



Protocols

Characterization of β -Amylase Alleles in 79 Barley Varieties With Pyrosequencing

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Abstract. β -Amylase is involved in the starch degradation process and therefore influences grain quality. Starch degradation efficiency is dependent on the enzyme thermostability during malting and mashing. Four alleles resulting in different enzyme thermostability are known. These alleles are distinguished by coding single nucleotide polymorphism (cSNP). Pyrosequencing was used for cSNP genotyping of β -amylase alleles in 79 spring barley varieties by using analyser PSQ MA96 System (Pyrosequencing, Biotage). A new cSNP was revealed by means of Pyrosequencing analysis of sequence flanking cSNP⁶⁹⁸, thus recognizing a fifth β -amylase allele. Pyrosequencing is a high-throughput, fast, and precise system for barley SNP genotyping.

Key words: barley, β -amylase, coding single nucleotide polymorphism, genetic resources, Pyrosequencing

Abbreviations: coding single nucleotide polymorphism, cSNP.

Introduction

Grain quality is an important factor in barley seed production. Characterising the grain qualitative parameters of barley genetic resources is important so that donors can be identified to improve traits required in malt production; for alternative grain end-use in feeding, paper, and textile industries (Petersen and Munck, 1993); and for human dietary programmes. Breeders traditionally characterise cultivars and breeding lines at the biochemical and phenotypic levels. These analyses are expensive and labour intensive, making it necessary to develop and use molecular markers for grain quality genes and to choose the most suitable screening method in terms of time and cost effectiveness. In this study, we used Pyrosequencing to characterize cSNP variation in the β -amylase gene *Bmy1* among 79 barley varieties.

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β -Amylase belongs to a group of diastatic enzymes (α -amylases, limit dextrinase, and α -glucosidase (Fincher and Stone, 1993). The combined activities of these enzymes are evaluated as diastatic power (DP), which is a direct measure of the total starch-degrading activity of barley malt. β -Amylase activity is highly correlated with DP (Evans et al., 1995) but is more thermolabile than other diastatic enzymes and therefore more prone to loss of activity during malting. Four allelic forms of β -amylase exhibiting different thermostability levels have been described (*Bmy1*-Sd1, *Bmy1*-Sd2L, *Bmy1*-Sd2H, and *Bmy1*-Sd3; Eglinton et al., 1998). The *Bmy1* locus is located on the long arm of chromosome 4H and contains 3 copies of the gene. The gene is expressed in mature grain and determines amylase activity (Erkkila et al., 1998), enzyme thermostability (Eglinton et al., 1998), isoenzyme form, and free-to-bound enzyme ratio. Paris et al. (2002) reported 2 cSNPs distinguishing *Bmy1* allelic forms. A second β -amylase locus (*Bmy2*), containing a single copy of the gene, has been mapped to chromosome 2H. The 2H-encoded enzyme is expressed in leaf and root tissues (Kreis et al., 1988; Sharp et al., 1988).

We used Pyrosequencing to study allelic variation at the *Bmy1* locus in 79 spring barley varieties. In addition to identifying the previously known alleles, we discovered a new cSNP at position 702 in the cDNA sequence during analysis of the cSNP⁶⁹⁸. Because Pyrosequencing is based on short fragment sequencing (≤ 20 bp), both SNPs were identified in a single analysis.

Material and Methods

Plant material

The collection of spring barley varieties derived from 15 countries was provided from genetic resources held by the Agricultural Research Institute Kromeriz, Ltd. (Table 1).

DNA extraction

Barley seeds were planted in a glasshouse, and genomic DNA was extracted from 14-day-old bulked leaves from 30 individual plants of each cultivar. DNA was extracted by using the CTAB protocol of Saghai-Marooif et al. (1984). DNA concentrations were estimated spectrophotometrically, and DNA quality was verified after electrophoretic separation on 0.8% agarose gel and visualisation by means of ethidium bromide staining under UV light. DNA was diluted to final concentrations of 100 ng/ μ L.

PCR amplification

The barley cDNA sequence (EMBL: D49999) was used as a standard reference. Two primer pairs were designed to amplify 2 fragments containing previously reported SNP variants (Table 2). Because these occur within coding regions of the gene, they are referred to as cSNPs. The primers were designed to be specific for fragments containing cSNPs G⁴⁹⁵→C or C⁶⁹⁸→T and had no homology to other parts of the barley genome. One primer of each pair was end-labelled by biotinylation and HPLC purified (Thermohybid). The sequences of the primers used

were forward 5'-BIO-gCTgTgACAgATgTATgCCgAT-3' and reverse 5'-TCC-gTggCTCTgAggATATgAT-3' to amplify a 145-bp fragment containing cSNP⁴⁹⁵ and forward 5'-gCCATCCTgAgTgggAATTTC-3' and reverse 5'-BIO CCC-gTTgTCCCTgAAgAATTg-3' to amplify an 83-bp fragment containing cSNP⁶⁹⁸. Forward and reverse primers did not form primer-dimers or hairpins.

PCR amplifications were performed in 50- μ L reaction volumes containing 1 \times buffer with 15 mM MgCl₂ (Boehringer), 200 μ M of each dNTP (Invitrogene), 0.2 μ M of each primer, 1 U of *Taq* DNA polymerase (Boehringer), and 100 ng of template DNA. PCR amplifications were carried out for 2 min at 94°C, followed by 45 cycles of 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C, with a final 5-min incubation at 72°C, using an MJ Research Thermocycler. Conditions were the same for both PCR primer pairs. PCR amplification quality was checked by means of electrophoresis on 2% agarose gel after staining with ethidium bromide.

Pyrosequencing

Designing the sequencing primers was necessary to avoid self-annealing and primer-dimer formation with the biotinylated PCR primer. The position of the sequencing primer is flexible within 5 bases of the SNP. Interpretation of the SNP can be difficult if one or both of the polymorphic bases form a homopolymer with adjacent bases. The melting temperature of the sequencing primers should be approximately 50°C.

The sequencing primers for cSNP⁴⁹⁵ and cSNP⁶⁹⁸ detection were reverse 5'-CACCAGCATCCAAgAA-3' and forward 5'-TgggAATTTCTAACgA-3', respectively. PCR products were the templates for Pyrosequencing reaction preparation, which used a Vacuum Prep Tool (Pyrosequencing, Biotage) following the manufacturer's instructions. Samples were analysed by using a Pyrosequencing analyser PSQ MA96 (Pyrosequencing, Biotage), and data were evaluated by using the manufacturer's software.

Results

β -Amylase alleles were characterized in the collection of 79 spring barleys (Table 1). We used Pyrosequencing to unambiguously genotype the cSNPs, distinguishing the 4 previously described *Bmy1* allelic forms (Table 2; Paris et al., 2002). The sequence of the short fragment containing cSNP⁶⁹⁸ also revealed a previously unknown G \rightarrow A SNP at position 702 that enabled the Sd1 allele to be further divided into a G/T/A haplotype (as in Milonov Nudum; Table 1) and a more common G/T/G haplotype, named allele Sd1b. The new cSNP⁷⁰² did not result in amino acid substitution in the protein, as both codons (GGA and GGG) encoded a glycine residue. Therefore, it is unlikely that alleles Sd1 and Sd1b differ in thermostability. The position of cSNP⁷⁰² 3 bases upstream of cSNP⁶⁹⁸ enabled us to multiplex the pyrosequence analysis (Figure 1).

Overall, 28 varieties (35%) had the G/T/G Sd1b haplotype, 26 (33%) had the C/T/A Sd2L haplotype, and 12 (15%) had the C/C/A Sd2H haplotype. One variety had the G/T/A Sd1 haplotype, and 11 (14%) had different alleles at one or two SNP positions, suggesting that these varieties are heterogeneous and carry more than one allele in their respective populations.

Table 1. The evaluation of the β -amylase thermostability test using the cSNP molecular markers.

Cultivar	Country ^a	Genotype cSNP ⁴⁹⁵ (G/C)	Genotype cSNP ⁶⁹⁸ (C/T)	Genotype cSNP ⁷⁰² (G/A) ^b	Allele	Thermostability
Merlin	CAN	G	T	G	Sd1b ^c	intermediate
No. 94609D7	DEU	G	T	G	Sd1b	intermediate
Ohara	AUT	G	T	G	Sd1b	intermediate
Selecta	AUT	G	T	G	Sd1b	intermediate
Margit	SWE	G	T	G	Sd1b	intermediate
M1070	USA	G	T	G	Sd1b	intermediate
M955	USA	G	T	G	Sd1b	intermediate
M635	USA	G	T	G	Sd1b	intermediate
M422	USA	G	T	G	Sd1b	intermediate
Harrington	CAN	G	T	G	Sd1b	intermediate
Olbram	CZE	G	T	G	Sd1b	intermediate
Buck	CAN	G	T	G	Sd1b	intermediate
HB803	CAN	G	T	G	Sd1b	intermediate
Ebsdorfer nackt	DEU	G	T	G	Sd1b	intermediate
Kavkazkyj Golozernyj	SUN	G	T	G	Sd1b	intermediate
KM2092	CZE	G	T	G	Sd1b	intermediate
KM2084	CZE	G	T	G	Sd1b	intermediate
KM2082/2	CZE	G	T	G	Sd1b	intermediate
Sabel	GBR	G	T	G	Sd1b	intermediate
Annabel	DEU	G	T	G	Sd1b	intermediate
Jersey	NLD	G	T	G	Sd1b	intermediate
Madonna	DEU	G	T	G	Sd1b	intermediate
Orbit	CSK	G	T	G	Sd1b	intermediate
Thuringia	DEU	G	T	G	Sd1b	intermediate
Barke	DEU	G	T	G	Sd1b	intermediate
Krona	DEU	G	T	G	Sd1b	intermediate
Scarlett	DEU	G	T	G	Sd1b	intermediate
Tolar	CZE	G	T	G	Sd1b	intermediate
CDC Candle	CAN	C	T	A	Sd2L	low
Primus	CZE	C	T	A	Sd2L	low
Jarek	CSK	C	T	A	Sd2L	low
Lyallpur3647	IND	C	T	A	Sd2L	low
Nudum7566	MNG	C	T	A	Sd2L	low
H2186	ETH	C	T	A	Sd2L	low
H2176	ETH	C	T	A	Sd2L	low
CI12953	ETH	C	T	A	Sd2L	low
Forum	CSK	C	T	A	Sd2L	low
Gopal	IND	C	T	A	Sd2L	low
Wabet	USA	C	T	A	Sd2L	low
Washonubet	USA	C	T	A	Sd2L	low
Wanupana	USA	C	T	A	Sd2L	low
Wapana	USA	C	T	A	Sd2L	low
Wanubet	USA	C	T	A	Sd2L	low
KM2001	CZE	C	T	A	Sd2L	low
KM2087	CZE	C	T	A	Sd2L	low
KM1057	CZE	C	T	A	Sd2L	low

Table 1 (concluded).

Cultivar	Country ^a	Genotype cSNP ⁴⁹⁵ (G/C)	Genotype cSNP ⁶⁹⁸ (C/T)	Genotype cSNP ⁷⁰² (G/A) ^b	Allele	Thermostability
Maridol	CZE	C	T	A	Sd2L	low
H2173	ETH	C	T	A	Sd2L	low
H2193	ETH	C	T	A	Sd2L	low
KM2283	CZE	C	T	A	Sd2L	low
Nordus	DEU	C	T	A	Sd2L	low
Pax	SVK	C	T	A	Sd2L	low
Pejas	CZE	C	T	A	Sd2L	low
Kompakt	SVK	C	T	A	Sd2L	low
Shimabara	PRK	C	C	A	Sd2H	high
Chiro Chinko	JPN	C	C	A	Sd2H	high
Nabavi	JPN	C	C	A	Sd2H	high
Ai Gan Qi	CHN	C	C	A	Sd2H	high
Milonov	CSK	G	T	A	Sd1	intermediate
Nudum						
Taiga	DEU	G/C	T	G/A	Sd1b/Sd2L	low
KM2082/1	CZE	G/C	T	G/A	Sd1b/Sd2L	low
KM2045	CZE	G/C	T	G/A	Sd1b/Sd2L	low
KM2037	CZE	G/C	T	G/A	Sd1b/Sd2L	low
KM2083	CZE	G/C	T	G/A	Sd1b/Sd2L	low
KM1771	CZE	G/C	T	G/A	Sd1b/Sd2L	low
KM2062	CZE	G/C	T	G/A	Sd1b/Sd2L	low
KM1910	CZE	G/C	T	G/A	Sd1b/Sd2L	low
Amulet	CSK	G/C	T	G/A	Sd1b/Sd2L	low
Galan	CSK	G/C	T	G/A	Sd1b/Sd2L	low
MK1332	MNG	C	C/T	A	Sd2H/Sd2L	intermediate?
Donors of thermostabile β-amylase						
Amagi Nijo	JPN	C	C	A	Sd2H	high
Haruna Nijo	JPN	C	C	A	Sd2H	high
Hoshimasari	JPN	C	C	A	Sd2H	high
Misato	JPN	C	C	A	Sd2H	high
Golden						
Masan	JPN	C	C	A	Sd2H	high
Naked1						
Dangomugi	JPN	C	C	A	Sd2H	high
Mochimugi	JPN	C	C	A	Sd2H	high
Sumire Mochi	JPN	C	C	A	Sd2H	high
Azuma Golden	JPN	C	T	A	Sd2L	low

^aCountry of origin or country from where cultivars have been obtained. AUT=Austria, CAN=Canada, CHN=China, CZE=Czech Republic, CSK=Former Czechoslovakia, DEU=Germany, ETH=Ethiopia, GBR=United Kingdom, IND=India, JPN=Japan, MNG=Mongolia, NLD=Netherlands, PRK=Korea (North), SVK=Slovakia, SWE=Sweden, SUN=Former Soviet Union.

^bNewly revealed cSNP.

^cNewly defined allele.

Nine Japanese cultivars were included in the collection as known donors of a thermostabile β -amylase. We confirmed that 8 Japanese cultivars carry the high-thermostabile allele (Sd2H), while Azuma Golden carries the Sd2L allele (Table 1). Four Asian cultivars (Shimabara, Chiro Chinko, Nabavi, and Ai Gan

Table 2. The cSNPs distinguishing the 4 allelic forms of *Bmy1* identified by Paris et al. (2002).

cSNP ⁴⁹⁵	cSNP ⁶⁹⁸	Allele	Thermostability
C	T	Sd2L	Low
G	T	Sd1	Intermediate
C	C	Sd2H	High
G	C	Sd3	High

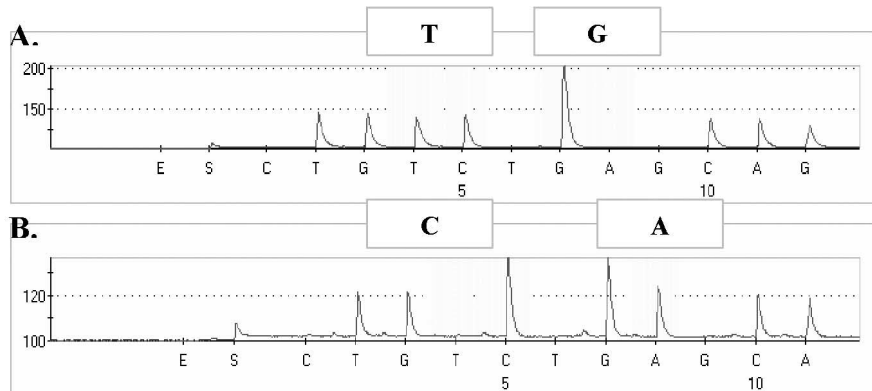


Figure 1. Example of multiplex pyrosequence analysis of cSNP⁶⁹⁸ (T/C) and cSNP⁷⁰² (G/A). The sequence to analyse was TGT/CCGGG/ACAGT (forward strand). Sample A has the sequence analysed TGTCGGGACAG. Sample B has the sequence analysed TGCCGGACAG.

Qi) also carry the Sd2H allele. Mongolian line MK1332 is heterogeneous and carries alleles Sd2L and Sd2H. The Czech good malting cultivar, Olbram, and poor malting cultivar, Tolar, have the Sd1b allele, while the good malting cultivar, Kompakt, has the Sd2L allele.

Discussion

β -Amylase thermostability is an important trait affecting barley grain quality and fermentation ability in the brewing process (Eglinton et al., 1998). Thermal inactivation of β -amylase at temperatures higher than 55°C is a serious problem in the brewing procedure; therefore, identifying barley genotypes that carry superior alleles will help to select improved cultivars in breeding programs focusing on higher malting quality. Barleys carrying low-thermostabile alleles might be used to develop new varieties suitable for alternative end uses (Paris et al., 2002).

On the basis of isoelectric focusing (IEF) analysis (Kihara et al., 1998), the A and C type cultivars carried the Sd2 forms of β -amylase, whereas B type cultivars were divided into 2 subtypes, B1 (Sd1) and B2 (Sd2). A and C types are equivalent to the Sd2H and Sd2L forms, respectively. Ma et al. (2002) presented a biochemical analysis based on IEF, which distinguishes Sd1 and Sd2 alleles. The authors suggested that this could be used as a biochemical marker to predict

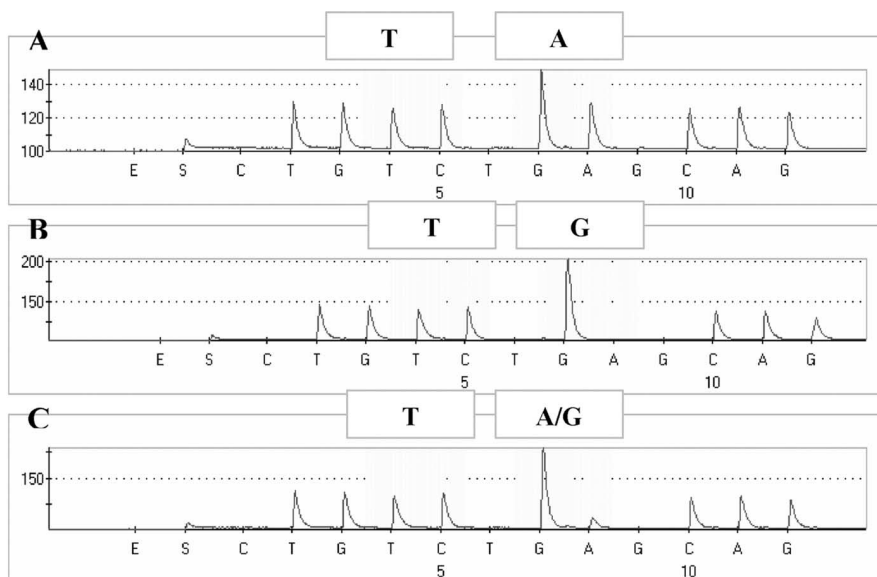


Figure 2. Example of cSNP analysis in a homopolymer region. The sequence to analyse was TGT/CCGGG/ACAGT (forward strand). The cSNP⁷⁰² G/A lies in a homopolymer region (in bold). Pyrosequencing precisely quantifies the number of G, with 2 G in the case of homozygote A and heterozygote G/A (samples A and C) and 3 G in the case of homozygote G (sample B).

malting quality in barley breeding. Kaneko et al. (2000) described an STS marker that distinguishes type A cultivars from most of the type B and type C cultivars except from some nonmalting barleys.

Paris et al. (2002) identified 2 cSNPs in *Bmy1*. Because these resulted in amino acid substitutions, this combined to give 4 β -amylase allelic forms (Sd2L, EMBL X52321; Sd1, Genbank AF300800; Sd3, Tr EMBL P82993; and Sd2H, GenBank D21349) that were directly linked to β -amylase thermostability (Paris et al., 2002). Such molecular markers are highly valuable for breeding because the recombination between the marker and the gene is eliminated.

These previously defined cSNP variants were used in this study to characterise a collection of spring barleys. Pyrosequencing is based on short fragment sequencing containing the target SNP, and this proved highly effective for classifying β -amylase alleles. The analysis system, including appropriate software, allowed rapid screening, with 96 samples and controls being analysed within 10 min. Reaction preparation of 96 samples using the Vacuum Prep Tool took a maximum of 30 min. Pyrosequencing allowed precise identification of homozygotes and heterozygotes even when the SNP lays in a homopolymer region in the sequence (Figure 2). In analyzing the novel A/G cSNP⁷⁰² during cSNP⁶⁹⁸ analysis (Figure 1), we showed the possibility of multiplexing the analysis if the positions of 2 analysed SNPs are in the short sequence.

We also confirmed that 8 of 9 Japanese cultivars, which were believed to be donors of a thermostable β -amylase allele, indeed carry the thermostable Sd2H allele. Azuma Golden carries the low-thermostable Sd2L allele. We identified 4

additional Asian cultivars that carry the high-thermostabile Sd2H allele. We found that Czech malting cultivars carry intermediate- and low-thermostabile β -amylases. The rest of the collection carries the low-thermostabile Sd2L allele and the newly identified Sd1b allele. Only one cultivar, Milonov Nudum, carries the intermediate-thermostabile Sd1 allele (Table 1). This suggests that introgression of the high-thermostabile allele into European material would be an effective means of increasing quality and could be readily achieved by selection with cSNP markers.

Breeding programmes in the last century tended to reduce biodiversity within cultivars. The spectrum of bred cultivars is therefore genetically similar. To provide new sources of genetic variation, it is necessary to preserve and characterise genetic resources, which form a gene pool for agricultural crops. Pyrosequencing allowed the rapid characterisation of a collection of 79 barleys and could easily be extended to a larger sample. Data obtained will help select genetic resources carrying variation in β -amylase thermostability for breeding programmes aiming to improve barley malting quality and grain qualitative parameters for alternative end-use.

Acknowledgments

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