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Seed Science Research / Volume 11 / Issue 04 / December 2001, pp 325 - 333

DOI: 10.1079/SSR200189, Published online: 22 February 2007

Link to this article: http://journals.cambridge.org/abstract_S0960258501000332

How to cite this article:

Woosuk Jung, Ronald W. Skadsen and David M. Peterson (2001). Characterization of a novel barley β -amylase gene expressed only during early grain development. Seed Science Research, 11, pp 325-333 doi:10.1079/SSR200189

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Characterization of a novel barley β -amylase gene expressed only during early grain development

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Abstract

A developmentally regulated, novel β -amylase gene (*ESDbamy*) from *Hordeum vulgare* L. has 76% amino acid similarity with a previously described barley β -amylase. However, the 3'-end sequences of these β -amylases were different. The glycine-rich repeats, which are signature sequences of endosperm-type β -amylases, are absent in this novel *ESDbamy* gene. *ESDbamy* is highly expressed in the caryopsis during early development; however, it is not expressed in other tissues. Early seed development-specific (ESD) β -amylase mRNA levels and enzyme activities were higher in a shrunk endosperm mutant, *seg8*, than in the wild type. *Seg8* has defective endosperm development, reduced starch synthesis and elevated monosaccharide concentrations.

Keywords: β -amylase; barley (*Hordeum vulgare* L.); grain development; shrunk endosperm mutant

Introduction

Barley β -amylase (1,4- α -D-glucan maltohydrolase; EC 3.2.1.2) is synthesized in the developing endosperm, where it is stored in an inactive form until germination (Shewry, 1993). The temporal synthesis profile of barley β -amylase is almost identical to that of hordeins, the major barley storage proteins (Giese and Hejgaard, 1984). At grain maturity, about half of the β -amylase is attached, apparently via disulphide bridges, to insoluble constituents of the starchy

endosperm. A large portion of bound β -amylase is associated with the periphery of starch granules (Sopanen and Laurière, 1989; Ziegler, 1999). In maize and rice, unlike barley, β -amylase is synthesized *de novo* during seed germination (Wang *et al.*, 1996, 1997).

Various β -amylases have been detected immunologically in developing and germinating caryopses and in vegetative tissues of barley. These may result from different β -amylase genes or post-translational modification and proteolytic cleavage (Shewry *et al.*, 1988). Aggregation with itself and with other proteins, including one of the major grain albumins (protein Z) has been reported (Nummi *et al.*, 1966; Hejgaard and Carlsen, 1977). Multiple β -amylases have been classified by their physical properties and expression patterns (LaBerge and Marchylo, 1983; Shewry *et al.*, 1988). During grain development, two peaks of β -amylase mRNA accumulation coincide with the accumulation of early and late isoforms (Shewry *et al.*, 1988). Different genes may encode isoforms during early development compared to those present in mature grain, and these genes may be differentially regulated (Kreis *et al.*, 1988; Shewry *et al.*, 1988).

In this report a novel barley β -amylase gene (*ESDbamy*) was identified that is highly expressed very early in grain development. This gene and its expression pattern are characterized in a barley shrunk endosperm mutant, *seg8*, and its wild-type counterpart, 60Ab1810–53 (CI 15686). *Seg8* is a developmental mutant with reduced endosperm cell number and early termination of starch synthesis (Djarot and Peterson, 1991). It is one of eight barley shrunk endosperm mutants with a monofactorial recessive inheritance pattern and a maternal effect on mutant gene expression (Jarvi and Eslick, 1975).

Materials and methods

Plant growth and samples

Grains of barley line 60Ab1810–53, *seg8* (Ramage and Crandall, 1981), and cultivars Klages and Betzes were

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supplied by Darrell Wesenberg, USDA-ARS, Aberdeen, ID, USA. Klages is a composite of multiple pedigree lines, including 60Ab1810–53 (Wesenberg *et al.*, 1974). Plants were grown in growth chambers and in a greenhouse. The growth chamber was kept at 18°C for a 16-h light period and at 15°C for an 8-h dark period. Illumination was supplied from cool white fluorescent and incandescent lamps providing a photon flux density of $600 \mu\text{E m}^{-2} \text{s}^{-1}$. The greenhouse photoperiod was maintained at 14 h with supplemental high-pressure sodium lights. Developing grains were collected from Klages and *seg8* plants for cDNA library construction and Northern blot analysis. Caryopses (excluding the lemma, palea and remnant anthers) were removed from developing spikes, frozen immediately in liquid nitrogen, and separated by size into two age groups, representing 3–6 and 7–12 d after anthesis (DAA). To determine the temporal expression pattern of β -amylases, grains of Klages and *seg8* were collected at 5, 9, 14, and 19 DAA, representing syncytial, late cellularization and early differentiation, starchy endosperm differentiation and aleurone mitosis stages, respectively (Brown *et al.*, 1994). The greatest portion of 5-DAA grain was maternal-origin tissues (pericarp, testa, nucellus, vascular bundle and chalaza) (Djarot and Peterson, 1991). Fully expanded green leaves of cv. Betzes and F_1 plants of Klages $\times$ *seg8* were used for genomic DNA analyses.

RNA isolation for cDNA synthesis and Northern blot analysis

Total RNAs were isolated as described from developing and germinating grains (Skadsen, 1993; Tibbot and Skadsen, 1996) and various vegetative tissues (Sambrook *et al.*, 1989): etiolated coleoptiles and roots from seedlings 7 d after imbibition (DAI), leaves and stems from 65-day-old plants, and flag leaves collected during the light and dark periods. RNA (25 μg) was separated on 1.1 % agarose-formaldehyde gels. Northern blot transfer, hybridization and autoradiography were conducted as described previously (Skadsen, 1993). Comparative autoradiographs using two probe sets were made by exposing films for the same duration in the same X-ray cassette. Autoradiographs were scanned densitometrically with a soft laser scanner (Biomed Instrument, Fullerton, CA, USA).

cDNA library construction and differential screening

Two micrograms of poly(A⁺) RNA, isolated from 3 to 6 DAA Klages grain, was used for cDNA synthesis using a RiboClone kit (Promega, Madison, WI, USA). cDNA was synthesized by priming with oligo-d(T)_{8–12}

and transcribing with AMV reverse transcriptase (Promega). *Eco*RI linkers were ligated and cDNAs were ligated into the *Eco*RI site of λ ZapII (Stratagene, La Jolla, CA, USA). The primary library was prepared using Gigapack packaging extracts as described by the manufacturer (Stratagene). About 30,000 pfu from the primary library were differentially screened. First, plaque lifts were hybridized with a *seg8* total cDNA probe, which was synthesized from the poly(A⁺) RNA of 3–6 DAA grains by oligo-d(T) primed reverse transcription in the presence of [α -³²P]dCTP. Hybridization conditions were the same as for the Northern blot analysis, but were carried out at 42°C for 16 h. Filters were washed twice in 2 $\times$ SSPE (solution of 150 mM NaCl, 10 mM NaH₂PO₄ and 1 mM EDTA, pH 7.4) and 0.1% sodium dodecyl sulphate (SDS) at 24°C and then twice in 0.2 $\times$ SSPE and 0.1% SDS at 42°C for 15 min each. After autoradiography, the *seg8* cDNA probe was completely removed as described (Sambrook *et al.*, 1989). Filters were then hybridized with a cDNA probe made from Klages grain cDNA as described above. Differentially hybridized plaques were purified, and plaques were converted to pBluescript SK plasmids by *in vivo* excision as described in the manufacturer's manual (Stratagene).

Probe preparation for Northern and Southern blot analysis

Three different β -amylase probes were designed to investigate the temporal expression of barley β -amylase (Fig. 1A). Both polymerase chain reactions (PCR) and random hexamer methods were used for probe synthesis. PCR was performed with GeneAmp PCR systems 9600 and 2400 (Perkin Elmer, Foster City, CA, USA). Synthesis of probes and routine amplification of partial β -amylase sequences were carried out with 35 cycles of denaturing at 94°C (30 s), primer annealing at 42°C (30 s) and extension at 72°C (30 s) with or without [α -³²P]dCTP. Probes were used for Northern, Southern and cDNA dot blot analyses. Barley actin (pACT10–4, GenBank accession U21907) and a cell division cycle protein gene CDC48 (pBCDC48, GenBank accession AF045927) were used as internal controls.

Genomic DNA isolation and Southern blot analysis

Genomic DNAs were extracted from Klages, *seg8*, an F_1 plant of Klages $\times$ *seg8* and Betzes, as described (Gawel and Jarret, 1991). Two sets of genomic DNA digestions were set up to check the genomic structure of the early seed development-specific (ESD) β -amylase gene. First, the genomic DNAs of Klages, *seg8* and an F_1 plant of Klages $\times$ *seg8* were digested with restriction enzymes

EcoRV and *XbaI* and separated on a 0.9% agarose Tris-acetate gel. The blot was hybridized with probe II (Fig. 1A), which detects both ESD and endosperm (END) β -amylase genes. Secondly, the genomic DNA of Betzes was digested with *BamHI*, and the blot was hybridized with probe III (Fig. 1A) to specifically detect the ESD β -amylase gene.

DNA dot blot analysis

Serial dilutions from 1 ng to 10 pg of denatured cDNAs of isolated candidates were applied to duplicate Nytran nylon membranes and baked at 80°C for 90 min. Hybridization was conducted at 40°C. Blots were washed twice in 2 $\times$ SSPE, 0.1% SDS solution at 24°C and then twice in 0.1 $\times$ SSPE, 0.1% SDS solution at 40°C for 15 min each. Filters were autoradiographed as described previously (Skadesen, 1993).

Sequence analysis

Clones from differential library screening were sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977) using Sequenase (US Biotechnical Corp, Cleveland, OH, USA), and by cycle sequencing with *TaqFS* polymerase (Perkin Elmer) and an automated DNA sequencer (ABI 373, Biotech Center, University of Wisconsin-Madison). Sequences were analysed using Genetics Computer Group (GCG, Madison, WI, USA) sequence analysis software (GCG version 8 for VMS). Evolutionary distance and phylogenetic analysis of β -amylase sequences were carried out using the AlignX (InforMax, Bethesda, MD, USA) program. Nucleotide sequences were used for the phylogenetic analysis.

β -Amylase activity assay and mono- and disaccharide analysis

Developing grain samples of the same lots that were used for Northern blot analysis were lyophilized. About 20–50 mg (dry weight) of developing caryopses were ground in liquid nitrogen using a pestle and mortar. The powder was divided into three or more aliquots and extracted for measuring total β -amylase activity using the Betamyl assay kit (Megazyme, Wicklow, Ireland) according to McCleary and Codd (1989). Mono- and disaccharides were extracted in triplicate with 80% (v/v) ethanol from the same grain samples used for β -amylase assay. The ethanol was evaporated, mono- and disaccharides were redissolved with water and separated on an HPLC CarboPac PA1 (Dionex, Sunnyvale, CA, USA) anion exchange column, and detected/quantified by pulsed amperometry (Im and Henson, 1995).

Results

Identification and sequence analysis of pEESbamy clone

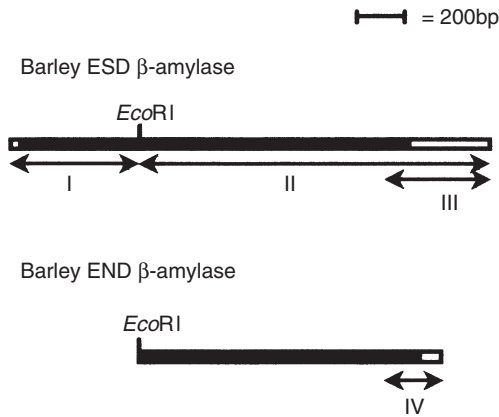
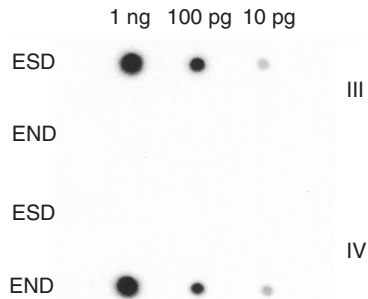
A β -amylase clone, pEESbamy, was isolated from differential library screens designed to find genes that are differentially regulated in the *seg8* mutant (Jung *et al.*, 1996, 1997). pEESbamy was a candidate that showed a quantitative difference between *seg8* and Klages developing grains. The pEESbamy insert, ESDbamy, was named 'barley early seed development-specific (ESD) β -amylase' because the corresponding mRNA showed a unique temporal expression pattern and deduced amino acid sequence (see below).

pEESbamy (GenBank accession No. AF012343) contained a 1888 bp insert, with a single open reading frame of 505 amino acids. The molecular mass of the deduced polypeptide was 57 kDa, and the isoelectric point was 6.2. A full coding sequence was judged to be included in pEESbamy by the presence of a 48-bp long 5' untranslated region (UTR) and a 326-bp 3' UTR downstream from the stop codon (data not shown).

A comparison of the deduced amino acid sequence of ESDbamy with other β -amylases is presented in Fig. 1B. ESDbamy has greater amino acid identity with β -amylases of rye, wheat and rice than with barley endosperm (END) β -amylase (GenBank X52321). Nucleotide and deduced amino acid sequences of ESDbamy were over 90% identical with those of wheat (GenBank X98504) and rye (GenBank Z11772) β -amylases in the coding regions, but only 76% identical with barley endosperm β -amylase.

The most pronounced difference is that ESDbamy lacks the glycine-rich repeats found in the C-terminal region of barley endosperm β -amylase (Fig. 1B), as do rye and wheat β -amylases (Sadowski *et al.*, 1993). The isoelectric focusing (IEF) pattern of the protein expressed in *E. coli* from a 3' end-truncated barley endosperm β -amylase clone was altered as compared to expression of the intact clone; its pattern was similar to that of β -amylase from early stage developing grains. Another observation was that glycine-rich repeats may be involved in thermostability of β -amylase in an *E. coli* system (Yoshigi *et al.*, 1995).

The catalytic sites of various β -amylases, around Asp104 and Glu183 (Nitta *et al.*, 1989; Toda *et al.*, 1993) in ESDbamy are conserved. The 5' and 3' UTRs of ESDbamy were highly homologous with the 5' and 3' UTRs of rye and wheat β -amylase clones. However, the 5' UTR was less homologous, and the 3' UTR of ESDbamy showed no homology with barley endosperm β -amylase or with rice, sweet potato and *Arabidopsis* β -amylase clones (data not shown).

A**C****B**

Barley ESD	1	MAGNMLANYVQVYVMLPLDVVSDNKEBKGEIRAHVKKL.TEAGV	45
Barley END		MEVNVKQNYVQVYVMLPLDASVNNREBKGEIRALQRLK.VEAGV	
Rye		MAGNMLANYVQVYVMLPLDVVSDNKEBKGEIRALQRLK.TEAGV	
Wheat		MAGNMLANYVQVYVMLPLDVVSDNKEBKGEIRALQRLK.TEAGV	
Rice		MAGNMLANYVQVYVMLPLDVVSDNKEBKGEIRALQRLK.TEAGV	
Sweet potato		MAPIPGVMEICNYVSLVYVMLPLGVNADNVFDPKKEVDELKQVK.AGCC	
Arabidopsis		MATNYNEKLLINYPVYVMLPLGVNVDNVPADPETLETQLKRLKEAGV	
Barley ESD	46	DGVVIYFWWGLVSGICEKAKHHTFYNHVFYLVHDARLQLQAIMSFHQCGG	95
Barley END		DGVVVDVWGLVSGICEKAKHHTFYNHVFYLVHDARLQLQAIMSFHQCGG	
Rye		DGVVIDVWGLVSGICEKAKHHTFYNHVFYLVHDARLQLQAIMSFHQCGG	
Wheat		DGVVIDVWGLVSGICEKAKHHTFYNHVFYLVHDARLQLQAIMSFHQCGG	
Rice		DGVVIDVWGLVSGICEKAKHHTFYNHVFYLVHDARLQLQAIMSFHQCGG	
Sweet potato		DGVVVDVWGLVSGICEKAKHHTFYNHVFYLVHDARLQLQAIMSFHQCGG	
Arabidopsis		DGVVVDVWGLVSGICEKAKHHTFYNHVFYLVHDARLQLQAIMSFHQCGG	
Barley ESD	96*	NVGDLFNIPIPIQWVRDVCATDPDIEYTNRRGARNIDYTLTGVDQDPLFHG	145
Barley END		NVGDVAVNIPIPIQWVRDVCATDPDIEYTNRRGARNIDYTLTGVDQDPLFHG	
Rye		NVGDVAVNIPIPIQWVRDVCATDPDIEYTNRRGARNIDYTLTGVDQDPLFHG	
Wheat		NVGDVAVNIPIPIQWVRDVCATDPDIEYTNRRGARNIDYTLTGVDQDPLFHG	
Rice		NVGDVAVNIPIPIQWVRDVCATDPDIEYTNRRGARNIDYTLTGVDQDPLFHG	
Sweet potato		NVGDVAVNIPIPIQWVRDVCATDPDIEYTNRRGARNIDYTLTGVDQDPLFHG	
Arabidopsis		NVGDVAVNIPIPIQWVRDVCATDPDIEYTNRRGARNIDYTLTGVDQDPLFHG	
Barley ESD	146	RTAVQMYADYVTSFRENKKFLDAGTIVDIEVGLCPAGEVRYPSYPSQSG	195
Barley END		RTAVQMYADYVTSFRENKKFLDAGTIVDIEVGLCPAGEVRYPSYPSQSG	
Rye		RTAVQMYADYVTSFRENKKFLDAGTIVDIEVGLCPAGEVRYPSYPSQSG	
Wheat		RTAVQMYADYVTSFRENKKFLDAGTIVDIEVGLCPAGEVRYPSYPSQSG	
Rice		RTAVQMYADYVTSFRENKKFLDAGTIVDIEVGLCPAGEVRYPSYPSQSG	
Sweet potato		RTAVQMYADYVTSFRENKKFLDAGTIVDIEVGLCPAGEVRYPSYPSQSG	
Arabidopsis		RTAVQMYADYVTSFRENKKFLDAGTIVDIEVGLCPAGEVRYPSYPSQSG	
Barley ESD	196	WVFFGIGEFICYDKYLEADSKAAAKAGHPDWELP.DDAGEYNDPEKTKQ	244
Barley END		WVFFGIGEFICYDKYLEADSKAAAKAGHPDWELP.DDAGEYNDPEKTKQ	
Rye		WVFFGIGEFICYDKYLEADSKAAAKAGHPDWELP.DDAGEYNDPEKTKQ	
Wheat		WVFFGIGEFICYDKYLEADSKAAAKAGHPDWELP.DDAGEYNDPEKTKQ	
Rice		WVFFGIGEFICYDKYLEADSKAAAKAGHPDWELP.DDAGEYNDPEKTKQ	
Sweet potato		WVFFGIGEFICYDKYLEADSKAAAKAGHPDWELP.DDAGEYNDPEKTKQ	
Arabidopsis		WVFFGIGEFICYDKYLEADSKAAAKAGHPDWELP.DDAGEYNDPEKTKQ	
Barley ESD	245	FFKENSTYLTKEKKEFLSWYSNKLKHGDKILDEANKVFLGCRVOLAIRK	294
Barley END		FFRDNGTYLSEKKEFLSWYSNKLKHGDKILDEANKVFLGCRVOLAIRK	
Rye		FFKENSTYLTKEKKEFLSWYSNKLKHGDKILDEANKVFLGCRVOLAIRK	
Wheat		FFRDNGTYLSEKKEFLSWYSNKLKHGDKILDEANKVFLGCRVOLAIRK	
Rice		FFRDNGTYLSEKKEFLSWYSNKLKHGDKILDEANKVFLGCRVOLAIRK	
Sweet potato		FFRDNGTYLSEKKEFLSWYSNKLKHGDKILDEANKVFLGCRVOLAIRK	
Arabidopsis		FFRDNGTYLSEKKEFLSWYSNKLKHGDKILDEANKVFLGCRVOLAIRK	
Barley ESD	295	SGIHWYVRVPNHAAELTAGYNLDDRDGYRTIARMLTRHASMNFQCAEM	344
Barley END		SGIHWYVRVPNHAAELTAGYNLDDRDGYRTIARMLTRHASMNFQCAEM	
Rye		SGIHWYVRVPNHAAELTAGYNLDDRDGYRTIARMLTRHASMNFQCAEM	
Wheat		SGIHWYVRVPNHAAELTAGYNLDDRDGYRTIARMLTRHASMNFQCAEM	
Rice		SGIHWYVRVPNHAAELTAGYNLDDRDGYRTIARMLTRHASMNFQCAEM	
Sweet potato		SGIHWYVRVPNHAAELTAGYNLDDRDGYRTIARMLTRHASMNFQCAEM	
Arabidopsis		SGIHWYVRVPNHAAELTAGYNLDDRDGYRTIARMLTRHASMNFQCAEM	
Barley ESD	345	RDSEQSEBAKSAFDELVQOVLGAGWREGLVACENALPRWDATAYNTILR	394
Barley END		RDLEQSSQAMSAFDELVQOVLGAGWREGLVACENALPRWDATAYNTILR	
Rye		RHSEQSEBAKSAFDELVQOVLGAGWREGLVACENALPRWDATAYNTILR	
Wheat		RDSEQSEBAKSAFDELVQOVLGAGWREGLVACENALPRWDATAYNTILR	
Rice		RDSEQSEBAKSAFDELVQOVLGAGWREGLVACENALPRWDATAYNTILR	
Sweet potato		RDSEQSEBAKSAFDELVQOVLGAGWREGLVACENALPRWDATAYNTILR	
Arabidopsis		KDTDNTABALSAPDELVQOVLGAGWREGLVACENALPRWDATAYNTILR	
Barley ESD	395	NARFKCHINENPPEHKLFCEFTYLRLSNELLKGONYATQTFVEKGHANLG	444
Barley END		NARFKCHINENPPEHKLFCEFTYLRLSNELLKGONYATQTFVEKGHANLG	
Rye		NARFKCHINENPPEHKLFCEFTYLRLSNELLKGONYATQTFVEKGHANLG	
Wheat		NARFKCHINENPPEHKLFCEFTYLRLSNELLKGONYATQTFVEKGHANLG	
Rice		NSREHCHINENPPEHKLFCEFTYLRLSNELLKGONYATQTFVEKGHANLG	
Sweet potato		KLRFNEVNLNGPEKLMSCFTYLRLSDLLQTDNFELEKKFKVKKHADDL	
Arabidopsis		NARFKCHINENPPEHKLFCEFTYLRLSNELLKGONYATQTFVEKGHANLG	
Barley ESD	445	HNPSV...DPVAPERSKPEMPIELILKAAQPKLEPFPFDKNTDLVKN	490
Barley END		RDPIV...DPMAPERSKPEMPIELILKAAQPKLEPFPFDKNTDLVKN	
Rye		HDPTV...DPVAPERSKPEMPIELILKAAQPKLEPFPFDKNTDLVKN	
Wheat		HDPSV...DPVAPERSKPEMPIELILKAAQPKLEPFPFDKNTDLVKN	
Rice		SATNV...DPLEPORSMPMPIELILKAAQPKLEPFPFDKNTDLVKN	
Sweet potato		PSPNAI...SPAVPERSKPEMPIELILKAAQPKLEPFPFDKNTDLVKN	
Arabidopsis		YCGDAAYGHEIVPPEKTSNSQLTLEDIAAQAQPS.GAKKWDSETDLKVDG	
Barley ESD	491	HTDVGDEVLAPVAV	505
Barley END		TGGMGQAEGPTCGMGQVKGPTGGMGQAEDPTSIGIGELPATM	
Rye		HTDVGDEVLAPVAV	
Wheat		HTDVGDEVLAPVAV	
Rice		HTDVGDEVLAPVAV	
Sweet potato		SNPFD	
Arabidopsis			

Developmental expression pattern of *pEESbamy* in *seg8* and its wild-type counterpart

Because of the similarity between *ESDbamy* and barley endosperm β -amylase coding regions, specific probes for each were synthesized to determine their respective expression patterns. Because there was no homology between their 3' UTRs, gene-specific probes were synthesized from these sequences (Fig. 1A). Cross-hybridization between the 3'-end probe of *ESDbamy* (III) and the barley endosperm β -amylase cDNA clone, p129 (IV), was checked at low stringency (40°C), using duplicate cDNA dot blots. Cross-hybridized signals were not detected in β -amylases and two controls (Fig. 1C).

In a Northern blot analysis, using RNA from four different stages, probe II, which recognizes the coding regions of both β -amylases, revealed a bimodal expression pattern, with peaks at 5 and 19 DAA (Fig. 2A). However, when gene-specific probes were used, expression was resolved into two distinct patterns (Fig. 2B and C). *ESDbamy* (probe III) was highly expressed at 5 DAA but not thereafter. Conversely, barley endosperm β -amylase gene (probe IV) was expressed at high levels at 14 DAA and 19 DAA; however, very weak but detectable signals of barley endosperm β -amylase gene transcripts were observed in 5 and 9 DAA developing caryopses. Thus, *ESDbamy* represents a β -amylase gene that is expressed only in very young caryopses. The expression of *ESDbamy* was higher in *seg8* than in line 60Ab1810-53 at 5 DAA (Fig. 2B and F). The expression of barley endosperm β -amylase gene was similar in both genotypes at 14 DAA and lower in *seg8* than in line 60Ab1810-53 at 19 DAA (Fig. 2C and F).

Clones for a barley actin (pACT10-4) and putative CDC48 (pBCDC48) were used as internal controls to monitor the general expression level of constitutive

genes (Fig. 2D and E). CDC48 has a constitutive expression pattern in all stages of grain development and other tissue samples from both *seg8* and line 60Ab1810-53. CDC48 mRNA levels declined slightly during development, but were equal in *seg8* and line 60Ab1810-53. Little actin mRNA was present in 5-DAA grain and leaves from either line, and significantly less actin mRNA was present in *seg8* than in 60Ab1810-53, the opposite of ESD β -amylase expression.

Expression of β -amylases from various tissues

Northern blot analysis was used to compare *ESDbamy* and barley endosperm β -amylase mRNA levels in various tissues that have been reported to have β -amylase enzyme activity. Neither of the β -amylase mRNAs were detected, even under low stringency hybridization conditions and after extended exposure, in 3-DAI germinating seeds, etiolated and fully expanded green leaves, and roots of 60Ab1810-53 (Fig. 2A, B and C), or in developing embryos isolated from 19-DAA caryopses (data not shown). The β -amylase mRNAs were also not detected in flag leaves collected during the light or dark period (data not shown). Thus, the expression of these β -amylase genes is specific to grain tissues.

β -Amylase enzyme activities in early seed development

β -Amylase enzyme activities of developing barley grains were measured to determine whether differences in mRNA levels are reflected at the enzyme level. β -Amylase activities in developing 5- and 9-DAA grains of *seg8* were about 37% and 48% higher than in line 60Ab1810-53 (Fig. 3A). This corresponded to the higher levels of *ESDbamy* expression that were

Figure 1. Specificity of probes and PILEUP analysis of amino acid sequences of various plant β -amylases. (A) Gene-specific probes were prepared from 3'-end sequences of early seed development-specific (ESD) and endosperm (END) β -amylase. A DNA clone coding for barley END β -amylase was isolated from barley cultivar Morex developing seed cDNA library. Solid bars represent coding regions and open bars represent 5' and 3' untranslated regions (UTRs). Probe II, which represents both ESD and END β -amylases, was prepared from a 1.3 kb *EcoRI* fragment of *ESDbamy* (region II). Probe III, which represents particularly the 3'-end region (region III) of ESD β -amylase (400 bp), was prepared by PCR with two sets of primers: upstream, BM3A: 5'-CACCGACCTGCCAGT and downstream, BM3B: 5'-GAACGGCAGTTCCAT. Probe IV, which represents the 3'-end region (region IV) of END β -amylase (209 bp), was prepared by PCR with two sets of primers: upstream, BAMY3B: 5'-CTCTGTCACTCAC and downstream, BAMY3C: 5'-GGCAGAGAAAGGTTTCTCTGTACA. (B) A comparison between the deduced amino acid sequences of the barley ESD β -amylase clone *pEESbamy* and other plant β -amylases. The PILEUP program (GCG, Version 8 for VMS) was used for multiple sequence alignment. Conserved sequences are shaded. Numbers represent nucleotides from the ATG start codon of barley ESD β -amylase. β -Amylase catalytic sites were detected by the MOTIFS program (GCG, version 8 for VMS) and are indicated by asterisks. The underlined italic letters represent glycine-rich repeats in the C-terminal end of barley END β -amylase. GenBank Accession numbers of each entry: barley ESD, AF012343; barley END, X52321; rye ubiquitous type (Rye 1), Z11772; wheat, X98504; rice, L10346; sweet potato, D01022; and *Arabidopsis*, M73467. (C) cDNA dot blot analyses demonstrating the specificity of β -amylase probes. Denatured cDNAs of ESD and END β -amylase were applied to duplicate nitrocellulose membranes. The duplicated blots were differentially screened with probe III and with probe IV.

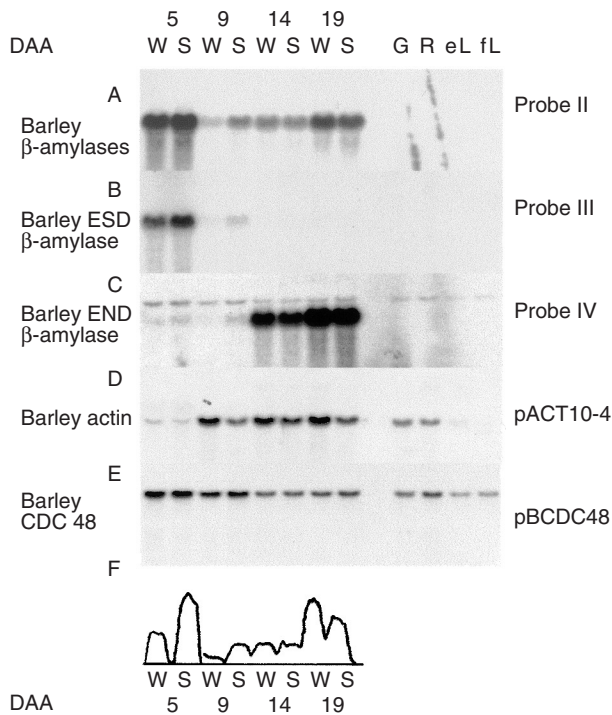


Figure 2. Temporal expression of barley early seed development-specific (ESD) and endosperm (END) β -amylases in developing line 60Ab1810-53 (W: wild type), *seg8* (S: shrunken endosperm mutant) barley grains and in vegetative tissues. (A) Probe II was hybridized to the blot. The blots were then screened with ESD and END β -amylase probes (probes III and IV) (B) and (C). Probes for barley actin gene (pACT10-4) and putative barley CDC48 (pBCDC48) were used as controls to assess general mRNA levels (D and E, respectively). Total RNAs were extracted from developing grains of 60Ab1810-53 and *seg8* at 5, 9, 14 and 19 DAA; and 3 DAI germinating grains (G), roots (R) and etiolated leaves (eL) from 7 DAI germinating seedlings, and fully expanded leaves (fL) of 60Ab1810-53. Faint bands above END β -amylase in (C) remained from previous hybridization of the blot with CDC48. (F) Laser scan densitometry reading of the X-ray film shown in (A).

found in *seg8* versus 60Ab1810-53 at 5 and 9 DAA (Fig. 2F). The decline in β -amylase activity from 5 to 9 DAA also corresponded to decreased *ESDbamy* expression at 9 DAA. The decrease in *ESDbamy* expression from 5 to 9 DAA was greater than the decrease in β -amylase activity, indicating that previously synthesized β -amylase may remain active for several days. Alternatively, some of the β -amylase activity at 9 DAA may result from early expression of endosperm (END) β -amylase gene.

Mono- and disaccharide sugars in developing seeds

Monosaccharide concentrations in developing grains were significantly higher (approximately twofold) in

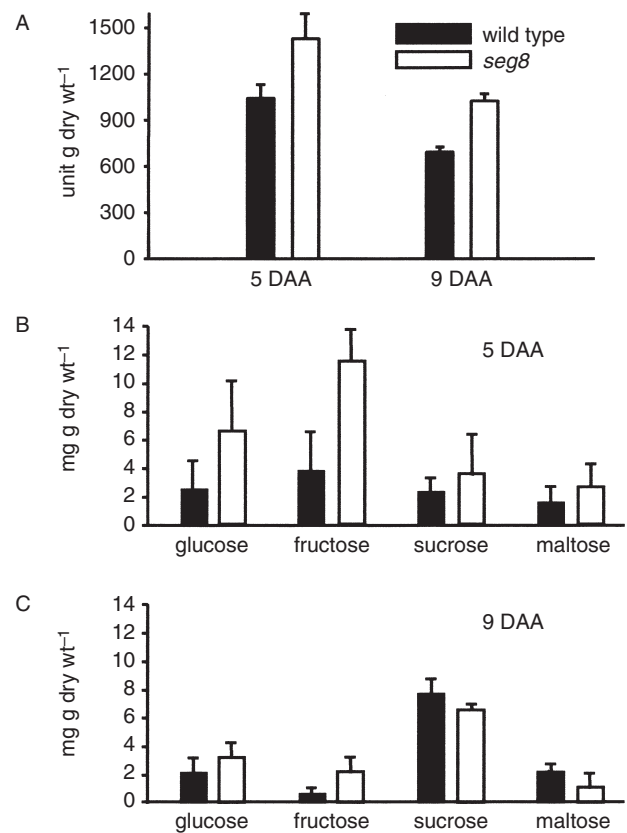


Figure 3. β -Amylase enzyme activities (A) and carbohydrate contents of 5- (B) and 9-DAA (C) developing grains of *seg8* and 60Ab1810-53. Error bars represent standard deviations.

seg8 than in wild type (60Ab1810-53) at 5 DAA, and total monosaccharide concentrations were higher than total disaccharides in both genotypes (Fig. 3B). At 9 DAA, sucrose concentrations had doubled, and monosaccharides had declined (Fig. 3C). Differences between *seg8* and 60Ab1810-53 were less apparent at 9 DAA than at 5 DAA.

Southern blot analyses and evolutionary analysis of barley ESD β -amylase gene

Genomic DNAs from Klages, *seg8* and an F_1 plant of Klages $\times$ *seg8* were digested with *EcoRV* and *XbaI* and analysed on Southern blots with probe II, which represents both ESD and barley endosperm β -amylase genes. Probe II was hybridized with two bands of 5.5 and 5.0 kb (Fig. 4A-C). Additionally, DNA from cv. Betzes was digested with *BamHI*, as reported by Kreis *et al.* (1988). When incubated with the ESD β -amylase gene-specific probe III, a single 10 kb band was hybridized (Fig. 4D).

Evolutionary distance and phylogenetic analysis using the nucleotide sequences of the full coding

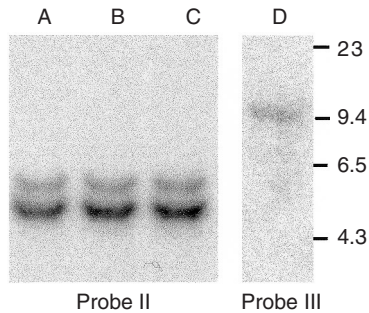


Figure 4. Southern blot analysis of barley ESD β -amylase in various barley cultivars. Fifteen micrograms of genomic DNA of Klages (A), *seg8* (B) and F_1 plant of Klages $\times$ *seg8* (C) were digested with *EcoRV* and *XbaI*, simultaneously (two enzymes were mixed and added to a restriction enzyme reaction). The blot was hybridized with probe II. This probe hybridized with two bands at 5.5 kb and at 5.0 kb. Genomic DNA of Betzes was digested with *BamHI* (D). This blot was screened with probe III. This ESD β -amylase gene-specific probe hybridized with a single 10 kb band.

regions showed that ESD β -amylase was most closely related to wheat and rye β -amylases (Rye 1) and was closely related to β -amylases of other members of the *Gramineae* family, such as maize and rice (Fig. 5). Two β -amylases that have glycine-rich repeats (barley endosperm β -amylase gene and Rye 2) (Kreis *et al.*, 1987; Rorat *et al.*, 1991) were identified as endosperm-type β -amylases. Barley endosperm β -amylase is more distantly related to the other β -amylases in monocotyledonous species.

Discussion

A barley shrunken endosperm mutant *seg8* has a severe defect in grain development and a maternal-effect inheritance pattern. However, because *seg8* shows normal germination and vegetative growth (Djarot and Peterson, 1991), the effect of the mutation may be confined to the endosperm. Starch synthesis is greatly reduced in *seg8*, and abnormal development of *seg8* grain is clearly recognizable as early as 5 DAA. The net accumulation of photosynthetic product in *seg8* grain ceases by about 12 DAA, and grains have only about 25% of the dry weight of wild-type grains at harvest (Djarot and Peterson, 1991). The elevated expression of ESD β -amylase gene in *seg8* relative to 60Ab1810–53 at 5 DAA coincides with the onset of the shrunken phenotype. This indicates a possible relationship between ESD β -amylase and the retarded mutant endosperm development. In contrast, barley endosperm β -amylase expression was identical in both genotypes at 14 DAA (Fig. 2C and F), a stage marked by a clear phenotypic difference in size and development.

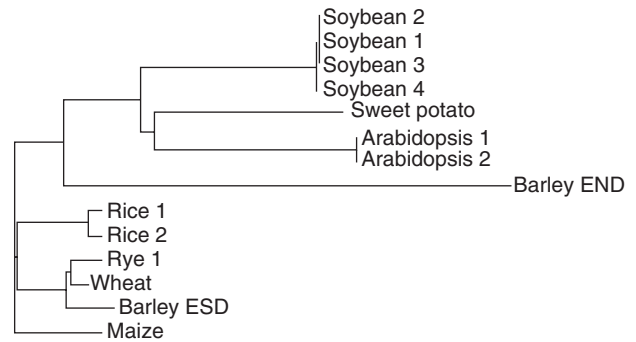


Figure 5. Phylogenetic dendrogram of the β -amylases from different species. Full-length clones of barley ESD (AF012343), barley END type (X52321), rye ubiquitous type (Rye 1, Z11772), wheat (Wheat, X98504), rice (Rice 1, L10345; Rice 2, L10346), maize (Z25871), sweet potato (D01022), soybean (Soybean 1, D50866; Soybean 2, AB004271; Soybean 3, X71419; Soybean 4, M92090) and *Arabidopsis* (Arabidopsis 1, D43783; Arabidopsis 2, M73467) were found in GenBank. The evolutionary distances and the phylogenetic dendrogram were analysed by the AlignX program (default setup) of InforMax (Bethesda, MD, USA). The partial sequence of rye endosperm-type β -amylase (Rye 2, X56785) was not included.

β -Amylase activities in early stage developing caryopses have been reported in barley (LaBerge and Marchylo, 1986), wheat (Daussant and Laurière, 1990), and rye (Daussant *et al.*, 1991). The novel barley ESD β -amylase gene may code for this well-known 'green' β -amylase present in the pericarp and/or testa of early stage developing caryopses. This 'green' β -amylase enzyme activity declined between 5 and 9 DAA (Fig. 3A), corresponding to a similar decline in ESD β -amylase mRNA levels. At 9 DAA, very little β -amylase mRNA was present, especially in wild-type barley (Fig. 2B). This suggests that the enzyme may be relatively long lived.

An elevated accumulation of sugars in developing grains of *seg8* (Fig. 3) may be caused by an inhibition of starch synthesis, and/or abnormal uptake of photosynthetic products by developing endosperm and embryo as active sink tissues. The defect in the starch synthesis mechanism of *seg8* is not fully understood. Previously, the net incorporation rate of [^{14} C]sucrose into starch was retarded in *seg8* at 8 and 12 DAA (Djarot and Peterson, 1991), but monosaccharide concentrations in very early stages of *seg8* developing grain were not reported.

The higher concentrations of glucose and fructose in *seg8* (Fig. 3) may result from blocked starch synthesis. In *Arabidopsis* and sweet potato, leaf β -amylase is induced by glucose, fructose and sucrose (Nakamura *et al.*, 1991; Ohto *et al.*, 1992; Mita *et al.*, 1995). Similarly, the higher expression of barley ESD

β -amylase in *seg8* may be related to its higher concentrations of glucose and fructose.

Specific expression patterns of ESD and barley endosperm β -amylase genes were detected in developing grain. Clearly, the expression patterns of these two starch hydrolase genes are differently regulated in temporal and spatial manner. Although very faint but detectable signals of barley endosperm β -amylase mRNA were detected in early stage developing caryopses, the major β -amylase gene expression at 5 and 9 DAA was *ESDbamy*.

Multiple isoforms of β -amylase have been identified from developmental stages in barley grain and vegetative tissues through analysis of salt-soluble proteins (LaBerge and Marchylo, 1986; Ainsworth *et al.*, 1987; Shewry *et al.*, 1988). However, ESD and barley endosperm β -amylase mRNAs were not detected in total RNA of vegetative tissues by Northern blot analyses. Previously, β -amylase expression was detected by dot blot analyses of shoot and leaf total RNA from the cultivar Sundance (Kreis *et al.*, 1987). However, our results imply that both ESD and barley endosperm β -amylase genes may not code for the 'ubiquitous' form of β -amylase, which is detected in vegetative tissues (Ziegler, 1999).

Two bands were hybridized with probe II in Southern blot analysis. The intensity of the 5.0-kb band was twofold higher than the 5.5-kb band, indicating the presence of about three β -amylase gene copies per haploid genome (Kreis *et al.*, 1988). The genomic DNA extracted from barley cultivar Betzes was digested with *Bam*HI restriction enzyme, as reported by Kreis *et al.* (1988) for Southern blot analysis. An important difference between these two Southern analyses is the origin of the probes. The probe (789 bp of *Acc*I fragment of barley endosperm β -amylase gene) used by Kreis *et al.* (1988) represents the middle coding sequence of a barley endosperm β -amylase clone (pc β C51) from nucleotide number 573 to 1362, the region that shows 84% identity at the nucleotide level with ESD β -amylase. The probe from the *Acc*I fragment hybridized with three bands: one at about 10 kb and two at 14 kb. However, probe III (Fig. 1A), which represents the 3' end of pESDbamy, hybridized only with the 10-kb band (Fig. 4). This band is thus specific for the ESD β -amylase gene.

We could not determine the function of ESD β -amylase. Starch granules present in outer layers of the pericarp in developing barley grains (MacGregor and Dushnicky, 1989) appeared to be digested gradually and used in pericarp cell elongation (Duffus and Cochrane, 1992). However, we do not know whether ESD β -amylase is functional for initial degradation of these starch granules. It is possible that the level of β -amylase may not exert a pronounced effect on early development, since mutants of rye and soybean that contain little active β -amylase grow normally

(Daussant *et al.*, 1981; Hildebrand and Hymowitz, 1981). Moreover, it has been reported that β -amylase may not actively attack native starch granules that were isolated from fully grown barley grains (Sun and Henson, 1991). However, these *in vitro* observations do not exclude the possibility that β -amylase could be involved in digestion of starch granules in young pericarp tissue.

Acknowledgements

The authors gratefully acknowledge the critical review and discussions of Drs Cynthia Henson and Patrick Masson. We thank Drs Evan Evans and Berne Jones for helpful advice. The authors also thank Keith Gilchrist, John Herbst, Charles Karpelenia, Michael O'Connor and Laurie Marinac for excellent technical support.

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