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## Characterisation of disproportionating enzyme from wheat endosperm

Received: 10 July 2005 / Accepted: 15 November 2005 / Published online: 7 December 2005  
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**Abstract** Disproportionating enzyme or D-enzyme (EC 2.4.1.25) is an  $\alpha$ -1,4 glucanotransferase which catalyses cleavage and transfer reactions involving  $\alpha$ -1,4 linked glucans altering (disproportionating) the chain length distribution of pools of oligosaccharides. While D-enzyme has been well characterised in some plants, e.g. potato and *Arabidopsis*, very little is known about its abundance and function in cereals which constitute the major source of starch worldwide. To address this we have investigated D-enzyme in wheat (*Triticum aestivum*). Two putative D-enzyme cDNA clones have been isolated from tissue-specific cDNA libraries. TaDPE1-e, from an endosperm cDNA library, encodes a putative polypeptide of 575 amino acid residues including a predicted transit peptide of 41 amino acids. The second cDNA clone, TaDPE1-l, from an *Aegilops tauschii* leaf cDNA library, encodes a putative polypeptide of 579 amino acids including a predicted transit peptide of 45 amino acids. The mature polypeptides TaDPE1-e and TaDPE1-l were calculated to be 59 and 60 kDa, respectively, and had 96% identity. The putative polypeptides had significant identity with deduced D-enzyme sequences from corn and rice, and all the expected conserved residues were present. Protein analysis revealed that D-enzyme is present in the amyloplast of developing endosperm and in the germinating seeds. D-enzyme was partially purified from wheat endosperm and shown to exhibit disproportionating activity in vitro

by cleaving maltotriose to produce glucose as well as being able to use maltoheptaose as the donor for the addition of glucans to the outer chains of glycogen and amylopectin.

**Keywords** Disproportionating enzyme · Wheat · Amyloplast · Starch synthesis

**Abbreviations** DP: Degree of polymerisation · dpa: Days post anthesis · GBSS: Granule bound starch synthase

### Introduction

Starch is present in a wide range of tissues of plants where it serves as a store of carbohydrate and energy. In leaves, starch is formed and degraded in a diurnal cycle that allows for the maintenance of a supply of carbon from the leaves when photosynthesis is inactive. In cereal grains, starch comprises approximately 65% of the dry weight of the cereal grain, and its purpose is to supply energy and carbon to support germination and emergence. Starch is composed of two classes of polymers of glucose: amylose and amylopectin, in an approximate ratio of 1:3. Both amylose and amylopectin consist of a backbone of  $\alpha$ -1,4 linked glucose residues, but, whereas amylose has a degree of polymerisation (DP) of 1,000–5,000 and few branch points (fewer than 0.5%  $\alpha$ -1,6 linkages), amylopectin is a very large molecule (DP 5,000–50,000) with frequent branches (3–4%  $\alpha$ -1,6 linkages).

Starch biosynthesis occurs in plastids and requires the concerted action of four different classes of enzymes (see Kossmann and Lloyd 2000; Smith 2001; Ball and Morell 2003; James et al. 2003; Tetlow et al. 2004 for reviews). ADP-glucose pyrophosphorylase (AGPase; EC 2.7.7.27) is regarded as the first committed step in the pathway producing the activated glucosyl precursor (ADP-glucose), which is the source of glucose for chain

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elongation and subsequent branching. Starch synthases (SS: EC 2.4.1.21) catalyse the elongation of the chains, granule bound starch synthase (GBSS) is involved in the synthesis of amylose, while the soluble starch synthases elongate glucan chains of amylopectin. Branch points are introduced by the action of branching enzymes (BE: EC 2.4.1.18). Genetic evidence demonstrates that the debranching enzymes isoamylase (EC 3.2.1.68) and pullulanase (EC 3.2.1.41) play a role in starch synthesis; however, their precise biochemical functions still have to be elucidated.

A variety of enzymatic systems with the capacity to degrade starch have been found in plants, including  $\alpha$ -amylases (EC 3.2.1.1),  $\beta$ -amylases (EC 3.2.1.1),  $\alpha$ -glucosidases, debranching enzymes, starch phosphorylase (EC 2.4.1.1.) and disproportionating enzyme (EC 2.4.1.25). Both forward and reverse genetics approaches have been used to define the individual contributions of these enzymes to starch degradation, primarily in leaves. In leaves,  $\beta$ -amylolysis appears to be the major route for starch degradation. In germinating seeds, a combination of  $\alpha$ -amylase,  $\beta$ -amylase, starch phosphorylase,  $\alpha$ -glucosidase and pullulanase ( $\beta$ -limit dextrinase) has been suggested to be responsible for starch degradation (see Lloyd et al. 2005; Smith et al. 2005 for reviews).

While the enzymes noted above have clear roles in either starch synthesis or degradation, there are ranges of enzymes whose properties do not unambiguously assign them a role in synthesis or degradation alone, and where the genetic evidence in favour of one role or the other is not complete. Such enzymes include starch phosphorylase, pullulanase and disproportionating enzyme (or D-enzyme).

Disproportionating enzyme is an  $\alpha$ -1,4 glucanotransferase which catalyses the cleavage of  $\alpha$ -1,4 glucosidic bonds of polyglucans (maltotriose or larger), transferring the glucosyl groups to the non-reducing end of another glucan chain or free glucose, and releasing either glucose or a glucan chain depending on the cleavage site. D-enzymes have been identified in a number of plant species and has been characterised most extensively in potato (Peat et al. 1956; Jones and Whelan 1969; Takaha et al. 1998a, b, 1993, 1996; Takaha and Smith 1999) and *Arabidopsis* (Critchley et al. 2001). Recently, an enzyme that was identified, based upon protein sequence similarity, as a second isoform of D-enzyme (DPE2) and characterised in both *Arabidopsis* and potato (Chia et al. 2004; Lu and Sharkey 2004; Lloyd et al. 2004). As this enzyme has the ability to use maltose as a donor molecule to transfer one glucose moiety to a polysaccharide, leading to release of the other moiety (Chia et al. 2004; Lloyd et al. 2004), it has now been reclassified as a transglucosidase (Lloyd et al. 2005; Smith et al. 2005). The bacterial disproportionating enzyme, MalQ, has been extensively studied and is involved in the degradation of maltose (Boos and Shuman 1998; Xavier et al. 1999).

The role of D-enzymes in higher plants has been examined in several systems, with quite different results. The identification of mutants in the alga *Chlamydomonas reinhardtii* D-enzyme with reduced starch synthesis and a causal lesion in D-enzyme suggest a critical role for the enzyme in amylopectin synthesis (Colleoni et al. 1999a, b). However, knock-out studies in the leaves and tubers of plants have not supported a role for D-enzyme in starch synthesis. Anti-sense inhibition of D-enzyme in potatoes, in which D-enzyme expression levels were reduced to approximately 1% of wild type, did not alter starch structure in the tuber (Takaha et al. 1998a). In *Arabidopsis*, loss of D-enzyme activity had no effect on starch synthesis, but starch degradation was impaired, raising the possibility that D-enzyme is involved in the metabolism of malto-oligosaccharides during starch degradation (Critchley et al. 2001). Loss of activity of the transglucosidase in both *Arabidopsis* and potato resulted in both maltose and starch excess phenotype (Chia et al. 2004; Lu and Sharkey 2004; Lloyd et al. 2004). The similarity in phenotype is striking as in *Arabidopsis*; the transglucosidase is cytosolic (Chia et al. 2004), while in potato it is plastidic (Lloyd et al. 2004). While the *Arabidopsis* and potato systems are now well characterised, very little is known about the role of D-enzyme in starch metabolism in the leaves and endosperm of cereals.

In this paper we report the identification of cDNAs encoding D-enzyme from hexaploid wheat (*Triticum aestivum*) and diploid *Aegilops tauschii*, the donor of the D-genome to wheat, and also a partial gene structure for the gene encoding D-enzyme from *A. tauschii*. The chromosomal location of this gene is defined and is syntenous with the location of the rice gene encoding the D-enzyme that has the highest identity with the gene identified in this study. Further, we show that the protein encoded by these sequences is present in the amyloplasts of developing endosperm and in germinating grain. A partially purified D-enzyme from the endosperm of developing grain was shown to have the ability to disproportionate maltotriose and to add glucans cleaved from maltoheptaose to the outer chains of glycogen, amylopectin and starch.

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## Materials and methods

### Plant material

All plants were grown in glasshouses. Endosperm from *T. aestivum* cv. Hartog was collected at 5, 8, 11, 15, 18, 21 and 25 days post anthesis (dpa), immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . *T. aestivum* cv. Fielder was used for the collection of endosperm 15–18 dpa and also frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . The spring wheat *T. aestivum* cv. Axona was used for amyloplast purification.

## Libraries used in screening for D-enzyme cDNA and genomic clones

The following cDNA libraries were used in this study: (a) endosperm cDNA library from wheat cultivar Wyuna (Li et al. 1999b), and (b) leaf cDNA library from *A. tauschii*, accession AUS 18913 (provided by Dr Evans Lagudah, CSIRO Plant Industry, Canberra, Australia). The leaf library was constructed using the same protocol as described in Lagudah et al. (1997) for a root cDNA library from *A. tauschii*. Genomic libraries from *A. tauschii* var. Strangulata (Rahman et al. 1997), *T. aestivum* cv. Rosella (Turner et al. 1999) and a large insert BAC library from *A. tauschii* AUS 18913 (Moulet et al. 1999) were also used.

The endosperm cDNA library was screened with two different probes. The first was amplified from an unpublished wheat EST that had significant homology to D-enzyme from potato, using the primers ZLWD1 and ZLWD2 (Table 1) yielding a 268 bp probe. The second probe was a potato D-enzyme cDNA (Genbank accession x68664).

A probe to screen the *A. tauschii* leaf library was made by amplifying a region of the endosperm D-enzyme cDNA using the following primers NBGen17 and NBGen20 (Table 1). This resulted in the generation of a 207 bp probe that encodes a region that is highly conserved in all D-enzymes described.

## Southern-blot analysis

DNA was isolated from leaves of *A. tauschii* var. Strangulata and *T. aestivum* cv. Chinese Spring based on a protocol used by Lagudah et al. (1991). For digests 10 µg of *A. tauschii* and 30 µg of Chinese Spring DNA were used, respectively.

## Extraction of soluble proteins from developing wheat endosperm

Total soluble protein was extracted by homogenising the developing endosperm in a microfuge tube with 1.5 volumes of extraction buffer (25 mM Tris-HCl, pH 7.5, 5 mM EDTA, 5 mM DTT, 1 mM Pefabloc, 20% (v/v) glycerol). Mature dried seed was broken up with a hammer and left in 1.5 volumes of extraction buffer

**Table 1** Sequence of primers used in this study

Primer	Sequence
ZLWD1	CACTCCAGCTACACAGAAAGG
ZLWD2	ACCCTATTATTCGGGGACAGG
NBGen17	GGACAATAATGGTTTCCCCAC
NBGen20	CTGCCCAAAAACCAGCAAGC
NBGen9	GAATTTGACACCGTTGCTAATCTG
NBGen16	GATTTCTGTTTGCCCAAACAT
NBNT32F	GATGCGTTCAAAGCTGTTG
NBNT32R	GAAGAAGTGCATCCCTGGAG

overnight at 4°C. The samples were pelleted by centrifugation at 11,000 g for 10 min at 4°C. The supernatant containing soluble protein was collected and the protein concentration determined by performing a Bradford Assay using BSA as a standard (Bradford 1976).

## Production of a polyclonal antibody raised against wheat endosperm D-enzyme

A synthetic peptide, SVGVGEDLPEGYEQM, from near the N-terminus of the mature endosperm polypeptide (TaDPE1-e), as deduced from the cDNA sequence, was used to produce a carrier-coupled peptide (DE1). Residues 2–15 of DE1 are conserved in TaDPE1-l. The peptide was conjugated to the carrier protein ovalbumin (OVA) using the heterobifunctional cross-linker 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS, Pierce Chemical Co.). The polyclonal antibody raised against the synthetic peptide was designated anti-DE1 and purified on an affinity column containing the synthetic peptide bound to Sulfolink® Coupling Gel (Pierce) using standard techniques (Harlow and Lane 1999).

## Localisation of D-enzyme to the wheat endosperm amyloplasts

Amyloplasts from spring wheat cultivar Axona were prepared as described by Tetlow et al. (2003) and were used at 0.9–1.1 mg ml<sup>-1</sup> protein content. Lysed plastid preparations were prepared by freezing and thawing samples three times using liquid nitrogen. Trypsin protection experiments were performed by incubating intact and lysed fractions on ice with 4 mg ml<sup>-1</sup> trypsin (Sigma, type XI from bovine pancreas) for 20 min. Following incubation, a solution of 12 mg ml<sup>-1</sup> trypsin inhibitor was added (Fluka, from soybean). Samples were then freeze-thawed, centrifuged to remove starch and cell debris (13,000 g for 5 min) and the supernatant immediately mixed with SDS-sample buffer and boiled for 2 min. Samples were run out on Invitrogen 4–12% Bis-Tris gradient gels in a MOPS-based buffer, following the manufacturer's instructions, transferred onto nitrocellulose membranes using standard techniques, and then probed with the anti-DE1 antibody. Blots were visualised using the alkaline phosphatase/BCIP/NBT system.

## Partial purification of endosperm D-enzyme of wheat

Developing endosperm (3 g) was collected from *T. aestivum* cv. Fielder at approximately 15–18 dpa. The endosperm was homogenised in a mortar and pestle with 1.5 volumes of ice-cold buffer (25 mM sodium acetate, 2 mM DTT, 1 mM Pefabloc). The soluble fraction was separated by centrifugation at 16,000 g for 10 min at 4°C. The volume of the soluble fraction was determined, and a 20% ammonium sulphate precipitation was per-

formed by slowly adding 3.93 M solution of ammonium sulphate with mixing over 20 min at 4°C, so that the final concentration of ammonium sulphate was 9.8 mM. The mixture was left at 4°C without stirring for 10 min. The pellet was separated from soluble protein by centrifugation at 16,000 g for 10 min at 4°C. The supernatant was retained and a 50% ammonium sulphate precipitation performed (as previously). The second pellet was separated (as previously) and resuspended in 500 µl size exclusion buffer (50 mM sodium acetate, pH 6.0, 2 mM DTT).

The resuspended pellet was semi-purified on a FPLC (Pharmacia) system using a Superdex 200 10/300 GL size exclusion column (Pharmacia). Fractions were eluted at 1 ml min<sup>-1</sup>, and every second fraction was analysed by Western blot analysis with the polyclonal antibody raised against the synthetic peptide DE1. The approximate molecular weight of native D-enzyme was estimated in comparison to molecular weight standards.

A Mono-Q 10/300 GL anion exchange column on a FPLC system was used to further purify D-enzyme. The fractions containing D-enzyme from the Superdex 200 (Pharmacia) size exclusion column were diluted to reduce the sodium acetate concentration by addition of buffer (20 mM sodium acetate, 2 mM DTT, pH 6.0) until the volume was 10 ml. The sample was injected onto a Mono-Q anion exchange column and eluted at 1 ml min<sup>-1</sup> with a NaCl gradient that increased from 0 to 500 mM over 20 min. Every second fraction was analysed by Western blot analysis using the anti-DE1 antibody to determine where D-enzyme was eluted from the gradient. One-twentieth of every second fraction from the Mono-Q column containing D-enzyme were separated on a SDS-PAGE and compared to the 50% ammonium sulphate fraction to determine the purity of the fractions. The gel was then stained with Coomassie R-250 (0.25% (w/v), 10% (v/v) acetic acid, 50% (v/v) ethanol) for 1 h and destained in a solution containing 50% (v/v) ethanol and 8.3% (v/v) acetic acid for 16 h.

### Gel electrophoresis and immunoblotting

SDS-PAGE and non-denaturing PAGE were carried out using 8% gels according to Laemmli (1970). A modified version of Towbin et al. (1979) was employed for immunoblotting.

### Zymogram analyses

Zymogram analyses were conducted as described by Colleoni et al. (1999b). The elongating activity of wheat endosperm D-enzyme was examined by running the partially purified D-enzyme extract in duplicate on 8% non-denaturing polyacrylamide gels containing either 0.1% (w/v) bovine liver glycogen (Sigma), 0.1% (w/v) potato amylopectin (Sigma) or 0.1% (w/v) potato starch (Sigma). Upon completion the duplicate lane was

excised and Western blot analysis was performed using the polyclonal antibody anti-DE1. The other lanes containing separated proteins were incubated twice for 15 min each at 37°C in a solution containing 100 mM Tris-HCl, pH 7.0, 1 mM MgCl<sub>2</sub>, 1 mM EDTA and 1 mM DTT. The gels were then incubated in the same solution to which 2 mM maltoheptaose (Sigma) had been added, for 16 h at 37°C. The gel was then briefly washed with distilled water to remove traces of DTT and stained with iodine stain (0.2% (w/v) iodine, 2% (w/v) potassium iodide).

The disproportionating activity of D-enzyme was examined by running semi-purified D-enzyme samples in duplicate on a non-denaturing gel. One of the lanes was analysed by Western blot analysis using the polyclonal antibody anti-DE1. The second lane was incubated in the dark at room temperature in an assay mix developed by Colleoni et al. (1999a) containing 3 mg ml<sup>-1</sup> maltotriose (Sigma), 200 mM Tris-HCl, pH 8.0, 1 mM EDTA, 42 mM MgCl<sub>2</sub>, 0.014% (w/v) NADP, 0.027% (w/v) NAD, 0.027% (w/v) MTT, 0.015% (w/v) PMS, 1.5 mM ATP, 1 U ml<sup>-1</sup> hexokinase, 0.5 U ml<sup>-1</sup> Glc-6-PDH. When D-enzyme produces glucose from maltotriose a blue band is seen on the gel as the glucose is converted to glucose-6-phosphate by hexokinase, which in turn is converted to 6-phosphogluconate by glucose-6-phosphate dehydrogenase. The 6-phosphogluconate in the presence of NAD<sup>+</sup> and NADP<sup>+</sup>, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and phenazine methylsulphate (PMS) produces formazan, which is blue in colour and precipitates in the gel at the site of D-enzyme activity.

### Glucose assay and capillary electrophoresis

A method adapted from Colleoni et al. (1999b) was used to determine the disproportionating and elongating capacity of D-enzyme in solution. Aliquots of 300 µl from two of the partially purified fractions containing D-enzyme activity were mixed with 3 µl of 100 mM maltotriose in 20 mM sodium acetate, pH 6.0 and 300 µl water was mixed with 3 µl of 100 mM maltotriose in 20 mM sodium acetate, pH 6.0 as a negative control. The tubes were incubated at 30°C for 1 h.

Aliquots of 250 µl from the maltotriose assays were dried under vacuum. The samples were labelled for analysis in a P/ACE 8000 capillary electrophoresis system with argon-laser LIF detection as described by O'Shea and Morell (1996).

## Results

### Isolation of D-enzyme cDNA sequences expressed in wheat endosperm and leaf

Two nucleic acid probes, as described in the [Materials and methods](#), were used to screen a *T. aestivum* cv.

Wyuna endosperm cDNA library. Sequencing of a positive clone revealed a cDNA (TaDPE1-e) of 2,110 bp (Genbank accession DQ068045). A putative ORF encoding a polypeptide of 575 amino acids was identified. The ORF had significant identity with D-enzymes from corn (81%—AY109140), rice (76%—NM\_193039), potato (66%—X68664), *Arabidopsis* (64%—At5g64860) and *C. reinhardtii* (49%). To determine if the putative polypeptide contained a plastid targeting sequence, the first 100 amino acids were analysed with ChloroP (Emanuelsson et al. 1999). The sequence was predicted to contain a transit peptide of 41 amino acids. The molecular mass of the mature polypeptide was estimated to be 59 kDa.

In order to establish whether genes encoding different isoforms of D-enzyme are expressed in different tissues of the wheat plant a leaf cDNA library from *A. tauschii*, the donor of the D genome of wheat, was screened, using a 207 bp fragment of TaDPE1-e as the probe. Analysis of a positive clone revealed a cDNA clone (TaDPE1-l) of 2,109 bp long encoding a putative polypeptide of 579 aa (Genbank accession DQ068046). The molecular mass was calculated to be 60 kDa, and analysis using ChloroP revealed a predicted transit peptide of 45 amino acids. At the nucleotide level the coding region and 3' UTR of TaDPE1-l had 97 and 95% identity with TaDPE1-e, respectively. The putative polypeptides encoded by TaDPE1-e and TaDPE1-l had 96% identity, suggesting that they are members of the same class of D-enzyme.

Analysis of the crystal structure of MalQ from *T. aquaticus* and subsequent sequence alignments identified four regions that are conserved in all  $\alpha$ -amylase superfamily enzymes and a fifth region encompassing the "250s loop" and flanking  $\beta$ -strands that is conserved in all D-enzyme and MalQ sequences (Przylas et al. 2000). The wheat sequences identified in this study contain all five of these conserved regions (Fig. 1), thus strengthening their identification as genes encoding D-enzymes.

A selection of sequences annotated as D-enzyme in the databases and MalQ from *Escherichia coli* were aligned using ClustalW (Thompson et al. 1994), and a phylogenetic tree was constructed (Fig. 2). The D-enzyme sequences were separated into two distinct groups with the bacterial MalQ being equidistant from both. The wheat endosperm and leaf D-enzymes were in a group with rice (NM\_193039), potato (X68664), *Arabidopsis* chromosome 5 D-enzyme and corn (AY109140) sequences. The second group contained the *Arabidopsis* chromosome 2 D-enzyme and the second potato D-enzyme (AY510449), which have recently been reclassified as transglucosidases (Lloyd et al. 2005; Smith et al. 2005), rice (NM\_186042 and BAD31425), corn (US patent 5994623 seq#2) and soybean sequences along with a wheat EST sequence. The identity between these two groups is low with the *Arabidopsis* chromosome 2 sequence only having 25–30% identity compared to the sequences in the first group. This suggests that there are two distinct classes of enzymes: class I which contains

true D-enzymes, that are unable to utilise maltose as the glucan donor, and class II that groups with the transglucosidases which are able to utilise maltose as the glucan donor. The proteins encoded by the wheat endosperm and leaf cDNAs identified in this study fall into class I and are thus likely to be members of a homoeologous family.

#### Gene structure of wheat D-enzyme

Screening of an *A. tauschii* genomic library, using the same probes used to screen the endosperm cDNA library from *T. aestivum* cv. Wyuna, identified one positive clone, encoding the 3' end of the gene. Despite extensive screening of available libraries no clones, which contained the 5' region of the gene were identified. A PCR-based approach, with primers designed against the cDNA sequences, was used to extend the coverage of the genomic sequence. The putative exon/intron structure of the sequence obtained (Genbank accession DQ068047) was determined in comparison to the cDNAs; ten exons and nine introns were identified. The exon/intron structure of the available *A. tauschii* D-enzyme sequence is very similar to that of the *Arabidopsis* (chromosome 5) and rice (NM\_193039 on chromosome 7) D-enzymes (Fig. 3). Based upon this similarity, it is expected that six more exons and introns remain to be identified in the *A. tauschii* D-enzyme genomic sequence. All attempts to amplify the remaining region using the PCR-based strategy have failed suggesting that there is a large intron between exons 6 and 7.

#### Chromosomal location and copy number

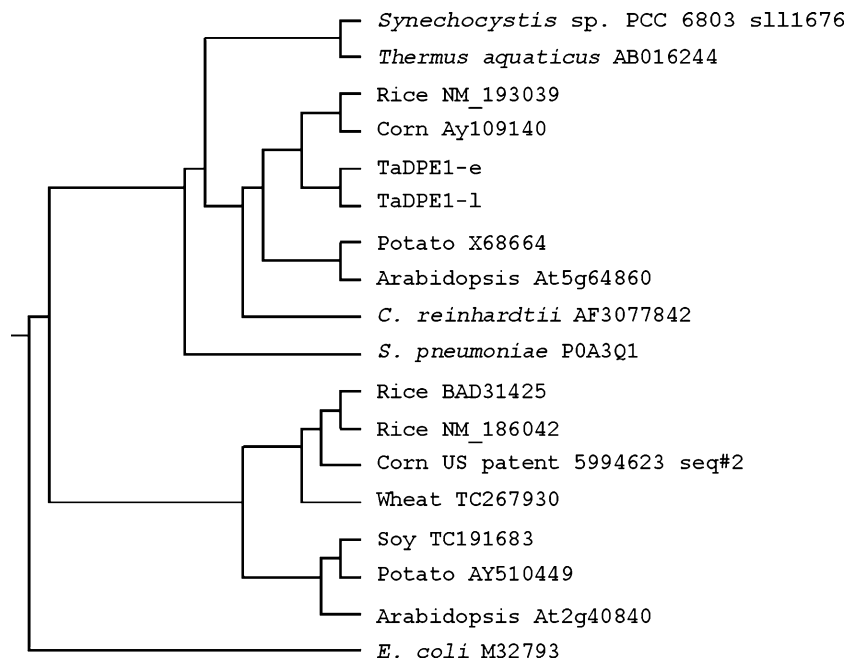
Primers were designed from the available exon sequences of the *A. tauschii* genomic clone to amplify the introns of D-enzyme to determine if genome-specific sequences could be identified. PCR analysis was performed using DNA from nullisomic to tetrasomic lines of *T. aestivum* cv. Chinese Spring (Sears and Miller, 1985). With one primer pair NBNT32F and NBNT32R (Table 1) amplification using euploid Chinese Spring genomic DNA as the template resulted in two PCR products (Fig. 4), while in line N2BT2D which lacked chromosome 2B (but had four copies of chromosome 2D) the lower band was absent (Fig. 4, lane N2BT2D) suggesting that it is specific for the gene encoding D-enzyme located on chromosome 2B. The upper band is the amplification product of both the A and D genomes as it was present when genomic DNA from lines N2AT2B (lacks chromosome 2A, but has four copies of 2B) and N2DT2B (lacks chromosome 2D, but has four copies of 2B) was used as the template (data not shown). The lower band was also absent when DNA from a line lacking the distal 50% of chromosome 2B is used as the template (Fig. 4, Lane 2BS-1) suggesting that the

	Region 1 ←-----→	Region 2 ←-----→	Region 3 ←-----→	Region 4 ←-----→	
	206	244 -----	287	335	387
<i>Synechocystis</i> 6803 s111676	RHIAIFGDLPIY	VGVPPDYFSATGQLWGNP	LDIVRIDHFRG	LIPVAEDLG	AVVYTGTHD
<i>T. aquaticus</i> AB016244	LGIRIIGDMPIF	VGVPPDYFSETGQRWGNP	FHLVRIDHFRG	VIVLAEDLG	VVYTGTHD
Rice NM 193039	LGISIMGDMPIY	VGVPPDAFSETGQLWNSP	YDEFRIDHFRG	INIIAEDLG	QVYTGTHD
Corn AY109140	LCISIMGDMPIY	VGVPPDAFSETGQLWNSP	YDEFRIDHFRG	IDIIAEDLG	QVYTGTHD
TaDPE1-e	LGISIMGDMPIY	VGVPPDAFSETGQLWNSP	YDEFRIDHFRG	INIIAEDLG	QVYTGTHD
TaDPE1-l	LGISIMGDMPIY	VGVPPDAFSETGQLWNSP	YDEFRIDHFRG	INIIAEDLG	QVYTGTHD
Potato X68664	KGISIMGDMPIY	VGVPPDAFSETGQLWNSP	YDEFRIDHFRG	INIIAEDLG	QVYTGTHD
Arabidopsis At5g64860	QGVDMGDMPIY	VGVPPDLFSETGQLWNSP	YDEFRIDHFRG	INIIAEDLG	QVYSGTHD
<i>C. reinhardtii</i> AF307842	KGIKIMGDMPIY	VGVPPDAFSATGQLWNSP	YDEFRIDHFRG	VPILAEDLG	CFVYPGTHD
<i>S. pneumoniae</i> P0A3Q1	NHIELIGDMPIY	VGVPPDAFSATGQLWNSP	YDEFRIDHFRG	INIIAEDLG	SVMYTGTHD
Rice BAD31425	KKVIVGDMPIY	VGAPPDYFDKNGQNWGFP	FTAYRIDHILG	MLACGEDLG	TVCAPSCHD
Rice NM-186042	KKVILKGDLPIG	VGAPPDYFDKNGQNWGFP	FTAYRIDHILG	MLACGEDLG	TVCAPSCHD
Corn US patent 5994623 seq#2	KNVILKGDLPIG	VGAPPDYFDKNGQNWGFP	FTAYRIDHILG	MLACGEDLG	TVCAPSCHD
Wheat TC174012	NKVILKGDLPIG	VGAPPDYFDKNGQNWGFP	FTAYRIDHILG	MLACGEDLG	TVCAPSCHD
Soy TC191683	KGVILKGDLPIG	VGAPPDYFDKNGQNWGFP	FTAYRIDHILG	MLACGEDLG	TVCAPSCHD
Potato AY510449	KGVVLKGDLPIG	VGAPPDYFDKNGQNWGFP	FTAYRIDHILG	MLACGEDLG	TVCAPSCHD
Arabidopsis At2g40840	KGVVLKGDLPIG	VGAPPDYFDKNGQNWGFP	FTAYRIDHILG	MLACGEDLG	TVCAPSCHD
<i>E. coli</i> M32793	MPIGLYRDLAVG	VGAPPDILGPLQNWGLP	CGALRIDHVMV	CMVIGEDLG	SMAVAATHD
	*	*	***	**	**

**Fig. 1** Conserved regions shared by D-enzyme and MalQ sequences. The four regions indicated by arrows are those that are common to all  $\alpha$ -amylase superfamily enzymes (Przylyas et al. 2000). The shaded grey region represents the “250s loop” and flanking  $\beta$ -strands, the dashed line indicates the residues that encompass the “250s loop” (Przylyas et al. 2000). Numbers above the alignment represent the position of the first residue of each

conserved region in the *T. aquaticus* D-enzyme sequence. Conserved active site residues are marked by asterisk below the alignment. Genbank accession numbers are given for each sequence, TC numbers are TIGR consensus sequences from <http://www.tigr.org/tdb/tgi/> for the respective organisms, and the *Synechocystis* sp. PCC6803 sequence is from Cyanobase <http://www.kazusa.or.jp/cyano/cyano.html>

**Fig. 2** Phylogenetic tree of D-enzyme-like sequences and MalQ from a number of organisms. The sequences were aligned using ClustalW, and the phylogenetic tree was drawn using the nearest neighbour-joining algorithm. Sequence annotations are as in Fig. 1. Note: the proteins encoded by *Arabidopsis* At2g40840 and Potato AY510449 have recently been reclassified as transglucosidases (Lloyd et al. 2005; Smith et al. 2005)



genomic D-enzyme clone is located in that region of the chromosome.

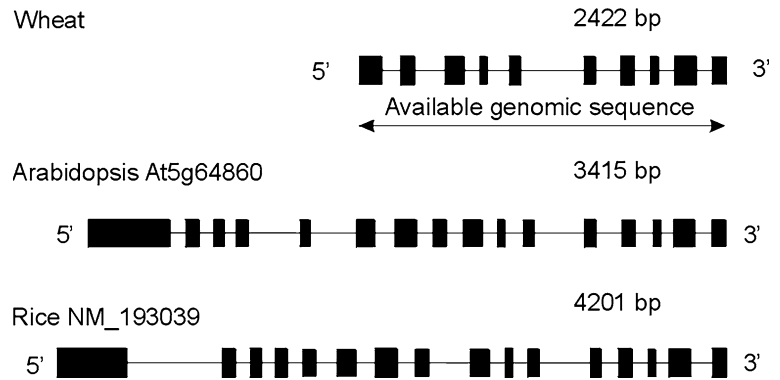
Southern blot analysis was performed to determine the copy number of D-enzyme within the wheat genome. Using a probe internal to the TaDPE1-e sequence only one band of approximately 800 bp hybridised with the probe when genomic DNA from *A. tauschii* was used as the template (data not shown). This suggests that only one copy of the gene encoding the D-enzyme sequence analysed here is present in each genome. Given the low

level of homology between the classes of D-enzymes in plants, the probe used in this study would be highly unlikely to cross-react with a gene encoding the second class of D-enzyme present in the genome.

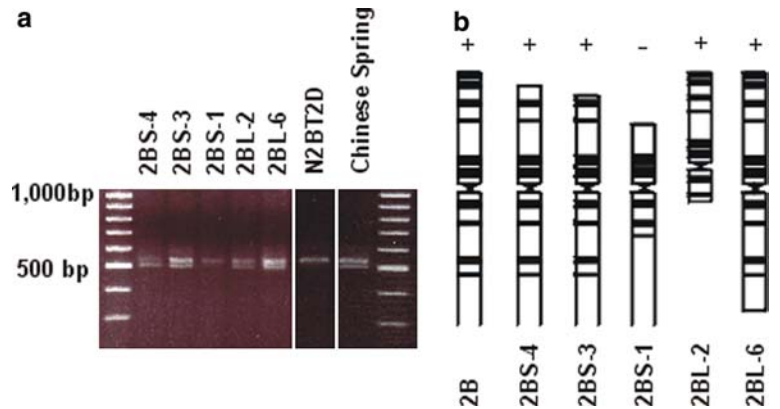
#### Localisation of D-enzyme

The polyclonal antisera raised against DE1 was used to probe a Western blot of a denaturing polyacrylamide

**Fig. 3** Comparison of partial *A. tauschii* D-enzyme gene structure with Arabidopsis and rice. Exons shaded boxes, introns continuous line



**Fig. 4 a** PCR analysis of Chinese Spring deletion lines of chromosome 2B. Deletions made at location 4, 3 and 1 on the short arm and locations 2 and 6 on the long arm (Endo and Gill 1996) were used in PCR to determine the location of D-enzyme on chromosome 2B of wheat. Those products missing the bottom band on the gel indicate that the gene is missing from the template. **b** The map of each of the deletion lines. + or - indicates the presence or absence of a PCR product



gel of soluble protein fractions from various wheat tissues for the presence of D-enzyme. The protein that cross-reacted most strongly with the antisera, in wheat endosperm samples, was approximately 65 kDa (Fig. 5), which is similar to the estimated molecular weight of 59 kDa of wheat endosperm D-enzyme. This protein is present in all stages of development analysed (Fig. 5). A protein cross-reacting with the anti-DE1 antisera is also found in germinating mature wheat seeds. The presence of D-enzyme in both developing and germinating seeds suggests that it may have a role in both starch synthesis and degradation in wheat.

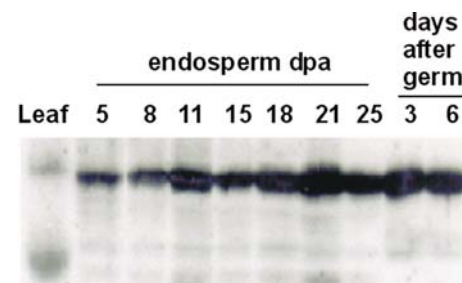
For D-enzyme to have a role in starch synthesis, in the endosperm, it must be localised within the amyloplast in developing endosperm. To test this, amyloplasts were isolated from developing endosperm and the sample subjected to a trypsin protection study. Western analysis of these samples using the antibody against D-enzyme detected a protein in the intact amyloplast samples but not in the samples that were lysed prior to the trypsin treatment (Fig. 6). This clearly demonstrates that D-enzyme is present within the amyloplast, the site of starch synthesis.

Despite the presence of transcripts for D-enzyme in leaves, as determined by RT-PCR (data not shown), it is unclear from the Western blots if the protein is present in leaves. In leaves the antibody detects two proteins at low levels, but neither of these are the same size as the D-enzyme detected in endosperm (Fig. 5). Thus, further

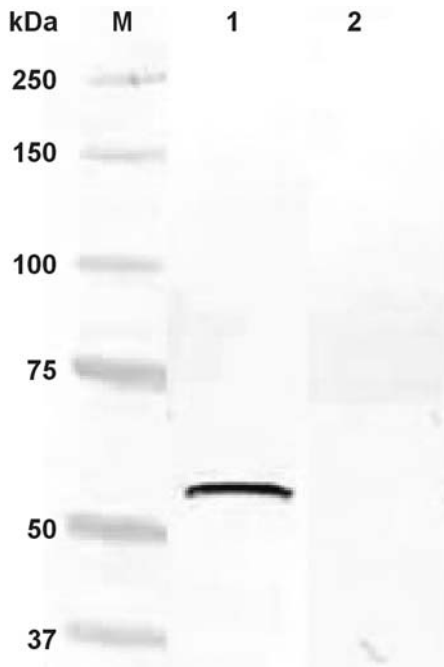
work is required to characterise the D-enzyme in the leaves of wheat plants.

#### Partial purification of wheat endosperm D-enzyme

Wheat endosperm D-enzyme was partially purified in order to remove residual enzymic activity of other enzymes, e.g.  $\alpha$ -amylase, using a three-step purification procedure as described in [Materials and methods](#). Analysis of the elution profile from the size exclusion column indicated that native D-enzyme had a molecular weight of approximately 54 kDa, suggesting it is a monomer. The fraction containing D-enzyme, from the



**Fig. 5** Immunoblot of soluble proteins from wheat leaves, endosperm and germinating seeds probed with the purified polyclonal antibody raised against DE1. The types of tissue are clearly marked on the figure. dpa = days post anthesis; days after germ = days after germination



**Fig. 6** Localisation of D-enzyme from wheat endosperm amyloplasts by immunoblot using the polyclonal antibody against DE1. Lane 1 shows reaction to a 60 kDa protein band in trypsin treated intact amyloplasts. Lane 2 shows no cross-reaction to lysed amyloplasts treated with trypsin

final purification step, was analysed by SDS-PAGE and contained approximately six proteins one of which was the size expected for D-enzyme (data not shown).

This partially purified extract was subjected to zymogram analysis to determine if the partially purified D-enzyme preparation contained polypeptides with the activities typical of disproportionating enzymes. The first assay involved incubating a native PAGE gel, on which the partially purified fraction had been separated, in a reaction mix containing maltotriose and assaying for glucose release. A dark band was seen (Fig. 7a, lane 1) indicating glucose was being produced by the 59 kDa band. Three gels containing substrate (glycogen, amylopectin and starch, respectively) were assayed for elongating activity by incubating in maltoheptaose and then staining with iodine stain. The expectation is that the presence of active D-enzyme would lead to elongation of the outer chains of the substrate by transfer of glucan from the maltoheptaose donor, and the elongated structure would be detected by iodine staining. In the glycogen gels a dark-brown band appeared (Fig. 7b, lane 1); in the amylopectin gel a red band appeared (Fig. 7c, lane 1) and in the starch gel a white band appeared (Fig. 7d, lane 1). The colours of the bands in all three gels corresponded to the activity seen in zymograms with the D-enzyme from *C. reinhardtii* (Colleoni et al. 1999b) and indicated alteration of the size of the outer chain of the glucans. Western blot analysis of all the zymograms using the polyclonal antibody raised against DE1 clearly indicates that a protein that migrates to the same point as the protein that has

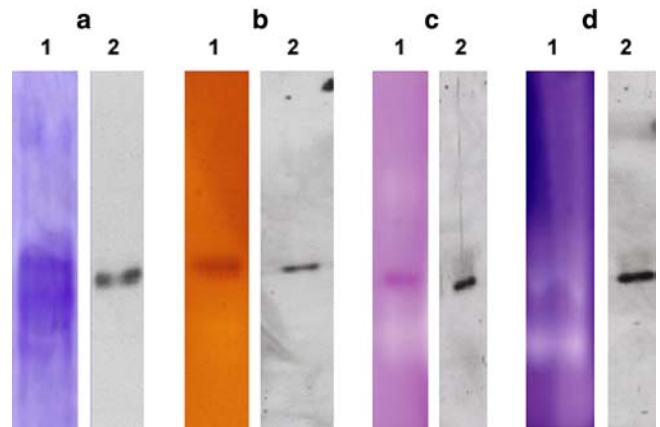
D-enzyme activity cross-reacts with this antibody (Fig. 7). These results demonstrate that a functional D-enzyme has been partially purified from wheat endosperm.

## Discussion

This paper provides three lines of evidence which indicate that disproportionating enzyme is present in the developing wheat grain. Firstly, a cDNA clone encoding a disproportionating enzyme-like sequence was identified in a library generated from wheat endosperm. Secondly, antibodies raised against a peptide sequence deduced from the endosperm cDNA sequence cross-react with a protein from the wheat endosperm which has further been shown to be located within the wheat amyloplast. Thirdly, these antibodies cross-react with a band of identical size which is capable of (a) producing glucose when provided with a malto-oligosaccharide substrate and (b) extending the external chains of amylopectin when incubated with maltoheptaose.

Phylogenetic analysis indicates there are two D-enzyme classes in plants

Comparison of the available gene sequences, and the sequences of the *Arabidopsis* and rice genomes, indicates that higher plants contain two classes of sequences, which have been annotated as disproportionating enzymes. The key reference genes for these classes are the genes present on chromosome 5 (At5g64860) and chromosome 2 (At2g40840) of *Arabidopsis*. For convenience, we denote the classes of genes as class I (genes clustering



**Fig. 7** Zymogram analyses of semi-pure Mono-Q fraction and corresponding immunoblots with the polyclonal antibody raised against DE1. **a** Glucose zymogram where a native gel minus substrate is incubated in 3 mg/ml maltotriose (1) and corresponding immunoblot for D-enzyme (2). **b** Glycogen zymograms whereby a native gel containing glycogen as a substrate is incubated with maltoheptaose (1) and corresponding immunoblot for D-enzyme (2). **c** Amylopectin zymogram (1) and immunoblot (2). **d** Starch zymogram (1) and corresponding immunoblot for D-enzyme (2)



with *Arabidopsis* chromosome 5 D-enzyme, which is unable to utilise maltose as a glucan donor), class II (genes clustering with the *Arabidopsis* chromosome 2 transglucosidase, which is able to utilise maltose as a glucan donor) and class III as bacterial D-enzymes homeologous with *E. coli* MalQ. Alignment of the two wheat cDNA sequences obtained in this study (TaDPE1-e and TaDPE1-l) with other D-enzyme sequences (Fig. 2) quite clearly places them in class I with maize (AY109140), rice (NM\_193039), potato and *Arabidopsis* chromosome 5 D-enzyme. The potato and *Arabidopsis* chromosome 5 D-enzymes have been extensively studied (Critchley et al. 2001; Takaha et al. 1998a, b, 1993, 1996). A BLASTN search with the two cDNA sequences identified a wheat EST sequence (TaGI TC 267930) which has only 24% identity with the wheat endosperm D-enzyme sequences identified in this study, and clusters in class II with the *Arabidopsis* chromosome 2 transglucosidase in phylogenetic analysis. This result strongly suggests that both genes encoding class I and II sequences are present and expressed in wheat. Both classes are also expressed in rice as ESTs corresponding to the putative genes encoding class I and II predicted from the sequenced genome have been identified.

The four conserved regions that are present in all members of the  $\alpha$ -amylase superfamily and the fifth region that is specific to D-enzymes (Przylas et al. 2000) were found to be conserved in all D-enzyme sequences analysed (Fig. 1). Further analysis of the sequences revealed that, in addition to the conserved regions, the seven active site residues that are conserved in all  $\alpha$ -amylases (Przylas et al. 2000) (Tyr59, Asp213, Arg291, Asp293, Glu340, His394 and Asp 395 in the *T. aquaticus* sequence) are also conserved in all the sequences in this alignment. Four other active site residues that are conserved in all D-enzyme and MalQ proteins, but not in other  $\alpha$ -amylase family members (Trp258, His294, Leu342 and Asn464 in the *T. aquaticus* sequence) are also present in all the sequences in this alignment adding further weight to their assignment as genes encoding D-enzymes.

#### Origins of the wheat D-enzyme cDNA sequences

The amino acid sequences of TaDPE1-e with TaDPE1-l are 96% identical, suggesting that they are members of a homeologous family rather than tissue-specific isoforms of the same enzyme, as these are typically less well conserved, e.g. the endosperm and leaf isoforms of GBSS from wheat only have 70% identity (Vrinten and Nakamura 2000). Comparison of TaDPE1-e and TaDPE1-l reveals two differences near the N-terminus. Firstly, there is a 5-amino acid insertion at position 37 in TaDPE1-l and secondly, an extra lysine at position 26 of TaDPE1-e. Both differences are within the putative plastid targeting sequences. The putative plastid targeting sequence of TaDPE1-l (45 amino acids) is the same size as the putative plastid targeting sequence of the chromosome 5 D-enzyme of *Arabidopsis* (Critchley et al.

2001). Throughout the rest of the sequence of TaDPE1-e and TaDPE1-l, there are only a small number of amino acid differences. As the TaDPE1-l sequence was obtained from an *A. tauschii* library, this sequence is encoded by a gene on the D-genome of wheat. There are two possibilities for the origins of the TaDPE1-e sequence. Firstly, it may be the product of a homeologous gene encoded by a locus on either the A or B genomes of wheat. Secondly, it may represent an alternative allele of the D-genome encoded TaDPE1-l locus; further work is required to resolve which of the two possibilities applies. Homeologous genes from the three wheat genomes typically have high levels of homology. For example, the A and D genome homeoforms of SBEIIa have 97% identity (Rahman et al. 2001), while the polypeptides encoded by the three homeoforms of GBSS have identities of over 97% (Yan et al. 2000).

#### The characterisation of the wheat D-enzyme gene from *A. tauschii*

Previous studies have shown that the starch biosynthetic genes of wheat, rice and *Arabidopsis* share very similar if not identical exon/intron structures, e.g. starch synthase I (Li et al. 1999a), starch synthase II (Li et al. 1999b), starch synthase III (Li et al. 2000), starch branching enzyme I (Rahman et al. 1999), starch branching enzyme IIa (Rahman et al. 2001) and isoamylase (Rahman et al. 2003). Use of the cDNA sequences for D-enzyme identified the exon/intron structure of the partial genomic sequence from *A. tauschii*, and ten exons and nine introns were identified (Fig. 3). This structure is very similar to the exon/intron structure of the genes encoding D-enzyme on rice chromosome 7 (NM\_193039) and *Arabidopsis* chromosome 5. Of the exons identified, all are exactly the same length as the corresponding exons in rice and *Arabidopsis*, and splicing occurs in the same homeologous codon for all three species. Intron sizes vary slightly with one significant difference being in intron 11 of wheat, which is approximately 100 bp longer than rice and *Arabidopsis*.

#### Chromosomal location of the class I wheat D-enzyme genes

Polymerase chain reaction primers that were able to distinguish the B homeoform from the A and D homeoform of D-enzyme in *T. aestivum* cv. Chinese Spring were used to locate it to the distal end of the short arm of chromosome 2B (Fig. 4). Using previously established syntenic relationships (Kurata et al. 1994) we predicted that the corresponding rice D-enzyme sequence would be located on chromosome 4a, 4b or 7. When the rice genome sequence became available this was confirmed, as the gene was located on chromosome 7, which is syntenic with chromosome 2 in wheat.

The expression profile of wheat D-enzyme in various tissues

Western analysis of developing endosperm (8–25 dpa) showed the presence of D-enzyme at all time points analysed (Fig. 5), and protease protection studies showed D-enzyme was located within the amyloplast (Fig. 6). The amyloplast location of D-enzyme in wheat and its expression during the time of active starch synthesis supports a role for D-enzyme in starch synthesis in cereal endosperm. However, the nature of any such role needs further work to be elucidated. Two hypotheses can be proposed. Firstly, the D-enzyme directly participates in amylopectin synthesis in a manner suggested by results in *Chlamydomonas* (Colleoni et al 1999a, b). Secondly, the role of D-enzyme in actively starch synthesising tissue, such as wheat endosperm, may be only indirectly associated with starch synthesis, for example, D-enzyme may be involved in the turnover of some fraction of the starch synthesised, for example, in the recovery of carbon and energy invested in the synthesis of the oligosaccharide released by the action of debranching enzymes during starch synthesis. D-enzyme was also detected in germinating seeds (Fig. 5), thus it may also play a role in mobilization of starch in germinating cereal grains as well as its apparent role during grain development. Future studies on lines that lack D-enzyme will help elucidate its role in starch synthesis and degradation in cereal grains.

#### Activity of D-enzyme

The analyses used by Colleoni et al (1999a; 1999b) to determine the function of *C. reinhardtii* D-enzyme were used. The partially purified D-enzyme from wheat shares the defining function of all D-enzymes, its ability to disproportionate maltotriose to produce glucose. Glucose production was shown by zymogram analysis when the partially purified extracts containing D-enzyme were incubated with maltotriose (Fig. 7a). The band displaying D-enzyme activity co-migrated with the band detected by immunoblot with the highly specific polyclonal antibody against DE1.

The ability of wheat D-enzyme to elongate malto-oligosaccharides was demonstrated by incubation of the D-enzyme containing fractions separated on native gels containing, glycogen, amylopectin or starch with maltoheptaose. After incubation and staining with iodine a brown band was revealed on the glycogen gel (Fig. 7b), indicating that a chain elongation reaction had occurred, generating chains of sufficient length to bind iodine strongly. This result was confirmed for the amylopectin containing zymogram where on addition of maltoheptaose, a dark-red band developed, again at a position co-migrating with the glucose-evolving activity on maltotriose addition, the chain elongation activity observed on incubation with glycogen and maltoheptaose, and co-migrating with a band revealed

by immunoblotting with anti-sera from an animal challenged with a synthetic peptide with a sequence deduced from the wheat D-enzyme cDNA sequences. The change from the dark-blue colour of the starch iodine complex to a dark-red coloured band at the site of D-enzyme activity shows a shift in the colour of the iodine polysaccharide complex, as noted by Colleoni et al. (1999a; 1999b) in their work with *C. reinhardtii*. As well as in zymogram analysis Colleoni et al. (1999b) examined the action of D-enzyme on amylopectin. The starch gel containing D-enzyme, when incubated with maltoheptaose produced a white band. Previously, a dark-red band has been observed when less D-enzyme was used. The dark-red band is representative of D-enzymes ability to elongate the outer chains of starch by adding malto-oligosaccharides derived from maltoheptaose to the non-reducing ends, as seen after incubation of D-enzyme with amylopectin. In the gel a white band is seen instead (Fig. 7d), which is believed to represent the ability of D-enzyme to degrade the starch when it is present in excess (Colleoni et al. 1999b). The demonstration of the ability of partially purified fractions to disproportionate maltotriose combined with its ability to elongate glycogen and amylopectin, and degrade starch clearly shows that this fraction contains an active D-enzyme.

This work has demonstrated the presence of an active D-enzyme in the developing endosperm of wheat. This is the first conclusive evidence for the presence of D-enzyme in cereal endosperm. The partially purified enzyme displays the classic function of an  $\alpha$ -1,4-glucanotransferase, the ability to both disproportionate and elongate malto-oligosaccharides. Using a combination of activity and immunological assays, we have shown that the native enzyme catalysed the expected reactions for a disproportionating enzyme. This enzyme was localised to the amyloplast, the site of starch synthesis and was shown to be present in the endosperm when starch is being synthesised. The next challenge will be to elucidate the function of D-enzyme in starch synthesis and/or degradation in cereal endosperm and germinating grain.

**Acknowledgements** NB was the recipient of a postgraduate scholarship from Biogemma. Dr Kevin Gale, CSIRO Plant Industry, is thanked for assistance with design of the synthetic peptide, and Malcolm Blundell for assistance with generation of the antibodies.

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