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Protein mobilization and malting-specific proteinase expression during barley germination☆

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ABSTRACT

Malting is a process of controlled germination and early seedling growth. With appropriate control of grain moisture, environmental conditions, germination time, and kilning conditions, maltsters produce malt with the composition and enzymatic activity needed for brewing. In this study we compared protein mobilization in a widely grown malting barley variety germinated under controlled malting regimens and in a laboratory incubator not optimized for malting. Analysis of malts produced under three regimes showed differences in nitrogen mobilization and expression profiles of some proteinase genes. Many transcript probes changed in abundance during malting, with transcripts from the commercial malting and micromalting series trending similarly. Fewer transcripts showed differential expression between the laboratory germination series and the malting series that corresponded to protein mobilization differentials. Expression differentials that matched protein mobilization differences were seen for previously identified germination proteinases as well as those not previously linked to seed germination. These expression differentials suggest that several proteinases may serve previously unrecognized functions in protein mobilization during germination and early seed growth. Similarity of malting quality results and gene expression profiles in the two malting environments suggest that barley germination in an optimized micromalter is a suitable model system for barley germination in a commercial malting process.

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1. Introduction

Seed germination is a coordinated sequence of events involving hydration of the dried seed, activation of cellular metabolism, followed by synthesis, secretion, and relocation of hydrolytic enzymes to storage and structural compartments in the seed, and subsequent degradation of seed macromolecules by newly synthesized and stored hydrolases. Degradation products are subsequently used by the embryonic axis to support growth of seedling shoots and roots. Since barley grain contains substantial amounts of starch, sugars are the predominant product formed during germination, followed by amino acids/peptides, cell wall fragments, and other minor components.

Malting is a process of controlled, partial germination of cereal seeds involving hydration (steeping), incubation in an environment conducive to seed germination and early seedling growth, and then heating and drying (kilning). Kilning stabilizes the partially germinated grain when it has reached the desired germination endpoint. Through selection of barley germplasm and careful control of the germination environment, maltsters are able to reliably produce malt with finely tuned characteristics. Experience and technological improvement have allowed maltsters to develop methods that yield a product with the desired composition of enzymes and solutes as well as an appropriate physical state that together meet the needs of their brewing customers.

Among germination-related hydrolytic processes, starch degradation has been most extensively studied. Starch hydrolysis generates 90–92% of solutes in wort (an aqueous extract of partially-germinated barley) with proteolysis contributing approximately 4% to wort solutes (Meilgaard, 1999). Starch hydrolysis is a relatively simple process effected by four enzymes (α-amylase, β-amylase, α-glucosidase, and limit dextrinase) acting

Abbreviations: CM, germination in a commercial malthouse; MM, germination in a micromalter; LG, germination in a laboratory incubator; DS, dry seed; OS, out-of-steep; D1 – D5, days (24 h increments) 1–5 after transfer of hydrated grain to germination conditions; QA, malting quality analysis; S/T, ratio of wort soluble protein: malt total protein; CCRU, Cereal Crops Research Unit; AMBA, American Malting Barley Association; DP, Diastatic power.

☆ Mention of trade names or commercial products in this paper is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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upon two forms of starch (amylose and amylopectin). Both the enzymes and substrates are well characterized biochemically and genetically. In contrast, proteolytic activities remain complex and unresolved. Zhang and Jones (1995) identified more than 40 proteinase activities in malt using 2-D zymography. While this is a large number compared to the number of starch-degrading enzymes, it is only a fraction of the total proteinase genes identified in the *Arabidopsis* or rice genomes, 826 and 678 proteinase genes respectively (van der Hoorn, 2008), with barley likely containing similar numbers. The range of potential proteinase substrates in the germinating barley grain is similarly large, with Finnie and Svensson (2009) identifying several hundred proteins in malt via proteomic analysis. With a few exceptions (Hordein degradation by some cysteine-class proteinases and β-amylase degradation by several serine-class proteinases (see below)), most studies on malt proteinases have used either synthetic peptides or model proteinase substrates (frequently readily available/readily digestible animal proteins such as casein or gelatin) such that details of malt proteolytic processes (substrates, specific proteinases involved, and potential regulation) are largely unknown.

Early work on peptide hydrolyzing enzymes (peptidases and endoproteinases) in malt largely focused on terminal carboxy- and amino-peptidases (Burger et al., 1968, Moeller et al., 1970, Mikola et al., 1972), identifying a number of enzymes in barley and especially barley malt that hydrolyzed C- and N-terminal amino acids from defined peptide substrates. With the recognition that complete protein hydrolysis to constituent amino acids most likely involved both initial endoproteolytic cleavage followed by subsequent action of terminal peptidases, research efforts included endoproteinases as well. Of the barley seed endoproteinases, the best characterized are the cysteine endoproteinases EP-A and EP-B (Koehler and Ho, 1988, 1990) and related enzymes (Poulle and Jones, 1988; Zhang and Jones, 1996). These cysteine proteinases have been shown to digest Hordein, the primary barley storage protein (Phillips and Wallace, 1989; Koehler and Ho, 1990), as well as other barley proteins. Cysteine endoproteinases are also involved in activation of β-amylase through a partial proteolysis of a low activity β-amylase preprotein to form a more active carbohydrase (Guerin et al., 1992), and have been suggested to activate limit dextrinase (Sissons, 1996). These proteinases have been characterized in detail, to the level of their substrate preferences at their active sites (Davy et al., 1998). Other barley proteinases have also been studied to lesser extents, with their roles in protein mobilization during malting correspondingly less well understood.

A second thiol peptidase, Aleurain, found in germinating barley, has been localized to the barley aleurone layer vacuoles, and is thought to be an aminopeptidase involved in processing of proteins secreted from the aleurone into the endosperm. As such, it is not thought to be directly involved in bulk protein mobilization from the endosperm during malting but rather to play an indirect role in protein mobilization through its involvement in secretion of protein hydrolases from the aleurone into the endosperm (Rogers et al., 1985; Holwerda et al., 1990; Holwerda and Rogers, 1992; Bethke et al., 1996).

A barley seed aspartic proteinase has been identified and localized in a number of barley tissues, although its expression is not responsive to gibberellic acid. It has been speculated to be involved in regulatory processes due to its limited hydrolytic specificity (Runeberg-Roos et al., 1991; Sarkkinen et al., 1992; Kervinen et al., 1993; Tormakangas et al., 1994).

Several isoforms of the metalloproteinase class of enzymes have been described (Fontanini and Jones, 2001) and have been implicated in proteolysis during mashing (Jones and Budde, 2005). The enzymes have not been fully characterized with regard to their physiological substrates and roles in establishing malt quality.

Two distinct serine endoproteinases have been purified and characterized (Terp et al., 2000; Fontanini and Jones, 2002). Both studies used model substrates, and did not identify *in vivo* substrates for the two serine endoproteinases. Jones and Budde (2005) reported little effect of addition of chemical inhibitors of serine-class endoproteinases during mashing on the levels of soluble protein in the resultant worts, and suggested at best a minor role in contributing to malting quality. In contrast, we have recently shown that several serine-class endoproteinases can degrade barley malt β-amylase (Schmitt and Marinac, 2008), a key carbohydrate degrading enzyme, although the extent to which this influences final malting quality is unclear.

In contrast to the classic target-oriented studies described above, several recent studies have used a different approach, microarray-based gene expression analysis, to identify barley genes linked to malting quality. The Affymetrix Barley1 gene chip (Close et al., 2004) allows parallel analysis of expression levels for over 22,000 probes derived from multiple international sources of >350,000 barley ESTs (from 84 cDNA libraries) and 1145 barley gene sequences. Use of the Barley1 gene chip allows sensitive analysis of differences in gene expression patterns and linkage of those expression differences to various treatments, physiological states, and pedigrees. Among the studies using the Barley1 gene chip, recent studies have found links between transcript levels for several proteases and malting quality. Potokina et al. (2004, 2006) reported that a QTL for malting quality (diastatic power, DP) coincided with a serine carboxypeptidase I (*Cxp1*) structural gene and its *cis* regulatory sequence. The authors also found links between a cysteine-class endoproteinase and several measures of malting quality. Lapitan et al. (2009) linked both the cysteine proteinase EP-B1 precursor and a serine carboxypeptidase 3 probe to DP. Munoz-Amatriain et al. (2010a) found that a cucumisin-like serine endoproteinase (Contig13847_s_at) was differentially expressed between winter-hardy and spring malting barleys. In another study, the same subtilisin-type serine probe (Contig13847_s_at) was strongly associated with malting quality (malt extract) improvements in newer malting barley varieties in the University of Minnesota breeding program when compared with older lines in the same program (Munoz-Amatriain et al., 2010b). These data suggest that several serine-class proteinases have a positive, although mechanistically unknown, effect on carbohydrate mobilization.

In this study, we use an analogous approach in following the expression patterns of a large number of barley genes as a common malting barley variety, Robust, germinates and undergoes early seedling growth in three growth regimes. Two of the environments, a commercial malthouse malting and a laboratory micromalter, have been optimized to produce barley malt, a partially germinated barley grain with specific properties meeting the needs of the brewing industry. The third environment, a laboratory incubator, was not controlled to produce desired malt specifications but rather was representative of conditions used in many fundamental studies of physiological or molecular biological processes occurring in germination and early seedling growth. Through this approach, we examine how the processes that lead to the specific malt phenotype may differ from those occurring under conditions used for examining traditional (non-malting) seed germination. Specifically, we compare expression profiles and malting quality metrics measured on the germinated grain from the three environments.

2. Materials and methods

2.1. Plant material

"Robust" barley, a 6-rowed malting variety, was obtained from a commercial lot produced at the Benson-Quinn Farms (Sisseton, S.D.).