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ORIGINAL PAPER

Development of DNA markers associated with beer foam stability for barley breeding

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Abstract Traits conferring brewing quality are important objectives in malting barley breeding. Beer foam stability is one of the more difficult traits to evaluate due to the requirement for a relatively large amount of grain to be malted and then the experimental costs for subsequent brewing trials. Consequently, foam stability tends to be evaluated with only advanced lines in the final stages of the breeding process. To simplify the evaluation and selection for this trait, efficient DNA makers were developed in this study. Previous studies have suggested that the level of both of the foam-associated proteins Z4 and Z7 were possible factors that influenced beer foam stability. To confirm the relationship between levels of these proteins in beer and foam stability, 24 beer samples prepared from malt made from 10 barley cultivars, were examined. Regression analyses suggested that beer proteins Z4 and Z7 could be positive and negative markers for beer foam stability, respectively. To develop DNA markers associated with contents of proteins Z4 and Z7 in barley grain, nucleotide sequence polymorphisms in barley cultivars in the upstream region of the translation initiation codon, where the promoter region might be located were compared. As a result, 5 and 23 nucleotide sequence polymorphisms were detected in protein Z4 and protein Z7, respectively. By using these polymorphisms, cleaved amplified polymorphic sequence (CAPS) markers were developed. The CAPS markers for proteins Z4 and Z7 were applied to classify the barley grain content of 23 barley cultivars into two protein Z4 (pZ4-H and pZ4-L) and three protein Z7 (the pZ7-H, pZ7-L and pZ7-L2) haplotypes, respectively. Barley cultivars with pZ4-H showed significantly higher levels of protein Z4 in grain, and those with pZ7-L and pZ7-L2 showed significantly lower levels of protein Z7 in grain. Beer foam stability in the cultivars with pZ4-H and pZ7-L was significantly higher than that with pZ4-L and pZ7-H, respectively. Our results indicate that these CAPS markers provide an efficient selection tool for beer foam stability in barley breeding programs.

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Introduction

Beer foam is an important quality trait for brewers. It can be characterized by a series of characteristics, such as stability, quantity, lacing, whiteness, creaminess, density, viscosity and strength (Bamforth 1985). Proteins (Dale and Young 1987; Siebert and Knudson 1989), hop iso-α-acids (Evans and Sheehan 2002; Simpson and Hughes 1994), polysaccharides (Evans et al. 1999a; Stowell 1985) and metal ions (Evans and Sheehan 2002) have been considered to contribute to beer foam stability. Among protein factors derived from barley malt, protein Z (Evans and Hajgaard 1999; Evans et al. 2003; Iimure et al. 2008; Maeda et al. 1991), lipid transfer protein 1 (LTP 1)

(Perrocheau et al. 2006; Sorensen et al. 1993; van Nierop et al. 2004) and barley dimeric alpha-amylase inhibitor 1 (BDAI-1) (Iimure et al. 2008; Okada et al. 2008) have been reported to be associated with foam stability.

Protein Z is a major beer protein and its positive relationship with beer foam stability has been widely discussed (Evans and Bamforth 2009). Protein Z is a small family of barley serine protease inhibitors (serpin). Some isoforms such as protein Z4, protein Z7 and protein Zx are included in the barley protein Z family (Rasmussen 1993; Roberts et al. 2003). However, previous reports have only observed protein Z4 and protein Z7 in beer (Iimure et al. 2008, 2010; Perrocheau et al. 2005, Evans et al. 1999a). Protein Z4 is a predominant isoform comprising 80% of the total protein Z content in barley grain and malt (Evans and Hajgaard 1999). Therefore, when most previous investigations have referred to protein Z in beer, it is suggested that they are mainly referring to protein Z4. Evans and Hajgaard (1999) also defined the fractions of proteins Z4 and Z7 in barley and malt. There is a free fraction of proteins Z4 and Z7 that is extractable with aqueous salt-solution. Reducing agents and presumably proteases release the bound fraction while the latent fraction (primarily in barley) can only be released with SDS and reducing agent. Total protein Z4 or protein Z7 was defined as the sum of the free, bound and latent fractions while the combined fraction was the sum of the free and bound fraction. Generally it is either the free or combined fractions that are quantitated and reported. Evans and Hajgaard (1999) observed in seven barley varieties that the average content of free protein Z4 was 30% (range 20–52) and 31% of protein Z7 (range 27–40). In malt, the average content of free protein Z4 rose to 58% (range 37–75) and protein Z7 was also 58% (range 42–72).

It is reasonably widely accepted that protein Z4 is a key beer foam-positive protein (Evans and Bamforth 2009). In comparison, protein Z7 may also play an important role in beer but it has not been well investigated with the exception of Evans and Hajgaard (1999) and Evans et al. (1999a). The removal of protein Z from beer by immunoaffinity treatment, only reduced of foam stability by a relatively minor 10% (Hollemans and Tonies 1989). Gibson et al. (1996) also reported that little difference was observed in foam stability between beers brewed with (normal) and without (deficient, cv. Pirkka) protein Z in malt. Pirkka was later found to contain a relatively high level of protein Z7 (Evans and Hajgaard 1999). Thus, the relationship between protein Z, particularly the relative contribution of protein Z4 to protein Z7, and beer foam stability is still unclear. Importantly, higher levels of malt proteins Z4 and Z7 result in their increased levels in beer (Evans and Bamforth 2009).

The malt content of proteins Z4 and Z7 are considered to be selectable indicators of beer foam stability for the breeding of malting barley (Evans and Bamforth 2009). It is, however, difficult to estimate the protein contents in malt of early hybrid generations due to the limited amount of seed which precludes malting and the potentially large environmental effects related to seed produced by single or limited numbers of plants as a result growing conditions. Therefore, application of DNA markers to select desirable alleles for proteins Z4 and Z7 may enable the selection of single plants without these limitations. For barley grain protein Z4 content, a restriction fragment length polymorphism (RFLP) marker has previously been developed (Kaneko et al. 1999). However, the RFLP marker evaluation requires a significant amount of DNA sample and complicated assessment procedures. In addition, Kaneko et al. (1999) did not investigate the relationship between protein Z4 genotype and beer foam stability. Recently, selection protocols for DNA markers based on single nucleotide polymorphism (SNP), such as cleaved amplified polymorphic sequences (CAPS), have been used in crop breeding due to their advantages with respect to amount of DNA required and the PCR-based co-dominant nature of the assay (Francia et al. 2005). CAPS markers have also been applied in barley breeding for the selection of several malting quality traits (Paris et al. 2002; Potokina et al. 2006).

In this study, 24 beer samples were prepared from the malt made from 10 barley cultivars. By using these beer samples, relationship between foam stability, and both proteins Z4 and Z7 in beer were evaluated by regression analysis. The selection efficiency of DNA markers developed from nucleotide sequences of proteins Z4 and Z7 were validated by using these beer samples and the markers were applied to assess seed from a range of other cultivars of known origin.

Materials and methods

Barley and malt samples

Beer was brewed from malt made from ten barley cultivars that had the quality characteristics shown in Table 1. Each malt sample was made from 75 kg barley grain by the method described in a previous report (Okada et al. 2008). To analyze the barley grain contents of proteins Z4 and Z7, 23 cultivars (the cultivar names shown in Fig. 2) were grown under the recommended management practices in Gunma prefecture, Japan, in 2000, 2004 and 2008. Kolbach Index (soluble nitrogen/total nitrogen × 100) was analyzed according to the standard method of the European Brewery Convention (1987). To investigate the genetic profile of proteins Z4 and Z7 in worldwide malting barley varieties, 64 cultivars collected from Japan, North America,

Table 1 Summary of the characteristics of the malt and beer samples

Cultivar	Area of production	Year	Kolbach Index[a]	Beer protein Z4 (μg/ml)	Beer protein Z7 (μg/ml)	Beer bitterness unit	NIBEM (s)	Protein Z4 genotype	Protein Z7 genotype
Cultivar A-1	Canada	2000	42.1	17.49	1.27	24.2	285	H[b]	L[c]
Cultivar A-2	Canada	2000	50.0	11.89	1.19	23.5	264	H	L
Cultivar A-3	Canada	2007	43.9	8.88	2.99	24.8	258	H	L
Cultivar A-4	Canada	2008	43.2	7.80	4.19	25.5	265	H	L
Cultivar B-1	Canada	2004	42.5	8.97	8.76	19.1	247	H	H
Cultivar B-2	Canada	2008	40.8	8.13	6.49	20.6	244	H	H
Cultivar B-3	Canada	2008	45.2	7.93	8.12	23.2	251	H	H
Cultivar C-1	Canada	2007	43.3	6.94	5.98	22.4	239	H	H
Cultivar C-2	Canada	2008	39.3	7.54	6.60	25.7	246	H	H
Cultivar D	Canada	2008	41.3	7.67	4.87	25.4	264	H	L
Cultivar E-1	Japan	2000	47.3	8.30	4.29	22.3	245	L	L
Cultivar E-2	Japan	2002	47.1	6.82	4.38	22.9	258	L	L
Cultivar E-3	Japan	2002	50.1	7.26	5.15	23.7	260	L	L
Cultivar E-4	Japan	2002	51.6	9.33	5.99	20.4	243	L	L
Cultivar F-1	Japan	2003	50.9	4.56	11.46	19.1	232	L	H
Cultivar F-2	Japan	2004	43.4	4.07	7.71	19.1	230	L	H
Cultivar F-3	Japan	2007	42.9	6.16	15.04	20.0	233	L	H
Cultivar F-4	Japan	2007	43.3	6.49	16.61	19.5	232	L	H
Cultivar G-1	Japan	2004	42.2	2.42	8.75	19.9	219	L	H
Cultivar G-2	Japan	2004	42.2	3.26	12.21	20.8	226	L	H
Cultivar H-1	Japan	2007	42.4	6.94	17.06	17.7	232	L	H
Cultivar H-2	Japan	2007	43.4	6.63	15.95	19.6	238	L	H
Cultivar I	Japan	2007	42.5	5.96	14.51	21.7	258	L	H
Cultivar J	Japan	2007	47.2	5.79	12.51	19.5	248	L	H
Mean			44.5	7.4	8.4	21.7	246.5		
Standard deviation			3.4	3.0	4.9	2.4	15.4		
Maximum			51.6	17.5	17.1	25.7	285.0		
Minimum			39.3	2.4	1.2	17.7	219.0		

[a] (Soluble nitrogen/malt total nitrogen) × 100

[b] H and L indicate pZ4-H and pZ4-L, respectively, in the genotypes of protein Z4

[c] H and L indicate pZ7-H and pZ7-L, respectively, in the genotypes of protein Z7

Australia and Europe were used. The cultivar names in Tables 1 and 3 can be disclosed to interested parties under a Material Transfer or Confidentiality Agreement.

Beer processing

The beer samples were prepared from barley malt (67% w/w of total raw materials), corn starch, rice and hops in a 400-l pilot scale plant according to the brewing protocol described in a previous report (Okada et al. 2008). The fermentation conditions were previously described by Okada et al. (2008). Beer foam stability of the resulting 24 beers was determined using a foam stability tester type NIBEM-T (Haffmans B. V., Venlo, Holland) according to the manufacturer's instructions.

Determination of proteins Z4 and Z7 concentrations

Proteins Z4 and Z7 contents in beer and barley grain were determined by enzyme-linked immunosorbent assay (ELISA) using each specific antibody. A quantitative sandwich ELISA for beer protein Z4 was conducted as follows. A purified rabbit anti-recombinant protein Z4 polyclonal antibody, which did not cross-react with protein Z7 (data not shown) was used as the primary antibody, and the Fab fragment of the rabbit anti-protein Z4 polyclonal antibody, which was conjugated to horse radish peroxidase, was used as the secondary antibody. The primary antibody diluted 1 in 1,000 with coating buffer (15 mM sodium carbonate and 35 mM sodium hydrogen carbonate, pH 9.6), was added to a 96-well EIA/RIA plate (Corning

Incorporated, USA), and then allowed to bind to the plate overnight at 4°C. Blocking was achieved at room temperature for 2 h with a buffer containing 10 g/l casein, 137 mM sodium chloride, 8 mM disodium hydrogenphosphate 12-water, 1 mM potassium dihydrogenphosphate. The beer samples were diluted 1 in 1,000 with "Can Get Signal Solution 1" (TOYOBO, Japan); where after 100 µl of sample was added to the coated wells in triplicate. After incubation at room temperature for 2 h, the wells were washed three times by washing buffer (20 mM Tris–HCl, 150 mM sodium chloride and 0.05% Tween 20, pH 7.5). Next, 100 µl of secondary antibody diluted 1 in 500 with "Can Get Signal Solution 2" (TOYOBO) was added to the wells. After incubation at room temperature for 2 h, the wells were again washed three times with washing buffer. The color development reaction was conducted by the peroxidase coloring kit (Sumitomo Bakelite, Japan). The absorbance at 492 nm was measured by a VARIOSKAN microtitre plate reader (Thermo Electron Corporation, Japan).

A semi-quantitative direct adsorption ELISA for barley grain protein Z4 was performed as follows. Barley protein extract was prepared from 50 mg milled barley grain in 1 ml phosphate buffered saline with 0.28% dithiothreitol to extract both the free and bound fractions. Proteins were extracted overnight at 4°C with mixing. After total protein concentration was determined by the Bradford method using bovine serum albumin as a standard (Bradford 1976), 100 µl of sample diluted 1 in 4,000 by coating buffer were added to a 96-well EIA/RIA plate in triplicate, and then the plate was incubated at 4°C overnight. The blocking reaction was conducted with the addition of 200 µl of blocking buffer in each well and incubating at room temperature for 2 h. After the well was washed twice with washing buffer, where after 100 µl of primary antibody, purified rabbit anti-protein Z4 polyclonal antibody diluted 1 in 1,000 with "Can Get Signal Solution 1" was added to the well, and then the plate was incubated at room temperature for 2 h. Following washing the plate with washing buffer three times, 100 µl of goat anti-rabbit IgG-AP (secondary antibody) (Santa Cruz Biotechnology, California, USA) diluted 1 in 1,000 with "Can Get Signal Solution 2" was added to the well. After the plate was incubated at room temperature for 2 h, the plate was again washed three times. The color development reaction was achieved by the addition of 150 µl of coloring solution (1 mg/ml disodium *p*-nitrophenyl phosphate hexahydrate in 10% diethanolamine). After color development the reaction was stopped by the addition of 3 M sodium hydroxide, the absorbance was measured at 405 nm by VARIOSKAN.

For quantitative ELISA of barley and beer protein Z7, rabbit anti-protein Z7 polyclonal antibody as the primary and rabbit anti-protein Z7 polyclonal antibody conjugated with biotin as the secondary antibody specific to barley and beer protein Z7 (Evans et al. 1999a) provided by Dr. E. Evans, Tasmania University, Australia was used. Protein Z7 ELISA was conducted according to Evans et al. (1999a) with minor modification. Barley protein extract (bound + free protein Z7) was prepared as described above. The barley protein extracts were diluted 1 in 1,000 with "Can Get Signal Solution 1". For beer protein Z7 assay, the beer sample was also diluted 1 in 500 with "Can Get Signal Solution 1". 100 µl of sample solution was then added to the plate coated in the primary antibody diluted 1 in 300 with coating buffer in triplicate. The plate was incubated for 2 h and washed three times with washing buffer. Subsequently, the secondary antibody diluted 1 in 5,000 by "Can Get Signal Solution 2" was added to the plate. The remainder of the procedure was completed as described by Evans et al. (1999a). The contents of barley grain proteins Z4 and Z7 were calculated by ng/µg protein to correct extraction efficiency and protein content in barley grain.

PCR and thermal asymmetric interlaced (TAIL) PCR

Total DNA was isolated from the leaf blade by the following protocol. A zirconia ball and 200 µl of extraction buffer (200 mM Tris–HCl, 250 mM sodium chloride, 25 mM ethylenediaminetetraacetate, pH 7.5) were added to 5 mm square piece of barley leaf, and then the solution was shaken at 1,200 rpm for 20 s using MULTI-BEADS SHOCKER MB-501 (YASUI KIKAI, Japan). Subsequently, the solution was incubated at 60°C for 30 min. After centrifugation at 1,200×*g* for 20 min, 150 µl of isopropanol was added to the supernatant. After centrifugation at 1,200×*g* for 20 min, 200 µl of 70% ethanol solution was added to precipitate DNA and mixed. Following centrifugation at 1,200×*g* for 20 min, and drying, the DNA was dissolved in 50 µl of sterilized water. PCR was conducted as follows. 2 µl of DNA solution, 1 µl of 10 mM primer solution, 10 µl of Premix Taq Ex Taq Version 2.0 (Takara Bio Inc., Japan) and 6 µl of sterilized water was mixed. The primer list is shown in Table 2. Subsequently, PCR was conducted using a thermal cycler, PTC-200 (MJ Research, Inc., USA) using the following program: 35 cycles at 94°C for 1 min, 62.5°C for 1 min and 72°C for 5 min, and 1 cycle at 72°C for 5 min. Thermal asymmetric interlaced (TAIL) PCR (Liu and Whittier 1995) was performed to isolate DNA fragments of the upstream region from the translation initiation codon for protein Z7. 2 µl of DNA solution, 1 µl of 100 µM random primer (Table 2), 4 µl of 1 µM specific primer 1 (Table 2), 10 µl of Premix Taq Ex Taq Version 2.0, and 3 µl of sterilized water were mixed, and then the first PCR was conducted using the following program: 1 cycle at

Table 2 Primer list for PCR

Primer name	Sequence
For protein Z4 sequence	
pZ4 Se-F	5'-GAGACGTGTAGTAATCTTCG-3'
pZ4 Se-R	5'-GCGAGCACAAATTGCACCACC-3'
For protein Z4 genotyping by CAPS marker	
pZ4 CAPS-F	5'-GGAGTATATGAGGGCTCGCG-3'
pZ4 CAPS-R	5'-CCCTTCGCGTAAGGAAGCTT-3'
For TAIL PCR	
Random primer	5'-GTNCGA(G/C)(A/T)CANA(A/T)GTT-3'
Specific primer-1	5'-CGTTGGTGGCAGCAGACTCGGGG-3'
Specific primer-2	5'-GGTCGGAGGAGATGGCGGAGGCG-3'
Specific primer-3	5'-GGTCGGTGGTGAGGGTGGTTGCCA-3'
For protein Z7 genotyping by CAPS marker	
pZ7 CAPS-F	5'-GGTCACATGACGTGTATTAATCTCC-3'
pZ7 CAPS-R	5'-CGTTGGTGGCAGCAGACTCGGGG-3'

94°C for 1 min and 95°C for 1 min, 5 cycles at 94°C for 1 min, 65°C for 1 min and 72°C for 3 min, 1 cycle at 94°C for 1 min, 30°C for 3 min and 72°C for 3 min, 15 cycles at 94°C for 0.5 min, 68°C for 1 min, 72°C for 3 min, 94°C for 0.5 min, 68°C for 1 min, 72°C for 3 min, 94°C for 0.5 min, 44°C for 1 min, and 72°C for 3 min, and 1 cycle at 72°C for 5 min. Subsequently, a second PCR was conducted by mixing 1 µl of the first PCR product diluted 50-fold, 3 µl of 100 µM random primer, 4 µl of 1 µM specific primer 2 (Table 2), 10 µl of Premix Taq Ex Taq Version 2.0, and 2 µl of sterilized water using the program as follows: 13 cycles at 94°C for 0.5 min, 68°C for 1 min, 72°C for 3 min, 94°C for 0.5 min, 68°C for 1 min, 72°C for 3 min, 94°C for 0.5 min, 44°C for 1 min, and 72°C for 3 min, and 1 cycle at 72°C for 5 min. A third PCR was conducted by mixing 1 µl of the second PCR product diluted tenfold, 3 µl of 100 µM random primer, 1 µl of 10 µM specific primer 3, 10 µl of Premix Taq Ex Taq Version 2.0, and 5 µl of sterilized water using the same program as the second PCR.

Statistical analyses

Statistics analysis was carried out using Microsoft Office Excel 2003 (Microsoft Corporation, USA).

Results

The relationships between beer protein Z4, protein Z7 and foam stability

The concentrations of beer proteins Z4 and Z7 were measured in 24 beer samples each brewed from a specific malt sample made from 10 barley cultivars (Table 1). The NIBEM value, beer protein Z4 and protein Z7 concentrations showed a wide range of variation between the beers. Variation in NIBEM value was observed between the beers from the same cultivars. This might be caused by the fact that many factors influencing foam stability such as malt Kolbach Index, beer protein Z4, protein Z7 and beer bitterness unit were different among the beer samples from same cultivars (Table 1). The relationships between NIBEM value and these protein contents were analyzed in Fig. 1. The correlation coefficients between NIBEM value and proteins Z4 and Z7 were significant at the 1% level. The coefficient between protein Z4 and NIBEM value was positive, while the relationship between protein Z7 and NIBEM value was negative. These results indicate that the level of protein Z4 in beer was a marker for improved foam stability, while protein Z7 was a negative marker for beer foam stability.

Genetic variation of proteins Z4 and Z7 contents in barley grain

The combined (free + bound) proteins Z4 and Z7 contents were measured in barley grain from 23 cultivars grown under the same management practices at Gumma, Japan, in 2000, 2004 and 2008 (Fig. 2). One-way analysis of variance for proteins Z4 and Z7 contents, using the three production years as replications, was used as an estimate for genotypic variation. This analysis showed that the genetic variation in grain proteins Z4 and Z7 contents between varieties was significant at the 1% level.

It was observed that the genotypic variation between cultivars for proteins Z4 and Z7 showed regional patterns for the cultivars assessed (Fig. 2). In the Japanese cultivars, protein Z4 content was lower in Haruna Nijo, Akagi Nijo, Golden Melon, Ryofu and Hokuiku39 (9.0–21.5 ng/µg protein) compared to Myogi Nijo, Satsuki Nijo and Hoshimasari (23.5–39.7 ng/µg protein). For the Australian cultivars, protein Z4 content was lower in Schooner, Clipper and Franklin (8.2–21.0 ng/µg protein) compared to Sloop SA and Prior (21.2–39.4 ng/µg protein). In European cultivars, protein Z4 content for Barke was slightly lower (10.4–22.1 ng/µg protein) than that in other cultivars (18.2–34.4 ng/µg protein). All the North American cultivars tested had relatively higher levels of barley grain protein Z4 (17.6–38.6 ng/µg protein). For protein Z7 content, the Japanese cultivars, Haruna Nijo, Ryofu, Hokuiku39, Myogi Nijo and Satsuki Nijo had higher levels (20.3–41.2 ng/µg protein). The North American cultivars, Harrington and CDC Copeland (13.2–22.0 ng/µg protein) showed relatively higher levels of protein Z7 content compared to the other North American cultivars. All of the Australian and European cultivars examined had

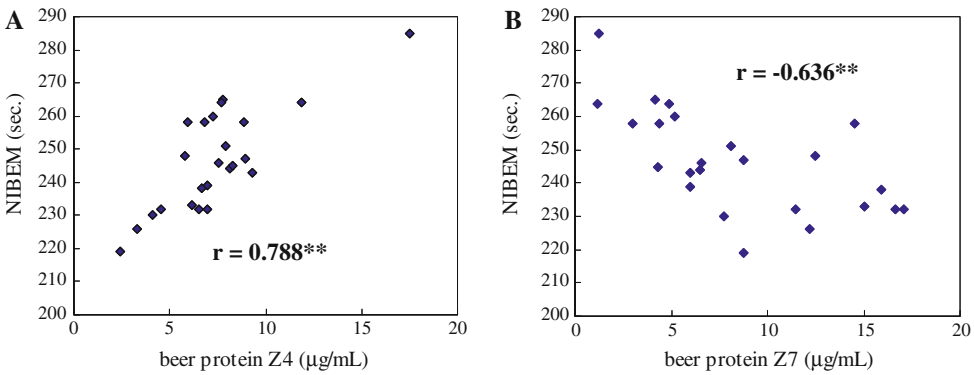

Fig. 1 The relationship between beer protein Z4, protein Z7 and foam stability ($n = 24$). **a** The relationship between beer protein Z4 and NIBEM, **b** the relationship between beer protein Z7 and NIBEM. $^{**}P < 0.01$

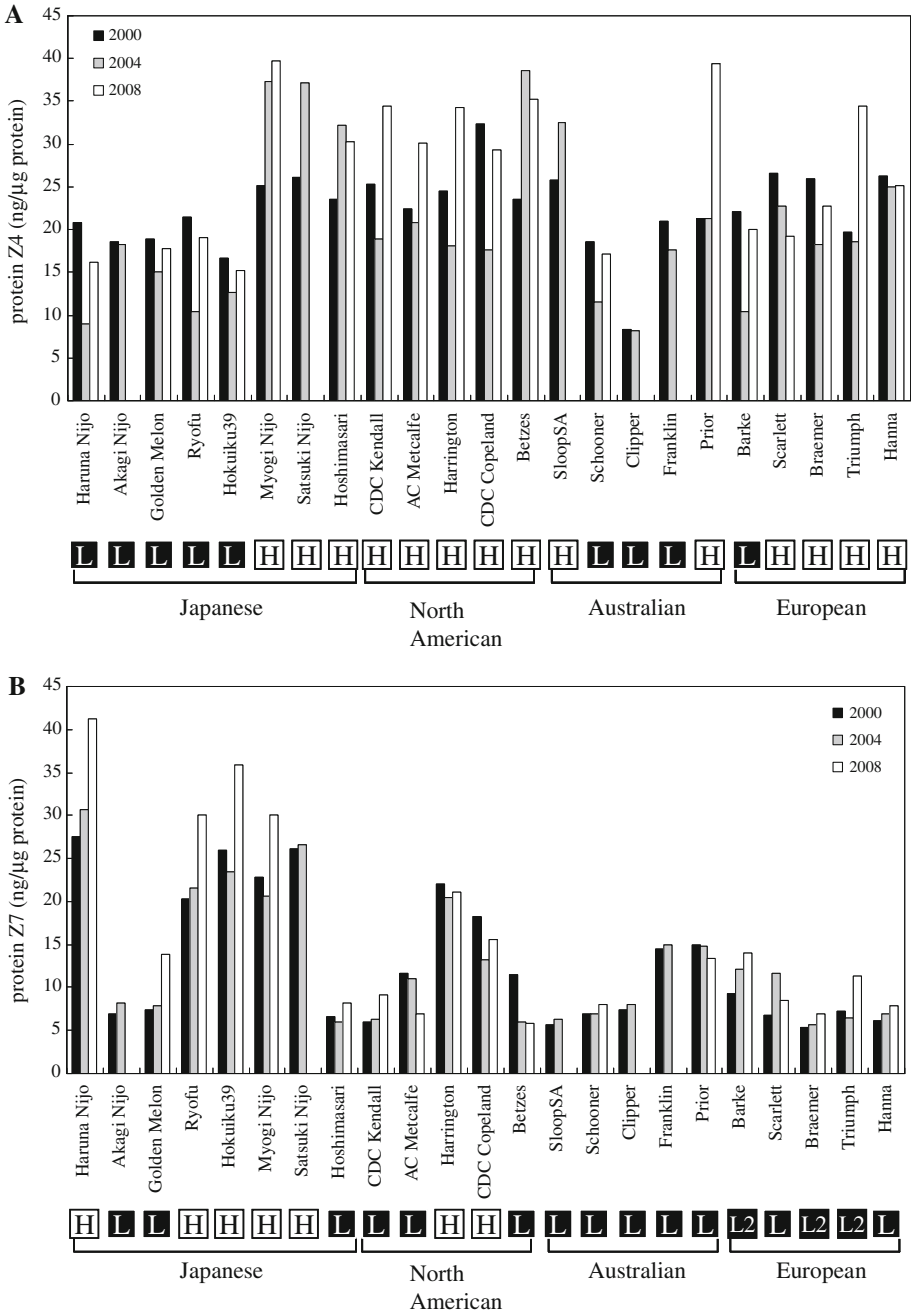

Fig. 2 Varietal variation of grain protein Z4 (**a**) and protein Z7 (**b**) contents in 23 malting barley cultivars. 2000, 2004 and 2008 indicate production years of the barley examined. Blank is not determined. **a** *L* and *H* indicate pZ4-L and pZ4-H in the genotype of protein Z4, respectively. **b** *L*, *L2* and *H* indicate pZ7-L, pZ7-L2 and pZ7-H in the genotype of protein Z7, respectively

comparatively lower levels in protein Z7 content (5.4–15.0 ng/μg protein). The levels of grain proteins Z4 and Z7 were not significantly correlated ($r = 0.071$), indicating that levels of protein Z4 and protein Z7 were independent. Consistent with this study, but for the combined fractions for malt, Evans et al. (1999b) also identified three apparent levels of protein Z4 (high, intermediate and low) and two apparent of levels of protein Z7 (high and low) between cultivars.

Construction of CAPS marker for barley grain protein Z4 content

Evans et al. (1999b) mapped quantitative trait loci for malt proteins Z4 and Z7 contents on the short arm of chromosome 4H and the long arm of chromosome 5H, respectively, where each structural gene was located. Based on these results, it was expected that single nucleotide polymorphisms (SNPs) could be identified in the proteins Z4 and Z7 structural genes or adjacent sequences that could then be tested as markers for barley grain proteins Z4 or Z7 contents. We focused on nucleotide sequences upstream of the translation initiation codon (ATG) for the development of CAPS markers for proteins Z4 and Z7 levels. For protein Z4, the 1,078 bp nucleotide sequence upstream from the translation initiation codon was available on the NCBI database (Genbank accession no. X51726). Based on this sequence, PCR primer sets (pZ4 Se-F and pZ4 Se-R) were designed (Table 2). Each of three barley cultivars with lower (Ryofu, Haruna Nijo and Barke) or higher (CDC Kendall, CDC Copeland and Harrington) protein Z4 content based on the results shown in Fig. 2 were selected to compare the nucleotide sequences of the PCR amplicons. The alignment of these nucleotide sequences revealed five nucleotide polymorphisms in the regions shown in Fig. 3a (M1, M2, M3, M4 and M5). Among these polymorphisms, four (M1, M2, M3 and M4) were considered efficient DNA markers to classify the six barley cultivars into two haplotypes, which corresponded to lower or higher protein Z4 contents in barley grain. Of these, M3 is contained within a restriction enzyme digestion site (*Acc*I: GTAGAC). The cultivars with lower grain protein Z4 content (pZ4-L), i.e., Ryofu, Haruna Nijo and Barke were digested by *Acc*I, but those with higher grain protein Z4 content (pZ4-H), i.e., CDC Kendall, CDC Copeland and Harrington were not digested by the enzyme.

Genomic DNA samples of the 23 cultivars shown in Fig. 2 were then used to amplify the upstream region for protein Z4 by primers pZ4 CAPS-F and pZ4 CAPS-R. The PCR products were digested by *Acc*I and applied to agarose gel electrophoresis (Fig. 4a). As a result, the cultivars tested were classified into pZ4-L ($n = 9$) and pZ4-H ($n = 14$). The mean values of the grain protein Z4 content by the digestion type were compared in each production year (Fig. 5a). In every production year, the average protein Z4 content in the pZ4-H haplotype was significantly higher than that in the pZ4-L. These results suggest that the CAPS marker M3 is effective for the estimation of protein Z4 content in barley grain.

Construction of CAPS marker for barley grain protein Z7 content

For protein Z7, only 29 bp of nucleotide sequence in the upstream region of the translation initiation codon was available in the NCBI database (Genbank accession no. X95277). An additional 307 bp nucleotide sequence was obtained by TAIL PCR using genomic DNA of CDC Kendall as a template. Based on this sequence, primer sets (pZ7 CAPS-F and pZ7 CAPS-R) to amplify the entire nucleotide sequence region were designed (Table 2). The cultivars with higher (Ryofu, Harrington and CDC Copeland) or lower (CDC Kendall, Akagi Nijo and Barke) grain protein Z7 content based on the results shown in Fig. 2 were selected for sequence comparison. The alignment of nucleotide sequences for their PCR amplicons (Fig. 3b) revealed 23 polymorphisms. Of these, 17 were specific to CDC Kendall and Akagi Nijo (Mka), 3 were specific to Barke (Mb), two were specific to CDC Kendall, Akagi Nijo and Barke (Mkab), and one was specific to Akagi Nijo and Barke (Mab), respectively. Among the polymorphisms found in the sequence, Mkab showed the highest capability for classification and divided six cultivars into two groups with higher (Ryofu, Harrington and CDC Copeland) and lower (CDC Kendall, Akagi Nijo and Barke) grain protein Z7. However, the Mkab markers could not be discriminated by the restriction enzymes tested in this study. Therefore, one Mka (Mka1) and one Mb (Mb1) were selected for the maker development. The PCR products containing the sequence shown in Fig. 3b were digested except for CDC Kendall and Akagi Nijo by *Bgl*II (Mka1), and Barke by *Hin*fI (Mb1). By using these marker combinations, six cultivars were classified into three haplotype groups, i.e., pZ7-H (Ryofu, Harrington and CDC Copeland), pZ7-L (Kendall and Akagi Nijo) and pZ7-L2 (Barke).

Genomic DNA samples of the 23 cultivars from Fig. 2 were amplified by the primers pZ7 CAPS-F and pZ7 CAPS-R. The PCR products were double digested by *Bgl*II and *Hin*fI, and applied to agarose gel electrophoresis (Fig. 4b). The cultivars tested were grouped into the pZ7-H ($n = 7$), pZ7-L ($n = 13$) and pZ7-L2 ($n = 3$). The mean values of the grain protein Z7 content by the digestion type were compared for each production year (Fig. 5b). In each production year, the average protein Z7 contents in the pZ7-L and pZ7-L2 were significantly lower than that for the pZ7-H. These results suggest that the CAPS markers,

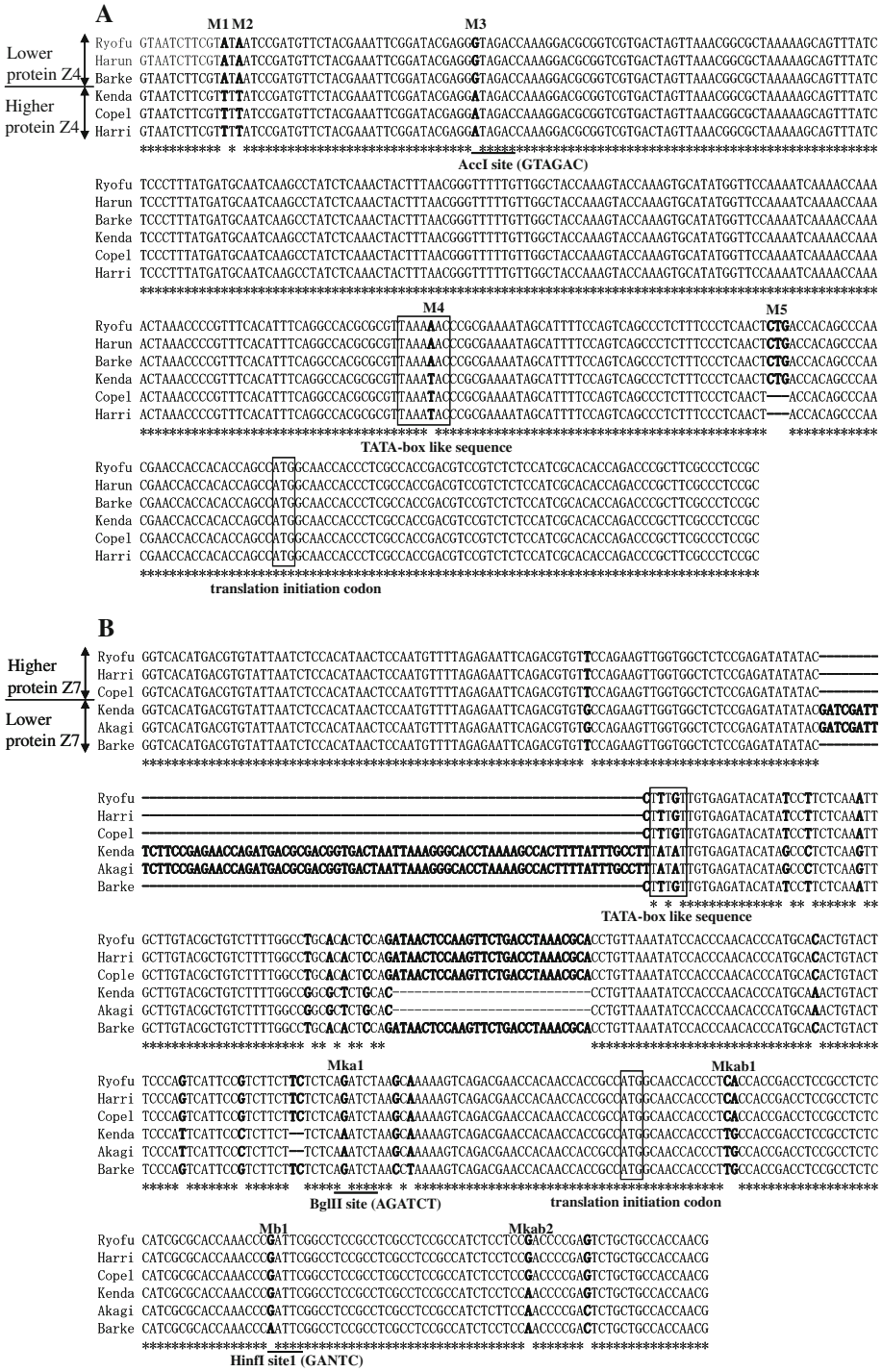

Fig. 3 Alignment of nucleotide sequences of the upstream region of the translation initiation codon for protein Z4 (**a**) and protein Z7 (**b**) for 6 cultivars with higher or lower protein Z4 and protein Z7 contents in barley grain

Mka1 and Mb1 are effective for the estimation of protein Z7 content in barley grain.

The relationships between genotypes of proteins Z4 and Z7, and beer foam stability

The malts from the ten cultivars (Table 1) used in the pilot brewing test were genotyped by the CAPS markers for proteins Z4 and Z7. These cultivars were classified into pZ4-L and pZ4-H for protein Z4, and pZ7-L and pZ7-H for protein Z7 according to the genotyping by the CAPS markers. The mean NIBEM values in each genotype were compared (Fig. 6). In these haplotype comparisons for proteins Z4 and Z7, the mean NIBEM value for the pZ4-H and pZ7-L was significantly higher than that in the pZ4-L and pZ7-H, respectively. These results suggest that the

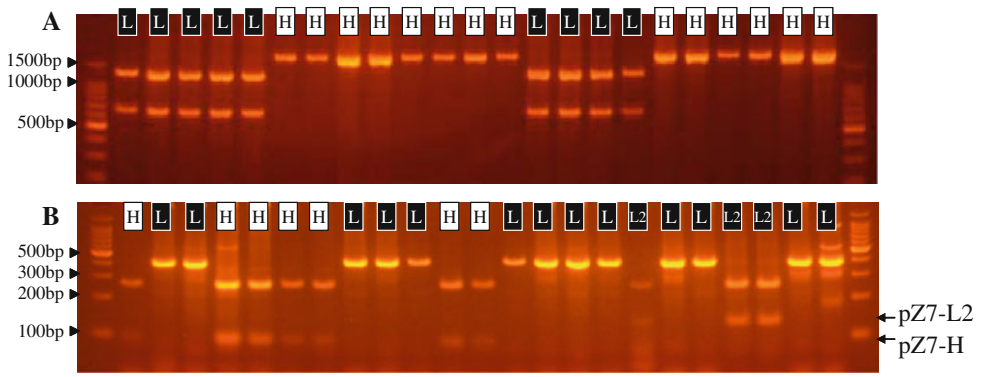

Fig. 4 The image of agarose gel electrophoresis of the PCR products treated with *Acc*I (for protein Z4) (**a**), and *Bgl*II and *Hin*fI (for protein Z7) (**b**). **a** *L* and *H* indicate pZ4-L and pZ4-H in the genotype of protein Z4, respectively. **b** *L*, *L2* and *H* indicate pZ7-L, pZ7-L2 and pZ7-H in the genotype of protein Z7, respectively. The molecular sizes of the amplified products of protein Z4 and protein Z7 were 1,994 and 401 bp for Ryofu, respectively. Digestion of the protein Z4 1,994 bp fragment by *Acc*I resulted in 1,317 and 677 bp restriction fragments for pZ4-L and 1,994 bp (not digested) for pZ4-H. Double digestion of the protein Z7 fragment by *Bgl*II and *Hin*fI resulted in 251, 91 and 59 bp restriction fragments for pZ7-H, 389 and 59 bp for pZ7-L and 251 and 150 bp for pZ7-L2

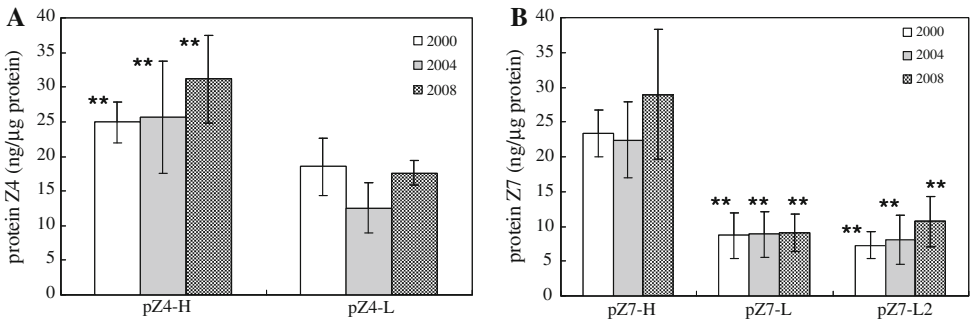

Fig. 5 Average protein Z4 (**a**) and protein Z7 (**b**) contents in protein Z4 genotypes of pZ4-L and pZ4-H, and protein Z7 genotypes of pZ7-H, pZ7-L and pZ7-L2. The 23 cultivars (the cultivar names shown in Fig. 2) grown under the recommended management practices in Gunma prefecture, Japan, in 2000, 2004 and 2008 were used for the analysis. $^{**}P < 0.01$ between pZ4-L and pZ4-H (**a**), pZ7-H and pZ7-L, and pZ7-H and pZ7-L2 (**b**) in each production year

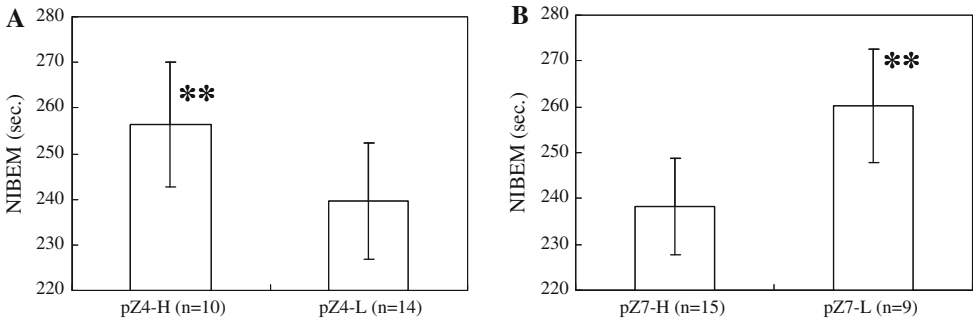

Fig. 6 Average NIBEM in pZ4-H and pZ4-L in protein Z4 (**a**), and pZ7-H and pZ7-L in protein Z7 (**b**) genotypes. The genotypes of protein Z4 and protein Z7 was determined for barley cultivars used in 24 beer samples shown in Table 1. Subsequently, The NIBEM values in each genotype were averaged and compared. $^{**}P < 0.01$

CAPS marker is valid for selecting varieties or breeding lines with potentially higher beer foam stability.

Proteins Z4 and Z7 haplotypes in worldwide malting barley varieties

To survey the potential level of beer foam stability for a selection of international malting barley varieties, 64 cultivars collected were classified into four groups based on the CAPS marker genotypes (Table 3). More than half of the Japanese cultivars were in Group IV (lower foam stability type), and only one was in Group I (higher foam stability type). Conversely, none of the North American cultivars surveyed were observed with the pZ4-L haplotype for protein Z4. Of the 12 cultivars, 5 were classified into Group III (medium foam stability type) in Australian cultivars. In contrast to the Japanese cultivars, more than half of the European cultivars were grouped into Group I, and

Table 3 Protein Z4 and protein Z7 genotypes in 64 malting barley cultivars

	Genotype			
Protein Z4 Protein Z7	pZ4-H pZ7-L or pZ7-L2 Group I	pZ4-H pZ7-H Group II	pZ4-L pZ7-L or pZ7-L2 Group III	pZ4-L pZ7-H Group IV
Japan	1	3	5	12
North America	5	6	0	0
Australia	2	3	5	2
Europe	13	0	6	1

only one cultivar was in Group IV. These results suggested that many Japanese cultivars have a disadvantage with respect to potential beer foam stability.

Discussion

As shown in Fig. 1a, beer foam stability (NIBEM) was significantly correlated with beer protein Z4 ($r = 0.788$, $R^2 = 0.621$). The result suggests that protein Z4 is a useful foam-positive marker. Evans et al. (1999a) also observed that the relationship between malt protein Z4 and beer foam stability was significant. The importance of protein Z4 with respect to foam stability was perhaps not surprising as Douma et al. (1997) and Maeda et al. (1991) suggested that a 40-kDa protein, or protein Z4, had the foam beneficial characteristics of having the highest surface viscosity and elasticity among beer proteins. On the other hand, Hollemans and Tonies (1989) had observed that the removal of the 40 kDa protein from beer resulted in only 10% reduction of foam stability indicating that factors other than protein Z were also important for foam stability. To confirm the relationship between foam stability and protein Z4 more directly, further investigations, such as estimating the effect of adding purified protein Z4 to beer, are necessary to confirm the "cause and effect" relationship between protein Z4 and improved beer foam stability.

The relationship between beer protein Z7 and foam stability was also significant but negative ($r = -0.636$, $R^2 = 0.405$) (Fig. 1b). Beer protein Z7 has not been well investigated except for Evans and Hajgaard (1999) and Evans et al. (1999a, b) and they indicated that no significant correlation was observed between foam stability and malt protein Z7. In their report, foam stability was determined by the Rudin method (Bishop et al. 1975) which was different from the NIBEM method used in this study. These methods place somewhat different emphasis on foam stability factors and give different results (as outlined by Evans et al. 2008). In this study protein Z7 was potentially a foam-negative marker (Fig. 1b), in contrast to Evans et al. (1999a) where the relationship with foam stability was not significant. The reason why protein Z4 and protein Z7 showed reverse effects on beer foam stability has not been resolved. This is despite proteins Z4 and Z7 having high sequence identity (72%, Dahl et al. 1996) in their amino acid sequences. Although these relationships between proteins Z4 and Z7 are practically useful for cultivar selection, they neither confirm nor deny a direct causal mechanism between these proteins and foam stability.

Evans and Hajgaard (1999) and Evans et al. (1999b) indicated that 80% of protein Z was of the protein Z4 isoform while 20% was protein Z7 on average. In this study, protein Z4 was also the dominant isoform constituting 63.7% of total protein Z on average (Fig. 2). However, the ratio of protein Z4 to protein Z7 was considerably different between cultivars. It was lower in Japanese cultivars such as Haruna Nijo (31.3%), Ryofu (40.9%) and Hokuiku39 (34.7%) than CDC Kendall (78.4%), SloopSA (82.9%) and Betzes (79.8%). The cultivars with lower ratio of protein Z4 to protein Z7 have the genotypes characterized as pZ4-L and pZ7-H, and the cultivars with a higher ratio have the genotypes of pZ4-H and pZ7-L. Based on these results, it is suggested that the ratio of protein Z4 to protein Z7 in barley grain was determined by the genotypes of proteins Z4 and Z7.

Figure 2 showed genetic variation in grain proteins Z4 and Z7 contents among 23 barley cultivars. Evans et al. (1999a, b) also revealed that proteins Z4 and Z7 contents in barley malt depended on barley cultivars, with three and two different groupings evident, respectively. Additionally, Evans et al. (1999b) identified the genomic regions associated with the malt proteins Z4 and Z7 contents, which located in or near structural genes of proteins Z4 and Z7, respectively, by QTL analysis. In this study, polymorphisms were found in the upstream sequence of the translation initiation codon for proteins Z4 and Z7. From these sequences, CAPS markers for grain proteins Z4 and Z7 contents were developed. It was assumed that the SNPs (M1, M2, M3 and M4) shown in Fig. 3a could be used to classify the level of protein Z4 content. Brandt et al. (1990) indicated a TATA box-like sequence 79 bp upstream from translation initiation codon for protein Z4. As shown in Fig. 3a, an SNP (M4) is positioned within the TATA box-like sequence, and might directly affect protein Z4 expression level during grain maturation. For protein Z7, a number of sequence polymorphisms were observed (Fig. 3b). Of these, Mka1 and Mb1 may be effective markers for the selection of barley cultivars with different levels of protein Z7. In the protein Z7 sequence shown in Fig. 3b, SNPs in the TATA box-like sequence specific to Kendall and Akagi Nijo (both with lower protein Z7 content) were observed 169 bp upstream from translation initiation codon. These SNPs might influence the expression

level of protein Z7. However, the TATA box-like sequence for Barke (lower protein Z7 content) was coincident with that for Ryofu and Harrington (both with higher protein Z7 content). The polymorphism(s), which alone control the protein Z7 content in Barke are still unknown, but this suggests that the mechanism to control protein Z7 content in Barke might be different from that in Kendall and Akagi Nijo.

Evans et al. (1999b) and Evans and Bamforth (2009) identified three categories of combined protein Z4 (low, intermediate and high) and two categories of combined protein Z7 (low and high) in barley malt. The high protein Z4 cultivars (pZ4-H), Alexis, Bonanza, Gairdner and Harrington, and low protein Z4 categories (pZ4-L) for Schooner, were coincident with the high and intermediate categories described by Evans et al. (1999a). In this study, low protein Z4 cultivars such as Pirkka (Europe), Morex and Karl (North America) were not assessed. Their classifications for protein Z7 were also coincident for Alexis, Harrington and Schooner. However, our CAPS marker identified Bonanza and Gairdner as pZ7-H, which was different from the classification by Evans et al. (1999b). This might have been because they categorized cultivars based on the malt protein Z7 content.

As shown in Fig. 6, the foam stability of the cultivars with the pZ4-H and pZ7-L was significantly higher than that with the pZ4-L and pZ7-H, respectively. The results demonstrate that the genotyping of proteins Z4 and Z7 by the CAPS markers was valid for selection of single plants and lines with higher foam stability in barley breeding. A number of reports suggested that beer proteins derived from barley malt, such as protein Z4 (Evans and Hajgaard 1999; Evans et al. 2003; Iimure et al. 2008; Maeda et al. 1991), LTP1 (Perrocheau et al. 2006; Sorensen et al. 1993; van Nierop et al. 2004) and BDAI-1 (Iimure et al. 2008; Okada et al. 2008) contribute to beer foam stability. However, an efficient DNA marker system for the selection of beer foam stability had not been developed until this study. This report therefore provides the first DNA marker assisted system for the selection of beer foam stability. By using the current marker system, the selection for beer foam stability can even be applied to F_2 populations.

These markers will however need further validation because, proteins Z4 and Z7 contents in barley grain may change under different environmental conditions (Evans et al. 1999b) and perhaps during malting. As shown in Fig. 2, the protein Z contents varied between growing years. However, the results shown in Figs. 2 and 5 also suggested that the genotypes of proteins Z4 and Z7 significantly influence these protein contents in the same environmental conditions. Therefore, both environmental and genetic effects are important for barley grain proteins Z4 and Z7 contents. To produce beer with higher foam stability, proteins Z4 and Z7 contents in grain and/or malt should be confirmed in terms of the relative levels of the protein Z forms (i.e., ELISA) in addition to the marker selection.

As shown in Table 3, more than half of the Japanese cultivars examined were classified into the pZ4-L and pZ7-H haplotypes. To develop cultivars with higher beer foam stability in Japanese breeding programs, parents with the pZ4-H and pZ7-L genotypes should be used for crossing. All 11 North American cultivars examined had the pZ4-H haplotype and 6 had the pZ7-H haplotype. Therefore, to improve beer foam stability, selection of the pZ7-L haplotype should be effective for North American breeding programs whereas the selection of pZ4-H would be a reasonable strategy in European breeding programs. Selection of both pZ4-H and pZ7-L genotypes maybe useful in Australian breeding programs. Importantly, the selection for specified haplotypes at proteins Z4 and Z7 will have the potential in improving beer foam stability early in barley breeding programs.

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