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Identification of SNPs and development of allele-specific PCR markers for γ-gliadin alleles in *Triticum aestivum*

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Abstract The coding regions of 28 entries of hexaploid wheat γ-gliadin genes, gene fragments or pseudogenes in GenBank were used for nucleotide alignment. These sequences could be divided into nine subgroups based on nucleotide variation. The chromosomal locations of five of the seven unassigned subgroups were identified through subgroup-specific polymerase chain reactions (PCR) using *Chinese Spring* group-1 nulli-tetrasomic lines. Multiple single nucleotide polymorphisms (SNPs) and small insertions/deletions were identified in each subgroup. With further mining from wheat expressed sequence tag databases and targeted DNA sequencing, two SNPs were confirmed and one SNP was discovered for genes at the *Gli-A1*, *Gli-B1* and *Gli-D1* loci. A modified allele-specific PCR procedure for assaying SNPs was used to generate dominant DNA markers based on these three SNPs. For each of these three SNPs, two allele-specific primer sets were used to test *Chinese Spring* and 52 commercial Australian wheat varieties representing a range of low-molecular-weight (LMW) alleles. PCR results indicated that all were positive with one of the primer sets and negative with the other, with the exception of three varieties containing the 1BL/1RS chromosomal translocation that were negative for both. Furthermore, markers GliA1.1, GliB1.1 and GliD1.1 were found to be correlated with *Glu-A3 a, b* or *c, Glu-B3 b, c, d* or *e* and *Glu-D3 a, b* or *e* LMW glutenin alleles, respectively. Markers GliA1.2, GliB1.2 and GliD1.2 were found to be correlated with the *Glu-A3 d* or *e, Glu-B3 a, g* or *h* and *Glu-D3 c* alleles, respectively. These results indicated that the γ-gliadin SNP markers could be used for detecting linked LMW glutenin subunit alleles that are important in determining the quality attributes of wheat products.

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Introduction

The most abundant storage proteins of wheat endosperm are gliadins and glutenins, representing about 80% of the total protein in the wheat grain (Shewry et al. 1997). Gliadins are normally monomeric proteins, while glutenins form polymeric structures. Gliadins are classified into three groups, – α/β, γ and ω – on the basis of their electrophoretic mobility in acidic polyacrylamide gel electrophoresis (A-PAGE, Bushuk and Zillman 1978; Autran et al. 1979; Wrigley et al. 1982; Metakovsky et al. 1984). Genes coding for most of the γ- and ω-gliadins are tightly clustered at three homoeologous loci, *Gli-A1, Gli-B1* and *Gli-D1*, on the short arms of chromosomes 1A, 1B and 1D, respectively. α/β-gliadins are encoded by tightly clustered genes at three homoeologous loci, *Gli-A2, Gli-B2* and *Gli-D2*, on the short arm of each group-6 chromosome (Metakovsky et al. 1984; Metakovsky 1991). The gluten polymer is composed mainly of high-molecular-weight (HMW) and low-molecular-weight (LMW) glutenin subunits (GS) linked by disulphide bonds (Shewry et al. 1989, 1992; MacRitchie 1992). The genes coding for HMW subunits are located on the long arms of chromosomes 1A, 1B and 1D at the *Glu-A1, Glu-B1* and *Glu-D1* loci, respectively (Payne 1987). The LMW subunits are encoded by genes on the short arm of group-1 chromosomes at the *Glu-A3, Glu-B3* and *Glu-D3* loci (Singh and Shepherd 1988). *Glu-3* loci are closely linked to the *Gli-1* loci (Singh and Shepherd 1988; Pogna et al. 1990).

HMW and LMW glutenins are the major determinants of the bread-making quality of common wheat (Payne et al. 1981, 1987; Gupta et al. 1989; Gupta and McRitchie 1994; Luo et al. 2001) and pasta-making quality of durum wheat (Pogna et al. 1990). Gliadin alleles at the *Gli-1* loci are useful genetic markers for dough quality because of their close linkage with *Glu-3* alleles (Redaelli et al.

1997). The selection of the most favourable *Glu-1* and *Glu-3* alleles is therefore a high priority in all wheat breeding programmes.

Sodium dodecyl sulfate (SDS)-PAGE and A-PAGE are currently the most widely used methods for determining the glutenin and gliadin composition of wheat (Bushuk and Zillman 1978; Payne et al. 1987; Gupta et al. 1989). Fractionation techniques with progressively greater discriminating power have also been applied to wheat protein analysis, such as two-dimensional electrophoresis (Jackson et al. 1983; Skylas et al. 2000) and reversed phase-high performance liquid chromatography (RP-HPLC) (Burnouf and Bietz 1984; Marchylo et al. 1989; Sutton 1991; Margiotta et al. 1993). Most techniques still require the selective extraction of proteins prior to separation, and there is the problem of overlapping fractions (Weegels et al. 1996).

Based on the gene sequence polymorphisms in wheat storage protein allelic loci, the polymerase chain reaction (PCR) has been applied to specifically detect the 1Dx5 HMW glutenin subunit (D'Ovidio and Anderson 1994), 1Dy12 (Smith et al. 1994) and 1Ax2*, 1Ax1 or 1Ax Null (De Bustos et al. 2000). A co-dominant marker for distinguishing 1Bx7 and 1Bx17 has also been developed (Ahmad 2000). A γ-gliadin PCR marker (D'Ovidio et al. 1990) and a LMW-GS PCR marker (D'Ovidio 1993) have been used to distinguish durum wheat cultivars according to their quality characteristics. Two microsatellite markers based on sequences linked to one LMW glutenin subunit and one γ-gliadin gene sequence have been developed as markers for use in mapping hexaploid wheat (Devos et al. 1995). A γ-gliadin PCR marker has been applied to distinguish closely related species; for example, spelt and hexaploid wheat (von Büren et al. 2000).

Single nucleotide polymorphisms (SNPs) and small insertions/deletions (InDels) are the most abundant form of DNA sequence variation in the genome of most organisms (Kwok et al. 1996; Kruglyak 1997). Sachidanandam et al. (2001) identified more than 1.4 million SNPs in the human genome. In wheat, some important genes, such as Rht1, Rht2 (linked to dwarfing, Peng et al. 1999) and puroindoline b (linked to grain texture, Giroux and Morris 1997), have been found to contain SNPs. Some allele-specific (AS) PCR markers have been applied to detect specific alleles at these loci based on the SNPs (Gale et al. 2001; Ellis et al. 2002). The rapid development of databases of wheat gene sequences and expressed sequence tags (ESTs) provides the opportunity to identify SNPs and InDels using existing sequence information. Here we describe the identification of SNPs in wheat γ-gliadin genes (*Gli-1*), which are closely linked to the LMW-GS genes (*Glu-3*), in order to obtain PCR-based markers for these bread wheat quality loci.

Materials and methods

Germplasm

Most of the wheat germplasm included in this study is maintained at CSIRO Plant Industry. Part of the collection is germplasm previously obtained from the Australian winter cereals collection in Tamworth, Australia. *Chinese Spring* euploid and its nullitetrasomic lines were kindly provided by Dr. E. Lagudah (CSIRO Plant Industry, Canberra) and derived from stocks described in Sears and Miller (1985).

Allelic composition at the Glu-3 loci by SDS-PAGE

Reduced and alkylated glutenin subunits were fractionated by one-step one-dimensional SDS-PAGE to determine the LMW-GS composition of the bread wheat studied. The monomeric proteins (gliadins, albumins and globulins) were first extracted with dimethyl sulphoxide (DMSO) and then with 70% ethanol. Polymeric proteins were solubilised from the remaining pellet by a further extraction with 70% ethanol and 5% β-mercaptoethanol and were alkylated with 10% 4-vinylpyridine as described previously by Gupta and MacRitchie (1991). Gels were run at constant voltage (200 V) for 4 h and then stained with Coomassie Brilliant Blue G-250 overnight following the method of Neuhoff et al. (1988). The gels were de-stained with distilled water overnight and then stored in 20% (w/v) ammonium sulphate solution at 4 °C. Nomenclature of allelic composition of the LMW glutenin subunits (*Glu-3* loci) was as described by Gupta and Shepherd (1990).

DNA isolation and PCR amplification

DNA was isolated from 3- to 5-day-old seedlings with a modified CTAB procedure (Gale et al. 2001). Amplification of subgroup-specific and allele specific DNA fragments was performed using Hotstar Taq polymerase (1 u; Qiagen, Valencia, Calif.) in 10 μl of reaction buffer (Qiagen, 1.5 m*M* $MgCl_2$) containing 20 ng genomic DNA, 100 μM of each dNTP and 5 pmol of each PCR primer. PCR was 95 °C for 3 min followed by 38 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min.

Chromosome assignment of subgroup-specific PCR markers

Assignment of subgroup-specific PCR markers to specific chromosomes was accomplished through PCR amplification using template DNA from each of the three *Chinese Spring* group 1 nullitetrasomic lines.

Sequencing of amplification products

Individual amplification products (2 μl) were treated with 0.25 u shrimp alkaline phosphatase and 2.5 u exonuclease I at 37 °C for 15 min in PCR reaction buffer (Qiagen, 1.5 m*M* $MgCl_2$). The PCR products were then sequenced using the appropriate PCR primers (3.2 pmol per reaction) using BigDye terminator mix (Applied Biosystems Foster City, Calif.) as per the manufacturer's instructions. The cycle sequencing reactions were performed on a FTS-1 Thermal Sequencer (Corbett Research) using 25 cycles of (96 °C for 30 s, 55 °C for 15 s, 60 °C for 4 min.) and five cycles of 96 °C for 30 s, 60 °C for 4 min. The extension products were precipitated by the addition of a 1/10 vol of sodium acetate (pH 5.2) and 2 vol. of ethanol and sequenced on an ABI PRISM 377XL Genetic Analyzer (Applied Biosystems) according to manufacturer's instructions.

Results

An alignment of γ-gliadin gene sequences

In order to obtain γ-gliadin gene sequences from GenBank, the coding region of a γ-gliadin gene, M13713 (Rafalski 1986) was chosen to perform a Blast search against the GenBank non-redundant DNA database. Twenty-eight γ-gliadin genes, gene fragments or pseudogenes were identified (Table 1). The coding regions of the genes, partial genes or pseudogenes were used for nucleotide alignment through the PILEUP programme (GCG software package, WebANGIS). Multiple polymorphisms were identified between these γ-gliadin genes or gene fragments, permitting the sequences to be divided into nine subgroups (Fig. 1).

Chromosomal location of genes

From Table 1 it was evident that chromosomal locations were not available for all nine sequence groups, although subgroups 2 and 6 had been localised previously (Devos et al. 1995; von Büren et al. 2000). No sequence was identified as being from the Gli-A1 locus. Therefore, several PCR primer sets specific for those undefined subgroups were designed (Table 2). *Chinese Spring* euploid and its group-1 nulli-tetrasomic lines were used as templates for amplification. The chromosomal locations of five of the seven unassigned subgroups were thereby identified (Table 1).

SNP identification and confirmation

Only one sequence (AF234646 from GenBank) was located on chromosome 1A. Therefore, the coding region of AF234646 was used to search the US Wheat Genome Project wheat EST database (Http://wheat.pw.usda.gov/wEST). Six EST fragments from about 58,000 sequences searched were found with very high sequence identity to AF234646. Seven single base-pair mismatches, one single base-pair insertion and one single base-pair deletion were detected in an alignment of 286 base pairs. However, no polymorphism occurred at the same position more than once. In order to confirm these polymorphisms, genomic

Table 1 Wheat γ-gliadin gene sequences and chromosomal locations

Sub-group	GenBank accession	Cultivar	Reference	ORF length	Chromosomal location
1	AF234646	Cheyenne	Anderson et al. 2001	858	1A
2	M13712	Yamhill	Rafalski 1986	987	1B[b]
	AJ389689	Chinese Spring	von Büren 2001	955	1B[c]
	AJ389690	Forno	von Büren 2001	907	1B[c]
	AJ389691	Galaxie	von Büren 2001	713	1B[c]
	AJ389692	Kanzer	von Büren 2001	712	1B[c]
	AJ389693	Ketepwa	von Büren 2001	949	1B[c]
	AJ389694	Neepawa	von Büren 2001	940	1B[c]
	AF234648	Cheyenne	Anderson et al. 2001	969	1B
3	M36999	Yamhill	Scheets and Hedgcoth 1988	909	1B
	AF234645	Cheyenne	Anderson et al. 2001	621	1B
	AF234647	Cheyenne	Anderson et al. 2001	909	1B
	AF234651	Cheyenne	Anderson et al. 2001	624	1B
4	M13713	Yamhill	Rafalski 1986	952	1B
5	D78183	1CW	Maruyama et al. 1998	840	1D
	AJ133613	–[a]	Arentz-Hansen et al. 2000	834	1D
	AF234649	Cheyenne	Anderson et al. 2001	897	1D
6	M16060	Newton	Scheets et al. 1985	753	1D[d]
	AF144104	Forno	von Büren et al. 2000	897	1D[d]
	AJ389666	CS	von Büren et al. 2000	757	1D[d]
	AJ389667	Ketepwa	von Büren et al. 2000	750	1D[d]
	AJ389668	Neepawa	von Büren et al. 2000	777	1D[d]
7	M16064	Yamhill	Sugiyama et al. 1986	894	1D
	AF175312	Yamhill	Arentz-Hansen et al. 2000	927	1D
	AF234650	Cheyenne	Anderson et al. 2001	984	1D
8	AF234643	Cheyenne	Anderson et al. 2001	771	
9	AF234642	Cheyenne	Anderson et al. 2001	725	
	AF234644	Cheyenne	Anderson et al. 2001	1014	

ORF open reading frame)
[a] Variety not mentioned
[b] Chromosome location known (Devos et al. 1995)
[c] Chromosome locations known (von Büren 2001)
[d] Chromosome locations known (von Büren et al. 2000)

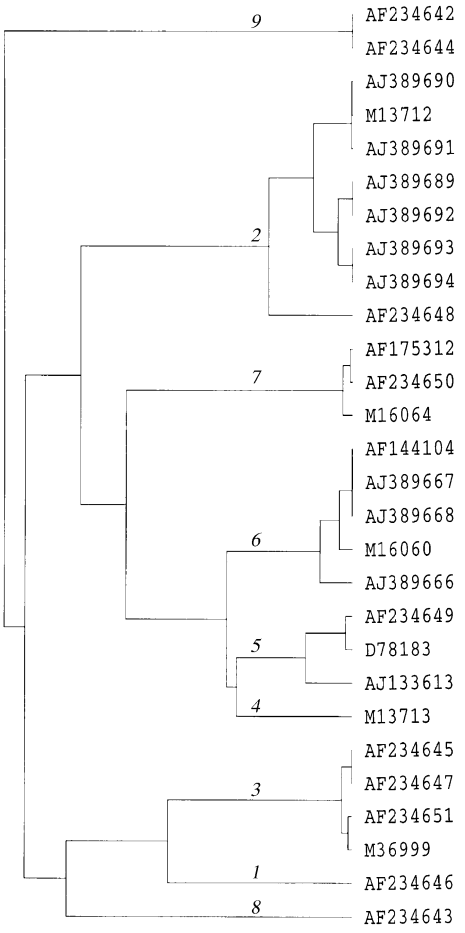

Fig. 1 Dendrogram of clustering γ-gliadin DNA sequences. The open reading frame of γ-gliadin genes or corresponding parts for partial gene sequences and pseudogenes were used together with the PILEUP program (GCG package on WebANGIS). *Numbers* indicate the corresponding subgroup in Table 1

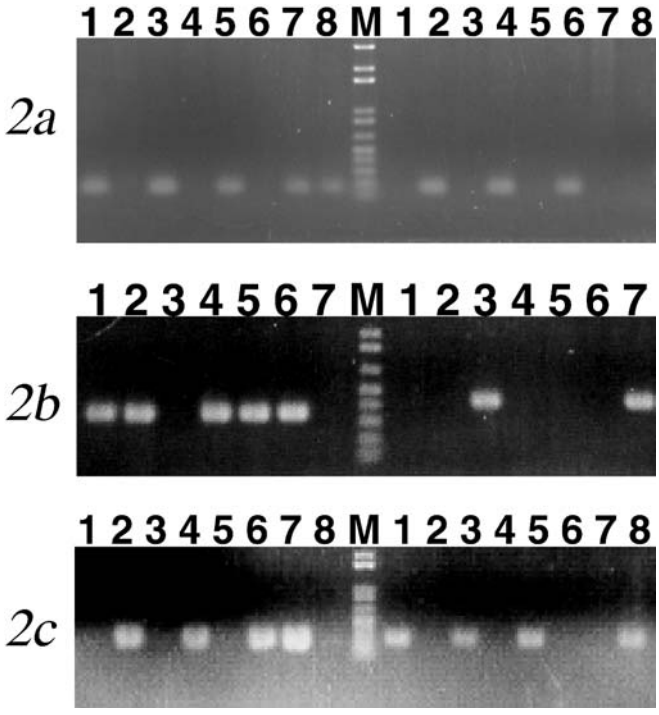

Fig. 2a–c Allele-specific (AS)-PCR markers based on gamma gliadin SNPs. **a** Products of AS-PCR using the markers GliA1.1 (*left side* of gel) and GliA1.2 (*right side* of gel). Wheat varieties used as PCR template were: *1 Sunstar*; *2 Sunelg*; *3 Chinese Spring*; *4 Darter*; *5 Osprey*; *6 Lark*; *7 Triller*; *8 Gordon* **b** Products of AS-PCR using the markers GliB1.1 (*left side* of gel) and GliB1.2 (*right side* of gel). Wheat varieties used as PCR template were: *1 Sunvale*; *2 Sunbri*; *3 Hartog*; *4 Rosella*; *5 Sunmist*; *6 Sunlin*; *7 Suneca* **c** Products of AS-PCR using the markers GliD1.1 (*left side* of gel) and GliD1.2 (*right side* of gel). Wheat varieties used as PCR template were: *1 Banks*; *2 Tailorbird*; *3 Gordon*; *4 Diamondbird*; *5 Tatiara*; *6 Lark*; *7 Warbler*; *8 Tern*. *M* 1-kb ladder marker (BRL)

DNA of *Chinese Spring* and five selected Australian wheat varieties (*Sunelg, Batavia, Sunstate, Banks* and *Darter*) were used as templates for amplification with the primer set GligF1/GligR1 (Table 2). PCR fragments were sequenced directly. The sequences were compared with AF234646 and the six wheat EST sequences. None of the original putative polymorphisms were confirmed. However, a new candidate SNP (a C to T substitution) was identified in two of the Australian varieties (*Sunelg* and *Darter*). The nucleotide change also resulted in a valine to isoleucine substitution in the predicted open reading frame. Based on this candidate SNP, two sets of primers, GligAF1/GligAR1 and GligAF2/GligAR1, were designed (Table 2). Primers were designed with the polymorphic nucleotide at the 3′ end of the forward primer plus an additional mismatch at the position 3 nucleotides from the 3′ end following the strategy of Kwok et al. (1990). PCR results indicated that the two primer sets could be used as dominant markers for detecting the SNP alleles (Fig. 2a). Varieties *Sunstar, Chinese Spring, Osprey, Triller* and *Gordon* were positive with the GligAF1/AR1 primer pair, and vars. *Sunelg, Darter* and *Lark* were positive with the GligAF2/AR1 primer combination. When 53 wheat varieties were tested, all were positive with either one or other of the two primer pairs (Fig. 2a, Table 3).

There are three subgroups of DNA sequences located on wheat chromosome 1B (Table 1). Other researchers have investigated one of these, subgroup 2. From the published sequences, a candidate SNP (a C to A substitution) in vars. *Soisson*, *Timgalen* (Devos et al. 1995) and *Cheyenne* (Anderson et al. 2001) could be confirmed. Since the sequence is a pseudogene, no protein was affected. Two primer sets (GligBF1/BR1 and GligBF2/BR1) were designed based on the candidate SNP (Table 2). The same design rules for the PCR primers were applied as for the GliA primers above. The results of using these primers showed that of the 53 varieties tested, 50 were positive with one or other of the two primer pairs, with the exception of three varieties (*Grebe*, *Triller* and *Warbler*) containing the 1B/1R wheat/rye translocation, which were negative for both markers (Fig. 2b, Table 3).

Three gamma gliadin subgroups were identified as being located on wheat chromosome 1D (Table 1). One of these, subgroup 5, was randomly selected for SNP

Table 2 Wheat γ-gliadin gene locus or allele specific PCR primers

Marker name	Primers	Primer sequence[a] (5′–3′)	Product length (bp)	Subgroup or sequence specific
Gli1.1	GligF1	GTCATTGGTGTCATCCCACG	286	Subgroup 1
	GligR1	GCACATGTTTGGAAGGGATC		
Gli1.2	GligF2	GAAGTGATTGCCAGGTGATG	277	Subgroup 3
	GligR2	GTGGAGCAGTCAGGTCGC		
Gli1.3	GligF3	AAGCGATTGCCAAGTGATGCG	347	Subgroup 4
	GligR3	CAATGCTGGCGACTATGGTA		
Gli1.4	GligF3	See above	287	Subgroup 5
	GligR4	ACACGTTGCACATGGTAGC		
Gli1.5	GligF3	See above	331	Subgroup 7
	GligR5	GCTGGCAAATGGTGCCGTC		
GliA1.1	GligAF1	CATAGCGTCGTGCATTCCAAC**G**	168	AF234646
	GligAR1	As GligR1		
GliA1.2	GligAF2	CATAGCGTCGTGCATTCCAAC**A**	168	New[b]
	GligAR1	See above		
GliB1.1	GligBF1	TGATCTGGCCACAAAGGG**A**	369	AF234648
	GligBR1	CATTGGCCACCAATTCCTGT		
GliB1.2	GligBF2	TGATCTGGCCACAAAGGG**C**	397	M13712
	GligBR1	See above		
GliD1.1	GligDF1	As GligF3	264	AJ133613, AF234649
	GligDR1	GTTTGCAACACC**A**ATGACGTA		
GliD1.2	GligDF1	See above	270	D78183
	GligDR2	GCAAGAGTTTGCAACAGC**G**		

[a] Bold, allele-specific SNPs; underlined, additional mismatched nucleotide
[b] New SNP reported in this paper

discovery. Four single base-pair mismatches, two small insertions and one small deletion were found among the three sequences representing the coding region. Three additional wheat EST sequences that belong to this subgroup were retrieved from the US Wheat Genome Project wheat EST database. Seven putative SNPs were identified in these three EST sequences. In order to confirm these polymorphisms, primer set GligF3/GligR4 (Table 2) was used to produce a single amplicon from the same six selected wheat varieties as above. The sequencing results confirmed one SNP from this 286-bp fragment although the original sequence alignment contained eight putative SNPs. Two allele-specific primer sets, GligDF1/GligDR1 and GligDF1/GligDR2, were designed. PCR results (Fig. 2c, Table 3) indicated that of the 53 cultivars tested, all were positive with either one or the other of the two primer pairs.

Linkage between SNP markers and designated *Glu-3* loci

A total of 53 wheat varieties was used to investigate the correlation between the genotyping results using the SNP-based markers and the linked *Glu-3* LMW-GS loci. All varieties were tested using the SNP-based markers and the results compared with the results of SDS-PAGE. All nine wheat varieties detected as having the *Glu-A3 d* and three varieties with *Glu-A3 e* alleles were positive for the GliA1.2 marker, while all 22 varieties with *Glu-A3 b*, 17 varieties with *Glu-A3 c*, one variety with *Glu-A3 a* and one variety with *Glu-A3 b* or*c* alleles were negative (Table 3). All 14 of the wheat varieties with the *Glu-B3 h* allele, the one variety with the *Glu-B3 g* allele and the one variety with *Glu-B3 a* were positive for the GliB1.2 marker, whereas 30 of the 31 wheat varieties (except cv. *Harrier*) with *Glu-B3 b*, the one variety with *Glu-B3 c*, the one variety with *Glu-B3 d* and the one variety with the *Glu-B3 e* allele were positive for GliB1.1 (Table 3). The three varieties with the 1BL/1RS translocation were negative for both the GliB1.1 and GliB1.2 markers (Table 3). Of the 12 wheat varieties (except cv. *Lark*) with *Glu-D3 c*, 11 were positive for the GliD1.2 marker, and all 34 varieties with *Glu-D3 b*, four varieties with *Glu-D3 a* and three varieties with *Glu-D3 e* alleles were positive for the GliD1.1 marker (Table 3). These results indicated that the γ-gliadin SNP markers could be used for detecting linked LMW glutenin subunit alleles.

Discussion

Single nucleotide polymorphisms

There are between 15 and 40 γ-gliadin genes in hexaploid wheat (Sabelli and Shewry 1991). In this study, 28 γ-gliadin sequences were classified into nine subgroups according to sequence similarity. Three subgroups were selected and used for the identification of SNPs and subsequent development of AS-PCR markers. The results indicated that different subgroups represent different genes and that there is sufficient sequence variation between γ-gliadin genes from different cultivars for marker development.

Sequence variation was identified within each subgroup and by comparison with homologous sequences from the US Wheat Genome Project wheat EST database.

Table 3 Correlation between wheat γ-gliadin gene PCR markers[a] and LMW-glutenin subunit alleles

Wheat variety	GliA1.1	*Glu-A3*	GliA1.2	GliB1.1	*Glu-B3*	GliB1.2	GliD1.1	*Glu-D3*	GliD1.2
Banks	+	b	–	+	b	–	–	c	+
Barunga	+	c	–	+	b	–	–	c	+
Batavia	+	c	–	+	b	–	–	c	+
Calingiri	–	d	+	+	d	–	+	a	–
Chinese Spring	+	a	–	–	a	+	+	a	–
Condor	+	b	–	+	b	–	+	b	–
Cunningham	+	c	–	+	b	–	+	b	–
Darter	–	d	+	+	b	–	+	b	–
Diamondbird	+	b	–	–	h	+	+	b	–
Frame	+	c	–	–	h	+	–	c	+
Goldmark	+	c	–	–	h	+	+	b	–
Gordon	+	c	–	+	b	–	–	c	+
Grebe	+	c	–	–	Sec–1	–	+	b	–
Halberd	–	e	+	+	c	–	–	c	+
Harrier	–	d	+	–	b	+	+	b	–
Hartog	+	b	–	–	h	+	+	e	–
Houtman	+	b	–	–	h	+	+	e	–
Janz	+	b	–	+	b	–	+	b	–
Kelalac	+	c	–	+	b	–	–	c	+
Kite	–	e	+	+	b	–	+	b	–
Lark	–	d	+	–	h	+	+	c	–
Miskle	+	b,c	–	+	b	–	+	b	–
Molineux	+	c	–	+	b	–	–	c	+
Nyabing	–	d	+	–	h	+	+	b	–
Osprey	+	c	–	+	b	–	+	b	–
Pelsart	+	b	–	+	b	–	+	b	–
Perenjori	–	d	+	+	b	–	+	a	–
Rosella	+	b	–	+	b	–	+	b	–
Silverstar	+	c	–	–	h	+	+	b	–
Sunbird	+	c	–	+	b	–	+	b	–
Sunbri	+	b	–	+	b	–	+	b	–
Sunbrook	+	b	–	–	h	+	+	b	–
Sunco	+	b	–	+	b	–	+	b	–
Suneca	–	d	+	–	h	+	+	e	–
Sunelg	–	e	+	+	b	–	+	b	–
Sunkota	+	b	–	+	b	–	+	b	–
Sunland	+	b	–	+	b	–	+	b	–
Sunlin	–	d	+	+	b	–	+	b	–
Sunmist	+	b	–	+	b	–	+	b	–
Sunstar	+	c	–	+	b	–	+	b	–
Sunstate	+	b	–	–	h	+	+	b	–
Sunvale	+	b	–	+	b	–	+	b	–
Swift	+	b	–	+	b	–	+	b	–
Tailorbird	+	b	–	–	h	+	+	b	–
Tasman	+	b	–	+	e	–	+	a	–
Tatiara	+	c	–	+	b	–	–	c	+
Tern	+	c	–	–	g	+	–	c	+
Triller	+	b	–	–	Sec–1	–	+	b	–
Vulcan	+	b	–	+	b	–	+	b	–
Warlber	+	b	–	–	Sec–1	–	+	b	–
Wilgoyne	–	d	+	–	h	+	+	b	–
Worrakatta	+	c	–	+	b	–	–	c	+
Wyuna	+	c	–	–	h	+	+	b	–

+, PCR product present; –, PCR product absent; Sec-1, cv. contains IB/IR wheat/rye translocation

Some of this variation will be directly attributable to sequencing errors or errors generated during cDNA synthesis or propagation in *Escherichia coli* (reverse transcriptase or DNA polymerase errors; Cooper and Krawczak 1995). In order to address this limitation of the data, a filter used by Picoult-Newberg et al. (1999) in human genome SNP mining was introduced into this investigation. The filter is that a sequence mismatch must occur in more than one sequence in a subgroup before it can be considered a high-quality candidate SNP. From the three randomly selected subgroups, a total of 2,885 bp of DNA sequence was investigated. However, only one high-quality candidate SNP was identified using this criterion. When two DNA fragments (286 bp and 287 bp) were re-sequenced, two more high-quality candidate SNPs were identified. The results of the current study indicated that the SNP rate in wheat γ-gliadin sequences is similar to that observed in the human genome

(approximately one SNP per kilobase, Cooper et al. 1985). Some of the putative polymorphisms observed in the database sequence information may be authentic, but could not be confirmed in the limited Australian germplasm analysed in this study.

SNPs-based primer design

The original AS-PCR technique utilises primers with specific mismatches at the 3′ end that allow preferential amplification of one allele relative to another (Ugozzoli and Wallance 1991). A significant problem with employing this method for SNP detection is that under normal PCR conditions different mismatches located at the 3′ end are extended with different efficiencies by *Taq* polymerase. Modified PCR procedures for assaying SNPs have been used to overcome this problem (Newton et al. 1989; Drenkard et al. 2000). The addition of an extra mismatch within the last four bases of the primer produces a significant reduction in the PCR product yield of the mismatched allele, but has a relatively minor effect on the amplification of the correct allele (Kwok et al. 1990). For the wheat γ-gliadin markers described in the current study, an additional mismatch at the position three bases from the 3′ end of one primer was shown to facilitate AS-PCR product accumulation, even if the other primer of the pair was conserved between the two alleles. In some cases, it was shown that an additional mismatch at the 3′ end of both primers was still sufficient to produce AS-PCR product. However, one primer (GligDR1), with a SNP located at the ninth base, with the third base extra mismatch at the 3′ end, still produced allele-specific PCR product. The results indicated that the effects of PCR primers designed with extra mismatches are complex. A semi-empirical approach to primer development is therefore required.

Application of γ-gliadin gene SNP-based AS-PCR markers

The methods normally used for detecting allelic variation of LMW glutenins and gliadins are currently SDS-PAGE, A-PAGE or RP-HPLC. Based on the three SNPs observed between different genes in each subgroup of γ-gliadin sequences, six AS-PCR markers were developed and found to give either positive or negative PCR results for all of the hexaploid wheat varieties tested except three. The exceptions were the three wheat varieties containing the 1BL/1RS translocation that gave negative results for markers GliB1.1 and GliB1.2. These results are not unexpected in view of the fact that the 1BL/1RS translocation lines do not contain the short arm of chromosome 1B. Therefore, the two primer pairs for each di-allelic SNP could be used in parallel, effectively as a co-dominant marker to detect heterozygous as well as homozygous wheat lines in a breeding programme.

Marker GliB1.2, derived from the sequence of a γ-gliadin pseudogene on chromosome 1B, also showed size variation in the positive wheat varieties (Fig. 2b) due to the presence of a polymorphic microsatellite in the sequence, as reported by Devos et al. (1995). Therefore, this marker can be utilised as a dominant marker in some cases and as a co-dominant marker in other cases. The advantage of dominant PCR markers is the potential to use a simple, high-throughput assay system such as solid phase amplification (Zhang et al. 2001) that permits colorimetric scoring of results in microtitre plates.

LMW-GS effects on dough resistance and extensibility have been determined (Gupta et al. 1989). The effect on dough quality, measured as R_{max}, was demonstrated for some allelic variants of LMW-GS in bread wheats (Gupta and MacRitchie 1994). Redaelli et al. (1997) found that null alleles at the Gli-D1/Glu-D3 loci have a strong positive influence on dough tenacity and a significant negative influence on dough extensibility when compared to alleles Gli-D1/Glu-D3b. Luo et al. (2001) suggest the optimal Glu-3 combination is Glu-A3d, Glu-B3b and Glu-D3b. γ-gliadin AS-PCR markers developed in this paper were shown to be linked to specific LMW glutenin subunit alleles. It is possible to use these markers to distinguish between *Glu-A3 d* and *Glu-A3 a*, *b* and *c*; *Glu-B3 b* and *Glu-B3 a*, *g* and *h*; *Glu-D3 b* and *Glu-D3 c*.

The limitation of using linked markers rather than 'perfect' markers for the LMW-GS alleles directly is that recombination may occur, as was observed for vars. *Harrier* (between GluB3 b and marker GliB1.1) and Lark (between GluD3c and marker GliD1.2) in this study. Clearly, prior analysis of parent lines by both PCR and SDS-PAGE is required to establish the most appropriate DNA markers for implementation in a specific breeding programme. Gliadin sequences were used for marker development in the current study due to the availability of a suitable sequence data set. In the future, further markers useful for tracing individual LMW-GS alleles in breeding programmes may be developed using further gliadin sequence information or by targeting the LMW-GS genes directly.

We have demonstrated that PCR primers based on the SNPs identified from sequence information could reliably amplify simple DNA products for the detection of specific alleles within a complex multicopy gene family in hexaploid wheat. PCR markers based on SNPs could potentially be developed for a large number of genes linked to quality and agronomic traits and thus greatly enhance the efficiency of marker-assisted breeding and genetic mapping in wheat. In the future these markers may be scored using high-throughput, array-based methods to permit the parallel application of many markers in a single operation.

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