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ORIGINAL PAPER

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Novel puroindoline and grain softness protein alleles in *Aegilops* species with the C, D, S, M and U genomes

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Abstract DNA from six hexaploid, tetraploid and diploid species of *Aegilops* with the C, D, S, M and U genomes was amplified with specific PCR primers to identify sequences encoding puroindolines (Pins) a and b and grain softness protein (GSP), all of which are encoded by genes at the *Ha* (hardness) locus, with *Ae. tauschii* (DD) and bread wheat (*T. aestivum*) (AABBDD) cv Hiline being studied as controls. Seven new allelic forms of *Pin a* and *Pin b* were identified, including forms with mutations within or close to the tryptophan motif. In addition, five new forms of GSP were detected. In all species both genomic DNA from leaves and cDNA from developing grain were analysed. This revealed the presence of both silent genes (with premature stop codons) and multiple genes, with the latter being confirmed by Southern blot analysis. Freeze fracture analysis demonstrated that all except one accession (*Ae. sharonensis*) were soft textured. However, this difference cannot be accounted for by the sequences of the *Pin* alleles present in this line.

Introduction

Grain texture, or hardness, is one of the main characteristics that determine the processing properties of wheat, affecting both the milling and bread-making properties (Pomeranz and Williams 1990). Thus, hard wheats require more energy to mill, yielding larger particles which have a higher degree of starch damage and better properties for bread making. It has been known for some time that hardness is mainly determined by a single locus, called *Ha*, located on the short arm of chromosome 5D (Law et al. 1978), although more recent work indicates the existence of further minor loci (Turner et al. 2004).

The major structural difference between grains of hard and soft-textured wheats is the degree of adhesion between the surface of the starch granules and the matrix (i.e. gluten) proteins in the mature cells of the starchy endosperm. Hence early attempts to explain the biochemical basis for hardness focussed on identifying differences between the surfaces of starch granules in hard and soft cultivars. This resulted in the identification of an M_r 15,000 protein present on the surface of starch granules prepared from soft but not hard-textured types of wheat and the hypothesis that this acted as an "non-stick" protein which prevented adhesion between the starch granule and the gluten matrix (Greenwell and Schofield 1986). It has since been shown that this protein, called "friabilin", actually comprises a mixture of components (Oda and Schofield 1997) including two major tryptophan-rich proteins called puroindolines (Pins) a and b (Blochet et al. 1993; Gautier et al. 1994). Furthermore, these two proteins are encoded by genes located at the *Ha* locus (Chantret et al. 2004), together with a gene encoding a third protein called "grain softness protein" (GSP) (Jolly et al. 1993, 1996).

The role of pins in determining grain texture is now well established, with grain hardness being determined by mutations which affect either the expression of the *Pin a* or *Pin b* genes (i.e. null alleles) or the amino acid sequence of the *Pin b* protein (Giroux and Morris 1997, 1998; Lillemo and Morris 2000; Turnbull et al. 2000; Morris 2002). Furthermore, direct evidence for their role has been provided by functional complementation, by expressing the *Pin a* and *Pin b* genes in transgenic rice (Krishnamurthy and Giroux 2001) and the *Pin b* gene in hard-textured bread wheat (Beecher et al. 2002). In both cases, the grain from the transgenic lines was softer than those from the controls.

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Pin a and *b* genes are not present on the A and B genomes of either bread wheat or tetraploid durum (pasta) wheat, with the result that the latter is ultra-hard. However, related genes are present in diploid species with genomes related to those present in bread wheat (Gautier et al. 2000; Tranquilli et al. 1999; Pogna et al. 2002; Lillemo et al. 2002; Massa and Morris 2004; Massa et al. 2004), and in barley, rye and oats (Gautier et al. 2000; Darlington et al. 2001; Beecher et al. 2001; Tanchak et al. 1998).

In contrast to the *Pin* genes, *GSP* genes do appear to be present on chromosomes 5A and 5B of bread wheat. However, deletion of these genes has no significant impact on grain texture (Tranquilli et al. 2002) and biochemical studies indicate that GSP may actually play a role in cell wall structure (Van den Bulck et al. 2002). *GSP* genes have also been characterised in diploid species, *T. monococcum* (AA) and *T. tauschii* (DD) (Chantret et al. 2004; Massa and Morris 2004; Massa et al. 2004).

These studies demonstrated that related diploid and possibly also tetraploid and hexaploid species form a potential source of novel pin variants which can be exploited to expand the range of texture in cultivated pasta and bread wheats. We have, therefore, analysed a number of diploid, tetraploid and hexaploid species with the C, D, S, M and U genomes in order to identify further allelic variants of all three genes at the *Ha* locus.

Materials and methods

Plant materials

Nine accessions of *Aegilops* spp. were obtained from the Genebank of the Chinese Academy of Science (CAAS) Institute of Crop Germplasm Resources and grown in the glasshouse at Rothamsted Research (Harpenden, UK). Seedlings were vernalised at 4°C for 4 weeks (for spring and weak winter types) or 8 weeks (winter types).

Sequence analysis

Genomic DNA was extracted from young leaf tissue using the CTAB method (Stacey and Isaac 1994). Total RNA for RT-PCR was isolated from 15 DPA endosperm (1–2 g) following the method of Chang et al. (1993). Ambion DNA-free Kit was used to ensure that the RNA samples were free from DNA contamination. First-strand cDNA synthesis was carried out with a SuperScript III Reverse Transcriptase Kit (Invitrogen).

Full-length *Pin a, Pin b* (both 447 bp) and *GSP* (495 bp) genes were amplified with gene-specific primers (Table 1). Reactions were performed in 50 µl containing ≈200 ng of genomic DNA or cDNA, 0.75 µM of each primer, 400 µM of each dNTP, 1× pfu DNA polymerase reaction buffer and 1 U of pfu DNA polymerase (Promega, Madison, WI, USA). The cycling conditions were 95°C for 3 min followed by 35 cycles of 95°C for 1 min, 58°C for 1 min, 72°C for 2 min and the extension of 72°C for 10 min. PCR products were analysed on 1.2% (w/v) agarose gels, stained with ethidium bromide and visualised by UV light. PCR products were purified from the gel using a QIAquick Gel Extraction Kit (Qiagen 28704). Sequencing reactions were performed with the BigDye Terminator Version 1.1 Cycle Sequencing Kit (ABI) and sequenced with an ABI 3730×1 sequencer (PerkinElmer Applied Biosystems Division, Foster City, CA, USA). Variants were confirmed by sequencing PCR products from two plants in both directions. All sequences were aligned by MacVector 7.0 including sequences from *T. aestivum* for comparison.

Southern blotting

Genomic DNA was completely digested by *Eco*RI which cuts outside the three genes. The *Pin a* plasmid MPL61.16.1 was digested by *Not*I and the *Pin b* plasmid MPL13.1.16 by *Eco*RI to give full constructs as positive controls. *GSP* plasmid pBx17HMW linearised by *Eco*RI was used as a positive control for detection of the *GSP* gene. Genomic (10–15 µg) and plasmid (5 pg) DNA were separated by electrophoresis on a 1.0% (w/v) agarose gel at 20 V for 40 h and transferred by capillary blotting onto positively charged nylon membrane (Roche Diagnostics GmbH, Lewes, East Sussex, UK) using the method of Sambrook et al. (1989). Blotting, hybridisation and chemiluminescent detection were carried out as described in the DIG System User's Guide for Filter Hybridisation (Roche, Welwyn Garden City, UK). The filters were probed with DIG-labelled full-length genes generated by PCR from the above plasmids (*Pin a* plasmid MPL61.16.1, *Pin b* plasmid MPL13.1.16 and *GSP* plasmid pBx17HMW) with the primers in Table 1.

Table 1 Gene-specific PCR primers for producing full-length sequences of the genes *Puroindoline a* (*Pin a*), *Puroindoline b* (*Pin b*) and *Grain Softness Protein* (*GSP*)

Gene	Forward primers	Reverse primers
Pina	5′-ATGAAGGCCCTCTTCCTCA-3′	5′-TCACCAGTAATAGCCAATAGTG-3′
Pinb	5′-ATGAAGACCTTATTCCTCCTAGC −3′	5′-TATAGATATCATCACCAGTAATAGCC-3′
GSP	5′-CATGAAGACCTTCTTCCTCC-3′	5′-TCACAAGTAATATCCGCTAG-3′

Western blotting

Total proteins were extracted from seeds and separated by SDS-PAGE using a Tricine Gel System as described by Shewry et al. (1995). Blotting was performed with nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA) as described by Fido et al. (1995). The membrane was incubated with the primary antibody (mouse anti-friabilin monoclonal antibody). Bound antibodies were detected by goat anti-mouse IgG coupled with alkaline phosphatase (Santa Cruz Biotechnology) and NBT/BCIP (nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt, Roche Diagnostics GmBH) as substrate.

Determination of grain texture by SEM

Mature dry seeds were quench-frozen in liquid nitrogen slush and transferred under vacuum to a Cryo SEM preparation chamber (Gatan Alto 2100). Samples were then fractured, etched by sublimation at −85°C for 2 min, sputter coated with gold and finally examined at 5–15 kV in a JEOL JSM-6360 LV scanning electron microscope. Images were taken at 400–4,000× magnification (Brennan et al. 1996).

Results and discussion

Novel alleles at the Pin a, Pin b and GSP loci

Seeds of six species of *Aegilops* were selected for analysis (Table 2). These include three diploids with the S genome (*Ae. longissima, Ae. sharonensis, Ae. bicornis*), one tetraploid with the U and S genomes (*Ae. kotschyi*), one tetraploid with the U and C genomes (*Ae. triuncialis*) and one hexaploid with the D, M and U genomes (*Ae. juvenalis*). Bread wheat (*T. aestivum*) cv Hiline and *Ae. tauschii*, which is the progenitor of the D genome of bread wheat, were included for comparative purposes. All of these species are known to be inbreeding and hence should be homozygous. However, in order to rule out the possibility of heterogeneity within accessions, it was decided to isolate genomic DNA (from young leaves) and cDNA (from mid-development grain) from the same single plants of each accession.

Gene-specific PCR primers for *Pin a* (Gautier et al. 1994), *Pin b* (Gautier et al. 1994) and *GSP* (Rahman et al. 1994; Turner et al. 1999) were successfully used to amplify genomic DNA and cDNA for *Pin b* and *GSP* from all lines and for *Pin a* from all lines except *Ae. bicornis* 022 and *Ae. longissima* 023. The sequences of the encoded *Pin* proteins were aligned with those reported for *T. aestivum* cv. Capitole (accession numbers X69913 and X69914 for *Pin a* and *Pin b*, respectively), and the sequences of the *GSP* proteins with those reported for the *GSP-D1* gene (Massa et al. 2004). The alignments are shown in Table 3 and the distribution of the alleles identified in the various accessions in Table 2.

Seven new allelic forms of *Pin a* and *Pin b* (called alleles 1–7, respectively) and five new allelic forms of GSP (alleles 1–5) were identified. In addition, the *Pin* a and GSP alleles present in *Ae. tauschii* were identical to *Pin a-D1d* and *GSP-D1d* alleles, respectively, which had previously been reported for bread wheat.

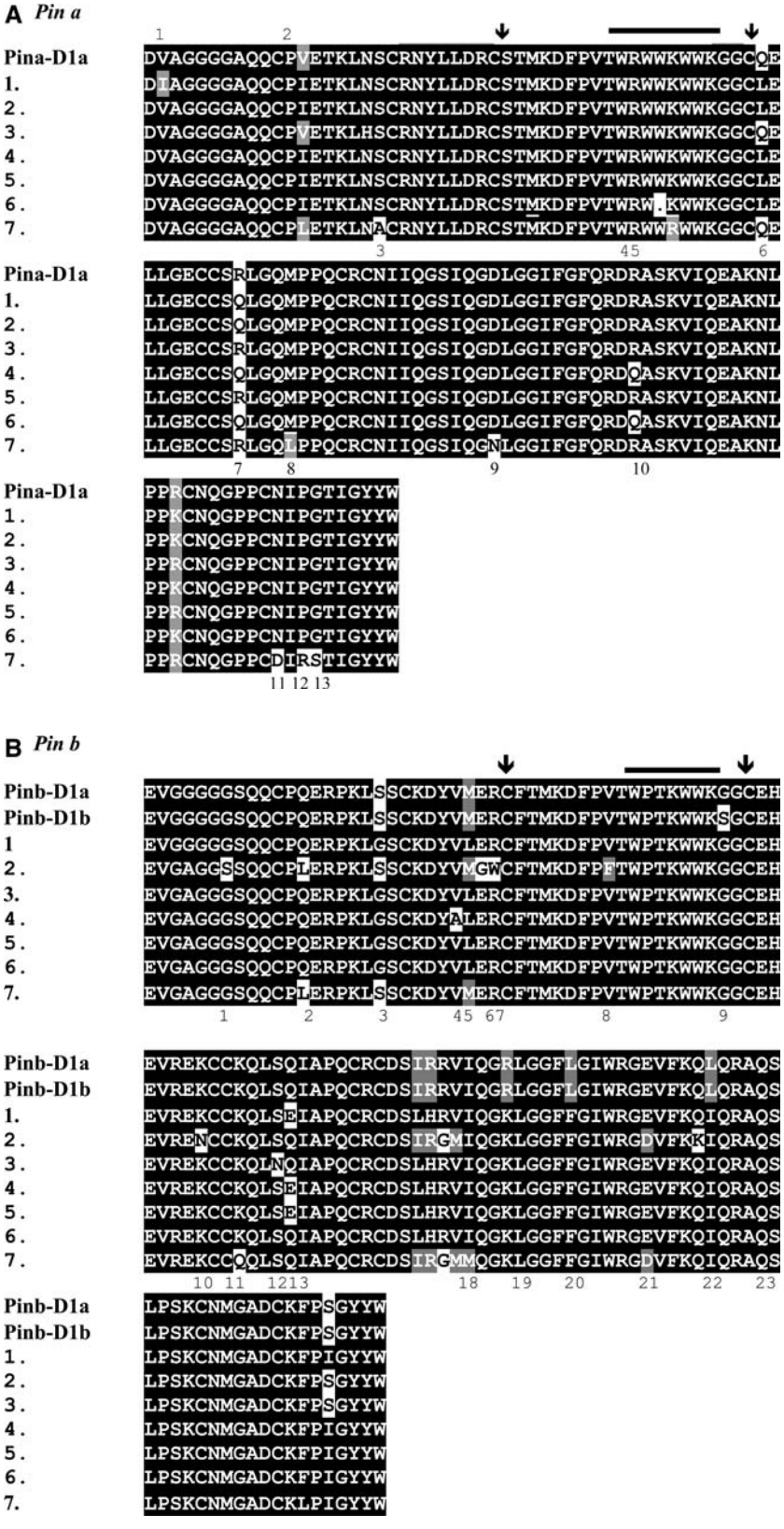
In most cases, identical sequences were determined from cDNA and genomic DNA, but where they differed the amplifications were repeated in order to confirm the different sequences and to look for additional sequences. In *Ae. kotschyi* (038, 039) this confirmed the different sequences initially determined for cDNA and genomic DNA for *Pin a* including the fact that the genomic sequences contained a premature stop codon and were presumably not expressed. However, additional genomic sequences corresponding to the expressed cDNAs were not identified. Similarly, the different genomic and cDNA sequences determined for *GSP* from *Ae kotschyi* 039 were confirmed. In three other cases (*Pin a* in *Ae. triuncialis*, *Pin b* in *Ae. juvenalis, GSP* in *Ae. longissima*), two genomic sequences were identified, one of which corresponded to the cDNA sequence. Hence it is possible that only single genes were expressed. Finally, in one case (*Pin b* in *Ae. kotschyi* 011) two cDNAs were iden-

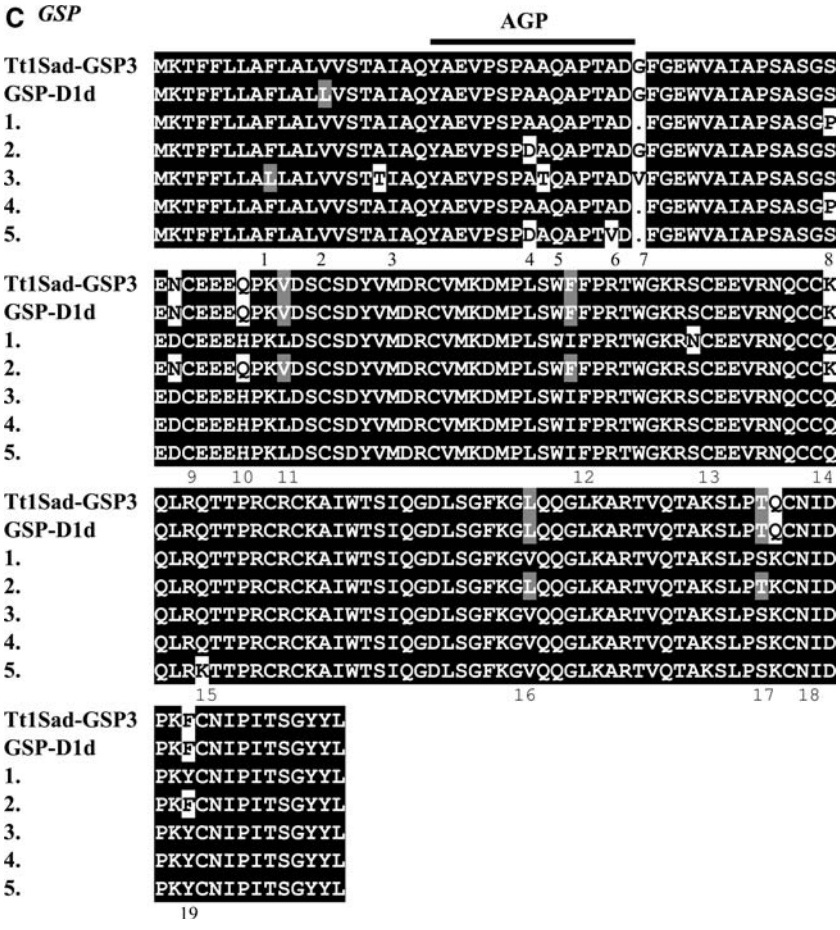
Table 2 Characteristics of the lines used and their *Pin a*, *Pin b* and *GSP* alleles

Species	Accession	Genomes	Habit	*Pin a* alleles	*Pin b* alleles	*GSP* alleles	Texture
Ae. kotschyi	Q03–011	UUSS	Spring	1	1, 2	1	Soft
Ae. kotschyi	Q03–038	UUSS	Weak winter	6 g*, 7c	5	4	Soft
Ae. kotschyi	Q03–039	UUSS	Weak winter	6 g*, 7c	4	3 g, 4c	Soft
Ae. triuncialis	Q03–012	UUCC	Weak winter	2 g, 3	7	2	Soft
Ae. longissima	Q03–023	SS	Winter	NA	6	2 g, 4	Soft
Ae. sharonensis	Q03–026	SS	Spring	5	6	5	Hard
Ae. bicornis	Q03–022	SS	Spring	NA	5	*GSP-D1b*	Soft
Ae. juvenalis	Q03–013	DDMMUU	Weak winter	4	3, 4 g	3	Soft
Ae. tauschii	Q03–002	DD	Weak winter	*Pin a-D1d*	2	*GSP-D1d*	Soft
T. aestivum cv Hiline	–	AABBDD	Spring	*Pin a-D1a*	*Pin b-D1b*	*GSP-D1a*	ND

NA not amplified with gene-specific primers, ND not determined
Alleles indicated as c were amplified from cDNA only and as g from genomic DNA only. Other alleles were amplified from both cDNA and gDNA. Alleles indicated * contain a premature stop codon

Table 3 Alignment of the deduced amino acid sequences of *Pin a* (A) *Pin b* (B) and *GSP* (C) alleles identified in the various *Aegilops* species. The sequences of the mature *Pin a* and *Pin b* proteins and the whole protein encoded by the GSP DNA are shown. The ''tryptophan motif'' is indicated by a bar in parts A and B and the two cysteine residues that form a disulphide bond to stablise the loop containing this motif by arrows (Cys28 and 48 in A, 29 and 48 in B). The sequence corresponding to the AGP is indicated in C





tified, each with a corresponding genomic DNA. This accession therefore provided the only evidence for the presence of two expressed *Pin* or *GSP* genes.

Sequences of Pin a, Pin b and GSP

Although a similar number of new allelic variants of each gene were detected, there were clearly differences in the numbers of substitutions that occurred between the alleles. Thus, differences between the *Pin a* alleles were observed at 13 positions, between the *Pin b* alleles at 24 positions and between the *GSP* alleles at 19 positions (three of which are within the putative signal peptide). The differences in frequency of substitutions between the *Pin a* and *Pin b* alleles are consistent with studies of bread wheat where *Pin b* occurs in at least six allelic forms (Morris 2002) but *Pin a* only in a single form.

The puroindoline sequences are characterised by the presence of a ''tryptophan motif'', comprising five tryptophan residues in *Pin a* (residues 38, 40, 41, 43, 44) and three in *Pin b* (residues 39, 43, 44) (see bars in Table 3a, b). These tryptophan residues are probably present in a surface loop stabilised by a disulphide bond (between Cys 28/Cys 48 in *Pin a*, Cys 29/Cys48 in *Pin b*, see Table 3a, b) and it has been suggested that they form the lipid-binding site (Kooijman et al. 1997). Two of the known *Pin b* mutations which are associated with grain hardness in bread wheat are present in this loop region (*Pin b-D1b*, Gly46Ser; *Pin b-D1d*, Trp44Arg) while two other ''hard'' alleles have stop codons in this region (*Pin b-D1e*, Trp39 stop; *Pin b-D1f*, Trp44 stop) and presumably correspond to silent genes (Morris 2002). The latter would result in the synthesis of truncated proteins if expressed.

It is therefore of interest that one of the new mutations identified in *Pin a* (allele 7) resulted in a Lys/Arg substitution within the tryptophan motif (position 42) while a *Pin b* mutation (allele 2) was adjacent to the tryptophan motif (Val37Phe). These mutations may clearly be of interest in relation to grain texture.

In contrast, the significance of mutations in GSP is more difficult to interpret as the biological role of this protein remains uncertain. The *N*-terminus of the mature *GSP* protein is not known but residues 1–20 may form a signal peptide. If so, residues 21–35, which correspond to the arabinogalactan-binding peptide (AGP), may also correspond to the *N*-terminus of the mature protein. Kieliszowski (2001) has reported that hydroxyprolines which are glycosylated with arabinogalactan polysaccharides are frequently separated by one to three amino acids, commonly Ser, Ala and Thr. The Ala/Thr mutation which was detected at position 9 of the AGP region in allele 3 would therefore not be expected to

affect any glycosylation. In contrast, it is possible that the Ala/Asp mutation present in alleles 2 and 5 could affect glycosylation.

Apart from the substitutions discussed above, it is notable that most of the mutations detected are essentially conservative in nature, involving residues which are similar in size and/or properties. For example, leucine/isoleucine/valine (all aliphatic) substitutions occur at seven positions and lysine/arginine/histidine (all basic) substitutions at four positions. Nine substitutions also involve combinations of glycine, alanine (both aliphatic), serine and threonine (both hydroxy).

Southern blotting

In several accessions the sequences of *Pins* or *GSPs* determined from genomic DNA and cDNA differed. The PCR and sequencing were initially repeated which confirmed the presence of differences in all cases. Southern blotting was therefore carried out to determine if multiple genes were present in these lines. Total genomic DNA was prepared and digested with *Eco*RI which cuts outside the coding regions of the *Pin* and GSP genes. The DNA was then separated by electrophoresis and hybridised with the appropriate probe. This showed that two hybridising fragments were present in all of the lines in which two DNA sequences were identified: for the *Pin a* gene in *Ae. kotschyi* (038 and 039) and *Ae. triuncialis*, the *Pin b* gene in *Ae. juvenalis* and *Ae.* kotschyi 011 and the GSP gene in *Ae. kotschyi* (039) and *Ae. longissima*. It should be noted that *Ae. kotschyi* and *Ae. triuncialis* are tetraploid while *Ae. juvenalis* is hexaploid. Hence, these species might be expected to have *Pin* and *GSP* genes on their individual genomes. In contrast, *Ae. longissima* is diploid and may therefore contain duplicated *GSP* genes on its single S genome. An analogous situation has been reported in barley in which the *Ha* locus comprises two *Pin b* genes, one *Pin a* gene and one *GSP* gene (Darlington et al. 2001; Caldwell et al. 2004).

Expression of Pin proteins and grain texture

The presence of *Pin* proteins in the grain of the lines was demonstrated by western blotting of protein extracted from crushed grain, using a commercially available monoclonal antibody which reacts with both *Pin a* and *Pin b*. In all cases, immunoreactive bands of the expected mobility were observed (not shown), which could correspond to *Pin a*, *Pin b* or both proteins.

Grain texture of wheat can be determined on a single seed basis using the Perten single kernel characterisation system (SKCS) or on milled flour as particle size index (PSI). However these standard technologies could not readily be applied to many of the lines as their seeds were small and difficult to isolate and mill. Hence, we decided to directly determine the degree of adhesion between the starch granules and matrix proteins by scanning electron microscopy (SEM) of freeze-fractured grain (Brennan et al. 1998; Beecher et al. 2002).

Control samples of bread wheat cvs Riband (soft) and Mercia (hard) were initially analysed. These showed clear differences, with the matrix proteins being cleanly separated from the surface of the starch granules in Riband (Fig. 1a) but adhering in Mercia (Fig. 1b). The nine *Aegilops* samples were then fractured in the same way and their surfaces compared. Eight of the samples were clearly similar to Riband (i.e. soft), as illustrated by *Ae. kotschyi* (038) in Fig. 1c. Only *Ae. sharonensis* showed adhesion of protein to the starch granule surface (Fig. 1d) indicating that the texture was harder.

Consideration of the sequences of the *Pin* alleles in *Ae. sharonensis* provides no explanation for this difference in texture. Only a single *Pin a* allele was identified from both genomic DNA and cDNA which is consistent with its diploid constitution. Although this allele (5) did not occur in any of the other accessions it differed from the *Pin-D1a* sequence by only two substitutions (Ile/Val at position 13, Leu/Gln at position 49), both of which also occur in other *Pin a* alleles (Ile/Val in alleles 1, 2, 4, 6; Leu/Gln in alleles 1, 2, 4, 6). Similarly, the single *Pin b* allele detected in both genomic and cDNA of *Ae. sharonensis* (allele 6) was also present in *Ae. longissima* which was clearly soft in texture.

Similarly, we have no explanation why the novel allelic forms of *Pin a* and *Pin b* detected in the other accessions appeared to have no impact on texture (i.e. all were soft), whereas all allelic forms of *Pin b* present in bread wheat are associated with hard texture with the exception of the wild type *Pin b-D1a* allele. We used PCR to amplify cDNA corresponding to *Pin b* from all accessions and to *Pin a* from all accessions except *Ae. longissima* and *Ae. bicornis*, demonstrating that these genes were expressed, and also used western blotting to demonstrate the presence of immunoreactive Pin protein in all lines. However, we did not determine the precise expression levels of the *Pin* genes which also could affect grain texture, or determine the locations of the proteins within the cells of the starchy endosperm or their binding properties. Further studies are clearly required to provide an explanation for our observations.

The frequent occurrence of new *Pin* and *GSP* alleles in the species reported here agrees with studies of *Ae. tauschii*. Analysis of only 50 accessions identified six *Pin a* alleles, four *Pin b* alleles and seven *GSP* alleles, only two of which (*Pina-D1a* and *Pinb-D1c*) had previously been detected in bread wheat (Massa and Morris 2004; Massa et al. 2004). However, they did not determine the textural characteristics of the grains.

Conclusions

It is concluded that the *Aegilops* species studied here are potentially valuable sources of novel *Pin* alleles which could be used to extend the range of textural charac-

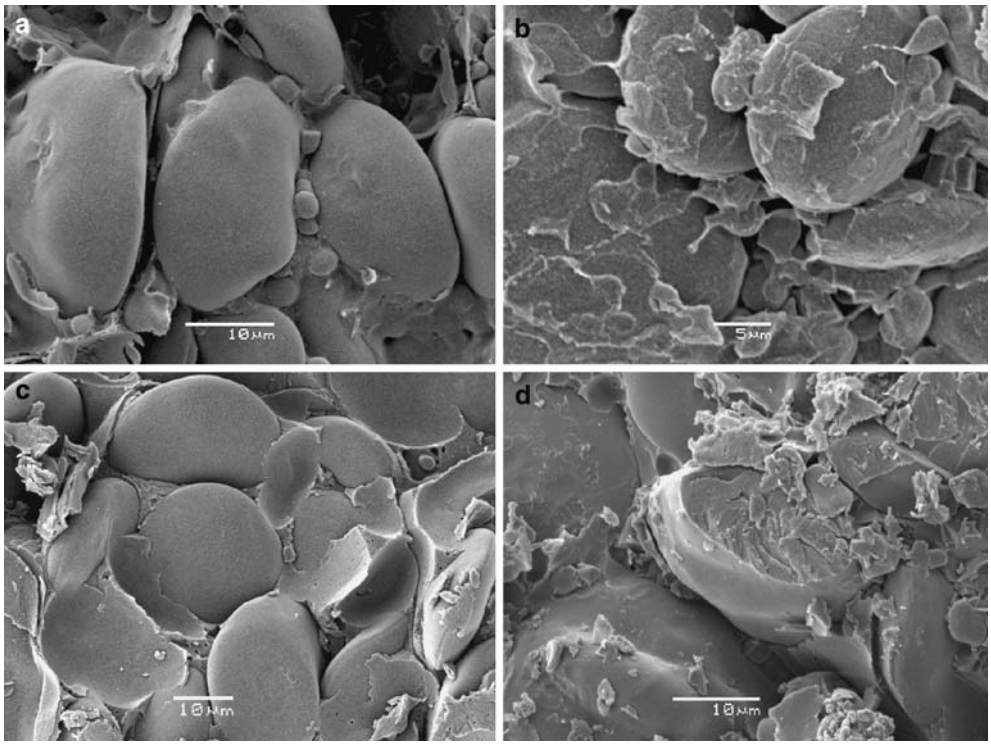


Fig. 1 Stereoscan electron microscopy of freeze-fractured grain of bread wheat cv Riband (soft) (**a**) and Mercia (hard) (**b**), *Ae. kotschyi* accession Q03–038 (**c**) and *Ae. sharonensis* (**d**)

teristics in bread and pasta wheats. However, these would need to be evaluated in bread or pasta wheat backgrounds (e.g. by transgenesis) as fine differences in texture cannot readily be determined in their endogenous backgrounds.

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