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Brief report
Special barley β-amylase allele in a Finnish landrace line HA52 with high grain enzyme activity

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Barley (*Hordeum vulgare* L.) grain mainly consists of starch, which provides energy during germination and seedling growth (for review, see MACGREGOR and FINCHER 1993). Starch degradation requires concerted action of limit dextrinase, β-amylase, α-glucosidase (SUN and HENSON 1990) and α-amylase. Liberation of maltose and limit dextrins from the nonreducing ends of starch is catalysed by β-amylase (1,4-α-D-glucan maltohydrolase, EC 3.2.1.2) (ROBYT and WHELAN 1968; SOPANEN and LAURIÈRE 1989). Being synthesised during grain development (KREIS et al. 1987), β-amylase is one of the major proteins found in the starchy endosperm (HEJGAARD and BOISEN 1980). The endosperm β-amylase gene (β-*amy1*) is located in chromosome 4H (KREIS et al. 1987). Another β-amylase gene (β-*amy2*), called ubiquitous, is located in chromosome 2H and the protein is found in leaves and roots (KREIS et al. 1988; SHARP et al. 1988).

Modern plant breeding has reduced the genetic variability in domesticated barley (THOMPSON et al. 1990; FORSTER et al. 1991) and declined landraces in Finland prior to about 1950 (AHOKAS 2000). Large genetic diversity is found in wild barley *H. vulgare* ssp. *spontaneum* (K. Koch) A. & Gr., abbreviated as *H. spontaneum* (AHOKAS 1982; ZHANG et al. 1993; SAGHAI MAROOF et al. 1995). High β-amylase trait was inherited in the backcrossed progeny of *H. spontaneum* and domesticated barley (AHOKAS and ERKKILÄ 1992). However, *H. spontaneum* was not widely used in breeding for several reasons such as shattering of spikelets, adverse growth rythm at high latitudes, and apparent low grain yield.

Because landraces are better adapted to local environments and are morphologically closer to desired domesticated types than wild barley, they are a more suitable source of genetic variation for barley breeding. Compared with the variation in wild barley grown in Finland (AHOKAS and NASKALI 1990), landraces had significantly higher mean activities for α- and β-amylase and for β-glucanase (AHOKAS and POUKKULA 1999). The line HA52 was selected from Finnish landraces (AHOKAS 1977), a diverse genetic resource (AHOKAS and MANNINEN 2001). Later, HA52 was found to have a high β-amylase activity (AHOKAS et al. 1996) and also a high thermostability (AHOKAS and MANNINEN 2000).

The result presented here is an extension previous studies of β-*amy1* alleles found in barley (ERKKILÄ et al. 1998; ERKKILÄ 1999). YOSHIGI et al. (1995) described an allele in cv. Haruna Nijo (EMBL–GenBank database accession number D49999). We have previously found two β-*amy1* alleles, one in cv. Adorra and another in *H. spontaneum* strain PI 296897 (EMBL–GenBank database accession numbers AF061203 and AF061204, ERKKILÄ et al. 1998). All the alleles differ from each other through their nucleotide sequences especially in intron III, but not significantly in the open reading frame (ERKKILÄ et al. 1998). Further studies showed that several barley cultivars, lines and wild strains had either a cv. Adorra-like, cv. Haruna Nijo-like or *H. spontaneum* PI 296897-like β-*amy1* allele (ERKKILÄ 1999). To study the β-*amy1* locus in the Finnish landrace barley line having high enzyme activity, the gene of the line HA52 was sequenced and compared with previously found alleles.

The β-*amy1* sequence in HA52 – The locus of β-*amy1* in the Finnish landrace line HA52 was PCR amplified from genomic DNA using primers based on the sequence of the cv. Haruna Nijo (YOSHIGI et al. 1995). DNA sequencing was performed on ALF DNA Sequencer (Pharmacia-LKB) and sequences were analysed with the PC/Gene software package (IntelliGenetics). The sequence of the HA52 β-*amy1* gene obtained was 4951 bp in length and included a promoter, seven exons and six introns, which is consistent with the schematic structure of all the alleles sequenced before (Fig. 1, YOSHIGI et al. 1995; ERKKILÄ et al. 1998). The β-*amy1* sequence of HA52 has an EMBL accession number AJ301645. Transcription initiation site is situated at position 1205 bp from the beginning of the clone.

Some promoter elements, such as TATA and CCAAT boxes, are common to many genes transcribed by polymerase II. In the HA52 β-*amy1* a

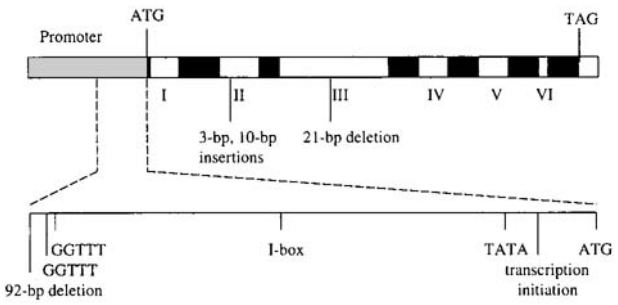

Fig. 1. Schematic presentation of β-*amy1* structural gene. Seven exons are marked with black boxes, and six introns with white boxes (numerals I–VI). Gray box, ATG and TAG indicate promoter, translational start and stop codons, respectively. The HA52-specific deletions and insertions are marked in introns II and III. A segment of promoter is enlarged showing the relative positions of ATG translation start site, transcription initiation site, TATA box, I-box, two GGTTT motives and HA52-specific 92-bp deletion.

TATA box is at − 31 bp, as can be expected, since in plants, it is normally between − 29 bp and − 33 bp (MESSING et al. 1983). Translation starts at position + 55 bp (Fig. 1) and has the same motive as common consensus sequence in plants, CCACCATG (KOZACK 1984).

Introns and insertions – Compared with the alleles of cv. Haruna Nijo, cv. Adorra, and *H. spontaneum* PI 296897 (YOSHIGI et al. 1995; ERKKILÄ et al. 1998), the HA52 β-*amy1* allele had several substitutions, and additionally, both single-base and short multiple-base deletions and insertions (Table 1). Major sequence differences between the four β-*amy1* alleles were in the promoter region, and in the introns II and III (Fig. 1). There were short insertions of 3 bp and of 10 bp close to each other in the intron II of HA52 (Fig. 2B). The 10-bp insertion contains a repeat, 5′-ATATTTA-3′, which is furthermore found once or twice in the introns III of the barleys studied so far. The intron II insertion in the HA52 β-*amy1* is 10 bp long and contains the ATATTTA repeat (Fig. 2B) and is also found twice in its intron III, hence suggesting a conversion at replication. GNIADKOWSKI et al. (1996) suggested that U-rich sequences stimulate splicing in RNA, regardless of their position within an intron. This holds in dicots, but the monocot splicing machinery is less dependent on UA composition (GOODALL and FILIPOWICZ 1991). Thus the short AT-rich insertion in HA52, a monocot, intron II has probably no effect on transcription.

Deletions – There is a deletion of 21 bp in the intron III of HA52 (Fig. 2C). In cv. Adorra, cv. Haruna Nijo and *H. spontaneum* this fragment contains a repeat, GGTGGG, which is found four times at the end of the ORF in all β-*amy1* alleles, also in HA52 allele. The longer fragment in the intron III may serve as a binding site for a negative transcription factor being a reason for the higher β-amylase activity in HA52 than the barleys having the G-rich repeat.

The 92-bp deletion in the promoter region of HA52 (Fig. 2A) is positioned 451 bp upstream from the TATA box (Fig. 1). OKADA et al. (2000) found two direct repeats in the same region in cv. Haruna Nijo. The other segments of the repeats are relatively close to the CCAAT box at − 194 bp. Only 15 bp upstream from the CCAAT HA52 has an I-box, defined as GATAA by TERZAGHI and CASHMORE (1995). Related motives to GATAA are found in many plant promoters, some of which are light regulated (reviewed by TERZAGHI and CASHMORE 1995). The I-box in β-*amy1* is 183 bp upstream from the TATA

Table 1. *Number of the bases involved in the unique alterations of the β-amy1 alleles in barley*

Barley	Total number of bases		
	In deletions	In insertions	In substitutions
Adorra	0	132	8
HA52	125	28	40
H. spontaneum	39	1	5
Haruna Nijo	25	0	7
	Number of bases		
	In the longest deletion	In the longest insertion	
Adorra	0	126	
HA52	92	10	
H. spontaneum	38	0	
Haruna Nijo	25	0	

A

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Adorra              -616 TTTTTTTGGCCCCC-GAAGCATATTCTTCCGGGAGCCAAATTGACATTCC
HA52                -524 TTTTTTTGGTCCCTGGAAGCATATTCTCCCTTGAGCCAAATT--------
H. spontaneum       -615 TTTTTTTGGCCCCC-GAAGCATATTCTTCCGGGAGCCAAATTGACATTCC
Haruna Nijo         -615 TTTTTTTGGCCCCC-GAAGCATATTCTTCCGGGAGCCAAATTGACATTCC

Adorra              -567 GGTCATGATGTGGCTTGGATCCCAAGTTAGTTATACAGATAAGGATATAT
HA52                     ----------------------------------------------------
H. spontaneum       -566 GGTCATGATGTGGCTTGGATCCCAAGTTAGTCATACAGATAAGGATATAT
Haruna Nijo         -566 GGTCATGATGTGGCTTGGATCCCAAGTTAGTCATACAGATAAGGATATAT

Adorra              -517 CTTACCTCAACCGAATCTAGGTTACAACAAGCTTAACACTCATGCATTAG
HA52                -482 ------------------------------AACATTCATGCATTAG
H. spontaneum       -516 CTTACCTCAACCGAATCTAGGTTACAACAAGCTTAACACTCATGCATTAG
Haruna Nijo         -516 CTTACCTCAACCGAATCTAGGTTACAACAAGCTTAACACTCATGCATTAG
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B

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Adorra               739 CTAGTTCTCTGATGCATAT-T---TATA----------TAGAAGTTCAAG
HA52                 736 CTAGTTCTCTGATGCATATATAGATATACATATTTAGATAGAAGTTCAAG
H. spontaneum        739 CTAGTTCTCTGATGCATAT-T---TATA----------TAGAAGTTCAAG
Haruna Nijo          739 CTAGTTCTCTGATGCATAT-T---TATA----------TAGAAGTTCAAG
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C

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Adorra               1982 TGCTTATGGAGAAAGGTGGGCTATGCATTTATACTTCAACAATAAGAATA
HA52                 1871 TGCTTATGGA--------------------TACTTCAACAATAAGAATA
H. spontaneum        1835 TGCTTATGGGGAAAGGTGGGCTATGCATTTATACTTCAACAATAAAAATA
Haruna Nijo          1877 TGCTTATGGGGAAAGGTGGGCTATGCATTTATACTTCAACAATAAAAATA
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Fig. 2A–C. Sections of nucleotide sequence alignments of β-*amy1* alleles of cv. Adorra, HA52, *H. spontaneum* PI 296897 and cv. Haruna Nijo. **A** A deletion of 92 bp in the promoter region of HA52. Palindromic sequences are in italics and inverted repeats are in boldface. **B** Insertions in intron II of HA52 with the AT-motive in boldface. **C** A deletion of 21 bp in intron III of HA52 with the bold GT-motive. The sequence in boldface in B also appears in intron III twice, and the bold sequence in C appears four times in towards the end of the seventh exon of the β-*amy1* gene.

box (Fig. 1). This is in agreement with many light-regulated ribulose-1,5-bisphosphate carboxylase genes having a single I-box 100–300 bp upstream from the TATA box (BORELLO et al. 1993).

The deleted fragment of 92 bp in the promoter of HA52 is relevant in the other β-*amy1* alleles. Three inverted repeats, four palindromic sequences, and seven hairpin loops were found from the 92 bp fragment in the promoter region in β-*amy1* of cv. Adorra, cv. Haruna Nijo and *H. spontaneum* PI 296897 (Fig. 2A and data not shown). This highly repetitive sequence in promoter region 482 bp upstream of the transcription initiation site (Fig. 1) is a putative binding site for a negative transcription factor. Its absence may contribute to the high β-amylase activity for grain mass in HA52, being 2.5 times that of cv. Haruna Nijo and 2.9 times that of cv. Adorra (AHOKAS and MANNINEN 2000).

GGTTT motive and substitutions – Two GGTTT motives were found at position −421 bp and −436 bp from transcription initiation site (Fig. 1). The GGTTT motive and additionally a GCCGC motive are critical for expression in endosperm and embryo in the maize *Adh1* (alcohol dehydrogenase 1) gene promoter expressed in transgenic rice (KYOZUKA et al. 1994). Because these motives are also required for expression in other tissues, KYOZUKA et al. (1994) assumed that there might be tissue-specific post-translational modifications of binding proteins or possibly additional promoter elements. These motives may also hold true for the *Adh1*-like genes in other monocots but not necessarily for other genes. Because no GCCGC motive was found in β-*amy1* gene, further investigations are needed to explore if the GGTTT motive (Fig. 1) alone is adequate for specifying the gene expression in endosperm. In β-*amy1* promoter,

OKADA et al. (2000) did not find any specific sequence similar to the endosperm box, which is known as a common sequence in the promoter region of prolamin genes and which seems to be associated with seed specific expression (HAMMOND-KOSACK et al. 1993).

Four single-base substitutions were found in the open reading frame of HA52. All of these led to amino acid substitutions. The amino acid substitutions in β-*amy1* of HA52 are unique compared with known amino acid sequences of cv. Adorra, cv. Haruna Nijo, and *H. spontaneum* PI 296897 namely Arg-115 → Cys, Asp-164 → Glu, Phe-246 → Leu, and Val-430 → Ala. None of these substitutions is found in conserved regions or in the vicinity of active sites. The amino acids at the positions 115, 164 and 430 are identical in cv. Harrington (cf. KANEKO et al. 2000) and in HA52. Because there is no β-amylase activity data from same trial, we can only speculate on the role of the two amino acid differences, Phe-246 → Leu and Thr-520 → Ala, between cv. Harrington and HA52. The promoter region and the introns probably have more effect on the β-amylase activity than amino acid substitutions, although these may affect secondary modifications or affinities of the protein.

The Canadian cv. Harrington has ancestors among Swedish and Norwegian landraces. There is a 25 % chance that cv. Harrington inherited its β-*amy1* allele from cv. Bjørneby, provided that there was no intragenic recombination. Cv. Bjørneby originated from the area of Trysil, Norway (HAUGUM 1940). Around 1600 Finns, especially from Savonia, a south-eastern province of Finland, immigrated to this area where they used slash-and-burn cultivation (HANSEN 1904; HÄMÄLÄINEN 1947). The Finns brought cereal seeds with them (NORDMANN 1888; HÄMÄLÄINEN 1947). Therefore, the cv. Bjørneby and the line HA52 may represent a common gene pool from SE Finland. Other similarities of their β-*amy1* genes would be worth comparing.

The promoter region lacking from the HA52 β-*amy1* allele provides grounds to postulate that this region serves as binding site for a negative regulation factor. The HA52-specific promoter and amino acid sequence may also cumulate in the β-amylase activity. Comparison between the different β-*amy1* alleles and β-amylase activities suggest that the promoter and the intron regions exert more influence on β-*amy1* gene expression than the observed amino acid changes. Further studies of the promoter region are needed to confirm present postulations and to determine specific regions of enhancing, repressing and endosperm-specific expression.

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