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ORIGINAL ARTICLE

Differential expression of two β-amylase genes (*Bmy1* and *Bmy2*) in developing and mature barley grain

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Abstract Two barley (*Hordeum vulgare* L.) β-amylase genes (*Bmy1* and *Bmy2*) were studied during the late maturation phase of grain development in four genotypes. The *Bmy1* and *Bmy2* DNA and amino acid sequences are extremely similar. The largest sequence differences are in the introns, seventh exon, and 3′ UTR. Accumulation of *Bmy2* mRNA was examined in developing grain at 17, 19, and 21 days after anthesis (DAA). One genotype, PI 296897, had significantly higher *Bmy2* RNA transcript accumulation than the other three genotypes at all developmental stages. All four genotypes had *Bmy2* mRNA levels decrease from 17 to 19 DAA, and remain the same from 19 to 21 DAA. Levels of *Bmy1* mRNA were twenty thousand to over one hundred thousand times more than *Bmy2* mRNA levels in genotypes Legacy, Harrington, and Ashqelon at all developmental stages and PI 296897 at 19 and 21 DAA. PI 296897 had five thousand times more *Bmy1* mRNA than *Bmy2* mRNA at 17 DAA. However, Bmy2 protein was not found at 17 DAA in any genotype. The presence of Bmy2 was immunologically detected at 19 DAA and was present in greater amounts at 21 DAA. Also, Bmy2 protein was found to be stored in mature grain and localized in the soluble fraction. However, Bmy1 protein was far more prevalent than Bmy2 at all developmental stages in all genotypes. Thus, the vast majority of β-amylase activity in developing and mature grain can be attributed to endosperm-specific β-amylase.

Keywords Grain development · RT-qPCR · Malting

Abbreviations

DAA	Days after anthesis
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SNP	Single nucleotide polymorphism
RER	Relative expression ratio

Introduction

β-Amylase (3.2.1.2) is a starch degrading enzyme that hydrolytically cleaves α-1,4-D-glucosidic bonds to liberate β-maltose from the non-reducing ends of a variety of polyglucans. In barley grain and malt (i.e., germinated and kilned), β-amylase has the highest activity of all of the amylolytic enzymes (Doehlert and Duke 1983; Duke and Henson 2009a; Henson and Duke 2008). There are two known genes encoding β-amylase in barley (*Hordeum*

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vulgare L.); *Bmy1* and *Bmy2*. The *Bmy1* gene encodes the 535 amino acid 'endosperm-specific' barley β-amylase located in the telomeric region on the long arm of chromosome 4H (Kreis et al. 1987, 1988; Nielsen et al. 1983; Powling et al. 1981; Yoshigi et al. 1994). In the mature grain, 'endosperm-specific' β-amylase is present in either a free (soluble) form, which is fully active, or a bound (insoluble) form, which is only fully activated upon conversion to the free form (Hara-Nishimura et al. 1986; Sopanen and Laurière 1989; Guerin et al. 1992). 'Endosperm-specific' β-amylase is one of the four barley malt enzymes involved in the fermentable sugar production during mashing, and of these four enzymes β-amylase best correlates with diastatic power which is a measurement of total amylolytic activity and an important determinant of malt quality (Duke and Henson 2009b; Henson and Duke 2008; Sun and Henson 1991). The barley *Bmy2* gene encodes a 505 amino acid protein and is located on the short arm of chromosome 2H (Clark et al. 2005; Jung et al. 2001; Kreis et al. 1988). This β-amylase is homologous to wheat and rye 'ubiquitous' β-amylase, and in barley this nomenclature has been perpetuated despite never being identified in any tissue other than the grain in early development. The deduced amino acid sequence of barley *Bmy2* shares over 90% identities with 'ubiquitous' β-amylases from other Triticeae species (Jung et al. 2001). The deduced amino acid sequences of barley *Bmy1* and *Bmy2* share 76 and 80% identities (Jung et al. 2001 and Clark et al. 2005, respectively).

Spatial and temporal expression of Triticeae 'ubiquitous' β-amylase differs from species to species. 'Ubiquitous' β-amylase is present in the outer pericarp, roots, leaves, flowers, and both developing and mature grain of wheat (*Triticum aestivum* L.) (Daussant and Laurière 1990; Wagner et al. 1999). However, in wild-type barley, β-amylase mRNA corresponding to *Bmy2* was only found in early grain development at 5 DAA and was not identified in germinating grain, roots, etiolated leaves, or fully grown leaves (Jung et al. 2001). In rye (*Secale cereal* L.), *Bmy2* mRNA and protein accumulation occurred early in grain development (Rorat et al. 1995). In both rye and barley, *Bmy2* mRNA accumulated at much lower levels than *Bmy1* (Jung et al. 2001; Rorat et al. 1995).

However, a recent study identified the *Bmy2* transcript in various tissues during grain development in barley (Radchuk et al. 2009). This study reported levels of *Bmy2* mRNA at around half the levels of *Bmy1* in the barley endosperm during late grain development. They concluded that the observed β-amylase activity during late grain development was the result of both *Bmy1* and *Bmy2* gene expression. This was the first report of barley *Bmy2* transcript accumulation this late in barley grain development. Radchuk et al. (2009) also showed that *Bmy2* mRNA was present in large amounts in the pericarp during early grain development, whereas the *Bmy1* transcript was not detected.

The research presented here was conducted in part to clarify the aforementioned conflicting research on barley *Bmy2* mRNA accumulation. In this report the *Bmy2* gene, from two cultivated and two wild (*Hordeum vulgare* ssp. *spontaneum*) barley genotypes, was sequenced and accumulation of *Bmy2* mRNA in developing grain at 17, 19, and 21 DAA was determined using relative reverse transcription quantitative real-time PCR (RT-qPCR). Also, the ratio of *Bmy1* to *Bmy2* RNA transcript accumulation is compared in all genotypes at 17, 19, and 21 DAA. Antibodies specific for Bmy1 and Bmy2 were used to determine the presence or absence of the specific gene products in the developing grain of each genotype at 17, 19, and 21 DAA and to determine whether the proteins were located in the bound or free fraction and to further enable us to determine the significance of any differences in *Bmy1* to *Bmy2* transcript accumulation.

Materials and methods

Plant material

Grain from Legacy, Harrington, Ashqelon, and PI 296897 were germinated in Petri dishes containing germination paper for 6 weeks at 1–2°C. Seedlings were transferred to pots containing a 1:1 ratio of peat moss and perlite. Plants were randomized and grown under greenhouse conditions with the photoperiod and temperatures of L12(20°C)/D12(15°C) for 4 weeks followed by L16(25°C)/D8(20°C) until grain was harvested at 17, 19, and 21 DAA, and maturity. Anthesis was considered to have occurred when the awn emerged from the boot. Developing grain was harvested between 8 and 11 o'clock in the morning. The top four grains from each spike were removed, dehulled, frozen in liquid nitrogen and stored at −80°C.

Relative RT-qPCR

Total RNA was isolated from the developing grain using the Concert™ Plant RNA Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's small scale RNA isolation protocol. RNA was purified further using the RNeasy® Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's RNA clean up protocol with on-column DNase I digestion and stored at −80°C. Total RNA integrity was determined using gel electrophoresis. Duplicate cDNA was generated using 1 μg of total RNA with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA).

Table 1 qPCR efficiencies and primer sequences

Gene	Efficiency[a]	Primer sequence (5′–3′, forward/reverse)
Bmy1[b]	2.0[c]	CCTGCCACCATGTAATGGAAC/AGGTTTCTCTGTCACACTCACACAC
Bmy2[d]	1.87[c]	GTGCATCACTGATGCAGTCTATGAG/TCCTTGGCGGCTTATTTCC
Actin[b]	1.95[e]	GGCATGGAGTCTTCTGGAATCC/CCACCACTGAGCACTATGTTTC
Cyclophilin[f]	1.96[e]	CCTGTCGTGTCGTCGGTCTAAA/ACGCAGATCCAGGAGCCTAAAG
Hsp70[f]	1.98[e]	CGACCAGGGCAACCGCACCAC/ACGGTGTTGATGGGGTTCATG

[a] qPCR efficiencies were determined using a dilution curve of four or five 5-fold dilutions with the formula: $E = 10^{-1/\text{slope}}$ (Pfaffl 2001; Rasmussen 2001)

[b] From Vinje et al. (2011)

[c] Plasmid DNA used as a template to determine qPCR efficiency

[d] From GenBank DQ889983

[e] Genomic DNA used as a template to determine qPCR efficiency

[f] From Burton et al. (2004)

Quantitative PCR reactions were conducted using either genomic DNA (PCR efficiencies), plasmid DNA (PCR efficiencies), or a 10-fold cDNA dilution in duplicate or triplicate using SYBR® *Premix Ex Taq*™ (Takara, Madison, WI, USA) according to the manufacturer's instructions using primers listed in Table 1. The relative expression ratio (RER) was calculated using the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmitgen 2001; Pfaffl 2001).

Reference gene transcripts were validated across all of the time points from each genotype and across all four genotypes using the *BestKeeper* program (Pfaffl et al. 2004). Coefficient of correlation to the *BestKeeper* index was $0.966 > r > 0.748$ for all samples at a p value of 0.001. The stable accumulation of transcripts from our reference genes allows for comparison of target gene levels across all genotypes and all time points.

Cloning and sequencing of *Bmy2*

Amplification of the *Bmy2* gene was performed using DNA isolated from mature barley grain and primers found in Table 2 under the following cycling conditions; 95°C for 5 min (1 cycle), 95°C for 45 s, 57°C for 30 s, 72°C for 4 min (35 cycles), with a final extension of 72°C for 7 min (1 cycle). Amplification products were separated using agarose gel electrophoresis, excised, purified, and cloned using the TOPO TA Cloning® Kit for Sequencing (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Sequencing reactions were conducted with primers found in Table 2 using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, Foster City, CA, USA) following the manufacturer's protocol. Reaction products were sequenced by the DNA Sequencing Facility at the University of Wisconsin Biotechnology Center (Madison, WI, USA).

Table 2 Cloning and sequencing primers for the *Bmy2* gene

Primer name	Primer sequence (5′–3′)	Function
Bmy2-F	CCAGTGTTTGTAGCCAGCAG	Cloning
Bmy2-R	TATACTGCTGAAAACTTCACGATCA	Cloning
Bmy2-1	CCCCACCAGACGTCTATCAT	Sequencing
Bmy2-2	TGCGGTTCGTGTAGAAAATG	Sequencing
Bmy2-3	CATCCCTGGCTCTGAGGATA	Sequencing
Bmy2-4	AACATGAAAAAGTTCTTGGATGC	Sequencing
Bmy2-5	ACCTTGTTTGCTTCGTCCAA	Sequencing
Bmy2-6	GCCGAAAGGTATCAACGAGA	Sequencing
Bmy2-7	AGCCAGGTTAGCATGCATTT	Sequencing
Bmy2-8	TCATCCACGACATCAGTGTG	Sequencing
Bmy2-9	CACTGATGTCGTGGATGAGG	Sequencing

Primers were created using Primer3 (http://frodo.wi.mit.edu/primer3)

Sequence chromatograms were viewed and edited using Chromas (Technelysium Pty. Ltd, 1988–2000). Contigs were assembled using the GeneStudio™ Contig Editor (Suwanee, GA, USA).

GenBank numbers for *Bmy2* DNA sequences are FJ936154–FJ936157.

DNA and amino acid alignments

DNA and amino acid alignments were created using Gene Studio™ Alignment Editor (Suwanee, GA, USA) using the default parameters in the slow/accurate pairwise alignment method.

Protein extraction, assay, and immunoblot

Total protein was extracted from developing and mature grain in buffer (50 mM Tris-base, 1 mM EDTA, 100 mM Cysteine, pH 8.0) according to Megazyme's instructions

(Betamyl Method, 12/04). Developing grain was initially ground in liquid nitrogen.

Soluble and insoluble proteins were extracted in six successive extractions. Five millilitres of soluble extraction buffer (50 mM Tris-base and 1 mM EDTA) were added to 500 mg of ground barley grain from Legacy, and incubated for 1 h at room temperature with vigorous vortexing every 10 min. Extracts were centrifuged for 10 min at 1,000×*g* and the supernatant removed. After five soluble extractions, cysteine (100 mM) was added to the extraction buffer and one additional extraction was performed.

Quantification of total protein was accomplished using the Coomassie Plus Assay Reagent following the manufacturer's instructions (Pierce Biotechnology, Rockford, IL, USA). In extracts containing cysteine, the samples were either diluted 10-fold or dialyzed (MWCO 8,000–10,000 Da) overnight at 4°C in 4 L of soluble extraction buffer (50 mM Tris-base and 1 mM EDTA).

Antibodies for protein immunoblots were raised against short peptide sequences from the C-terminal end of both *β*-amylases (AnaSpec, Fremont, CA, USA). Peptide sequence used for the production of 'endosperm-specific' *β*-amylase antibodies was QVKGPTGGMGGQAEDPTS. Peptide sequence used for the production of antibodies from the *β*-amylase encoded by the *Bmy2* gene was PVKDHTDVV-DEALLAP. Protein immunoblots were conducted using a 1:20,000 dilution of Bmy1 polyclonal anti-sera or 1:10,000 dilution of Bmy2 polyclonal anti-sera and probed with an Immun-Star™ GAR-HRP Conjugate dilution of 1:50,000 (Bio-Rad, Hercules, CA, USA). The Immun-Star™ WesternC™ Chemiluminescent Kit was used to detect Bmy1 and Bmy2 (Bio-Rad, Hercules, CA, USA). The chemiluminescent signal was captured using the UVP AutoChemi™ System (Upland, CA, USA).

Multiple immunoblots were conducted for each genotype and time point. Figures 5 and 6 depict a characteristic sample of each genotype and time point.

Statistical analysis

The Fisher's least significant difference (LSD) test was conducted in Figs. 2 and 3 using SAS 9.1 (Cary, NC, USA). The alpha level set at 0.05. The LSD analysis was considered significant at the $P < 0.05$ level.

Results and discussion

DNA and amino sequence analysis

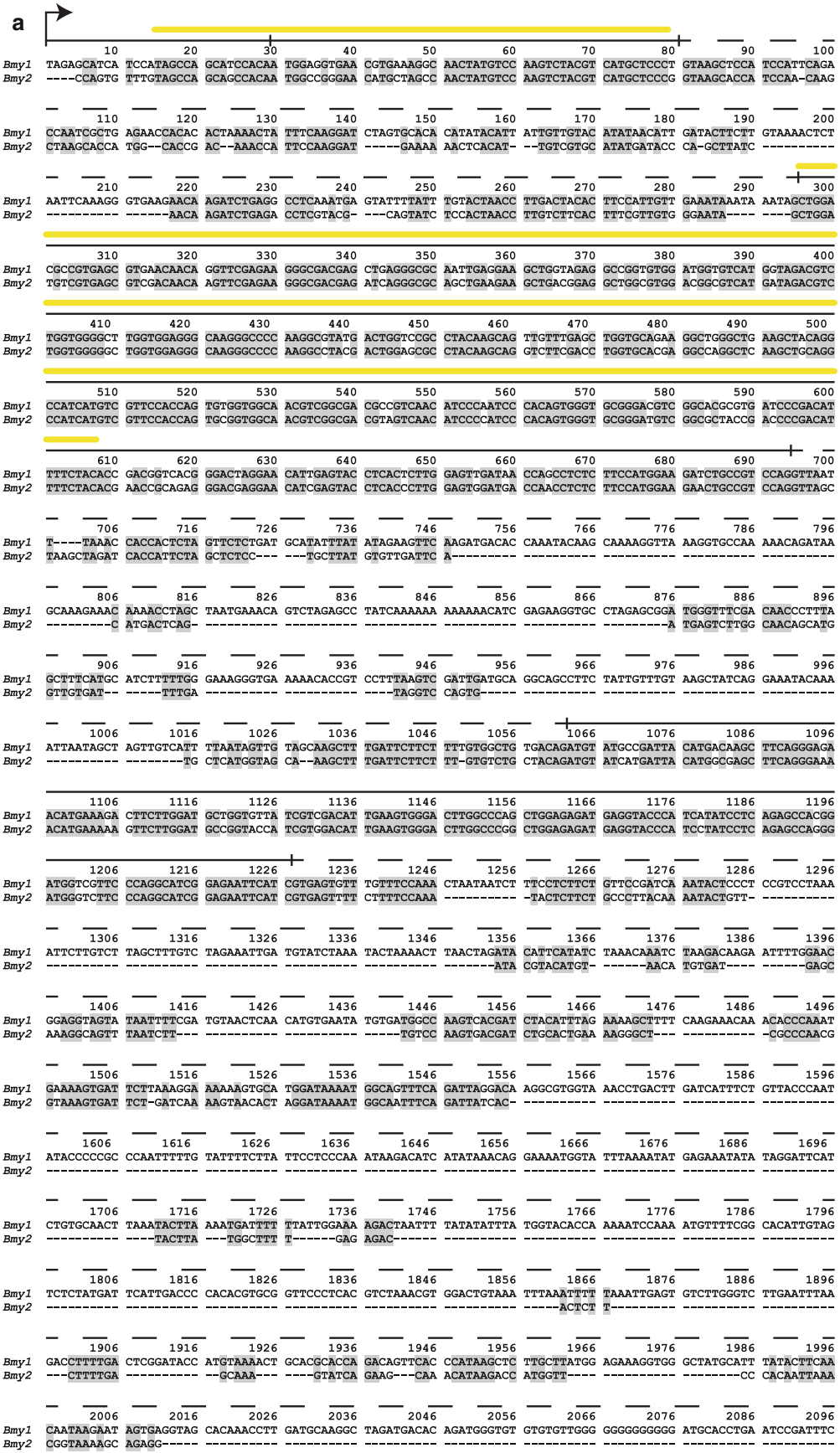
The DNA and deduced amino acid sequences of *Bmy1* and *Bmy2* from the genotype Legacy is very similar (Fig. 1). The deduced amino acid sequences from the Legacy *Bmy1* and *Bmy2* genes share 83% identities which is similar to the previous reports of 76 and 80% identities (Jung et al. 2001 and Clark et al. 2005, respectively). However, it is important to note that after amino acid 488, only one amino acid aligns between Bmy1 and Bmy2 (Fig. 1b). This is largely due to the lack of the glycine rich C-terminal end in Bmy2. The introns are the most dissimilar regions between *Bmy1* and *Bmy2*. The seventh exon and 3′ UTR are the only coding regions with sequences sufficiently dissimilar to create gene specific primers (Fig. 1a). *Bmy1* qPCR primers span the seventh exon and 3′ UTR, whereas the *Bmy2* qPCR primers amplify a region entirely within the 3′ UTR (Red and blue arrows in Fig. 1a).

Fig. 1 Sequence alignment of Legacy *Bmy1* (FJ161080) and *Bmy2* (FJ936156). **a** DNA sequence alignment from 5′ UTR to 3′ UTR. Transcription start site is denoted by an *arrow* at 0. Exons and UTRs are denoted by *solid black lines* above the sequence. Introns are denoted by *perforated black lines* above the sequence. Exon/intron boundaries are denoted by a *vertical black line*. Stop codons are in *boxes*. *Yellow bar* above sequence denotes where the *Bmy2* probe (EST HZ50o15) used by Radchuk et al. (2009) aligns with *Bmy1*. *Red arrows* denote location of *Bmy1* RT-qPCR primers used in this study. *Blue arrows* denote location of *Bmy2* RT-qPCR primers used in this study. **b** Deduced amino acid sequence alignment. Identical sequences are *shaded gray*. *Dashes* represent gaps ▶

The alignment of full length *Bmy2* DNA sequences from genotypes Legacy, Harrington, Ashqelon, and PI 296897 revealed eight polymorphisms (Supplemental Fig. 1). Of the eight polymorphisms, there were six single nucleotide polymorphisms (SNPs), one 1-bp indel, and one region in Harrington that contained eight deletions and three SNPs. Ashqelon and PI 296897 had the exact same sequence and differed from Legacy in four positions. There were only two SNPs located within the exons and both were synonymous. The first synonymous SNP was located in the second exon at nucleotide 281, and the second synonymous SNP was located in the sixth exon at nucleotide 2328. All four genotypes studied here had the exact same-deduced Bmy2 amino acid sequence as the malt cv. Morex (Clark et al. 2005). In contrast, there have been numerous amino acid substitutions reported in the 'endosperm-specific' *β*-amylase, encoded by *Bmy1*, which also has the highly polymorphic third intron (Clark et al. 2003; Eglinton et al. 1998; Erkkilä et al. 1998; Erkkilä 1999; Erkkilä and Ahokas 2001; Filichkin et al. 2010; Ma et al. 2000, 2001; Sjakste and Zhuk 2006; Vinje et al. 2010, 2011).

Bmy2 mRNA levels during grain development

In this work, *Bmy2* mRNA levels were measured, using RT-qPCR, during the late maturation stage of grain development in all four genotypes (Fig. 2). Legacy was the calibrator for all three developmental stages and, thus, has a


a
Bmy1 TAGAGCATCA TCCATAGCCA GCATCCACAA TGGAGGTGAA CGTGAAAGGC AACTATGTCC AAGTCTACGT CATGCTCCCT GTAAGCTCCA TCCATTCAGA
Bmy2 ----CCAGTG TTTGTAGCCA GCAGCCACAA TGGCCGGGAA CATGCTAGCC AACTATGTCC AAGTCTACGT CATGCTCCCG GTAAGCACCA TCCAA-CAAG
Bmy1 CCAATCGCTG AGAACCACAC ACTAAAACTA TTTCAAGGAT CTAGTGCACA CATATACATT ATTGTTGTAC ATATAACATT GATACTTCTT GTAAAACTCT
Bmy2 CTAAGCACCA TGG--CACCG AC--AAACCA TTCCAAGGAT -----GAAAA AACTCACAT- --TGTCGTGC ATATGATACC CA-GCTTATC ----------
Bmy1 AATTCAAAGG GTGAAGAACA AGATCTGAGG CCTCAAATGA GTATTTTATT TGTACTAACC TTGACTACAC TTCCATTGTT GAAATAAATA AATAGCTGGA
Bmy2 ---------- ------AACA AGATCTGAGA CCTCGTACG- ---CAGTATC TCCACTAACC TTGTCTTCAC TTTCGTTGTG GGAATA---- ----GCTGGA
Bmy1 CGCCGTGAGC GTGAACAACA GGTTCGAGAA GGGCGACGAG CTGAGGGCGC AATTGAGGAA GCTGGTAGAG GCCGGTGTGG ATGGTGTCAT GGTAGACGTC
Bmy2 TGTCGTGAGC GTCGACAACA AGTTCGAGAA GGGCGACGAG ATCAGGGCGC AGCTGAAGAA GCTGACGGAG GCTGGCGTGG ACGGCGTCAT GATAGACGTC
Bmy1 TGGTGGGGCT TGGTGGAGGG CAAGGGCCCC AAGGCGTATG ACTGGTCCGC CTACAAGCAG TTGTTTGAGC TGGTGCAGAA GGCTGGGCTG AAGCTACAGG
Bmy2 TGGTGGGGGC TGGTGGAGGG CAAGGGCCCC AAGGCCTACG ACTGGAGCGC CTACAAGCAG GTCTTCGACC TGGTGCACGA GGCCAGGCTC AAGCTGCAGG
Bmy1 CCATCATGTC GTTCCACCAG TGTGGTGGCA ACGTCGGCGA CGCCGTCAAC ATCCCAATCC CACAGTGGGT GCGGGACGTC GGCACGCGTG ATCCCGACAT
Bmy2 CCATCATGTC GTTCCACCAG TGCGGTGGCA ACGTCGGCGA CGTAGTCAAC ATCCCCATCC CACAGTGGGT GCGGGATGTC GGCGCTACCG ACCCCGACAT
Bmy1 TTTCTACACC GACGGTCACG GGACTAGGAA CATTGAGTAC CTCACTCTTG GAGTTGATAA CCAGCCTCTC TTCCATGGAA GATCTGCCGT CCAGGTTAAT
Bmy2 TTTCTACACG AACCGCAGAG GGACGAGGAA CATCGAGTAC CTCACCCTTG GAGTGGATGA CCAACCTCTC TTCCATGGAA GAACTGCCGT CCAGGTTAGC
Bmy1 T----TAAAC CACCACTCTA GTTCTCTGAT GCATATTTAT ATAGAAGTTC AAGATGACAC CAAATACAAG CAAAAGGTTA AAGGTGCCAA AAACAGATAA
Bmy2 TAAGCTAGAT CACCATTCTA GCTCTCC--- ---TGCTTAT GTGTTGATTC A--------- ---------- ---------- ---------- ----------
Bmy1 GCAAAGAAAC AAAACCTAGC TAATGAAACA GTCTAGAGCC TATCAAAAAA AAAAAACATC GAGAAGGTGC CTAGAGCGGA TGGGTTTCGA CAACCCTTTA
Bmy2 ---------C ATGACTCAG- ---------- ---------- ---------- ---------- ---------- ---------A TGAGTCTTGG CAACAGCATG
Bmy1 GCTTTCATGC ATCTTTTTGG GAAAGGGTGA AAAACACCGT CCTTTAAGTC GATTGATGCA GGCAGCCTTC TATTGTTTGT AAGCTATCAG GAAATACAAA
Bmy2 GTTGTGAT-- -----TTTGA ---------- ---------- ----TAGGTC CAGTG----- ---------- ---------- ---------- ----------
Bmy1 ATTAATAGCT AGTTGTCATT TTAATAGTTG TAGCAAGCTT TGATTCTTCT TTTGTGGCTG TGACAGATGT ATCCCGATTA CATGACAAGC TTCAGGGAGA
Bmy2 ---------- --------TG CTCATGGTAG CA--AAGCTT TGATTCTTCT TT-GTGTCTG CTACAGATGT ATCATGATTA CATGGCGAGC TTCAGGGAAA
Bmy1 ACATGAAAGA CTTCTTGGAT GCTGGTGTTA TCGTCGACAT TGAAGTGGGA CTTGGCCCAG CTGGAGAGAT GAGGTACCCA TCATATCCTC AGAGCCACGG
Bmy2 ACATGAAAAA GTTCTTGGAT GCCGGTACCA TCGTGGACAT TGAAGTGGGA CTTGGCCCGG CTGGAGAGAT GAGGTACCCA TCCTATCCTC AGAGCCAGGG
Bmy1 ATGGTCGTTC CCAGGCATCG GAGAATTCAT CGTGAGTGTT TGTTTCCAAA CTAATAATCT TTCCTCTTCT GTTCCGATCA AATACTCCCT CCGTCCTAAA
Bmy2 ATGGGTCTTC CCAGGCATCG GAGAATTCAT CGTGAGTTTT CTTTTCCAAA ---------- -TACTCTTCT GCCCTTACAA AATACTGTT- ----------
Bmy1 ATTCTTGTCT TAGCTTTGTC TAGAAATTGA TGTATCTAAA TACTAAAACT TAACTAGATA CATTCATATC TAAACAAATC TAAGACAAGA ATTTTGGAAC
Bmy2 ---------- ---------- ---------- ---------- ---------- -------ATA CGTACATGT- ------AACA TGTGAT---- ------GAGC
Bmy1 GGAGGTAGTA TAATTTTCGA TGTAACTCAA CATGTGAATA TGTGATGGCC AAGTCACGAT CTACATTTAG AAAAGCTTTT CAAGAAACAA ACACCCAAAT
Bmy2 AAAGGCAGTT TAATCTT--- ---------- ---------- -----TGTCC AAGTGACGAT CTGCACTGAA AAGGGCT--- ---------- -CGCCCAACG
Bmy1 GAAAAGTGAT TCTTAAAGGA AAAAAGTGCA TGGATAAAAT GGCAGTTTCA GATTAGGACA AGGCGTGGTA AACCTGACTT GATCATTTCT GTTACCCAAT
Bmy2 GTAAAGTGAT TCT-GATCAA AAGTAACACT AGGATAAAAT GGCAATTTCA GATTATCAC- ---------- ---------- ---------- ----------
Bmy1 ATACCCCCGC CCAATTTTTG TATTTTCTTA TTCCTCCCAA ATAAGACATC ATATAAACAG GAAAATGGTA TTTAAAATAT GAGAAATATA TAGGATTCAT
Bmy2 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Bmy1 CTGTGCAACT TAAATACTTA AAATGATTTT TTATTGGAAA AGACTAATTT TATATATTTA TGGTACACCA AAAATCCAAA ATGTTTTCGG CACATTGTAG
Bmy2 ---------- ----TACTTA ---TGGCTTT T------GAG AGAC------ ---------- ---------- ---------- ---------- ----------
Bmy1 TCTCTATGAT TCATTGACCC CACACGTGCG GTTCCCTCAC GTCTAAACGT GGACTGTAAA TTTAAATTTT TAAATTGAGT GTCTTGGGTC TTGAATTTAA
Bmy2 ---------- ---------- ---------- ---------- ---------- ---------- -----ACTCT T--------- ---------- ----------
Bmy1 GACCTTTTGA CTCGGATACC ATGTAAAACT GCACGCACCA GACAGTTCAC CCATAAGCTC TTGCTTATGG AGAAAGGTGG GCTATGCATT TATACTTCAA
Bmy2 ---CTTTTGA ---------- --GCAAA--- ----GTATCA GAAG---CAA ACATAAGACC ATGGTT---- ---------- ---------CC CACAATTAAA
Bmy1 CAATAAGAAT AGTGAGGTAG CACAAACCTT GATGCAAGGC TAGATGACAC AGATGGGTGT GTGTGTTGGG GGGGGGGGGG ATGCACCTGA ATCCGATTTC
Bmy2 CGGTAAAAGC AGAGG----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

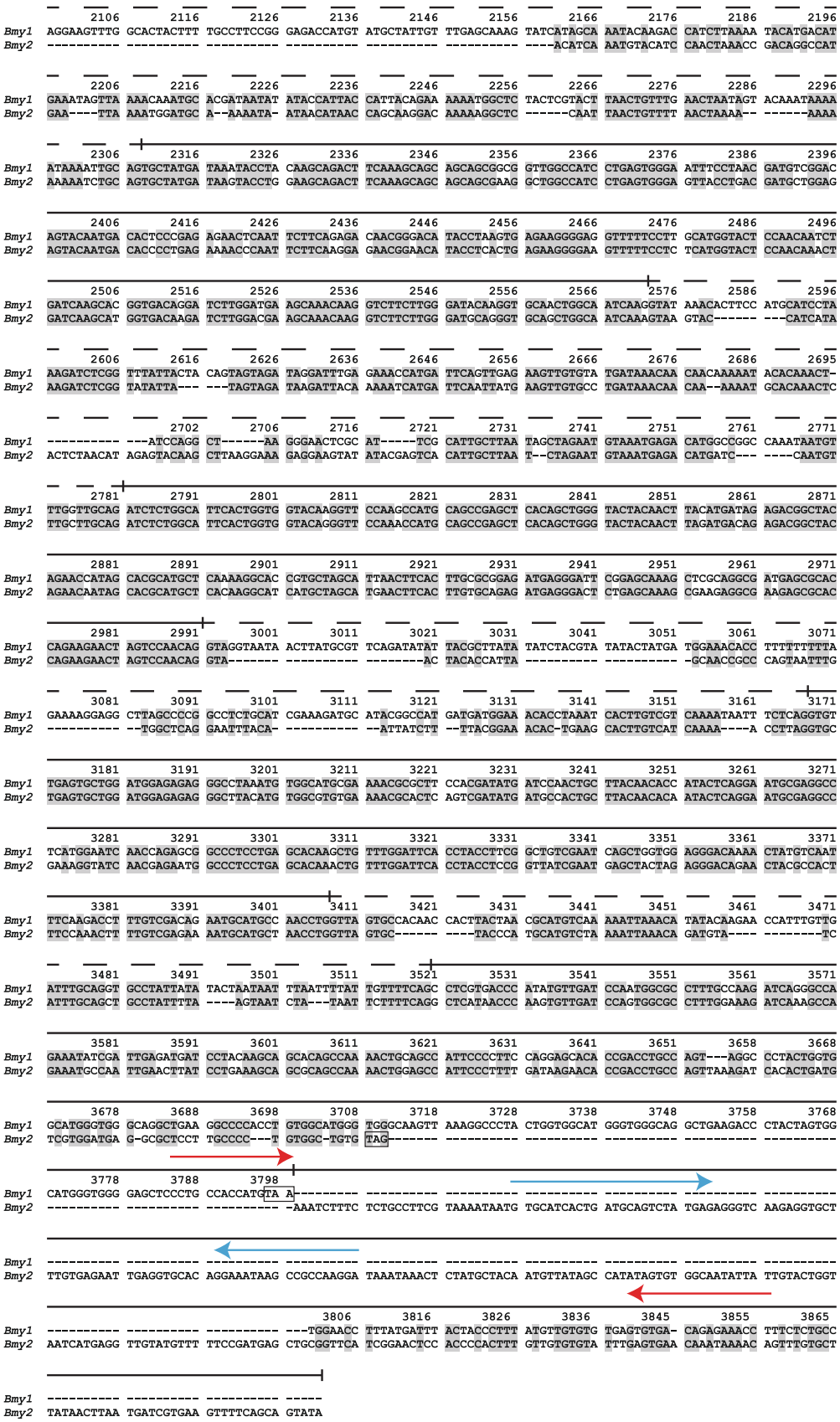


Fig. 1 continued

b

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                10         20         30         40         50
Bmy1   MEVNVKGNYV QVYVMLPLDA VSVNNRFEKG DELRAQLRKL VEAGVDGVMV
Bmy2   MAGNMLANYV QVYVMLPLDV VSVDNKFEKG DEIRAQLKKL TEAGVDGVMI
               60         70         80         90        100
Bmy1   DVWWGLVEGK GPKAYDWSAY KQLFELVQKA GLKLQAIMSF HQCGGNVGDA
Bmy2   DVWWGLVEGK GPKAYDWSAY KQVFDLVHEA RLKLQAIMSF HQCGGNVGDV
              110        120        130        140        150
Bmy1   VNIPIPQWVR DVGTRDPDIF YTDGHGTRNI EYLTLGVDNQ PLFHGRSAVQ
Bmy2   VNIPIPQWVR DVGATDPDIF YTNRRGTRNI EYLTLGVDDQ PLFHGRTAVQ
              160        170        180        190        200
Bmy1   MYADYMTSFR ENMKDFLDAG VIVDIEVGLG PAGEMRYPSY PQSHGWSFPG
Bmy2   MYHDYMASFR ENMKKFLDAG TIVDIEVGLG PAGEMRYPSY PQSQGWVFPG
              210        220        230        240        250
Bmy1   IGEFICYDKY LQADFKAAAA AVGHPEWEFP NDVGQYNDTP ERTQFFRDNG
Bmy2   IGEFICYDKY LEADFKAAAA KAGHPEWELP DDAGEYNDTP EKTQFFKENG
              260        270        280        290        300
Bmy1   TYLSEKGRFF LAWYSNNLIK HGDRILDEAN KVFLGYKVQL AIKISGIHWW
Bmy2   TYLTEKGKFF LSWYSNKLIK HGDKILDEAN KVFLGCRVQL AIKISGIHWW
              310        320        330        340        350
Bmy1   YKVPSHAAEL TAGYYNLHDR DGYRTIARML KRHRASINFT CAEMRDSEQS
Bmy2   YRVPNHAAEL TAGYYNLDDR DGYRTIARML TRHHASMNFT CAEMRDSEQS
              360        370        380        390        400
Bmy1   SQAMSAPEEL VQQVLSAGWR EGLNVACENA LPRYDPTAYN TILRNARPHG
Bmy2   EEAKSAPEEL VQQVLSAGWR EGLHVACENA LSRYDATAYN TILRNARPKG
              410        420        430        440        450
Bmy1   INQSGPPEHK LFGFTYLRLS NQLVEGQNYV NFKTFVDRMH ANLPRDPYVD
Bmy2   INENGPPEHK LFGFTYLRLS NELLEGQNYA TFQTFVEKMH ANLAHNPSVD
              460        470        480        490        500
Bmy1   PMAPLPRSGP EISIEMILQA AQPKLQPFPF QEHTDLPVGP TGGMGGQAEG
Bmy2   PVAPLERSKP EMPIELILKA AQPKLEPFPF DKNTDLPVKD HTDVVDEALL
              510        520        530
Bmy1   PTCGMGGQVK GPTGGMGGQA EDPTSGMGGE LPATM 535
Bmy2   APVAV----- ---------- ---------- ----- 505
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Fig. 1 continued

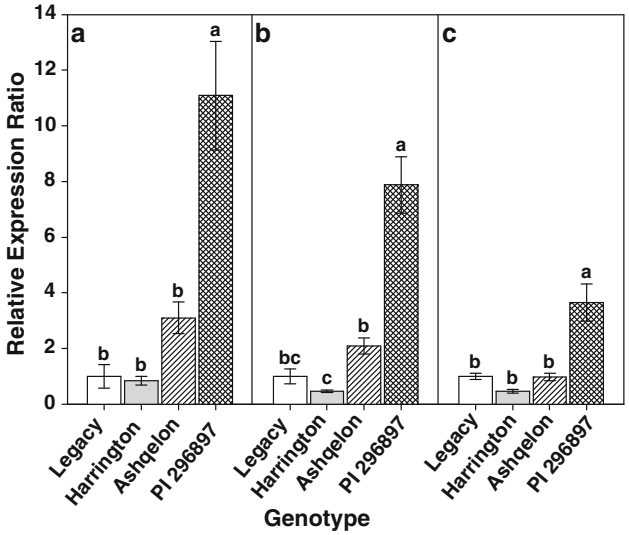

Fig. 2 Relative expression ratio of *Bmy2* in developing grain at 17, 19, and 21 days after anthesis (DAA) from Legacy, Harrington, Ashqelon, and PI 296897. Genotypes are calibrated to Legacy. **a** 17 DAA, **b** 19 DAA, **c** 21 DAA. *Bars* within the *same panel* that have *different letters* are significantly different ($P < 0.05$). *Bars* represent standard errors

RER of 1.0. *Bmy2* mRNA levels in PI 296897 were significantly higher than the *Bmy2* mRNA levels in Legacy, Harrington, and Ashqelon at 17, 19, and 21 DAA (Fig. 2). At 17 DAA, PI 296897 has 11.1-, 13.3-, and 3.6-fold more *Bmy2* transcript accumulation than Legacy, Harrington, and Ashqelon, respectively. *Bmy2* mRNA levels in PI 296897 decrease relative to Legacy at 19 and 21 DAA. In barley, *Bmy2* mRNA is present in high levels at 5 DAA and observed as late as 12 DAA (Jung et al. 2001; Shewry et al. 1988). In rye, *Bmy2* mRNA was detected in very small amounts at 10, 15, 20, and 25 days after pollination (Rorat et al. 1995). In a rye mutant, deficient in 'endosperm-specific' β-amylase, the *Bmy2* mRNA levels were 3.8- and 7.6-fold higher than the wild-type line, at 10 and 15 days after pollination, respectively (Rorat et al. 1995). However, the β-amylase deficient mutant did not have any observable *Bmy1* transcript present. In wheat, one of the two 'ubiquitous' β-amylase transcripts was detected in small amounts during late grain development and the other 'ubiquitous' β-amylase transcript was detected in larger amounts in grain development and germination (Wagner et al. 1999). Jung et al. (2001) did not find any barley *Bmy2* transcript after 9 DAA using RNA blots. The data reported here were collected using RT-qPCR which is much more sensitive than RNA blotting, and could be a reason for the detection of *Bmy2* mRNA when others had not. However, it is important to note that our data show relative expression ratios between four genotypes as opposed to absolute quantities of each transcript. The raw qPCR data indicate the *Bmy2* transcript levels in all four genotypes are very low (data not shown).

Bmy2 transcript levels were calibrated to 19 DAA for each genotype to determine the temporal accumulation of *Bmy2* mRNA from 17 to 21 DAA (Fig. 3). All four genotypes had a decrease in *Bmy2* transcript levels from 17 to 19 DAA with decreases of 3.3-, 4.4-, 4.4-, and 4.2-fold for Legacy, Harrington, Ashqelon, and PI 296897, respectively, although the change in Legacy was not statistically significant. There were no significant changes in *Bmy2* mRNA levels from 19 to 21 DAA for any genotype. The decrease in *Bmy2* mRNA levels from 17 to 19 DAA supports the previous reports of decreasing *Bmy2* mRNA as grain development proceeds (Jung et al. 2001; Rorat et al. 1995).

Accumulation of *Bmy1* and *Bmy2* mRNA

The ratio of *Bmy1* to *Bmy2* mRNA levels was determined for each genotype at three stages in grain development (Fig. 4). Levels of *Bmy1* mRNA were between twenty thousand and over one hundred thousand times higher than *Bmy2* mRNA levels in Legacy, Harrington, and Ashqelon at all developmental stages. Legacy is a U.S. six-row cultivar and Harrington is a Canadian two-row cultivar. Both cultivars are widely used in malting for brewing and have high diastatic power and malt extract values (Duke and Henson 2009b). The wild barleys used in this study have high protein content per unit weight (Vinje et al. 2011) making them unsuitable for malting and brewing. PI 296897, which had the highest *Bmy2* mRNA levels (Fig. 2), had around five thousand times more *Bmy1* than *Bmy2* mRNA levels at 17 DAA (Fig. 4). However, *Bmy1* mRNA levels of PI 296897 at 19 DAA are about thirteen thousand

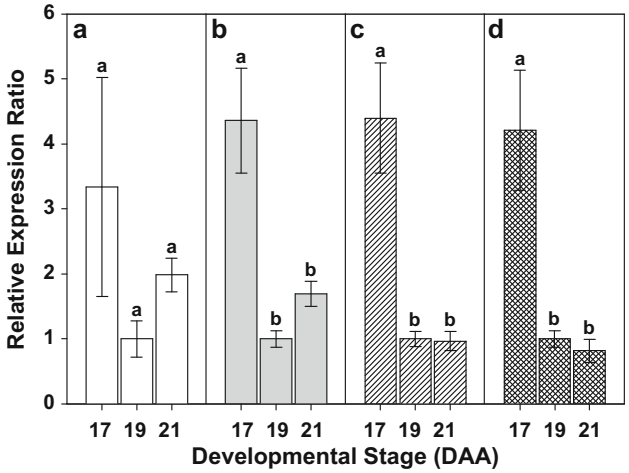

Fig. 3 Relative expression ratio of *Bmy2* mRNA in developing grain at 17, 19, and 21 days after anthesis (DAA) from Legacy, Harrington, Ashqelon, and PI 296897. Each genotype is calibrated to 19 DAA. **a** Legacy, **b** Harrington, **c** Ashqelon, **d** PI 296897. *Bars* within the *same panel* that have *different letters* are significantly different ($P < 0.05$). *Bars* represent standard errors

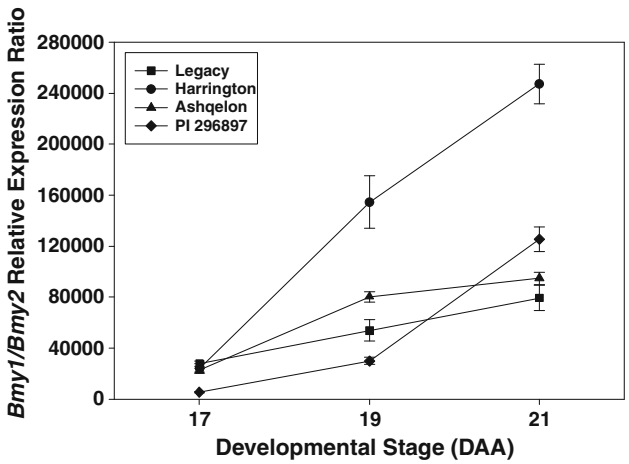

Fig. 4 *Bmy1*/*Bmy2* relative expression ratio in developing grain at 17, 19, and 21 days after anthesis from Legacy, Harrington, Ashqelon, and PI 296897. *Bars* represent standard error

times more than *Bmy2* and at 21 DAA the ratio of *Bmy1* to *Bmy2* mRNA is over one hundred thousand. This is vastly different than a recent report that found *Bmy2* expressed in the endosperm of developing barley grain at about half the rate as *Bmy1* (Radchuk et al. 2009) and more in line with the studies of Jung et al. (2001) and Rorat et al. (1995). When studying barley *Bmy1* and *Bmy2*, it is imperative that gene-specific probes or primers be used. Jung et al. (2001) created a *Bmy2* probe using 1.3 kb that aligned to *Bmy1* with a high degree of identity, and identified β-amylase RNA in early and late grain development. However during later grain development, the hybridization of the 1.3 kb *Bmy2* probe was markedly weaker than when a *Bmy1*-specific probe was used indicating that the *Bmy2* probe was hybridizing weakly to the abundant *Bmy1* mRNA. The specificity of the qPCR primers used in this research was accomplished by creating the forward *Bmy1* primer to align within a region in the seventh exon that does not have any significant similarities with the *Bmy2* gene and the *Bmy2* primer set was created in the 3′ UTR also in a region that did not share any significant similarities with *Bmy1* (Fig. 1a and Table 1). Also, qPCR reactions using the *Bmy1* primer set with a plasmid containing the *Bmy2* gene and vice versa were conducted that determined specificity (data not shown). Radchuk et al. (2009) used the reference EST HZ50o15 to represent *Bmy2* in their macroarray study, which is 424 bp and aligns to the first 700 bp of the Legacy *Bmy1* coding region with 83% identities (Yellow bar in Fig. 1a). It is possible that the high levels of *Bmy2* transcript reported by Radchuk et al. (2009) in the endosperm during late grain development were caused by partial hybridization of highly abundant *Bmy1* mRNA to their *Bmy2* sequence used on the macroarray.

Accumulation of Bmy1 and Bmy2 protein

The presence of Bmy2 during grain development was detected using Bmy2-specific antibodies (Fig. 5). This, as far as we know, is the first documentation of barley Bmy2 protein in any tissue. Bmy2 protein was not observed at 17 DAA despite the *Bmy2* mRNA levels being at their highest at 17 DAA (Fig. 3). At 19 DAA, Bmy2 protein was observed at very low levels. Similarly, the gene product of the barley *sbeIIb* gene was not detected until 5 days after the maximal mRNA accumulation was observed (Mutisya et al. 2003). In rye, Rorat et al. (1995) observed an increase in Bmy2 protein levels 20 days after pollination despite a decrease in *Bmy2* transcript levels from 10 to 30 days after pollination. Barley *Bmy2* mRNA levels decrease from 17 to 19 DAA and remain the same at 21 DAA, whereas the Bmy2 protein levels increase from 19 to 21 DAA (Figs. 3, 5). The amount of Bmy2 increases from 21 DAA to maturity indicates that, perhaps, *Bmy2* expression is bi-modal and expression is re-initiated in the developing grain after 21 DAA. Low levels of Bmy2 protein are not unexpected due to the extremely low levels of *Bmy2* mRNA (Fig. 5). Low levels of Bmy2 protein are observed in Legacy, Harrington, and Ashqelon at 21 DAA. Radchuk et al. (2009) identified *Bmy2* mRNA in the pericarp and endosperm, but did not identify *Bmy1* mRNA in the pericarp. Jung et al. (2001) also found *Bmy2* mRNA in developing barley grain (5 DAA) that contained tissue predominantly of maternal-origin, including pericarp. It appears that the *Bmy2* expression that occurs early in the grain development is caused by maternal tissue-specific gene expression, whereas the

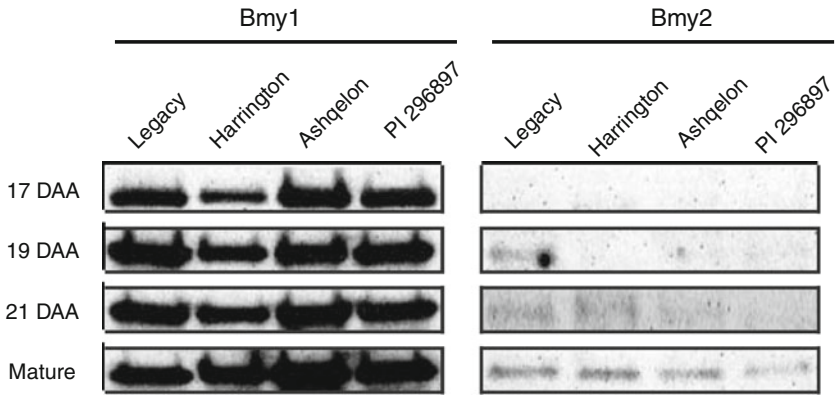

Fig. 5 Bmy1 and Bmy2 protein levels in developing grain at 17, 19, and 21 days after anthesis (DAA), and maturity from Legacy, Harrington, Ashqelon, and PI 296897. Five micrograms of total protein is loaded in each lane. All exposure times were 120 s, except for Bmy2 at 21 DAA which was 180 s

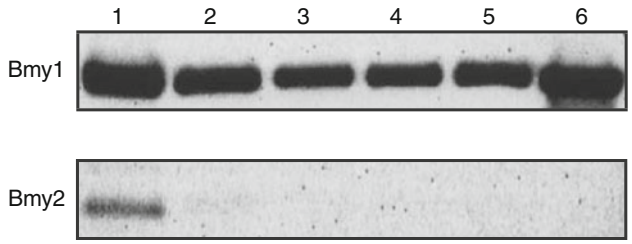

Fig. 6 Bmy1 and Bmy2 protein levels in the soluble and insoluble fractions of mature barley grain. Six successive 1 h extractions with *Lanes 1* through *5* depict salt extractions. *Lane 6* is an extraction with the addition of 100 mM cysteine. Six micrograms of total protein is loaded in each lane. 500 s exposure time

Bmy2 that was observed to accumulate in the mature grain could be derived from maternal and/or filial tissue-specific gene expression.

Mature grain was found to contain more Bmy2 protein than all other developmental stages studied (Fig. 5). β-Amylase is stored in mature barley grain as either bound (insoluble) or free (soluble) β-amylase, where free β-amylase is converted to bound β-amylase during the desiccation phase of grain development (Hara-Nishimura et al. 1986). During germination the bound form is released by the reduction of disulphide bonds or via proteolytic cleavage resulting in a completely active enzyme (Guerin et al. 1992; Sopanen and Laurière 1989). In this work, Bmy2 was found to be located only in the soluble fraction of the mature barley grain (Fig. 6). It was previously assumed that Bmy1 was the only β-amylase that was stored, but these data show that Bmy2 as well as Bmy1 is stored in the mature grain.

Low levels of Bmy2 observed in the developing and mature grain likely preclude the Bmy2 protein from having a significant contribution to the overall β-amylase activity in the developing and mature grain. By far most of the β-amylase present in both the developing and mature grain is Bmy1 (Fig. 5). The prediction by Radchuk et al. (2009) is that Bmy2 may be contributing to as much as half of the β-amylase activity in developing and mature grain could only be true, if Bmy2 had an extraordinarily higher K_{cat} than Bmy1. Our data indicate that Bmy1 is by far the major contributor to β-amylase activity based upon the considerably larger quantities of protein observed. However, the presence of the Bmy2 protein, albeit small, during development and maturity is interesting and requires further examination to determine if the *Bmy2* gene has a role during development and/or germination of barley grain.

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