THE ROYAL SOCIETY

Published online 15 May 2003

Do the different parental 'heteromes' cause genomic shock in newly formed allopolyploids?

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Allopolyploidy, the joining of two parental genomes in a polyploid organism with diploid meiosis, is an important mechanism of reticulate evolution. While many successful long-established allopolyploids are known, those formed recently undergo an instability phase whose basis is now being characterized. We describe observations made with the *Arabidopsis* system that include phenotypic instability, gene silencing and activation, and methylation changes. We present a model based on the epigenetic destabilization of genomic repeats, which in the parents are heterochromatinized and suppressed. We hypothesize that loss of epigenetic suppression of these sequences, here defined as the heterome, results in genomic instability including silencing of single-copy genes.

Keywords: genome stability; hybridization; polyploidy; DNA methylation

1. INTRODUCTION

Interspecific hybrids between distant relatives are usually sterile. In 1917, a botanist named Winge speculated that speciation could occur by interspecific hybridization followed by chromosome doubling (Winge 1917). Winge believed that hybrid sterility was caused by unbalanced chromosome sets. He reasoned that upon doubling, a proper pairing partner would be available to each chromosome resulting in fertility. Since then, this prediction has been experimentally verified many times. In addition, allopolyploids, hybrid species that contain two or more diploid sets of parental genomes, are common in nature (Soltis & Soltis 1993; Leitch & Bennett 1997; Rieseberg 2001). Other 'diploid' species, such as maize, display the unmistakable footprint of a diploidized allotetraploid genome (Gaut *et al.* 2000). Therefore, allopolyploidy is an important mechanism of reticulate evolution.

Allopolyploids are a special type of hybrid. The two parental genomes in an allopolyploid, defined as homeologous, undergo limited intergenomic recombination and thus maintain their integrity through sexual generations. Therefore, karyotypic stability and, probably, heterosis are achieved at the expense of the evolutionary flexibility provided by unhindered recombination of the parental genomes (Comai 2000; Rieseberg 2001). By contrast, diploid hybrids recombine the two parental genomes, generating progeny with countless combinations of parental chromosomal segments, which can be fixed by selection (Ungerer *et al.* 1998). The forced maintenance of both parental genomes should limit the evolutionary flexibility of allopolyploid hybrids, but their ubiquitous distribution implies otherwise.

Artificially made allopolyploids are called synthetic and typically they lack the phenotypic and genotypic stability of established allopolyploids (Soltis & Soltis 1995; Pikaard 1999; Comai 2000). These differences are difficult to explain by classical genetic rules. Presumably, the instabilities that are manifested by recently formed allopolyploids are mitigated in the process of evolutionary adaptation that gives rise to stable species. But, the steps through which adaptation proceeds remain obscure. Barbara McClintock believed that genomic incompatibilities unmasked by interspecific hybridization are among the causes of genomic shock, a preprogrammed response to stress (McClintock 1984). More specifically, she pointed to the difference in repeated elements between the two parental genomes as a cause of genomic shock, suggesting that hybridization 'initiated mobilities of these elements'.

Since McClintock's insight, the understanding of transposons has increased greatly. It is now recognized that the many repeats in eukaryotic genomes arose by transposition and that these suppressed repeats form the bulk of heterochromatin. The maintenance of heterochromatin in a suppressed status depends on the proper functioning of silencing pathways (Volpe *et al.* 2002). We propose the name 'heterome' for the DNA elements that are part of heterochromatin, and the term 'heteromics' for their study. A major point of this review is the discussion of the role that parental heteromes might play in the outcome of allopolyploidization.

2. STUDIES ON SYNTHETIC ALLOPOLYPLOIDS

We are using an allopolyploid system based on the model plant *Arabidopsis* to explore the role of genetic and epigenetic regulation. *Arabidopsis suecica* ($2n = 2x = 26$) is a natural allopolyploid whose maternal parent is *A. thaliana* ($2n = 2x = 10$) and whose paternal parent is probably

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One contribution of 21 to a Discussion Meeting Issue 'Mechanisms regulating gene flow in flowering plants'.

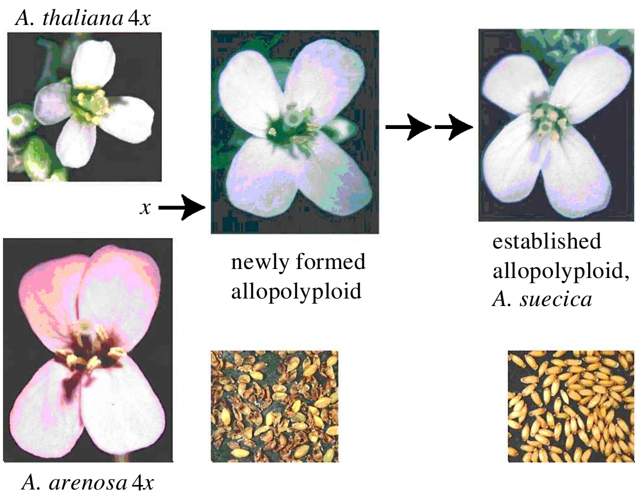

Figure 1. Formation of *Arabidopsis* allotetraploids.

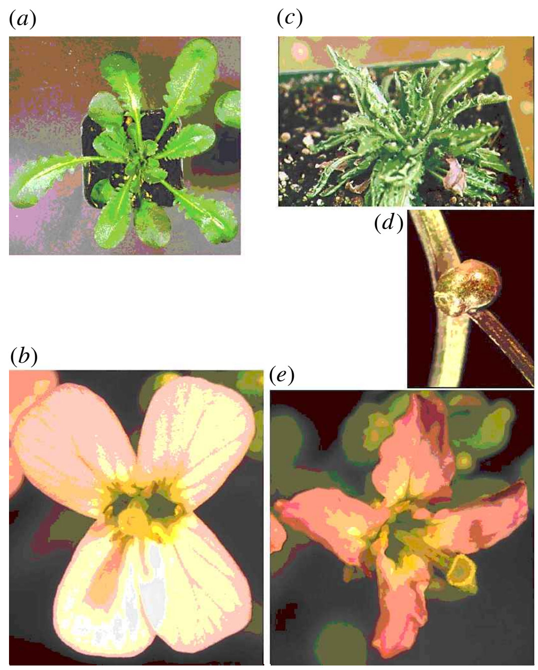

Figure 2. Abnormal phenotypic responses to azadC treatment. (*a*), (*b*) Controls (no azadC); (*c*), (*d*) and (*e*) azadC-treated.

A. arenosa (also known as *Cardaminopsis arenosa*, $2n = 2x,4x = 16,32$ (Hylander 1957; Loeve 1961)), as demonstrated by molecular analyses (Mummenhoff & Hurka 1995; O'Kane *et al.* 1996). The chromosomal make-up of *A. suecica* has been ascertained by FISH to consist of 10 chromosomes that hybridize to the *A. thaliana* 180 bp centromeric repeat and 16 chromosomes that hybridize to the *A. arenosa* 180 bp centromeric repeat (Kamm *et al.* 1995). To compare this established species with a newly generated one, we produced synthetic *A. suecica*-like allopolyploids (figure 1; Comai *et al.* 2000). These allopolyploids were characterized for phenotype, chromosomal number, gene expression and DNA methylation pattern at randomly selected loci. These analyses demonstrated epigenetic gene silencing (Comai *et al.* 2000; Madlung *et al.* 2002), which is also found in *A. suecica* (Lee & Chen 2001). Unexpected phenotypes (Schranz & Osborn 2000) and widespread changes in gene expression have been documented in other synthetic allopolyploids (Kashkush *et al.* 2002). Less is known about genomic instability and DNA rearrangements. Although synthetic hybrids of wheat display frequent loss of DNA sequences (Ozkan & Feldman 2001; Shaked *et al.* 2001), similar widespread losses have not been documented in other allopolyploids (Liu *et al.* 2001). Below, we use our own data with *Arabidopsis* synthetic allopolyploids to exemplify the results on phenotypic, transcriptional and genomic changes.

(a) *Changes in phenotype*

The phenotypic instability of the synthetic *Arabidopsis* allopolyploids is manifested by sterility, embryonic lethality and wide variation in phenotypes even within the same plant (Comai *et al.* 2000). Surprisingly, this phenotypic instability was greatly increased by treatment with the demethylating agent azadC. Diploid and tetraploid *A. thaliana* and *A. suecica* treated with azadC grew normally, *A. arenosa* displayed relatively less frequent (22% of the treated individuals) and less severe abnormalities, whereas the allotetraploids consistently produced greatly abnormal phenotypes (60–90%, depending on the line; see figure 2 (Madlung *et al.* 2002)). Some of the displayed abnormalities were also seen in untreated allotetraploids but at much reduced severity. The most common abnormality was a semi-dwarf phenotype that displayed many secondary inflorescences with shorter and zigzag internodes. Other frequent abnormalities included fasciation of the shoots and homeotic transformations of the flowers, including dipetalous and tetrapetalous phylloid flowers, *apetala*-like flowers, open carpels or cauliflower-like inflorescences. Abnormal and normal body sectors sometimes appeared on the same plants. Other examples of phenotypic abnormalities included dwarfism, aberrant branching patterns and tumour formation. Often these phenotypes affected only lateral inflorescences and showed occasional reversion during branching or further apical growth. In certain cases, abnormalities changed in intensity along the axis of growth (Madlung *et al.* 2002).

Although azadC does not induce instability in *A. thaliana*, mutations that more profoundly affect chromatin regulation, such as *ddm1* and antisense-*MET1* transgenes, cause comparable abnormalities. These abnormalities have been attributed to the silencing of genes controlling development, such as the *SUPERMAN* gene, whose silencing was shown to be associated with hypermethylation (Jacobsen & Meyerowitz 1997).

(b) *Changes in gene expression*

We compared gene expression in the allopolyploids and in the parents. We used lines with the same ploidy: the parents were autotetraploid, whereas the progeny were allotetraploid. We employed AFLP-cDNA analysis, a PCR-based method that displays random restriction enzyme fragments of cDNA on denaturing polyacrylamide gels. The analyses revealed several products that, although present in the parents and in an artificial reconstruction of hybrid mRNA, were absent in the F_2 hybrids (Madlung *et al.* 2002). In a first study (Comai *et al.* 2000), by RT-PCR analyses we confirmed the silencing of three genes out of about 20 differential AFLP-cDNA candidates

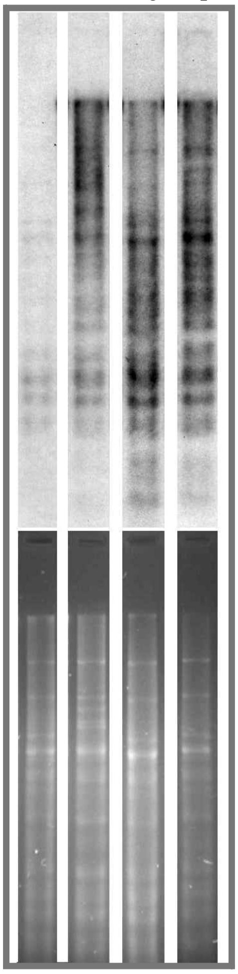

Figure 3. Changes in methylation and copy number of the K7 repeat. The silenced sequence K7, which is related to transposons, was used as a probe in a Southern blot analysis of parental and allopolyploid DNA. Top, hybridization of K7 probe; bottom, gel with stained MspI-digested DNA. At, *Arabidopsis thaliana*; Aa, *A. arenosa*; allos, allopolyploid F_1 and its F_2 progeny. The shift to lower molecular weight fragments in the F_1 indicates a loss of cytosine methylation. Note that methylation is partially regained in the F_2 concomitantly with an increase in copy number of the K7 element.

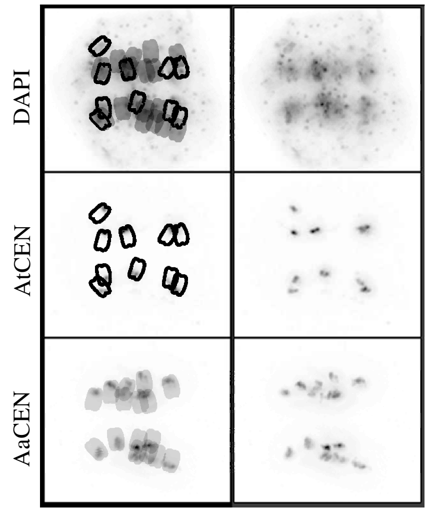

Figure 4. Meiotic anaphase I karyotype of allopolyploid *Arabidopsis*. Ten *A. thaliana* chromosomes (stained by the AtCEN probe) and 16 *A. arenosa* chromosomes (stained with the AaCEN probe) are present in these allopolyploids. In addition, the position of the separated bivalents (At- and AaCEN signals are arranged symmetrically) indicates homologous pairing. The drawing provides a schematic interpretation of the chromosome positions.

examined (*ca.* 1% of the total genes). Later studies confirmed this level of silencing. BLASTN analyses and Southern blotting revealed that two of these genes contain repeated DNA. In the case of a gene called K7, the repeated element was determined to be a 'solo LTR', a retrotransposon-related element. Interestingly, comparison of cytosine methylation in these families of LTRs by HpaII and MspI restriction digestion revealed decreased methylation at 5′-cytosine-any nucleotide-guanine-3′ sites in the allopolyploids (figure 3).

Was the azadC-caused phenotypic instability associated with increased changes in gene expression? We profiled azadC-treated allopolyploids and found that 3% of their genes were silenced. By contrast, no silencing was observed when the treated and untreated *A. thaliana* plants were compared (Madlung *et al.* 2002). Therefore, azadC increases both phenotypic end-expression instability, suggesting a relationship between the two.

Are changes always in the silencing direction? mRNA profiling of untreated allopolyploids suggested that about 1 out of 10 changes is an activation. In collaboration with the laboratories of Rob Martienssen at Cold Spring Harbor Laboratory and of Jeff Chen at Texas A&M University, we are using genome-tiled microarrays (representing both genic and intergenic regions) and gene-based oligonucleotide microarrays (representing known or putative genes) to address this question. Preliminary results suggest that some of the activated transcripts correspond to transposons that were silenced or expressed at low levels in the parents and became highly expressed in the allopolyploids.

A major symptom of instability is the frequent failure of pollen and embryos in the allopolyploids. Although variable, all lines display significant levels of infertility and inviability. Embryonic inviability is manifested already in the F_1 zygotes, of which only 5% form viable seeds. We have demonstrated by FISH with species-specific centromeric probes that the majority of meioses involve proper homologous pairing of the chromosomes (figure 4; Comai *et al.* 2003). We are probing this system for abnormal expression. Our results indicate that certain retroelements are activated in the embryo or endosperm of the F_1 progeny (C. Josefsson, unpublished data).

(c) *Changes in DNA methylation*

To test the hypothesis that allotetraploidization results in partial loss of epigenetic gene regulation affecting genome methylation and gene expression, we investigated methylation patterns of untreated allopolyploids. We found no gross changes between parents and F_3 allotetraploids in overall 5′-cytosine-guanine-3′ DNA methylation at *TaqI* restriction sites (Madlung *et al.* 2002). To sample

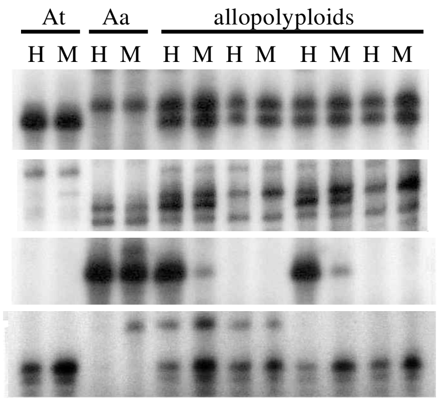

Figure 5. Alteration of DNA methylation detected by MSAP profiling. Changes in DNA methylation are displayed by the amplification of restriction fragments generated by digestion with the methylation sensitive isoschizomeric (ccgg) enzymes HpaII (H, c<u>c</u>gg) and MspI (M, <u>c</u>cgg; sensitive methylated is underlined). At, *Arabidopsis thaliana*; Aa, *A. arenosa*.

defined sequence sites for methylation changes we employed MSAP analysis, which revealed frequent changes in F_4 allotetraploids, involving both increases and decreases in methylation but no overall hyper- or hypomethylation. Out of the 623 products scored, 52 (8%) showed polymorphic methylation patterns between the parents and the F_3 progeny. Of these, 40 were invariable across the four F_3 individuals analysed, while 12 varied in pattern between the allotetraploids. Depending on their status in the progeny, the first 40 were classified into either a demethylation or a hypermethylation group. Twenty-five out of 40 (62%) underwent demethylation, while 15 out of 40 (37%) displayed an increase in methylation levels (figure 5; Madlung *et al.* 2002). These results indicate that allopolyploidization is accompanied by frequent changes in epigenetic imprints.

Interestingly, many of the changes in methylation state were observed in different individual siblings, indicating that at least some methylation changes were either inherited or were locus-specific. Close to half of the sequenced differential MSAP DNA fragments did not have a match in the sequence databases: they could represent heterochromatic regions that either have not been sequenced or that are unique to *A. arenosa*. These results indicate that a considerable fraction of the genome undergoes epigenetic remodelling. Nevertheless, there are fewer alterations than with epigenetic mutants of *Arabidopsis*, such as *ddm1*. The application of microarray analysis to DNA methylation has been demonstrated using probes that represent methylated and unmethylated DNA fractions (Van Steensel *et al.* 2001). We are now using this tool in collaboration with the Rob Martienssen laboratory (Cold Spring Harbor Laboratory) to survey the dynamics of methylation changes on a genome-wide scale.

(d) *Changes in genome structure*

We are exploring the effect of allopolyploidization on genome structure using cytology and molecular markers analysis. Our preliminary results indicate that rearrangements can occur in certain lineages. The nature of these rearrangements is being investigated and some cases appear to be consistent with DNA transposition.

3. MECHANISMS

A critical survey of the mechanisms that may play a regulatory part in auto- and allopolyploids was recently published by Osborn *et al.* (2003). Here, we consider a subsest of these possible mechanisms: the effect of mismatched parental contributions on chromatin regulation and the incompatibility of different heteromes. The models presented here are a reinterpretation, one genetic and the other epigenetic, of a mechanism proposed by Dobzhansky to explain hybrid inviability (Dobzhansky 1937; Orr & Presgraves 2000). According to Dobzhansky, while certain genes are neutral or advantageous in their homologous species, they are deleterious in a hybrid genetic background due to the accumulation of incompatible features since divergence of the two taxa.

(a) *Mismatched, divergent subunit of complexes*

Interacting proteins have regions that precisely fit each other and must evolve coordinately. Upon speciation, orthologous proteins diverge in synchrony with the interacting partners. Upon hybridization, mismatched proteins must interact and may function aberrantly because even slight alterations in the structure of regulatory complexes may impair them. A deficiency in recombination, chromatin remodelling, repression of transposons or DNA methylation could have severe consequences.

Loss of nuclear homeostasis may cause fluctuations of certain regulatory components in threshold-sensitive pathways and generate phenotypes that appear or are epigenetic. In *Drosophila* and *Arabidopsis*, impairment of HSP90, a chaperone that interacts with many proteins, released cryptic phenotypic variation affecting development (Rutherford & Lindquist 1998; Queitsch *et al.* 2002). This finding indicates that HSP90 functions as a cellular capacitor to suppress variation. Surprisingly, in *Drosphila*, loss of HSP90 capacitor function resulted in epigenetic resetting of chromatin (Sollars *et al.* 2002). Similar malfunctions may be triggered by hybridization as in the case of the loss of DNA methylation in marsupial hybrids, which was accompanied by a burst of retrotransposition (O'Neill *et al.* 1998).

There is no evidence for the existence of speciation-derived incompatibilities in chromatin and gene regulatory proteins. Rapid divergence between orthologous proteins in *A. thaliana* and *A. arenosa* has been demonstrated by characterization of the centromeric histone H3 HTR12 (Talbert *et al.* 2002). This chromatin protein displays dramatic divergence in the aminoterminal region indicative of adaptive evolution. The two forms of this protein, however, coexist in *Arabidopsis* allopolyploids and may form dimers. Because of the recent and ongoing discovery of chromatin regulatory factors, a comprehensive examination of their evolution during speciation is not yet available. Therefore, we cannot rule out the possibility that some proteins of this type may be subject to adaptive evolution resulting in interspecific incompatibilities.

(b) *Epigenetic interactions: homology-dependent gene silencing*

Differences in gene regulation are thought to be a major source of incompatibilities between species. The establishment of different silencing patterns could play a major part

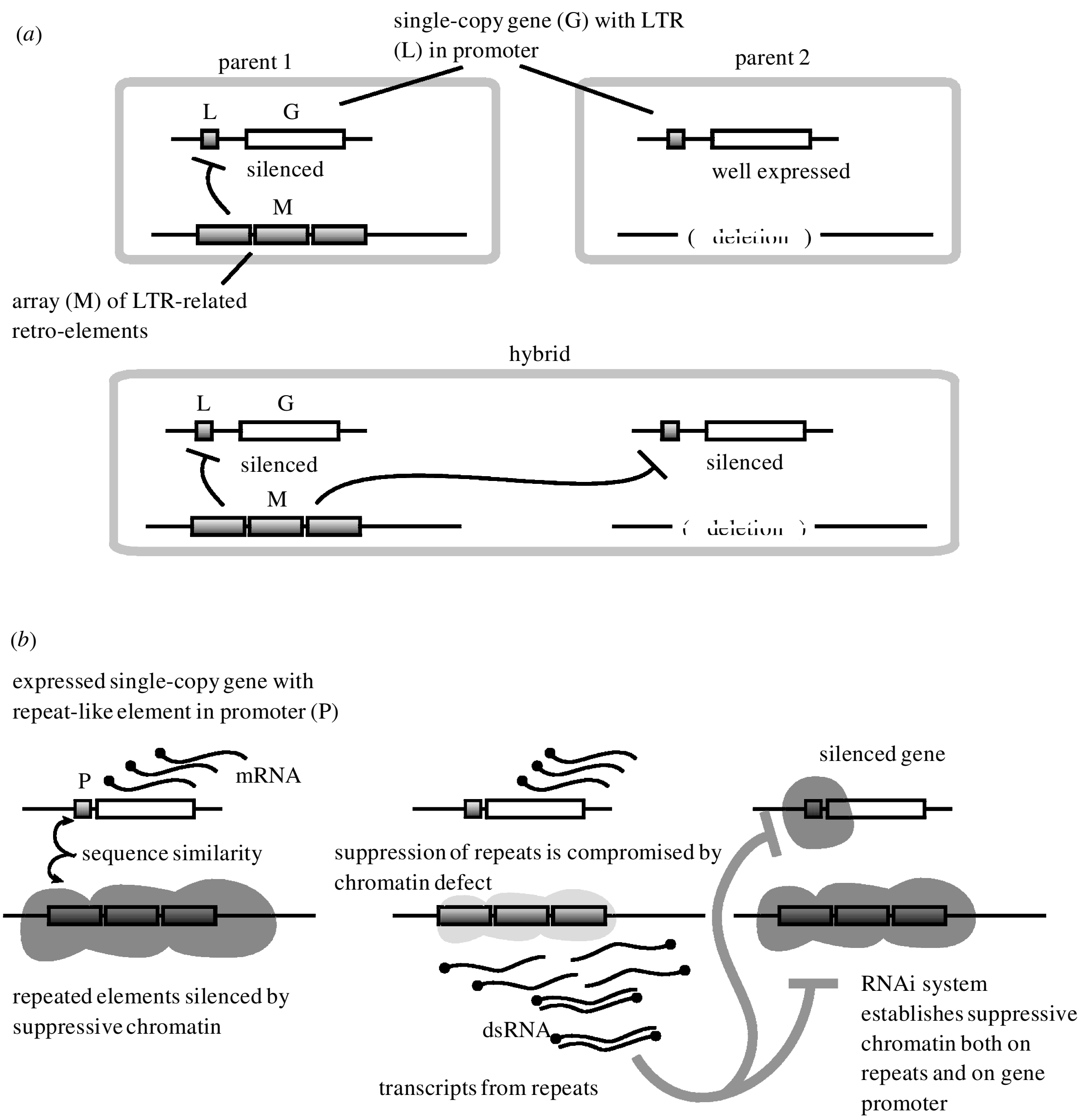

Figure 6. Models for gene silencing. (*a*) Polymorphism of parental species for silencing repeats leads to silencing in the hybrid of a gene that was active in one of the parents. (*b*) Activation of heterochromatic repeats. A well-expressed gene contains a promoter element with sequence similar to that of repressed heterochromatic repeats. The repeats are activated by a genomic shock leading to production of RNA and, possibly, transposition. Transcription of the repeats forms dsRNA, triggering the RNAi genome defence that leads to silencing of the repeats, but also to silencing of the cognate gene. Although the illustration depicts a case of transcriptional silencing by repressive chromatin, post-transcriptional silencing could also be involved.

in species differences in gene regulation. Gene silencing involves the suppression of one gene by another homologous gene (Vaucheret *et al.* 2001; Matzke *et al.* 2002). Genes that have silencing properties are usually repeated. At least two mechanisms of gene silencing have been described; one transcriptional, often associated with methylation of promoter sequences, and one post-transcriptional, associated with the sensing of dsRNAs and the activation of the ancient RNAi regulatory pathway leading to RNA turnover or translational inhibition. However, the distinction between these two mechanisms is becoming blurred (Pal-Bhadra *et al.* 2002), dsRNA can cause methylation of promoters (Mette *et al.* 2000) and trigger the heterochromatization of centromeric repeats (Volpe *et al.* 2002). In the latter case, the heterochromatic state of fission yeast centromeric repeat was shown to depend on the RNAi pathway, suggesting the repeats may trigger their own heterochromatization by the production of dsRNA. Thus, production of dsRNA by one cluster of repeated elements can silence isolated cognate elements dispersed through the genome.

Gene silencing could have a significant impact on genome regulation of interspecies hybrids. Different species form different genomic arrangements through evolution. Diverged genomes with different silencing and expression patterns may exhibit incompatibilities when joined. For example, transposons inserted next to genes can alter their expression pattern (Martienssen 1998; Hamdi *et al.* 2000; Kashkush *et al.* 2003). The regulatory novelty provided by the transposon can be recruited to build promoters with new advantageous specificities. For example, Alu repeats and retrotransposon LTRs are commonly associated with cellular genes and are thought to contribute regulatory variety (Wessler *et al.* 1995; McDonald *et al.* 1997;

Hamdi *et al.* 2000). In different species, different elements may become free or subject to silencing through random variation and selection of favourable states.

The mechanics of these incompatibilities can be illustrated with the following example (figure 6*a*). Consider the hypothetical case of a LTR (L) inserted next to a 'cellular' gene (G). An unlinked locus (M) with multiple copies of the cognate full-length retrotransposon may exert a silencing action on L and cause it to become heterochromatic, silencing G. In some progeny, M may be deleted, reactivating G and causing L to become a new regulatory element. These lineages may diverge further and eventually speciate. The different states of the L–G system would conflict if the two species formed a hybrid. The M locus in one species genome would probably exert its silencing effect on both L homeologous loci, silencing the one that was active and the associated G gene. A similar situation (although not involving LTRs) was described for the PAI genes in *Arabidopsis* accessions (Melquist *et al.* 1999). Widespread silencing may occur when transposons become activated in newly formed allopolyploids: the connected plant defence response that attempts to silence transposons may be responsible for single-copy gene silencing (figure 6*b*; see also Kashkush *et al.* 2003). The susceptibility of single-copy genes to silencing mechanisms targeted against transposons would stem from the presence of sequences similar to transposons within the genes themselves.

Evidence consistent with the above model is emerging: we and others have demonstrated frequent gene silencing and chromatin remodelling in newly made allopolyploids (Comai *et al.* 2000; Ozkan *et al.* 2001; Kashkush *et al.* 2002; Madlung *et al.* 2002) as well as transposon activation (C. Josefsson and L. Comai, unpublished data; Kashkush *et al.* 2003; Madlung *et al.* 2003). Furthermore, by microarray analysis we have documented frequent differences in gene expression between the *Arabidopsis* species that are the allopolyploid parents.

4. CONCLUSION

Allopolyploids are the main conduit of reticulate evolution in angiosperms, accomplishing horizontal gene flow through the combination of two diverged genomes in one nucleus. The study of *Arabidopsis* and other human-made allopolyploids indicates that the bottleneck of instability associated with polyploidization has a major epigenetic component. Elucidating the molecular mechanisms affecting allopolyploidization will not only help us to understand this important evolutionary pathway but will also shed light on how genomes balance their euchromatic component, the genes, with their heterochromatic component, the repeats.

This work was supported in part by a USDA-NRICGP grant to L.C. and by a grant from the National Science Foundation Plant Genome Research Programme (NSF 0077774). We thank Paul Talbert for help with the Delta Vision microscope and stimulating discussions, and our collaborators in the NSF project 'Functional Genomics of Plant Polyploids' for stimulating discussions and comments. We gratefully acknowledge the help of Steve Reynolds with plant growth and care.

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Discussion

Z. J. Chen (*Department of Soil and Crop Science, Texas A&M University, TX, USA*). Were the methylation differences observed in the knob region between *A. thaliana* and *C. cuenosa*, resulting from knob sequence divergence (i.e. hybridization ability difference)?

L. Comai. It is a possibility. However, the probes are methylated versus unmethylated DNA and we measured the ratio of hybridization.

GLOSSARY

AFLP: amplified fragment length polymorphism
azadC: 5-aza-2-deoxycytidine
BLASTN: basic local alignment search tool—nucleotide
dsRNA: double-stranded RNA
FISH: fluorescent *in situ* hybridization
LTR: long terminal repeat
MSAP: methylation sensitive amplified polymorphism
RNAi: RNA interference