Barley β-amylase 1 (*Bmy1*) intron III classification and association with enzyme activity and thermostability

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Abstract

Polymorphisms in intron III of barley (*Hordeum vulgare* L.) endosperm-specific β-amylase (*Bmy1*) have been associated with β-amylase activity and thermostability and are thought to have potential as a selective marker for breeding elite malting cultivars. The third intron of *Bmy1* was sequenced in thirty-nine barley accessions. Four alleles were identified based upon insertion/deletions (indels) of 126-bp, 38-bp, 11-bp, and 21-bp. The *Bmy1.a* allele has the 126-bp, 38-bp, and 21-bp indels. *Bmy1.b* only has the 38-bp indel. *Bmy1.c* has the 38-bp, 11-bp, and 21-bp indels. *Bmy1.d*, found in only one accession, is missing the 126-bp and 38-bp indel. Thirty-nine accessions were assayed for β-amylase activity and thermostability (percent remaining after exposure to 60°C for 10 minutes). Accessions with the *Bmy1.a*, *Bmy1.b*, *Bmy1.c*, and *Bmy1.d* alleles had averages of 26.7, 23.8, 38.5, and 56.1% residual activity, respectively. Each allele had statistically different thermostabilities according to the Kruskal-Wallis test. Accessions with *Bmy1.a*, *Bmy1.b*, *Bmy1.c*, *Bmy1.d* alleles had activity averages of 1181, 1460, 1058, and 2739 U/g flour, respectively. Each allele had statistically different activities using the Kruskal-Wallis test, except between *Bmy1.a* and *Bmy1.c*. These data support the use of intron III as a selective tool. However, when the data within each intron allele are analyzed, the conclusions are different. Using the least significant difference (LSD) test on the activities and thermostabilities within each allele (i.e. *Bmy1.a*, *Bmy1.b*, and *Bmy.c*), statistically significant differences were found. Therefore, we conclude that the third intron is not a reliable marker for use in selecting elite malting cultivars.

Introduction

β-Amylase (3.2.1.2) is a starch degrading enzyme that is very important to the malting and brewing industry because it is one of the four main enzymes involved in fermentable sugar production during the mashing process (Sun and Henson, 1991). Diastatic power (DP) is an official international measure of malt quality (Kunze, 1999) and it measures the contribution of all amylolytic enzymes involved in starch degradation. β-Amylase has also been shown to be the major contributor to DP (Delcour and Verschaeve, 1987). Researchers have been studying β-amylase in hopes of finding selective markers for breeding elite malting barley varieties.

Erkkila *et al*. (1998) identified three distinct *Bmy1* alleles (Haruna Nijo-like, Adorra-like, and PI 296897-like), in cultivated barley (*Hordeum vulgare*) and wild barley (*Hordeum vulgare* ssp. *spontaneum*), based upon two indels in *Bmy1* intron III. Erkkila (1999) determined the mean activity for β-amylase in crude extracts of cultivars with the Haruna Nijo-like allele had 1.3 times more activity than the Adorra-like allele. The PI 296897-like allele had 2.1 times more activity than the cultivars with the Adorra-like allele.

Gunkel *et al*. (2002) correlated the intron III alleles with β-amylase thermostability. They showed that cultivars with non-thermostable β-amylase contained the 126-bp indel in *Bmy1* intron III whereas the thermostable β-amylases did not contain the 126-bp indel. They correlated the presence of the 126-bp indel with inferior β-amylase thermostability. The inferior β-amylase thermostability lowered concentrations of maltose in wort, which was associated with a lower apparent attenuation limit (AAL). AAL is a measure of the capacity of yeast to convert wort into alcohol.

Coventry *et al*. (2003) correlated the presence of the 126-bp indel with lower DP, lower β-amylase activity, and inferior thermostability. Their intron III PCR marker was used to distinguish between the presence and absence of the 126-bp indel at the *Bmy1* locus and they concluded that it was effective for screening for β-amylase thermostability and activity as well as DP.

Logically, if one can find simple and reliable markers (i.e. PCR) to select for β-amylase activity, β-amylase thermostability, DP, and AAL, without actually measuring them, then it could lead to more efficient and cost effective breeding of elite malting quality barley.

Materials and Methods

Bmy1 from thirty-nine wild and cultivated barley accessions were cloned and sequenced, using conventional techniques, and classified into four alleles.

The barley seeds had their hull removed and were ground to flour. β-Amylase was extracted from 150 mg of flour following manufacturer's instructions (Megazyme). Three separate extractions were performed for each accession. The extracts were diluted 1250-fold in a dilution buffer. The diluted extract (10 µl) was sub-sampled four times. The sub-samples were placed in a 96-well PCR plate. Prior to the β-amylase activity assay the plate containing the diluted extract was incubated in an iCycler Thermal Cycler at 40°C or 60°C for 10 min and then an additional 1 min at 40°C before conducting the assay at 40°C. The *p*-nitrophenyl maltopentaoside substrate was pre-incubated at 40°C and 10 µl was added to the pre-incubated diluted extract. The assays were run for 5 and 10 minutes, during which time rates were previously determined to be linear. The reaction was stopped by adding 150 µl of 1% Trizma® base. The absorbance was read at 400 nm on a microplate spectrophotometer. Activity rates were determined by using the equation in the Betamyl method handbook.

The percent residual activity was determined by dividing the β-amylase activity after pre-incubating at 60°C by the β-amylase activity after pre-incubating at 40°C and multiplying by 100.

All statistics were accomplished using SAS 9.1. The LSD test was conducted using the GLM procedure. The Kruskal-Wallis test was conducted using the NPAR1WAY procedure.

Results

The *Bmy1* intron III sequences were grouped into one of four intronic alleles. Alleles were determined by the presence or absence of four indels: 126-bp, 38-bp, 11-bp, and 21-bp (Table 1).

Table 1. *Bmy1* intron III alleles based on the four largest indels.

Allele	Indel			
	126-bp	38-bp	11-bp	21-bp
Bmy1.a	+	+	-	+
Bmy1.b	-	+	-	-
Bmy1.c	-	+	+	+
Bmy1.d	-	-	+	+

The average β-amylase activity for each allele was 1181±358, 1460±433, 1057±367, and 2739±42 U/g of flour for *Bmy1.a*, *Bmy1.b*, *Bmy1.c*, and *Bmy1.d*, respectively (Table 2, Figure 1). The β-amylase activity of *Bmy1.a* is statistically different from *Bmy1.b* and *Bmy1.d* but not from *Bmy1.c*, according to the Kruskal-Wallis test (Table 3). The β-amylase activity levels of *Bmy1.b*, *Bmy1.c*, and *Bmy1.d*, are all statistically different from one another (Table 3).

Table 2. Mean±SD, range, and sample number of barley β-amylase activities (U/g flour), and thermostabilities (% residual activity) for all four *Bmy1* intron III alleles.

β-Amylase		Allele			
		Bmy1.a	*Bmy1.b*	*Bmy1.c*	*Bmy1.d*
Activity	Mean±SD	1181±358	1460±433	1057±367	2739±42
	Min.	368	409	543	2701
	Max.	1879	2088	1761	2784
	Samples (*n*)	51	28	15	3
Thermostability	Mean±SD	26.7±10.1	23.8±2.8	38.5±9.7	56.1±1.1
	Min.	3.3	18.2	21.2	55.34
	Max.	37.1	27.4	50.7	56.9
	Samples (*n*)	52	27	17	2

Results cont.

Table 3. Kruskal-Wallis test of barley β-amylase activities and thermostabilities amongst *Bmy1* intron III alleles.

Assay	Compared Alleles	*H* statistic	Probability
β-Amylase activity	*Bmy1.a* vs. *Bmy1.b*	11.7	0.0006
	Bmy1.a vs. *Bmy1.c*	1.79	0.1806
	Bmy1.a vs. *Bmy1.d*	8.3	0.0039
	Bmy1.b vs. *Bmy1.c*	8.9	0.0029
	Bmy1.b vs. *Bmy1.d*	7.9	0.005
	Bmy1.c vs. *Bmy1.d*	7.1	0.0077
	All	22.8	<0.0001
β-Amylase thermostability	*Bmy1.a* vs. *Bmy1.b*	15.5	< 0.0001
	Bmy1.a vs. *Bmy1.c*	11.6	0.0006
	Bmy1.a vs. *Bmy1.d*	5.7	0.0172
	Bmy1.b vs. *Bmy1.c*	19.1	< 0.0001
	Bmy1.b vs. *Bmy1.d*	5.4	0.0201
	Bmy1.c vs. *Bmy1.d*	5.1	0.0239
	All	34	< 0.0001

The enzyme activities of accessions within each allele were compared using the LSD test (Table 4). There are twelve statistically different groups of means for the twenty *Bmy1.a* accessions. The twelve accessions of *Bmy1.b* have six statistically different groups. *Bmy1.c* is composed of six accessions with five statistically different groups. The LSD values for *Bmy1.a*, *Bmy1.b*, and *Bmy1.c* were 81.4, 236, and 102, respectively.

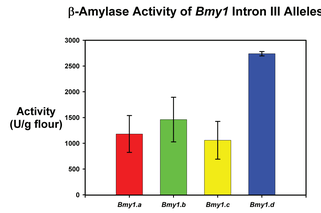

Figure 1. Barley β-amylase activity (U/g flour) of 39 accessions broken up into four intronic alleles based upon four indels (126-bp, 38-bp, 11-bp, and 21-bp). Bmy1.a (+++) is represented by 20 accessions (n=51). Bmy1.b (-+--) is represented by 12 accessions (n=28). Bmy1.c (-+++) is represented by 6 accessions (n=15). Bmy1.d (--++) is represented by the only accession known to date with this allele (n=3). Error bars represent standard deviation.

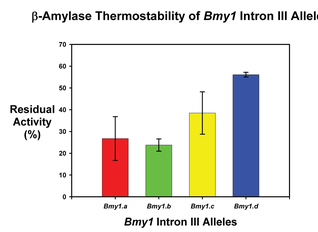

Figure 2. Barley β-amylase thermostability (% residual activity) of 39 accessions broken up into four intronic alleles based upon four indels (126-bp, 38-bp, 11-bp, and 21-bp). Bmy1.a (+++) is represented by 20 accessions (n=52). Bmy1.b (+--) is represented by 12 accessions (n=27). Bmy1.c (-+++) is represented by 6 accessions (n=17). Bmy1.d (--++) is represented by the only accession known to date with this allele (n=2). Error bars represent standard deviation.

The average β-amylase thermostabilities of the four intron III alleles range from 23.8 ± 2.8 to 56.1 ± 1.1% residual activity after treatment at 60°C with the minimum residual activity of all the samples being 3.3 and the maximum being 56.9% (Table 2, Figure 2). The residual activities of the *Bmy1.a*, *Bmy1.b*, *Bmy1.c*, and *Bmy1.d* alleles are 26.7±10.1, 23.8±2.8, 38.5±9.7, 56.1±1.1% respectively and are, according to the Kruskal-Wallis test, all significantly different than each other (Tables 2 and 3).

LSD analysis was used to determine if there were any differences among the accessions of each allele. The *Bmy1.a* allele had an LSD value of 2.7 and was found to have eleven statistically different groups amongst twenty accessions, with four accessions being the maximum for one group. There were five statistically different groups amongst twelve accessions for *Bmy1.b* using an LSD value of 2.5. *Bmy1.c* had six accessions and four statistically different groups using an LSD of 4.2 (Table 5).

Discussion

Selective Marker Potential of *Bmy1* Intron III

The *Bmy1* intron III indels have the potential to be exploited as a PCR-based screen for elite malting cultivars based on the Kruskal-Wallis test, but when further statistical scrutiny is employed the potential disappears. The *Bmy1.d* allele, represented by PI 296897, would have potential as a selective marker except it has only been found in one wild barley accession and has not been found in cultivated barley. The β-amylase activity within *Bmy1.a* ranges from 417 to 1819 U/g flour, with twelve statistically different groupings (Table 4). The ranges of β-amylase activity in *Bmy1.b* and *Bmy1.d* are 628 to 1736 and 417 to 1991 U/g flour, respectively. If the *Bmy1* intron III indels were used as a marker to select for high β-amylase activity, there would be insufficient information to determine if the β-amylase activity was actually high or low. Therefore, we have concluded that the *Bmy1* intron III indels are not effective markers for selecting lines with high β-amylase activity.

Table 4. LSD analysis of β-amylase activities (U/g flour) for alleles *Bmy1.a*, *Bmy1.b*, and *Bmy1.c*. Accessions within alleles that have different *t* group letters are significantly different (α=0.05). *Bmy1.a* LSD=81.4, *Bmy1.b* LSD=236, *Bmy1.c* LSD=102.

Allele	Mean	n	Accession	*t* grouping
Bmy1.a	1819	2	Legacy	a
	1683	3	Lacey	b
	1661	2	Morex	b
	1547	2	B1602	c
	1368	2	Tradition	d
	1363	3	B1614	d e
	1339	3	Sisler	d e f
	1338	2	Stander	d e f
	1326	2	Foster	d e f
	1283	3	Drummond	e f g
	1262	3	Baronesse	f g
	1230	3	Excel	g h
	1172	2	C-14	h i
	1124	3	Bor Mashash 17-5	i
	946	3	Robust	j
	936	2	86ab536	j
	88	3	Talpiyyot 14-20	j
	668	3	Mt. Hermon 1-13	k
	646	2	Adorra	k
	417	3	Steptoe	l
Bmy1.b	1991	2	Garnet	a
	1835	2	Klages	a b
	1815	3	Merit	a b
	1761	2	B1202	a b c
	1668	2	Stratus	b c d
	1554	3	Shuna 1-32	c d
	1515	2	Metcalfe	d
	1459	3	Harrington	d
	1443	3	B1215	d
	974	2	Amman 7-33	e
	879	2	Mt. Meron 9-53	e
	417	2	Strider	f
Bmy1.c	1736	2	Dana 17-43	a
	1339	2	Shuna 20-14	b
	1127	2	Ashqelon 28-3	c
	1044	3	Damon 11-11	c
	803	3	Safi 19-16	d
	628	3	Wadi Musa 18-21	e

Table 5. LSD analysis of β-amylase thermostabilities (% residual activity) for alleles *Bmy1.a*, *Bmy1.b*, and *Bmy1.c*. Accessions within alleles that have different *t* group letters are significantly different (α=0.05). *Bmy1.a* LSD=2.7, *Bmy1.b* LSD=2.5, *Bmy1.c* LSD=4.2.

Allele	Mean	n	Accession	*t* grouping
Bmy1.a	36	3	Stander	a
	35.4	2	Tradition	a b
	34.4	3	B1614	a b c
	33.3	3	Bor Mashash 17-15	a b c d
	33.3	3	Talpiyyot 14-20	a b c d
	33.2	2	Lacey	b c d
	32.1	3	Excel	c d e
	32.1	3	Legacy	c d e
	31.8	2	Foster	c d e f
	31.3	3	B1602	d e f g
	30.1	2	Mt. Hermon 1-13	e f g
	29.1	2	Drummond	f g h
	29	3	Sisler	f g h
	26.9	2	Robust	h
	26.9	3	86ab536	h
	22.8	3	Steptoe	i
	21.4	2	Morex	i
	8.2	2	Adorra	j
	4.3	3	Baronesse	k
	4.1	3	C-14	k
Bmy1.b	26.5	3	Strider	a
	26.2	2	Metcalfe	a b
	26	2	Amman 7-33	a b
	25.9	2	B1215	a b
	25.3	2	Klages	a b
	24.8	3	Harrington	a b
	24.5	2	Garnet	a b
	23.9	3	Shuna 1-32	b c
	21.8	3	Merit	c d
	20.4	2	Stratus	d e
	19.2	2	B1202	e
	18.8	2	Mt. Meron 9-53	e
Bmy1.c	49.1	3	Damon 11-11	a
	46.6	3	Ashqelon 28-3	a
	41.6	2	Shuna 20-14	b
	40.9	3	Safi 19-16	b
	30.5	3	Dana 17-43	c
	23.2	3	Wadi Musa 18-21	d

The mean thermostabilities of each of the four intron III alleles, according to the Kruskal-Wallis test, are significantly different from each other (Table 3). Similar to the β-amylase activity data, the thermostability data have a large range within any given allele, except *Bmy1.d* (only one accession). The maximum value in the *Bmy1.a* allele is 36%, whereas the maximum value for the *Bmy1.b* allele is 26.5%. The *Bmy1.a* mean is significantly different from the *Bmy1.b* mean using the Kruskal-Wallis test. However, the minimum values for the *Bmy1.a* allele is 4%, whereas the minimum value for the *Bmy1.b* allele is 18.8%. There is no way to determine if the thermostability is going to be at the high end or the low end of these ranges by just examining the third intron. Therefore, we conclude that using the third intron as a marker to select for β-amylase thermostability will not provide consistent or reliable results.

A more effective way to screen germplasm for β-amylase thermostability would be to use a two point assay as that Eglington *et al*. (1998) proposed. We propose using a high throughput 96-well plate method, as done in this study, to determine the β-amylase activity and thermostability from barley grain as a selective tool as opposed to using assays based on the indels or other motifs in the third intron of *Bmy1*.

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