Theor Appl Genet (2004) 109: 1525–1535
DOI 10.1007/s00122-004-1776-8

ORIGINAL PAPER

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Dissemination of the highly expressed Bx7 glutenin subunit (*Glu-B1al* allele) in wheat as revealed by novel PCR markers and RP-HPLC

Received: 4 May 2004 / Accepted: 16 July 2004 / Published online: 31 August 2004

Abstract Increased expression of the high molecular weight glutenin subunit (HMW-GS) Bx7 is associated with improved dough strength of wheat (*Triticum aestivum* L.) flour. Several cultivars and landraces of widely different genetic backgrounds from around the world have now been found to contain this so-called 'over-expressing' allelic form of the Bx7 subunit encoded by *Glu-B1al*. Using three methods of identification, SDS-PAGE, RP-HPLC and PCR marker analysis, as well as pedigree information, we have traced the distribution and source of this allele from a Uruguayan landrace, Americano 44D, in the mid-nineteenth century. Results are supported by knowledge of the movement of wheat lines with migrants. All cultivars possessing the *Glu-B1al* allele can be identified by the following attributes: (1) the elution of the By sub-unit peak before the Dx sub-unit peak by RP-HPLC, (2) high expression levels of Bx7 (>39% Mol % Bx), (3) a 43 bp insertion in the matrix-attachment region (MAR) upstream of the gene promoter relative to Bx7 and an 18 bp nucleotide duplication in the coding region of the gene. Evidence is presented indicating that these 18 and 43 bp sequence insertions are not causal for the high expression levels of Bx7 as they were also found to be present in a small number of hexaploid species, including Chinese Spring, and species expressing *Glu-B1ak* and *Glu-B1a* alleles. In addition, these sequence inserts were found in different isolates of the tetraploid wheat, *T. turgidum*, indicating that these insertion/deletion events occurred prior to hexaploidization.

Communicated by C. Möllers

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Introduction

Gluten proteins play a key role in the determination of dough and bread-making quality. This is due to the interaction between the high and low molecular weight glutenins and gliadins, facilitated by complex inter- and intra-molecular covalent and non-covalent bonding. Different roles can be attributed to the glutenins, according to their size and structure. The high molecular weight (HMW) glutenins form the polypeptide network responsible for the visco-elastic properties of dough. The low molecular weight glutenin subunits (LMW-GS) function as chain terminators or extenders according to the number of cysteine residues available for disulfide bonding (Greenfield et al. 1998). Those LMW-GS associated with branch extension, in conjunction with HMW glutenins, are thought to increase the polymer size and confer dough strength (Pogna et al. 1996; Lafiandra et al. 1999). The HMW glutenin subunits are encoded by tightly-linked "x" and "y" type genes at the *Glu-A1*, *Glu-B1* and *Glu-D1* loci on the long arms of chromosomes 1A, 1B and 1D, respectively (Payne et al. 1987.) The LMW glutenin subunits are encoded by genes at the *Glu-A3*, *Glu-B3* and *Glu-D3* loci on the short arms of chromosomes 1A, 1B and 1D, respectively (Gupta and Shepherd 1990.) Work using transgenic wheat lines showed that the insertion of multiple copies of the gene encoding for the HMW-glutenin subunit Dx5 caused significant increases in dough strength due to manipulation of the glutenin backbone (Rooke et al.1999; Popineau et al. 2001.) Likewise, naturally occurring over-expression of the *Glu-B1*-encoded subunit Bx7 ($Bx7^{OE}$), designated *Glu-B1al*, has also been found to confer improved dough

strength in a number of cultivars and landraces (Marchylo et al. 1992; Lukow et al. 1992; D'Ovidio et al. 1997). This association has recently been shown for a range of Australian and North American cultivars and breeding lines (Butow et al. 2002, 2003a, b; Radovanovic et al. 2002), and in old Hungarian varieties and landraces (Juhász et al. 2003a, b).

SDS-PAGE is used routinely to identify HMW glutenin subunits and a visual estimate of expression levels can be achieved, as first noted by Ng et al. (1989). However, reversed-phase (RP)-HPLC is the only method to date which provides both a qualitative and quantitative analysis of glutenin subunits (Marchylo et al. 1989). This is particularly useful in the case of the *Glu-B1*-encoded subunit Bx7, in which only a small difference in electrophoretic mobility exists between subunit Bx7 and Bx7* (Bx7 being slightly larger than Bx7*); these subunits cannot be differentiated on the basis of elution time using RP-HPLC (Marchylo et al. 1992). Quantitative analysis of these two subunits using RP-HPLC, however, revealed that the proportion of subunit Bx7 relative to the total amount of HMW-GS was significantly higher than for subunit Bx7*. Subunit Bx7* has been found in combination with subunits By9, By8, or a variant designated as subunit By8*, whereas subunit Bx7 was found in combination with By8 or By8* only (Marchylo et al. 1992). More recent molecular approaches have identified DNA markers for both the coding region (Butow et al. 2003a) and matrix attachment region (MAR) (Butow et al. 2003b; Radovanovic and Cloutier 2003; Juhász et al. 2003b) of the $Bx7^{OE}$ encoding gene.

In order to trace the dispersal of a particular gene, an insight into the worldwide transfer of germplasm prior to the twentieth century is useful. Landraces from all the major wheat-producing regions have contributed to the varieties currently grown by farmers in the developing world. Germplasm exchange dates to the sixteenth century when European settlers migrated from Spain to South America and Mexico. In countries such as Argentina and Uruguay agriculture was not important until the end of the nineteenth century when migrants from Europe (coming from Russia, Hungary and Germany) brought new wheat landraces such as Barleta, Russo and Hungaro to Uruguay and Argentina. At the close of the nineteenth century, scientific breeding programs began to develop throughout the wheat-producing regions of the world. Landraces that were first used by plant breeders before 1920, and still figure heavily in the pedigrees of current bread wheats, include Sheriff's Squarehead, Zeeuwse Witte, Turkey, Blount's Lambrigg, Purple Straw, and Fife. An analysis of the pedigrees of European germplasm reveals obvious differences between the origin of the first cultivated *Triticum aestivum* genotypes in Western and Eastern Europe. Sherriff's Squarehead, one of the earliest products of modern plant breeding, originated in Great Britain in the mid- to late nineteenth century and along with its descendants, became a cornerstone of the early French, Belgian, German, Dutch, Swedish, and (indirectly) Italian breeding programs (Lupton 1987). The East-Central European farmers mainly used the breeding stocks available from the Carpathian basin and the surrounding area, for example, Galicia (West Ukraine, Lelley and Mandy 1963). Red Fife, also called Fife, Canadian Fife, and Scotch Fife in North America, originated in Galicia under the name Halychanka and was shipped to David Fife in Ontario in 1842 (Symko 1999). Its success allowed the use of this wheat as the basis of the North American spring wheats (e.g. Marquis), the bread wheats grown today in the developing world and an important part of the Eastern European germplasm.

The aim of this study was to identify the source of the *Glu-B1al* allele in modern cultivars by taking into account the available pedigree and historical information. A further aim of this work was to develop markers which, in conjunction with qualitative and quantitative protein information, would aid in the identification of the *Glu-B1al* allele in lines of unknown origin and in tracing the correct lineage of the gene.

Materials and methods

Materials

A total of 75 cultivars and landraces of hexaploid wheat, *T. aestivum* (AABBDD) and 65 tetraploid wheat lines, *T. turgidium* (AABB) were obtained from AWB Ltd., the Australian Winter Cereals Collection (AWCC, Tamworth, Australia), Crop and Food Science (Christchurch, New Zealand), CSIRO wheat seed collections, ARI HAS Martonvasar (Hungary), INRA, and the USDA seed bank. Pedigree information was obtained from the International Wheat Information System (Fox et al. 1997) and personal communications with Dr. Lindsay O'Brien and Dr. David Bonnet.

SDS-PAGE analysis of HMW-GS

Crushed grain (10 mg) was initially extracted at 65°C for 30 min with 200 µl SDS extraction buffer (62.5 mM Tris, 4% (w/v) SDS, 10% (v/v) glycerol, 0.001% bromophenol blue and 2% (v/v) 2-mercaptoethanol, pH 6.8). After centrifugation at 14,000 rpm for 10 min, 10 µl of supernatant was loaded onto the upper 4% SDS-PAGE stacking gel and the proteins were separated on a 10% SDS-PAGE resolving gel at 200 V for 4 h (Lawrence and Shepherd 1980). The gels were then stained overnight with Neuhoff stain (Neuhoff et al. 1988).

RP-HPLC analysis of HMW-GS

Following milling of the grain, the flour was extracted sequentially for gliadins and glutenins and the HMW-GS composition was analyzed by RP-HPLC (Marchylo et al. 1989) as modified by Larroque et al. (2001). The Mol% Bx value was calculated to quantify the amount of subunit

Bx7 using the area of each sub-unit peak relative to the respective molecular weight value (Butow et al. 2003a).

Wheat DNA extraction

Genomic DNA was extracted from 3 to 6 day-old hypocotyls (10 mg) of germinating seeds or from 50 mg of flour using a rapid isolation technique (Stewart and Via 1993).

Scoring of the Bx7 coding region (BxFp) and matrix attachment region DNA markers

PCR was performed in a reaction volume of 20 µl using 60 ng of genomic DNA, 1 U HotStar *Taq* DNA polymerase (Qiagen), in 1× PCR buffer (Qiagen, containing 1.5 mM $MgCl_2$), 200 µM dNTP mix and 10 ρmol of each primer. Primers for the coding region marker (BxFp) were: forward 5′-CAAGGGCAACCAGGGTAC-3′ and reverse 5′-AGAGTTCTATCACTGCCTGGT-3′. This primer pair consists of a novel forward primer based on the repetitive domain of the coding region and the same reverse primer designed by Ma et al. (2003) and used by Butow et al. (2003a) to identify lines showing over-expression of Bx7.

Utilizing the sequence of the Bx7 gene from the Canadian cultivar Glenlea (Patent no. US 6 177 612 B1), the following primers were designed to amplify the MAR from wheat cultivars and landraces: forward 5′-CCTCAG-CATGCAAACATGCAGC-3′ and reverse 5′-CTGAAACCTTTGGCCAGTCATGTC-3′.

Amplification conditions for the PCR reaction were an initial cycle at 95°C for 5 min, followed by 38 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 1 min, followed by a final extension at 72°C for 5.25 min.

Results

Markers used to differentiate *Glu-B1* alleles

A novel coding-region marker, BxFp

We have shown previously (Butow et al. 2003a) that wheat cultivars and lines with the *Glu-B1al* allele can be differentiated using the Bx coding-region PCR marker of Ma et al. (2003), established around an 18-bp insertion, where lines with this allele produce two bands at ~670 and ~770 bp compared to lines with the *Glu-B1b* allele that yield fragments of ~650 and 750 bp. In this study, a new DNA marker (BxFp) was developed (Fig. 1) whereby one primer was designed to prime at multiple sites within the repetitive region of the gene, and the other was Bx specific (Ma et al. 2003), thus giving a more complex pattern; five to seven fragments are amplified falling in three clear groups, 1 (one band, ~800–900 bp), 2 (two to four bands, 400–600 bp) and 3 (two bands, 200–300 bp). All these bands identify Bx alleles that code for glutenin products, while no products were obtained with a *Glu-B1* null line (data not shown). This enabled easier identification of lines, especially on the basis of the smaller fragments where the shift in mobility was higher. The BxFp marker again indicated the 18 bp shift differentiating *Glu-B1al* alleles from *Glu-B1b* alleles (for example CD87 and Frame respectively, Fig. 1). Moreover, the cultivar Sunstate, which carries the *Glu-B1i* allele coding for the 17+18 glutenin subunits, showed a different pattern of five fragments (Fig. 1) as compared to seven fragments for Bx7-containing lines (Fig. 1). Thus, the difference in nucleotide sequences between different cultivars was made readily discernible with the new "fingerprint" marker for the coding region of the Bx7 gene (Fig. 1). We have also used this marker to differentiate other *Glu-B1* alleles (B.J. Butow, in preparation).

A co-dominant MAR marker

A co-dominant PCR marker, designed to amplify the Bx MAR region 750 bp upstream of the coding region, indicated that all cultivars and landraces showing a phenotype of increased Bx7 expression produced a fragment of ~563 bp, indicating the presence of a 43 bp insertion in the MAR of the gene of these lines (for example, Bankuti 1201 B35, Fig. 2) relative to that in the gene of lines without the *Glu-B1al* (520 bp product). This has been shown previously only in the Canadian cultivar Glenlea (containing the *Glu-B1al* allele) and its derivative progeny lines using a primer pair designed to produce a dominant marker fragment of 1,116 bp (Radovanovic and Cloutier 2003). The Bx MAR marker was also found to give a discrete band at ~800 bp for cultivars expressing

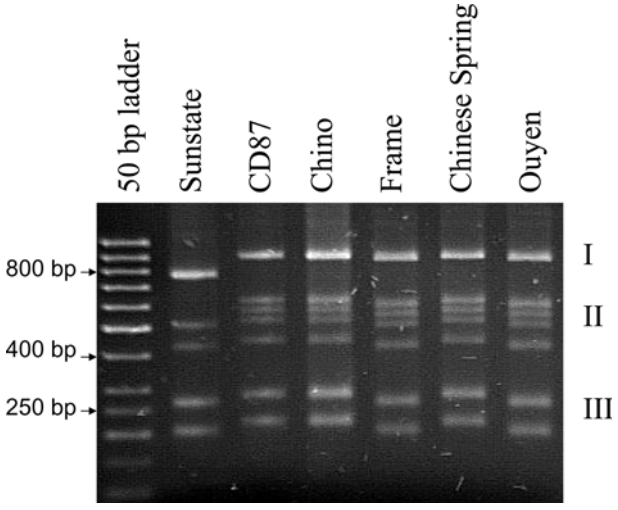

Fig. 1 Comparison of PCR amplicons derived using a *Glu-B1* coding region marker (BxFp) for different cultivars: Sunstate (Bx17), CD87 ($Bx7^{OE}$), Chino (Bx7), Frame (Bx7), Chinese Spring (Bx7), Ouyen (Bx7). Although Chino and Chinese Spring do not possess the *Glu-B1al* allele, they have a similar PCR pattern with that of lines with this allele, due to an 18 bp insert in the coding region

Bx20, such as Olympic (Fig. 2). It is possible that this ~200 bp size shift could correspond to the transposition event in the promoter region of the North American cultivar Bidi (Bx20) reported by Anderson et al. (1998).

Allelic variation at *Glu-B1*

The polymorphic nature of the *Glu-B1* locus is well known (Anderson et al. 1998) and as many as 59 allelic variations have been found to date (McIntosh et al. 2003; Xu et al. 2004). Although in this research we were predominantly interested in the *Glu-B1al* allele encoding the $Bx7^{OE}$ + By8 subunits, use of DNA markers together with RP-HPLC has enabled us to differentiate Bx7 subunits with and without sequence insertions and with varying HPLC elution profiles of the corresponding By subunit pair. Table 1 outlines eight allelic variants found for the *Glu-B1* Bx7 subunit in this study. Examples of each gene type are given, but only those in group 1 show quantitative increased expression of Bx7 (Table 1). Cultivars and landraces (e.g. Barletta) in group 2 (Table 1) showed an 18 bp insertion with the BxFp marker and elution of the By subunit before the Dx subunit, but no insertion of the 43 bp fragment in the MAR nor increased expression levels of Bx7. The RP-HPLC profile of 7+8* types (Fig. 3) indicated that as observed previously, the By8* subunit eluted before the Dx2 subunit for both groups 1 and 2 of Table 1. However, Barletta (Fig. 3B) is shown as an example of a group 2 type of 7+8*, where there is no increase in expression level of Bx7, unlike Chara (a group 1 type; Fig. 3A). Cultivars with a Bx7+By8 subunit combination (Rosella, Fig. 3C) show elution of By8 after the Dx2 subunit in HPLC and no increase in the amount of Bx subunit. Norstar, a group 3 cultivar (Table 1) with similar traits to group 2 cultivars (regarding HPLC elution of the By peak, 18 bp insertion in the coding region and no increase in Bx expression levels) also was shown to have the Bx MAR 43 bp insert. The electrophoretic mobility of the Bx subunit in Norstar (Bx7*) (group 3) has been found to be slightly greater than that of other Bx7 subunits (Marchylo et al. 1992) found in cultivars of groups 1, 2, 4, 6 and 7. Group 5 cultivars (Table 1), such as Hobbit, are null for the By subunit and were found to have both the 18 and 43 bp insertions with no increase in expression levels of Bx7. Chinese Spring (and Chino) fall into a yet another different allelic grouping (group 6) as they both have the 18 bp coding region insertion, but otherwise share the same Bx7 phenotype as *Glu-B1b* cultivars (group 4, Table 1). This difference was picked up by the BxFp marker (Fig. 1) and was originally discussed by Anderson and Greene (1989). Chinese Spring is believed to be named after the spring wheat Chino ("Chinese" in Spanish), which also has the same 18 bp insert in the coding region as *Glu-B1al* lines (Fig. 1), although neither Chino nor Chinese Spring has the 43 bp insert in the MAR region (Fig. 2). The final two allelic variants do not have insertions in the coding region or MAR, but in group 7, Bx7 is paired with By9, which is eluted prior to subunit Dx (e.g. Cheyenne). In group 8, the Bx7 subunit differs slightly from that of the group 6 lines in that it has a slightly higher electrophoretic mobility (e.g. Fiorello; Marchylo et al. 1992).

Dispersal of wheat cultivars and lines showing high expression of Bx7

A survey of wheat cultivars and landraces from around the world revealed representation of the *Glu-B1al* allele in eight countries spread over four continents (Table 2). The Mol% Bx values obtained were found to be similar (±0.65%) to the estimated values given by the percentage of the Bx subunit within the total HMW glutenin subunits (Gianibelli et al. 2002; Marchylo et al. 1992).

The presence of different biotypes was quite common, especially in old varieties, such as Sinvalocho or Bankuti 1201, where biotypes with different *Glu-B1* alleles were found (results not shown). Similarly, several Fife populations were found to be heterozygous at the *Glu-B1* locus (Table 3). Table 2 also shows examples of Argentinean landraces (e.g. Barletta) and a US landrace (Kanred) which did not have the *Glu-B1al* allele. The landraces, Klein Universal II No. 7485 and Grajo, together with the cultivar

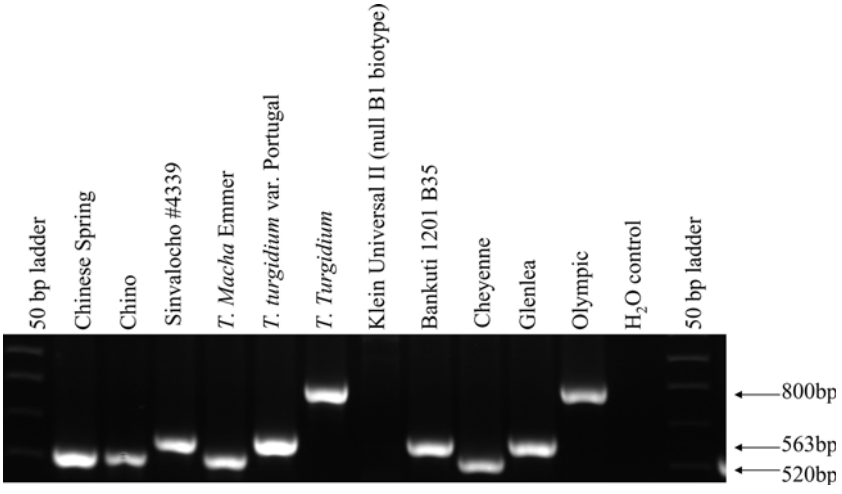

Fig. 2 Comparison of PCR amplicons derived using a MAR primer pair to identify lines expressing Bx20, $Bx7^{OE}$ and Bx7 using the following control lines (HMW-GS in *parenthesis*): Klein Universal II (null *Glu-B1* biotype), Bankuti 1201 B35 ($Bx7^{OE}$), Cheyenne (Bx7), Glenlea ($Bx7^{OE}$) and Olympic (Bx20)

Table 1 Bx7-type alleles encoded at *Glu-B1*. The RP-HPLC elution position of the By8 (elution after the Dx subunit) and By8* (elution before the Dx subunit) peaks is given as indicated in Fig. 3. Note that the presence of an 18 bp insertion in the coding region causes a shift in all the bands indicated (group I, II and III in Fig. 1). The MAR marker approximate fragment sizes reflect the presence or absence of the 43 bp MAR insertion – no insertion, *ND Glu-B1* pair not designated

Group	*Glu-B1* allele	Bx+By glutenin subunits	RP-HPLC elution position of By sub-unit peak relative to Dx sub-unit peak	BxFp marker: insertion size (bp)	MAR marker: fragment size (bp)	Example
1	al	7+8*	Before	18	563	Klein Universal Sinvalocho 4339 Tezanos Pintos Precoz Glenlea CD87 Bankuti 1201 B35
2	ND	7+8*	Before	18	520	Barletta Kanred Sinvalocho 1250 Retacon INTA Hungaro Naposta
3	ak	7*+8*	Before	18	563	Norstar
4	b	7+8	After	–	520	Frame Janz
5	a	7 (null By8)	Absent	18	563	Fife Tuscan Hobbit Galahad
6	ND	7+8	After	18	520	Chinese Spring Chino
7	c	7+9	Before	–	520	Cheyenne Marquis
8	u	7*+8	After	–	520	Fiorello

Glenlea, were also found to carry the 43 bp MAR insertion. Presence of the *Glu-B1al* allele in these wheat cultivars/landraces was confirmed by RP-HPLC analysis (Table 2). Use of the Bx MAR marker enabled the elucidation of biotypes or misclassification of lines which, by SDS-PAGE, were considered to have the *Glu-B1al* allele. For example, when the Bx MAR marker was used to test two accessions of the Argentinean cultivar Sinvalocho, an amplicon 43 bp larger than that obtained from Sinvalocho no. 1250 was produced with Sinvalocho no. 4339 (Fig. 2), the presence of the *Glu-B1al* allele in this line was then verified by RP-HPLC (Table 2).

Source of *Glu-B1al*

By analyzing the pedigree (Fox et al. 1997) of the lines detailed in Table 2 containing the *Glu-B1al* allele, Tezanos Pintos Precoz (TPP), an Argentinean cultivar from the Experimental Station of Tezanos Pinto, was found to be the common predecessor for all the lines listed, except for the Hungarian and Israeli landraces. TPP was shown to come originally from Argentinean wheat crosses between Barletta, Kanred, Chino and Klein Universal II (Fig. 4) (Fox et al. 1997). Although the BxFp coding-region PCR marker showed that the first three of these lines contained the 18 bp insertion indicative of the *Glu-B1al* allele, no 43 bp insertion in the MAR was evident (Table 2). Furthermore, the RP-HPLC results confirmed that the expression levels did not exceed 39% (Mol% Bx) and therefore that neither Barletta, Kanred and Chino could be sources of the $Bx7^{OE}$ subunit. Thus, by use of two different PCR markers for Bx7 encoding alleles, it was ascertained that Klein Universal II was the original traceable cultivar with the *Glu-B1al* allele.

Linkage of Bx7 insertions and the over-expressing phenotype

All modern wheat varieties in the "New World" countries of North America, Australia and New Zealand have a strong presence of Fife, or a Fife derivative, in their lineages. Investigations were therefore made regarding the presence of the *Glu-B1al* allele in a collection of Fife lines. Thirty-three different sources of Fife, from various international cereal collections, were checked using SDS-PAGE and PCR analysis (Table 3). RP-HPLC was carried out on half seeds of those lines that were clearly *Glu-B1b* or appeared to have more intense staining on SDS-PAGE, and thus potentially carried the *Glu-B1al* allele. However, the results indicated that none of the Fife

Table 2 Survey of the presence of the *Glu-B1al* allele (indicated by Mol% Bx >39%) in cultivars and landraces of worldwide origin. Sources of seed: AWCC Australian winter cereals collection, NWMMP National wheat molecular marker program; AWB Australian wheat board, ARIHAS Agricultural Research Institute of the Hungarian Academy of Sciences. Intense staining of the Bx7 sub-unit (Y yes; N no) with SDS-PAGE is qualitatively indicative of the presence of $Bx7^{OE}$. Approximate fragment sizes for the MAR marker reflect the presence or absence of the 43 bp MAR insertion

Origin	Name	Accession no.	Source of seed	Release date	SDS-PAGE: intense staining of Bx7 subunit	MAR marker: fragment size (bp)	RP-HPLC: Mol% Bx	References
Argentina	Barletta	1874	AWCC	1915	Y	520	31.55	
	Barletta	1875	AWCC	1915	Y	520	31.58	
	Barletta	1876	AWCC	1915	Y	520	31.12	
	Buck Nandu	21877 6 WG83	AWCC	1975	Y	563	48.08	Gianibelli et al. 2002
	Buck Pucara	21879	AWCC	1980	Y	563	48.63	Gianibelli et al. 2002
	Chino	26162	AWCC		N	520	37.60	
	Hungaro	7300	AWCC			520	32.48	
	Klein Atlas	16106	AWCC	1967	Y	563	48.35	Gianibelli et al. 2002
	Klein Fortin	22076	AWCC	1974	Y	560	50.00	Gianibelli et al. 2002
	Klein Sendero	21877 16 WG8	AWCC	1971	Y	563	48.08	Gianibelli et al. 2002
	Klein Toledo	19925	AWCC	1972	Y	563	46.26	Gianibelli et al. 2002
	Klein Universal	7459	AWCC	1922	Y	563	53.61	
	Klein Universal	7483	AWCC	1922	Y	563	51.81	Gianibelli et al. 2002
	Pampa INTA	24400	AWCC	1984	Y	563	44.54	Gianibelli et al. 2002
	Retacon INTA	24410	AWCC	1984		520	38.05	
	Tezanos Pintos Precoz		AWCC	1956	Y	563	48.83	
	Sinvalocho	4339	AWCC	1939	Y	563	45.98	
	Sinvalocho	1250	AWCC	1939	Y	520	33.94	
	Victoria INTA	23519	AWCC	1978	Y	563	46.69	Gianibelli et al. 2002
Australia	CD87		NWMMP		N	563	43.61	Butow et al. 2003a
	Chara		AWB	1999	Y	563	47.13	Butow et al. 2002
	Kukri		AWB	1999	Y	563	46.64	Butow et al. 2002
Canada	Biggar		Lukov	1989	Y	563	42.50	Marchylo et al. 1992
	Bluesky		Lukov	1987	Y	563	39.50	Marchylo et al. 1992
	Glenlea		Lukov	1972	Y	563	48.07	Marchylo et al. 1992
	Laura		Lukov	1976	Y	563	43.02	Radovanovich et al. 2002; Marchylo et al. 1992
	Norstar		Lukov	1977	N	563	21.87	Marchylo et al. 1992
	Roblin		Lukov	1986	Y	563	40.66	Marchylo et al. 1992
	Wildcat		Lukov	1987	Y	563	39.70	Marchylo et al. 1992
Hungary	Bankuti 1201	B28	ARIHAS	1931	Y	563	52.30	Juhász et al. 2003a
		B25	ARIHAS	1931	Y	563	51.51	Juhász et al. 2003b
		B88	ARIHAS	1931	Y	563	46.81	
Israel	TAA36		Feldman		Y	563	48.81	Lukov et al. 1992
Mexico	Emu	17224	AWCC	1974		563		
	Grajo	17229	AWCC	1973		563		
	Mexico		CSIRO			563	52.48	
	Red River 68		AWB	1968	Y	563	45.00	D'Ovidio et al. 1997
	Tobari F 66			1966		563		
New Zealand	Endeavour			1994		563		Cornish et al. 1999
	Otane	23362	AWCC	1985	Y	563	48.56	Sutton 1991
USA	Kanred	2701	AWCC	1917	Y	520	25.01	Symko 1999

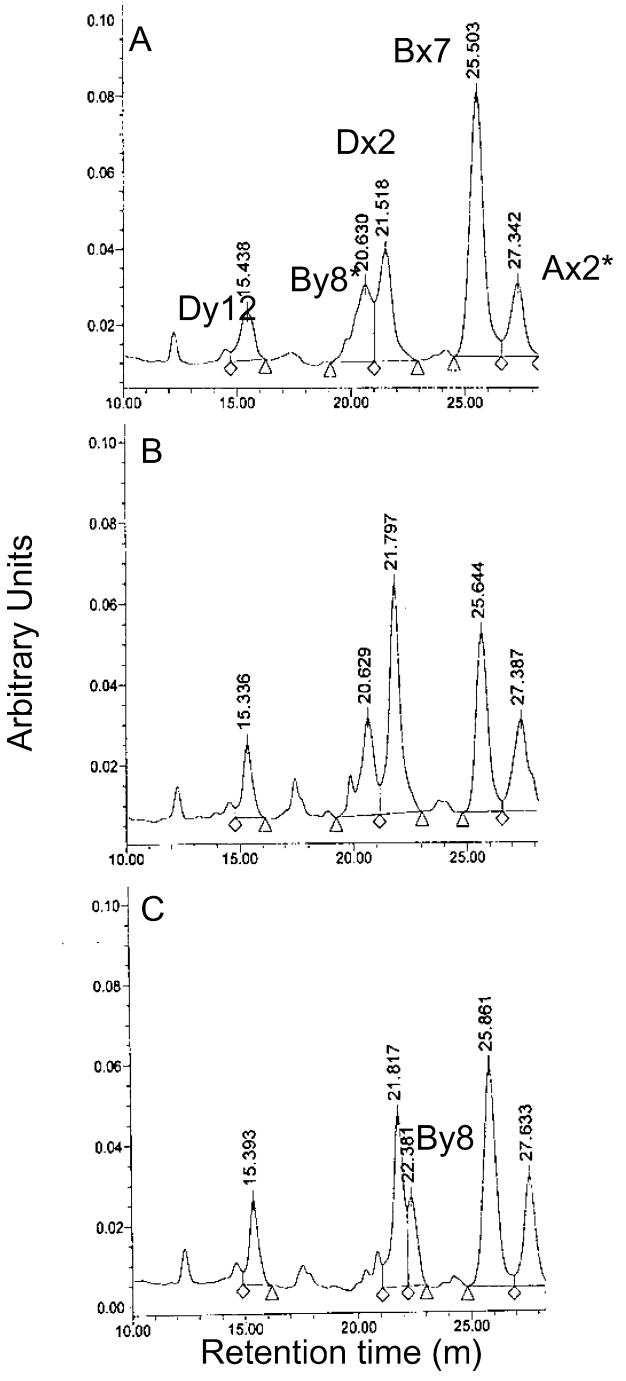

Fig. 3A–C RP-HPLC elution profiles [*y axis* in arbitrary units and *x axis* representing elution time (min)] indicate differences between (**A**) the 7+8* allelic pair (*Glu-B1al*) for lines with the $Bx7^{OE}$ subunit (e.g. Chara) (**B**) the 7+8* allelic pair (no designated gene code) for lines without the $Bx7^{OE}$ subunit (e.g. Barletta) and (**C**) the 7+8 allelic pair (e.g. Rosella). In 7+8* lines, By8* elutes before the Dx subunit; subunit Bx in (**A**) is more highly expressed (i.e. $Bx7^{OE}$) than in (**B**)

lines possessed the *Glu-B1al* allele (Table 3). Ten individual seeds were tested from each Fife line and heterogeneity was observed at all three *Glu-1* loci (~25% of the lines contained a mixture of *Glu-1* alleles), indicating that many Fife lines are in fact populations; some of the lines also showed heterogeneity for gliadins and LMW-GS. One of these heterogeneous populations was Fife Tuscan. Both of the New Zealand Fife Tuscan lines (AUS nos. 3028 and 2410) were initially thought to have the *Glu-B1al* allele, as the BxFp coding region and MAR markers indicated an 18 and a 43 bp insertion respectively (Table 3). However, RP-HPLC results showed that there was no quantitative indication of high expression of the Bx7 subunit and indicated the absence of the By8 subunit. Confirmation that lines with a *Glu-B1a* (null By8) composition exhibit 18 and 43 bp insertions in the coding region and MAR respectively was shown with control lines, Hobbit and Galahad (group 5; Table 1). This is of practical importance because it demonstrates that neither the BxFp nor the Bx MAR marker are a perfect marker for the *Glu-B1al* allele, and shows that increased expression is unlikely to be caused by the insertions in these regions.

That no Fife populations were found to contain the *Glu-B1al* allele was subsequently explained when investigations into the source of Fife, the Ukrainian landrace Halychanka (Symko 1999), did not show evidence of the presence of the *Glu-B1al* allele and was in fact shown to contain *Glu-B1c* (Bx7+By9). This was by far the most common allele found (64%) at *Glu-B1* for the Fife lines investigated.

A common thread between tetraploids and hexaploids?

The origin of cultivated tetraploid wheat in the Levantine corridor and cultivated hexaploid wheat southwest of the Caspian Sea is comprehensively explained by Feldman (2001). The tetraploid, *T. turgidium* var. *dicoccoides*, is the immediate progenitor of most cultivated wheat and the donor of the B genome in cultivated tetraploids and hexaploids (Levy et al. 1988). Thus, to investigate the presence of the *Glu-B1a1* allele pre- or post-hexaploidization, we conducted a survey of progenitor species using SDS-PAGE and PCR analysis using the BxFp coding region and Bx MAR markers. RP-HPLC analysis was performed on specific lines. Several lines of evidence showed that the *Glu-B1* alleles found in hexaploid wheat also exist in some tetraploid species. For example, the *Glu-B1a* allele (By8 null) was found in *T. turgidium* "Russian perennial" AUS 3301 as indicated by the presence of the 18 bp insertion in the coding region, the 43 bp insertion in the MAR and the absence of the By8 subunit (SDS-PAGE). The MAR marker has been found to clearly differentiate Bx20 (Fig. 2) in addition to lines with and without the presence of *Glu-B1al* and this marker confirmed the presence of the Bx20 subunit in other *T. turgidium* sub-species (Fig. 2). Most significantly, we found that one *T. turgidium* species isolate (var. Portugal 170 AUS 5523) showed direct similarities with lines carrying the *Glu-B1al* allele (Fig. 2); that is, intense staining of the Bx7 subunit with SDS-PAGE and the 18 and 43 bp insertions in the coding region and MAR

Table 3 Fife cultivars and populations investigated as potential sources of the *Glu-B1al* allele. Results are shown for ten single seeds tested by SDS-PAGE. The MAR marker was tested on eight replicate DNAs, each comprising three pooled seeds. Results with the MAR show the approximate fragment size referring to the presence (563 bp) or absence (520 bp) of the 43 bp MAR insertion; the presence of both products (520/563 bp) indicates a heterozygous population. The presence of an 800-bp fragment (200 bp insertion in the MAR) is indicative of the Bx20 subunit

Line	Accession no.	Source	Origin	SDS-PAGE *Glu-B1*	MAR marker: fragment size (bp)	Mol% Bx
Saskatchewan Fife	12092	AWCC	CAN	7+9	520	
White Fife	12101	AWCC	CAN	17+18/20	520/800	
Jones Fife	90115	AWCC	USA	6+8	520	
Powers' Fife	10770	AWCC	USA	7+9	520	
Red Fife	10765	AWCC	CAN	7+9	520	
Biffens White Fife	47	AWCC	USA	7+8	520/563	37.29
White Fife	1657	AWCC	CAN	7+9	520/563	
Improved Fife	2635	AWCC	USA	7+9	520	
Red Fife	3238	AWCC	CAN	7+9	520	
Red Fife	3239	AWCC	CAN	17+18/7+8	520/563	
Red Fife	3240	AWCC	CAN	7+9	520	
Red Fife	7188	AWCC	CAN	7+9	520	
Fife	2409	AWCC	CAN	7+9	520/563	
Fife Tuscan	2410	AWCC	NZL	7	563	
Fife	15696	AWCC	CAN	7+9	520	
Powers Fife	3179	AWCC	USA	7+9	520	
Fife Tuscan	3028	AWCC	NZL	7/7+9	520/563	
Red Fife	8091	INRA	CAN	7+9	520	
Early Red Fife	8092	INRA	CAN	7+9	520	
White Fife	13384	INRA	CAN	7+9	520	
Jones Fife	3947	INRA	USA	6+8	520	
Fife Tuscan	3418	Crop and Food	NZL	7+9	520	
Fife Tuscan	PI 283868	USDA	NZL	17+18	520	
Red Fife	PI 348919	USDA	AUS	7+9	520	
Rysting	PI 565341	USDA	USA	7+8	520	34.98
Fife	PI 283820	USDA	AUS	7+9	520	
Fife Tuscan	PI 266864	USDA	NZL	7+9	520	
White Fife	Cltr. 4412	USDA	CAN	6+8	520/563	
Early Red Fife	Cltr. 4932	USDA	CAN	7+8	520	37.54
White Fife	PI 61345	USDA	USA	7+9	520	
Jones Fife	Cltr. 4468	USDA	USA	7+9	520	
Burgoyne Fife	PI 58559	USDA	UK	7+9	520	
Carlson's Fife	Cltr. 11922	USDA	USA	7+8	520	37.06

respectively. We are currently establishing a method for quantifying HMW subunits in lines containing null alleles and tetraploid species to enable a direct quantitative comparison between lines containing five HMW glutenin sub-units (hexaploids) with three and two HMW glutenin sub-units (tetraploids). However, we can deduce from the

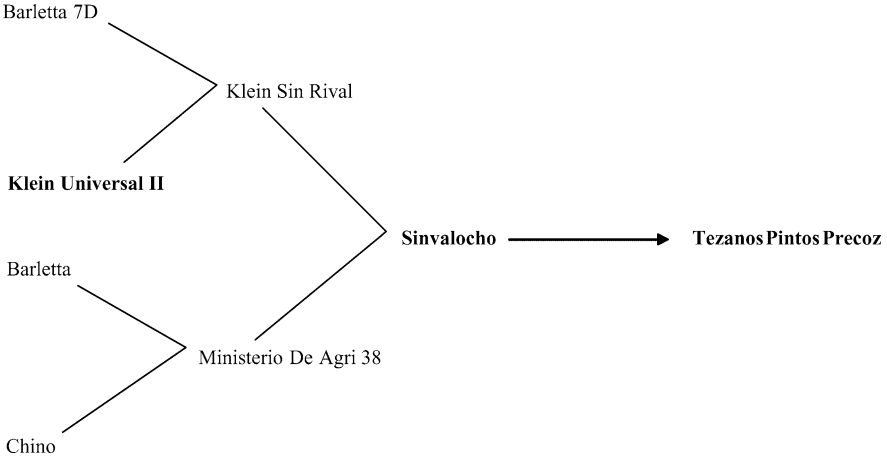

Fig. 4 Pedigree of Tezanos Pintos Precoz, the source of the *Glu-B1al* allele found in current Australian, Canadian, New Zealand and U.S. strong wheat cultivars (adapted from Fox et al. 1997). *Bold lettering* indicates the deduced $Bx7^{OE}$ subunit source

elution profile of *T. turgidium* that By8 is present and not By8*. We cannot therefore be conclusive about the expression levels at *Glu-B1* in *T. turgidium* (var. Portugal 170 AUS no. 5523), but it appears likely that identical or similar insertion sequences to those found in hexaploid cultivars, such as Glenlea, existed prior to hexaploidization.

Discussion

This survey indicated that the *Glu-B1al* allele in modern cultivated wheat was disseminated from the Argentinean landrace Klein Universal II. In turn, this originated from the Uruguayan landrace Americano 44D of unknown origin that was used by the German wheat breeder E. Klein in breeding early Argentinean lines (Smale and McBride 1996). Klein began scientific plant breeding in 1914 in Uruguay (Nisi and Antonelli 2001); the pedigrees of his early releases contain more than 50 varieties of distinct origins including selections from Uruguayan populations such as Universal II. Prior to this, it is thought that this landrace was brought to South America from Europe during the early twentieth century. Americano 44D, and ultimately TPP, was originally used as a source of rust resistance in both North and South American breeding programs. It is still considered to be an important source of durable resistance to leaf rust (van Ginkel and Rajaram 1993).

In Australia, the cultivars Kukri and Chara, together with the breeding line CD87, carry the *Glu-B1al* allele showing increased expression of the Bx7 subunit. We have traced the source of *Glu-B1al* from these cultivars to Tobari 66, a daughter line of TPP. Tobari 66 is a Bajio selection from CIMMYT and is one of the ten most common parents of Canadian and US wheat (Mercado et al. 1996). This, in effect, represents the introduction of the allele subunit into North America and Oceania. The *Glu-B1al* allele in Kukri was derived from a cross involving the Mexican cultivar Grajo (Table 2) a daughter line of Tobari 66. By contrast, *Glu-B1al* in CD87 (and subsequently its daughter line, Chara), originated from a cross between Condor and Emu, (the latter also being a Mexican Red Spring variety with TPP in its background) and was shown to have the 43 bp insert in the MAR (Table 2). The value of the *Glu-B1al* allele in Australian germplasm has recently been demonstrated for a DH population (CD87 × Katepwa) in which segregation of *Glu-B1al* was positively linked to variation in dough strength (Butow et al. 2003a, b).

The origin of the *Glu-B1al* allele in the Hungarian landrace Bankuti 1201 cannot be explained directly by Argentinean ancestors. Bankuti 1201 was extremely important in Hungarian wheat breeding. It was developed by Laszlo Baross from a cross between the Canadian spring wheat Marquis and Bankuti 5, which was selected from the landrace Tiszavideki, and the variety was registered in 1931. The storage protein composition of Bankuti 1201 is characteristic of a population; only 38% of the lines possess the *Glu-B1al* allele whereas the majority of the lines express subunits encoded by the *Glu-B1c* allele (59.6%) and 1.9% possess the *Glu-B1d* allele (subunits 6+8) (Juhász et al. 2003a.) This heterogeneity is most likely a result of its pedigree whereby one of the parents, Bankuti 5, was selected from the landrace Tiszavideki, which also shows population-like characteristics. The other parent, Marquis, has the subunits encoded by the *Glu-B1c* allele, although based on Symko (1999) and Rajháthy (1961) it also showed heterogeneous properties. Marquis was developed by A.P. Sanders from a cross between Hard Red Calcutta (an Indian landrace) and Red Fife in 1892.

The historical Hungarian landraces also have a close connection with germplasm from Galicia (part of the Austro-Hungarian Empire during the mid- to late nineteenth century). Hungarian wheat breeding can be traced back to the beginning of the nineteenth century, when weather conditions (in Western Hungary) favoured the growth of Western European wheat varieties, while in the dryer eastern part, landraces of unknown origin were bred. However, as the drought of 1863 had an enormous impact on Hungarian wheat breeding, seed was imported from Galicia for re-planting. Due to the extreme dry conditions, the newcomer wheats adapted fast to the micro-climate of the Carpathian Basin (Lelley and Rajháthy 1955; Mokry 1875) and were collected as landraces at the end of the nineteenth century. These landraces were then used for making further selections and crosses.

Norstar is a Canadian variety, which does not have the *Glu-B1al* allele, yet it does show certain traits in common with lines that do (Table 1). In addition to having a strong Fife element in its background (Fox et al. 1997), Norstar also has landrace ancestors from the European former Soviet Union, such as Lutescens, Erythrospermum, Turkey and Odessa, which may also serve as a source of the *Glu-B1c* allele. However, although we have not conducted an exhaustive survey, of the landraces we have investigated so far, we have not found indications of a 43 bp insert in the MAR in these lines. These Eastern European landraces may also be the source of the *Glu-B1al* allele found in the Israeli and Hungarian landraces (Table 2) and which were transported to Argentina/Uruguay before arriving in Mexico for dispersal to Canada, the US, Australia and New Zealand. In an investigation of the taxonomical name of Americano 44D, we found that this genotype, together with other Uruguayan wheat genotypes, is classified as *T. aestivum* L. ssp. *aristatum* var. *ferrugineum*. Interestingly, a search for var. *ferugineum* in the Eastern European germplasm revealed that some landraces from Hungary originate from the same subtaxa. Although Bankuti 1201 is classified as *T. aestivum* L. ssp. *aristatum* var. *erythrospermum*, some landraces from the same Tisza region are *ferrugineum*. The origin of these *ferrugineum* landraces, and the landraces Galitckaya (Galicia) and Halychanka, was the Ukraine. Further indications of the Eastern European origin of the Uruguayan and Argentinean genotypes are given in http://www.cimmyt.org/research/economics/map/facts_trends/

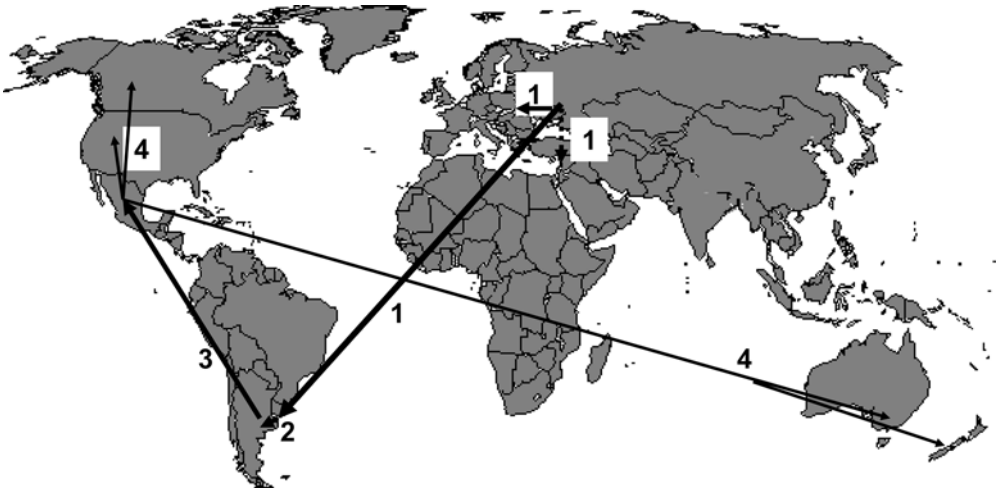

Fig. 5 Schematic diagram summarizing the proposed gene flow and worldwide dissemination of the *Glu-B1al* allele. Routes: *1* Galicia to Uruguay, and possibly Hungary and Israel (the latter being represented by a *dotted line* due to unknown origin); *2* Uruguay to Argentina; *3* Argentina to Mexico; *4* Mexico to Canada, the US, Australia and New Zealand

wft. Based on this information, both the South American and Middle American regions used landraces from Galicia and the former Soviet Union regions. A summary of the dispersal route of the *Glu-B1al* allele is shown in Fig. 5.

The phenotypes of cultivars designated in groups 2, 3, 5, and 6 in Table 1 provided evidence that the presence of either 18 or 43 bp sequence insertions are not causal for the high expression levels for the $Bx7^{OE}$ subunit. This work has also brought to light the existence of two new types of *Glu-B1* allele according to gene sequence differences, thus differentiating group 2 types from those of group 1, and group 6 types from those of group 4 (Table 1). The functional significance of these differences has not been studied in depth.

This study extensively utilized a marker designed to amplify a 43 bp insertion found within the MAR of lines with the *Glu-B1al* allele. The origin of this insertion appears to be due to sequence duplication; the 43 bp sequence located at position 181624–181663 (GenBank AY368373) was found to be a tandem duplication in the Glenlea sequence (Patent No. US 6 177 612 B1). There has been some speculation as to the role of the MAR in controlling gene expression; it is thought that the MAR anchors chromatin to a nuclear protein scaffold enabling organization of active chromatin domains (Holmes-Davis and Comai 1998.) The role of the 43 bp insertion in increasing expression levels of the Bx7 subunit though is unclear in light of the finding that wheat lines other than those with *Glu-B1al* had the insertion too. Possibly there is a further co-regulatory factor in *Glu-B1al* lines linking the 43 bp insertion to expression efficiency. Work is currently underway to investigate whether gene duplication is thecause of the high expression levels of the $Bx7^{OE}$ subunit, as first described by D'Ovidio et al. 1997. Gene duplication in Glenlea has recently been confirmed (S. Cloutier et al., personal communication). It is unknown as yet whether Norstar and the null By8 cultivars (which contain the MAR duplication) contain the same Bx7 gene as found in *Glu-B1al* lines, but as a single copy.

Conclusion

The *Glu-B1* locus appears to be more diverse than other *Glu-1* loci as indicated by the presence of short insertions/deletions within the MAR and coding regions, a possible gene duplication event and significant genetic variation in expression levels of sub-unit Bx7. At least some of these indicators are present in some tetraploid accessions, such as *T. turgidium*. By using a combination of approaches we have traced the common pedigree of lines with high expression of Bx7, and also shown that using DNA markers or SDS-PAGE alone for genotyping can lead to misleading results. This is of practical importance when selection for the *Glu-B1al* allele, a significant source of increased dough strength, is desired in wheat breeding programs.

Acknowledgements We thank Dr. M. Feldman and Dr. O. Lukow for providing some of the material for this study. We thank Lynnette Rampling for her excellent technical assistance and Dr. Lindsay O'Brien and Dr. David Bonnet for informative discussions. Initial discussions with Dr. Matthew Morell and Dr. Frank Bekes are also gratefully acknowledged. This work was carried out as part of the Graingene research program; Graingene is a research consortium between Australian Wheat Board Ltd, the CSIRO, the Grains Research and Development Corporation and Syngenta Seeds.

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