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A spelt-specific γ-gliadin gene: discovery and detection

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Abstract Polymerase chain reaction (PCR) primers GAG5 and GAG6 were designed based on published γ-gliadin gene sequences and applied to 35 cultivars of closely related spelt (*Triticum spelta* L.) and hexaploid wheat (*T. aestivum* L.). Eight tetraploid durum wheat (*T. durum* Desf.) cultivars were included in the analysis. The obtained PCR products originated from two γ-gliadin genes which were mapped to homeologous chromosomes 1B and 1D and termed GAG56B and GAG56D, respectively. Two alleles of GAG56D differing in a 9-bp deletion/duplication and single nucleotide polymorphism were found. The 18 spelts tested and wheat cultivar 'Chinese Spring' were discovered to carry a previously unknown γ-gliadin gene, while 16 wheat cultivars possessed its longer, already published allele. Two PCR-based detection systems for the diagnostic alleles were developed and applied. The occurrence of two alleles of GAG56B among the investigated durum wheats correlated with their expression of gluten quality markers γ-gliadins 42 or 45.

Key words Spelt · Wheat · γ-Gliadins · PCR · Molecular markers

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Introduction

Spelt (*T. spelta* L.) and hexaploid wheat (*T. aestivum* L.) are closely related and interbreeding species within the *Triticum* genus; some authors even regard them as subspecies of *T. aestivum* (Abdel-Aal et al. 1996; Sears 1974). Assumed to be an immediate progenitor of modern bread wheat, spelt is nevertheless easily recognized morphologically, e.g. by longer, lax spikes or tougher glumes (Winzeler and Rüegger 1990). However, general molecular markers for distinguishing the two hexaploid cereals are still not established, despite efforts to do so using prolamin storage protein patterns (Abdel-Aal et al. 1996; Federmann et al. 1992; Harsch et al. 1997; Radic et al. 1997). Spelt, in former times the predominant grain cultivated in central Europe, has gained renewed interest as an undemanding crop suitable for climates too harsh for other cultivated plants. While it is also viewed as a potential alternative for patients of coeliac disease or wheat allergies, so far there is no scientific reason to treat spelt any differently than wheat with respect to these medical conditions (Abdel-Aal et al. 1996; Forssell and Wieser 1995; Friedman et al. 1994; Maruyama et al. 1998; Tanabe et al. 1996).

The prolamin storage proteins form a very polymorphic family which is present in all cereal species. They are thought to have a common evolutionary origin (Kreis et al. 1985a, b; Shewry and Tatham 1990). Based on their molecular nature, they can be subdivided into a sulfur-rich [α/β- and γ-gliadins, low-molecular-weight (LMW) glutenins], a sulfur-poor (ω-gliadins) and a high-molecular-weight (HMW) group (HMW glutenins). Traditionally, the prolamins have been classified primarily as gliadins and glutenins based on solubility and then divided further into the mentioned fractions by electrophoretic mobility (Osborne 1924). They are encoded by intron-less genes at three major locations in all three chromosome sets: HMW glutenins on the long arms of chromosomes of homeologous group 1 (*Glu-1* loci), LMW glutenins, γ- and ω-gliadins on the short arms of group 1 (*Glu-3* and *Gli-1* loci, respectively) and α/β-gliadins on

the short arms of group 6 (*Gli-2* loci) (Payne 1987). Quite a number of prolamin gene and protein sequences have been published. Of ω-gliadins however, no sequence information beyond N-terminal peptides and repeat structures is known (Kasarda et al. 1983). The γ-gliadins in particular consist of a proline-rich, N-terminal domain mainly composed of repeats and a proline-poor, C-terminal domain containing the evolutionarily conserved regions A, B and C (Kreis et al. 1985a, b).

HMW and LMW glutenins have been attributed a major role in determining the bread- and pasta-making quality of common and durum wheat (*T. durum* Desf.), respectively (Pogna et al. 1990; Shewry et al. 1995). The relevant HMW and LMW glutenin subunits can be detected using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). On the other hand, gliadins are the triggering agents in coeliac disease (Sturgess et al. 1991), and various prolamin fractions are antigens in wheat allergy (Maruyama et al. 1998). ω-Gliadins are targeted in the routine enzyme-linked immunosorbent assay (ELISA)-analysis of gluten contamination in "gluten-free" products (Skerritt and Hill 1990). The prolamin protein fingerprints obtained with acidic polyacrylamide gel electrophoresis (APAGE), SDS-PAGE or high-performance liquid chromatography (HPLC) are utilized for cultivar identification (Ng et al. 1988; Payne and Lawrence 1983).

The polymerase chain reaction (PCR) has been applied to species identification in cereals (Ko et al. 1994), detection of wheat contamination (Allmann et al. 1993) or *T. aestivum* adulteration of pasta (Bryan et al. 1998). In connection with prolamins, PCR has been developed to detect the 1Dx5 HMW glutenin subunit gene associated with good bread-making quality (D'Ovidio and Anderson 1994) or the γ-gliadin and LMW glutenin genomic markers strongly correlated with rheological properties of durum wheat gluten (D'Ovidio 1993; D'Ovidio et al. 1990). Moreover, PCR of prolamin genes has been used as a tool in cloning and research (D'Ovidio et al. 1991; D'Ovidio et al. 1992a, b; Lafiandra et al. 1997; Maruyama et al. 1998). Here we report the discovery of a spelt-specific γ-gliadin gene using a PCR-analytical approach. The gene appears to be an allele of a published nucleotide sequence. The occurrence of both alleles among 35 hexaploid cultivars was studied. Two PCR-based detection systems were designed.

Materials and Methods

Biocomputing

Nucleotide and protein sequence analysis was performed using the Wisconsin Package Version 9.1 [Genetics Computer Group (GCG), Madison, Wis.]. PCR primers were designed with the Oligo 5.0 (Molecular Biology Insights, Plymouth, Minn.) and Primer Designer for Windows 3.0 (sci-ed Software, Durham, N.C.) software.

Triticum cultivars

Thirty-five hexaploid *Triticum* cultivars were used in this work: 11 spelts, seven spelt-wheat crosses and 17 wheats. The spelt (*T. spelta* L.) cultivars were 'Altgold' [abbreviated AD, origin: Switzerland (CH)], 'Bauländer Spelz' [BS, Germany (D)], 'Ebners Rotkorn' [ER, Austria (A)], 'Fuggers Babenhauser' (FB, D), 'Oberkulmer' (OK, CH), 'Ostar' (OR, CH), 'Ostro' (OO, CH), 'Schwabenkorn' (SK, D), 'Steiners Roter Tiroler' (SR, A), 'Vögelers Dinkel' (VD, D) and 'Waggerhauser Hohenheimer' (WH, D). Spelt-wheat crosses were 'Balmegg' (BG, CH), 'Frankenkorn' (FK, D), 'Hércule' [HE, Belgium (B)], 'Hubel' (HL, CH), 'Redouté' (RE, B), 'Rouquin' (RN, B) and 'Sertel' (SL, CH). The wheat (*T. aestivum* L.) cultivars used were 'Ami' [AI, France (F)], 'Arbola' (AB, CH), 'Arina' (AA, CH), 'Bernina' (BA, CH), 'Boval' (BL, CH), 'Camino' (CO, CH), 'Chinese Spring' (CS, China), 'Forno' (FO, CH), 'Galaxie' (GE, F), 'Kanzler' (KR, D), 'Kolibri' (KI, D), 'Katepwa' [KA, Canada (CAN)], 'Lona' (LA, CH), 'Neepawa' (NA, CAN), 'Ramosa' (RA, CH), 'Tamaro' (TO, CH) and 'Toronit' (TT, CH). Gene localization was performed on a set of five nullisomic-tetrasomic lines of 'Chinese Spring' (Sears 1974): nullisomic-1A-tetrasomic-1B (N1A/T1B), N1B/T1A, N1D/T1B, N6A/T6D and N6B/T6A. The following eight durum wheat (*T. durum* Desf.) cultivars were used as tetraploid references: 'Astrodur' (AR, D), 'DT433' (DT, CAN), 'Duilio' [DO, Italy (I)], 'Fortore' (FE, I), 'Gianni' (GI, I), 'Primadur' (PR, F), 'Rugby' (RY, USA) and 'Wascana' (WA, CAN).

DNA extraction

Genomic DNA was prepared from *Triticum* plants using a CTAB method adapted from Richards et al. (1994). One gram of leaves harvested 3–4 weeks after sowing yielded 600–800 µg of DNA. No effort was made to remove RNA.

PCR

One hundred nanograms of genomic DNA was subjected to PCR in a 50-µl reaction volume containing 1 unit (u) of *Taq* polymerase (Promega), 1× *Taq* PCR buffer (Promega), 2 ng/µl of bovine serum albumin, 1–2.5 m*M* $MgCl_2$ (see Table 1 for individual concentrations), 200 µ*M* of each deoxyribonucleotide and 500 n*M* of each oligonucleotide primer (Microsynth, Balgach, Switzerland). PCR was run on either Progene (Techne) or Mastercycler (Eppendorf) thermocyclers as follows: an initial denaturation step of 3 min at 94°C was followed by 45 cycles of denaturation for 20 s at 96°C, annealing and extension as indicated in detail in Table 1, and concluded with a final extension step for 3 min at 72°C. PCR products were analyzed on 1.5% or 2.5% agarose gels. Sequencing of PCR fragments was done with the corresponding PCR primers using fluorescence-labelled dideoxynucleotides on a ABI PRISM 377 DNA Sequencer (Perkin Elmer, carried out by Microsynth).

Restriction analysis

One unit of endonuclease was added directly to 10 µl of amplified PCR products and incubated at the appropriate temperature for 2 h. Of *Bst*4CI (SibEnzyme, Novosibirsk, Russia) in particular, 0.5 U per potential restriction site (i. e. 0.5 U and 1.0 U for GAG17/18 and GAG15/16 products, respectively) was added to 10 µl of amplified PCR products and digested for 2 h at 65°C. Restriction digests were analyzed on 2.5% agarose gels.

Results

PCR primer design

With the intention of finding potential differences in gliadin gene patterns between spelt (*T. spelta* L.) and wheat (*T. aestivum* L.), we wanted to construct PCR primer

Table 1 PCR primer sequences, primer positions, product lengths and reaction variables. Mismatches in GAG56D-specific primer sequences GAG15 – GAG19 as compared to accession M13712 (GAG56B allele) are shown in bold print. Primer positions refer to GenBank accessions M16060 (wheat allele) and AF120267 (spelt allele). Resulting product lengths (wheat and spelt fragments, respectively), used magnesium ion concentration as well as temperature and duration of annealing and extension steps of the applied PCR program are indicated for the primer pair named in the first column

Primer	Sequence (5'–3')	Position on M16060 (wheat)	Position on AF120267 (spelt)	Product length (bp)	[Mg^{2+}] (m*M*)	Annealing and extension
GAG05	ACAAT GGCCA CAACA ACAAC	132–151	100–119	659/n. a.[b]	2.5	68°C, 2 min 40 s
GAG06	TGCCC TGRCC CTGGR C	775–790	n.s.[a]			(2-step PCR)
GAG15	GCAAC CACAA **C**AAC**A** ATTT**T** C**T**	387–408	355–376	359/350	2.5	65°C, 1 min 20 s
GAG16	GATAT AGTGG CAGCA GGATA **T**G	724–745	683–704			72°C, 1 min 20 s
GAG17	GATCT GGCCA CAAAG CG**A**TT	573–592	541–560	188/179	2.5	68°C, 1 min 20 s
GAG18	CCACC TGTTG **C**TGCT GATAT AG	739–760	698–719			(2-step PCR)
GAG19	G**C**ATC AAATC CAAGT ACGT**A** GT	6–27	n.s.	755/n.a.	1.0	62°C, 1 min 20 s
GAG18	See above					72°C, 1 min 20 s

[a] Not sequenced
[b] Not applicable

pairs on conserved regions of published gliadin gene sequences and to apply them to genomic DNA of both *Triticum* species. As no ω-gliadin gene sequences have been published so far, our efforts were restricted to α/β- and γ-gliadin gene sequences. Starting our investigations with the homeologous chromosome group 1-encoded γ-gliadins (Payne 1987), we did find 12 entries of γ-gliadin genes or gene fragments (including pseudogenes) in the GenBank. The entries were tentatively divided into two groups A and B, based on nucleotide alignment of the coding regions using the PILEUP program (GCG software package). Group A comprised accession numbers (acn.) J01309 (Bartels and Thompson 1983), M11077 (Okita et al. 1985), M11335 (Okita 1984) and M11336 (Okita et al. 1985), while group B consisted of acns. D78183 (Maruyama et al. 1998), M13712, M13713 (Rafalski 1986), M16060 (Scheets et al. 1985), M16064 (Sugiyama et al. 1986), M36999 (Scheets and Hedgcoth 1988) and X53412 (D'Ovidio et al. 1991). Entry X04532 (Rustembekov et al. 1986) was assigned to neither group and was not used further in this work. A total of four primer pairs (two on each group) were designed on conserved regions of γ-gliadin genes. This report focusses on results obtained with primer pair GAG5 and GAG6 (see Table 1 for primer sequences and positions) on group B. Mixed bases were introduced into primer GAG6 to partially accomodate ambiguities. However, GAG6 still only fit three of the seven aligned sequences of group B, whereas primer GAG5 matched five of them. In combination, primer pair GAG5 and GAG6 were a perfect fit for two published sequences, acns. M16060 and M13712.

PCR analysis using primer pair GAG5 and GAG6

PCR parameters (see Materials and Methods and Table 1) for primers GAG5 and GAG6 were optimized using genomic DNA of wheat cultivar 'Forno'. The PCR system was then applied to a selection of five spelt, five bread wheat and four durum wheat cultivars (Fig. 1). All 10 hexaploid cultivars produced a band around 650 bp and, with the exception of cvs 'Bernina' and 'Boval', one between 750 and 800 bp. Whereas wheat cv 'Bernina' did not give rise to a PCR fragment in the latter size class, cv 'Boval' produced a double band. The four tested durum wheats, on the other hand, each yielded only one PCR fragment, which was between 750 and 800 bp. When we later included the remaining 25 hexaploid and four durum cultivars in the GAG5/6-PCR analysis (data not shown), we found that all 35 hexaploid cultivars but none of the eight tetraploid durum wheat cultivars produced a band around 650 bp. As seen with cv 'Bernina', spelt-wheat cross cultivar 'Hubel' and wheat cultivars 'Arbola' and 'Toronit' did not result in any amplification product in the 750- to 800-bp region, all other hexaploid cultivars exhibited one or two products larger than 750 bp. The eight durums produced exclusively one band between 750 and 800 bp. Closer in-

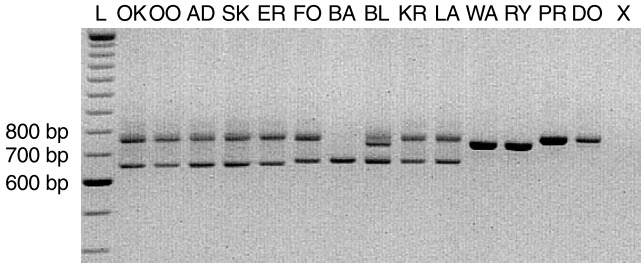

Fig. 1 PCR using primers GAG5 and GAG6 applied to selected *Triticum* cultivars. The band at around 650 bp was consistently a little shorter in spelt (*lanes OK – ER*) than in hexaploid wheat (*FO – LA*) and never present in durum wheat (*WA – DO*). A larger fragment of 750–800 bp was obtained for all cultivars except 'Bernina' (BA). Assay conditions: 1.5% agarose gel; 16 µl of PCR products. *L* 100-bp ladder (bolder 600-bp band), *X* negative control. Two-letter abbreviations are as defined in Materials and Methods

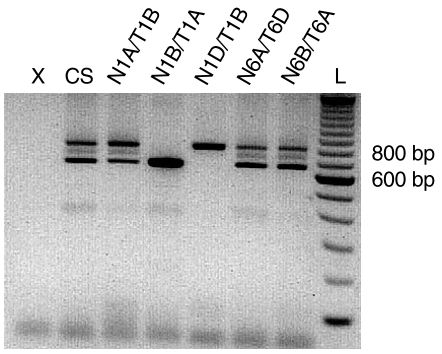

Fig. 2 Localization of GAG5/6-PCR products using nullisomic-tetrasomic lines of cv 'Chinese Spring'. Three fragments were found for euploid 'Chinese Spring' (*CS*). The largest band was missing in nullisomic-1B-tetrasomic-1 A (*N1B/T1A*), while the 650-bp band was not found for nullisomic-1D-tetrasomic-1B (*N1D/T1B*). The weak intermediate fragment was missing in both lines concerned. Assay conditions: 1.5% agarose gel; 16 µl of PCR products; *X* Negative control, *L* 100-bp ladder (bolder 600-bp band). Two-letter abbreviations are as defined in Materials and Methods

spection of the PCR products at around 650 bp revealed that the five fragments originating from spelt DNA were consistently a little shorter than their counterparts amplified from wheat DNA (Fig. 1). As this appeared to be the kind of species-specific difference we sought, the PCR products amplified with primers GAG5 and GAG6 were further investigated.

Localization

As none of the tetraploid wheat cultivars produced a 650-bp band, we suspected the 650-bp fragment to originate from the D genome. On the other hand, wheat cultivar 'Bernina' did not result in any PCR products in the 750- to 800-bp range. This pointed to chromosome 1B as the template location of these fragments since cv 'Bernina' was known through our own results (see below) and those of Schlegel (1997) to contain a rye-wheat chromosome 1RS.1BL translocation. Both presumptions were confirmed by applying the GAG5/6-PCR system to DNA of nullisomic-tetrasomic lines of cv 'Chinese Spring' (Sears 1974) (Fig. 2). Euploid 'Chinese Spring' produced three bands: one at 650 bp, one between 700 and 800 bp, and one larger than 800 bp (lane CS). The PCR product at 650 bp was missing only in the line deficient of chromosome 1D (lane N1D/T1B), whereas the largest band did not appear in nullisomic-1B-tetrasomic-1A (lane N1B/T1A). The intermediate product was missing in both nullisomic lines involved, apparently depending on the presence of both flanking products. We concluded from this finding that the intermediate band was a hybrid PCR product formed by heterogenous annealing of single-stranded PCR fragments from each of the actual PCR products. Hybrid products were thus a possible explanation whenever more than two products appeared in this PCR system, e.g. as for cv 'Boval' in Fig. 1. Extrapolating the location evidence, it seemed probable that cvs 'Hubel', 'Arbola' and 'Toronit', which only yielded the smaller 650-bp PCR product, also possessed a 1RS.1BL translocation. This was confirmed by results obtained when the cultivars in question were tested with an experimental, ω-secalin-specific (Hull et al. 1991) PCR system (data not shown). Cultivars 'Hubel', 'Arbola', 'Bernina' and 'Toronit' all gave rise to the desired fragment of about 1.1 kb, while cvs 'Forno' and 'Tamaro' [also named in Schlegel (1997) as carriers of a 1RS.1BL translocation] neither yielded a PCR product of appropriate size nor did they fail to give two fragments using the GAG5/6-PCR system. For brevity in the context of this work, the two genes yielding products with PCR primers GAG5 and GAG6 are referred to as GAG56B for the longer, chromosome 1B-encoded fragment and GAG56D for the 650-bp, chromosome 1D-encoded fragment.

γ-Gliadin alleles

In order to discover the molecular reason behind the length polymorphism found in the GAG56D fragment between the spelt and the wheat cultivars, we sequenced representative fragments amplified from spelt cultivar 'Oberkulmer' and wheat cultivar 'Forno' using the PCR primers. The nucleotide sequences obtained were very similar to each other and, by comparison with known genes, undoubtedly part of γ-gliadin genes. The 'Forno' allele was identical to part of acn. M16060 [genomic clone pW10 isolated from cv 'Yamhill' (Scheets et al. 1985)], one of two sequences that PCR primers GAG5 and GAG6 matched. The reason for the small-sized difference was revealed when both sequences were aligned (Fig. 3). The tandemly repeated nonamer CAAGAACAA (encoding QEQ) in the conserved region B of the C-terminal domain (Kreis et al. 1985a, b) of the 'Forno' sequence occurred only in single copy in the 'Oberkulmer' allele. The only other difference between the two sequences was a single nucleotide polymorphism 24 nucleotides upstream from the beginning of the nonamer.

Fig. 3 Sequence alignment of the region that varies between the two alleles of GAG56D. The spelt allele (*OK* for cv 'Oberkulmer') is aligned *above* the wheat allele (*FO* for cv 'Forno'). The duplicated/deleted region is *underlined*. The associated single nucleotide polymorphism and the resulting diagnostic *Bst*4CI restriction site are shown in *bold print*. Nucleotide numbering refers to GenBank accessions AF120267 and M16060, respectively

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OK    630 TCCAT ACCGT CATACATTCCATCATCATGCAG.........CAAGAACAACAACAAGGC 679
          |||||||| |||||||||||||||||||||||||         ||||||||||||||||||
FO    662 TCCATACC A TCATACATTCCATCATCATGCAGCAAGAACAACAAGAACAACAACAAGGC 720
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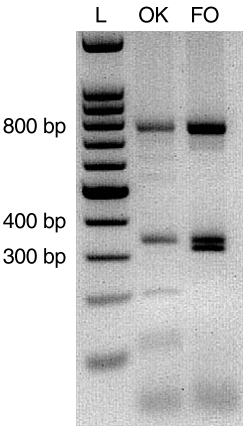

Fig. 4 Restriction of GAG5/6-PCR products with *Bst*4CI. The GAG56D fragment (originally at 650 bp) of cv 'Oberkulmer' (*OK*) was digested into three fragments each of a of distinct size, while its wheat counterpart amplified from cv 'Forno' (*FO*) was cut to two pieces of about equal size. The larger GAG56B fragment was not restricted in either case. Assay conditions: 1.5% agarose gel, 20 µl of digested PCR products. *L* 100-bp ladder (bolder 500-bp band)

In place of the A in the wheat sequence, we found a G in the spelt counterpart, changing an ATC-isoleucine to an GTC-valine codon. More importantly for this work, it creates an additional *Bst*4CI restriction site in the 'Oberkulmer' allele (a common site lies 196 bp upstream). Restriction of the GAG5/6 PCR products with endonuclease *Bst*4CI provided the experimental confirmation for this (Fig. 4). While the band of cv 'Forno' (lane FO) was cut about in half, the GAG56D product originating from cv 'Oberkulmer' (lane OK) was reduced to three fragments of about 350, 200 and 100 bp, respectively. Both GAG56B fragments were not restricted. The 5'-ends of the coding regions of GAG56D were sequenced using the GAG19/18-PCR system (see Table 1). Primer GAG19 was set within the 45 bp of the 5' non-coding region of acn. M16060, and primer GAG18 was designed as part of a GAG56D-specific detection system (see below). Nucleotide sequences upstream of the just described polymorphisms proved to be identical to acn. M16060 in fragments obtained from both cvs 'Forno' and 'Oberkulmer'.

The GAG56B fragments of cv 'Forno' and durum wheats 'Duilio' and 'Wascana' were partially sequenced as well. They were also amplified from γ-gliadin gene templates. Apparently, they were alleles of acn. M13712 [genomic clone L311A from wheat cultivar 'Yamhill' (Rafalski 1986)], a pseudogene and the second published γ-gliadin gene sequence PCR primers GAG5 and GAG6 fitted perfectly. The length polymorphism originated from a variable number of CAA-glutamine codons in a poly-Q stretch also at the end of conserved region B (Kreis et al. 1985a, b). The alleles found in 'Duilio' and 'Wascana' contained 18 and 10 glutamine codons, respectively, as opposed to 15 in acn. M13712. Sequencing of the Forno fragment was incomplete in that region. The second of two premature stop-codons of M13712 (localized immediately 5' of the poly-Q stretch) was conserved in both 'Duilio' and 'Wascana', while the first was not found in either of them. An additional TAA-stop-codon was found in the 'Duilio' fragment. Neither acn. M13712 nor any of the sequenced fragments appeared to have a *Bst*4CI restriction site. However, as this was not the core issue of this work, sequencing of the GAG56B fragments was not pursued further, and sequences were left partially ambiguous. Simply judging from GAG5/6-PCR product length, there have to exist at least three different alleles of this pseudogene besides acn. M13712: the ones found in cvs 'Wascana', 'Duilio' (Fig. 1, lanes WA and DO) and 'Chinese Spring' (Fig. 2), respectively. The hexaploid cultivars in Fig. 1 appeared to produce the same fragment length as cv 'Duilio', but this remained inconclusive. Among the investigated eight durum cultivars, cv 'Rugby' (Fig. 1, lane RY) yielded the short 'Wascana'-sized fragment, while cvs 'Astrodur', 'DT433', 'Primadur' (Fig. 1, lane PR), 'Fortore' and 'Gianni' all produced the same fragment as cv 'Duilio'. This distribution correlated with the occurrence of γ-gliadins 42 and 45 (as detected by APAGE; data not shown), respectively, which in turn are markers strongly linked to poor and good pasta-making quality of durum wheat cultivars (Kosmolak et al. 1980).

Specific detection of GAG56D polymorphism

In order to simplify the analysis, to emphasize the size difference and to avoid possible ambiguities arising from alleles of GAG56B, a GAG56D-specific PCR system amplifying a shorter product had to be designed. Two upper (GAG15 and GAG17) and two lower (GAG16 and GAG18) PCR primers were set on nucleotide polymorphisms between GAG56D and GAG56B (see Table 1, mismatches depicted in bold print). Primer GAG15 differed at four positions and the other three primers at one position each compared to the sequence of acn. M13712. All four combinations of the primers were evaluated using genomic DNA of cv 'Forno' and gave products of desired size. Restriction analysis with *Bst*4CI, *Nsi*I and *Bsm*I was used to exclude fragments originating from GAG56B. PCR with primer combinations GAG15/16 and GAG17/18 were further optimized. The annealing and extension time (2-step PCR) using primers GAG17 and GAG18 had to be reduced to 1 min and 20 s to eliminate weakly appearing, larger fragments (most likely) of GAG56B. Both PCR systems including subsequent restriction with *Bst*4CI were applied to the entire panel of 43 *Triticum* cultivars (Figs. 5 and 6). All hexaploid cultivars produced the sought products of about 200 and 350 bp, respectively. Some of the durum wheat cultivars yielded the just mentioned unspecific fragments using the GAG17/18-PCR system (Fig. 5A, lanes AR – WA). However, no amplification products were detected from any of the eight durum wheats when primers GAG15 and GAG16 were used (Fig. 6A, lanes AR – WA). With both systems, PCR products obtained from all spelts, spelt-wheat crosses (Figs. 5A and 6A, lanes AD – SL and AD – RN, respectively) and, unexpectedly, from wheat cv 'Chinese Spring' (lane CS) were shorter compared to the wheat fragments (lanes AI – TT and AI –NA, respectively). This correlated perfectly with results obtained by digestion of the PCR products with *Bst*4CI

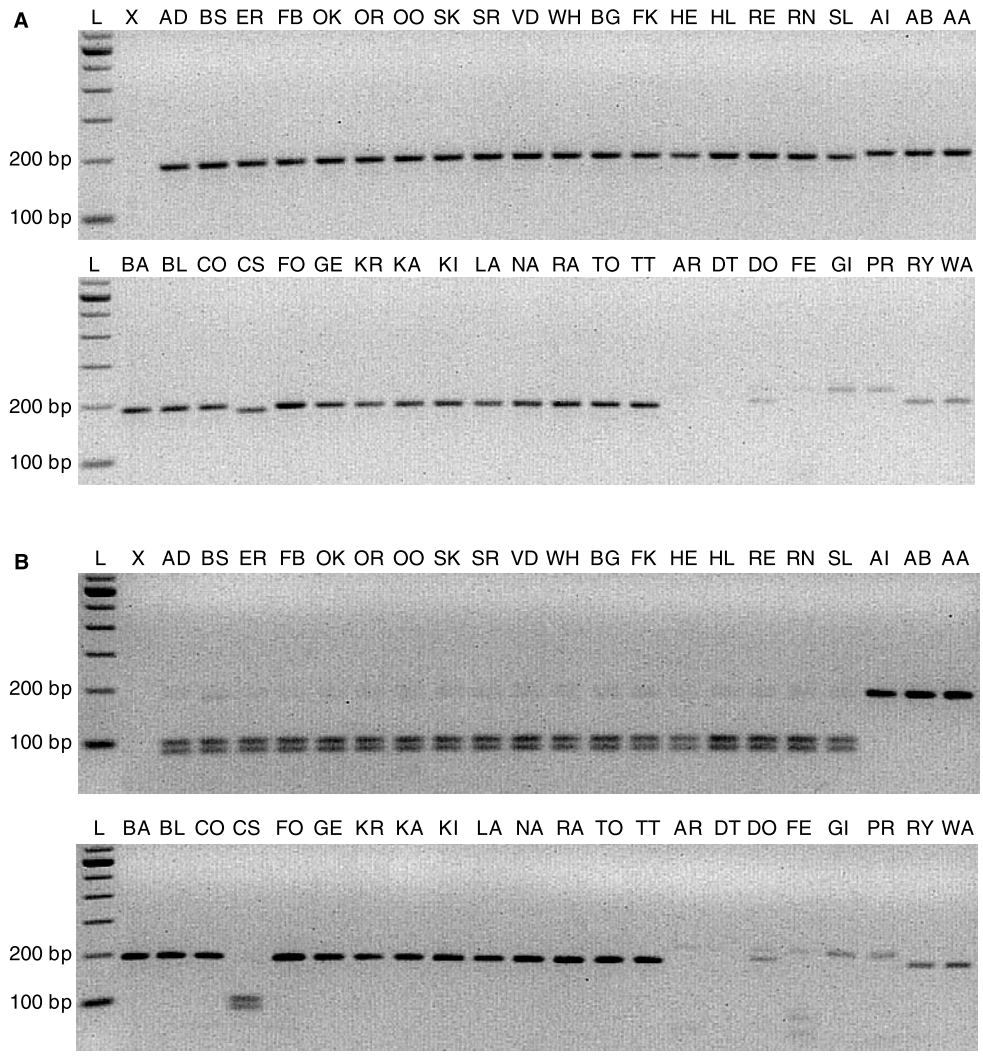

Fig. 5A, B PCR using primers GAG17 and GAG18 and restriction with *Bst*4CI. **A** PCR using GAG17 and GAG18 was applied to all 43 *Triticum* cultivars under investigation. All hexaploid cultivars (*AD – TT*) produced a fragment of about 200 bp amplified from GAG56D. Some of the durum cultivars yielded an unspecific band originating from GAG56B (*AR – WA*). The PCR products of all spelts (*AD – WH*), spelt-wheat crosses (*BG – SL*) and wheat cv 'Chinese Spring' (*CS*) were shorter than the ones of hexaploid wheat (*AI – TT*). **B** The PCR products obtained with GAG 17 and GAG18 were restricted with *Bst*4CI. The products amplified from spelts (*AD – WH*), spelt-wheat crosses (*BG – SL*) and wheat cv 'Chinese Spring' (*CS*) were cut into two fragments of (calculated) 97 and 82 bp, respectively, while the bread wheat bands (*AI – TT*) and unspecific durum bands (*AR – WA*) remained intact. Assay conditions: 2.5% agarose gels, 10 µl of PCR products or restricted PCR products. *L* 100-bp ladder (bolder 600-bp band), *X* negative control. Two-letter abbreviations are as defined in Materials and Methods

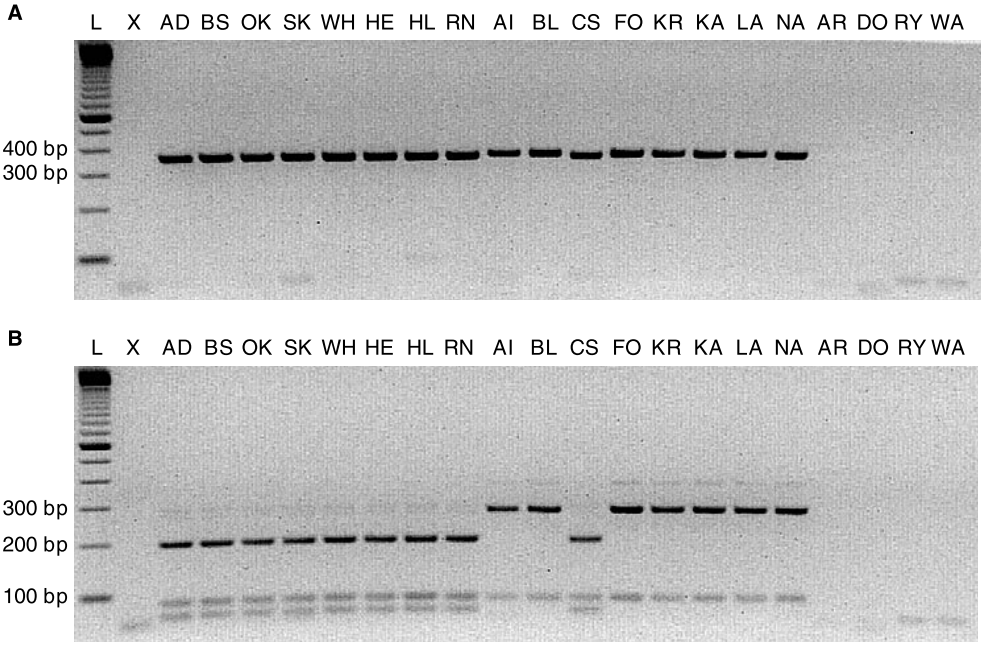

Fig. 6A, B PCR using primers GAG15 and GAG16 and restriction with *Bst*4CI. **A** PCR using GAG15 and GAG16 was applied to a representative set of 20 *Triticum* cultivars. The 16 hexaploid cultivars (*AD – NA*) each yielded a product of about 350 bp, while no fragments were amplified by the durum wheats (*AR – WA*). **B** The GAG15/16 fragments were digested with *Bst*4CI. Fragments amplified from spelts and spelt-wheat crosses (*AD – RN*) as well as wheat cv 'Chinese Spring' (*CS*) were cut twice into bands of (calculated) 196, 87 and 67 bp, respectively. The PCR products obtained from wheat were restricted only once giving fragments of 272 and 87 bp, respectively. Assay conditions: 2.5% agarose gels, 10 µl of PCR products or digested PCR products. *L* 100-bp ladder (bolder 600-bp band), *X* negative control. Two-letter abbreviations are as defined in Materials and Methods

(Figs. 5B and 6B). The spelt, spelt-wheat cross and 'Chinese Spring' PCR products all contained the additional restriction site as compared to corresponding wheat fragments, i.e. one site versus none in the GAG17/18- and two sites versus one in the GAG15/16-PCR product.

Discussion

PCR primers GAG5 and GAG6 were designed on published γ-gliadin gene sequences. Applied to genomic DNA of 43 hexaploid and tetraploid *Triticum* cultivars, they produced one to three PCR fragments which were found to originate from two γ-gliadin genes localized to chromosomes 1D and 1B and subsequently referred to as GAG56D and GAG56B, respectively. The occurrence of the two alleles of GAG56D strongly correlated with the phenotypic designations spelt and wheat. Two GAG56D-specific detection systems were developed and applied. Two different alleles of GAG56B correlating with the appearance of γ-gliadins 42 and 45 (Kosmolak et al. 1980), respectively, were found among the eight investigated durum wheat cultivars.

Through this work, we have demonstrated the feasibility of our approach and how well-suited PCR analysis can be in gliadin research. A possible pitfall, however, is the appearance of hybrid bands without actual genomic template from as few as two very similar PCR products (e.g. exhibited by cv 'Chinese Spring' in Fig. 2). This would probably disable PCR-fingerprinting of gliadin genes analogous to gliadin protein analysis with APAGE (Ng et al. 1988). Primers GAG5 and GAG6 amplified fragments of alleles of the genes the primers were designed on, but no completely new genes were discovered. With the GAG56D-specific PCR systems, we also showed that it is possible to target one specific gliadin gene among the large number occurring in any given variety (Anderson and Greene 1997; Payne 1987) as was previously demonstrated for HMW and LMW glutenin genes (D'Ovidio and Anderson 1994; D'Ovidio and Porceddu 1996).

Two polymorphisms were found by sequencing the GAG56D fragments of spelt cultivar 'Oberkulmer' and wheat cultivar 'Forno', a 9-bp deletion/duplication and a single nucleotide change. When the 35 hexaploid cultivars were tested with the GAG56D-specific detection systems that we developed, we found that all contained only one of these two alleles. Both mutations occurred exclusively coupled (Figs. 5 and 6). It was rather surprising not to find more alleles considering the very polymorphic nature of the gliadins (Shewry and Tatham 1990). The 'Forno' allele [identical to genomic clone pW10 (acn. M16060) from wheat cultivar 'Yamhill' (Scheets et al. 1985)] was found in all wheat cultivars except 'Chinese Spring', regardless of phenotypic features such as sowing season (e.g. cvs 'Forno' versus 'Lona') or bread-making quality (cvs 'Neepawa' versus 'Camino'). All spelt and spelt-wheat cross cultivars, on the other hand, carried the allele characterized from cv 'Oberkulmer'. From the agronomic point of view, the investigated spelt-wheat crosses are considered spelts that contain wheat introgression to improve field performance. Therefore, the occurrence of the two alleles of GAG56D correlated perfectly with the agronomic use of the terms "spelt" and "wheat" with the exception of wheat cv 'Chinese Spring' carrying the spelt allele. If we regard spelt as the progenitor of modern bread wheat (Sears 1974), the spelt allele could represent the ancestral gene from which the wheat counterpart evolved. 'Chinese Spring', an old landrace, would also contain the original allele. On the other hand, the occurrence of these two alleles might be evidence for two different origins of hexaploid wheat as suggested by Talbert et al. (1998). Testing of a larger, geographically more diverse selection of bread wheat cultivars and different varieties of D-genome donor *T. tauschii* could offer answers supporting either hypothesis and lead to the discovery of more alleles of GAG56D.

Coupled with the *Bst*4CI restriction, the two GAG56D-specific PCR systems provide a rapid and unequivocal screening method for the two alleles. The GAG17/18 system (Fig. 5) is faster and more economical due to shorter PCR cycles and less endonuclease required for the single restriction site of the PCR product, but it has the drawback that some tetraploid cultivars produce a GAG56B fragment, given the absence of a competing GAG56D template. Sequencing of the 'Wascana' allele indicated a perfect match for primer GAG17. Therefore, it was no surprise that cvs 'Wascana' and 'Rugby', which carry this allele, yielded the strongest of the undesired signals. Primers GAG15 and GAG16 provide a more selective, but also more time- and enzyme-consuming detection system (Fig. 6). Finally, it is no problem to scale down either PCR reaction volume to save reagents in extensive screenings.

The GAG56B fragments were found to be alleles of clone L311A (acn. M13712), a γ-gliadin pseudogene located in the 5'-half of a 10-kb genomic clone isolated from wheat cultivar 'Yamhill' (Rafalski 1986). The functional γ-gliadin gene in the 3'-half, clone L311B (acn. M13713), is therefore also located on chromosome 1B. Our results contradict a report (Schlegel 1997) that cvs 'Forno' and 'Tamaro' carried a rye-wheat 1RS.1BL translocation (besides cv 'Bernina'). Both cultivars in question amplified a GAG56B fragment and did not produce a positive signal using a ω-secalin PCR. Instead it appeared that cvs 'Hubel', 'Arbola' and 'Toronit' possessed the translocation. At least three alleles in addition to acn. M13712 were detected. Preliminary sequencing indicated that the length polymorphism mainly stemmed from a variable number of glutamine codons in a poly-Q stretch at the end of region B (Kreis et al. 1985a, b). Only two alleles occurred among the tested durum cultivars. The longer and shorter allele correlated with the appearance of γ-gliadins 45 and 42, which in turn are markers for good and bad gluten performance, respectively (Kosmolak et al. 1980). Thus, the GAG56B alleles proved to be yet another molecular marker for pasta-

making quality of durum wheats (D'Ovidio and Porceddu 1996; D'Ovidio et al. 1990; Pogna et al. 1990). The testing of durum cultivars that possess neither the LMW-1 nor LMW-2 glutenin subunit alleles genotype [e.g. cv 'Dritto' (D'Ovidio and Porceddu 1996)] could yield additional alleles of GAG56B. Moreover, extended analysis (using a GAG56B-specific PCR system) of the allele distribution of this rather polymorphic gene among di-, tetra- and hexaploid *Triticum* species could help in solving the long-disputed identity of the B-genome donor to allopolyploids (Nath et al. 1984; Sears 1974).

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