EXPRESSION OF DUPLICATED GENES IN A POLYPLOID PLANT IN RESPONSE TO ABIOTIC STRESS

by

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ABSTRACT

Polyploidy, or genome doubling, is an important process in plant evolution that has effects on phenotypes, such as changes in flowering time. Allopolyploidy can result in considerable genetic changes including alterations to genome structure, DNA methylation patterns, and gene expression. Although the expression of duplicated genes in polyploid plants has been extensively studied, little is known about the effects of abiotic stress conditions on homeologous gene expression. In this thesis, I examined the expression of 30 homeologous gene pairs in response to five abiotic stress treatments, using a single strand conformation polymorphism (SSCP) assay in allotetraploid *Gossypium hirsutum*. Twenty-two genes showed stress-induced changes in the expression ratio of the two homeologs, and eight genes showed reciprocal expression changes in response to different abiotic stress treatments, suggesting quantitative subfunctionalization. I also examined the expression of ten homeologous gene pairs in response to three abiotic stress treatments in a synthetic *Gossypium* allotetraploid. Eight genes showed stress-induced expression changes. Comparison of the expression changes showed that there was little correspondence in the stress-induced homeolog expression patterns between the natural and synthetic *Gossypium* polyploids. The results of this study indicate that abiotic stress conditions can have considerable effects on expression of homeologous genes. Some of those expression changes might help plants survive abiotic stresses.

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Introduction

Polyploids are produced by the duplication of a single genome (autopolyploid) or by the combination of two genomes from different organisms (allopolyploid) in a common nucleus (Chen and Ni, 2006), and in natural populations, estimates indicated that allopolyploidy was more prevalent than autopolyploidy (Levin, 2002). Studies showed that over 70% of angiosperms have at least one event of genome doubling during their evolutionary history and many diploid species are in fact ancient polyploids (Levin, 2002; Simillion et al., 2002; Blanc et al., 2004; reviewed in Hegarty et al., 2008). Many important agricultural crops are polyploid plants, such as wheat, canola, soybean, potatoes, sugarcane, and cotton (Wendel, 2000). Polyploidy can contribute to the rapid emergence of new species because different chromosome numbers could cause reproductive isolation between the polyploids and their parents (Tate et al., 2005). The increased heterozygosity in allopolyploids could help them occupy broader niches and colonize areas where the diploids are not found (Stebbins, 1950; Stebbins, 1985). Polyploidy can result in novel phenotypes, such as changes in flowering time (Schranz and Osborn, 2000).

Polyploidy can result in extensive genetic and epigenetic changes, which have been shown in resynthesized polyploids (e.g., Liu et al., 1998; Shaked et al., 2001; Kashkush et al., 2002). Studies in resynthesized allopolyploid *Brassica* species and

wheat showed that there were loss and gain of many DNA restriction fragments, both in the coding and noncoding regions, in the generations immediately following polyploid formation (Feldman et al., 1997; Liu et al., 1998; Ozkan et al., 2001; Shaked et al., 2001; Kashkush et al., 2002), likely caused by rearrangements (Gaeta et al., 2008). Previous studies also revealed frequent epigenetic changes, including DNA methylation, histone modification and chromatin packaging in newly synthesized allopolyploids of *Arabidopsis*, *Brassica*, and wheat (Song et al., 1995; Liu et al., 1998; Shaked et al., 2001; Kashkush et al., 2002; Madlung et al., 2002).

Besides genetic and epigenetic changes, polyploidy can result in changes to duplicate gene expression. Studies of duplicate gene expression in allopolyploids and their progenitors have shown biased expression patterns and silencing of duplicate genes in newly created synthetic *Brassica napus*, wheat, *Arabidopsis suecica*, and *Senecio cambrensis* (Comai et al., 2000; Kashkush et al., 2002; Wang et al., 2004; Hegarty et al., 2005; Albertin et al., 2006) as well as in natural polyploids (Lee and Chen, 2001; Bottley et al., 2006). The fates of homeologs (genes duplicated by polyploidy), after polyploidization usually falls into one of four categories: non-functionalization, where one homeolog is silenced or completely lost; neofunctionalization, where one homeolog gains a new function; subfunctionalization, where the expression or function is partitioned between the homeologs so that both copies are needed by the organisms, or retention of both copies for dosage or other reasons (Ohno., 1970; Force et al., 1999; Lynch and Force, 2000).

Cotton (*Gossypium*) is a useful group for studying polyploidy. There are approximately 40 diploid and 5 natural polyploid *Gossypium* species recognized (Percival et al., 1999; Wendel et al., 1999; Liu et al., 2001). All five polyploids are allotetraploids (AADD; 2n=4x=52) which originated by hybridization between an A genome diploid (genome AA; 2n=26) and a D genome diploid (genome DD; 2n=26) about 1.5 million years ago (Senchina et al., 2003; Brubaker and Wendel., 1994; Bourland and Wendel., 1999). Homeologous genes in polyploid cotton are designated At and Dt for the genes derived from the A-genome diploid progenitor and the D-genome progenitor, respectively. Homeologous gene expression changes have been found in polyploid *Gossypium hirsutum* (Adams et al., 2003; Liu and Adams, 2007; Hovav et al., 2008; Flagel et al., 2008; Chaudhary et al., 2009). For example Adams et al. (2003) showed there was organ-specific silencing of *AdhA* (alcohol dehydrogenase gene A) homeologs in *Gossypium hirsutum*, and Hovav et al. (2008) used homeolog-specific microarrays to demonstrate that around 30% of homeologs showed biased expressed in natural allopolyploid cotton. Also in new synthetic allopolyploid cotton, biased expression and silencing of the homeologous genes were found (Adams et al., 2004; Chaudhary et al., 2009).

There can be changes in homeologous gene expression patterns in polyploid plants in response to abiotic stresses. Liu and Adams (2007) found that in *Gossypium hirsutum* under water-submersion treatment, At copy (derived from the A genome parent) of the

AdhA gene was silenced in hypocotyls and roots, and under cold-stress treatment the Dt copy (derived from the D genome parent) was silenced in hypocotyls, which together showed reciprocal silencing of the *AdhA* homeologs and indicated subfunctionalization. This was the first and only study so far of homeologous gene expression in response to abiotic stresses. Thus, the extent of homeologous gene expression changes in response to abiotic stresses remains nearly unknown.

In this study I investigated the expression of pairs of homeologs in response to abiotic stresses in natural allotetraploid *Gossypium hirsutum* and a newly synthesized allotetraploid, designated 2(A2D3). I designed a series of experiments to determine: (1) whether changes in the expression levels of homeologs, relative to each other, under abiotic stresses is a common phenomenon in *Gossypium hirsutum*; (2) what kinds of stress-specific and organ-specific patterns are observed; (3) whether changes in the expression levels of homeologs under abiotic stresses can occur in a newly synthesized allotetraploid.

2 MATERIALS AND METHODS

2.1 Gene choices, sequence alignments, phylogenetic analyses, and primer design

The stress regulated genes in cotton were retrieved from Zhao et al. (2003) and Qin et al. (2004), Wang et al. (2007), Ni et al. (2008) and Guo et al. (2009) and the stress regulated genes in *Arabidopsis thaliana* were retrieved from Swindell (2006) and Kilian et al. (2007) through NCBI. BLAST searches of NCBI were used to identify cotton homologs of the stress-regulated genes in *Arabidopsis*. Sequences were aligned in ClustalX and analyzed with parsimony analysis using PAUP 4.0 with default settings. From the phylogenetic trees the A_t and D_t homeologs were identified or inferred when possible, and other paralogs were also identified. Primers were designed to amplify about 200 bp products that contain several SNPs (single nucleotide polymorphisms) which can be used to distinguish homeologs with SSCP (Single Strand Conformation Polymorphism). There were no introns in the amplified regions so that amplification from genomic DNA could be used as a control for band locations on the gels.

2.2 Plant materials and stress treatments

Gossypium hirsutum cultivar TM1 (Texas Marker-1), and a synthetic allopolyploid created by doubling the chromosomes of a *G. arboreum* × *G. davidsonii* F_1 hybrid (studied in Adams et al., 2004) were used for expression experiments. For *Gossypium hirsutum* cultivar TM1, the stress treatments were separated into two sets. The first set included water-submersion stress, cold stress and control plants as grown in Liu and Adams (2007), and the second set included heat stress, salt stress, drought stress and control plants. For each stress treatment and control, there were three replicates, and for each replicate, there were six plants. The seedlings were grown in a peat-vermiculite soil mixture under fixed day/night (16 hour day/ 8 hour night) and temperatures of 24℃. For water-submersion stress, the seedlings were completely submerged under distilled water for 2 days to simulate flooding; for cold-stress treatment, the seedlings were put in a cold room (4℃) for 2 days; both stress treatments were done in Liu and Adams (2007). For heat-stress treatment the seedlings were put in a growth chamber at 43℃ for 2 days, which is an intermediate temperature between 41℃ (Law et al., 2001) and 45℃(Fender and O'Connell, 1989); for drought-stress treatment, the seedlings were removed from the pots and placed on a dry filter paper for 2 days, as done in Jin and Liu (2007); for salt-stress treatment, the roots of the seedlings were submerged in an aqueous soil-extract solution with 400 mM NaCl for 2 days, as performed in Jin and Liu (2007). All the above stresses were performed from 6 to 8 days post-planting (dpp). For the stress treatments, all the other conditions of plant growth conditions were the same as the controls. After the stress

treatments, the abnormal seedlings (compared with the other seedlings in the replicate) were discarded; this was never more than 2 out of 6 seedlings per replicate. For the synthetic *Gossypium* allopolyploids, plant growth, cold stress, heat stress and salt stress were performed from 6 to 8 days post-planting (dpp) as above.

2.3 Nucleic acid extraction, reverse transcription and polymerase chain reaction

Genomic DNA was extracted with the QIAGEN DNeasy Plant Mini Kit according to the manufacturer's instructions, and RNA was extracted from tissues with a hot borate method (Adams et al., 2003). DNA and RNA concentrations and purities were measured with a NanoDrop spectrophotometer. RNA quality was checked on an agarose gel to identify strong ribosomal RNA bands.

RNAs were treated with DNaseI to remove genomic DNA before reverse transcription, and single-stranded cDNA was synthesized by M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Negative controls were made with DEPC-treated water instead of reverse transcriptase to check for any remaining genomic DNA.

PCR conditions were optimized with genomic DNA for each primer. PCR reactions were performed in a reaction mixture (10 µl) containing 1µl of template (genomic DNA or cDNA), 1 µl of 10X PCR buffer, 0.1 µl of 2.5 mM dNTPs, 0.5 µl of each 0.4

uM forward and reverse primer, 0.1 µl of Paq polymerase (Stratagene) and 6.8 µl of ddH_2O. 35 cycles of PCR were carried out, and the conditions for each cycle were as follows: 30 seconds at 94℃, 30 seconds at the optimized annealing temperature, 1 minute at 72℃. PCR products were run on 2% agarose gels (0.5×TBE buffer) for band separation and stained with ethidium bromide for visualization.

2.4 SSCP (Single Strand Conformation Polymorphism)

After PCR amplification, QIAGEN quick spin columns were used for cleaning PCR products to remove primers, and around 2-4 ng of clean, double-stranded PCR product was used for SSCP labeling reactions. The labeling reactions were performed in a reaction mixture (10 µl) containing 4 ng of double-stranded PCR product (diluted to 6 µl with water), 0.5 µl (10 uM stock) primer, 1.0 µl 10 X PCR buffer, 0.8 µl (2.5mM) dNTPs, 0.5 µl (50mM) $MgCl_2$, 1.04 µl dH2O, 0.08 µl Taq polymerase and 0.08 µl (32P) dCTP, 3000 Ci/mmol stock. The labeling conditions were as follows: 2 minutes at 94℃; 25 cycles of 30 sec at 94℃, 30 sec at 54-57℃ (depending on predicted primer Tm), 1 min at 72℃; a final extension for 5 minutes at 72℃

After labeling, the samples were denatured by mixing 2 ul of PCR product with 8 µl of denaturation solution (0.05% bromophenol blue, 0.05% xylene cyanol, 95% formamide, 20 mM EDTA), heated to 95℃ for 5 minutes, then snap cooled on ice until loaded on the gel. Up to 4 µl of each sample was loaded into polyacrymide gels

(25% MDE® gel solution, from Lonza, 0.1 X TBE, 0.033% g/v ammonium persulfate and 0.05% v/v TEMED). Electrophoresis was performed in 0.5 X TBE at 8W for approximately 20 hours at room temperature. Following electrophoresis, the gels were dried and placed on phosphorimaging screens for 2 days and then the images were visualized on a Bio-Rad phosporimager. Band intensities of homeologs were measured using Quantity One software (Bio-Rad) with background corrections.

2.5 DNA sequencing to identify homeologs

After PCR amplification, the products were purified by QIAGEN columns, and then loaded into the SSCP gel without isotope labeling. After the electrophoresis was finished, the gel was fixed in 10% acetic acid for 30 min, washed with distilled water three times, and then incubated in silver staining solution (0.1% silver nitrate and 0.15% formaldehyde) for 30 min, briefly washed with distilled water, and incubated in pre-cooled developing solution (3% Na_2CO_3, 0.15% formaldehyde, 0.024% Na_2SO_3). When clear bands were seen, the developing solution was poured out and 10% acetic acid was added to stop development.

For DNA sequencing, a single silver-stained band was directly excised from the gel and was vigorously rinsed with 500 μl extractant (2.5% sodium thiosulfate, 1% potassium hexacyanoferrate) for 5 min to remove the silver from gel. The rinsing process was repeated five times until the gel became transparent. After another rinse with distilled water, the DNA was extracted from the gel with 30 μl of distilled water

at 80℃ for 15 min. The DNA was re-amplified with a 20 cycle PCR and subjected to Sanger sequencing on an ABI 3730 with ABI Big Dye Version 3.1 (Applied Biosystems) at the Nucleic Acid Protein Service Unit at the University of British Columbia.

2.6 Statistical analysis

SSCP data from three replicates, or in a few cases two replicates, were analyzed for each stress treatment and organ type. T tests, one-way ANOVA, and two-way ANOVA tests, performed with R software, were used for determining significant differences in expression ratios (the percentage of transcripts derived form the At homeolog vs the Dt homoelog) between normal plants and abiotic stress treated plants. The stress-specific significant changes were determined by t-tests, and organ-specific significant differences were determined by one-way ANOVA and two-way ANOVA tests. 5% false discovery rate (FDR) was used to correct the multiple testing results.

3 RESULTS

3.1 Identification of duplicated genes in *Gossypium* species

I assayed homeologous gene expression in response to abiotic stress conditions using a total of 30 gene pairs. Genes and their functions or putative functions are listed in Table 1. I used 12 genes from polyploid *Gossypium* species (*G. hirsutum* and *G. barbadense*) that had previously been shown to be up- or down-regulated by one or more abiotic stresses (Zhao et al., 2003; Qin et al., 2004; Wang et al., 2007; Ni et al., 2008; Guo et al., 2009). Also I used cotton homologs of 18 genes known to be up- or down-regulated in *Arabidopsis thaliana* in response to abiotic stresses, obtained from microarray studies (William, 2006; Kilian et al., 2007). The cotton homologs were identified by using tblastx searches of expressed sequence tags (ESTs) from GenBank with the gene from *Arabidopsis* as a query. I attempted to find homologs from polyploid *G. hirsutum*, the A-genome diploid *G. arboretum* which is similar to the A-genome progenitor, and the D-genome diploid *G. raimondii* which is similar to the D-genome progenitor.

Paralogs and homeologs of each gene within *Gossypium* were identified by constructing phylogenetic trees from sequence alignments. Paralogs were identified by separate and distinct clades in the trees. Homeologs, designated At for the one

derived from the A-genome parent and Dt for the one derived from the D-genome parent, were identified by finding *G. hirsutum* sequences that branch with sequences from the diploids *G. raimondii* and *G. arboreum*, indicating Dt and At homeologs, respectively. In some cases a sequence from one of the diploids, more often *G. arboreum*, was not available due to the lower number of available ESTs, and the corresponding homeolog from *G. hirsutum* was inferred by the phylogenetic tree structure.

Two examples of the phylogenetic trees are shown in Figure 1. In Figure 1A, AV823701 is a drought, cold and salt stress responsive gene from *Arabidopsis thaliana*, which was defined as an outgroup together with sequences from *Brassica napus* and *Populus trichocarpa*. The topology of the phylogenetic tree suggests the relationship between the sequences from the A-genome diploid and its counterpart A-subgenome (At) of the allotetraploid as well as the relationship between sequences from the D-genome diploid and its counterpart D-subgenome (Dt) from the allotetraploid. The sequence alignment in Figure 1C showed a high similarity between the At and Dt homeologs except for several SNPs (single nucleotide polymorphisms), which were used for SSCP (next section). In Figure 1B, AY572463 is a salt-stress related gene from *Gossypium hirsutum* and a paralog was used in my study. The homeologous relationships among the cotton sequences were inferred by the placement of the *G. hirsutum* sequences compared with the sequences from the diploids.

3.2 Duplicate gene expression in response to cold and water submersion stresses in *G. hirsutum*

To study the expression of 30 homeologous gene pairs, the single stranded confirmation polymorphism (SSCP) method was used (Cronn and Adams, 2003). SSCP can separate duplicated genes with very similar sequences and the technique has been used previously for assaying homeologous gene expression (e.g., Adams et al., 2003; Pumphrey et al., 2009). PCR primers were designed to co-amplify the two homeologs from *G. hirsutum*, a region of the genes with SNPs between the two homeologs (Figure 1; Table 2). RT-PCR was conducted for each gene pair and the products were separated by SSCP. For each gene, the SSCP gel conditions were optimized by adjusting the running time and urea concentration (Table 2) so that two bands were visible for each gene, one for each homeolog, when a single labeling primer was used (or four bands, corresponding to both strands if two labeling primers were used). Figure 2 shows examples of SSCP gels. The two bands were quantified with a PhosphorImager and sequenced to determine which was the At copy vs. the Dt copy

The stress treatments of *G. hirsutum* seedlings were divided into two sets. The first set included assaying homeologous gene expression patterns in response to cold stress and water submersion stress as a follow-up to Liu and Adams (2007), with more genes, using RNAs from that study. Among the 30 homoeologus gene pairs that were

examined by SSCP separation and PhosphorImager quantification, 15 gene pairs showed significant changes in the At/Dt expression ratio in response to stress treatments in at least one organ, among which 10 genes showed changes in At/Dt expression in at least one organ under cold stress treatment and 10 gene pairs showed changes in At/Dt expression in at least one organ after the water-submersion stress treatment. This indicates that the changes in expression patterns of homeologous genes varied under different stress treatments. Five genes showed significant responses to both cold and water submersion stresses. Among the three different organ types examined, 11 genes showed changes in the At/Dt expression ratio in hypocotyls under at least one stress treatment, seven genes in cotyledons, and seven genes in roots. Three genes showed changes in all three organs, four genes showed changes in two organs and eight genes showed changes in one organ under at least one stress treatment.

Among the 12 homeologous genes that showed changes in the At/Dt expression ratio in response to cold and water submersion stresses in at least one organ, 5 genes showed large changes, where the difference between stress and control is more than 20% (for example, 60:40 vs. 80:20) after abiotic stress treatments (Figure 3). Among the largest changes, gene 7 (an aminomethyltransferase) showed a great increase in the At/Dt expression ratio in hypocotyls under cold stress treatment and the Dt copy was almost silenced (At/Dt ratio 90:10) compared with equal expression of both copies under normal conditions. The other gene with the largest change was

gene 9 (coenzyme binding function) that showed a dramatic decrease in the At/Dt expression ratio in hypocotyls under cold stress treatment because the expression of At copy was strongly depressed (At/Dt ratio 15:85) compared with preferential expression of the At copy under normal conditions (At/Dt ratio 65:35). Another example of a gene with a large change in expression in response to the stresses was gene 14 (a MAP kinase) that showed a considerable decrease in the At/Dt ratio in cotyledons under water-submersion stress treatment from preferential expression of the At copy (At/Dt ratio 74:26) under normal conditions to almost equal expression of both copies under water submersion stress. Two genes showed reciprocal changes in the At/Dt expression ratio; that is, an increase under one stress and a decrease under the other stress. Gene 11 (a peptide methionine sulfoxide reductase) showed reciprocal changes in the At/Dt expression ratios in response to cold and water submersion stresses: there was an increase in the At/Dt ratio after cold treatment in hypocotyls (83:17, from 65:35 under normal conditions) and a decrease in the At/Dt ratios after water submersion treatment in hypocotyls (to almost 50:50). Also, gene 20 (a late embryogenesis-abundant protein) also showed reciprocal changes in the At/Dt expression ratio in response to these two stresses, where in cotyledons the expression ratio greatly increased (80:20) after cold stress compared to normal conditions (53:47) but decreased after water-submersion stress (40:60).

3.3 Duplicate gene expression in response to heat, salt and drought stresses in *G. hirsutum*

After having documented many genes with changes in the At/Dt expression ratio in response to cold and water submersion stresses, we decided to examine three other abiotic stresses. The second set of stresses included three abiotic stress treatments: heat, salt and drought stress. The expression patterns of 30 homeologous genes were examined in three organ types of *G. hirsutum* seedlings (Figure 4). Twenty genes showed changes in the At/Dt expression ratio in at least one organ after one of the abiotic stress treatments. Among the 20 homeologous genes, 14 gene pair showed changes in at least one organ under the heat stress treatment, 15 genes showed changes under the salt stress treatment, and 10 genes showed changes under the drought stress treatment. Also the expression patterns of the homeologous genes in response to heat, salt and drought stresses were compared in different organ types. Eleven gene pairs showed changes in the At/Dt expression ratio in hypocotyls under at least one stress treatment, 14 gene pairs in cotyledons, and 10 gene pairs in roots.

Of the 19 homeologous genes that showed significant changes in the At/Dt expression ratio in response to abiotic stresses, 10 genes showed large (over 20 percent; e.g, 60:40 to 80:20) changes after abiotic stress treatments. Among them, two genes showed dramatic increases (genes 10 and 20). Gene 10 (a phenylcoumaran benzylic ether reductase-like protein) showed large increases in the At/Dt ratio under different stresses. In hypocotyls and cotyledons, under heat stress treatment, expression was

greatly biased towards the At copy (At/Dt ratio 82:18) compared with strongly biased Dt expression (At/Dt ratio 20:80) under normal conditions. In roots, under drought stress treatment, there was also a very large increase (At/Dt ratio 85:15) compared with normal conditions (At/Dt ratio 18:82). Gene 20 (a late embryogenesis abundant protein) showed large increases in the At/Dt ratio from biased expression of the Dt copy (At/Dt ratio 30:70) to preferential expression of the At copy (At/Dt ratio 62:38) in all three organs after heat stress treatment. Three genes showed reciprocal expression changes in response to stress treatments. Gene 19 (a zinc finger family protein) showed reciprocal expression ratio changes in cotyledons in response to heat stress and salt stress treatments: there was an increase in the At/Dt ratio after heat stress treatment (70:30, from 40:60 under normal conditions) and a decrease after salt stress treatment (At/Dt ratio 17:83). Gene 15 (MAP kinase 6) also showed reciprocal expression ratio changes in cotyledons: there was an increase after heat stress treatment (75:25, from 45:55 under normal conditions), and decreases after salt stress treatment (At/Dt ratio 37:63) and drought stress treatment (At/Dt ratio 32:68). Finally gene 14 (a MAP kinase) showed reciprocal changes under salt stress among different organs: there was an increase in cotyledons (At/Dt ratio 35:65 from 10:90 under normal conditions) and a decrease in roots (At/Dt ratio 15:85 from 31:69 under normal conditions).

3.4 Comparisons of duplicate gene expression in response to five abiotic stresses

Overall, among the 30 homeologous genes examined after abiotic stress treatments, 22 genes showed significant changes in the At/Dt expression ratio after abiotic stress treatments in at least one organ type compared with the untreated control plants. Cold, water-submersion, heat, salt and drought stresses induced 10, 10, 14, 15, and 10 homeologous gene expression ratio changes, respectively, in at least one organ, which indicates that heat and salt stresses had the largest effect on duplicate gene expression levels. Among the three different organ types examined, 17 genes showed changes in At/Dt expression ratio after at least one stress treatment in hypocotyls, compared with 16 genes in cotyledons and 13 genes in roots.

Among the five abiotic stress treatments there was variation in the number of genes that showed organ-specific changes: 4 homeologous gene pairs (genes 6, 9, 11, and 19) after cold stress, 6 homeologous genes (genes 6, 11, 12, 16, 18, and 19) after water-submersion stress, 3 homeologous genes (gene 8, 10, and 15) after heat stress, 2 homeologous genes (genes 6 and 20) after salt stress, and 4 homeologous genes (genes 9, 10, 17, and 26) after drought stress. Overall, 7% (under salt stress treatment, 2 out of 30 genes) to 20% (under water-submersion stress treatment, 6 out of 30 genes) of the homeologous genes were influenced in an organ-specific manner under abiotic stress treatments, and 7% (2 in 30 genes) showed organ-specific, reciprocal, homeologous expression changes (organ-specific quantitative subfunctionalization)

under abiotic stress treatments. These results indicate that abiotic stress-induced, organ-specific, expression changes and quantitative subfunctionalization may be a common phenomenon in polyploid *G. hirsutum*.

Further analyses of the expression pattern changes of the 30 homeologous genes are shown in Table 3. Seven genes showed reciprocal At/Dt ratio changes in one organ after different abiotic stress (stress specific) treatments ("+" and "—" in same row: genes 4, 10, 11, 14, 15, 19 and 20), and 3 genes showed reciprocal At/Dt ratio changes among different organs (organ-specific) after one stress treatment ("+" and "—" in same column: gene 3 under water-submersion stress, gene 11 under cold stress and water-submersion stress, and gene 14 under salt stress). Two genes showed both the stress-specific and organ-specific reciprocal At/Dt ratio changes (genes 11 and 14).

All comparisons of results from the abiotic stress treatments above were made between the stressed and the untreated control plants from within a set. The absolute At/Dt ratios between sets could not be compared because the plants in the two sets were grown at the different times. Thus, all of the comparisons of the five stress treatments were relative comparisons based on comparing the stress treatment with the corresponding control grown under normal conditions, not the stress treatments compared directly to each other. There were a few genes that showed differences in homeologous gene expression under untreated normal conditions between the two sets. The inconsistency might be explained by differences in the developmental stages of

the plants upon tissue harvesting between the two sets, perhaps caused by differences in seed germination rates. Homeologous gene expression can vary during organ development, as shown in Liu and Adams (2007).

3.5 Duplicate gene expression in response to abiotic stresses in a synthetic *Gossypium* allotetraploid

Synthetic allopolypoids are useful for examining genetic changes that take place immediately upon and within a few generations after allopolyploidy. The expression patterns of 10 homeologous genes that showed significant changes after one or more abiotic stress treatment in natural allopolyploid *G. hirsutum* were examined in a synthetic allopolyploid, designated 2 (A2D3), under salt, cold and heat stress treatments (Figure 5). Eight genes showed changes in the At/Dt expression ratio in at least one organ after one or more abiotic stress treatments. Among the 8 genes, 7 genes showed expression changes under salt stress treatment in at least one organ, while 3 genes were affected by cold stress treatment, and 2 by heat stress treatment. In hypocotyls, 6 genes showed changes in response to at least one abiotic stress treatment, in cotyledons it was 5 genes, but in root no genes showed changes in At/Dt expression ratios, surprisingly.

Among the 8 homeologous genes that had At/Dt ratio changes, only 2 of them showed large changes (20% or greater; for example, 60:40 to 80:20) in response to stress. Gene 8 (GDSL-motif lipase) showed a considerable decrease in the At/Dt ratio in

hypocotyls after heat stress treatment (At/Dt ratio 27:73) compared with the normal conditions (At/Dt ratio 55:45) in the synthetic allopolyploid, whereas in natural allopolyploid *G. hirsutum*, there were dramatic decreases in At/Dt ratio that happened in cotyledons under both heat (At/Dt ratio 38:62) and salt (At/Dt ratio 40:60) stresses compared with normal conditions (At/Dt ratio 62:38). Gene 20 (late embryogenesis-abundant protein) showed large increases under salt stress treatment in hypocotyls (At/Dt ratio 74:26) and cotyledons (At/Dt ratio 68:32) compared with normal conditions (At/Dt ratio 48:52), which was similar to the expression pattern changes in natural allopolyploid *G. hirsutum* under heat stress treatment, but the dramatic changes in response to cold and heat stress treatments seen in natural *G. hirsutum* were not found in the synthetic allopolyploid.

Comparison of the expression patterns of the 10 homeologous genes between the natural and synthetic *Gossypium* allopolyploids showed that only 2 genes with parallel expression changes under certain stress treatments in certain organs: gene 12 showed a decrease in the At/Dt ratio under salt stress in cotyledons, both in natural and synthetic *Gossypium*, but changes in other organs and stress treatments were not in parallel; and gene 20 showed increases in the At/Dt ratio under salt stress in hypocotyls and cotyledons in both natural and synthetic *Gossypium*, but changes in other organs and stress treatments were in parallel. All the other genes showed different expression patterns between natural and synthetic *Gossypium* allopolyploids.

Another interesting finding is the large differences in the At/Dt ratios in all organs under all stresses between the synthetic and natural allopolyploids for gene 4 and gene 12. Gene 4 (ATP synthase Delta Chain) showed great down-regulation or even silencing of the At copy in all the organs under all the stresses in the synthetic allopolyploid compared with the almost equal expression of both copies in natural *G. hirsutum.* Gene 12 (ethylene responsive element binding protein) also showed considerable down-regulation of the At copy (although not as dramatic as gene 4) in the synthetic allopolyploid compared with the almost equal expression of both copies in natural *G. hirsutum*.

4 Discussion

4.1 Abiotic stress induced changes in homeologous gene expression and stress-specific quantitative subfunctionalization

In my project, five abiotic stresses were performed for the natural allopolyploid *G. hirsutum*, and the At/Dt expression ratio was compared with untreated control plants grown under normal conditions. Overall, in the 30 homeologous gene pairs examined by SSCP, 22 gene pairs (73%) showed At/Dt expression ratio changes under at least one stress treatment in at least one organ compared with the controls. Given the fact that all of the genes used in my study are abiotic stress-regulated or homologs of genes regulated by at least one abiotic stress, 73% might not be generally applicable when applied to the whole allopolyploid *G. hirsutum* transcriptome, but still we can get the sense that abiotic stress-induced duplicate gene expression changes are likely to be a major phenomenon in natural allopolyploid cotton. Among the 22 gene pairs that showed expression changes under abiotic stresses, 12 gene pairs had large changes, defined as an expression ratio difference of more than 20% (e.g., 60:40 to 80:20) compared with the control. Seven of those homeologous gene pairs had reciprocal expression changes where the At/Dt expression ratio was considerably up-regulated under one stress treatment and down-regulated under another stress treatment in the same organ compared with the control. An increase in the level of

expression of one duplicate gene relative to the other has been described as quantitative subfunctionalization (Force et al., 1999; Duarte et al., 2006). Quantitative subfunctionalization differs from qualitative subfunctionalization, where expression or function of duplicated genes is completely partitioned between the duplicates (Force et al., 1999; Hahn, 2009).

The abiotic stress treatments used in my study had effects on homeologous gene expression that varied by organ type. I found that 13 out of 30 homeologous gene pairs examined had organ-specific responses to the stress treatments. Among the 13 genes, 2 genes (gene 11 and gene 14) showed reciprocal changes in the At/Dt ratio among different organs under the same stress treatment, that is, in one organ there was an increase in the At/Dt ratio and there was a decrease in another organ after the same abiotic stress treatment, which indicates organ-specific quantitative subfunctionalization. Overall there were a total of 8 genes that showed quantitative subfunctionalization (3 organ-specific and 7 stress-specific, with two genes showing both).

In the only previous study of the effects of abiotic stress treatments on homeologous gene expression in a polyploid plant, qualitative subfunctionalization in response to stress conditions was discovered (Liu and Adams, 2007). In that study, one homeolog of the alcohol dehydrogenase gene *AdhA* was expressed in hypocotyls under water-submersion treatment and only the other copy was expressed in

hypocotyls under cold stress treatment (Liu and Adams, 2007). In my study I found many cases of stress-specific quantitative subfunctionalization, but no cases of qualitative subfunctionalization. These results suggest that stress induced quantitative subfunctionalization could be a prevalent phenomenon while stress-specific qualitative subfunctionalization could be relatively rare and may appear only for a small percentage of genes under certain organ and stress combinations. My findings of altered homeologous gene expression and quantitative subfunctionalization in response to abiotic stress treatments demonstrate that abiotic stresses can have a major influence on the expression of duplicated genes, which may help lead to the preservation of duplicated genes over evolutionary time.

In addition to the changes in homeologous gene expression in response to abiotic stress treatments, that were in some cases organ-specific, I also found evidence for organ-specific variation in the expression of the two homeologs in *G. hirsutum* under normal conditions. These results are consistent with previous studies of polyploid cotton and other polyploids (e.g., Adams et al., 2003; Adams et al., 2004; Hovav et al., 2007; Chaudhary et al., 2009). Cases of qualitative subfunctionalization were rare in the previous studies. Adams et al. (2003) showed two cases of organ-specific qualitative subfunctionalization out of 40 genes examined, and Hovav et al. (2007) showed 4 cases of developmental stage-specific subfunctionalization in 1484 genes in a microarray study. These results indicate that qualitative subfunctionalization probably is not a prevalent phenomenon in *G. hirsutum.*

The homeologous gene expression changes in response to abiotic stresses in *G.hirsutum* could have evolved after the polyploidy event that happened about 1.5 million years ago. It has been proposed that gene duplication can create a redundant locus and more mutations that were "forbidden" in the previous singletons are now allowed in polyploids because of relaxed selective constraints (Ohno, 1970; Lynch and Katju, 2004). In natural allopolyploid *G. hirsutum*, during the time since the polyploidy event genes could accumulate mutations in regulatory elements which could provide selective advantages in response to changing environments. Those mutations that were helpful in accommodating the organisms to the new and changing environmental stress conditions could be possibly fixed. That could be one explanation for the significant changes in the At/Dt ratios of some genes in response to abiotic stress treatments in the natural *Gossypium* polyploids.

4.2 Effects of abiotic stress on gene expression in synthetic vs. natural allopolyploids

In my experiments stress-induced homeologous gene expression changes were also found in the new synthetic *Gossypium* polyploids, where 8 of 10 genes examined showed changes in expression patterns in response to one or more abiotic stress treatments. Among the 10 genes examined, only 2 of them had similar expression pattern changes under certain stress treatments in certain organs between the two

genotypes, while the other 8 genes showed different expression pattern changes in response to stress treatments. The plants were grown under the same controlled environmental conditions and the stress treatments were done using the same conditions; thus, experimental variability is unlikely to account for the differences. Overall, there does not appear to be much correspondence between the stress-induced changes in homeologous gene expression in the synthetic and natural allopolyploids. This finding suggests that the phenomenon is not caused by merger of the A and D genomes in allopolyploid cotton *per se*. Instead it is likely that that the process is largely random in terms of which genes are affected. It is possible that the allopolyploidization process could have played a role in the homeologous gene expression changes in response to abiotic stress treatments in the synthetic polyploid. Previous studies have shown that gene silencing and biased expression can occur following the allopolyploidization in new synthetic *Gossypium* polyploids (Adams et al., 2004; Chaudhary et al., 2009). Also gene expression changes were also found in synthetic allotetraploid *Arabidopsis* and wheat (Madlung et al., 2002; Kashkush et al., 2002). These observations suggest that the immediate genetic or epigenetic changes caused by allopolyploidization might play a role in the stress-induced homeologous gene expression changes found in the synthetic allopolyploid.

My study did not determine if the stress-responsiveness of the eight homeolog pairs occurred after polyploid formation or it was present in the diploids from which the synthetic polyploid was derived. It could be interesting in the future to study gene

expression patterns under abiotic stress treatments in diploid parents compared with the interspecific diploid hybrids, and make comparisons among the diploids, diploid hybrids and the synthetic polyploids; such experiments could provide more information on the specific roles that genome merger and duplication played in stress-induced expression pattern changes.

4.3 Possible mechanisms of stress-induced expression changes in homeologous genes

Although the mechanisms for organ-specific and stress-induced expression alteration in natural and new synthetic allopolyploid cotton are not well understood, there could be many mechanisms responsible for these phenomena, such as *cis*-regulatory variation, regulatory network changes, genomic changes, and epigenetic changes (Birchler et al., 2003; Comai et al., 2000, Osborn et al., 2003; Riddle and Birchler, 2003). Below I discuss each of these possible factors in turn.

Cis-regulatory elements are major constituents effecting gene expression and they are predominantly located within the 5' upstream region of genes. Several studies have shown a positive correlation between *cis*-element variation and duplicate gene expression divergence. Haberer et al. (2004) showed that in *Arabidopsis thaliana* there was a continuous divergence within the regulatory regions after duplication, and Evangelisti and Wagner (2004) found both loss and gain of transcription factor

binding sites in yeast after gene duplication. The changes in the *cis*-regulatory elements played a significant role in duplicate gene divergence and could be an explanation for the expression differences between duplicate genes. That possibility was further supported by microarray studies in *Gossypium* that demonstrated that variation in gene expression following genome merger was the likely the result of *cis*-regulatory element changes (Flagel et al., 2008; Chaudhary et al., 2009). The relationship between *cis*-element changes and stress responsive duplicate genes has been investigated in *Arabidopsis thaliana*, Walter et al. (2007) found the density of *cis*-elements increased with the differential gene expression diversity in response to stresses, and Zou et al. (2009) showed that differences in *cis*-elements between duplicate genes played a role in expression variation in response to stresses. These observations from other studies collectively indicate that *cis*-regulatory elements may contribute to homeologous gene expression changes in response to abiotic stress treatments in the *Gossypium* polyploids examined in my study.

The expression of most genes depends on networks of regulators, such as transcription factors (Birchler et al., 2001; Osborn et al., 2003). In diploids, the regulation network is complex, but it is even more complex in the allopolyploids. For example in an allotetraploid, all of the regulators derived from the two diploids can interact to affect gene expression. The functioning of the regulators in allopolyploids depends on not only the two regulation networks, but also the interactions between the two networks (Osborn et al., 2003); thus allopolyploidy can result in changes in regulation networks

compared with the diploid progenitors. The changing regulation networks could in part explain some of the expression changes of duplicate genes in allopolyploids, and might be responsible for some of the expression patterns documented in this study.

There is evidence for rapid genetic changes in resynthesized allopolyploids of *Brassica* species (Song et al., 1995; Gaeta et al., 2008) and wheat (Liu et al., 1998; Ozkan et al., 2001; Shaked et al., 2001; Kashkush et al., 2002) where loss and gain of many DNA restriction fragments were found. Chromosome rearrangements, gene loss, point mutation, and gene conversion could be responsible for these observations (Song et al., 1995). Gene losses have been reported in synthetic wheat allopolyploids. Although most of the gene losses in newly synthesized allopolyploids had no homology to known genes and were probably noncoding sequences (Feldman et al., 1997; Liu et al., 1998; Ozkan et al., 2001; Shaked et al., 2001), Kashkush et al. (2002) found one gene (RAIF32) which was identical to wheat *Acc-2* gene was missing from tetraploid wheat. Chromosome rearrangements have been reported in synthetic *Nicotiana tabacum* and *Brassica napus* allopolyploids (Lim et al., 2006; Gaeta et al., 2007) and reciprocal exchanges between homeologs have been detected in *Brassica* allopolyploids (Gaeta et al., 2007). In contrast to other polyploid systems that have been studied, cotton does not show gene loss or chromosome rearrangements (Liu et al., 2001); thus such phenomena are unlikely to be responsible for the gene expression patterns shown here in synthetic cotton allopolyploids.

Also epigenetic changes could play a role in the duplicate gene expression changes in polyploids such as histone modifications and DNA methylation (Osborn et al., 2003; Chen., 2007). Studies revealed that there were changes in DNA methylation patterns between allopolyploid and the diploid parents in *Spartina* (Salmon et al., 2005) and synthetic *Arabidopsis* allotetraploids (Madlung et al., 2002). Notably, treatment of aza-dC, a chemical inhibitor for DNA methylation, reactivated some genes that were active in parent plants and fully or partially silenced in synthetic *Arabiodopsis* allotetraploid (Madlung et al., 2002). Chen et al. (2008) showed many previously identified cases of genome specific gene silencing were caused or related to methylation by RNAi of *met1*, which is a cytosine methyltransferase. These studies demonstrated that epigenetic changes could in part explain the expression changes of duplicate genes in allopolyploids compared with their progenitors. Although rapid epigenetic changes were not found in synthetic *Gossypium* allopolyploids (Liu et al., 2001), epigenetic changes could still in part explain the homeolog expression changes in other allopolyploids compared with their progenitors.

4.4 Conclusions

In this study, I found that abiotic stresses can have a considerable effect on homeologous gene expression both in natural and synthetic *Gossypium* polyploids. In

natural *G. hirsutum*, different expression pattern changes were found among the 30 homeologous genes in response to abiotic stress treatments. Also for some genes, there were different expression changes under different abiotic stress treatments. Even under the same abiotic stress treatment, different expression changes were found among organ types. In natural *G. hirsutum* I found several cases of stress-induced quantitative subfunctionalization, suggesting that stress-induced quantitative subfunctionalization is relatively common. In the synthetic *Gossypium* polyploids, I also found stress-specific and organ specific expression changes in response to abiotic stress treatments, and there was little correspondence in the homeologous gene expression changes in response to abiotic stresses in natural and synthetic *Gossypium* polyploids.

Gene #	Function or putative function	Accession#
1	Ultraviolet-B-repressible protein	DV850210
2	photosystem II subunit X	ES839555
3	photosystem II subunit X	DN780680
4	ATP Synthase Delta Chain	AI728201
5	ATP Synthase Delta Chain	ES811921
6	Aminomethyltransferase	ES804546
7	Aminomethyltransferase	DW242796
8	GDSL-motif lipase	ES845029
9	Coenzyme binding	DN780968
10	Phenylcoumaran benzylic ether reductase-like protein	DW484270
11*	Peptide methionine sulfoxide reductase	DW227959
12*	Ethylene responsive element binding protein ERF2	ES809854
13*	Ethylene responsive element binding protein ERF2	ES837957
14*	MAP kinase	DT558090
15*	MAP kinase 6	EX167377
16*	Alcohol dehydrogenase B	AF226633
17*	Alcohol dehydrogenase C	AF036575
18*	Alcohol dehydrogenase D	AF250204
19	Zinc finger family protein	DT571829
20	Group 4 late embryogenesis-abundant protein	DW514110
21	Unknown function	DW505445
22	Cold regulated plasma membrane	DW504511
23	Unknown function	DW505445
24*	Ethylene responsive element binding protein ERF2	DT459748
25*	Peptide methionine sulfoxide reductase	DW487846
26	Manganese ion binding	DR454806
27	Aminomethyltransferase	DT545047
28	Transferase, transferring hexosyl groups	DW482030
29*	Zinc finger transcription factor	AY887895
30*	Homeobox protein	EF151309

Table 1 List of *Gossypium* genes assayed for homeologous gene expression

Accession # refers to a GenBank accession ID. One accession number is provided per gene.

*Paralogs of stress related genes from polyploid *Gossypium* (Zhao et al., 2003; Qin et al., 2004; Wang et al., 2007; Ni et al., 2008; Guo et al., 2009). The other genes are homologs of stress regulated genes from *Arabidopsis thaliana* (William., 2006; Kilian et al., 2007).

Gene #	Forward Primer	Reverse Primer	Urea%
1	GCTCCAACAATGGCGACCAGAA	GGGCGGTGTTAAAAGACCCAAC	5%
2	CTGAGGCATTCTTTAAGCCACT	GACACACCAATTACGGCTCCAA	5%
3	CAAGGAAAAGGCGGTGACGGGG	AGGTCCGCTTGACAGGGTCGAA	5%
4	CCAACAACACCCTTGATGCCAC	TGTAAACCAACTCGAACTCTTT	5%
5	GTCCAACAACACCCTTGATGCC	GCCAGCTCTGTATCCGTCAGCT	5%
6	GAAGTCTTTGGCAACTTGGGCA	CCACCATTCTCCCTGCAATTCA	2%
7	GGCAATGACATGGAACAACACA	GGCTAAATCCTCCGCTGGTGAT	5%
8	ACAACAGCAAGCGAAGCATGTT	TCAAAGATTACGAAGTTGTT	2%
9	GTTGATCCGAAGAGTAGGCACA	GGAATTGGAATAGGGCGACC	5%
10	ATGGCTGAAAAGAGCAAGGTTT	GTACATATCCCCTAACAACAAA	5%
11	GGAATGATGTGGGAACACAGTA	TGCTGGTGATATTCCTCTGCTC	2%
12	ATCAATTGTTCCGACTATGGCT	CAACAGCTCGTCCGACAAGGTC	5%
13	CTGCGAGAGCTTATGATGCTGA	ATAGACGTAGGAACAGGGTCCA	5%
14	GTACTTTCTATATCAGATTTTA	ATGCACATAGTCTCTTCCAGGG	2%
15	ATCTTACGTGGGCTGAAGTATA	CGATCCATCAATTCCATAAAAA	2%
16	GAGGGCGTGACTGATCTAAAGC	GGCGACCTGACCGACATGGACG	5%
17	TCATGTTCTACCTATCTTCACC	CCTGGCCGACATGGACAACAGT	2%
18	TGTGGGTGAGGGTGTGACAC	TAGAGATGCCACAGCTGAGAAT	5%
19	ATGGCATCGAAGTTGTGTGACTC	AGCAAGAGGGTTGGCGGAGTGGA	5%
20	GGGATAGCATCAGGGACAATAC	GAGAGGGATAGTGAAGTTCCCC	2%
21	ATGTTTGAGCATAAGGAACTTG	AATGAGAGTGAGAATAAGATAG	2%
22	GATTGGCTGGAACTTCCTGGAG	CAAGCAGGGTAAACCAACAGAA	5%
23	TCAGTGGTTTTCATTCTGCTTA	GATTAAGATGGAAGGGATAAAG	2%
24	ATGGTCAAGCTGAGAAATGTG	CTCGTTAGGGAAGTTCACCTT	5%
25	TCTAGGCTTCGGAGCCAACAAC	CCGTACACACGTCTTCATAGCT	5%
26	ACACCAGCCTTTTCGGCTCAAT	TTGCCTGCATTGATTTGGAAAT	5%
27	ATCATGGACTCAACTGTGAATT	CAATGTGAGCCAGATCCTTATC	2%
28	TACTTGGACGGCGACATTCAGG	GCAAATGGGGTAGGTTGTGTGA	5%
29	ATTTCCGTATGTACGAGTTCAA	ACAACAAGTACCGTCCTTGCAT	5%
30	GGGGAGGATGATTTATCAGATG	AGCTTTTGGTTCTGAGCTTGGA	5%

Table 2 Primers used for gene amplification and urea concentration for SSCP. Primer sequences are listed 5' to 3'.

Gene1	CS	WS	HS	SS	DS	Gene11	CS	WS	HS	SS	DS	Gene21	CS	WS	HS	SS	DS
Hypocotyl	0	0	0	0	0	Hypocotyl	+	−	0	0	0	Hypocotyl	0	0	0	0	0
Cotyledon	+	0	0	0	0	Cotyledon	+	+	0	0	−	Cotyledon	0	0	0	0	0
Root	0	0	0	0	0	Root	−	0	0	0	0	Root	0	0	0	−	0
Gene2						Gene12						Gene22					
Hypocotyl	0	0	0	0	0	Hypocotyl	0	0	0	0	0	Hypocotyl	+	0	0	+	0
Cotyledon	0	0	0	0	0	Cotyledon	0	0	−	−	0	Cotyledon	0	0	0	0	0
Root	0	0	0	0	0	Root	0	0	0	0	0	Root	0	0	0	0	−
Gene3						Gene13						Gene23					
Hypocotyl	0	−	0	0	0	Hypocotyl	0	0	0	0	0	Hypocotyl	0	0	0	+	0
Cotyledon	+	+	+	0	+	Cotyledon	0	0	0	0	0	Cotyledon	0	0	+	0	0
Root	0	0	0	0	0	Root	0	0	0	0	0	Root	0	0	0	0	−
Gene4						Gene14						Gene24					
Hypocotyl	0	+	−	−	−	Hypocotyl	0	0	+	0	0	Hypocotyl	−	0	0	0	0
Cotyledon		0	0	0	0	Cotyledon	0	−	+	+	+	Cotyledon	0	0	0	0	0
Root	−	0	0	0	0	Root	0	−	0	−	0	Root	0	0	0	0	0
Gene5						Gene15						Gene25					
Hypocotyl	0	0	+	0	0	Hypocotyl	0	0	0	0	0	Hypocotyl	0	0	0	0	0
Cotyledon	0	0	0	0	0	Cotyledon	0	0	+	−	−	Cotyledon	0	0	0	0	0
Root	0	0	0	0	0	Root	0	+	0	−	0	Root	0	0	0	0	0
Gene6						Gene16						Gene26					
Hypocotyl	0	0	0	+	0	Hypocotyl	0	0	+	0	0	Hypocotyl	0	0	0	0	0
Cotyledon	0	0	0	0	0	Cotyledon	0	0	0	0	+	Cotyledon	0	0	−	0	0
Root	0	0	0	0	0	Root	0	+	+	+	0	Root	0	0	0	0	0
Gene7						Gene17						Gene27					
Hypocotyl	+	0	0	+	0	Hypocotyl	0	0	−	0	0	Hypocotyl	0	0	0	0	0
Cotyledon	+	0	0	0	+	Cotyledon	0	0	0	0	0	Cotyledon	0	0	0	0	0
Root	+	0	0	0	0	Root	0	0	0	−	0	Root	0	0	0	0	0
Gene8						Gene18						Gene28					
Hypocotyl	0	0	0	0	0	Hypocotyl	0	0	0	0	0	Hypocotyl	+	0	0	0	0
Cotyledon	0	0	−	−	0	Cotyledon	0	0	0	0	0	Cotyledon	0	0	0	−	0
Root	0	0	0	0	0	Root	0	0	0	0	0	Root	0	0	0	−	0
Gene9						Gene19						Gene29					
Hypocotyl	−	0	0	0	0	Hypocotyl	0	0	0	0	0	Hypocotyl	0	0	0	0	0
Cotyledon	0	+	0	0	0	Cotyledon	0	0	+	−	0	Cotyledon	0	0	0	0	0
Root	0	0	0	0	0	Root	0	0	0	0	0	Root	0	0	0	0	0
Gene10						Gene20						Gene30					
Hypocotyl	0	−	+	0	0	Hypocotyl	+	0	+	+	0	Hypocotyl	0	−	0	0	0
Cotyledon	0	0	+	0	0	Cotyledon	+	−	+	+	0	Cotyledon	0	0	0	0	0
Root	0	0	0	0	+	Root	+	0	+	0	0	Root	0	0	0	0	0

Table 3 At/Dt expression ratio changes between the stress treatment and normal conditions in *G. hirsutum*. "+" indicates an increase in the At/Dt ratio after stress treatment; "-" indicates a decrease in the At/Dt ratio after stress treatment; "0" indicates there is no statistical difference in the At/Dt ratio between the stress treatment and the untreated plants.

Figure 1 Phylogenetic trees and homeologous gene alignments

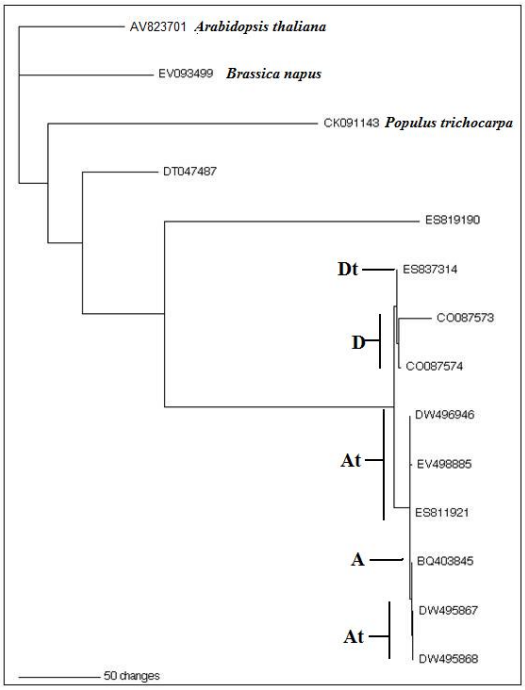
(A) Phylogenetic analysis of gene5 resulting from parsimony analysis, rooted with genes from *A. thaliana*, *B. napus* and *P. trichocarpa*. CO087574 and CO087573 are from *Gossypium raimondii* (DD genome diploid), and BG403845 is from *Gossypium arboreum* (AA genome diploid). All the other cotton sequences were from *G. hirsutum*.

(B) Phylogenetic analysis of gene 24 resulting from parsimony analysis, rooted with genes from *A. thaliana*, *B. napus* and *P. trichocarpa*. BG443984 is from *Gossypium arboreum* (AA genome diploid), CO081291,CO112016 and CO115074 is from *Gossypium raimondii* (DD genome diploid). All of the rest of the cotton sequences are from *G. hirsutum***.**

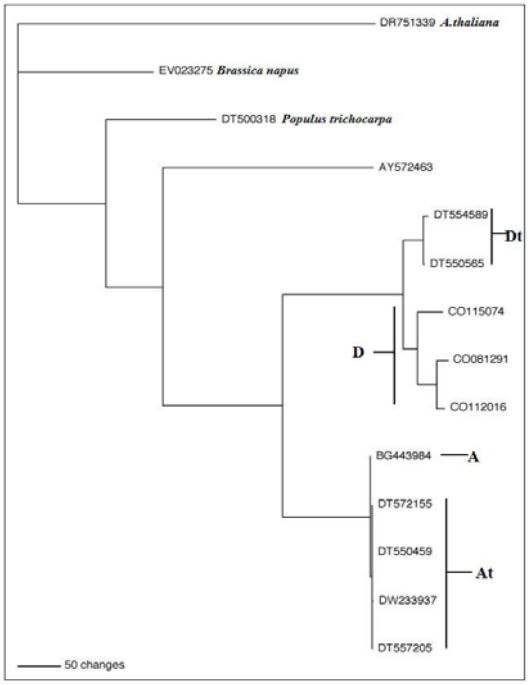
(C) Sequence alignment of a region of the At and Dt copies from gene 5. (D) Sequence alignment of a region of the At and Dt copies from gene 24.

Figure 1

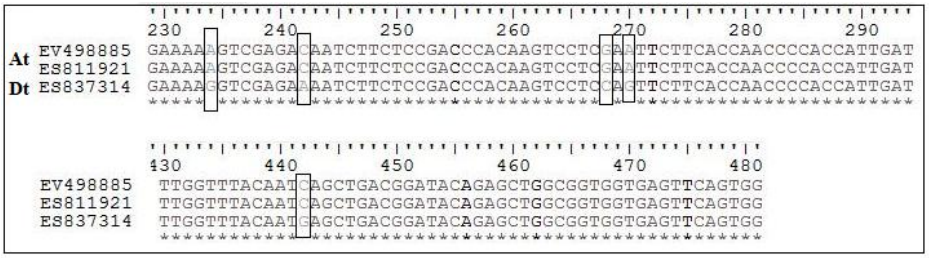
A



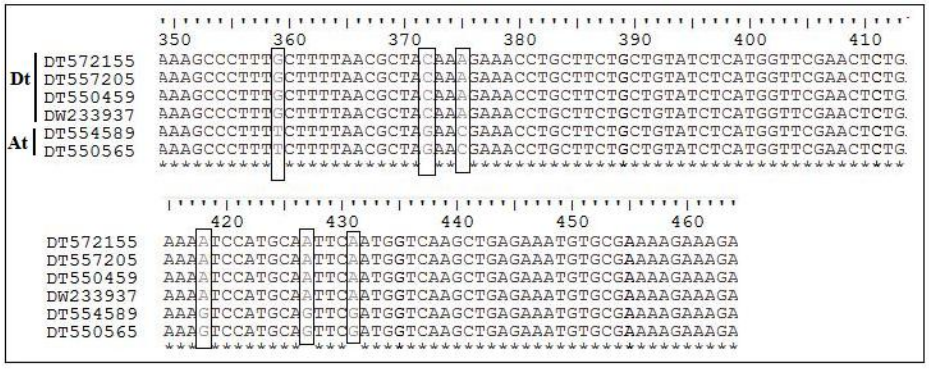
B



C



D



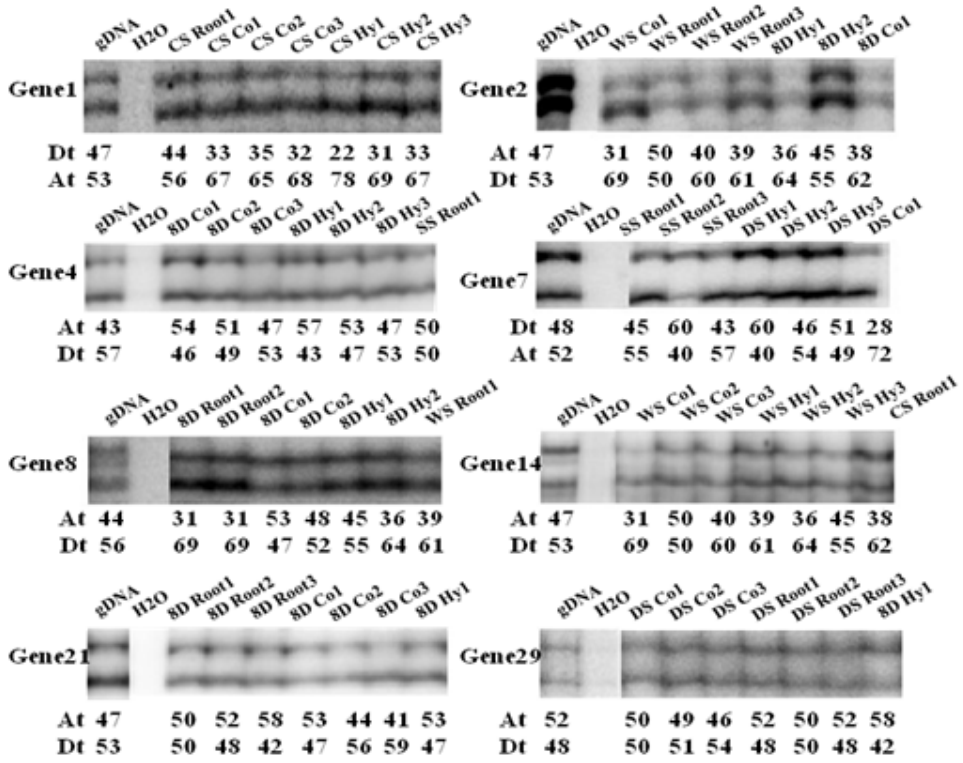


Figure 2 Examples of SSCP gel pictures. "At" and "Dt" indicate two homeologous genes from the A or D genome of the natural allopolyploid *G.hirsutum*. The numbers indicate transcript percentages as determined by the PhosphorImager. gDNA lane is the band position control and the H_2O lane is the PCR contamination control. CS indicates cold stress, WS indicates water-submersion stress, DS indicates drought stress,, SS indicates salt stress, 8D indicates untreated plants at 8 days. Hy indicates hypocotyls, and Co indicates cotyledons. The number 1, 2, 3 after the organ indicates replicates 1, 2, and 3, respectively.

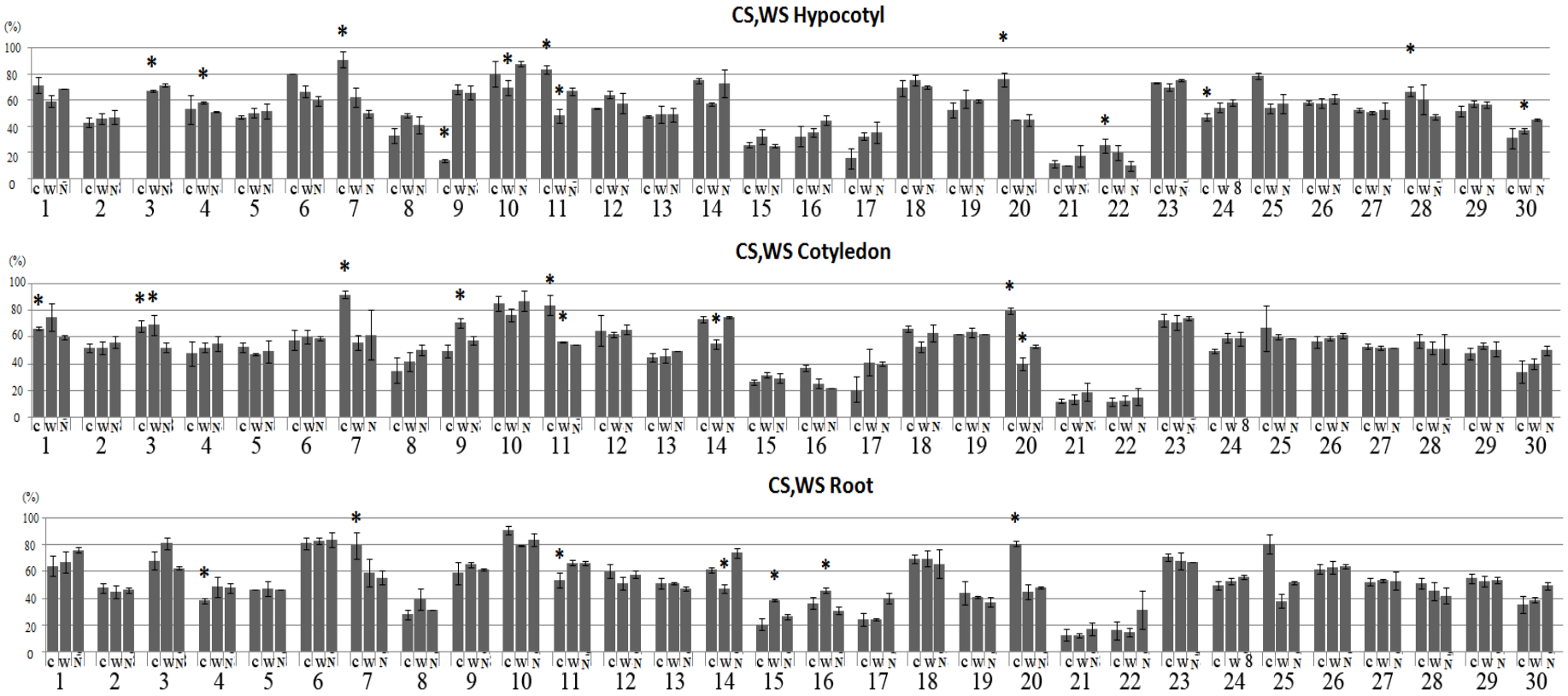
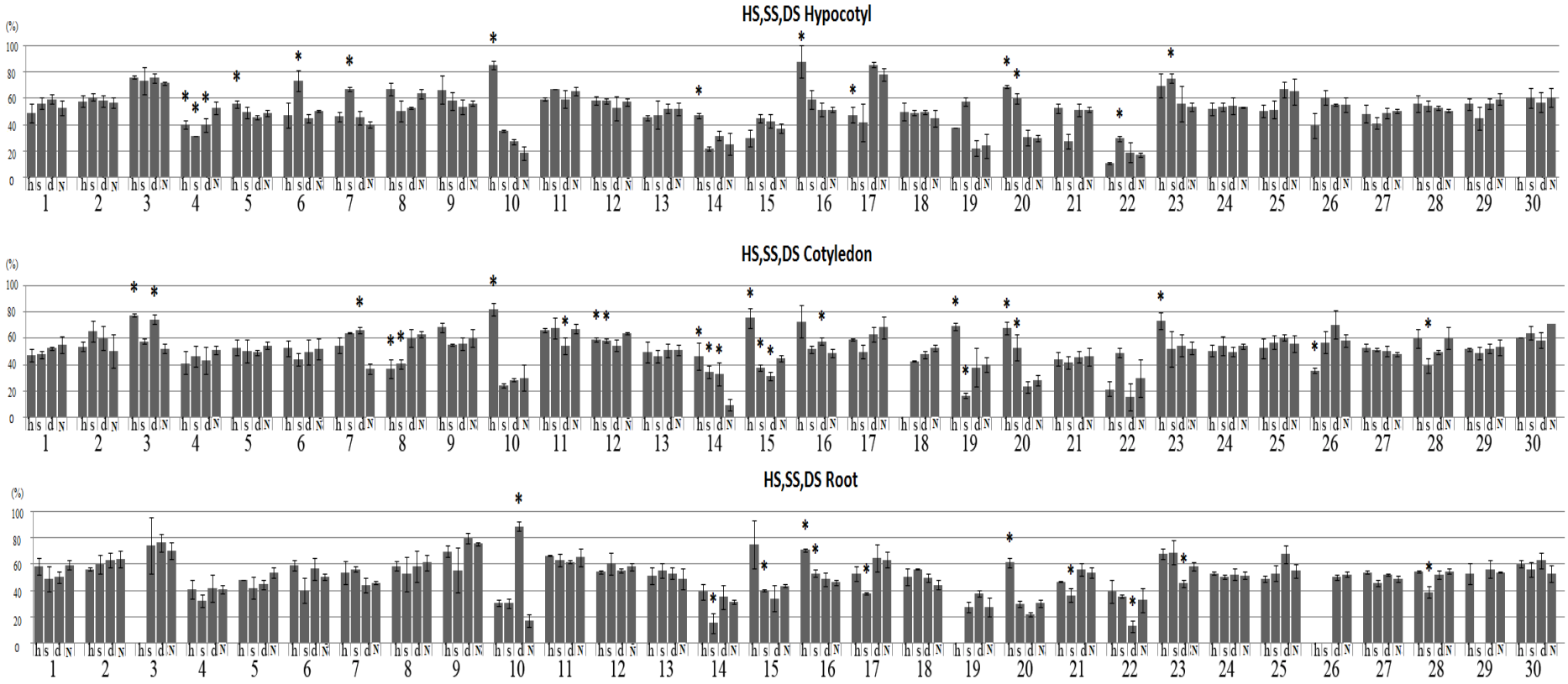


Figure 3 Ratios of At/Dt expression vary under cold stress and water-submersion stress in *G. hirsutum*

Graphs showing the percentage of transcripts derived from the At homeolog (y axis) in seedling organs of *G. hirsutum*. "C" indicates cold stress, "W" indicates water submersion, and "N" means untreated control plants. Error bars represent standard deviations among replicates. The treatments with "*" mean a statistically significant difference compared with untreated plants, detected by T-tests ($P<0.05$).



Fi Figure 4 Ratios of At/Dt expression vary under heat stress, salt stress and drought stress in *G. hirsutum*

Graphs showing the percentage of transcripts derived from the At homeolog (y axis) in seedling organs of *G. hirsutum*. “H” indicates heat stress, “S” indicates salt stress, “D” indicates drought stress, and “N” means untreated control plants. Error bars represent standard deviations among replicates. The treatments with “*” mean a statistically significant difference compared with untreated plants, detected by T-tests ($P<0.05$).

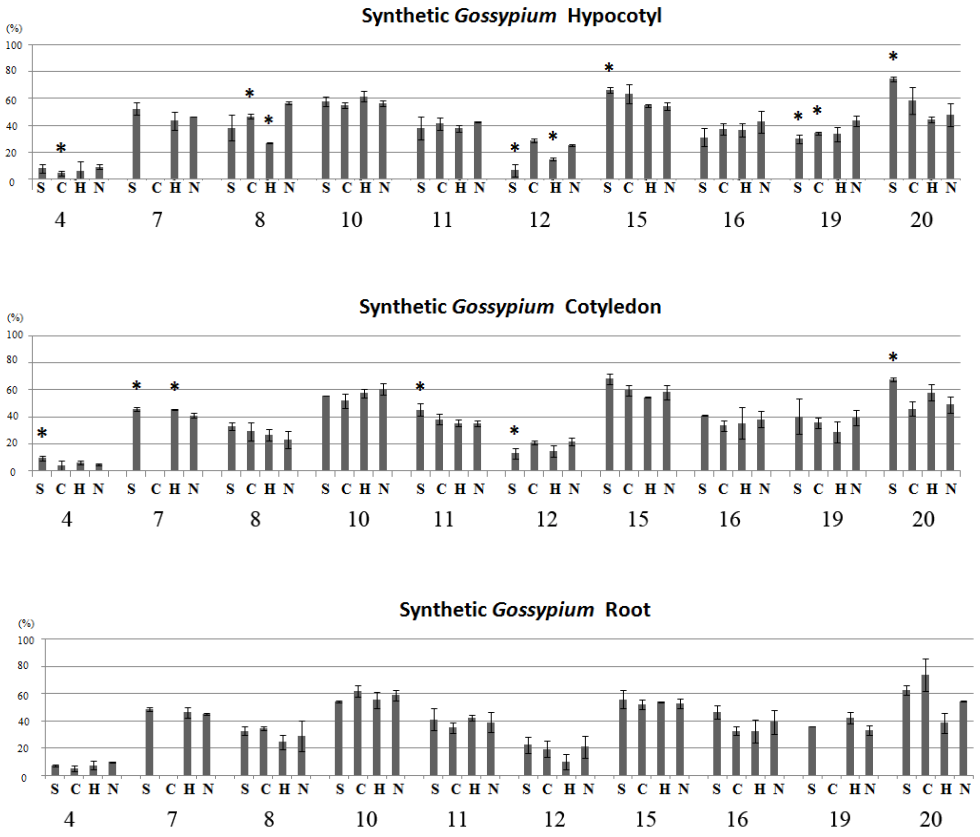


Figure 5 Ratios of At/Dt Expression vary under salt stress, cold stress and heat stress in a synthetic *Gossypium* allopolyploid.

Graphs showing the percentage of transcripts derived from the At homeolog (y axis) in seedling organs of the synthetic *Gossypium* allopolyploid. "S" indicates salt stress, "C" indicates cold stress, "H" indicates heat stress, and "N" means untreated control plants. Error bars indicate standard deviations among replicates. The treatments with "*" indicate a significant difference compared with control plants, detected by T-tests ($P<0.05$).

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