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ORIGINAL PAPER

Genetic variation of *Bmy1* alleles in barley (*Hordeum vulgare* L.) investigated by CAPS analysis

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Abstract The enzyme β-amylase is one of the most important hydrolytic enzymes in the grain of malting barley and is encoded by the gene *Bmy1*. To learn more about its structure and function, a total of 657 barley accessions including 541 *Hordeum vulgare* ssp. *vulgare* (HV), and 116 *H. vulgare* ssp. *spontaneum* (HS) were selected for the cleaved amplified polymorphic sequence (CAPS) analysis. These materials, covering all the 16 kinds of β-amylase phenotypes screened from more than 8,500 accessions of the world barley germplasm, were classified into 13 CAPS types in the present study. A combined assay of phenotypes and CAPS types revealed extensive genetic variation at the *Bmy1* locus, and in total 23 *Bmy1* allele types were identified. The newly identified alleles (A-I-11, A-II-6, A-II-7, A-II-10, B-I-3, B-I-12 and B-I-13) provided us with a novel resource for barley breeding and *Bmy1* study. In HV barley, six out of seven major allele types (C-II-1, B-II-2, B-Ia-3, A-II-5, A-II-6, and A-II-7) were shared with HS barley; the B-I-8 allele, which was predominant in north European cultivated barley, was found to be unique. Remarkably, very low *Bmy1* genetic variation was detected in Tibetan barleys, which puts the validity of the hypothesis that Tibet is one of the original centers of cultivated barley into question.

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Introduction

Barley is the major material for beer production. In the grain of malting barley, β-amylase (α-1,4-glucan maltohydrolase; EC 3.2.1.2) is one of the most important starch degrading enzymes. During malting, not only β-amylase activity but also its thermostability plays an important role, because β-amylase is more thermolabile than other component enzymes of "Diastatic Power". A close correlation has been revealed between thermostability and the apparent attenuation limit (AAL), which is a major malting quality parameter (Eglinton et al. 1998; Kihara et al. 1998, 1999). β-amylase in the endosperm of barley grain is encoded by *Bmy1* locus, which is located on the long arm of chromosome 4H (Nielsen et al. 1983; Kreis et al. 1988; Netsvetaev 1992). By thermostability analysis, the β-amylase is classified into three distinct types: A, B and C, possessing high, medium and low thermostability, respectively (Kihara et al. 1998, 1999). In addition to thermostability type, isoelectric focusing (IEF) pattern is another important property of β-amylase. By IEF analysis, two β-amylase

IEF patterns (Sd1 and Sd2) were found in HV barley (cultivated barley), while HS barley (the wild ancestor) contains an additional rare pattern Sd3 (Eglinton et al. 1998). It has been genetically confirmed that both thermostability types and IEF patterns of β-amylase were controlled by the *Bmy1* gene (Kaneko et al. 2000, 2001b; Ma et al. 2001; Li et al. 2002).

In our previous studies (Zhang et al. 2004a, b), polymorphisms of β-amylase were investigated by thermostability and IEF analysis in 8,270 accessions of HV barley, 174 accessions of HS barley and 43 accessions of HA (*Hordeum vulgare* ssp. *agriocrithon*) barley, collected from different regions of the world. In both cultivated barley and its wild relatives, three main β-amylase thermostability types (A high; B medium; C low) and three major IEF patterns (I, Ia, II) were observed. By combined analysis of the thermostability types and IEF patterns, 16 β-amylase phenotypes were identified in total, including A-I, A^{+}-II, A-Ia, A-II, A-IIa, B-I, B-Ia, B-II, A–B-IIb, A–B-IIc, A–B-III, C-I, C-Ia, C-II, C-IV and β-amylase-less (Zhang et al. 2004a, b). Our classification system of barley β-amylase is much more detailed and comprehensive than the previous one, which included only Sd1 (equal to B-I), Sd2H (A-II), Sd2L (C-II) and Sd3 types (Eglinton et al. 1998).

We have also reported β-amylase polymorphisms in 19 species (27 taxa, 337 accessions) of wild barley, including *H. bulbosum, H. murinum*, *H. marinum*, *H. brachyantherum*, *H. jubatum*, *H. chilense* and *H. roshevitzii*, etc (Zhang et al. 2004b). Our results also suggested that β-amylase IEF pattern together with thermostability type could be a good assistant marker in the phylogenetic analysis of *Hordeum* genus. Barley β-amylase has the following properties: first, it has relative high genetic diversity; secondly, both thermostability type and IEF pattern are determined by the single complex *Bmy1* locus, which makes it relatively easy for further genetic analysis; thirdly, β-amylase IEF pattern and thermostability type are not influenced by the growth environment and can be neutrally selected by so far breeding method except for several modern malting barleys; finally, mutations on *Bmy1* are independent from adaptation of barley, since even the β-amylase-less mutants could grow healthily (Takeda et al. 1998).

In recognition of the fact that β-amylase contributes to overall malting quality of barley, extensive efforts have been made on studies concerning β-amylase activity, thermostability, IEF pattern, gene and protein structure. The genomic DNA or cDNA sequences of *Bmy1* alleles from Haruna Nijo (Yoshigi et al. 1995), Adorra (Erkkilä et al. 1998), HA52 (Erkkilä and Ahokas 2001), Schooner (Ma et al. 2001) and Harrington (Li et al. 2002) have been reported. The factors that influence β-amylase thermostability type and IEF pattern were investigated (Yoshigi et al. 1995; Kaneko et al. 2000; Ma et al. 2001, 2002; Li et al. 2002). Useful PCR specific marker and CAPS marker have been developed and applied for evaluation of *Bmy1* alleles (Erkkilä 1999; Paris et al. 2002; Malysheva et al. 2004; Sjakste and Röder 2004). However, most of these studies were limited to Sd2H (A-II), Sd1 (B-I), Sd2L (C-II) and Sd3 types.

The present study is a part of our systematic research of barley β-amylase. Thermostability and IEF analysis of β-amylase that we have used previously have several advantages. For example, they are fast, relatively inexpensive and suitable for evaluating large number of germplasm. To learn more about structure and function of the *Bmy1* gene, in this study we applied CAPS analysis, which is a PCR based highly reliable method for the further examination The main feature of the method is based on the detection of the restriction site polymorphisms of the amplified fragment. The previous CAPS assay method (Paris et al. 2002), which is based on a *Msp*I restriction site polymorphism in exon IV region of the *Bmy1* gene, is not effective in distinguishing the *Bmy1* alleles with novel phenotypes that we reported, and significant structural alterations were observed in non-coding regions, especially intron III (Erkkilä et al. 1998; Sjakste and Röder 2004; Sjakste and Zhuk 2006). Accordingly, new CAPS markers were used and full length genomic DNA of the *Bmy1* gene containing seven exons and six introns was investigated in present assay.

The objectives were: to make a primary DNA structure comparison of different *Bmy1* alleles in both cultivated and wild barley; to verify our former β-amylase classification system; and to select typical materials for further DNA sequence and amino acid sequence analysis. In addition, since high genetic polymorphisms were detected in Tibetan wild barley (Zhang et al. 1994; Yin et al. 2003), Tibet was proposed to be one of the original centers of cultivated barley (Zhang et al. 1994). In the present study, we included 40 HS accessions from Tibet, and the genetic diversity of *Bmy1* in Tibetan wild barley was evaluated and reported here for the first time.

Materials and methods

Plant materials

In total 657 barley accessions were used in this study. Their origin, β-amylase thermostability type and IEF pattern are shown in Tables 1 and 2. In cultivated barley

Table 1 *H. vulgare* ssp. *vulgare* accessions used in this study

Thermostability type	A+	A			A–B		B			C				Bmy-less	Total
IEF pattern	II	Ia	II	IIa	IIb	IIc	I	Ia	II	I	Ia	II	IV		
Japan			46						2			1			49
Korean peninsula			45					1	2						48
China	2		121	2				3	2					2	132
Mongolia		1	9				1	3							14
Nepal and Bhutan			20					11						1	32
India and Pakistan			8			1		2							11
Afghanistan			15		1										16
Iran and Iraq			11					6				8			25
Former USSR (East European plain)[a]							1	3							4
Former USSR (Caucasus)[b]											2	3			5
Former USSR (Uzbekistan)									1						1
Turkey and Syria			3					9	6	4		8			30
Europe			23				21	10	9		1	17	1		82
North Africa[c]			27					4	2		2	4			39
Ethiopia			7					1				36			44
The Americas[d]			1				3	2	1		1	1			9
Total	2	1	336	2	1	1	26	55	25	4	6	78	1	3	541

[a] Russia and Lithuania

[b] Azerbaijan and Georgia

[c] Egypt, Algeria, Tunisia, Libya and Morocco

[d] USA, Canada and Guatemala

Table 2 *H. vulgare* ssp. *spontaneum* accessions used in this study

Thermostability type	A		A–B	B			C	Total
IEF pattern	I	II	III	I	Ia	II	II	
Afghanistan	7	6		1				14
Iran and Iraq		11	1	7				19
Morocco					1	2	3	6
Pakistan		1						1
Syria					1			1
Former USSR	5	4		8	7		3	27
China (Tibet)[a]		35			5			40
Turkey	1			1	1	1		4
Unknown				3	1			4
Total	13[b]	57	1[b]	20	16	3	6	116

[a] The serial number of the HS accessions that from Tibet: ZYM 0001, 0002, 0003, 0005, 0006, 0007, 0010, 0012, 0013, 0019, 0020, 0021, 0022, 0025, 0028, 0029, 0036, 0040, 0041, 0047, 0051, 0071, 0083, 0095, 0115, 0121, 0136, 0149, 0169, 0185, 0194, 0199, 0210, 0215, 0223, 0227, 0237, 0974, 0976, 0983

[b] β-amylase phenotypes that were not found in cultivated barley

(Table 1), most accessions were landraces. Among them, 274 accessions were the core collection of the Research Institute for Bioresources, Okayama University, which were selected by different collection regions and morphological characteristics. In wild barley, 116 HS accessions were included (Table 2), and they mainly originated from the former USSR, Iran, Iraq, Afghanistan and Tibet. The newly acquired 40 HS accessions that were from different areas of Tibet were provided by the Institute of Crop Germplasm Resources, Chinese Academic of Agricultural Sciences. Details on the materials are available upon request.

β-amylase phenotype analysis

The β-amylase thermostability types and IEF patterns of newly acquired Tibetan wild barley accessions were assayed and classified according to the methods described previously (Zhang et al. 2004a).

Genomic DNA isolation

Barley seeds were sown on Petri dishes and cultivated at 20°C in the dark for about 1 week. For each sample, an etiolated leaf (approximately 50 mg) of single seedling was used for DNA extraction by SDS-isopropanol method.

PCR amplification

PCR primers were designed according to the genomic DNA sequence of the *Bmy1* gene in Haruna Nijo (Genbank D49999). The forward primer b-amy-5-4(5′-ATCATCCATAGCCAGCATCCACAATGGAGG-3′) was from 23-bp upstream of the translational start codon ATG, the reverse primer b-amy-3-1(5′-AACCCGGGCTTTTCAGAACACACACAAGTG-3′) was from 91-bp downstream of the translational stop

codon TAA. In addition, one alternative reverse primer, b-amy-A9(5′-CAGAGAAAGGTTTCTCTG TCACACTCACAC-3′, from 35-bp downstream of the translational stop codon) was designed. The PCR fragment sizes amplified by these two primer pairs were 3,801 and 3,745 bp, respectively, and this small difference made no noticeable change in the following restriction assay. PCR was carried out in 50 μl reaction volumes containing one unit of *Ex Taq* polymerase (Takara Shuzo co., Japan), 5 μl of 10× *Ex Taq* buffer, 200 μM of dNTPs, 0.2 μM of each primer, and about 50 ng of template DNA. PCR was performed on a thermal cycler (GeneAmp PCR System 9700, ABI) with the following cycling parameters: one cycle of 3 min at 94°C; 30 cycles of 1 min at 94°C, 2 min at 60°C, 5 min at 68°C; and a final extension of 7 min at 68°C followed by a hold at 4°C. Then, 5 μl PCR product of each sample were electrophoresed in 1% agarose gels and visualized by staining with ethidium bromide.

Restriction analysis

By computational analysis of published *Bmy1* genomic DNA sequences and a preliminary digestion test in small samples, six restriction enzymes were selected, namely, *Dde*I (Toyobo Co., Osaka, Japan), *Hinf*I, *Hae*III, *Bgl*II, *Dra*I (Takara Shuzo Co., Shiga, Japan) and *Taq*I (Gibco, BRL, Japan). The reason for selecting these enzymes is that their digestion could at least unambiguously distinguish the major *Bmy1* alleles. Information of the restriction analysis based on *Bmy1* genomic DNA sequences from Haruna Nijo (Genbank D49999) and Adorra (Genbank AF061203) were shown in Table 3; restriction sites were located in both extrons and introns. The 20 μl reaction mixture contained 5 μl of PCR product, ten units of restriction enzyme, 2 μl of 10× buffer (supplied with each enzyme) and sterilized MilliQ water. These reactions were performed in an incubator over night at 37°C, except *Taq*I digestion, which was performed in a water bath over night at 65°C. After the digestion, cleaved fragments were detected in 3% NuSieve agarose gels (BioWhittaker Molecular Applications, Rockland, USA). The polymorphisms of band patterns were analyzed and CAPS types were classified. In this assay, faint bands (usually less than 100 bp) were not included.

Statistical analysis

Gene diversity (H) of *Bmy1* locus was evaluated using the gene diversity index (Nei 1973): $H = 1 - \sum p_i^2$, in which p_i is the frequency of the *i*th allele of the locus.

Results

β-amylase phenotypes in Tibetan wild barley

Among the 40 accessions of Tibetan HS, 35 accessions showed β-amylase thermostability type A and IEF

Table 3 Restriction analysis based on the genomic DNA sequence of *Bmy1* in Haruna Nijo (A-II) and Adorra (C-II)

Cultivar and genbank no.	Restriction enzyme and restriction site	Number of restriction site	Position of restriction site (bp)[a]
Haruna Nijo (D49999)	*Bgl*II (A/GATCT)	5	192, **651,** 1760, 2463, 2639
	*Dde*I (C/TNAG)	13	79, 196, **312**, **1159**, 1685, 1965, **2232**, **2315**, 2568, 2941, 3022, **3114**, **3157**
	*Dra*I (TTT/AAA)	3	673, 1723, 1731
	*Hae*III (GG/CC)	19	201, **342**, **397**, **471**, **1126**, 1293, 1690, **2226**, 2615, 2619, 2951, 2976, **3052**, **3128**, **3151**, **3427**, **3516**, **3549**, **3582**
	*Hinf*I (G/ANTC)	10	1013, 1354, 1657, 1769, 1956, 2510, **2797**, **3136**, **3175**, **3197**
	*Taq*I (T/CGA)	9	**295**, 830, 858, 920, **1105**, 2960, **3195**, **3244**, **3437**
Adorra (AF061203)	*Bgl*II (A/GATCT)	4	192, **651**, 2571, 2747
	*Dde*I (C/TNAG)	13	79, 196, **312**, **1153**, 1274, 1345, **2340**, **2423**, 2676, 3049, 3130, **3222**, **3265**
	*Dra*I (TTT/AAA)	4	673, 1639, 1829, 1837
	*Hae*III (GG/CC)	18	201, **342**, **397**, **471**, **1120**, 1413, **2334**, 2723, 2727, **3059**, 3084, 3160, **3236**, **3259**, **3535**, **3624**, **3657**, **3690**
	*Hinf*I (G/ANTC)	9	1007, 1474, 1660, 1774, 1875, 2618, **3244**, **3283**, **3305**
	*Taq*I (T/CGA)	9	**295**, 852, 914, **1099**, 1382, 3068, **3303**, **3352**, **3545**

Restriction sites that located in the exons were in blot font

[a] Analyzed lengths of *Bmy1* sequence in Haruna Nijo and Adorra were 3,658 and 3,766 bp, respectively. They were from the translational start codon ATG to stop codon TAA

pattern II, and the other five accessions showed thermostability type B and IEF pattern Ia (Table 2). Thus, A-II type was predominant in Tibetan HS barley.

PCR amplification

Among the 657 studied barley accessions, the *Bmy1* gene of 645 accessions was specifically amplified by PCR using the primer pair of b-amy-5-4 and b-amy-3-1, single bands were observed at about 3.8 Kb. The other seven accessions were only amplified using the primer pair of b-amy-5-4 and b-amy-A9. The remaining five accessions, including three β-amylase-less mutants and two B-I type accessions, failed to be amplified using either of the primer pairs in two independent experiments.

Cleaved fragment polymorphism

Cleaved fragment polymorphisms of PCR-amplified products digested by the six different restriction enzymes are shown in Fig. 1a–f, respectively. In summary, four to nine band patterns were identified when digested with different restriction enzymes: *Taq*I digestion showed four kinds of band patterns (Fig. 1f); *Bgl*II digestion detected five band patterns (Fig. 1a); both *Dde*I and *Dra*I digestions revealed seven band patterns

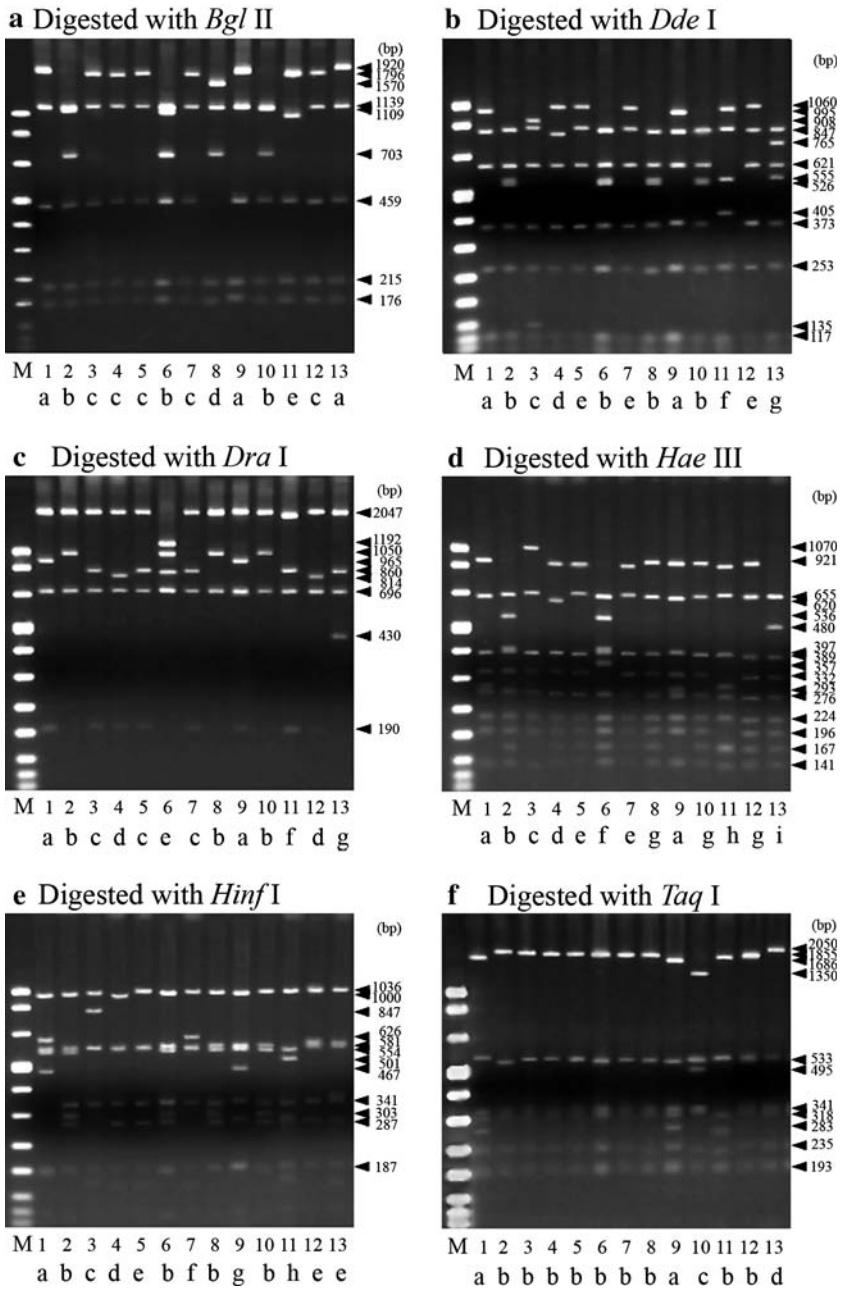

Fig. 1 Cleaved fragment polymorphisms of *Bmy1* alleles digested by six kinds of restriction enzymes, respectively. *1* Schooner (C-II); *2* Haruna Nijo (A-II); *3* Harrington (B-I); *4* OUU094 (C-Ia); *5* NI100 (B-Ia); *6* CAS178 (A-II); *7* OUT226 (C-I); *8* OUI021 (A-II); *9* Robust (B-II); *10* OUH609 (A-II); *11* OUH644 (A-I); *12* OUH602 (B-I); *13* OUH730 (B-I); *M* molecular marker VIII (Roche, Germany). *Lines 1–9* were HV accessions, and *10–13* were HS accessions. The letters from *a* to *i* denote the different band patterns. The indicated fragment sizes were inferred from *Bmy1* genomic DNA sequences of Haruna Nijo (Genbank D49999), HA52 (Genbank AJ301645) and Adorra (Genbank AF061203)

(Fig. 1b, c); *Hinf*I and *Hae*III digestion showed eight and nine band patterns (Fig. 1d, e), respectively. According to Fig. 1c (*Dra*I digestion), the sizes of total fragments of different patterns could easily be reckoned. The result revealed a large variation in gene length: the accession OU094 had the shortest length about 3,740 bp, and the accession OUH730 had the longest length about 4,000 bp, the difference was more than 200 bp. As shown in Fig. 1, all the band pattern polymorphisms could be represented by 13 barley accessions, which were Schooner (C-II type), Haruna Nijo (A-II), Harrington (B-I), OUU094 (C-Ia), NI100 (B-Ia), CAS178 (A-II), OUT226 (C-I), OUI021 (A-II), Robust (B-II), OUH609 (A-II), OUH644 (A-I), OUH602 (B-I) and OUH730 (B-I).

CAPS typing and classification of *Bmy1* alleles

According to the band pattern polymorphisms of these six restriction enzymes, CAPS typing result of all analyzed materials are summarized in Table 4, and totally 13 kinds of CAPS types were classified. Among them, CAPS types 4, 8 and 9 were observed in HV accessions only, and CAPS types 10–13 were found in HS accessions only. The other six CAPS types (1–3, 5–7) were common in both cultivated barley and wild barley.

Further, the integrated information of thermostability types, IEF patterns and CAPS types of examined barley accessions are shown in Tables 5 and 6. *Bmy1* alleles were classified based on these three characteristics, and frequencies of different alleles were computed. Of the HV accessions (Table 5), 16 *Bmy1* allele types were identified, and among them A-II-5, A-II-6, A-II-7, B-I-8, B-Ia, B-II-2, C-II-1 were seven major allele types. The other nine allele types (A-Ia-3, A-IIa-5, A^+-II-5, A–B-IIb-5, A–B-IIc-6, C-I-9, C-Ia-4, C-IV-1 and *Bmy1*-less) were rare and also unique in cultivated barley. Of the HS accessions (Table 6), 13 *Bmy1* allele types were detected, which were A-I-5, A-I-11, A-II-5, A-II-6, A-II-7, A-II-10, A–B-III-5, B-I-3, B-I-12, B-I-13, B-Ia-3, B-II-2, C-II-1. As denoted in Table 6, seven allele types (A-I-5, A-I-11, A-II-10, A–B-III-5, B-I-3, B-I-12 and B-I-13) were only detected in HS accessions. Noticeably, almost all the major allele types in cultivated barley were observed in wild barley except the B-I-8 allele. The *Bmy1* diversity indexes in HV and HS barley were 0.782 and 0.837, respectively. HS barley showed higher *Bmy1* diversity index than that of cultivated barley.

Of the 40 Tibetan HS accessions, 31 accessions had the A-II-5 allele, four had the A-II-6 allele and five had B-Ia-3 allele. In wild barley, the A-II-6 type allele was detected in Tibetan HS only.

Overall, 23 kinds of *Bmy1* alleles were revealed in *Hordeum vulgare* L., namely A-I-5 (OUH630, the representative accession), A-I-11 (OUH644), A-Ia-3 (Mo007), A-IIa-5 (CAS140), A^+-II-5 (CS188), A-II-5 (Haruna Nijo), A-II-6 (CAS178), A-II-7 (OUI021), A-II-10 (OUH609), A–B-IIb-5 (OUI429), A–B-IIc-6 (OUI462), A–B-III-5 (OUH760), B-I-3 (OUH696), B-I-8 (Harrington), B-I-12 (OUH602), B-I-13 (OUH730), B-Ia-3 (NI100), B-II-2 (Robust), C-I-9 (OUT226), C-Ia-4 (OUU094), C-II-1 (Schooner), C-IV-1 (OUU421) and *Bmy1*-less (OUC355). Importantly, seven allele types (A-I-11, A-II-6, A-II-7, A-II-10, B-I-3, B-I-12 and B-I-13) were newly identified by the present CAPS analysis.

Table 4 CAPS typing of *Bmy1* alleles based on the band pattern polymorphisms of six restriction enzymes in barley (*H. vulgare* L.)

CAPS type	*Bgl*II	*Dde*I	*Dra*I	*Hae*III	*Hinf*I	*Taq*I
1	a	a	a	a	a	a
2	a	a	a	a	g	a
3	c	e	c	e	e	b
4[a]	c	d	d	d	d	b
5	b	b	b	b	b	b
6	b	b	e	f	b	b
7	d	b	b	g	b	b
8[a]	c	c	c	c	c	b
9[a]	c	e	c	e	f	b
10[b]	b	b	b	g	b	c
11[b]	e	f	f	h	h	b
12[b]	c	e	d	g	e	b
13[b]	a	g	g	i	e	d

[a] CAPS types that were only found in HV accessions

[b] CAPS types that were only found in HS accessions

Morphological characteristics and geographical distribution of three subgroups of A-II type cultivated barley

Previously (Zhang et al. 2004a), we demonstrated that A-II, B-I, B-Ia, B-II and C-II were five major phenotypes covering more than 99% of the cultivated barley accessions, and A-II type accessions had the highest frequency at 52%. We reported the relationships between β-amylase phenotypes and morphological characteristics of barley, and confirmed a clear geographical differentiation of these five phenotypes. In the present CAPS analysis, 336 accessions of A-II type cultivated barley were divided into three subgroups: CAPS type 5, 6 and 7 (Table 5). Since A-II is the only phenotype that showed CAPS type polymorphism in cultivated barley, we also investigated the morphological characteristics and the geographical distribution of these three subgroups. Our result revealed that: the

Table 5 Classification of *Bmy1* alleles in HV barley based on thermostability types, IEF patterns and CAPS types

β-amylase phenotype	No.	CAPS type								
		1	2	3	4	5	6	7	8	9
A-Ia	1			1[a]						
A-IIa	2					2[a]				
A^{+}-II	2					2[a]				
A-II	336					178	146	12		
A–B-IIb	1					1[a]				
A–B-IIc	1						1[a]			
B-I	26 (1)								25[a]	
B-Ia	55			55						
B-II	25		25							
C-I	4									4[a]
C-Ia	6				6[a]					
C-II	78	78								
C-IV	1	1[a]								
β-amy-less	3 (3)									
Total	541(4)	79	25	56	6	183	147	12	25	4
Frequency		0.146	0.046	0.104	0.011	0.338	0.272	0.022	0.046	0.007

Number of accessions, which β-amylase gene could not be amplified by PCR using selected primer pairs, is given within parenthesis

[a] *Bmy1* allele types that were only found in HV accessions

Table 6 Classification of *Bmy1* alleles in HS barley based on thermostability types, IEF patterns and CAPS types

β-amylase phenotype	No.	CAPS type											
		1	2	3	4	5	6	7	8	9	10	11	12	13
A-I	13					3[a]						10[a]		
A-II	57					36	2	16			3[a]			
A–B-III	1					1[a]								
B-I	20 (1)			12[a]									6[a]	1[a]
B-Ia	16			16										
B-II	3		3											
C-II	6	6												
Total	116 (1)	6	3	28		40	2	16			3	10	6	1
Frequency		0.052	0.026	0.241		0.345	0.017	0.138			0.026	0.086	0.052	0.009

Number of accessions, which β-amylase gene could not be amplified by PCR using selected primer pairs, is given within parenthesis

[a] *Bmy1* allele types that were only found in HS accessions

A-II-5 allele was found in both two rowed (13%) and six rowed (87%), hulled (48%) and naked (52%), E (57%) and W (43%) type brittleness barley; the A-II-6 allele was mostly detected in six rowed (99%), E type brittleness (91%) and hulled (75%) barley; cultivated barleys carrying the A-II-7 allele were all six rowed, hulled and W type brittleness without exception; long rachilla hair barley accessions were overwhelming (more than 90%) in all these three subgroups.

The geographical distribution of these three *Bmy1* alleles is shown in Fig. 2. On the whole, A-II-5 and A-II-6 type alleles were widely distributed in the world, while A-II-7 type showed a limited distribution range.

In East Asia (Japan, Korean Peninsula and China) where about 90% accessions were of the A-II type (Zhang et al. 2004a), only the A-II-5 and A-II-6 type alleles were observed, and in general, frequency of these two allele types were almost equal in this region. However, a definite distribution difference was found within China. In southwestern China (Tibet and its vicinity), 100% of the A-II type accessions were of the A-II-5 allele. In contrast, 95% of the A-II type barley accessions from the middle and downstream drainage region of the Changjiang River (Hubei, Jiangxi and Jiangsu provinces) and the southeastern coastal zone of China (Zhejiang, Fujian, Guangdong and Guangxi provinces) were of the A-II-6 type allele. In the Yellow River drainage area (Gansu, Shanxi, Shannxi, Henan and Shandong province) and north eastern China, the frequencies of the A-II-5 and A-II-6 allele were almost equivalent.

The balance of A-II-5 and A-II-6 type alleles was also observed in the Korean Peninsula and northwestern Japan, and no clear difference was observed between North and South Korean barleys. In southwestern Japan, A-II-6 allele became more prevalent (Fig. 2), resembling that found in southeastern China. Outside of East Asia, barley accessions with the A-II-6

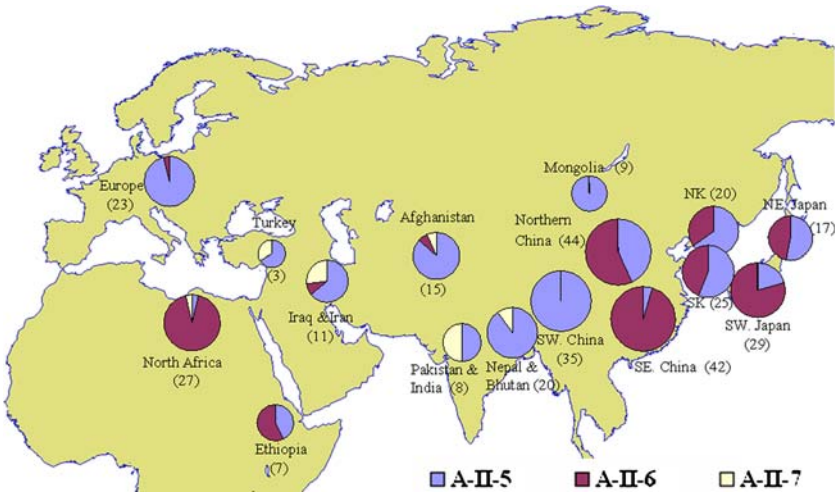

Fig. 2 Geographical distribution of A-II-5, A-II-6 and A-II-7 *Bmy1* allele types in cultivated barley. Values in *parenthesis* indicate number of accessions examined; *SE China* southeastern China; *SW China* southwestern China; *NK* northern Korean Peninsula; *SK* southern Korean Peninsula; *SW Japan* southwestern Japan; *NE Japan* northeastern Japan; North Africa: Egypt, Algeria, Tunisia, Libya, Morocco

type allele were seldom observed in Afghanistan, Iran and Europe. In North Africa and Ethiopia, the percentage of A-II-6 type allele was unexpectedly high, covering 93 and 57% of the analyzed local A-II type barleys, respectively.

Barley accessions with A-II-7 type allele were frequently detected in the Middle East, southern and central Asia, but were absent in East Asia and Europe. It also appeared at a low frequency in North Africa.

Discussion

Our previous works (Zhang et al. 2004a, b) confirmed that barley β-amylase possessed three main thermostability types (A, B and C) and three major IEF patterns (I, Ia and II). By combination of these three thermostability types and three IEF patterns, theoretically there could be nine kinds of possible β-amylase phenotypes (A-I, A-Ia, A-II, B-I, B-Ia, B-II, C-I, C-Ia and C-II). All these β-amylase types have been detected by our extensive investigations (Zhang et al. 2004a, b). The present study revealed that most of the *Bmy1* alleles of these nine β-amylase phenotypes could be effectively discriminated by CAPS analysis (Tables 5, 6). The different CAPS types of various alleles indicate clear differences in DNA structure. Thus, our CAPS data further confirmed our β-amylase classification system at molecular level.

As mentioned previously, A-II-5, A-II-6, A-II-7, B-I-8, B-Ia, B-II-2 and C-II-1 were seven major allele types in HV barley. All these allele types except the B-I-8 were observed in HS barley, and the genetic variation of *Bmy1* in HS barley was higher than that of cultivated barley. This result reconfirms our suggestion that the main differentiation of β-amylase preceded the domestication of barley (Zhang et al. 2004b), and indicates that cultivated barley originated from different wild barley ancestors. Although barley accessions with B-I type β-amylase were common in HS barley, their allele types (B-I-3, B-I-12 and B-I-13) differ from that of HV barley (B-I-8). The B-I type cultivated barley was mostly restricted to the former USSR and Europe (Zhang et al. 2004a), and was predominant in North Europe (Kaneko et al. 2002). Therefore, we propose that the B-I-8 type allele probably appeared after the domestication of barley, but further studies are needed.

To discriminate specific restriction sites detected in the present CAPS assay, published *Bmy*1 genomic DNA sequences of Adorra, Haruna Nijo and HA52 (Genbank AJ301645) were used as templates, comparisons and discussions were made accordingly.

As shown in Table 4 and Fig. 1, the C-II-1 and B-II-2 type alleles exhibited the same band patterns in five out of the six restrictions, and only the *Hinf* I digestion (Fig. 1e) could distinguish these two kinds of alleles (the C-II-1 allele with the band of 626 bp, but without the bands of 287 and 339 bp), implying their close relationship in evolution. Using the *Bmy1* genomic DNA sequence of Adorra as template, an additional restriction site was detected in B-II-2 allele by computational analysis. This specific site was at position 2905 of genomic DNA (exon V), and a T to C (GATTT to G/ATTC) nucleotide substitution at 1040 of cDNA could be inferred. This cDNA substitution could result in a L (leucine) to S (serine) amino acid substitution at position 347. Here, we propose that the L347S substitution was responsible for the significant higher thermostability of B-II type β-amylase than that of C-II type, since it could increase the enzyme's thermostability index T_{50} by 2.1°C (Ma et al. 2001).

Erkkilä et al. (1998) first reported a 126 bp insertion/deletion event (indel) in the 5′-proximal end of intron III.

And the 126 bp insertion was proposed to identify the *Bmy1*-Sd2L (C-II) allele, encoding a low-thermostability β-amylase (Erkkilä et al. 1998; Kaneko et al. 2000). In the present restriction assay, the 126 bp insertion is located between the first and second *Hinf*I restriction site (1,007 and 1474 bp, Table 3) of Adorra. These two restriction sites could generate a 467 bp fragment in digestion, while corresponding restriction sites in the *Bmy1* alleles that without the 126 bp insertion generated 341 bp fragments (Fig. 1e). Because the C-II-1, C-IV-1 and B-II-2 type alleles all exhibited the 467 bp fragment, this result clearly indicates that the 126 bp insertion in intron III exists not only in the C-II-1 (Sd2L) type allele but also in the C-IV-1 and the B-II-2 (middle thermostability) type alleles. On the other hand, the absence of the 126 bp indel was detected in alleles encoding various thermostability types (C, B, A–B, A and A^+) from lowest to highest. Since β-amylase thermostability is correlated with malting quality of barley (Eglinton et al. 1998; Kihara et al. 1998, 1999), we propose that the absence of 126 bp indel could not be an indicator of high malting quality. Malting quality data reported by Sjakste and Zhuk (2006) supports this proposal, as the absence of the 126 bp indel was observed in low, middle and high malting quality barley accessions.

Comparison results of the restriction band patterns among the A-II-5, A-II-6 and A-II-7 alleles, encoding high thermostability β-amylase (Fig. 1; Table 4), indicate a high similarity in their gene structure, and suggest a common ancestor of these three alleles. As shown in Fig. 1a (*Bgl*II digestion), the A-II-7 (OUI021) type allele had the unique 1,570 bp band but without the 459 bp band. By comparing with the genomic DNA sequence of Haruna Nijo, we found that a restriction site (A/GATCT) at 651 bp (exon II) was absent in the A-II-7 allele. To the best of our knowledge, this mutation was reported here for the first time. The relevant cDNA substitution was from position 436–441, and possibly brought an amino acid substitution at 146 or 147 residue. In addition, *Hae*III digestion of the A-II-7 allele revealed another absence of restriction site at 1,690 bp (intron III). Of the A-II-6 (CAS178) type allele, the unique 1,192 bp band generated by *Dra*I digestion (Fig. 1c) was caused by an additional restriction site at 2,923 bp (intron V). Because all three alleles encode high thermostability β-amylase, we propose that barley accessions possessing any of these three alleles are suitable materials for malting barley breeding.

Paris et al. (2002) reported a high-throughput CAPS assay, enabling broader discrimination between lower thermostability alleles (Sd2L and Sd1) and higher thermostability alleles (Sd2H and Sd3). This method was developed based on a *Msp*I restriction site polymorphism, caused by a single nucleotide polymorphism (SNP) at 698 of cDNA ($C^{698} \rightarrow T$). The presence of the C^{698} mutation was reported in all cases linked to a 6 + 1 bp deletion event in the 3′ region of intron III (Sjakste and Röder 2004; Malysheva et al. 2004). However, in our present study, which was based on extensive investigations of β-amylase phenotypes of world barley (Zhang et al. 2004a, b), two novel alleles A–B-IIb-5 (OUI429) and A–B-IIc-6 (OUI462) carrying the *Msp*I restriction site did not show high thermostability (data not shown). We suggest that, in rare cases, the linkage between genotype C^{698} and high thermostability could be interfered with by other nucleotide alteration in the *Bmy*1 gene.

Using the genomic DNA sequence of HA52 (B-I-8 type) as template, three specific restriction sites of *Dde*I, *Hae*III and *Hinf*I were revealed in the B-Ia-3 type allele, and they were located in intron III (absent, 1,303 bp), III (additional, 1,301 bp) and IV (additional, 2,487 bp), respectively.

In addition, Tables 4 and 5 also revealed that some of the *Bmy1* alleles with different thermostability types and/or IEF patterns were not distinguished by CAPS analysis using the six selected restriction enzymes. This result suggests a rather high structural similarity and close relationship among these alleles. Hereby, we speculate that the rare *Bmy1* allele C-IV-1 was a mutation of the C-II-1 allele; the A-Ia-3 type allele derived from the B-Ia-3 allele; the A–B-IIc-6 type allele was from the A-II-6 type allele; the A^+-II-5, A-IIa-5, A–B-IIb-5 and A–B-III-5 type alleles were derivatives of the major A-II-5 type allele. Further studies, such as full length sequencing of these alleles are absolutely necessary to reveal their structural variation. The barley accessions with novel β-amylase phenotypes are excellent materials for *Bmy1* study.

As we described previously, the length of the *Bmy1* gene changed largely in different type alleles. It is hard to make a reliable predication of specific restriction sites without a suitable template DNA. Therefore, the specific restriction sites of the rest novel alleles (such as OUH730) were not discussed in this paper. After the full length sequencing of different alleles, we will present a definite and detailed report in a later paper.

Ma et al. (2001, 2002) investigated the effects of barley β-amylase amino acid substitutions in positions 115, 165, 233, 347 and 430 by site-directed mutagenesis. They reported that an R (arginine) 115 C (cysteine) mutation was responsible for the difference in kinetic properties, and it was also responsible for the two distinct IEF band patterns, while A (alanine) 233 and

S347 were responsible for enhancing thermostability. In one of the latest reports about *Bmy1* gene (Malysheva and Röder 2006), five SNP sites corresponding to substitutions in the five amino acid positions were genotyped in 493 cultivated barley accessions, and a total of six different *Bmy1* haplotypes were identified, namely *Bmy1*-Sd1a, *Bmy1*-Sd1b, *Bmy1*-Sd2L, *Bmy1*-Sd2H, *Bmy1*-Sd3 and *Bmy1*-Sd4. Comparing these results with our classification of *Bmy1* alleles, it could be confirmed that the B-I-8, C-II-1, A-II-5 and B-II-2 correspond to the Sd1a, Sd2L, Sd2H and Sd4 alleles, respectively. Since our studies revealed a more complex β-amylase classification system, we speculate that some undetected β-amylase amino acid substitutions are responsible for the novel thermostability types (A^+ and A–B) or IEF patterns (Ia, IIa, IIb, IIc, III and IV). In another recent report (Sjakste and Zhuk 2006), data on polymorphisms of the intron III were largely extended, and high levels of inter- and intra-haplotype variability were demonstrated. Due to the high complexity of the of *Bmy*1 gene, we propose that more interesting findings could be expected.

The role of Tibetan barley in the origin and evolution of cultivated barley has been an important issue for a long time. A number of studies reported the high genetic variation in Tibetan barley (Xu 1982; Dai and Zhang 1989; Zhang and Dai 1991; Ma 2000). Zhang et al. (1992) examined the polymorphisms of ribosomal DNA in cultivated barley from Tibet, Ethiopia and 36 other countries. They reported a clear differentiation of two distinct barley groups: an Oriental group represented by the samples from China and Tibet; and an Occidental group, represented by the samples from Ethiopia and other countries. Thus, they proposed Tibet as the center of Oriental barley and Ethiopia as the center of Occidental barley, and this hypothesis was supported by several later reports (Zhang et al. 1994; Yin et al. 2003).

In the case of β-amylase analysis, nearly 90% of the accessions were of the A-II type in China and East Asia, while 97% of the Ethiopian accessions were of the C-II type (Zhang et al. 2004a). This result is consistent with the clear differentiation of Oriental and Occidental barley groups. However, the general differences between "Oriental barley" and "Occidental barley" are not direct evidence that the "Oriental barley" originated in Oriental regions. Our further analysis revealed that the A-II-6 allele, which was common in East Asian barley, was widely distributed in North African barley, and also existed in Ethiopian barley (Fig. 2). Barley accessions with A-II-5 or A-II-6 allele in these west regions mostly showed "Oriental barley" characteristics: long rachilla hair and E type rachis brittleness (Takahashi 1955, 1987). Based on the same Oriental barley characteristics and the high homologous DNA structure at *Bmy1* locus, we propose that Ethiopian and North African barleys carrying A-II-6 type allele have a significant relationship with East Asian barley, and they were not independently domesticated. In Fig. 2, a migration route of A-II-6 type barley could be inferred as most likely from North Africa via Iraq, Iran, and Afghanistan to East Asia, and unlikely through southern Asia and Tibet, because A-II-6 allele were absent in the cultivated barley in these regions. Taketa et al. (2004) examined the molecular variation of the marker sKT7 that tightly linked to the naked caryopsis gene (*nud*), revealed that naked domesticated accessions from different regions of the world have extremely homogeneous DNA sequences at the *sKT7* locus, supporting the monophyletic origin of naked barley. Ethiopian and Himalayan naked barleys had an identical *sKT7* sequence, excluding the possibility of their independent origins. Besides, geographical distribution of *sKT7* alleles showed that the haplotype of allele *sKT7 IV* in China, Korea, and Japan was different from that of Himalaya naked barley. They proposed that naked barley in China, Korea, and Japan was probably introduced from Afghanistan through the Silk Road, independently of the migration of naked barley into the Himalayan regions. Our results support their proposals. In addition, since the A-II-6 allele widely distributed in North Africa, and RFLF and chloroplast DNA microsatellite data supported Morocco as an origin center of barley (Molina-Cano et al. 1999, 2005). Therefore, we proposed that North Africa is a candidate of the origin of the A-II-6 type cultivated barley. The reason why we did not find the A-II-6 allele in HS barley in North Africa may be due to the very limited HS barley resources we have in this region.

The classical concept maintains that a center of origin of a crop is the region where the wild ancestor and the domesticated form co-exist (Bakhteyev 1964), and the center of origin should have the greatest genetic diversity of the crop (Harlan 1992). When evaluating Tibetan barley by *Bmy1* locus previously, we discovered that more than 99% of Tibetan cultivated barleys were of the A-II type β-amylase in more than one thousand cultivated barley accessions (Zhang et al. 2004a). Those barley material were collected from Tibet and its surrounding regions (Qinghai, Sichuan, and Yunnan provinces), which were the traditional activity area of Tibetan people. Most of the barley accessions were Tibetian "Qingke" barley (six rowed and naked). In the present study, 35 barley accessions from these regions were randomly selected for CAPS

analysis and revealed that they were all of the A-II-5 allele. This result suggests that genetic variation of *Bmy1* allele is very low in Tibetan cultivated barley. Although the very special β-amylase mutants with superior thermostability A^{+}-II type (CS188, Kaneko et al. 2001a) and *Bmy1*-less alleles (Kihara et al. 1999) were found in Tibetan barley, our CAPS data and sequence data (unpublished) indicate that they are mutations of A-II-5 allele, probably caused by the special environment and climate in Tibet.

In Tibetan HS barley, the A-II-5, A-II-6, and B-Ia-3 allele type were observed in the present study. Since, the A-II-5 allele type was predominant in Tibetan HV and HS barleys, and the A-II-6 type allele was only detected in Tibetan HS barley for the present, there is the possibility that cultivated barley with the A-II-5 or A-II-6 allele originated in Tibet and migrated to other regions. However, this possibility could be largely rejected for the following reasons: the A-II-5 and A-II-6 alleles were widely distributed in the world (Fig. 2), and culture history in the western regions (around 8,000 years B.C., Harlan and Zohary 1966) is much longer than that in Tibet (about 1,500 years B.C., Fu et al. 2000b). Archaeological evidence strongly supports that Tibetan ancient cultivated barley was introduced (Fu et al. 2000a). On the assumption that cultivated barley with A-II-6 allele was originated in Tibet, the A-II-6 allele should be readily observed in cultivated barley in Tibet and its surrounding regions, but this has not been seen (Fig. 2). Besides, cultivated barleys with B-Ia-3 or A-II-7 allele were pervasive in the regions to the west of Tibet, but no A-II-7 and B-Ia-3 allele were detected in Tibetan cultivated barley. As we know, in the high mountain Tibet area where barley is the staple food source for humans, naked barley is preferred to hulled one because it is easy to prepare. As described previously, all A-II-7 type accessions were hulled barley. Therefore, it is reasonable that cultivated barley with A-II-7 allele was not introduced into Tibet. For the same reason, the B-Ia-3 type allele was not observed in Tibetan cultivated barley (Zhang et al. 2004a). This fact suggests that there is a bottleneck effect on Tibetan barley origin. In addition, AFLP data of HV, HS, and HA barleys (Badr et al. 2000) also excluded the possibility that the Himalayan region was a center of domestication, and suggested that the Himalayas can be considered a center of diversification of cultivated barley. Taking into account all these facts, the hypothesis that Tibet is the center of origin for the cultivated barley in the Oriental region needs more discussion.

In conclusion, our present study revealed an extensive genetic variation in barley *Bmy1* locus, where at least 23 kinds of allele types exist. The newly identified alleles, especially those with high thermostability would provide us novel resources for malting barley breeding. Further study on the DNA sequence of these alleles and amino acid sequence of these various β-amylase isozymes would help us to understand more about the mechanism of β-amylase thermostability and gain more information about the phylogenic relationship of world barley.

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