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Biological relevance of polyploidy: ecology to genomics
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Exploring the genomic mysteries of polyploidy in cotton

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For several years allopolyploid cottons have been the subject of evolutionary investigations into the genomic mysteries of polyploidy. An array of genomic interactions have been documented, including interlocus concerted evolution, differential rates of genomic evolution and intergenomic sequence transfer. Substantial alterations in gene expression have occurred in response to allopolyploidization, including gene silencing and expression changes that vary by organ. Some of the molecular phenomena occurring in polyploids appear to be non-Mendelian. Many of the genomic and expression alterations have occurred on an evolutionary timescale, whereas others reflect more immediate consequences of genomic merger. © 2004 The Linnean Society of London, *Biological Journal of the Linnean Society,* 2004, **82**, 573–581.

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INTRODUCTION

Because polyploidy has been so central to plant evolution, it is important to understand the suite of molecular evolutionary phenomena associated with genome doubling as well as any functional consequences thereof. Toward this end the use of model plant systems is particularly valuable, especially to distinguish processes that might characterize the earliest stages of polyploid formation from those that are responsible for longer-term evolutionary change. One of the best models in this respect is in the cotton genus (*Gossypium*), which provides one of the textbook examples of allopolyploid speciation. Here we present a synopsis of recent efforts to understand the allopolyploid genome of *Gossypium*, with a focus on the evolutionary consequences of gene and genome doubling, and gene expression alterations that accompany or follow genome merger.

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ORIGIN OF ALLOPOLYPLOID COTTON AND BIOGEOGRAPHICAL DISTRIBUTION

As documented by extensive molecular systematic investigations, the origin of allopolyploid *Gossypium* (Fig. 1) traces to the mid-Pleistocene, about 1.5 Mya (Wendel, 1989; Cronn *et al*., 2002; Senchina *et al*., 2003; Wendel & Cronn, 2003), following a remarkable *trans*-oceanic dispersal of an Asiatic 'A-genome' species to the New World. The A-genome species hybridized with a native New World, 'D-genome' diploid, with the D-genome serving as the paternal parent and the A-genome serving as the maternal parent (Wendel, 1989; Small & Wendel, 1999). Evidence indicates that *G. raimondii* is the closest living relative of the D-genome donor, whereas the two extant A-genome species, *G. arboreum* and *G. herbaceum*, are phylogenetically sister to each other and hence equidistant from the A genome of allopolyploid cotton (Endrizzi, Turcotte & Kohel, 1985; Wendel & Cronn, 2003). Thus, the actual parents of the allopolyploids are extinct, and reference to their parentage is more appropriately framed in terms of closest living descendants of the donor species.

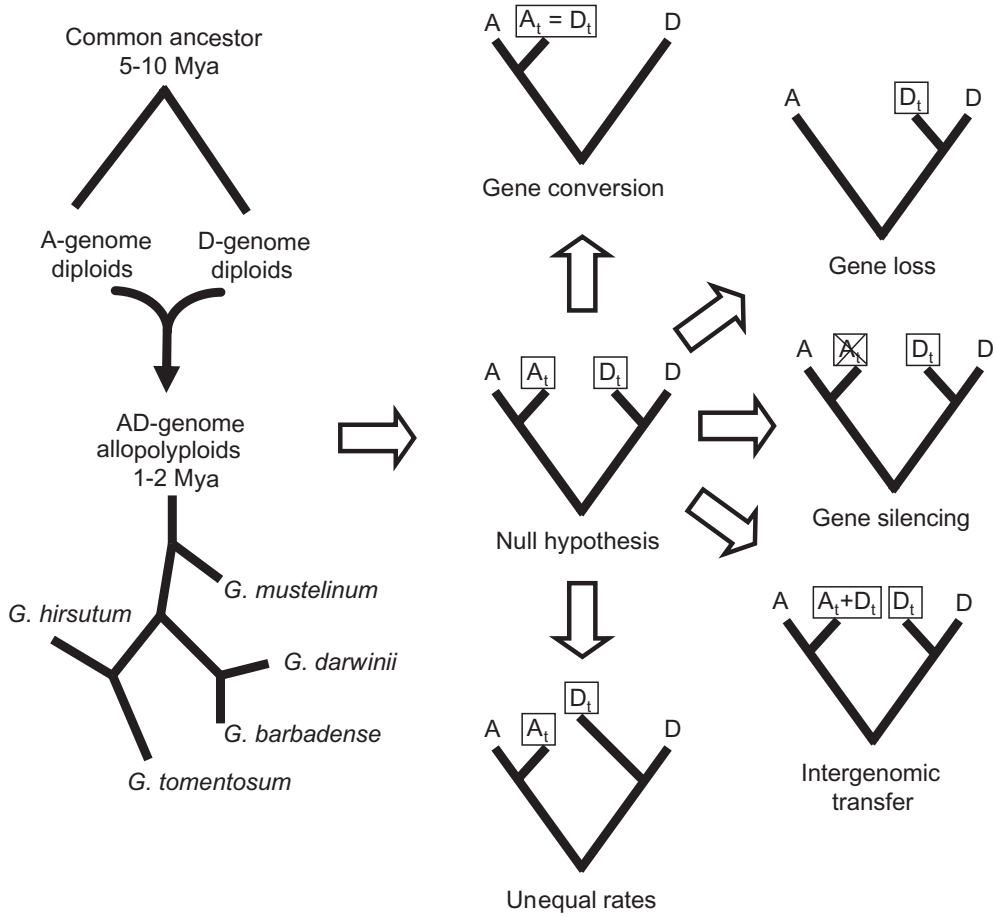

Figure 1. Phylogenetic history of diploid and allopolyploid *Gossypium* species (left) and various possibilities for subsequent gene and genome evolution (right). Allopolyploid cottons formed in the Americas approximately 1.5 Mya between an A-genome diploid and a D-genome diploid. At formation, allopolyploids are expected to have duplicated copies (A_t and D_t) of most single-copy and low-copy genes and duplicated suites of repetitive DNAs. In the absence of mutation or selection, homoeologous copies are expected to evolve at equivalent rates and independently of one another, such that they are phylogenetically sister to their counterparts from the progenitor diploids rather than to each other (centre). This expectation provides a convenient null hypothesis for diagnosing molecular evolutionary phenomena that accompany genome doubling, such as gene conversion, accelerated evolutionary rates, transfer of sequences between genomes, and gene silencing or gene loss.

Following polyploidization, there was subsequent radiation into five recognized species: *G. hirsutum* (Upland cotton), *G. barbadense* (Pima cotton, Sea Island cotton), *G. darwinii*, *G. tomentosum* and *G. mustelinum* (Fig. 1; Wendel, Rowley & Stewart, 1994). *Gossypium hirsutum* presently is responsible for over 90% of the cotton crop internationally, having spread from its original home in Mesoamerica to over 50 countries in both hemispheres (Brubaker & Wendel, 1994; Brubaker, Bourland & Wendel, 1999).

Allopolyploidy in *Gossypium* led to the establishment of a new and successful clade as well as the apparent invasion of a new ecological niche. In contrast to the majority of diploid species within this genus, allopolyploid species typically occur in coastal habitats, at least those forms that arguably are truly wild (see also Brubaker & Wendel, 1994). Thus, among the five allopolyploid species, two are completely restricted to near coastlines, in that they are island endemics (*G. darwinii* from the Galapagos Islands and *G. tomentosum* from the Hawaiian Islands), and for two others (*G. barbadense* and *G. hirsutum*), wild forms occur in littoral habitats ringing the Gulf of Mexico, north-west South America and even distant Pacific Islands. This ecological innovation is envisaged to have not only permitted the initial establishment of the nascent polyploid lineage, but is also suggested to have provided a means for the rapid dispersal of the salt-water-tolerant seeds. By this means, perhaps, the mobile shorelines of the Pleistocene facilitated exploi-

Table 1. Molecular phenomena that characterize duplicate gene evolution in allopolyploid *Gossypium*

Molecular phenomenon	References
Interlocus concerted evolution	Wendel *et al*. (1995)
Independent sequence evolution	Cronn *et al*. (1999), Senchina *et al*. (2003)
Unequal rates of sequence evolution	Small *et al*. (1999a), Liu *et al*. (2001a), Small & Wendel (2002)
Accelerated rates of sequence evolution	Small *et al*. (1998)
Gene silencing	Adams *et al*. (2003)
Biased expression	Adams *et al*. (2003)
Organ-specific differences in expression	Adams *et al*. (2003)

tation of a new ecological niche, and hence colonization of the New World tropics. Polyploidy has been proposed to have been a driving force in the postglacial colonization of the arctic (Brochmann *et al*., 2004 – this issue).

GENOMIC INTERACTIONS

Polyploid cottons show a diversity of genomic responses to genome doubling, including interactions between the two genomes in the polyploid nucleus. Clues to the nature of these interactions are evidenced in the results of several recent investigations (Table 1). In a study of experimental backcross populations between *G. hirsutum* and *G. barbadense*, Jiang *et al*. (2000) noted large deficiencies of donor parent (*G. barbadense*) transmission for some chromosomal regions. They attributed this to epistatic interactions affecting chromatin transmission, a high proportion of which were caused by interactions between alleles contributed by the two genomes. Other data show that dispersed repetitive elements have become mobilized as a consequence of polyploidization in cotton, possibly leading to novel regulatory changes or gene functions. The studies of Zhao *et al*. (1998) and Hanson *et al*. (1998) are noteworthy in this respect; using fluorescent *in situ* hybridization, they showed that dispersed repetitive sequences that are A-genome-specific at the diploid level have colonized the D-genome in natural *Gossypium* polyploids. Similarly, Hanson *et al*. (2000) showed that a family of *copia*-like retrotransposable elements 'horizontally' transferred across genomes following allopolyploid formation. These and other studies highlight the possibility of transposable element spread across genomes following polyploid formation and raise the possibility that this process has played a role in diversification and adaptation.

In *Gossypium* these intergenomic interactions appear to arise on an evolutionary timescale as opposed to being an immediate consequence of hybridization and polyploidization. Liu *et al*. (2001a) used AFLP analysis to evaluate the extent of fragment additivity in nine sets of newly synthesized allotetraploid and allohexaploid *Gossypium*. Approximately 22 000 genomic loci were examined, yet fragment additivity was observed in nearly all cases, even when methylation-sensitive and -insensitive isoschizomers were used. These indications of genomic additivity and epigenetic stasis during allopolyploid formation provide a contrast to recent evidence from several synthetic plant allopolyploids, most notably wheat (reviewed in Levy & Feldman, 2004 – this issue), *Brassica* (Song *et al*., 1995 and Lukens *et al*., 2004; Pires *et al*., 2004 – both this issue) and an *Arabidopsis* polyploid (Madlung *et al*., 2002), in which rapid and unexplained genomic changes, as well as methylation changes, have been reported. In addition, the data contrast with the foregoing account of repetitive DNAs in natural *Gossypium* allopolyploids, some of which are subject to non-Mendelian molecular evolutionary phenomena such as interlocus concerted evolution and intergenomic colonization. Collectively, these and other recent studies have drawn attention to the 'dynamic' nature (Soltis & Soltis, 1995) of polyploids, and emphasized the relatively poorly understood and sometimes non-Mendelian mechanisms that may characterize gene and genome evolution in polyploids.

EVOLUTION OF GENES DUPLICATED BY POLYPLOIDY

The most immediate and important genomic consequence of allopolyploid formation in *Gossypium* was simultaneous duplication of all nuclear genes. Theory suggests at least four possible outcomes of gene duplications. Duplicated genes may maintain their original function. Alternatively, relaxation of selection could allow divergence between the duplicates and the acquisition of new function (Ohno, 1970; Ferris & Whitt, 1979; Li, 1985; Hughes, 1994; Hughes *et al*., 2000; Lynch & Conery, 2000; Lynch & Force, 2000) Indeed, polyploidization is widely perceived to provide the raw material for the origin of physiological, ecological and morphological novelty (Stebbins, 1950; Lewis, 1980; Grant, 1981; Levin, 1983; Barrier *et al*., 1999; Schranz & Osborn, 2000). Another alternative is that one member of the duplicated gene pair will become silenced and ultimately degenerate as a pseudogene (Lynch & Conery, 2000; Wendel, 2000). Recently a fourth possible fate has been proposed: aggregate function and/or expression pattern may be partitioned between the two duplicates, termed sub-

functionalization (Force *et al*., 1999; Lynch & Force, 2000).

From a phylogenetic perspective, these various fates of gene duplication may be partially modelled as shown in Figure 1. The null hypothesis for sequence evolution in allopolyploids derives from the organismal history; if both duplicated genes evolve independently and at equal rates following allopolyploid formation, then each homoeologue should be phylogenetically sister to its orthologue from a diploid cotton, rather than to the other homoeologue. Similarly, if rates of sequence evolution are similar at the diploid and allopolyploid level, branch lengths for the two A-genome sequences (one from the diploid, the other from the allopolyploid) should be similar, as they should for the two D-genome sequences (Fig. 1, centre). The utility of the null hypothesis lies in its falsification; if homoeologous sequences interact, for example (Fig. 1, top), a different tree may be recovered, or if there is strong directional selection or pseudogenization (Fig. 1, bottom), rate inequalities may become evident. Additional possibilities include silencing or loss of one of the duplicated copies (Fig. 1, top right and middle right) and transfer of sequences from one genome to the other (Fig. 1, bottom right).

Recently, there have been several tests of the null expectations of independence and rate equality in allopolyploid *Gossypium* as well as in other plants (reviewed in Wendel, 2000). Wendel, Schnabel & Seelanan (1995) demonstrated interaction among the 18S–26S ribosomal genes that exist at multiple loci in the A- and D-genomes (Ji *et al*., 1999). Specifically, instead of evolving independently, as expected if they were sequestered in separate genomes of diploid plants, repeats at the different loci in allopolyploid cotton become homogenized to the same sequence (either 'A-like' or 'D-like') by one or more processes of concerted evolution (reviewed by Elder & Turner, 1995). In four of the five allopolyploid species, interlocus homogenization has created exclusively D-genome-like rDNAs, whereas in *G. mustelinum* nearly all rDNA repeats have been homogenized to an A-like form. This example showed that since polyploid formation in the Pleistocene, some 3800 repeats, each approximately 10 kb in length, were 'overwritten' with the alternative form originating from the other parental genome, probably through unequal crossing over or gene conversion. Moreover, interlocus concerted evolution was bi-directional, operating in different directions in different allopolyploid lineages. Interlocus concerted evolution of rDNA has also been documented in other plants, including *Nicotiana* (Kovarik *et al*., 2004 – this issue) and *Glycine* (Doyle *et al*., 2004 – this issue). What mechanisms underlie rDNA interactions? Unequal crossing-over and gene conversion are likely candidates, and interchromosomal exchanges are probably facilitated by the near-telomeric location of the rDNAs in some plants including *Gossypium* (Wendel, 2000).

This demonstration that some repeated sequences could interact across genomes in the allopolyploid nucleus led to additional investigations of the scope of the phenomenon. In an analogous study, Cronn *et al*. (1996) showed that the duplicated arrays of tandemly repeated 5S rDNA genes are not homogenized by concerted evolutionary forces in the allopolyploid, in contrast to the 18S–26S arrays. Similarly, low-copy nuclear genes duplicated by allopolyploidy largely evolve independently of one another in the polyploid nucleus (Cronn, Small & Wendel, 1999; Senchina *et al*., 2003). To date, there has been no convincing demonstration of interlocus gene conversion for single-copy or low-copy nuclear genes in cotton.

DIFFERENTIAL EVOLUTION OF GENES IN COHABITING GENOMES

Following the union of two genomes into a single nucleus as a consequence of allopolyploidization it is expected that over time some genes will become pseudogenes whereas others may diverge and acquire new function, as discussed above. On average, however, one would expect that these and other phenomena that impact the molecular evolution of genes would be equally distributed in the two allopolyploid genomes. This leads to a useful null hypothesis, i.e. that evolutionary rates will be equivalent for duplicated homoeologues. A corollary expectation is that both gene copies will accumulate infraspecific diversity at an equivalent rate. The model may be helpful in informing a search for the underlying explanation for differential evolutionary rates or different levels of diversity when these are observed. For example, if one homoeologue becomes pseudogenized while the other remains under purifying selection, then nucleotide diversity is expected to increase in the former locus at a faster rate than in the latter. The fact that duplicated genes reside in the same nucleus greatly simplifies the challenge of isolating potentially important genomic forces from population-level factors that might affect patterns of diversity, such as breeding system or effective population size. Because population-level factors are neutral with respect to the two homoeologues, observed differences in diversity are more easily attributed to genetic or genomic processes.

Gossypium allopolyploids offer a powerful model for these explorations, particularly inasmuch as the two genomes are known to be largely co-linear. In two independent phylogenetic analyses (Small *et al*., 1998; Liu *et al*., 2001b), D-genome sequences in the allopolyploids were found to have longer branches (i.e. faster

evolutionary rates) than their homoeologous A-genome sequences. A more direct test of the null hypothesis of rate equivalence for homoeologous genes is provided by measures of nucleotide diversity levels. If evolutionary forces are equivalent for duplicated genes, mutations should accumulate randomly with respect to homoeologue, and hence in a survey of allelic polymorphism in a sample of individuals, the number of alleles detected should be approximately equal for the two gene copies. This was the approach used by Small, Ryburn & Wendel (1999) in a study of approximately 1 kb of sequence from the alcohol dehydrogenase A gene (*adhA*) for 22 accessions (44 alleles per genome) of *G. hirsutum* and for five accessions (ten alleles per genome) of *G. barbadense*. In both allopolyploid species, estimates of nucleotide diversity were higher for *adhA* from the D-genome than from the A-genome, by a factor of two or more. In a follow-up study, in which a 1.3-kb section was sequenced of a second alcohol dehydrogenase gene (*adhC*) with a faster overall evolutionary rate, the same conclusion was even more emphatically reached (Small & Wendel, 2002). These observations collectively suggest that there has been an overall acceleration in evolutionary rate in the D-genome relative to the A-genome of allopolyploid *Gossypium*. Although this rate enhancement is not always observed (Cronn *et al*., 1999; Senchina *et al*., 2003), the emerging picture is that evolutionary forces operating on the two genomes may be different. At present, the responsible forces and underlying molecular mechanisms are obscure.

EXPRESSION AND SILENCING OF GENES DUPLICATED BY POLYPLOIDY

In addition to evolutionary changes in gene and genome structure, a key component of polyploid evolution concerns the consequences of genome doubling on gene expression. We have begun to explore the expression of homoeologous gene pairs in several genotypes of allopolyploid *Gossypium*, including both natural and newly created synthetic allopolyploids. In principle, both genomes in an allotetraploid could contribute equally to the total transcriptome or, alternatively, there may be preferential transcription of one genome due to intergenomic interactions that could bias the transcription machinery in unknown ways toward expression of one genome or the other. We have examined expression of 40 homoeologous gene pairs in ovules of the natural allotetraploid *G. hirsutum* (cultivated upland cotton) to determine if genes duplicated by polyploidy are expressed at equal levels or if there is silencing or biased expression. Genes selected were shown to be single copy, and orthologous and homoeologous relationships were established by phylogenetic analyses (Cronn *et al*., 1999; Small & Wendel, 2000; Senchina *et al*., 2003). Assays of transcript accumulation by cDNA-SSCP (Cronn & Adams, 2003) revealed silencing or biased expression of one homoeologue (see Fig. 2A,B) for ten of 40 genes examined (Adams *et al*., 2003). There was no obvious bias for expression from one genome or the other in the polyploid nucleus.

Having sampled one organ (ovules) initially, we decided to extend the expression assays to other organs of *G. hirsutum* to determine if there is organ-specific partitioning of homoeologous gene expression. Expression assays of 18 genes in several organs revealed that individual genes may exhibit strong expression biases and vary greatly in this respect (Adams *et al*., 2003). Almost one-third of the genes examined revealed appreciable bias toward one homoeologue or the other in at least one organ. Transcript levels for the two members of each gene pair vary considerably by gene and, unexpectedly, by organ type. Some genes, for example the alcohol dehydrogenase gene *adhA*, showed organ-specific, reciprocal silencing of alternative homoeologues, where there is minimal to no transcription of one member of a duplicated gene pair in some organs and a similar absence

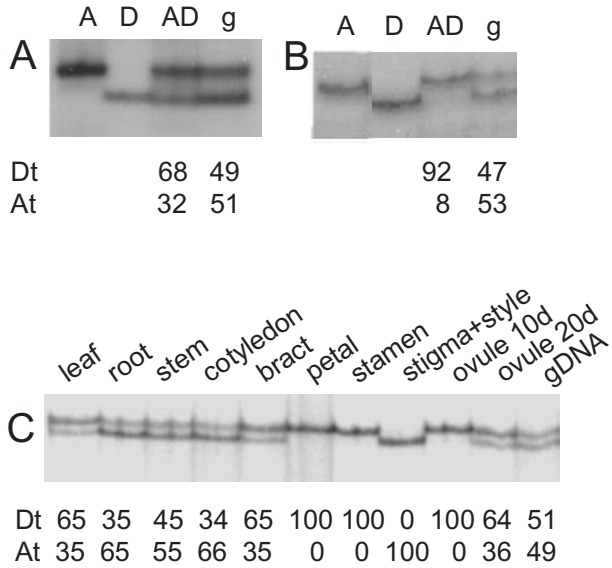

Figure 2. Analysis of homoeologue expression in *G. hirsutum*, using cDNA-SSCP. A, B; Four lanes are shown in each panel: A, *G. herbaceum*; D, *G. raimondii*; AD, *G. hirsutum*; g, a genomic DNA control (from *G. hirsutum*). A, Gene D7, a root hair defective 3 homologue that shows about 2 : 1 expression bias toward the A_t homoeologue. B, Alcohol dehydrogenase D (*adhD*), showing an A_t/D_t expression ratio of greater than 9 : 1. C, Analysis of alcohol dehydrogenase A (*adhA*) expression in several organs of *G. hirsutum.* At and Dt indicate transcript levels from the A and D genomes. Data are from Adams *et al*. (2003).

of expression of its duplicate in other parts of the plant (Fig. 2C). Floral organs showed particularly interesting expression patterns, with major differences between stamens and stigmas + styles. These results show that the genomic response to polyploidy varies in different parts of the plant, varies widely among genes, and can be developmentally regulated in previously unanticipated ways.

The process of subfunctionalization, partitioning of function and/or expression pattern between two duplicate genes, is conceived to be one that operates on an evolutionary timescale, requiring fixation of mutations in regulatory regions or functional domains such that complementary degenerate mutations arise (Force *et al*., 1999; Lynch & Force, 2000). The data discussed above for alcohol dehydrogenase A (*adhA*) suggest that the homoeologous gene pairs have undergone subfunctionalization sometime during the evolution of the cotton polyploids in the last 1.5 million years (see also comments in Kellogg, 2003). This is probably the most evolutionarily recent example of subfunctionalization to be reported.

In contrast to gene silencing that is reciprocal by organ type, our findings of biased expression (i.e. transcript accumulation) of homoeologues relative to each other that varies by organ type raise questions as to its biological meaning. Biased expression levels could indicate the contributions of each homoeologue to the transcriptome in all cell types within an organ. Alternatively, because organs are composed of multiple cell types, the observation of biased expression could be due to silencing of one homoeologue in some cell types and expression in others within the organ. It will be interesting to examine expression in single cell types in the future.

To investigate gene expression changes that arise during or soon after polyploidization, we have examined expression of a few homoeologous gene pairs in synthetic cotton allotetraploids. Silencing and biased expression were detected in an AD synthetic allotetraploid (Adams *et al*., 2003). Surprisingly, two genes (including *adhA*) showed reciprocal patterns of silencing and biased expression (see comments in Otto, 2003). Examination of homoeologue expression in three independently created synthetic allotetraploids revealed similarities in *adhA* silencing between the two AD synthetic genotypes, but different silencing patterns than to the AG synthetic (K. L. Adams, unpubl. data). The *adhA* silencing patterns in both synthetics mirrored those in the natural AD allotetraploid *G. hirsutum*. Overall, there appear to be factors causing similar *adhA* expression patterns in independently created AD tetraploids, suggesting that chromosomal context might play a role in gene silencing.

We are currently undertaking a comparative genomic study of gene expression and silencing in multiple organs of a synthetic allopolyploid to estimate the genomic extent of homoeologue silencing in different organs. We and others (Chen *et al*., 2004 – this issue) are currently performing global surveys of polyploid gene expression using oligonucleotide-based microarrays. Such genomic level approaches are likely to yield major new insights into the effects of allopolyploidization on gene expression.

What kind of molecular mechanisms might be causing the observed gene silencing patterns in polyploid cottons? Gene silencing in synthetic polyploids is likely to be epigenetic in nature, as discussed by Adams *et al*. (2003). Possible epigenetic mechanisms for homoeologue silencing include hypermethylation of cytosines, histone modifications including deacetylation and methylation, and aspects of higher order chromatin structure; these mechanisms are likely to be interrelated (Wolffe & Matzke, 1999; Richards & Elgin, 2002). Gene silencing caused by DNA cytosine methylation was shown for a gene in polyploid *Arabidopsis suecica* (Lee & Chen, 2001). Models for gene silencing involving repeats and LTRs of retroelements have been proposed (Comai *et al*., 2003). Recently, it was shown that antisense transcripts generated by readout synthesis of a retrotransposon caused silencing of adjacent genes (Kashkush, Feldman & Levy, 2003). It is also possible that small RNAs (Marker *et al*., 2002) and RNA interference (RNAi) play a role in gene silencing in polyploids (see also Comai *et al*., 2003).

Why are genes silenced in polyploids? Some genes may be silenced for dosage reasons, as discussed in Osborn *et al*. (2003). Alternatively, if homoeologous gene sequences have different amino acid sequences, one homoeologue might interact better with other proteins in multisubunit complexes (Comai, 2000). For example, all of the maternal genes whose sequences have co-adapted to each other might be expressed and the paternal copies silenced. Multi-subunit complexes in mitochondria and chloroplasts are composed of subunits derived from genes in the nuclear genome and the organellar genome. Because organelles are usually uniparentally inherited (often from the maternal parent), there could be silencing of genes derived from the parent that did not donate the organelles; we are beginning to explore this interesting possibility. It is possible that various negative cyto-nuclear interactions might influence expression and silencing of homoeologous genes. In this regard it will be especially interesting to examine polyploids derived from reciprocal crosses. By contrast, some expression variation may be functionally and selectively immaterial, reflecting instead an evolutionarily more passive side-effect of higher-order mechanistic processes that perhaps are global in scope. The example of retrotranspo-

son activation in synthetic wheat polyploids and silencing of adjacent genes (Kashkush *et al*., 2003) illustrates the role that transposons can play in causing gene silencing. As empirical data accumulate in *Gossypium* and other model plant allopolyploids, the scope of factors and mechanisms causing expression alteration and gene silencing will become clearer.

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