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Allopolyploidy alters gene expression in the highly stable hexaploid wheat

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Abstract

Hexaploid wheat (*Triticum aestivum*) contains triplicated genomes derived from three distinct species. To better understand how different genomes are coordinated in the same nucleus of the hexaploid wheat, we globally compared gene expression of a synthetic hexaploid wheat with its diploid (*Aegilops tauschii*) and tetraploid (*T. turgidum*) parents by cDNA-AFLP display. The results suggested that the expression of a significant fraction of genes was altered in the synthetic hexaploid; most appeared to be diminished and some were activated. We characterized nine cDNA clones in details. Cytogenetic as well as genomic sequence analyses indicated that the gene silencing was not due to chromosome/DNA loss but was caused by gene regulation. Northern and RT-PCR divided these genes into three groups: (I) four genes were down-regulated nonspecifically, likely involving both parental orthologues; (II) four genes were down-regulated in an orthologue-dependent manner; (III) one gene was activated specifically in the synthetic hexaploid wheat. These genes were often altered non-randomly in different synthetic hexaploids as well as natural hexaploid wheat, suggesting that many of the gene expression changes were intrinsically associated with polyploidy.

Introduction

Polyploidization plays an important role in plant evolution. According to Averett (1980), at least 70% of angiosperm species have undergone a polyploidization event in their evolutionary history. In addition to well-established polyploids such as wheat, oat, tobacco, potato, banana, sugarcane, cotton, and certain *Brassica* species, RFLP studies in maize, soybean, and many classical diploid *Brassica* species have detected genome-wide duplications, suggesting that these plants were ancient polyploids (reviewed by Wendel, 2000). Comparative genomic studies at the nucleotide and amino acid sequence levels suggested that the *Arabidopsis* and tomato genomes were duplicated at least once (Ku *et al.*, 2000; Blanc *et al.*, 2000). Even *Saccharomyces cerevisiae* may be a segmental tetraploid (Wolfe and Shields, 1997).

After the initial genome doubling, the newly formed polyploid undergoes a speciation process that is driven by a combined force of chromosomal repatterning, gene deletion, mutation, suppression of gene expression (silencing), or acquisition of new gene function (for review see Wendel, 2000). Several studies in *Brassica* (Song *et al.*, 1995) and wheat (Liu *et al.*, 1998; Ozkan *et al.*, 2001; Shaked *et al.*, 2001) suggested that genomic changes such as genomic DNA elimination occur rapidly during the formation of these allopolyploid species. Three recent reports indicate that allopolyploidy in *Arabidopsis* (Comai *et al.*, 2000; Lee and Chen, 2001; Madlung *et al.*, 2002) and Triticeae (Kashkush *et al.*, 2002) is associated with gene silencing. These demonstrate that substantial molecular changes can occur early during polyploidization.

However, newly synthesized allotetraploid and allohexaploid cotton did not exhibit rapid genomic changes (Liu *et al.*, 2001). Earlier studies on isozymes in hexaploid wheat suggested that gene silencing was

The nucleotide sequence data reported will appear in the EMBL/GenBank Nucleotide Sequence Databases under the accession numbers 00000–00000.

rare in hexaploid wheat (Hart, 1979, 1996). These discrepancies call for more investigations before genomic changes and gene silencing can be generalized. In addition, mechanisms underlying these changes are poorly understood. For example, McClintock (1984) proposed that the merging of two distinct genomes in the same nucleus during the formation of an allopolyploid presents a major 'genomic shock' to which plants respond with a variety of genomic restructuring. Newly synthesized allopolyploids often are unstable, exhibiting aberrant meiosis that leads to progeny with a wide range genome restructuring including deletions, translocations, inversions, and duplications of chromosomal segments or entire chromosomes. It is not known whether the genome instability triggers the genomic sequence elimination and gene silencing observed in new synthetic amphiploids. Indeed, the materials used in these molecular studies are known to display genome instability (Comai *et al.*, 2000; Kashkush *et al.*, 2002).

One of the best-known allpolyploid complexes exists in the Triticeae. Among the roughly 30 species of *Aegilops* and *Triticum*, 75% are natural allotetraploids or allohexaploids (Sakamoto, 1973; Gill and Friebe, in press). One of the most remarkable polyploids is the young hexaploid *Triticum aestivum*, also called common wheat or bread wheat. *T. aestivum* has a genome composition of AABBDD that arose by two rounds of genome duplications. The first round of genome doubling produced *T. turgidum*, a tetraploid wheat with a genome composition of AABB in which *T. urartu* (AA) donated the A genome (Dvorak, 1998; Gill and Friebe, in press). The B genome donor is extinct, but it was thought to be an ancestor of *Aegilops speltoides* (SS). *T. turgidum* can no longer be synthesized, because numerous differences exist between the B and S genomes (Dvorak, 1998). Hybrids between *T. urartu* and *Ae. speltoides* are highly unstable (B.R. Friebe and B.S. Gill, unpublished results). Fossil evidence and molecular studies demonstrate that the first *T. aestivum* plant arose about 8000 years ago upon a hybridization between *T. turgidum* and *A. tauschii* (DD) (Heun *et al.*, 1997; Huang *et al.*, 2002 and the references cited therein). *Triticum turgidum* and *A. tauschii* can be readily crossed to produce synthetic *T. aestivum* that is identical to the natural common wheat. The pairing between homoeologous chromosomes is completely suppressed in *T. aestivum* due to the presence of two major pairing homoeologous genes *Ph1* and *Ph2* (McFadden and Sears, 1946), resulting in typical disomic meiosis. This is distinct from other allopolyploid plants that display genomic instability because of mispairing of homoeologous chromosomes (Song *et al.*, 1995; Comai *et al.*, 2000; Kashkush *et al.*, 2002). Thus, hexaploid wheat provides a unique system for the investigation of the polyploidization process.

To determine the effect of allopolyploidy on gene expression in the absence of genome instability, we compared gene expression profiles in synthetic and natural hexaploid wheat with parental *A. tauschii* and *T. turgidum* lines. Our results indicate that a significant fraction of parental genes was suppressed in the hexaploid, while a smaller number of genes were activated. Four of the five genes tested were suppressed in both synthetic and natural hexaploid wheat, indicative of changes intrinsically associated with hexaploid wheat.

Materials and methods

Plant materials

The plant materials used for this study include two independent synthetic hexaploids (in the 6th-7th generations), their parental lines and Chinese Spring (CS). The first synthetic hexaploid (AABBDD, accession number TA4152L3) was derived from the parental lines *Aegilops tauschii* (genome DD, accession number TA1651) and the Mexican durum wheat cultivar Altar 84 (*Triticum turgidum*, genome AABB, accession number TA2970). Both parents were maintained by inbreeding and were expected to be homozygous, as *T. turgidum* and *A. tauschii* are strictly self-pollinating species. The second synthetic hexaploid (accession number TA4152L26) was derived from a cross between the parental lines *A. tauschii* (accession number TA2454) and the Mexican durum wheat Aco89 (*T. turgidum*, accession number TA4185). The synthetic hexaploids were produced by Mujeeb-Kazi at CIMMYT and maintained in the Wheat Genetics Resource Center (WGRC) at Kansas State University. The *A. tauschii* lines had been self-pollinated for numerous generations in WGRC prior to the crosses with Mexican durum wheat. All seeds used in this study were obtained from WGRC. Plants were grown in a growth chamber at 20 °C.

Cytogenetic procedures

C-banding and chromosome identification were according to Gill (1991). Meiotic metaphase I pair-

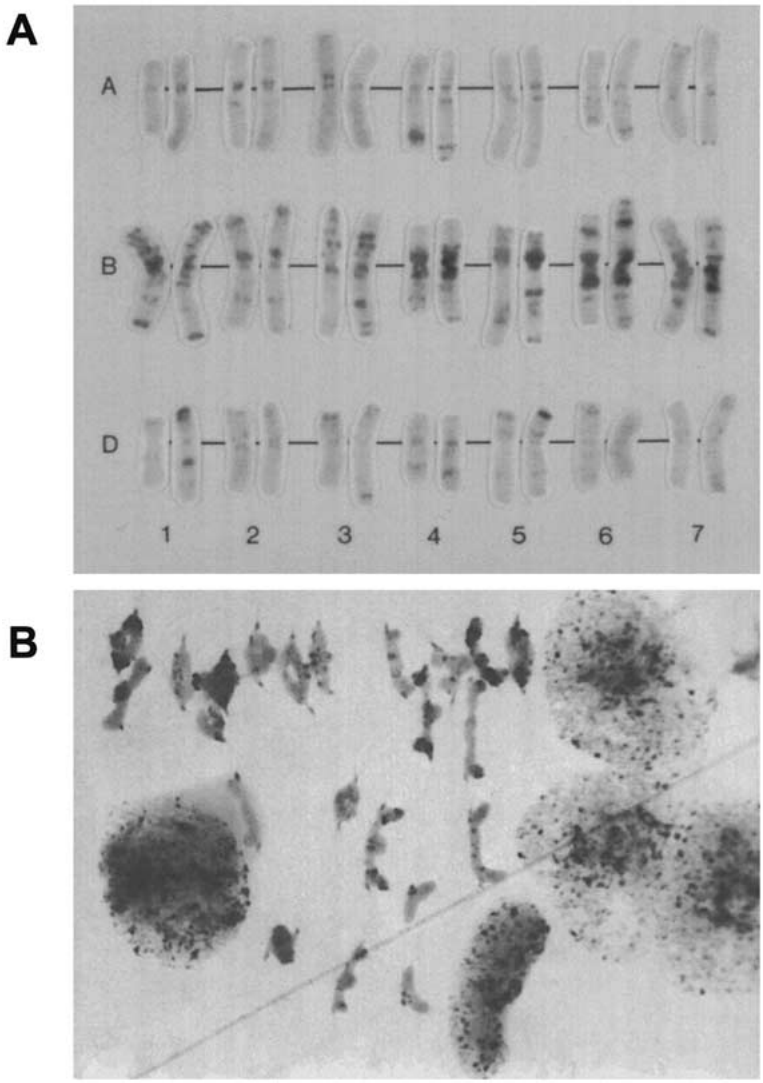

Figure 1. Cytogenetic comparisons of synthetic hexaploid with common wheat cultivar Opata. A. Comparison of C-banded mitotic metaphase chromosomes of the synthetic hexaploid (shown on the right) with the corresponding chromosomes of Opata (shown on the left); note that the overall chromosome morphology and C-banding patterns of the chromosomes of synthetic hexaploid wheat are very similar to the wheat cultivar Opata. B. C-banded meiotic metaphase I chromosomes of the F_1 hybrid TA4152L3 × Opata; note that the A-, B-, and D-genome chromosomes of the synthetic hexaploid show normal diploid-like bivalent pairing with the corresponding chromosomes of cv. Opata ruling out any structural rearrangements during the formation of synthetic hexaploid.

ing was analyzed in C-banded pollen mother cells (PMCs).

RNA extraction, cDNA synthesis and cDNA-AFLP display

Total RNA was extracted from leaves of one-month old seedlings. mRNA was isolated from 300 μg of total RNA by using the polyATtract mRNA isolation system (Promega, Madison, WI). First- and second-strand cDNA was synthesized from 4 mg μRNA by using a cDNA synthesis kit (Clontech, Palo Alto, CA). The cDNA was then digested with *Mse*I and *Apo*I, and subjected to AFLP analysis according to Bachem *et al.* (1996) and Durrant *et al.* (2000). Sequences of the adaptors, pre-amplification primers and selective amplification primers used for AFLP reaction were according to Durrant *et al.* (2000). cDNA-AFLP display products were resolved on a 6% denaturing polyacry-

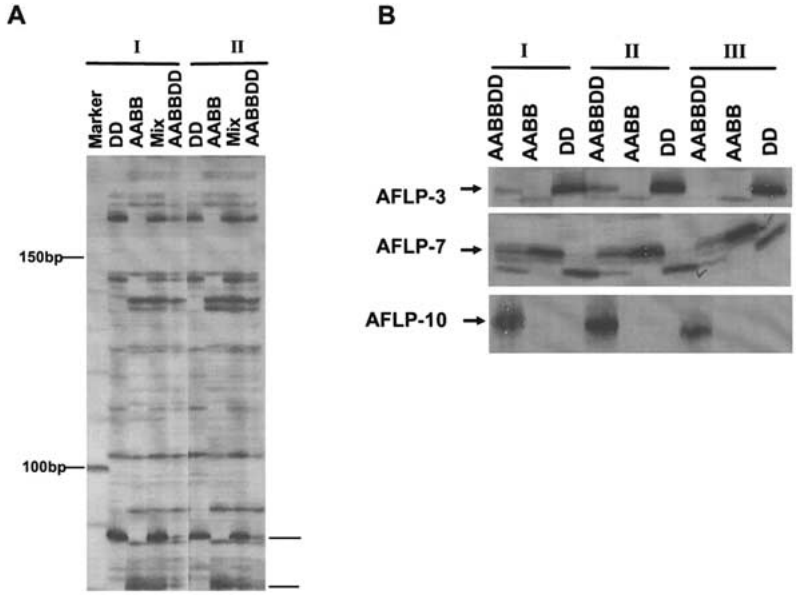

Figure 2. cDNA-AFLP display of gene expression in synthetic wheat. A. A typical cDNA-AFLP gel picture displaying cDNA from *A. tauschii* (DD), *T. turgidum* (AABB), the synthetic hexaploid (AABBDD), and parental cDNA mixed at an 1:1 ratio. mRNA was isolated from three independent sets of plant materials (I, II, and III). Arrow heads indicate differentially expressed bands. B. A close-up view of three differentially expressed bands.

lamide gel, and radiolabeled DNA fragments were visualized by autoradiography.

Differentially expressed bands were excised from the gel by aligning with markings on the X-ray film and eluted in 100 μl H_2O for at least one hour. The eluted DNA was PCR-amplified with AFLP pre-amplification primers. The PCR products were cloned with the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced. The confirmed cDNA sequences were compared with the GenBank database by using BlastX and BlastN.

RNA and DNA gel blot analyses

Total RNA (10 μg) was separated on a denaturing agarose gel, transferred onto a nylon membrane (Hybond-N^+; Amersham, UK). RNA gel blots were performed in NorthernMax Prehyb/Hyb buffer (Ambion, Austin, TX) at 42 °C overnight. Southern blots were hybridized to radiolabeled probes in 6× SSPE at 65 °C and washed to 0.3× SSC at 65 °C (Sambrook *et al.* 1989). The probes for AFLP-1, -3, -5, -9, -10, and -33 were PCR-amplified from plasmid clones by using T7 and M13 reverse primers, and the PCR products were radiolabeled with the DecAprime random priming DNA labeling kit (Ambion).

Reverse transcriptase (RT)-PCR and genomic PCR analyses

Total RNA (5 μg) was treated with RQ1 RNase-Free DNase (Promega, Madison, WI), and cDNA was synthesized by using AMV reverse transcriptase and RNaseH in a final volume of 25 μl. One μl of cDNA or 100 ng genomic DNA template was PCR-amplified with 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Sequences of specific primers for AFLP-3, 9, 10, 11, 23, 33, 36 and the actin cDNA are as follows: AFLP-3, forward 5′-CATGGGTATTAGGAGTTG-3′ and reverse 5′-ATGTGTCTGAATATCTCT-3′; AFLP-9, forward 5′-GCAATGGTAGGACAGATC-3′ and reverse 5′-CATCATTTATCCAGAAAC-3′; AFLP-10, forward 5′-TATAGTAGCCAAATACAC-3′ and reverse 5′-TCATCGTGACTAATAATG-3′; AFLP-11, forward 5′- CAAAATCCGACCACAGCA-3′ and reverse 5′-CACTGATTTGTTTAGTTC-3′; AFLP-23, forward 5′-TAGTGAAGAGCGGCCATG-3′ and reverse 5′-CTCACGGCACCTTCTGAA-3′; AFLP-33, forward 5′-TGAAGAGAGAGGTACTTG-3′ and reverse 5′-CTGGATCTGCTGCTTGAC-3′; AFLP-36, forward 5′-CTTTTACCTGGTAACTGG-3′ and reverse 5′-ATCTCAAGTACCAGCATG-3′; actin, forward 5′-GGCACACTGGTGTCATGG-3′ and reverse 5′-

CTCCATGTCATCCCAGTT-3′. Each forward primer, except for AFLP-33, was end-labeled with [γ-^{33}P]ATP by T4 kinase (New England Biolabs, Beverly, MA). After amplification, PCR products were resolved in a 1% agarose gel or a 6% polyacrylamide sequencing gel.

Competitive RT-PCR was carried out according to Imaizumi *et al.* (2000). The firefly luciferase gene (Promega) was amplified as a DNA competitor with long primers containing both the *LUC* and AFLP-23 cDNA sequences. A common forward primer, 5′-TAGTGAAGAGCGGCCATGCTGGAGAGCAACT-GCAT-3′, and two specific reverse primers, 5′-GTGGACGATTCTCTGCCAGAGTTCATGATCAG-TGC-3′ (AFLP-23D) and 5′-GTGGATGATTCTCT-GCCGGAGTTCATGATCAGTGC-3′ (AFLP-23A), were used to amplify competitors for AFLP-23D and AFLP-23A, respectively. The competitors were added at concentrations from 10^{-4} to 10^{2} pg to RT reactions prior to PCR amplification with 35 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s. AFLP-23D and AFLP-23A were amplified with a common forward primer, 5′-TAGTGAAGAGCGGCCATG-3′, and two specific reverse primers, 5′-GTGGACGATTCTCTGCCA-3′ and 5′-GTGGATGATTCTCTGCCG-3′, respectively. PCR products were separated in a 1.5% agarose gel.

Results

Characterization of synthetic hexaploid wheat

Most of the experiments described in this report, unless indicated otherwise, used the synthetic hexaploid (accession number TA4152L3), its parental lines *A. tauschii* (accession number TA1651) and *T. turgidum* Altar 84 (accession number TA2970). This synthetic line has well documented pedigree and has been used widely for the generation of an extensive genetic linkage map of common wheat (Nelson *et al.*, 1995). The formation of hexaploid wheat through the hybridization of *T. turgidum* and *A. tauschii* was first established by McFadden and Sears (1944, 1946) and is now a routine practice that produces fully fertile plants. To demonstrate the presence of 21 chromosomes from the A, B, D genomes in the synthetic hexaploid wheat (TA4152L3) and determine their similarities to those in known common wheat (cv. Opata), we performed C-banding analysis. This technique produces a diagnostic pattern of dark and light bands along the chromosomes that is chromosome-specific and permits identification of all 21 chromosome pairs of wheat (Gill, 1991). The C-banding pattern of the synthetic hexaploid chromosomes was highly similar to the corresponding chromosomes of Opata (Figure 1A) and other wheat cultivars (Friebe and Gill, 1994). Only 16 polymorphic differences in C-banding patterns were observed between the A-, B-, and D-genome chromosomes of the synthetic hexaploid and Opata. Similar polymorphic differences in C-banding patterns were commonly observed between different wheat cultivars (Friebe and Gill, 1994). The C-banding patterns of the D-genome chromosomes of the synthetic hexaploid are similar to the corresponding chromosomes of the D genome donor species *Aegilops tauschii* (Friebe *et al.*, 1992). The presence of a prominent telomeric C-band in the short arm of chromosome 5D in the synthetic hexaploid indicates the presence of a large number of 18–26S rDNA repeats in this region which is characteristic of *A. tauschii*. However, the copy number of these repeats is drastically reduced in *T. aestivum* cultivars (Mukai *et al.*, 1991). Some of the chromosomes in the haploid complement appeared to be larger than their homoeologous chromosomes in the natural hexaploid, although the significance is unknown.

The presence of *Ph1* and *Ph2* genes enables true disomic chromosomal pairing in all *T. aestivum* lines, including synthetic hexaploid (McFadden and Sears, 1946). To further verify the integrity of the chromosome complement of the synthetic hexaploid, meiotic metaphase I pairing was analyzed in the F_1 testcross of TA4152L3 × Opata. The chromosomes showed normal diploid-like bivalent pairing in 47 of the 55 pollen mother cells (PMCs) analyzed (21^{II}, 0.85; Figure 1B). In four PMCs the chromosomes paired as 20 bivalents plus 2 univalents ($20^{II} + 2^{I}$, 0.07; data not shown) and in six PMCs the chromosomes paired as 19 bivalents plus one quadrivalent ($19^{II} + 1^{IV}$, 0.11; data not shown). The C-banding pattern of the occasional quadrivalent suggested that Opata and the synthetic hexaploid differ by one reciprocal translocation probably involving a pair of A-genome chromosomes. This is not unexpected, because translocation differences are common between different hexaploid wheat cultivars (Friebe and Gill, 1994). Thus, the cytogenetic data revealed no major chromosomal rearrangements between the chromosome complement of the synthetic hexaploid and common wheat cultivars. Most importantly, no mispairing between homoeologous chromosomes was observed, demonstrating a disomic meiotic

behavior of synthetic hexaploid chromosomes. Taken together, we conclude that the synthetic hexaploid behaved like a true diploid and its chromosome complement was intact.

The synthetic lines were completely stable, and their morphologies were highly similar to common wheat except for spike traits such as fragile rachis and tough glumes (*tg*) that were inherited from *A. tauschii*. Mutations at these loci occurred during domestication leading to tough spike and free threshing spike of modern bread wheat. The synthetic hexaploid plants were fully fertile and exhibited no lethality. The synthetic hexaploid seedlings were more vigorous and larger than both parents. Unlike the *A. tauschii* parental lines that developed trichomes on leaves, the two *T. turgidum* parents and the synthetic hexaploids showed no trichome development (data not shown). Spikes of the synthetic hexaploid were more similar to *T. turgidum* than the *A. tauschii* parent although they show longer rachis internodes. The durum parent was glaucous, whereas the *A. tauschii* parent and synthetic hexaploid were completely non-glaucous. Chromosome 2D is known to carry $W2^1$, a homoeoallelic suppressor of the glaucousness gene *W1* on 2B (McIntosh *et al.* 1998). The synthetic hexaploid also resembled the *T. turgidum* parent in its spring-type flowering behavior, presumably because of the dominant *Vrn* genes in the durum wheat.

Suppressed gene expression in synthetic hexaploid wheat

To globally compare gene expression between hexaploid wheat and its D-genome and AB-genome donors, we used cDNA-AFLP display (Bachem *et al.*, 1996) to examine gene expression profiles in the synthetic hexaploid (TA4152L3) and the parental lines *A. tauschii* (TA1651) and *T. turgidum* Altar 84 (TA2970) at the seedling stage. To ensure that the observed differential gene expression between synthetic hexaploid and parental lines was not caused by sampling variations, RNA samples from three sets of seedlings that were grown independently were used for cDNA-AFLP display. Figure 2 shows autoradiograms of typical cDNA-AFLP gels. Each cDNA fragment (band) represents a unique mRNA species. The expression pattern was highly reproducible among three replicates. Only bands detected by all three replicates were scored. On average, each primer combination displayed ca. 50–70 cDNA fragments ranging from 50 bp to 400 bp in either parent. These fragments were highly polymorphic between the tetraploid (AABB) and diploid (DD) parents. Parental bands were completely additive in the mixed cDNA control, indicating that the presence of parental cDNA species did not result in a competition in PCR reaction (Figure 2A). About 64% of the fragments detected in the D parent were absent in the AB parent, and vice versa. By using 47 primer combinations, 2800 bands were scored, among which 1050 were unique to the D-genome parent, 1150 were unique to the AB-genome parent, and 600 were shared fragments (Table 1). Only polymorphic cDNA fragments were used for hexaploid-parent comparison. Monomorphic cDNA fragments were not informative and were thus ignored. For most polymorphic cDNA species, the synthetic hexaploid and parental plants showed similar expression. However, 168 of the 2200 polymorphic fragments (7.7%) displayed altered expression in the hexaploid line. One hundred twenty two D-genome mRNA species (11.6% of the polymorphic D fragments) and 38 AB-genome mRNA species (3.3% of the polymorphic AB fragments) showed greatly reduced or no expression in the synthetic hexaploid. Only eight mRNA species (0.4%) were more abundant in the synthetic hexaploid compared with the parental plants. These observations were consistent in all three replicates.

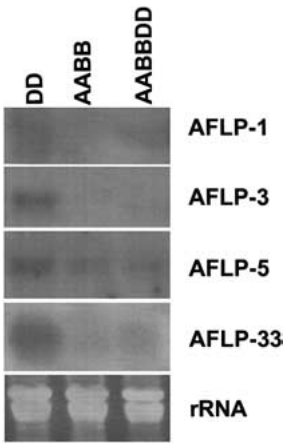

Figure 3. Northern analysis of group I gene expression. Total RNA (10 μg/lane) from *A. tauschii* (DD), durum wheat (AABB) and the synthetic hexaploid (AABBDD) was fractionated on a denaturing agarose gel, transferred onto nylon membranes, and replicated filters were hybridized with the indicated probes. Ethidium bromide staining of rRNA indicates equal loading of RNA.

Table 1. cDNA-AFLP display summary.

	Origin		
	DD	AABB	total
Bands scored	1650	1750	2800
Bands polymorphic between two parents	1050	1150	2200
Bands reduced or missing in hexaploid	122 (11.6%)[a]	38 (3.3%)	160 (7.3%)
Bands induced in hexaploid		8 (0.4%)	

[a]Percentage of reduced/induced bands was calculated based on polymorphic bands.

Characterization of differentially regulated genes

AFLP fragments were excised from the gel and cloned into the TOPO TA cloning vector. Nine cDNA clones were selected for further verification. cDNA-AFLP suggested that eight of these genes were suppressed and one was induced in the synthetic hexaploid. Northern analysis indicated that AFLP-3 and AFLP-33 were highly expressed in the diploid parent *A. tauschii* but reduced or completely silenced in the hexaploid (Figure 3). AFLP-1 and AFLP-5 showed reduced expression in the hexaploid. Although these differences are quantitative, they were verified in repeated Northern analyses. Interestingly, very little orthologous transcripts were detected in the *T. turgidum* parent, suggesting a suppressed expression of the orthologous genes. Alternatively, the probes might have failed to cross-hybridize with orthologous genes, although this is unlikely (see discussion). These genes (AFLP-1, AFLP-3, AFLP-5, and AFLP-33) are designated group I genes. No cDNA clones isolated from *T. turgidum* belonged to this group.

The other four putative down-regulated genes were either not detected or showed similar transcript levels in the hexaploid and parental plants in Northern analysis. Figure 4A shows the Northern analysis of two of these genes. Because Northern analysis does not discriminate transcripts from different orthologues, we could not conclude whether the genes examined were not suppressed or they were suppressed in an orthologue-specific manner in the synthetic hexaploid plants. To determine if any of these genes were reduced in expression in an orthologue-specific manner, we designed PCR primers according to the ends of the cloned cDNA fragments and conducted RT-PCR analysis. In the absence of reverse transcriptase, no PCR products were detected, ruling out genomic DNA contamination. RT-PCR products for three of the four genes, AFLP-9, 11, 36 showed a size polymorphism between *T. turgidum* and *A. tauschii* orthologues (Figure 4B). The expression of the respective *T. turgidum* genes was dramatically reduced in the synthetic hexaploid, while the expression of *A. tauschii* orthologues was not affected in the hexaploid for the three genes. Initial RT-PCR failed to produce a cDNA length polymorphism for AFLP-23 (Figure 4B), and the parental and synthetic hexaploid lines produced a similar level of total transcripts. We isolated and sequenced a corresponding cDNA fragment from *T. turgidum* plants homologous to AFLP-23. A single *T. turgidum* cDNA species was detected, and it contained seven single nucleotide substitutions compared with AFLP-23 that is of *A. tauschii* origin. The *T. turgidum* gene was carried by the A genome (see Figure 5B) and is thus called AFLP-23A, whereas the D genome copy is designated AFLP-23D. Respective gene-specific primers were designed for the AFLP-23A and AFLP-23D transcripts. Competitive RT-PCR (Imaizumi *et al.*, 2000) showed that AFLP-23D gene expression was reduced ca. 10-fold in the synthetic hexaploid plants compared to the *A. tauschii* parent, whereas the AFLP-23A gene was not affected in the hexaploid wheat (Figure 4C). Thus, the four genes (AFLP-9, -11, -23, and -36) had undergone selective suppression, in which only one of the orthologous genes showed reduced expression in the hexaploid compared with its expression in parental plants. An alternative interpretation is that competition in PCR might have caused differential amplification of orthologous transcripts for genes corresponding to AFLP-9, -11, and -36. However, we view this as unlikely for the following reasons. First, at least for AFLP-36, both parental orthologues were amplified equally when genomic DNA was used as template (Figure 5). Secondly, a primer bias toward the constitutively expressed orthologues is unlikely, because primers were designed for perfect base-pairing with the down-regulated transcripts. A remote possibility may be that unforeseen factors could have impeded the amplification of the AFLP-9 and AFLP-11 cDNAs

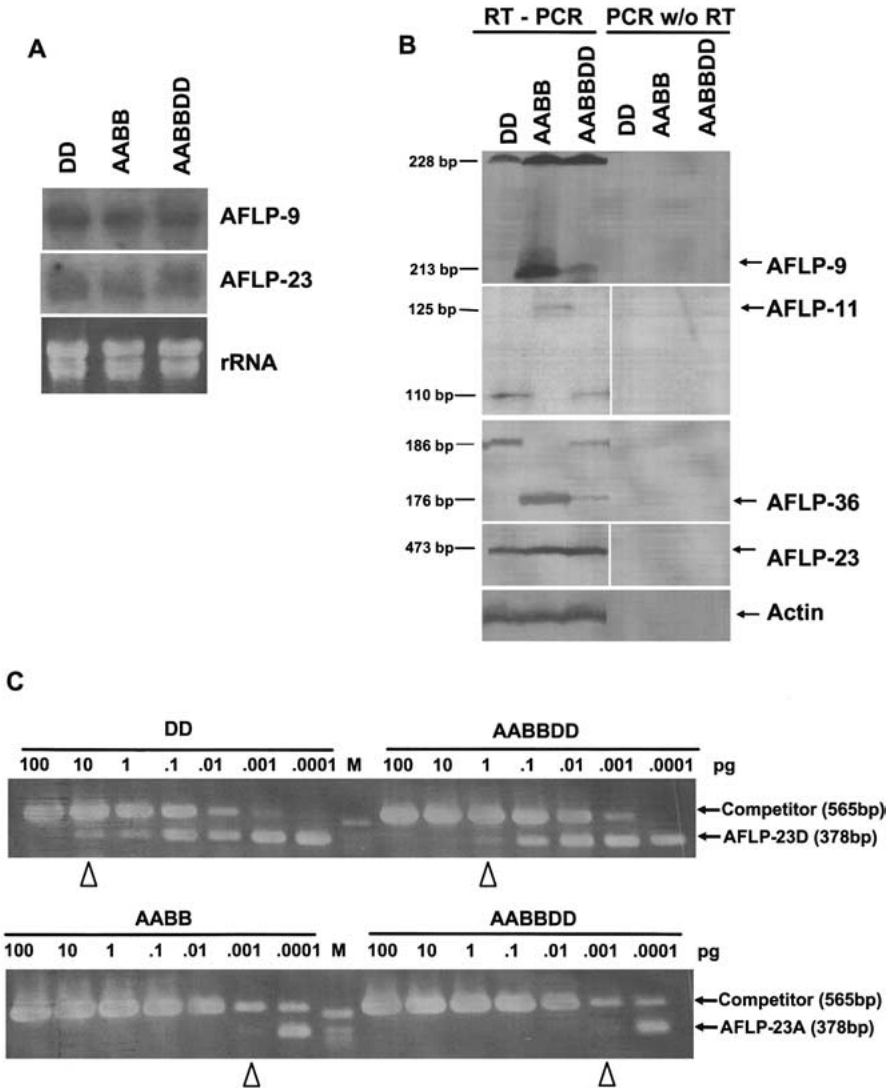

Figure 4. Northern and RT-PCR analyses of group II gene expression. A. Northern analysis of AFLP-9 and AFLP-23 in synthetic hexaploid (AABBDD) and parental lines (AABB and DD). RNA blots were hybridized with cloned cDNA fragments as indicated. B. RT-PCR analysis of group II gene expression. Radiolabeled RT-PCR products were fractionated through a sequencing gel, and the gel was exposed to X-ray film. Actin cDNA was amplified as a control for a constitutively expressed gene. Arrows indicate differentially expressed orthologues. For control, PCR reactions without reverse transcriptase were performed to rule out possible genomic DNA contamination (w/o RT). C. Competitive RT-PCR analysis of AFLP-23 expression. Top panel, amplification with the primers and competitor designed for AFLP-23D; lower panel, amplification with the primers and competitor designed for AFLP-23A. Triangles indicate minimal concentrations of competitors that inhibit the amplification of target transcripts. The numbers indicate the amount of competitor DNA used in the assay. PCR without reverse transcriptase did not produce any products (not shown). Lane M contains DNA markers.

in the RT-PCR experiments. Given consistent results from both cDNA-AFLP and RT-PCR experiments, we conclude that overall these four genes were selectively down-regulated in the synthetic hexaploid wheat. We designate these as group II genes. They include one *A. tauschii* and three *T. turgidum* genes.

We next examined whether the orthologous transcript species detected for group II genes were encoded by homoeologous chromosomes. PCR primers for AFLP-9 and -11 failed to amplify a product from any genomic DNA templates, perhaps because of the presence of introns in the templates. Amplification of genomic DNA from the synthetic hexaploid, its parental plants, and Chinese Spring with AFLP-36 primers produced PCR products identical to corresponding RT-PCR products (Figure 5). The AFLP-36

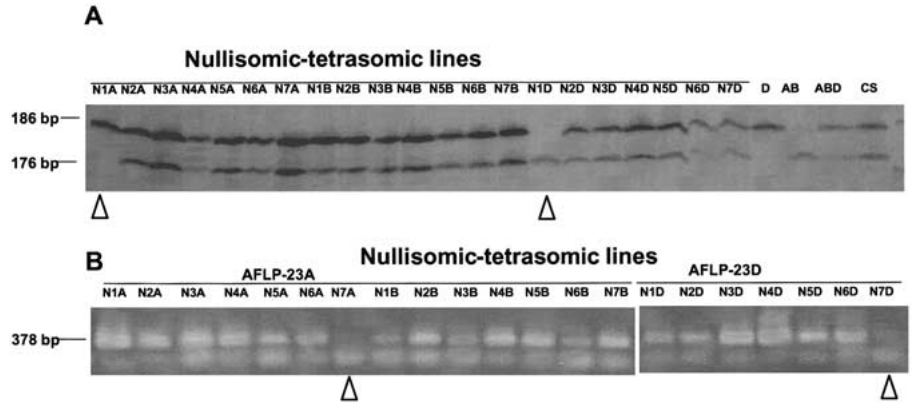

Figure 5. Mapping of group II gene orthologues by using nulli-tetras lines. A. mapping of AFLP-36 orthologues. B. mapping of AFLP-23 orthologues. Genomic DNA from nulli-tetras lines (N1A represents nullisomic for chromosome 1A, etc.) was PCR-amplified with appropriate primers. *A. tauschii* (D), *T. turgidum* (AB) and the wild-type Chinese Spring (ABD) were used as controls. Triangles indicate nulli-tetras lines lacking one of the orthologous bands.

orthologues were placed on chromosomes by using Chinese Spring nullisomic-tetrasomics (nulli-tetras; Sears, 1966) lines (Figure 5). Of the two PCR products detected, the *A. tauschii* (DD) origin fragment (the upper band) was missing in nullisomic 1D, whereas *T. turgidum* origin fragment (the lower band) was missing in nullisomic 1A. Thus, the orthologous genes detected by PCR were AFLP-36A and AFLP-36D that were mapped to homoeologous chromosomes 1A and 1D, respectively. A similar method was used to map AFLP-23. The two orthologues for AFLP-23, AFLP-23A and AFLP-23D were mapped to chromosomes 7A and 7D, respectively. AFLP-36A and AFLP-23D were selectively suppressed in the synthetic hexaploid compared with their respective donor plants. Interestingly, the AFLP-23B and AFLP-36B homoeoalleles were not amplified with the primers used, indicating that either the primers were too specific for the amplification of B homoeoalleles or that the B homoeoalleles had been deleted. Nucleotide sequences of AFLP-23A and AFLP-23D, and AFLP-36A and AFLP-36D were 99% and 97% identical, respectively, with a few base pairs of insertions/deletions or single base pair substitutions.

Genes specifically activated in the synthetic hexaploid line were designated group III genes. cDNA-AFLP results suggested that these genes were rare. AFLP-10 was the only gene of this group to be examined in detail. Northern analysis using the cloned AFLP-10 fragment as a probe indicated that transcripts accumulated at a very low level in the two parental lines but were abundant in the synthetic hexaploid plants (Figure 6A). PCR primers were designed from the AFLP-10 cDNA ends for RT-PCR and genomic PCR analyses. In addition to the activated allele in the hexaploid, RT-PCR revealed an orthologous transcript species that accumulated equally in *A. tauschii* and the synthetic hexaploid. Comparison of genomic PCR products with RT-PCR indicated that the strong expression of AFLP-10 in the hexaploid plants was a result of specific activation of a previously silenced *T. turgidum* gene (Figure 6B). The weak hybridization signal in *T. turgidum* detected by Northern analysis may be caused by the expression of a homologous gene. In fact, Southern analysis suggested that AFLP-10 belongs to a small gene family (data not shown).

Thus, of all the nine genes for which the expression could be detected by either Northern analysis or RT-PCR, the altered gene expression in synthetic hexaploid wheat was confirmed. The results validate our conclusion from the cDNA-AFLP display experiment. Table 2 summarizes the expression analysis and sequence homologies of the nine genes with known proteins or DNA sequences in the database. Seven cDNA clones shared significant homology with known sequences with a wide range of functions. The homology of AFLP-33 to histone H2A promoter sequence is not understood, but this sequence is apparently transcribed.

Alteration of gene expression is nonrandom and occurs in the natural hexaploid wheat

We next tested if any of the gene silencing or activation described above was genotype-specific. Besides the synthetic and parental lines used for cDNA-AFLP

Table 2. Summary of three groups of differentially expressed genes.

Clone	Expression pattern	Orthologue expression	Putative function[a]	E-value
Group I				
AFLP-1	DD>AABBDD	Low in 4× and 6×	D83391 *Zea mays* uroporphyrinogen III methylase	8^{e-50}
AFLP-3	DD>AABBDD	Low in 4× and 6×	Unknown	
AFLP-5	DD>AABBDD	Low in 4× and 6×	AY054525 *A. thaliana* ABC transporter	7^{e-84}
AFLP-33	DD>AABBDD	Low in 4× and 6×	X94693 *T. aestivum* histone H2A promoter	7^{e-38}
Group II				
AFLP-9	AABB>AABBDD	Not silenced	AY063063 *A. thaliana* Phe-tRNA synthetase	1^{e-57}
AFLP-11	AABB>AABBDD	Not silenced	X75089 *T. aestivum* *petF* gene for ferredoxin	9^{e-29}
AFLP-23	DD>AABBDD	Not silenced	AF323103 *M. truncatula* protein phosphatase	6^{e-23}
AFLP-36	AABB>AABBDD	Not silenced	Unknown	
Group III				
AFLP-10	AABBDD-specific	Not affected	U32429 *T. aestivum* sulfur-rich/thionin-like protein	4^{e-30}

[a]Putative gene function as suggested by BLASTX searches.

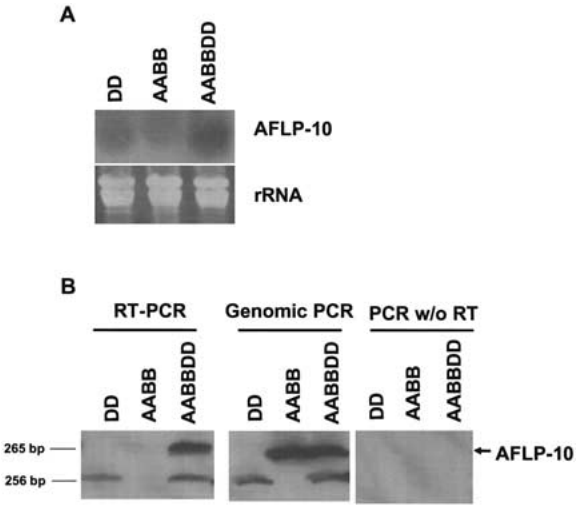

Figure 6. Activation of a group III gene in synthetic hexaploid wheat. A. Northern analysis of transcripts in synthetic hexaploid (AABBDD), *A. tauschii* (DD) and *T. turgidum* (AABB). Ethidium bromide staining of rRNA indicates equal loading of RNA. B. RT-PCR (left) and genomic DNA PCR (center) of the AFLP-10 gene. PCR reactions with RNA but no reverse transcriptase were performed to rule out possible genomic DNA contamination in RT-PCR (right).

display, we compared a second synthetic hexaploid line (AABBDD; accession number TA4152 L26) and its parental lines *A. tauschii* (DD; accession number TA2454) and Mexican durum wheat cultivar Aco89 (AABB; accession number TA4185). In addition, we also examined gene expression in Chinese Spring (CS), a natural hexaploid wheat. Figure 7A indicates that the two *A. tauschii* accessions showed a high level of transcripts corresponding to AFLP-3 and AFLP-33, while the two durum wheat and synthetic hexaploid produced little detectable transcripts. Thus, the genes corresponding to AFLP-3 and AFLP-33 were silenced in the hexaploid wheat in a genotype-independent manner. Similarly, RT-PCR showed that the *T. turgidum* transcripts corresponding to AFLP-9 and AFLP-36 were reduced in both synthetic hexaploid lines compared with the tetraploid parents (Figure 7B). These genes were also down-regulated in Chinese Spring (Figure 7). Assuming that the expression of 10% of the genes is randomly altered in a hexaploid wheat, observing such a change for any given gene in three independent hexaploid wheat genotypes will have a probability of 0.001. Observing

such a change in the same four of nine genes examined in all three wheat lines will have a probability of 2.6×10^{-11}. The probably will be even lower if the frequency of gene expression alteration is less than 10%. Therefore, we conclude that a significant portion of the genes are down-regulated non-randomly in synthetic as well as natural hexaploid wheat. In contrast, the activation of AFLP-10 in the hexaploid wheat was specific to TA4152 L3 (Altar84 × TA1641), but

Gene deletions are not the cause of decreased gene expression

Most of the down-regulated not in TA4152L26 (Aco89 × TA2454) or Chinese Spring (Figure 7A). genes described in this report showed a reduction rather than a complete loss of transcripts, suggesting that reduced gene expression level instead of gene deletion was the cause of silencing. Genomic PCR and Southern analyses were conducted to further test whether the synthetic hexaploid carried deletions or chromosome rearrangements at the differentially expressed loci. Genomic DNA from the synthetic hexaploid (AABBDD; accession number TA4152L3), the durum (AABB; accession number TA2970), and *A. tauschii* (DD; accession number TA1651) parents was PCR-amplified with the primers derived from AFLP-3 and AFLP-33 (Figure 8). The parental (*A. tauschii*) genomic fragments for both genes were present in the synthetic hexaploid. We did not detect a genomic sequence from the durum parent with the PCR primers used, which maybe caused by the primer specificity or gene deletion in the durum plant. Similarly, Southern analyses of AFLP-5 and AFLP-9 showed that all parental genomic fragments were accounted for in the synthetic hexaploid, and no new DNA fragments were detected. Thus, at least with the four genes examined, we did not find any evidence of genomic sequence changes.

Discussion

Early studies of isozymes in hexaploid wheat indicated that most enzymes examined showed similar expression from three orthologues, and gene silencing was rare (Hart, 1979, 1996). However, the expression of high-molecular-weight (HMW) glutenin genes encoded by the A-genome, but not B- or D-genomes, is frequently suppressed in tetraploid and hexaploid wheat (Galili and Feldman, 1983, 1984; Feldman *et al.* 1986). A caveat with these results is that the results may be biased toward the enzymes or storage proteins chosen in the studies. Our cDNA-AFLP display combined with Northern and RT-PCR analyses provide a better assessment of gene expression in the hexaploid wheat. Up to 7.7% of the parental cDNA-AFLP fragments were altered in the synthetic line, suggesting that a significant fraction of the genome is altered. Northern and RT-PCR results with the selected genes were completely consistent with cDNA-AFLP results, indicating that the cDNA-AFLP results were highly reliable. It is interesting that *A. tauschii* genes were affected much more frequently than *T. turgidum* genes, although the latter carried twice as many genes. This correlated with the gross morphology of hexaploid wheat that is more similar to *Triticum* than *Aegilops*. A significant fraction of these genes was also down-regulated in Chinese Spring. Thus, we conclude that the alteration of gene expression is frequent in both synthetic and natural hexaploid wheat.

Several possibilities can be proposed for the altered gene expression in the hexaploid wheat. Allopolyploidy may impact gene expression by: increased genome size and gene number; interaction of different genomes in the same nucleus; interaction of the nuclear genome with a new cytoplasmic genome (Gill, 1991).

Increased ploidy is known to cause both gene silencing and gene activation in yeast (Galitski *et al.*, 1999) and gene silencing in *Arabidopsis* (Mittelsten-Scheid *et al.*, 1996; Lee and Chen, 2001; Madlung *et al.*, 2002) and maize (Guo *et al.*, 1996). Group I gene silencing appears to be associated with tetraploid and hexaploid plants. All group I genes are expressed in *A. tauschii*, but few orthologous transcripts were detected in *T. turgidum* and *T. aestivum* (Figure 3). This suggests that the orthologous genes might have been silenced in *T. turgidum* in the previous round of polyploidization, presumably because of the increased ploidy. It is unlikely that the probes might have failed to cross-hybridize with orthologous genes. In fact, the AFLP-5 and AFLP-33 probes detected homologous sequences in *T. turgidum* in Southern blot analysis (Figure 8; P. He and J.-M. Zhou, unpublished results). A previous round of gene silencing in tetraploid wheat might explain the relatively low frequency of AB-origin genes being affected in hexaploid wheat (Table 1). Alternatively, the preferential down-regulation of D genome genes may reflect the incompatibility between the *A. tauschii* nuclear genome and *T. turgidum* cytoplasm. It is unlikely, however, that *T. turgidum*

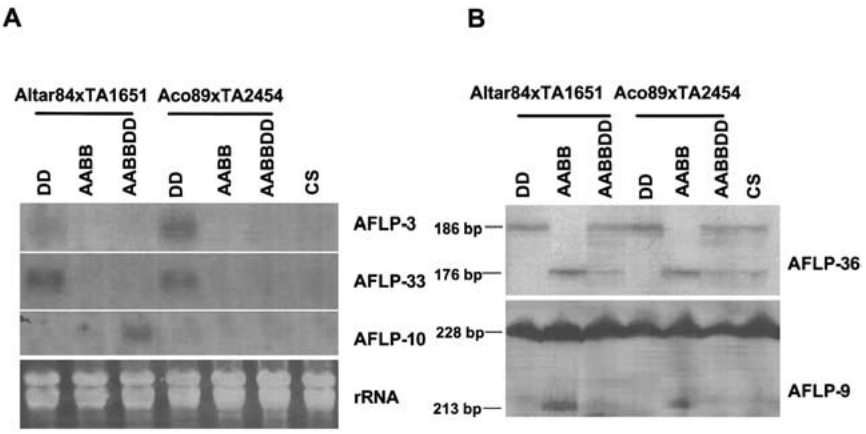

Figure 7. Gene expression changes are nonrandom and occur in a natural hexaploid. A. Northern analysis of AFLP-3, AFLP-33, and AFLP-10 in the two synthetic hexaploid lines, their respective parental lines, and Chinese Spring. B. RT-PCR analysis of AFLP-9 and AFLP-36 in the two synthetic hexaploid lines, their respective parental lines, and Chinese Spring. Actin cDNA was amplified as constitutive control. PCR reactions without reverse transcriptase did not produce any products (not shown).

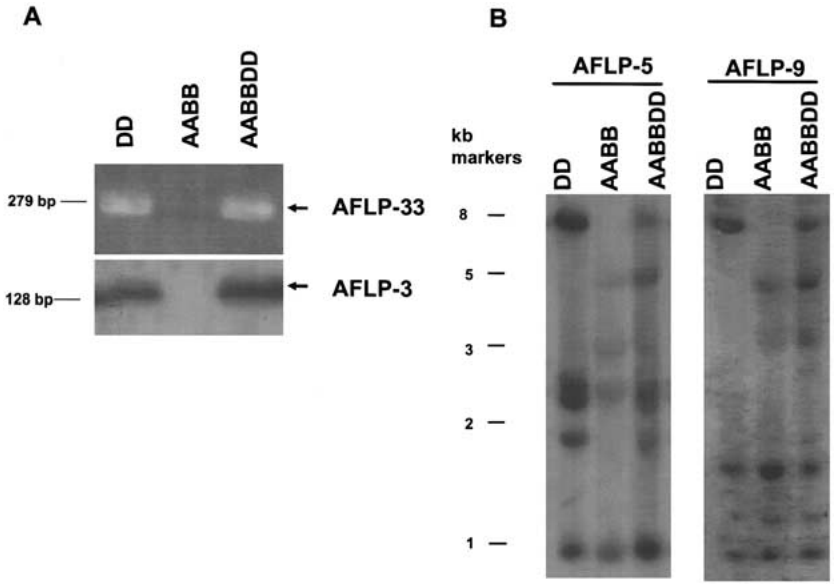

Figure 8. Lack of genomic changes at the differentially expressed loci. A. Genomic PCR of AFLP-3 and AFLP-33. PCR products were resolved on an agarose gel (AFLP-33) or a sequencing gel (AFLP-3). B. Southern analysis of AFLP-5 and AFLP-9. Genomic DNA from the synthetic hexaploid (AABBDD) and parental lines (AABB and DD) was digested with *Eco*RI before fractionation on an agarose gel. Southern blots were hybridized with the indicated probes.

plants carried deletions at the orthologous loci, because homologous sequences of AFLP-5 and AFLP-33 were detected in both *A. tauschii* and *T. turgidum* (Figure 8; P. He and J.-M. Zhou, unpublished results).

In contrast to group I, group II gene expression was affected for only one of the parental genes. Three of the four group II genes were of the *T. turgidum* origin. Therefore, it is unlikely that their down-regulation is caused by a nuclear-cytoplasmic genome interaction. These genes were down-regulated in an orthologue-specific manner, and this can not be explained by the increased ploidy level. Instead, group II genes may be regulated by interactions between different subgenomes in the same nucleus. Loss and activation of gene expression have been reported in the newly synthesized allotetraploid Triticeae derived from a cross between *Aegilops sharonensis* ($S^{sh}S^{sh}$) and *Triticum monococcum* ssp. *aegilopoides* (A^mA^m).

Some of these alterations are accounted for by gene loss (Kashkush *et al.*, 2002). In contrast, gene loss did not appear to be the cause of altered gene expression in the synthetic hexaploid wheat. First, most of the cDNA-AFLP fragments exhibited reduced, instead of a lack of expression in the hexaploid plants. Second, Southern blot and PCR analyses of genomic DNA indicated that at least the five genes examined (AFLP-3, -5, -9, -10, and -33) did not show detectable genomic changes. It remains to be determined if rapid genomic restructuring occur in the hexaploid wheat.

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