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ORIGINAL PAPER

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Coding region single nucleotide polymorphism in the barley low-pI, α-amylase gene *Amy32b*

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Abstract Barley α-amylase variability influences the quality of barley grain in the brewing, feed and food industries. α-Amylase proteins are encoded by multigene families in cereals, and this study focused on the barley *Amy32b* gene. We identified coding region single nucleotide polymorphism (cSNP) and insertion/deletion variation in DNA sequences, which resulted in amino acid substitution and stop codon formation, respectively. The substitution affected the β1 strand in domain C, whereas the stop codon removed the β5 strand. Possible effects of these changes on the protein are discussed. A cSNP in the coding region of the *Amy32b* gene was used as a specific marker to map *Amy32b* loci on chromosome 7H.

Keywords α-Amylase · *Amy32b* · Barley · Coding region single nucleotide polymorphism · Linkage map · Protein sequence

Introduction

α-Amylases (α-1,4-D-glucan glucanohydrolase, EC 3.2.1.1) hydrolyze internal α-1,4-glucosidic bonds in starch, maltodextrins and maltooligosaccharides (Yamamoto 1995). In barley, isozymes of α-amylase are classified on the basis of their isoelectric points (pIs) into two groups (low and high pI). Each group consists of multiple isozymes (Muthurkrishnan et al. 1984). Several nomenclatures for α-amylase isoforms and their genes are found in the literature. Jones and Jacobsen (1991) classed Amy1 as a group of low-pI α-amylases and Amy2 as a group of high-pI α-amylases. Rogers (1985) described high-pI and low-pI α-amylases as AmyA and AmyB, respectively.

Huang et al. (1992) divided α-amylase genes in Triticae into three subfamilies (Amy1, Amy2 and Amy3), grouping Amy1 and Amy2 into class AmyA and Amy3 into class AmyB. An additional subfamily of α-amylase genes (Amy3) has been identified in wheat (Huang et al. 1992). Some authors (Whittier et al. 1987; Rogers and Milliman 1984b) named low-pI isoenzyme genes as type A and high pI as type B. However, most authors (e.g. Knox et al. 1987; Zwickert et al. 1996; Rondenburg et al. 2000) refer to isoenzyme gene families as Amy1 (high pI = α-amylase2) and Amy2 (low pI = α-amylase1), and we use this nomenclature in this paper.

The *Amy1* locus is located on chromosome 6H, whereas *Amy2* is on chromosome 7H (Brown and Jacobsen 1982). The total number of α-amylase gene copies on each chromosome is not known with certainty. However, Muthurkrishanan et al. (1984) reported that there were three or more genes on 7H chromosome, whereas chromosome 6H carried six or more genes.

The *Amy32b* gene was characterised by Whittier et al. (1987). It encodes a low-pI isozyme and is expressed in barley aleurone cells under the control of the plant hormones gibberellic acid and abscisic acid. We revealed a G/A coding region single nucleotide polymorphism (cSNP) in the fourth exon of the gene by sequencing. The cSNP distinguished cultivars among a studied collection of 70 spring barleys (Table 1). We herein describe the cSNP in relation to deduced amino acid sequence, align it with other DNA and protein sequences of barley α-amylases and discuss the possible impact of the amino acid substitution on the protein. We also describe an

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Table 1 Collection of 70 spring barley genotypes evaluated based on *Amy32b* coding region single nucleotide polymorphism (*cSNP*) (G/A) assay

Cultivar	Kernel type[a]	Country[b]	*Amy32b* cSNP (G/A)	Cultivar	Kernel type[a]	Country[b]	*Amy32b* cSNP (G/A)	Cultivar	Kernel type[a]	Country[b]	*Amy32b* cSNP (G/A)
Ai Gan Qi	nud	CHN	A								
Amulet	cov	CSK	A	KM2001	nud	CZE	A	MK1332	nud	MNG	A
Annabel	cov	DEU	A	KM2037	nud	CZE	A	Nabavi	nud	JPN	A
Barke	cov	DEU	A	KM2045	nud	CZE	A	No. 94609-D7	cov	DEU	A
Buck	nud	CAN	A	KM2062	nud	CZE	A	Nordus	cov	DEU	A
CDC Candle	nud, waxy	CAN	G	KM2082/1	nud	CZE	A	Nudum 7566	nud	MNG	A
CI12953	nud	ETH	A	KM2082/2	nud	CZE	A	Ohara	cov	AUT	A
Ebsdorfer nackt	nud	DEU	G	KM2083	nud	CZE	A	Olbram	cov	CZE	A
Forum	cov	CSK	A	KM2084	nud	CZE	A	Orbit	cov	CSK	A
Galan	cov	CSK	A	VKM2087	nud	CZE	A	Pax	cov	SVK	A
Gopal	nud	IND	G	KM2092	nud	CZE	A	Pejas	cov	CZE	A
H2173	nud	ETH	A	KM2283	nud	CZE	A	Primus	cov	CZE	A
H2176	nud	ETH	A	Kompakt	cov	SVK	A	Sabel	cov	GBR	A
H2186	nud	ETH	A	Krona	cov	DEU	A	Scarlett	cov	DEU	A
H2193	nud	ETH	A	Lyallpur 3647	nud	IND	G	Selecta	cov	AUT	A
Harrington	cov	CAN	A	M1070	cov, lpa	USA	A	Shimabara	nud	PRK	G
HB803	nud, waxy	CAN	G	M422	cov, lpa	USA	A	Taiga	nud	DEU	A
Chiro Chinko	nud	JPN	A	M635	cov, lpa	USA	A	Thuringia	cov	DEU	A
Jarek	cov	CSK	A	M955	cov, lpa	USA	A	Tolar	cov	CZE	A
Jersey	cov	NLD	A	Madonna	cov	DEU	A	Wabet	cov, waxy	USA	A
Kavkazyj Golozernyj	nud	SUN	G	Margit	cov	SWE	A	Wanubet	nud, waxy	USA	G
KM1057	nud	CZE	A	Maridol	cov	CZE	A	Wanupana	ud, waxy	USA	G
KM1771	nud	CZE	A	Merlin	nud, waxy	CAN	G	Wapana	cov, waxy	USA	A
KM1910	nud	CZE	A	Milonov nudum	nud	CSK	A	Washonubet	nud, waxy	USA	G

[a] *cov* Covered, *nud* nudum (hull-less), *lpa* low-phytic acid mutants

[b] Country 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* Great Britain, *IND* India, *JPN* Japan, *MNG* Mongolia, *NLD* Netherlands, *PRK* Korea (North), *SVK* Slovak Republic, *SWE* Sweden, *SUN* Former Soviet Union

additional insertion/deletion event affecting the length of the protein and establish the map location of the *Amy32b* locus on 7H chromosome.

Materials and methods

Plant material

The collection of 70 spring barley varieties used in this study derived from 16 countries were provided from genetic resources held by the Agricultural Research Institute, Kromeriz (Table 1).

A population of 141 F_2 plants from a 'Nordus' × 'CDC Candle' cross was used for mapping. 'Nordus' is a covered (*Nud*) spring malting cultivar from Germany (NORDSAAT Saatzuchtgesellschaft), registered for cultivation in the Czech Republic in 1998. 'CDC Candle' is a waxy hull-less (or naked, *nud*) spring barley, bred at the CDC Saskatoon, Canada, and registered in 1995 (labelling No HB 313 waxy). Wheat–barley whole chromosome addition lines (provided by JIC, Norwich, UK) were used to confirm the chromosomal location of the *Amy32b* gene.

DNA extraction

The DNA from young leaves was extracted using the DNeasy 96 kit (Qiagen, Hilden, Germany). The extraction protocol followed the manufacturer's recommendations apart from the first step, in which 50 mg of frozen leaves were directly transferred into the tubes containing carbide beads and mixture of the AP1 Buffer, RnaseA and DX Reagent (Qiagen).

PCR amplification

The 719 bp of PCR product for sequencing analysis, which included 297 bp of exon 4, was amplified by PCR using primers at positions 1750 bp and 2469 bp (including primer sequences) in the *Amy32b* genomic sequence (GenBank accession: X05166). Primer sequences were as follows: 5′-GGCGACGGTAAGCCCAACTA-3′ (forward) and 5′-GGTGGATGAGTGGAGGCTTC-3′(reverse). The total PCR volume was 20 µl containing one-time buffer with 10 mM $MgCl_2$ (Invitrogene, UK), each dNTP at 200 µM (Gibco–BRL), each primer at 0.1 μ M, and 1 U *Pfu* polymerase (TaKaRa). The PCR amplification was carried out for 2 min at 94°C, followed by 30 cycles of 30 s 94°C, 30 s 58°C, 1 min 72°C, with a final 5 min incubation at 72°C using UNOII thermocycler (Schöeller Instruments).

The 205-bp fragment for cSNP detection was generated using primer pair 5′AGCTCAGCCTCGGTCTCAGT 3′ (forward) 5′ACGTAGGCGTCTCCTTCGTG 3′ (reverse). The PCR amplification was performed in 20-µl reaction volumes containing one-time buffer with 15 mM $MgCl_2$ (Qiagen), each dNTP at 200 μ M (Gibco–BRL), each primer at 0.1 μ M, 1 U *Taq* DNA polymerase (Qiagen) and 100 ng template DNA. The PCR amplification was carried out for 2 min at 94°C, followed by 30 cycles of 30 s 94°C, 30 s 58°C, 30 s 72°C, with final 5 min incubation at 72°C using a thermocycler UNOII (Schöeller Instruments).

Sequencing analysis

The 719-bp PCR products of G and A haplotypes ('CDC Candle' and 'Nordus', respectively) were purified using a PCR purification kit (Qiagen). Sequence analysis was performed by the dye terminator method (BigDye, version 3.0) using an ABI PRISM 3100 sequencer system (Applied Biosystems, Warrington, UK) using appropriate forward and reverse primers. Both DNA strands of PCR products were sequenced three times. The sequences of both haplotypes were named as SEQG and SEQA.

cSNP detection

The 205-bp amplified region comprises parts of intron 3 and exon 4 and contains a G ('CDC Candle')/A('Nordus') SNP. The presence of a G creates an *Xho*I restriction site that was used to score the cSNP. Cleaved products (174-bp and 29-bp bands) corresponded to G haplotypes, uncleaved (205 bp) were A haplotypes.

Multiple alignment analysis

Known α-amylase (*Amy2*) genomic DNA sequence (HVMY32B = GenBank accession: X05166, Whittier et al. 1987) and cDNA (BLYAMYAA = GenBank accession: J01236, Rogers and Milliman 1984a; BLYAMY2A = GenBank accession: M17127, Knox et al. 1987) were aligned with sequenced 719-bp PCR fragments of G and A haplotypes (SEQG, SEQA).

Translated SEQG and SEQA and protein primary sequences of low-pI and high-pI α-amylases HVMY32B (GenBank accession: X05166, Whittier et al. 1987), BLYAMYAA (GenBank accession: M17127, Knox et al. 1987), BLYAMY2A (GenBank accession: M17127, Knox et al. 1987) and HVAMY56 (X15227, Rahmatullah et al. 1989), HVAMY152 (GenBank accession: X15226, Rahmatullah et al. 1989) were also aligned. For these purposes we used T-Coffee, version 1.41, in Clustal format (Notredame et al. 2000).

Linkage map construction

Markers within approximately 15 cM of the *HvAMY2* simple sequence repeat (SSR) locus (clone E, BLYAMYAA, Ramsay et al. 2000) were selected from pub-

lished maps. 'Nordus' and 'CDC Candle' were screened for polymorphism, and this resulted in five SSR markers (Bmag0321, Ebmac0785, GMS46, Ebmac0764 and Bmag0120 from Ramsay et al. 2000), one cleaved amplified polymorphic sequence/sequence-tagged site (MWG2031) marker and three sequence-characterized amplified regions (KT1, KT2 and KT3), which were described and developed by Kikuchi et al. (2003). The character of grain (covered *Nud* or naked *nud*) was determined from F_2 plants. All the molecular markers used were co-dominant, whereas the phenotype marker *nud* was recessive. The linkage map was constructed using MapManager QTX, version 0.23 (Manly et al. 2001), and map distances were calculated using the Kosambi function.

Results

cSNP detection among 70 barleys

All 70 barley genotypes were tested using the cSNP detection assays with *Xho*I restriction endonuclease. All the covered barleys and 27 hull-less barleys possessed the non-cleaved allele (A haplotype). The remaining five hull-less (two landraces and three Asian cultivars) and all six waxy hull-less barleys had the cleaved allele (G haplotype, see Table 1).

DNA and protein sequences multiple alignment

We aligned known genomic DNA and cDNA sequences of barley *Amy2* group genes with the sequenced 719-bp *Amy32b* PCR fragments generated from G and A haplotypes ('CDC Candle' and 'Nordus', respectively). Sequences of 'CDC Candle' and 'Nordus' differed by a single nucleotide substitution in exon 4 (Fig. 1a, box 1). The similarity of the sequenced fragments with *Amy32b* genomic sequence (HVMY32B) was 99%, with clone cDNA sequence BLYAMYAA (clone E) and BLYAMY2A (clone p155.3) the similarities were 92% and 84%, respectively. HVMY32B differed from other sequences by a single base insertion/deletion event that created a stop codon (Fig. 1a, box 2).

We also compared the protein sequences of barley low-pI and high-pI α-amylases. Deduced protein sequence (HVMY32B) encoded by *Amy32b* was 98% similar to sequences of low-pI α-amylase BLYAMYAA, 91% to BLYAMY2A (low pI) and 75% to high-pI isozymes (HVAMY56 and HVAMY152) (Fig. 1b). The translated SEQG and SEQA from 'CDC Candle' and 'Nordus' clearly group with other low-pI proteins. The cSNP difference between 'CDC Candle' and 'Nordus' results in a glutamic acid (E)-to-lysine (K) substitution. This acidic to basic change lies in the β1 strand region (Fig. 1b, Box 1). The stop codon in HVMY32B truncates the protein and removes the β5 strand (Fig. 1b, box 2).

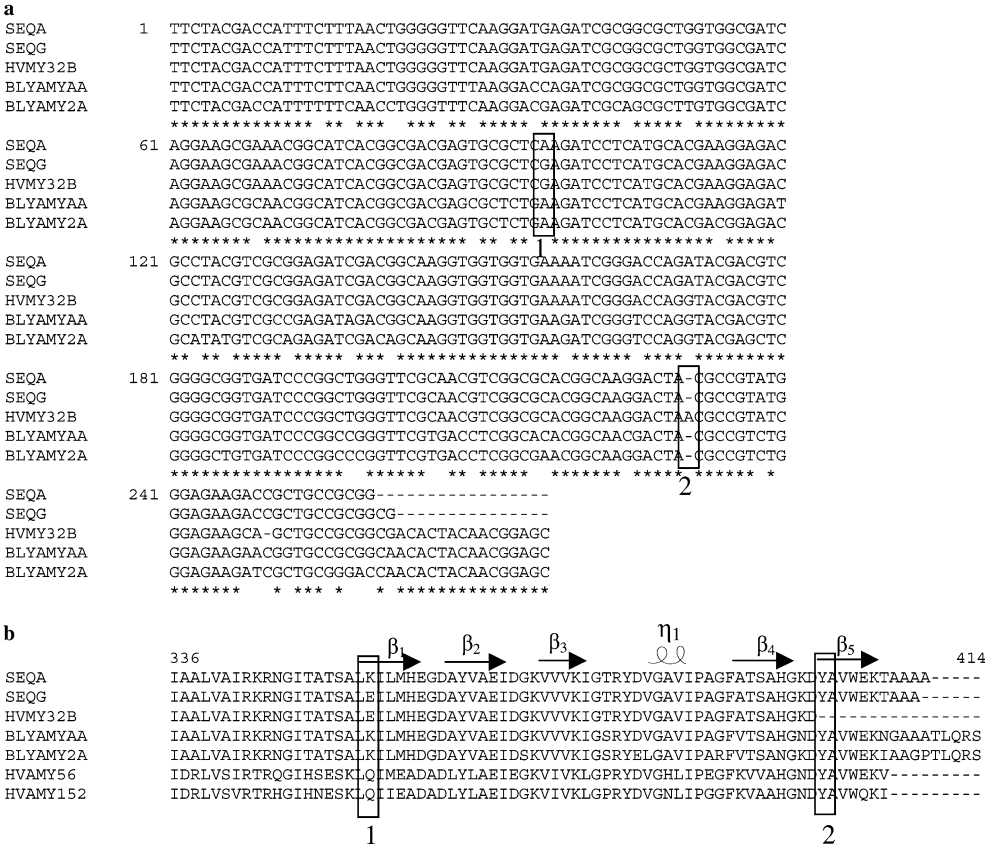

Fig. 1 **a** Alignment of exon 4 sequences of *Amy2* genes. *Box 1* shows the G/A coding region single nucleotide polymorphism (cSNP). *Box 2* shows a 1-bp insertion/deletion polymorphism. **b** Domain C alignments of Amy2 and Amy1 α-amylase proteins. The *arrows* indicate β strands, *helix* indicates an α *helix*. *Box 1* shows the position of the glutamic acid (E)–to-lysine (K) substitution resulting from the cSNP. *Box 2* shows the truncation of the HVMY32B protein by a stop codon formed by the insertion/deletion polymorphism. Sequences are: SEQG-sequence of G haplotype ('CDC Candle'), SEQA-sequence of A haplotype ('Nordus'), HVMY32B-*Amy32b* (low pI), BLYAMYAA-clone E (low pI), BLYAMY2A-clone p155.3 (low pI), HVAMY56-clone gRAmy152 (high pI); HVAMY152-clone gRAmy152 (high pI)

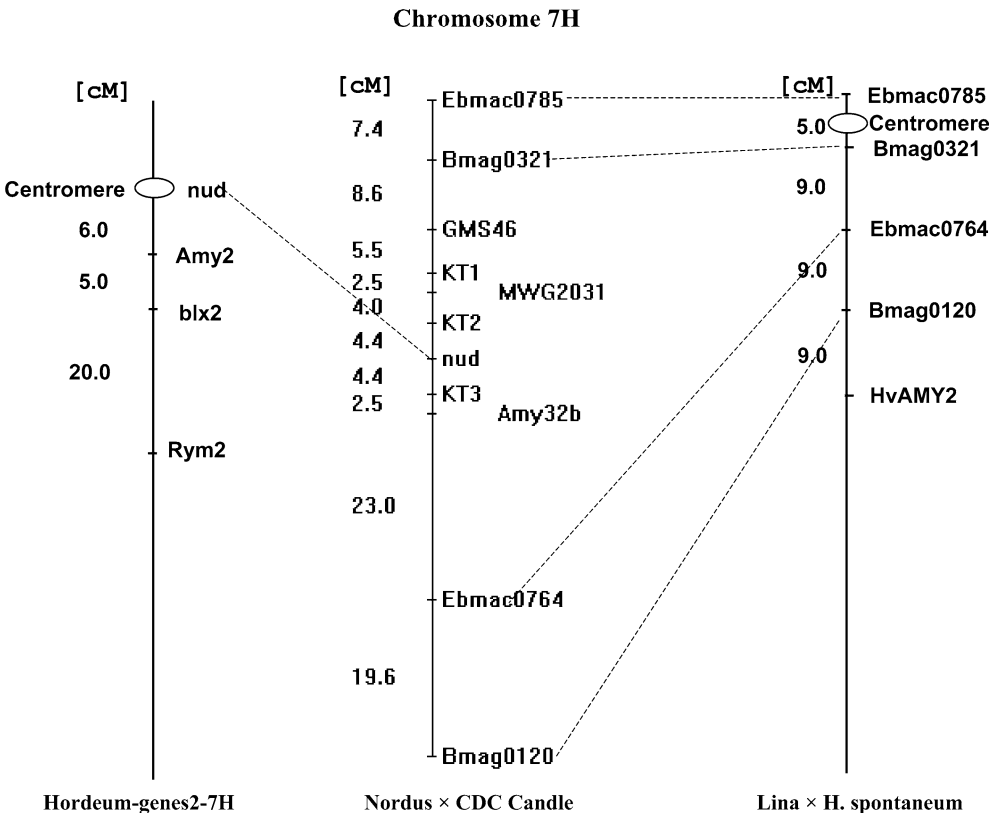

Fig. 2 Barley 7H linkage maps. From left to right, the morphological marker map of Franckowiak (1997), the 'Nordus' × 'CDC Candle' map and the SSR-based 7H chromosome map of Ramsay et al. (2000). The positions of common SSRs and the *nud* marker are connected by *dotted lines*

Genetic mapping of *Amy32b*

We first used *Amy32b*-specific primers on DNA from wheat–barley chromosome addition lines. The specific PCR product was generated only from the barley chromosome 7H addition line. Having confirmed the gene was located on chromosome 7H, *Amy23b* was mapped in the 'Nordus' × 'CDC Candle' cross using *Xho*I digestion of a 205-bp amplicon containing the cSNP. Nine additional molecular markers and the naked seed (*nud*) morphological marker were also scored. The resulting linkage map is shown in Fig. 2. *Amy32b* was mapped 2.5 cM distal to KT3 and 6.9 cM distal to *nud*. Comparison to the barley 7H map of Ramsay et al. (2000) showed that the common SSR markers occurred in the same order but were separated by greater genetic distances in the 'Nordus' × 'CDC Candle' cross (Fig. 2).

Discussion

A number of cDNA clones of α-amylases have been isolated and sequenced. The sequence homology within the coding region is 90–95% within the high- and low-pI groups and about 75% between groups (Jones and Jacobsen 1991).

The genomic DNA sequence of *Amy32b* (X05166, Whittier et al. 1987) was used for this study. The *Amy32b* gene product has previously been described as a low-pI type (Whittier et al. 1987) and as a high-pI type (Rogers and Milliman 1984b). The alignments in Fig. 1b suggest that the low-pI characterization is correct.

The cSNP (G/A) polymorphism that distinguishes 'CDC Candle' and 'Nordus' resulted in a E-to-K substitution at position 355 in the Amy32b protein. This is within the region of domain C that forms the β1 strand (Robert et al. 2003; Fig. 1b). Domain C is important in determining enzyme-binding efficiency for starch granules, but comparison of different sequences (Fig. 1b) shows this region to be variable among barley α-amylases. The E355-to-K355 substitution might influence the structure of domain C, but based on the known role of domain C in substrate recognition and binding (Robert et al. 2003), the amino acid sequence in this region seems not to be a critical site for sugar binding or substrate recognition; however, the possible conformation change might influence substrate binding site two amino acids upstream. If the E-to-K substitution affected enzyme activity, this would be expected to have a quantitative effect on final enzyme activity as all the α-amylase genes of each pI group are coordinately expressed in germinating seed (Whittier et al. 1987; Jones and Jacobsen 1991). The insertion/deletion change in *Amy32b* relative to the other sequences (Fig. 1a, b) has a more obvious impact, as it results in a premature stop codon that removes the β5 strand of domain C. The domain C isozyme structure of *Amy32b* (X01566, Whittier et al. 1987) has only four β strands and one α helix. Such a protein is likely to be nonfunctional, as it was reported that the highly conserved region 401VWEK404 (see Fig. 1b) was

important for structural stability (Tibbot et al. 2002). It was observed that low-pI amylases lacking these residues had low-to-no measurable activity. Moreover, Tyrosin Y399 is a sugar-binding site (Robert et al. 2003), which is also deleted in the protein with removed β5 strand. Based on abovementioned findings we can conclude that the SEQA ('Nordus') and SEQG ('CDC Candle') sequences of *Amy32b* probably present the functional alleles of the gene (Fig. 1b).

The *Amy2* genes encoding low-pI isozymes have previously been located on chromosome 7H (Brown and Jacobsen 1982). This is also the case for *Amy32b* (Fig. 2). The overall structure of the map was similar to previous studies with the *nud* gene being close to the centromere (Franckowiak 1997; Becker and Heun 1995; Qi et al. 1998) and closely linked to the KT markers (Kikuchi et al. 2003). The linkage distance between *Amy2* (clone E, HVAMYAA) and *nud* on HordeumGenes2-7H map (Franckowiak 1997) is 6.0 cM compared to 6.9 cM for *nud* to *Amy32b* (Fig. 2). When compared to the SSR map of Ramsay et al. (2000), SSRs flanking the centromere behaved similarly, but Ebmac0764 and Bmag0120 were much further away. This is attributable to a difference in the distribution of recombination between the barley cross ('Nordus' × 'CDC Candle') and the barley × *H. spontaneum* cross (Ramsay et al. 2000). The most significant difference is the position of *Amy32b* compared to that of *HvAMY2* (Fig. 2). This suggests that *Amy* genes occupy two distinct loci on 7H. We could not include the SSR marker Hvamy2 (clone E, HVAMYAA) in our mapping study because of a lack of polymorphism. To confirm this we intend to develop a new polymorphic marker for *HvAMY2* or to map both genes in other crosses where both are polymorphic.

We can conclude that *Amy32b* is a member of group of α-amylase genes localised on 7H chromosome and is closely linked to the *nud* locus and possibly linked to a second low-pI gene (*HvAMY2*). The characterised *Amy32b* cSNP probably has a low effect on final quantitative parameters of α-amylase; however, it can be a useful molecular marker for studying barley diversity and for mapping studies. The insertion/deletion affecting the β5 strand can be used as a molecular marker for detection of functional or nonfunctional alleles.

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