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DHPLC Scoring of a SNP between Promoter Sequences of HMW Glutenin x-type Alleles at the *Glu-D1* Locus in Wheat

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The promoter regions of HMW glutenin x-type genes at the *Glu-D1* locus were surveyed for SNPs within a subpopulation of German bread wheat cultivars. On the basis of the promoter sequences of HMW glutenin subunit genes *Glu-A1-x1*, *Glu-A1-x2*, *Glu-B1-x1*, *Glu-B1-x7*, *Glu-D1-x2,* and *Glu-D1-x5*, an amplification refractory mutation system assay was designed to selectively amplify Dx-specific PCR fragments. Comparative sequence analysis among seven *Glu-D1-x2* and seven *Glu-D1-x5* wheat cultivars only confirmed a G−A transition in the promoter sequence to be a true polymorphism. SNP scoring by DHPLC of 95 German bread wheat cultivars, with the exception of cv. Anemos, showed that the transition completely agreed with the presence of HMW glutenin subunits 1Dx5 + 1Dy10 in SDS−PAGE. Therefore, the developed DHPLC assay is suitable for high-throughput genotyping to assist the selection of HMW glutenin genes in wheat quality breeding programs.

KEYWORDS: Wheat; *Triticum aestivum*; breadmaking quality; glutenin; single-nucleotide polymorphism; DHPLC

INTRODUCTION

Wheat HMW glutenin subunits are encoded by genes at the *Glu-1* loci on the long arm of group 1 chromosomes, and their allelic variation significantly influences breadmaking quality. Each locus contains an x- and y-type HMW glutenin subunit gene, which are similar in structure: a large central domain with repeated short motifs is surrounded by unique N- and C-terminal sequences. The ‘good-quality’ HMW glutenin subunits 1Dx5 + 1Dy10 are associated with stronger doughs and the allelic pair 1Dx2 + 1Dy12 with weaker doughs (*1*). The most widespread standard technique assisting in selection for breadmaking quality in wheat breeding programs is the separation of HMW glutenin subunits by SDS−PAGE (*2*, *3*). However, this technique is not amenable for automated high-throughput allele typing.

PCR-based genotyping systems are fast and reliable alternatives for the study of wheat endosperm protein genes. SNPs are of particular interest since they represent the most frequent variations in the genome of any organism and can be simply treated as diallelic markers. However, genome-specific amplification of DNA sequences is complicated in bread wheat because genetic information is usually triplicated (2n = 6x = 42; A, B, and D genomes, each with seven pairs of chromosomes, and derived from three different related grass ancestors). Thus, specific PCR primers could amplify sequences from more than one genome, yielding a mixture of homologous DNA fragments. Therefore, allele-specific PCR (AS−PCR), which uses 3′ terminal bases of primers for allele discrimination, display a low efficiency in wheat compared to diploid species (*4*). Additionally, reaction conditions can hardly be standardized for a high-throughput screening.

These problems can be circumvented by the application of DHPLC (*5*), which is based on the detection of heteroduplexes in PCR products by ion-paired reversed-phase chromatography under partially denaturing conditions. A major advantage of DHPLC in polyploid genomes is the high flexibility in primer design, because sizes of PCR fragments in a range of 200−500 bp can be analyzed and detection of heteroduplexes is not affected by the SNP position within the DNA fragment. Therefore, the amplification of unique sequences from either A, B, or D genome in wheat will be facilitated. Additionally, DHPLC represents a fully automated high-throughput SNP genotyping technique due to rapid sample preparation, lack of requirement for post-PCR modifications, autosampling, short run times, and computer-assisted allele calling.

In this study, a SNP in the promoter region of HMW glutenin subunit x-type alleles at the *Glu-D1* locus in wheat was validated and a DHPLC assay was developed for SNP scoring in a set of German wheat cultivars.

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Table 1. HMW Glutenin Subunits at the *Glu-D1* Locus of Selected German Wheat Cultivars

	cultivar	*Glu-D1* subunits		cultivar	*Glu-D1* subunits		cultivar	*Glu-D1* subunits
1	Agent	2+12	33	Estica	2+12	65	Olivin	5+10
2	Alidos	5+10	34	Eta	5+10	66	Ortler	2+12
3	Altos	5+10	35	Fasan	5+10	67	Pagode	2+12
4	Andros	5+10	36	Flair	5+10	68	Passat	5+10
5	Anemos	2+12	37	Florida	5+10	69	Pegassos	5+10
6	Apollo	2+12	38	Futur	2+12	70	Perdix	5+10
7	Applaus	2+12	39	Glockner	5+10	71	Petrus	2+12
8	Ares	2+12	40	Gorbi	2+12	72	Picolo	5+10
9	Aristos	5+10	41	Habicht	5+10	73	Piko	2+12
10	Armada	2+12	42	Herzog	2+12	74	Previa	2+12
11	Aron	5+10	43	Hybnos 1[a]	2+12; 3+12	75	Quattro	2+12
12	Astron	5+10	44	Hybnos 2[a]	2+12; 3+12	76	Ramiro	5+10
13	Atlantis	2+12	45	Ibis	5+10	77	Ranger	3+12
14	Batis	5+10	46	Idol	5+10	78	Redford	5+10
15	Bold	5+10	47	Kalistos	5+10	79	Rektor	5+10
16	Borenos	2+12	48	Kanzler	2+12	80	Ritmo	3+12
17	Borneo	5+10	49	Karpos	5+10	81	Semper	2+12
18	Bussard	5+10	50	Kontrast	5+10	82	Skater	2+12
19	Caesar	2+12	51	Kornett	5+10	83	Star	5+10
20	Cardos	5+10	52	Korund	5+10	84	Tambor	5+10
21	Cheyenne	5+10	53	Kris	2+12	85	Tarso	5+10
22	Clan	2+12	54	Kronjuwel	2+12	86	Terrier	5+10
23	Clever	3+12	55	Lindos	5+10	87	Thasos	5+10
24	Club	5+10	56	Maverick	5+10	88	Toni	2+12
25	Combi	5+10	57	Melon	2+12	89	Toronto	5+10
26	Contra	2+12	58	Mikon	5+10	90	Tower	2+12
27	Corvus	5+10	59	Miras	5+10	91	Trakos	5+10
28	Dekan	5+10	60	Monopol	5+10	92	Velos	5+10
29	Devon	5+10	61	Motiv	2+12	93	Winni	5+10
30	Dream	5+10	62	Munk	5+10	94	Xanthos	5+10
31	Drifter	2+12	63	Nandu	5+10	95	Zentos	5+10
32	Ebi	5+10	64	Naxos	5+10	96	Combo[b]	

[a] Hybrid wheat varieties. [b] Durum wheat cultivar; *Glu-D1* absent.

MATERIALS AND METHODS

Plant Material. SNP scoring was carried out in a sample of 95 common wheats (**Table 1**). Data for HMW glutenin subunit constitutions were obtained from the Section for Seed Certification of the Bavarian State Research Center for Agronomy and the GrainGenes database (http://wheat.pw.usda.gov). Three nulli-tetrasomics (N1AT1B, N1BT1A, and N1DT1A) and two ditelosomics (Dt1DS and Dt1DL) of *T. aestivum* cv. Chinese Spring were used for chromosome arm assignment of PCR products. Absence of a band in a particular nullitetrasomic line indicates the chromosome harboring the corresponding sequence, whereas presence of a band in a ditelosomic line indicates the chromosome arm carrying the marker locus. Durum wheat cultivar Combo was used as negative control for D genome-specific PCR amplification.

DNA Isolation. Genomic DNA from kernels was extracted and purified using a modified CTAB standard protocol (*6*). After overnight incubation in 1 mL of 1× CTAB buffer [0.7 M NaCl, 100 mM Tris-Cl (pH 7.5), 20 mM EDTA (pH 8.0), and 50 μL of proteinase K (20 mg/mL)] at room temperature, a single kernel was crushed and further incubated for 1 h at 60 °C. The samples were extracted twice with 1 volume of chloroform/isoamyl alcohol (24:1); genomic DNA was precipitated with 1 volume of 2-propanol and washed with 1 mL of 75% ethanol. DNA pellets were dried at room temperature, rehydrated in 100 μL of H_2O, and stored at −20 °C until use.

Polymerase Chain Reaction. PCR was carried out on a GeneAmp PCR System 9600 (Perkin-Elmer) in 20-μL reaction volumes containing 50 ng of genomic DNA, 0.2 μM each of primers Dx-f1 5′-CGTGT-TGCTGGAAATCCAAC-3′ and Dx-r1 5′-TTTGTTTAGCCTAAGAA-GAAAGCAC-3′, 0.2 mM dNTP, and 0.5 U of *Taq* DNA polymerase (Qiagen) in 1× PCR reaction buffer supplied by the manufacturer. PCR products were amplified using the following conditions: Initial denaturation for 2 min, followed by 34 cycles of 30 s at 95 °C, 1 min at 56 °C, and 1 min at 72 °C, final extension for 10 min at 72 °C.

Sequence Analysis. Direct sequencing of PCR products was performed from both ends with primers Dx5-f1 and Dx5-r1 on an ABI377 platform (Applied Biosystems) using standard dye terminator chemistry (Amersham-Pharmacia Biotech). Editing of DNA sequences was performed with Sequence Navigator software (Applied Biosystems), and multiple sequence alignment tool ClustalW (*7*; http://www.ebi.ac.uk/clustalw) was used for SNP evaluation.

DHPLC Analysis. DHPLC analysis was carried out on a fully automated Helix System (Varian, Inc.), which uses ion-paired reversed-phase chromatography to separate out DNA fragments. Prior to DHPLC analysis, PCR products from a reference variety with known allele constitution were added in equimolar amounts to PCR products from all test samples, denatured at 95 °C for 3 min, and reannealed over 30 min by decreasing the temperature from 95 to 60 °C to allow heteroduplex formation. The mixtures were automatically loaded on the column with an autosampler. At a critical denaturing temperature, homo- and heteroduplexes were released off the column at different times. The mobile phase consisted of two eluents (pH 7.0): buffer A contained 0.1 M TEAA, buffer B contained 0.1 M TEAA, 25% acetonitrile. DNA fragments were eluted with a linear acetonitrile gradient of 2% per min at a flow rate of 0.45 mL/min and detected by 260 nm absorbance. The analysis took 8 min, including column regeneration and reequilibration to the starting conditions. DHPLC data were managed with Star Chromatography Workstation Version 5 (Varian, Inc.).

RESULTS AND DISCUSSION

Partial Amplification of the Promoter Region from HMW Glutenin x-type Genes at the *Glu-D1 Locus.* Two criteria for the selection of primers were considered: fragment length and genome specificity of the potential amplicon. As a default for subsequent DHPLC analysis, the optimal fragment length for SNP identification should be 200−500 bp, since an increase in fragment size will reduce DHPLC sensitivity and extend the time of elution, whereas short fragments are difficult to analyze due to narrow differences of melting points between homo- and

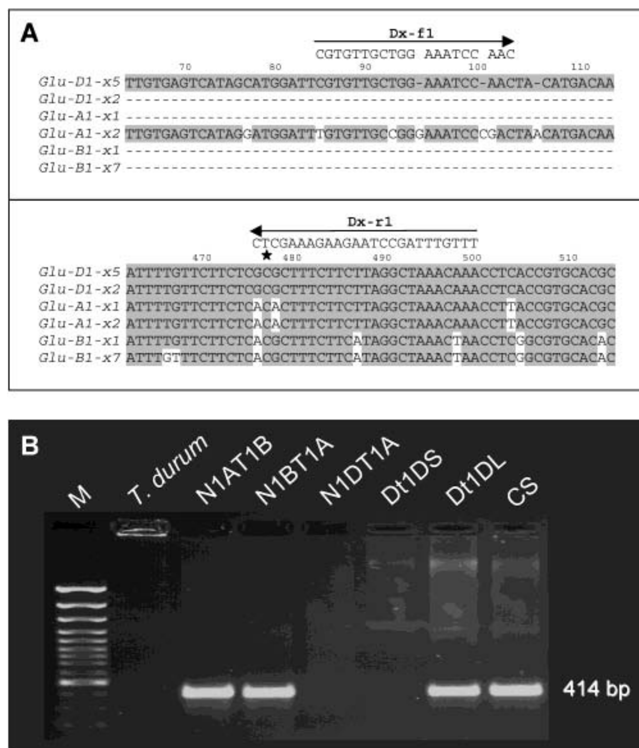

Figure 1. Specific amplification of the promoter region from HMW glutenin x-type genes at the *Glu-D1* locus. (A) Primer binding sites and amplification refractory mutation system. The 3′-subterminal mismatching nucleotide of the primer Dx-r1 is indicated by an asterisk. (B) Use of *T. durum* cv. Combo, nullitetrasomic, and ditelosomic lines of *T. aestivum* Chinese Spring for examination of genome-specific amplification. Designations of investigated lines are given above each lane. (M) 100-bp ladder, (CS) Chinese Spring.

heteroduplex molecules. To achieve genome-specific amplification, promoter sequences of *Glu-A1-x1* (*8*; GenBank Accession No. X61009), *Glu-A1-x2* (*9*; M22208), *Glu-B1-x1* (*10*; Y10954), *Glu-B1-x7* (*9*; X13927), *Glu-D1-x2* (*11*; X03346), and *Glu-D1-x5* (*12*; X12928) were compared among each other. This alignment clearly revealed polymorphisms between the promoter sequences from HMW glutenin x-type genes from A, B, and D genomes to be useful for the construction of genome-specific PCR primers (**Figure 1A**). Complete sequence information for all glutenin alleles was available for the 3′-terminal promoter region only, wherefore the reverse primer was selected to be responsible for the suppression of fragment amplification from A and B genomes. Therefore, the 3′-end of the reverse primer was fixed to a nucleotide position, where promoter sequences from *Glu-D1* alleles differ from that of both *Glu-A1* and *Glu-B1* alleles. In addition, the power of the oligonucleotide for allele discrimination was enhanced by introducing an artificial mismatch at the 3′-subterminal base (*13*). The deliberate C:T mismatch combined with the mismatched 3′-residue (C:A) successfully suppressed amplification of HMW glutenin x-type gene promoters from A and B genomes as demonstrated by PCR analysis of tetraploid *T. durum* cv. Combo, consisting of A and B genomes only (**Figure 1B**). The lack of amplification of the 414-bp PCR fragment in nullitetrasomic line N1DT1A of *T. aestivum* Chinese Spring proved its allocation to chromsome 1D. Traced back to amplification in ditelosomic line Dt1DL of *T. aestivum* Chinese Spring, the position of the promoter-specific marker was refined to the long arm of chromosome 1D.

Sequence Analysis for SNP Validation. A ClustalW alignment was performed with partial *Glu-D1-x2* and *Glu-D1-x5* promoter sequences from public databases, which correspond to the amplified 414-bp PCR fragment (**Figure 2A**). Two single-base insertions/deletions at positions 143 and 169 and one SNP at position 219 were found. Two sets of genotypes each of seven wheat cultivars carrying the allelic protein pairs Dx5 + Dy10 and Dx2 + Dy12, respectively, were used for sequencing in order to ensure accurate SNP validation. Comparative analysis revealed no sequence variations within the two subsets, whereas between the two subsets, only the G−A transversion at position 219 was confirmed (**Figure 2B**). Moreover, the additionally gained sequence information for the *Glu-D1-x2* alleles did not provide new polymorphism.

DHPLC Analysis for SNP Scoring. For SNP scoring the DHPLC technique was applied, which is based on the differential retention of homo- and heteroduplex DNA molecules under conditions of partial heat denaturation. Since wheat cultivars are homozygous, heteroduplexes were detected by mixing PCR products from cv. Monopol (promoter amplicon from *Glu-D1-x5* allele was defined as 'reference' allele) in an equimolar ratio with PCR products from all wheat samples. Initially, the mixtures were analyzed at the critical temperature of 58 °C, as predicted by the melting algorithm available at http://insertion.stanford.edu/melt.html. For optimal resolution of homo- and heteroduplexes, a temperature of 57 °C was determined by varying the predicted mobile-phase temperature in 1 °C increments. At this temperature, the homoduplex elution profiles were clearly distinct from the profiles associated with heteroduplex DNA molecules, formed upon mixing promoter amplicons from *Glu-D1-x5* (cv. Monopol) and *Glu-D1-x2* (cv. Apollo) alleles (**Figure 3**). SNP scoring within a panel of 95 German bread wheat cultivars (**Table 1**) revealed that all cultivars carrying 1Dx5 + 1Dy10 always showed the single-peak pattern of homoduplex DNA molecules. Furthermore, heteroduplex elution profiles were found for all cultivars carrying either 1Dx2 + 1Dy12 or 1Dx3 + 1Dy12 allele combinations, with the exception of spring wheat cultivar Anemos, which is known to carry the 1Dx2 + 1Dy12 combination but showing the homoduplex elution profile. Considering SDS−PAGE results only, one would expect that cv. Anemos will show poor breadmaking properties, but it is a high-quality bread wheat. In contrast, the classification by SNP is in agreement with the phenotype. Missclassification of HMW glutenin subunits in SDS−PAGE can occur due to discrepancies in their relative mobility as already described for the *Glu-D1*-encoded subunits (*14*−*16*).

Several AS−PCR assays which are based on oligonucleotides targeting the SNP with their 3′-end were already developed for the detection of HMW glutenin x-type genes at the *Glu-D1* locus of wheat (*17*−*19*). The detection of SNPs using DHPLC analysis of PCR products is superior to AS−PCR, especially in polyploid genomes such as wheat, which is composed of highly homologous sequences. In AS−PCR, amplification and detection of an allelic variant usually must be performed in a single step, whereas they are decoupled in the DHPLC-based SNP detection system. This enabled the high flexibility in designing specific primers to distinguish the highly homologous alleles of the complex HMW glutenin family, because the SNP did not have to be involved in the amplification step.

Besides the *Glu-D1* locus, alleles at *Glu-A1* and *Glu-B1* loci are firmly associated with breadmaking quality. Therefore, a set of SNPs has to be developed for all relevant glutenin subunits to provide a powerful tool for marker-assisted selection of breadmaking properties in breeding programs. To meet the requirements, we found that the cost-effective and high-

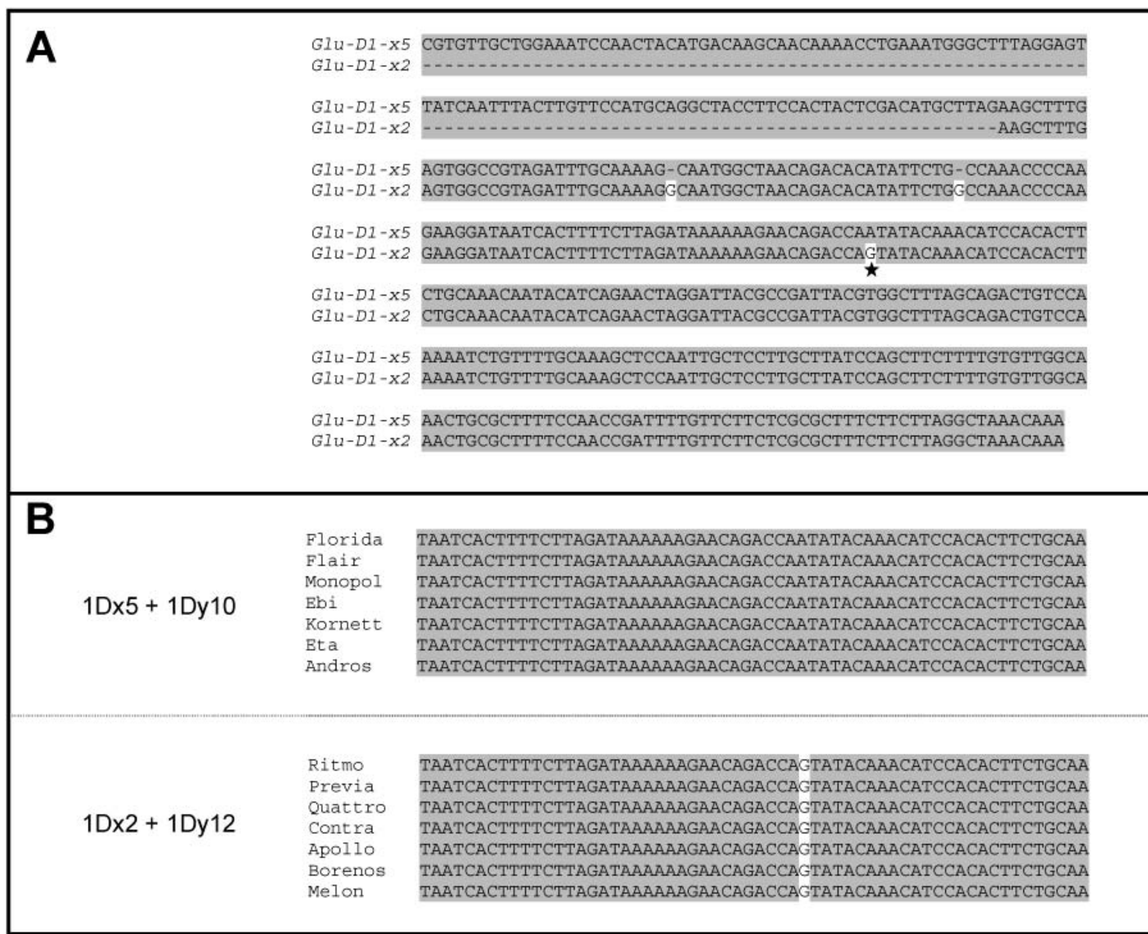

Figure 2. Comparative analyses of promoter sequences from HMW glutenin x-type genes at the *Glu-D1* locus. (A) *Glu-D1-x5* and *Glu-D1-x2* from EMBL database. The position of the confirmed SNP is indicated by an asterisk. (B) Partial sequences of promoter amplicons from 1Dx5 + 1Dy10 and 1Dx2 + 1Dy12 wheat genotypes.

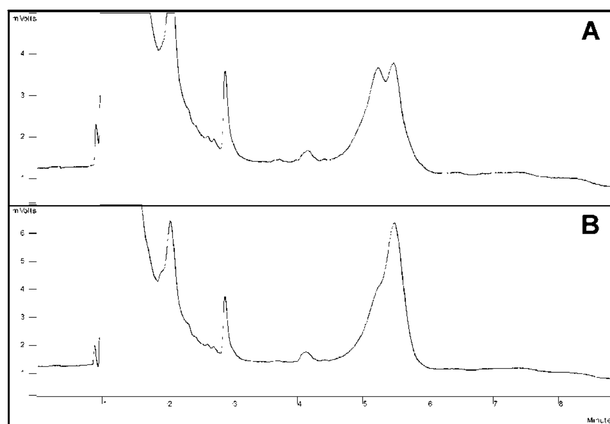

Figure 3. DHPLC elution profiles of (A) heteroduplex and (B) homoduplex DNA molecules formed with amplicon mixtures from cvs. Monopol (*Glu-D1-x5*)/Apollo (*Glu-D1-x2*) and Monopol (*Glu-D1-x5*)/Monopol (*Glu-D1-x5*), respectively. An additional peak with a shorter retention time was seen in the heterozygous sample.

throughput genotyping DHPLC technique is particularly suitable for routine diagnosis of SNPs in homologous HMW glutenin gene sequences.

ABBREVIATIONS USED

CTAB, hexadecyltrimethylammonium bromide; cv., cultivar; DHPLC, denaturing high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; HMW, high molecular weight; NaCl, sodium chloride; PCR, Polymerase Chain Reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SNP, single-nucleotide polymorphism; TEAA, triethylammonium acetate; Tris, tris(hydroxymethyl)aminomethane.

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