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## PCR Markers for Selection of the *Bmy1* Locus that encodes Beta-amylase in Barley Grain

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## Introduction

Barley *beta*-amylase catalyses the release of maltose from the non-reducing ends of starch chains and related compounds. There are three *beta*-amylase genes in the barley genome (Kreis et al, 1987), which encode at least five different forms of the enzyme in different tissues and at different developmental stages (Shewry et al, 1988). Given its importance in malting, most research has concentrated on the *Bmy1* gene which encodes the *beta*-amylase produced in the barley grain (Kreis et al, 1987; Li et al, 2001).

Four cDNAs of *Bmy1* have been isolated from different barley cultivars which encode the Sd1 or Sd2 forms of *beta*-amylase (Kreis et al, 1987; Yoshigi et al, 1994, 1995; Erkkila et al, 1998; Li et al, 2001). The *Bmy1* gene is located on the long arm of chromosome 4H (Kleinhofs et al, 1993; Li et al, 2001). This locus determines *beta*-amylase activity (Erkkila et al, 1998), enzyme thermostability (Eglinton et al, 1998), isoenzyme form and free/bound enzyme ratio (Li et al, 2001). In the present study, we investigated genetic diversity at the *Bmy1* locus among 106 barley cultivars/lines using PCR markers based on primers that were designed from the cloned cDNAs or gene sequences of *Bmy1*.

## Materials and Methods

One hundred and six barley cultivars/lines from a collection at the Crop Development Centre, University of Saskatchewan were evaluated. These cultivars/lines originated from Canada, the USA, Europe, Mexico, Japan, Australia and China. DNA from each cultivar/line was extracted from young leaves using a modified micro-CTAB method (Procunier et al, 1990).

Four cDNA sequences were aligned using the software DNAMAN. Based on the alignments and on differences in the DNA sequences that resulted in amino acid substitutions, allele-specific PCR primers were designed with the software DNASTAR. Two primer pairs were designed to amplify across a microsatellite and a palindromic insertion in intron III of *Bmy1* respectively (Erkkila et al, 1998; Yoshigi et al, 1995). Products of amplification around the microsatellite were separated on a sequencing gel containing 6% polyacrylamide, 7M urea and 1x TBE at 85 W constant power for 3 h (BioRad sequencing system). The gel was fixed, stained and dried using a DNA silver staining kit (Promega). NTSYSpc (Version 2.0) was used to calculate the genetic similarity (Jaccard's coefficient) of the *Bmy1* sequences and for cluster analysis (Unweighted Paired Group Method using Arithmetic averages).

## Results

### *Alignment of the cDNA sequences of Bmy1*

The four cDNA sequences of *Bmy1* were aligned using DNAMAN software. There were thirteen nucleotide substitutions among the four *Bmy1* cDNAs. This equals one replacement in every 123 nucleotides of the *Bmy1* cDNA sequence. Six nucleotide substitutions resulted in amino acid changes. PCR primers were designed to amplify the specific allele of each amino acid substitution.

### *Amplifying specific alleles of Bmy1 from barley cultivars/lines*

PCR reaction conditions were optimized for each primer pair to amplify the specific allele. Six allele-specific primer pairs were used to amplify portions of the *Bmy1* locus from 106 barley cultivars/lines. The two alleles, represented by either presence or absence of the amplification product, were amplified for each primer pair among the 106 cultivars/lines. These six primer pairs (R115, E165, V233, S347, A430 and M527) amplified a PCR product from 58, 49, 46, 39, 51 and 55 of the 106 cultivars/lines, respectively. Except for the Ser347 mutation detected in Haruna Nijo, the two alleles were fairly evenly distributed among the 106 cultivars/lines.

Based on the sequence around the palindromic insertion in intron III of *Bmy1* (Erkillä et al, 1998), a pair of primers (designated Bmyintron3) was developed. Similarly a pair of primers for the microsatellite in intron III of *Bmy1* (Erkillä et al, 1998; Yoshigi et al, 1995) was designed and designated as BmyMicros. Two alleles were amplified using the Bmyintron3 primer pair.

Five alleles were revealed for the microsatellite including a null allele. Forty-nine cultivars/lines had the null allele. The frequencies of the other four alleles (from the smallest to the largest) were 14% (8), 38% (22), 34% (20) and 14% (7) among the other 57 cultivars/lines.

### *Genetic similarity at the Bmy1 locus*

Genetic similarity at the *Bmy1* locus using the eight markers was calculated using NTSYS (version 2.0). Cluster analysis divided the 106 barley cultivars/lines into two large groups of 48 and 58 cultivars/lines. Genetic similarity of the two groups was less than 0.1. All cultivars/lines known to carry the Sd1 form were located in one group while those known to carry the Sd2 form (Eglinton et al, 1995, 1997) were in the other. Further analysis revealed that the division of the two groups was consistent with the Arginine115/Cysteine substitution detected between the Sd1 and Sd2 forms of *beta*-amylase (Li et al, 2001). The Sd1 group had a Cysteine at position 115 of the *Bmy1* gene, while the Sd2 group had an Arginine at that position.

There was a low level of sequence variation at the *Bmy1* locus in the Sd1 group. All Sd1 type cultivars/lines had the short allele for the intron III insertion and failed to amplify the microsatellite in intron III. Furthermore, the Glu165 and Ala430 mutations were conserved in most of the cultivars in the Sd1 group. Nevertheless, the Met527/Iso substitution showed some variation among the cultivars in the Sd1 group. In contrast, the Sd2 group showed more divergence at the *Bmy1* locus. Four alleles for the microsatellite and two alleles for the intron III insertion were detected among the Sd2 cultivars/lines. Polymorphisms were also evident among the Sd2 group for the primers BmyV233, BmyS347 and BmyM527. All the Japanese cultivars and two Canadian lines HB334 and HB345 formed a subgroup in the Sd2 group. Cultivars/lines in this subgroup carried the same allele as Haruna Nijo, which has high enzyme activity and thermostability (Eglinton et al, 1998). The microsatellite was the only polymorphic marker in this subgroup.

Two isoenzymes of *beta*-amylase have been identified by Isoelectric focusing (IEF) in cultivated barley grain (Evans et al, 1997; Forster et al, 1991; Allison, 1973) and malt (Allison and Swanston, 1974; Swanston, 1979). These isoenzymes are referred to as Sd1 (starch degrading enzyme) and Sd2. A third isoenzyme (Sd3) was recently found in *Hordeum spontaneum* (Eglinton et al, 1998). In the present study, 106 barley cultivars/lines could be separated into two groups using DNA polymorphisms in the *Bmy1* gene. The two groups were consistent with the isoenzyme forms revealed by IEF analysis, Sd1 and Sd2. There was more variation at the *Bmy1* locus among cultivars/lines with the Sd2 isoenzyme than those carrying Sd1. This is consistent with results for *beta*-amylase thermostability, in which only one Sd1 *beta*-amylase allele was found but two Sd2 alleles (*Bmy1*-Sd2L and -Sd2H) were found (Eglinton et al, 1998). The deduced amino acid and nucleotide sequences of the Sd2 form of *beta*-amylase were the same or very similar to those of *Hordeum spontaneum* (the ancestor of cultivated barley). These findings add further support to our earlier conclusion (Li et al, 2001), that the Sd2 form of *beta*-amylase is the wild-type allele while Sd1 is a more recent allele.

The *Bmy1* locus controls isoenzyme form, free/bound enzyme ratio, enzyme activity and *beta*-amylase thermostability (Li et al, 2001; Erkkila et al, 1998; Eglinton et al, 1998). Sd1 and Sd2 *beta*-amylases exhibit different immunological properties and inhibitor protein Z affinities (Evans et al, 1995; 1997). Attempts to relate isoenzyme form to enzyme activity have been inconclusive (Evans et al, 1995; Swanston, 1979). Obviously, different mechanisms exist that determine enzyme activity and isoenzyme form. In a recent study, we showed that a single amino acid substitution (Arg115/Cys) determines isoenzyme form and the binding of the enzyme with an inhibitor of *beta*-amylase (Li et al, 2001).

Erkkilä *et al* (1998) proposed that a 126-bp palindromic insertion in intron III of the *Bmy1* gene determines *beta*-amylase activity. The results presented here do not support this assumption. This insertion was not associated with either enzyme activity or the isoenzyme type among 106 barley cultivars/lines. For example, the cultivars Hiproly and Haruna Nijo both have the Sd2 form of *beta*-amylase that has high enzyme activity (Eglinton *et al*, 1997; Kreis *et al*, 1987; Yoshigi *et al*, 1994), but in the present study they varied in the presence of the intron III insertion. Similarly, neither Harrington nor Haruna Nijo have the insertion but they have different isoenzyme forms (Eglinton *et al*, 1995; Li *et al*, 2001). More research is required to determine the mechanism of enzyme activity. An insertion detected in the promoter region (Erkkilä *et al*, 1998) may play a role in controlling enzyme activity.

Genetic diversity within *beta*-amylase of cultivated barley has been investigated based on thermostability of the enzyme (Eglinton *et al*, 1998; Kihara *et al*, 1998, 1999). Eglinton *et al*. (1998) classified *beta*-amylase as *Bmy1*-Sd1, -Sd2H and -Sd2L by combining analysis of the isoenzyme forms and enzyme thermostability following analysis of a limited number of cultivars. The Sd1 form of *beta*-amylase showed intermediate thermostability while the Sd2H and Sd2L forms exhibited the highest and lowest thermostability respectively. Kihara *et al*. (1998, 1999) investigated geographical variation in *beta*-amylase thermostability. They classified *beta*-amylases as types A, B and C with high, intermediate and low thermostability respectively based on a study of 274 barley cultivars from East Asia (Japan, the Korean Peninsula, China and Nepal), North America, North Africa, Southwest Asia, Turkey, Europe and Australia. Isoelectric focusing analysis indicated that the A and C types all carried the Sd2 form of *beta*-amylase whereas B type cultivars were divided into two subtypes B1 (Sd1) and B2 (Sd2). Thus, A and C types were equivalent to the Sd2H and Sd2L forms respectively and subtype B1 was the same as Sd1. All Sd1 genotypes exhibited intermediate thermostability whereas Sd2 genotypes exhibited high, intermediate or low thermostability. This is again consistent with the present study based on the DNA polymorphisms of the *Bmy1* gene in which Sd1 alleles were more conserved than Sd2 alleles.

Recent Japanese malting barley cultivars exhibit high thermostability while most current European, North American and Australian cultivars exhibit intermediate or low thermostability (although some cultivars from these areas were found to have high thermostability Kihara *et al*, 1998, 1999). In the present study, the seven Japanese cultivars (Haruna Nijo, Tochikei, Mokkei, Hokuika, Amonori, Kojita and Pryota) and two Canadian lines HB334 and HB345 formed a subgroup in the Sd2 isoenzyme group. The *Bmy1* gene of these cultivars/lines had the same alleles as the cultivar Haruna Nijo (with high enzyme activity and high thermostability) for seven of the eight primers used in the present study. Polymorphism among these was only detected using the primer pair that amplified around the microsatellite. In these cultivars/lines Arg115 is conserved, thus all have the Sd2 form of *beta*-amylase. Amino acids at Asp165, Ala233, Ser347, Val430 and Met527 of the *Bmy1* gene are also conserved in these cultivars. By comparing the amino acid sequences of *Bmy1* genes, it has been suggested that Ala 233 and Ser347 may be related to thermostability of the enzyme (Eglinton *et al*, 1998). *In vitro* mutation analysis showed that four amino acid substitutions (Ser295Ala, Ile297Val, Ser351Pro and Ala376Ser) increased *beta*-amylase thermostability (Mikami *et al*, 1999). Thus, the high *beta*-amylase thermostability in the Japanese cultivars may be due to interactions

of several amino acid substitutions. At present we do not know which amino acid substitutions play a more important role in determining thermostability. Nevertheless, the present study has shown that Ser347 in Haruna Nijo is a rare mutation among the 106 barley cultivars/lines. The reason why HB334 and HB345 (two hulless feed barley lines) carry the same allele as the Japanese cultivars is under investigation. Both lines have complex pedigrees which may involve some Japanese parents.

All Japanese cultivars with high thermostability have the Sd2 form of *beta*-amylase. Thus the PCR primer pairs BmyR115, BmyE165 and BmyA430 developed in the present study can be used to select for a *Bmy1* allele with high thermostability and activity from the Japanese parent in crosses between Japanese material and cultivars carrying the Sd1 form. Our results also indicate that all Sd2 cultivars/lines that have *beta*-amylase with high thermostability have a short allele of the *Bmy1* intron III. Thus the BmyIntron3 primer pair can be used to select for alleles of the *Bmy1* gene with high thermostability and activity in a cross between Japanese lines and other cultivars/lines with the Sd2 form having intermediate or low thermostability. The multiple-allele microsatellite would also be a useful marker to detect polymorphism between different Sd2 forms of *beta*-amylase.

In conclusion, eight PCR primer pairs were designed based on amino acid substitutions or sequence variations in the *Bmy1* gene. Genetic diversity of the *Bmy1* gene was studied among 106 barley cultivars/lines. These primers could be used to classify the cultivars/lines into two large groups. Results were consistent with those from studies of *beta*-amylase isoenzyme polymorphism and thermostability. It is clear that an amino acid substitution (Arg115/Cys) determines isoenzyme type, but it is not clear what causes differences in enzyme activity and thermostability. Nevertheless, the present study provides PCR primers that can be used to select for *Bmy1* alleles with high thermostability and activity in a marker-assisted breeding program.

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