Hordein quantification by image analysis showed a statistically significant difference in the distribution of C hordein in the cheek parts of the caryopses at 35 dap, with more C hordein being present in the sub-aleurone region (between 0 and 990 µm from the surface) in caryopses grown under long days than in those grown under short days ($P < 0.05$, $n = 6$; Fig. 3A–C). However, no significant differences in the distribution of B hordein were observed. Few statistically significant differences in the distributions of B and C hordein in the younger caryopses were found (Fig. 3A–D) and neither antibody showed differences in hordein distribution in the dorsal parts of the caryopses (data not presented). However, the analyses did show that the B and C hordeins labelled by the antibodies had different accumulation patterns in the later stages of grain filling, with B hordein ceasing to accumulate after 28 dap.
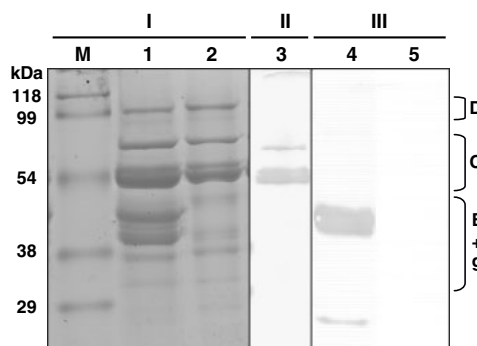
Figure 1. SDS-PAGE (I) and immunoblots of total hordein extracts using monoclonal antibodies IFRN0614 (II) and BII (III). Lanes: molecular marker (M), cv. Barke (1, 3 and 4), cv. Risø56 (2 and 5).

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I/3
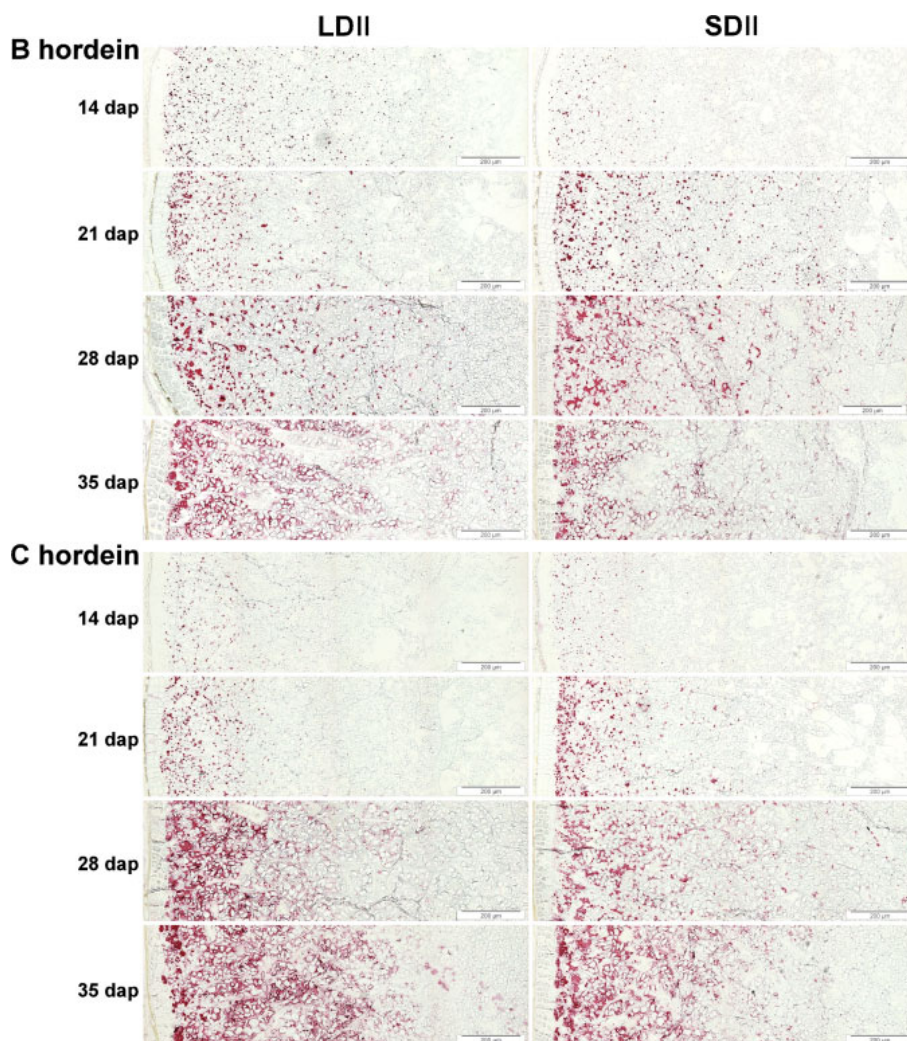
Figure 2. B and C hordeins in the cheek part of the developing main shoot head barley caryopses from experiments LDII and SDII labelled with antibodies BII and IFRN0614, respectively. Each picture is a compilation of four micrographs taken successively from the aleurone (left in the pictures) towards the centre of the grain (right in the pictures). Scale bar: 200 μm. For statistical analysis, micrographs of six cheek parts per experiment and antibody were examined. dap, days after pollination.

Composition of mature grains

The proportions of grains >2.5 mm (considered acceptable for malting) were high indicating successful grain filling under both photoperiods (Table 1). No significant daylength-related differences were observed in the 1000 grain weights or the contents of grain components including protein, starch and β-glucan (Table 1).

Hordein localisation in mature grain

The hordein distribution in mature grain was initially determined by sequential abrasion (pearling). The total protein contents of pearled grains decreased with increasing abrasion rates with the greatest decrease in protein content being observed after removal of 20–25% of the dry weight (data not shown). Visual inspection showed that abrasion to this extent removed the embryo and part of the aleurone and sub-aleurone layers.

Hordeins were extracted in two sequential fractions from whole grains and from samples produced by abrasion (Fig. 4). The first fraction was extracted in 500 mL L^{-1} 1-propanol without a reducing agent and consisted mostly of monomeric B and C hordeins. The second fraction was extracted in 500 mL L^{-1} 1-propanol containing 10 g L^{-1} DTT and contained B and D hordein subunits released from polymers by reduction of inter-chain disulfide bonds. However, some D hordein was also extracted without DTT in the 1-propanol-soluble fraction. Furthermore, some C hordein was present in the 1-propanol+DTT-soluble fraction and may have been entrapped in the B+D hordein polymers.
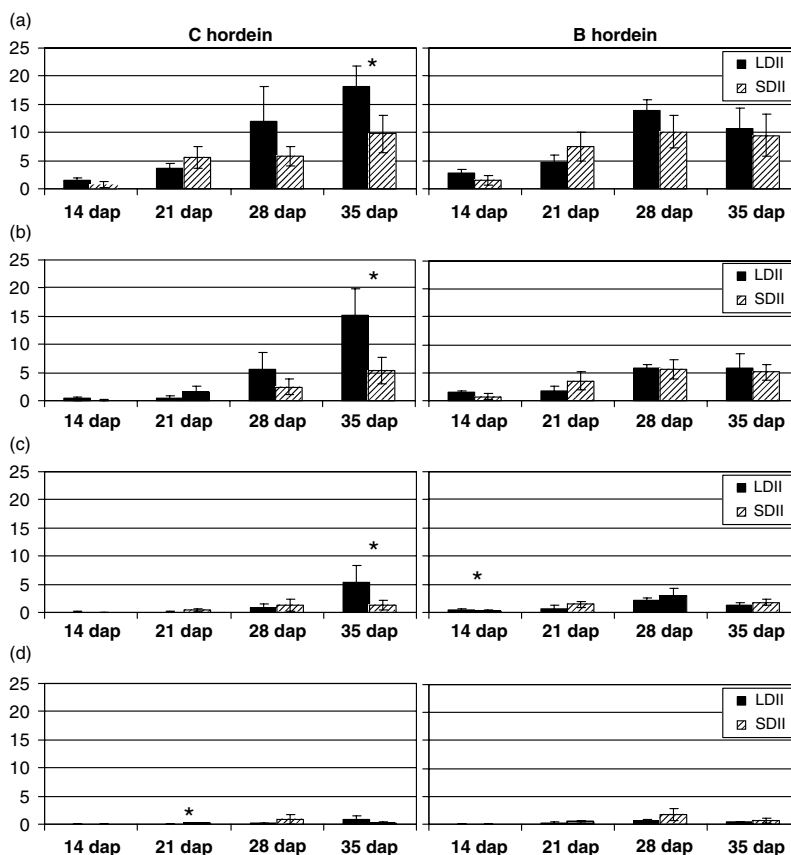
Figure 3. C and B hordein localisation in the cheek parts of main shoot head barley caryopses during development in experiments LDII and SDII. Proportions of BII or IFRN0614 antibody-labelled areas of imaged endosperm area. A = 0–310 µm, B = 310–650 µm C = 650–990 µm and D = 990–1330 µm deep from the surface of the grain. Error bars represent the 95% confidence intervals ($n = 6$). *Statistically significant difference ($P < 0.05$) between long- and short-day samples. dap, days after pollination.

Table 1. Analysis of the barley grains >2.5 mm

Grains (%) and analysis	Long-day conditions	CI	Short-day conditions	CI
Grains >2.5 mm (%)	99.7	–	93.7	–
Analysis (grains >2.5 mm)				
Moisture (g kg⁻¹)	104.1	±0.3ᵃ	105.6	±1.0ᵇ
Starch (g kg⁻¹ db)	607.6	±9.4ᵃ	628.0	±16.2ᵃ
Protein (g kg⁻¹ db)	107.8	±1.8ᵃ	112.6	±9.5ᵃ
β-Glucan (g kg⁻¹ db)	46.0	±1.5ᵃ	42.8	±3.7ᵃ
1000 grain weight (g db)	58.3	±1.4ᵃ	55.3	±5.0ᵃ

Values are averages of triplicates of two replicate experiments ($n = 6$). CI, 95% confidence interval.
ᵃ,ᵇ Different superscript letters in the same row indicate a statistically significant difference ($P < 0.05$) between samples.
db, dry basis.

The proportions of B, C and D hordeins in the 1-propanol- and 1-propanol+DTT-soluble fractions were determined by densitometric analysis of SDS-PAGE separations. The ratios of hordeins in each fraction were also calculated (Table 2). This showed only few statistically significant differences between the outer and central parts of the grain in 1-propanol-soluble hordein fractions ($P < 0.05$, $n = 6$; Table 2).

By contrast, the proportions of hordeins in the 1-propanol+DTT-soluble fractions showed strong, statistically significant differences. The proportion of B hordein was significantly lower in all abraded samples than in the intact grains ($P < 0.05$, $n = 6$; Table 2). Significantly greater proportions of D hordein were present in the pearled grains than in the intact ones ($P < 0.05$). Similarly to B and D hordeins, the proportions of C hordein differed between intact and abraded grains, being lower in the 16–17% and 26–28% pearled grains compared to the intact ones ($P < 0.05$). However, at the pearling rate of 36–39% the proportion of C hordein was significantly higher in long day grown grains than in the corresponding short day grown grains ($P < 0.05$). Additionally, the ratios of B:C hordein at the pearling rates of 26–28% and 36–39% were significantly lower for grains grown under long days ($P < 0.05$, $n = 6$; Table 2), while no clear differences were observed in the ratios of B:D and C:D hordein in the 1-propanol+DTT-soluble hordein fraction.
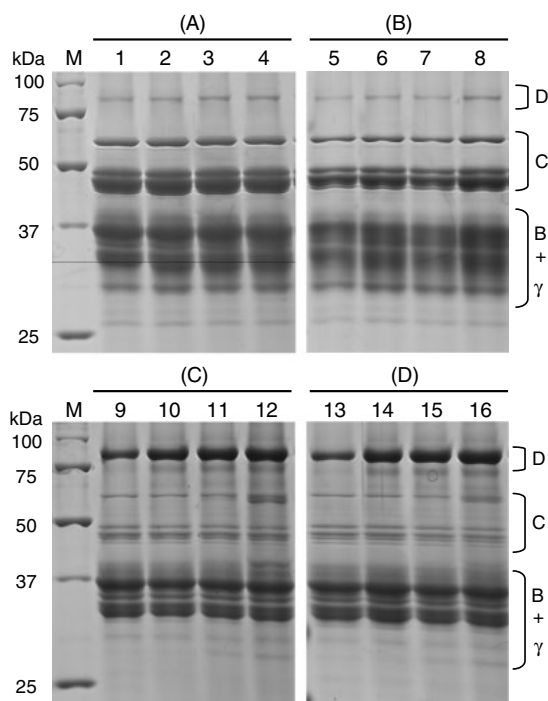
Figure 4. 1-Propanol (A and B) and 1-propanol+DTT-soluble (C and D) hordeins of intact and pearled barley grains grown in the long photoperiod (A and C) and in the short photoperiod (B and D) separated in 12% Tris-borate gels (pH 8.9). Lanes: molecular marker (M), intact grains (1, 5, 9, 13), grains with pearling rate of 16–17% (2, 6, 10, 14), 26–28% (3, 7, 11, 15) and 36–39% (4, 8, 12, 16), respectively. The images are examples of SDS-PAGE separations analysed statistically.

Water uptake during malting and friability of malt

Visual inspection was used to estimate the proportion of germinated grains during malting. All the samples reached acceptable level of malting having 100% of grains germinated by 72 h from the beginning of malting. Analysis of samples taken after 48 h of malting (i.e. after steeping) showed that the grains grown under long days hydrated faster than grains grown under short days (Fig. 5A). At this stage of malting, over 50% of the grains grown under long photoperiod showed endosperm hydration of 25% or more, while only 15% of the grains grown under short days had reached the same degree of hydration. The short day grown grains still showed lower degrees of hydration after 72 and 96 h (Fig. 5A and B) but after 144 h were equally hydrated with c. 20% of grains from both treatments showing endosperm hydration of 50% or more (Fig. 5B).

Determination of the friability of the micromalted and dried grains showed that the long-day grown samples had undergone more extensive endosperm degradation during malting (Fig. 5C). The values for friability and partially modified grains (PUG) also showed higher standard deviations for the short day grown grains which was probably due to the slower hydration. However, only the PUG values differed significantly between long and short day grown grains with the latter showing poorer malting quality with a greater proportion of less modified grains ($P < 0.05$,

$n = 6$). The PUG values were higher than usual for micromalted grain which could have resulted from downscaling of the analysis.

DISCUSSION
Hordein localisation during grain filling and in the mature grains

Labelling of grain sections with antibodies was used to determine the locations of hordeins and to compare the relative amounts between grains grown under different regimes. The determination of hordein localisation by abrasion of mature grains is less accurate than by immunolabelling as it is affected by the shape of the barley grains, resulting in greater abrasion of the ends and no abrasion within the ventral groove. Nevertheless, it allows the production of sufficient amounts of fractions to analyse and quantify hordeins.

Immunolabelling of sections from developing grains showed a similar time course for hordein accumulation during grain development to that reported previously. Møgelsvang and Simpson[25] reported hordein accumulation from 11 to 39 dap with the greatest rate from 23 to 29 dap. The most notable difference in hordein localisation related to photoperiod was that C hordein was more concentrated in the outer layers of the endosperm and also extended deeper into the endosperm in developing caryopses grown under long-day conditions.

Determination of hordein location by abrasion of mature grains showed that the distribution of total protein was similar irrespective of growth conditions. In general, the localisation of hordeins agreed with the observations by Shewry et al.[13] and Tesci et al.,[14] with B hordein being concentrated in the sub-aleurone layer and D hordein in the more central cells of the starchy endosperm. However, differences related to daylength were also observed in that long-day grains contained proportionally more C hordein and had a lower ratio of B : C hordeins in the 1-propanol+DTT-soluble hordein fraction derived from the more central part of the grain. The C hordein present in this fraction was probably entrapped by polymeric forms of B and D hordein, and it can be hypothesised that entrapped C hordein could have different physical properties compared to the 1-propanol-soluble, non-trapped C hordein.

Our results therefore indicate that differences in daylength result in differences in the localisation of hordeins. In particular, C hordein was located deeper within the endosperm of long day grains.

Imbibition and endosperm modification during malting of long and short day grown grains

Although there were no significant difference between the protein contents of the long and short day grains, there were differences in malting characteristics. Long day grains showed faster hydration and more uniform modification of endosperm than short day grains. This is consistent with the fact that C hordein was located deeper in the endosperm, indicating that C hordeins could affect the water uptake especially in the steeping phase of the malting. In previous studies, a high total content of C hordein was similarly associated with greater water absorption during malting and better malting quality.[5,16]

Thus, although our results indicate that C hordein may affect water uptake, this remains to be confirmed and the mechanism elucidated. On the one hand, C hordein could make the sub-aleurone more water permeable by restricting the formation of

Table 2. Compositions of 1-propanol- and 1-propanol+DTT-soluble hordein fractions and hordein ratios in intact and gradually abraded grains grown in long- and short-day conditions

	Intact grains	Pearling rate		
		16–17%	26–28%	36–39%
Propanol-soluble hordeins				
B hordein (%)				
Long-day	64.0 ± 1.7[a]	65.7 ± 1.2[ab]	65.4 ± 1.5[ab]	65.5 ± 1.6[ab]
Short-day	66.0 ± 1.7[ab]	67.4 ± 1.1[b]	66.8 ± 1.5[ab]	66.8 ± 0.6[ab]
C hordein (%)				
Long-day	35.0 ± 1.9[a]	33.3 ± 1.3[a]	33.1 ± 2.0[a]	32.9 ± 2.0[a]
Short-day	32.9 ± 1.5[a]	31.6 ± 1.0[a]	32.0 ± 1.4[a]	31.8 ± 0.6[a]
D hordein (%)				
Long-day	1.0 ± 0.3[a]	1.0 ± 0.3[a]	1.4 ± 0.5[a]	1.6 ± 0.5[a]
Short-day	1.1 ± 0.2[a]	1.0 ± 0.1[a]	1.2 ± 0.2[a]	1.4 ± 0.1[a]
B : C hordein ratio				
Long-day	1.8 ± 0.2[a]	2.0 ± 0.1[a]	2.0 ± 0.2[a]	2.0 ± 0.2[a]
Short-day	2.0 ± 0.1[a]	2.1 ± 0.1[a]	2.1 ± 0.1[a]	2.1 ± 0.1[a]
B : D hordein ratio				
Long-day	70.8 ± 16.0[a]	71.2 ± 16.4[a]	51.7 ± 13.9[a]	45.3 ± 11.3[a]
Short-day	65.6 ± 15.0[a]	69.2 ± 9.8[a]	57.4 ± 11.2[a]	46.7 ± 4.6[a]
C : D hordein ratio				
Long-day	39.5 ± 11.2[b]	36.4 ± 9.5[ab]	27.0 ± 8.9[ab]	23.2 ± 6.9[a]
Short-day	32.3 ± 6.1[ab]	32.2 ± 3.4[ab]	27.2 ± 3.4[ab]	22.2 ± 2.0[a]
Propanol+DTT -soluble hordeins				
B hordein (%)				
Long-day	75.5 ± 1.0[d]	63.6 ± 1.4[abc]	62.2 ± 1.8[ab]	59.9 ± 2.1[a]
Short-day	76.8 ± 2.2[d]	66.6 ± 1.9[c]	65.6 ± 1.7[bc]	63.5 ± 1.9[abc]
C hordein (%)				
Long-day	10.9 ± 0.5[d]	8.6 ± 0.7[abc]	8.4 ± 0.4[abc]	9.6 ± 0.7[cd]
Short-day	10.7 ± 0.5[d]	8.4 ± 0.9[abc]	7.1 ± 0.5[a]	7.8 ± 0.6[ab]
D hordein (%)				
Long-day	13.6 ± 1.3[a]	27.8 ± 1.3[bc]	29.5 ± 1.8[c]	30.5 ± 2.7[c]
Short-day	12.6 ± 2.0[a]	25.0 ± 1.9[b]	27.3 ± 1.9[bc]	28.7 ± 1.6[bc]
B : C hordein ratio				
Long-day	7.0 ± 0.3[ab]	7.4 ± 0.8[ab]	7.4 ± 0.4[ab]	6.3 ± 0.3[a]
Short-day	7.2 ± 0.5[ab]	8.0 ± 0.9[bc]	9.2 ± 0.5[c]	8.2 ± 0.8[bc]
B : D hordein ratio				
Long-day	5.6 ± 0.6[b]	2.3 ± 0.2[a]	2.1 ± 0.2[a]	2.0 ± 0.3[a]
Short-day	6.4 ± 1.3[b]	2.7 ± 0.2[a]	2.4 ± 0.2[a]	2.2 ± 0.2[a]
C : D hordein ratio				
Long-day	0.8 ± 0.1[b]	0.3 ± 0.0[a]	0.3 ± 0.0[a]	0.3 ± 0.1[a]
Short-day	0.9 ± 0.1[b]	0.3 ± 0.0[a]	0.3 ± 0.0[a]	0.3 ± 0.0[a]

Results are the means of triplicates of two separate experiments ±95% confidence interval ($n = 6$).
[a–d] Mean values within the same hordein proportion or ratio with different superscript letters differ significantly from each other ($P < 0.05$).

interchain disulfide bonds in B hordeins.[5] On the other hand, C hordein may itself form a barrier limiting grain hydration and endosperm hydrolysis.[17] Other studies have also shown that the amount and distribution of B hordein also affects water uptake.[16]

phase was correlated positively with a deeper location of C hordein.

CONCLUSIONS

Our studies conducted in controlled environment cabinets demonstrated an effect of environment (daylength) on the localisation of C hordein in the barley grain. This difference in hordein localisation was also associated with differences in malting quality; in particular, water uptake in the steeping

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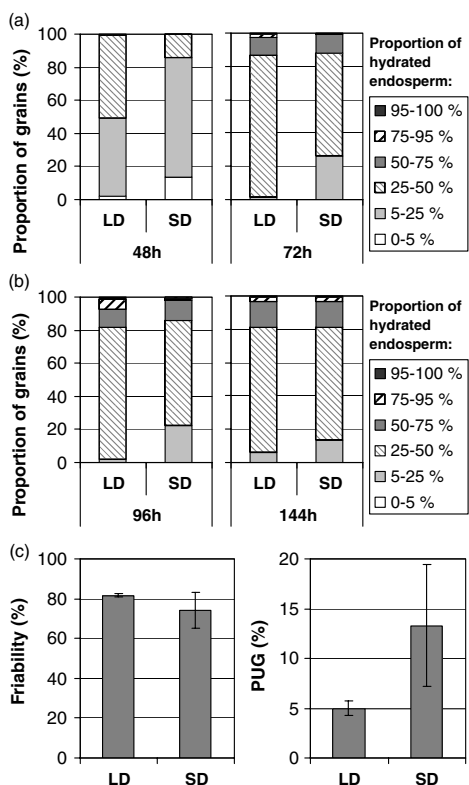
Figure 5. (A and B) Water uptake of barley grains after 48, 72, 96 and 144 h of malting. Results are based on two replicate experiments represented by 200 grains/sampling point. (C) Friability and PUG using a modified friabilimeter method. Results are calculated from three replicate measurements of two experiments and error bars indicate the 95% confidence intervals ($n = 6$).

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Influence of sulphur application on hordein composition and malting quality of barley (*Hordeum vulgare* L.) in Northern European growing conditions

Influence of Sulphur Application on Hordein Composition and Malting Quality of Barley (*Hordeum vulgare* L.) in Northern European Growing Conditions

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ABSTRACT

The influence of sulphur (S) application on yield formation, hordein composition and malting quality of 2-rowed spring barley (*Hordeum vulgare* L.) was studied in Nordic conditions for the first time. In a greenhouse experiment, S deficiency was indicated when 10 mg S/kg soil or less was available, by an increase in the malate:sulphate ratio in leaves. The contents of aspartic acid and cystine in grains increased and decreased, respectively. Also a substantial decrease of total hordein and the proportion of B hordein, and an increase in the proportion of C hordein were associated with S deficiency. The effect of S was further assessed in field experiments by applying N and S in four different combinations prior to sowing. No apparent S deficiency occurred in the field conditions based on the malate:sulphate and grain N:S ratio. However, in a site where the grain S content was slightly but significantly increased by S application a change in hordein composition and malting quality was observed. Thus even in S-sufficient conditions, the end use quality of malting barley may be affected by S application.

KEYWORDS

barley; nitrogen; sulphur; malting quality

1 INTRODUCTION

The availability of nitrogen (N) and sulphur (S) influences essentially the yield formation of cereals. By regulating the gene expression of prolamin storage proteins, they also affect the grain protein composition (Halford and Shewry, 2007). The interest especially in the effect of S on yield formation and grain quality has risen due to reduced amounts of atmospheric S deposition, decreased use of traditional ammonium-containing fertilizers and increased removal of minerals due to higher crop yields (Zhao et al., 1999).

In barley grains, alcohol-soluble and proline-rich proteins called hordeins act as a major store of N and S, which are made available to the growing seedling during germination (Giese et al., 1983; Shewry et al., 1985). Hordeins are classified by their amino acid composition and molecular size. B and C hordeins account for the majority of the grain hordein while D hordeins with high molecular weight and γ hordeins are minor components (Shewry, 1992). B and D hordeins are rich in S-containing amino acids whereas C hordein contains no cysteine and only few methionine residues (Shewry, 1992). Protein and especially the hordein content of barley grain correlate positively with grain N content (Giese et al., 1983; Kirkman et al., 1982). The hordein composition is, in turn, influenced by the availability of S. In S-deficient barley grains, the proportion of C hordein is typically increased mainly due to decreased accumulation of S-rich B and D hordeins (Rahman et al., 1983; Shewry et al., 1983). A similar effect on hordein composition has been shown in conditions with limited S supply due to high N availability and increased hordein content (Giese et al., 1983; Kirkman et al., 1982).

In yield formation, N and S are known to be closely interacting nutrients. In barley, high yields are achieved only when both nutrients are not limited (Reisenauer and Dickson, 1961; Randall and Wrigley, 1986). By increasing N availability alone, kernel size and weight are, for example, decreased due to limited supply of S (Reisenauer and Dickson, 1961). The

yield-increasing effect of S addition has been observed in some cases but only at S deficient sites (Zhao et al., 2006).

Although a high concentration of available N supports high yield potential, it may lead to excessive grain protein content if the yield potential is not realised, e.g. due to drought or disease (Rajala et al., 2007). High grain N levels reduce malting quality by leading to higher malt and wort N content and lower fermentable extract yield (Agu, 2003). Sulphur additions, in turn, have been associated with improved endosperm modification during malting and decreased β-glucan concentration in wort. Also, the concentration of the precursor of dimethyl sulphide, a major flavour component in lager beers, correlates positively with grain S concentration (Zhao et al., 2006).

Several chemical analyses have been performed on barley leaves and grains for diagnosis of S deficiency. These include the amount of total S, sulphate and glutathione, N:S ratio and proportion of total S as sulphate (reviewed by Blake-Kalff et al., 2000). In addition, the ratio of malate and sulphate used to verify S deficiency in wheat (even before anthesis) can be applied also to barley (Carver, 2005; Zhao et al., 2005). In this method, concentrations of malate and sulphate in leaf samples are determined. In leaves there is an inverse relationship between malate and sulphate concentrations. This is because malate maintains the cytoplasmic pH in response to the accumulation of ions. When the concentration of sulphate increases, that of malate decreases and vice versa (Blake-Kalff et al., 2000). In other words, the smaller the value of the malate:sulphate ratio, the higher the S availability at the time of sampling.

Finland is the most northern country where malting barley is grown. The importance of S application rates in malting barley quality was studied here for the first time in boreal growing conditions. The main objective of the greenhouse and field experiments was to clarify the associations between N and S application rates, barley hordein content and malting quality. The aim of the greenhouse experiment was to show the effect of S alone on growth and amino acid and hordein composition The purpose

of the field experiments was to demonstrate the influence of S on hordein composition and malting quality at two N application levels. Also the use of the malate:sulphate ratio in determining S deficiency before anthesis was evaluated in both experiments.

2 MATERIALS AND METHODS

2.1 Barley material

2.1.1 Pot-scale experiments

To study the effect of S application rate, spring-type 2-rowed malting barley (*Hordeum vulgare* L.) cultivar Scarlett was grown under controlled conditions in plastic pots containing 9 kg of nutrient-poor quartz sand (92% with grain diameter <0.5 mm). The following levels of nutrients were added to pots prior to sowing (mg): 1000 N [as $Ca(NO_3)_2$ and NH_4NO_3], 400 P ($Na_2HPO_4 * 2 H_2O$), 2000 K (KCl/K_2SO_4), 200 Mg ($MgCl_2$), 10 Mn ($MnCl_2$), 15 Fe ($FeCl_3$), 10 Zn ($ZnCl_2$), 5 Cu [$Cu(CH_3COO)_2 * H_2O$], 4 Mo ($Na_2MoO_4 *2 H_2O$) and 8 B (H_3BO_3). One sixth of the N was applied as NH_4. Sulphur application rates were 0, 5, 10, 20, 40 and 80 mg S supplied as K_2SO_4/kg of quartz sand. Each S-treatment was applied to 15 pots. Twenty-five seeds were sown per pot, and after seedling emergence, the plant number was thinned to 19 seedlings per pot. Pots were placed outdoors in a greenhouse with net walls and a glass roof in a completely randomized design. At the tillering stage an additional dose of 1000 mg N was applied per pot. Plants were watered frequently with deionized water. Aphids were controlled with deltamethrin spray when necessary.

Plants of 3 replicate pots per S application rate were collected to assess above-ground phytomass (g/plant) at tillering, at the beginning of stem elongation and at the flag leaf stage. Plant samples were dried for 48h at 60°C and weighed. From these same plant samples, the malate:sulphate ratio was determined except that at the flag leaf stage, the flag leaf and the second uppermost leaf were used for the assessment of the malate:sulphate ratio. Due to poor growth at 0 mg S/kg soil, total above-ground phytomass was used to analyse malate:sulphate ratio at all three stages.

Mature plant stand samples were collected by cutting the plants at the soil surface (6 replicate pots per each S application rate), oven drying for 48h at 60°C and weighing. The yield was hand-threshed. Barley grown without S application failed to produce any grain. The following parameters were measured: total above-ground phytomass (g/plant), yield (g/plant), single grain weight, ear number per plant, and grain number per ear. Vegetative mass (g/plant) was calculated as the difference between plant above-ground phytomass and yield.

2.1.2 Field experiments

Four spring-type 2-rowed malting barley cultivars (Barke, Prestige, Saana and Scarlett) were grown in the field in three locations in southern Finland (Vihti (60°25' N 24°19' E), Jokioinen (60°48' N 23°29' E) and Inkoo (60°03' N 24°00' E)) during growing seasons 2004 and 2005. Both N and S were applied as a compound fertilizer with other nutrients before sowing. N was in the form of ammonium and nitrate (45:55 ratio) and S as gypsum comprising 20% and 6% of total fertilizer amount, respectively. Four different fertilizer combinations with varying N and S contents were used: 60 kg N + 0 kg S/ha, 60 kg N + 18 kg S/ha, 90 kg N + 0 kg S/ha, and 90 kg N + 27 kg S/ha. Standard agricultural practices including weed and disease control were performed. Each fertilizer combination with each cultivar was cultivated in four separate replicate plots, i.e. 64 samples were grown in each of three locations in two years giving 384 samples in total. Trial setup was a split-split-plot experimental design, for which the cultivar was the main plot, N-rate was sub-plot and S-rate was sub-sub-plot factor.

2.2 Methods

2.2.1 Malate-sulphate test

The malate:sulphate ratio, indicating the degree of S deprivation, was analysed from the plant samples taken during vegetative growth. In the greenhouse experiment, plant samples were collected as described in section 2.1.1 at tillering, at the beginning of stem elongation and at the flag leaf stage. Samples representing three replicate pots were pooled before analysing the malate:sulphate ratio. In the field trials, plant

samples for the malate-sulphate test were taken at the beginning of the tillering phase and the samples of four replicate plots were analysed separately except for the samples from Vihti in 2004 which were combined as one sample. Malate:sulphate ratios of the samples were analysed at Hill Court Farm Research (UK) according to the method described by Blake-Kalff et al. (2000).

2.2.2 Standard barley analyses

The protein content of greenhouse-grown barley grains was analysed using the Kjeldahl method (AOAC Official methods 1980: 14.068). For characterization of field-grown barley, methods recommended by European Brewing Convention were used in analysing germinative capacity (Analytica-EBC: 3.5.1), pre-germinated barley kernels (3.8.1), the grain size distribution (3.11.1), visual examination of damaged barley kernels (3.11.2) as well as the contents of moisture and protein by near-infrared spectroscopy (3.13) with simultaneous analysis of starch content (EBC, 1998). The samples of four replicate plots were analysed separately.

2.2.3 Amino acid analysis

Total amino acid analysis including both peptide-bound and free amino acids was performed with a Biochrom 20 amino acid analyser (Biochrom Ltd., Cambridge, England) using sodium citrate buffer. Grains from two replicate pots of the greenhouse-grown barley were analysed separately (EC, 1998).

2.2.4 Analysis of S content

The S contents of field-grown barley grains were analysed at Soil Analyses Service Ltd. (Mikkeli, Finland) using a routine method consisting of wet combustion in HNO_3, pressure cooking at 140°C and analysis using inductively-coupled plasma atomic emission spectrophotometry (ICP-AES). Grains from four replicate plots were analysed separately.

2.2.5 Micromalting and malt analyses

Grain samples of 1 kg (sieved > 2.5 mm) were micromalted either at LP Research Centre Ltd. (Lahti, Finland) or at Raisio Malt (Raisio, Finland). Steeping conditions were optimized by crop year in order to reach the target moisture of 46-47% at the end of steeping. In all maltings, the first and the second wet steep each lasted for 6-8h with the intervening dry steep of 16 h. For the samples of crop 2005, an additional dry steep (16h) and wet steep (2h) were required. Steeping was carried out at 13-15°C, and moisture after steeping was analysed by weighing. The germination phase lasted for 5 days at 14-16°C. As an exception, Barke grown in Jokioinen 2005 was germinated for 6 days. Grains were kilned for 22h to a final temperature of 82-83°C. The malting process was monitored by analysing malting losses as described in Holopainen et al. (2005).

Malts were characterized by performing the following standard malt analyses according to Analytica-EBC (1998): friability (4.15), extract (fine; 4.5.1), pH, colour (4.7.2), viscosity (4.8), soluble N and Kolbach index (4.9.1), free amino N (FAN; 4.10), and β-glucan (4.16.2). Grains from four replicate plots were malted and analysed as separate samples.

2.2.6   Hordein   quantification   by   SDS-PAGE
Hordein fractions soluble without and with reducing agent were extracted from 150 mg ground grain in triplicate using a modified Osborne fractionation method described in Holopainen et al. (2005) with the following modifications: a protease inhibitor tablet (Complete Mini, Roche) was added to 0.5 M sodium chloride solution and the extractions with 0.5 M sodium chloride, water and 50% (v/v) 1-propanol were carried out with constant shaking at room temperature. 50 µL aliquots of the propanol-containing extracts were dried under vacuum in a Speedvac centrifuge evaporator (Savant Instruments Inc., Holbrook, NY, USA) and were resuspended in a sample buffer (62.5 mM Tris-HCl buffer, pH 6.8, 20% (v/v) glycerol, 2.0% (v/v) SDS, 5% 2-mercapthoethanol, and 0.05% (w/v) bromophenol blue). Protein contents of the samples were determined with the 2D Quant protein kit (Amersham Biosciences).

Samples were boiled for 3 min and centrifuged at 12000 g for 10 min at 4°C. Hordeins were separated on precast 12% Tris-HCl Ready Gels (Bio-Rad Laboratories, Hercules, CA, USA) using 192mM glycine, 25mM Trizma base and 0.1% (w/v) SDS as a running buffer. Fixation, staining and densitometric scanning of the gels was carried out as described in Holopainen et al. (2012) using ethanol instead of methanol in staining and rinsing solutions. Hordeins in the gels were quantified based on the band intensity and protein content of the hordein extract. Content and composition of hordeins were analysed in grains from two replicate plots and results were calculated as averages of triplicate extractions.

2.2.7 Statistical analysis

Data of the greenhouse experiment representing two replicate pots were statistically analysed using one-way ANOVA with Tukey´s HDS post-hoc test ($p < 0.05$; IBM SPSS Statistics software for Windows v20.0 or earlier; IBM Corporation, Somers, NY, USA).

Data on the effect of N and S application rates on yield, malting process and quality and hordein parameters in field conditions were statistically analysed using the following linear mixed model based on experimental design:

$$y_{ijklm} = \mu + \text{trial}_i + \text{block(trial)}_{ij} + CV_k + CV \times \text{trial}_{ik} + CV \times \text{block(trial)}_{ijk} + N_l + N \times \text{trial}_{il} + N \times CV_{kl} + N \times CV \times \text{trial}_{ikl} + N \times CV \times \text{block(trial)}_{ijkl} + S_m + S \times \text{trial}_{im} + S \times CV_{km} + S \times CV \times \text{trial}_{ikm} + S \times N_{lm} + S \times N \times \text{trial}_{ilm} + S \times CV \times N_{klm} + S \times CV \times N \times \text{trial}_{iklm} + \varepsilon_{ijklm}$$

where μ is intercept, trial_i, CV_k, N_l, and S_m are fixed main effects of trial (location and year were combined as one factor, trial, i=1,...,6), cultivar (k=Barke, Prestige, Saana and Scarlett), N-rate (l=60 kg N, 90 kg N) and S-application (m=no,yes), respectively. $CV \times \text{trial}_{ik}$, $N \times \text{trial}_{il}$, $N \times CV_{kl}$, $N \times CV \times \text{trial}_{ikl}$, $S \times \text{trial}_{im}$, $S \times CV_{km}$, $S \times CV \times \text{trial}_{ikm}$, $S \times N_{lm}$, $S \times N \times \text{trial}_{ilm}$, $S \times CV \times N_{klm}$, $S \times CV \times N \times \text{trial}_{iklm}$ are fixed interaction effects. The model included the following random effects: block(trial)_{ij} is the effect of block,

CV*block(trial)$_{ijk}$ is the main-plot error term, N×CV×block(trial)$_{ijkl}$ is the sub-plot error term and ε$_{ijklm}$ is the term of residual or sub-sub-plot error.

Statistical analysis of yield parameters covered all trials, cultivars and replicate plots (exp. 1, n=384). Malting and hordein parameters were examined in selected varieties from three trials over four (exp. 2, n=128) and two replicate plots (exp. 4, n=64), respectively. It was not possible to estimate all interactions in the analysis of malting parameters. Parameters were estimated after the effect of trial and all interactions with a trial were defined as random effects. This model uses CV*trial$_{ik}$ as main-plot error, N*trial$_{il}$ as sub-plot error and S*trial$_{im}$ as sub-sub-plot error. In the analysis of the hordein parameters, the model was simplified removing cultivar-by-N, cultivar-by-S and cultivar-by-N-by-S interactions from the model. In addition, all trials were analysed separately by the traditional mixed model for a split-split-plot design (malting and hordein parameters only; exp. 3 and 5). Methods used assume that the data are normally distributed. Graphical methods were used to check normality. Generally, proportional data needed arc-sine transformation before normality. All mixed models were fit using SAS/MIXED software (SAS v9.3; SAS Institute Inc., Cary, NC, USA) with restricted maximum likelihood (REML) estimation method.

3 RESULTS

3.1 The influence of S availability on pot-grown barley

In greenhouse conditions, S availability influenced several parameters (Table 1). The phytomass of plants grown with 0 mg S/kg soil were significantly lower (p<0.05) already at the tillering stage and that of the plants with 5 mg S/kg soil at flag leaf stage in comparison to higher S applications (Table 1A). Sulphur deficiency was indicated by a high malate:sulphate ratio, which decreased with increasing S availability. Plants grown with 0 and 5 mg S/kg soil had the highest values of malate:sulphate ratios at all stages, but at shoot elongation and flag leaf stage also plants with 10 mg S/kg soil had higher malate:sulphate ratios compared to plants with higher S application levels. At flag leaf stage, an

increase in S application from 10 to 20 mg S or higher per kg soil resulted in lower malate-sulphate ratio without affecting phytomass.

The S application rate had no effect on the final vegetative masses (Table 1B). Plants grown without S application did not produce ears. Only the plants grown with 5 mg S/kg soil produced significantly ($p<0.05$) smaller yield, smaller grains, and fewer ears per plant compared to those grown with higher S application rates. Grain number per ear did not change with S application.

The amount of S applied did not influence significantly the N content of barley grains (Table 1C). However, the contents of several individual amino acids were associated with the amount of S applied. Contents of cystine, glycine, isoleucine, leucine, serine, threonine and tyrosine were significantly higher and the content of aspartic acid lower ($p<0.05$) in the grains grown with 10 mg S/kg soil compared to those grown with 5 mg S/kg soil. The amount of S-containing cystine residues increased significantly also when S application was increased from 10 to 20 mg/kg soil. Likewise, the content of methionine increased with S availability, but more gradually compared to that of cystine. Only few differences in the contents of other amino acid were observed between S application rates of 10, 20 and 40 mg S/kg soil. The increase in S availability from 40 to 80 mg S/kg soil was associated with a significant increase ($p<0.05$) in the amount of glutamic acid, glycine, isoleucine, serine, threonine and tyrosine as well as in the total content of amino acids.

Table 1 A) Growth parameters, B) yield parameters and C) grain composition of barley plants (cv. Scarlett) in pot-scale experiment with five rates of S applied. Values represent averages of plants or grains from two replicate pots except for malate:sulphate ratio which was analysed of pooled sample of three pots. (*on next page*)

	S application rate (mg S/kg soil)					
	0	**5**	**10**	**20**	**40**	**80**
A **Growth parameters**						
Phytomass (g/pot)						
tillering stage	1 a [a)]	2.4 b	3.2 bc	3.3 bc	3.3 bc	3.6 c
shoot elongation stage	3.4 a	19.9 b	21.3 b	21.5 b	22.8 b	23.5 b
flag leaf stage	7.1 a	46.4 b	63 c	65.5 c	64.6 c	64.5 c
Malate:sulphate ratio						
tillering stage	48.0	12.1	2.3	2.5	2.5	2.7
shoot elongation stage	155	139	32.2	7.1	4.7	7.6
flag leaf stage	70.1	66.2	18.0	2.0	0.8	1.3
B **Yield parameters**						
Vegetative mass (g/plant)	- [b)]	3.4 a	4.2 a	3.7 a	4.3 a	4.2 a
Yield (g/plant)	-	1.8 a	3.4 c	3.6 d	3.6 d	3.2 b
Single grain weight (mg)	-	31.2 a	41.4 b	45.6 bc	45 bc	48.6 c
No of ears/plant	-	3.3 a	4.5 ab	5.2 b	5.1 b	5.1 b
No of grains/ear	-	17.7 a	18.2 a	15.2 a	15.9 a	13 a
C **Grain composition**						
Grain N (% db)	-	1.86 a	1.88 a	1.72 a	1.70 a	1.99 a
Amino acids (mg/g db)		110 a	110 a	107 a	111 a	126 b
Alanine	-	4.1 a	4.3 ab	4.5 ab	4.7 bc	5.2 c
Arginine	-	6.2 a	5.2 a	5.2 a	5.3 a	5.9 a
Aspartic acid	-	16.4 b	7.7 a	6.4 a	6.6 a	7.1 a
Cystine	-	0.9 a	1.3 b	2.3 c	2.4 c	2.6 c
Glutamic acid	-	29.5 ab	31.0 ab	27.2 a	28.5 a	33.4 b
Glycine	-	3.6 a	4.1 b	4.6 bc	4.7 c	5.3 d
Histidine	-	2.3 a	2.6 ab	2.6 ab	2.7 b	3 b
Isoleucine	-	3.1 a	3.5 b	3.7 b	3.8 b	4.4 c
Leucine	-	6.1 a	7.2 b	7.8 b	8 bc	9.1 c
Lysine	-	3.7 a	3.8 ab	3.9 ab	3.9 ab	4.3 b
Methionine	-	0.9 a	1.4 ab	1.9 ab	2 bc	2.1 c
Phenylalanine	-	6.3 ab	7.0 b	6.1 a	5.9 a	7.0 b
Proline	-	11.2 a	12.7 ab	11 a	12.7 ab	14.2 b
Serine	-	4.5 a	4.9 b	4.8 b	4.9 b	5.6 c
Threonine	-	2.9 a	3.6 b	3.6 b	3.7 b	4.3 c
Tyrosine	-	3.1 a	3.6 b	3.8 b	3.8 b	4.4 c
Valine	-	4.5 a	5.1 ab	5.9 bc	6.1 bc	6.3 c

[a)] Different letters within a same row indicate statistically significant difference (p<0.05) between samples based on Tukey's HSD test.
[b)] No ears were produced without S application.

The hordein composition of barley cv. Scarlett grains clearly changed with increasing S application level (Figure 1). The total hordein content as well as the proportions of B and D hordein increased significantly (p<0.05) with higher S application rates (Figure 1A). The content of C hordein in propanol- and propanol+DTT-soluble fractions, in turn, decreased significantly (p<0.05) when more S was available. The most considerable changes in hordein amounts occurred when the S application rates increased from 5 to 10 and 20 mg S/kg soil, although B hordein content increased further when more S was applied. The increase in the amount of B and D hordeins and the decrease in C hordein at these S application rates were clear and significant (p<0.05) also when the amounts of hordein groups were examined as proportions of total hordein (Figure 1B). However, only minor changes were observed in hordein composition between the S applications of 20 to 80 mg S/kg soil.
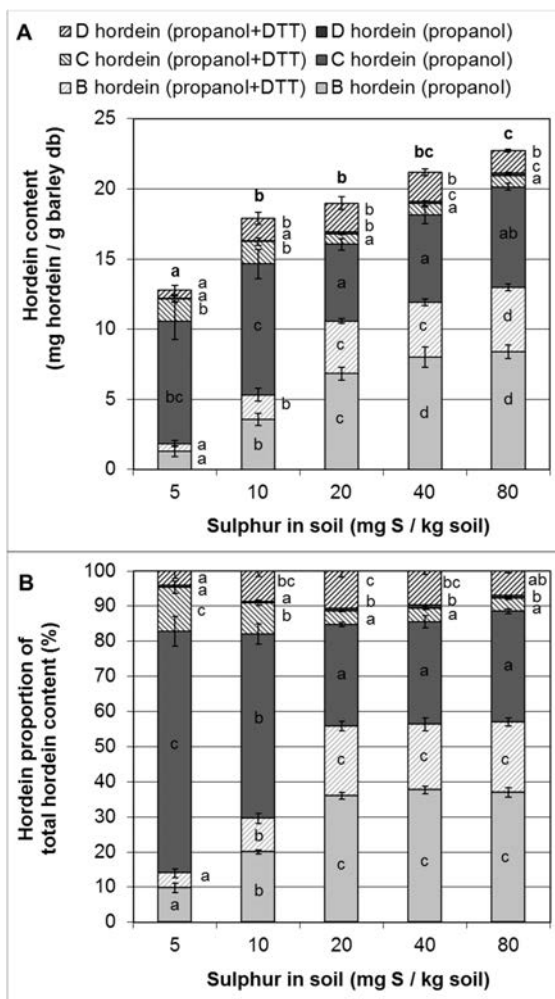
Figure 1 Hordein contents (A) and composition (B) in the barley kernels from pot grown plants (cv. Scarlett) with different S application levels in soil. Error bars represent the 95% confidence intervals (n=6). Different letters within and on top of the columns indicate significant differences (p<0.05) in contents of hordein fractions or total hordein between S treatments based on Tukey's HSD test.

3.2 Yield parameters of field-grown barley

On examination of the whole sample set of 384 subjects (exp. 1), yield, starch and protein content and proportion of grains >2.5 mm showed significant trial × cultivar interaction ($p<0.001$, Table 2). No influence of S application was found for yield, while starch content was reduced by the main effect of S ($p<0.01$) by 0.2%. The effect of S application rate was observed as trial × S interaction for protein content and proportion of grains > 2.5 mm ($p<0.05$). A closer inspection of the effect of S on protein content or proportion of grains >2.5 mm revealed that the significant S-related differences occurred only in one or two of the six trials.

Nitrogen influenced yield, starch and protein content with changes +8.5%, -0.7%, and +5.8%, respectively ($p<0.001$, Table 2). Trial × N interaction was detected for proportion of grains > 2.5 mm ($p<0.05$). In most trials, N application reduced the grain size.

Table 2 A) ANOVA table and B) estimated means of yield parameters (n=384, exp. 1). [a]

A

A	DF	Yield P	Starch P	Protein P	Grains > 2.5 mm P
Trial (T)	5	<0.001	<0.001	<0.001	<0.001
Cultivar (CV)	3	0.07	<0.001	0.01	<0.001
Nitrogen (N)	1	<0.001	<0.001	<0.001	0.04
Sulphur (S)	1	0.34	<0.01	0.04	0.14
T × CV	15	0.02	<0.001	<0.01	<0.001
T × N	5	0.54	0.25	0.13	0.01
T × S	5	0.32	0.31	0.01	0.04
CV × N	3	0.59	0.93	0.76	0.13
CV × S	3	0.79	0.80	0.95	0.83
N × S	1	0.99	0.92	0.16	0.19

B		S [b]		N [c]	
		0	6	60	90
Yield (kg/ha)		4911	4942	4726	5126
SE [d]		83		85	
Starch (% dm)		63.1	63.2	63.4	62.9
SE		0.084		0.090	
Protein (% dm)					
2004	Jokioinen	11.3	10.9		
	Vihti	9.5	9.6		
	Inkoo	10.7	10.7	10.4	11.0
2005	Jokioinen	10.8	10.7		
	Vihti	10.5	10.4		
	Inkoo	11.7	11.8		
SE		0.25		0.26	
Grains > 2.5 mm (%)					
2004	Jokioinen	87.4	89.4	90.1	86.6
	Vihti	91.1	90.9	91.1	90.8
	Inkoo	95.0	95.3	95.8	94.5
2005	Jokioinen	95.0	94.8	95.3	94.5
	Vihti	97.3	97.3	96.8	97.7
	Inkoo	97.7	97.7	97.7	97.7
SE		0.19		0.22	

[a] Interactions of three or four factors were not significant (p<0.05).
[b] S application 0 or 6% of total fertilizer amount applied
[c] N application 60 or 90 kg N/ha
[d] standard error

The malate:sulphate ratio, which was measured from all samples except for those from Inkoo in 2004 and Vihti in 2005 (n=286), was significantly affected by all main factors and showed trial × cultivar, trial × S, cultivar × S, and trial × N interactions (p<0.01, Table 3). The effect of S application on malate:sulphate ratio was consistent for all trials and cultivars and was highest in Jokioinen in 2005 (48.4%) and on Barke (41.7%).

Based on the yield and protein content, germinative capacity and proportions of split and pre-germinated kernels, a sub-set of 128 samples was selected for analysis of grain S content and malting quality. This sample set included all fertilizer combinations and four replicate plots of varieties Barke, Prestige, Saana and Scarlett grown in Vihti in 2004 (n=64), Barke and Saana grown in Jokioinen (n=32) and Barke and Prestige grown in Inkoo in 2005 (n=32). In this sub-set (exp. 2), the grain N:S ratio was significantly decreased by the main effect of S application (p<0.01,Table 3), but grain S content was significantly affected only by cultivar (data not shown). However, a trial-wise examination (exp. 3) revealed that the effect of S application on grain S content was significant in Vihti in 2004 (p<0.001) with an increase of 9.0% (Table 3). Grain S content was increased also by the main effect of N in both Vihti in 2004 and Jokioinen in 2005.

Table 3 A) ANOVA table and B) estimated means of malate:sulphate (n=256; exp.1), grain N:S ratio (n=128; exp. 2) and grain S content (n=64/32/32, for Vihti, Jokioinen and Inkoo, respectively; exp. 3). [a] (*on next page*)

A	DF	Malate: sulphate ratio P	Grain N:S ratio P	Grain S 2004 Vihti P	Grain S 2005 Jokioinen P	Inkoo P
T	3	<0.001	n.d.	n.d.	n.d.	n.d.
CV	3	0.02	<0.001	<0.001	0.01	0.18
N	1	<0.001	0.87	<0.001	0.02	0.07
S	1	<0.001	<0.01	<0.001	0.17	0.74
T × CV	9	<0.001	n.d.	n.d.	n.d.	n.d.
T × N	3	0.01	n.d.	n.d.	n.d.	n.d.
T × S	3	<0.001	n.d.	n.d.	n.d.	n.d.
CV × N	3	0.53	0.65	0.66	0.21	0.78
CV × S	3	<0.01	0.90	0.81	0.40	0.74
N × S	1	0.57	0.83	1.00	0.77	0.11
CV × N × S	3	0.15	0.34	0.25	0.77	0.03

B		S [b] 0	6	N [c] 60	90
Malate:sulphate ratio					
	Jokioinen	2.5	1.8	2.4	1.9
2004	Vihti	4.0	3.3	3.8	3.5
	Inkoo	n.d.	n.d.	n.d.	n.d.
	Jokioinen	3.6	1.9	3.2	2.3
2005	Vihti	n.d.	n.d.	n.d.	n.d.
	Inkoo	3.5	2.2	3.1	2.6
	SE [d]	0.67		0.68	
	Barke	3.4	2.0		
Cultivar	Prestige	3.3	2.2	3.1	2.6
	Saana	3.4	2.6		
	Scarlett	3.5	2.4		
	SE	0.38		0.40	
Grain N:S ratio		14.9	14.4	14.6	14.6
	SE	0.075		0.075	
Grain S (g/kg dm)					
2004	Vihti	1.01	1.06	0.99	1.08
	SE	0.013		0.013	
2005	Jokioinen	1.23	1.26	1.21	1.28
	SE	0.024		0.025	
2005	Inkoo	1.27	1.28	1.25	1.29
	SE	0.014		0.016	

[a] Interactions of three or four factors other than presented were not significant (p<0.05).
[b] [c] [d] see Table 2

3.3 Malting quality parameters of field-grown barley

No consistent patterns in malting process or quality parameters related to S application were observed in the set of 128 samples (exp.2, data not shown). Because the effect of S application on grain S content was observed only in Vihti in 2004, the malting parameters were examined in corresponding samples (n=64; exp. 3). The main detectable effect of S was an increase in soluble N by 2.3% and cultivar × S interaction was observed for FAN and colour of Congress wort (p<0.05, Table 4). However, the effects were not the same or significant in each cultivar. FAN and colour of Congress wort increased in Barke and Prestige and decreased in Scarlett while no effect was observed in Saana. Changes in malting loss parameters showing effects of cultivar × S or cultivar × N × S interaction were not consistent over varieties either (data not shown).

The main effect of N was observed in friability, extract, Kolbach index, and β-glucan content and viscosity of wort with change by -4.8%, -0.7%, -4.3%, +32.0%, and +0.6%, respectively (exp. 3, data not shown). In addition, cultivar by N interaction was observed on root loss, pH (data not shown), FAN and colour of wort (Table 4). No differences related to S or N application were found in the proportion of germinated grain after 2 days of malting.

Table 4 A) ANOVA table and B) estimated means of malting process and quality parameters of grain grown in Vihti in 2004 (n=64; exp. 4).

A	DF	Soluble N P	FAN P	Colour P
CV	3	0.11	<0.001	<0.001
N	1	<0.001	<0.01	0.08
S	1	0.03	0.20	0.02
CV × N	3	0.37	0.05	<0.01
CV × S	3	0.46	0.03	0.04
N × S	1	0.33	0.54	0.22
CV × N × S	3	0.91	0.06	0.14

B		S [a]		N [b]	
		0	6	60	90
Soluble N (mg / 100 g dm)		655	670	639	686
	SE [c]		6.0		6.0
FAN (mg/l)					
	Barke	158	161	159	160
Cultivar	Prestige	160	170	164	166
	Saana	158	156	155	159
	Scarlett	176	173	168	181
	SE		2.6		2.6
Colour (°EBC)					
	Barke	2.6	2.8	2.7	2.7
Cultivar	Prestige	3.6	3.8	3.9	3.6
	Saana	3.0	3.1	3.0	3.1
	Scarlett	3.0	2.8	3.0	2.8
	SE		0.17		0.17

[a) b) c)] see Table 2

3.4 Hordein composition of field-grown barley

Application rate of S had no significant effect on hordein composition based on statistical analysis of 64 samples representing two replicate plots selected from the set of 128 samples (exp. 4, data not shown). Thus the influence of S application on the hordein parameters was examined with grains grown in Vihti in 2004 (n=32; exp. 5), which responded to S application with an increase in grain S content. The increase in total hordein content by 7.6% (p<0.01; Table 5) was explained mostly by the

increase in the amount of propanol-soluble B hordein (data not shown). The main effect of S increased also proportions of total and propanol-soluble B and decreased the proportion of total C hordeins (p<0.01, Table 5). Except for Saana, the cultivar × S interaction caused a clear decrease in propanol+DTT-soluble C hordein, which was decreased also by N × S interaction. The effect of N application showed cultivar-dependency in the total hordein content and proportions of propanol-soluble B and propanol+DTT-soluble C hordein.

Table 5 A) ANOVA table and B) estimated means of hordein parameters hordein parameters of samples grown in Vihti in 2004 (n=32, exp. 5).

A		Total hordein content	Proportions of hordeins total hordein content			
			B hordein		C hordein	
			Total	propanol-soluble	Total	propanol+DTT-soluble
	DF	P	P	P	P	P
CV	3	0.01	<0.01	<0.001	<0.01	0.12
N	1	0.06	0.23	0.02	0.22	<0.01
S	1	<0.01	<0.01	<0.001	<0.01	<0.01
CV × N	3	<0.01	0.32	<0.01	0.19	0.02
CV × S	3	0.08	0.57	0.26	0.54	0.02
N × S	1	0.52	0.29	0.93	0.21	0.01
CV × N × S	3	0.07	0.52	0.21	0.57	0.05

B		S [a]		N [b]	
		0	6	60	90
Total hordein (µg/mg dm)					
	Barke			10.5	15.2
Cultivar	Prestige	11.8	12.7	13.1	10.2
	Saana			13.4	15.7
	Scarlett			10.4	9.5
	SE [c]	0.62		0.76	
Proportions of hordeins of total hordein (%)					
Total B hordein		71.5	73.1	72.7	71.8
	SE	0.76		0.79	
Propanol-soluble B hordein					
	Barke			51.9	53.2
Cultivar	Prestige	43.6	45.9	40.8	39.8
	Saana			39.7	39.9
	Scarlett			43.6	48.9
	SE	0.73		1.00	
Total C hordein		23.2	21.7	22.1	22.8
	SE	0.72		0.73	
Propanol+DTT-soluble C hordein					
	Barke	4.6	1.8	4.4	2.0
Cultivar	Prestige	3.4	2.8	3.2	3.0
	Saana	4.3	4.3	4.3	4.3
	Scarlett	3.6	2.5	3.7	2.3
	SE	0.41		0.41	

[a b c] see Table 2

4 DISCUSSION

4.1 Implications of S availability effects in the greenhouse experiment

Sulphur is known to be one of the essential nutrients required for plant growth and development (Randall and Wrigley, 1986). Its importance in growth and yield formation of barley plants was demonstrated in the current study in a pot-scale experiment, where plants deprived of S were not capable of producing ears. Growth of generative plant parts, grains, is generally reported to be more sensitive to S deficiency than vegetative growth (Eriksen and Mortensen, 2002; Zhao et al., 1999). Apparently, vegetative growth is less restricted by availability of S as its deficiency likely increases as the growing season progresses. Hence, potential S deficiencies in the field may be difficult to detect at early growth stages, that is, crops can grow vigorously, but close to grain filling S sources begin to be limiting and the lack of S depresses the formation and realization of yield. Differences in vegetative and generative growth responses to low S availability are likely to be partly due to poor redistribution of S from vegetative plant parts to grains (Hocking, 1994).

In the current study, most differences in growth, yield and grain content parameters were observed between the three lowest S application levels (5, 10 and 20 mg S/kg soil). Based on the results of Shewry et al. (1983), the significant increase in the amount of cystine, formed by the oxidation of sulphydryl groups of two cysteine residues, clearly suggested an essential increase in S availability between these S application rates. Methionine, which is synthesized from cysteine, showed a more gradual increase with S availability. The increase in cystine was accompanied by a decrease in the amount of aspartic acid between application rates of 5 and 10 mg S/kg soil. Similar changes in the amounts of amino acids (fast increase in cysteine, more gradual increase in methionine and fast decrease of aspartic acid) related to S availability have been reported by Mortensen et al. (1992). Asparagine serves as a storage form of non-protein N in S-deficient barley, when protein synthesis is limited (Shewry et al., 1983). During hydrolysis of proteins and peptides for assessing amino acid composition, asparagine is deaminated resulting in aspartic

acid. The decreased amount of aspartic acid thus indicated improved protein synthesis when 10 mg of S or more was added per kg of soil. The amount of cystine levelled off only after addition of 20 mg S/kg soil, which showed that the synthesis of cysteine was enhanced by the higher S application rate even after protein synthesis was no longer limited by S availability. The balancing role of this non-protein N pool is likely to explain why the grain N content did not show differences related to S application rate.

An increase in total hordein content followed from the improved rate of protein synthesis indicated by the decrease in aspartic acid content. The proportions of S-rich B hordein and S-poor C hordein were clearly linked to the amount of cystine. The significant decrease in C hordein content between application rates of 10 and 20 mg S/kg soil revealed the essential change and critical level of S availability, as C hordein is known to be a N sink when S is limiting (Rahman et al., 1983). C hordein content did not change further with higher S application rates. The proportional changes in hordein composition between these fertilizer application rates were well in line with the results of Shewry et al. (1983) on barley grown at low and high application levels of S. It can therefore be deduced that 10 and 20 mg S/kg soil represented S-deficient and S-sufficient conditions, respectively. Possibly, the hordein composition did not change further at higher S application rates due to the limiting N availability in the pot-scale experiment.

When considering the response of yield, grain N and amino acid content to S application rate, amino acid content was far more responsive to S availability than grain N content or yield. The increase in S application rate from 10 to 20 mg S/kg, which represents the potential range of S availability in agricultural soils, produced a 6% increase in yield in barley. In contrast the proportional changes in S-containing amino acids and hordeins were greater; cystine, methionine and B hordein concentrations were increased by 75, 29 and 99%, respectively, and C hordein content was decreased by 43%.

In this study, the higher S availability of the plants grown in the greenhouse trial with 10 or 20 mg S/kg soil or more, was indicated by the clear decrease and levelling off observed in the malate:sulphate ratios. The malate:sulphate ratio of leaves during the vegetative growth phase was in line with the results on grain amino acid and hordein content. The higher malate:sulphate ratios, independent of S application rate at the shoot elongation stage, were probably due to a transient S deficiency occurring during rapid vegetative growth before anthesis (Blake-Kalff et al., 2001). The results of Zhao et al. (2005) and Carver (2005) have shown that a limit malate:sulphate ratio for S deficiency of barley cannot perhaps be unambiguously set. This may be due to the dependency of the malate:sulphate ratio in barley on growing season, soil type or cultivar.

4.2 Evaluation of S deficiency in the field experiments
In field conditions, the malate:sulphate ratio correlated negatively with S application, but the changes were not as considerable as those observed at pot-scale between S additions of 10 and 20 mg/kg soil. Furthermore, although S-application led to a decrease in grain N:S ratio in three trials and an increase in grain S concentrations in Vihti in 2004, neither of them showed S deficiency according to definitions of Randall and Wrigley (1986) and Withers et al. (1995). The increase in yield usually observed as a response to S application, if S is limiting (Reisenauer and Dickson, 1961; Zhao et al., 2006), was not observed either. Only the decrease of grain size in four trial sites due to higher N application could correspond to slight S deficiency, and grain size is affected by several other variables (Reisenauer and Dickson, 1961). Thus there was no apparent S-deficiency in the field trial sites in this study. The examination of malting process, quality and hordein parameters over three trials (exp. 2 and 4) did not reveal S-related differences. Based on data of Zhao et al. (2006), this is an indication of sufficient or only marginally deficient availability of S.

NPK-fertilizers with an S content of 2-3% have been widely used in Finland since the 1980s. Based on the current results, S-fortified fertilizers are probably sufficient to fulfil the S demand of barley in the

field conditions typical of southern Finland. Nevertheless, the risk of severe S deficiencies causing yield reductions is potentially higher in very coarse sandy soils.

4.3 Comparison of effects of S and N applications on hordein composition and malting quality, when S is sufficiently available

Although S supply in the trial sites of this study was not limiting, several effects of S application on grain composition were observed. Firstly, the decrease in grain N:S ratio observed in three trials, showed that grain S status may be affected by S application despite the lack of change in grain protein or S content. Grain N:S ratio has been shown to decrease due to the dilution effect of increased yield under limited N availability (Zhao et al., 2006), but no influence of S application on yield was found in this study. Furthermore, higher N application increased yield and both grain N and S content without changing their ratio. This indicates that N was not limiting.

Responses of malting quality parameters to S application were observed in Vihti in 2004, where the grain S content increased with S application. The soluble N content of malt increased with S application, although to a lesser extent than by N application. The S response for FAN and wort colour was cultivar-dependent, and these parameters were clearly linked. FAN is likely to influence wort colour through Maillard reactions of amino acids during mashing. Interestingly, there was no linkage between responses of FAN and wort colour to N application. The different effects of S and N application on wort colour may be due to differences in amino acid composition. The results suggest that S availability potentially affects malting quality through products of proteolysis, even when clear S deficiency does not occur, and that cultivars differ in their response to increased S availability. Wort colour is an important quality parameter in the lager-dominated brewing industry. A better understanding of the dependency of colour development on growing conditions and cultivar would serve both the barley breeding and brewing industry.

N application was, as expected, related to changes that compromise the malting quality. The decreased friability could be explained by the harder and not easily modified endosperm texture associated with increased protein or hordein content of barley grains (Buiatti et al., 2009). The lower extract yield could be a consequence of the inverse relation of protein and starch content and the decreased degree of modification due to hard endosperm (Buiatti et al., 2009). The higher amounts of soluble N and FAN in wort are related to the increase in barley protein content (Agu, 2003). However, the proportion of soluble to total N in malt (or Kolbach index) decreased with N application rate, probably due to the lower modification leading to increased malt protein content.

Consistent with earlier studies, both S and N application were found to increase total hordein content (Buiatti et al., 2009; Giese et al., 1983; Kirkman et al., 1982; Shewry et al., 1983). Also according to earlier findings, the proportions of B and C hordein increased and decreased with higher S application rate (Shewry et al., 1983). Nitrogen application did not affect the total proportions of C and B hordein, but did influence, in a cultivar-dependent manner, those B and C hordein fractions that responded to S application. In line with the current results, the response of malting quality to different N application rates has been found to be cultivar-dependent (Wang et al., 2007). Current results suggest that the effect of S on hordein composition and malting quality is also dependent on cultivar to some extent.

Even though S additions have been shown to have little impact on malting quality at sites sufficient or marginally deficient in S, the loss of B and D hordeins due to S deprivation has been suspected to affect the malting quality (Shewry et al., 1985; Zhao et al., 2006). In this study, S application at a site with no severe S deficiency was found to impact hordein composition, increasing the amount of S-containing B hordein and protein degradation products in malt. It can be hypothesised that the increase in the degradation products of B hordein could result in the observed differences in malting quality parameters.

5 CONCLUSIONS

In the current study, a clear influence of S availability on malate:sulphate ratio, amino acid and hordein composition was demonstrated in greenhouse-grown barley grains, and S deficiency occurred when 10 mg S/kg soil or less was available. In field conditions, S availability affected the malate:sulphate ratio and grain N:S ratio, but these did not indicate any apparent S deficiency suggesting that soil S was probably sufficient in the trial sites. However, in a site where grain S was slightly but significantly increased by S application, a change in hordein composition and malting quality was observed. This indicates that even in S-sufficient conditions, the end-use quality of malting barley may be affected by S application, but the effect is most probably cultivar-dependent. Moreover, the observed S responses were in accordance with those reported earlier on hordein composition. This indicates that e.g. more intensive growth rhythm induced in northern growing conditions does not alter greatly the effect of S on grain composition.

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Milling, water uptake and modification properties of different barley (*Hordeum vulgare* L.) lots in relation to grain composition and structure

Milling, Water Uptake, and Modification Properties of Different Barley (*Hordeum vulgare* L.) Lots in Relation to Grain Composition and Structure

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ABSTRACT: Milling properties, water uptake, and modification in malting were studied in 14 barley (*Hordeum vulgare* L.) lots from two consecutive crop years. In all barley lots studied, grains with lower β-glucan and protein content and higher starch content produced finer flours upon milling. Grains with lower β-glucan content also hydrated more rapidly during steeping. A detailed study of two cultivars from two crop years indicated that similar environmental conditions could induce a higher β-glucan content and concentration of aggregated B hordein in the peripheral endosperm and a lower proportion of C hordein entrapped among aggregated hordeins deeper within the endosperm. These characteristics were associated with production of coarser flours during milling as well as with slower water uptake and lower modification. However, the data do not distinguish between the effect of β-glucan content and that of hordein localization. Distribution of β-glucan or total protein within the kernel was not linked to hydration or modification.

KEYWORDS: *barley (Hordeum vulgare), milling, water uptake, hordein, starch, β-glucan*

■ INTRODUCTION

Barley (*Hordeum vulgare* L.) is a globally important crop plant not only because of its annual production quantities but also due to its wide cultivation area, from equatorial to nearly circumpolar latitudes. Barley is mainly exploited as feed or as a raw material for malt production. Interest in its use in other industrial food applications has recently grown as barley has the potential to be used as an alternative to more commonly used cereals. In particular, the high content of soluble dietary fiber, mixed-linkage $(1\rightarrow3, 1\rightarrow4)$-$\beta$-D-glucan ($\beta$-glucan), has helped promote the status of barley as a food ingredient.[1]

The starchy endosperm, the predominant part of cereal grains, is an important determinant for the structural characteristics of grain. The starchy endosperm comprises approximately 75% of the weight of the barley grain.[2] Even though starch granules form the majority of the starchy endosperm tissue, proteins within endosperm cells and β-glucan in endosperm cell walls also determine the structure and processing characteristics of barley grains.[3,4] Mixed-linkage β-glucan polymers comprise approximately 75% of the cell walls of barley starchy endosperm, arabinoxylan being a minor constituent.[7] Inside the starchy endosperm cells, protein forms a matrix surrounding starch granules. It consists mostly of alcohol-soluble storage proteins, hordeins, which are evolutionarily related to gliadins and glutenins in wheat.[5] Hordeins are classified by their molecular size and amino acid composition into B, C, D, and γ hordeins. C hordein, which lacks cysteine residues, and most of the B hordein are present in monomeric form or as single polypeptide subunits. D hordein and the remaining B hordein are present in polymeric form as aggregates of polypeptide subunits linked by interchain disulfide bonds.[6]

Although β-glucan and total protein concentrations are known to influence barley processing behavior, more detailed compositional analysis is required to explain the observed physical properties of the grain. High β-glucan content has been associated with hard, steely grains, high power consumption in milling, and inadequate endosperm modification during malting due to slow water uptake.[3,4,8] Therefore, knowledge of the processing properties of barley rich in β-glucan, such as waxy barley varieties with starch consisting mainly of amylopectin, is essential to maximize their use, for example, in the milling industry. Besides the content of β-glucan, grain hardness is also affected by the degree of packing of endosperm cells, cell wall thickness, and the amount of protein bound to the surface of starch granules.[3,9,10] Total protein content has also proven to be an insufficient parameter for describing the malting quality of the barley grain.[9,11] The hydration characteristics of barley grains have, for example, been shown to correlate with the composition or localization of hordeins.[12−14]

The influence of hordein and β-glucan distribution in the endosperm on the processing behavior of grain is not yet sufficiently understood. The major aim of this study was therefore to clarify the role of both components in water uptake and milling behavior of native and malted grains. For this purpose, a barley sample set with differing composition was

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Table 1. Composition of Whole Grain Barley Samples

variety	crop year	ear type[a]	starch (% db[b])	protein (% db)	β-glucan (% db)	raw fat (% db)	ash (% db)
feed type varieties							
Pilvi	2006	6r	67.8	11.7	3.4	3.0	2.8
	2007	6r	67.9	11.5	2.9	2.9	2.9
Vilde	2006	6r	67.8	10.6	4.3	3.0	2.9
	2007	6r	65.0	11.2	3.8	3.0	2.7
Voitto	2006	6r	71.8	9.7	3.9	3.2	2.4
	2007	6r	69.5	9.7	3.3	3.0	2.7
malting type varieties							
Saana	2006	2r	66.3	12.5	4.8	3.2	2.4
	2007	2r	64.9	12.3	4.0	3.0	2.6
Scarlett	2006	2r	65.2	12.5	5.5	2.9	2.2
	2007	2r	64.8	11.8	5.1	3.0	2.4
SW Makof	2006	2r	65.9	12.0	4.3	3.6	2.4
	2007	2r	66.5	11.8	3.1	3.0	2.6
waxy variety							
Magdalena	2006	2r	61.2	12.3	5.7	3.0	2.6
	2007	2r	62.9	11.7	4.6	3.2	2.8
u[c]			5.6	0.9	0.1	0.2	0.1

[a]2r, 2-row; 6r, 6-row. [b]Dry basis. [c]Standard uncertainty corresponding to one standard deviation.

selected, and associations between the chemical composition, structure, and processing behavior were studied.

■ MATERIALS AND METHODS

Barley Material. The barley material consisted of 14 lots representing seven spring barley cultivars: three 6-row feed type varieties (Pilvi, Vilde, Voitto), three 2-row malting type varieties (Saana, Scarlett, SW Makof), and one 2-row waxy variety (Magdalena). Each variety was grown in the same field in Hauho (southern Finland, 60°48′ N, 23°30′ E) in 2006 and 2007. The preceding crop was the same for all lots. Fertilizer (N, K) was placed in the seed bed at sowing. Samples of 6-row and 2-row barley were from the separate trials in which 50 and 80 kg N/ha were applied, respectively. Weeds and plant diseases were controlled with herbicides and fungicides.

Composition Analysis. Moisture, protein, lipids, and ash contents were analyzed using methods described in Nordlund et al.[15] Starch quantification was carried out in triplicate using an enzymatic–spectrophotometric method.[16] β-Glucan contents were determined in triplicate with a fluorometric method based on Calcofluor binding, according to the protocol recommended by the European Brewing Convention (Analytica-EBC: 3.10.2).[17]

Milling. Whole-grain barley samples were milled with a fine impact mill (100 UPZ-lb, Hosokawa Alpine, Augsburg, Germany) with stainless steel pin disk grinders using maximum speed (17700 rpm; tip speed 180 m/s) for the pin disk. Particle size distributions of pin-milled and two commercial barley flours were determined in triplicate using a set of 11 sieves with apertures from 1250 to 75 μm.

Water Uptake during Malting and Friability of Malts. Sixty gram lots of barley were malted in duplicate using a Joe White micromalting unit (Joe White Maltings Pty. Ltd., Adelaide, Australia). Malting consisted of steeping for a total of 48 h (8 h wet, 16 h dry, 8 h wet, 16 h dry) at 14 °C, germination for 5 days at 15 °C, and kilning for 19 h to a final temperature of 80 °C. Water uptake during malting was measured by weighing the duplicate grain lots after 24, 48, and 72 h of malting. Distribution of water in endosperm was determined according to the method described in detail in Holopainen et al.[14] For this purpose, grain samples were taken after steeping or 48 h of malting. Grains were classified into four groups based on the proportion of hydrated endosperm.

The friability of the malt samples was determined using the EBC method (Analytica-EBC: 4.15)[17] optimized for smaller sample size according to Holopainen et al.[14] using a malt with friability of 84.9 as a

standard. The analysis was performed in triplicate for each sample. Due to down-scaling of the standard procedure, the numerical values for friability are not directly comparable to the standard EBC method, but results describe the relative friability of the samples assayed.

Localization of Grain Components by an Abrasive Method. To study the localization of β-glucan, total protein, and hordeins, grain batches of 100 g were pearled using a custom-made barley dehuller-pearler (VTT Technical Research Centre of Finland). The grain material was pearled for 60 s four times consecutively. This resulted in grain samples with 13–17, 21–24, 29–33, and 35–40% of the outer layers removed. Protein and β-glucan contents of the pearled grains were determined in triplicate as described above.

For localization of salt-soluble proteins and hordeins, the amounts of these protein fractions were analyzed from both intact and pearled barley kernels. Protein fractions were extracted from 150 mg of ground grain in triplicate using a modified Osborne fractionation method described in Holopainen et al.[11] with the following modifications: a protease inhibitor tablet (Complete Mini, Roche) was added into 0.5 M sodium chloride solution, and the extractions with 0.5 M sodium chloride, water, and 50% (v/v) 1-propanol were carried out with constant shaking at room temperature.

Protein contents of these extracts were determined with a 2D Quant protein kit (Amersham Biosciences). NaCl extracts containing albumins and globulins were analyzed as such. Two hundred microliters of the 1-propanol- and the 1-propanol+dithiothreitol (DTT)-soluble hordein fractions were precipitated in 4 volumes of acetone at −20 °C and resuspended in 100 μL of rehydration solution containing 7 M urea, 2 M thiourea, and 0.5% (v/v) Triton-X-100 (BDH, Dorset, UK) prior to protein assay. The total hordein content per dry matter of barley was defined as the sum of the two hordein extracts.

For SDS-PAGE, a 25 μL aliquot of each triplicate 1-propanol- or 1-propanol+DTT-soluble hordein fraction was precipitated as described above and resuspended in 40 μL of sample buffer (62.5 mM Tris-HCl buffer (pH 6.8), 20% (v/v) glycerol, 2.0% (v/v) SDS, 0.05% (w/v) bromophenol blue, and 5% (v/v) mercaptoethanol) for 16 h at 4 °C. Samples were then boiled for 3 min, centrifuged (20800g, 10 min, 4 °C) and separated in 12% Tris-borate gels (pH 8.9) using 0.125 M Tris-borate with 0.1% (v/v) SDS (pH 8.9) as a running buffer.[18] Gels were rinsed, stained with Coomassie Brilliant Blue G 250 (Fluka, Buchs, Switzerland), and fixed according to the method of Neuhoff et al.[19] using ethanol instead of methanol in both staining and washing solutions. After fixation, gels were scanned with GS-710 calibrated

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Table 2. Correlations between Barley Grain Components and Grain Processing Parameters Calculated as Spearman Correlation Coefficients ($n = 14$)

	starch	protein	β-glucan	raw fat	ash
grain components					
starch	1.00				
protein	−0.74**[a]	1.00			
β-glucan	−0.63*	0.48	1.00		
raw fat	0.12	0.00	0.07	1.00	
ash	0.03	−0.33	−0.53	−0.31	1.00
proportion of size fractions of whole grain flour					
>633 μm	−0.55*	0.24	0.48	−0.16	0.09
355−<633 μm	−0.80**	0.50	0.81**	−0.05	−0.19
180−<355 μm	−0.23	0.02	0.45	−0.23	−0.07
132−<180 μm	0.81**	−0.61*	−0.58*	0.23	0.05
95−<132 μm	0.86**	−0.46	−0.66*	−0.05	0.05
75−<95 μm	0.41	0.06	−0.39	0.39	−0.11
<75 μm	0.23	−0.19	−0.63*	−0.07	0.30
water uptake from the beginning of malting					
24 h	0.36	−0.37	−0.68*	−0.41	0.41
48 h	0.41	−0.37	−0.61*	−0.41	0.25
72 h	0.11	−0.38	−0.52	−0.39	0.49
distribution of water in grains after 48 h of malting					
0−25%	0.18	−0.25	−0.19	0.09	0.37
25−50%	−0.25	0.13	0.42	−0.27	−0.24
50−75%	0.33	−0.14	−0.51	0.23	0.22
75−100%	−0.05	0.17	−0.10	0.27	−0.11
modification during malting					
friability	0.62*	−0.33	−0.42	0.29	−0.36

[a]Significance level: *, $p < 0.05$; **, $p < 0.01$.

imaging densitometer (Bio-Rad Laboratories, Hercules, CA, USA). B, C, and D hordeins separated in the gels were quantified using QuantityOne software (Bio-Rad) based on the band intensity and protein content of the hordein extracts. The average content of each hordein fraction and their relative proportions were calculated on the basis of the results of three replicate extracts.

Statistical Analysis. The results were calculated as averages of the replicates. The results of the composition analysis, excluding β-glucan contents, were presented with the standard uncertainty corresponding to one standard deviation calculated from the extended uncertainty of measurement of each validated method. For the β-glucan results, respective standard uncertainty was calculated from the 95% confidence interval. For the water uptake results, pooled standard deviations were calculated on the basis of two replicate micromaltings. When applicable, the results were examined statistically using correlation analysis and analysis of variance (ANOVA) with Tukey's honestly significant difference posthoc test (IBM SPSS Statistics software for Windows v20.0; IBM Corp., Somers, NY, USA).

■ RESULTS AND DISCUSSION

Composition of Different Barley Lots. For comparison with processing properties, the chemical composition of the barley samples was analyzed (Table 1). The starch contents varied from 61.2 to 71.8%, and the feed varieties had somewhat higher contents (65.0−71.8%) compared to other samples. The starch contents of the feed varieties were higher than reported earlier, which may be explained by their lower nitrogen fertilizer application rate in this study.[20−22] The lower starch content of the waxy barley variety in comparison to the other varieties is in accordance with earlier literature.[21,22]

Protein contents of the samples ranged from 9.7 to 12.5% (Table 1). The variation in protein content was a little lower compared to the data of Holtekjølen et al.,[22] which included 29 hulled barley samples with protein contents from 8.4 to 14.5%.

In the current study, lower nitrogen application to feed varieties resulted in lower protein contents compared to the malting type varieties. Higher protein contents of the malting barley varieties were most likely due to unrealized yield potential resulting in excessive nitrogen availability.

The variation in β-glucan content (2.9−5.7%; Table 1) was consistent with results published earlier.[23,24] Both genotype and growing conditions are known to influence β-glucan content.[23,25,26] The 6-row ear type and waxy barley varieties have been associated with low and high contents of β-glucan, respectively,[23,27] and similar results were obtained in the current study. In addition to genotypic association, each cultivar showed also crop year dependency, the β-glucan contents of each variety being substantially higher in 2006 than in 2007. The ranges of raw fat and ash contents were similar to those reported earlier.[20,28]

The correlation coefficients calculated between the main grain components showed significant negative correlations between starch and protein contents as well as starch and β-glucan contents (Table 2; $p < 0.01$ and $p < 0.05$, $n = 14$). Both of these correlations are in line with earlier studies (e.g., Holtekjølen et al.[22]). Protein and β-glucan contents did not show a positive correlation as in previous studies.[22] Raw fat and ash contents showed no significant correlations with other components. Overall, the compositional variation in the sample set used was considered to be adequate and suitable for further investigation of the relationship between composition and processing behavior of grains.

Association of Milling Behavior with Grain Composition. The dissociation of endosperm into flour particles was studied by analyzing particle size distribution of flours produced by pin milling. All of the studied barley samples yielded flour

Table 3. Cumulative Particle Size Distribution of Flours Calculated as Averages of Triplicate Sieving Tests

variety	crop year	proportion of particles (% w/w) smaller than					
		633 μm	355 μm	180 μm	132 μm	95 μm	75 μm
feed/starch type varieties							
Pilvi	2006	97.5 bcd[a]	87.6 fg	65.7 de	57.9 ef	46.9 b	5.8 efgh
	2007	96.9 bcd	87.0 ef	67.1 e	59.8 g	49.4 de	6.5 h
Vilde	2006	97.8 cd	88.0 fg	66.5 e	58.1 f	47.1 bc	4.7 defg
	2007	97.8 cd	86.9 def	65.0 de	57.3 ef	49.0 cde	10.8 i
Voitto	2006	97.8 cd	88.3 fg	67.0 e	58.2 fg	46.5 b	4.1 bcde
	2007	96.4 b	86.1 cde	65.1 de	57.3 ef	47.6 bcd	4.2 cdef
malting type varieties							
Saana	2006	97.5 bcd	85.4 c	63.6 cd	56.3 de	46.9 b	3.0 abcd
	2007	97.0 bcd	85.5 cd	65.1 de	57.4 ef	47.4 bc	4.8 efgh
Scarlett	2006	96.7 bc	81.1 b	57.8 b	51.2 c	42.6 a	2.6 abc
	2007	97.8 cd	85.5 cde	62.8 c	55.3 d	46.1 b	4.2 cdef
SW Makof	2006	97.1 bcd	85.0 c	66.5 e	58.8 fg	50.6 ef	5.9 fgh
	2007	97.9 d	88.5 g	69.8 f	62.4 h	51.8 f	6.2 gh
waxy variety							
Magdalena	2006	92.9 a	72.3 a	51.7 a	46.8 a	41.9 a	1.6 a
	2007	96.7 b	80.4 b	55.8 b	49.0 b	42.2 a	2.4 ab
commercial barley flours							
flour I		99.9	98.0	80.9	71.7	63.7	5.9
flour II		99.8	91.2	55.5	46.3	39.2	3.7

[a]Different letters indicate statistically significant difference ($p < 0.05$) between samples based on Tukey's HSD test.

with similar, bimodal particle size distribution with peaks at 75−95 and 250−355 μm (data not shown). However, certain differences between samples were observed in the cumulative particle size distributions (Table 3). The results were comparable to particle size distribution of commercial barley flours available in Finland. Compared to other samples, the milling of the grains of waxy variety Magdalena resulted in coarser flour containing significantly fewer particles smaller than 132 μm ($p < 0.05$, $n = 3$). Excluding samples of Voitto, each variety produced finer flour from the 2007 crop than from the 2006 crop. This difference was observed at least in one of the cumulative proportions of particles smaller than 132 μm ($p < 0.05$, $n = 3$). Nair et al.[31] have shown that the flours produced from harder grains contain more particles >106 μm than the flours from grains with softer structure. Therefore, the grains from crop 2006 may have been harder compared to those grown in 2007.

For examination of the effect of composition on milling properties, particle size distributions of flours were compared with the results of composition analysis. The composition analysis had revealed that starch content correlated negatively with protein and β-glucan contents, which, however, were not positively correlated with each other (Table 2). It was therefore considered reasonable to examine associations of starch with β-glucan content and protein content separately. Higher starch and lower protein and β-glucan contents were significantly linked to a higher fragility of the grain as indicated by the increased formation of flour particles of size from 132 to 180 μm upon milling (Table 2). Also, the proportion of the finer flour particles (from 95 to 132 μm) correlated positively with starch content and negatively with β-glucan content. Negative correlations were observed between the proportion of coarser flour particles (from 355 to 633 μm) and starch and β-glucan contents. Moreover, negative correlations were found between the proportion of the finest flour and β-glucan content as well as between the proportion of the coarsest flour and starch content. These results suggest that starch, protein, and β-glucan

contents each affect the dissociation of the endosperm into flour particles.

The current results are consistent with the previous findings that starch content correlates negatively and β-glucan content positively with milling energy and kernel hardness.[4,8,29,30] Additionally, finer flours are produced from softer grains.[31] The more fragile structure of the grains with higher starch content could be a consequence of their lower β-glucan content (Table 2). The lower β-glucan content might indicate thinner cell walls and/or larger cell size causing a more easily breakable endosperm. Accordingly, thicker cell walls have been associated with hard-structured, steely barley grains.[10] However, varieties representing both low and high β-glucan levels showed crop year-related differences in flour coarseness, indicating that also factors besides β-glucan content affected grain milling properties and the hardness of grains. This is in line with the fact that a relationship between β-glucan content and grain hardness has not been found in all studies.[30,32]

In the sample set studied, also a lower protein content (associated with higher starch content) was linked to higher grain fragility in milling. Accordingly, protein content correlates positively with grain hardness.[3,29] Protein influences grain hardness via two mechanisms, both related to the dissociation of starch granules from the protein matrix during milling. First, a discontinuous or less compact protein matrix surrounding the starch granules is associated with softer grain structure.[3,10] Second, limited adhesion of protein to starch granules has been linked to a more fragile endosperm structure.[9,10] A correlation between protein content and milling energy or grain hardness has, however, not been observed in all studies, probably because grain hardness is associated also with β-glucan content, as described above.[4,30,32] It can be concluded therefore that barley grain composition greatly defines its milling properties, although the structure of endosperm must also be considered.

Association of Water Uptake and Endosperm Modification with Grain Composition. Water uptake was monitored during malting by measuring the increase in the

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water content of the grains (Table 4). Water uptake was faster in the samples grown in 2007 than in the samples of 2006. This

Table 4. Water Uptake of Barley Grains Measured as Water Content at 24, 48, and 72 h from the Beginning of Malting and the Friability of Final Malts[a]

| variety	crop year	water content from beginning of malting (%; w/w)			friability[b] (%)
		24 h	48 h	72 h	
feed type varieties					
Pilvi	2006	38.2	44.4	45.1	63.3 c[c]
	2007	42.4	46.4	46.8	65.2 cd
Vilde	2006	38.2	43.9	45.3	68.3 de
	2007	42.6	47.1	47.2	73.3 fg
Voitto	2006	38.6	44.6	45.1	80.4 ij
	2007	42.2	47.7	47.9	89.1 l
malting type varieties					
Saana	2006	38.3	44.8	44.8	78.1 hi
	2007	42.1	46.7	47.0	74.8 fgh
Scarlett	2006	36.6	44.3	45.1	71.0 ef
	2007	38.7	44.4	45.2	76.0 ghi
SW Makof	2006	36.0	42.6	45.1	85.7 kl
	2007	38.6	44.4	45.4	83.3 jk
waxy variety					
Magdalena	2006	35.0	41.7	45.2	41.3 a
	2007	37.7	43.9	45.9	51.2 b
s_p[d]		0.8	0.5	0.3	

[a]Results on water uptake are expressed as means of two replicate malting lots and friability values as averages of triplicate analyses. [b]Relative value of friability. [c]Different letters indicate statistically significant difference ($p < 0.05$; $n = 3$) between samples based on Tukey's HSD test. [d]Pooled standard deviation.

difference in water uptake was observed in all varieties after 24 and 48 h of malting, except for samples of Scarlett, in which there was no difference between the crop years at the 48 h time point. After the first day of germination (72 h from the beginning of malting), all of the samples had reached at least a water content of 45%, which is considered optimal for germination. Distribution of water in the grains was analyzed after 48 h of malting (Figure 1). By that time point, hydration had proceeded further in the samples of Vilde, Voitto, Scarlett, and Magdalena grains from the 2007 crop compared to those from 2006. This difference was observed in the proportion of grains having 50−75 and/or 75−100% of endosperm hydrated. In addition, the water content of grains after 24 and 48 h of malting correlated positively with the proportion of grains having 50−75% of endosperm hydrated after 48 h of malting ($p < 0.05$, $n = 14$, data not shown).

Similarly to the results of Gamlath et al.,[4] hydration of the grains at the time points of 24 and 48 h correlated negatively with β-glucan content (Table 2; $p < 0.05$, $n = 14$). Due to higher β-glucan content, grains grown in 2006 may have had a harder structure, which is known to be negatively correlated with water uptake.[3] However, β-glucan did not explain all of the variation observed in the extent of hydration, and no significant correlations between the distribution of water and grain composition were observed (Table 2). Therefore, water uptake was probably regulated also by packing or distribution of components in the endosperm rather than by the chemical composition only, as shown by, for example, Brennan et al.[33]
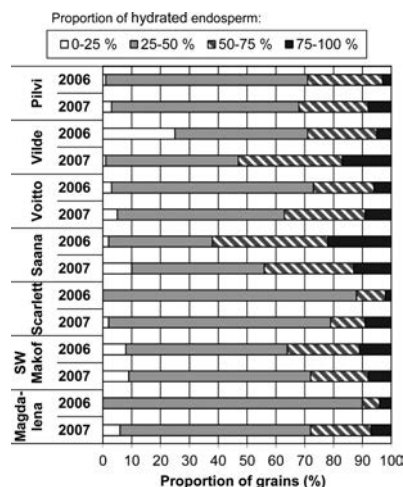
Figure 1. Distribution of water in barley grains after 48 h of malting. Results are based on 100 grains/sample.

and Chandra et al.[3] Although the steeping phase in the malting process represents a discontinuous hydration method, the effect of β-glucan content on water uptake observed in this study is likely to be applicable to other procedures requiring entry of water into grain.

The extent of endosperm modification during malting was determined by analyzing friability, the tendency of the endosperm to break into flour in a specified milling process. The higher the friability value, the more extensively the endosperm has been modified during malting, mostly with respect to degradation of cell walls and protein matrix.[34,35] Quite large variation in the friability values was observed among the barley samples in this study (Table 4). Malts of the samples of malting type variety SW Makof and feed variety Voitto were the most friable. SW Makof is known to have high activities of hydrolyzing enzymes, leading to release of high concentrations of fermentable sugars for distilling processes. The lower friability values of the other malting barley varieties were explained by high protein contents, which are known to cause insufficient modification.[36] The good modification of the Voitto samples was likely a consequence of low protein content.

The samples of waxy barley variety Magdalena had the lowest friability values (Table 4; $p < 0.05$, $n = 3$). This variety also showed high water uptake as mentioned above. The rapid hydration of waxy barley grains has been linked to starch composition, as low-amylose starch is known to differ from regular barley starch with respect to its physicochemical properties.[37,38] Although water uptake is a fundamental requirement for endosperm modification by enabling the synthesis and activity of hydrolyzing enzymes, fast hydration does not necessarily ensure good friability. Modification is also greatly dependent on hydrolytic enzyme activities not analyzed in this study.[33] Enzyme activities, the rate of enzyme activation, and the accessibility of the substrate to the enzyme may vary due to genetic background.[24,33]

Starch content correlated positively with friability (Table 2; $p < 0.05$, $n = 14$). Due to the inverse relationship of starch and protein contents of the grains, higher starch content could be associated with higher modification because of a more easily

hydrolyzed, less dense protein matrix surrounding starch granules.[3] No significant correlation between protein content and friability was found. A negative association between protein content and friability is usually observed in studies of malting barley varieties and feed-type varieties.[11,33,36,39] The lack of correlation may be due to different nitrogen application given to 2-row and 6-row barley varieties.

The association between crop year and friability was not consistent; friability was higher in 2007 samples except for those of Pilvi, Saana, and SW Makof. This was probably due to varietal differences related, for example, to enzymatic hydrolysis of the endosperm during germination. Furthermore, no direct correlation was found between barley β-glucan content and friability in this study, although β-glucan content has been linked to grain hardness, which is known to correlate negatively with endosperm modification and friability.[29,30] In the case of β-glucan, the role of enzyme action during germination is significant, and the amount of β-glucan left in barley malt has been shown to correlate more strongly with friability than the β-glucan content of unmalted barley.[40]

Association of Grain Processing Behavior with Localization of β-Glucan, Total Protein, and Hordeins. The distribution of different components within the grain was examined by progressively pearling grains of the varieties Scarlett and Vilde from crop years 2006 and 2007 and analyzing the composition of the pearled kernels. In all samples, protein content decreased gradually toward the center of the grain at pearling rates >15% (Figure 2a). β-Glucan, in turn, was enriched in the remaining kernel up to a pearling rate of 30%. Similar changes in the protein and β-glucan contents of the pearled kernels have been linked to removal of the protein-rich aleurone layer and enrichment of subaleurone and starchy endosperm cell walls rich in β-glucan.[38,41] Grains of Scarlett samples had consistently higher contents of protein and β-glucan in the endosperm at all pearling rates compared to samples of Vilde, indicating a less fragile endosperm structure, which may explain the coarser flour produced from sample lots of Scarlett. Higher protein contents in the endosperm of grains of Scarlett samples were probably a consequence of the higher nitrogen fertilizer application given for this cultivar. The distribution of total protein or β-glucan showed no clear association with water uptake or friability.

The decrease in concentrations of water- and salt-soluble proteins (globulins and albumins) was more pronounced toward the center of the grain relative to the total protein content (Figure 2b). Hordein was rather evenly distributed in the grains of Scarlett samples, but in the grains of Vilde samples it decreased at the pearling rates of 20−30% (Figure 2c). Distribution of these protein fractions is in line with the known location of hordeins in the subaleurone and starchy endosperm cells and globulins and albumins in the aleurone layer.[42] Clear correlations between the distribution of these protein fractions and processing behavior including milling, water uptake, and friability were not observed.

Hordeins were extracted from whole and pearled grains sequentially in 50% (v/v) 1-propanol first without and thereafter with DTT for the reduction of interchain disulfide bonds of B and D hordein aggregates (Table 5). Irrespective of the pearling rate, most of the hordeins were monomeric B and C hordeins. Some C hordein was extracted after reduction, showing its entrapment among the aggregated B and D hordeins. The inner endosperm (pearling rate approximately 30%) of Vilde and Scarlett grown in 2007 contained
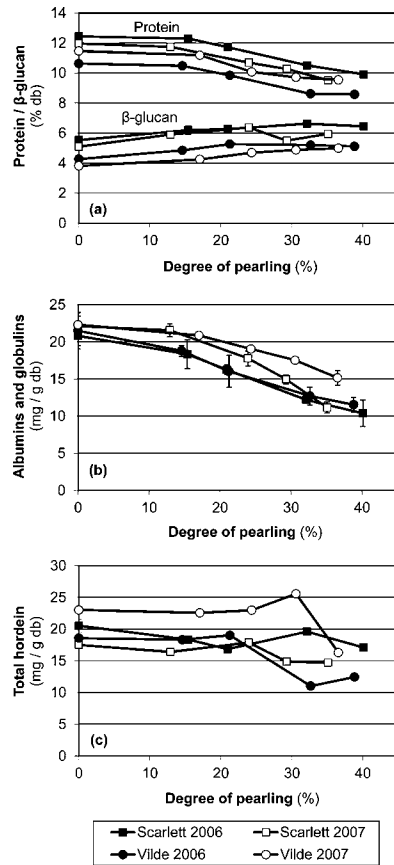
Figure 2. Effect of pearling on content of (a) protein and β-glucan, (b) water- and salt-soluble proteins or albumins and globulins, and (c) total hordeins in barley grains. Analyses and extractions were performed in triplicate. Error bars indicate the 95% confidence intervals ($n = 3$).

proportionally less 1-propanol-soluble C hordein and more 1-propanol+DTT-soluble B, C, and D hordeins in the endosperm compared to the samples harvested in 2006 (Table 5; $p < 0.05$, $n = 3$). Moreover, 1-propanol+DTT-soluble B hordein was more concentrated in the peripheral endosperm in the grains of both varieties grown in 2006.

Both the hordein localization and β-glucan content in the grains of Scarlett and Vilde were associated with the crop year, suggesting that their formation during grain filling could be affected by similar environmental conditions. Furthermore, distribution of hordein fractions and β-glucan was associated with processability in the samples of these two cultivars. In both cases there was a lower β-glucan content, localization of aggregated hordeins (with entrapped C hordein) within the more central endosperm, and nonentrapped C hordein located less deeply. These characteristics correlated positively with the proportion of fine flour after milling, water uptake after 24 h of malting, and proportion of hydrated endosperm after 48 h of malting and friability.

Table 5. Proportions of 1-Propanol- and 1-Propanol+DTT-Soluble B, C, and D Hordeins of Total Hordein Content in Intact and Gradually Pearled Grains of Cultivars Scarlett and Vilde Grown in 2006 and 2007.[a]

sample	intact grains	pearling rate			
		13–17%	21–24%	29–33%	35–40%
1-propanol-soluble hordeins					
B hordein (% (w/w db) of total hordein)					
Scarlett 2006	40.2 a[b]	42.3 ab	48.2 d	49.0 d	46.8 cd
Scarlett 2007	41.6 a	45.0 bc	46.9 cd	48.6 d	49.7 d
Vilde 2006	40.1 a	41.2 ab	46.9 c	62.6 e	55.8 d
Vilde 2007	39.8 a	40.4 a	42.5 b	46.3 c	54.1 d
C hordein (% (w/w db) of total hordein)					
Scarlett 2006	34.1 bc	37.9 d	44.5 e	43.8 e	42.8 e
Scarlett 2007	30.6 a	32.8 ab	35.1 bcd	36.2 cd	36.6 cd
Vilde 2006	18.4 a	20.3 b	23.2 c	29.3 d	27.6 d
Vilde 2007	21.9 bc	20.6 b	22.0 bc	23.2 c	28.5 d
D hordein (% (w/w db) of total hordein)					
Scarlett 2006	0.6 a	0.5 a	0.7 a	0.8 a	0.7 a
Scarlett 2007	0.4 a	0.4 a	0.5 a	0.6 a	0.8 a
Vilde 2006	1.0 a	1.1 ab	1.1 ab	1.6 b	1.2 ab
Vilde 2007	0.8 a	0.6 a	0.6 a	1.1 ab	0.8 a
1-propanol+DTT-soluble hordeins					
B hordein (% (w/w db) of total hordein)					
Scarlett 2006	15.8 h	10.7 f	3.5 a	3.0 a	4.6 b
Scarlett 2007	17.2 i	13.0 g	9.5 e	7.7 d	6.3 c
Vilde 2006	31.3 f	26.9 d	20.0 c	4.3 a	10.3 b
Vilde 2007	28.9 e	29.5 ef	26.1 d	20.8 c	11.8 b
C hordein (% (w/w db) of total hordein)					
Scarlett 2006	4.7 e	3.9 de	1.3 a	1.3 a	1.9 ab
Scarlett 2007	4.6 e	3.4 d	3.2 cd	2.4 bc	2.4 bc
Vilde 2006	3.1 cd	3.3 cd	2.8 cd	0.6 a	1.5 ab
Vilde 2007	3.3 cd	3.5 d	3.0 cd	2.6 c	1.7 b
D hordein (% (w/w db) of total hordein)					
Scarlett 2006	4.7 cd	4.7 cd	1.7 a	2.1 a	3.2 b
Scarlett 2007	5.6 e	5.3 de	4.8 cde	4.6 cd	4.2 c
Vilde 2006	6.1 c	7.1 c	6.0 c	1.6 a	3.6 ab
Vilde 2007	5.3 bc	5.3 bc	5.9 c	6.1 c	3.1 ab

[a]Values are expressed as means of triplicate hordein extractions. [b]Proportions of the same variety followed by different letters differ significantly from each other ($p < 0.05$) based on Tukey's HSD test.

Although the effect of hordein localization and β-glucan content on processing behavior cannot be fully separated on the basis of the current results, several earlier studies suggest that hordeins influence grain properties. For example, the concentration of aggregated B hordein in the peripheral endosperm has been associated with impaired malting quality,[43] and the deeper localization of C hordein entrapped by aggregated B and D hordeins improved water uptake during steeping.[14] Moreover, this minor proportion of C hordein might assist hydration and modification, restricting the aggregation of B hordein or changing the number and orientation of disulfide bonds between aggregated hordeins.[12,44] The nonentrapped C hordein, representing the majority of the C hordein, could in turn limit water uptake and endosperm modification on the basis of its hydrophobic structure, effect on endosperm hardness, and slow hydrolysis during malting.[44–46] Hordeins could also influence milling properties, because steely grains, which generally produce coarser flour upon milling, are rich in C hordein.[31,45] Therefore, a lower proportion of nonentrapped C hordein within the central starchy endosperm could be expected to facilitate dissociation of the protein matrix and starch granules into finer flour particles. However, the more specific mechanisms by which the degree of hordein aggregation affects properties of barley endosperm such as dissociation of flour particles, hydration, or modification during germination are not known.

This study has shown that the processing properties (milling behavior, water uptake, and modification) of barley grains are associated not only with their main constituents (starch and protein) but also with β-glucan content. The distribution of hordein within the grain could also contribute to grain structure and processing performance. These results further indicate that understanding the role and biochemical action of macromolecules located inside cells, as well as macromolecules that make up cell walls, is necessary to accurately predict the functional performance of individual lots of barley grains.

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Endosperm structure affects the malting quality of barley (*Hordeum vulgare* L.)

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Endosperm Structure Affects the Malting Quality of Barley (*Hordeum vulgare* L.)

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Twenty-seven barley (*Hordeum vulgare* L.) samples collected from growing sites in Scandinavia in 2001 and 2002 were examined to study the effect of endosperm structure on malting behavior. Samples were micromalted, and several malt characteristics were measured. Samples were classified as having a mealier or steelier endosperm on the basis of light transflectance (LTm). Because endosperm structure is greatly dependent on protein content, three barley sample pairs with similar protein contents were chosen for further analysis. During malting, the steelier barley samples produced less root mass, but showed higher respiration losses and higher activities of starch-hydrolyzing enzymes. Malts made from steelier barley had a less friable structure, with more urea-soluble D hordein and more free amino nitrogen and soluble protein. The reason for these differences may lie in the structure or localization of the hordeins as well as the possible effects of endosperm packing on water uptake and movement of enzymes.

INTRODUCTION

To produce malt of good quality, the cell walls of the endosperm and a part of the small starch granules and surrounding protein matrix should be broken down during the malting process (*1*). This degradation of endosperm reserves involves coincident action of the enzymes hydrolyzing protein, starch, and cell wall structures. These enzymes are synthesized, or activated in the aleurone and scutellar cells, and secreted to starchy endosperm. In addition to the embryonic gibberellin-activated signal transduction pathways, breakdown of endosperm reserves is controlled by the structural pattern of tissues and stored macromolecules (*2*).

Good malting barley varieties should have an endosperm structure that is easy to modify and a good enzyme-synthesizing capacity to ensure fast modification. These quality parameters are, however, seldom analyzed when barley is purchased for malting purposes. Barley lots for malting purposes are currently purchased on the basis of total protein content, although mealiness has also traditionally been tested for in, for example, Great Britain. A suitable protein content of barley is known to be favorable for malting, whereas grains with a high protein

content are often steely and are therefore a malting quality risk (*3*). However, barley samples of the same variety with similar protein contents may show very different modification patterns depending on crop year and growth environment. Good and poor malting quality barley cultivars, with similar protein contents, have been noted to differ in starch–protein adhesion and the patterns of fracture through the endosperm (*4*). These differences appear to relate to the properties of the endosperm storage proteins, hordeins in the case of barley, rather than total protein amount.

Grain hardness is related to the packing of the endosperm and affects endosperm modification because the dense structure of a steely endosperm limits water uptake and passage of hydrolyzing enzymes (*5*). Hardness of barley grains may also be linked to the protein–starch association as in wheat (*6, 7*). Recently, grain hardness has been considered to be the most important variable for describing malting performance, despite the facts that a low correlation with chemical and physical grain characteristics has been previously noted and that this cannot be used as a single factor in the prediction of malting quality (*8–10*). For malting and brewing purposes, a method for quantifying the structure of the endosperm is available. This LTm method is based on the use of a light transflectance meter, which assesses the density of endosperm structure by its ability to transmit and reflect light (*11*).

This study was undertaken to investigate and explain the effect of endosperm structure on modification and malting

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behavior between barley lots of the same variety. For this purpose, different compositional, structural, and enzymatic properties of 27 barley samples and their corresponding malted samples were compared. Three sample pairs with similar protein contents were studied in more detail.

MATERIALS AND METHODS

Plant Material. Twenty-seven barley (*Hordeum vulgare* L.) samples of the varieties Barke, Scarlett, Wikingett, and Luberon were collected from different growing sites in Scandinavia in 2001 and 2002.

Barley Analyses. Protein and starch contents of the samples were determined by using near-infrared analysis (Foss Tecator, Infratec 1241 grain analyzer). Germinative capacity, kernel size distribution, and moisture of the samples were determined according to recommended methods of the European Brewing Convention (*12*). The relative proportions of steeliness and mealiness and the homogeneity of the endosperm were estimated with a light transflectance meter and are indicated as mean LTm values of the samples (*11*). The endosperm structure of 97 grains of each sample batch was analyzed. Chandra et al. (*11*) defined grains having average LTm values of <200 mV as mealy and those having LTm values of ≥300 mV as steely.

Micromalting Process and Malt Analyses. Grain samples of 1 kg (>2.5 mm screen) were micromalted (Joe White Malting System) at LP Research Centre Ltd., Lahti, Finland. The malting procedure consisted of steeping at 13−15 °C for 2 days, germination for 5 days at 14 °C, and kilning for 22 h to a final temperature of 82 °C to produce malts with ~4% moisture. The moisture contents of the samples after steeping were determined by weighing. After kilning, the rootlets were removed. Weight losses (dry basis) caused by respiration and removal of rootlets were determined by weighing. Respiration losses were calculated as differences of total weight loss and weight loss in removing rootlets.

Malt samples were analyzed using the following EBC-recommended methods: moisture, fine/coarse extract, friability, modification, homogeneity, total protein, soluble nitrogen, protein solubility as Kolbach index, free amino nitrogen, wort β-glucan, wort color, and wort viscosity (*12*). Megazyme assay kits (Megazyme, Co. Wicklow, Ireland) were used to determine the activities of the following enzymes in malts: α-amylase (*13*; with modification of the extraction time to 30 min), free β-amylase (*14*, *15*), total and free limit dextrinase [*16*; with modification of the concentration of dithiothreitol (DTT) in extraction buffer to 62 mM], and β-glucanase (*17*, *18*). The endo-β-xylanase activity of malt samples was measured viscometrically according to the method described by Autio et al. (*19*).

Assay of Endopeptidase Activity. The crude endopeptidase extraction procedure was modified from the method of Zhang and Jones (*20*). Extracts were prepared by extracting 2.00 g of ground barley or malt with 9 mL of 50 mM sodium acetate buffer containing 2 mM cysteine with constant magnetic stirring at 4 °C for 1 h. After extraction, extracts were centrifuged (1590*g*, 15 min, 4 °C).

To determine the proportion of cysteine proteinases of the total endopeptidase activity, cysteine proteinases were inhibited by the specific inhibitor *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane, E-64 (E3132, Sigma Chemical Co., St. Louis, MO). The inhibitor was added to crude enzyme extracts to give a final concentration of 24.9 μM (*21*). Extracts were incubated for 30 min at 4 °C before the assay. E-64-inhibited endopeptidase activity was defined as cysteine proteinase activity.

The endopeptidase activity was determined with the Megazyme assay kit using azo-casein substrate. The activity of endopeptidases was detected spectrophotometrically against reaction blank at 440 nm. Each assay was done in triplicate. For statistical analysis, initial hydrolysis rates between time points of 0 and 10 min were calculated.

Hordein Extraction Procedure. Barley and malt samples (>2.5 mm) were ground in a sample mill (Pulverisette 14, Fritsch) equipped with a 0.5 mm sieve. The moisture contents of the barley and malt samples were determined as described in *Analytica EBC* (*12*).

The extraction procedure used was modified from that of Marchylo et al. (*22*) and was done in triplicate for each sample. Ground grain and malt (0.2 g) were extracted sequentially at 60 °C for 30 min with

vortexing at 10 min intervals, followed by centrifugation (10000*g*, 15 min, 4 °C) using 1.2 mL of the following extracting solutions: (1) 0.5 M sodium chloride (twice), (2) water, (3) 50% 1-propanol (HPLC grade, Rathburn) (twice), and (4) 50% 1-propanol containing 1% DTT. The residual samples were extracted overnight at room temperature with 1.2 mL of 8 M urea containing 1% DTT and centrifuged at room temperature (20200*g*, 15 min). The supernatants were stored at −20 °C and passed through 0.45 μm GHP Minispike filters (Pall Gelman Laboratory, Ann Arbor, MI) before RP-HPLC analysis.

RP-HPLC Separation of Hordeins. The protein extracts were analyzed in triplicate with an HT-Alliance 2795 HPLC chromatograph (Waters Associates, Inc., Milford, MA) including a Waters 996 photodiode array detector. A 250 mm × 4.6 mm i.d. SynChropak RP-P column [C18, 300 Å, 6.5 μm particle size (Waters)] preceded by a 4.0 μm Novapak C18 Guard-Pak precolumn (Waters) was used for separation. The column and the sample compartment temperatures were 25 and 20 °C, respectively. The injection volume was 25 μL. Running solvents consisted of water and acetonitrile (HPLC grade, Rathburn), each containing 0.1% trifluoroacetic acid (HPLC grade, Fluka). Elution was carried out by using a gradient extending from 31.2 to 54.0% acetonitrile in 105 min followed by a 20 min washing step. The initial conditions were restored, and the column was equilibrated for 10 min. Flow rate was 1 mL/min, and detection was at 210 nm. Data were collected and analyzed with Millenium[32] software (Waters). Hordein amounts are presented as chromatogram areas in arbitrary units (AU).

Analysis of Grain Microstructure. Twenty grains of each sample were prepared for microstructure analysis. A 2−3 mm thick section was cut from the middle of each grain, fixed in 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0), dehydrated in a graded ethanol series, and embedded in hydroxyethyl methylacrylate as recommended by the manufacturer (Leica Historesin embedding kit, Heidelberg, Germany). Polymerized samples were sectioned (2 μm sections) in a rotary microtome HM 355 (Microm Laborgeräte GmbH, Walldorf, Germany) using a steel knife. The sections were transferred onto glass slides and stained with Acid Fuchsin and Calcofluor White or Light Green and Lugol's iodine solution (*23*−*25*).

Acid Fuchsin and Calcofluor White. Sections were pretreated in 2,4-dinitrophenylhydrazin and in 0.5% periodic acid for staining. Protein was stained with aqueous 0.1% (w/v) Acid Fuchsin for 1 min (Gurr, BDH Ltd., Poole, U.K.), and β-glucan was stained with aqueous 0.01% (w/v) Calcofluor White for 1 min (fluorescent brightener 28, Aldrich, Germany). In exciting light (excitation, 330−385 nm; fluorescence, >420 nm) intact cell walls stained with Calcofluor appear blue and proteins stained with Acid Fuchsin appear red. Starch is unstained and appears black.

Light Green and Iodine Staining. Protein was stained with aqueous 0.1% (w/v) Light Green for 1 min (Gurr, BDH Ltd.) and starch with 1:10 diluted Lugol's iodine solution (I_2, 0.33%, w/v; and KI, 0.67%, w/v). Light Green stains protein green. Iodine stains the amylose component of starch blue and amylopectin brown.

The samples were examined with an Olympus BX-50 microscope (Tokyo, Japan). Micrographs were obtained using a SensiCam PCO CCD camera (Kelheim, Germany) and the AnalySIS 3.0 image analysis program (Soft Imaging System, Münster, Germany). The micrographs shown were chosen to represent the average of the 20 grains analyzed of each sample.

Statistical Analysis. The statistical significance of the differences between the barley samples representing different endosperm structure as well as the differences in endopeptidase activities, hordein composition, and LTm values between the sample pairs chosen were calculated. Mean values were compared by two-tailed Student *t* test, and the differences were considered to be significant when $p < 0.05$.

RESULTS

The 27 barley samples of four different cultivars (Barke, Luberon, Scarlett, and Wikingett) from different growing sites in Scandinavia were arranged into two groups according endosperm structure as measured with a light transflectance meter. In 2001, samples with LTm mean values >210 mV were classified as "steelier" and those with values <160 mV as

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Table 1. Barley and Malt Characteristics of Samples Classified as Steelier or Mealy in 2001 and 2002[a]

	2001			2002		
	steelier[b] (n = 4)	mealier (n = 6)	c	steelier (n = 11)	mealier (n = 6)	c
barley						
protein (% db[d])	12.0 ± 1.0	9.9 ± 1.3	*	11.4 ± 0.6	10.3 ± 0.6	*
starch (% db)	63.4 ± 1.2	64.7 ± 1.2	ns	63.9 ± 0.7	63.2 ± 1.1	ns
germination capacity (%)	99.0 ± 1.4	99.2 ± 0.8	ns	98.7 ± 0.5	98.7 ± 1.0	ns
grains over 2.8 mm (%)	69.1 ± 11.4	81.6 ± 5.6	*	41.2 ± 15.3	92.0 ± 5.3	*
LTm, mean (mV)	327 ± 66	106 ± 49	*	213 ± 34	52 ± 16	*
malt						
friability (%)	71 ± 10	93 ± 6	*	78 ± 4	91 ± 4	*
soluble N (mg/100 g)	890 ± 52	742 ± 72	*	880 ± 66	746 ± 52	*
free amino N (mg/L)	205 ± 16	176 ± 16	*	221 ± 20	185 ± 16	*
extract (%/average)	83.0 ± 0.7	84.3 ± 1.1	ns	82.7 ± 0.5	84.1 ± 1.1	*
wort color (°EBC)	4.5 ± 0.4	2.8 ± 0.3	*	4.5 ± 0.4	2.8 ± 0.3	*

[a] Results are expressed as means ± SD. [b] Refers to the endosperm structure as measured by LTm. In 2001, samples with LTm mean values >230 mV were classified as "steelier" and those with LTm values <160 mV as "mealy". In 2002, "steelier" samples had LTm values >150 mV and "mealy" samples <100 mV. [c] Statistical significance: ns, not significant; *, significant for $p < 0.05$. [d] Dry basis.

Table 2. Barley Characteristics

	Barke 2001 1A[a]	Barke 2001 1B	Barke 2002 2A	Barke 2002 2B	Scarlett 2002 3A	Scarlett 2002 3B	
moisture (%)	11.7	12.8	9.9	9.7	10.1	9.6	± 0.12[b]
protein (% db[c])	10.8	11.3	11.2	11.0	11.2	10.6	± 0.09
starch (% db)	64.8	64.0	64.2	62.7	64.2	62.5	± 1.62
grains over 2.8 mm (%)	80.9	84.7	47.2	95.4	70.8	97.5	nd[d]

[a] The A samples had a steelier endosperm than the B samples. [b] Standard deviation of the standard malt sample. [c] Dry basis. [d] Not determined.

Table 3. Endosperm Structure of Barley Samples as LTm Values[a]

	Barke 2001 1A (n = 97)	Barke 2001 1B (n = 97)	Barke 2002 2A (n = 96)	Barke 2002 2B (n = 97)	Scarlett 2002 3A (n = 97)	Scarlett 2002 3B (n = 97)
LTm (mV)	233 ± 109	160 ± 110	226 ± 118	64 ± 88	239 ± 102	73 ± 74
mealy grains[b] (%)	46	71	43	91	42	94
steely grains[c] (%)	25	10	31	5	24	3

[a] Results are expressed as means ± SD. For differentiation of A and B samples, see **Table 2**. [b] LTm value < 200 mV. [c] LTm value ≥ 300 mV.

"mealy". In 2002, barley samples were in general mealier than in 2001, and therefore samples with an LTm value >150 mV were included in the "steelier" group and those with an LTm mean value <100 mV were classified as mealy. As a result, four samples were classified as steelier and six samples as mealy in 2001. In 2002, 11 samples were classified as steelier and six samples as mealy.

Endosperm structure was related to several barley and malt characteristics (**Table 1**). The steelier barley samples had higher protein content, smaller grain size, lower friability, darker wort color, higher soluble nitrogen content, and higher free amino nitrogen content than the mealy barley samples. All of these differences were statistically significant at the 95.0% confidence level ($p < 0.05$). Some of the malting quality parameters, such as grain size, were also affected by the cultivar (data not shown). However, in general, the effect of growth site was much stronger than that of the cultivar. The majority of the analysis parameters, especially endosperm structure, are greatly affected by protein content. To eliminate the effect of protein content only three barley sample pairs with similar protein contents of 11% were studied in detail. Three steelier barley samples (A) and three mealy barley samples (B) were selected as follows: Barke 2001

Table 4. Moisture after Steeping and Weight Losses in Malting Process[a]

	Barke 2001 1A	Barke 2001 1B	Barke 2002 2A	Barke 2002 2B	Scarlett 2002 3A	Scarlett 2002 3B
moisture (%)	41.4	41.7	44.1	44.8	45.8	45.6
respiration loss (%)	5.4	4.0	9.1	3.2	5.2	4.2
loss in rootlet removal (%)	3.3	3.7	4.5	4.7	3.8	4.2
total weight loss	8.6	7.7	13.6	7.9	9.0	8.4

[a] A and B samples as in **Table 2**.

(samples 1A and 1B), Barke 2002 (2A and 2B), and Scarlett 2002 (3A and 3B).

Barley Analyses. Despite the similar protein contents (**Table 2**), the differences in LTm values were significant ($p < 0.001$) between barley sample pairs chosen. Samples 1B, 2B, and 3B consisted mostly of grains with a mealy endosperm, whereas samples 1A, 2A, and 3A were less homogeneous and had a steelier endosperm structure (**Table 3**). On average, the mealy B samples had slightly lower starch contents than the steelier A samples.

Table 5. Malt Characteristics[a]

	Barke 2001 1A	Barke 2001 1B	Barke 2002 2A	Barke 2002 2B	Scarlett 2002 3A	Scarlett 2002 3B	
friability (%)	85	92	86	92	78	90	± 1.02[b]
modification (%)	96	99	97	97	92	95	± 3.25
homogeneity (%)	81	93	84	85	76	83	± 4.86
protein (% db[c])	10.4	10.8	11.3	10.4	11.2	10.1	± 0.17
soluble N (mg/100 g)	828	741	910	719	866	815	± 0.76
Kolbach index (% soluble protein/total protein)	50	43	50	43	48	50	± 0.91
free amino N (mg/L)	193	160	237	167	220	199	± 3.62
α-amylase (units/g db)	240	216	340	255	306	314	± 13.2
β-amylase (units/g db)	942	984	941	943	745	678	± 45.1
total limit dextrinase (APU/kg db)	491	477	689	553	600	530	nd[d]
free limit dextrinase (APU/kg db)	61	45	69	44	71	68	nd
xylanase (VU/g db)	28 ± 0.4	11 ± 0.5	41 ± 2.2	11 ± 0.4	46 ± 3.6	16 ± 1.5	nd
β-glucanase 30°C (units/g db)	518	505	573	524	590	590	± 19.1
extract (%/average)	83.6	82.9	82.5	82.1	83.5	84.8	± 0.3
wort β-glucan (mg/L)	142	72	88	219	255	186	± 20.81
wort color (°EBC)	4.4	2.5	4.7	2.5	4.4	2.8	± 0.12
wort viscosity (mPa.s)	1.45	1.46	1.42	1.47	1.46	1.49	± 0.01

[a] A and B samples as in **Table 2**. [b] Standard deviation of the standard malt sample. [c] Dry basis. [d] Not determined.

Malting Process, Malt Characteristics, and Enzyme Activities. The steelier barley samples showed higher respiration losses but lower losses due to rootlet removal than the mealy samples (**Table 4**). The total weight loss was higher in steelier samples compared with mealy samples. The moisture contents determined after steeping did not show any differences between mealy and steelier samples.

Results of malt analyses are presented in **Table 5**. The friabilities of the mealy samples were higher than those of the steelier ones. The differences in homogeneity and Calcofluor modification were less evident. During malting, the protein content of the mealy barley samples decreased slightly more than that of the steelier samples. However, malts produced from steelier barley contained more soluble and free amino nitrogen. The degree of proteolysis was also reflected in the wort color, which was higher for the steelier samples. In Barke, α-amylase, β-glucanase, and xylanase activities were slightly higher in the steelier than in the mealy sample pairs. In all steelier samples

examined, the total and free limit dextrinase activities were higher than in the mealy samples, whereas differences in β-amylase activities were ambiguous. Wort viscosity was lower for all steelier samples, but the extract and the β-glucan content of the wort did not correlate with steeliness.

Total endopeptidase activities of unmalted barley samples were very low, and the differences between samples were not statistically significant (data not presented). Even though activities of different malts showed similar patterns, there were noticeable and statistically significant ($p < 0.01$) differences between mealy and steelier Barke samples (**Figure 1**). The specific cysteine proteinase inhibitor E-64 reduced greatly the total endopeptidase activities (**Figure 2**). Differences in E-64-inhibited activities between sample pairs 2A and 2B and 3A and 3B were statistically significant ($p < 0.001$).

Hordein Patterns. Total hordein amounts of the samples varied slightly, showing no clear correlation with steeliness (**Figure 3A,B**). The steelier barley samples 2A and 3A contained significantly more hordein than their mealy sample pairs ($p < 0.01$ for both sample pairs). B hordein was present in all barley
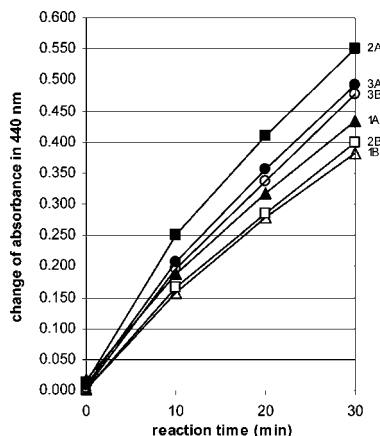

Figure 1. Total endopeptidase activities in malt samples (mean, $n = 3$). Standard deviations of absorbance values varied between 0.001 and 0.010. Samples: (▲) 1A Barke 2001; (△) 1B Barke 2001; (■) 2A Barke 2002; (□) 2B Barke 2002; (●) 3A Scarlett 2002; (○) 3B Scarlett 2002. The steelier A samples are indicated by solid symbols and the mealy B samples by open symbols.
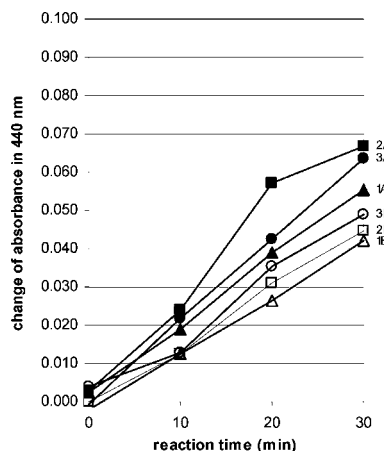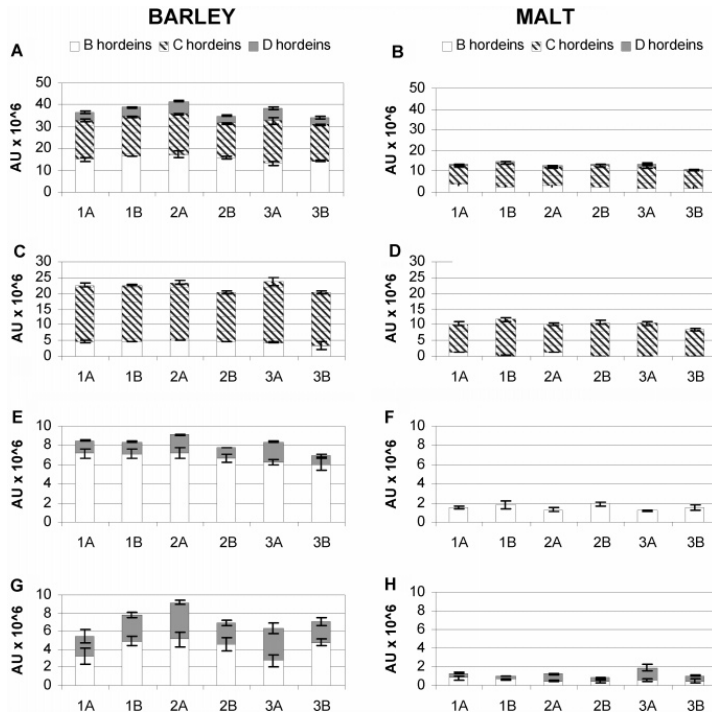

Figure 2. E-64 inhibited endopeptidase activities in malt samples (mean, $n = 3$). Standard deviations of absorbance values varied between 0.000 and 0.003. Samples are as in **Figure 1**.

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Figure 3. Total hordein composition in barley and malt samples and hordein composition in the extraction fractions in arbitrary units (AU) (means, $n = 9$): (**A**, **B**) total hordein composition; (**C**, **D**) propanol-soluble hordeins; (**E**, **F**) propanol–DTT-soluble hordeins; (**G**, **H**) urea–DTT-soluble hordeins. Samples: 1A Barke 2001, 1B Barke 2001, 2A Barke 2002, 2B Barke 2002, 3A Scarlett 2002, and 3B Scarlett 2002. The A samples had a steelier endosperm structure and the B samples a mealy structure.
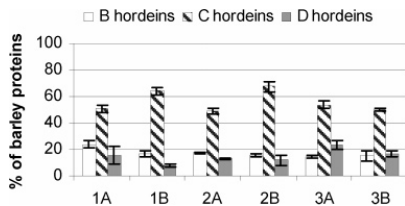
Figure 4. Hordeins in malt samples as proportions of respective barley hordein amount (means, $n = 9$). Samples are as in **Figure 3**.

and malt extracts, and C hordein was present only in propanol extracts. D hordein was present in propanol–DTT and urea–DTT extracts of barley samples but only in urea–DTT extracts of malt samples (**Figure 3C–H**). Some differences in the solubility of hordeins were observed between steelier and mealy samples. The steelier barley samples 2A and 3A contained more C hordein ($p < 0.01$ and $p < 0.05$, respectively), and both propanol–DTT-soluble ($p < 0.001$ for both sample pairs) and urea–DTT-soluble ($p < 0.01$ and $p < 0.05$, respectively) D hordein than their mealy sample pairs. Differences in B hordein solubilities were not significant.

During malting, C hordein was proportionally the least degraded hordein in all samples, whereas D hordein was the most degraded during malting in all Barke samples and B hordein was the most degraded in Scarlett samples (**Figure 4**). Steelier Barke malt samples contained significantly more D hordein ($p < 0.05$ and $p < 0.01$, respectively) and B hordein ($p < 0.05$ for both) than their mealy sample pairs. The difference in D hordein amounts in Scarlett malt samples was not

statistically significant. In the steelier Barke malt samples equal amounts of B hordein were present in the propanol extracts, whereas in the other malt samples propanol-soluble B hordein was hardly present (**Figure 3D**). C hordein amounts were lower in steelier Barke malt samples ($p < 0.01$ and $p < 0.05$, respectively), but higher in steelier Scarlett samples ($p < 0.01$) than in their corresponding mealy sample pairs.

Grain Microstructure. On the basis of the visual examination of 20 grains of each sample, there was a clear difference in the structure of cell walls of starchy endosperm between the two cultivars Barke and Scarlett. In Barke barley (**Figure 5A**) the cell walls in the starchy endosperm appeared to be thinner than in Scarlett and usually already partially degraded in the area surrounding the crease. In Scarlett (**Figure 5B**) the cell walls appeared to be thicker and the grains had an even cell wall thickness throughout the endosperm. No systematic differences in cell wall structure of aleurone layer or starchy endosperm were observed between the mealy and steelier pairs of barley grains (**Figure 5C,D**). The packing of endosperm cells with starch granules and protein matrix was also similar in all samples, and no systematic differences between the two cultivars or the mealy and steelier sample pairs could be seen (**Figure 5E,F**). The subaleurone cells contained mostly small, B-type starch granules, whereas the cells of the middle part of the endosperm were evenly packed with small and large starch granules surrounded by protein matrix.

DISCUSSION

A large barley sample collection grown in 2001 and 2002 was analyzed for its barley and malt characteristics, and six of
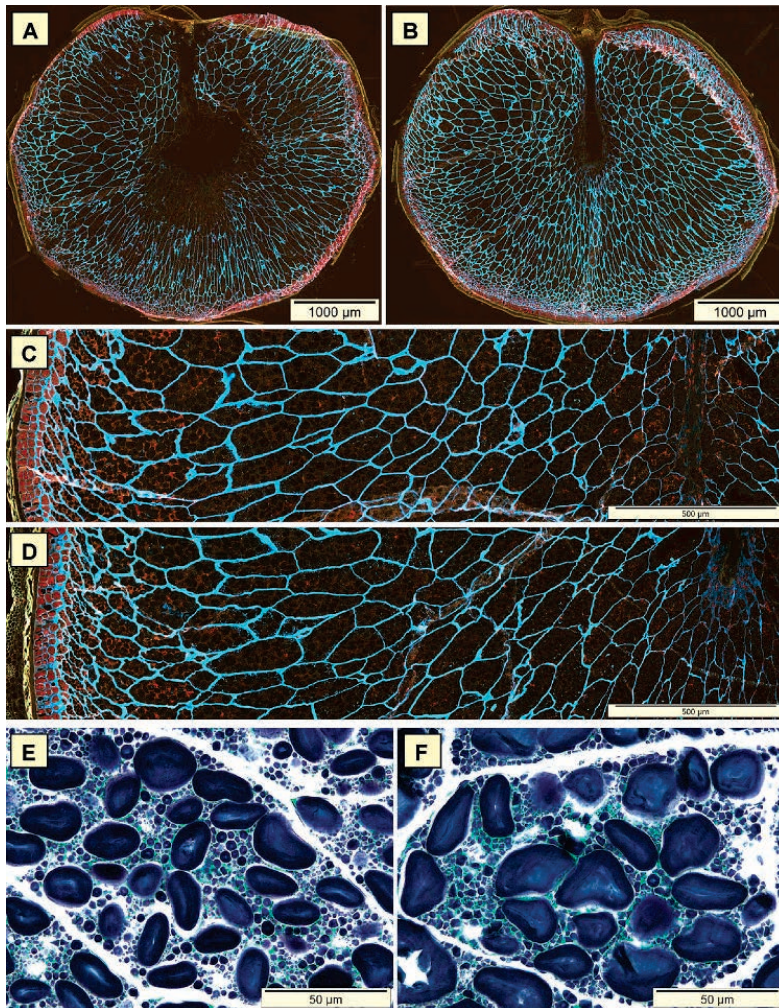
Figure 5. Micrographs of cross sections of the barley samples: (**A**–**D**) stained with Acid Fuchsin and Calcofluor to show protein (red) and cell walls (light blue); (**E**, **F**) stained with Light Green and iodine to show protein (green) and starch (blue-violet); (**A**) 1B Barke 2001; (**B**) 3B Scarlett 2002; (**C**) 3A Scarlett; (**D**) 3B Scarlett 2002; (**E**) 2A Barke 2002; (**F**) 2B Barke 2002.

the samples were chosen for further analysis. According to our results the endosperm structure measured by LTm analysis is an essential factor of malting performance as mealy and steelier samples consistently had different characteristics in several analyses. Steeliness was related to higher starch content, even though this correlation was not observed in results concerning the whole sample collection. This difference in starch content could not be detected in the microstructure, but it may be a consequence of the denser packing of a steelier than a mealy endosperm.

All samples were micromalted using the same malting schedule. The weight loss during the malting process showed that even though the steelier samples had a higher respiration rate and a higher total malting loss, the growth of their rootlets was lower than in the mealy samples. Recently, low respiratory losses have been linked to a low grain protein content (*26*), but our results suggest that endosperm structure may also affect respiratory losses. Apparently the high respiration rate of steelier barley was not related to the growth of the embryo but possibly

to an increased requirement of energy for endosperm degradation caused by the tight structure of endosperm. Wallwork et al. (*27*) reported higher total malting loss for heat-treated barley than controls.

The malts made of steelier barley were less modified than the mealy samples, on the basis of friability parameters. The Barke samples could, however, all be defined as well modified, whereas the steelier Scarlett sample was slightly undermodified according to the friability values. The corresponding difference in Calcofluor modification was slightly smaller, and there was no difference at all between mealy and steelier Barke 2002 samples. Whereas the Calcofluor method is based on the detection of unhydrolyzed β-glucan, the friability test measures the hardness of the malted grain. It may be concluded that malts made of steelier barley had a harder, less friable, structure, although they were not necessarily undermodified in terms of β-glucan degradation. The worts from the steelier samples also had a darker color, which according to Palmer (*28*) is a sign of uneven pattern of modification.

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The average decrease in protein content during malting was larger for the mealy samples. This observation is consistent with earlier studies showing that the hydrolysis of storage proteins proceeds more rapidly in a soft-textured endosperm than in a hard one (7). In germinating barley, the hydrolyzed protein is directed to the growing roots and shoot. Because the rootlets were removed after malting, the decrease in protein content of the mealy samples can be attributed to the more extensive rootlet growth compared with the steelier samples. However, the steelier malt samples contained a higher amount of free amino acids and soluble nitrogen than the mealy samples. The free amino nitrogen and soluble protein were extracted from the malt using a temperature-programmed mash procedure by which the temperature was maintained at 45 °C for 30 min, allowing the action of proteolytic enzymes. The difference in proteolysis between mealy and steelier samples could therefore be a consequence of the mashing procedure as well as the malting procedure. It is possible that the steelier samples contained more slowly hydrolyzable storage proteins that were not hydrolyzed until during mashing. This difference can be only partially explained by the small differences in endopeptidase activities, most of which consisted, expectedly, of cysteine proteinase activities (29). It has been stated that differences in rates of hordein degradation refer more to differences in hordein structures than to the amount of endopeptidases (30). It is also possible that the spatial patterns of endoproteolytic activity development or substrate localization differed between mealy and steelier sample pairs. Additionally, the differences in protein degradation could be caused by differential activity of carboxypeptidases needed for further degradation of peptides (31, 32).

Visually recognized steely grains have been found to contain more nitrogen, especially hordein proteins, than mealy grains (33). However, in this study the mealy and steelier grain samples had equal protein contents, and only minor differences were observed in total protein or hordein contents. Steelier barley and malt samples did not differ systematically in their total hordein composition from mealy samples, but certain differences in hordein classes and solubilities were detected.

Both steelier Barke malt samples contained more D hordein than the corresponding mealy samples. In Scarlett samples this difference was not statistically significant. This result could be associated with the incompelete degradation of D hordein in steely kernels proposed in earlier studies (5). Malted samples contained only urea−DTT-soluble D hordein, although barley samples contained both propanol−DTT- and urea−DTT-soluble D hordein. This may be an indication that propanol−DTT-soluble D hordein is more easily degraded than urea−DTT-soluble D hordein. In addition, there were fewer propanol−DTT- and more urea−DTT-soluble D hordeins left in steelier Barke malt samples, possibly indicating that the D hordeins remaining after malting in steelier samples were those most difficult to degrade. It remains to be explained whether the detected differences in hordein solubilities were a reason for, or a consequence of, the differences in the endosperm structure and malting performance. The properties of storage proteins have indeed been considered to be a more important factor influencing endosperm structure than the total protein content of the grain (4). Both C hordein and gel protein composed of B and D hordeins are suggested to restrict hydration and access of hydrolases (34−37).

Overall, the degradation of hordeins during malting proceeded in the order observed in earlier studies, with D hordeins being hydrolyzed more readily than B hordeins and C hordeins being the most resistant to degradation (22, 38, 39). Barke 2002 and

Scarlett 2002 barley samples had many similarities in hordein composition, whereas, after malting, Barke 2001 and 2002 samples had most in common with each other. This could be an indication of varietal characteristics, which appear in malting performance. For example, differences in hordein degradation patterns can be explained by varietal preferences in hordein degradation during malting. In this study, the D hordein in Barke was more susceptible to degradation than the D hordein in Scarlett. These differences in substrate preferences may be linked to differences in the proteolytic enzyme spectrum. Scarlett was found to lack one proteolytic enzyme activity that was clearly observed in Barke using edestin gel electrophoresis at pH 5.0 (Holopainen and Wilhelmson, unpublished results).

In earlier studies, β-amylase activity has been linked to steeliness and total protein and hordein contents (33). In this study, a relationship between free β-amylase activity and steeliness was not observed. However, the α-amylase and limit dextrinase activities were in general higher in the steelier than in the mealy samples of the large sample collection analyzed (data not shown). The activities of these starch-hydrolyzing enzymes are controlled by gibberellic acid in the germinating grain, and their increased activities may be linked to the higher respiration losses observed in the steelier grains.

The relationship between β-glucanase activity and steeliness was not clear. Recently, malt β-glucanase activity was found to correlate positively with Kolbach index and negatively with viscosity (40). These relationships between malt quality parameters were also observed in this study, but their possible association with grain hardness remains to be determined. Xylanases, hydrolyzing cell wall arabinoxylans, are released from the aleurone cells during the final stage of endosperm mobilization and linked to the gibberellin-controlled programmed cell death of aleurone cells (41−43). Higher activities of xylanases may be an indication of a further proceeded programmed cell death in steely grains. Xylanase activities may also be partly of microbiological origin.

The differences in steeliness were not clearly seen in the microscopic examination of grain microstructure; that is, mealy and steelier barley grains did not differ in cell wall thickness or in packing of endosperm cells. This is consistent with an earlier observation that the modification rate of barley grain does not correlate with cell wall thickness, although thicker cell walls may be more resistant to enzymic degradation than thinner ones (1). However, the differences observed in biochemical analyses between malts of cultivars Barke and Scarlett may be partially explained by the difference in the cell wall thickness. Cell wall degradation before maturation in the area surrounding the crease seems to be a varietal characteristic of Barke. However, its significance for endosperm modification cannot be estimated on the basis of these results.

In conclusion, a clear relationship between the structure of endosperm and the malting quality was observed in this study. Endosperm structure determined by the LTm method proved to be linked to many differences in malting performance. The steelier samples showed higher respiration loss during malting, higher activities of starch-hydrolyzing enzymes, and possibly a further proceeded programmed cell death. Malts made from steelier barley had a less friable structure, with more slowly degradable, urea-soluble D hordein depending on cultivar. Steelier barley produced less root mass during malting, and more free amino nitrogen and soluble protein were released during mashing. Hordein degradation was also at least partially a cultivar-dependent feature. On the basis of these results, it seems that grains with steelier structured endosperms have an equal

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or better capacity to produce enzymes hydrolyzing starch, protein, and cell wall than mealy grains. Despite this, endosperm modification is slower in steelier grains than in mealy samples. The fundamental reason for this difference may lie in the structure or localization of the hordeins as well as the possible effects of endosperm packing on water uptake and movement of enzymes. Further experiments are needed to clarify this aspect.

ABBREVIATIONS USED

AU, arbitrary units; EBC, European Brewing Convention; DTT, dithiothreitol; LTm, light transflectance meter.

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Title	**Composition and structure of barley (*Hordeum vulgare* L.) grain in relation to end uses**
Author(s)	Ulla Holopainen-Mantila
Abstract	Barley (*Hordeum vulgare* L.) is a globally important grain crop. The composition and structure of barley grain is under genotypic and environmental control during grain development, when storage compounds (mainly starch and protein), are accumulated. Grain structure plays a significant role in malting and feed- and food-processing quality of barley. Hordeins, the major storage proteins in barley grains, are centrally located in the endosperm forming a matrix surrounding starch granules, but their role in the structural properties of barley grain is not completely understood. Thus, the main aim of the current study was to demonstrate the role of hordeins in barley grain structure. The dependence of the grain structure on the growth environment, in particular with respect to day-length and sulphur application relevant to northern growing conditions, was studied. The effects of the grain structure on end use properties in milling as well as in hydration and modification during malting were characterized.

The longer photoperiod typical to latitudes in Southern Finland resulted in a C hordein fraction, entrapped by aggregated B and D hordeins, being more deeply located in the endosperm of barley cultivar Barke. Thus the impact of the growing environment on hordein deposition during grain filling was observed both at the tissue and subcellular level. However, the mechanism behind the differential accumulation of C hordein remains unclear. The deeper localization of entrapped C hordein was linked to improved hydration of grains during malting in three barley cultivars. Thus, the role of the subaleurone region in barley grain was found to be significant with respect to end use quality. Moreover, the results suggest that the growing environment affects the end-use properties of barley and that especially the northern growing conditions have a positive impact on barley processing quality.

The influence of sulphur application on hordein composition in the Northern European growing conditions was demonstrated for the first time. Asparagine and C hordein served as nitrogen storage pools when the S application rate was lower than 20 mg S / kg soil, whereas total hordein and B hordein contents increased with higher S application rates. The current study also showed that even when sulphur is sufficiently available in field conditions, the hordein composition may react to sulphur application. The observed sulphur responses were in accordance with those reported earlier for hordein composition. This indicates that the more intensive growth rhythm induced in northern growing conditions does not alter greatly the effect of sulphur on grain composition.

The current study confirmed that the main grain components: starch, protein and -glucan, influence grain processing properties including milling, hydration and endosperm modification. However, their influence on endosperm texture (hardness or steeliness), which also affects the performance of barley grains in these processes, cannot be directly derived or estimated on the basis of the grain composition. The results obtained suggest that hordeins should also be taken into account in the evaluation of the processing behaviour of barley grains. |
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Nimeke	**Ohran (Hordeum vulgare L.) jyvän rakenteen ja koostumuksen yhteys käyttötarkoituksiin**
Tekijä(t)	Ulla Holopainen-Mantila
Tiivistelmä	Ohra (*Hordeum vulgare* L.) on maailmanlaajuisesti tärkeä viljelykasvi. Ohran jyvän koostumus ja rakenne muodostuvat jyvän kehityksen aikana perimän ja ympäristön ohjaamina, kun varastoaineet, pääasiassa tärkkelys ja proteiini, kertyvät jyvään. Jyvän rakenteella on tärkeä rooli ohran mallastus-, rehu- ja elintarvikekäytössä. Hordeiinit, ohran jyvän pääasialliset varastoproteiinit, sijaitsevat keskeisellä paikalla endospermisolukossa tärkkelysjyvästen ympärillä. Hordeiinien merkitystä ohran jyvän rakenneominaisuuksille ei kuitenkaan täysin ymmärretä. Tämän tutkimuksen päätarkoitus oli osoittaa, miten hordeiinit vaikuttavat jyvän rakenteeseen. Tutkimuksessa selvitettiin jyvän rakenteen riippuvuutta kasvuympäristöstä, erityisesti liittyen pohjoisille kasvuolosuhteille tyypilliseen päivänpituuteen ja rikkilannoitukseen. Jyvän rakenteen vaikutusta sen loppukäyttöominaisuuksiin tutkittiin jauhatuksessa sekä jyvän vedenotossa ja rakenteen muokkautumisessa mallastuksen aikana. Etelä-Suomen leveyspiireille tyypillinen pitempi päivänpituus tuotti Barke-lajikkeessa aggregoituneiden B- ja D-hordeiinien ympäröimän C-hordeiinijakeen, joka sijaitsi syvemmällä endospermissä. Siten kasvuympäristön vaikutus hordeiinien sijoittumiseen jyvän täyttymisen aikana havaittiin sekä solukkotasolla että solujen sisällä. C-hordeiinin poikkeavan kertymisen aiheuttava mekanismi ei kuitenkaan ole selvä. Kolmella ohralajikkeella C-hordeiinin sijainti syvemmällä jyvässä oli yhteydessä jyvien parempaan vedenottoon mallastuksessa. Tulokset korostavat ohran jyvän subaleuronikerroksen merkitystä suhteessa jyvän loppukäyttölaatuun. Lisäksi tulosten perusteella kasvuympäristö vaikuttaa ohran loppukäyttöominaisuuksiin ja erityisesti pohjoisilla kasvuolosuhteilla on myönteinen vaikutus ohran prosessointilaatuun.

Tässä työssä osoitettiin ensimmäistä kertaa rikkilannoituksen vaikutus ohran jyvän hordeiinikoostumukseen Pohjois-Euroopan kasvuoloissa. Asparagiini ja C-hordeiini varastoivat typpeä ohran jyvässä, kun rikkilannoitus oli matalampi kuin 20 mg S / kg maata. Korkeammilla rikkilannoitustasoilla kokonais- ja B-hordeiinipitoisuudet puolestaan nousivat. Tutkimuksessa näytettiin, että rikkilannoitus voi vaikuttaa hordeiinikoostumukseen pelto-olosuhteissa, vaikka rikkiä olisikin riittävästi saatavilla. Havaitut rikkivasteet olivat yhdenmukaisia aiemmin raportoitujen tulosten kanssa. Tämä osoitti, että pohjoisten kasvuolosuhteiden kiivaampi kasvurytmi ei muuta merkittävästi rikin vaikutusta jyvän koostumukseen.

Tutkimus vahvisti, että jyvän pääkomponentit, tärkkelys, proteiini ja -glukaani, vaikuttavat jyvän prosessointiominaisuuksiin jauhatuksessa sekä mallastuksen aikaisessa kostumisessa ja muokkautumisessa. Näiden komponenttien vaikutus endospermin rakenteeseen (kovuuteen tai lasimaisuuteen) ei kuitenkaan ole pääteltävissä suoraan jyvän koostumuksesta. Saatujen tulosten perusteella hordeiinit tulisi huomioida ohran jyvien prosessikäyttäytymisen arvioinnissa. |
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Composition and structure of barley (*Hordeum vulgare L.*) grain in relation to end uses

Today, barley (*Hordeum vulgare* L.) is a globally significant crop plant. Barley grains are mainly exploited as feed or as a raw material for malt production, but the use of barley as a food ingredient is increasing. Grain structure is known to play an important role in processing quality of barley. The composition and structure of barley grain are formed under genotypic and environmental control during grain development, when storage compounds, mainly starch and protein, are accumulated.

Hordeins, the major storage proteins in barley grains, are centrally located in the endosperm forming a matrix surrounding starch granules. However, their significance for the structural properties of barley grain is not completely understood. Thus, the main aim of this thesis was to demonstrate the role of hordeins in barley grain structure. The dependence of the grain structure on the growth environment, in particular with respect to day-length and sulphur application relevant to northern growing conditions, was studied. Furthermore, the effects of the grain structure on end use properties in milling as well as in hydration and modification during malting were characterized.