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Locus-specific primers for LMW glutenin genes on each of the group 1 chromosomes of hexaploid wheat

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Abstract To reveal the chromosomal location of three known low-molecular-weight (LMW) glutenin genes in wheat, we designed and used three sets of sequence-specific primers in polymerase chain reactions (PCR) on 'Chinese Spring' and its derived group 1 aneuploid nullisomic-tetrasomic stocks. Two sets proved to be chromosome specific and amplified sequences from the *Glu-A3* and *Glu-D3* loci, respectively. The third set was apparently composed of conserved sequences as it produced PCR products in each of the aneuploids. Two of these products were cloned, and their sequences differed from the known LMW glutenin genes at several positions. Again, primer sets specific for these sequences were designed. One set was directed to the *Glu-A3* locus, the second set resulted in two PCR products differing in length, one of which was located on chromosome 1B and the other on 1D. Primer sets constructed for the latter two sequences were specific for the *Glu-B3* and *Glu-D3* loci, respectively. Hence, primer sets specific for each of the three homoeologous chromosomes of the group 1 (1A, 1B, 1D) are available. In addition, these locus-specific primers were assayed for their ability to distinguish among wheat cultivars. PCR products amplified with one of the *Glu-A3*-specific primer sets showed length polymorphisms in various wheat varieties. Varieties carrying the 1RS.1BL translocated chromosomes could be recognized by the absence of a PCR product when the *Glu-B3* primer set was used. These results suggest that PCR with locus-specific primers can be useful in the molecular genetic analysis of hexaploid wheat.

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Introduction

Gluten, an intricate protein complex in the endosperm of common wheat (*Triticum aestivum* L.) consists of two major fractions: gliadins and glutenins. Gliadins are monomeric proteins, whereas glutenins are aggregates of high-molecular-weight (HMW) and low-molecular-weight (LMW) subunits held together by disulphide linkage (reviewed by Payne 1987). Due to variations in the electrophoretic patterns of LMW subunits of glutenin among varieties, Singh and Shepherd (1988) were able to map this multigene family at the *Glu-3* loci, closely linked to the *Gli-1* loci for ω- and γ-gliadins located on the short arms of the group 1 chromosomes. There have been a number of studies on the effects of LMW subunits on flour properties (Payne et al. 1987; Gupta et al. 1989, 1994). Payne et al. (1987) suggested that the positive effects on dough quality associated with the *Gli-1*/*Glu-3*alleles were due to the *Glu-3* alleles. Therefore, it would be worthwhile to investigate the individual *Glu-3* alleles in detail.

Over the past few years, more efforts have been focused on analysis at the molecular level. Some cDNA clones have been isolated that encode LMW glutenin components (Bartels and Thompson 1983; Okita et al. 1985). gDNA clones containing LMW glutenin genes were isolated by Pitts et al. (1988) and Colot et al. (1989). In the latter investigation the chromosomal location of the sequence was also determined. D'Ovidio et al. (1992) cloned a polymerase chain reaction (PCR) fragment corresponding to a LMW glutenin gene from *Triticum durum*. This direct cloning of genomic sequences amplified by PCR (Saiki et al. 1988) opens a new opportunity for the characterization of different allelic forms and the detailed study of genetic polymorphisms (D'Ovidio et al. 1991). DNA sequence analysis of these clones showed that the coding regions are uninter-

rupted by introns and possess a proline- and glutamine-rich domain encoded by a tandem array of irregular repeats followed by a unique sequence (C-domain) often interspersed with several stretches of glutamine codons (Colot et al. 1989).

In the study presented here we used sequence data from the previously published LMW glutenin sequences to construct sequence-specific primers. It was then determined whether these primers were chromosome-specific (and hence locus-specific) by using them in PCR on the group 1 nullisomic-tetrasomic lines of hexaploid wheat. When the primer set was not chromosome-specific, PCR products were cloned and sequenced. This revealed novel chromosome-specific LMW glutenin sequences that, in turn, made it possible to design new locus-specific primers. The obtained *Glu-A3*-, *Glu-B3*- and *Glu-D3*- specific PCR markers on each of the group 1 chromosomes were tested for their ability to detect length polymorphisms of the amplified sequences in various hexaploid wheat varieties as observed by agarose gel electrophoresis.

Materials and methods

Plant material

Aneuploid 'Chinese Spring' stocks were developed by Sears (1966). The bread wheat varieties 'Alba', 'Cappelle-Desprez', 'Disponent', 'Ergo', 'Neuzucht', 'Reliance', 'Salzmünder' and 'Sportsman' were kindly provided by G. De Wever (Veredelingsstation van Heverlee, Linter, Belgium); 'Cheyenne', 'Gabo' and 'Hope' were generously provided by Dr. R. Koebner (John Innes Centre for Plant Science Research, Norwich, England).

DNA isolation

Genomic DNA was prepared using the following procedure: 10 mg fresh weight of young leaf tissue was put in a 1.5-ml tube, frozen under liquid nitrogen and ground with a sealed disposable pipette tip that had been placed onto an automatic screwdriver. One milliliter of warm (55°C) extraction buffer (100 m*M* TRIS-HCl pH 9.5, 1.4 *M* NaCl, 20 m*M* EDTA, 2% CTAB, 0.2% thioglycerol, 2% PVP-40, 0.1% vitamin C) was added followed by 400 µl chloroform:isoamyl alcohol (24:1). This mix was incubated at 55°C for 10 min. After centrifugation (5 min. at 13000 rpm), the supernatant was treated with 100 µg RNase A for 15 min at 37°C. After the addition of 500 µl ice-cold isopropanol, DNA was precipitated on ice for 10 min and then centrifuged (10 min at 13000 rpm). The pellet was washed with 1 ml cold ethanol (70%) and dissolved in 100 µl TE-buffer (10 m*M* TRIS-HCl, 1 m*M* EDTA, pH 8) and stored at –20°C until use.

Polymerase chain reactions and primers

DNA sequences were compared by using the multiple alignment programme of the Genmon (version 4.3) software package (GBF, Braunschweig, Germany). Oligonucleotides were synthesized with an Applied Biosystems (Foster City, Calif.) 381 A DNA synthesizer. PCR reactions contained 10 m*M* TRIS-HCl pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 200 µ*M* of each dNTP, 1 µ*M* of each primer, 2 units *AmpliTaq* DNA polymerase (Perkin Elmer Cetus, Norwall, Conn.) and 50 ng genomic DNA in a total volume of 100 µl. The reaction was overlaid with mineral oil and subjected to 1 cycle of 1 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 59°C, 90 s at 72°C on a TRIO-thermoblock (Biometra, Göttingen, Germany). Aliquots of the reaction mixture (10 µl), was electrophoretically analysed on 1.5% agarose gels (Biozym, Landgraaf, The Netherlands) and, in one case (primer set O11BD), on a 2% MetaPhor agarose gel (FMC, Rockland, Me.). Following ethidium bromide staining, DNA was visualized under UV light. The gels in Figs. 3–7 are presented as negative images.

Cloning and sequencing PCR products

The final extension step from the amplification protocol was omitted to leave blunt-end PCR products (Haqqi 1992). The PCR products were selectively precipitated by adding 0.6 volumes of polyethylene glycol mix (20% PEG 6000, 2.5 m*M* NaCl). After a 30-min precipitation on ice, the samples were spun at 13000 rpm for 30 min, and the supernatant was carefully removed. Pellets were washed twice with 70% ethanol and dissolved in water. Ligation was carried out as modified from Liu and Schwarz (1992) as follows: a reaction volume of 20 µl contained 200 ng PCR product, 80 ng of *Sma*I-cut pUC19 vector, 2 units of T4 DNA ligase (Promega, Madison, Wis.), 3.3 m*M* TRIS acetate (pH 7.9), 1 m*M* Mg-acetate, 6.6 m*M* K-acetate, 0.05 m*M* dithiothreitol, 0.04 m*M* rATP and 5 units *Sma*I restriction endonuclease (Promega). This ligation mixture was incubated at 22°C for 5 h. Half of it was used to transform *E. coli* strain WK6 (Zell and Fritz 1987). DNA sequencing of the cloned fragments was done on Qiagen tip-20-(Qiagen, Hilden, Germany) purified plasmids using cycle sequencing with dye terminators (Applied Biosystems). The same protocol was used to sequence purified locus-specific PCR products directly. Sequence analysis was done on a 373 A DNA sequencer (Applied Biosystems).

Results

PCR with LMW glutenin sequence-specific primers

After comparison of the published LMW glutenin gene sequences of hexaploid wheat, three sets (P1, O1 and C1) of sequence-specific primers were constructed (Table 1). The rationale in designing the primers was based on the premise that the 3′-terminal positions ought to be unique among the known *Glu-3* sequences and preferably among some of the preceding nucleotides as well (Fig. 1A and B). They were designed within the coding region such that their PCR products should contain the whole repeat domain and a large part of the C-domain (Fig. 2). These primer sets resulted in clear amplification products in 'Chinese Spring' and were used in aneuploid analysis (Fig. 3). The specific primer set for the sequence published by Pitts et al. (1988) yielded a PCR product only in those lines where the 1A chromosome pair is present (Fig. 3, P1: lanes c–g), and hence these primers must be considered to be specific for chromosome 1A and in particular for the *Glu-A3* locus. The predicted length of the reaction product for the set O1 was 695 bp (based on B11-33, a cDNA clone from 'Cheyenne', published by Okita et al. 1985). A band of approximately this size was the most prominent band and was present in all of the nullisomic-tetrasomic lines of the group 1 chromosomes (Fig. 3, O1). Hence, these primers apparently represent conserved sequences and give products with at least two of the homoeologous group 1 chromo-

Table 1 Primer sequences designed during this study, target sequences from which they were derived and chromosomal locations determined. Nucleotide sequences are deposited under EMBL, Genbank and DDBJ accession numbers X84887, X84959, X84960 and X84961

Primer set	Primer sequences	Target sequence	Chromosomal location (locus)
P1	P15: GCGCAAATTTCACAGCAACA P13: TGGCACACTAGTGGTGGTTTC	Pitts et al. 1988	1A (*Glu-A3*)
O1	O15: TTTGCCCTCATCGCCGTTGT O13: TGCTGTTGAGGTTGTTGGAAAGA	Okita et al. 1985	Group 1 (*Glu-3*)
C1	C15: CAGATGGAGACTAGATGCATCC C13: ACTGCACATCGTTGGCAGGA	Colot et al. 1989	1D (*Glu-D3*)[a]
O11A	O11A5: CGCCGTTGTGGCGACAAGTA O11A3: GTTCTTGTAGGATGATGGAGTAGG	Set O1 PCR product N1DT1A	1A (*Glu-A3*)
O11BD	O11BD5: CCACATCCCTAGCTTGGAGAA O13: TGCTGTTGAGGTTGTTGGAAAGA	Set O1 PCR product N1AT1B	1B (*Glu-B3*) + 1D (*Glu-D3*)
O11B	O11B5: GGTACCAACAACAACAACCC O11B3: GTTGCTGCTGAGGTTGGTTC	Set O11BD PCR product N1DT1B	1B (*Glu-B3*)
O11D	O11BD5: CCACATCCCTAGCTTGGAGAA O11D3: ATGGTATTTGTTGTTGCGGA	Set O11BD PCR product N1BT1D	1D (*Glu-D3*)

[a] In the case of primer set C1 the chromosomal location of the target gene was also determined by Colot et al. (1989)

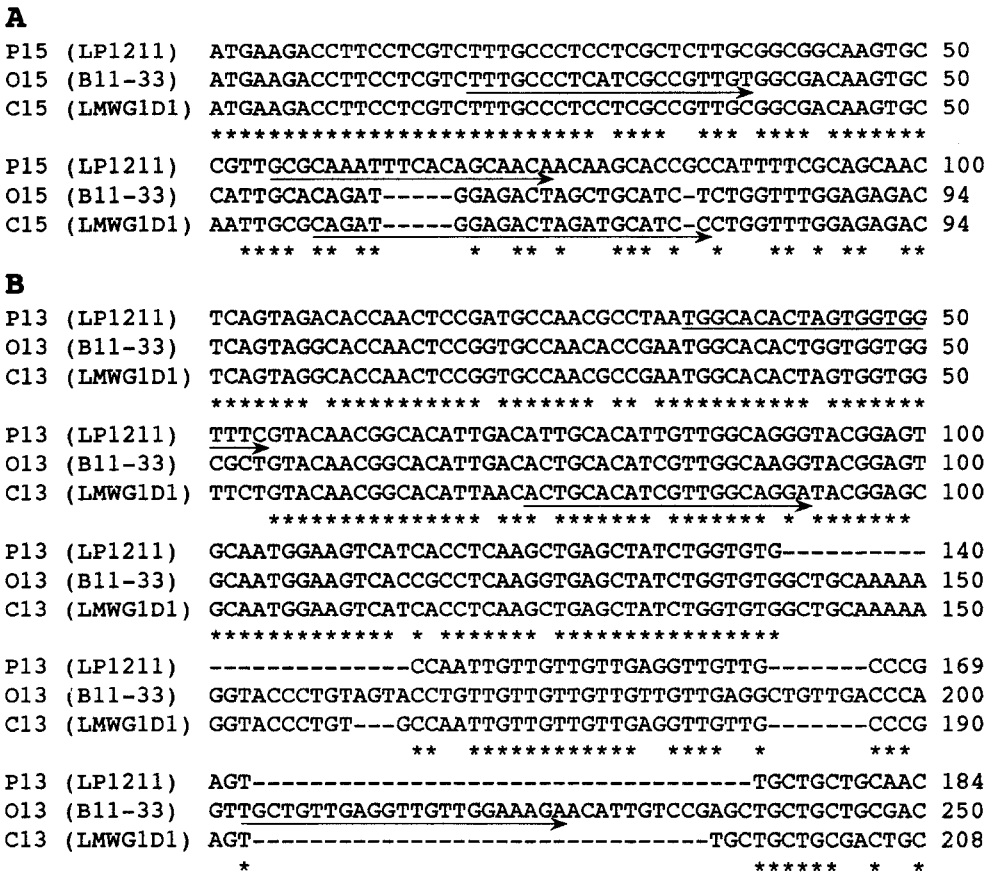

Fig. 1 A, B Designed sequence-specific primer sets P1 (P15+P13), O1 (O15+O13) and C1 (C15+C13) – see also Fig. 2. Parts of three known LMW glutenin sequences, LP1211 (Pitts et al. 1988), B11-33 (Okita et al. 1985) and LMWG1D1 (Colot et al. 1989), are aligned. Identical sequences are indicated by *asterisks*, *dashes* represent deletions. **A** 5′ region of the LMW glutenin genes: primers P15 (LP1211), O15 (B11-33) and C15 (LMWG1D1) are *underlined*. *Numbering* is from the initiation codon. **B** 3′ region of the LMW glutenin genes: primers P13 (LP1211), O13 (B11-33) and C13 (LMWG1D1) are *underlined*. The non-coding strand is presented (5′≤3′), numbering is from the sequence complementary to the stop triplet

somes. In addition, a much weaker 1B-specific band about 100 bp longer appeared in the N1AT1B, N1DT1B and also, but hardly visible, in the N1AT1D, N1DT1A and disomic 'Chinese Spring' lines (Fig. 3, O1: lanes b, e and a, f, g). Set C1 showed amplification of a chromosome 1D-specific region since the amplification product is missing in the two nullisomic 1D lines (Fig. 3, C1: lane e and f). This result is in agreement with that of Colot et al. (1989) who mapped this sequence on chromosome 1D by Southern blot analysis in conjunction with aneuploid analysis.

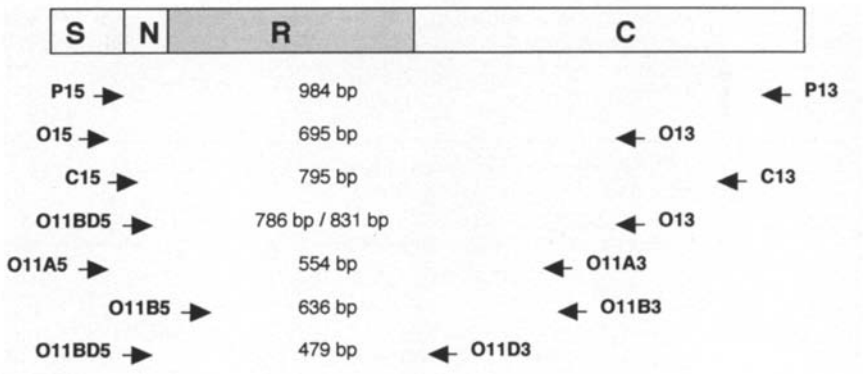

Fig. 2 Diagram of the coding region of LMW glutenin genes (*S* signal peptide, *N* NH_2-domain, *R* variable repeat domain, *C* C-domain). The positions of the primers used for PCR analysis are indicated by *arrows* (see Table 1 for primer sequences). For every primer set, the expected (sets P1, O1 and C1) or observed length (sets O11BD, O11A, O11B and O11D) of the amplification products is shown

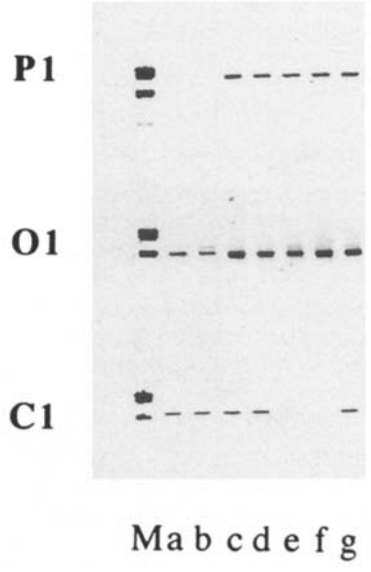

Fig. 3 Agarose gel (1.5%) of PCR amplification products using primer sets P1 (at the *top*), O1 (*middle*) and C1 (at the *bottom*). *Track M* marker with fragments of 1408 bp+1195 bp, 739 bp+692 bp and 283 bp; 'Chinese Spring' nullisomic-tetrasomic lines: *a* N1AT1D, *b* N1AT1B, *c* N1BT1A, *d* N1BT1D, *e* N1DT1B, *f* N1DT1A, *g* 'Chinese Spring'

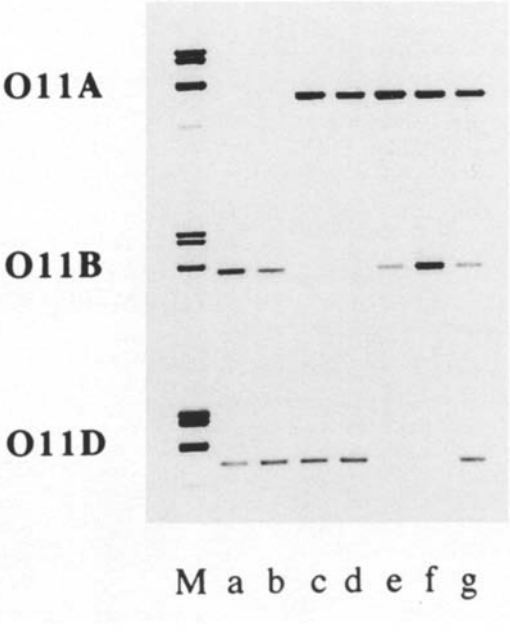

Fig. 4 Amplification using *Glu-A3*-, *Glu-B3*- and *Glu-D3*-specific primer sets O11A, O11B and O11D on nullisomic-tetrasomic lines. *Lane M* marker with fragments of 1408 bp, 1195 bp, 739 bp + 692 bp and 283 bp; 'Chinese Spring' nullisomic-tetrasomic lines: *a* N1AT1B, *b* N1AT1D, *c* N1BT1A, *d* N1BT1D, *e* N1DT1A, *f* N1DT1B, *g* 'Chinese Spring'

Construction of new *Glu-3*-specific primer sets

The PCR products amplified by the conserved primer set O1 in N1AT1B and N1DT1A were precipitated and blunt-end cloned into the *Sma*I site of pUC19. With the N1DT1A product, two clones were selected that contained an insert of 689 bp with an identical sequence, but differing from any published *Glu-3* sequence. A specific set of primers (Table 1: set O11A) was designed, and aneuploid analysis showed (Fig. 4, O11A) that they amplified a chromosome 1A-specific (and hence *Glu-A3* specific) fragment. The cloning of the N1AT1B amplification product resulted in a single clone that contained 341 bp of the fragment. Apparently the cloned fragment had undergone rearrangements and deletions since, for example, the primer binding site for O13 was missing. Nevertheless, the sequence downstream of primer O15 was similar to, but still different from, B11-33. This led to the construction of primer O11BD5 (Table 1) lying in front of the repeat domain (Fig. 2). This primer together with primer O13 produced two products of different lengths. The aneuploid analysis (Fig. 5) showed that the smaller band of 786 bp was 1B-specific and that the 45-bp larger fragment was 1D-specific. After cloning and sequencing the 1B-specific PCR product, we were able to design a set of primers specific for this sequence on chromosome 1B (Table 1: set O11B). Primer O11B5 is situated at the beginning of the repetitive domain (Fig. 2). The *Glu-B3* specificity was confirmed

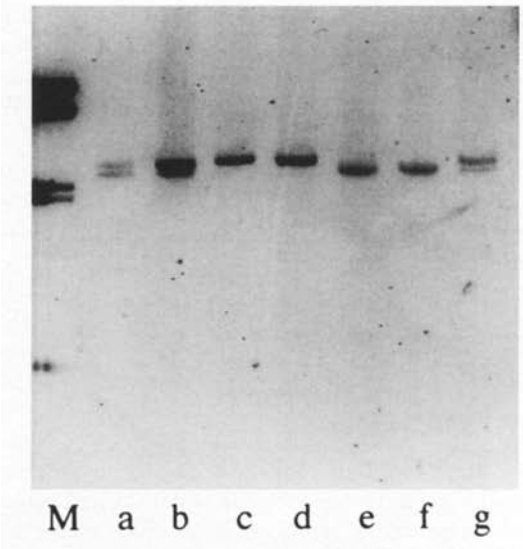

Fig. 5 Amplification of nullisomic-tetrasomic DNA using primer O11BD5 coupled with primer O13. *Lane M* marker with fragments of 1408 bp, 1195 bp, 739 bp, 692 bp and 283 bp; *a* N1AT1B, *b* N1AT1D, *c* N1BT1A, *d* N1BT1D, *e* N1DT1A, *f* N1DT1B, *g* 'Chinese Spring'. Results are shown on a 2% MetaPhor agarose gel

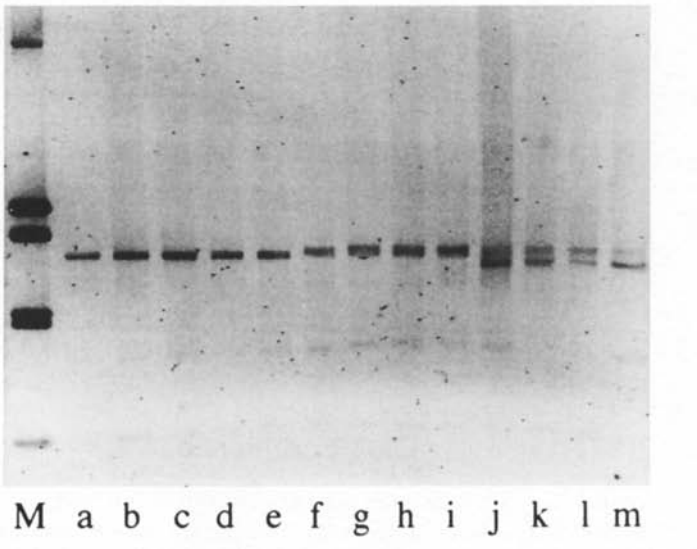

Fig. 6 Allelic variation among hexaploid wheat varieties detected with primer set P1, directed to *Glu-A3*. *Lane M* marker with fragments of 1408 bp, 1195 bp, 739 bp, 692 bp and 283 bp; *lanes a-p* wheat varieties: *a* 'Chinese Spring', *b* 'Alba', *c* 'Ergo', *d* 'Mezöhegyesi', *e* 'Cheyenne', *f* 'Gabo', *g* 'Hope', *h* 'Canthatch', *i* 'Reliance', *j* 'Cappelle-Desprez', *k* 'Disponent', *l* 'Neuzucht', *m* 'Sportsman'

by aneuploid analysis (Fig. 4, O11B). The 1D-specific product from N1BT1D (Fig. 5) was sequenced directly. The specific primer O11D3 (Table 1), used together with primer O11BD5, was designed to amplify the repetitive domain only (Fig. 2). This primer combination gave an amplification product that could be shown to be specific for *Glu-D3* (Fig. 4, O11D). In that way locus specific amplification was shown to be possible even if only one of the two primers was designed to be locus specific.

Amplification and variation among hexaploid wheat cultivars

The five locus-specific primer sets (Table 1) were tested for their ability to generate polymorphic bands among 19 diverse hexaploid wheat cultivars. Primer set P1, appeared to be directed towards a polymorphic *Glu-A3* locus as the amplified DNA fragments identified insertion/deletion polymorphisms on agarose gel electrophoresis (Fig. 6). Primer set P1 is based on a gDNA clone from the variety 'Yamhill' (Pitts et al. 1988), and PCR should give a 984-bp product (Fig. 2). We show differentiation among 13 cultivars in Fig. 6. Three amplification patterns of alleles can be distinguished: (1) some varieties give indeed one product (lanes a–e); (2) 4 others show a larger, somewhat diffuse band, which therefore may consist of doublets of bands (lanes f–i); (3) the last 4 lanes show varieties with two distinct amplification products. The PCR products amplified by the other four primer sets did not show size polymorphisms. In the case of set O11B, however, polymorphism was observed as the presence or absence of the band

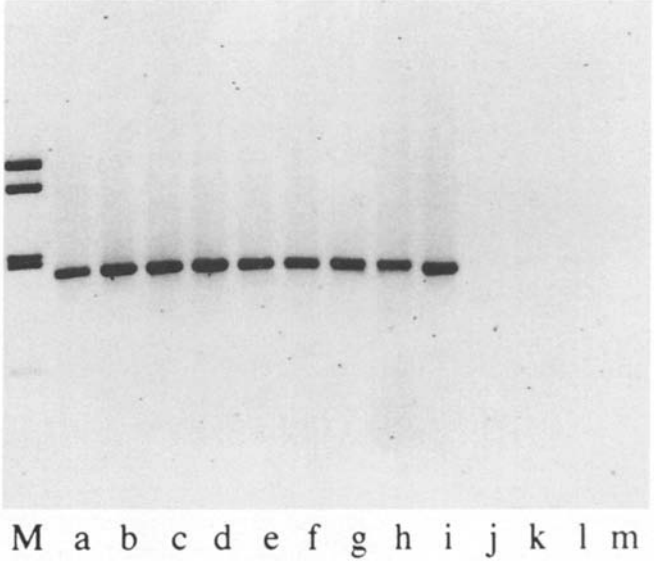

Fig. 7 PCR on different wheat varieties using primer set O11B, specific for *Glu-B3*. The amplification product is absent in those varieties carrying the 1RS.1BL translocation (*lanes j, k, l, m*). *Lane M* marker with fragments of 1408 bp, 1195 bp, 739 bp, 692 bp and 283 bp; *lanes a–m* wheat varieties: *a* 'Chinese Spring', *b* 'Alba', *c* 'Canthatch', *d* 'Cappelle-Desprez', *e* 'Ergo', *f* 'Gabo', *g* 'Mezöhegyesi', *h* 'Reliance', *i* 'Sportsman', *j* 'Disponent', *k* 'Neuzucht', *l* 'Salzmünder', *m* 'Kavkaz'

(Fig. 7). The varieties where no amplification product was produced contained the 1RS.1BL translocation and consequently should not bear the *Glu-B3* locus. This again proves the reliability of this locus-specific primer set.

Discussion

We wished to test whether it is possible to design locus-specific (and hence chromosome-specific) primer sets that can be used in a PCR for genome analysis of hexaploid wheat. The LMW glutenin genes were chosen as a model system because both sequence information is available and these genes show allelic length polymorphisms at the protein level (Payne et al. 1987; Singh and Shepherd 1988; Gupta et al. 1994). Primers were designed that flank the area that was likely to be of variable length, a methodology analogous to that of D'Ovidio et al. (1990) and Weining and Langridge (1991) who derived primer sequences from γ-gliadin and α-amylase genes, respectively, to detect size polymorphisms in cereals. D'Ovidio (1993) reported the identification of two amplification products from LMW glutenin sequences in durum wheat, one of which showed length polymorphism and was localized on chromosome 1B. In addition, D'Ovidio et al. (1994) used locus-specific primers to detect allelic variants of high-molecular-weight glutenin subunits at the *Glu-D1* locus. They showed that length polymorphisms were due to a variation in the length of the repetitive domain of these proteins.

In our study, PCR with sequence-specific primers on aneuploid wheat stocks provided a simple and fast method for the determination of the chromosomal location of known sequences. Similarly to allele-specific PCR, the primers were designed such that at least the 3′ ultimate base of the primer is placed at a site of DNA sequence difference in the target sequence of the genome (Wu et al. 1989). In this way, the *Glu-3* sequences published by Pitts et al. (1988) and Colot et al. (1989) could be mapped on chromosomes 1A and 1D, respectively. The chromosomal location of the B11-33 sequence, published by Okita et al. (1985), could not be determined because the sequence-specific primer set O1 produced PCR products in each of the group 1 aneuploids. This shows that sequence-specific primers based on known sequences are not always locus-specific. This is especially true for complex multigene families as the LMW glutenins. Hence, in such cases, it is also necessary to gain information about unknown sequences of the homoeologous loci to design truly sequence-specific primers. In this work, PCR products generated with primer set O1 in N1AT1B and N1DT1A were cloned. This revealed novel *Glu-3* sequences and led to the construction of locus-specific primer sets for LMW glutenin genes on each of the group 1 chromosomes. Remarkably, the B11-33 sequence was not found among the cloned sequences, but this may be due to the rather low cloning efficiency of the PCR products. Nevertheless, the design of yet other primers specific for the B11-33 sequence is envisageable after comparison with the novel LMW glutenin sequences, although this has not been done in this study.

Amplification length polymorphisms seen among the homoeologous *Glu-3*loci are summarized in Fig. 2. With the exception of products generated with primer sets P1 and C1, all of the PCR products were sequenced. These sequences are deposited in the EMBL, Genbank and DDBJ nucleotide sequence databases under accession numbers X84887, X84959, X84960 and X84961. It is clear that differences are mainly due to variations in length of the proline- and glutamine-rich repeat domain of the LMW glutenin genes. In particular, primer set O11BD amplified two LMW glutenin fragments differing in length, these are encoded by the B and D genomes respectively. These sequences seem to be very homologous, with the main variation being found in the repeat domain, due to the occurrence of several single base differences. The larger 1D specific product carries two insertions in the repeat domain that are responsible for the length polymorphism. This shows that locus-specific primers are useful for detecting intergenomic variation and can be applied to study the relationship between the A, B and D genomes.

Only one of the five locus-specific primer sets showed clear allelic variation in the amplification pattern among some wheat cultivars. However, in as much as the primers amplify a unique product, more allelic variation may be found by direct sequencing of the PCR products. The comparison of the LMW glutenin gene sequences isolated in this way from genotypes with different quality characteristics should help to relate the presence of certain alleles with dough quality.

The locus-specific primer sets are to be considered as sequence-tagged-sites (STS), as proposed by Olson et al. (1989). An STS is a short, unique sequence that can be amplified by PCR and that identifies a known location on a chromosome. The simple interpretation of single-locus markers make them superior to multilocus DNA marker types, especially for map construction. Tragoonrung et al. (1992) and Talbert et al. (1994) used STS-PCR products as molecular markers in barley and wheat to replace restriction fragment length polymorphisms (RFLPs) because of the ease and speed of PCR analysis and the facility of distribution of primer sequence data. Also, the fact that repeated DNA sequences can be mapped by PCR (Rogowsky et al. 1992) argues in favor of PCR as a tool in genetic analysis.

With the *Glu-B3*-specific PCR markers we were able to distinguish between wheat varieties having 1RS.1BL translocated chromosomes and varieties that do not have it. This observation not only confirms the high specificity and reliability of these markers but also shows that the PCR markers can be of use for wheat breeding, as well as in the development of wheat-rye recombinants (Rogowsky et al. 1993).

It is noteworthy that locus-specific amplification was obtained for LMW glutenin genes belonging to a multigene family, characterized by high homology between member genes. The results obtained in this complex situation let us suppose that the present strategy to develop locus-specific primer sets can be applied to virtually all sequenced wheat genes.

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