Gene Loss, Silencing and Activation in a Newly Synthesized Wheat Allotetraploid

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ABSTRACT

We analyzed the events that affect gene structure and expression in the early stages of allopolyploidy in wheat. The transcriptome response was studied by analyzing 3072 transcripts in the first generation of a synthetic allotetraploid (genome $S^lS^lA^mA^m$), which resembles tetraploid wheat (genome BBAA), and in its two diploid progenitors *Aegilops sharonensis* (S^lS^l) and *Triticum monococcum* ssp. *aegilopoides* (A^mA^m). The expression of 60 out of 3072 transcripts was reproducibly altered in the allotetraploid: 48 transcripts disappeared and 12 were activated. Transcript disappearance was caused by gene silencing or by gene loss. Gene silencing affected one or both homeologous loci and was associated in part with cytosine methylation. Gene loss or methylation had occurred already in the F_1 intergeneric hybrid or in the allotetraploid, depending on the locus. The silenced/lost genes included rRNA genes and genes involved in metabolism, disease resistance, and cell cycle regulation. The activated genes with a known function were all retroelements. These findings show that wide hybridization and chromosome doubling affect gene expression via genetic and epigenetic alterations immediately upon allopolyploid formation. These events contribute to the genetic diploidization of newly formed allopolyploids.

ONE of the important insights derived from genome sequencing projects in eukaryotes is that species that were considered typical diploids (*e.g.*, yeast, humans, and Arabidopsis) are in fact ancient polyploids (paleopolyploids) that have undergone one or more rounds of chromosome doubling during their evolution but nevertheless behave cytologically as diploids (see review by Wolfe 2001). Presumably, diploidization of polyploids enables the organism to deal, on one hand, with genome stability and fertility via proper chromosome pairing and segregation (chromosome diploidization) and, on the other hand, with gene redundancy through genetic diploidization (*e.g.*, gene loss and silencing). Other events such as gene dosage compensation and gene activation are probably also necessary for the harmonious expression of different genomes. Most plant species have undergone one or more polyploidization events during their history (Leitch and Bennett 1997), including Arabidopsis, which is probably an ancient tetraploid (Arabidopsis Genome Initiative 2000). The duplication of genomes, either of the same (autopolyploidy) or more frequently of diverged genomes (allopolyploidy or amphiploidy), is therefore a major force of evolution that affects genome size and gene copy number (Soltis and Soltis 2000; Wendel 2000). To establish themselves as successful species, newly formed allopolyploids must overcome the reduced fertility (Comai *et al.* 2000; Ozkan *et al.* 2001) that usually occurs in the first generations following chromosome doubling, resulting in part from improper chromosome pairing and segregation. Rearrangements in noncoding genomic DNA that were found in newly synthesized allopolyploids (Feldman *et al.* 1997; Liu *et al.* 1998; Ozkan *et al.* 2001; Shaked *et al.* 2001) may contribute to such a stabilization process. Another way to enable harmonious cohabitation of two different genomes in the same nucleus is through epigenetic changes and through the regulation of gene expression. One of the best-characterized examples of such regulation is the silencing of transcription of the rRNA genes of one of the parental sets in amphiploids (for review see Pikaard 1999). Pioneer work done in transgenic plants (Matzke *et al.* 1989; Elkind *et al.* 1990; Napoli *et al.* 1990) showed that gene silencing occurred in duplicated genes (transgenes *vs.* the similar endogenous gene). It was also shown that a change in ploidy might affect transgene silencing in Arabidopsis (Scheid *et al.* 1996). However, only a few works have addressed the response of the transcriptome to whole genome duplication. In yeast, the work of Galitski *et al.* (1999), using cDNA microarrays, showed ploidy-regulated activation and silencing of genes, including genes that are related to cell growth and development. In plants, Guo *et al.* (1996) have analyzed the transcript level of 18 specific genes in a maize ploidy series of 1x to 4x. They found that most genes had the mRNA level expected for their ploidy with a few exceptions where gene expression was up- or downregulated. Two recent works in synthetic and in natural Arabidopsis allotetraploids (Comai *et al.* 2000 and Lee and Chen 2001, respectively), involving a genome-wide analysis of gene expression using cDNA-amplified fragment length polymorphism (AFLP), reported on epigenetic

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changes and on gene silencing. Patterns of DNA methylation in and around the silenced genes were described (Lee and Chen 2001). Understanding which types of genes are affected and which mechanisms are involved in ploidy regulation of gene expression remains a major challenge. Such an understanding is relevant to the formation and diploidization of new polyploid species as well as to cellular processes involving changes in ploidy, *e.g.*, endoreduplication in particular cell types and in certain types of cancer.

The wheat (Aegilops-Triticum) group offers an ideal system for studying the evolution of polyploids because several species are young, such as the hexaploid bread wheat, which is only ~8500 years old, because most diploid progenitors are known and because allopolyploids can be easily synthesized (Feldman 2001). In previous works, we analyzed a random set of genomic loci in wheat and we showed that DNA elimination and methylation are rapid responses of the genome to wide hybridization and chromosome doubling (Ozkan *et al.* 2001; Shaked *et al.* 2001). The loci analyzed had no similarity to known genes, with the exception of retroelements, and were probably noncoding. In this work, we have focused on the fate of genes during the early stages of allopolyploidy. Gene expression of an unbiased set of wheat genes was analyzed in the first generation of a synthetic allotetraploid and in its two diploid progenitors. We report on rapid gene loss, either in the F_1 intergeneric hybrid or after chromosome doubling, on gene silencing, in part associated with cytosine methylation, and on the transcriptional activation of retroelements. These events contribute to genetic diploidization of a large number of loci in the very early stages of amphiploidy.

MATERIALS AND METHODS

Plant material: The plant material for the present study consists of the newly synthesized allotetraploid (genome $S^lS^lA^mA^m$) that is related to tetraploid wheat (genome BBAA) and its diploid progenitors *Aegilops sharonensis* (TH02; genome S^lS^l) and *Triticum monococcum* ssp. *aegilopoides* (TMB02; genome A^mA^m). The synthetic allotetraploid was obtained after colchicine treatment of the F_1 plants. In this work, the S_1 allotetraploid, namely, the first generation after chromosome doubling, was used. Production of the allotetraploid is described in Ozkan *et al.* (2001). Only those plants having the expected karyotype, namely, 28 chromosomes in mitotic metaphase and 14 bivalents in meiotic metaphase, were used in this study. The parental lines were tested for homozygosity using 23 restriction fragment length polymorphism markers with at least two markers per chromosomal arm. No heterozygotes were found (data not shown).

DNA analysis: Genomic DNA was extracted from young fresh leaves by the cetyltrimethylammonium bromide method (Kidwell and Osborn 1992). Southern blot analysis followed essentially the method described by Liu *et al.* (1997). Analysis of DNA similarity was performed using the BLAST package 2.0 on the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST/). In addition, sequences were annotated using the LabOnWeb software of Compugen (http://www.labonweb.com/).

Gene expression analysis: Total RNA was isolated from fresh leaves of seedlings 2 weeks after germination and from root tips of seedlings 2 days after germination using the TRI (St. Paul) reagent method (Chomczynski 1993). First and second strand cDNA synthesis was carried out according to standard protocols (Sambrook *et al.* 1989). The resulting double-stranded cDNA was phenol extracted, ethanol precipitated, and resuspended in a final volume of 40 μl ddH_2O. Half of this volume was checked on gel and if the expected smear between 100 and 3000 bp was observed, the rest of the cDNA was subjected to the standard AFLP analysis (Vos *et al.* 1995). Fragments that showed qualitative gene expression alterations in the amphiploid (appearance or disappearance of bands compared to the diploid parents) were excised from the sequencing cDNA-AFLP gel and reamplified using the following PCR conditions: 3 min at 94°, 30 sec at 94°, 1 min at 56°, and 1 min at 72°, followed by 34 cycles. Each cDNA-AFLP gel was done in duplicate observing full reproducibility between replicas in the internal part of the gel that was used for band scoring (the upper and lower parts of the gel give poor band resolution). To ensure that there is no DNA contamination in our RNA samples, a negative control was prepared without reverse transcriptase. A clear cDNA-AFLP gel with no bands was obtained (data not shown). The RNA-AFLP-isolated fragments (RAIFs) were sequenced and then ligated into pGEM-T easy vector (Promega, Madison, WI) and transformed into the XL-1 blue *Escherichia coli* strain (Stratagene, La Jolla, CA). They were resequenced using universal T7 and/or T3 primers. Reverse-Northern analysis was performed as described (Zegzouti *et al.* 1997) with minor modifications. Each RAIF was reamplified and run on a 1.5% agarose gel and transferred to a nylon membrane. The cDNA was ^{32}P-labeled as described (Feinberg and Vogelstein 1983) and was hybridized to a Hybond N^+ membrane (Amersham, Buckinghamshire, UK) according to the manufacturer's recommendations. The reverse-Northern blot experiments were repeated twice using different RNA sources of the parental lines and the allotetraploid. Reverse transcriptase (RT)-PCR was performed according to the Super-Script One-Step RT-PCR kit (GIBCO-BRL, Gaithersburg, MD) using the following PCR conditions: 3 min at 94°, 30 sec at 94°, 1 min at 52°, and 1 min at 72°, followed by 34 cycles. The primer pair for the NBS/LRR resistance gene RPM1 are forward 5′-GG GATCGAGGAGAACAAGGA-3′ and reverse 5′-GCAACCCAGTG AGGCTATTA-3′. The amplified products were analyzed in 1.5% agarose gel.

RESULTS

Detection of gene expression alterations in the newly synthesized allotetraploid by cDNA-AFLP: cDNA-AFLP band patterns were compared between the synthetic allotetraploid and its two homozygous diploid parents *Ae. sharonensis* (genome S^lS^l) and *T. monococcum* ssp. *aegilopoides* (genome A^mA^m). A gene expression alteration event was scored only when a new band, which is absent in the diploid parents, appeared in the allotetraploid or, conversely, when a band present in the diploids disappeared in the allotetraploid. We monitored only qualitative and not quantitative differences. Figure 1 shows the cDNA-AFLP patterns detected in the parents and allotetraploid. Note that bands were scored only in the high-resolution part of the gel, namely the middle part,

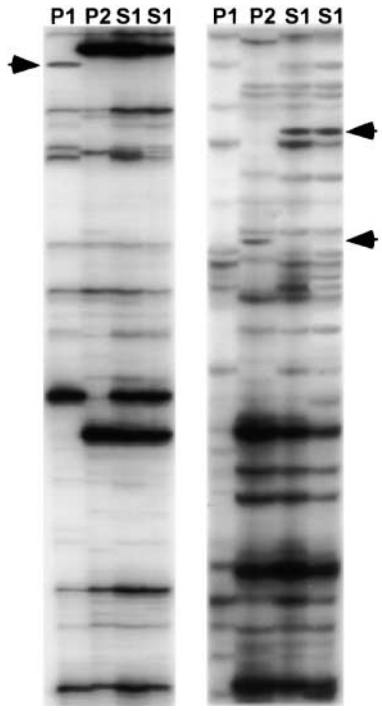

FIGURE 1.—cDNA-AFLP patterns detected in the two diploid parents, *Ae. sharonensis* (P_1), and *T. monococcum* ssp. *aegilopoides* (P_2), and the first generation of the derived synthetic allotetraploid (S_1). (Left) The primer combination used is *Eco*RI-5′-GACTGCGTACCAATTCACT-3′/*Mse*I-5′-GATGAGTCCTGAGTAACAT-3′. (Right) The primer combination used is 5′-GACTGCGTACCAATTCACT-3′/*Mse*I-5′-GATGAGTCCTGAGTAACTT-3′ (Vos *et al.* 1995). The arrows show examples of differential fragments between the parents and the allotetraploid in the high-resolution part of the gel.

where banding patterns were fully reproducible (data not shown). In leaves, 1872 transcripts were examined using 37 pairs of selective primers in the cDNA-AFLP assay (Table 1). Out of these, 1422 transcripts were monomorphic and 450 were polymorphic between the parental lines. Of the 1872 transcripts analyzed, 39 (2.1%) showed alteration in expression in the allotetraploid. Of these 39 transcripts, 29 (74.3%) disappeared in the allotetraploid and 10 (25.7%) new transcripts appeared in the allotetraploid. In root tips, 1200 transcripts were examined using 25 pairs of selective primers (Table 1). Of these 1200 transcripts, 920 were monomorphic and 280 were polymorphic between the parental lines. Of the 1200 transcripts, 21 (1.8%) showed alteration in gene expression in the allotetraploid. Out of these, 19 transcripts disappeared (90.5%) and two new transcripts appeared (9.5%) in the allotetraploid.

Combining the leaf and the root-tip data together, 60 out of 3072 transcripts (2.0%) were altered in their expression pattern in the allotetraploid. Note that this is an underestimate as alterations in monomorphic loci could be detected only when both parental-type transcripts disappeared. Of the altered transcripts, 48 (80%) disappeared in the allotetraploid and 12 (20%) were new transcripts (Table 1).

Characterization of the cDNA-AFLP fragments subjected to gene expression alterations: All the 60 cDNA-AFLP fragments subjected to gene expression alteration (see Table 1) were isolated, sequenced, and cloned. Clear sequences were obtained for 48 fragments. Table 2 summarizes the molecular characterization of the loci subjected to gene expression alterations. Forty-two transcripts (RAIF1-42) were expressed in one or two parental lines and could not be detected in the allotetraploid, and six loci (RAIF43-48) were expressed only in the allotetraploid (Table 2).

Among the 42 silenced genes, sequence analysis showed that five transcripts were not similar to any known gene (RAIF1–3 and RAIF10–11); eight transcripts showed high similarity to the open reading frame (ORF) for putative proteins (RAIF4–6 and RAIF12–16); seven were similar to rice or wheat expressed sequence tags (ESTs; RAIF7, RAIF19–23, and RAIF37); two transcripts showed high similarity to retrotransposons (RAIF9, RAIF33, and RAIF38); two transcripts (RAIF17–18) had a high similarity to ORFs flanked by autonomous replicated se-

TABLE 1

cDNA-AFLP analysis in an allotetraploid and its diploid progenitors

		Expression alteration in allopolyploid[b]		
Tissue	Total no. of transcripts[a]	Transcript absent in allopolyploid (%)	New transcript in allopolyploid (%)	Total alterations (%)
Leaves	1872	29 (1.6)	10 (0.5)	39 (2.1)
Root tips	1200	19 (1.6)	2 (0.2)	21 (1.8)
Total	3072	48 (1.6)	12 (0.4)	60 (2.0)

[a] Each cDNA-AFLP band was considered as a different transcript. The 1872 leaf transcripts are derived from 37 pairs of selective primers and the 1200 root-tip transcripts are from 25 pairs of selective primers. Monomorphic transcripts (similar in both parents) were scored only once.

[b] Only qualitative alterations were scored, namely, disappearance of parental transcripts in the allotetraploid or appearance of a new transcript in the allotetraploid. The percentage of altered bands is out of the total number of transcripts.

TABLE 2

Molecular characterization of cDNA clones obtained by cDNA-AFLP

Clone identification	cDNA AFLP pattern[a]			Sequence similarity[b]
	S^lS^l	A^mA^m	$S^lS^lA^mA^m$	
RAIF1–3	+	−	−	None
RAIF4–6	+	−	−	ORF (putative proteins)
RAIF7	+	−	−	Rice EST
RAIF8	+	−	−	*Ae. speltoides* genome-specific DNA
RAIF9	+	−	−	Wheat Wis 2-1A retrotransposon
RAIF10–11	−	+	−	None
RAIF12–16	−	+	−	ORF (putative proteins)
RAIF17–18	−	+	−	Wheat DNA for ORF flanked by ARS
RAIF19–23	−	+	−	Wheat EST
RAIF24–26	−	+	−	Rye 26S rRNA
RAIF27	−	+	−	Gene cluster 21-kb wheat chloroplast DNA
RAIF28	−	+	−	Wheat rubisco-large subunit
RAIF29	−	+	−	Wheat mitochondrion rrn26 gene for rRNA
RAIF30	−	+	−	Rice alpha 2 subunit of 20S proteasome
RAIF31	−	+	−	*Agropyron cristatum*-RAPD marker
RAIF32	−	+	−	Wheat acetyl-coenzyme A carboxylase
RAIF33	−	+	−	Wheat retrotransposon Tar1
RAIF34	−	+	−	Barley NBS/LRR resistance gene RPM1
RAIF35	−	+	−	Human cell cycle regulatory protein p95
RAIF36	+	+	−	ORF (putative proteins)
RAIF37	+	+	−	Wheat EST
RAIF38	+	+	−	*Arabidopsis thaliana* putative retroelement
RAIF39	+	+	−	Succinyl-diaminopimelate desuccinylase
RAIF40	+	+	−	*A. thaliana* Ring-h2 finger protein
RAIF41–42	+	+	−	*A. thaliana* acylaminoacyl-peptidase
RAIF43–44	−	−	+	None
RAIF45	−	−	+	Maize retrotransposon (gag)
RAIF46–48	−	−	+	Wheat Wis2-1A retrotransposon

RAPD, randomly amplified polymorphic DNA.

[a] +, transcript present; −, transcript absent.

[b] The search was done using the BLAST package. Similarity was considered as significant for E-values $< e^{-10}$.

quences (ARS); three transcripts corresponded to nuclear 26S ribosomal RNA genes (RAIF24–26); three transcripts showed high similarity to organellar genes (RAIF27–29); RAIF30 showed high similarity to the rice alpha 2 subunit of 20S proteasome; and one transcript (RAIF32) was identical to the *Acc-2* gene, GenBank accession no. U39321 (Faris *et al.* 2001). RAIF34 showed high similarity to the barley NBS/LRR powdery mildew resistance protein RPM1 and the RAIF35 transcript showed high similarity to the human cell cycle regulatory protein p95. RAIF39 was similar to the Arabidopsis succinyl-diaminopimelate desuccinylase, RAIF40 was similar to the Arabidopsis Ring-h2 finger protein, and RAIF41–42 were similar to the Arabidopsis acylaminoacyl-peptidase.

Among the six transcripts (RAIF43–48) that were expressed only in the allotetraploid (Table 2), two transcripts had no similarity to known genes (RAIF43 and RAIF44) and four transcripts showed high similarity to retroelements (RAIF45–48).

Reverse-Northern blot and RT-PCR validation of the cDNA-AFLP detected expression alteration: To further analyze the cDNA-AFLP results and to avoid possible artifacts, the RAIFs were subjected to reverse-Northern blot analysis and RT-PCR. For example, differential cDNA-AFLP patterns between the two parents could result from one of the two following possibilities: the homeologous transcripts are expressed in both parents but sequence polymorphism gives rise to a differential migration pattern or, alternatively, the transcript is expressed in only one of the parents. Methods based on hybridization, such as the reverse-Northern blots, can help distinguish between these possibilities. Examples of reverse-Northern blots are shown in Figure 2A. In lanes 1, 2, and 7 a transcript was observed in both parents and was missing in the amphiploid, and in lanes 8 and 9 a transcript was observed in only one of the parents and was missing in the amphiploid. No signal could be detected with lanes 3, 4, and 6. In lanes 5 and 10, hybridization was observed in both parents and in the amphiploids.

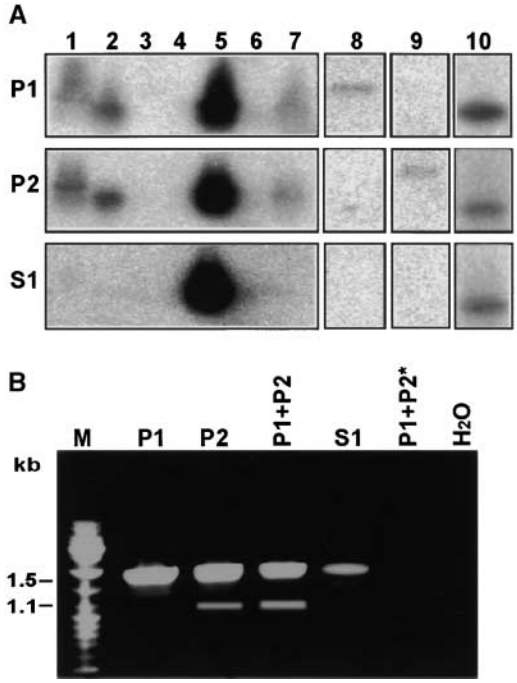

FIGURE 2.—Analysis of expression patterns. (A) Reverse-Northern blot validation of the cDNA-AFLP-detected gene expression alterations. Each of the cDNA-AFLP fragments was PCR amplified, loaded on an agarose gel, and transferred to a nylon membrane. Triplicate membranes were prepared and each membrane was hybridized to ^{32}P-labeled total cDNA of *Ae. sharonensis* (P_1), *T. monococcum* ssp. *aegilopoides* (P_2), and the synthetic allotetraploid (S_1), respectively. Lanes 1–10 correspond to (starting from lane 1) RAIF37, RAIF42, RAIF13, RAIF11, RAIF25, RAIF43, RAIF40, RAIF3, RAIF15, RAIF34, respectively (see Table 2 for gene annotations). (B) RT-PCR analysis of the NBS/LRR resistance gene RPM1 (RAIF34). The expected fragment (1.1 kb) is present only in P_2 and is absent in the allotetraploid (S_1). Lane $P_1 + P_2^*$ is a mixture of the cDNA templates of the two parents used as a control to ensure similar competition conditions. Two negative controls were used: $P_1 + P_2^*$ is a mixture of the RNA of the two parents after RNAase treatment and in the H_2O lane water was used as a template for the RT-PCR reaction. Lane M is a 1-kb DNA ladder molecular weight marker (MBI-Fermentas).

In these cases it is not possible to determine whether silencing occurred in one of the parents; therefore RT-PCR was used whenever possible to check the cDNA-AFLP data. Table 3 summarizes the validation results of the loci subjected to gene expression alterations. Six different classes of reverse-Northern blot hybridization patterns have been detected (Table 3, classes A–F). Interestingly, the cDNA-AFLP method could identify only six genes (Table 2, RAIFs 36–40) for which both parental transcripts were silenced in the allotetraploid. The use of a hybridization-based method supports the cDNA-AFLP data and further shows that this group (Table 3, class A) in fact comprises 13 genes. This suggests that some of the differential cDNA-AFLP fragments in the parents were obtained because of sequence polymorphism rather than differential expression. Genes in classes B and C showed differential patterns with both the cDNA-AFLP and the hybridization methods, validating the cDNA-AFLP data and suggesting differential expression in the parents and silencing of the active homeologue in the amphiploid. Genes in class D, namely genes that were not expressed in both parents but were activated in the amphiploid, support the cDNA-AFLP data, except for RAIF43 (class F), which was apparently below expression level detection by reverse-Northern analysis. Genes in class E show the same hybridization patterns in both parental lines and in the allotetraploid (see lanes 5 and 10 in Figure 2A). This is an apparent discrepancy with the cDNA-AFLP data, suggesting either a cDNA-AFLP artifact or the inability of hybridization-based methods to differentiate between the two parents. To distinguish between these two possibilities, five genes of class E were tested for further validation by RT-PCR analyses. Figure 2B shows RT-PCR validation of RAIF34, the NBS/LRR resistance gene RPM1 (see Table 2 and lane 10 in Figure 2A). According to the cDNA-AFLP data (Table 2), silencing of the *T. monococcum* homeoallele occurred. The same pattern of silencing was obtained by RT-PCR: the 1.1-kb expected band was amplified only in *T. monococcum* and was absent in the allotetraploid. Similarly, silencing was confirmed in all five RT-PCR-tested transcripts (Table 3, class E) according to the same pattern as found by cDNA-AFLP. This suggests that genes in class E are expressed in both parents (this could not be seen by cDNA-AFLP only because of sequence polymorphism between the homeoalleles) and silenced in only one of them (this cannot be seen by reverse-Northern blot only because of hybridization to both homeoalleles). No signal could be detected in four clones (class F), suggesting a weak expression level. Overall, both reverse-Northern and RT-PCR data confirmed the robustness of the cDNA-AFLP method and enabled further characterization of gene expression.

Southern blot analysis of the transcripts showing altered expression: To test whether alterations in gene expression were associated with DNA rearrangements or with changes in cytosine methylation, a Southern blot analysis was performed for 12 RAIFs, corresponding to transcripts that disappeared in the amphiploid. This analysis included both parents, the F_1 hybrid, and the first generation of the amphiploid, following chromosome doubling of the F_1 and the use of six different enzymes (*Hpa*II, *Msp*I, *Eco*RI, *Dra*I, *Eco*RV, and *Hin*dIII). Results are summarized in Table 4. One fragment, RAIF8, was a high-copy gene and Southern analysis was not informative. Transcript disappearance was associated with cytosine methylation in the allotetraploid in 4 out of the 12 loci analyzed. Figure 3A shows cytosine methylation patterns using the two isoschizomers *Hpa*II and *Msp*I and RAIF34 as a probe (see description in Tables 2 and 3). Alterations in cytosine methylation at this locus are probably due to hypermethylation of both cytosines in the *Hpa*II and *Msp*I restriction sites, as suggested by the

TABLE 4

Southern blot analysis in an allotetraploid and its diploid progenitors

Probe[a]	Origin of the transcript	Copy no.	Alteration of the hybridization pattern	
			Type[b]	Timing[c]
RAIF3	*sharonensis*	Low	Cytosine methylation	S_1
RAIF8	*sharonensis*	High	ND	
RAIF13	*aegilopoides*	Low	Gene loss	F_1
RAIF16	*aegilopoides*	Low	Gene loss	F_1
RAIF17	*aegilopoides*	Low	No alteration	
RAIF18	*aegilopoides*	Low	No alteration	
RAIF30	*aegilopoides*	Low	Cytosine methylation	S_1
RAIF31	*aegilopoides*	Low	No alteration	
RAIF32	*aegilopoides*	Low	Gene loss	S_1
RAIF34	*aegilopoides*	Low	Cytosine methylation	S_1
RAIF35	*aegilopoides*	Low	No alteration	
RAIF40	Both parents	Low	Cytosine methylation	F_1

ND, no data.

[a] Twelve transcripts that were expressed in one or both diploid parents and disappeared in the first generation of the allotetraploid (S_1) were used as probes in Southern blots (see details in Table 2).

[b] No alteration, no deviation from additivity between the parents, F_1, and S_1 amphiploid. Gene loss was considered when all enzymes (*Hpa*II, *Msp*I, *Eco*RI, *Dra*I, *Eco*RV, and *Hin*dIII) tested showed band disappearance.

[c] Generation when the alteration occurred. Cases with no alteration (−) are not relevant. Alterations occurring in F_1 were maintained in S_1.

shift of the lower band, higher in the gel (Figure 3A), and by the lack of evidence for DNA rearrangements when using other restriction enzymes such as *Dra*I (Figure 3B) and other enzymes (*Eco*RV, *Eco*RI, and *Hin*dIII). This change in methylation did not occur in the F_1 but only after chromosome doubling. For the other probes that showed methylation changes, the alteration occurred also after chromosome doubling (RAIF3 and RAIF30) or already in the F_1 hybrid (RAIF40; Table 4). By contrast, the hybridization pattern of the same blot as shown in Figure 3A with probe RAIF16 showed no evidence for changes in methylation but rather suggested that the *T. monococcum* ssp. *aegilopoides* fragment was eliminated in the F_1 hybrid (Figure 3C). Further evidence for gene loss was obtained when using the same probe with *Eco*RI (Figures 3D) or other enzymes (data not shown). Results similar to those of RAIF16 were obtained for RAIF13 (data not shown). The hybridization pattern obtained using the RAIF32 probe also showed evidence of DNA elimination (Figure 3, E and F) but in this case gene loss occurred after chromosome doubling.

With other probes used in the Southern blot analysis, namely, RAIF8, RAIF17, RAIF18, RAIF31, and RAIF35, we found no deviation from additivity in the Southern blot hybridization patterns between parents, F_1, and amphiploids, suggesting that gene silencing in these cases is not associated with gene loss or with cytosine methylation at the restriction sites analyzed.

TABLE 3

Reverse-Northern blot analysis in an allotetraploid and its diploid progenitors

Class	Reverse-Northern hybridization[a]			Clones[b]	No. of loci
	S^lS^l	A^mA^m	$S^lS^lA^mA^m$		
A	+	+	−	RAIF4–5, RAIF8, RAIF14, RAIF16, RAIF30, RAIF32, RAIF36–38, RAIF40–42	13
B	+	−	−	RAIF1–3, RAIF6	4
C	−	+	−	RAIF10, RAIF12, RAIF15, RAIF17–23, RAIF35	11
D	−	−	+	RAIF44–48	5
E	+	+	+	RAIF7,[c] RAIF9,[c] RAIF24–29, RAIF33,[c] RAIF34,[c] RAIF39[c]	11
F	−	−	−	RAIF11, RAIF13, RAIF31, RAIF43	4

[a] Hybridization was (+) or was not (−) detected.

[b] See details of each clone in Table 2.

[c] Showed the RAIFs that were also tested by RT-PCR (see RESULTS).

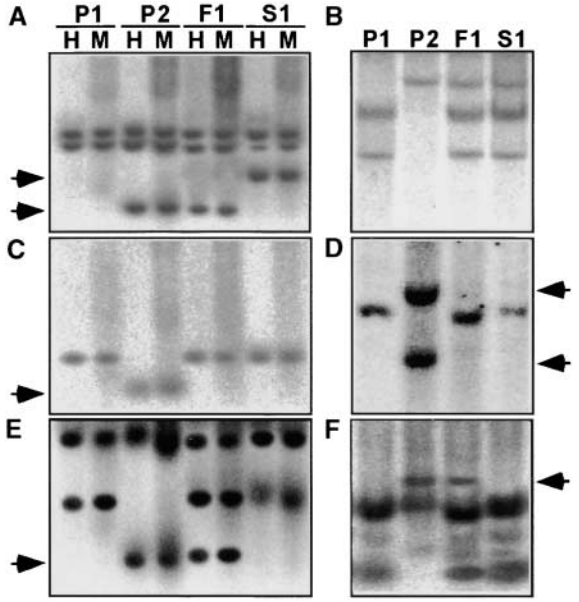

FIGURE 3.—Southern blot analysis of three genes that showed an alteration in gene expression in the F_1 hybrid and the first generation allotetraploid (S_1) of the cross between *Ae. sharonensis* (P_1) and *T. monococcum* ssp. *aegilopoides* (P_2). (A, C, and E) DNA samples of P_1, P_2, F_1, and S_1 digested with the two isoschizomers *Hpa*II (H) and *Msp*I (M) loaded on the gel side by side and hybridized with ^{32}P-labeled probes RAIF34, RAIF16, and RAIF32, respectively. (B, D, and F) DNA samples probed with RAIF34, RAIF16, and RAIF32, respectively, after digestion with *Dra*I (B) and *Eco*RI (D and F). See Table 2 for probe description.

DISCUSSION

In this work, we showed that gene loss, silencing, and activation occur in the early stages of allopolyploid formation. This is the first report showing that genes can be lost from one parent's genome as early as the F_1 intergeneric hybrid or just after chromosome doubling. Gene loss is an irreversible process, in contrast to epigenetic alterations that can further change during evolution. Gene silencing associated with cytosine methylation was shown recently in newly synthesized and natural amphiploids in Arabidopsis (COMAI *et al.* 2000; LEE and CHEN 2001). We show here that methylation in or near genes can occur already in the F_1 hybrid or soon after chromosome doubling. The combined use of cDNA-AFLP, reverse-Northern analysis, and RT-PCR showed that amphiploidy can trigger silencing of one or both homeologous alleles or activation of new transcripts. This suggests that both wide hybridization and allopolyploidy can trigger changes in gene structure and expression through deletion, silencing, or activation of one or both homeoalleles. These changes contribute to the rapid genetic diploidization of the new species and to novel types of expression profiles.

Gene loss: Evidence that gene loss has occurred throughout wheat evolution has already been reported (GORNICKI *et al.* 1997; GAUTIER *et al.* 2000; FEUILLET *et al.* 2001). In previous works, we found that DNA elimination in newly synthesized amphiploids involved sequences that had no homology to known genes and were probably noncoding sequences (FELDMAN *et al.* 1997; LIU *et al.* 1998; OZKAN *et al.* 2001; SHAKED *et al.* 2001). Here we show that genes can be lost as early as in the F_1 hybrid or in the first generation of an amphiploid. This suggests that some of the previously reported cases of gene loss (GORNICKI *et al.* 1997; GAUTIER *et al.* 2000; FEUILLET *et al.* 2001) did not occur on an evolutionary scale but rather early on in the amphiploid's life. In support of this hypothesis, we note that one of the eliminated genes we found (RAIF32) is identical in sequence to the wheat acetyl-coenzyme A carboxylase *Acc-2* gene (FARIS *et al.* 2001). The *Acc-2* gene was recently shown to be missing from genome A of tetraploid wheat (genome BBAA) while it was present as a single gene in the genome of each of the three diploid progenitors of hexaploid (bread) wheat (FARIS *et al.* 2001). Similarly, in the synthetic allotetraploid analyzed here, elimination occurred in genome A^m, which is very close to genome A of domesticated wheat, thus mimicking the natural event. Although we do not have an accurate quantitative estimate of the percentage of transcript disappearance that can be explained by gene loss, we found three such events (RAIF13, RAIF16, and RAIF32) out of 12 probes analyzed by Southern blot, suggesting that gene loss is a significant factor that causes transcript disappearance in nascent amphiploids. In summary, gene loss can be induced by wide hybridization (in the F_1) or by chromosome doubling soon after the formation of the allopolyploid. This contributes irreversibly to various aspects of diploidization of the new amphiploids: genetic diploidization of gene expression, which may lead to a more harmonious genetic activity. In addition, gene loss in F_1 or in the first allopolyploid generation may contribute to instantaneous cytological diploidization by increasing the physical divergence of homeologous chromosomes, as was suggested for elimination of noncoding DNA (OZKAN *et al.* 2001).

Gene silencing: We refer to gene silencing for all the transcript disappearance events where there is no evidence for gene loss. Southern blot analysis showed that 9 out of 12 probes were not involved in any genetic rearrangements. Assuming that ~75% of the 48 lost transcripts disappeared because of gene silencing, this means that silencing occurred in ~1% of all the transcripts. This ratio is probably an underestimate because of the 3072 bands analyzed only 730 were polymorphic and silencing of a monomorphic transcript (as seen by cDNA-AFLP) would be unnoticed if it occurred in only one of the parents. Therefore, the actual ratio of silenced genes can be only roughly estimated to be in the 1–5% range. This estimate is in the same order of magnitude as found in Arabidopsis (COMAI *et al.* 2000; LEE and CHEN 2001). Interestingly, silencing can affect one or both homeoalleles at similar frequencies, as de-

termined by the combined cDNA-AFLP, reverse-Northern, and RT-PCR data. For the nine silenced genes analyzed by Southern blot, four (RAIF3, RAIF30, RAIF34, and RAIF40) showed evidence for *de novo* cytosine methylation of an unmethylated parent in the amphiploid (Figure 3A), while in the other five loci (RAIF8, RAIF17, RAIF18, RAIF31, and RAIF35) no evidence was found for methylation or DNA rearrangements. The mechanisms for this transcriptional or post-transcriptional gene silencing is still unknown. While gene loss is an irreversible event, gene silencing is potentially reversible. In addition, methylated genes may become hot spots for future mutations (Chan *et al.* 2001).

Sequence analysis of the transcripts that disappeared in the amphiploid: The spectrum of the lost/silenced genes did not show a trend for a particular class: genes involved in metabolism, disease resistance, cell cycle regulation, and retrotransposons were affected. The finding of silencing of rRNA genes (RAIF24–26) is consistent with previous reports on polyploid wheat on dominance of the nuclear organizer from the genome of one of the progenitors (Sasakuma *et al.* 1995) and with reports on Arabidopsis amphiploids (Chen *et al.* 1998). The presence of organellar genes among the disappearing transcripts suggests that some nonpolyadenylated transcripts escaped the mRNA selection through oligo(dT) priming during reverse transcription. In this group (RAIF27–29), which can be considered as a positive control, the paternal gene was always missing in the amphiploid, as expected for maternal inheritance of the organelles. The spectrum of the genes whose expression was affected by polyploidy was broad, possibly because of both direct and indirect effects on a variety of functions. Future work should help determine whether such events are fortuitous and generate random variation or whether they contribute to the fitness of the newly formed amphiploid.

Conclusions: This work shows that qualitative (on/off) alterations in gene expression occur for a significant fraction (~1–5%) of the genes in a newly synthesized amphiploid of wheat. It is probable that an even bigger fraction of the genome is affected in a quantitative manner. The response of the wheat transcriptome to allopolyploidy has similarities to that of Arabidopsis (silencing and cytosine methylation) but has also notable differences, such as gene loss, suggesting interspecific differences. Finally, we conclude that wide hybridization and/or chromosome doubling triggers a genomic shock whose outcome is gene loss, gene silencing, and as shown previously, elimination of noncoding DNA (Ozkan *et al.* 2001; Shaked *et al.* 2001). Taken together, these events increase the divergence of the parental genomes and thus contribute to the rapid genetic diploidization of the new amphiploid. Unlike chromosomal diploidization, which contributes to gamete fertility and disomic inheritance and thus increases fitness, the adaptive role of genetic diploidization, shown here through gene loss and silencing, is not clear. Does it increase fitness through reduction of gene redundancy and by making possible the harmonious coexistence of two different genomes, or are gene loss and gene silencing fortuitous events induced by some genomic stress? On the one hand, amphiploidy provides a buffer that enables toleration of a broad range of random genetic and epigenetic alterations (some of which have occurred already in the hybrid and are unrelated to gene dosage), supporting the "neutral" theory. On the other hand, the response of the wheat genome to amphiploidy is rapid, well orchestrated, and reproducible, supporting McClintock's view of a genomic shock response that "initiates a highly programmed sequence of events within the cell that serves to cushion the effect of the shock" (McClintock 1984).

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