Current Biology 21, 551–556, April 12, 2011 ©2011 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2011.02.016

Report

Transcriptomic Shock Generates Evolutionary Novelty in a Newly Formed, Natural Allopolyploid Plant

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Summary

New hybrid species might be expected to show patterns of gene expression intermediate to those shown by parental species [1, 2]. "Transcriptomic shock" may also occur, in which gene expression is disrupted; this may be further modified by whole genome duplication (causing allopolyploidy) [3–16]. "Shock" can include instantaneous partitioning of gene expression between parental copies of genes among tissues [16–19]. These effects have not previously been studied at a population level in a natural allopolyploid plant species. Here, we survey tissue-specific expression of 144 duplicated gene pairs derived from different parental species (homeologs) in two natural populations of 40-generation-old allotetraploid *Tragopogon miscellus* (Asteraceae) plants. We compare these results with patterns of allelic expression in both in vitro "hybrids" and hand-crossed F_1 hybrids between the parental diploids *T. dubius* and *T. pratensis*, and with patterns of homeolog expression in synthetic (S_1) allotetraploids. Partitioning of expression was frequent in natural allopolyploids, but F_1 hybrids and S_1 allopolyploids showed less partitioning of expression than the natural allopolyploids and the in vitro "hybrids" of diploid parents. Our results suggest that regulation of gene expression is relaxed in a concerted manner upon hybridization, and new patterns of partitioned expression subsequently emerge over the generations following allopolyploidization.

Results and Discussion

Variation in Relative Gene-Copy Expression

Changes in patterns of parental gene expression are frequently observed in hybrids and allopolyploids; this phenomenon has been termed transcriptomic shock [3–13, 16]. Study of natural transcriptomic shock in the wild is hampered by the rarity of known, recently formed polyploid species that still co-occur with their parental species. *Tragopogon miscellus* (Tm) (Asteraceae) is a young natural allotetraploid species that formed multiple times during the past 80 years in the NW USA from the diploids *T. dubius* (Td) and *T. pratensis* (Tp), which were introduced from Europe and remain extant in areas of polyploid formation [20]. Allopolyploid populations formed reciprocally, with an immediate and conspicuous phenotypic difference: populations with Td as the maternal parent have inflorescences with long ligules while those with Tp as the maternal parent have inflorescences with short ligules (see Figure 1A).

To explore transcriptomic shock in the formation and early generations of allopolyploidy in *T. miscellus* populations, and unravel the effects of hybridization and whole-genome duplication, we investigated tissue-specific Td and Tp gene expression in: the diploid parents Td and Tp, as 1:1 mixes of cDNA from five pairs (designating these as in vitro "hybrids," showing simple additivity of parental gene expression); true synthetic diploid F_1 hybrids (n = 6); synthetic first-generation (S_1) allopolyploids (n = 6); and in two naturally occurring Tm allopolyploid populations of reciprocal origin (n = 10+8). We examined expression of 144 gene pairs: 126 with quantitative Sequenom MassARRAY allelotyping assays previously developed using 454 and Illumina sequencing data [21], and 18 using previously developed qualitative cleaved amplified polymorphic sequence (CAPS) assays [22–24]. We examined the expression of both copies of these genes in transcriptomes of up to seven tissues of each of 40 plants. Example data for four genes are shown in Figure 2.

For many genes, we found cases of lack of expression of a homeolog in all tissues of individual plants (e.g., gene 07259_1424 in four short-liguled allopolyploid plants, Figure 2A). These cases of nonfunctionalization [17] were excluded from our analysis of tissue-specific transcriptomic shock. They are likely a consequence of a genomic change. Consistent with this hypothesis a Sequenom analysis on genomic DNA of a sample (n = 168) of these cases in Tm showed that 89.9% had one homeolog missing from the genome. This corresponds with previous studies showing losses of homeologs in Tm [22–24].

To understand differences among the plant groups in the variability of relative expression of Td and Tp genes, we calculated the standard deviation of expression for every Sequenom gene assay within each tissue sample transcriptome and used these statistics to generate the boxplots shown in Figure 1B. The five plant groups showed a significant difference (Wilcoxon test $X^2 = 62.49$, $p < 0.0001$). Nonparametric comparisons of each pair of plant groups using the Wilcoxon method showed a small difference between the F_1 hybrids and S_1 allopolyploids ($Z = 3.07$, $p < 0.0021$), no significant difference between diploid in vitro "hybrids" and the two populations of natural allopolyploids ($p > 0.5$ in all three comparisons), and a significant difference in every other comparison ($p < 0.0001$ in six comparisons). We also made a similar comparison of the variation of tissue samples within

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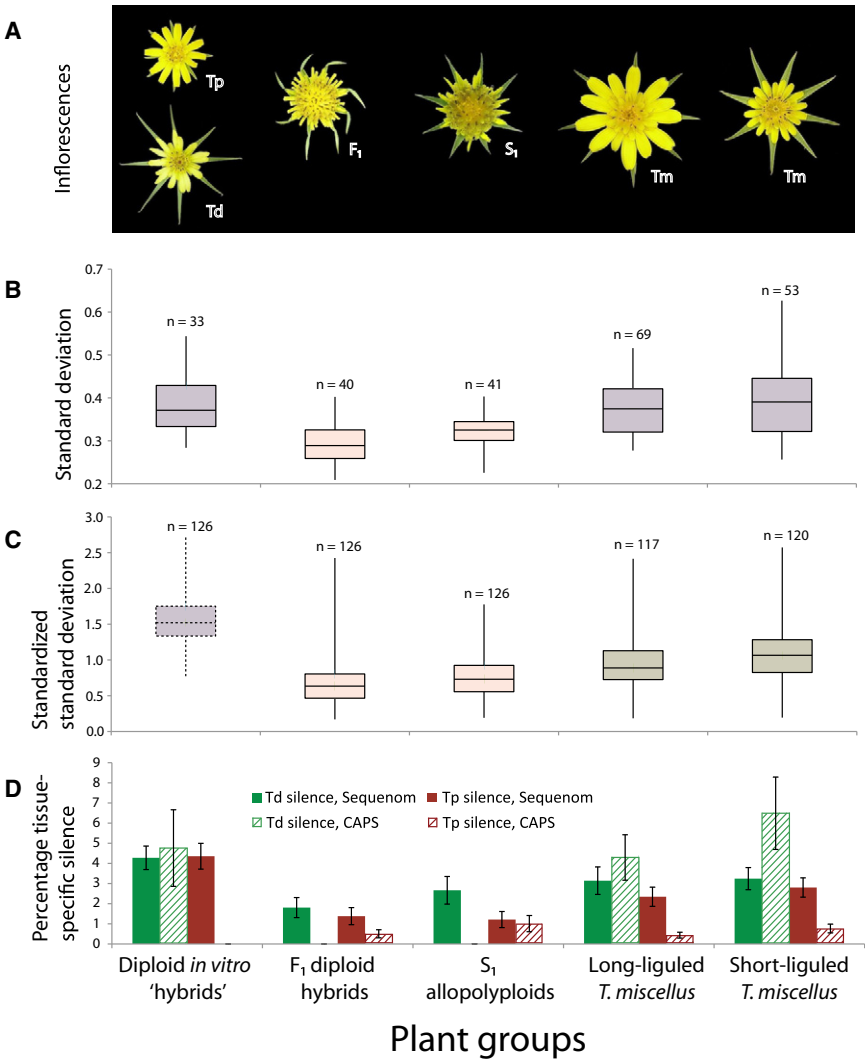

Figure 1. Transcriptomic Shock in *Tragopogon*

(A) Examples of inflorescences from the groups of *Tragopogon* plants sampled. Identity of plants is shown by abbreviations used in the main text (Tp = *T. pratensis*, Td = *T. dubius*, Tm = *T. miscellus*, F_1 = diploid hybrid between Tp and Td, S_1 = first-generation allopolyploid produced between Tp and Td).

(B) Variation in quantitative results for relative allele/homeolog expression (excluding cases of apparent nonfunctionalization) from Sequenom data, based on 126 genes; showing boxplots of standard deviation among genes within samples. Number of samples within each group is shown above boxplots; box fill colors indicate plant groups that differed with a significance greater than p = 0.0001.

(C) Variation in quantitative results for relative allele/homeolog expression (excluding cases of apparent nonfunctionalization) from Sequenom data, showing boxplots of standardized standard deviation among samples within genes. Number of genes analyzed in each group is shown above boxplots; box fill colors indicate plant groups that differed with a significance greater than p = 0.0001. The standard deviation of relative gene expression among samples in diploid in vitro "hybrids" may have been increased by pipetting errors.

(D) Mean percentage of tissues showing tissue-specific silence of alleles/homeologs in a series of *Tragopogon* diploids and polyploids for 126 genes assayed using Sequenom and 18 genes assayed using CAPS. Error bars show standard error.

genes for each group (Figure 1C), following the same method as that above, except that we standardized the standard deviation for each gene within each plant group by the average standard deviation shown by each gene across all samples. This showed a similar pattern: the five plant groups differed significantly (Wilcoxon test $X^2 = 278.03$, $p < 0.0001$), due to highly significant differences ($p < 0.0001$) between all pairs of groups except F_1s and S_1s ($Z = 2.79$, $p = 0.0052$) and the two natural Tm populations ($Z = 2.85$, $p = 0.0043$). The high standard deviation shown by diploid in vitro "hybrids" among tissues within genes (Figure 1C) may have been increased by small pipetting errors that shifted the overall ratio of the two transcriptomes in each sample away from 1:1, but this factor would not affect the measurement of standard deviation among genes within tissue samples (Figure 1B).

These results suggest that F_1 hybrids have lower variation of relative Td:Tp expression levels among plants and tissues than is observed when cDNAs of the parental diploids are mixed (in vitro "hybrids"). Yet after 40 generations following allopolyploidization, variation in relative Td:Tp expression is observed among plants and tissues in natural Tm populations. This difference in variation of expression between the groups occurred despite the fact that the six F_1 and six S_1 plants were derived from a total of eight unique crosses (i.e., between different parental combinations, see Table S1 available online), whereas the 18 Tm plants studied were from two natural populations, each of which appears to have its origin in a single cross [25].

Tissue-Specific Silence in Gene-Copy Expression

Of particular interest are cases where relative expression of gene copies is so skewed that one copy is not detected at all in the transcriptome of a tissue; we call this tissue-specific silence (TSS). Taking Sequenom and CAPS assays together, the mean percentage of assays showing TSS per gene was highest in the diploid in vitro "hybrids" (Figure 1D; in Wilcoxon matched pair analysis for diploid data compared with the four other groups, TSS was more frequent for diploids with $p < 0.0001$ in all four comparisons except for that with short-liguled Tm, where $p = 0.0243$). F_1 hybrids and S_1 allopolyploids had the lowest frequency of TSS and these two plant groups did not differ significantly. The two natural allopolyploid populations both had more frequent TSS than the F_1s and S_1s ($p < 0.0001$), and TSS differed in frequency between the two natural Tm populations ($p = 0.0416$). In no group was there a significant difference in the frequency of TSS of Td versus Tp homeologs. Thus, it appears that in diploid parental species it is common for a gene to be entirely unexpressed in some of the tissues examined, but these genes are globally activated by hybridization, such that copies from both parents are

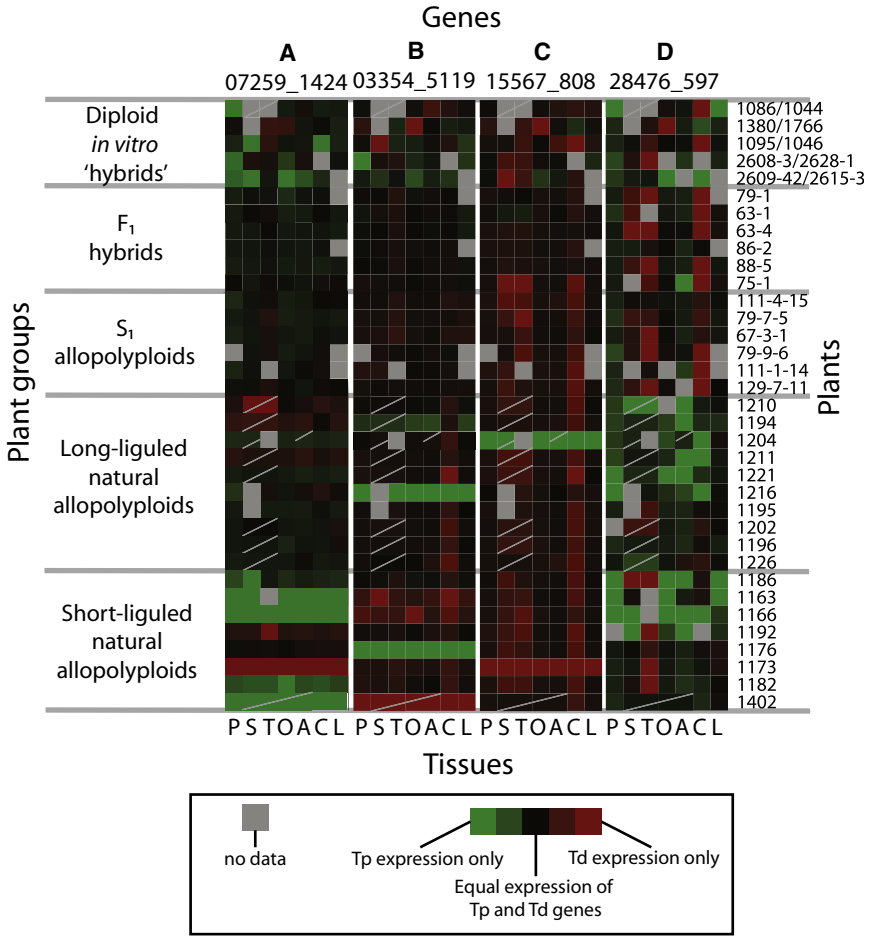

Figure 2. Examples of Results for Individual Genes

(A–D) Tissue-specific relative expression of gene copies derived from *T. dubius* and *T. pratensis* in diploid in vitro "hybrids," F_1 hybrids, S_1 synthetic allopolyploids, and natural populations of *T. miscellus*. Results for four example gene pairs are shown, measured using Sequenom analysis (the full data set of 126 Sequenom assays and 18 CAPS gene assays are shown in Figure S1). Columns represent tissues, and lines represent plants. Columns are grouped by gene, and rows are in plant groups. Tissue abbreviations are as follows: P = Phyllary, S = Style, T = Stigma, O = Ovary, A = Pappus, C = Corolla, L = Leaf. Colors show relative tissue-specific expression of Td and Tp gene copies (see legend). Cells joined by diagonal gray lines represent groups of tissues that were assayed together. The *Arabidopsis thaliana* homologs of the four genes shown are: (A) Histidine kinase 3; (B) a remorin family protein; (C) a haloacid dehalogenase-like hydrolase family protein; (D) a metal ion binding protein.

expressed. In many genes, tissue-specific silencing of one homeolog occurs in the first 40 generations of allopolyploidy (while total silencing of both copies of a gene in the same tissue is rare). The Sequenom and CAPS assays gave the same general pattern with small differences likely due to differences in sample size and genes sampled (Figure 1D).

We then asked whether the same genes showed TSS in the diploid in vitro "hybrids," F_1 hybrids, S_1 allopolyploids, and natural allopolyploids. There was a significant correlation between the percentage of TSS shown by individual genes in the diploid in vitro "hybrids" and in the natural allopolyploids ($R^2 = 0.307$, $F = 48.23$, $p < 0.0001$), for 111 genes assayed using Sequenom, which were expressed in at least one tissue in every diploid in vitro "hybrid." There was a weaker correlation between F_1s and natural allopolyploids ($R^2 = 0.097$, $F = 11.73$, $p < 0.0009$) and between S_1s and natural allopolyploids ($R^2 = 0.106$, $F = 12.92$, $p < 0.0005$). Therefore, the same genes tended to show TSS in the diploid in vitro "hybrids" and natural allopolyploids despite loss of TSS upon hybridization. It must be emphasized that in the diploids TSS involves total nonexpression of that gene in a tissue, whereas in allopolyploids exhibiting TSS, the expression of one homeologous gene copy is retained.

While there are general trends across all genes, five genes that we studied showed patterns that are found with some regularity among plants after hybridization and/or whole-genome duplication (Figures 2B–2D; Figure S2). For example, gene 15567_808 (Figure 2C), a putative haloacid dehalogenase-like hydrolase family protein, showed zero TSS in all but one F_1 hybrid, but the pattern of tissue-specific expression found in this one hybrid – Td bias in the stigma, style and corolla – was also found in most of the synthetic and natural allopolyploids examined. Two genes showed patterns of TSS that are found across all groups (diploid through natural allopolyploids) (Figures S2D and S2E). Two genes showed patterns of tissue-specific expression that were to some extent found in diploid in vitro "hybrids," F_1 hybrids, and S_1 allopolyploids but absent in natural Tm populations (Figure 2D; Figure S2G): for example, a metal ion-binding homolog 28476_597 (Figure 2D) tended to show high relative expression of the Td copy in the stigma and corolla of diploid in vitro "hybrids," F_1 and S_1 plants, but this expression pattern was not present in the majority of the natural 40-generation-old allopolyploids.

Transcriptomic Shock as a Reduction in Tissue-Specificity of Gene Expression

The general trends of our results suggest that transcriptomic shock upon hybridization [26] includes the activation of allele/homeolog expression in all tissues, causing a loss of tissue-specific expression patterns seen in the diploid parents. Such activation has been shown for repetitive and transposable elements [26–29], but has seldom been considered in terms of the tissue-specific activation of protein-coding genes. A rare example is in the derepression of Polycomb group proteins in hybrid endosperm [30]. Activation of homeologs has also been found occasionally in cotton F_1 hybrids and allopolyploids by Chaudhary et al. [7], who termed it "transcriptional neofunctionalization." Here, we show this to be widespread in *Tragopogon*.

Our findings may fit a newly proposed transcriptomic shock scenario in which activity of small interfering RNA molecules, which influence gene expression, is temporarily lost in F_1 hybrids and early allopolyploids, but restored as subsequent

generations stabilize [31, 32]. Novel expression in hybrids could also be due to trans-activation between the two parental genomes, whereby a regulatory element produced by one parental genome activates gene expression in the other genome; perhaps this is uncoupled in subsequent generations.

Previous studies in domesticated cotton allopolyploids, using methods that distinguish among tissues and between homeologs, have led to the conclusion that allopolyploidization causes "an immediate, massive, and saltational disruption of ancestral expression patterns" [7, 14–16, 33]. Alterations in gene expression have also occurred upon allopolyploidization in *Arabidopsis* [4, 5, 9, 12], wheat [3, 10, 13, 34, 35], and *Brassica* [11]. Our results shed new light upon these disrupted expression patterns in a wild, nonmodel plant.

Our results might appear to contrast with those of a study in the young natural allohexaploid *Senecio cambrensis*, where hybridization was found to be the most influential step with respect to the transcriptome [8]. Gene expression changes that occurred over five generations of a synthetic allopolyploid line and over the ~100-year existence of the natural allopolyploid *Senecio cambrensis* were smaller than those that occurred at the time of hybridization [8]. However, the *Senecio* experiment did not distinguish between homeologs or among tissues, and we therefore do not know whether expression changes occurring upon hybridization were due to loss of tissue-specific expression patterns. Regardless, the results for *Senecio* might be expected to differ from those reported here because in *Senecio* the hybridization step was between a diploid and a tetraploid, not between two diploids as in *Tragopogon*. Hence, in *Senecio* the F_1 hybrids were triploids, which are likely to have genome dosage effects [36].

Frequency of Expression Subfunctionalization

We also examined a special category of TSS, reciprocal tissue-specific silence of homeologs, that may be indicative of rapid subfunctionalization, the partitioning of multiple functions of an ancestral gene between its duplicate descendents [16]. In the in vitro "hybrids" of *Tragopogon* diploid transcriptomes, we found 26 cases of reciprocal TSS, where a gene was not expressed by one diploid parent in a certain tissue but not expressed by the other diploid parent in another tissue (3.97% of 655 plant × gene combinations examined that did not show apparent nonfunctionalization). In contrast, we found just six cases of reciprocal TSS in F_1 hybrids (0.74% of 807 plant × gene combinations), zero cases in S_1s (0% of 707), and eight cases in natural allopolyploids (0.37% of 2152; for an example identified using CAPS, see Figures S2F and S3). Thus, the activation of genes by transcriptomic shock seems to cause lower reciprocal TSS after hybridization than we might expect if gene expression were strictly additive of that in parental diploids.

Models of subfunctionalization involving tissue-specific expression tend to assume identical expression patterns of ancestral and newly duplicated genes [17]. Under such a condition, reciprocal TSS has been shown to occur in F_1 hybrids [15], synthetic allopolyploids [14, 16], and allopolyploid domesticated cotton [16]. However, the data presented here show that different tissue-specific patterns of homolog expression are common in the diploid parents of Tm. The data also suggest that the activation of genes by transcriptomic shock seems to cause lower reciprocal TSS after hybridization than we might expect if gene expression were strictly additive of that in parental diploids. This suggests that instantaneous subfunctionalization is the exception, not the norm, in the evolution of gene expression in new allopolyploids.

Conclusions

The patterns of transcriptomic shock shown here are likely to affect profoundly the evolutionary success of the natural populations of allopolyploid *Tragopogon miscellus*. If genes in the diploid parental species have finely tuned patterns of tissue-specific expression, disruption of these patterns could have negative fitness consequences in an unchanging environment, but might be highly beneficial after a long-range migration, such as that undergone by *Tragopogon* species when introduced to the NW USA from Europe [37, 38]. Current models for the evolution of genetic complexity and diversity rely upon gene and genome duplication [19, 39–42]; divergence in the location or timing of gene expression is likely to be one possible early step in the functional divergence of duplicated genes [17, 42, 43]. In light of these models, the data presented here suggest that rather than being a saltational leap to a new fitness peak, allopolyploidization, for the majority of genes, provides the genetic and transcriptomic resources for novel trajectories of evolution, by activation of gene expression (as well as by genetic redundancy at all loci). Even though allopolyploid formation inevitably involves a genetic bottleneck, subsequent generations display diverse patterns of tissue-specific gene expression, whose phenotypic effects may be exposed to natural selection and thus gradually lead the new allopolyploid species to new adaptive peaks.

Experimental Procedures

Seed Sources

Seeds were collected from natural populations of *T. miscellus* (Tm) of independent and reciprocal origin: the short-liguled form (with *T. pratensis* [Tp] as the maternal parent) from Moscow, ID and the long-liguled form (with *T. dubius* [Td] as the maternal parent) from the only known natural population, which is found nearby in Pullman, WA (for Soltis and Soltis collection numbers, see Table S1). Samples of Td were obtained from Pullman, WA; Palouse, WA; and Spokane, WA, and those of Tp were obtained from Moscow, ID and Spangle, WA (see Table S1). These seeds were grown in the greenhouse (at Washington State University, Pullman, WA) and allowed to self-fertilize for one generation. After self-fertilization, seeds were germinated and grown under controlled conditions in a greenhouse at the University of Florida (Gainesville, FL). Five plants of each diploid parent and ten plants of each Tm population were used in the experiment.

Six F_1 hybrids and six first-generation synthetic allopolyploids (S_1s) formed between Td and Tp, were grown in the same greenhouse [44]. The crosses that gave rise to these synthetic lines are shown in Table S1. Five of the F_1s and S_1s were Tp × Td crosses, and one F_1 and S_1 were a Td × Tp cross. The latter cross had a much lower success rate in terms of viable progeny than the former [44]. The synthetic allopolyploids were produced using colchicine treatment of F_1 hybrids [44]. These hybrids were different individuals to those included as F_1 hybrids in this experiment. In only one case was an F_1 from exactly the same parental diploid pair as an S_1, due to the difficulties of successfully inducing whole genome duplication with colchicine and nurturing the new allopolyploid to flowering.

Tissue Dissection and RNA Extraction

Leaf and inflorescence tissue was collected from all plants and flash frozen in liquid nitrogen [23]. Inflorescences were dissected on dry ice into up to six tissue types: corolla, pappus, ovary, stigma, style, and phyllary. Sometimes the quantities of stigma and style tissues available were not sufficient to provide the quantity of RNA needed, so the two tissues were combined. Tissues were ground with a mortar and pestle at −80°C, and RNA was extracted using the RNAeasy kit with on-column DNase digestion from QIAGEN (Valencia, CA). First-strand cDNA synthesis was carried out on 500 ng of RNA using Superscript II reverse-transcriptase (Invitrogen, Carlsbad, CA) and polyT primers. In the CAPS analyses, cDNAs from diploids

were analyzed separately and the data combined for the analyses (below); in the Sequenom analyses, cDNAs for specific tissues were combined from Tp and Td to make in vitro "hybrids," allowing quantification of the relative expression levels of homologs.

Allele/Homeolog Assays

We wished to characterize the expression of genes derived from Td and Tp in tissues of the five plant groups. In diploid hybrids these genes are present as alleles, but in allopolyploids they are present as homeologs. Allele/homeolog expression was analyzed in the tissue transcriptomes isolated above, using 18 CAPS markers (including one set of homeolog-specific PCR primers) and 128 Sequenom MassARRAY assays. The CAPS assays were carried out on tissue transcriptomes of all the plants mentioned above, and the Sequenom assays were carried out on the same set of transcriptomes with the omission of those from two short-liguled Tm plants. Below we outline how the assays were implemented.

For the CAPS markers, alleles/homeologs were amplified by PCR from the cDNA using 10 primer sets from Tate et al. [23], seven primer sets from Buggs et al. [24], and one primer set from Tate et al. [22]. Putative identities of these genes are provided in the original papers cited. The PCR products were digested using the enzymes and conditions specified in the above publications, which cut only one of the two alleles/homeologs, due to a single nucleotide difference in the enzyme cut site between the two alleles/homeologs. CAPS and allele/homeolog-specific PCR products were visualized on high-resolution 4% Metaphor agarose (Lonza, Allendale, NJ) gels. Qualitative results were scored as 0 for expression of both alleles/homeologs, 1 for expression of only the Tp allele/homeolog and −1 for the expression of only the Td allele/homeolog.

Sequenom MassARRAY assays were developed as described in Buggs et al. [21]. In this technology, a short section of DNA containing a SNP is amplified from an individual by PCR. This is followed by a single-base primer extension reaction over the SNP being assayed, using nucleotides of modified mass. The different SNP-alleles therefore produce oligonucleotides with mass differences that can be detected using Matrix-Assisted Laser Desorption / Ionization Time-Of-Flight mass spectrometry. This provides a trace in which peak heights correspond to the frequency of each different oligonucleotide, allowing measurement of the relative frequency of two SNP-alleles at one locus. This can be used to measure quantitative gene expression [45]. Up to 40 different assays can be multiplexed in one reaction mix if primers are designed to give unique mass ranges for each single-base-extended primer. We identified single base-pair differences between homologous genes in Td and Tp using 454 and Illumina sequencing and designed Sequenom assays to measure the expression of 139 gene pairs as alleles/homeologs in the plant groups in this study [21].

The Sequenom assays were carried out at Iowa State University. The accuracy of our assays in measuring relative expression of alleles/homeologs was checked by running three replicates of a *T. miscellus* leaf transcriptome that had been Illumina deep-sequenced [21]. Where the three replicates gave a result in agreement with the Illumina count data, the assays were deemed to be accurate. Where there were insufficient Illumina count data, the accuracy of the assays was checked using genomic DNA from six F_1 hybids between Td and Tp and mixes of Td and Tp genomic DNA in ratios of 1:3, 1:1, and 3:1. Where the majority of these gave an expected result, the assays were deemed to be accurate. By the first method, 111 assays were found to be accurate and a further 17 by the second method, giving a total of 128 working assays. Two of these were not used: one because one diploid was polymorphic, and one because the assay failed to work on a homeolog that had not been covered in the Illumina run.

Where one homeolog was found to be silenced in all tissues of an individual Tm plant by Sequenom analysis, genomic DNA was extracted from that plant using a modified CTAB protocol [46], and Sequenom analysis carried out, to test for genomic loss of that homeolog. Only 73 of the Sequenom assays that had worked on cDNA worked consistently on genomic DNA, probably due to intron splicing.

Analysis

Raw Sequenom MassARRAY allelotyping data consist of areas under a mass-spectrometer peak for expression of each allele/homeolog. We converted the data to quantitative measures of relative expression of the two alleles/homeologs, where 0 represents equal expression of both alleles/homeologs, 1 represents expression of only the Tp allele/homeolog and −1 represents the expression of only the Td allele/homeolog. Results from both CAPS and Sequenom gene expression analyses on tissue-specific cDNAs were clustered separately using Cluster 3.0 [47], using a hierarchical centered Pearson correlation with average linkage. The two clustered datasets were visualized in Java Treeview 1.1.4r3 [48] (Figure S1).

To compare variation in relative gene expression among the plant groups (parental diploid in vitro "hybrids," F_1 hybrids, S_1 allopolyploids, long-liguled natural allopolyploids, and short-liguled allopolyploids), we calculated the standard deviation of expression both among genes within tissue transcriptomes (Figure 1B) and among tissue transcriptomes within genes (Figure 1C). The former statistics did not include variation caused by small pipetting errors in the production of the diploid in vitro mixes, whereas the latter inevitably did. Differences between the mean standard errors of each group were tested in JMP using Wilcoxon tests among all groups and nonparametric comparisons for each pair of groups using the Wilcoxon method.

For the data sets from both CAPS and Sequenom methods we calculated the mean percentage of tissue-specific assays from each of the five plant groups that displays tissue-specific silence (TSS; Figure 1D). Only a 1 or −1 in the Sequenom result for an assay was scored as nonexpression of one gene copy. If all Sequenom assays for a plant yielded a result of all 1 or all −1, except for a single tissue, we counted this as a putative nonfunctionalization, as comparison with results from assays on genomic DNA showed that occasional false positives occurred, spuriously showing slight expression of one homeolog in only one tissue where that homeolog had been lost from the genome. The TSS data were analyzed in JMP using Wilcoxon matched pair analysis and bivariate fits.

Supplemental Information

Supplemental Information includes three figures and one table and can be found with this article online at doi:10.1016/j.cub.2011.02.016.

Acknowledgments

Funding for this research was provided by the University of Florida and NSF grants DEB-0614421 to P.S. Soltis and D.S.S., MCB-0346437 to P.S. Soltis, D.S.S., and J.A.T., DEB-0919254 to P.S. Soltis, D.S.S., and W.B.B., and DEB-0919348 to P.S. Schnable. R.J.A.B. has been supported since March 2010 by NERC Fellowship NE/G01504X/1. We thank two anonymous reviewers for helpful comments.

Received: January 6, 2011
Revised: February 10, 2011
Accepted: February 14, 2011
Published online: March 17, 2011

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