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Evolution and Expression of Homeologous Loci in *Tragopogon miscellus* (Asteraceae), a Recent and Reciprocally Formed Allopolyploid

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ABSTRACT

On both recent and ancient time scales, polyploidy (genome doubling) has been a significant evolutionary force in plants. Here, we examined multiple individuals from reciprocally formed populations of *Tragopogon miscellus*, an allotetraploid that formed repeatedly within the last 80 years from the diploids *T. dubius* and *T. pratensis*. Using cDNA–AFLPs followed by genomic and cDNA cleaved amplified polymorphic sequence (CAPS) analyses, we found differences in the evolution and expression of homeologous loci in *T. miscellus*. Fragment variation within *T. miscellus,* possibly attributable to reciprocal formation, comprised 0.6% of the cDNA–AFLP bands. Genomic and cDNA CAPS analyses of 10 candidate genes revealed that only one "transcript-derived fragment" (TDF44) showed differential expression of parental homeologs in *T. miscellus*; the *T. pratensis* homeolog was preferentially expressed by most polyploids in both populations. Most of the cDNA–AFLP polymorphisms apparently resulted from loss of parental fragments in the polyploids. Importantly, changes at the genomic level have occurred stochastically among individuals within the independently formed populations. Synthetic F_1 hybrids between putative diploid progenitors are additive of their parental genomes, suggesting that polyploidization rather than hybridization induces genomic changes in Tragopogon.

POLYPLOIDY, or genome doubling, has been an important process in many eukaryotic lineages, particularly in flowering plants. Recent genome-level studies have revealed that even the model "diploid" plant *Arabidopsis thaliana* has undergone several rounds of whole-genome duplication (Vision *et al.* 2000; Blanc *et al.* 2003; Bowers *et al.* 2003; Blanc and Wolfe 2004b). Many polyploid plants are allopolyploids, having arisen through hybridization between genetically distinct entities (usually species) and chromosome doubling. This combination of divergent but compatible genomes in allopolyploids may provide a novel substrate for evolutionary processes (Stebbins 1950; Levin 1983; Grant 2002; Osborn *et al.* 2003). As all genes in allopolyploids are duplicated, a number of possibilities exist for the evolutionary fate of the homeologs (genes duplicated by polyploidy). Theory predicts three potential outcomes for these duplicated genes: (1) both copies are retained and remain functional, (2) one copy retains the original function while the other copy is lost or silenced, or (3) the two copies diverge such that each copy assumes only a part of the original gene function (subfunctionalization) or one copy acquires a new function (neofunctionalization) (Ohno 1970; Lynch and Conery 2000; Prince and Pickett 2002).

In recent years, studies of genome evolution and gene expression have been in the foreground of polyploidy research (Leitch and Bennett 1997; Soltis and Soltis 1999; Wendel 2000; Liu and Wendel 2003). Much of this work has been conducted on Arabidopsis (Comai *et al.* 2000; Lee and Chen 2001), Brassica (Lukens *et al.* 2004, 2006; Pires *et al.* 2004b), and crop plants, such as wheat (Feldman *et al.* 1997; Ozkan *et al.* 2001; Shaked *et al.* 2001; Kashkush *et al.* 2002, 2003) and cotton (Zhao *et al.* 1998; Liu *et al.* 2001; Adams *et al.* 2003). Among the significant findings from these studies are that polyploids may experience rapid genomic rearrangements (Song *et al.* 1995; Ozkan *et al.* 2001; Lukens *et al.* 2004, 2006), gene loss (Shaked *et al.* 2001; Kashkush *et al.* 2002; Lukens *et al.* 2006), or gene silencing (particularly due to epigenetic mechanisms, such as DNA methylation) (Comai *et al.* 2000; Lee and Chen 2001; Lukens *et al.* 2006).

Despite these important insights, however, little is known about genome evolution and gene expression changes in natural polyploid populations. These data are beginning to emerge for some natural systems, including the Glycine polyploids (Joly *et al.* 2004) and

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Spartina hybrids (Salmon *et al.* 2005). In a study of rDNA evolution and expression in the allotetraploids *Glycine tomentella* and *G. tabacina*, Joly *et al.* (2004) found that most natural allopolyploids possessed only one parental repeat type in their genomes. For polyploids maintaining both copies, preferential expression of one homeolog was detected in most cases. Synthetic polyploids and some artificial diploid hybrids did not exhibit preferential expression of parental rDNA, suggesting that neither polyploidization nor hybridization immediately resulted in nucleolar dominance in Glycine (Joly *et al.* 2004). In the salt marsh grass Spartina, recent work has shown that methylation differences exist in the independently formed F_1 hybrids *Spartina* × *neyrautii* and *S.* × *townsendii* (the latter of which gave rise to the invasive allopolyploid *S. anglica*) (Salmon *et al.* 2005). Although these studies of Glycine and Spartina have yielded important evolutionary insights into natural polyploids, a fundamental question yet to be addressed is whether individuals within polyploid populations show similar or divergent patterns of genome evolution and gene expression. Further, although many natural polyploids have likely formed multiple times (Soltis and Soltis 1993, 1999), little is known about the consequences of recurrent formation on polyploid genome evolution and gene expression.

A unique system for the study of recent and recurrent allopolyploidy in natural populations is provided by the genus Tragopogon (Asteraceae). This system represents a classic example of instantaneous speciation via polyploidy. Following the introduction of three diploid ($2n = 12$) species (*Tragopogon dubius*, *T. pratensis*, and *T. porrifolius*) from Europe to western North America during the early 1900s, two allotetraploid species (*T. mirus* and *T. miscellus*) formed (Ownbey 1950). The ancestries of both allotetraploids were confirmed through flavonoid, isozymic, and DNA studies (Ownbey and McCollum 1953, 1954; Brehm and Ownbey 1965; Roose and Gottlieb 1976; Soltis and Soltis 1989; Soltis *et al.* 1995; Cook *et al.* 1998). Molecular data indicate that *T. miscellus* and *T. mirus* have formed repeatedly, perhaps as many as 21 and 13 times, respectively, in just the past 80 years (reviewed in Soltis *et al.* 2004). *T. dubius* and *T. pratensis* are the diploid progenitors of *T. miscellus*, while *T. dubius* and *T. porrifolius* are the parents of *T. mirus* (Figure 1A). *T. miscellus* is of particular interest because it has formed reciprocally, and the reciprocally formed lineages can be readily distinguished morphologically: when *T. pratensis* is the maternal parent, *T. miscellus* has short-ligulate ray flowers, and when *T. dubius* is the maternal parent, *T. miscellus* has long-ligulate ray flowers (Ownbey 1950; Ownbey and McCollum 1953). Only one existing population of *T. miscellus* is long liguled (Pullman, WA); all others (∼40) are short liguled (Novak *et al.* 1991).

In this study, we examine the evolution and expression of homeologous loci in multiple individuals of

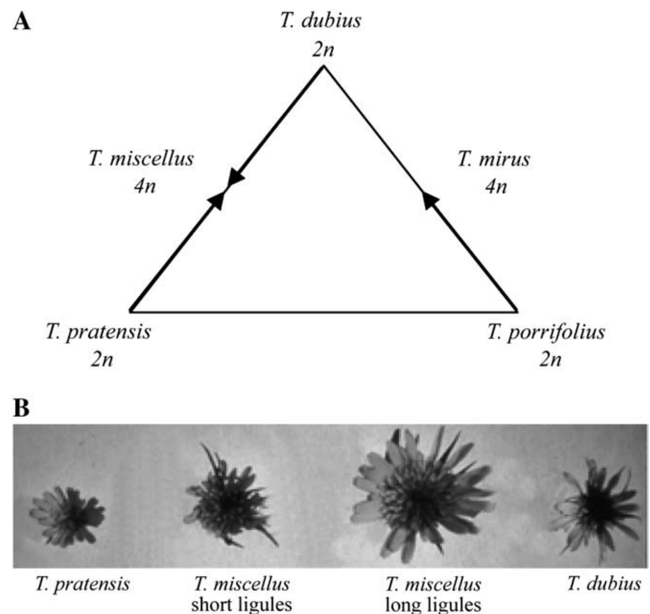

Figure 1.—Origins of the New World Tragopogon allotetraploids. (A) Following the introduction of three diploid species (*T. dubius*, *T. porrifolius*, and *T. pratensis*) to the northwestern United States in the early 1900s, two polyploid species (*T. miscellus* and *T. mirus*) formed recurrently. The direction of the arrow indicates the maternal progenitor. (B) Populations of *T. miscellus* that differ in maternal parentage can be readily distinguished by their inflorescence morphology. *T. pratensis* is the maternal parent of the short-liguled form, and *T. dubius* is the maternal parent of the long-liguled form of *T. miscellus*.

T. miscellus from reciprocally formed populations, compared to the diploid progenitors, *T. dubius* and *T. pratensis*. Our main objectives were to identify genomic changes and expression differences in *T. miscellus* relative to its diploid progenitors, to determine the identity of the genes that showed these changes and to assess whether multiple individuals within and between the reciprocally formed populations showed similar patterns of genome evolution and gene expression. To address these issues, we employed a two-step approach. We first used cDNA–amplified fragment length polymorphisms (cDNA–AFLPs) (Bachem *et al.* 1996) to identify potentially differentially expressed genes. This approach has been used successfully in several polyploid systems, including Arabidopsis (Comai *et al.* 2000; Lee and Chen 2001), cotton (Adams *et al.* 2004), and wheat (Kashkush *et al.* 2002; He *et al.* 2003), and it has proven to be particularly useful in nonmodel systems that lack developed genomic resources. However, a shortcoming of this method is that fragment differences (*i.e.*, absent fragments) on a cDNA–AFLP gel may result from true expression differences, sequence polymorphisms, or gene or homeolog loss and these sources of variation cannot be differentiated from one another merely by the cDNA–AFLP gel banding patterns (see Wang *et al.* 2005). Because of this limitation, we also isolated

several polymorphic cDNA–AFLP fragments and investigated these candidate genes using both genomic and cDNA cleaved amplified polymorphic sequence (CAPS) analysis (Konieczny and Ausubel 1993). With this follow-up method, changes at the genomic level can be readily distinguished from true expression differences.

MATERIALS AND METHODS

Plant materials: Field-collected seeds were grown in the greenhouse (at Washington State University, Pullman, WA) and allowed to self-fertilize for one generation to reduce individual allelic variation and maternal effects. The plants used in this study were derived from these S_1 seed, which were germinated and grown under controlled conditions in a greenhouse at the University of Florida (Gainesville, FL). We analyzed individuals from two populations of *T. miscellus* of reciprocal origin: the short-liguled form from Moscow, Idaho (Soltis and Soltis collection no. 2604), and the long-liguled form from Pullman, Washington (Soltis and Soltis collection no. 2605). One population of each parental species, *T. dubius* (Pullman, WA; Soltis and Soltis collection no. 2613) and *T. pratensis* (Moscow, ID; Soltis and Soltis collection no. 2608), which represent the most likely progenitor genotypes for these *T. miscellus* populations (Soltis *et al.* 1995), were also sampled.

F_1 hybrids were created by crossing *T. pratensis* and *T. dubius* plants grown in the greenhouse. These plants were grown to maturity from seed in the greenhouse at the University of Florida. To induce flowering, mature plants (~6 months old) were sent from the University of Florida to Washington State University in October 2004 and overwintered in an unheated glasshouse for a period of 3 months. Following this cold treatment, the plants were returned to the University of Florida and were again placed in a greenhouse under standard conditions. After ~2 weeks, most plants began to bolt, and within 1 month, the first heads were produced. The parental plants crossed were *T. pratensis* (Moscow, 2608-11) and *T. dubius* (Pullman, 2613-41) and *T. pratensis* (Spangle, 2609-28) and *T. dubius* (Spokane, 2615-22). In both cases, *T. pratensis* served as the maternal progenitor. These plants were chosen to generate hybrids that would represent the natural *T. miscellus* genotype from Moscow and an additional independently formed *T. miscellus* genotype. Heads were bagged with glassine envelopes after emasculation (Fahselt *et al.* 1976) and after pollination, which occurred 2–3 days following emasculation. Seed from these crosses were germinated following standard protocols. Progeny from the crosses were screened using diagnostic rDNA markers (Kovarík *et al.* 2005) to determine if individual plants were hybrids or selfed maternal plants.

To investigate the utility of cDNA–AFLPs in Tragopogon, we conducted a preliminary study for which a single individual from each diploid and tetraploid population was included. From this initial screen, several polymorphic fragments were characterized and subsequently analyzed for 10 individuals from each population using both genomic and cDNA–CAPS analyses (see *CAPS analyses*). An expanded cDNA–AFLP study of these same populations used six individuals from each diploid and tetraploid population (see *cDNA–AFLP display and identification of polymorphic fragments*), including the same individuals from the preliminary analysis. The individuals examined for comparative expression analyses were germinated at the same time and grown under uniform conditions, and the same tissue type was collected from them concurrently.

cDNA–AFLP display and identification of polymorphic fragments: Leaf tissue was collected from seedlings 4 weeks after germination and frozen in liquid nitrogen. Total RNA was extracted using Trizol reagent (Invitrogen, San Diego) following the manufacturer's instructions. Messenger RNA isolation, cDNA synthesis, and cDNA–AFLP techniques were performed as previously described (Lee and Chen 2001), except that here we used [^{33}P]dATP to label the *Eco*RI primer. Thirty-four primer combinations (*Eco*RI and *Mse*I) were used in the selective amplification reactions for the first screening. The primer combinations used in the preliminary screen and those used in the expanded study are listed in supplemental Table 1 at http://www.genetics.org/supplemental/. The selective amplification reactions were run on polyacrylamide gels, and the resulting cDNA–AFLP bands were scored as monomorphic (present in all individuals) or polymorphic (absent in at least one individual).

To determine the putative identity of selected polymorphic fragments, we excised and sequenced the transcript-derived fragments (TDFs) as previously described (Lee and Chen 2001). Approximately 80 polymorphic bands were cut from the polyacrylamide gels, reamplified using the same set of AFLP primers, and cloned using Promega (Madison, WI) pGEM-T easy vector following manufacturer's instructions. Ten clones from each reaction were sequenced.

The resulting sequences were subjected to BLAST searches against online databases. For most fragments, we used two search strategies. First, the sequences were submitted to a BLAST search against The *Arabidopsis* Information Resource (TAIR) database (http://www.arabidopsis.org/), which contains annotations for the Arabidopsis genome. Second, the sequences were subjected to a BLAST search against the NCBI EST database (http://www.ncbi.nlm.nih.gov/), which contains ~110,000 Lactuca and Helianthus expressed sequence tags (ESTs) resulting from the Compositae Genome Project (http://compgenomics.ucdavis.edu/). If putative homologs from Lactuca or Helianthus were returned, then these sequences were submitted to TAIR for another round of annotated searches.

To determine if cDNA–AFLP variation could be detected among individuals from the diploid and polyploid populations, we conducted an expanded cDNA–AFLP analysis, which included six individuals from each diploid and tetraploid population. Again, frozen leaf tissue from young plants was used and RNA extracted as described. Twelve selective primer pairs were used (the most variable as determined from our initial screen) for these individuals, and the fragments were separated on polyacrylamide gels followed by silver staining using standard protocols. The resulting cDNA–AFLP fragments were scored and tabulated as described previously. We further characterized cDNA–AFLP fragment variation among individuals within the diploid and polyploid populations by calculating "within-population fragment variation." This measure was determined by calculating the percentage of individuals that were polymorphic at each fragment locus averaged over all fragments produced in that population.

CAPS analyses: To determine if cDNA–AFLP fragment polymorphisms resulted from changes at the genomic or transcriptomic level, we conducted genomic and cDNA CAPS analyses. In CAPS analysis, amplified PCR products are digested with diagnostic restriction enzymes, separated by agarose gel electrophoresis, stained, and visualized. For both genomic and cDNA CAPS analyses, we designed primers for 20 of the 80 sequenced TDFs using the web-based program Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Rozen and Skaletsky 1997). These TDFs were selected because they showed the most interesting cDNA–AFLP patterns (*e.g.*, missing fragments or novel fragments in the

allopolyploids). In some cases, we used the Lactuca or Helianthus ESTs from our BLAST searches to design primers that would include a larger region of the gene and/or a region that would include introns. Primer sequences are given in supplemental Table 2 at http://www.genetics.org/supplemental/.

Genomic DNA was extracted using a modified CTAB protocol (Doyle and Doyle 1987). For each of the 20 TDFs, genomic fragments were amplified in a 25-μl volume with 50 ng template, 10× buffer, 1.5 mm $MgCl_2$, 0.4 mm dNTPs, 0.2 μm each primer, and 0.5 unit Taq polymerase. Thermal cycling conditions were as follows: 94° for 2 min, followed by 35 cycles of 94° for 30 sec, 52°–54° for 30 sec, 72° for 1 min, and a final 5-min extension at 72°. Products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized by UV on a transilluminator. Sephadex G-50 (Fisher Scientific) columns were used to purify the PCR products. Cycle sequencing was performed using Big Dye (ABI Prism) terminator chemistry, and the products were separated on an ABI 377 DNA sequencer at the Institute of Mammalian Genetics, University of Florida. Homologous sequences for *T. dubius* and *T. pratensis* were aligned by eye in Sequencher v. 4.1.4 (Gene Codes, Ann Arbor, MI) and inspected for diagnostic restriction sites between the species (using the "cut map" function in Sequencher and selecting all restriction enzymes). Diagnostic restriction sites were identified for 10 of the 20 TDFs. Several additional TDFs contained single nucleotide polymorphisms between *T. dubius* and *T. pratensis*, but these did not occur at restriction sites and were therefore not pursued further.

Genomic CAPS analyses: Genomic DNA was extracted from 10 individuals from each diploid and tetraploid population as described. PCR amplification and digests for all individuals for each gene region were performed twice to determine potential bias in PCR amplification. Likewise, an additional set of primers was designed for each region to verify the results. Mutations that occur at the priming sites (in either the diploids or the polyploids) would result in differing amplification and digestion patterns. Given that the *T. miscellus* polyploids are <80 years old, we expected that novel mutations in the allotetraploids that might cause mis-priming would be rare. Because *T. dubius* and *T. pratensis* have fairly divergent genomes (Mavrodiev *et al.* 2005), we anticipated that mis-priming in the polyploids resulting from sequence differences between the two parents would be more plausible. In most cases, the first set of primers was designed from only one diploid species (*i.e.*, the individual fragment isolated from the cDNA–AFLP gel), while the second primer set was designed from sequences of both diploid progenitors. PCR and digests were conducted for this second set using the same conditions as the first. Genomic digests were carried out in a 10-μl volume, containing 1× buffer (New England Biolabs, Beverly, MA), 1 μl PCR product, 2–10 units of restriction enzyme (New England Biolabs), and 100 μg/ml bovine serum albumin (when required), and were allowed to incubate at the appropriate temperature for 3 hr. Digested products were separated on either a 2% agarose gel or 3–4% Metaphor (Cambrex) agarose gel, stained with SybrGold (Molecular Probes, Eugene, OR), and visualized on a transilluminator.

To determine if genomic changes might occur as early as the F_1 generation, we conducted genomic CAPS analyses for these same genes on 11 F_1 hybrid individuals that resulted from the two independent crosses between *T. pratensis* and *T. dubius* described earlier.

cDNA CAPS analyses: For the same set of TDFs for which genomic CAPS analyses were performed, we also conducted cDNA CAPS analyses. Different patterns in the cDNA and genomic CAPS analyses would distinguish expression differences from alterations at the genomic level. Total RNA was extracted from frozen leaf tissue using the RNeasy plant minikit (QIAGEN, Chatsworth, CA) with optional on-column DNase digestion and was quantified using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). First-strand cDNA synthesis was carried out on 200 ng total RNA using Superscript II reverse transcriptase (Invitrogen) and a poly(T) (T17) primer. Using the same primer combinations as the genomic CAPS analyses, RT–PCR was performed using one-twentieth cDNA template from the first-strand synthesis reaction, 10× buffer, 1.5 mm $MgCl_2$, 0.4 mm dNTPs, 0.2 μm each primer, and 0.5 unit Taq polymerase. Thermal cycling conditions were as follows: 94° for 2 min, followed by 30–35 cycles of 94° for 1 min, 52°–54° for 45 sec, 72° for 1 min, and a final 5-min extension at 72°. For each set of reactions, negative (without reverse transcriptase) and positive (glyceraldehyde 3-phosphate dehydrogenase, a housekeeping gene; Harris and Water 1976) controls were included. Amplified RT–PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized by UV on a transilluminator. RT–PCR products were digested and analyzed as described for the genomic CAPS analyses.

RESULTS

cDNA–AFLP polymorphism in populations of *T. miscellus* and identification of putatively differentially expressed genes: To investigate changes in the evolution and expression of homeologous loci in *T. miscellus* relative to its parents, we used cDNA–AFLPs as a first step toward identifying candidate genes. From our initial screen using 34 *Eco*RI/*Mse*I primer pairs on four individuals, 1262 fragments were surveyed, and of these, 556 were monomorphic (44.1%) and 706 were polymorphic (55.9%) among *T. miscellus*, *T. dubius*, and *T. pratensis*. Missing cDNA–AFLP bands in either one or both of the reciprocally formed polyploids accounted for 13.5% (171 bands) of the total fragments, while novel cDNA–AFLP bands in the polyploids composed 4.0% (50 bands) of the total fragments. Shared fragments with a maternal origin in the polyploids were 3.7% (47 fragments) of the total bands produced in the preliminary study, while fragments with a paternal origin composed 4.1% (52 fragments) of the bands.

On the basis of the larger sampling, which included six individuals from each diploid and tetraploid population and for which we used a subset (12) of the original 34 primer pairs, 610 fragments were scored, and of these, 216 were monomorphic (35.4%) and 394 were polymorphic (64.6%) (Table 1, Figure 2). Polymorphisms were detected among individuals within the diploid and polyploid populations. Within-population variation was calculated as the percentage of individuals that lacked a fragment at each fragment locus, averaged over all fragment loci produced by that population. In *T. dubius*, within-population variation was highest at 7.4%, while *T. pratensis* was 3.8%. Within the short-liguled (Moscow) population of *T. miscellus*, fragment variation was 2.5%, while in the long-liguled (Pullman) population of *T. miscellus*, fragment variation was 5.8%

TABLE 1

Summary of cDNA–AFLP population study

Species	Population	No. of individuals	No. of amplified bands	Polymorphic "fragment loci" (%)	% within-population variation[a]	Maternal fragments	Paternal fragments (%)	Novel fragments (%)
T. pratensis	Moscow, ID (2608)	6	456	68 (14.9)	3.8	—	—	—
T. miscellus	Moscow, ID (2604)	6	506	50 (9.9)	2.5	0	3 (0.6)	5 (∼1)
T. miscellus	Pullman, WA (2605)	6	539	116 (21.5)	5.8	0	3 (0.6)	5 (∼1)
T. dubius	Pullman, WA (2613)	6	432	109 (25.2)	7.4	—	—	—
Total		24	610	394 (64.6)				

[a] Calculated as the percentage of individuals polymorphic at each fragment locus averaged over all fragments produced in that population.

(Table 1). In contrast to the results from the preliminary study, additional sampling revealed that most of the maternal and paternal patterns were not uniform within populations and were therefore not consistent between the reciprocally formed populations. No bands uniformly corresponding to a strict maternal origin were detected in either *T. miscellus* population, but three fragments (0.6% of fragments produced) in each reciprocally formed population were shared with the respective paternal progenitor. Five novel bands (∼1% of fragments

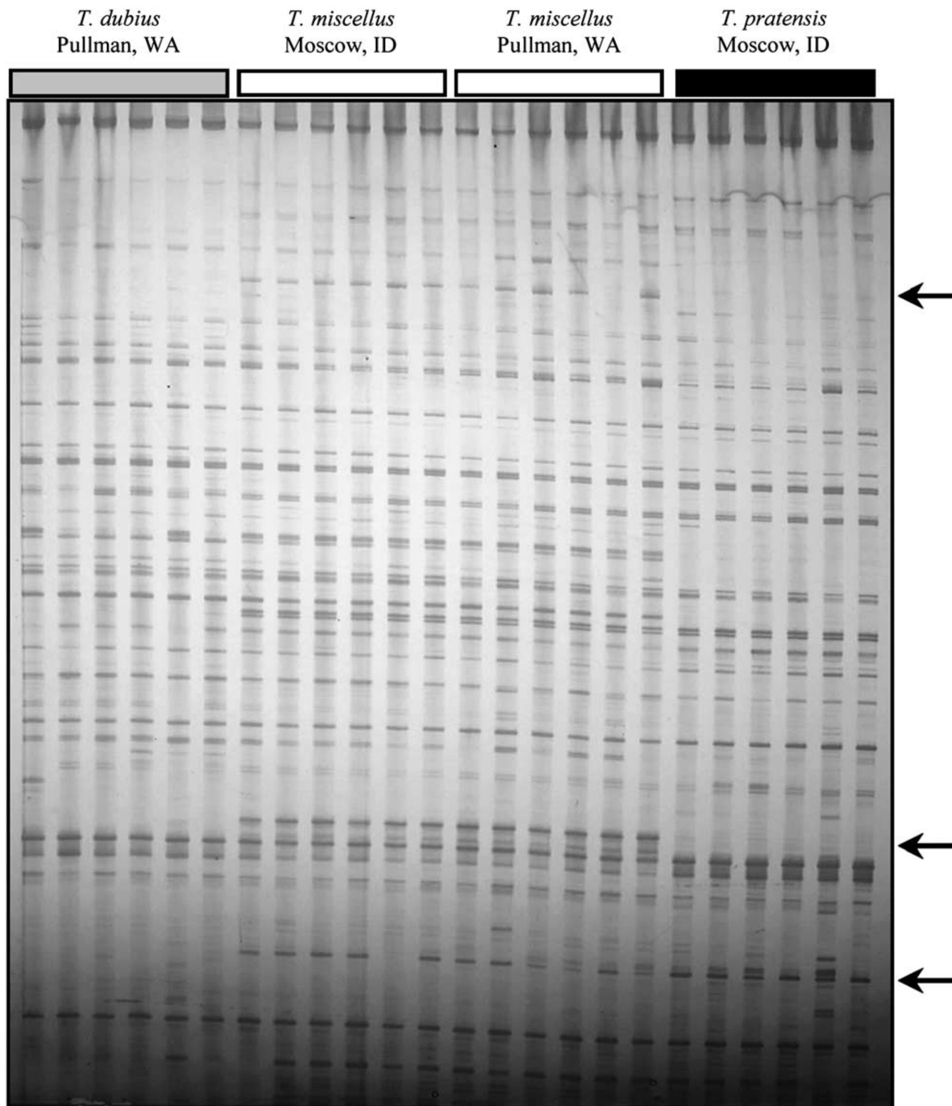

Figure 2.—cDNA–AFLP display of gene expression in Tragopogon allotetraploids and their diploid progenitors. *T. dubius* (Pullman, 2×), lanes 1–6; *T. miscellus* (Moscow, 4×), lanes 7–12; *T. miscellus* (Pullman, 4×), lanes 13–18; *T. pratensis* (Moscow, 2×), lanes 19–24. Arrows indicate examples of polymorphic fragments that were examined with genomic and cDNA CAPS analysis. The primer combination used was *Eco*RI–AA/*Mse*I–CTT.

TABLE 2

Putative identities for a subset of polymorphic cDNA–AFLP fragments

Cloned fragment ID	cDNA–AFLP pattern[a]				Putative protein	Nucleotide similarity to *A. thaliana* (%)/*E*-value
	Tp	Tm	Tm′	Td		
TDF7	+	+	+	–	Casein kinase	88/1.9*e*-7
TDF12	–	+	+	+	Leucine-rich repeat transmembrane protein kinase	61/0.02
TDF13	–	–	+	+	Peroxidase	69/8.5*e*-20
TDF17.4	–	+	–	+	Polyubiquitin	79/1.5*e*-33
TDF31	+	+	+	–	ABC transporter family protein	74/9.4*e*-18
TDF36.3	+	+	–	–	Thioredoxin M-type 1	65/1.5*e*-5
TDF44	+	–	–	–	Leucine-rich repeat transmembrane protein kinase	72/1.8*e*-30
TDF46	–	–	–	+	Protein phosphatase 2C family protein	84/9.7*e*-8
TDF47	–	–	–	+	TATA-box-binding protein-interacting protein	73/3.4*e*-6
TDF53	–	–	+	+	Expressed protein	67/1.1*e*-14
TDF69.8	+	+	–	–	Expressed protein	69/2.8*e*-30
TDF72.1	+	+	–	–	MADS-box protein	64/4.3*e*-12
TDF62	+	–	+	–	Auxin conjugate hydrolase	70/1.7*e*-30
TDF72.3	+	+	–	–	Putative adenine–DNA glycosylase	65/0.037
TDF74	+	–	+	–	Transducin family protein	64/1.5*e*-4
TDF75	–	+	+	–	Putrescine-binding periplasmic protein	73/5.8*e*-11
TDF80.10	+	–	+	–	Fructose–bisphosphate aldolase	77/2.9*e*-36
TDF85	–	+	–	–	β-fructosidase	69/2.2*e*-10
TDF90	–	–	–	+	Small GTP-binding protein	77/2.4*e*-37
TDF97	+	–	+	–	bZIP transcription factor family protein	81/1.3*e*-7

[a] +, fragment present; –, fragment absent.

Tp, *T. pratensis*, Moscow ID (2608); Tm, *T. miscellus*, Moscow, ID (2604); Tm′, *T. miscellus*, Pullman, WA (2605); Td, *T. dubius*, Pullman, WA (2613).

produced) that were not present in either diploid progenitor appeared in individuals from both allotetraploid populations (Figure 2).

Approximately 50% of the cloned fragments from the initial screen showed high sequence identity, in terms of *E*-value ($< 2 \times 10^{-10}$) and nucleotide equivalence (85–95% identical), to ESTs in the Compositae database (http://compgenomics.ucdavis.edu/). The remaining fragments showed moderate -to-low levels (*E*-value <1–2) of sequence identity to ESTs from other taxa including Glycine (Fabaceae), Solanum (Solanaceae), and Stevia (Asteraceae). BLAST searches against the TAIR database typically produced matches of higher sequence identities than those against the NCBI database. A subset of the polymorphic fragments identified is shown in Table 2. The TDFs matched several genes of known function that belong to various gene classes, including several transcription factors and kinases. These potentially differentially expressed genes appear to be involved in diverse cellular processes, such as carbohydrate metabolism, signal transduction, protein transport and degradation, and cell division. Interestingly, we found two polymorphic fragments that belong to the same gene class (TDF12 and TDF44; leucine-rich-repeat transmembrane protein kinase), although they corresponded to different loci in the *A. thaliana* genome. These fragments were identified on different gels using different selective amplification primers. Approximately 15% of the fragments did not match any plant sequence in the available databases and may be too short in length to produce either a match or AFLP artifacts.

Rapid and stochastic changes in homeologous loci in *T. miscellus*: The genes analyzed with CAPS analysis, the enzymes used for each gene, and the approximate product sizes for the digested genomic and cDNA amplifications for the diploid progenitors, *T. dubius* and *T. pratensis*, are listed in Table 3. The results of the genomic and cDNA CAPS analyses for these 10 genes are shown in Figure 3. For 7 of 10 genes surveyed in this study, at least one allopolyploid individual is missing one parental homeolog or a fragment of this homeolog from its genome (Table 4). To determine if the missing parental fragments resulted from a mutation at the restriction site, we sequenced the PCR products for the *T. miscellus* individuals that were not additive of their parental genomic fragments. For all individuals sequenced, there were no nucleotide changes in the polyploid sequences when compared to the parental fragments either at the diagnostic restriction site or along the length of the fragment that was sequenced. Further, the *T. miscellus* individuals that were missing one parental homeolog did not display any nucleotide polymorphisms in the chromatograms at any of the sites where the parental sequences differ (in contrast to the other "additive" individuals, which showed double

TABLE 3

Homeologous loci examined in *T. miscellus* with genomic and cDNA CAPS analysis

Fragment	Diagnostic restriction enzyme	Genomic fragment sizes[a] (bp)		cDNA fragment sizes[a] (bp)	
		T. dubius	*T. pratensis*	*T. dubius*	*T. pratensis*
TDF7	*Bst*NI	447	413	114	77
			37		37
TDF17.4	*Sau*3AI	108	132	108	132
		81	81	81	81
		41	41	41	41
		24	18	24	18
		18		18	
TDF36.3	*Dde*I	88	164	88	164
		72		72	
TDF44	*Hpy*CH4IV	393	219	339	191
	(*Mae*II)	91	170		148
			72		
			21		
TDF46	*Nla*III	186	277	186	277
		94		94	
TDF62	*Sau*3AI	266	371	185	291
		106	97	106	97
		97		97	
TDF72.3	*Aci*I	289	220	209	140
			69		69
TDF74	*Nla*III	345	403	54	80
		88	51	45	45
		51	8	26	
TDF85	*Alu*I	305	270	303	264
		92	145		39
			39		
TDF90	*Bsm*AI	222	222	222	222
		110	125	110	125
		13		13	

[a] Following digestion with diagnostic restriction enzyme.

peaks at all positions where the parents differ). Only for TDF17.4, TDF46, and TDF85 are both parental copies maintained in all allopolyploid individuals of *T. miscellus*. For the remaining genes, genomic CAPS analysis revealed much diversity in terms of genomic changes in these loci among individuals from the polyploid populations (Figure 3). For several fragments (TDF7, TDF36.3, TDF44, TDF72.3), a few *T. miscellus* individuals have apparently lost the *T. dubius* homeolog, while the *T. pratensis* copy was eliminated less frequently (TDF44, TDF74). The pattern of genomic change is much more striking for other genes. For TDF90, some individuals from each polyploid population retained both parental copies, and others lost one or the other homeolog (more frequently, the *T. dubius* homeolog). Across the 10 loci examined, individuals from the short-liguled population of *T. miscellus* lost parental homeologs more frequently (almost twice as many) than individuals from the long-liguled population (Table 4). Within each population, some individuals retained both parental copies for all genes, while others lost one parental homeolog or the other for as many as three genes (Table 4, *e.g.*, individual 2604-22).

Conflicting digestion patterns in the genomic CAPS analysis (using two different sets of PCR amplification primers) occurred in only one case. For TDF74, when the first primer set (TDF74-F1/TDF74-R1) was used, only the *T. pratensis* copy was present in each *T. miscellus* individual from Pullman. However, with the second primer set (TDF74-F2/TDF74-R2), both parental copies of TDF74 were detected in the genomic CAPS analysis for these same individuals, indicating that a mutation exists in these individuals at the priming site of the first primer set. The *T. miscellus* individuals from Moscow displayed the same pattern for both primer sets.

F_1 hybrids are additive of their parental genomes: Genomic CAPS analyses for the synthetic F_1 hybrids show that the individuals resulting from two independent crosses between *T. pratensis* and *T. dubius* are additive of their progenitors for the genes analyzed (Figure 4). These results provide a critical framework for the interpretation of the genomic changes observed in the young polyploid populations (*i.e.*, compare Figure 3 to Figure 4).

Genomic change *vs.* differential expression of homeologous loci: For 8 of the 10 genes surveyed here,

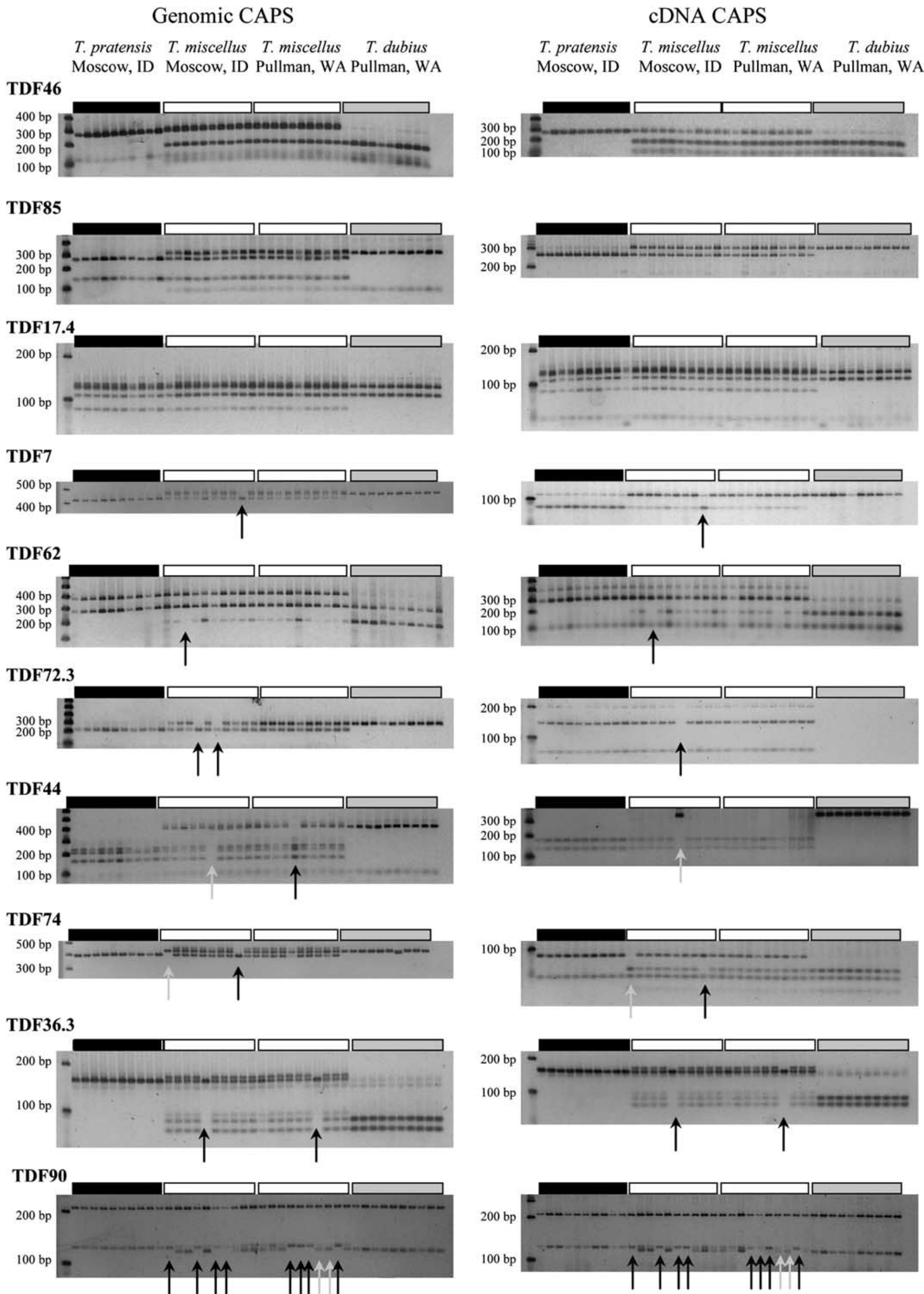

FIGURE 3.—Genomic and cDNA CAPS analyses for 10 candidate genes in individuals of allotetraploid *T. miscellus* from reciprocally formed populations. Arrows indicate the parental origin of the homeolog retained or expressed in the allopolyploid individual when one parental copy was missing.

the cDNA CAPS results match those of the genomic CAPS analyses, indicating no qualitative changes in expression for these genes. Expression differences between *T. miscellus* individuals and the diploid progenitors, as well as among the allopolyploid individuals, were evident for TDF72.3 and TDF44 (Figure 3). For TDF72.3, no expression was detected for the *T. dubius* individuals analyzed, despite repeated RT–PCR amplifications with different pools of cDNA, nor was TDF72.3 expression detected for one individual of *T. miscellus* (2604-22) from Moscow, despite the amplification of the fragment in genomic CAPS analysis in all *T. dubius* individuals and despite the retention of the *T. pratensis* homeolog in *T. miscellus* (Figure 3). The other *T. miscellus* individuals expressed the *T. pratensis* TDF72.3 homeolog only. For TDF44, the *T. miscellus* individuals expressed only one parental homeolog or the other. One polyploid individual (2604-22, the same individual that did not express either parental copy for TDF72.3) expressed the *T. dubius* homeolog, while all other

TABLE 4

Retention of homeologous loci in individuals of *T. miscellus* on the basis of genomic CAPS analysis

T. miscellus individual[a]	TDF46[b]	TDF85	TDF17.4	TDF7	TDF62	TDF72.3	TDF44	TDF74	TDF36.3	TDF90
2604-4	—	—	—	—	—	—	—	D	—	P
2604-10	—	—	—	—	—	—	—	—	—	—
2604-11	—	—	—	—	P	—	—	—	—	—
2604-15	—	—	—	—	—	P	—	—	—	P
2604-20	—	—	—	—	—	—	—	—	P	—
2604-22	—	—	—	—	—	P	D	—	—	P
2604-24	—	—	—	—	—	—	—	—	—	P
2604-31	—	—	—	—	—	—	—	—	—	—
2604-35	—	—	—	P	—	—	—	P	—	—
2604-43	—	—	—	—	—	—	—	—	—	—
2605-4	—	—	—	—	—	—	—	—	—	—
2605-5	—	—	—	—	—	—	—	—	—	—
2605-7	—	—	—	—	—	—	—	—	—	—
2605-13	—	—	—	—	—	—	—	—	—	P
2605-14	—	—	—	—	—	—	P	—	—	P
2605-24	—	—	—	—	—	—	—	—	—	P
2605-28	—	—	—	—	—	—	—	—	P	D
2605-29	—	—	—	—	—	—	—	—	—	D
2605-42	—	—	—	—	—	—	—	—	—	P
2605-46	—	—	—	—	—	—	—	—	—	—

[a] Individuals are listed in the order in which they appear in Figure 3 (from left to right); 2604 individuals are from Moscow, Idaho, and 2605 individuals are from Pullman, Washington.

[b] —, both parental copies detected in genome; D, *T. dubius* detected only; P, *T. pratensis* detected only.

individuals in both allotetraploid populations expressed the *T. pratensis* copy.

DISCUSSION

cDNA–AFLP variation within and between the diploid progenitor populations: cDNA–AFLPs provide a useful tool for detecting potentially differentially expressed genes in polyploid systems, particularly those that lack developed genomic resources. However, because missing fragments on the gel may result from differences at the genomic level (*e.g.*, sequence polymorphism, gene, or homeolog loss), the results must be interpreted cautiously and followed up with additional methods, such as CAPS analysis (see Wang *et al.* 2005). Despite this potential shortcoming of cDNA–AFLPs, this method provides a relatively fast and inexpensive means to identify candidate genes for further study.

The high level of genetic differentiation detected by cDNA–AFLPs between the diploid progenitor species *T. dubius* and *T. pratensis* (38.4% in the preliminary study and 35.2% in the extended study) is consistent with previous Tragopogon studies based on allozymes, RAPDs, and chloroplast DNA restriction site data, which showed wide divergence among the *T. dubius*, *T. pratensis*, and *T. porrifolius* genomes (Roose and Gottlieb 1976; Soltis and Soltis 1989; Soltis *et al.* 1995; Cook *et al.* 1998). Recent phylogenetic analyses of Tragopogon using internal and external transcribed spacer sequence data also indicate that *T. dubius* and *T. pratensis* are well differentiated, with these two species placed in separate major clades, further attesting to their divergent evolutionary histories (Mavrodiev *et al.* 2005). The majority of the polymorphic cDNA–AFLP fragments between these two species likely result from differences at the primary sequence level. However, real expression differences must account for at least some of the cDNA–AFLP variation detected, because we were able to verify expression differences between *T. pratensis* and *T. dubius* for TDF72.3 in the cDNA CAPS analysis (Figure 3).

Rapid and stochastic genomic changes in *T. miscellus*: On the basis of both the cDNA–AFLP and the CAPS data, a number of differences in the evolution and expression of homeologous loci are apparent within and between the reciprocally formed allotetraploid populations. In particular, the genomic CAPS data indicate stochastic small-scale losses of homeologous loci. Previous genomic work on the Tragopogon allopolyploids, using fluorescent *in situ* hybridization of rDNA and short, tandem centromeric and telomeric repeats, indicated that no large-scale genomic rearrangements or losses had occurred (Pires *et al.* 2004a). Genome size estimates (Pires *et al.* 2004a) for the Moscow ($4C = 20.30 \pm 1.50$ pg) and Pullman ($4C = 20.99 \pm 1.14$ pg) populations of *T. miscellus*, while within the range of the sum of the diploid progenitors *T. dubius* ($4C = 10.83 \pm 0.65$ pg, 11.76 ± 0.79 pg) and *T. pratensis*

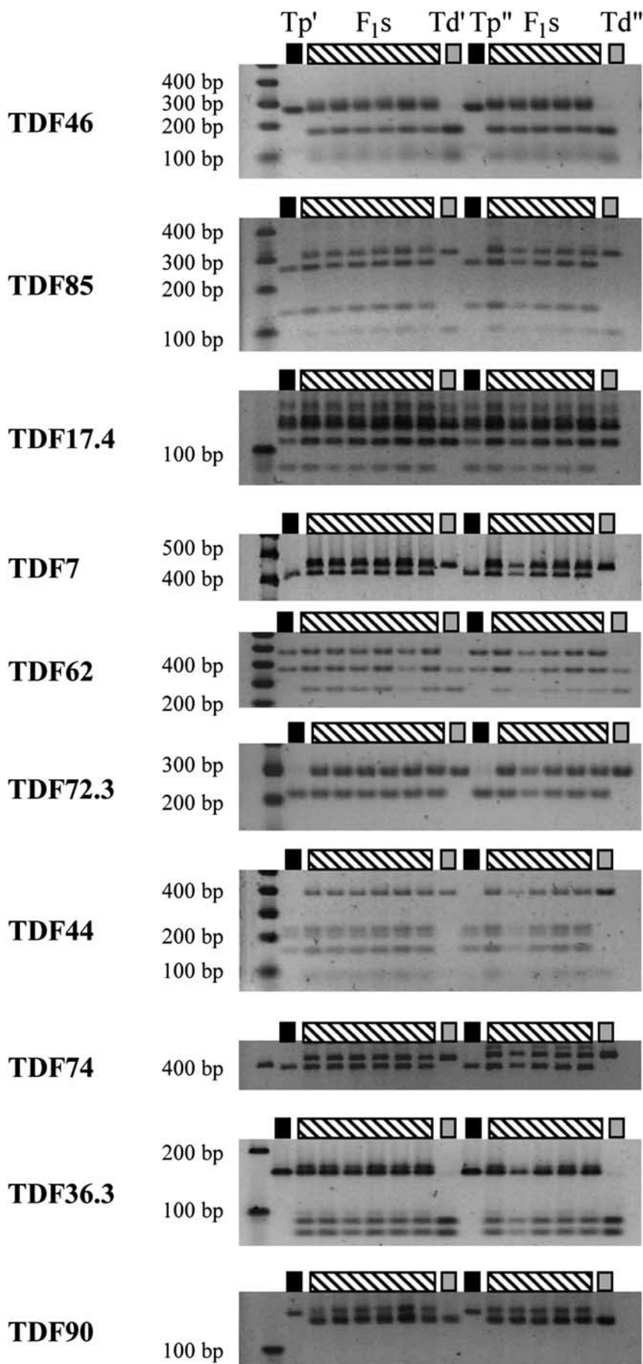

Figure 4.—Genomic CAPS analyses for two sets of Tragopogon F_1 hybrids and their progenitors. Tp′, *T. pratensis* (Spangle, 2609-28); Td′, *T. dubius* (Spokane, 2615-22); Tp′′, *T. pratensis* (Moscow, 2608-11); Td′′, *T. dubius* (Pullman, 2613-41). For both sets of hybrids, *T. pratensis* was the maternal parent and *T. dubius* the paternal parent.

($4C$ = 12.44 ± 0.87 pg), were slightly less than the estimate for another short-liguled *T. miscellus* population from Spangle, Washington (4C = 21.76 pg ± 0.87). Genome downsizing is a well-documented phenomenon for other polyploid groups (Leitch and Bennett 2004). For most of the genes surveyed here, the pattern of loss appears to be stochastic, although we currently lack a genomic framework for identifying the relative locations of these loci. Furthermore, the size of the fragments lost is not known. The losses detected could be small regions of the individual genes, entire genes, or short chromosomal fragments.

The parental homeolog that is more often lost in both populations of *T. miscellus* is the *T. dubius* copy. The *T. pratensis* homeolog was lost in only three cases (TDF44, TDF74, and TDF90; Figure 3). This result agrees with previous rDNA studies, which found that the number of *T. dubius* repeats has been reduced in the genomes of both *T. mirus* and *T. miscellus* (Kovarík *et al.* 2005). Concerted evolution has apparently not acted to completion in these young polyploids, however, as some *T. dubius* rDNA units are still maintained and expressed. Furthermore, both reciprocally formed *T. miscellus* populations have lost the *T. dubius* units, suggesting a lack of a cytoplasmic effect on rDNA parental copy number (Kovarík *et al.* 2005). Directed silencing of one parental genome has been demonstrated in allopolyploids of *A. suecica*, which has as its parents *A. thaliana* and *A. arenosa* (Wang *et al.* 2004). In *A. suecica*, genes from the *A. thaliana* genome are often silenced by DNA methylation. These genes were reactivated in *ddm-1* and *met1*-RNAi transgenic mutants (Wang *et al.* 2004). In contrast to this system, however, where the genes of one progenitor are silenced by epigenetic mechanisms, the Tragopogon allopolyploids appear to be eliminating homeologous loci, at least for most of the genes examined here.

Other polyploid systems, most notably synthetic polyploids of Brassica (Song *et al.* 1995) and allohexaploid wheat (Feldman *et al.* 1997; Shaked *et al.* 2001; Kashkush *et al.* 2002; Levy and Feldman 2004), have also shown rapid genomic changes. Synthetic allopolyploids of Brassica showed considerable changes in parental fragments at the F_5 generation (Song *et al.* 1995) on the basis of RFLPs. Comparing the synthetic Brassica polyploids with the natural Tragopogon polyploids, we find similarities in that frequent and rapid changes in parental fragments have occurred in the allopolyploids formed from distantly related diploid progenitors. However, while *T. dubius* homeologs are more frequently eliminated than the *T. pratensis* homeologs, there does not appear to be a significant directional aspect (in terms of the maternal or paternal progenitor) to loss in the reciprocally formed populations of *T. miscellus*, at least for the genes examined in detail for this study, because individuals from these two populations of separate origin show similar patterns of homeolog loss and expression.

The data for *T. miscellus* allopolyploids, suggesting rapid loss of genes or gene fragments, seem similar to comparable reports of rapid gene loss in synthetic wheat polyploids (Feldman *et al.* 1997; Liu *et al.* 1998; Ozkan *et al.* 2001; Shaked *et al.* 2001; Kashkush *et al.* 2002). In

wheat, the same low-copy (most likely noncoding) sequences were eliminated in natural and early generation synthetic allohexaploids, suggesting that genome evolution is reproducible in these wheat allopolyploid species (Feldman *et al.* 1997; Ozkan *et al.* 2001; Shaked *et al.* 2001). Additional studies showed that gene loss could occur as early as the F_1 or first amphiploid generation in synthetic wheat tetraploids (Kashkush *et al.* 2002). The loss of homeologs in Tragopogon has apparently occurred rapidly, as these polyploids were formed <80 years ago. Synthetic F_1 hybrids between *T. pratensis* and *T. dubius* are additive of their parental fragments for the same loci investigated for the *T. miscellus* polypoids (Figure 4), suggesting that polyploidization rather than hybridization may be acting as a "genomic shock" (McClintock 1984). In Tragopogon, the loss of homeologous loci seems to be stochastic among individuals within and between polyploid populations (Table 4). Moreover, the homeologs that have been purged from the *T. miscellus* genomes appear to be functional genes that are involved in essential developmental and physiological processes (Table 2). In *A. thaliana*, which is known to be an ancient polyploid, certain classes of genes have been retained in duplicate (*e.g.*, genes with functions in transcription and signal transduction), while others (*e.g.*, DNA repair) have been preferentially lost (Blanc and Wolfe 2004a).

The mechanism responsible for the loss of homeologs from the *T. miscellus* allopolyploid genomes is currently unknown. However, Ownbey (1950), who constructed the first karyotypes for *T. mirus* and *T. miscellus*, noted that, while both species primarily form bivalents at metaphase I of meiosis, multivalent formation was quite frequent. Interestingly, in F_1 hybrids between *T. dubius* and *T. pratensis*, Ownbey (1950) observed an occasional pair of univalents and a ring of four chromosomes. On the basis of Ownbey's accounts, Roose and Gottlieb (1976) and Soltis *et al.* (1995) proposed that recombination among parental chromosomes was responsible for the nonadditive patterns that they observed in some *T. miscellus* allozyme profiles. Even with only occasional multivalent formation, sporadic loss of homeologous loci via recombination is plausible. Given the evidence of loss that we detected in the *T. miscellus* allopolyploids, multivalent formation may be more frequent than previously appreciated (or was at least frequent in early generations) and, at present, is the most likely mechanism responsible for homeolog loss in these polyploids. Determining how early (*i.e.*, at what generation) this loss might occur in synthetic allopolyploids of *T. miscellus* will be of particular interest.

Genomic changes *vs.* differential expression in *T. miscellus* relative to its diploid progenitors: For most of the genes examined here, genome-level changes appear to be responsible for most of the differences observed in *T. miscellus* relative to its diploid progenitors on the basis of cDNA–AFLPs. This finding demonstrates both the potential power and the shortcoming of cDNA–AFLPs. For one gene (TDF44), we found differences between the genomic CAPS and cDNA CAPS analyses, which together indicate silencing of one parental homeolog in the allopolyploid individuals. Although most of the "expression" differences between polyploids and their diploid parents result from genomic changes, other genes not studied here may be subject to epigenetic phenomena including DNA, RNA, and chromatin-mediated processes (Chen and Ni 2006).

Conclusions: The Tragopogon allotetraploids occupy an important position in the continuum of polyploid formation because they are natural and established allopolyploids that are still young (<80 years, perhaps closer to 60). Given that these plants appear to be biennials, the time frame involved may be fewer than 30–40 generations. The allotetraploid individuals of *T. miscellus* show frequent and stochastic elimination of homeologous loci, with recombination of the parental homeologs the most likely mechanism. This loss of genetic material agrees with earlier allozyme studies (Roose and Gottlieb 1976), as well as with more recent genome size estimates for these same populations of *T. miscellus* (Pires *et al.* 2004a), which indicate genome downsizing. McClintock (1984) introduced the idea of "genomic shock" in which the genome reorganizes as a response to events such as mutagenesis, transposable elements, or hybridization. On the basis of our data to date, we can rule out hybridization-mediated events as being directly responsible for the differences between polyploids and their diploid parents. Clearly, these successful Tragopogon allopolyploids have been able to contend with the genomic effects of both wide hybridization (between divergent progenitors) and chromosome doubling. In this system, it will be particularly important to determine when genomic changes occur during the evolution of these polyploids and to assess further the genomic consequences of additional polyploidization events that produced multiple lineages of the short-liguled *T. miscellus*.

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