Theor Appl Genet (2004) 109: 1295–1302
DOI 10.1007/s00122-004-1743-4

ORIGINAL PAPER

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Genome-specific primer sets for starch biosynthesis genes in wheat

Received: 22 March 2004 / Accepted: 1 June 2004 / Published online: 31 August 2004

Abstract Common wheat (*Triticum aestivum* L., 2*n*=6*x*=42) is an allohexaploid composed of three closely related genomes, designated A, B, and D. Genetic analysis in wheat is complicated, as most genes are present in triplicated sets located in the same chromosomal regions of homoeologous chromosomes. The goal of this report was to use genomic information gathered from wheat–rice sequence comparison to develop genome-specific primer sets for five genes involved in starch biosynthesis. Intron locations in wheat were inferred through the alignment of wheat cDNA sequences with rice genomic sequence. Exon-anchored primers, which amplify across introns, allowed the sequencing of introns from the three genomes for each gene. Sequence variation within introns among the three wheat genomes provided the basis for genome-specific primer design. For three genes, ADP-glucose pyrophosphorylase (*Agp-L*), sucrose transporter (*SUT*), and waxy (*Wx*), genome-specific primer sets were developed for all three genomes. Genome-specific primers were developed for two of the three genomes for *Agp-S* and starch synthase I (*SsI*). Thus, 13 of 15 possible genome-specific primer sets were developed using this strategy. Seven genome-specific primer combinations were used to amplify alleles in hexaploid wheat lines for sequence comparison. Three single nucleotide polymorphisms (SNPs) were identified in a comparison of 5,093 bp among a minimum of ten wheat accessions. Two of these SNPs could be converted into cleaved amplified polymorphism sequence (CAPS) markers. Our results indicated that the design of genome-specific primer sets using intron-based sequence differences has a high probability of success, while the identification of polymorphism among alleles within a genome may be a challenge.

Communicated by P. Shewry

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Introduction

Common wheat is economically one of the most important crops. The chromosome complement of common wheat is composed of three closely related genomes, designated A, B, and D. The allohexaploid nature of wheat provides unique experimental challenges and opportunities. Most wheat genes are present in triplicated sets, usually located in the same chromosomal regions of homoeologous chromosomes (Akhunov et al. 2003). This genetic redundancy has made it difficult to follow specific loci in genetic and breeding projects. The development of genome-specific markers is hence an important goal to sustain advances in wheat genetics and breeding. Polymerase chain reaction (PCR)-based markers, such as those employing simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs), can potentially be genome-specific, allowing hexaploid wheat to be treated as a diploid in genetic analysis. Numerous genome-specific SSR markers have been developed for hexaploid wheat (Pestsova et al. 2000; Röder et al. 1995; US Wheat and Barley Scab Initiative 2001). Their advantage is that they are highly polymorphic, even though wheat suffers generally from low levels of molecular polymorphism. Their disadvantage is that they are not suitable for very high-throughput detection assays. In contrast, numerous high-throughput detection assays have been developed for SNPs, and hence, the development of wheat genome-specific SNP markers is a logical goal.

Because of the great abundance of repetitive sequence in the wheat genome, the development of genome-specific PCR primers is usually most successful if genic regions are targeted in primer design. Introns and untranslated regions are usually more polymorphic than exons (Haga et al. 2002). Allele-specific markers based on intron differ-

ences have been made in tomato, sunflower, and maize (Chetelat et al. 1995; Hongtrakul et al. 1998; Esen and Bandaranayake 1998). Amplification of genomic DNA using exon-anchored primers that amplify across one or more introns allows cloning and sequencing of introns and the development of allele-specific markers (e.g., Van Campenhout et al. 2003).

In the absence of extensive wheat genomic sequence, the location of introns in wheat genes can be inferred by alignment of wheat cDNA sequences and expressed sequence tags (ESTs) with the rice genomic sequence, since the intron positions are highly conserved between wheat genes and those of its Triticeae relatives and rice genes (Dubcovsky et al. 2001; Ramakrishna et al. 2002). Such alignments allow the design of primer sets, which amplify one or more introns from genomic DNA of each wheat genome. Sequence variation within introns among the three wheat genomes can then be exploited to design genome-specific primer sets.

The enzyme that controls the rate-limiting step in the starch biosynthetic pathway (for reviews see Hannah 1997; Preiss 1997), ADP-glucose pyrophosphorylase (AGP), is the product of the *Agp-L* and *Agp-S* subunits. Modifications in *Agp-L* can lead to measurable changes in starch biosynthesis and grain yield. The presence of the modified maize transgene (*Sh26sh*) in wheat and rice resulted in increased seed yield in greenhouse experiments (Smidansky et al. 2002, 2003). Several other enzymes also have essential roles in starch biosynthesis in wheat. Sucrose transporter (SUT) controls sucrose uptake and downloading into sink cells (Lemoine 2000). The production of the starch components, amylose and amylopectin, is controlled by granule-bound starch synthase (GBSSI, also known as waxy) and soluble starch synthases (SSI and SSII), respectively (Shure et al. 1983).

In this study, we have evaluated the strategy outlined above for the development of genome-specific primers for five genes involved in starch biosynthesis in wheat. Additionally, genome-specific primer sets were used to amplify alleles in a set of hexaploid wheat lines for sequence comparison, and subsequent design of PCR-based screening techniques.

Table 1 Conserved exon-anchored and genome-specific primers for starch biosynthetic genes, with requisite amplification parameters and product sizes

Gene name	Sequence (5′→3′)		PCR conditions[a]	Product length
	Forward	Reverse		
Agp-L				
Conserved 1[b]	TTCCGCGGAACAGCGGATGCC	GAAGACATAGACTCCCATAG	PCR 50	
Conserved 2[b]	CAAAGAAGCGATCATTTCGC	TTTATTTACACGAACATCCC	PCR 50	
A-specific	CAAAGAAGCGATCATTTCGC	TTAGATTATTAAAGTAGTGATC	PCR 50	926
B-specific	ATCTGGATACTTGTACATCTGC	GAAGACATAGACTCCCATAG	PCR 50	672
D-specific	CACATGACCCTCCTTGCACCTC	GAAGACATAGACTCCCATAG	PCR 50	688
Agp-S				
Conserved	CCTTCACCAATAACACTGTCC	CATTCAGGCACACAGAGAAAC	PCR 55	
B-specific	CCGATAACGTATGACTCGAC	CATTCAGGCACACAGAGAAAC	PCR 50	695
D-specific	GCAAATCCCCTTTCTTACGAT	CATTCAGGCACACAGAGAAAC	Touchdown	712
Wx				
A-specific	TCGCTCTGCATATCAATTTTGC	GGAACTGGCAAGAAGGACTG	PCR 55	1,022
B-specific	GCGTCGTCTCCGAGGTACAC	GTCGAAGGACGACTTGAACC	PCR 60[c]	870
D-specific	CCATGGCCGTAAGCTAGAC	GTCGAAGGACGACTTGAACC	PCR 60[c]	1,124
SUT				
Conserved	TGCGCAAATCTGAAAGGCGC	GTAGATCTCACGACCCATCC	PCR 57	
A-specific	AGTCCTTTTTTTCGGTGTGAA	GTAGATCTCACGACCCATCC	PCR 55	369
B-specific	CACTCCCTAGTGTTTATTAT	GTAGATCTCACGACCCATCC	PCR 55	485
D-specific	ACCTGGTCCTTTTTTCCGTG	GTAGATCTCACGACCCATCC	PCR 55	379
SsI				
Conserved	TGAATGTCGACATCGCGGTT	AAAGGAGAGGAGGGTACAGG	PCR 60	
A-specific	TGCACAAGTGTCGAACAAGG	AAAGGAGAGGAGGGTACAGG	PCR 55	247
B-specific	TATCGTCACAAAGGGGCACT	AAAGGAGAGGAGGGTACAGG	PCR 55	445

[a]*PCR* 50: 30X (94°C–1 min, 50°C–1 min, 72°C–1.2 min); PCR 55: 30X (94°C–1 min, 55°C–1 min, 72°C–1.2 min); PCR 57: 30X (94°C–1 min, 57°C–1 min, 72°C–1.2 min); PCR 60: 30X (94°C–1 min, 60°C–1 min, 72°C–1.2 min); Touchdown: 15X (94°C–1 min, 65°C–dec 1°C/cycle, 72°C–1.2 min) + 30X (94°C–1 min, 45°C–1 min, 72°C–1.2 min). Each PCR program starts with 4 min–94°C and ends with 7 min–72°C

[b]Conserved 1 primer set amplifies chromosome 1B and 1D products only, while conserved 2 primer set amplifies a chromosome 1A product

[c]TaKaRa LA *Taq* produced better amplification than our standard Promega *Taq* polymerase for this primer set

Materials and methods

Design of exon-anchored primers based on wheat–rice synteny

The wheat starch biosynthetic genes selected for this study were AGP large and small subunits (*Agp-L*, *Agp-S*), sucrose transporter *(SUT*), waxy (*Wx*), and soluble starch synthase I (*SsI*). cDNA sequences for these genes were obtained from GenBank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov), while for *Wx*, the complete genomic sequence is available (Murai et al. 1999). BLAST searches with each cDNA were conducted using the Wu-BLAST, version 2.0, program: *blastn* and database: All Rice BAC and PAC Sequences in GenBank (The Institute for Genomic Research, http://www.tigr.org/tdb/e2 k1/osa1/). Default settings were used for the BLAST search. The BAC/PAC with the highest score and mapping to the rice chromosome syntenic with the wheat location was selected for predicting intron–exon junctions in wheat. Exon-anchored primers were designed to span one or more intron regions. Between five and ten primer pairs were designed for each cDNA. Primers were screened in several combinations for the ability to amplify the postulated products. PCR was performed on genomic DNA of the variety Chinese Spring and the appropriate nulli-tetrasomic (N-T) mapping stocks (Sears 1954). This allowed the amplified products to be assigned to a specific chromosome in a specific genome. As more than one product was amplified from Chinese Spring, it was necessary to clone each specific product prior to sequencing. This was done using the pGEM-T vector (Promega). Clones were identified by PCR with exon-anchored primers and purified for sequencing with Wizard Plus SV Minipreps (Promega). Sequencing was performed at the Washington State University Sequencing Lab.

Genome-specific primer design

Alignments of sequence obtained from Chinese Spring identified differences between genomic sequences that were exploited for the design of genome-specific primers. Putative genome-specific primers were paired with one of the original exon-anchored primers. PCR was performed on DNA from Chinese Spring and the appropriate N-T lines to verify genome specificity. Sequences of the exon-anchored and genome-specific primer sets are listed in Table 1.

SNP identification

Genome-specific primers were used to amplify portions of specific starch biosynthesis alleles in a set of 10–14 diverse wheat accessions (Table 2). Sequence alignments were screened for SNPs between accessions. SNPs were screened for restriction sites to convert to cleaved amplified polymorphic sequence (CAPS) markers where possible.

Table 2 Wheat accessions used for sequence survey of genome-specific starch biosynthetic alleles

Accessions	Sequenced Alleles						
	Agp-L			*Agp-S*		*SUT*	*Wx*
	A	B	D	B	D	D	A
Reeder	x	x		x	x	x	x
McNeal			x	x	x	x	x
Hi-line	x	x	x	x	x	x	x
Russ		x	x	x	x	x	x
Era	x	x	x	x	x	x	x
Thatcher	x	x	x	x	x	x	x
Red Fife	x	x	x	x	x	x	x
Red Egyptian	x	x	x	x	x	x	x
Jackson	x	x	x				x
Bigsky	x						
Knox			x	x	x		
Asosan				x	x		x
Trumbull	x	x	x	x	x		
Purplestraw			x	x	x	x	
PI 191100		x	x	x	x	x	x
PI 430130				x	x		x

Results

ADP-glucose pyrophosphorylase-L

The design of genome-specific primer sets is illustrated with the wheat *Agp-L* locus. Of the 2,040 bases of wheat cDNA sequence (GenBank accession: Z21969), bases 183–1750 were aligned with rice genomic sequence. Significant homology (BLAST score: 690) was observed with a rice BAC clone 10A19I on rice chromosome 1 (Fig. 1). Primer sets were designed to span one or more introns based on this alignment. Additional primer sets were designed from the cDNA sequence in the area that did not align with rice (represented by dotted lines in Fig. 1). Five forward and five reverse primers were designed. Primers 3 and 4 amplifed two products in Chinese Spring and N-T N1A-T1D (Fig. 2a). These can be assigned to chromosomes 1B and 1D, respectively, based on their absence in the relevant N-T lines. These DNAs were cloned, sequenced, and aligned, revealing that a 26-bp insertion/deletion (indel) event distinguishes the 1B and 1D sequences. Primer 3D (located within this insertion), used in combination with primer 4 amplified template from Chinese Spring, N1A-T1D, and N1B-T1D, but not N1D-T1B, so that this primer pair is D genome-specific (Fig. 2a). Primer 3B, spanning the deletion, in combination with primer 4, amplified a product from Chinese Spring, N1A-T1D, and N1D-T1B, but not N1B-T1D, so that this primer set is B genome-specific (Fig. 2a). Primers 7 and 10 amplified two products in Chinese

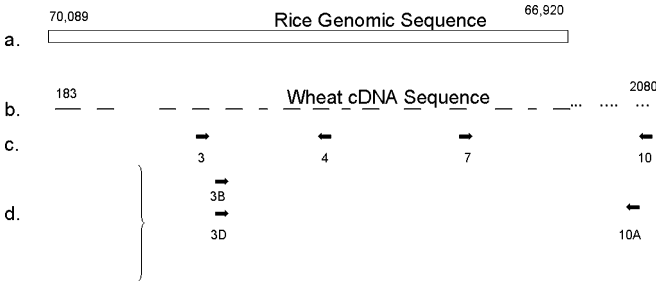

Fig. 1 Diagram of alignment between wheat *Agp-L* cDNA sequence and rice genomic sequence showing locations of conserved and genome-specific primer sets **a** A portion of rice BAC clone 10A19I (GenBank accession: AC007858). **b** Wheat *Agp-L* cDNA sequence (GenBank accession: Z21969). The *gaps* represent introns. The *dotted lines* represent wheat sequence that did not align with the rice genomic sequence. Intron locations for this portion of the sequence are based on wheat genomic sequence obtained in the present experiments. **c** Location of conserved primer sets. **d** Location of genome-specific primer sets

Spring, one of which is A genome-specific. The sequences of these two products differ by a 76-bp insertion in the A-genome-specific product. Primer set 7 and 10A (located in the insertion) amplifies from Chinese Spring, N1B-T1D and N1D-T1B but not from N1A-T1D, identifying this primer pair as A genome-specific (Fig. 2a).

The three genome-specific primer sets were used to amplify DNA from a set of 12 wheat accessions. A total of 926, 672, and 688 bases of sequence were obtained for the A, B, and D genomes, respectively. No sequence variation was observed for either the A or D genome sequences, but one SNP was observed for the B-specific sequence. This polymorphism defined a *Hin*dIII restriction site in the intron adjacent to the one used for primer design, allowing for the development of a CAPS marker to distinguish the alternative alleles (Fig. 2b).

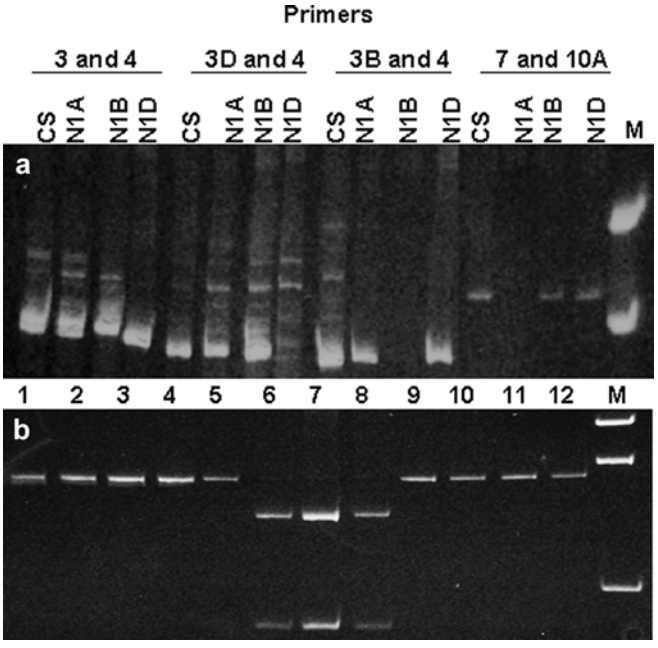

Fig. 2 **a** Gel electrophoresis of genome-specific primer sets developed for the *Agp-L* locus. Each primer pair was used to amplify Chinese Spring (*CS*), N1A-T1D (*N1A*), N1B-T1D (*N1B*), and N1D-T1B (*N1D*), respectively. Primer pairs: exon-anchored primers 3 and 4, D-genome-specific primers 3D and 4, B-genome-specific primers 3B and 4, and A-genome-specific primers 7 and 10A. M-restriction fragments of 1,769 and 676 bp from pUC18 digested with *Rsa*I. The locations of the primers used for amplification are shown in Fig. 1. **b** Gel of *Hin*dIII polymorphism detected in B-genome allele of *Agp-L* among a set of wheat accessions. *Lanes 1–8* B-genome allele amplified with primers 3B and 4 and digested with *Hin*dIII. *Lanes 9–12* B-genome allele amplified with primers 3B and 4, undigested. *Lane 1* Russ, *lane 2* Hi-Line, *lane 3* Thatcher, *lane 4* Era, *lane 5* Red Fife, *lane 6* Reeder, *lane 7* Jackson, *lane 8* PI191100, *lane 9* Hi-Line, *lane 10* Era, *lane 11* Reeder, *lane 12* PI 191100. M-restriction fragments of 1,769, 676, and 241 bp from pUC18 digested with *Rsa*I

ADP-glucose pyrophosphorylase-S

Bases 29–1438 of the 1,747-bp wheat cDNA sequence of AGP small subunit (*Agp-S*) (GenBank accession: X66080) aligned with rice chromosome 8 BAC P0410E11 (GenBank accession: AP004459). Exon-anchored primers were designed which amplified three products from Chinese Spring DNA. These three products were assigned to chromosomes 7A, 7B, and 7D (Fig. 3a). Sequencing revealed that a 28-bp indel differentiated the 7A from the 7B amplicon. Part of this indel distinguished the 7D product from those of 7A (17 bp) and 7B (11 bp). Genome-specific primers for 7B and 7D alleles of *Agp-S* were designed based on this indel sequence (Fig. 3b, c), but although several potential 7A-specific primers were designed, none proved to be genome-specific. No sequence variation was uncovered in 694 bp of the 7B locus (14 wheat accessions), or in 712 bp of the 7D locus (13 wheat accessions).

Sucrose transporter

The three homeologous versions of *SUT*, have been identified by Aoki et al. 2002. While alignment of the three sequences identified exonic differences between genomes, rice genome comparison was needed to identify intron locations. BLAST results identified significant homology between bases 898 and 1847 of the 2,134-bp *SUT*1A cDNA and rice chromosome 3 BAC OSJNBa 0091P11 (GenBank: accession AC073556). Exon-anchored primers amplified three products from Chinese Spring DNA, which were assigned to chromosomes 4A, 4B, and 4D (Fig. 4a). Sequencing revealed several indels between the 4A, 4B, and 4D loci. These differences could be used to design genome-specific primers for the 4A, 4B, and 4D *SUT* loci (Fig. 4b). No sequence differences were revealed among ten wheat accessions over the 379 bp portion of the *SUT* D-genome locus.

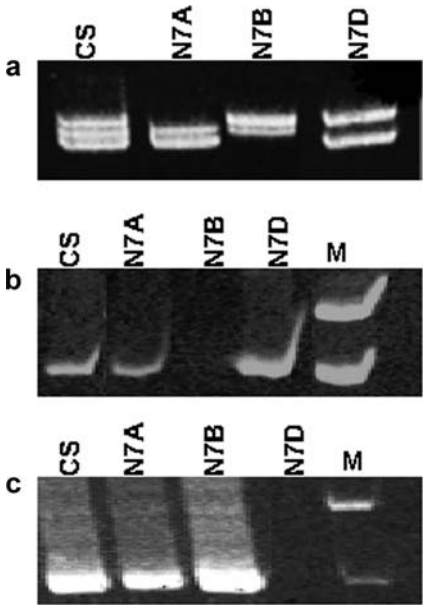

Fig. 3 Gel electrophoresis of genome-specific primer sets developed for the *Agp-S* locus. Each primer pair was used to amplify Chinese Spring (*CS*), N7A-T7B (*N7A*), N7B-T7A (*N7B*), and N7D-T7A (*N7D*), respectively. M-restriction fragments of 1,769 and 676 bp from pUC18 digested with *Rsa*I. Amplification with **a** exon-anchored primers, **b** B-genome-specific primers, and **c** D-genome-specific primers

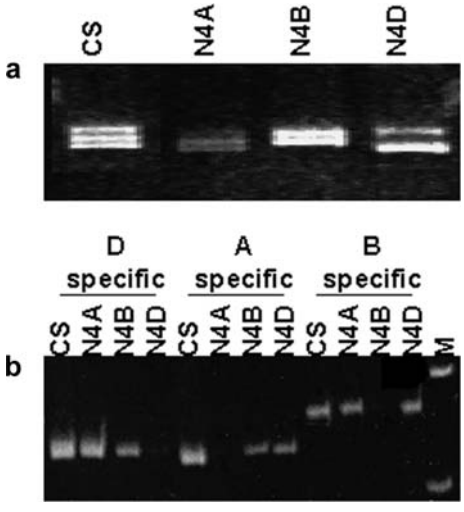

Fig. 4 Gel electrophoresis of genome-specific primer sets developed for the *SUT* locus. Each primer pair was used to amplify Chinese Spring (*CS*), N4A-T4D (*N4A*), N4B-T4D (*N4B*), and N4D-T4B (*N4D*), respectively. M-restriction fragments of 676 and 241 bp from pUC18 digested with *Rsa*I. Amplification with **a** exon-anchored primers and **b** A-, B-, and D-genome-specific primers

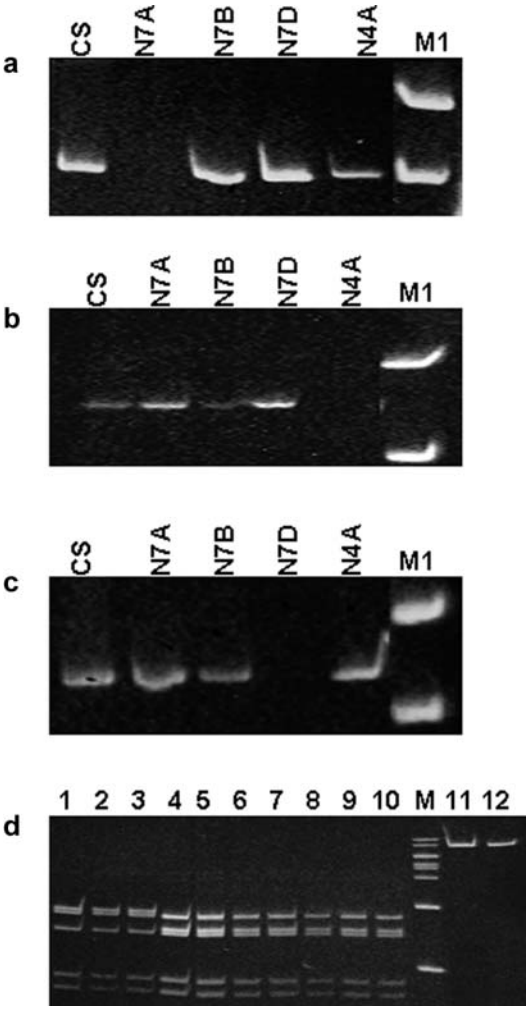

Fig. 5 Gel electrophoresis of genome-specific primer sets developed for the *Wx* locus. Each primer pair was used to amplify Chinese Spring (*CS*), N7A-T7B(*N7A*), N7B-T7A (N7B), N7D-T7A (*N7D*), and N4A-T4D (*N4A*), respectively. Amplification with **a** A-genome-specific primers. M1-Restriction fragments of 1,769 and 676 bp from pUC18 digested with *Rsa*I, **b** B-genome-specific primers. (Note the missing band is located on chromosome 4A due to a translocation of a segment of chromosome 7B to 4A.) M1-restriction fragments of 1,769 and 676 bp from pUC18 digested with *Rsa*I. **c** D-genome-specific primers. M1-restriction fragments of 1,769 and 676 bp from pUC18 digested with *Rsa*I. **d** Gel of *Hpy* 99I polymorphism detected in the A-genome allele of *Waxy* among a set of wheat accessions. *Lanes 1-10* A-genome allele amplified and digested with *Hpy* 99I. *Lanes 11-12* A-genome allele undigested. *Lane 1* Era, *lane 2* Thatcher, *lane 3* Russ, *lane 4* Red Egyptian, *lane 5* Jackson, *lane 6* Hi-Line, *lane 7* McNeal, *lane 8* Reeder, *lane 9* PI191100, *lane 10* PI430130, *lane 11* Hi-Line, *lane 12* Thatcher. M-markers: 1,000 bp (double band), 750, 500 (double band), 300, 150, and 50 bp

Waxy

Complete genomic sequences of the three *Wx* loci of wheat are available (Murai et al. 1999). Genome-specific primers for *Wx-A1*, *Wx-B1*, and *WX-D1* were designed from these published sequences (Fig. 5a–c). The *Wx-B1* locus maps to a segment of chromosome 7B translocated to chromosome 4A. A 1,022-bp portion of the *Wx-A1* allele was sequenced from 12 wheat accessions, and two SNPs were identified, both within exon 6. One SNP characterized an *Hpy* 99I restriction site that allowed the design of a CAPS marker (Fig. 5d).

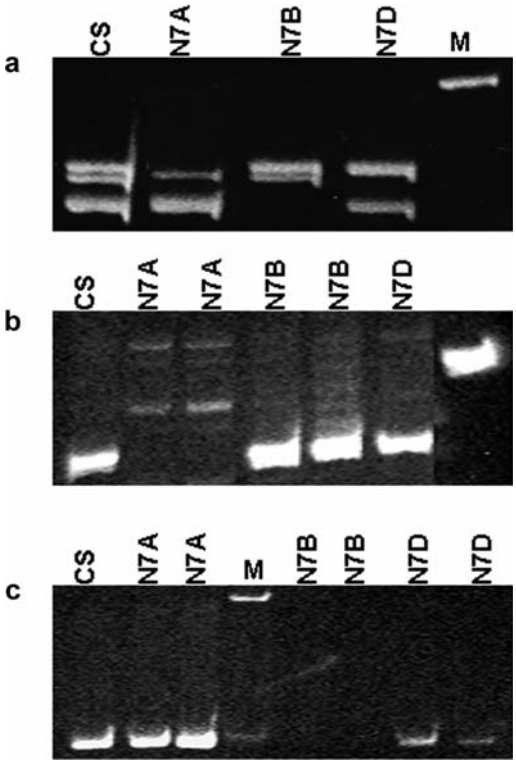

Fig. 6 Gel electrophoresis of genome-specific primer sets developed for the *SsI* locus. Each primer pair was used to amplify Chinese Spring (*CS*), N7A-T7B (*N7A*), N7B-T7A (*N7B*), and N7D-T7A (*N7D*), respectively. Amplification with **a** exon-anchored primers. M-Restriction fragment of 241 bp from pUC18 digested with *Rsa*I. **b** A-genome-specific primers. M-restriction fragment of 241 bp from pUC18 digested with *Rsa*I. **c** B-genome-specific primers. M-restriction fragments of 676 and 241 bp from pUC18 digested with *Rsa*I

Starch synthase I

Bases 1–1460 of the 2,591 bp of the wheat soluble *SsI* cDNA aligned with the rice chromosome 6 BAC clone P0681F10 (GenBank accession: AB026295). Exon-anchored primers amplified three products mapping to chromosomes 7A, 7B, and 7D. A 15-base indel was used to design a 7A-specific primer, while a 97-base indel was used to design a 7B-specific primer (Fig. 6a, b).

Discussion

The objective of this work was to assess the feasibility of designing genome-specific primer sets for genes involved in starch biosynthesis in wheat. Such PCR primer sets would in effect allow hexaploid wheat to be treated as a diploid species in regard to DNA-based molecular analysis of these genes. Most of the sequence data available for wheat are cDNA based, and variability between and within genomes in these exon-derived sequences is limited. However, alignment of wheat cDNA sequence with rice genomic sequence allows identification of intron positions, and subsequent design of exon-anchored primer sets that amplified DNA from one or more introns. Such primer sets were used in this experiment to amplify the starch biosynthesis-related genes from the three wheat genomes for design of genome-specific primer sets.

All of the wheat cDNA sequences tested aligned with rice genomic sequence. The numbers of introns identified by the wheat–rice comparisons were 17, 8, 6, and 13 for *Agp-L*, *Agp-S*, *SUT*, and *SsI*, respectively. This is a minimal estimate of the number of introns per gene, in that portions of the *Agp-L*, *Agp-S*, *SUT*, and *SsI* cDNA sequences did not show sufficient homology to rice for alignment. Previous results (Aoki et al. 2002) showed a total of 12 introns in the wheat *SUT* gene compared to 13 in rice; however, the rice BAC identified with our BLAST search contained only a portion of the complete rice gene. Previously published wheat *Wx* genomic sequence showed a total of ten introns (Murai et al. 1999).

We attempted to develop genome-specific primer sets for *Agp-L*, *Agp-S*, *SUT*, *Wx*, and *SsI*, using the exon–intron junctions predicted from wheat–rice comparisons. We successfully designed genome-specific primer sets for all three genomes for *Agp-L*, *SUT*, and *Wx*, but were only able to obtain genome-specific primer sets for the B and D genomes for *Agp-S* and the A and B genomes for *SsI*. Thus, 13 of the 15 possible genome-specific primer sets were developed. All possibilities for exon-anchored amplification and intron-based primer design were not exhausted for the A-genome *Agp-S* gene. We tested six exon-anchored primer sets for the *Agp-S* locus, but only two differentiated the amplified products of the A genome relative to the B and D genomes. Ten primer sets, designed from intronic sequence differences, failed to specifically amplify the A genome. A similar situation occurred with *SsI*. It is possible that additional effort would yield the remaining genome-specific primer sets. However, it is also clear that some loci will be more amenable to this strategy than others.

Wheat genome-specific PCR primers can be used for various purposes, such as amplifying specific genomic sequences from hexaploid wheat for direct comparison between wheat and its diploid progenitors. For instance, Talbert et al. (1998) identified a D-genome-specific primer set among a set of several dozen primers designed from an *Aegilops tauschii* genomic library (Gill et al. 1991). This primer set was used to amplify and sequence a locus from wheat and its D-genome progenitor, *Ae. tauschii*. Haplotype comparison revealed that two distinct alleles from *Ae. tauschii* were also present in the wheat D genome, suggesting more than one source for the hexaploid wheat D-genome gene pool. The development of genome-specific primer sets for an array of important genes will provide an opportunity to more precisely determine the evolutionary history of wheat and the sources of existing variability.

In addition to utility in evolutionary studies, genome-specific primer sets would be ideal for monitoring segregating alleles in breeding populations. Their utility in this regard, depends upon nucleotide sequence polymorphism among alleles. We analyzed a total of 5,093 bp

from 10–14 wheat accessions (depending on the gene surveyed) for nucleotide sequence polymorphism. A total of three SNPs were identified. One was in an intron, while the two identified in the *Wx* gene were within a single exon. This amount of polymorphism, 1 SNP per 1.7 kb, is slightly lower than reported in previous studies. A frequency of 1 SNP/kb was observed by Bryan et al. (1999) in a group of seven accessions, including European wheat varieties, Chinese Spring, and a synthetic wheat. Somers et al. (2003) found 1 SNP per 540 bases in analyzing wheat EST sequences from seven cultivars. Blake et al. (1999) analyzed DNA sequence amplified by sequence-tagged-site (STS) primer sets for 14 single-copy loci in the B genome and nine loci in the D genome from four to nine accessions (depending on locus surveyed). A minimal estimate of SNP frequency, calculated using only parsimonious sites, was 1.1 and 0.4 per 1,000 bases for the B and D genomes, respectively. All of the previous studies and the present one assayed fewer than 15 accessions. Thus, only the most frequently occurring SNPs would be observed. Sequence analysis of more accessions may reveal additional SNPs and allow a more precise estimate of SNP frequency in hexaploid wheat.

It is difficult to make conclusions regarding the degree of polymorphisms in exons and introns based on the data we have obtained due both to limited numbers of nucleotides surveyed and to the nature of the genes we analyzed. Starch biosynthesis is clearly essential to wheat grain development and thus, it is likely that allelic variation may be constrained. Indeed, Whitt et al. (2002) examining six maize starch biosynthesis genes, including three that we studied (*Agp-L, Agp-S*, and *Wx*), found relatively low nucleotide diversity and significant evidence of selection for these genes. In general, the present study suggests that the development of genome-specific primer sets, using the rice alignment strategy will be relatively straightforward while identification of useful polymorphisms will require considerable effort.

Acknowledgements This publication is based on work supported by the National Science Foundation Grant No. DBI0321757 and USDA-IFAFS Project No. 2001-52100-11293. The authors gratefully acknowledge the assistance of Jason Cook, Kelly Hansen, Megan Hartzell, and Steve Morris.

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