Technical Note

Development of genome-specific primers for homoeologous genes in allopolyploid species: the waxy and starch synthase II genes in allohexaploid wheat (Triticum aestivum L.) as examples

Xiu-Qiang Huang and Anita Brûlé-Babel*

Abstract

Background: In allopolyploid crops, homoeologous genes in different genomes exhibit a very high sequence similarity, especially in the coding regions of genes. This makes it difficult to design genome-specific primers to amplify individual genes from different genomes. Development of genome-specific primers for agronomically important genes in allopolyploid crops is very important and useful not only for the study of sequence diversity and association mapping of genes in natural populations, but also for the development of gene-based functional markers for marker-assisted breeding. Here we report on a useful approach for the development of genome-specific primers in allohexaploid wheat.

Findings: In the present study, three genome-specific primer sets for the waxy (Wx) genes and four genome-specific primer sets for the starch synthase II (SSII) genes were developed mainly from single nucleotide polymorphisms (SNPs) and/or insertions or deletions (Indels) in introns and intron-exon junctions. The size of a single PCR product ranged from 750 bp to 1657 bp. The total length of amplified PCR products by these genome-specific primer sets accounted for 72.6%-87.0% of the Wx genes and 59.5%-61.6% of the SSII genes. Five genome-specific primer sets for the Wx genes (one for Wx-7A, three for Wx-4A and one for Wx-7D) could distinguish the wild type wheat and partial waxy wheat lines. These genome-specific primer sets for the Wx and SSII genes produced amplifications in hexaploid wheat, cultivated durum wheat, and Aegilops tauschii accessions, but failed to generate amplification in the majority of wild diploid and tetraploid accessions.

Conclusions: For the first time, we report on the development of genome-specific primers from three homoeologous Wx and SSII genes covering the majority of the genes in allohexaploid wheat. These genome-specific primers are being used for the study of sequence diversity and association mapping of the three homoeologous Wx and SSII genes in natural populations of both hexaploid wheat and cultivated tetraploid wheat. The strategies used in this paper can be used to develop genome-specific primers for homoeologous genes in any allopolyploid species. They may be also suitable for (i) the development of gene-specific primers for duplicated paralogous genes in any diploid species, and (ii) the development of allele-specific primers at the same gene locus.

Background

Polyploidy is recognized as a major force in plant speciation, with approximately 70% of flowering plants being polyploid [1]. Polyploids are divided into autoploids and allopolyploids. Allopolyploids contain two or more sets of related chromosomes that are brought together into the same nucleus by interspecific hybridization and followed by spontaneous chromosome doubling or unreduced gametes in nature [2]. Allopolyploids are preponderant in nature and include the world's most important crops such as cotton, canola, oat and wheat. In allopolyploid crops, homoeologous genes in different genomes exhibit a very high sequence similarity, especially in the coding regions of genes [3,4]. This makes it difficult to
design genome-specific primers to amplify individual genes from different genomes for sequence diversity and association mapping. Development of genome-specific primers for agronomically important genes in allotetraploid crops will facilitate not only the study of sequence diversity and association mapping of genes in natural populations, but also the development of gene-based functional markers for marker-assisted breeding. Here we describe a useful approach for the development of genome-specific primers in allotetraploid species.

Bread wheat (Triticum aestivum L.) is an allohexaploid species, consisting of three sets of subgenomes (A, B, and D), each of which contains seven pairs of homoeologous chromosomes. Its grain is the staple food for 35% of the world’s population [8,9]. The biosynthesis of starch is the major determinant of yield in wheat grains, because starch accounts for about 70% of grain dry weight [5]. Starch is composed of approximately 75% amylopectin and 25% amylose. Amylose is a relatively less-branched α-(1,4)-linked glucose polymer, whereas amylopectin is a branched glucose polymer in which α-(1,4) -linked polymer are connected by α-(1,6)-linkages. Starch synthesis involves a series of biosynthetic enzymes including ADP-glucose pyrophosphorylase (AGP), starch synthases (SS) and starch branching enzymes (SBE). Two distinct types of starch synthases, isoforms of granule-bound starch synthase (GBSSI, also known as Waxy) and soluble starch synthases (SSI, SSII and SSIII), are involved in the conversion of ADP-glucose to the starch polymers. The GBSSI is the major enzyme responsible for amylose production, whereas the soluble starch synthases with starch branching enzymes are thought to be involved in amylopectin synthesis [6]. Starch synthase II is thought to be involved in elongating the short chains of amylopectin [6]. SSII has a gene dosage effect on grain weight in wheat [7]. It is known that three homoeologous SSII genes, SSII-A, -B and -D, coding SGP (starch granule protein-1) -A1, -B1, and -D1, are located on chromosome arms 7AS, 7BS and 7DS respectively [12,15], respectively. To date, three homoeologous genomic sequences have been isolated and cloned for the other genes involved in starch synthesis [12].

The sequence diversity and association mapping of starch biosynthesis genes has been conducted in diploid cereal crops such as barley [16-18], maize [19,20], rice [21,22], sorghum [23] and foxtail millet [24]. The allohexaploid nature of common wheat and the homoeologous genes from the genomes A, B and D with a high level of sequence similarities in the coding regions make it very difficult to design genome-specific primers [3,4]. Development of one primer set from any region of gene in each genome based on large InDels (insertions or deletions) is not difficult. However, it is more challenging to develop many genome-specific primer sets from the homoeologous sequence regions, which cover the entire genes. Blake et al. [25] developed genome-specific primers for the Wx genes, however, only one primer set was designed for each genome and three genome-specific primers were not developed from the homoeologous sequence regions. Genome-specific primers for the SSII genes have not been reported so far. The objectives of the present study were to (i) develop genome-specific primers from the homoeologous sequence regions of both the Wx and SSII genes, (ii) determine whether genome-specific primers for the Wx genes were able to distinguish the wild type wheat and partial waxy wheat lines, and (iii) determine whether genome-specific primers were able to amplify the Wx and SSII genes in its progenitors, tetraploid wheat lines and diploid species.

Results and Discussion
Sequence similarities of the Wx and SSII genes
Genomic sequences of the Wx and SSII genes have been isolated by Murai et al. [13] and Shimbata et al. [15], respectively. Three homoeologous Wx genes consist of 11 exons and 10 introns. The length of their genomic sequences ranges from 2781 bp for Wx-7A (AB019622) to 2862 bp for Wx-7D (AB019624). Three homoeologous SSII genes contain 8 exons and 7 introns. The length of their genomic sequences ranges from 6566 bp for SSII-7B (AB201446) to 6775 bp for SSII-7D (AB201447). The coding regions (CDs) of three Wx genes range in length from 1815 bp to 1818 bp, while the length of the CDs of three SSII genes varies from 2397 to 2400 bp. Sequence alignment indicated that sequence similarities of the CDs were more than 95.0% among the three homoeologous Wx genes and over 96.0% among the three homoeologous SSII genes. Therefore, it is very difficult to design more genome-specific primer sets from the CDs to cover the Wx and SSII genes.

In general, more genetic variations occur in intron regions than in exon regions in grass genomes [26]. Sequence alignment of the intron regions showed that
intron sequence similarities ranged from 68.9% to 74.4% among the three homoeologous \textit{Wx} genes and from 68.7% to 82.1% among the three homoeologous \textit{SSII} genes. Many SNPs (single nucleotide polymorphisms) and/or InDels (insertions or deletions) were detected in the intron regions (Figure 1). This provides strategies to design genome-specific primer sets from the SNPs and/or Indels of the intron regions.

Development of genome-specific primers for the \textit{Wx} and \textit{SSII} genes

Genome-specific primers are required to investigate nucleotide diversity of the three homoeologous \textit{Wx} and \textit{SSII} genes and to conduct association analysis between the three homoeologous \textit{Wx} and \textit{SSII} genes and grain yield-related traits. To be useful, the primers should be developed to meet the following criteria: (1) the amplified fragment must be genome-specific; (2) the fragment length should be from 800 bp to 1500 bp for the complete sequencing using both forward and reverse primers by means of ABI 3730 × l DNA Analyzer (Applied Biosystems, CA); (3) the neighboring amplicons should overlap by 80 bp to 100 bp, so that multiple sequences of two or more amplicons can be assembled together after direct sequencing of the amplicons.

Multiple mismatches especially a mismatch of the 3' terminus of a primer between one genome sequence and sequences of other two genomes were required to obtain the genome specificity of the primer. After sequence alignment, both forward and reverse primers were manually designed by picking primers ending with a mismatch (SNP) at the 3' terminus. Primer3 \texttt{http://frodo.wi.mit.edu/primer3/} were used to check general parameters of manually picked primers. Melting temperature (Tm) of both primers ranging from 59°C to 61°C was adjusted by adding or deleting nucleotides at the 5' terminus of primers. Primer length from 17 nt to 25 nt worked well. Other parameters were used as default. Annealing temperature 60°C was used for PCR.

Only one genome-specific primer based on one mismatch at the 3' terminus in a primer set and both genome-specific forward and reverse primers based on mismatch(s) in the middle or beginning of primers did not guarantee genome-specific amplification. In the absence of a perfect match between a primer and a template, primer-template mismatch amplification may be generated in the first cycle of PCR (e.g. nulli-tetrasomic lines). Huang and Cloutier [3] used BAC DNA as template and primer-template mismatch PCR to identify a number of genes for low molecular weight glutenin sub-units (LMW-GS) in hexaploid wheat. In Figure 1, when a \textit{SSII}-7A gene-specific forward primer \textit{SSII}-7A-F3 from Intron III combined with a reverse primer \textit{SSII}-7A-R3 from Exon V with one mismatch T/C at the fifth position from the 3'-terminus of the primer, a weak fragment appeared in N7AT7D (Figure 2). This fragment was most likely amplified from the \textit{SSII} gene on chromosomes 7D by primer-template mismatch PCR. When the specific forward primer \textit{SSII}-7A-F3 combined with a specific reverse primer \textit{SSII}-7A-R3a from Exon V with one mismatch T/C at the 3'-terminus of the primer, a specific fragment was produced from chromosome 7A (Figures 1 and 2).

![Figure 1 Alignment of partial genomic DNA sequences of three homoeologous \textit{SSII} genes from hexaploid wheat indicating the position of genome-specific primers. The exon and intron regions are shown with gray and white color, respectively. Deletions are indicated by dashes. The sequence of the forward primer and complementary sequences of the reverse primers are boxed.](image-url)
For the three homoeologous SSII genes, four genome-specific primer sets for all three genomes were designed (Table 1 and Figure 4). The forward primers of primer set 2 were located in Exon II, while the reverse primers were designed from Intron III. The forward primers of primer set 3 were developed from Intron III. The reverse primers of primer set 3 were located in Exon V (SSII-7A-R3a) and Intron V (SSII-7B-R3 and SSII-7D-R3), respectively. The forward primers of primer set 4 were developed from Intron IV (SSII-7A-F4a) or Exon V (SSII-7B-F4a and SSII-7D-F4), whereas the reverse primers were located in Intron VII. Both the forward and reverse primers of primer set 5 were designed from Intron VII. The size of a single PCR product ranges from 883 bp to 1657 bp. The genome specificity of these four primer sets were confirmed through amplifying genomic DNA of CS, N7AT7D, N7BT7D and N7DT7B (Figure 4). The total length of amplified PCR products by the four primer sets covered 59.5% of the SSII-7B gene, 60.3% of the SSII-7D gene and 61.6% of the SSII-7A gene.

These genome-specific primer sets for the three homoeologous Wx and SSII genes were used to amplify 90 bread wheat genotypes, which were originated from Canada, USA, UK and Germany. Single PCR products were recovered in all 90 wheat genotypes by using each of the genome-specific primer sets (data not shown). The result indicated that the regions used to design the primers were conserved and no genetic variation occurred in these regions during wheat domestication and artificial selection in wheat breeding. These genome-specific primers are being used for the study of sequence diversity and association mapping of the three homoeologous Wx and SSII genes in natural populations of hexaploid wheat.

Distinguishing the wild type wheat and partial waxy wheat lines using genome-specific primers for the Wx genes

It has been reported that wheat varieties Sturdy and Fujimi Komugi carry the null Wx-A1 allele (Wx-A1b) [27-29] (Table 2). Gabo, Gamenya and Santanta possess the null Wx-B1 allele (Wx-B1b). The Chinese wheat variety Bai Hao has the null allele (Wx-D1b) at the Wx-D1 locus, whereas the Japanese wheat variety Kanto 107 carries the double null alleles Wx-A1b and Wx-B1b and Wx-D1b [30]. Morris and Konzak [31] developed soft and hard homoyzous waxy wheat lines, NSGC 8645 (PI 612545) and NSGC 8646 (PI 612546), which both carry the triple null alleles Wx-A1b, Wx-B1b and Wx-D1b. The nine genome-specific primer sets for the three homoeologous Wx genes were used to amplify these partial waxy wheat genotypes and breeding lines. It was found that one out of three 7A-specific primer sets, Wx-7A-F1/Wx-7A-R1a, could distinguish 72.6% of the Wx-4A gene, 82.9% of the Wx-7A gene and 87.0% of the Wx-7D gene.

All primer sequences and amplicon information for the three homoeologous Wx and SSII genes are listed in Table 1. For the three homoeologous Wx genes, three genome-specific primer sets for all three genomes were developed (Table 1 and Figure 3). The forward primers of primer set 1 were located in Intron I (Wx-7A-F1 and Wx-4A-F1) or at Exon 1/Intron I boundary (Wx-7D-F1), while the reverse primers were designed from Intron V (Wx-4A-R1 and Wx-7D-R1) or Intron V/Exon VI junction (Wx-7A-R1a). The forward primers of primer set 2 were developed from Intron V (Wx-7A-F2), Intron IV (Wx-4A-F2), and Exon V (Wx-7D-F2a), respectively. The reverse primers of primer set 2 were located in Intron VIII (Wx-7A-R2a), Intron VII (Wx-4A-R2), and Exon VIII (Wx-7D-R2a), respectively. The forward primers of primer set 3 were developed from Intron VI (Wx-7A-F3) or Intron VII/Intron VII junction (Wx-7A-F3 and Wx-7D-F3), whereas the reverse primers of primer set 3 were located in Intron X (Wx-7A-R3), Intron IX (Wx-4A-R3), and Exon X1 (Wx-7D-R3a), respectively. The size of a single PCR product varies from 731 bp to 1038 bp (Figure 3). The genome specificity of these nine primer sets were determined through amplifying genomic DNA of Chinese Spring (CS), N7AT7D, N7BT7D and N7DT7B. M = 1 kb plus DNA ladder.

Figure 2 A comparison between unspecific (SSII-7A-F3/SSII-7A-R3) and specific (SSII-7A-F3/SSII-7A-R3a) primer sets. Each primer set was used to amplify Chinese Spring (CS), N7AT7D, N7BT7D and N7DT7B. M = 1 kb plus DNA ladder.

Wx and SSII diversity and association mapping of the three homoeologous Wx and SSII genes were used to amplify 90 bread wheat genotypes, which were originated from Canada, USA, UK and Germany. Single PCR products were recovered in all 90 wheat genotypes by using each of the genome-specific primer sets (data not shown). The result indicated that the regions used to design the primers were conserved and no genetic variation occurred in these regions during wheat domestication and artificial selection in wheat breeding. These genome-specific primers are being used for the study of sequence diversity and association mapping of the three homoeologous Wx and SSII genes in natural populations of hexaploid wheat.
Table 1: Genome-specific primer sets for the three homoeologous Wx and SSII genes.

| Primer name   | Primer sequence (5’-3’) | Tm (°C) | Size (bp) | Chromosomal location |
|---------------|-------------------------|---------|-----------|---------------------|
| Wx-7A-F1      | GTAAGCTTGCGCCACGTGC     | 60      | 950       | 7A                  |
| Wx-7A-R1a     | GGATGCGAAGATGCCACCTA    |         |           |                     |
| Wx-7A-F2      | CGCTCTGCAATACATTCTTTGC  |         |           |                     |
| Wx-7A-R2a     | ATATGCAAAGAGGTTGAGGAAC  | 60      | 1038      | 7A                  |
| Wx-7A-R3      | CGGCCCTTCACTTATAGTTG    | 60      | 750       | 7A                  |
| Wx-7A-F3      | CTGGTACGATCGACCGACAT    |         |           |                     |
| Wx-4A-F1      | AGCTGACCACTGCTTCTTCTGA  |         |           |                     |
| Wx-4A-R1      | GCCGCTCTATAGTGCCACAC    | 60      | 854       | 4A                  |
| Wx-4A-R2      | TCAAACACACCCAGCAGCTA    |         |           |                     |
| Wx-4A-R3      | CGATGCGAAGGTTGAGGAAC    | 60      | 943       | 4A                  |
| Wx-7D-F1      | CCATATGCTTACAGCTAGGA    |         |           |                     |
| Wx-7D-R1      | CCAGAAATGTTAGCTTCTCTT  | 60      | 978       | 7D                  |
| Wx-7D-F2a     | AACTACGATCCACATGGCATCAC |         |           |                     |
| Wx-7D-R2a     | GCTGGGGTCGAGACTTA       | 60      | 938       | 7D                  |
| Wx-7D-R3a     | CCACACACCCACACCATGAG    | 60      | 731       | 4A                  |
| SSII-7A-F2    | CCAAACACAGATACAGTGAAC   |         |           |                     |
| SSII-7A-R2    | CGATGCGAAGGTTGAGGAAC    | 60      | 953       | 7A                  |
| SSII-7A-F3    | GCATGCGAAGGTTGAGGAAC    |         |           |                     |
| SSII-7A-R3a   | CCTGACGCGTGCGAACATG     | 60      | 883       | 7A                  |
| SSII-7A-F4a   | AGCCAATGCAATCGAAAGG     |         |           |                     |
| SSII-7A-R4a   | GCTGGGTAGTAGGGGGGAAT    | 60      | 1657      | 7A                  |
| SSII-7A-F5a   | TGACATCGCTGCTGAGAT      | 60      | 947       | 7B                  |
| SSII-7A-R5    | CGATGCGAAGGTTGAGGAAC    |         |           |                     |
| SSII-7B-F2    | ATCGACACGTGCTGAGAT      | 60      | 947       | 7B                  |
| SSII-7B-R2    | TGACATCGCTGCTGAGAT      | 60      | 947       | 7B                  |
| SSII-7B-F3    | GCATGCGAAGGTTGAGGAAC    |         |           |                     |
| SSII-7B-R3    | CGATGCGAAGGTTGAGGAAC    | 60      | 947       | 7B                  |
| SSII-7B-F4a   | CATTGACGCTCTCCTTCC      |         |           |                     |
| SSII-7B-R4a   | TGACATCGCTGCTGAGAT      | 60      | 947       | 7B                  |
| SSII-7B-F5    | ACACATCGCATGCCAGACG     | 60      | 947       | 7B                  |
| SSII-7B-R5    | TGACATCGCTGCTGAGAT      | 60      | 947       | 7B                  |
| SSII-7D-F2a   | GTCAGGACGCTGGAAAGA      |         |           |                     |
| SSII-7D-R2a   | CGCGAAACTAGCCTCCAATC    | 60      | 1596      | 7B                  |
| SSII-7D-R3    | CGCGAAACTAGCCTCCAATC    | 60      | 973       | 7B                  |
| SSII-7D-F3    | AGTTGACGGAGGCGGTCAG     | 60      | 949       | 7B                  |
| SSII-7D-R4    | ATGAGGAGGAGGAGGAGGTTG   | 60      | 973       | 7B                  |
| SSII-7D-F5    | TGAGGAGGAGGCGGATAGA     | 60      | 949       | 7B                  |
| SSII-7D-R5    | GCACAGACACTGACCTCAG     | 60      | 987       | 7B                  |

The forward and reverse primer pairs are listed together for each fragment amplified.
alleles for marker-assisted selection in the wheat breeding programs because of the absence of PCR products in the partial waxy wheat lines. Co-dominant markers have been developed for the selection of all the three null alleles at the Wx loci [27,32]. However, the primers for the most co-dominant markers were not genome-specific.

Amplification of the Wx and SSII genes in diploid and tetraploid progenitors of hexaploid wheat

Bread wheat (Triticum aestivum L.) is an allohexaploid (2n = 6x = 42) species with three (A, B and D) genomes. It originated from two natural hybridization events. The first one involved in the hybridization between T. urartu Thum. (A\textsuperscript{u}A\textsuperscript{m}) and an unknown species (BB) closely related to Aegilops speltoides Tausch. (SS), generating wild emmer T. turgidum ssp. dicocoides (Körn. ex Aschers. & Graebn.) Thell. (AABB). The second event occurred between the domesticated emmer T. turgidum ssp. dicoccon Schrank (AABB) and Ae. tauschii Coss. (DD), resulting in the formation the hexaploid wheat T. aestivum L. (AABBDD) [33-35]. The first event happened less than half a million years ago, while the second event occurred only 8,000 years ago [33]. T. monococcum L. (A\textsuperscript{m}A\textsuperscript{u}), closely related to T. urartu, includes the wild einkorn, T. monococcum ssp. aegilopoides (syn. T. boeoticum) and the cultivated einkorn T. monococcum ssp. monoccocum. T. turgidum ssp. durum Desf. is another domesticated form of T. turgidum ssp. dicocoides [34,35].

These genome-specific primer sets for the three Wx and SSII genes were used to amplify 18 diploid (Triticum and Aegilops species) and 13 tetraploid (wild and domesticated forms) progenitors of hexaploid wheat (Additional file 1). It was found that the A genome specific primer sets designed from hexaploid wheat failed to yield any amplification products in most of the accessions of T. monococcum and T. boeoticum (the A\textsuperscript{m} genome), and T. urartu (the A\textsuperscript{u} genome), even though the A\textsuperscript{u} genome has been widely accepted as the A-genome donor of hexaploid wheat [33,34]. These results indicate significant sequence differences in the intron regions of Wx and SSII genes between the A\textsuperscript{m} (A\textsuperscript{u}) genome and the A genome of bread wheat. The sequence differences could be insertion(s) and/or deletion(s). The most B genome-specific primer sets designed from hexaploid wheat could not generate amplification products in four accessions of Ae. speltoides (the S genome) (Additional file 1). The only primer set SSII-7B-F2/SSII-7B-R2 amplified two PCR fragments in one Ae. speltoides accession (PI 499261) and one fragment in other three Ae. speltoides accessions (Figure 6). These results also reveal sequence differences in the intron regions of Wx and SSII genes between the S genome and the B genome of bread wheat. Four D genome-specific primer sets for the SSII-7D genes could amplify PCR products with no length polymorphism.
from all six accessions of *Ae. tauschii*, the D genome donor of hexaploid wheat, whereas only one D genome-specific primer set Wx-7D-F2a/Wx-7D-R2a for the Wx-7D gene amplified a uniform fragment from six *Ae. tauschii* accessions, which was identical to that of hexaploid wheat (Additional file 1).

The identical PCR products amplified by A and B genome-specific primer sets for the Wx and SSII genes from hexaploid wheat were detected in 10 accessions of domesticated tetraploid wheat *T. turgidum* ssp. *dicoccon* and *T. turgidum* ssp. *durum*. This indicates that very high conservation of the Wx and SSII genes exists in the homologous A (B) genomes between cultivated durum and hexaploid wheat and these genome-specific primers can be used for the study of sequence diversity and association mapping of the three homoeologous Wx and SSII genes in natural populations of domesticated tetraploid wheat. However, no amplicon was observed in three accessions of wild tetraploid wheat *T. turgidum* ssp. *dicoccoides* (Additional file 1). These results indicate greater genetic variation in the Wx and SSII genes of the wild tetraploid wheat. It was interesting to find that PCR products were amplified in the accessions of *T. monococcum* and *T. boeoticum* (the A<sub>m</sub> genome), and *T. urartu* (the A<sup>u</sup> genome) by B genome specific primer sets (Wx-4A-F1/Wx-4A-R1 and SSII-7B-F2/SSII-7B-R2, Figure 6) and D

---

**Figure 4** Genome-specific PCR amplification for the three homoeologous SSII loci. Each primer set was used to amplify Chinese Spring (CS, lane 1), N7AT7D (lane 2) and N7BT7D (lane 3) and N7DT7B (lane 4). M = 1 kb plus DNA ladder. PCR amplification with (A) 7A-specific primer sets, (B) 7B-specific primer sets, and (C) 7D-specific primer sets.
genome-specific primer set SSII-7D-F3/SSII-7D-R3 (Additional file 1). This might be caused by mismatch PCRs between template DNA and primers [3].

Huang and Cloutier [4] reported that the same type LMW-GS genes are highly conserved in the homologous A (B) genomes between durum and hexaploid wheat, as well as in the homologous D genomes between hexaploid wheat and Aegilops tauschii. Recent studies indicated that intergenic sequences in the Glu-3 regions are not conserved between the A\textsuperscript{m} genome of T. monococcum and the A genome of durum and bread wheats [36,37]. In the present study, we found very high conservation of Wx and SSII genes in the homologous A (B) genomes between durum and hexaploid wheats, as well as of SSII-7D gene between hexaploid wheat and Aegilops tauschii. Large variations in the intron regions of Wx and SSII genes were observed in the wild diploid and tetraploid progenitors of hexaploid wheat. Therefore, in order to be able to compare genetic variation between the wild diploid/tetraploid progenitors and hexaploid wheat at the sequence level, primers can be developed from the conserved domains of exon regions after sequence alignment. The wild relatives of bread wheat containing novel alleles of Wx and SSII genes could be used as a valuable source for new genetic variation with the potential to improve wheat starch properties and starch contents.

Conclusions
In summary, this paper describes a detailed and useful method for the development of genome-specific primers for the three homoeologous Wx and SSII genes in allohexaploid wheat. These genome-specific primers can be used for the study of sequence diversity and association mapping of the three homoeologous Wx and SSII genes in natural populations of both hexaploid wheat and cultivated tetraploid wheat. The strategies used in this paper can be used to develop genome-specific primers for agronomically important genes in any allopolyploid crops. They may be also suitable for (i) the development of gene-specific primers for duplicated paralogous genes in any diploid species, and (ii) the development of allele-specific primers at the same gene locus.

Methods
Plant materials and DNA isolation
Four nulli-tetrasomic (NT) lines (N4AT4D, N7AT7D, N7BT7D and N7DT7B) of 'Chinese Spring' (CS) were kindly provided by the Wheat Genetic and Genomic Resources Center (WGGRC), Department of Plant
Pathology, Kansas State University, which were originally obtained from Dr. E.R. Sears [38]. These NT lines were used for chromosome assignment of PCR products.

Partial waxy wheat cultivars and breeding lines from different countries of the world (Table 2) were analyzed using the genome-specific primers for the Wx genes. These included the cultivars Fujimi Komugi and K107 from Japan; Bai-Huo and Chinese Spring from China; Gabo and Gamenya from Australia; NSGC 8645, NSGC 8646, Sturdy and Santanta from the United States. Seed of these lines was obtained from the Germplasm Resources Information Network (GRIN), USDA-ARS.

A total of 18 diploid *Triticum* and *Aegilops* species as well as 13 tetraploid wheat lines (*T. turgidum*, 2n = 4x = 14, AABBA) were used to study the genetic variations of the Wx and SSII genes (Table 2). The diploid *Triticum* and *Aegilops* species included two *T. monococcum* and three *T. boeoticum* (2n = 2x = 14, A⁺A⁻), three *T. urartu* (2n = 2x = 14, A⁺A⁺), six *Ae. tauschii* (2n = 2x = 14, DD), and four *Ae. speltoides* (2n = 2x = 14, SS) accessions, whose genomes are closely related to A-, B-, and D-genome of the cultivated wheat (*T. aestivum*, 2n = 6x = 42, AABBDD), respectively [34]. These *Triticum* and *Aegilops* accessions were obtained from the Germplasm Resources Information Network (GRIN), USDA-ARS and the Plant Gene Resources of Canada (PGRC), Agriculture and Agri-Food Canada, respectively.

Total genomic DNA was extracted from young leaf tissue and frozen in liquid nitrogen, as previously described by Huang et al. [39]. The DNA was diluted to a concentration of 50 ng/μL before use for PCR.

**Figure 5** PCR amplification for distinguishing null alleles of the waxy genes and wild type in wheat. (A) PCR results with primer set Wx-7A-F1 and Wx-7A-R1a to distinguish the wild type (Wx-A1a) and null allele (Wx-A1b) of the Wx-A1 gene. (B) PCR results with primer set Wx-7B-F2 and Wx-7B-R2 to distinguish the wild type (Wx-B1a) and null allele (Wx-B1b) of the Wx-B1 gene. (C) PCR results with primer set Wx-7D-F3 and Wx-7D-R3a to distinguish the wild type (Wx-D1a) and null allele (Wx-D1b) of the Wx-D1 gene. M = 1 kb plus DNA ladder. Lanes: 1, CS, 2, Sturdy; 3, Fujimi Komugi; 4, Gabo; 5, Gamenya; 6, Santanta; 7, Bai Huo; 8, Kanto 107; 9, NSGC 8645; 10, NSGC 8646.

**Figure 6** PCR amplification of 18 diploid (*Triticum* and *Aegilops* species) and 13 tetraploid (wild and domesticated forms) progenitors of hexaploid wheat using the primer set SSII-7B-F2/SSII-7B-R2. M = 1 kb plus DNA ladder. Lanes 1-2, *T. monococcum* accessions; lanes 3-5, *T. boeoticum* accessions; lanes 6-8, *T. urartu* accessions; lanes 9-13, *T. turgidum* ssp. *durum* accessions; lanes 14-18, *T. turgidum* ssp. *dicoccon* accessions; lanes 19-21, *T. turgidum* ssp. *dicoccoides* accessions; lanes 22-25, *Ae. speltoides* accessions; lanes 26-31, *Ae. tauschii* accessions.

**Primer design and PCR amplification**

Genomic sequences of the three Wx (AB019622, AB019623 and AB019624) and three SSII genes (AB201445, AB201446 and AB201447), which were isolated by Murai et al. [13] and Shimbata et al. [15], respectively, were retrieved from the GenBank database http://www.ncbi.nlm.nih.gov/Genbank. Multiple alignment of nucleotide sequences were conducted by Bioedit 7.0 [40].

Primers were designed by eye based on the sequence polymorphism and general primer-picking parameters were checked using Primer3 http://frodo.wi.mit.edu/primer3/.

PCR reactions contained 200 ng template DNA, 1 x PCR buffer (Fermentas, 10 mM Tris-HCl pH 8.8, 50 mM KCl, and 0.08% Nonidet P40), 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.6 μM of each primer, 1 U Taq DNA polymerase (recombinant, Fermentas) in a total volume of 25 μL. The PCR reaction was carried out in a MyCycler Thermo Cycler (Bio-Rad Laboratories Inc., Hercules, CA). After 5 min of initial denaturation at 94°C, 35 cycles were performed with 30 sec at 95°C, 30 sec at 60°C, 90 sec at 72°C and a final extension step of 10 min at 72°C. PCR products were separated on a 1% agarose gel in 0.5 x TBE buffer. The gel was photographed using a Molecular Imager Gel Doc XR system (Bio-Rad Laboratories Inc., Hercules, CA).
Additional material

Additional file 1 Summary of PCR analysis in 31 diploid and tetraploid progenitors of wheat. PCR analysis in 31 diploid and tetraploid progenitors of wheat using genome-specific primer sets for the Wx and SSI genes.

Abbreviations
AGP: ADP-glucose pyrophosphorylase; BAC: bacterial artificial chromosome; CS: Chinese Spring; DNP: deoxynivalenolucetate triphosphate; GBSS: granule-bound starch synthase; Indel: insertion or deletion; LMW-GS: low molecular weight glutenin subunit; NT: nulli-tetrasomic; PCR: polymerase chain reaction; SBE: starch branching enzyme; SGP: starch granule protein; SNP: single nucleotide polymorphism; SS: starch synthases; TBE: tris borate EDTA; Tm: melting temperature; Wx: Waxy.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
XQH designed and carried out the experiments, collected materials, performed the sequence alignment, the primer design and the data analysis, and drafted the manuscript. ABB conceived the study and revised the manuscript. Both authors read and approved the final manuscript.

Acknowledgements
We thank Dr. J. Raupp, the Wheat Genetic and Genomic Resources Center (WGGRC), Department of Plant Pathology, Kansas State University for providing the nulli-tetrasomic lines of ‘Chinese Spring’ (CS). The seed of accessions with PI and CN numbers was kindly supplied by Dr. H. Bockelman of the Germplasm Resources Information Network (GRIN), USDA-ARS and D. Kessler of the Plant Gene Resources of Canada (PGRC), Agriculture and Agri-Food Canada, respectively. This work was supported by NSERC (National Science and Engineering Research Council of Canada) and Husky Energy Inc.

Author Details
Department of Plant Science, University of Manitoba, 66 Dafoe Road, Winnipeg, Manitoba R3T 2N2, Canada

Received: 12 May 2010 Accepted: 24 May 2010

References
1. Masterson J: Stomatal size in fossil plants: evidence for polyplody in majority of angiosperms. Science 1994, 264:421-423.
2. Jauhar PP: Meiotic restitution in wheat polyploids (amphiphloids): A potent evolutionary force. J Hered 2007, 98:188-193.
3. Huang XQ, Cloutier S: Hemi-nested touchdown PCR combined with primer-template mismatch PCR for rapid isolation and sequencing of low molecular weight glutenin subunit gene family from a hexaploid wheat BAC library. BMC Genomics 2007, 8:18.
4. Huang XQ, Cloutier S: Molecular characterization and genomic organization of low molecular weight glutenin subunit genes at the Glu-3 loci in hexaploid wheat (Triticum aestivum L.). Theor Appl Genet 2008, 116:953-966.
5. Ernes MJ, Bowsher CG, Hedley C, Burrell MM, Scrase-Field ESF, D. Kessler of the Plant Gene Resources of Canada (PGRC), Agriculture and Agri-Food Canada, respectively. This work was supported by NSERC (National Science and Engineering Research Council of Canada) and Husky Energy Inc.
6. James MG, Denny K, Myers AM: Starch synthesis in the cereal endosperm. Curr Opin Plant Biol 2003, 6:215-222.
7. Konik-Rose C, Thistleton J, Chanvrier H, Tan J, Hallay P, Gidley M, Kosar-Hashemi B, Wang H, Larroque O, Ikee J, McMaugh S, Regina A, Rahman S, Morell M, Li Z: Effects of starch synthase IIa gene dosage on protein and starch in endosperm of wheat. Theor Appl Genet 2007, 115:1053-1065.
8. Nakamura T, Yamamoto M, Hirano H, Hidaka S, Nagamine T: Production of waxy (amylose-free) wheats. Mol Gen Genet 1995, 248:253-259.
9. Yamamoto M, Fujita S, Hayakawa K, Matsuki J: Genetic elimination of a starch granule protein, SGP-1, of wheat generates an altered starch with apparent high amylose. Theor Appl Genet 2000, 101:21-29.
10. Nakamura T, Shimbara T, Vrienten P, Saito M, Yonemar J, Seto Y, Yasuda H, Takahama M: Sweet wheat. Genes Genet Syst 2006, 81:361-365.
11. Li Z, Chu X, Mouille G, Yan L, Kosar-Hashemi B, Hey S, Napi er J, Shewry P, Clarke B, Appel R, Morell MK, Rahman S: The localization and expression of the class II starch synthases of wheat. Plant Physiol 1999, 120:1147-1156.
12. Li Z, Sun F, Xu S, Chu X, Mukai Y, Yamamoto M, Ali S, Rampling L, Kosar-Hashemi B, Rahman S, Morell MK: The structural organisation of the gene encoding class II starch synthase of wheat and barley and the evolution of the genes encoding starch synthases in plants. Funct Integr Genomics 2003, 3:76-85.
13. Murai J, Taira T, Ohta D: Isolation and characterization of the three Waxy genes encoding the granule-bound starch synthase in hexaploid wheat. Gene 1999, 234:71-79.
14. Vrienten PL, Nakamura T, Yamamori M: Molecular characterization of waxy mutations in wheat. Mol Gen Genet 1999, 261:463-471.
15. Shimbara T, Nakamura T, Vrienten P, Saito M, Yonemaru J, Seto Y, Yasuda H: Mutations in wheat starch synthase II genes and PCR-based selection of a SGP-1 null line. Theor Appl Genet 2005, 111:1072-1079.
16. Morrell PL, Lundy KE, Clegg MT: Distinct geographic patterns of genetic diversity are maintained in wild barley (Hordeum vulgare ssp. spontaneum) despite migration. Proc Natl Acad Sci USA 2003, 100:10812-10817.
17. Morrell PL, Toleno DM, Lundy KE, Clegg MT: Low levels of linkage disequilibrium in wild barley (Hordeum vulgare ssp. spontaneum) despite high rates of self-fertilization. Proc Natl Acad Sci USA 2005, 102:2442-2447.
18. Kilian B, Opitz H, Kohl J, von Haeseler A, Barale F, Deusch O, Brandolini A, Yulec C, Martin W, Salamin F: Haplotypic structure at seven barley genes: relevance to gene pool bottlenecks, phylogeny of ear type and site of barley domestication. Mol Gen Genomics 2006, 276:230-241.
19. Whitt SR, Wilson LM, Tenaillon ML, Gaul B, Buckler ES: Genetic diversity and selection in the maize starch pathway. Proc Natl Acad Sci USA 2002, 99:12969-12972.
20. Wilson LM, Whitt SR, Ibáñez AM, Rochefer TR, Goodman MM, Buckler ES: Dissection of maize kernel composition and starch production by candidate gene association. Plant Cell 2004, 16:2719-2733.
21. Chen MH, Bergman C, Pinson S, Felli-strom R: Waxy gene haplotypes: Associations with apparent amylose content and the effect by the environment in an international rice germplasm collection. J Cereal Sci 2008, 47:536-545.
22. Olsen KM, Cai cedo AL, Polato N, McCul ung A, McCouch S, Purugganan MD: Selection under domestication: Evidence for a sweep in the rice waxy genomic region. Genetics 2006, 173:975-983.
23. Hamblin MT, Fernandez MG5, Tuina rra MR, Rooney WL, Kresovich S: Sequence variation at candidate loci in the starch metabolism pathway in Sorghum: Prospects for linkage disequilibrium mapping. Crop Sci 2007, 47(5):1525-534.
24. Van K, Onoda S, Kim MY, Kim KD, Lee S-H: Allelic variation of the Waxy gene in foxtail millet [Setaria italica (L.) P. Beauv.] by single nucleotide polymorphisms. Mol Genet Genomics 2008, 279:255-266.
25. Blake NK, Sherrman JD, Dvorak J, Alt bert LE: Genome specific primer sets for starch biosynthesis genes in wheat. Theor Appl Genet 2004, 109:1295-1302.
26. Ramakrishna W, Dubcovsky J, Park YJ, Busco C, Ebermayer J, San Miguel P, Bennetzen JL: Different types and rates of genome evolution detected by comparative sequence analysis of orthologous segments from four cereal genomes. Genetics 2002, 162:1389-1400.
27. Nakamura T, Vrienten P, Saito M, Konda M: Rapid classification of partial waxy wheats using PCR-based markers. Genome 2002, 45:1150-1156.
28. Saito M, Konda M, Vrienten P, Nakamura K, Nakamura T: Molecular comparison of waxy null alleles in common wheat and identification of a unique null allele. Theor Appl Genet 2004, 108:1205-1211.
29. Yamamoto M, Nakamura T, Endo TR: Waxy protein deficiency and chromosomal location of coding genes in common wheat. Theor Appl Genet 1994, 89:179-184.
30. Yamamoto M, Quyh N: Differential effects of Wx-A1, -B1 and -D1 protein deficiencies on apparent amylose content and starch pasting properties in common wheat. Theor Appl Genet 2000, 100:52-38.
31. Morris CF, Konzak CF: Registration of hard and soft homozygous waxy wheat germplasm. Crop Sci 2001, 41:934.

Published: 24 May 2010
32. Saito M, Vrinten P, Ishikawa G, Graybosch R, Nakamura T: A novel codominant marker for selection of the null Wx-B1 allele in wheat breeding programs. Mol Breed 2009, 23:209-217.

33. Huang S, Sirkhachomkrit A, Su X, Faris J, Gill B, Haselkorn R, Gornicki P: Genes encoding plastid acetyl-CoA carboxylase and 3-phosphoglycerate kinase of the Triticum/Aegilops complex and the evolutionary history of polyploid wheat. Proc Natl Acad Sci USA 2002, 99:8133-8138.

34. Petersen G, Seberg O, Yde M, Berthelsen K: Phylogenetic relationships of Triticum and Aegilops and evidence for the origin of the A, B, and D genomes of common wheat (Triticum aestivum). Mol Phylogenet Evol 2006, 39:70-82.

35. Salamini F, Ozkam H, Brandolini A, Schäfer-Pregl R, Martin W: Genetics and geography of wild cereal domestication in the Near East. Nat Rev Genet 2002, 3:429-441.

36. Wicker T, Yahiaoui N, Guyot R, Schlegenhauf E, Liu ZD, Dubcovsky J, Keller B: Rapid genome divergence at orthologous low molecular weight glutenin loci of the A and A′ genomes of wheat. Plant Cell 2003, 15:1186-1197.

37. Wang ZN, Huang XQ, Cloutier S: Recruitment of closely linked genes for divergent functions: the seed storage protein (Glu-3) and powdery mildew (Pm3) genes in wheat (Triticum aestivum).L, Funct Integr Genomics 2010, 10:241-251.

38. Sears ER: The aneuploids of common wheat. Mo Agric Exp Str Res Bull 1954, 572:1-58.

39. Huang XQ, Zeller FJ, Hsam SLK, Wenzel G, Mohler V: Chromosomal location of AFLP markers in common wheat (Triticum aestivum) utilizing nulli-tetrasomic stocks. Genome 2000, 43:298-305.

40. Hall TA: BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 1999, 41:95-98.

doi: 10.1186/1756-0500-3-140
Cite this article as: Huang and Brûlé-Babel, Development of genome-specific primers for homoeologous genes in allopolyploid species: the waxy and starch synthase II genes in allohexaploid wheat (Triticum aestivum L) as examples BMC Research Notes 2010, 3:140