Genetic Variation in Barley (1→3,1→4)-β-Glucan Endohydrolases

Submitted by

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Abstract

Complete starchy endosperm cell wall degradation of (1→3,1→4)-β-glucan (β-glucan) during malting is essential to prevent problems associated with high wort viscosity and chill haze in beer. Cell wall β-glucan is hydrolysed by two (1→3,1→4)-β-glucanase (β-glucanase) isoforms in the early stages of germination. The EI and EII β-glucanase isoenzymes are relatively thermolabile resulting in significant activity losses during kilning and complete activity loss during mashing at high temperatures. The current study aimed to identify and characterise novel β-glucanase alleles in elite and exotic germplasm conferring increased thermostability.

An allele mining approach was selected to examine 57 *Hordeum vulgare* ssp. *spontaneum* accessions from Israel and 80 elite varieties sourced globally. The exon regions of the two β-glucanase genes *HvGlb*1 and *HvGlb*2 were amplified and sequenced identifying five new EI allozymes and 13 new EII allozymes. Significantly more allelic variation was identified in *HvGlb*2 predominantly from *H. spontaneum* accessions. Changes in enzyme structure and stability caused by amino acid substitutions were examined using predictive modelling techniques to prioritise alleles for biochemical characterisation. Two EI and four EII allozymes identified in *H. spontaneum* were predicted as likely to possess increased thermostability and were selected for biochemical analysis.

Selected alleles were heterologously expressed in *Escherichia coli* to produce tagged recombinant proteins that were purified for use in kinetic and thermostability assays. Allozyme characterisation identified no significant difference in catalytic efficiency for EI or EII and no improvement in EI thermostability. The four EII alleles predicted to confer increased thermostability exhibited significantly more residual β-glucanase activity following five minutes of heat treatment. However, only one EII allozyme, EII-l, exhibited increased β-glucanase activity at elevated temperatures. The functional significance of the three amino acid differences between the novel β-glucanase allozyme and the reference EII-a were examined in combinatorial mutations of EII-a using site-directed mutagenesis. Two of the three amino acids were shown to be responsible for the increase in β-glucanase thermostability.

EII-a and EII-l were further examined in conditions similar to commercial processes to validate the increase in EII thermostability conferred by the EII-l allele. Recombinant β-glucanase activity was examined in a simulated barley mash at 65°C to mimic commercial mashing conditions. Additionally, the irreversible thermal inactivation of the endogenous β-glucanase activity in green malt from an F_2 population derived from elite by wild barley cross was also assayed. EII-l consistently demonstrated approximately 10% more residual β-glucanase activity than the reference EII-a in both experiments. Therefore, EII-l is a promising new resource for genetic improvement of barley β-glucanase and a viable target for routine selection in the development of new malting varieties.

Expected Publications

Lauer, J.C., Cu, S., Burton, R.A., Eglinton, J.K., (Under Review). Variation in barley (1→3,1→4)-β-glucan endohydrolases reveals novel allozymes with increased thermostability. Theor Appl Genet.

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Lauer, J.C., Cu, S., Burton, R.A., Eglinton, J.K., (To be submitted). Validation of Novel Barley (1→3,1→4)-β-Glucan Endohydrolase Allele Confer Increased Enzyme Thermostability. J. I Brewing.

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Abbreviations and Acronyms

Å	Angstrom
AGRF	Australian Genome Research Facility
AMY	*Bacillus amyloliquefaciens*
ANOVA	Analysis of variance
ASBC	American Society of Brewing Chemists
BC	Backcross
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
cv.	Cultivar
EI	(1→3,1→4)-β-Glucan Endohydrolase isoenzyme 1
EII	(1→3,1→4)-β-Glucan Endohydrolase isoenzyme 2
EBC	European Brewery Convention
GA	Gibberellic acid
GBS	Genotyping-by-sequencing
GM	Genetically modified
HMW	High molecular weight
Hv	*Hordeum vulgare*
ICARDA	International Centre for Agricultural Research in the Dry Area
INDEL	Insert-deletion
IOB	Institute of Brewing
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria broth
LSD	Least significant difference
MAC	*Bacillus macerans*
MAS	Marker assisted selection

MBIBTC	Malting and Brewing Industry Barley Technical Committee
M_r	Molecular weights
NIL	Near isogenic line
ORF	Open reading frame
PCR	Polymerase Chain Reaction
pHABAH	*p*-hydroxybenzoic acid hydrazide
pI	Isoelectric points
QTL	Quantitative trait loci
SNP	Single nucleotide polymorphism
T_{50}	Half-life at elevated temperature
UNEAK	Universal Network Enabled Analysis Kit
ΔG	Gibbs free energy

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Degradation of (1→3,1→4)-β-glucan during endosperm modification is essential for malt quality and brewing practices. Residual malt β-glucan impacts the brewing process by increasing wort viscosity, contributing to reduced filtration efficiency (Bamforth 2000; Ullrich, 2011). (1→3,1→4)-β-Glucan endohydrolase is responsible for the hydrolysis of β-glucan, however natural variation in the β-glucanase enzyme is unexplored as an opportunity to improve barley quality. This analysis of current literature summarises the role of β-glucanase in malting and brewing. The malting process is described with specific focus on endosperm modification and cell wall degradation. The impact of β-glucanase in the mashing stage of the brewing processes is explained. The biochemistry, genetics and quantitative trait loci (QTL) mapping of variation in β-glucanase activity are reviewed. Finally, the success and limitations of previous research investigating β-glucanase for malt and brewing quality improvement is examined.

1.2 Barley grain structure

Knowledge of the internal structure of the barley grain is important for understanding germination processes. The major grain components are the husk, starchy endosperm, aleurone layers, scutellar epithelium and the embryo. The external husk encloses the mature grain protecting the internal components from the environment (Figure 1-1). Lining the inside of the husk is the aleurone tissue that surrounds the starchy endosperm. The aleurone is composed of three layers of living cells which synthesise hydrolytic enzymes required for degrading the starchy endosperm (Bacic and Stone, 1981). The scutellar epithelium layer also synthesises hydrolytic enzymes and separates the starchy endosperm from the embryo.

During germination the hydrolytic enzymes begin to degrade the starchy endosperm releasing sugars and amino acids that are translocated to the growing embryo. The starchy endosperm is compartmentalised by a network of cell walls that enclose the starch and protein matrix. The endosperm cell walls must first be degraded to allow the synthesised enzymes access to starch and protein.

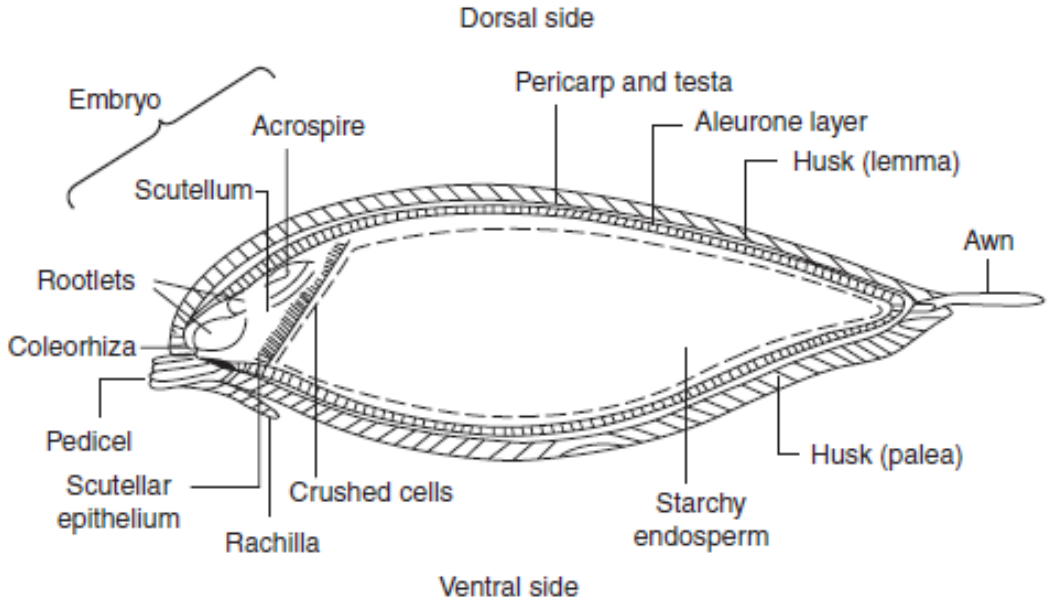

Figure 1-1. The major components of barley grain (MacLeod, 2004).

1.2.1 Endosperm cell walls

Cell walls provide the skeletal framework and intercellular cohesiveness in the starchy endosperm. The endosperm cell walls predominantly consist of the two polysaccharides arabinoxylan and (1→3,1→4)-β-glucan (β-glucan). Minor cell wall components are also present including cellulose, glucomannan and proteins (Fincher, 1975). Arabinoxylans are abundant in the aleurone layer, contributing to ~71% of the walls, compared to ~26% β-glucan (Fincher, 1975). However, in the starchy endosperm β-glucan and arabinoxylan contribute ~70% and ~ 20% to walls respectively and thus rapid and complete degradation of β-glucan is desired by maltsters and brewers (Bacic and Stone, 1981).

1.2.2 Cell wall (1→3,1→4)-β-glucans

β-Glucans are water soluble linear polymers of differing size and structure (Woodward et al., 1983b). The long chain polysaccharides are formed by (1→4)- and (1→3)-linkages of β-glucopyranosyl monomers at a ratio of 2.2-2.6:1 (Fincher and Stone, 2004). Generally (1→4)-β-glucosyl residues are polymerised in sections of two or three and are interrupted by individual (1→3)-β-glucosyl residues (Figure 1-2). (1→4)-β-Glucosyl residues can also form extended continuous linkages up to 10 units (Woodward et al., 1983a). β-Glucan varies in the degree of polymerisation and it is common for high molecular weight (HMW) β-glucans to form chains of up to 1000 β-glucosyl residues (Woodward et al., 1983b).

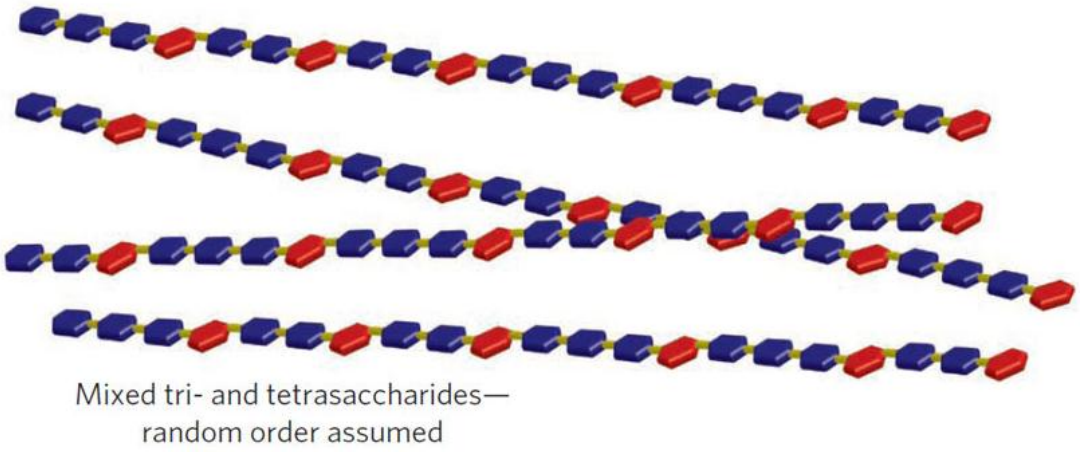

Figure 1-2. Structure of the (1→3,1→4)-β-glucan polysaccharide. (1→3)-β-Glucosyl residues are indicated in red and (1→4)-β-glucosyl residues in blue (Burton et al., 2010).

The asymmetrical conformation of HMW β-glucans and their ability to dissolve in water enable them to form gel-like matrices (Woodward et al., 1983b). These gel-like structures allow the walls in the endosperm to remain strong and pliable and allow the transfer of nutrients through the porous walls. As a result, highly viscous solutions emerge when the grain is imbibed that cause problems in the filtration stages of brewing.

1.3 Malt

Malted barley is required for brewing as it provides the necessary enzymes, substrates and nutrients for yeast to ferment sugars and produce alcohol. The grain is modified through a malting process exposing starch and proteins for further degradation during the mashing process (Figure 1-3).

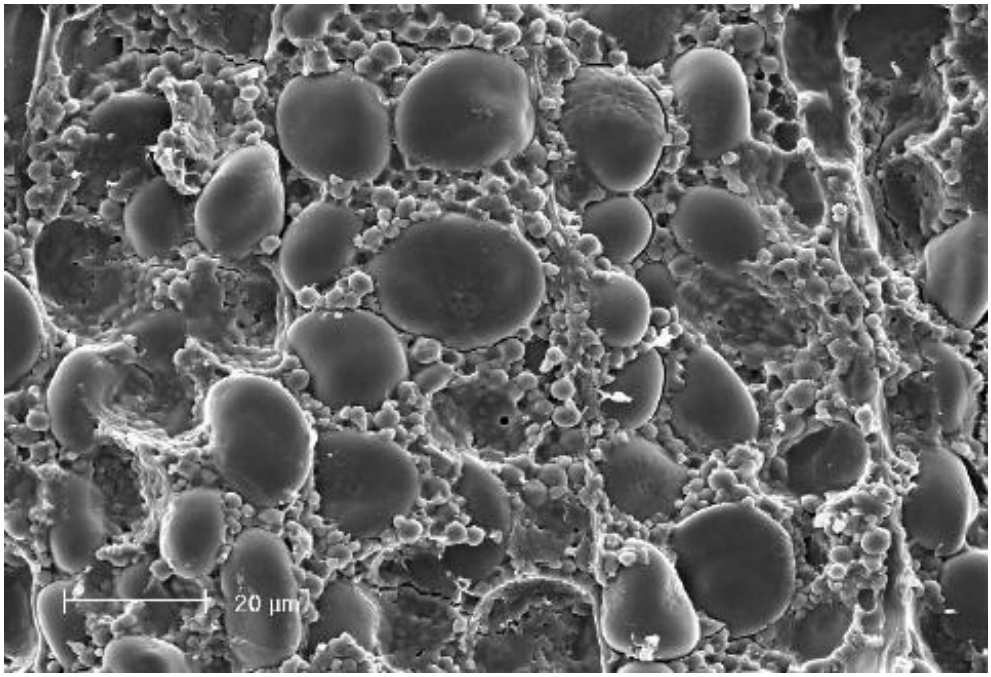

Figure 1-3. A scanning electron micrograph of the starchy endosperm of barley showing starch granules of various sizes embedded in a matrix containing numerous protein bodies (Black, 2001).

Malting is fundamentally controlled germination that physically and chemically modifies the structure of the grain to achieve two outcomes: degradation of starchy endosperm cell walls and the synthesis and uniform mobilisation of hydrolytic enzymes. There are three steps involved in the malting process, outlined in Figure 1-4.

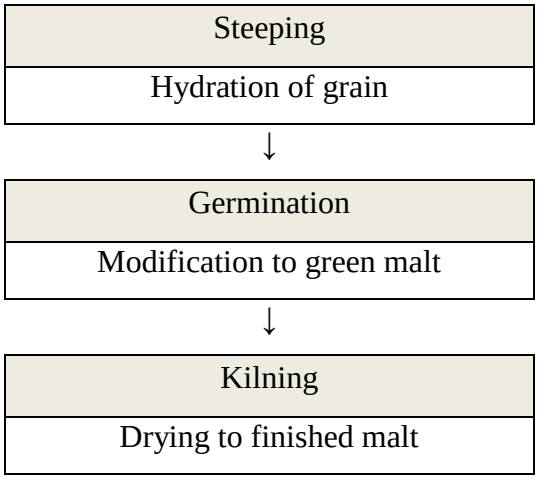

Figure 1-4. Schematic outline of the malting process.

Malting begins with the steeping process by soaking the grain in water with interval air rests under controlled temperatures of 14 – 18°C. This hydrates the grain, increasing moisture from ~ 10% up to ~ 42 – 45% over 48 hours (Bamforth, 2000; Ullrich, 2011). Germination requires a minimum moisture content of 35% to ensure the uniform dispersal of hydrolytic enzymes (Ullrich, 2011). The conditions are adjusted once germination begins and it is controlled by maintaining the temperature at 16 – 20°C and moisture levels at ~ 20% for a period of 3 – 5 days (Bamforth, 2000; Briggs, 1998 ; Kunze, 1996; MacLeod, 2004; Ullrich, 2011).

Germination modifies the endosperm by initiating enzyme synthesis triggered by gibberellic acid (GA), cell wall degradation and enzyme mobilisation. Hydrolysis of cell walls by β-glucanase and xylanase to a lesser extent allows the mobilisation of diastatic enzymes throughout the endosperm and the breakdown of the protein matrix by proteases providing access for α- and β-amylase, and limit dextrinase to starch granules (Table 1-1) (MacLeod, 2004). Incomplete endosperm modification restricts enzymes access to starch and proteins, through the intact cell walls, while excess germination causes over modification and premature starch degradation thus reducing malt extract.

Table 1-1. Enzymes and their function in endosperm modification (Ullrich, 2011)

Purpose	Enzyme	Function
Diastatic	α-amylase	Hydrolysis of internal linkages of starch granules
	β-amylase	Releasing maltose from the reducing end of starch
	Limit dextrinase	Hydrolysis of branched starch amylopectin
Cell wall degrading	β-glucanase	Hydrolysis of β-glucan
	Xylanase	Hydrolysis of arabinoxylans
Protein degrading	Exo- and endo-protease	Hydrolysis of protein matrix

Kilning is the final stage of malting that terminates germination by drying that reduces grain moisture and maintains active enzymes. Green malt is treated with a hot air flow that gradually reduces the moisture content to 5% in 24 hours (Briggs, 1998; Kunze, 1996; Ullrich, 2011). Preserving enzyme activity is important for final malt quality for brewing purposes. However, the partial loss of enzyme activity during kilning is unavoidable and for β-glucanase the loss varies from 8% - 71.5% of activity (Bamforth, 2000; Briggs, 1998;

Kunze, 1996; MacLeod, 2004; Ullrich, 2011; Georg-Kraemer et al., 2004). The final kilned malt is friable, aromatic, coloured, shelf stable and contains the hydrolytic enzymes and modified endosperm required for brewing (Nischwitz et al.,1999).

1.3.1 Malt Quality

Malt quality for brewing purposes is determined using recommended analysis methods set by; the Institute of Brewing (IOB); European Brewery Convention (EBC); and the American Society of Brewing Chemists (ASBC) (Briggs, 1998; Kunze, 1996; Ullrich, 2011). These methods assess malt quality for key characteristics that are summarised in Table 1-2.

Table 1-2. Summary of the key characteristics for malt quality assessment

Parameters	Definition
Hot water extract	Percentage of extract of malt on a dry basis
Wort viscosity	Level of high molecular weight material solubilised in wort including residual β-glucan
Kolbach Index	Ratio of soluble to total nitrogen of malt protein
Soluble protein	Level of soluble nitrogen
Total protein	Level of total nitrogen
Wort β-glucan	Total (1→3,1→4)-β-glucan content in wort
Fermentability	The level of fermentable sugars obtained from the malt.
Free Amino Nitrogen	Measure of free amino acid composition in wort
Diastatic Power	Capacity of malt enzymes to hydrolyse starch
Friability	Direct measure of malt modification
α-amylase	Activity of α-amylase extracted from malt
β-amylase	Activity of β-amylase extracted from malt
Wort colour	Wort colour intensity from the degree of Maillard browning of malt

It is critical that optimum endosperm modification is reached to produce malt with high malting quality characteristics. Enzyme activity is important in barley breeding and malt quality programs because it is the enzyme activity that determines the quality of the malt and ultimately, beer quality.

1.4 The brewing process

The brewing process is a hot water extraction of malt sugars that are subsequently fermented by yeast to produce beer. The efficiency of beer production is affected by the quality of the malt used and poor malt can often lead to increased viscosity and filtration problems throughout the process (Briggs, 1998; Kunze, 1996; Lewis and Young, 2001). Figure 1-5 indicates the steps involved the brewing process.

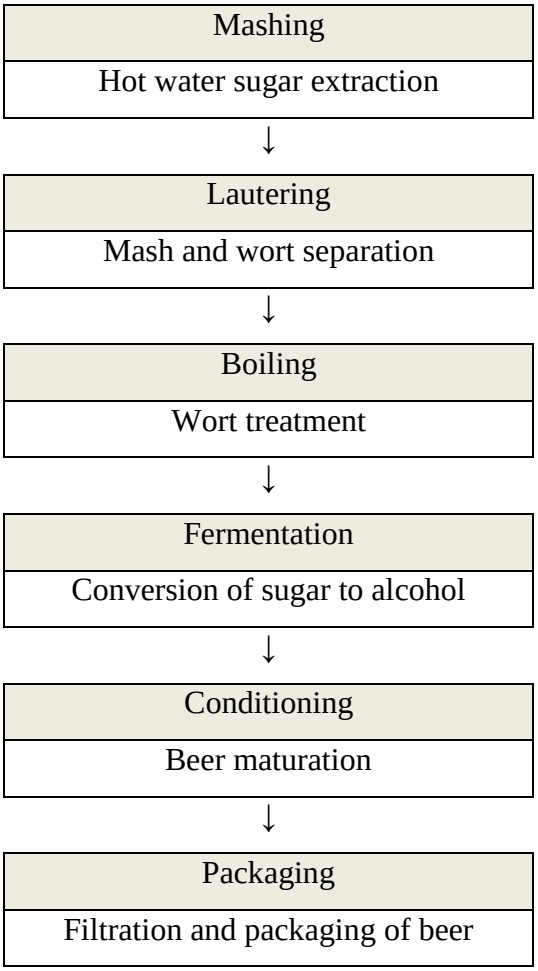

Figure 1-5. Schematic outline of the brewing process.

The brewing process begins with a hot water extraction. Milled malt is added to water and the mashing temperature gradually increases from 40 to 75°C or begins at 65°C, depending on the brewer (Bamforth, 2000; Kunze, 1996; Lewis and Young, 2001). As the mash reaches 70°C the starch gelatinises allowing more efficient hydrolysis (Kunze, 1996; Lewis and Young, 2001). During the mashing process there is minimal malt β-glucan degradation because β-glucanase activity is rapidly lost as the mashing temperature increases (Bamforth and Martin, 1983; Loi et al., 1987). Residual β-glucan solubilises in the mash increasing wort viscosity

that negatively affects malt extract yield, wort separation, wort filtration, beer filtration and beer clarity caused by haze formation (Kanauchi and Bamforth, 2008; Kunze, 1996; Palmer and Agu, 1999; Ullrich, 2011; Robinson et al., 2007).

1.5 (1→3,1→4)-β-glucan endohydrolases

Complete cell wall degradation is one of the major requirements of the malting process. β-Glucan is hydrolysed sequentially by three different classes of β-glucanases including (1→3)- and (1→4)-β-glucan exohydrolases and (1→3,1→4)-β-glucan endohydrolases (EC 3.1.2.73). The most important class impacting malt quality are the (1→3,1→4)-β-glucan endohydrolases responsible for the primary degradation of the high molecular (1→3,1→4)-β-glucan. The exohydrolases further reduce the products released by the endohydrolases to monosaccharides.

1.5.1 Biochemistry of (1→3,1→4)-β-glucanase

β-Glucanase catalyses the hydrolysis of specific linkages within mixed linked β-glucan. (1→4)-β-Glucosyl linkages are hydrolysed where there is an adjacent (1→3)- and (1→4)-β-glucosyl residue on the reducing end as shown in Figure 1-6 (Varghese et al., 1994; Woodward et al., 1982b). The hydrolysed linkages release characteristic tri- and tetrasaccharides reducing β-glucan molecular weight (Woodward et al., 1982b).

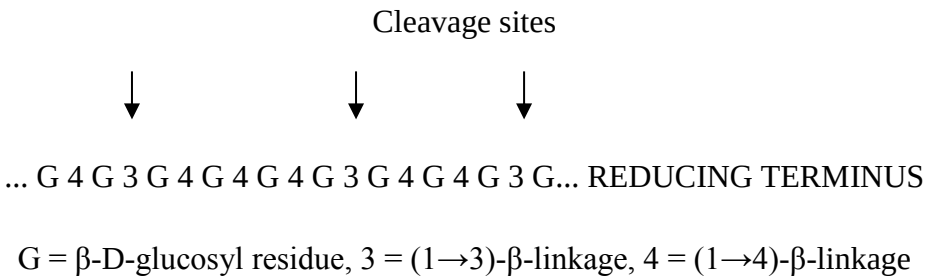

Figure 1-6. Schematic diagram of the cleavage sites of (1→3,1→4)-β-glucanase in (1→3,1→4)-β-glucan.

The two isoenzymes of β-glucanase EI and EII are present during the early stages of germination (Woodward and Fincher, 1982a). Expression of the isoenzymes is tissue-specific with EI synthesised predominantly in the scutellum and a little in the aleurone layer and EII is synthesised exclusively in the aleurone layer (Wolf, 1992; Slakeski and Fincher, 1992b). Both EI and EII mRNA are synthesised during early germination from days 1 – 3 (Slakeski and Fincher, 1992a). As germination continues mRNA activity decreases and EI appears in young leaves and roots after 8 days, with little evidence of EII in these tissues (Slakeski and Fincher, 1992a). Additionally, there is a difference in response to GA. The expression of EII is stimulated by GA but repressed by abscisic acid, compared to EI that shows little response to both hormones suggesting EI is also important in the later stages of growth.

EI and EII have different molecular weights (M_r), isoelectric points (pI), kinetic properties, carbohydrate contents and thermostabilities as shown in Table 1-3. EII is significantly more thermostable than EI and also hydrolyses β-glucan at a slightly faster rate. The differences between EI and EII are believed to be partially due to the *N*-glycosylation site located at Asn^{190} of the amino acid sequence in EII (Doan and Fincher, 1992). Doan and Fincher (1992) determined that EII contained 3.6% carbohydrate by weight that effectively increased the thermostability of EII compared to EI that has <1% glycosylation. The optimum temperatures for EI and EII enzyme activity are approximately 37°C and 45°C respectively (Woodward and Fincher, 1982a). Woodward and Fincher (1982a) investigated the enzyme activity levels of EI and EII and found that EI retained <50% activity compared with EII, which lost approximately 15% when assayed at 40°C for 15 minutes. The loss of EI and EII enzyme activity above 40°C after 15 minutes coincides with EI β-glucanase activity lost after kilning and EII lost as a result of high mashing temperatures.

Table 1-3. Properties of (1→3;1→4)-β-glucanase isoenzymes EI and EII[a]

Property	Isoenzyme EI	Isoenzyme EII
Mr	28,000	33,000
pI	8.5	>10.0
V_{max}	7.06 x 10^3µmol/min	11.62 x 10^3 µmol/min
K_m	3.0 mg/mL	3.4 mg/mL
K_{cat}	118.3 sec^{-1}	193.0 sec^{-1}
Optimum pH	4.7	4.7
Optimum temperature	~ 37°C	45°C
Carbohydrate content	<1%	3.6%

a Values sourced from Woodward and Fincher 1982a, Woodward and Fincher 1982b, Slakeski et al., 1990.

1.5.2 (1→3,1→4)-β-glucanase genetics

β-Glucanase isoenzymes EI and EII are encoded by *HvGlb*1 and *HvGlb*2 respectively. *HvGlb*1 is located on the short arm of chromosome 1H and *Hv*G*lb*2 is located on the long arm of chromosome 7H as shown in Figure 1-7 (Kleinhofs et al., 1993; Loi et al,. 1988). The *HvGlb*1 and *HvGlb*2 genes have been sequenced and the exon regions share 92% sequence identity (Slakeski et al., 1990). Both genes have exon lengths of 1,002 base pairs with a single large intron of different lengths that interrupts the coding regions. The coding regions translate to 334 amino acids with a 28-residue signal peptide and a mature protein of 306 amino acids that is entirely encoded by the second exon (Doan and Fincher, 1992; Litts et al., 1990; Loi et al., 1988; Slakeski et al., 1990; Wolf, 1991; Woodward and Fincher, 1982a).

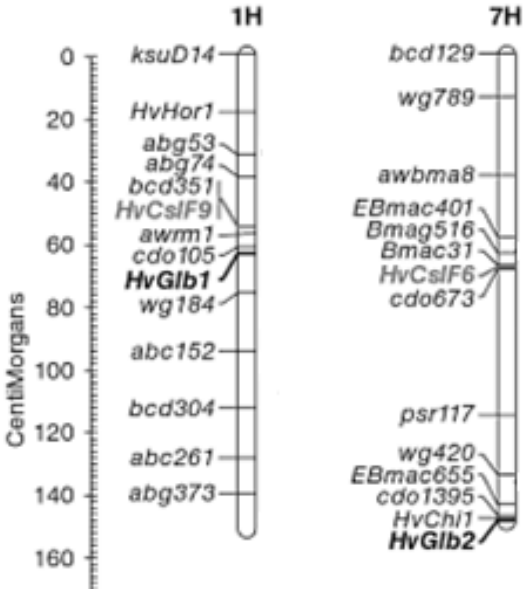

Figure 1-7. Location of *Hv*G*lb*1 and *Hv*G*lb*2 on chromosomes 1H and 7H. Adapted from Burton et al., (2008).

The 3D structure of the EII protein has been solved to a resolution of 2.0Å (Figure 1-8) (Chen et al., 1993; Müller, 1998; Varghese et al., 1994). The major feature of β-glucanase is the deep cleft that runs across the surface (Varghese et al., 1994). This substrate binding cleft is approximately 40Å long and contains 6-8 glucosyl binding sub sites. The structure adopts a $(\beta/\alpha)_8$ barrel conformation common to polysaccharide hydrolases and the enzyme belongs to the GH17 glycoside hydrolase family (Henrissat et al., 1998). The 3D structure of EII has been used as a model to guide attempts to increase enzyme thermostability using protein engineering and transgenic techniques (Stewart et al., 2001). These experiments are discussed in section 1.6.1.

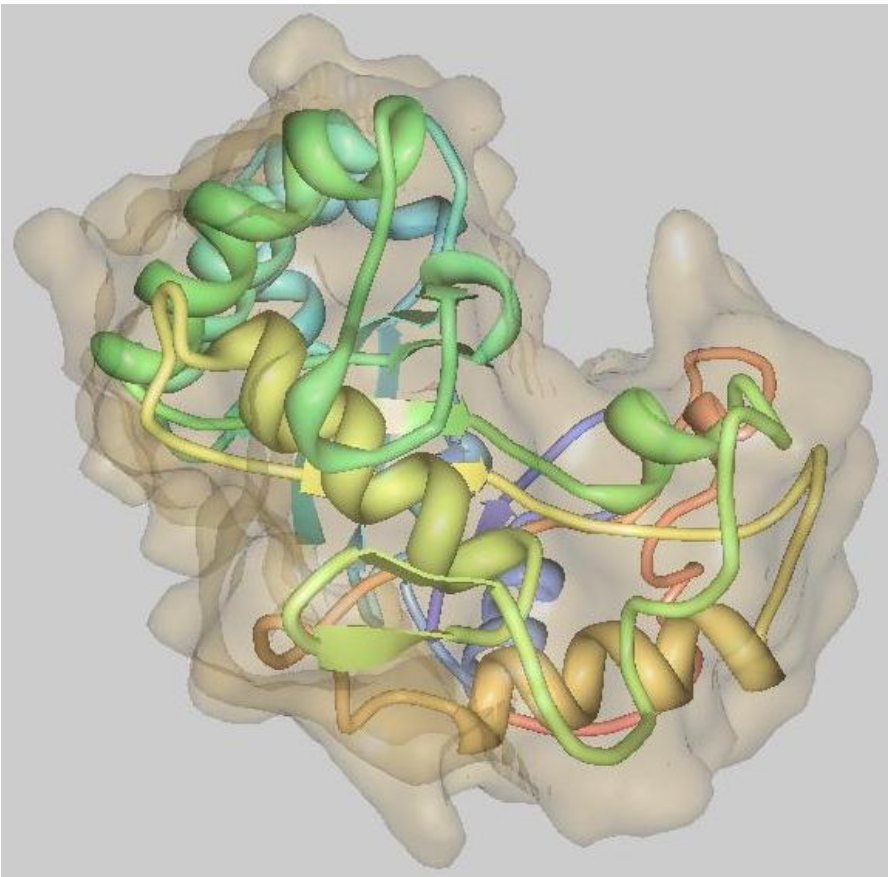

Figure 1-8. Three-dimensional structure of EII (Varghese et al. 1994).

1.5.3 Quantitative trait loci mapping of (1→3,1→4)-β-glucanase

QTL mapping allows for identification of the genetic factors that influence malt quality traits (Zhang and Li, 2009). QTL reported for β-glucanase are limited but there are a growing number of QTL for malt quality traits affected by β-glucanase activity including β-glucan content and viscosity. A summary of published studies identifying QTL for β-glucan content, viscosity and β-glucanase activity are presented in Table 1-4.

The significance of QTL mapping of β-glucanase activity is that QTL coincident with *HvGlb*1 or *HvGlb*2 potentially support the presence of alternative alleles. QTL in other regions indicate the influence of epistatic factors, confounding effects, or involvement of unknown genes or regulatory factors. The QTL listed in Table 1-4 are not limited to chromosome 1H and 7H, indicating that the *HvGlb*1 and *HvGlb*2 genes do not exclusively control enzyme activity. This indicates that there are other factors involved that affect the expression of *HvGlb*1 and *HvGlb*2. An additional factor affecting the detection of QTL at *HvGlb* loci is that the parents must differ in β-glucanase sequence, which is not always likely.

Table 1-4. Summary of QTL identified for β-glucanase activity, β-glucan content and viscosity traits

Traits	Parental populations	No. of QTL	Chromosome	References
Grain β-glucan	Steptoe/Morex	3	1H, 2H	Han et al., 1995
Grain β-glucan	CDC Bold/TR251	7	2H, 3H, 5H, 6H, 7H	Li et al., 2008
Grain β-glucan	Nure/Tremois	2	1H	Laido et al., 2009
Malt β-glucan	Steptoe/Morex	6	1H, 3H, 4H, 7H	Han et al., 1995
Malt β-glucan	Steptoe/Morex	2	7H	Han et al., 2004
Wort β-glucan	Dicktoo/Morex	2	5H	Oziel et al., 1996
Wort β-glucan	Harrington/ TR306	3	3H, 5H, 6H	Mather et al., 1997
Wort β-glucan	Harrington/ TR306	3	3H, 5H, 6H	Igartua et al., 2000
Wort β-glucan	VB9524/ ND11231*12	4	2H, 3H, 5H, 7H	Emebiri et al., 2004
Wort β-glucan	Arapiles/ Franklin	1	1HC	Panozzo et al., 2007
Wort β-glucan	Alexis/Sloop	3	1HC, 2HS, 3HL	Panozzo et al., 2007
Wort β-glucan	Mikamo/ Harrington	3	5H, 6H	Zhou et al., 2012
Wort Viscosity	Blenheim/ E224	4	2H, 3H, 4H, 5H	Thomas et al., 1996
Wort Viscosity	Harrington/ TR306	3	3H, 5H, 6H, 7H	Mather et al., 1997
Wort Viscosity	VB9524/ ND11231*12	4	2H, 3H, 4H	Emebiri et al., 2004
Wort Viscosity	Arapiles/ Franklin	1	1HC	Panozzo et al., 2007
Wort Viscosity	Alexis/Sloop	3	1HC, 2HS, 3HL	Panozzo et al., 2007
Wort Viscosity	Scarlett/ ISR42-8	7	1HS, 1HL, 2HS, 3HS, 3HL, 5HL, 6HL	von Korff et al., 2008

Traits	Parental populations	No. of QTL	Chromosome	References
Wort Viscosity	Nure/Tremois	2	1H, 5H	Laido et al., 2009
Wort Viscosity	Scarlett/S42IL	4	1H, 6H	Schmalenbach et al., 2009
Wort Viscosity	Mikamo/ Harrington	4	2HS, 2HL, 3H, 5H	Zhou et al., 2012
Green malt β-glucanase	Steptoe/Morex	3	1H, 4H, 5H	Han et al., 1995
Malt β-glucanase	Steptoe/Morex	5	1H, 5H, 7H	Han et al., 1995
Green malt β-glucanase	Steptoe/Morex	3	4H, 5H, 7H	Zwickert-Menteur et al., 1996
Malt β-glucanase	Steptoe/Morex	3	1H, 5H, 7H	Zwickert-Menteur et al., 1996
Malt β-glucanase	VB9524/ ND11231*12	4	1H, 3H, 5H, 7H	Emebiri et al., 2004
Malt β-glucanase	Arapiles/ Franklin	1	1H	Panozzo et al., 2007
Malt β-glucanase	Alexis/Sloop	2	1H, 4H	Panozzo et al., 2007

Two strong separate QTL regions for malt β-glucan and β-glucanase have been detected on chromosome 7HS in the Steptoe/Morex population. This region provides a number of complex malt quality QTL including malt-extract content, α-amylase and diastatic power (Han et al., 1995; Hayes et al., 1993). Two studies by Han et al. (1995) and (2004) examined the Steptoe/Morex population and located the QTL for malt β-glucan and β-glucanase on 7H. The strong QTL was attributed to Morex alleles, which was also the case in the Dickoo/Morex population (Oziel et al., 1996). However, these QTL were not coincident with *HvGlb*2 loci.

The 7HS QTL region also includes a grain β-glucan QTL in the CDC Bold/TR251 population that was attributed to CDC Bold alleles (Li et al., 2008; Wong et al., 2015). The 7HS QTL region is not associated with the location of *HvGlb2*, indicating that there are other significant factors contributing to the expression of β-glucanase activity. Li et al., (2008) also found that the magnitude of the grain β-glucan QTL significantly varied between trials indicating that environmental factors influence important malt quality traits.

1.6 Improving β-glucanase for malting and brewing

Attempts at improving barley β-glucanase activity for malt and brewing practices have taken different approaches using molecular biology and transgenic methods. Early attempts focused on increasing barley EII thermostability using site-directed mutagenesis and expression of a hybrid bacillus thermostable β-glucanase during germination (Horvath et al., 2001; Jensen et al., 1996; Stewart et al., 2001). More recently Han et al., (2016) increased expression of barley EII in germinating grain. These methods focus on transgenic approaches to improve barley β-glucanase. Another approach that has not been fully explored is the examination of genetic variation of barley β-glucanase in exotic barley germplasm. Genetic variation has been identified in genetically diverse wild barley material, however this variation has not been characterised (Jin et al., 2011; Nevo et al., 1979).

1.6.1 Protein engineering of thermostable β-glucanase

Fundamental knowledge of barley β-glucanases have allowed researchers to improve β-glucanase thermostability in germinating grain using protein engineering and transformation techniques. Protein engineering was used to increase EII β-glucanase thermostability where Stewart et al., (2001) mutated barley EII using site-directed mutagenesis to induce an amino acid substitution at residue 300 from histidine to proline (H300P). The amino acid substitution targeted the –COOH loop that has been identified as the region of initial protein unfolding (Varghese et al., 1994). H300P was selected in an effort to decrease conformational entropy and was predicted to reduce the flexibility of the C-terminus. EII thermostability was successfully increased through this amino acid substitution that resulted in a T_{50} value 3.7°C higher than the recombinant EII wild type. In simulated mashing trials the mutated enzyme was three times more active at 48°C after 50 minutes of incubation.

A different approach was taken in the development of a thermostable hybrid β-glucanase derived from two *Bacillus* species that was successfully transformed into barley (Horvath et al., 2001; Jensen et al., 1996; Olsen et al., 1991). The structure of bacterial β-glucanases differ from the barley β-glucanase $(\beta/\alpha)_8$ barrel conformation and instead are formed by two β-sheets (Keitel et al., 1993). The development of the hybrid β-glucanase came from the selection of *Bacillus amyloliquefaciens* (AMY) and *B. macerans* (MAC) (Olsen et al., 1991). MAC was selected for thermostability and AMY was selected for better tolerance in acidic environments. Together MAC and AMY were used to produce a hybrid thermostable β-glucanase that would theoretically better survive mashing conditions. The hybrid β-glucanase was demonstrated to be more thermostable than the parental isoforms and was hardly affected after 60 minutes at 65°C and at pH 4.1, and significantly reduced β-glucan levels in a laboratory mash procedure. Jensen et al., (1996) successfully expressed the hybrid β-glucanase in germinating barley and Horvath et al., (2001) used the *Hor3-1* promoter to over express the hybrid β-glucanase in barley. The success of genetically engineered alternatives for β-glucanase improvement are evident, however they have not been released as consumer perceptions of genetically modified food is not currently positive (Bamforth, 2009; Scheffler and Bamforth, 2005).

1.6.2 Processing aids

Industrial production of exogenous thermostable fungal and bacterial β-glucanase for use in brewing has been achieved to aid the degradation of β-glucan in poorly modified malt (Scheffler and Bamforth, 2004). Incomplete endosperm modification can be treated by two different methods. One approach is to begin mashing at lower temperatures for longer to allow β-glucanase to degrade β-glucan before increasing the temperature and gelatinising the starch. This approach decreases brewery productivity and is therefore not commercially desirable. An alternative is to add commercial heat stable exogenous enzymes to aid the degradation of β-glucan (Scheffler and Bamforth, 2004). While these enzymes are very effective in reducing wort viscosity and increasing malt extract, this approach represents additional cost and customer perception of the use of recombinant enzymes in beer production is considered negative in some markets (Scheffler and Bamforth, 2004; Ullrich, 2011).

1.6.3 Wild barley as a source of genetic variation

Alternative approaches to improving malt quality include investigating diverse genetic variation of β-glucanase in wild barley, *Hordeum vulgare* spp. *spontaneum*. *H. spontaneum* is the recognised progenitor of cultivated barley with the ability to produce fertile hybrids with cultivated barley (Nevo et al., 1979). Investigations of wild barley from Israel regarding enzymes of interest to malt quality, including α-amylase, β-amylase and β-glucanase by Ahokas and Naskali (1990) found substantial variation in activity compared to a cultivated variety. Jin et al., (2011) surveyed wild barley originating from Tibet and also found wide genetic variation in the *HvGlb*1 gene. Variation of EI in wild barley and cultivated varieties was investigated using isoelectric focusing and immunoblotting in a study by MacLeod et al., (1991). The D13 variant of EI has a shifted isoelectric point from 8.5 to 8.0, but the biochemical properties or potential influence on malting quality of the D13 allele have not been characterised to date.

Investigation of EI variation in Tibetan wild barley found 10 polymorphism sites in *HvGlb*1 and a wider diversity of haplotype than cultivated barley (Jin et al., 2011). The conclusion of this study suggested Tibetan barley is a potentially rich source of novel or rare alleles, however the effect of the polymorphisms on amino acid sequence or enzyme structure and function was not investigated. To date there is no literature reporting genetic variation of *HvGlb*2.

1.7 Conclusion

It is important for barley breeding programs to produce malting barley varieties that ensure complete and uniform modification of the starchy endosperm during the malting process. The degree of endosperm modification is analysed to ensure high quality malt with high malt extract yield and minimal residual β-glucan is produced for use in brewing. β-Glucan residue in wort increases viscosity, thus reducing brewery efficiency and increasing chill haze in beer. The activity of β-glucanase throughout the malting and mashing process is critical for the complete degradation of β-glucan.

Previous studies have identified the importance of β-glucanase in malt quality. There is collective evidence that β-glucanase is related to other important malt quality traits, from the analysis of malt quality performance and from the identification of QTL. However, there has been very little research conducted to identify and characterise new alleles. The methods available for improving β-glucanase in malting and brewing are limited and shifting towards the exploration of naturally occurring allelic variation in genetically diverse wild barley. This provides opportunities for allele mining to enable identification of novel and desirable variation in β-glucanase activity and thermostability.

This study includes approaches to examine allelic variation in barley β-glucanase in elite and exotic germplasm. This will provide insight into the natural variation of the β-glucanase genes *HvGlb*1 and *HvGlb*2. The kinetic and thermostable properties of novel allelic forms of the β-glucanase enzymes will be characterised and novel alleles with increased β-glucanase thermostability will be examined in validation experiments.

CHAPTER 2

VARIATION IN BARLEY (1→3,1→4)-β-GLUCAN ENDOHYDROLASES REVEALS NOVEL ALLOZYMES WITH INCREASED THERMOSTABILITY

Statement of Authorship

Title of Paper	Variation in barley (1→3,1→4)-β-glucan endohydrolases reveals novel allozymes with increased thermostability
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Juanita Lauer, Suong Cu, Rachel Burton, Jason Eglinton This is an experimental manuscript reporting new allelic variation in barley (1→3,1→4)-β-glucan endohydrolases identified in elite and exotic germplasm. The corresponding allozymes were examined in prediction analysis to identify candidate alleles of interest for characterisation.

Principle Author

Name of Principal Author (Candidate)	Juanita Lauer		
Contribution to the Paper	Designed and performed experiments, analysed data and wrote manuscript		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	15/8/2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Suong Cu		
Contribution to the Paper	Supervised experimental design, data analysis and interpretation Edited the manuscript. I hereby certify that the statement of contribution is accurate.		
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Contribution to the Paper	Supervised experimental design, data analysis and interpretation. Edited the manuscript. I hereby certify that the statement of contribution is accurate.		
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Contribution to the Paper	Conceived the project. Supervised experimental design, data analysis and interpretation. Edited the manuscript. I hereby certify that the statement of contribution is accurate.		
Signature		Date	11/8/2016

Variation in barley (1→3,1→4)-β-glucan endohydrolases reveals novel allozymes with increased thermostability

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2.0 Abstract

Rapid and reliable degradation of (1→3,1→4)-β-glucan to produce low viscosity wort is an essential requirement for malting barley. The (1→3,1→4)-β-glucan endohyrolases are responsible for the primary hydrolysis of cell wall β-glucan. The variation in β-glucanase genes *HvGlb*1 and *HvGlb*2 that encode EI and EII respectively were examined in elite and exotic germplasm. Six EI and 14 EII allozymes were identified and significant variation was found in β-glucanase from *Hordeum vulgare* ssp. *spontaneum* (wild barley), the progenitor of modern cultivated barley. Allozymes were examined using prediction methods; the change in Gibbs free energy of the identified amino acid substitutions to predict changes in enzyme stability and homology modelling to examine the structure of the novel allozymes using the existing solved EII structure. Two EI and four EII allozymes in wild barley accessions were predicted to have improved barley β-glucanase thermostability. One novel EII candidate was identified in existing backcross lines with contrasting *HvGlb*2 alleles from wild barley and cv Flagship. The contrasting alleles in selected near isogenic lines were examined in β-glucanase thermostability analyses. The EII from wild barley exhibited a significant increase in β-glucanase thermostability conferred by the novel *HvGlb*2 allele. Increased β-glucanase thermostability is heritable and candidates identified in wild barley could improve malting and brewing quality in new varieties.

2.1 Introduction

Complete modification of endosperm cell walls is essential in malting barley to reduce wort viscosity and chill haze. The (1→3,1→4)-β-glucan endohydrolases (β-glucanase EC 3.2.1.73) are synthesised during germination and are responsible for hydrolysing long chain (1→3,1→4)-β-glucan (β-glucan) in the starchy endosperm cell walls. The tri- and tetra-saccharides released by the endo β-glucanases are further reduced to glucose monomers by exo β-glucanases. Incomplete degradation of cell walls results in the solubilisation of residual malt β-glucan during mashing that reduces hot water extract and increases wort viscosity, significantly reducing filtration efficiency (Evans et al., 1999; Stuart et al., 1988). The reduction of wort β-glucan is a challenge as barley β-glucanases are relatively thermolabile and rapidly denature during kilning and mashing. Currently, recombinant bacterial and fungal β-glucanases that are more thermostable than barley β-glucanases are available to reduce viscosity and aid filtration at an extra cost to brewers (Bamforth, 2009).

The fundamental characteristics of the enzymes and the structure of the genes encoding β-glucanases are well characterised and their role in malt and brewing quality is understood. β-Glucanases are synthesised in the form of two isoenzymes EI and EII. EI is synthesised predominantly in the scutellum layer and increases in young tissue during seedling development, whilst EII is exclusively synthesised in the aleurone layer during germination (Slakeski and Fincher, 1992; Wolf, 1992). The structure of EII is slightly different from EI with the addition of a glycosylation site that partially contributes to the increased thermostability of EII (Doan and Fincher, 1992). The predicted optimum temperatures for EI and EII are 37°C and 45°C, respectively and as a result, the role of the isoenzymes during germination is altered (Woodward and Fincher 1982a). The high kilning temperatures has a greater detrimental effect on EI leaving only EII present in finished malt (Loi et al., 1987). Average losses of β-glucanase activity range from 55% to 62% in malt varieties due to the loss of EI during kilning (Georg-Kraemer et al., 2004; Loi et al., 1987; Zwickert-Menteur et al., 1996). In addition, in a 65°C mash β-glucanase is required to function at temperatures far greater than optimum resulting in significant activity losses in the first 10 minutes without any influence on reducing wort β-glucan (Loi et al., 1987). Knowledge of genetic variation within EI and EII in relation to β-glucanase specific activity and thermostability between cultivars and genetically diverse wild barley is limited.

Wild barley (*Hordeum vulgare* ssp. *spontaneum*) is the progenitor of domesticated barley and is a source of novel alleles for malt quality improvement (Eglinton et al., 1998). A survey of wild barley accessions identified significant variation in β-glucanase activity (Ahokas and Naskali, 1990), however assessment of enzyme levels in wild barley is potentially confounded by large differences in grain size and composition such that variation may not relate to novel β-glucanase alleles. Jin et al., (2011) examined *HvGlb*1 sequence variation in 80 cultivated varieties and 80 Tibetan wild barley accessions using single stranded conformation polymorphism identifying 32 and 34 single nucleotide polymorphisms (SNP) respectively in *HvGlb*1 of which five were unique to Tibetan wild barley. In the same study a significant correlation was shown between genomic *HvGlb*1 polymorphisms and malt β-glucan levels using a subset of 34 cultivated varieties however the experimental approach did not allow for individual alleles to be defined or specifically linked to phenotypic variation.

These previous studies have highlighted the degree of variation in β-glucanase activity present in the primary gene pool of barley and suggest this may be conferred by allelic variation within *HvGlb*1 or *HvGlb*2. Published sequence analysis of *HvGlb*1 is limited to two complete gene sequences from the varieties Klages and Northrup King 1558 which exhibit no difference in amino acid sequence, and a single complete gene sequence of *HvGlb*2 from Northrup King 1558 (Litts et al., 1990; Slakeski et al., 1990; Wolf, 1991). The three dimensional structure of EII has been solved (Müller et al., 1998) which provides an opportunity to predict the effect of amino acid changes on the structure and stability of the enzyme by homology modelling . This study examines the sequence variation of *HvGlb*1 and *HvGlb*2 in a diverse collection of barley germplasm and novel allozymes were assessed using protein prediction techniques. An existing set of backcross (BC) lines contrasting for *HvGlb*2 were identified and examined for validation of phenotypic effects in a common genetic background.

2.2 Materials and Methods

2.2.1 Germplasm

80 genotypes were selected to represent a broad range of barley germplasm comprising malting and feed varieties from Australia, North America, Europe, Japan and China as well as advanced breeding lines from Australia, the International Centre for Agricultural Research in the Dry Areas (ICARDA), North America and North Africa (Table 6-1). 57 wild barley accessions described by Nevo et al., (1979) were also examined (Table 6-2).

2.2.2 PCR primers and protocol

Genomic DNA was extracted and purified from leaf tissues taken from two week old seedlings as described by Karakousis and Langridge, (2003). PCR primers were designed to target the two exon regions of the published *HvGlb*1 (Genbank: X56260) and *HvGlb*2 (Genbank: M62740) sequences from cv Northrup King 1558, using the Geneious R6 software (Kearse et al., 2012) to target the two exon regions of *HvGlb*1 and *HvGlb*2 for amplification and sequencing (Table 2-1)

Table 2-1. PCR primer sequences for amplifying *HvGlb*1 and *HvGlb*2 exon regions

Gene	Exon region	Primer pair sequences
*HvGlb*1	1st exon	CGAGTGGATTGGACCGAACT/ TGCACCCAATCACTTCAAGAGA
	2nd exon	GCCGCCATTCTTGCTTATTGG/ GTATAGCAAGCCACTACTCGC
*HvGlb*2	1st exon	AGACGTGAACACATCCGGAC/ TTTGGGGCGAGAGAGAAAGG
	2nd exon	CAATGGAAGGAACAGTACTGCTAC/ GAGGAGGTAATTAAGAGGCTCTCC

PCR amplifications were conducted in 25µL total volume reactions with approximately 25ng of genomic DNA, 1X PCR Buffer, 200µM of each dNTP, 1.5mM MgCl2, 400nM of each primer and 5 units of Tfi DNA Polymerase. Reactions were cycled under the following conditions: initial denaturation at 94°C for 2 minutes, with 35 cycles of denaturation at 94°C for 30 seconds, annealing at 58.5°C for 30 seconds, and extension for 72°C for 90 seconds. Final extension was at 72°C for 10 minutes with reactions held at 15°C until removed from the thermocycler. PCR products were purified and sequenced by the Australian Genome Research Facility (AGRF, Adelaide South Australia).

2.2.3 Sequence analysis

Sequence reads were assembled against the reference sequences using Geneious R6 (Kearse et al., 2012). Exon only sequences were aligned and translated to amino acid sequence. Novel EI and EII haplotypes were identified by amino acid substitutions.

2.2.4 Homology modelling and structural analysis

The putative amino acid sequences of novel Glb1 and Glb2 alleles were used to generate three dimensional structures using the existing EII structure as the template (Müller et al., 1998). Modeller 9.12 was used to generate the 3D structure for each allele (Sali and Blundell, 1993). Models were generated for each allele and amino acid substitutions were visualised to examine conformational entropy, hydrogen bonding and van der Waals forces using the PyMol graphic tool (The PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC),. The change in Gibbs free energy (ΔG) for each amino acid substitution was calculated to determine changes in stability using PoP MuSiC 2.1 (Dehouck et al., 2011).

2.2.5 Backcross Lines

An existing set of wild barley backcross lines were genotyped using the PCR primers described above to determine the presence of novel alleles at the *Glb*2 locus. BC lines were selected based on similarity of wild barley introgression segments and contrasting *Glb*2 alleles. Four BC lines containing wild barley introgression segments at the *Glb*2 locus conferring the *Glb*2-c allele were selected and four BC lines containing the Flagship *Glb*2-a allele were selected for analysis of β-glucanase thermostability.

The BC lines had been developed with an F_1 hybrid between the wild barley accessions CPI77146-32 and CPI71283-48 (Nevo *et al.* 1979) backcrossed twice to the variety Flagship and the progeny progressed as unselected bulk populations with single plant selections taken from the BC_2F_3 generation then grown as single rows in the field. 270 lines were selected to eliminate brittle rachis and genotyped with Genotyping-by-Sequencing (GBS) using the double digest PstI/MspI method as previously described (Poland *et al.* 2012). The GBS libraries were prepared and sequenced on a single lane of an Illumina HiSeq 2000. Bi-allelic SNP markers were called using the Tassel UNEAK pipeline (Lu *et al.* 2013). GBS marker positions were estimated from BLAST searches against the published anchored barley genome finger-printed contigs.

2.2.6 Green malt preparation

Grain samples of the selected BC lines were cleaned on a 2.5mm sieve. Germinations were performed using the European Brewing Convention (EBC) method 3.6.2 (EBC 1997). Samples were immediately placed in a -20°C freezer followed by freeze drying. Freeze dried green malt samples were ground to flour using an electric coffee grinder.

2.2.7 β-Glucanase thermostability assays

β-Glucanase thermostability was determined using two methods: (i) to examine activity at elevated temperatures and (ii) to examine residual activity after irreversible thermal inactivation. β-Glucanase activity in green malt was determined using the Azo-Barley glucan Megazyme malt β-glucanase assay kit (Megazyme Ltd. Ireland) (McCleary and Shameer, 1987). The assay volume was reduced by half with three replicates and randomised (Zwickert-Menteur et al., 1996). β-Glucanase activity at an elevated temperature was

determined from the difference between activity at 30°C and 55°C. β-glucanase thermostability was expressed as a percentage of activity at 30°C. Irreversible thermal inactivation of β-glucanase activity was determined following enzyme heat treatment at 55°C for 5 minutes and immediately placed on ice. β-glucanase activity before and after heat treatment was assayed at 30°C and the irreversible thermal inactivation was calculated as a percentage of initial activity. Results were analysed by t-test for statistical significance.

2.3 Results

2.3.1 Identification of novel *HvGlb*1 and *HvGlb*2 alleles

*HvGlb*1 and *HvGlb*2 exon regions were amplified from genomic DNA of 137 barley cultivars, advanced breeding lines and wild barley accessions (Tables 6-1 and 6-2). *HvGlb*1 and *HvGlb*2 have 73 bp and 932 bp in the first and second exons respectively, with a total open reading frame of 1,002 bp encoding 334 amino acids for each isoenzyme. Within the 137 individuals examined there was no sequence variation in the first 28 amino acids which is a signal peptide. Variation was identified within the remaining 306 amino acids that form the β-glucanase enzyme.

Wild barley accessions exhibited greater genetic variation in *HvGlb*1 and *HvGlb*2 than cultivated barley (Table 2-2). The EI-a allozyme was the most common form of EI and was found in 92.5% of the germplasm (Table 2-3). EI-a has previously been published and was selected as the reference sequence for amino acid substitution comparisons (Litts et al., 1990; Slakeski et al., 1990). A total of 26 unique alleles were identified in *HvGlb*1and only five of these occurred in cultivated barley. The majority of the sequence polymorphisms were silent and corresponded to only six unique enzyme sequences. Among all of the cultivated varieties examined only one EI sequence was detected, landraces exhibited two new EI sequences, while three novel EI sequences were confined to wild barley. *HvGlb*2 exhibited a similar level of SNP variation and allele number in cultivated and wild barley but a significantly higher level of variation at the protein level. Amino acid substitutions occurred at 13 locations in the enzyme and 13 novel allozymes were identified, 11 of which were exclusive to wild barley.

Table 2-2. SNP and amino acid substitutions identified in *HvGlb*1 and *HvGlb*2 from 137 barley genotypes

Gene	Germplasm Source	SNP	Alleles	AA substitutions	Allozymes
	Cultivated Varieties	9	5	2	3
*HvGlb*1	Wild Barley accessions	23	22	3	4
	Total	25	26	5	6
	Cultivated Varieties	14	3	6	3
*HvGlb*2	Wild Barley accessions	32	28	10	13
	Total	38	28	13	14

Three EII allozymes defined by six SNPs were identified in cultivated barley. EII-a and EII-b accounted for 98.75% of EII allozymes identified in cultivated barley and only a single landrace carried the common wild barley EII-c allozyme (Table 2-4). The sequence of EII-a has previously been published and was selected as the reference (Wolf, 1992). The variation in wild barley *HvGlb*2 alleles was considerably larger with 13.8% higher SNP frequency. Twelve new EII allozymes corresponding to different combinations of the 10 amino acid substitutions were identified in wild barley. The alleles EII-d through to EII-n were exclusive to wild barley and four of these rare alleles were identified in single individuals.

Table 2-3. Amino acid substitutions identified in β-glucanase isoenzyme EI. Bold values indicate a predicted increase in stability

EI	**No. of individuals**	**T17S**	**A95T**	**Q110H**	**Q282E**	**L293I**	**Allele ΔΔG (kJ/mol)**
EI-a	127	T	A	Q	Q	L	Reference
EI-b	7				E		**-0.377**
EI-c	1		T				**-2.594**
EI-d	1			H			3.473
EI-e	1					I	3.849
EI-f	1	S					3.556

Table 2-4. Amino acid substitutions identified in β-glucanase isoenzyme EII. ΔΔG is given for each substitution and allele. Bold values indicate a predicted increase in stability

EII	No. Of individuals	S20G	G44S	S84T	A98G	R100Q	V114A	A149V	N162S	Q204H	G219A	A246T	E271K	D284E	ΔΔG by allele (kJ/mol)
EII-a	51	S	G	S	A	R	V	A	N	Q	G	A	E	D	Reference
EII-b	29	G	S				A	V	S		A				5.774
EII-c	22	G									A				**-1.674**
EII-d	4						A				A				**-3.849**
EII-e	7	G					A				A				**-1.506**
EII-f	7	G													2.343
EII-g	5						A								0.167
EII-h	2	G					A								2.510
EII-i	1	G								H					1.464
EII-j	2	G											K		4.937
EII-k	1				G		A								2.469
EII-l	1	G				Q								E	**-0.084**
EII-m	1	G									A	T			0.795
EII-n	4	G		T							A	T			1.715
ΔΔG by Substitution (kJ/mol)		2.343	1.464	0.920	2.301	**-1.130**	0.167	3.640	2.176	-0.879	**-4.017**	2.469	2.594	**-1.297**	

2.3.2 Structural analysis of new allelic forms of EI and EII

EI-b and EI-c from wild barley exhibited EI with decreased ΔG values associated with the glutamine to glutamic acid change at residue 282 and alanine to threonine substitution at residue 95 (Table 2-3). The ΔG of A95T in EI-c is predicted to have the greatest impact on improving EI thermostability. The amino acid substitutions in EI-d, EI-e and EI-f all have increased ΔG values indicating that these substitutions are likely to decrease β-glucanase thermostability.

The ΔG predictions for amino acid changes in EII identified four substitutions and four allozymes with increased thermostability (Table 2-4). The amino acid substitution of glycine to alanine at residue 219 had the greatest decrease in ΔG that is predicted to have the greatest increase in β-glucanase thermostability. G219A occurred in six new forms of EII, however five of these six also had the serine to glycine substitution at residue 20 that was predicted to confer decreased thermostability from the increased ΔG. The G219A substitution was identified in EII-c, EII-d and EII-e and was predicted to confer increased overall β-glucanase thermostability. The fourth form of EII with improved thermostability was EII-l which has higher predicted thermostability from combination of the two different amino acid substitutions arginine to glutamine at residue 100 and aspartic acid to glutamic acid at residue 284. EII -l similar to EII-c and EII-e also contains the predicted negative S20G substitution.

Homology modelling was used to visually examine amino acid substitutions to aid the identification of favourable substitutions with improved β-glucanase thermostability. EI and EII share 92% sequence identity and were both aligned to the EII template (Müller et al., 1998). EI variation was limited, however EI-c was predicted to have the greatest increase in thermostability (Figure 2-1). The A95T substitution in EI-c is located in the loop before the α4-helix and is situated two residues away from the hydrogen donor for the catalytic binding of (1→3,1→4)-β-glucan at glutamic acid residue 93 (Tsai et al., 2008). The extended side chain of threonine and the additional polar site allows for an additional hydrogen bond that may result in reduced surface hydrophobicity and increased stability.

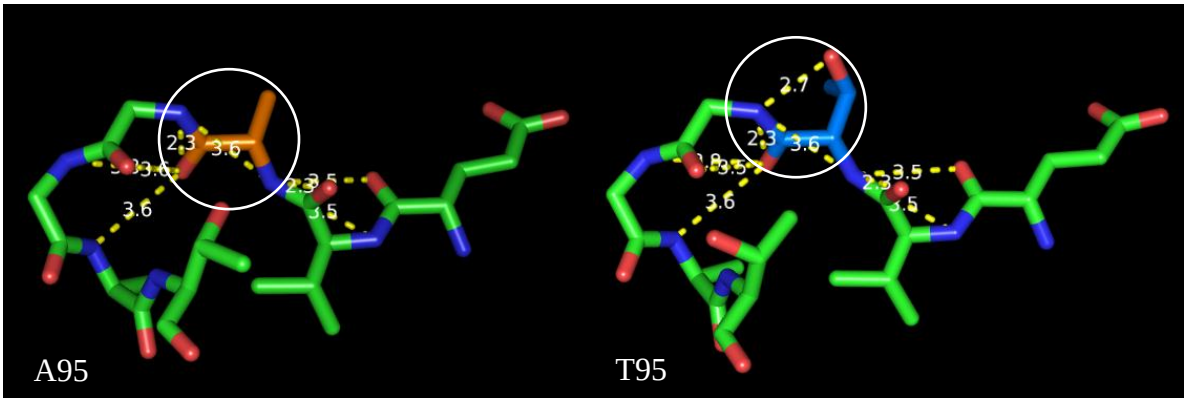

Figure 2-1. Representation of the amino acid substitution A95T identified in β-glucanase isoenzyme EI (green). EI wild type residue A95 is orange and the corresponding amino acid substitution T95 is blue. Nitrogen and oxygen atoms are coloured dark blue and red respectively. Hydrogen bonds from the substituted residue are illustrated by yellow broken lines with distances labelled in angstrom units.

G219A was predicted to have the greatest increase in protein thermostability from the ΔG calculations. The G219A substitution is located in the α6-helix and the change to alanine increases the hydrophobicity of residue 219 (Figure 2-2a). The S20G substitution is located in the α1-helix and loses two hydrogen bonds (Figure 2-2b). The allele with the best predicted combination of amino acid substitutions is EII-d that contains the favourable G219A without the unfavourable S20G substitution.

The greatest increase in protein stability was generated by the G219A substitution located in α_6-helix that increased the hydrophobicity of residue 219 (Figure 2-2a). The replacement of a glycine residue within an α-helix reduces flexibility and the entropy of the unfolded state resulting in an increase of overall EII stability (Matthews et al., 1987). The opposite occurs with the S20G substitution located in the α_1-helix. G20 increases flexibility in the α_1-helix and results in the loss of two hydrogen bonds. The allele with the best combination of amino acid substitutions is *HvGlb*2-d because it contains the favourable G219A without the unfavourable S20G substitution.

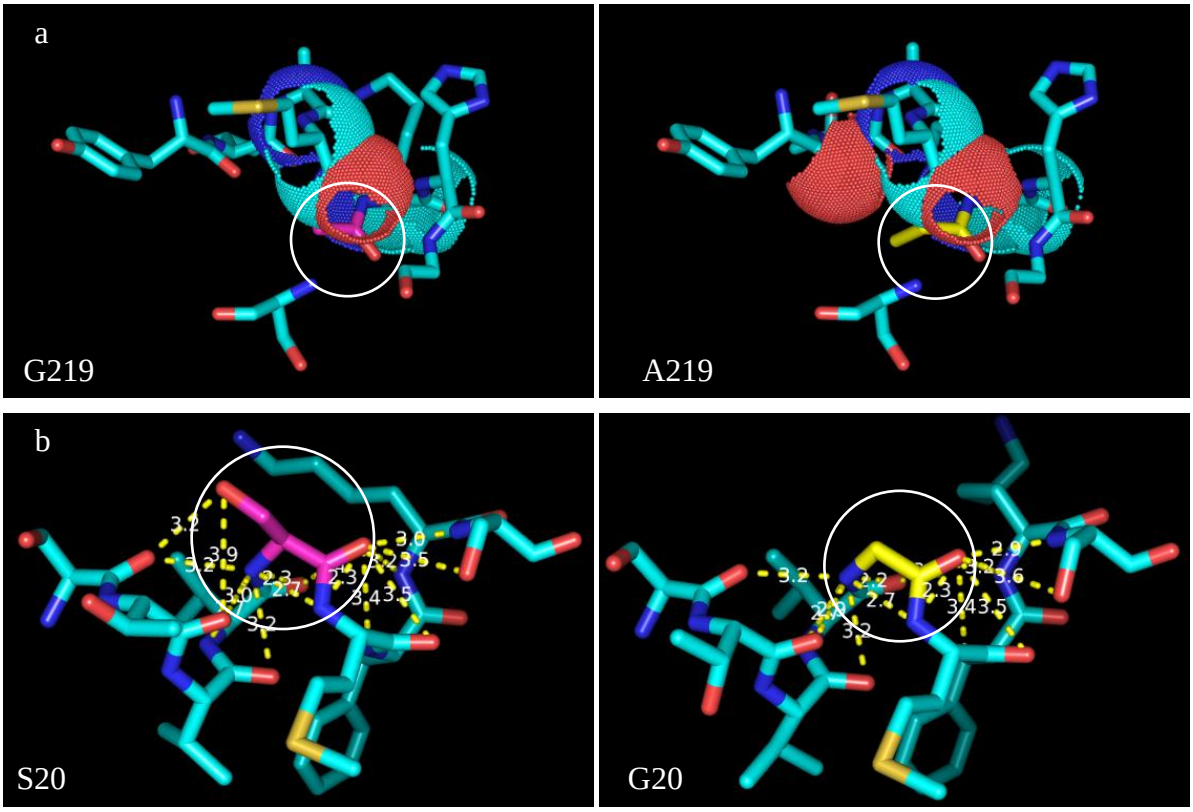

Figure 2-2. Representation of amino acid substitutions a) G219A and b) S20G identified in β-glucanase isoenzyme EII (cyan). EII wild type residues G219 and S20 are pink and corresponding amino acid substitutions A219 and G20 are yellow. Nitrogen and oxygen atoms are coloured dark blue and red respectively. Van der Waal forces are illustrated by dot clouds to 3Å from the substituted residue. Hydrogen bonds from the substituted residue are illustrated by yellow broken lines with distances labelled in angstrom units.

2.3.3 Analysis of backcross lines

Three parents from an existing set of BC lines were identified with *HvGlb*2 variation. Two wild barley accessions CPI71283-48 and CPI77146-32 contain EII-c and cv Flagship carries EII-a. GBS markers close to the *HvGlb*2 locus from the Flagship backcross population were identified by blasting the marker sequences with the Genbank *HvGlb*2 accession M62740. The blast analysis identified three markers closely linked to the *Glb*2 locus and these were used to confirm the wild barley introgressions at the *Glb*2 locus. Only four lines were identified with the novel *HvGlb*2-c allele which is significantly lower than the 21 lines expected based on 12.5% allele frequency in a BC_2 population. Based on flanking marker polymorphisms all four lines inherited the *HvGlb*2-c allele from the wild parent CPI71283-48. Four backcross lines were selected with the *HvGlb*2-a allele from cv Flagship and in combination they closely matched the genetic composition of the four test lines (Table 2-5). Only 7 of the 2,085 polymorphic GBS markers (0.3%) were present in all four test lines and

absent from the control group (Table 2-5). A single line, BX04S;033-012 exhibited no wild barley marker alleles and was also included as a control.

Table 2-5. Genetic Structure of BC lines

		No. of wild marker alleles	Percentage wild marker alleles	Approximate segment size at *HvGlb*2 (cM)
Test lines	**BX04S;032-005**	71	3.4%	137.0 – 140.9
	BX04S;046-036	143	6.9%	131.6 – 140.9
	BX04S;050-033	44	2.1%	131.6 – 140.9
	BX04S;051-031	156	7.5%	131.6 – 140.9
Control lines	**BX04S;033-012**	0	0%	
	BX04S;053-013	19	0.9%	
	BX04S;079-046	70	3.3%	
	BX04S;082-030	62	3.0%	
Combined test lines		355	17.0%	
Combined control lines		156	7.4%	
Unique and present in all test lines		7	0.3%	

2.3.4 EII thermostability variation

Three parents from an existing set of backcross lines were identified with *HvGlb*2 variation. Two wild barley accessions CPI71283-48 and CPI77146-32 contain EII-c and cv Flagship carries EII-a. β-glucanase thermostability was examined in green malt samples of the parents and selected backcross lines to determine if allelic variation conferred a difference in β-glucanase thermostability. Thermostability of Flagship and wild barley was determined from β-glucanase activity assayed at 55°C expressed as a percentage of the activity measured at 30°C. Significant variation in β-glucanase thermostability was identified between the parents and BC lines contrasting for EII-a and EII-c (Figure 2-3). EII-c from wild barley was significantly ($p<0.001$) more thermostable than EII-a and exhibited 8% higher enzyme activity at 55°C. The composition and grain size difference between Flagship and wild barley can potentially confound the results, to avoid this BC lines were examined. EII-c from BC lines was significantly ($p<0.001$) more thermostable than BC lines from EII-a and exhibited 4.2% higher enzyme activity at 55°C.

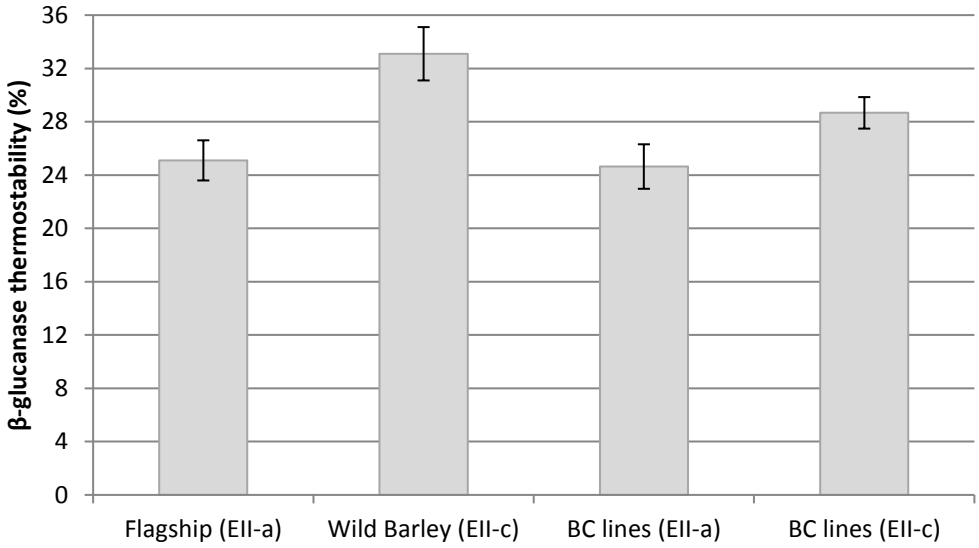

Figure 2-3. β-Glucanase thermostability was measured as the percentage of β-glucanase activity in Flagship, wild barley and BC lines contrasting for EII-a and EII-c. β-Glucanase was extracted from green malt germinated for four days and assayed at 30°C and 55°C for 15 minutes. Data points represent the mean of four replicates. Error bars indicate the standard deviation.

Irreversible thermal inactivation of β-glucanase was also examined in the BC lines contrasting for EII-a and EII-c (Figure 2-4). The percentage of residual β-glucanase activity after 5 minutes of heat treatment at 55°C was determined. EII-c was significantly ($p<0.01$) more thermostable than EII-a and exhibited 3.5% more residual activity.

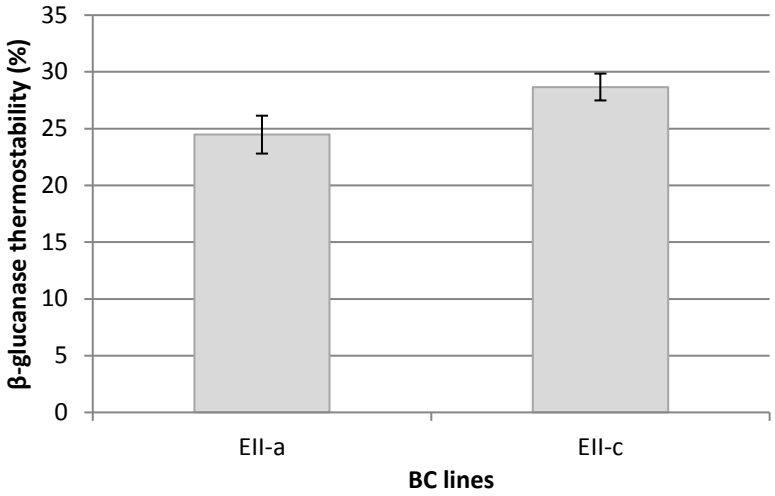

Figure 2-4. Irreversible thermal inactivation of β-glucanase activity in BC lines contrasting in EII-a and EII-c. β-Glucanase was extracted from green malt germinated for four days and samples were heated at 55°C for 5 minutes. Initial and residual β-glucanase activity was assayed 30°C and the percentage of residual activity was calculated. Data points represent the mean of four replicates. Error bars indicate the standard deviation.

2.4 Discussion

β-Glucanase efficiency is important for complete degradation of endosperm cell wall β-glucan during malting, however malt β-glucanase has limited impact on reducing wort β-glucan (Loi et al., 1987). Previous research has identified variation between cultivars and an effect of environmental conditions on β-glucanase activity from green and kilned malt but there has been limited research on the variation in β-glucanase thermostability (Barber et al., 1994; Georg-Kraemer et al., 2004). Jensen et al., (1996) produced transgenic barley expressing a thermostable hybrid bacterial β-glucanase during germination to increase the survival of the β-glucanase in malt. Alternatively, Stewart et al., (2001) engineered a recombinant thermostable mutant EII with a single amino acid substitution, histidine to proline at residue 300 that exhibited an increase in T_{50} of 3.7°C above the recombinant wild type from 47.5°C to 51.2°C. The introduction of proline at residue 300 stabilises the C-terminus reducing the entropy of unfolding. Alternatively to the transgenic approaches to improving barley β-glucanase thermostability, the identification of non-plant thermostable β-glucanases in bacteria and fungi have been utilised as processing aids in brewing for many years (Teng et al., 2006). Here we have explored the natural genetic variation in *HvGlb*1 and *HvGlb*2 providing potential for barley β-glucanase thermostability improvement sourced from the wild progenitor *Hordeum vulgare* ssp. *spontaneum*.

The most significant variation was identified in *HvGlb*2 from wild barley. Limited variation was identified in *HvGlb*2 from the other germplasm sources and in *HvGlb*1 from all germplasm. Restricted allelic variation in *HvGlb*1 in both cultivated and exotic sources suggests that EI has a more fixed primary structure linked to a specific purpose in germination and plant growth. EI is synthesised in the scutellum layer reaching optimum levels by 48 hours of germination. Increases in EI mRNA have been detected 3 to 6 days in young roots and 8 to 20 days in young leaves after germination (Slakeski and Fincher, 1992). In contrast EII is synthesised exclusively in the aleurone layer during germination reaching optimum levels by 72-96 hours (Edmunds et al., 1994; Slakeski and Fincher, 1992). One significant difference between EI and EII is their predicted optimum temperatures of 37°C and 45°C respectively (Woodward and Fincher, 1982a). Enzyme optimum temperature is primarily influenced by the protein structure which is determined by peptide sequence and protein folding.

EI and EII share 92% sequence identity with only 25 amino acid differences occurring within the protein (Slakeski et al., 1990). EI has more polar uncharged residues compared to EII, whilst EII has more hydrophobic and charged polar residues. As a result, EII has a stronger hydrophobic core and more negative surface charges that are typical characteristics of a thermophile enzyme. Generally thermophiles have more negative surface charges that allow them to function more efficiently at higher temperatures as well as viscous solutions than mesophilic enzymes (Querol et al., 1996). The 25 amino acid differences between EI and EII thus do not appear to be random but are very specific. The thermophilic nature of EII allows greater survival under kilning and mashing conditions than for EI. The action of EI is important for supplying initial energy in the early stages of germination and seedling tissue development, whilst EII is solely required for releasing the energy reserves during the shorter germination phase in a viscous starchy environment. The restricted role of EII could be a factor underlying the significant allelic variation identified in wild barley, since variation in EII may be less detrimental to successful seedling establishment and growth when compared to the more serious detrimental effects that EI variation could cause.

This study identified two forms of EI and four forms of EII with greater predicted thermostability than the references EI-a and EII-a. Calculations of ΔG were used as a tool to aid the prediction of changes in protein thermostability caused by amino acid substitution. EI-b and EI-c were identified as the best candidates for improved EI thermostability from ΔG

calculations. MacLeod et al., (1991) has previously described an alternative form of EI that has been identified in this study as EI-b. EI isoelectric points (pI) were examined in selected wild barley accessions and an EI variant named D-13, with a slightly more acidic pI was identified. The results from the current study explain the pI shift in D-13 due to the amino acid change of Q282E in EI-b. The Q282E substitution is located in a loop near the enzyme surface next to a substrate binding residue, lysine 283 in the c-terminus region. The change from glutamine to glutamic acid increases the surface charge due to the carboxylic acid in the side chain. Overall, EI-c was likely to possess the greatest predicted increase in thermostability of the novel EI identified (Figure 2-1). EI-c has the A95T substitution that is located two amino acids downstream from glutamic acid at residue 93, the expected hydrogen donor in the hydrolytic step (Tsai et al., 2008). The increased stability of EI-c is likely to be due to the change in amino acid polarity from hydrophobic to polar uncharged so close to the catalytic site. The longer side chain of threonine may also affect substrate binding. Significant losses of EI and variable lower losses of EII occur during kilning (Georg-Kraemer et al., 2004). EI-b and EI-c identified from wild barley may have improved β-glucanase stability, however the predicted increase may not be significant enough to survive kilning temperatures.

EII-c, EII-d, EII-e and EII-l were identified as the best candidates for improved EII thermostability from ΔG calculations. This is largely due to the uniform presence of the G219A substitution and the combined substitutions R100Q and D284E identified in EII-l. The G219A substitution is predicted to improve stability of the α6-helix since the replacement of the glycine reduces its flexibility (Figure 2-2a) (Matthews et al., 1987). In contrast, S20G introduces a glycine residue into the α_1-helix that has the opposite effect, increasing helix flexibility and reducing thermostability (Figure 2-2b). S20G was the most commonly occurring substitution identified in 10 of the 13 novel EII and two of the three EII candidates with G219A, and EII-l that all have the S20G substitution. EII-l was predicted to have increased thermostability accruing from two substitutions R100Q and D284E that together may have an additive effect. The R100Q substitution has a structure change from a positive charge to a shorter side chain that is a polar with no charged residue. The D284E substitution is located in a loop next to the leucine 283 substrate binding residue on the surface of the substrate binding cavity. The glutamic acid at residue in EII-l and has an additional hydrogen bond that may be the result of the increased side chain length and the predicted increase in thermostability. EII allozymes conferring greater malt β-glucanase thermostability could reduce wort viscosity and the need for exogenous enzyme additions during brewing to

improve filtration efficiency. Improving barley β-glucanase catalytic efficiency and increasing thermostability would extend mash survival time and improve wort viscosity.

The cultivated barley gene pool is limited to EI-a, EII-a and EII-b alleles with the exception of three landraces. EII-c identified as a candidate with increased β-glucanase thermostability was examined in an existing subset of BC lines and their parents; wild barley accessions CPI77146-32 and CPI71283-48 and Flagship. β-glucanase activity in the wild barley accessions was significantly more thermostable than Flagship and exhibited 8% more activity at higher than optimal temperature (Figure 2-3). However, as wild barley is different in grain size and composition to the malt variety Flagship the results could be potentially confounded by factors not related to allelic *HvGlb*2 variation. To control for these possible confounding factors BC lines with contrasting EII-a and EII-c β-glucanase were examined. BC lines with EII-c had significantly higher β-glucanase thermostability with 4.2% more activity at 55°C than EII-a. The irreversible thermal inactivation of EII in BC lines demonstrated a significant increase in residual β-glucanase activity from BC lines with EII-c. EII-c had 3.4% higher residual activity that indicates the refolding of EII-c is more energetically favourable than that of EII-a (Figure 2-4). The increase in β-glucanase thermostability attributed from EII-c validates the predictions made from the structural analysis of the G219A substitution. The novel forms of EI and EII predicted to have increased β-glucanase thermostability are exclusive to wild barley and the results indicate that the increase in β-glucanase thermostability identified in the parents and BC lines is hereditary.

This study has identified significant genetic variation within elite and exotic germplasm, particularly in wild barley *HvGlb*2 alleles. Amino acid sequence variation was further examined using homology modelling to identify candidates for future characterisation experiments. Significant β-glucanase thermostability improvement conferred by contrasting EII allozymes in preliminary experiments has been characterised. There is now an opportunity to determine the basic kinetic and thermostability characteristics of the novel forms of β-glucanase EI and EII to specifically relate structural to functional changes in the enzyme. Assessing the potential for the new forms of β-glucanase to improve brewing quality in future barley varieties will require a range of considerations. It is possible that malting and brewing experiments under standard processing conditions may identify differences in wort viscosity, β-glucan content or β-glucan composition. However modern malting barley varieties do not exhibit limitations in these traits when samples of good physical grain quality are processed

correctly. It may be possible that any commercial advantages offered by the new forms of β-glucanase are more evident in suboptimal scenarios. Opportunities that could be explored include testing under modified malt with higher β-glucan content, higher mashing temperatures, high raw barley adjunct inclusion, and backcrossing the new alleles into feed barley with known elevated wort β-glucan. Significant effects from the new wild barley derived alleles in these situations could support changes to barley breeding, malting and brewing process conditions.

CHAPTER 3

NOVEL BARLEY (1→3,1→4)-β-GLUCAN ENDOHYDROLASE ALLELES CONFER INCREASED ENZYME THERMOSTABILITY

Statement of Authorship

Title of Paper	Novel barley (1→3,1→4)-β-Glucan Endohydrolase Alleles Confer Increased Enzyme Thermostability
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Juanita Lauer, Suong Cu, Rachel Burton, Jason Eglinton This is an experimental manuscript characterising the kinetic and thermostability properties of selected barley (1→3,1→4)-β-glucan endohydrolase allozymes. One novel EII allozyme was identified with significantly higher thermostability than the reference EII. The amino acid substitution differences between the reference EII and the novel EII were further examined creating several mutants using site-directed mutagenesis. The EII mutants were examined in thermostability experiments to identify the amino acid responsible for the significant thermostability increase.

Principle Author

Name of Principal Author (Candidate)	Juanita Lauer		
Contribution to the Paper	Designed and performed experiments, analysed data and wrote manuscript		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper		
Signature		Date	15/8/2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Kuok Yap		
Contribution to the Paper	Designed and created site-directed mutants. I hereby certify that the statement of contribution is accurate.		
Signature		Date	11/8/2016

Name of Co-Author	Suong Cu		
Contribution to the Paper	Supervised experimental design, data analysis and interpretation Edited the manuscript. I hereby certify that the statement of contribution is accurate.		
Signature		Date	15/8/2016

Name of Co-Author	Rachel Burton		
Contribution to the Paper	Supervised experimental design, data analysis and interpretation. Edited the manuscript. I hereby certify that the statement of contribution is accurate.		
Signature		Date	15/8/2016

Name of Co-Author	Jason Eglinton		
Contribution to the Paper	Conceived the project. Supervised experimental design, data analysis and interpretation. Edited the manuscript. I hereby certify that the statement of contribution is accurate.		
Signature		Date	11/8/2016

Novel Barley (1→3,1→4)-β-Glucan Endohydrolase Alleles Confer Increased Enzyme Thermostability

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3.0 Abstract

Barley (1→3,1→4)-β-glucan endohydrolases (β-glucanases; EI and EII) are primarily responsible for hydrolysing high molecular weight (1→3,1→4)-β-glucans (β-glucan) during germination. Incomplete endosperm modification during malting results in residual β-glucan that can contribute to increased wort viscosity and beer chill haze. Four newly identified forms of EI and EII and the reference enzymes EI-a and EII-a were expressed in Escherichia coli and the recombinant proteins were characterised for enzyme kinetics and thermostability. EI and EII variants that exhibited higher residual β-glucanase activity than EI-a and EII-a after heat treatment also exhibited increased substrate affinity and decreased turnover rates. The novel EII-l form exhibited significantly increased thermostability than the reference EII-a when activity was measured at elevated temperature. EII-l exhibited a T_{50} value 1.3°C higher than EII-a that indicates the temperature at which 50% of β-glucanase activity remains. The irreversible thermal inactivation difference between EII-a and EII-l after 5 minutes of heat treatment at 56°C was 11.9%. The functional significance of the three amino acid differences between EII-a and EII-l was examined by making combinatorial mutations in EII-a using site-directed mutagenesis. The S20G and D284E amino acid substitutions were shown to be responsible for the increase in EII-1 thermostability.

3.1 Introduction

Efficient hydrolysis of cell wall (1→3,1→4)-β-glucan (β-glucan) is essential when malting barley to ensure complete endosperm modification. Incomplete modification can lead to residual malt β-glucan that is problematic to brewers as it can limit hot water extract, increase wort viscosity and contribute to beer chill haze (Evans et al., 1999; Stuart et al., 1988). Increased wort viscosity results from the residual high molecular weight β-glucans rapidly solubilising in the mash causing β-glucan gelation thus reducing filtration efficiency (Stewart et al., 1998; Stuart et al., 1988). High molecular weight β-glucans are primarily hydrolysed by (1→3,1→4)-β- glucan endohydrolases (β-glucanase EC 3.2.1.73) that are relatively thermolabile in comparison to the other malt hydrolases α-amylase, β-amylase and limit dextrinase. The β-glucanases are partially denatured during kilning in malting and significantly denatured after 10 minutes of mashing at 65°C during brewing (Loi et al., 1987). As a result barley β-glucanases cannot reduce the β-glucans at high mashing temperatures.

*HvGlb*1 and *HvGlb*2 encode the two barley β-glucanase isoenzymes EI and EII respectively that have been thoroughly characterised (Woodward and Fincher, 1982a). The two isoenzymes share 92% sequence identity and differ by 25 amino acids (Litts et al., 1990; Slakeski et al., 1990; Wolf, 1991). Examination of amino acid differences indicated that EII has significantly more surface charge and a stronger hydrophobic core than EI (Lauer et al., 2017a). It is known that EII has significantly greater thermostability than EI with optimum temperatures at approximately 45°C and 37°C respectively (Woodward and Fincher, 1982a), with the difference in thermostability initially attributed to an additional glycosylation site in EII (Doan and Fincher, 1992). EI is the more thermolabile of the two isoenzymes and denatures during kilning (Loi et al., 1987). EII partially survives the kilning process and remains active in 45°C mashes, however it rapidly loses activity in mashes starting at higher temperatures above optimum temperature, limiting the reduction of wort β-glucan (Loi et al., 1987).

Wild barley (*Hordeum vulgare* spp. *spontaneum*) is the progenitor of cultivated barley and is an excellent resource for the genetic improvement of new varieties (Ellis et al., 2000). Previously Jin et al., (2011) examined the *HvGlb*1 genomic sequence in 80 cultivated varieties and 80 Tibetan wild barley accessions. The Tibetan wild barley lines were examined using the single-strand conformation polymorphism method that identified 34 single nucleotide polymorphisms (SNPs) (Jin et al., 2011). Five of these SNPs were unique to Tibetan wild barley, however the experimental method was unable to establish a link between the SNPs identified to possible phenotypic variation. These SNPs were identified in a gene fragment named P5 and were found to correlate (30%, $p<0.01$) with malt β-glucan content variation, but it was not stated whether this fragment was situated in the exon or intron regions. It was also unclear if these SNPs identified in cultivated varieties or Tibetan wild barley resulted in any new allozymes.

Allelic variation in β-glucanase has recently been characterised in elite and exotic germplasm and the majority of this variation was identified in wild barley accessions (Lauer et al., 2017a). This genetic survey was conducted by sequencing the exon regions and allowed the identification of 25 SNPs in *HvGlb*1. The SNPs resulted in few amino acid substitutions and were limited to five new allozymes; three in wild barley accessions and two in landraces. The new allozymes were found in only 11 individual accessions compared to 92.5% of germplasm that shared a common allozyme. Only one new allozyme, EI-b, was identified in multiple

wild barley accessions and was predicted by homology modelling to have increased β-glucanase stability. The nucleotide sequence examination of the exon regions in *HvGlb*2 identified 38 SNPs that resulted in 13 new allozymes; 11 of these were exclusive to wild barley accessions. Four of the new allozymes identified were predicted to have increased stability and one of these four was identified in 39% of the wild barley accessions examined (Lauer et al., 2017a).

The experimental work described in this study examined the thermostability and kinetic properties of selected EI and EII allozymes identified and described by Lauer et al., (2017a). Genes corresponding to the selected allozymes were cloned, heterologously expressed in *Escherichia coli*, and the proteins purified and tested using reducing sugar assays. The kinetic analysis of recombinant EI and EII proteins did not identify any significant differences when compared to the reference proteins. Examination of EI thermostability identified one EI variant with increased residual β-glucanase activity after heat treatment. One EII allozyme with significantly increased β-glucanase thermostability above the reference was further examined and the amino acid substitutions that contributed to the increased thermostability were identified. The results demonstrate the potential for increasing malting barley β-glucanase thermostability with a new form of EII identified from wild barley to improve brewing efficiency.

3.2 Materials and Methods

3.2.1 Materials

Oligonucleotide primers, vectors and chemically competent *E. coli* cells were purchased from Invitrogen, Life Technologies (Carlsbad, USA). Reagent-grade chemicals were purchased from Sigma-Aldrich (St. Louis, USA). Megazyme medium viscosity barley (1→3,1→4)-β-glucan (Lot No.: 90802, Mp: 167000 g/mol Mw: 245 kDa, Mn: 122000, intrinsic viscosity (η): 3.32 dL/g,) was purchased from Deltagen (Kilsyth, Australia). Genomic DNA extractions were performed as previously described (Lauer et al., 2017a).

3.2.2 Amplification, Cloning and Heterologous Expression of *HvGlb*1 and *HvGlb*2 alleles

Exon regions of *HvGlb*1 and *HvGlb*2 were amplified from genomic DNA. Primers were used as nested primers to differentiate between *HvGlb*1 and *HvGlb*2 (Lauer et al., 2017a). Exon primers were designed using the published *HvGlb*1 and *HvGlb*2 sequences and Geneious R6 (Kearse et al., 2012) exclusively targeting the second exon (Table 3-1). Thrombin cleavage sites were included in the forward primers. PCR amplification was conducted using Phusion High Fidelity following the manufacturer's instructions (Thermo-Fisher, Waltham, USA), PCR fragments were purified from 1% agarose gels using the NucleoSpin Gel and PCR kit (Scientifix, Springvale, Australia).

Table 3-1. PCR primer sequences for amplifying full-length open reading frames (ORF). Thrombin cleavage sites are underlined

Gene	Primers	Primer pair sequences
*HvGlb*1	Nested	GCCGCCATTCTTGCTTATTGG/ GTATAGCAAGCCACTACTCGC
	ORF	TTAGTTCCGCGGGGTTCTATCGGGGTGTGCTACG/ GTATAGCAAGCCACTACTCGC
		TTAGTTCCGCGGGGTTCTATCGGGGTGTGCTATG/ GTATAGCAAGCCACTACTCGC
*HvGlb*2	Nested	CAATGGAAGGAACAGTACTGCTAC/ GAGGAGGTAATTAAGAGGCTCTCC
	ORF	TTAGTTCCGCGGGGTTCTATCGGGGTGTGCTACG/ TCAGAAGTTGATGGGGTAGAC

*HvGlb*1 and *HvGlb*2 fragments were inserted by TA cloning into the pCR8 host vector (Gateway cloning system) and orientation was checked by restriction enzyme digest and sequenced by the Australian Genome Research Facility (AGRF, Adelaide, Australia). Correct constructs were transformed into One Shot Mach1 chemically competent *E. coli* cells. Cells were centrifuged and resuspended in xTractor buffer (Clontech, Mountain View, USA) followed by centrifugation at 7,500g for 10 minutes. Plasmids were purified from 1% agarose gels using the Isolate II Plasmid mini kit (Bioline, London, U.K.). Constructs were transferred from the pCR8 vector to the pDEST17 expression vector (Gateway cloning system) using LR Clonase II (Thermo-Fisher, Waltham, USA). The pDEST17 vector contains a 6x His tag at the N-terminus for purification by metal affinity chromatography. The pDEST17 constructs were transformed into One Shot BL21 star (DE3) chemically competent *E. coli* cells.

Expression conditions were as follows; BL21 star cells were grown in Luria Broth (LB) with 200µg/mL ampicillin at 37°C and 220 rpm until the absorbance reached 0.5-0.7 at OD_{600}. The temperature was reduced to 23°C and isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.25mM. The cells were grown for 16 hours at 23°C and harvested by centrifugation at 7,500g for 10 minutes. Cells were centrifuged and resuspended in xTractor Buffer (Clontech) with added lysozyme and DNaseI at room temperature for 30-60 minutes followed by centrifugation at 7,500g for 10 minutes. The soluble fraction was purified by metal affinity using TALON cobalt resin (Clontech) and eluted with Imidazole (20mM Tris-HCl; 500mM NaCl; 10% Glycerol (v/v); 0.05% Tween 20 (v/v); 200mM Imidazole, pH 7.4). The eluted protein was desalted and concentrated using a 10K Amicon Ultra-4 centrifugal filter (Merck Millipore Ltd, Billerica, USA). Recombinant β-glucanases used in kinetics experiments were exchanged into 50mM Tris-HCl, pH 8 and further purified by ion exchange using HiTrap SP HP columns (GE Healthcare Life Sciences, Chicago, USA) and the ÄKTAstart chromatography system. The purified β-glucanases were desalted and concentrated using a 10K Amicon Ultra-4 centrifugal filter (Merck Millipore Ltd, Billerica, USA) and exchanged into 50mM sodium acetate, pH 5.0. β-Glucanase concentrations were determined colormetically using the Pierce Coomassie (Bradford) Kit (Thermo-Fisher) (Bradford, 1976) and standardised in 2µg/µL bovine serum albumin (BSA) in 50mM sodium acetate, pH 5.0. Recombinant β-glucanases used in thermostability experiments were exchanged into 1X Thrombin Cleavage Buffer (20mM Tris-HCl; 150mM NaCl; 2.5mM $CaCl_2$, pH 8.4) in preparation for 6x His tag removal by thrombin cleavage.

3.2.3 Site-Directed Mutagenesis of EII-a

EII-l amino acid substitutions S20G, R100Q and D284E were introduced into the pDEST17EII-a construct using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) to generate seven EII-l variants with all possible EII-l amino acid combinations. Primer pairs were designed according to manufacturer's primer design recommendation (Table 3-2). Mutagenesis reactions were conducted following the manufacturer's protocol. Plasmids were purified and sequenced by AGRF (Adelaide, Australia) to confirm each mutation. EII-l variants were heterologously expressed in *E. coli* and purified as described above.

Table 3-2. Primer pair nucleotide sequences for site-directed mutagenesis

Amino acid substitution	Primer pair sequences
EII-a S20G	CGAGCACCGTCGTGGGCATGTTCAAGTCC/ GGACTTGAACATGCCCACGACGGTGCTCG
EII-a R100Q	CGGCGCCACCCAGAACCTCGTCC/ GGACGAGGTTCTGGGTGGCGCCG
EII-a D284E	TCAACGAGAACCAGAAGGAGAGCGGCGTGG/ CCACGCCGCTCTCTTTCTGGTTCTCGTTGA

3.2.4 Thrombin cleavage of 6x His tag

6x His tag removal was conducted in 1mL volume reactions with approximately 50µL of TALON purified recombinant β-glucanase concentrate, 1X Thrombin Cleavage Buffer (20mM Tris-HCl; 150mM NaCl; 2.5mM $CaCl_2$, pH 8.4), 0.05U/µL Thrombin from Human Plasma (Sigma Aldrich); 25% (v/v) Glycerol. Thrombin reactions were left at 37°C for a minimum of 16 hours. Reactions were chilled and added to TALON cobalt resin (Clonetech) to bind all 6x His tags at 4°C for 30 minutes on a rotator. Samples were centrifuged at 700g for 5 minutes and the supernatant was added to a pre-prepared 50% slurry of p-Aminobenzamidine-Agarose (Sigma Aldrich) and Thrombin Binding Buffer (20mM Tris-HCl; 500mM NaCl, pH 7.4) to remove thrombin at 4°C overnight on a rotator. Samples were centrifuged at 700g for 5 minutes and supernatant was desalted and concentrated using a 10K Amicon Ultra-4 centrifugal filter (Merck Millipore Ltd) and exchanged into 50mM sodium acetate, pH 5.0. Samples were quantified and stored as described above.

3.2.5 Enzyme activity assay

β-Glucanase activity was determined using the reducing sugar assay method as described by Mellitzer et al., (2012). For the substrate conversion recombinant β-glucanases (0.1ng/µL) were incubated with medium viscosity barley (1→3,1→4)-β-glucan over a range of substrate concentrations from 0.8µM – 16.5µM at 37°C for 20 minutes in 50mM Sodium Acetate, pH 5.0 with BSA at a final concentration of 0.4µg/µL. The optimal assay conditions for examining the kinetic properties were 37°C in 50mM sodium acetate, pH 5.0 and the reactions were terminated by incubating at 95°C for 5 minutes. The detection of the β-glucan reducing ends was achieved using *p*-hydroxybenzoic acid hydrazide (pHBAH) as described by Mellitzer et al., (2012).

Two methods were used to examine thermostability of recombinant β-glucanases; (i) T_{50} values were determined by incubating β-glucanases (0.2ng/μL) with 10μM medium viscosity barley (1→3,1→4)-β-glucan at a range of temperatures between 37°C to 65°C for 15 minutes in 50mM sodium acetate, pH 5.0 and BSA at a final concentration of 0.4μg/μL. Plotted temperature curves were used to determine the temperature at which 50% of β-glucanase activity remained, (ii) irreversible thermal inactivation was used to examine β-glucanase activity before and after 5 minutes of heat treatment at 50°C for EI and 56°C for EII. β-Glucanase activity was assayed at 37°C for 15 minutes in 50mM sodium acetate, pH 5.0 and BSA at a final concentration of 0.4μg/μL.

3.2.6 Modelling of amino acid substitutions

Amino acid substitutions were examined using the PyMol graphic tool (The PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC) using methods previously described (Lauer et al., 2017a).

3.2.7 Data Analysis

Microsoft Excel Spreadsheet Software (Microsoft, USA) was used for data analysis of β-glucanase activity, thermostability, statistics and generating Lineweaver-Burk plots to determine the kinetic parameters K_m, V_{max} and k_{cat}. An analysis of variance (ANOVA) was used in statistical analysis of β-glucanase activity, kinetic properties and thermostability data and the least significant difference (LSD) was calculated.

3.3 Results and Discussion

3.3.1 Expression and purification of recombinant EI and EII allozymes

Six novel forms of EI and 14 forms of EII have recently been identified and described (Lauer et al., 2017a). The *HvGlb*1 and *HvGlb*2 β-glucanase genes share 92% sequence identity and consist of two exons separated by a single intron (Slakeski et al., 1990). The 306 amino acid sequences of the mature EI and EII proteins encoded by *HvGlb*1 and *HvGlb*2 respectively are located exclusively in the second exon, whilst the signal peptide is encoded by the first exon. Therefore, the second exon was amplified from genomic DNA for heterologous s expression of EI and EII. Four variants of EI and EII and the EI-a and EII-a reference sequences (Litts et

al., 1990; Slakeski et al., 1990; Wolf, 1992) were successfully cloned in the pDEST17 vector and were expressed in *E. coli*. The new forms of EI differ by single amino acid substitutions to the reference sequence as shown in Table 3-3.

Table 3-3. EI amino acid substitutions compared to the reference EI-a

EI	T17S	A95T	Q110H	Q282E
EI-a	T	A	Q	Q
EI-b				E
EI-c		T		
EI-d			H	
EI-f	S			

Table modified from Lauer et al., (2017a).

The new forms of EII differ from the reference sequence by two or three amino acid substitutions as shown in Table 3-4. Thrombin cleavage sites were introduced via the pDEST17 vector for optional removal of the 6x His tag located at the N-terminus of the expressed protein. Recombinant β-glucanases were purified from crude bacterial extracts by cobalt metal affinity chromatography. β-Glucanase protein for kinetic experiments was further purified by cation exchange. Cation exchange was not required for β-glucanase protein used for thermostability experiments as the 6x His tag was found to reduce β-glucanase thermostability (data not shown), therefore the tags were removed using thrombin and proteins subsequently treated as described in the Materials and Methods. The 6x His tag did not interfere with substrate binding and thus was not removed from the β-glucanase proteins used in kinetic analyses.

Table 3-4. EII amino acid substitutions compared to the reference EII-a

EII	S20G	R100Q	V114A	G219A	D284E
EII-a	S	R	V	G	D
EII-c	G			A	
EII-d			A	A	
EII-e	G		A	A	
EII-l	G	Q			E

Table modified from Lauer et al., (2017a).

Purified recombinant EII-a was first examined with and without the 6x His tag attached at the N-terminus. The 6x His tag incorporated into the pDEST17 vector adds 22 amino acids to the protein before the thrombin cleavage site. EII-a exhibited an increase in T_{50} after His tag removal. The differences in recombinant EII-a thermostability due to the presence or absence of the His tag reported in this study are similar to findings in previous publications on native EII[5] and recombinant EII proteins (Table 3-5) (Stewart et al., 2001). The native EII extracted from Clipper and the recombinant EII previously cloned from Himalaya into the pET3a-HT expression vector were both confirmed by sequencing to also be EII-a (Lauer et al., 2017a; Stewart et al., 2001; Woodward and Fincher, 1982a). The T_{50} of the recombinant EII examined by Stewart et al., (2001) was 47.5°C, which is consistent with the recombinant EII-a with the His tag attached examined in this study. In comparison, the T_{50} of native EII was similar to the T_{50} of recombinant EII-a without the 6x His tag (Woodward and Fincher, 1982a). The k_{cat} values reported here for the reference EI-a and EII-a allozymes were 265 sec^{-1} and 359 sec^{-1} respectively. These are higher turnover rates than reported in previous publications where the k_{cat} values of native EI and EII proteins were 118 sec^{-1} and 193 sec^{-1} respectively.[5] This range of values could possibly to be due to differences in the molecular weight of the β-glucan substrate used in the assays.

Table 3-5. Comparison of native and recombinant EII T_{50} values

EII	T_{50}
Native EII[a]	51°C
Recombinant EII (+his)[b]	47.5°C
Recombinant EII-a (+his)	47.8°C
Recombinant EII-a (-his)	51.2°C

[a] From Woodward and Fincher, 1982a, [b] From Stewart et al., (2001)

3.3.2 Kinetic properties and thermostability of EI

The EI-a reference sequence published by Slakeski et al., (1990) has been identified in 92.5% of germplasm examined to date (Lauer et al., 2017a). The kinetic properties of the EI variants in comparison to the commonly occurring EI-a reference are presented in Table 3-6. Under optimal conditions at 37°C, pH 5.0, EI-b and EI-f exhibited similar kinetic properties and no significant differences to EI-a were identified. All EI allozymes shared a similar substrate affinity, however the k_{cat} value for EI-d revealed a significantly slower turnover rate than EI-a.

The enzyme catalytic efficiencies as defined by k_{cat}/K_m for EI-c and EI-d were significantly lower than EI-a by 37.1% and 47.4% respectively.

Table 3-6. Kinetic constants and thermostability of EI

	K_m (μM)	k_{cat} (sec^{-1})	k_{cat}/K_m (μM^{-1} sec^{-1})	T_{50}
EI-a	27.4	264.7	9.7	46.4°C
EI-b	22.6	227.6	10.1	46.3°C
EI-c	26.2	161.6	6.1*	46.7°C
EI-d	21.7	110.2*	5.1*	ND [a]
EI-f	28.1	265.9	9.5	ND [a]

[a] ND = not determined. Significant differences = * ($p<0.05$)

A significantly slower turnover rate and catalytic efficiency was exhibited by EI-d due to the Q110H substitution (Figure 1; red). Q110H introduces a His residue into an α-helix and forms an extra hydrogen bond linking to the backbone of the helix (Gregoret and Sauer, 1998). The additional hydrogen bond in the α-helix increases rigidity that may disrupt the catalytic site causing the decrease in k_{cat} and affecting catalytic efficiency (Figure 3-1) (Armstrong and Baldwin, 1993). EI-c exhibited similar kinetic properties to EI-d in comparison to EI-a due to the A95T substitution (Figure 3-1; orange). The change from Ala to Thr changes the polarity of the residue at position 95 and creates a hydrogen bond near Glu93 that has been proposed as a catalytic hydrogen donor (Figure 3-1; yellow) (Tsai et al., 2008). The extra hydrogen bond in A95T may cause disruption to the binding of β-glucan during hydrolysis and thus a slower turnover rate affecting catalytic efficiency (Armstrong and Baldwin, 1993). However, EI-c exhibited 6.8% more residual β-glucanase activity than EI-a in thermostability testing.

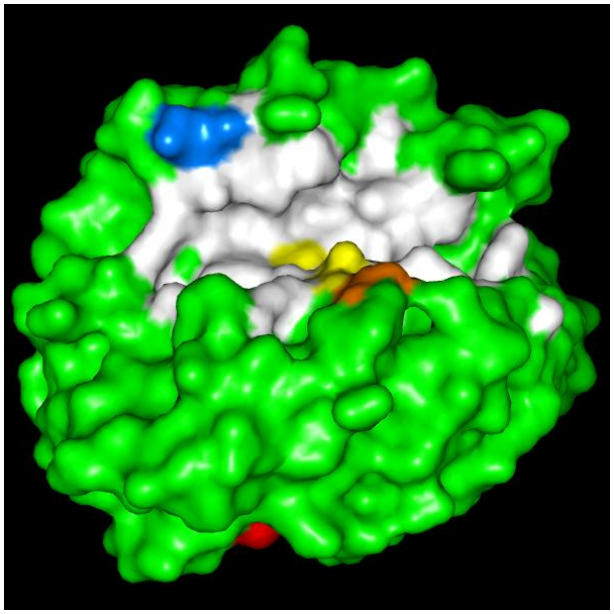

Figure 3-1. Three dimensional surface model of EI (green). T95 is shown in orange, H110 is shown in red and E282 is shown in blue. Substrate binding residues are shown in white and catalytic residues 93 and 232 are shown in yellow (Tsai et al., 2008).

EI-a, EI-b and EI-c were selected for thermostability analyses based on previous predictions for enzyme stability (Lauer et al., 2017a). To assess the thermostability of each β-glucanase, activity was assayed at a range of elevated temperatures to determine the T_{50} values that represent the temperature at which 50% activity remains (Table 3-6). The T_{50} values of the EI allozymes were similar with no statistically significant differences found. The irreversible thermal inactivation of EI allozymes was also determined by comparing the initial β-glucanase activity assayed at 37°C with activity after 5 minutes of heat treatment at 50°C (Figure 3-2). After 5 minutes of heat treatment significant differences ($p<0.001$) between EI allozymes in residual β-glucanase activity were identified. EI-c exhibited 6.8% higher residual β-glucanase activity than EI-a, while EI-b exhibited 5.5% lower residual β-glucanase activity. β-Glucanase thermostability of the novel forms of EI examined is unlikely to improve enzyme survival during the kilning process as a significant increase in thermostability similar to EII would be required to avoid total EI loss.

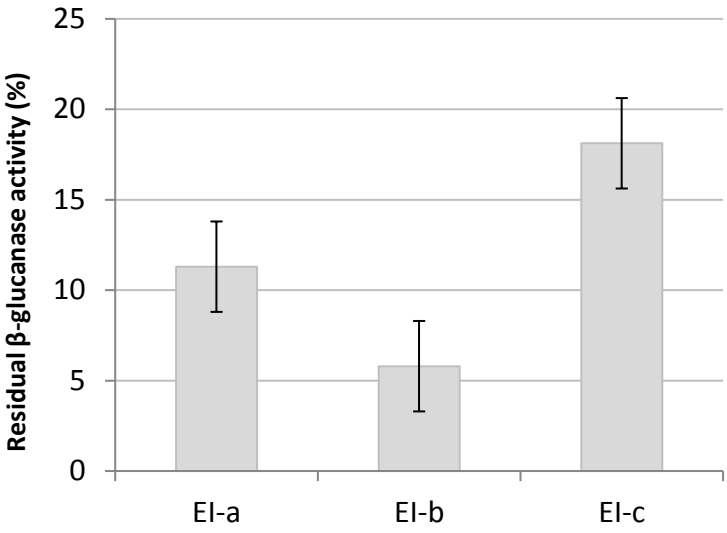

Figure 3-2. Irreversible thermal inactivation of recombinant EI β-glucanases. β-Glucanases were heat treated at 50°C for 5 minutes. Initial and heat treated β-glucanase activity was measured by assaying with medium viscosity barley (1→3,1→4)-β-glucan at 37°C. The percentage of residual activity was calculated. Error bars represent the LSD of three replicates.

EI-b and EI-c were originally predicted to be more likely to have greater β-glucanase stability than the reference EI-a (Lauer et al., 2017a), but no significant differences were identified experimentally for activity at elevated temperatures by any of the EI variants (Table 3-6). However, significant differences were identified in the irreversible thermal inactivation experiments that revealed a significant decrease in residual β-glucanase activity for EI-b (Figure 3-2). The Q282E substitution in EI-b was predicted to provide a minor increase in stability as a change from Gln to Glu significantly increases the entropy of protein refolding (Figure 3-1; blue) (Lauer et al., 2017a). The thermal inactivation of β-glucanase has been shown to initiate at the C-terminus where the protein starts to unfold (Stewart et al., 2001; Varghese et al., 1994). The EI and EII C-terminal segment consists of the last 30 amino acids in the peptide chain with an undefined flexible structure (Varghese et al., 1994). The Q282E substitution occurs in this C-terminal segment close to the substrate binding cleft (Figure 3-1; white and blue). The substitution of Gln to Glu in EI-b essentially increases the charge of residue 282. This may cause alternative interactions to occur within the C-terminal segment resulting in destabilisation of protein folding.

3.3.3 Kinetic properties and thermostability of EII

The EII-a reference sequence published by Wolf (1992) has been identified in 62.5% of cultivated barley and one wild barley accession of the germplasm examined to date (Lauer et al., 2017a). The kinetic properties of the EII variants are presented in Table 3-7. Under optimal conditions at 37°C, pH 5.0, EII-a and EII-c shared similar kinetic properties that were not statistically different. The amino acid differences between EII-a and EII-c, S20G and G219A occur in α-helices and involve a Gly residue that is known to increase conformational flexibility of an α-helix (Matthews et al., 1987). The G219A substitution favours increased protein stability because Ala reduces conformational flexibility, stabilising the α-helix. The kinetic results also suggest that the S20G substitution in EII is favoured. S20G was predicted to decrease protein stability because of the positive change in Gibbs free energy associated with Ser at position 20 (Lauer et al., 2017a). The short polar side-chain of Ser may be less favourable in the α^1-helix of EII than Gly. The combination of S20G and G219A did not significantly affect EII kinetic properties or EII activity at elevated temperature, however the slight improvement in conformational refolding of EII-c resulted in higher residual β-glucanase activity than for EII-a.

Table 3-7. Kinetic constants and thermostability of EII

	K_m (µM)	k_{cat} (sec^{-1})	k_{cat}/K_m (μM^{-1} sec^{-1})	T_{50}
EII-a	37.8	358.9	9.5	51.9°C
EII-c	32.2	306.1	9.5	52.0°C
EII-d	17.4*	154.8*	8.8*	51.8°C
EII-e	12.6***	114.1**	9.2*	52.1°C
EII-l	23.1**	189.6***	8.2**	53.2°C***

Significant differences = * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$)

EII-d, EII-e and EII-l exhibited significantly lower K_m and k_{cat} values that reflected significantly lower β-glucanase catalytic efficiency compared to EII-a (Table 3-7). The significant differences in substrate affinity and turnover rate identified between EII-c, EII-d and EII-e were caused by the presence or absence of the V114A substitution. EII-d and EII-e both have the V114A substitution and all three EII variants also have the G219A substitution (Table 3-4). The K_m and k_{cat} values for EII-d were 2-fold lower compared to those of EII-c caused by the V114A substitution. The effect on kinetic parameters due to V114A is further evident in EII-e that exhibited nearly 3-fold lower K_m and k_{cat} values, but still maintained

greater catalytic efficiency due to the presence of the S20G substitution. The V114A substitution is located in an α-helix that is not close to the active site (Figure 3-3; teal). Val to Ala substitutions in α-helices are generally considered to be favourable since it is likely that the shorter Ala side-chain alters the packing of the α-helix and causes the enzyme to bind more tightly to the substrate (Gregoret and Sauer, 1998).

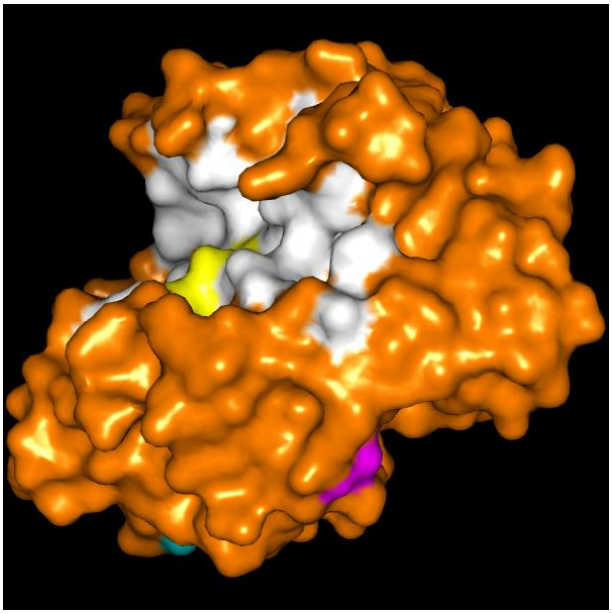

Figure 3-3. Three dimensional surface model of EII-d (orange). A114 is shown in teal and A219 is shown in magenta. Substrate binding residues are shown in white and catalytic binding residues 93 and 232 are shown in yellow (Tsai et al., 2008).

EII-l exhibited significantly increased substrate affinity, decreased turnover rate and a lower catalytic efficiency of 189.6 sec^{-1} compared to 358.9 sec^{-1} exhibited by EII-a (Table 3-7). The significant decrease in turnover rate and catalytic efficiency is likely to be caused by the R100Q amino acid substitution (Figure 3-4; red). This is located on the surface of the enzyme seven amino acids downstream from the putative Glu hydrogen donor at position 93 (Figure 3-4; yellow) (Tsai et al., 2008). The loss of the positive charge from Arg is likely to decrease the electrostatic potential of the binding site causing the significantly decreased k_{cat} value and reduced activity at elevated temperature (Gribenko et al., 2009). EII mutants carrying R100Q exhibited a significant decrease in β-glucanase activity at elevated temperatures and a slight increase in residual β-glucanase activity after irreversible thermal inactivation. R100Q was predicted to improve EII stability because of the negative change in Gibbs free energy favouring the substitution (Lauer et al., 2017a), however the change in electrostatic potential negatively affects enzyme activity and reduces EII thermostability.

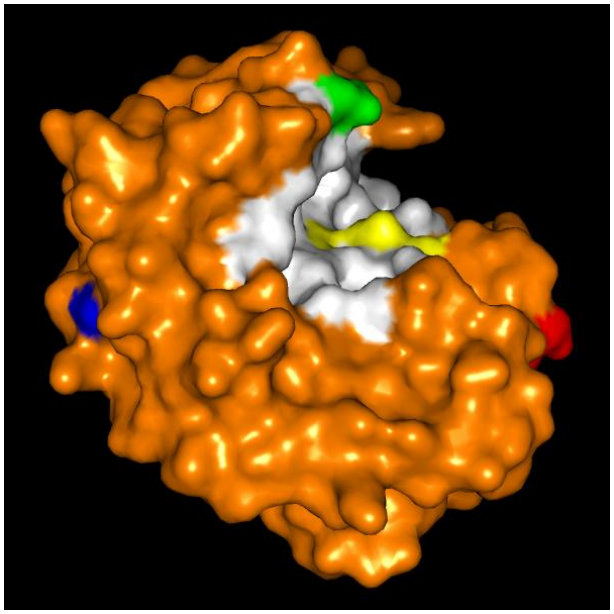

Figure 3-4. Three dimensional surface model of EII-l (orange).G20 is shown in blue, Q100 is shown in red and E284 is shown in green. Substrate binding residues are shown in white and catalytic residues 93 and 232 are shown in yellow (Tsai et al., 2008).

Analysis of EII variants showed that EII-l exhibits significantly increased β-glucanase thermostability. EII-l was significantly more thermostable and exhibited a T_{50} value 1.3°C higher than EII-a (Table 3-7). The other forms of EII were not significantly different to EII-a and exhibited similar T_{50} values. The irreversible thermal inactivation of EII allozymes was determined from β-glucanase activity assayed at 37°C before and after 5 minutes of heat treatment at 56°C (Figure 3-5). Significant differences were identified and all EII variants performed better than EII-a such that EII-c, EII-d, EII-e, and EII-l exhibited significantly higher residual β-glucanase activity by 6.6%, 4.2%, 9.3% and 11.9% respectively.

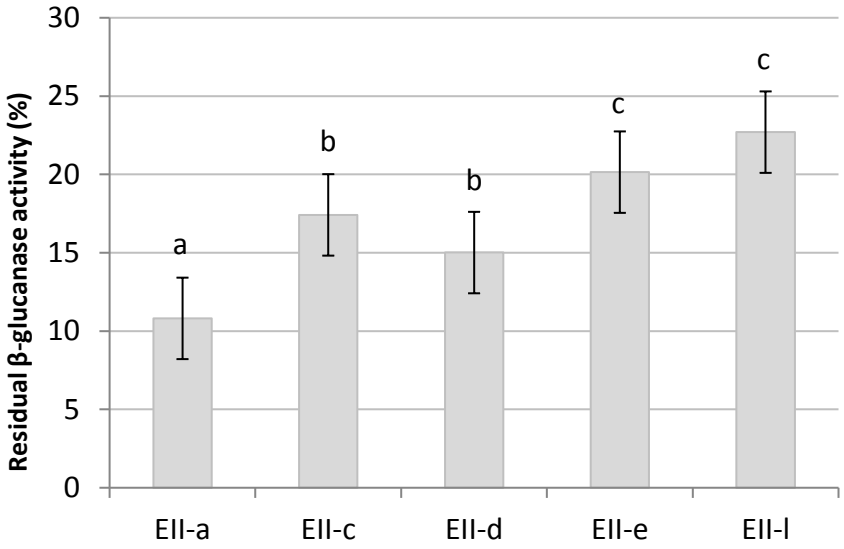

Figure 3-5. Irreversible thermal inactivation of recombinant EII β-glucanases. β-Glucanases were heat treated at 56°C for 5 minutes. Initial and heat treated β-glucanase activity was measured by assaying with medium viscosity barley (1→3,1→4)-β-glucan at 37°C. The percentage of residual activity was calculated. Error bars represent the LSD of three replicates.

The EII variants examined in the current study were previously predicted to have greater structural stability than the reference EII-a (Lauer et al., 2017a), however EII-l was the only allozyme that was significantly different to EII-a for both T_{50} and irreversible thermal inactivation. The S20G, V114A and G219A substitutions combined in EII-c, EII-d and EII-e provided no significant improvement in EII activity at elevated temperatures compared to EII-a. However, significant differences were identified in the irreversible thermal inactivation of EII-c, EII-d and EII-e that all exhibited increased in residual β-glucanase activity in comparison to EII-a (Figure 3-5). The significance of the increased thermostability after irreversible thermal inactivation has the potential benefit of increasing enzyme survival during the kilning process by retaining more than 36% of β-glucanase activity in finished malt.[4] It is also possible that malt with the EII-l form of β-glucanase has the extra potential to provide not only higher β-glucanase levels but also longer surviving β-glucanase activity in high temperature mashes.

3.3.4 The contribution of individual amino acid substitutions to enzyme stability

The effects of the three amino acid substitutions S20G, R100Q and D284E carried by EII-l on enzyme thermostability were examined in all possible combinations. Seven mutants of EII-a in the pDEST17 vector were developed using site-directed mutagenesis. These β-glucanase mutants were expressed and purified as previously described and thermostability was examined. T_{50} determinations for the seven mutants identified the S20G+D284E combination as contributing the greatest increase to EII-l thermostability (Figure 3-6a). S20G+D284E, the triple mutant and EII-l had T_{50} values that were significantly higher than all other EII variants examined. EII mutants with D284E and R100Q+D284E also demonstrated higher T_{50} values than EII-a. However, R100Q and S20G+R100Q EII mutants that did not have D284E exhibited significantly lower T_{50} values than EII-a. The irreversible thermal inactivation of EII mutants was determined as described for the EII variants (Figure 3-6b). D284E and mutants with S20G+ D284E revealed significantly more residual β-glucanase activity than all other EII mutants. R100Q+D284E exhibited reduced residual β-glucanase activity compared to the mutants containing D284E by 2.5% less than the triple mutant. R100Q+D284E was statistically similar to S20G and exhibited significantly higher residual β-glucanase activity than EII-a as did R100Q and S20G+R100Q.

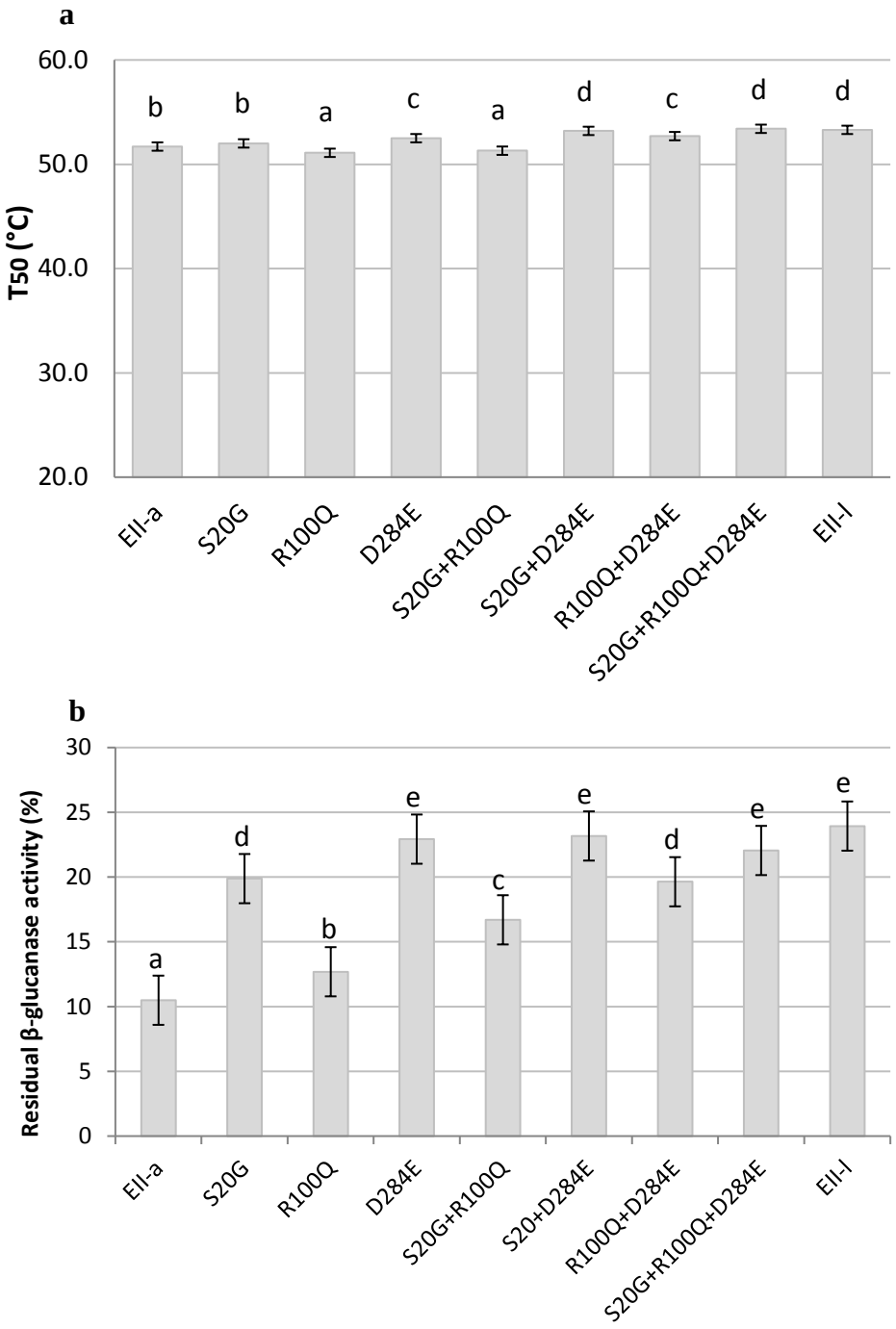

Figure 3-6. a) T_{50} determination of recombinant EII-l mutant β-glucanase. The enzyme was assayed with medium viscosity barley (1→3,1→4)-β-glucan at a range of temperatures for 15 minutes and β-glucanase activity was measured. T_{50} represents the temperature at which 50% of β-glucanase activity remains. Error bars represent the LSD of three replicates. **b)** Irreversible thermal inactivation of recombinant EII-l mutant β-glucanase. The β-Glucanase was heat treated at 56°C for 5 minutes. Initial and heat treated β-glucanase activity was measured by assaying with medium viscosity barley (1→3,1→4)-β-glucan at 37°C. The percentage of residual activity was calculated. Error bars represent the LSD of three replicates.

3.3.5 The D284E amino acid substitution

The substitution combination of S20G (Figure 3-4; blue) and D284E (Figure 3-4; green) exhibited the greatest increase in EII thermostability regardless of the presence of the R100Q (Figure 3-4; red) substitution. The irreversible thermal inactivation of EII mutants revealed that the D284E substitution increased residual β-glucanase activity to levels higher than obtained with G219A, which was predicted to have the greatest increase in β-glucanase stability (Lauer et al., 2017a). The change from glutamine to glutamic acid increases side chain length and forms an extra hydrogen bond in the loop of the C-terminus where residue 284 is located (Figure 3-4; green). The additional hydrogen bond improves thermal stability and the entropy of refolding potential of EII-l (Vogt et al., 1997).

The D284E substitution provided the greatest increase in EII thermostability and this was enhanced by the addition of the S20G substitution (Figure 3-7). The increased side-chain length of the glutamic acid residue at position 284 creates an additional hydrogen bond in EII-l (Figure 3-7), located in the C-terminus loop near the substrate binding cleft (Figure 3-4; white) and significantly improves the refolding of EII (Tsai et al., 2008). The EII mutants with the D284E substitution, with the exception of R100Q+D284E, exhibited similar residual β-glucanase activity (Figure 3-6b). Together S20G and D284E have an additive effect significantly increasing β-glucanase activity at elevated temperatures in the presence or absence of R100Q. S20G and D284E are located close together in the flexible N- and C-terminal regions of the EII peptide (Matthews et al., 1987). The proximity of the two substitutions to one side of the binding cleft is likely to reduce the flexibility of the N- and C-terminus regions and the slight increase in structural rigidity thereby increases thermostability (Figure 3-4; blue and green). Substitutions that increase structural rigidity will generally favour an increase in thermostability compared to flexible structures that favour enzyme activity (Mahanta et al., 2015; Shoichet et al., 1995). The results from this study are consistent with the observation of a trade-off effect between enzyme activity and thermostability (Mahanta et al., 2015).

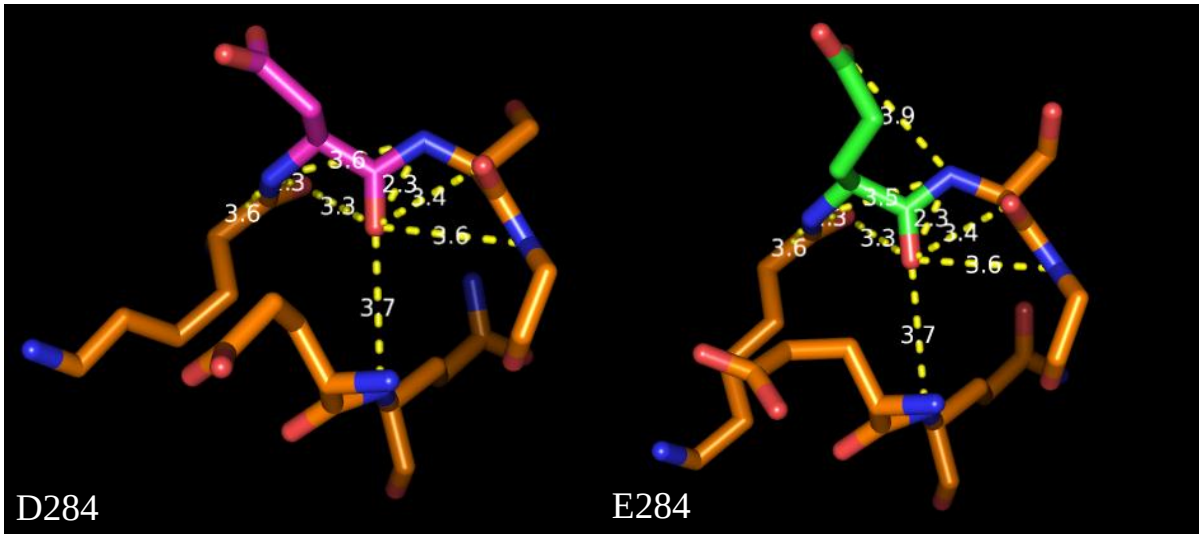

Figure 3-7. Representation of amino acid substitution D284E identified in EII (orange). EII wild type residue D284 is shown in pink and the corresponding E284 amino acid substitution is shown in green. Nitrogen and oxygen atoms are coloured dark blue and red respectively. Additional hydrogen bonds facilitated by the substituted residue are illustrated by yellow broken lines with distances labelled in angstrom units.

Barley β-glucanase is essential for complete endosperm modification in the malting process. The degradation of cell wall β-glucan by the β-glucanase enzymes is required to allow the key hydrolytic enzymes such as α- and β-amylase access to starch. Incomplete endosperm modification leads to residual malt β-glucan solubilising in the wort, increasing the viscosity and reducing filtration efficiency (Evans et al., 1999). The thermolabile nature of β-glucanase compared to α- and β-amylase causes a rapid loss of β-glucanase activity at high temperatures required for starch gelatinisation in mashing (Loi et al., 1987). Therefore, an increase in barley β-glucanase thermostability may assist viscosity reduction when using malt that is not highly modified or when raw barley is used as an adjunct.

This study has described the characterisation of the natural variation in β-glucanase previously identified in wild barley. The thermostability analysis of new forms of EI and EII revealed small but significant differences. EII-l exhibited an increase of 1.3°C in β-glucanase thermostability and 11.9% higher residual β-glucanase activity after heat treatment compared to EII-a. However, it was also demonstrated that the EI and EII variants exhibiting higher residual β-glucanase activity after irreversible thermal inactivation also displayed lower catalytic efficiency suggesting that the reduction in the entropy of β-glucanase refolding may be at the expense of reduced enzyme catalytic efficiency.

The novel β-glucanase alleles identified in wild barley have the potential to improve process efficiency in brewing based on characterisation of fundamental enzyme properties. Commercial beer production uses malt with a range of modification levels, diverse temperature regimes during mashing, and filtration equipment with differing sensitivity to residual β-glucan and elevated viscosity. Determining the commercial relevance of the novel β-glucanase allele will require its incorporation into a contemporary genetic background and determination of malting and brewing quality characteristics under a range of industrial conditions. The description of the effects of specific amino acid substitutions on structural stability and catalytic efficiency will inform further allele mining and rational design of β-glucanase with improved functional characteristics.

CHAPTER 4

VALIDATION OF NOVEL BARLEY (1→3,1→4)-β-GLUCAN ENDOHYDROLASE WITH INCREASED THERMOSTABILITY

Statement of Authorship

Title of Paper	Validation of Novel barley (1→3,1→4)-β-Glucan Endohydrolases with Increased Thermostability
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Juanita Lauer, Suong Cu, Rachel Burton, Jason Eglinton This is an experimental manuscript extending the examination of the novel EII-l β-glucanase. Two experimental approaches were selected to compare the reference EII-a and EII-l allozymes in conditions similar to commercial processes.

Principle Author

Name of Principal Author (Candidate)	Juanita Lauer		
Contribution to the Paper	Designed and performed experiments, analysed data and wrote manuscript		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	15/8/2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Suong Cu		
Contribution to the Paper	Supervised experimental design, data analysis and interpretation Edited the manuscript. I hereby certify that the statement of contribution is accurate.		
Signature		Date	15/8/2016

Name of Co-Author	Rachel Burton		
Contribution to the Paper	Supervised experimental design, data analysis and interpretation. Edited the manuscript. I hereby certify that the statement of contribution is accurate.		
Signature		Date	15/8/2016

Name of Co-Author	Jason Eglinton		
Contribution to the Paper	Conceived the project. Supervised experimental design, data analysis and interpretation. Edited the manuscript. I hereby certify that the statement of contribution is accurate.		
Signature		Date	11/8/2016

Validation of Novel Barley (1→3,1→4)-β-glucan Endohydrolase with Increased Thermostability

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4.0 Abstract

Barley (1→3,1→4)-β-glucan endohydrolases (β-glucanases) are essential for the complete degradation of (1→3,1→4)-β-glucan (β-glucan) in starchy endosperm cell walls in malt. Problems associated with residual malt β-glucan include increased wort viscosity that reduces filtration efficiency and causes chill haze in beer. Degradation of β-glucan during malting cannot be further increased by β-glucanase due to significant activity losses during kilning and mashing. Recently, a novel β-glucanase allele was identified in wild barley that exhibited a significant increase in β-glucanase thermostability *in vitro* based on testing of the recombinant enzyme in purified form. The current study extends this fundamental research by examining the new allele in conditions similar to commercial processes. Recombinant EII-l activity was examined in a complex biochemical matrix of a simulated barley mash at 65°C. Additionally, the irreversible thermal inactivation of the endogenous EII-l in green malt from a population derived from elite by wild barley cross was also assayed. EII-l exhibited 10% more residual β-glucanase activity than the reference EII in both experiments validating EII-l as a new source of genetic variation for improved malt quality. The detailed characterisation of the novel allele makes it viable for commercial application and routine selection in barley breeding.

4.1 Introduction

Complete endosperm cell wall degradation is important in malting to reduce (1→3,1→4)-β-glucan (β-glucan) to acceptable levels for brewing (Loi et al., 1987). Residual malt β-glucan can cause increased wort viscosity that contributes to reduced filtration efficiency and chill haze in beer (Evans et al., 1999; Stewart et al., 1998). β-Glucan is an asymmetric polysaccharide comprising (1→3)- and (1→4)-linked β-glucosyl residues and is a major component in the barley endosperm contributing 70% of total cell wall polysaccharides (Fincher, 1975). β-Glucan hydrolysis occurs during germination by (1→3,1→4)-β-glucan endohydrolase (β-glucanase) (EC 3.2.1.73) producing tri- and tetrasaccharides that are further reduced by β-glucan exohydrolase and β-glucosidase enzymes (Hrmova et al., 1996).

Barley β-glucanase has two isoforms, EI and EII that are relatively thermolabile in comparison to the key starch hydrolases; α-amylase, β-amylase and limit-dextrinase. EI activity is primarily lost during kilning and the remaining EII activity is significantly lost in the first 10 minutes of mashing (Bamforth and Martin, 1983; Georg-Kraemer et al., 2004; Loi

et al., 1987; Zwickert-Menteur et al., 1996). The early losses of β-glucanase leave no opportunity for the endogenous β-glucanases to reduce residual malt β-glucan in the wort. Residual malt β-glucan can be present in the distal regions of well-modified malts and in inhomogeneous malts caused by variable cell wall degradation (Scheffler and Bamforth, 2005).

Previous studies have successfully improved β-glucanase thermostability and expressed in germinating barley using protein engineering techniques. A hybrid bacterial β-glucanase was engineered from *Bacillus amyloliquefaciens* and *B. macerans* with increased thermostability designed to survive mashing conditions (Olsen et al., 1991). The gene encoding the thermostable hybrid β-glucanase was transformed into barley and successfully over-expressed in germinating grain (Jensen et al., 1996) and driven by the *Hor3-1* D hordein gene promoter (Horvath et al., 2001). In a different approach, site-directed mutagenesis of EII to improve β-glucanase thermostability was achieved (Stewart et al., 2001) and the variant was successfully expressed in transgenic lines and tested in green malt. Thus, genetically engineered alternatives for barley β-glucanase improvement have been successful, however heat-stable exogenous bacterial and fungal β-glucanases added into the mash are more acceptable to the consumer (Scheffler and Bamforth, 2005).

Recently new forms of EI and EII were identified in wild barley accessions (*Hordeum vulgare* ssp. *spontaneum*) and cloned for characterisation of recombinant enzymes in biochemical analyses (Lauer et al., 2017a; Lauer et al., 2017b). Four EII variants exhibited significant increases in residual β-glucanase activity after heat treatment compared to the reference form of EII previously identified in 65% of the cultivated barley germplasm examined. One of the novel EII variants named EII-l exhibited a significant increase of 1.3°C in T_{50} as represented by the temperature at which 50% of the activity remains and a significant increase of approximately 10% in residual β-glucanase activity after 5 minutes of heat treatment over the reference EII-a (Lauer et al., 2017b).

The current study validates the increased thermostability exhibited by the novel EII-l β-glucanase from wild barley for commercial application, using two approaches. The first approach tested the EII-a and EII-l recombinant enzymes produced in *Escherichia coli* in a simulated barley mash at 65°C to monitor the progressive loss of EII-a and EII-l β-glucanase

activity. The barley mash simulates the complex biochemical matrix mimicking a commercial mashing environment. The second approach examined endogenous activity of EII-a and EII-l in a population derived from an elite by wild barley cross in a bulked segregant analysis. The bulked segregant analysis method is a rapid approach to examine a gene of interest in a randomised genetic background that is suitable for the examination of novel alleles mined from wild barley (Michelmore et al. 1991). In this situation EII-l must consistently exhibit improved thermostability to achieve validation.

4.2 Materials and Methods

4.2.1 Characterisation of recombinant EII-a and EII-l under simulated mashing conditions

4.2.1.1 Plant materials

Australian cv Commander was grown in Charlick, South Australia in 2015. Grain was assayed for protein and β-glucan content, which were 11% and 4% respectively, and screened at 2.5mm. Barley flour was produced using a Bühler-Miag mill with a 0.2mm screen to fine grind according to European Brewing Convention (EBC) method 4.6 (EBC 1997).

4.2.1.2 Preparation of recombinant EII-a and EII-l

Previously prepared pDEST17 EII-a and EII-l constructs were expressed in BL21 star (DE3) and purified by cobalt metal affinity chromatography (Lauer et al., 2017b). The 6x His tag was removed and the purified protein was desalted and concentrated using a 10K Amicon Ultra-4 centrifugal filter (Merck Millipore Ltd, Billerica, USA). The full purification procedure is as described previously by Lauer et al., (2017b).

4.2.1.3 Simulated barley mash at 65°C

Barley mash was produced using a ratio of 1:5 barley flour and 50mM sodium acetate buffer, pH 5.0. The procedure to generate the simulated barley mash heated at 65°C is as follows: 50mM sodium acetate buffer, pH 5.0 was preheated and stored at 65°C. A 2.4g aliquot of barley flour was added to a conical flask and preheated and stored at 65°C. A 12mL volume of preheated 50mM sodium acetate buffer, pH 5.0 was added to the barley flour and mixed at 300rpm on a plate shaker in an incubator at 65°C. After 5 minutes, recombinant enzyme was

added to a final concentration of 2µg/mL. A negative control using 50mM sodium acetate buffer, pH 5.0 instead of recombinant enzyme was included. Aliquots of 1mL of the barley mash were collected after 1, 2, 4, 6, 8, 10, 12 and 15 minutes and immediately stored on ice. Samples were centrifuged for 10 minutes at 5,000g and the supernatant was divided into two replicates to measure β-glucanase activity.

4.2.1.4 β-Glucanase activity assay

Recombinant β-glucanase activity from the barley mash aliquots was determined using the Azo-Barley glucan Megazyme malt β-glucanase assay kit (Megazyme Ltd. Ireland) (McCleary and Shameer, 1987). β-Glucanase activity was measured at 30°C using Azo-Barley glucan and precipitate B solution. The assays were reduced to half volumes consisting of two replicates that were randomised when assayed (Zwickert-Menteur et al., 1996). Initial β-glucanase activity was determined from aliquots taken after 1 minute of incubation. The progressive loss of β-glucanase activities from the remaining time points were expressed as a percentage of the initial β-glucanase activity measured after 1 minute.

4.2.2 Characterisation of endogenous EII-a and EII-l extracted from F_2 green malt in a bulked segregant analysis

4.2.2.1 Development of F_2 population

Novel EII-l and the reference EII-a β-glucanase alleles were previously identified in wild barley accession CPI77137-85 (Nevo et al., 1979) and cv Flagship, respectively and that both carry the EI-a β-glucanase allele (Lauer et al., 2017a). CPI77137-85 was crossed into Flagship and the F_1 was self-pollinated to produce F_2 seed. All plants were grown under glasshouse conditions.

4.2.2.2 Petri dish germinations for green malt production

Two hundred randomly selected F_2 seeds were germinated over two petri dishes for six days at 18°C following the European Brewing Convention (EBC) method 3.6.2 (EBC, 1997) for green malt production. After germination the green malt samples were collected and stored at -20°C before processing to prepare for DNA and β-glucanase activity extraction from shoots and grain, respectively.

4.2.2.3 Marker assisted segregation of endogenous EII-a and EII-l homozygous groups

Shoots and grains from frozen green malt samples were separated and freeze dried. DNA was extracted from shoots from single seeds as described by Karakousis and Langridge (2003). The Kompetitive Allele Specific PCR (KASP™) genotyping system (LGC, Teddington, United Kingdom) was used to screen the extracted DNA to segregate the corresponding grains into EII-a and EII-l homozygous groups. Primers were designed following the manufacturer's instructions targeting the single nucleotide polymorphism (SNP) that caused the amino acid substitution at residue 20 exclusively identifying the EII-a and EII-l alleles (Table 4-1). PCR reactions were designed using LCG Genomics Kraken software and conducted following the manufacturer's protocol. The homozygous and heterozygous genotype calls were clustered manually and viewed using LCG Genomics SNP viewer software.

Table 4-1. Primer nucleotide sequences for KASP genotyping

Primer name	Primer sequence
Forward - A1	GAAGGTGACCAAGTTCATGCTCCCGTTGGACTTGAACATGCC
Forward - A2	GAAGGTCGGAGTCAACGGATTATCCCGTTGGACTTGAACATGCT
Reverse - C1	GCTACGGCATGAGCGCCAACAA

4.2.2.4 Irreversible thermal inactivation of endogenous EII-a and EII-l β-glucanase activity

The homozygous grain samples identified by the KASP genotyping system were treated as individuals, representing single grain green malts and were individually ground to a powder using a Qiagen retsch mill for the bulked segregant analysis. Genotypes identified as heterozygous or missing were not included in the analysis. β-Glucanase from each single grain green malt was extracted in 1mL of Megazyme malt β-glucanase assay kit extraction buffer (Megazyme Ltd. Ireland) (McCleary and Shameer, 1987). Extraction volumes were halved to examine β-glucanase activity before and after heat treatment. Heat treatment of extracted β-glucanase was conducted at 56°C for 5 minutes and samples immediately chilled on ice. β-Glucanase activity was measured as described previously in section 4.2.1.4. Residual β-glucanase activity was calculated as a percentage of initial activity per individual malt and pooled by EII-a and EII-l homozygous groups for the bulked segregant analysis.

4.3 Results

4.3.1 Progressive loss of recombinant EII-a and EII-l under simulated mashing conditions at 65°C

Recombinant EII-a and EII-l were examined in conditions similar to a 65°C industrial mash. Aliquots of barley mash were taken each minute from 1 to 15 minutes after the addition of the recombinant enzymes to examine the percentage of progressive loss of EII-a and EII-l activity at 65°C (Figure. 4-1). EII-a and EII-l exhibited similar residual β-glucanase activities initially, but differences in residual β-glucanase activity became evident at the four minute time point increasing from 7% to 10.8% at the eight minute time point. At the 6 and 8 minute time points EII-l exhibited 36.5% and 26.7% residual β-glucanase activity on average, respectively at approximately 10% higher than EII-a. At 10 minutes the difference between EII-a and EII-l residual β-glucanase activity began to decrease at a faster rate declining from 10.8% to 8.3% and continuing to decrease to 5.9% and 2.9% at 12 and 15 minutes respectively.

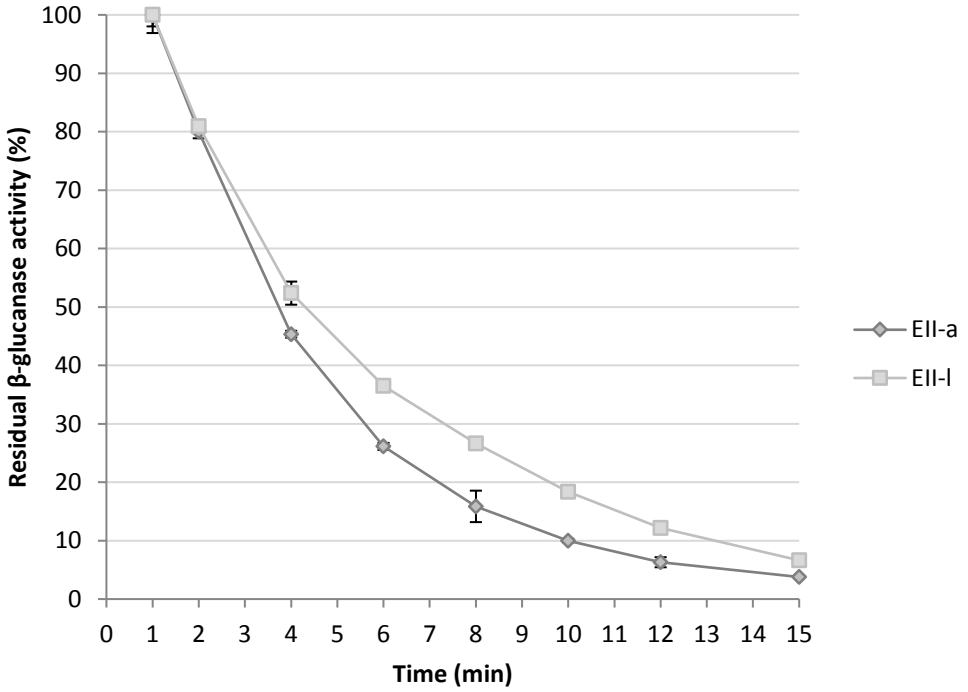

Figure. 4-1. Irreversible thermal inactivation of recombinant EII β-glucanases in a simulated barley mash. Recombinant EII-a and EII-l were added to pre-heated barley mash at 65°C. Sample aliquots were taken over 15 minutes. Progressive loss of residual β-glucanase activity over time was measured using Megazyme Azo-barley β-glucan at 30°C. The percentage of residual activity was calculated from the starting β-glucanase activity after 1 minute. Error bars represent the standard deviation of two replicates.

4.3.2 Marker assisted segregation of F_2 seeds homozygous for EII-a and EII-l

Primers designed for marker assisted selection of EII-a and EII-l homozygotes genotypes targeted the A:G SNP causing the amino acid change at residue 20. Homozygous EII-a and EII-l genotypes were identified by pairs of adenine and guanine nucleotides, respectively. The marker successfully called 181 reads from the 200 DNA samples. Homozygous genotypes identified for EII-a and EII-l were 35 and 31, respectively.

4.3.3 Bulked segregant analysis of the irreversible thermal inactivation of endogenous EII-a and EII-l extracted from green malt

Single F_2 green malt grains selected for analysis all carried EI-a and were separated into EII-a and EII-l homozygous groups. The 200 germinated grains were segregated into 35 grains homozygous for EII-a, 31 grains homozygous for EII-l, 19 grains were not determined and the remaining 115 grains were heterozygous. Grains were assayed individually and results were pooled together to examine the difference in β-glucanase irreversible thermal inactivation in a bulked segregant analysis. Irreversible thermal inactivation of total β-glucanase was determined as the percentage of residual β-glucanase activity after 5 minutes of heat treatment at 56°C as compared to the initial activity (Figure. 4-2). The EII-a and EII-l homozygous groups were significantly different ($p<0.001$) and EII-l exhibited 9.9% more residual β-glucanase activity than EII-a.

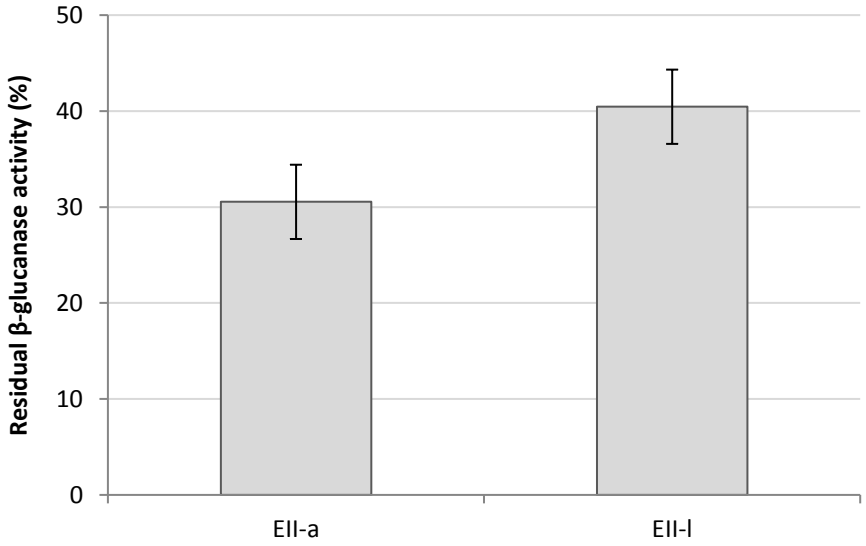

Figure. 4-2. Bulked segregant analysis of irreversible thermal inactivation of EII-a and EII-l from green malt. Endogenous β-glucanases were extracted and heat treated at 56°C for 5 minutes prior to assaying initial and remaining β-glucanase activity with Megazyme Azo-barley β-glucan at 30°C. The percentage of residual activity was calculated per sample and the final results were pooled for comparison. Error bars represent $LSD_{(0.05)}$ and assay error was 4.12%.

4.4 Discussion

Progressive loss of β-glucanase activity was examined in a barley mash at 65°C to demonstrate the improved thermostability of the EII-l allozyme under simulated commercial mashing conditions. Barley flour was used for the simulated mash instead of malt flour to provide a high molecular weight β-glucan substrate without interference from endogenous β-glucanases that would develop during the malting process. The simulated mashing environment provides proteins, sugars and polyols that mimic commercial mashing conditions. The complex biochemical matrix of the mashing environment has been shown to increase β-glucanase survival at higher temperatures and prolong activity compared to *in vitro* experiments that consist of substrate β-glucan in presence of bovine serum albumin (Bamforth and Martin, 1983).

Recombinant EII-l produced in *E.coli* was previously characterised and demonstrated a significant increase in β-glucanase thermostability compared to the reference, EII-a (Lauer et al., 2017b). EII-l exhibited a T_{50} value 1.3°C higher and 11.9% more residual β-glucanase activity after irreversible thermal inactivation at 56°C for 5 minutes than EII-a. In this study,

recombinant EII-a and EII-l in the simulated barley mash retained 26.1% and 36.5% residual β-glucanase activity respectively after the 6 minute time point (Figure. 4-1). The 10% difference in residual β-glucanase activity between the two allozymes observed in the barley mash from 6 to 8 minutes was consistent with the behaviour of recombinant β-glucanases examined in the previous study (Lauer et al., 2017b). The reduced difference in residual activity in the last 5 minutes is likely to be due to the presence of less substrate allowing fewer enzyme-substrate complex formations resulting in faster degradation at increased temperatures (data not shown).

The considerable differences in grain composition and germination behaviour are confounding factors in the direct comparison between cultivated and wild barley. To minimise these confounding effects the bulked segregant analysis approach was selected as these factors are managed by randomising the genetic background. Bulked segregant analysis is a rapid experimental approach for examining genes of interest compared to the lengthy development of near isogenic lines (Michelmore et al., 1991). Marker assisted selection of a single target gene allows the segregation of seed samples into homozygous genotypes for the bulked segregant analysis. EII-a and EII-l allozymes differ by three amino acid substitutions, S20G, R100Q and D284E (Lauer et al., 2017b). The A:G SNP causing the S20G substitution was successful in the genotyping process to segregate EII-a and EII-l homozygous seeds for β-glucanase activity analysis.

Endogenous EII-a and EII-l were examined in green malt from an F_2 population derived from the Australian malting variety Flagship and wild barley accession CPI77137-85 (Nevo et al., 1979). β-Glucanase activity extracted from green malt contains both EI and EII isozymes. EI is more thermolabile than EII and is denatured during kilning, so as a result EI is present only in the initial measurements of green malt β-glucanase activity and since both population parents carried the same form of EI results were randomised by the bulked segregation. The difference observed between the EII-a and EII-l homozygous groups is primarily due to structural differences (Lauer et al., 2017a).

Endogenous EII-l from green malt exhibited 9.9% more residual β-glucanase activity than EII-a (Figure. 4-2). This is slightly lower than the 11.9% difference observed between recombinant EII-l and EII-a previously reported (Lauer et al., 2017b). The irreversible thermal

inactivation of β-glucanase examines the refolding after heat treatment which mimics the heating process during kilning. EI is completely lost in the early stages of the kilning and on average 40% of EII survives the full kilning process (Bamforth and Martin, 1983; Georg-Kraemer et al., 2004; Loi et al., 1987; Zwickert-Menteur et al., 1996). The forms of EII that have previously been reported are likely to be either EII-a and EII-b, the two main forms of EII that have been identified in 98.8% of cultivated barley (Lauer et al., 2017a). Thus the 10% increase in residual β-glucanase activity exhibited by endogenous EII-l has the potential to improve EII levels in finished malt above the current 40% average conferred by the common alleles. Higher levels of a thermostable β-glucanase in finished malt could achieve more cell wall β-glucan degradation during mashing, further reduce wort viscosity and improve brew house efficiency. Final validation could be achieved by monitoring the survival of β-glucanase activity in elite lines carrying the novel allele during the malting process and by monitoring wort viscosity in pilot mashing experiments at 65°C.

4.5 Conclusion

The current study validates the improved thermostability of the novel EII-l β-glucanase allele from wild barley over the reference EII-a. The novel β-glucanase allele consistently demonstrated an approximate 10% increase in residual β-glucanase activity in two experimental approaches. Increased barley β-glucanase thermostability has the potential to improve β-glucanase survival in finished malt, improve brew house efficiency and reduce the need for the addition of exogenous β-glucanases. Variety evaluation and barley breeding have a strong focus on identifying material that does not achieve acceptable cell wall degradation and low wort viscosity, however variety development has not had the benefit of novel alleles conferring improvement in fundamental biochemical properties of β-glucanase. The detailed characterisation of the novel β-glucanase allele suggests it will be an ideal candidate for introgression into elite germplasm for the development of new varieties.

CHAPTER 5

DISCUSSION

Complete degradation of starchy endosperm cell wall (1→3,1→4)-β-glucan during germination is an essential requirement in barley varieties selected for malting and brewing. Residual malt β-glucan from incomplete degradation of endosperm cell walls during the malting process is associated with increased wort viscosity that can slow filtration and reduce brew house efficiency. New barley varieties commercially released in Australia intended for malting are extensively evaluated by the Malting and Brewing Industry Barley Technical Committee (MBIBTC) before achieving malting accreditation from Barley Australia. The MBIBTC evaluates the nominated barley varieties following industry procedures to examine; malt extract, diastatic power, Kolbach Index, viscosity, wort β-glucan and apparent attenuation limit. Failure for the barley variety to fulfil the malting specifications consistently over two seasons of commercial scale evaluation will result in a downgraded recommendation to food or feed barley.

The Australian variety Hindmarsh that was commercially released in 2007 is a recent example of a variety intended for malting that failed malt accreditation in 2010. Malting accreditation was not recommended because of unacceptably high wort β-glucan and viscosity levels in the final year of evaluation (Baker, 2010). Hindmarsh had proven to be strong agronomically, performing well in lower rainfall regions, and it continues to be adopted strongly by farmers with over two million tonnes of production in each of the 2013, 2014 and 2015 seasons (Eglinton pers comm). Although not accredited as a malting variety, Hindmarsh has found some market acceptance in high adjunct brewing and is suitable for Japanese shochu production (Seednet, 2011). As a result, Hindmarsh grain prices are usually $5 - $10 per tonne above feed and $20 - $30 lower than the currently preferred malting varieties, Commander, Buloke and Scope (AWB, 2016). On the assumption that 50% of production achieves first grade malt specifications these values equate to $20 - $30 million direct losses in farm gate value. The reliable production of malt with low β-glucan could prevent problems associated with borderline viscosity rather than managing viscosity by changing brew house processes or by adding exogenous enzymes, and this would be of value to the industry. Consistently acceptable viscosity levels could potentially be achieved if defined and specific genetic variation governing this trait was available for efficient selection in breeding programs.

The focus of this study was the identification and characterisation of novel barley (1→3,1→4)-β-glucan endohydrolase (β-glucanase) alleles from *H. spontaneum*. Currently, β-glucanase alleles are not targeted in breeding for malting barley, however β-glucanase activity

is commonly assayed. The potential outcome of this study was to identify novel β-glucanase alleles with economic significance for implementation into breeding programs. Genetic diversity of cultivated barley has been significantly reduced through domestication limiting the potential for genetic gain in many traits within elite germplasm. *H. spontaneum* has become a reliable genetic resource for allele mining of rare alleles for the genetic improvement of cultivated barley. Past research has identified a novel thermostable β-amylase allele in *H. spontaneum* which has been implemented into breeding programs for malt quality improvement (Eglinton et al., 2008; von Korff et al., 2008). Significant variation in β-glucanase activity and other malt quality traits have also previously been identified in *H. spontaneum* (Ahokas and Naskali, 1990). However, phenotyping for complex malting quality traits in *H. spontaneum* is likely to be confounded by gross differences in grain morphology and germination behaviour that can obscure the significant variation in the trait of interest. Methodologies that counter these effects have been developed using Advanced Backcross populations and Nested Associated Mapping populations that manage the effect of genetic background. The development of these populations involve the introgression of novel alleles from *H. spontaneum* donor lines into elite breeding lines for high resolution quantitative trait loci (QTL) discovery (Tanksley and Nelson, 1996; Yu et al., 2008). The F_1 progeny produced from the elite and *H. spontaneum* lines are backcrossed into the elite breeding line for two to three generations to reduce the frequency of undesirable donor alleles followed by serial self-fertilisation or doubled haploid production to produce homozygous lines. QTL discovery methods are able to exploit novel alleles from exotic germplasm in the mapping populations for crop improvement. However, the development of these populations are costly and time consuming due to the number of lines generated and limit the number of *H. spontaneum* accessions that can be exploited. Consequently, only a fraction of the genetic diversity of *H. spontaneum* is examined increasing the possibility of missing rare alleles.

A different approach is the examination of a gene of interest by conducting a sequence survey, which is less restricted by sample size. The sequence survey is most powerful when the target gene and protein has been well characterised, such as for β-glucanase. This study examined the exon regions of *HvGlb*1 and *HvGlb*2 in cultivated barley and *H. spontaneum* accessions for the presence of novel β-glucanase alleles. *H. spontaneum* accessions exhibited significantly more polymorphisms than cultivated barley, which is consistent with the loss of genetic diversity through domestication by traditional breeding methods. However, there were fewer polymorphisms identified in *HvGlb*1 in both cultivated barley and *H. spontaneum* accessions compared to *HvGlb*2. The differences in frequency observed in nucleotide and

amino acid sequence variation resulted in a total of 20 β-glucanase alleles for potential characterisation.

The sequence survey approach applied to *HvGlb*1 and *HvGlb*2 could be extended to include intron and promoter regions that may be important in influencing the expression levels of the active enzyme. Polymorphisms in intron regions generally occur more frequently than in coding regions with a higher likelihood of insertions and deletions. Differences in the intron region of *HvGlb*1 have been identified previously (Jin et al., 2011), however the influence of the *HvGlb*1 and *HvGlb*2 intron regions on enzyme expression remain to be examined. Additionally, expanding the geographical diversity of the *H. spontaneum* accessions from the Fertile Crescent with selections from North Africa, Central Asia and the Caucasus region would increase the probability of identifying additional rare alleles (Steffenson et al., 2007).

Identification of large numbers of sequence variants from a sequence survey necessitates the use of predictive modelling tools to prioritise alleles for functional characterisation; these are particularly useful when the structure of the target protein has been solved. Predictive protein modelling tools enable the visual examination of protein structure by homology modelling and stability predictions from calculations of Gibbs free energy changes upon the entropy of protein folding. The combination of structural and stability prediction tools increase the accuracy of the prediction analysis. The current study correctly predicted protein stability changes in EI-c, EII-c, EII-d, EII-e and EII-l. These allozymes all exhibited increased β-glucanase activity after heat treatment in comparison to the reference allozyme, however, only EII-l exhibited improved thermostability assayed at elevated temperatures. An effect on EI-b stability was not accurately predicted, and this was manifested as a decrease in β-glucanase activity after heat treatment. Incorrect predictions of functional variation limit the success of mining useful new alleles from sequence based surveys. The incomplete understanding of amino acid substitution effects on structure and function is also a significant limitation for genetically modified (GM) and gene editing approaches generating new variation. GM barley varieties are not accepted by brewing companies in any country, however it is possible that new gene editing methods, such as those based on CRISPR/Cas9, may produce lines that will be considered as non-GMOs in some jurisdictions. The current study demonstrates that a degree of redundancy is required when using predictive functional analyses as complete dependence on such tools is not reliable even for very well characterised enzymes such as β-glucanase.

Bulked segregant analysis is a rapid approach for the examination of genes of interest in a diverse randomised genetic background. This approach was sufficient for the limited germplasm available here for analysis of the novel β-glucanase allele. This validation approach could be improved by monitoring the progressive loss of EII-a and EII-l activity in kilned malt to allow estimation of the residual β-glucanase activity in finished malt. Further validation of EII-l thermostability in pilot malting and brewing experiments using near isogenic lines (NILs) from an Advanced Backcross population or elite breeding lines would confirm the commercial benefits of the novel β-glucanase allele. However, the development of a new Advanced Backcross population or elite breeding lines with the EII-l β-glucanase allele would be required.

The opportunity for breeders to select for a combination of favourable *HvGlb*1 and *HvGlb*2 alleles has the potential to alleviate brew house viscosity problems. In the experiments described here the variation characterised in *HvGlb*1 is unlikely to improve EI β-glucanase survival during the kilning process compared to the EII variants tested. Additionally, no significant improvements in catalytic efficiency were identified in either EI or EII. The functional characterisation of EI and EII variants displayed a trade-off between catalytic efficiency and thermostability. EII-b, identified exclusively in cultivated barley, was predicted to be less thermostable than the reference and therefore was not selected for biochemical characterisation. However, the trade-off effect between catalytic efficiency and thermostability suggests that EII-b could be a possible alternative allele with improved catalytic efficiency, and this could be tested in further work. If found to be the case this potentially offers a choice for breeders to select for novel EII β-glucanase alleles that have separate outcomes. One choice could improve β-glucanase catalytic efficiency allowing for the removal of residual malt β-glucan whilst the other could improve β-glucanase thermostability for longer activity in the wort to reduce residual malt β-glucan, or the β-glucan from raw barley in adjunct brewing, and thus ultimately viscosity. Improving the overall catalytic efficiency of EI and EII could improve homogeneity of cell wall β-glucan degradation during malting. This could reduce the need for maltsters to blend malt batches to achieve β-glucan specifications set by brewers and prevent the reoccurrence of a situation similar to that which affected the malting accreditation of Hindmarsh, which carries the *HvGlb2* gene encoding the EII-a enzyme.

The variation in EII β-glucanase identified in this study offers breeders a choice to select for catalytic efficiency or increased thermostability for β-glucanase improvement. Managing increased wort viscosity in the brew house comes at a cost of extended filtration time or from the addition of exogenous β-glucanase. The EII-l protein was demonstrated to offer an improvement in thermostability over the common EII-a allozyme. The use of a barley mash at 65°C simulated a brew house mashing environment using barley flour as the β-glucan substrate to compare the two recombinant β-glucanases. This result was extended to confirm that the increased thermostability observed for endogenously expressed enzyme was replicated using a bulked segregant population to compare the two alleles. This first report of functional differences in allelic forms of β-glucanase has prompted the University of Adelaide breeding program to introgress the EII-l allele into a range of elite genetic backgrounds. The resulting lines will be characterised through industry standard malting and brewing tests as well as modified mashing conditions as the next step in determining the commercial potential of the new variant. The diagnostic molecular markers developed in this study provide an opportunity for efficient early generation selection for β-glucanase alleles which could offer a significant advantage compared to current standard practice of measuring wort viscosity and β-glucan content at later stages of variety development.

CHAPTER 6

SUPPLEMENTARY MATERIAL

Table 6-1. Identity and origin of cultivated barley screened for variation in *HvGlb*1 and *HvGlb*2

Cultivated Barley	*HvGlb*1	*HvGlb*2	Cultivated Barley	*HvGlb*1	*HvGlb*2
AC Metcalfe (Canada)	*Glb*1-a	*Glb*2-a	Dhow (Australia)	*Glb*1-a	*Glb*2-b
Admiral (Australia)	*Glb*1-a	*Glb*2-a	ER/Apm (ICARDA)	*Glb*1-a	*Glb*2-b
Alexis (Germany)	*Glb*1-a	*Glb*2-a	Finniss (Australia)	*Glb*1-a	*Glb*2-a
Amaji Nijo (Japan)	*Glb*1-a	*Glb*2-a	Fitzroy (Australia)	*Glb*1-a	*Glb*2-b
Arapiles (Australia)	*Glb*1-a	*Glb*2-a	Flagship (Australia)	*Glb*1-a	*Glb*2-a
Arta (Syria)	*Glb*1-a	*Glb*2-a	Fleet (Australia)	*Glb*1-a	*Glb*2-a
Barque (Australia)	*Glb*1-a	*Glb*2-a	Franklin (Australia)	*Glb*1-a	*Glb*2-b
Barque-73 (Australia)	*Glb*1-a	*Glb*2-a	Gairdner (Australia)	*Glb*1-a	*Glb*2-a
Baudin (Australia)	*Glb*1-a	*Glb*2-b	Galleon (Australia)	*Glb*1-a	*Glb*2-a
Buloke (Australia)	*Glb*1-a	*Glb*2-b	Golden Promise (MLNS)	*Glb*1-a	*Glb*2-a
Capstan (Australia)	*Glb*1-a	*Glb*2-a	GrangeR (Australia)	*Glb*1-a	*Glb*2-b
CDC Kindersley (Canada)	*Glb*1-a	*Glb*2-a	Halcyon (UK)	*Glb*1-a	*Glb*2-a
Chameleon (Denmark)	*Glb*1-a	*Glb*2-a	Hamelin (Australia)	*Glb*1-a	*Glb*2-a
Chebec (Australia)	*Glb*1-a	*Glb*2-a	Harmal-02//Esp/1808-4L (ICARDA)	*Glb*1-a	*Glb*2-a
Cheerio (Denmark)	*Glb*1-a	*Glb*2-b	Harrington (Canada)	*Glb*1-a	*Glb*2-a
Chieftain (UK)	*Glb*1-a	*Glb*2-a	Haruna Nijo (Japan)	*Glb*1-a	*Glb*2-a
Clipper (Australia)	*Glb*1-a	*Glb*2-a	Henley (France)	*Glb*1-a	*Glb*2-b
CM72 (North Africa)	*Glb*1-a	*Glb*2-a	Hindmarsh (Australia)	*Glb*1-a	*Glb*2-a
Commander (Australia)	*Glb*1-a	*Glb*2-a	ICARDI SN3-26 (ICARDA)	*Glb*1-a	*Glb*2-a
Compass (Australia)	*Glb*1-a	*Glb*2-b	Kaputar (Australia)	*Glb*1-a	*Glb*2-b
Dash (UK)	*Glb*1-a	*Glb*2-b	Keel (Australia)	*Glb*1-a	*Glb*2-b

Cultivated Barley	*HvGlb*1	*HvGlb*2	Cultivated Barley	*HvGlb*1	*HvGlb*2
Lodamai (China)	*Glb*1-a	*Glb*2-a	Steptoe (USA)	*Glb*1-a	*Glb*2-a
Major (Canada)	*Glb*1-a	*Glb*2-b	Stirling (Australia)	*Glb*1-a	*Glb*2-a
Manley (Canada)	*Glb*1-a	*Glb*2-a	Sumire Mochi (China)	*Glb*1-d	*Glb*2-a
Morex (USA)	*Glb*1-a	*Glb*2-a	Tadmor (Syria)	*Glb*1-a	*Glb*2-c
Mundah (Australia)	*Glb*1-a	*Glb*2-b	Tallon (Australia)	*Glb*1-a	*Glb*2-b
Navigator (Australia)	*Glb*1-a	*Glb*2-b	Torrens (Australia)	*Glb*1-a	*Glb*2-b
ND24260-1 (USA)	*Glb*1-a	*Glb*2-a	Tremois (Spain)	*Glb*1-a	*Glb*2-a
Nure (Italy)	*Glb*1-a	*Glb*2-a	Triumph (Germany)	*Glb*1-a	*Glb*2-b
Oxford (UK)	*Glb*1-a	*Glb*2-a	Unicorn (Japan)	*Glb*1-a	*Glb*2-a
Parent19 (ICARDA)	*Glb*1-a	*Glb*2-a	VB9104 (Australia)	*Glb*1-a	*Glb*2-b
Quench (UK)	*Glb*1-a	*Glb*2-b	Vlamingh (Australia)	*Glb*1-a	*Glb*2-a
Sahara3771 (North Africa)	*Glb*1-e	*Glb*2-a	Westminster (UK)	*Glb*1-a	*Glb*2-a
Scarlett (Germany)	*Glb*1-a	*Glb*2-a	WI2585 (Australia)	*Glb*1-a	*Glb*2-a
Schooner (Australia)	*Glb*1-a	*Glb*2-a	Wimmera (Australia)	*Glb*1-a	*Glb*2-a
Scope (Australia)	*Glb*1-a	*Glb*2-b	Yagan (Mexico)	*Glb*1-a	*Glb*2-b
Skiff (Australia)	*Glb*1-a	*Glb*2-b	Zau7 (China)	*Glb*1-a	*Glb*2-b
Skipper (Australia)	*Glb*1-a	*Glb*2-b	Zhepi1-1 (China)	*Glb*1-a	*Glb*2-b
Sloop (Australia)	*Glb*1-a	*Glb*2-a	Zhepi4 (China)	*Glb*1-a	*Glb*2-b
SloopSA (Australia)	*Glb*1-a	*Glb*2-a	Zhoungdamei (China)	*Glb*1-a	*Glb*2-b

Table 6-2 Wild barley accessions screened for variation in *HvGlb*1 and *HvGlb*2 (Nevo *et al.* 1979)

Site in Israel	Site/ Family	Accession Number	Site in Israel	Site/ Family	Accession Number
Akhziv	24/7	CPI77127-7	Rosh Pinna A	5/36	CPI77140-36
Akhziv	24/46	CPI77127-46	Rosh Pinna B	5/53	CPI77140-53
Akhziv	24/50	CPI77127-50	Talpiyyot	14/2	CPI77144-2
Akhziv	24/55	CPI77127-55	Talpiyyot	14/25	CPI77144-25
Atlit	25/28	CPI77129-28	Tel Hay A	4/2	CPI77145-2
Atlit	25/37	CPI77129-37	Tel Hay	87	CPI77145-87
Atlit	25/49	CPI77129-49	Afiq	3/1	CPI77128-1
Atlit	25/50	CPI77129-50	Afiq	3/41	CPI77128-41
Atlit	25/54	CPI77129-54	Bet Shean	21/7	CPI77131-7
Atlit	25/55	CPI77129-55	Bet Shean	21/34	CPI77131-34
Caesarea	26/4	CPI77132-4	Mehola	22/7	CPI77137-7
Caesarea	26/38	CPI77132-38	Mehola	22/53	CPI77137-53
Caesarea	26/44	CPI77132-44	Mehola	22/85	CPI77137-85
Herzliyya	27/14	CPI77134-14	Tabigha	7/13	CPI77143-13
Herzliyya	27/19	CPI77134-19	Tabigha	7/18	CPI77143-18
Herzliyya	27/24	CPI77134-24	Tabigha	7/19	CPI77143-19
Bar Giyyora	13/8	CPI71283-8	Tabigha	7/25	CPI77143-25
Bar Giyyora	13/10	CPI71283-10	Tabigha	7/31	CPI77143-31
Bar Giyyora	13/20	CPI71283-20	Tabigha	7/48a	CPI77143-48a
Bar Giyyora A	13/25	CPI71283-25	Revivim	18/30	CPI77139-30
Bar Giyyora A	13/27	CPI71283-27	Revivim	18/35	CPI77139-35

Site in Israel	Site/ Family	Accession Number	Site in Israel	Site/ Family	Accession Number
Bar Giyyora A	13/42	CPI71283-42	Sede Boqer	20/14	CPI77141-14
Bar Giyyora B	13/48	CPI71283-48	Sede Boqer	20/27	CPI77141-27
Damon	11/13	CPI71284-13	Tel Shoqet	16/32	CPI77146-32
Damon	11/48	CPI71284-48	Tel Shoqet	16/33	CPI77146-33
Damon	11/52	CPI71284-52	Wadi Qilt	23/1	CPI77135-1
Eyzariya	15/25	CPI77130-25	Wadi Qilt	23/40	CPI77135-40
Maalot	10/17	CPI77136-17	Yeroham	19/5	CPI77147-5
Mt. Meron	9/20	CPI77138-20			

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