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Polymorphisms in the *α-amy1* gene of wild and cultivated barley revealed by the polymerase chain reaction

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Abstract α-Amylases are the key enzymes involved in the hydrolysis of starch in plants. The polymerase chain reaction (PCR) was used to detect polymorphisms in the length of amplified sequences between the annealing sites of two primers derived from published *α-amy1* gene sequences in barley. These two primers (Bsw1 and Bsw7), flanking the promoter region and the first exon, amplified two PCR fragments in barley. One of the amplified products, with the expected length of 820 bp, appeared together with another shorter PCR band of around 750 bp. This 750-bp fragment seems to be derived from an α-amylase gene not reported previously. Both of the PCR products could be amplified from the two-rowed barley varieties tested, including cv Himalaya from which the sequence information was obtained. Five of the six-rowed barley varieties also have the two PCR fragments whereas another two have only the long fragment. These two fragments seem to be unique to barley, neither of them could be amplified from other cereals; for example, wheat, rye or sorghum. These two α-amylase fragments were mapped to the long arm of 6H, the location of the *α-amy1* genes, using wheat-barley addition lines. Amplification of genomic DNA from wild barley accessions with primers Bsw1 and Bsw7 indicated that both of the fragments could be present, or the long and short fragments could be present alone. The results also demonstrated that the genes specifying these two fragments could be independent from each other in barley. The conserved banding pattern of these two fragments in the two-rowed barley varieties implies that artificial selection from these genes may have played an important role in the evolution of cultivated barley from wild barley.

Key words α-amylase · Barley · DNA polymorphism Evolution · Polymerase chain reaction

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Introduction

α-Amylases (EC 3.2.1.1) are endoglycolytic enzymes that play an important role in cereal seed germination. They are primarily responsible for the hydrolysis of the starchy endosperm, producing the sugars needed to sustain the growth of the emerging seedling (Fincher 1989). The α-amylase genes, encoding several α-amylase isozymes, comprise multigene families in wheat and barley. The high pI, α-amylase2-isozymes encoded by the *α-amy1* genes predominate during germination (Henry 1989). In barley, α-amylase2 has been shown to have a specific activity on amylose more than twice that of the low pI α-amylase1 encoded by *α-amy2* genes (Macgregor and Morgan 1992). As many as 12–14 *α-amy1* and 10–11 *α-amy2* genes were found in wheat by hybridization to α-amylase cDNA probes (Martienssen 1986). In Southern hybridizations a large number of bands appeared and these have been correlated to genes on group 5, 6 and 7 chromosomes in wheat and 6H and 7H in barley (Muthukrishnan et al. 1984; Lazarus et al. 1985; Baulcombe et al. 1987).

Several barley α-amylase cDNA and genomic DNA clones have been isolated and sequenced in different laboratories (Chandler et al. 1984; Lazarus et al. 1985; Rogers 1985; Baulcombe et al. 1987; Knox et al. 1987; Huttly et al. 1988; Khursheed and Rogers 1988). Cross-cultivar comparison of three genomic clones of high pI α-amylase revealed that sequence divergence between member loci of multigene families is much greater than that between the same gene from two cultivars (Brown 1992). Holwerda et al. (1993) observed large differences with restriction fragment length polymorphisms (RFLPs) between a number of multigene families in barley (*Hordeum vulgare*) and wild barley (*H. spontaneum*). They found that high pI α-amylase, alcohol dehydrogenase, and hordein C gene families all showed high levels of variation whereas the

low pI α-amylase and *waxy* gene families were more conserved.

In the present study we have analysed polymorphism in the region spanning the promoter and the first exon of the high pI α-amylase in cultivated and wild barleys with the polymerase chain reaction (PCR). The results indicate that this region is conserved in the barley varieties as well as in the wild barley accessions from Israel. The wild barleys appear to possess higher DNA divergence in the region of the high pI α-amylase genes than that in cultivated barley.

Materials and methods

Plant material

Wheat, barley and rye varieties were supplied by the Australian Winter Cereal Collection (Tamworth). The sorghum variety was from Dr. R. Henzell (Hermitage Research Station). The wild barley accessions were from Dr. A. H. D. Brown (CSIRO, Canberra). The wheat-barley addition lines described by Islam et al. (1981) were kindly provided by Drs. K. W. Shepherd and A. K. R. M. Islam (Waite Institute). The nomenclature for the barley chromosomes is based upon the equivalent wheat homoeologous group (traditional barley chromosomes numbers are given in parentheses): 2H (2), 3H (3), 4H (4), 5H (7), 6H (6), and 7H (1).

DNA isolation

The procedure for genomic DNA isolation was a modification of a previously-described method (Weining et al. 1993). DNA was isolated on a small scale from 200 mg of young leaves. The leaves were ground to a powder in microfuge tubes under liquid nitrogen. The powder was then mixed with 0.6 ml of extraction buffer (3% sarkosyl, 0.1 M Tris-HCl, 10 mM EDTA, pH 8.0) and, subsequently, with 0.6 ml of phenol/chloroform (1:1). The whole mixture was shaken for 20 to 30 s and the aqueous phase recovered after centrifugation. The phenol/chloroform extraction was repeated and the DNA precipitated with ethanol.

PCRs and primers

All oligonucleotide primers were synthesized on an Applied Biosystems 381A DNA synthesizer. The polymerase chain reactions were carried out in a 25-µl volume containing 0.5 µg of genomic DNA template, 0.2 µM of each primer, 200 µM each of dATP, dCTP, dGTP and dTTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin and 0.5 units of *Taq* polymerase (Perkin Elmer Cetus). The PCR was performed in a Thermal Cycler (Perkin Elmer Cetus). The reaction commenced with 3 min at 94 °C, followed by 35 cycles of 1.0 min at 94 °C, 2 min at 55 °C and 2 min at 72 °C, terminated with 5 min at 72 °C. One fifth of the PCR products were analysed on 2% agarose gels and visualised under UV light following ethidium bromide staining.

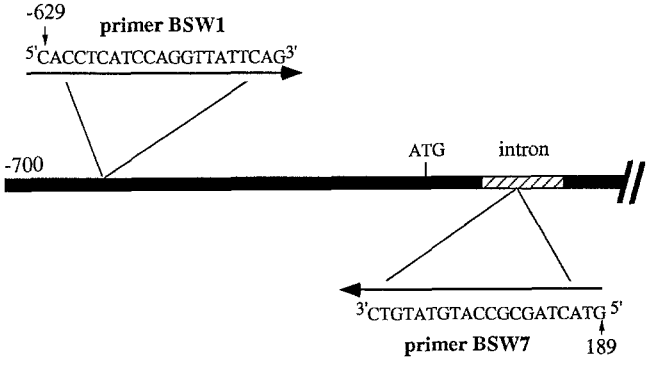

Fig. 1 Schematic representation of the location of the α-amylase primers used in this study. The sequences of primers Bsw1 and Bsw7 were based on the sequence of Amy 6-4 (Khursheed and Rogers 1988) and their numbering system is followed here

Results and discussion

There are varing degrees of homology between different members of the α-amylase gene families (Chandler et al. 1984;; Lazarus et al. 1985; Baulcombe et al. 1987; Knox et al. 1987; Huttly et al. 1988) resulting in a large number of bands in Southern hybridisations with α-amylase gene probes. α-Amylase primers derived from one cereal species often amplify more than one fragment in other species. This phenomenon is not confined to primers from the conserved coding region (Weining and Langridge 1991; Weining and Henry, unpublished data). A common feature of the primers studied previously is that they are located in the coding region of the gene. In this study, we focused our attention on DNA polymorphism in the promoter region. The two primers used are based on the sequences of a high pI α-amylase gene (Amy 6-4) (Khursheed and Rogers 1988). The sequences of the primers and the location of the primers relevant to the high pI α-amylase gene sequence are shown in Fig. 1. The 5′ primer (Bsw1) was cho-

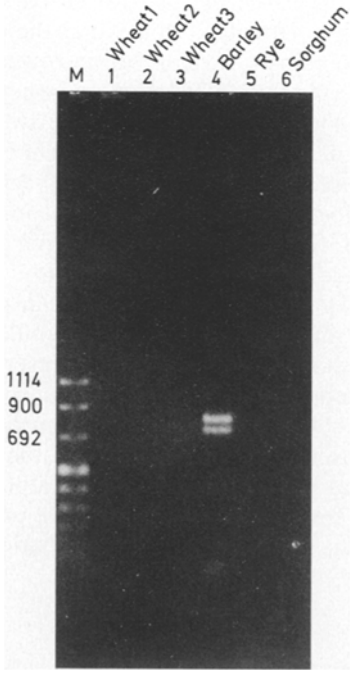

Fig. 2 Amplification of cereal DNA using α-amylase primers Bsw1 and Bsw7. The template DNA was from wheat: cv Chinese Spring in *lane 1*, cv Hartog in *lane 2* and cv Molineux in *lane 3*; barley (cv Betzes) in *lane 4*; rye (cv S. A. Rye) in *lane 5* and sorghum (cv Tam 422) in *lane 6*, respectively. DNA size markers are shown in *lane M*. The sizes of the marker bands are given on the left in base pairs. The PCR products were fractionated on a 1.5% agarose gel

sen to hybridise with sequences upstream of the promoter and other gibberellic acid- and abscisic acid-responsive elements, whereas the 3′ primer (Bsw7) is homologous to the sequences in the first intron (Rogers 1985; Khursheed and Rogers 1988; Jacobsen and Close 1991) to obtain barley-specific as well as high pI α-amylase-gene-specific PCR products.

Figure 2 shows the amplification products generated when the two primers, Bsw1 and Bsw7, were used with genomic DNAs from barley (cv Betzes) and other cereals: wheat (cv Chinese Spring; cv Hartog and cv Molineux), rye (cv S. A. Rye) and sorghum (cv Tam 422). The predicted length of the reaction product for barley α-amylase (*α-amy1*) is 820 bp. A band of approximately 820 bp was present in barley (lane 4, Fig. 2) but absent in wheat, rye and sorghum (lanes 1–3, 5 and 6, Fig. 2). Another unexpected product, around 750 bp, was also amplified in barley (cv Betzes; lane 4, Fig. 2). This lower band was not observed in any of the other cereals.

Genetic mapping of these two α-amylase bands was carried out with the barley addition line series (Islam et al. 1981). Both of the two PCR bands amplified in barley (cv Betzes) could be mapped to the long arm of barley chromosome 6H (compare lanes 1, 2 and 7, Fig. 3). The high pI α-amylase genes have been mapped onto the same barley chromosome arm by Southern hybridisation and isozyme analysis (Brown and Jacobson,1982; Muthukrishnan et al. 1984).

The barley varieties analysed in this study included both two-rowed and six-rowed types (Table 1); 14 were two-rowed whereas seven were six-rowed and their origins were quite diverse. Both of the PCR products present in cv Betzes could be amplified in all of the two-rowed barley varieties (lanes 1–14, Fig. 4). An important feature here is that the naked cultivar Himalaya, from which the sequence information was obtained, also has both the short fragment and the long one (lanes 8, Fig. 4). Five of the six-rowed barley varieties contained both of the PCR fragments (lanes 16, 17, 18, 20 and 21, Fig. 4) whereas the other two,

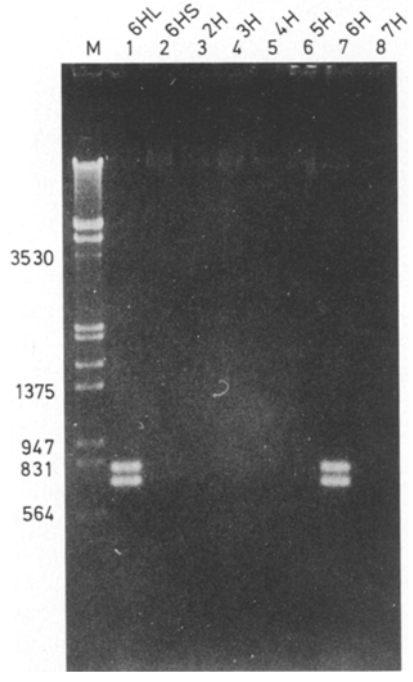

Fig. 3 Mapping of PCR products using wheat-barley addition lines. The template DNA was from 6H long arm in *lane 1*, 6H short arm in *lane 2* and the barley addition lines 2H to 7H, in *lanes 3 to 8*, respectively. DNA size markers are shown in *lane M*. The sizes of the marker bands are given on the left in base pairs. The PCR products were fractionated on a 1.5% agarose gel

Table 1 *H. vulgare* varieties and *H. spontaneum* accessions used for α-amylase polymorphism investigations

Barley varieties		
Code	Name	Origin /source
Two rowed		
1	Betzes	Europe
2	Blenheim	Europe
3	Cameo	Europe
4	Clipper	Australia
5	Gimpel	Europe
6	Grimmett	Australia
7	Harrington	Canada
8	Himalaya	Asia
9	Koru	Europe
10	Prior	Europe
11	Schooner	Australia
12	Stirling	Australia
13	Tallon	Australia
14	Triumph	Europe
Six rowed		
15	Bonneville	USA
16	Khemus	Bulgaria
17	Tapgolbori	Korea
18	Plana	Germany
19	Barbican	China
20	Kerabyg	Denmark
21	Batna	Algeria

Wild barley accessions		
Code	Site	Accession no.
1	Bar Giyyora	5
2	Bar Giyyora	10
3	Bar Giyyora	20
4	Bar Giyyora	48
5	Damon	13
6	Damon	22
7	Damon	23
8	Damon	37
9	Atlit	31
10	Atlit	2
11	Atlit	44
12	Atlit	50
13	Wadi Qilt	23
14	Wadi Qilt	31
15	Wadi Qilt	40
16	Wadi Qilt	55
17	Mehola	7
18	Mehola	26
19	Mehola	31
20	Mehola	53
21	Sede Boker	21
22	Sede Boker	27
23	Sede Boker	36

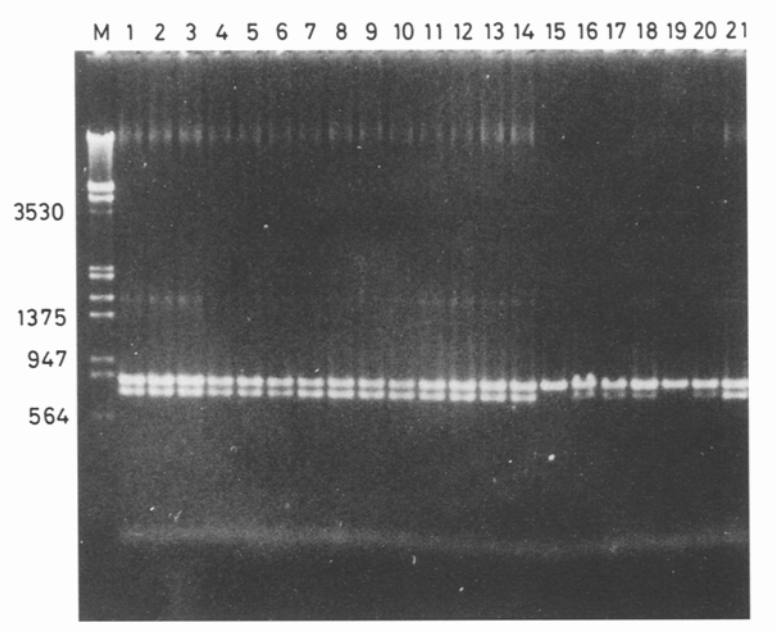

Fig. 4 Amplification of DNA from barley varieties using primers Bsw1 and Bsw7. DNA was amplified from a series of 2-rowed and 6-rowed barley varieties (Table 1) using primers Bsw1 and Bsw7. Lane 1, Betzes; *lane 2*, Blenhein; *lane 3*, Cameo; *lane 4*, Clipper; *lane 5*, Gimpel; *lane 6*, Grimmett; *lane 7*, Harrington; *lane 8*, Himalaya; *lane 9*, Koru; *lane 10*, Prior; *lane 11*, Schooner; *lane 12*, Stirling; *lane 13*, Tallon; *lane 14*, Trumph; *lane 15*, Bonneville; *lane 16*, Khemus; *lane 17*, Tapgolbori; *lane 18*, Plana; *lane 19*, Barbican; *lane 20*, Kerabyg; *lane 21*, Batna. DNA size marker (*lane M*) is shown on left with the sizes of the bands given in base pairs. The PCR products were fractionated on a 1.5% agarose gel

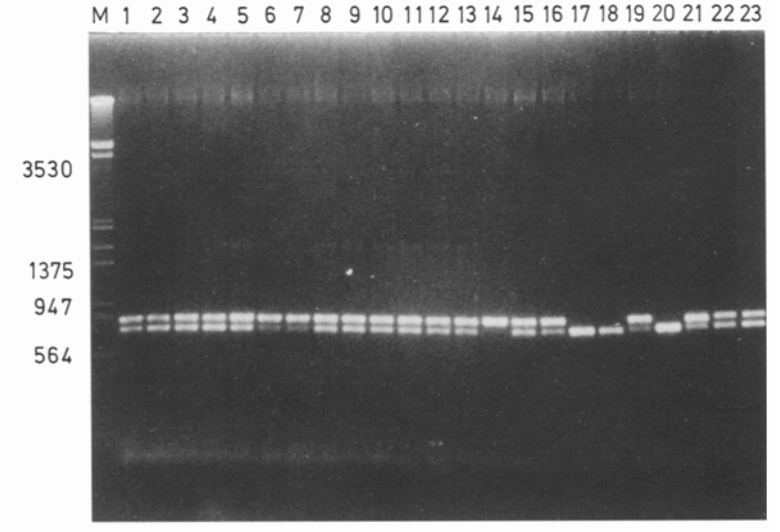

Fig. 5 Amplification of DNA from wild barleys using primers Bsw1 and Bsw7. DNA was amplified from a series of wild barley accessions (Table 1) using primers Bsw1 and Bsw7. *Lanes 1–23*, wild barley accessions 1–23 (code number as in Table 1). DNA size marker (*lane M*) is shown on left with the sizes of the bands given in base pairs. The PCR products were fractionated on a 1.5% agarose gel

one from the United States and the other China, were shown to have only the long fragment (lanes 15 and 19, Fig. 4).

Wild barleys (*H. spontaneum*), the progenitors of cultivated barley, were also tested with primer Bsw1 and Bsw7 (Table 1). The wild barley accessions were collected from 23 different habitats in Israel (A. H. D. Brown, personal communication). The amplification results with these accessions are presented in Fig. 5. A majority of the wild barleys, 18 out of 23, appeared to have both of the fragments (lanes 1–13, 15, 16, 21, 22 and 23, Fig. 5). Three accessions of wild barley contained only the short product (lanes 17, 18 and 20, Fig. 5) while two had only the longer fragment (lanes 14 and 19, Fig. 5).

The results clearly indicate that these two PCR fragments are barley specific and are well conserved in the two-rowed barley varieties. It seems that majority of the six-rowed barley varieties (5 out of 7) are similar to the two-rowed. However, we have not found a cultivated barley variety which contains only the short fragment.

The major difference between wild barley and cultivated barley is that the short fragment is present alone in a few wild barleys (3 out of 23) but not in cultivated barleys. Apparently, human selection has played an important role in fixing this DNA polymorphism. However, the exact mechanism of this process is unclear. The reason for the two-rowed barley varieties all having both of the PCR fragments instead of the three combinations present in wild barley is not clear. It could be that all of the two-rowed barley varieties have a common genetic origin or the *α-amy1* genes in two-rowed barley might be associated with a trait of agricultural importance. If it is the latter case, malting quality is unlikely to be a candidate as the non-malting barley varieties also contain both of the fragments. As wild barleys have been incorporated in numerous barley breeding programs, the various forms of *α-amy1* genes in wild barley could be easily introduced into cultivated barley. It is more reasonable to assume that *α-amy1* gene may be linked to a character which has been selectable by conven-

tional agricultural practise. It has been reported that the barley cultivar development program in North America has a longer history for six-rowed barley than for two-rowed barley (Martin et al. 1991). This may partially explain why the six-rowed barley contains more different forms of the *α-amy1* genes.

The results suggest that the wild barley possess a higher degree of polymorphism in *α-amy1* genes than that in barley cultivars. A higher genetic diversity of α-amylase isozymes was found in wild barley compared to cultivated barley (Brown and Jacobson 1982). It has also been shown before that wild barleys display a significantly wider range of α-amylase activities than cultivated barleys (Ahokas and Naskali 1990). The data presented here, at the DNA level through PCR, is in agreement with the biochemical and genetic analysis of Brown and Jacobson (1982) as well as that of Ahokas and Naskali (1990).

There have been numerous studies on the levels of genetic polymorphism between cultivated barley and its wild progenitor. Generally, a reduced genetic diversity has been observed under domestication (for a review, see Nevo 1992). The result presented here supports this conclusion.

It seems that the two PCR fragments were generated from two different *α-amy1* genes. Their presence in cultivated or wild barley is independent from each other. Different mechanisms have been proposed to account for the various forms of α-amylase isozymes, either due to the existence of families of α-amylase genes differing in DNA sequence to various extents (Khursheed and Rogers 1988) or to post-translational modifications (Sogaard et al. 1991). The two *α-amy1* genes observed in this study fit the first model.

The general advantages of PCR over isozymes and Southerns, such as cost and speed, have been discussed previously (D'Ovidio et al. 1990; Weining and Langridge 1991). With carefully-selected primers in the α-amylase genes, PCR can be used to target a specific region, or a specific gene family without the interference encountered in isozyme or Southern analysis. Furthermore, as demonstrated in this study, PCR can reveal DNA polymorphism which is impossible or difficult to detect through conventional approaches.

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