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Allelic Diversity of a Beer Haze Active Protein Gene in Cultivated and Tibetan Wild Barley and Development of Allelic Specific Markers

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ABSTRACT: The formation of haze is a serious quality problem in beer production. It has been shown that the use of silica elute (SE)−ve malt (absence of molecular weight (MW) ∼14000 Da) for brewing can improve haze stability in the resultant beer, and the protein was identified as a barley trypsin inhibitor of the chloroform/methanol type (BTI-CMe). The objectives of this study were to determine (1) the allelic diversity of the gene controlling BTI-CMe in cultivated and Tibetan wild barley and (2) allele-specific (AS) markers for screening SE protein type. A survey of 172 Tibetan annual wild barley accessions and 71 cultivated barley genotypes was conducted, and 104 wild accessions and 35 cultivated genotypes were identified as SE+ve and 68 wild accessions and 36 cultivated genotypes as SE−ve. The allelic diversity of the gene controlling BTI-CMe was investigated by cloning, alignment, and association analysis. It was found that there were significant differences between the SE+ve and SE−ve types in single-nucleotide polymorphisms at 234 (SNP_{234}), SNP_{313}, and SNP_{385}. Furthermore, two sets of AS markers were developed to screen SE protein type based on SNP_{313}. AS-PCR had results very similar to those obtained by immunoblot method. Mapping analysis showed that the gene controlling the MW∼14 kDa band was located on the short arm of chromosome 3H, at the position of marker BPB-0527 (33.302 cM) in the Franklin/Yerong DH population.

KEYWORDS: association analysis, haze protein, gene location, genetic diversity, Tibetan annual wild barley

INTRODUCTION

The first characteristic that a beer drinker sees with his or her eyes is the beer's clarity; hazes give a message to consumers that the beer has been contaminated or aged.[1] There are a number of factors that can affect haze formation in beer, but the factor most commonly associated with haze formation is the interaction between haze active proteins and certain polyphenols.[2−4]

During the colloidal stabilization of beer, silica is one of the common materials used to remove haze active proteins to reduce the formation of haze. The silica eluate (SE) proteins appear to be B and C hordeins, and a protein of MW ∼ 14000 Da. It was interestingly found that some varieties contained the MW ∼ 14000 band (SE+ve), whereas other varieties did not have the band (SE−ve).[5] The pilot brewing trials showed that the use of SE−ve malt in brewing could improve haze stability in the resultant beer.[5] N-Terminal sequence analysis of the tryptic peptides from SE+ve and SE−ve varieties showed that the SE protein had homology with the barley trypsin inhibitor of the chloroform/methanol type (BTI-CMe). Cloning of the BTI-CMe protein demonstrated that both SE−ve and SE+ve barley varieties contained a BTI-CMe protein family member, but the sequence differed between the two types of barleys.[6]

Interval mapping analysis showed that the gene controlling SE protein was located on the short arm of chromosome 3H.[7] The corresponding gene for BTI-CMe is also located on chromosome 3H.[8,9] BTI-CMe has been found to be polymorphic,[10] with five allelic variants so far being identified in both *Hordeum vulgare* and *Hordeum spontaneum*, including (1) BTI-CMe1; (2) BTI-CMe2, which consists of three variants, BTI-CMe2.1, BTI-CMe2.2, and BTI-CMe2.3; (3) BTI-CMe3, which consists of two variants, BTI-CMe3.1 and BTI-CMe3.2;[10,11,12] (4) BTI-CMe4; and (5) BTI-CMe5.[11]

The genotypic difference in SE+ve and SE−ve was examined by Robinson et al.[7] using 219 Australian and international barley varieties. It was found that 181 varieties belonged to SE+ve and only 38 varieties belonged to SE−ve varieties. Obviously, a wide range of variation in this trait was useful for genetic improvement.[13] In this, the potential use of novel variants in the wild barley germplasm would be exploited for gains in barley improvement. Tibetan wild annual barley, as the progenitor of cultivated barley,[14] was considered to have a wide biochemical, morphological, and physiological diversity.[15] Recently, wider variation in Tibetan wild barley than in cultivated barley was reported for salinity tolerance,[16] phytic acid content,[17] and β-amylase activity.[14] Thus, it was of interest to determine if wild barley has further variation for the BTI-CMe haze active protein.

As SE−ve malt is favorable for brewing in terms of improving beer haze stability,[5] malt barley breeders include the SE−ve trait in future malting barley varieties. However, the immunoblot analysis that is now available for screening the SE protein type has a number of disadvantages. These include difficulty of antibody preparation and relatively time-consuming experimental procedures. Therefore, alternative methods of identifying SE−ve type that are simple, cheap, and effective are required. On the basis of the immunoblot test, molecular markers such as SNPs, to be developed in this study, would effectively and efficiently select for this character and be easily integrated into a modern breeding program using marker-assisted selection for multiple traits.

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In this study, we examined the allelic diversity of haze active protein in cultivated and Tibetan wild barley and developed molecular markers for screening of SE protein type.

MATERIALS AND METHODS

Plant Materials. For immunoblotting analysis, 172 Tibetan annual wild barley accessions, including 120 two-rowed accessions (*H. vulgare* L. ssp. *spontaneum*) and 52 six-rowed accessions (*H. vulgare* L. ssp. *agriocrithum*), and 71 cultivated barley genotypes (*H. vulgare* L. ssp. *vulgare*), collected from different countries (Japan, 14; Canada, 7; United Kingdom, 3; Australia, 4; China, 43) were used. Tibetan annual wild barleys were collected from the Qinghai-Tibet Plateau of China. All accessions were planted in early November 2008, in adjacent plots of a field in the experimental farm of Zhejiang University, and each genotype or accession consisted of a 2 m length row, with three replications. Barley cultivation was conducted in accordance with conventional local agronomic practices. At maturity, the grains were harvested and stored at 4 °C, before being milled with a Cyclone mill (Cole-Parmer, Vemon Hills, IL) before analysis.[17]

For BTI-CMe cloning, total genomic DNA was extracted from the seedlings of 37 (14 SE+ve, 23 SE−ve) barley varieties or accessions, using Universal Genomic DNA Extraction Kit ver. 3.0 (TaKaRa, Dalian Bio-Co., China) following the manufacturer's instructions.

The SE polymorphism was screened in 95 lines from the Yerong (SE−ve)/Franklin (SE+ve) mapping population which was kindly supplied by Dr. M. Zhou, University of Tasmania, Australia. Franklin is an Australian two-rowed malting barley, and Yerong is an Australian six-rowed feed variety. Total genomic DNA was extracted from the 95 DH lines for analysis.

Identification of the SE Protein. SE polymorphism in cultivated and Tibetan wild barleys was analyzed according to the method of Evans et al.[5] The polyclonal antibody (anti-SE) was kindly provided by Dr. Evans of University of Tasmania, Hobart, Australia. Barley sample (0.3 g) was extracted using 5 mL of SDS-PAGE sample buffer (5 M urea, 4% SDS, Tris buffer, pH 8.0) containing 1% (v/v) 2-mercaptoethanol. SDS-PAGE was carried out as described by Laemmli,[18] with a resolving gel (15% T, 2.7% C) and a stacking gel (4% T, 2.7% C), using an electrophoresis unit (Bio-Rad, Hercules, CA). The gels were run at a constant voltage of 110 V, for approximately 1.5 h. After electrophoresis, the separated proteins were transferred to nitrocellulose transfer membrane (0.2 mm pore size, Whatman, USA), and the immunoblots were run for 15 min at a constant 15 V (Bio-Rad Trans-Blot Semi-Dry Cell and System). After transfer, the membrane was incubated with the polyclonal antibody (anti-SE) in 3% (w/v) nonfat milk/Tris-HCl−Tween 20 (TTBS). Goat anti-rabbit horseradish peroxidase (GAR-HRP) antibody (Gene Tex, USA) was used as the secondary antibody. The immunoblots were developed using the Immun-Star Western C Kit (Bio-Rad) following the manufacturer's instructions.

Sequencing and Polymorphism Identification of the Gene of BTI-CMe. On the basis of the published genomic sequence of the *H. vulgare Itr1* gene (*HvITR1* X65875) (for expressed protein CMe this was between nucleotides 2394 and 2877 of the 5463 nucleotide sequence),[19] PCR primers were designed with Primer 5.0. The primers were forward primer (F), 5′-CAACTAACAGAAAGTCAGAAAGCAC-3′, and reverse primer (R), 5′-CACAATACTGAAAATACTCTGATGC-3′. The PCR reactions were completed as follows: 2.5 μL of 10× TransTaq HiFi buffer I (200 mM Tris-HCl (pH 8.4), 100 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$, 200 mM KCl), 2 μL of 2.5 mM dNTPs, 0.5 μL of 10 μM forward primers, 0.5 μL of 10 μM reverse primer, 0.5 μL of 5 units μL^{-1} of TransTaq polymerase High Fidelity (Beijing TransGen Biotech Co., Ltd. China), and 1 μL of 50 ng of genomic DNA. All amplifications were performed on a DNA Engine Dyad thermal cycler (Bio-Rad) under the following conditions: an initial 5 min of denaturation at 95 °C, followed by 32 cycles of 30 s at 95 °C, 30 s at 61.4 °C, and 30 s at 72 °C, and then 10 min at 72 °C for a final extension. After the PCR product was purified, DNA sequencing was performed on an ABI 3100 automated sequencer following the manufacturer's instructions (Applied Biosystems, Foster City, CA). PCR products were sequenced from both strands.

Allele-Specific PCR (AS-PCR). To identify the type of SE protein, we designed primer pairs using the software Primer 5.0 based on the SNPs of the gene coding BTI-CMe. In general, if there is a noncomplementary base pair with the template of the 3′ end in one or both of the forward and reverse primers, the polymerization reaction will not be carried out or the rate will be greatly reduced. However, sometimes the mismatch of one base alone does not reliably distinguish between two alleles. To reduce the chance of primers annealing to the untargeted template, we employed the addition of a designed mismatch at the 3′ end of SNP. This kind of marker has been called a single-nucleotide amplified polymorphism (SNAP).[20] Two sets of the primers were designed. The primers were forward primer (AS-F), 5′-CAACTAACAGAAAGTCAGAAAGCAC-3′; reverse primer 1 (AS-R1), 5′-AGGGCAGTTGGGCGAA-3′; and reverse primer 2 (AS-R2), 5′-AGGGCAGTTGGGCGTA-3′.

The PCR reactions were completed as follows: 2.5 μL of 10× TransTaq HiFi buffer I (200 mM Tris-HCl (pH 8.4), 100 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$, 200 mM KCl), 2 μL of 2.5 mM dNTPs, 0.5 μL of 10 μM forward primers, 0.5 μL of 10 μM reverse primer, 0.5 μL of 5 units μL^{-1} of TransTaq polymerase High Fidelity (Beijing TransGen Biotech Co., Ltd.), and 1 μL of 50 ng of genomic DNA. All amplifications were performed on a DNA Engine Dyad thermal cycler (Bio-Rad) under the following conditions: an initial 5 min of denaturation at 95 °C, followed by 32 cycles of 30 s at 95 °C, 30 s at 62 °C, and 30 s at 72 °C, and then 10 min at 72 °C for a final extension. The PCR products were analyzed by 1% agarose gel electrophoresis.

Genetic Analysis of the SE Trait. The SE polymorphism was screened by AS-PCR (primers AS-F and AS-R1) in the 95 lines of the Franklin (SE+ve)/Yerong (SE−ve) mapping population. The population comprised 177 doubled haploid lines, and a linkage map for the population was constructed using 496 Diversity Array Technology (DArT) and 28 microsatellite markers.[21]

The sequences of forward and reverse strands were assembled by the SEQMAN program (DNAstar, Madison, WI). All of the sequences obtained were aligned by ClustalX version 2 (http://www.clustal.org/), and the single-nucleotide polymorphisms (SNPs) were detected. Multiple DNA and amino acid sequence alignments were performed using the GENEDOC program. The association analysis between nucleotide polymorphisms and different traits of SE protein were performed with Trait Analysis by Association, Evolution and Linkage, version tassel 2.1_standalone software (TASSEL). The significance (P value) and the proportion (R^2) of total variation were calculated for each marker by applying the General Linear Model (GLM). Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 4.[22] Three-dimensional (3D) structure models of BTI-CMe in 37 barley accessions were predicted with the Swiss model.[23,24]

RESULTS

SE Polymorphism in Cultivated and Tibetan Wild Barleys. SDS-PAGE immunoblot analysis, using SE antibody, detected many bands that ranged from 30 to 80 kDa in the examined cultivated and Tibetan wild barleys (Figure 1). These higher molecular weight bands may be parts of B, C, and D hordeins.[5,25] There was an interesting band of about 14 kDa. A polymorphism was observed for the band in some accessions or varieties that were SE+ve, whereas in other accessions that were SE−ve it was absent. All Tibetan annual wild barley accessions and cultivated barley genotypes were screened using immunoblot analysis. It

was found that among Tibetan annual wild barley 104 accessions were identified as SE+ve and 68 as SE−ve. Among cultivated barley genotypes, 35 were identified as SE+ve and 36 as SE−ve.

Allelic Diversity of BTI-CMe. Using the F and R primers, designed from the *Itr1* gene, the CDS of *HVITR1* were cloned (products = CMe) in 37 barley genotypes or wild accessions, and the PCR products were purified and then sequenced. Among these accessions, 14 were previously identified as SE+ve (9 were Tibetan wild barley, and 5 were cultivated barley) and 23 as SE−ve (11 were Tibetan wild barley, and 12 were cultivated barley).

All 37 sequences were aligned by ClustalX. In the coding sequence of the *HVITR1* gene, 22 SNPs were detected in the 447 bp region (Table 1). On the basis of the polymorphism analysis, 6 haplotypes were detected among the 37 accessions (Figure 2). Hap 1 and Hap 2 were identified as SE+ve, and haplotypes 3−6 were SE−ve. The difference between SE+ve and SE−ve is caused by SNP_{234}, SNP_{313}, and SNP_{385}. The association between the type of SE protein and SNPs was analyzed by TASSEL. It was found that the R^2 of SNP_{234}, SNP_{313}, and SNP_{385} was 0.64. This means these three SNPs could explain 64% of the total genetic variation of the MW ~ 14 kDa protein. Hence, they are ideal for the development of the molecular markers for marker-assisted selection.

Allele-Specific PCR Detection of SE Polymorphism. Two sets of AS markers were used to screen 91 accessions that were previously identified as SE+ve or SE−ve. In Figure 3, the left five lanes were screened by marker AS-R1: SE+ve belonged to the band of 455 bp (identified with an arrow), and SE−ve was without the 455 bp band. The right five lanes were screened by AS-R2, and the results were just the opposite, with Yerong (having the 455 bp band) being identified as SE−ve type and Franklin (without the 455 bp band) being identified as SE+ve type.

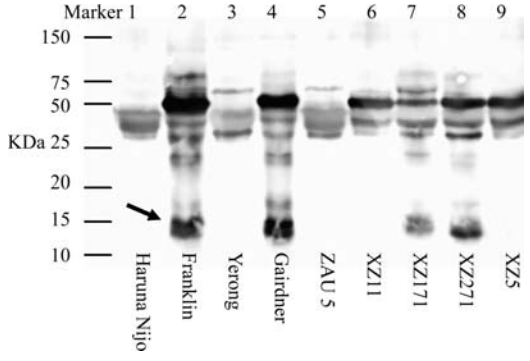

Figure 1. SDS-PAGE immunoblot analysis of total protein extracts from barley using the anti-SE antibody. The arrow indicates the MW ~ 14000 SE protein. Lanes: 1−5, cultivated barley; 6−9, Tibetan annual wild barley.

In AS-R2 screening, the conformity between AS-R2 screening and immunoblot results was 93.4%. In AS-R1 screening, the conformity between immunoblot and AS-PCR deducing results was up to 97.8%, and only two accessions showed a difference between the two analyses. The difference might be caused by the contamination of seed powder, as Robinson et al.[7] showed only 4% contamination could be detectable. It may be suggested that the two accessions should be SE−ve type and the AS-R1 marker is quite valid.

Mapping of SE Trait. SNP_{313} (A/T) was used to genotype 95 lines of the Yerong (SE−ve)/Franklin (SE+ve) DH population with AS-F and AS-R1 as primers. The results were scored using "0" or "1" scale, where "1" means there is a 455 bp PCR product in the line (being positive for the presence of the MW ~ 14 kDa SE protein), and "0" means there is no 455 bp PCR product in the lines The variation in the presence or absence of the SE protein was mapped to the short arm of chromosome 3H (Figure 3). The SE trait was located at the position of marker BPB-0527 (33.302 cM).

The chromosomal location of the SE trait, identified by AS-PCR in the current study, is consistent with the result Robinson et al.[7] (Figure 4) reported, using immunoblot analysis in the Chebec/Harrington mapping population. It is proved that the AS marker (AS-R1) is correct. Moreover, Robinson et al.[7] found that there was a larger distance between PSR1196/ABC171/PSR1316 and BCD089. In this study, the distance between BPB-0527 and BPB-1814 was much closer.

DISCUSSION

The SE protein types of Haruna Nijo (SE−ve), Franklin (SE+ve), Gairdner (SE+ve), and Golden Promise (SE+ve) have already been reported in previous studies,[7] and the results of the current study are essentially consistent with the earlier results. This indicated that the immunoblotting screening protocol we used in the current study was correct and effective. Robinson et al.[7] examined 219 Australian and international barley varieties and found that 83% of tested barley genotypes belonged to SE+ve and 17% belonged to SE−ve. However, the current study showed that the proportion of SE−ve in Tibetan wild barley was up to 39.5%, suggesting that the SE−ve protein type in Tibetan wild barley has higher frequency than in cultivated barley. It was reported that the absence of the SE+ve protein was favorable for improving beer haze stability.[5] Therefore, the wild

Table 1. Single-Nucleotide Polymorphisms (SNPs) in the Coding Sequence Region and Haplotype Pattern of *HVITR1* Gene Derived from 37 Barley Accessions

	22	27	78	82	83	96	101	151	211	**234**	262	298	299	**313**	**385**	403	407	443	446
Hap1	A	C	T	A	T	A	A	G	C	**T**	C	G	C	**T**	**G**	G	G	T	A
Hap2	C	G	G	T	C	G	C	A	C	**T**	C	G	C	**T**	**G**	G	G	T	A
Hap3	A	C	G	T	C	G	C	A	T	**C**	G	C	G	**A**	**T**	G	G	T	A
Hap4	A	C	G	T	C	G	C	A	C	**C**	C	G	C	**A**	**T**	C	C	C	C
Hap5	C	C	G	T	C	G	C	A	T	**C**	C	G	C	**A**	**T**	C	C	C	C
Hap6	A	C	G	T	C	G	C	A	T	**C**	C	G	C	**A**	**T**	C	C	C	C
R^{2a}	0.13	0.27	0.23	0.23	0.23	0.23	0.23	0.23	0.46	**0.64**	0.16	0.16	0.16	**0.64**	**0.64**	0.21	0.21	0.21	0.21
P^b value	*	***	**	**	**	**	**	**	***	***	*	*	*	***	***	**	**	**	**

[a] R^2 refers to genetic variation. [b] Significance of difference between the two accession classes: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

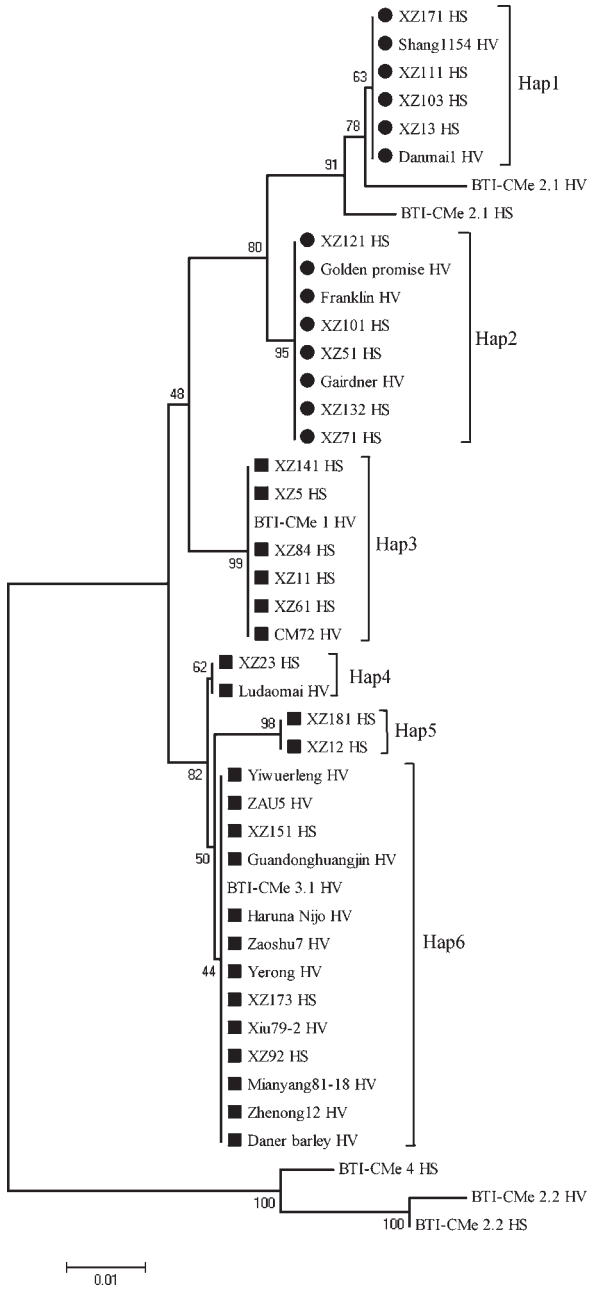

Figure 2. Phylogram created using the MEGA4 program to show the relationship between the CDS of *HVITR1* in the SE−ve and SE+ve accessions and known BTI-CMe variants obtained from the NCBI database. HV after the accession indicates BTI-CMe sequences from *H. vulgare*; HS after the accession indicates sequences from *H. spontaneum*. ■ indicates the accession has been identified as SE−ve, and ● indicates the accession has been identified as SE+ve.

barley may provide the elite germplasm for improving the malt quality of cultivated barley.

In the analysis of the allelic diversity of the gene controlling the MW ~ 14 kDa protein, we detected many SNPs and designed two sets of AS markers. The screening result obtained by using AS markers was consistent with that of immunoblot analysis. AS-PCR analysis is more easily accomplished than immunoblot analysis. Moreover, AS-PCR analysis requires only a small amount of tissue DNA and may provide direct information on the genetic

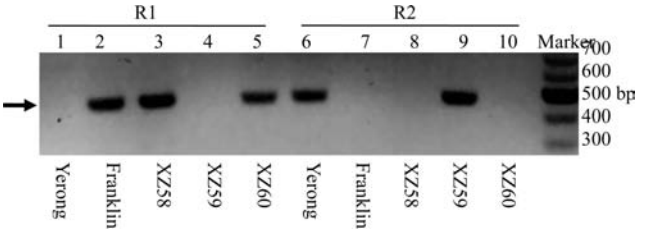

Figure 3. Test of the AS markers in accessions that have been screened by Western blotting. Lanes: 1−5, screened by AS-R1; 6−10, screened by AS-R2.

composition of examined plants. In addition, when the SE−ve trait marker is used in conjunction with other DNA markers in an integrated marker-assisted selection barley breeding system, rapid and efficient selection of the breeds would result. Obviously, it is an ideal method. The inclusion of the SE−ve trait in new malting barley varieties would enable breeders to develop the varieties that produce malt with reduced potential for haze formation in beer.

In the NCBI database (http://www.ncbi.nlm.nih.gov), several allelic variants of BTI-CMe have been published: BTI-CMe1 (AJ222977) (*H. vulgare*), BTI-CMe2.1 (AJ251931 and AJ222974) (*H. vulgare* and *H. spontaneum*, respectively), BTI-CMe2.2 (AJ223458 and AJ222975) (*H. vulgare* and *H. spontaneum*, respectively), BTI-CMe3.1 (AJ222978) (*H. vulgare*), and BTI-CMe4 (AJ222976) (*H. spontaneum*). In the current study, a phylogram was developed using the MEGA4 program to determine the relationship between the SE protein type and known BTI-CMe variants obtained from the NCBI database (Figure 2). It was revealed that the SE+ve type was associated with the BTI-CMe2.1 variant, but not with the BTI-CMe1 variant, whereas other BTI-CMe variants may be identified as SE−ve type.

The amino acid sequences of BTI-CMe were investigated by using the GENEDOC program. It was found that SNP_{234} is the same sense mutation, whereas SNP_{313} and SNP_{385} are mis-sense mutations. SNP_{313} in SE+ve variants translates serine 105, whereas in SE−ve variants it translates threonine 105. These two amino acids belong to polar but uncharged amino acid R groups. SNP_{385} in SE+ve variants translates glycine 129 and in SE−ve variants translates tryptophan 129. These two amino acids belong to nonpolar (hydrophobic) amino acid R groups.

The 3D structure model of SE+ve and SE−ve proteins was predicted using the Swiss model to explain why the change of these two amino acids would result in the difference in beer colloidal stabilization. All 37 amino acid sequences were aligned with bifunctional α-amylase/trypsin inhibitor from ragi seeds (RBI) and may be classified into three kinds (Figure 5). BTI-CMe proteins target exogenous α-amylases, trypsin, and other serine proteases, as well as proteins with no known in vitro activity.[25,26] Thus, the presumed model is reliable to some extent, as the functions are similar for the two types of proteins. The sequence identities of RBI and haplotype 1, haplotype 2, and SE−ve type BTI-CMe are 54.1, 55.7, and 56.6%, respectively. However, the available results have not illustrated the reason why such a structural change may improve haze stability in the resultant beer through using SE−ve barley for brewing. Although it was reported that polyphenol and haze active proteins are related to haze formation,[3] it is still unclear which domain in protein is haze active. Further investigations are required to determine the mechanism of how BTI-CMe protein promotes haze formation. Moreover, there are two critical questions to be answered in future investigations.

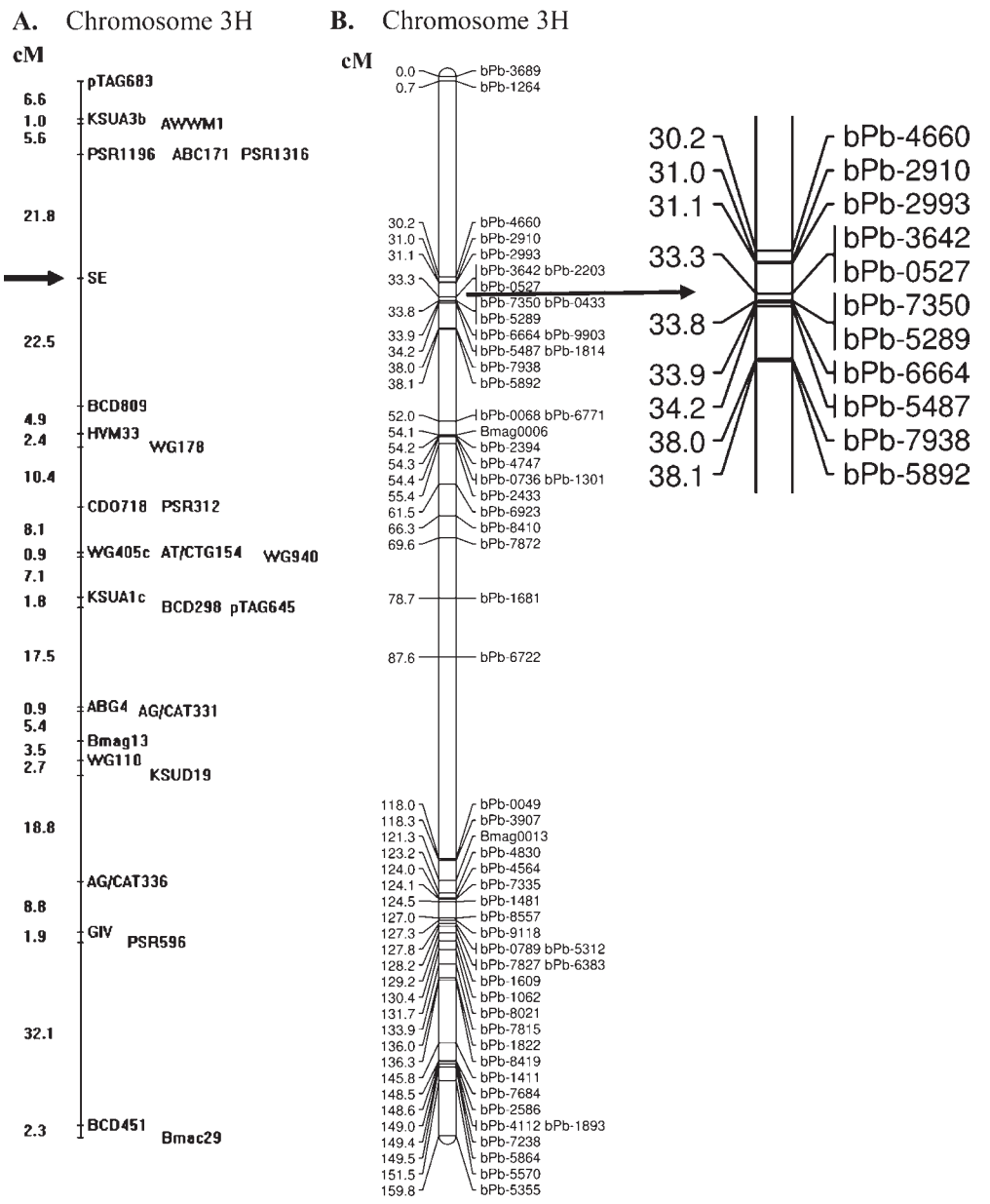

Figure 4. Map location of the SE trait on chromosome 3H: (A) in the Chebec/Harrington population;[7] (B) in the Yerong/Franklin population.[21] In panel B, the prefix "bPb" signifies a DArT marker; the other markers on the map are microsatellites.

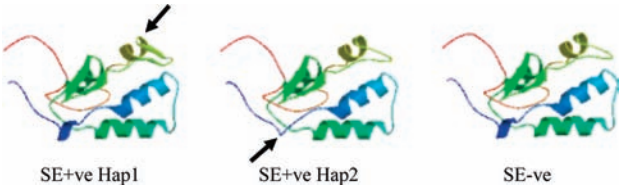

Figure 5. Three-dimensional structure model of BTI-CMe in 37 barley accessions predicted by Swiss model. The arrow indicates the differences between SE+ve and SE−ve.

First, do all of the BTI-CMe haplotypes cause similar levels of haze formation in beer among the six BTI-CMe haplotypes? Second, do one or more SE−ve haplotypes show a better haze stability than the haplotype initially described by Evans?[5]

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