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Chromosomal assignment of gene sequences coding for protein disulphide isomerase (PDI) in wheat

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Abstract Three different probes, obtained by PCR amplification and labelling of different segments of a PDI cDNA clone from common wheat, were used to identify and assign to wheat chromosomes the gene sequences coding for protein disulphide isomerase (PDI). One of these probes, containing the whole coding region except for a short segment coding for the C-terminal sequence, displayed defined and specific RFLP patterns. PDI gene sequences were consequently assigned to wheat chromosome arms 4BS, 4DS, 4AL and 1BS by Southern hybridisation of *Eco*RI- *Hin*dIII- and *Bam*HI-digested total DNA of nulli-tetrasomic and di-telosomic lines of Chinese Spring. This probe was also employed for assessing the restriction fragment length polymorphism in several hexaploid and tetraploid cultivated wheats. These showed considerable conservation at PDI loci; in fact polymorphism was only observed for the chromosome 1B fragment.

Key words Protein disulphide isomerase · Wheat · RFLPs · Chromosomal location

Introduction

Protein disulphide isomerase (PDI) (EC 5.3.4.1) is a luminal endo-membrane protein that catalyses thiol/disulphide exchange reactions, leading to the formation of the disulphide bonds that stabilise the tertiary and quaternary structures of many proteins processed through the endo-membrane system (Freedman 1989; Noiva and Lennarz 1992).

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PDI has been studied both structurally and functionally in a variety of animal species (Freedman et al. 1994). The vertebrate enzyme is a dimer consisting of two identical sub-units of about 57 kDa, whose cDNA and genomic sequences have been cloned (Edman et al. 1985; Pihlajanieni et al. 1987; Parkonnen et al. 1988; Freedman et al. 1994). PDI contains two nearly identical, putative active-site regions with a consensus sequence Ala-Pro-Trp-Cys-Gly-His-Cys-Lys, closely related to the active site of the thioredoxins (Edman et al. 1985). In addition to its primary role in disulphide bond formation, PDI also performs two additional functions, as a sub-unit of more complex enzyme systems. It is the beta-subunit of prolyl 4-hydroxylase that adds hydroxyl groups to some secretory proteins (Pihlajanieni et al. 1987), and a component of the triglyceride transfer complex that catalyses the incorporation of lipids into nascent core lipoproteins (Wetterau et al. 1990, 1991).

Little is known about plant PDIs. PDI cDNA sequences have been cloned from species like alfalfa (Shorrosh and Dixon 1991), barley (Chen and Hayes 1994), wheat (Shimoni et al. 1995 a) and maize (Li and Larkins 1996). They all contain a C-terminal KDEL signal sequence for endoplasmic reticulum (ER) retention (Denecke et al. 1992), a putative N-glycosylation site and the two thioredoxin-like catalytic sites, also present in the vertebrate and yeast PDIs (Noiva and Lennarz 1992). Studies on the expression and intracellular localisation of this enzyme in wheat and maize (Shimoni et al. 1995 b; Li and Larkins 1996) indicate that it may play an important role in the folding of plant secretory proteins, and in particular in the formation of protein bodies in the seed endosperm. Bulleid and Freedman (1988) showed that PDI is able to catalyse the formation of disulphide bonds in a gamma-gliadin synthesised in vitro. Storage protein folding and disulphide bond formation is assumed to take place within the ER lumen, although the precise mechanisms involved, and the role of other proteins

such as molecular chaperones, are not fully understood (Shewry et al. 1995). The presence of PDI in wheat endosperm was first demonstrated by Roden et al. (1982). More recently, Shimoni et al. (1995 b) described it as a 60-kDa glycoprotein, being among the most abundant proteins within the ER of developing grains. Here, in comparison with embryos, leaves and roots, it is up-regulated at early stages of development and precedes the onset of storage protein accumulation by several days.

As part of a study on the structure and expression of genes coding for PDI in wheat, their chromosomal location was determined using wheat aneuploids. The RFLP pattern of PDI genes was also analysed in different hexaploid and tetraploid cultivated wheats.

Materials and methods

Plant material

The nullisomic-tetrasomic (NT) and ditelosomic (DT) lines of *Triticum aestivum* L. cv Chinese Spring (CS) produced by Sears (1966) were used for chromosome assignment. A total of 70 hexaploid and tetraploid cultivated wheats were then analysed for detecting polymorphism. The hexaploid material included 20 common wheat (*T. aestivum* L.) cultivars from Italy, Canada, USA, France and Russia, three accessions each of *Triticum spelta* L., *Triticum sphaerococcum* Perc. and *Triticum compactum* Host., and one of *Triticum vavilovii* Tuman and *Triticum macha* Dekr. et Men. The tetraploid material comprised 30 durum wheat (*Triticum durum* Desf.) cultivars from Italy, USA, France, Turkey and Syria, three accessions of *Triticum dicoccum* Schubl., and two accessions each of *Triticum carthlicum* Nevski, *Triticum polonicum* L. and *Triticum turanicum* Jakubz.

DNA extraction and Southern analysis

Genomic DNA was isolated either from 5 g of fresh leaves (D'Ovidio et al. 1992) or from 0.5 g of fresh or frozen material (Dellaporta et al. 1983). Ten micrograms of DNA per line were digested with *Eco*RI, *Bam*HI and *Hin*dIII, electrophoresed in 1% agarose gels for 16–18 h at 40 V, before being transferred to a Hybond N+ nylon membrane (Amersham). Pre-hybridization and hybridisation reactions were carried out at 65°C in a solution containing 5 × SSC, 0.5% (w/v) sodium dodecyl sulphate (SDS), 0.1% N-lauroylsarcosine, 1% (w/v) blocking reagent (Boehringer). Filters were pre-hybridized for 4 h and hybridised for 16–20 h with a digoxigenin-labelled cDNA clone of wheat PDI (GeneBank Acc. No. U11496, Shimoni et al. 1995 a) and washed twice at room temperature in 2 × SSC and 0.1% SDS (w/v) for 5 min and twice at 65°C in 0.1 × SSC and 0.1% SDS for 15 min each. Immunological detection was performed following the methodology described by Lu et al. (1994). To remove the hybridised probe, the filters were incubated twice in 0.2 N NaOH, 0.5% SDS at 45°C for 15 min, then rinsed in 2 × SSC.

Digoxigenin labelling by PCR

Digoxigenin-11-dUTP was incorporated into DNA by PCR. Three pairs of oligonucleotide sequences were used as PCR primers for probe labelling:

(1) T3 = 5′-ATTAACCCTCACTAAAG-3′;
T7 = 5′-AATACGACTCACTATAG-3′;
(2) PDI-F1 = 5′-CATGGCGATCTCCAAGGTCTGG-3′;
PDI-R1 = 5′-GTTGGTTGTTGGTTGAGAGCAGGG-3′;
(3) PDI-F1 = 5′-CATGGCGATCTCCAAGGTCTGG-3′;
PDI-R3 = 5′-CATCAGCTGTTAGAGCAGCCGCG-3′.

The first pair of primers (T3–T7) amplified the whole cDNA clone of wheat PDI (1796 bp) (Shimoni et al. 1995 a), whereas the other two were synthesised to amplify different regions of the same cDNA clone. The second set of primers corresponds to the 36–58 (PDI-F1)-bp and 1695–1672 (PDI-R1)-bp positions, including the whole coding and part of the 3′ untranslated regions and lacking a short segment flanking the polyA tail. The target of the third set (PDI-F1 = 36–58 bp, PDI-R3 = 1485–1465 bp) was the whole coding region with the exclusion of about 100 bp comprising the sequence for KDEL, the signal for ER retention. The three labelled probes were designated as probe 1, probe 2 and probe 3. The reaction mixtures (100 µl) consisted of two units of *Taq* DNA polymerase (Polymed), 1 × reaction buffer (Polymed), 1.5 mM $MgCl_2$, 50 µM of each dATP, dCTP and dGTP, 45 µM of dTTP, 5 µM of Dig-11-UTP (Digoxigenin-11-uridine-5′-triphosphate), 0.25 µM of each primer and 50 ng of template plasmid DNA. DNA was subjected to an initial denaturation step at 94°C for 2 min; the amplification conditions were for 35 cycles at 92°C for 1 min, 60°C (55°C for the T3 and T7 combination) for 2 min, and 72°C for 2 min followed by a final incubation step at 72°C for 5 min.

Results

Chromosomal assignment

DNA from CS and from its nulli-tetrasomic lines was initially digested with *Bam*HI and hybridised with PDI probe 1. In spite of the high-stringency conditions employed in the hybridisation, the restriction fragment patterns had a high background and showed several weak hybridising bands, which prevented unequivocal chromosomal assignment. In order to select the probe/enzyme combination giving a better hybridisation pattern, DNA from some common and durum wheats was digested with *Eco*RI, *Hin*dIII, *Bam*HI, *Eco*RV, *Dra*I and *Pst*I restriction enzymes and hybridised with three different probes, as reported in Materials and methods. Results of Southern analyses, using *Hin*dIII and *Eco*RI restriction enzymes, indicated that probe 3 displayed a well-defined and specific pattern. It gave a strong hybridisation signal only to a limited number of fragments (from two to four, depending on the enzyme and the genotype used), whereas probes 1 and 2 hybridised with several bands, most of them with lower hybridisation intensity. These results were confirmed with all the restriction enzymes employed.

PDI gene sequences were consequently assigned to wheat chromosomes by Southern hybridisation of *Eco*RI-, *Hin*dIII- and *Bam*HI-digested total DNA of CS and its nulli-tetrasomic lines using probe 3. Since only nulli-tetrasomic lines of homoeologous groups 1 and 4 differed in their pattern from CS, further analyses were confined to di-telosomic lines of these

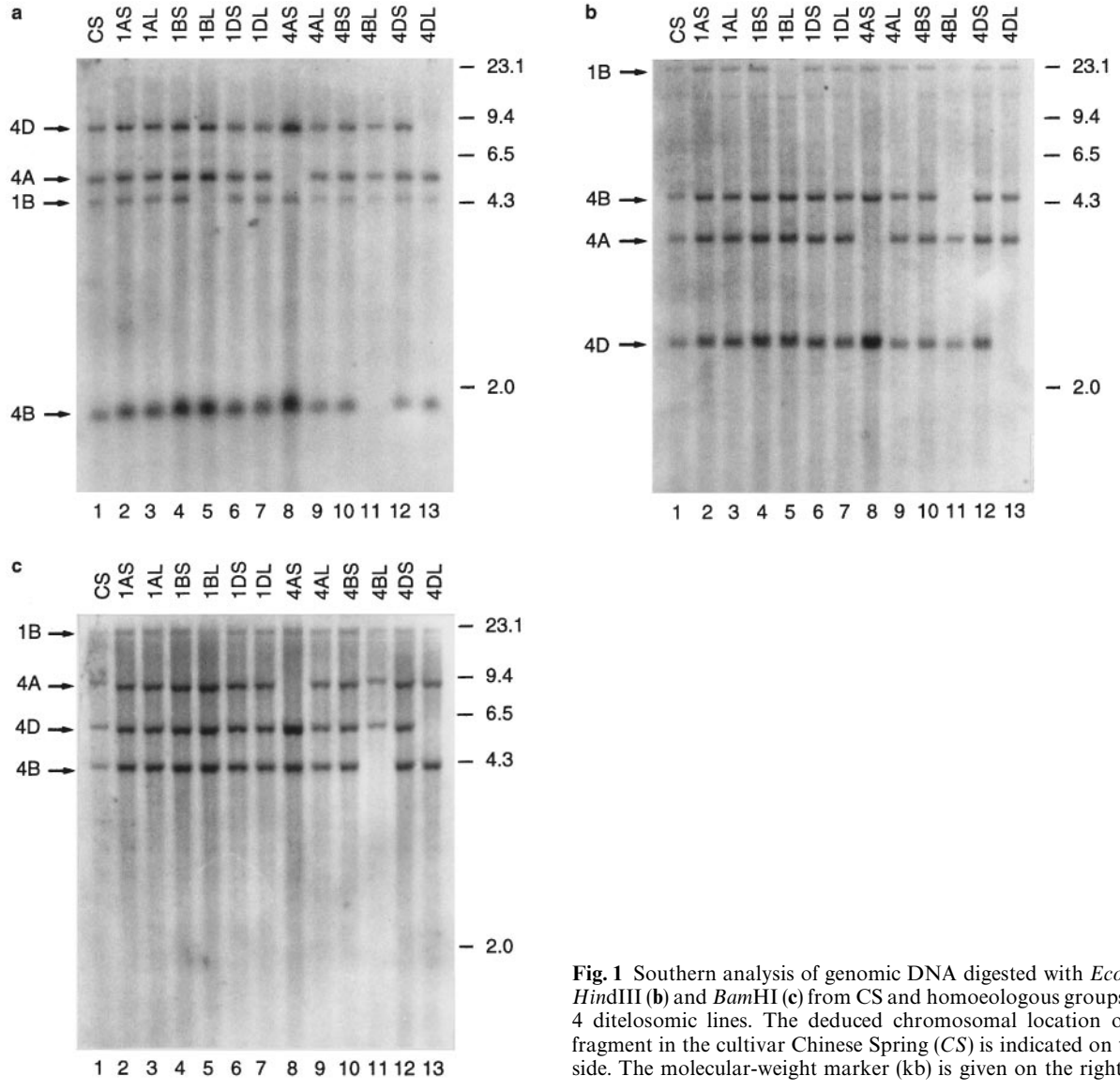

Fig. 1 Southern analysis of genomic DNA digested with *Eco*RI (**a**), *Hin*dIII (**b**) and *Bam*HI (**c**) from CS and homoeologous groups 1 and 4 ditelosomic lines. The deduced chromosomal location of each fragment in the cultivar Chinese Spring (*CS*) is indicated on the left side. The molecular-weight marker (kb) is given on the right side

homoeologous groups (Fig. 1). When hybridised to *Eco*RI-digested DNA from CS, the PDI clone displayed a pattern of four fragments, with estimated sizes of 9.3-, 5.3-, 4.6- and 1.8-kb (Fig. 1 a) Each of these fragments corresponds to a gene sequence located on chromosome arms 4DS (lane 13), 4AL (lane 8), 1BS (lane 5) and 4BS (lane 11), respectively. The chromosomal location of PDI gene sequences was further confirmed by Southern analyses of *Hin*dIII- and *Bam*HI-digested DNAs (Fig. 1 b and c). Both restriction enzymes gave a strong hybridisation pattern of three fragments, corresponding to related sequences on homoeologous group-4 chromosomes (lanes 8, 11 and 13 in Fig. 1b and 1c). The estimated size of the three fragments was 4.5-, 3.7- and 2.5-kb after *Hin*dIII digestion and 8.9-, 5.5- and 4.2-kb after *Bam*HI digestion. In addition, *Hin*dIII and *Bam*HI RFLP patterns indicated that the PDI cDNA clone hybridised also to a 23-kb fourth fragment, located on chromosome arm 1BS (lane 5 in Fig. 1b and c).

RFLP variation

The variation for RFLP patterns of PDI gene sequences in different lines of cultivated hexaploid and tetraploid wheat was assessed using *Eco*RI-digested DNAs.

Three different restriction patterns were exhibited by the hexaploid genotypes analysed (Fig. 2 a). Restriction fragments located on homoeologous group 4 were highly conserved, with some variation only in the 1B chromosome fragment. Some hexaploid wheats had patterns identical to the one in CS (e.g. lanes 1 and 5 in Fig. 2 a), whereas others showed a smaller (e.g. lanes 2–4), or absent (e.g. lanes 6–8), 1B fragment. The three

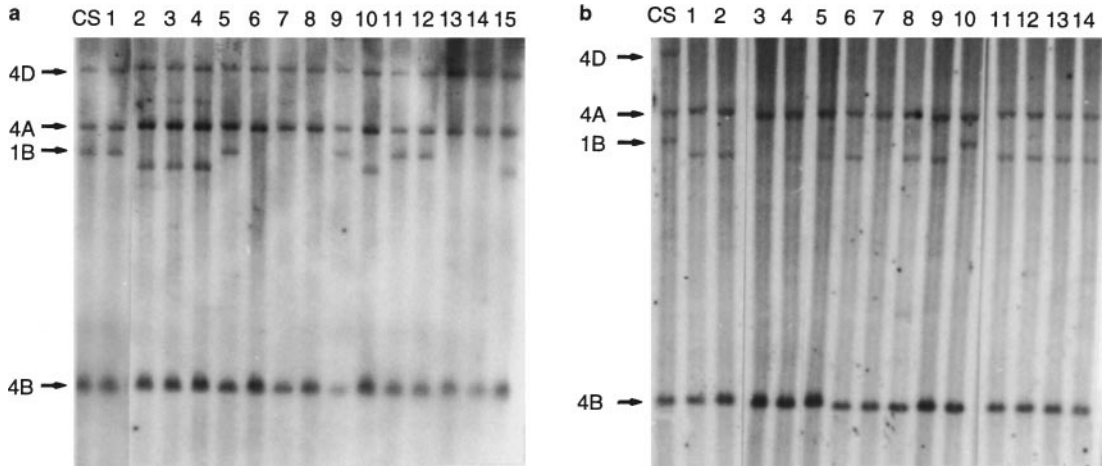

Fig. 2 Southern analysis of genomic DNA digested with *Eco*RI from CS and some representative lines of hexaploid (genome AABBDD) and tetraploid (AABB) cultivated wheats (**a** and **b**). **a** *1–8 = T. aestivum*; *9–10 = T. spelta*; *11 = T. compactum*; *12 = T. vavilovii*; *13–14 = T. sphaerococcum*; *15 = T. macha*. **b** *1–9 = T. durum*; *10 = T. carthlicum*; *11–12 = T. dicoccum*; *13 = T. polonicum*; *14 = T. turanicum*. The deduced chromosomal location of each band in the cultivar Chinese Spring (*CS*) is indicated on the left side

Table 1 Absolute frequency distribution of PDI RFLP patterns in the 70 hexaploid and tetraploid cultivated wheats analyzed

Species	Pattern		
	1B large	1B small	1B absent
Hexaploids			
T. aestivum	6	8	6
T. spelta	1	2	
T. sphaerococcum	1		2
T. compactum	1	1	1
T. vavilovii	1		
T. macha		1	
Tetraploids			
T. durum	2	20	8
T. dicoccum		3	
T. polonicum		2	
T. turanicum		2	
T. carthlicum	2		

different patterns were equally distributed among all the hexaploids analysed (Table 1). The patterns of cultivated tetraploids were also highly conserved (Fig. 2 b). Obviously they differed from the hexaploids in the lack of the fragment located on the D genome. More than 66% of *T. durum* cultivars possessed the RFLP pattern with the smaller 1B fragment, whereas only two out of the 30 cultivars analysed showed the larger 1B fragment (Table 1). The most frequently detected durum wheat pattern was also displayed by all the accessions of *T. dicoccum* (lanes 11 and 12 in Fig. 2 b), *T. polonicum* (lane 13) and *T. turanicun* (lane 14), whereas the *T. carthlicum* accessions showed the smaller 1B hybridisation fragment (lane 10).

Discussion

Three different probes, obtained by PCR amplification and labelling of different segments of the same cDNA clone (Shimoni et al. 1995 a), were used in order to identify and locate PDI gene sequences in the common and durum wheat chromosomes. One of these probes (probe 3), containing the whole coding region except for a short segment (about 100 bp) coding for the C-terminal sequence, produced well-defined hybridisation patterns. It hybridised to four *Eco*RI fragments of Chinese Spring (CS). The same number of hybridisation fragments was observed with CS DNA digested either with *Hin*dIII and *Bam*HI, but one of them was very large (about 23 kb). Southern analysis of aneuploid lines of CS indicated that each fragment corresponds to a gene sequence located in wheat chromosome arms 4BS, 4DS, 4AL and 1BS. Location of a PDI gene sequence on the long arm of chromosome 4A, instead of the short arm as in the B and D genomes, can be explained by the pericentric inversion which occurred in this chromosome (Devos et al. 1995; Mickelson-Young et al. 1995; Nelson et al. 1995). The other two probes displayed more complex patterns, independently of the enzyme employed. They showed non-specific hybridisation, with several weakly reacting fragments, possibly due to the presence, in the clone used, of a sequence coding for KDEL, a tetrapeptide characteristic of many ER resident proteins (Denecke et al. 1992).

The number of PDI gene sequences identified in the hexaploid wheat *T. aestivum*, one in each of the A and D genomes and two in the B genome, is in line with

results obtained in other diploid plant species. Alfalfa (Shorrosh and Dixon 1991) and maize (Li and Larkins 1996) PDI are, in fact, encoded by single-copy sequences, whereas two independent PDI loci were detected in barley (Chen and Hayes 1994). Analysing the Brookhaven recombinant inbreed lines, Li and Larkins (1996) found the maize PDI gene to be located on the short arm of chromosome 4, between the zein locus *zpr10* and the *adh2* locus. It is interesting to note that genes coding for alcohol dehydrogenase (*adh*) are also located on the short arms of chromosomes 4B and 4D and on the long arm of chromosome 4A, within conserved regions potentially orthologous in wheat and maize (Ahn et al. 1993).

The RFLP for PDI, assessed in different accessions and lines of hexaploid and tetraploid cultivated species, indicated that the restriction fragments located on homoeologous group 4 were highly conserved and that polymorphism occurred only at the 1B locus. Further investigations would be necessary to determine the origin of the PDI gene sequence located on chromosome 1B of polyploid species and, in particular, to ascertain whether it derives from a wild diploid species related to the B genome or is the result of different events (duplication and translocation) that took place at the polyploid level.

The results concerning the chromosomal location and the variation in RFLP patterns of PDI gene sequences suggest that there could be up to four or three different forms of the enzyme respectively, in common and durum wheat. Shimoni et al. (1995 b) identified one of these forms in CS and demonstrated that it is a 60-kDa glycoprotein, representing one of the most abundant proteins within the ER of developing grains. At present it is not known whether the four or three genes detected in common and durum wheat, respectively, are all functionally active and/or differ in their coding regions and in flanking sequences involved in the regulation of gene expression. Elucidation of differences among the gene sequences from different genomes and their importance in the developmental and tissue-specific regulation of PDI genes in wheat will be the subject of further research.

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