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Stochastic and Epigenetic Changes of Gene Expression in Arabidopsis Polyploids

Jianlin Wang,* Lu Tian,* Andreas Madlung,†,‡ Hyeon-Se Lee,* Meng Chen,* Jinsuk J. Lee,* Brian Watson,† Trevor Kagochi,‡ Luca Comai† and Z. Jeffrey Chen*,[1]

**Genetics Program and Department of Soil and Crop Sciences, Texas A&M University, College Station, Texas 77843-2474, †Department of Biology, University of Washington, Seattle, Washington 98195-5325 and ‡Department of Biology, University of Puget Sound, Tacoma, Washington 98416*

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ABSTRACT

Polyploidization is an abrupt speciation mechanism for eukaryotes and is especially common in plants. However, little is known about patterns and mechanisms of gene regulation during early stages of polyploid formation. Here we analyzed differential expression patterns of the progenitors' genes among successive selfing generations and independent lineages. The synthetic Arabidopsis allotetraploid lines were produced by a genetic cross between *A. thaliana* and *A. arenosa* autotetraploids. We found that some progenitors' genes are differentially expressed in early generations, whereas other genes are silenced in late generations or among different siblings within a selfing generation, suggesting that the silencing of progenitors' genes is rapidly and/or stochastically established. Moreover, a subset of genes is affected in autotetraploid and multiple independent allotetraploid lines and in *A. suecica*, a natural allotetraploid derived from *A. thaliana* and *A. arenosa*, indicating locus-specific susceptibility to ploidy-dependent gene regulation. The role of DNA methylation in silencing progenitors' genes is tested in DNA-hypomethylation transgenic lines of *A. suecica* using RNA interference (RNAi). Two silenced genes are reactivated in both *ddm1*- and *met1*-RNAi lines, consistent with the demethylation of centromeric repeats and gene-specific regions in the genome. A rapid and stochastic process of differential gene expression is reinforced by epigenetic regulation during polyploid formation and evolution.

OVER 70% of flowering plants are polyploids and many model organisms, including Arabidopsis and maize, are of polyploid origin (Lewis 1980; Masterson 1994; Leitch and Bennett 1997; Arabidopsis Genome Initiative 2000; Gaut 2001). Ancestral genome duplication is also observed in yeast, *Caenorhabditis elegans*, and Drosophila, which contains ~30–45% of the duplicate genes (Ohno 1970; Wolfe and Shields 1997; Rubin *et al.* 2000; McLysaght *et al.* 2002; Langkjaer *et al.* 2003). Whole-genome duplication results in autopolyploidy (duplication of a diploid genome) or allopolyploidy (combination of two or more divergent genomes). In animals interspecific hybrids are sterile (Clarke 1984; O'Neill *et al.* 1998; Vrana *et al.* 2000) and no general mechanism is available to produce disomic polyploids (Orr 1990). In plants allopolyploids can be formed via chromosome doubling of interspecific hybrids or fertilization of unreduced gametes (Grant 1981; Thompson and Lumaret 1992). Many important crops such as wheat, cotton, and canola are allopolyploids (Heyne 1987; Hilu 1993; Masterson 1994). Thus, polyploid formation is a major evolutionary feature in many plant (Grant 1981; Masterson 1994; Otto and Whitton 2000) and some animal (Bogart 1980; Schultz 1980; Becak and Becak 1998) species.

The evolutionary success of allopolyploid plants is thought to be associated with changes in genome organization and gene expression (Soltis and Soltis 1995; Wendel 2000; Liu and Wendel 2002; Osborn *et al.* 2003). The combination of two genomes may create interactions that give rise to novel gene expression. As a result, polyploid species often display new traits and genetic variability (Lewis 1980; Levin 1983; Ramsey and Schemske 1998). The heterozygosity and hybrid vigor resulting from the combination of two different genomes is maintained in self-pollinating allopolyploids. Furthermore, additional set(s) of genomes may free some genes from the pressure of natural selection and allow them to develop separate functions (Ferris and Whitt 1976; Bailey *et al.* 1978). Indeed, some genes originating from different progenitors are expressed in specific tissues or at different developmental stages, as demonstrated in cotton (Adams *et al.* 2003).

Arabidopsis is an attractive model system to elucidate fundamental biological processes associated with evolutionary consequences and molecular mechanisms of polyploid formation. The Arabidopsis genome is sequenced (Arabidopsis Genome Initiative 2000), and powerful reverse and forward genetics are available. New polyploids are easily created and propagated (Comai *et*

[1]*Corresponding author:* Molecular Genetics/MS 2474, Department of Soil and Crop Sciences, Texas A&M University, College Station, TX 77843-2474. E-mail: zjchen@tamu.edu

al. 2000; Bushell *et al.* 2003; Chen *et al.* 2004). Moreover, duplicate genes can be silenced in Arabidopsis polyploids using the RNA interference (RNAi) approach (Waterhouse *et al.* 2001; Lawrence and Pikaard 2003), providing valuable resources to elucidate mechanisms for establishing and maintaining the expression status of the progenitors' genes in polyploids.

Rapid changes in genome structure and gene expression have been documented in a variety of polyploid plants, including Arabidopsis, Brassica, cotton, and wheat (Comai *et al.* 2000; Wendel 2000; Lee and Chen 2001; Kashkush *et al.* 2002, 2003; Madlung *et al.* 2002; Adams *et al.* 2003; He *et al.* 2003; Osborn *et al.* 2003), of which many are not caused by genetic mutations. However, little is known about when and how the differential expression patterns of progenitor genes are established, because natural accessions of polyploids are presumably old and "established" and the exact progenitors are often unknown, except for a few newly formed polyploid species such as Tragopogon (Cook and Soltis 1999; Soltis and Soltis 1999) and *Spartina anglica* (Raybould *et al.* 1991). In this study, we compared and analyzed gene expression patterns in both natural and newly formed Arabidopsis allotetraploid lines. We designed a series of experiments to determine: (1) when the differential expression patterns are established in subsequent selfing generations after polyploid formation; (2) whether the same set of genes is silenced within a lineage or among different lineages of the new allopolyploids; and (3) how the silenced genes are maintained. We analyzed and compared gene expression patterns in synthetic allotetraplods in several successive generations and across several independent lines derived from the same parents. Silencing of selected genes occurred independently in different lines, suggesting that certain loci may intrinsically be more susceptible to epigenetic modifications. Remarkably, independent silencing was observed in the natural *Arabidopsis suecica*, whose progenitors, while clearly the same species, were unlikely to be genotypically related to the parents of the synthetic allotetraploids. The epigenetic nature of the silencing was indicated by the reactivation of these genes concurrent with RNAi inhibition of either *MET1* or *DDM1*. Taken together, these results suggest that a subset of genes is susceptible to epigenetic remodeling induced by allopolyploidization.

MATERIALS AND METHODS

Plant material: The synthetic *A. suecica* lines were produced by pollinating an autotetraploid *A. thaliana* [Landsberg *erecta* (L*er*), spontaneously tetraploidized during tissue culturing, accession no. CS3900 in the Arabidopsis Biological Resource Center (ABRC); $2n = 4x = 20$], with autotetraploid *A. arenosa* (accession no. 3901; $2n = 4x = 32$), as previously described (Chen *et al.* 1998; Comai *et al.* 2000). One of the four independent allotetraploid lines (605A or Allo745) was selfed for two generations (S2–S3) and then crossed with the natural *A. suecica* (pollen donor), which resulted in increased seed yield. Allo745 and the other three lines (Allo733, -738, and -747) were selfed continuously to generation S5. The accession number of the natural *A. suecica* used is 94-10-085-10 from Finland (O'Kane *et al.* 1995) and available from the ABRC (accession no. CS22505). Seeds harvested from Allo745 of F_1–F_4 generations were germinated on Murashige/Skoog medium (Sigma, St. Louis), and plants were grown in a growth chamber (24°/20° day/night and 16 hr of light/day). Seedlings at 3–4 weeks old were subjected to DNA and RNA analyses. Two sets of amplified fragment length polymorphism (AFLP)-cDNA displays were performed: the first set involved RNAs prepared from Allo745 in four generations (S2–S5); the second set involved RNAs prepared from Allo733, -738, -745, and -747, all in generation S5. For AFLP-cDNA display and RT-PCR analyses, leaves collected from 10 to 15 plants were pooled for RNA extraction, except for the RT-PCR analysis in individual plants in which each plant was randomly selected in generation S5 (Allo745).

All lines including diploid and tetraploid lines of *A. thaliana*, as well as plants of *A. arenosa*, and *A. suecica*, were grown as described (Madlung *et al.* 2002). The AFLP-cDNA display was performed as previously described (Lee and Chen 2001; Madlung *et al.* 2002). After amplification, the products were resolved in a 6% polyacrylamide gel as previously described (Chen and Pikaard 1997a). For each primer pair, at least two independent reactions using the same RNA samples were performed to ensure reproducibility. The cDNA fragments that displayed differential patterns were excised and eluted in TE and reamplified using the same primer pairs as in display analysis. Sequencing reactions were performed using the purified PCR products as template in the dideoxy chain termination method in an ABI 377 sequencer. For doublets amplified, individual cDNA fragments were cloned using pGEM vector (Promega, Madison, WI) and sequenced. Approximately 10–20 inserts from each gene were sequenced from each locus. Sequences were aligned using Clustal W software (Thompson *et al.* 1994).

Production of RNAi lines in Arabidopsis polyploids: DNA fragments were amplified from the 3′ regions of *DDM1* (310 bp, AF143940) and *MET1* (402 bp, L10692), respectively. Primer sequences for *DDM1* were 5′-ACTGGCGTTGCTTA AGGAAGATGA-3′ and 5′-CCTCCCAGCCAAAAGTAACCG ATC-3′, and for *MET1*, 5′-TAACGGCTCTGGAAACTGATG TTGAA-3′ and 5′-CAAGACCTATATCAGGATCCCCACCA-3′. The gene fragments were subcloned into a pART27 vector as previously described (Waterhouse *et al.* 2001). We used seed-derived calli to transform *A. suecica*, because it is difficult to induce *A. suecica* to flower. In brief, *A. suecica* (9502) seeds were sterilized and transferred onto Murashige/Skoog medium (Sigma, St. Louis) containing 0.5 μg/liter 2, 4-D and 0.05 μg/liter Kinetin and cultured for 30 days in the dark at 28° for callus induction. The fast-growing calli were inoculated with Agrobacterium (strain LAB4404) containing the Ti plasmid 35S::ddm1-RNAi or 35S::met1-RNAi and co-cultured at 25° for 36 hr. To induce shoot growth, the calli were transferred onto a medium (3.2 g/liter B5 medium in 20 g/liter agar plus 0.5 g/liter MES, 0.5 μg/liter 2, 4-D, 0.05 μg/liter kinetin, 0.1 μg/liter α-napthalene acetic acid, and 2 μg/liter benzyl amino purine) supplemented with 30 μg/liter kanamycin and 500 μg/liter carbercilin. Greenish calli resistant to kanamycin usually developed after 1 month of culture in light and were subcultured on fresh media until transgenic plants were regenerated. The T_1 transgenic plants containing transgenes were grown in a growth chamber and selfed to T_2 generations for DNA and RNA analysis as described below.

DNA, RNA, and RT-PCR analyses: Total genomic DNA and RNA isolation and DNA blot analyses were performed as previously described (Chen *et al.* 1998; Lee and Chen 2001; Madlung *et al.* 2002). RNA was isolated from leaf tissue of pools,

unless noted otherwise, of ~10 plants (3–4 weeks old) using the Trizol method (Invitrogen, San Diego). To remove genomic DNA, 3 μg of RNA were treated with DNase I and subsequently used for cDNA production using 100 pmol of N6 random primers and 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen). RNA blot analysis was carried out using a previously published protocol (Tian and Chen 2001). RT-PCR was performed according to the SuperScript One-Step RT-PCR kit (GIBCO BRL, Gaithersburg, MD) with 40 cycles of 94° for 15 sec, 50°–65° for 30 sec, and 72° for 1 min. The actin gene, *Act2* (An *et al.* 1996), was used as a control for PCR quantification.

RESULTS

Differential gene expression in natural and newly formed Arabidopsis polyploid lines: To determine the timing of establishment of differential expression patterns, we generated synthetic *A. suecica*-like allotetraploid lines ($2n = 4x = 26$) via interspecific hybridization between autotetraploid *A. thaliana* ($2n = 4x = 20$) and *A. arenosa* ($2n = 4x = 32$; Comai *et al.* 2000; Lee and Chen 2001), resulting in genome duplication and allopolyploidization. Both *A. thaliana* diploid and autotetraploid lines were also included in the analysis as progenitors and as a comparison of gene expression changes for autopolyploids and allopolyploids. AFLP-cDNA display (Madlung *et al.* 2002; Comai *et al.* 2003b) was performed using RNA prepared from pooled plants obtained in four selfing generations of an allotetraploid that was synthesized from *A. thaliana* and *A. arenosa* (Figure 1a). The line (605A) was crossed with a natural allotetraploid, *A. suecica*, in the third generation. The S1 and S4 plants showed the expected set of 13 chromosome pairs (Comai *et al.* 2000, 2003b), indicating stable chromosomal inheritance. The phenotypic variation observed in plants in selfing generations (Figure 1a and our unpublished data) could be associated with changes in gene expression. Indeed, differential gene expression patterns were detected in these newly formed allotetraploids. Some genes were expressed in the parents and remained silenced in the progeny for three or more selfing generations (Figure 1b, arrows, left), suggesting rapid establishment of a stable differential regulatory state. Other genes were expressed in only a few generations (small arrows, right), indicating that the expression of these genes in new allotetraploid lines is stochastic in selfing progeny (see below). Furthermore, novel expression patterns were detected, presumably due to reactivation of genes that were not expressed in both parents (Figure 1b, large arrow, right). In a survey of ~2430 cDNA fragments, we identified ~11% that displayed changes in S2–S4 generations relative to the two parents. Among them, ~4% of the changes were related to *A. thaliana*, ~5% to *A. arenosa*, 1% to both parents, and ~1% to neither parent (or novel expression). The proportion of differentially expressed genes in the new allotetraploid lines was higher than that found in the natural *A. suecica* lines (Lee and Chen 2001), which may reflect, at least in part, a plastic nature of gene

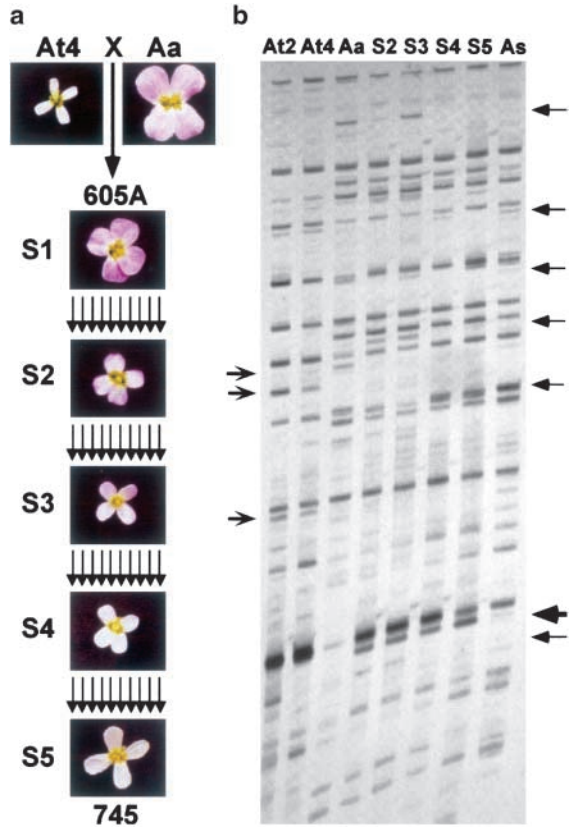

Figure 1.—Newly formed Arabidopsis allotetrapolyploids and differential gene expression. (a) A selfing scheme (from S1 to S5) for one of the newly formed Arabidopsis polyploids (605A or 745). A typical flower is shown in each generation, although variation in plant morphology and flowers exists among siblings within a progeny array. It is notable that the flower morphology in S5 resembles that of natural *A. suecica*. At4, *A. thaliana* (L*er*) autotetraploid (ABRC accession no. CS3900); Aa, *A. arenosa* autotetraploid (accession no. 3901). (b) AFLP-cDNA display results were obtained using RNA samples isolated from *A. thaliana* diploid (At2), autotetraploid (At4), *A. arenosa* (Aa), generations S2–S5 of newly formed allotetraploid lines, and the natural *A. suecica* (As) line. Arrows indicate gene silencing (left), novel expression patterns (large arrow, right), and random changes in gene expression in different generations (small arrows, right), respectively.

expression in the new allotetraploid lines. We cloned and sequenced a subset of 43 candidate genes that show putative novel expression or differential expression patterns originating from either of the progenitors (Table 1). The candidate genes identified encode proteins for transposons, cell division, cell metabolism, protein transport, signal transduction pathways, and unknown functions. Using locus identifications, the candidate genes were mapped among five chromosomes of *A. thaliana*; there was no indication of clustering silenced genes in a chromatin domain (data not shown), which does not preclude the chromatin effects on some specific regions.

Rapid and stochastic effects of polyploidization on the differential expression of progenitor genes: We designed primers specific to a subset of the *A. thaliana*

TABLE 1

A set of candidate genes detected by AFLP-cDNA display shows differential expression patterns in new *A. suecica* lines

Clone ID	Putative origin of silencing	At2	At4	Aa	S2	S3	S4	S5	As	Gene ID	Gene name
E-AC/1/2X/1	At	+	+	0	+	+	−	+	0	F24J35.19	Peptide transporter
E-AC/3/2X/1	At	+	+	0	0	0	−	0	0	F617.9	Protein transporter (PT)
E-AC/4/2X/1	At	+	+	−	−	−	−	−	−	T9N14.8	Cryptozoic factor, putative
E-AC/4/2X/2	At	+	+	0	+	+	−	+	−	AT4G28710	Myosin heavy chain-like protein
E-AC/8/2X/1	At	+	+	−	−	−	−	−	−	F22C12.7	Hypothetical protein
E-AG/6/2X/1	At	+	+	−	−	−	+	−	+	F1M20.33	Myb-like DNA-binding domain
E-AT/1/2X/1	At	+	+	−	+	+	−	+	−	F28C11	Similarity to peroxidase isozyme
E-AT/1/2X/2	At	+	+	0	−	−	−	−	−	T27G7.16	Unknown protein
E-AT/7/2X/3	At	+	+	0	+	+	0	+	0	F19K23.19	Similar to peroxidase isozyme
E-TT/2/2X/1	At	+	+	−	−	−	−	−	−	MYH9.6	Putative protein
E-TT/2/2X/2	At	+	+	0	−	−	−	−	+	F26P21.170	Putative protein (PP1)
E-TT/2/2X/3	At	+	+	0	−	−	−	0	−	F6N7.24	Putative protein
E-AC/7/4X/1	At	+	+	0	0	0	−	0	0	T21H19.200	Putative protein KIAA1012 protein
E-AT/1/4X/2	At	+	+	−	+	+	−	+	−	T23K8.10	Similarity to serine threonine kinase (STK)
E-TC/4/2X/1	At	+	+	−	+	+	−	+	−	MLJ15.9	Light-regulated protein
E-AC/7/F3/1	At	+	+	0	0	+	0	−	+	F7A10.1	Unknown protein
E-AC/5/2X/1	At	+	0	+	+	+	+	+	+	MYH9.8	Cellulose synthase catalytic subunit
E-AT/1/2X/3	At	+	−	−	−	−	−	−	−	F17I14.150	NAD-dependent malate dehydrogenase
E-TG/6/2X/1	At	+	−	+	+	+	+	+	−	T25K16.6	DNA-binding protein (DBP)
E-AG/6/2X/3	At	+	+	+	+	+	0	+	+	F14N12.2	Putative lipase
E-AA/5/CA/1	At	+	+	−	−	−	−	−	+	F22D16.7	Glutathione *S*-transferase (GST)
E-TG/6/CA/1	At	+	−	+	+	+	+	+	−	F10M23.190	Putative protein (PP2)
E-TT/4/4X/1	At	0	+	+	+	+	+	+	−	MBM17.52	Similarity to SNF2/RAD54 family (RAD54)
E-AC/5/4X/2	At	0	+	0	+	+	+	+	−	T4O12.9	Vacuolor sorting protein 35
E-AT/6/4X/2	At	0	+	+	+	+	+	+	+	MPA24.12	Nuclear matrix constituent protein 1
E-TA/6/4X/1	At	0	+	+	0	+	+	+	+	F16L2.60	Kinesin-related protein
E-AG/3/CA/1	Aa	0	0	+	+	+	0	0	0	F10K1.19	Fructose-2,6-bisphosphatase
E-TG/1/CA/1	Aa	0	0	+	+	0	0	0	0	F5M15.5	Hypothetical protein
E-AA/3/CA/1	Aa	0	0	+	+	+	−	+	0	K14A17.7	E2, ubiquitin-conjugating enzyme
E-AA/4/CA/1	Aa	−	−	+	+	+	−	−	−	MJL12.21	Rotamase FKBP
E-AT/7/CA/3	Aa	0	0	+	+	0	+	0	+	T24D18.11	Unknown protein
E-TC/2/CA/1	Aa	0	0	+	+	+	0	0	0	T9J14.13	Putative ribosomal protein
E-TG/2/CA/1	Aa	0	0	+	+	0	+	+	+	F1B16.11	Chloroplast 50S ribosomal protein
E-TG/3/CA/1	Aa	0	0	+	+	+	+	+	0	T9G5.7	Copia-type polyprotein
E-AC/8/F2/2	Aa	0	0	+	+	+	+	+	0	F1P2.90	Endochitinase-like protein
E-AC/7/Se/2	Aa	0	0	+	0	0	+	+	+	F2A10.1	Unknown protein
E-AA/5/F2/2	Novel	−	−	−	+	+	−	−	−	F5A8.1	Similar to Rubisco small unit
E-TC/3/F2/1	Novel	0	0	0	+	+	+	+	0	MCO15.7	Putative protein
E-TC/3/F2/2	Novel	0	0	0	+	+	0	+	0	T22P15.2	Hypothetical protein
E-TG/1/F2/2	Novel	0	0	0	+	+	+	+	−	LHI6299	Similarity to recN gene
E-TT/3/F2/1	Novel	0	0	0	+	+	+	+	0	MKP6.31	Hypothetical protein
E-TT/3/F2/2	Novel	0	0	0	+	+	0	0	0	T3K9.18	Hypothetical protein
E-TT/1/F3/1	Novel	0	0	0	0	+	0	0	0	T27G7.20	Mg-chelatase, putative

At, *A. thaliana*; At2, *A. thaliana* diploid; At4, *A. thaliana* autotetraploid; Aa, *A. arenosa*; As, *A. suecica*; S2–5, selfing generations 2, 3, 4, and 5; Novel, expression is present in polyploids but absent in either parent; 0, −, and + indicate no expression, very low, and very high levels of expression detected, respectively.

gene sequences at the 3′ ends so that only *A. thaliana* transcripts are amplified for the majority of genes tested using genomic and RT-PCR analyses (Table 2). Alternatively, the primer pairs amplify transcripts from both parents but the amplified products are polymorphic. Several differential expression patterns were detected among selfing generations of Allo745 and one generation of outcrossing (S4; Figure 2). First, expression of the *A. thaliana* gene encoding a protein transporter (PT) was undetectable in the natural and newly formed allotetraploid line, confirming the data obtained from the AFLP-cDNA display and indicating rapid establishment of a silenced status. Second, transcripts of *A. thaliana* genes encoding a DNA binding protein (DBP) and

TABLE 2

Primer sequences and expected sizes of amplified products for the set of genes studied using genomic and RT-PCR analyses

Gene-symbol	Expected size of cDNA	Expected size of genomic DNA	Primers
PT	400	796	F-5′-GACGCCATTGATTCCGTCGT
			R-5′-CCCTTAACATGCCAATGCAA
PP2	900	1500	F-5′-GGGCACGAGCTAGAAAACGA
			R-5′-GGCCTTGGAGATGGAATTGG
STK	902	902	F-5′-GTGTCGCGAAGCTGACTGAT
			R-5′-GGATCGATCAAAACCCAAAC
DBP	980	1080	F-5′-GCAGCTACATTCGCTGCTTCG
			R-5′-TCCAATCGAAGCCTTTTGCAG
GST	731	930	F-5′-ACCCAGCTTCCACAGCCACTA
			R-5′-AAATCAAACACTCGGCAGCA
PP1	804	1000	F-5′-CATGGCTGCTGAGGCTGCTA
			R-5′-GCACAAAATGCCCACAACCA
RAD54	190	190	F-5′-CTTCTTCTCACTCTTCTCTCCA
			R-5′-AAGAGGGAGGCTGTCTAGGA

PT, protein transporter; PP2, putative protein 2; STK, serine threonine kinase; DBP, DNA-binding protein; GST, glutathione *S*-transferase; PP1, putative protein 1; RAD54, SNF2/RAD54 family protein. The GenBank accession numbers of the genes are shown in Table 1.

putative protein 1 (PP1) were detected in newly formed lines but were undetectable in natural allotetraploids although both genes were present (Figure 2), suggesting that either it takes more than five generations of selfing to establish this differential expression pattern or the parental genotypes have a strong effect on the regulatory outcome. Absence of *DPB* and *PP1* transcripts in a new autotetraploid line (At4) followed by reactivation in the allotetraploids may suggest a different mechanism for gene expression changes between autoploidy and alloploidy. Third, the *A. thaliana* genes encoding glutathione *S*-transferase (GST) and putative protein 2 (PP2)

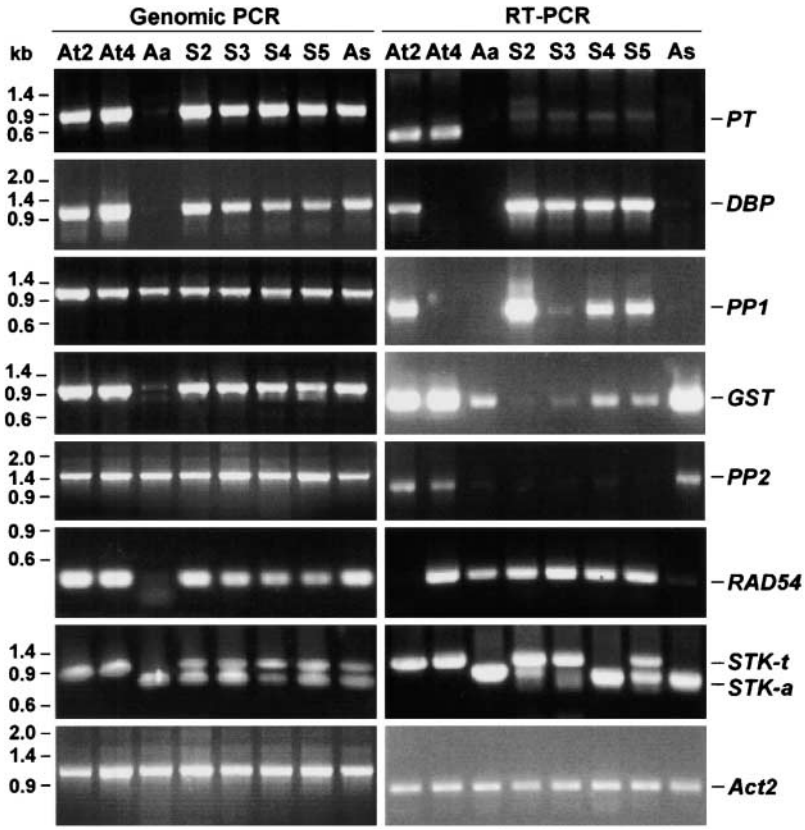

FIGURE 2.—Activation and silencing of progenitor genes in S2–S5 selfing generations of newly formed Arabidopsis polyploids (Allo745). The majority of primer pairs used in genomic and RT-PCR analyses was specific to *A. thaliana* genes (Table 2). PCR amplification using genomic DNA as templates is shown at the left, whereas RT-PCR analysis is shown at the right. PT, protein transporter; PP1 and -2, putative protein 1 and 2; DBP, DNA binding protein; GST, glutathione *S*-transferase; RAD54, similarity to SNF2/RAD54 family; STK, serine-threonine kinase (the suffix "t" or "a" indicates that the locus origin is from *thaliana* or *arenosa*, respectively). Actin gene (*Act2*) was used as a control. Size differences between amplified genomic and RT-PCR products are due to the presence of introns in some genes.

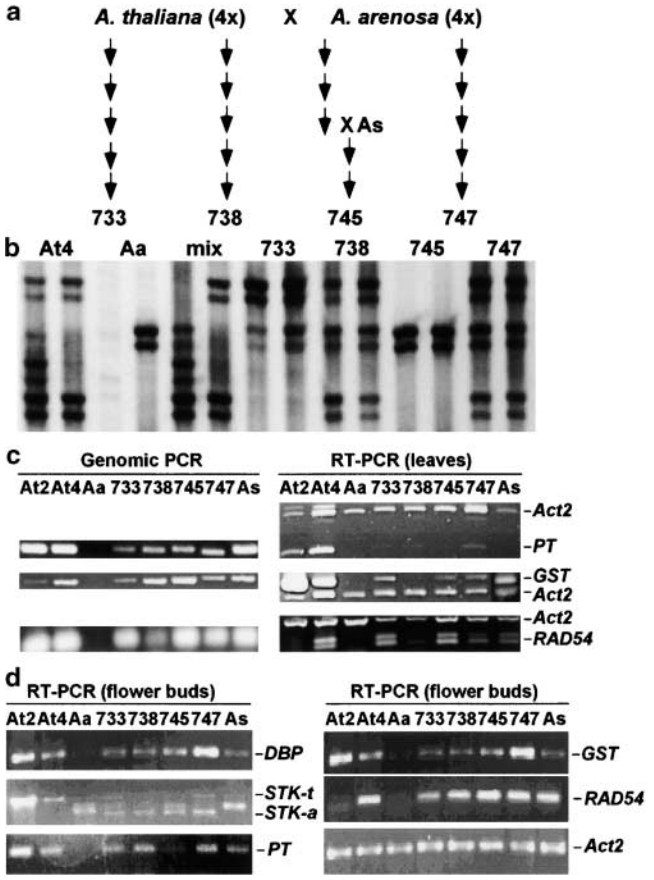

FIGURE 3.—Expression analysis of independently synthesized allopolyploid lines. (a) Four independent allotetraploid lines were produced by pollinating emasculated *A. thaliana* (L*er*) tetraploid with pollen collected from *A. arenosa*. Three individual S1 plants were self-pollinated to the fifth generation (S5) and one line (Allo745) was crossed with natural *A. suecica* (As) in S3 and selfed to S5. (b) AFLP-cDNA display was performed in the four allotetraploid lines (Allo733, -738, -745, -747), two parents, and a mix containing an equal amount of the RNAs from the two parents. The AFLP-cDNA analyses were replicated in each line using separate cDNA templates. (c) Differential expression patterns of a subset of genes in four independent lineages. Multiplex RT-PCR was performed for four genes that were found to be differentially regulated in different selfing lineages. Both the gene of interest and *Act2* (as an internal control) were spontaneously amplified in each reaction. Amplification of the gene fragments using genomic DNA templates is shown at the left. The same set of primers used in Figure 2 was used for this study. (d) RT-PCR was performed in RNA samples prepared from flower buds. The same sets of primers and controls were used as shown in c and in Figure 2.

were poorly expressed in newly formed allotetraploid line but highly expressed in the natural line and *A. thaliana* diploid and autotetraploid, suggesting downregulation of the genes in early generations and reactivation of the genes in late generations. Fourth, *SNF/RAD54* expression was observed in newly formed polyploids. The SNF/RAD54 gene, a member of the SNF subfamily (EISEN *et al.* 1995), is involved in both DNA repair and transcriptional regulation (COLE *et al.* 1989; MIYAGAWA *et al.* 2002). *RAD54* was not expressed in the diploid Arabidopsis ecotype L*er* (At2), only poorly expressed in natural *A. suecica* (As), but highly expressed in the newly formed autotetraploid (At4) and S2–S4 generations of allotetraploids as well as the allotetraploid outcrossed to natural *A. suecica* (S4). The *RAD54* expression patterns coincide with unstable chromosome behaviors observed in newly formed polyploids (both auto- and allotetraploids; COMAI *et al.* 2000). Finally, expression states of the progenitor genes changed in selfing generations. Either one of the parental loci or both *STK* loci were expressed among four selfing progeny. In genomic PCR reactions, both sets of progenitor genes were amplified in newly formed and natural *A. suecica* lines, ruling out a possibility of sequence elimination or interchromosomal DNA recombination. These data suggest that the differential expression states of duplicate genes are either rapidly or gradually established during polyploidization.

TABLE 3

Changes in gene expression between the inbred tetraploid *A. thaliana* parent and the four independent allotetraploid lines using AFLP-cDNA analysis

Category[a]	Behavior of AFLP-cDNA products in allopolyploids	%
1	New in one or more lines	0.3
2	Missing in one or more lines	2.1
3	Missing in two or more lines	1.3
4	Much stronger in one or more lines	0.4
5	Much weaker in one or more lines	0.2

[a] Products on the gel were categorized as genes that were activated (1); silenced in any of the four lines (2); silenced in at least two lines, indicating nonrandom silencing (3); and up- or downregulated, respectively (4, 5).

Is the same set of genes subjected to silencing in successive selfing generations and multiple independent lineages? Given the silencing frequency we observed, if all genes are equally likely to be silenced, independent allopolyploid lines should show silencing of mostly different genes. However, that is not the case. We tested which genes are silenced in four independently synthesized allotetraploid lineages (Allo733, -738, -745, and -747) (Figure 3, a and b) and analyzed ~3640 AFLP-cDNA products (Table 3). Here we excluded gene expression differences between the allotetraploids and *A. arenosa* parent to avoid heterozygosity in the outcrossing *A. areonsa* (MADLUNG *et al.* 2002). We found that ~3% are subjected to differential expression; 2.1% were silenced in at least one of the four independent lines, while ~0.4 and ~0.2% of the genes were strongly up- or downregulated, respectively. Significantly, ~1.3% of genes were silenced in more than one independent line, indicating that "choice" of silencing is not random.

We further investigated whether a similar set of genes

differentially regulated in successive selfing generations is also subjected to silencing in four independent lineages (Figure 3c). First, the PT expression was very low in all of the four different lines, with the lowest levels in Allo745. Second, the *RAD54* expression was low in the *A. thaliana* diploid and natural *A. suecica* but high in the tetraploid progenitor, very low in *A. arenosa*, and variable in the four new allotetraploid lines. Finally, a strong attenuation of GST expression was observed in all allotetraploid lines (regardless of outcrossing in Allo745) compared to the diploid and tetraploid *A. thaliana* lines. No GST transcript was detected in synthetic line Allo738. In comparing these results with that observed in selfing generations, it is clear that a similar set of genes is subjected to differential regulation in both successive selfing generations and independent lines of newly formed allotetraploids.

Differential expression of progenitors' genes may lead to subfunctionalization of homeologous or duplicate genes (Lynch and Force 2000; Adams *et al.* 2003). To test this, we examined the expression patterns of these genes in flower buds (Figure 3d) as well as in leaves (Figures 2 and 3c). Similar stochastic expression patterns were observed for the majority of genes tested. In addition, the expression of *GST*, *RAD54*, *STK-t*, and *STK-a* loci was slightly variable between flower buds and leaves in some of the lines tested.

How is differential gene expression established? Ribosomal RNA genes subjected to nucleolar dominance are selectively silenced in allopolyploids, although it takes two generations to complete the silencing process (Chen *et al.* 1998). Is the differential expression pattern selectively or stochastically established among different individuals or siblings within the same generation? We examined the expression patterns of eight plants randomly selected in the fifth generation (S5; Figure 4). The serine-threonine kinase (STK) gene was coexpressed in the pooled plants in generation S5. However, each individual displayed an expression pattern different from that of the progenitors (Figure 4a). There was a silencing trend for the *A. thaliana STK-t* locus in generation 4, because *STK-t* was poorly expressed in seven of eight plants; one plant displayed a high level of *STK-t* expression. This trend may contribute to the silencing pattern observed in late generations and natural *A. suecica* lines, in which no *STK-t* expression was detected among 10 different plants (data not shown). If a stochastic silencing mechanism is at work, the *STK-t* locus should be expressed in at least some lines. A similar behavior was observed in the study of the *A. thaliana* DBP gene, which was expressed in pooled plants in all of the generations examined. However, the *A. thaliana* DBP was silenced in two of the eight plants in the S5 generation and natural *A. suecica* lines (Figure 4b). Although the expression of the DBP and STK genes was dynamic and stochastic, *RAD54* expression was stable among different siblings in the same generation (Figure 4c). *A. arenosa* is a natural autotetraploid that displays some chromosomal abnormality during meiosis (Comai *et al.* 2000), showing a relatively high level of *RAD54* expression. But only a trace amount of *RAD54* transcript was detected in natural *A. suecica*. We extended the *RAD54* analysis to three independent synthetic allotetraploid lines and independently grown *A. arenosa* and *A. suecica* (Figure 3c). We also included in the analysis *A. thaliana* diploids (1 L*er* and 1 Col) and autotetraploid lines (2 L*er* and 1 Col) that had been derived simultaneously from colchicine-treated parents (data not shown). *RAD54* expression was moderately to strongly active in all lines except the original diploid L*er*.

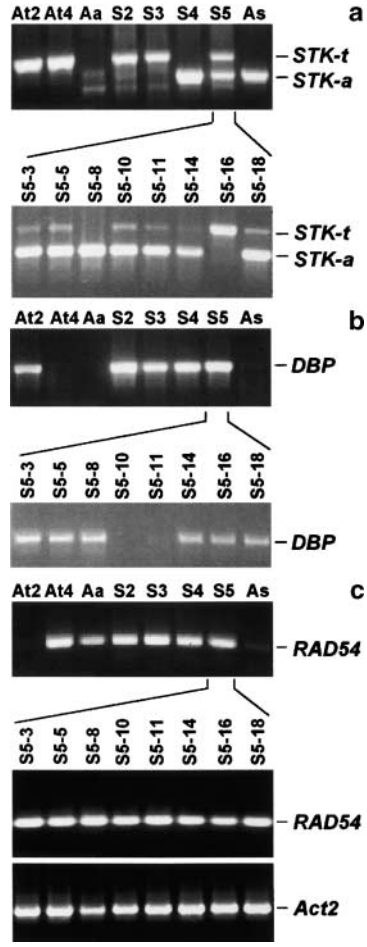

Figure 4.—Stochastic expression patterns of progenitor genes within a selfing generation. (a) RT-PCR analysis of *STK* in pooled plants of four selfing generations and among eight siblings in generation 5 in Allo745. For simplicity, the same set of RT-PCR results obtained from pooled plants (shown in Figure 2) were used in this figure. Within the fifth generation, eight individual siblings (S5-3, -5, -8, -10, -11, -14, -16, and -18) were used for RT-PCR analysis. (b and c) RT-PCR analysis of *DBP* (b) and *RAD54* (c) was performed using the same set of plants as described above. RT-PCR of *Act2* is shown as an internal control.

Silenced genes are maintained by DNA methylation: Transcriptional activation of mobile elements is observed in newly formed Arabidopsis and wheat poly-

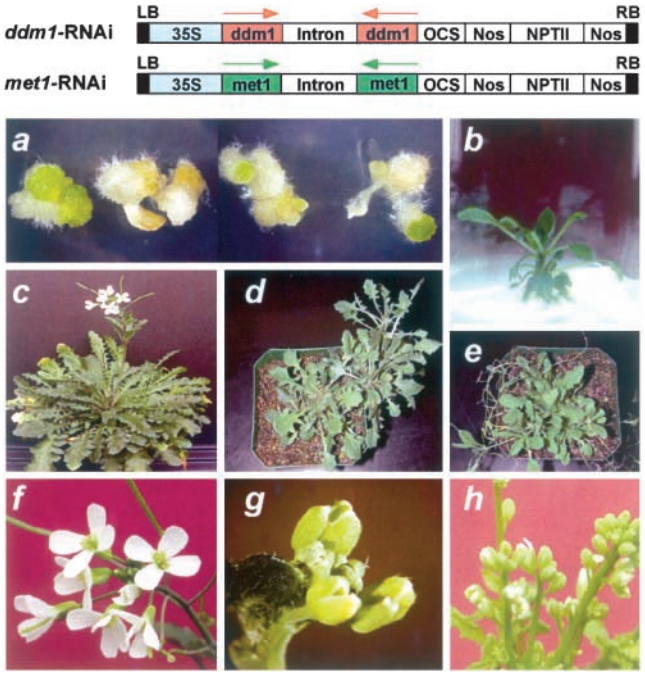

FIGURE 5.—Transformation and regeneration of demethylation *A. suecica* plants using RNAi. The RNAi was constructed using a pART27 vector (WATERHOUSE *et al.* 2001). PCR-amplified sequences of *DDM1* and *MET1* were used (see MATERIALS AND METHODS). (a) *A. suecica* seeds were germinated for callus induction (~4 weeks). (b) Four-week-old calli were incubated with Agrobacterium strain LAB4404 containing a construct containing a selectable marker (*NPTII*) and a double-stranded *ddm1* or *met1* (36 hr). The regeneration time of transgenic plants was ~3 months. (c and f) *A. suecica* lines as controls. (d and g) *ddm1*-RNAi lines. (e and h) *met1*-RNAi lines. The RNAi lines displayed some abnormal developmental and floral phenotypes (d, e, g, and h).

ploids (COMAI *et al.* 2000; MADLUNG *et al.* 2002; KASHKUSH *et al.* 2003), which may alter the expression of neighboring genes in the genome (KASHKUSH *et al.* 2003). Moreover, the newly formed *A. suecica* lines are susceptible to the treatment with 5′-aza 2′-deoxycytosine (aza-dC; MADLUNG *et al.* 2002), a suicide inhibitor of DNA methyltransferases (HAAF 1995). The silenced rRNA or protein-coding genes are shown to be reactivated by the aza-dC treatment (CHEN and PIKAARD 1997b; LEE and CHEN 2001; MADLUNG *et al.* 2002). However, aza-dC has toxic effects on cellular growth and development (HAAF 1995). To study a causal relationship between DNA methylation and silencing of duplicate genes, we employed RNAi (WATERHOUSE *et al.* 2001) to silence *AtDDM1* or *AtMET1* in natural *A. suecica* lines. Multiple independent lines were generated for each construct, although a single representative plant was shown for each transgenic line (Figure 5). Control (wild type) plants were regenerated through the same tissue-culture process using the pART27 vector (WATERHOUSE *et al.* 2001) alone. Compared to the control plants (Figure 5, c and f), both *ddm1*- and *met1*-RNAi *A. suecica* lines showed phenotypic abnormalities, suggesting that DNA methylation is important to plant growth and development (VONGS *et al.* 1993; MARTIENSSEN and RICHARDS 1995; FINNEGAN *et al.* 1996; RONEMUS *et al.* 1996). The abnormal phenotypes included development of aerial rosettes, fusion of flower organs, and a delay in flowering time. The *met1*-RNAi lines displayed more severe phenotypes than *ddm1*-RNAi lines.

We further examined whether the phenotypes were stable in selfing generations and whether the expression of target genes was affected in the RNAi lines. Seedling phenotypes in the T_2 generation (Figure 6a) were consistent with those in the T_1 generation (Figure 5). Furthermore, using RT-PCR analysis, we found that the expression of endogenous target genes (both *DDM1* and *MET1*) was dramatically reduced; <5% of transcripts were detected in independent RNAi lines compared to the control (Wt) and wild-type (WT) plants (Figure 6, b and c). Similar levels of *DDM1* or *MET1* expression were detected in both WT and Wt plants, ruling out a possibility that target genes may be suppressed through a tissue-culture process. It is notable that *DDM1* expression levels were much higher in *A. arenosa* and control plants than in *A. thaliana*, suggesting a high requirement of methylation-dependent gene regulation in auto- and allotetraploid plants. However, the expression of target genes was greatly reduced in both *ddm1*- and *met1*-RNAi *A. suecica* lines, although they displayed different phenotypes (Figures 5 and 6a), suggesting that *DDM1* and *MET1* may have overlapping but different functions in regulating the DNA methylation process. Moreover, *A. arenosa-DDM1* (*AaDDM1*) that was highly expressed in *A. arenosa* and *A. suecica* was greatly repressed in the *ddm1*-RNAi *A. suecica* lines (Figure 6b).

We selected two lines from each transgenic strain (for each construct) for further analysis, namely *ddm1*-RNAi #3 and #7 and *met1*-RNAi #1 and #12 in T_2 generation. To determine whether DNA methylation was affected in the RNAi lines, we analyzed methylation patterns of *Msp*I- and *Hpa*II-digested genomic DNA. *Hpa*II is inhibited from cleavage by methylation of either the outer or the inner cytosine of the CCGG site, whereas *Msp*I cleavage is not inhibited by methylation of the inner cytosine but partially inhibited if the outer cytosine is methylated (MCCLELLAND *et al.* 1994). Both *ddm1*- and *met1*-RNAi lines showed decreased levels of DNA methylation in centromeric repeats, gene-specific regions (Figure 7, a–d), and rDNA (data not shown). DNA methylation was less severely reduced in the *ddm1*-RNAi lines than in the *met1*-RNAi lines (Figure 7d), suggesting that *AtDDM1* and/or *AaDDM1* loci may still be expressed in the *ddm1*-RNAi lines. Alternatively, the RNAi may not work as efficiently for *DDM1*. Same *Msp*I restriction patterns were detected between the control and RNAi lines (Figure 7, c and d), suggesting that non-CG methylation is not affected in the RNAi lines.

Importantly, two silenced genes (*RAD54* and *PP1*)

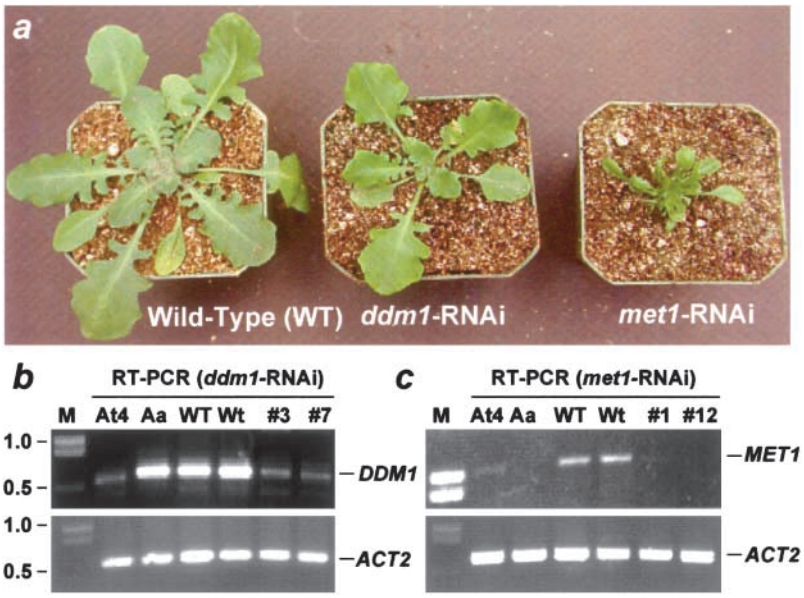

FIGURE 6.—Inheritance of phenotypes and downregulation of endogenous *DDM1* and *MET1* expression in T_2 generations of *ddm1*- and *met1*-RNAi plants. (a) Seedling (3 weeks old) phenotypes of *A. suecica* control (WT), *ddm1*-, and *met1*-RNAi lines. (b) Downregulation of endogenous *DDM1* expression in two independent *ddm1*-RNAi lines (nos. 3 and 7). At4, *A. thaliana* autotetraploid; Aa, *A. arenosa*; WT, *A. suecica*; Wt, *A. suecica* transformed with the pART27 vector (WATERHOUSE *et al.* 2001). (c) Suppression of endogenous *MET1* expression in two independent *met1*-RNAi lines (nos. 1 and 12). Only <5% of endogenous *DDM1* and *MET1* mRNAs were detected in the *ddm1*- and *met1*-RNAi lines, respectively. The expression of *Actin 2* (*ACT2*) was used as a control for RNA loading and RT-PCR analysis.

were partially reactivated in both *ddm1*- and *met1*-RNAi lines (Figure 7, f and g), regardless of relatively small effects on CpG methylation in the centromeric repeats in the *ddm1*-RNAi lines (Figure 7d). The levels of *PP1* reactivation were correlated with low and high levels of demethylation in the *ddm1*- and *met1*-RNAi lines, respectively (Figure 7, d and g). Furthermore, *RAD54* derepression in *ddm1*- and *met1*-RNAi lines was correlated with the demethylation of promoter sequences in this locus (Figure 7, a and c). Compared to the methylation patterns in a wild-type plant, cytosines in three CG sites located within a 1.4-kb region upstream of the ATG

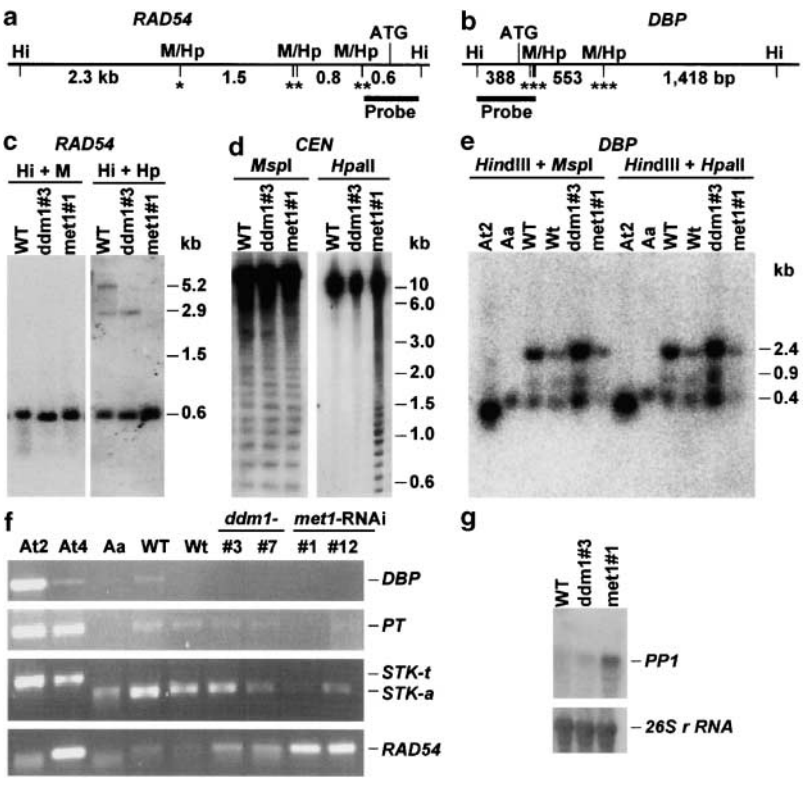

FIGURE 7.—Demethylation and reactivation of *RAD54* and *PP1* in *ddm1*- and *met1*-RNAi lines of natural *A. suecica*. (a and b) Diagrams of *RAD54* (a) and *DBP* (b) genomic fragments. The restriction sites of *Hin*dIII (Hi) and *Msp*I (M) or *Hpa*II (Hp) are shown. One, two, and three asterisks indicate CG methylation in the wild type, wild type and *ddm1*-RNAi, and all three lines, respectively. (c and d) DNA blot analyses of *A. suecica*, *ddm1*-, and *met1*-RNAi lines. The DNA was digested by *Hin*dIII and then by either *Msp*I or *Hpa*II and subjected to agarose gel electrophoresis. The DNA was transferred to a blot that was hybridized with an *AtRAD54* promoter fragment (−600 to ATG codon) as the probe (c). The blot was stripped of the probe and hybridized with the probe derived from a 180-bp centromere repeat (d). (e) Another blot containing *Hin*dIII and *Msp*I- or *Hpa*II-digested DNA was hybridized with a probe derived from the promoter region of DBP. At2, *A. thaliana* diploid; At4, *A. thaliana* autotetraploid; Aa, *A. arenosa*; WT, *A. suecica*; Wt, *A. suecica* transformed with the binary vector pAR27. (f) Multiplex RT-PCR analysis of four silenced genes in *ddm1*- and *met1*-RNAi *A. suecica* lines. The silenced *RAD54* was derepressed in RNAi-demethylation lines. However, silenced *DBP*, *PT*, *STK-t* were not derepressed in the RNAi lines. (g) RNA blot analysis showed reactivation of *PP1* in the RNAi-demethylation lines. A low level of reactivation might be correlated with a low level of demethylation in the *ddm1*-RNAi line (compare d and g). The RNA blot was rehybridized with a 26S-rRNA gene repeat as a control.

codon (Figure 7a) remained methylated in the *ddm1*-RNAi line, whereas cytosines in all CG sites were demethylated in the *met1*-RNAi line. In the *ddm1*-RNAi line, demethylation occurred only in the cytosine of the CG site located at ~2.9 kb upstream of the ATG codon.

Three silenced genes (*DBP*, *PT*, and *STK-t*) tested were not reactivated in either *ddm1*- or *met1*-RNAi lines (Figure 7f). The silenced *DBP* was heavily methylated in the *ddm1*- and *met1*-RNAi lines, as well as in the control plants (Figure 7, b and e), indicating that RNAi does not completely disrupt the expression or function of the target genes (*i.e.*, *DDM1* and *MET1*). Alternatively, other chromatin factors (*e.g.*, histone methylation or deacetylation) or mechanisms such as paramutation (Mittelsten Scheid *et al.* 2003) and RNAi may account for the differential expression of homeologous genes in allopolyploids (Osborn *et al.* 2003).

DISCUSSION

The fate of homeologous genes during polyploid formation: We estimate that ~3–11% of the progenitors' genes are susceptible to changes in expression in new allotetraploids. The lower number may be underestimated because gene expression differences between the allotetraploid and the *A. arenosa* parent were excluded due to heterozygous alleles in the outcrossing *A. arenosa* genome. The upper number may be overestimated because we scored all polymorphic bands between an allotetraploid and the two parents, including *A. arenosa*. It is notable that either the *A. thaliana* or the *A. arenosa* parent contributed to an equal amount of expression changes (4–5%) in the allotetraploid progeny. The majority of changes were associated with cDNA fragments present in only one of the two parents, suggesting that the genes highly expressed in one parent may be susceptible to changes during allopolyploidization. However, the data obtained from AFLP-cDNA analysis need to be carefully interpreted. For example, the number of amplified polymorphic fragments varied from one primer pair to another, suggesting that the technique is dependent on the efficiency of detecting restriction polymorphisms among different samples. Moreover, some "false positives" may be associated with PCR amplifications. Thus, differentially expressed genes detected in AFLP-cDNA display should be verified using at least one independent method such as RT-PCR, sequencing, or single-strand confirmation polymorphism analysis (Lee and Chen 2001; Adams *et al.* 2003).

Duplicate genes and genomes provide new genetic material for evolution (Ohno 1970; Lynch and Conery 2000; Wolfe 2001; Liu and Wendel 2002; Wolfe and Li 2003) by gaining new functions or functional divergence. However, the fate of homeologous genes in the allopolyploids is poorly understood. An appealing hypothesis suggests that the expression of homeologous genes is reprogrammed during early stages of polyploid formation and thus affects the genetic redundancy resulting from the combination of two genomes in a polyploid cell (Osborn *et al.* 2003). Moreover, increase in gene or genome dosage may result in overexpression of genes that are associated with disease syndromes or abnormal development in animals (Lengauer *et al.* 2000; Emanuel and Shaikh 2002). Thus, some homeologous genes are silenced or downregulated, whereas others are coexpressed and/or upregulated to meet the needs of protein synthesis and cellular functions in polyploid cells (Lee and Chen 2001). Which homeologous loci are subjected to silencing or activation? In nucleolar dominance (Reeder and Roan 1984; Pikaard 1999), only one set of parental rRNA genes is transcribed in an interspecific hybrid or allotetraploid. There is a preference for the expression of progenitors' rRNA genes (Chen and Pikaard 1997b), which exhibits natural variation in the choice of silencing (Pontes *et al.* 2003). The silenced loci are partially reactivated during flower development (Chen and Pikaard 1997b). For protein-coding genes, we found that 26 of 43 (~60%) candidate genes that are highly expressed in *A. thaliana* diploid or autotetraploid are subjected to downregulation in at least one generation or an allotetraploid line. Moreover, a few homeologous genes are randomly selected for silencing. Either of the progenitor's loci (*STK-t* and *STK-a*) in *A. suecica* can be switched "on" or "off" in five successive selfing generations and among siblings within a selfing generation, suggesting a stochastic effect on the expression of some homeologous loci. For loci such as *PT* and *PP2*, the decision on choosing which locus to silence is made early during polyploid formation so that the silencing is observed immediately after polyploidization. For *STK*, this process is gradually established with a trend of preferentially expressing the *STK-a* locus among eight siblings in the S5 generation (Figure 4a), which is reminiscent of the expression of the *A. arenosa* locus (*STK-a*) in the natural *A. suecica* line tested. It is notable that outcrossing to natural *A. suecica* had a little effect on the expression of the genes studied (Figures 2–4), suggesting that the gene expression states are stable after they are established.

Although the process of establishing an expression state of homeologous loci is stochastic, this process affects only a subset of genes in the homeologous genomes. Significantly, we have shown that the same set of genes (*PT*, *GST*, and *RAD54*) exhibiting differential expression in selfing generations and siblings in Allo745 display similar activating and silencing patterns in four independently derived allotetraploid lineages (Figure 3c). Moreover, another subset of genes, including *DBP*, *PP1*, and *RAD54* (Figure 2) and several others (Table 1), show expression differences between the diploid and isogenic autotetraploid lines, suggesting dosage-dependent gene regulation (Birchler 2001). The auto- and allopolyploidization may have different effects on gene regulation, because the two sets of genes (although

in a small sample) only partially overlap. Taken together, the data suggest that a subset of homeologous genes is susceptible to epigenetic modulation during polyploidization (see below).

It has been demonstrated that homeologous alleles for some loci, such as *AdhA* or *AdhD* in cotton allotetraploids (*Gossypium hirsutum* L.; Adams *et al.* 2003), show alternate expression patterns in different tissues, suggesting subfunctionalization (Lynch and Force 2000; Adams *et al.* 2003) of the homeologous genes after polyploidization. The timing of silencing establishment is hard to determine in that study, because the "new" allopolyploid cotton lines used were generated in the 1940s (Beasley 1940) and the number of subsequent generations is unknown. A similar phenomenon, called neo-functionalization, has also been observed for duplicate genes in ancient polyploid or paleo-polyploid species such as maize (Chandler *et al.* 1989; Mena *et al.* 1996). The R and B genes are derived from gene duplication events in maize and are involved in the same anthocyanin biosynthesis pathway. Some B alleles can substitute for R function, and only one of the two (B or R) is required for pigment production in a specific tissue (Chandler *et al.* 1989). We find that a few genes displayed different expression patterns in leaves and flower buds, which may be subjected to tissue-specific and/or developmental regulation (Chen and Pikaard 1997b; Adams *et al.* 2003).

How is gene silencing or activation established and maintained? Although molecules, factors, and/or signals involved in establishing an expression status of homeologous genes remain elusive, differential regulation of homeologous genes in polyploids may be a response to the genomic stress induced by two divergent genomes in the same cell nuclei. We speculate that initial signals triggered by homeologous genome interactions include species-specific factors and/or sequences, DNA repair and recombination, and/or RNA-mediated mechanisms. *RAD54* overexpression is associated with newly formed autotetraploids and allotetraploids, which may provide a repair mechanism for correcting intra- and intergenomic exchanges induced in new polyploids. RNA-mediated gene regulation is related to dosage-dependent transgene expression in Drosophila (Pal-Bhadra *et al.* 2002) and Arabidopsis (Mittelsten Scheid *et al.* 1996). It is conceivable that a similar mechanism may be involved in the control of endogenous duplicate or homeologous genes in polyploids. Moreover, RNA-mediated process is involved in DNA methylation and chromatin modifications (Mette *et al.* 2000; Matzke *et al.* 2001), which may be intervened by species-specific factors and DNA sequences from the two related species that diverged 5.8 million years ago (Koch *et al.* 2000).

DNA methylation and histone modifications serve as epigenetic marks in the maintenance mechanism for the silenced genes in allopolyploids (Chen and Pikaard 1997a; Lee and Chen 2001; Lawrence *et al.* 2004). Chemical inhibitors for DNA methyltransferases or histone deacetylases derepress the silenced rRNA genes (Chen and Pikaard 1997a; Lawrence *et al.* 2004), protein-coding genes (Lee and Chen 2001), and transposons (Madlung *et al.* 2002). However, chemicals such as aza-dC are known to have toxic effects on cellular development (Haaf 1995). It is difficult to discern causal effects using chemical inhibitor assays. Here we have generated loss-of-function *A. suecica* lines in *DDM1* and *MET1* using seed-induced callus transformation and RNAi. The "dominant negative" strategy is especially useful for silencing endogenous duplicate genes in polyploids (Lawrence and Pikaard 2003) because genetic redundancy in polyploids makes it impractical to produce recessive mutations for two homeologous loci. In this study, overexpressing double-stranded RNA corresponding to a target gene (*DDM1* or *MET1*) caused a dramatic reduction in the expression of *DDM1* or *MET1* and loss of DNA methylation in centromeric and some specific loci. As a result, the silenced genes (*RAD54* and *PP1*) are reactivated in the *ddm1*- and *met1*-RNAi lines. However, three of five silenced genes tested remain repressed in *ddm1*- and *met1*-RNAi lines, suggesting that silencing of endogenous duplicate genes is independent of DNA methylation. Moreover, the degree of reactivation varies in *ddm1*- and *met1*-RNAi lines, which probably correlates with the variability of downregulating endogenous target genes in the RNAi lines. Alternatively, *DDM1* and *MET1* may have slightly different effects on silenced homeologous loci. Indeed, *ddm1* is considered to be an epi-mutator that induces other epigenetic lesions maintained in the absence of mutations in *ddm1* (Soppe *et al.* 2000; Stokes *et al.* 2002; Stokes and Richards 2002). It will be interesting to know how DNA methylation affects the silencing of a subset set of genes in RNAi lines and in successive selfing generations of new allotetraploids.

Differential epigenetic modifications of homeologous genes in polyploids may play an important yet unrecognized evolutionary role. A mechanism for rapid and stochastic establishment of genome-specific gene expression may control the expression of duplicate genes in polyploids, leading to natural variation and evolutionary opportunities for adaptive selection and domestication (Grant 1981; Ramsey and Schemske 1998; Wendel 2000). The homeologous genes can provide extra settings of gene control in response to changes in environmental cues and developmental programs, because the best combination of gene expression patterns may be selected. This is consistent with the "rheostat" model proposed for the function of multiple copies of *FLC* in Brassica compared to Arabidopsis (Michaels and Amasino 1999).

Alternatively, polyploidization may induce "genomic shock" as predicted by McClintock (1984), leading to extensive reprogramming of the genome with concur-

rent large-scale activation of transposons (McClintock 1984; Comai *et al.* 2003a). Although a few mobile elements were detected in new allotetraploids (Comai *et al.* 2000), reactivation of transposons was not commonly observed in this and previous studies (Lee and Chen 2001). However, extensive epigenetic modifications may be associated with the expression of genes involved in various regulatory networks in new polyploid cells. Answers to these questions should come from genome-wide expression analyses of new allotetraploid lines.

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