Dominance and Sexual Dimorphism Pervade the Salix purpurea L. Transcriptome

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Abstract

The heritability of gene expression is critical in understanding heterosis and is dependent on allele-specific regulation by local and remote factors in the genome. We used RNA-Seq to test whether variation in gene expression among F1 and F2 intraspecific Salix purpurea progeny is attributable to cis- and trans-regulatory divergence. We assessed the mode of inheritance based on gene expression levels and allele-specific expression for F1 and F2 intraspecific progeny in two distinct tissue types: shoot tip and stem internode. In addition, we explored sexually dimorphic patterns of inheritance and regulatory divergence among F1 progeny individuals. We show that in S. purpurea intraspecific crosses, gene expression inheritance largely exhibits a maternal dominant pattern, regardless of tissue type or pedigree. A significantly greater number of cis- and trans-regulated genes coincided with upregulation of the maternal parent allele in the progeny, irrespective of the magnitude, whereas the paternal allele was higher expressed for genes showing cis/trans or compensatory regulation. Importantly, consistent with previous genetic mapping results for sex in shrub willow, we have delimited sex-biased gene expression to a 2 Mb pericentromeric region on S. purpurea chr15 and further refined the sex determination region. Altogether, our results offer insight into the inheritance of gene expression in S. purpurea as well as evidence of sexually dimorphic expression which may have contributed to the evolution of dioecy in Salix.

Key words: differential expression, dioecy, regulatory divergence, sex determination, shrub willow, ZW system.

Introduction

Allele-specific expression (ASE) reflects the regulatory status of each parent allele inherited in an individual and has become an informative phenotype for biologists in understanding nonadditive phenotypic expression (Stupar and Springer 2006). Without further knowledge of parent pedigree, ASE can only be considered for sites that differ between the parents of an F1 cross, whereby a single copy of each homozygous parent allele exists in a heterozygous state in the F1 hybrid. For any biallelic site, the normalized expression ratio of the female parent (P1) allele and the male parent (P2) allele is contrasted to the same ASE ratio (P1H/P2H) in the hybrid. Statistically significant deviations of ASE in the F1 from the expected contribution of each parent (P = 0.5) are based on binomial exact tests, which lay the groundwork for the estimation of cis-regulatory divergent gene expression. Despite the fact that the extent of regulatory divergence is largely dependent on predetermined global significance thresholds (i.e., False Discovery Rate, FDR), the overall patterns of divergent expression do not drastically change (Suvorov et al. 2013). These patterns are broadly subject to sequence variation observed in the domains of local cis-regulatory elements or remote trans-acting factors.

There is evidence that nonadditive gene expression can confer novel transgressive phenotypes in hybrids (Springer and Stupar 2007) and is alleged to be a major driver of hybrid speciation (Rieseberg et al. 2003). For instance, in interspecific crosses of Drosophilia melanogaster and D. simulans, cis-effects were shown to account for a majority of regulatory divergent
expression (Wittkopp et al. 2008a), whereas trans-effects accounted for a higher proportion of expression variation between parents of the same species (Wittkopp et al. 2004, 2008b). Hybridization can introduce substantial divergence in offspring gene expression when compared with that of the parents (McManus et al. 2010). Such a merger provides new allelic variation within the regulatory domains of genes (e.g., promotors) as well as new targets of trans-acting factors (e.g., transcription factors). Although mutations in cis-regulatory elements have been shown to account for evolutionarily significant phenotypic change, trans-regulatory evolution can also affect adaptive morphological change (Wittkopp and Kalay 2011).

Depending on the effective population size, the effects of cis-mutations on gene expression are generally considered to be less deleterious as they only affect a single gene and are more likely to become fixed, whereas trans-effects can alter the expression of a number of genes (pleiotropy) and are more likely to be subject to purifying selection (McManus et al. 2010). Consequently, the conservation of gene expression (P1 ≈ H ≈ P2) should be less pronounced in wide hybrids, but more common with inbreeding or sib-mating, because the transcriptional activity of ASE is simply a function of the two cis-regulatory parent alleles in a common trans-regulated background. Nevertheless, studies of ASE in intraspecific progeny derived from closely related parents have attributed parental expression divergence to both cis- and trans-regulatory components (Bell et al. 2013; Suvorov et al. 2013; Combes et al. 2015).

A bulk of ASE work in plants have used hybrids derived from inbred parents to study the effects of hybridization on gene expression (Stupar and Springer 2006; Song et al. 2013). Although there are notable exceptions (Bell et al. 2013), there is a general lack of understanding on the evolution of gene expression with regards to the hybridization of heterozygous parents from natural, obligate outcrossing populations. Previous expression studies in dioecious shrub willow (Salix spp., Salicaceae) have predominantly focused on correlating functional variation of candidate gene family members to lignocellulosic composition traits in contrasting pedigrees (Puckett et al. 2012; Serapiglia et al. 2012). In this study, we examined the variation in transcriptome-wide expression within and among full-sib F1 and F2 intraspecific families generated from heterozygous parents collected from naturalized S. purpurea L. populations.

Shrub willow has been bred as a dedicated energy crop since the early 1970’s with the goal of producing fast-growing bioenergy feedstock cultivars that are high-yielding, genetically diverse, pest and disease resistant, and able to grow on marginal land without competing with food crops (Stoof et al. 2015). The heterogeneity and adaptive plasticity of Salix spp. provides an abundant germplasm pool for trait improvement and phylogenetic characterization (Hanley and Karp 2014). Hybridization is a key component in the development of shrub willow bioenergy crops, as hybrids often display heterosis for yield (Fabio et al. 2017a, 2017b). While significant improvements in biomass yield has been realized in interspecific crosses of Salix (Kopp et al. 2001; Cameron et al. 2008), heterosis is more pronounced in triploid progeny derived from the hybridization of diploid and tetraploid parents (Smart and Cameron 2008; Serapiglia et al. 2014a; Carlson and Smart 2016). These high-yielding triploid shrub willow outperform foundation commercial cultivars and show promise for the future of the biomass production industry (Serapiglia et al. 2014b; Fabio et al. 2017a, 2017b).

With the public release of the S. purpurea genome reference assembly (phytozome.jgi.doe.gov; last accessed September 10, 2017), Salix has become a powerful model to study the genomic basis of heterosis in dioecious species. Salix purpurea has a relatively compact genome (≈400 Mb) with ~37,500 primary gene models and ~65,000 alternatively spliced isoforms (Smart et al., in prep). Although the genome of S. purpurea is remarkably collinear to that of Populus trichocarpa (Berlin et al. 2010), major differences in the overall arrangement and abundance of coding and noncoding DNA (Hou et al. 2016) has radically affected the ecology, habit, and reproductive paths since the Salicoid duplication and divergence of the genera (Rodgers-Melnick et al. 2012). For instance, the sex determining region (SDR) of P. trichocarpa resides within a peritelomeric region on Populus chr19 (Yin et al. 2008; McKown et al. 2017), whereas the SDR of Salix spp. has been mapped to a pericentromeric region on Salix chr15 (Pucholt et al. 2015; Zhou et al. 2017). In addition to contrasting genomic locations of the SDR, S. purpurea exhibits a ZW sex determining system with heterogametic females (Zhou et al. 2017) and P. trichocarpa, an XY system with heterogametic males (Tuskan et al. 2012). To date, the mechanism of sex determination in the Salicaceae has not been completely resolved.

The main objectives of this study were 1) to test for differential gene expression among the shoot tip and internode transcriptome of segregating F1 and F2 intraspecific S. purpurea family progeny, 2) to categorize gene expression by modes of inheritance, 3) to assess the magnitude and direction of regulatory divergent expression, and 4) to examine the regulatory components of sexually dimorphic gene expression that may have contributed to the evolution of dioecy in Salix.

Materials and Methods
Plant Material and Growing Conditions
The full-sib intraspecific F1 S. purpurea family was generated from a cross between the female clone 94006 and the male clone 94001, collected from a population of S. purpurea in Central New York. Two F1 siblings from this family were selected and crossed (9882-41 × 9882-34) to generate the F2 population (supplementary fig. S1A, Supplementary Material online). Dormant first-year postcoppice vegetative shoots of
all family parents and progeny were taken from nursery beds in the winter of 2013. Cuttings of equal length (20 cm) and diameter were cut from shoots and planted into 2.5 l containers filled with Farfard PV-1 potting media and grown under environmentally controlled greenhouse conditions with supplemental lighting provided on a 14 h day:10 h night regimen with a max daytime temperature of 26 °C and a nighttime temperature of 18 °C. All plots were completely randomized over five replicate blocks. Liquid fertilizer (Peter’s 15-16-17 Peat-Lite Special; Scotts Miracle-Gro Company, Marysville, Ohio, U.S.A.) was applied weekly at 100 ppm after the third week from planting cuttings, until the study was terminated. Herein, we refer to parents of the F1 and F2 families by their clone identifiers and discriminate the female and male parents as P1 and P2, respectively.

**Determination of Ploidy Level**

The relative DNA content (pg 2 C value⁻¹) of family parents and progeny was determined by flow cytometric analysis using young leaf material harvested from actively growing shoots in greenhouse conditions. Analysis of 50 mg of mature leaf tissue from parental genotypes and selected progeny was performed at the Flow Cytometry and Imaging Core Laboratory at Virginia Mason Research Center in Seattle, WA as was previously described (Serapiglia et al. 2014b). A minimum of four replicates of all samples were independently assessed using either the diploid *S. purpurea* female genome reference clone 94006 or the diploid *S. purpurea* male clone 94001 as an internal standard. Diploid parent clones from multiple runs were averaged and then divided by the value of the check for that run. This factor was then multiplied by each sample value within the same run as the check. When a clone was analyzed more than once, 2C values were averaged. All parents and progeny described in this study are diploid (2n = 38) according to flow cytometric and genetic marker analysis (Argus 1997; Serapiglia et al. 2014b).

**RNA Sample Preparation and Sequencing**

Total RNA was extracted from three biological replicates of ten random family progeny and their parents for the F1 and F2 families. Both shoot tip and internode tissues from each individual were collected and processed using the Spectrum™ Total Plant RNA Kit with DNase digestion (Sigma, St. Louis, MO), following the manufacturers procedures. Cold-ethanol precipitations were performed by addition of 10 µl acetic acid and 280 µl 100% cold ethanol to 100 µl eluate and placed in −80 °C for at least 3 h. Samples were centrifuged at 17,000 × g for 30 min at 4 °C, washed with 80% cold ethanol, then centrifuged at 17,000 × g for 20 min at 4 °C. After centrifugation, the ethanol supernatant was discarded and the pellet resuspended in ribonuclease-free 10 mM Tris–HCl (pH = 8). Quantification of sample quality and concentration was performed using the Experion RNA StdSens kit (Bio-Rad Laboratories, Inc., Hercules, CA), following manufacturers’ procedures.

Independent extractions were performed on three replicate plants of each of ten progeny individuals within each family and subsequently pooled in equal concentrations. For each tissue type, three RNA-Seq libraries were constructed representing the female parent, the male parent, and a pool of ten progeny. In addition, for comparisons between progeny within the F1 family, ten F1 progeny were individually barcoded and sequenced. Libraries were constructed using the NEBNext Ultra Directional RNA Library Prep Kit and sequenced on the Illumina platform (1x100 bp) at the J. Craig Venter Institute (Rockville, MD).

Shoot tips were defined as the shoot axis that is the most distal part of a shoot system, comprised of a shoot apical meristem and the youngest leaf primordia. Stem internodes were defined as the cardinal organ part that is the part of a shoot axis between two nodes of the axis.

**Read Filtering, Alignment, and Variant Discovery**

Low-coverage paired-end genomic DNA sequencing of the parental lines for the F1 and F2 families was performed to validate variants from RNA-Seq data. Biallelic SNPs were used to quantify allele-specific expression and regulatory divergence within and among intraspecific family progeny. Parent DNA libraries were sequenced (Illumina HiSeq 2 × 101) and aligned to the *S. purpurea* v1 reference genome using BWA-MEM (-M -R) (Li and Durbin 2009). Subsequent SAM files were sorted, marked for duplicates, and indexed in Picard (broadinstitute.github.io/picard). Indel realignment and variant calling was performed using HaplotypeCaller (emit_conf 10, call_conf = 30) in the Genome Analysis Toolkit (GATK) (DePristo et al. 2011). For all samples, RNA-Seq reads were trimmed (min length = 50) and mapped (length = 0.8, similarity = 0.9) to the *S. purpurea* v1 reference genome using CLC Genomics Workbench (www.qiagenbioinformatics.com; last accessed September 10, 2017). The *S. purpurea* L. v1.0 genome reference assembly and annotation is available online via the Joint Genome Institute Comparative Plant Genomics Portal, Phytozome v12 (phytozome.jgi.doe.gov).

**Gene Expression Inheritance Classifications**

To determine the mode of inheritance for genes, the number of RNA-Seq reads mapped to individual genes was counted for each of the female (P1) and male (P2) parents and progeny (H). Expression levels were compared based on normalized read counts using the edgeR package (Robinson et al. 2010). Differentially expressed genes were determined using an exact test implemented in edgeR for negative-binomially distributed counts (disp = 0.1, FDR = 0.005). We used a custom R script to sort genes into the following six inheritance categories: 1) P1-dominant: H ≈ P1 and H ≠ P2, 2) P2-dominant: H ≈ P2 and H ≠ P1, 3) additive: P1 < H < P2 or
Inheritance was determined as the Euclidean distance \( L^2 \) between vectors \( m_x \) and \( f_y \), such that: \( L^2 = (p, q) = \left[ (|m_x| - |f_x|)^2 + (|m_y| - |f_y|)^2 \right]^{0.5} \), where \( m_x \) is the male coordinate derived from \( \log_2(m/p_1) \), \( m_y \) is the male coordinate derived from \( \log_2(m/p_2) \), \( f_x \) is the female coordinate derived from \( \log_2(f/p_1) \), and \( f_y \) is the female coordinate derived from \( \log_2(f/p_2) \). \( p \) is the squared absolute difference of the vectors \((|m_x|, |f_x|)\) and \((|m_y|, |f_y|)\), and \( q \) is the squared absolute difference of the vectors \((|m_x|, |f_x|)\) and \((|m_y|, |f_y|)\).

### Regulatory Divergence Classifications

For regulatory divergence classification of genes, the sequence reads in progeny need to be assigned to their parental origins. For each gene, the expression levels of the two parental alleles were estimated based on the nucleotide allele counts across all SNP sites detected based on parent DNA libraries (described above), and where the nucleotide alleles present in each of the parents are distinct and therefore allow unambiguous assignment of parental origins. Categories of regulatory functions considered conserved, compensatory, ambiguous, cis, trans, cis + trans, and cis \( \times \) trans, were sorted using R scripts, as described in Landry et al. (2005) and McManus et al. (2010). Regulatory divergence assignments were based on two sets of tests: 1) a binomial exact test between P1 and P2 in the parents and between P1H and P2H in the progeny, and 2) Fisher’s Exact Test on P1, P2, P1H, and P2H (supplementary table S1, Supplementary Material online). The percent divergence due to cis- and trans-effects were calculated such that % cis \( = \frac{[\text{cis}] / ([\text{cis}] + \text{trans})] \times 100 \) and % trans \( = \frac{[\text{trans}] / ([\text{cis}] + [\text{trans}]]) \times 100 \), where \( \text{cis} = \log_2(P1H/P2H) \) and \( \text{trans} = \log_2(P1H/P2H) - \log_2(P1/P2) \).

### Tests for Differential Expression

All statistical analyses were performed in the open-source statistical computing environment, R (Team 2015). Tests for differential expression were conducted in the package edgeR (Robinson et al. 2010). Normalization factors and dispersion estimates (robust = T) were computed prior to tests for differential expression. A general linear model was used to fit normalized count data using glmFit and glmLRT to conduct likelihood ratio tests for the model coefficients. Tests for paired comparisons were conducted to investigate the effect of tissue type over the individuals within the F1 family, using an additive linear model with clone as a blocking factor \((y \sim 0 + \text{clone} + \text{tissue})\). In order to test for differential expression by sex \((y \sim \text{pedigree} + \text{sex})\) in the F1 family, a conservative and robust quasi-likelihood model using tag-wise dispersion estimates was used to fit the data with glmQLFit (robust = T). For tests of differential expression, genes were only considered to be significant at a False Discovery Rate of 0.05. To explore variation in gene content, we considered the total number of genes expressed per sample library (transcriptome-normalized expression), without inferring relative expression per gene copy or per cell, as is described in Coate and Doyle (2010). A summary of analyses and corresponding libraries used is in supplementary table S2, Supplementary Material online.

### Gene Ontology

Gene ontology (GO) enrichment was performed in agriGO (Du et al. 2010) using the subset of the *S. purpurea* v1 transcriptome (reference set) that passed filtering prior to tests of differential expression. Only significant ontologies were reported from query lists. For *S. purpurea* gene annotations which encode for hypothetical proteins, gene models and associated GO-terms were inferred using the best-hit (BLASTP E-value \( \leq 0.1 \)) to *Populus trichocarpa* (Phytozome v10.3 annotation) and *Arabidopsis* (TAIR10 and Araport11 annotations) proteome.

### Results

#### Transcriptome Analysis

In order to define the factors contributing to variation in global expression among the F1 and F2 parents and progeny, the RNA-Seq data were subjected to multidimensional scaling (MDS) analysis, considering only genes with a sum cpm-normalized expression >1.0 for \( \geq 50\% \) of the samples (supplementary fig. S1B, Supplementary Material online). As expected, the parent transcriptomes of the F1 were the most distantly clustered, because they are the least related in this study. The first MDS dimension clustered samples based entirely on tissue type, and the second dimension split F1 and F2 parents by sex, then by pedigree. Although 9882-41 and 9882-34 are F1 siblings, their gene expression levels clustered more towards the parent of the same sex. The pedigrees, ploidy levels, and read mapping statistics are summarized in supplementary table S3, Supplementary Material online.

### Inheritance of Gene Expression

The mode of gene expression inheritance assessed in F1 and F2 intraspecific progeny was based on two distinct tissue types: shoot tip and stem internode. Differentially expressed genes were defined as having normalized expression levels significantly higher or lower for treatment comparisons at an FDR of 0.005. In general, gene expression in both the F1 and F2 family was largely conserved, yet expression differences between the F1 parents was far more pronounced than among the
F1 progeny and the F1 midparent. Likewise, the parents of the F2 family had dramatically fewer differentially expressed genes than among the parents of the F1, whereas gene expression in the F2 was considerably more conserved than in the F1. Regardless of the tissue type, <0.07% of all differentially expressed genes in the F1 and F2 could be classified as having an additive mode of expression inheritance. Among differentially expressed genes, the greatest proportion were classified as dominant (fig. 1); accounting for 95% and 84% of all nonadditive (i.e., dominant, overdominant, and underdominant) F1 gene expression and 94% and 96% F2 gene expression in the internode and shoot tip transcriptome, respectively (table 1).

Further, both the F1 and F2 families showed a significantly greater proportion of dominant expression biased in the direction of the maternal parent (P1 dominant). The most extreme case of maternal dominance was identified in the F1 shoot tip transcriptome (845 P1-dominant genes) and F2 internode transcriptome (711 P1-dominant genes). The total number of maternally dominant genes in common among the F1 and F2 shoot tip (99) or among the F1 and F2 internode (92) transcriptomes was relatively greater than paternally dominant genes shared among the F1 and F2 shoot tip (27) or internode (13) transcriptomes. In addition to the extensive expression-level dominance present within these families, >10-fold the number of transgressive genes in the F1 shoot tip transcriptome were classified as underdominant (209), compared with the F2 shoot tip transcriptome (20). Likewise, there were fewer genes with overdominant expression in the F2 shoot tip transcriptome (53) than in the F1 shoot tip transcriptome (53).

There was a relatively large subset of genes differentially expressed between parents and progeny of the F1 family (supplementary table S4, Supplementary Material online). Many of these genes were classified as transcriptional regulators and hypothetical proteins with domains of unknown function (DUF). Gene ontology (GO) term analysis for P1-dominant genes in internode tissues showed significant enrichment for response to stimulus, catalytic activity and binding, as well as the cellular components intracellular membrane-bound organelle and intracellular part. Significant GO enrichment for P1-dominant genes in the shoot tip was similar to those in the internode for biological processes and cellular components, but included unique molecular functions of
catalytic and UDP-glycosyltransferase activity. Enrichment of P2-dominant genes in internode tissues included response to stimulus, transcription regulator activity, and cell wall components, whereas postembryonic development, programmed cell death, binding, and catalytic activity were over-represented in shoot tip tissue.

ASE Analysis

In order to discern the overall proportion of cis- and trans-regulation of gene expression in *S. purpurea*, ASE tests were conducted using RNA-Seq expression data which was based on biallelic sites called from DNA-Seq and RNA-Seq of the parents. For both families, the regulation of gene expression was primarily conserved, regardless of the tissue type assayed (fig. 1). However, for those genes showing nonconserved regulatory classes, moderate proportions of pure trans-regulated gene expression were identified in the F1 shoot tip (187, 11%) and internode (85, 14.5%) transcriptome, but substantially less in the F2 shoot tip (15, 2.5%) and internode (28, 2.6%) transcriptome (table 1). The proportion of ASE in the F1 shoot tip transcriptome was nearly twice that of the F1 internode transcriptome, yet there was no difference among F2 tissues. On average, the F1 had a greater number of SNPs per gene than the F2 (fig. 2; supplementary table S5, Supplementary Material online). As the number of SNPs per gene increased, the log2 (P1/P2) expression ratio decreased. The only major discrepancy in the average number of SNPs per gene was between F1 tissues for those genes showing cis + trans regulatory interactions.

A significantly greater number genes showing cis- and trans-regulation in the F1 coincided with upregulation of the maternal P1 allele, irrespective of the magnitude, whereas the paternal P2 allele was higher expressed for genes showing cis × trans or compensatory regulation. Compensatory regulation accounted for 13% (512) of gene expression in the F1 shoot tip transcriptome, but only 0.7% (38) in the internode transcriptome. Without considering conserved or ambiguous classes in the F1 shoot tip transcriptome, 30% exhibited compensatory patterns and 8.3% with cis × trans regulation, compared with 13.5% and 11% of genes with pure cis- and trans-regulation, respectively. Further analysis of the F1 revealed significantly greater levels of nonadditive and regulatory divergent expression in the shoot tip transcriptome compared with the internode transcriptome. Among the unpoled libraries of F1 progeny individuals (supplementary fig. S2, Supplementary Material online), the degree of mid-parent differential expression corresponded linearly to the variation in regulatory divergent expression, whereby increased levels of compensatory expression coincided with increased levels of cis × trans regulation (supplementary fig. S3, Supplementary Material online). However, compensatory regulation was negligible in the F2 family and did not show significant differences by tissue type.

### Table 1

Summary of Gene Expression Inheritance and Regulatory Divergence Classifications of *Salix purpurea* F1 and F2 Families

| Class                  | F1 Family 10X-082 | F2 Family 10X-317 |
|------------------------|------------------|------------------|
| **Expression Inheritance** | Internode | Shoot Tip | Internode | Shoot Tip |
| P1 dominant            | 586            | 2.14%         | 845        | 3.10%      |
| P2 dominant            | 350            | 1.28%         | 587        | 2.15%      |
| Overdominant           | 34             | 0.12%         | 53         | 0.19%      |
| Underdominant          | 10             | 0.04%         | 209        | 0.77%      |
| Additive               | 20             | 0.07%         | 13         | 0.05%      |
| Conserved              | 26,441         | 96.4%         | 25,545     | 93.7%      |
| **Regulatory Divergence** |               |                |               |            |
| cis only               | 145            | 2.80%         | 231        | 5.90%      |
| trans only             | 85             | 1.60%         | 187        | 4.80%      |
| cis + trans            | 5              | 0.10%         | 13         | 0.30%      |
| cis × trans            | 19             | 0.40%         | 140        | 3.60%      |
| Compensatory           | 38             | 0.70%         | 512        | 13.0%      |
| Ambiguous              | 833            | 16.1%         | 811        | 20.7%      |
| Conserved              | 4,063          | 78.3%         | 2,033      | 51.8%      |
| **Total**              | 5,188          | 78.3%         | 3,927      | 72.1%      |

**Legend:**
- **cis** only: cis-regulation only
- **trans** only: trans-regulation only
- **cis + trans**: cis + trans-regulation
- **cis × trans**: compensatory regulation
- **Compensatory**: compensation
- **Ambiguous**: ambiguous

**Note:** The total number and percentage of genes among those classified within the F1 and F2 shoot tip and internode transcriptome are partitioned by their inheritance and regulatory divergence classes (False Discovery Rate < 0.005). Numbers in boldface indicate significant (P < 0.01) deviations from a 1:1 ratio according to a χ² test.
normalization and filtering for low expression, we identified a total of 262 genes as differentially expressed between the F1 shoot tip and internode transcriptome. A subset of 46 genes were compiled (supplementary table S6, Supplementary Material online) that were highly expressed in internode tissues with log2 fold-differences ranging from 3.2 (SapurV1A.0130s0080) to 6.0 (SapurV1A.0216s0270). Genes encoding for fasciclin-like arabinogalactan (FLA)
proteins were most represented. A total of 20 FLAs from the 57 FLA gene family members were identified as differentially expressed (supplementary fig. S4, Supplementary Material online), all of which are annotated FLA11 or FLA12 with a single FLA2 representative (SapurV1A.0054s0430). GO term analyses of internode predominant genes showed significant enrichment for cell wall and biosynthetic processes.

Sex-Biased Gene Expression

In order to test for differential expression between male and female S. purpurea, we utilized the shoot tip transcriptome of three male and three female F1 individuals as well as their parents, such that each sex was represented by four related individuals. We identified a total of 315 genes in the F1 shoot tip transcriptome as having significant sex-biased expression (fig. 3A and table 2). In stark contrast, there were no genes in the F1 internode transcriptome that showed significant sex-biased expression. Of the 315 sex-biased genes, 62 map to S. purpurea chr15. In addition, 77 genes with best BLAST hits to P. trichocarpa v3 chr15 orthologues accounted for 24% of the sex-biased genes identified (fig. 3B). From this list, 231 genes were more highly expressed in females and 84 were more highly expressed in males, indicating that a significantly higher proportion of the shoot tip transcriptome is female-biased than male-biased (fig. 3C). Nearly all female-biased genes reported in this study localize to Salix chr15 or unplaced scaffolds syntenic to Populus chr15. By contrast, male-biased genes were not predominantly on S. purpurea chr15, many mapped to S. purpurea chr19, especially for genes with higher gene expression.
### Table 2

Sex-Biased Gene Information

| Salix purpurea v1 Gene Model | Chr or Scaffold | Functional Annotation | Homolog | log\(_2\) | \(-\log_{10}(P)\) |
|------------------------------|----------------|-----------------------|----------|-----------|-----------------|
| **Up in Females**            |                |                       |          |           |                 |
| SapurV1A.0582s0010           | 582            | NIPA, interacting partner of ALK | Potri.015G052400 | 6.7  | 42.6           |
| SapurV1A.2504s00020          | 2504           | AGL98, agamosome 98-like | AT5G39810 | 7.7  | 39.9           |
| SapurV1A.4040s00010          | 4040           | Di-glucose binding, kinesin | Potri.001G436200 | 3.3  | 27.3           |
| SapurV1A.0301s0070           | 15             | REM1, reproductive meristem 1 | Potri.009G103300 | 8.8  | 25.3           |
| SapurV1A.2504s00010          | 2504           | GAPI-anchored protein | Potri.015G040900 | 6.0  | 24.6           |
| SapurV1A.0301s01060          | 15             | DR1/INF-YB, TBP-associated | Potri.015G052800 | 4.7  | 21.0           |
| SapurV1A.0301s01070          | 15             | BRCa, fragile-X-F-associated | Potri.015G050300 | 6.8  | 20.8           |
| SapurV1A.0475s01100          | 15             | peptidase M508-like protein | Potri.015G045900 | 9.8  | 18.2           |
| SapurV1A.1892s00010          | 15             | GPI-anchored protein Potri.015G040900 | 5.9  | 11.7       |
| SapurV1A.0301s01100          | 15             | DR1/NF-YB, TBP-associated | Potri.015G052800 | 4.7  | 21.0           |
| SapurV1A.0301s01200          | 15             | BRCa, fragile-X-F-associated | Potri.015G050300 | 6.8  | 20.8           |
| SapurV1A.0475s01100          | 15             | peptidase M508-like protein | Potri.015G045900 | 9.8  | 18.2           |
| SapurV1A.2504s00010          | 2504           | GAPI-anchored protein | Potri.015G040900 | 6.0  | 24.6           |
| SapurV1A.2524s00010          | 2524           | PHYB, phytochrome protein B | Potri.008G105200 | 4.1  | 13.7           |
| SapurV1A.2212s00030          | 2212           | activating signal cointegrator 1, 3 | Potri.015G056700 | 5.5  | 12.5           |
| SapurV1A.4394s00010          | 4349           | SCD1, cytokinesis-defective 1 | Potri.015G049500 | 5.1  | 17.0           |
| SapurV1A.212s00000           | 212            | activating signal cointegrator 1, 3 | Potri.015G056700 | 5.5  | 12.5           |
| SapurV1A.0582s00600          | 582            | GUS2/HPSE1, heparanase 1-like | Potri.015G049100 | 5.0  | 8.3            |
| SapurV1A.0530s00900          | 15             | LRK10, serine/threonine kinase | Potri.015G044800 | 4.7  | 9.2            |
| SapurV1A.0178s01100          | 15             | CaS, extracellular Ca\(^{2+}\) receptor | Potri.015G048400 | 5.9  | 9.2            |
| SapurV1A.0107s01100          | 3              | IMPA-2, importin alpha 2 | Potri.005G020400 | 4.5  | 7.5            |
| SapurV1A.0107s00700          | 3              | IMPA-2, importin alpha 2 | Potri.005G020400 | 4.5  | 7.5            |
| SapurV1A.2772s00010          | 1765           | fertility restorer (Rf) | Potri.015G047900 | 5.2  | 9.4            |
| SapurV1A.2524s00010          | 2524           | PHYB, phytochrome protein B | Potri.008G105200 | 4.1  | 13.7           |
| SapurV1A.0530s00900          | 15             | LRK10, serine/threonine kinase | Potri.015G044800 | 4.7  | 9.2            |
| SapurV1A.1538s00200          | 15             | CaS, extracellular Ca\(^{2+}\) receptor | Potri.015G048400 | 5.9  | 9.2            |
| SapurV1A.0530s00400          | 15             | peptidase M508 Potri.015G045900 | 1.3  | 3.9         |
| SapurV1A.1254s00400          | 1254           | suppressor of protein silencing | Potri.015G036400 | 1.4  | 3.9            |
| SapurV1A.2212s00030          | 2212           | activating signal cointegrator 1, 3 | Potri.015G056700 | 5.5  | 12.5           |
| Up in Males                  |                |                       |          |           |                 |
| SapurV1A.0934s0010           | 15             | RPS3, 40S ribosomal protein S3-1 | Potri.015G071700 | 1.9  | 6.4            |
| SapurV1A.0830s0010           | 830            | NIPA, interacting partner of ALK | Potri.015G052400 | 6.1  | 21.0           |
| SapurV1A.0934s00600          | 934            | NIPA, interacting partner of ALK | Potri.015G052400 | 6.1  | 21.0           |
| SapurV1A.3555s0010           | 3555           | delta-ADR, AP-3 complex delta-1 | Potri.015G045600 | 2.5  | 6.5            |
| SapurV1A.1254s00400          | 1254           | AMY-1, associate of c-MYC | Potri.015G036400 | 1.4  | 3.9            |
| SapurV1A.2772s00010          | 2772           | RPL19e/EMB2386, ribosomal 19e | Potri.015G037100 | 4.7  | 9.2            |
| SapurV1A.0582s01000          | 582            | LAP4, less-adhesive pollen 4 | Potri.015G048400 | 5.2  | 9.4            |
| SapurV1A.0582s00400          | 582            | meiotic endonuclease, putative | Potri.015G049800 | 6.7  | 5.5            |
| SapurV1A.2353s01100          | 2535           | suppressor of protein silencing | Potri.015G047900 | 2.6  | 5.4            |
| SapurV1A.0178s01600          | 1765           | fertility restorer (Rf) | Potri.015G036400 | 1.4  | 3.9            |
| SapurV1A.1596s00500          | 15             | DYNLL1, dynein light chain 1-like | Potri.015G067900 | 4.6  | 8.7            |
| SapurV1A.0307s00600          | 19             | PIF1, phytochrome interacting 1 | Potri.008G203700 | 7.3  | 4.6            |

**NOTE.**—Rows within the table are ordered by the \(-\log_{10}(P)\) significance of each respective gene. *Salix v1* homologs are reported as the best BLAST hit (E-value \(\leq 0.01\)) to the *Populus trichocarpa* v3 or *Arabidopsis TAIR* v10 proteome.
expression in males. Over half of the sex-biased genes on *S. purpurea* chr19 which were highly expressed in males encode proteins related to signaling and response (e.g., ankyrin repeat, patatin phospholipase, and nucleotide-binding site leucine-rich repeat proteins), but do not appear to localize to any particular region of the chromosome.

There were two primary gene clusters on *S. purpurea* chr15 that were significantly enriched for sex-biased expression span chr15: 11.4–12.3 Mb and chr15: 13.6–14.6 Mb (fig. 3D); the latter with more sex-biased genes and with higher significance than the former. Differentially expressed genes located on unplaced *Salix* scaffolds 265, 582, 830, 1765, 2212, 2504, and 4349 align to *S. purpurea* chr15 gene clusters and corresponding regions on *Populus* chr15. For those genes located on unplaced *Salix* scaffolds and chr15 which have high identity to *Populus* chr15 homologs, when ordered according to *Populus* chr15 positions, a substantial number of those genes appear to be duplicated within one or the other cluster.

Sex determination regions of dioecious species are often highly polymorphic, so we investigated the presence and absence of gene expression specific to F1 females or males. Complete absence of expression in males was observed for *REM1* (SapurV1A.0301s0070), reticulon *RTNLB9* (SapurV1A.0530s0130), peptidase *M50B* (SapurV1A.0475s0170), chaperonin *TCP-1* (SapurV1A.1538s0020), *PMEI1* (SapurV1A.1210s0090), a gar2-related 18S preribosomal assembly protein (SapurV1A.0178s0110), terpene synthase *TPS21* (SapurV1A.1522s0030), and AGI98 (SapurV1A.2504s0020); all of which are located within or align to the pericentromeric SDR on *S. purpurea* chr15. Complete loss or very low-levels of gene expression in females was accompanied by correspondingly low-levels in males; nearly 10% of gene models were filtered from analyses for this reason.

Sex-biased genes highly expressed in females were enriched for GO terms in the biological processes of signaling, signal transmission and transduction, cation binding, and ion binding, as well as the molecular functions of copper ion binding, magnesium ion binding, signal transducer activity, and lyase activity. Genes showing higher expression in males were enriched for cell death, death, apoptosis, and programmed cell death, the molecular functions of ATP-binding, structural constituent of the ribosome, and structural molecule activity, and the cellular components of intracellular organelle, ribonucleoprotein complex, and ribosome.

Sexually Dimorphic Inheritance of Gene Expression

Although we were not able to identify sex-biased expression in the F1 internode transcriptome, considerable variation within the shoot tip transcriptome offered us a unique opportunity to dissect the heritable components of sexually dimorphic patterns of expression. Genes considered to exhibit sexually dimorphic inheritance were only reported for those with a significant nonadditive or transgressive inheritance class for at least one sex. Genes with expression inheritance classifications that did not show significant sex dimorphism were classified as having same-sex inheritance. Although a majority of the shoot tip transcriptome retained same-sex inheritance, 3.8% (1,055 genes) displayed sexually dimorphic patterns of inheritance (fig. 4, supplementary table S7, Supplementary Material online). While there were no significant differences in the median expression level for genes with same-sex inheritance, the expression levels of dimorphic genes were significantly greater in females than in males (fig. 4A). Broadly, sexually dimorphic inheritance in the F1 shoot tip transcriptome was associated with a greater number of genes with conserved expression in males (65%) (fig. 4B) and nonadditive expression in females (75%) (fig. 4C). In addition, for those genes with sexually dimorphic inheritance, there was a marginally greater number of P2-dominant genes (174) in males compared with P1-dominant genes (148), whereas the opposite was found in females. Nearly five-times the number of dimorphic genes in females were classified as P1-dominant (499) compared with P2-dominant (119).

The most drastic instances of sex dimorphism were for genes with transgressive (overdominant and underdominant) expression inheritance. Of the 175 overdominant genes in females, 167 had conserved expression in males, with only eight showing same-sex inheritance (supplementary table S7, Supplementary Material online). Further, of the 33 genes with an underdominant mode of expression inheritance, all were restricted to males. Relative to the results of the pooled F1 family inheritance classifications (table 1), additive inheritance plays a negligible role in both the female (7) and male (1) shoot tip transcriptome.

In order to quantify the extent of dimorphism for each gene, the magnitude of dimorphic expression was calculated as the Euclidean distance between male and female vectors on the same Cartesian plane of expression levels (supplementary fig. S5, Supplementary Material online). The direction and magnitude of dimorphic gene expression in the F1 shoot tip transcriptome is illustrated in figure 4D. The contribution of nonadditive inheritance on dimorphic gene expression was examined directly, because gene expression inheritance in an individual is simply a function of the difference in progeny and parent expression ratios. This method demonstrates that there was a positive relationship between the magnitude of dimorphic inheritance and midparent differential expression. In F1 males, sexually dimorphic genes with conserved inheritance exhibited significantly greater differences in the magnitude of differential expression than genes with nonadditive expression. However, dimorphic genes with conserved inheritance in females (and nonadditive inheritance patterns in males) exhibited a significantly lower magnitude of expression than genes with nonadditive expression (fig. 4E). Likewise, the absolute magnitude of genes showing conserved expression...
in males was not significantly different from the magnitude of nonadditive expression in females, signifying that a higher frequency of sexually dimorphic genes with conserved inheritance in males coincided with nonadditive gene expression in females. Simply, as the distance between female and male vectors (magnitude) increased, the frequency of nonadditive and conserved patterns of inheritance increased, respectively.

Sexually Dimorphic ASE

To test the hypothesis that sex dimorphic gene expression was attributable differences in regulation, we compared the magnitude of cis- and trans-regulation of genes expressed predominately in either females or males. In order to determine the extent to which patterns of regulatory divergent expression varies for the same gene, we contrasted the magnitude and direction of regulatory divergence and the proportion of their effects, based solely on sex in the F1. Sexually dimorphic ASE was considered to be the total number of genes that significantly differ by regulatory class between three F1 male individuals and three F1 female individuals. Genes assigned regulatory classes which did not differ between males and females were characterized as having same-sex ASE. For each gene showing significant regulatory divergence in either sex, these results illustrate the total number of genes that show contrasting or sexually dimorphic regulatory patterns. Only genes from the ASE analysis which were unique to either females or males were considered.

In the shoot tip transcriptome, a total of 297 genes displayed sexually dimorphic ASE (table 3 and fig. 5), where 143 had higher expression levels in F1 males and 154 were expressed greater in F1 females. For genes showing sexually dimorphic ASE, conserved or cis-regulation in females coincided with cis/trans or compensatory regulation for the same genes in males. For instance, there were significantly greater numbers of dimorphic genes expressed in the male internode transcriptome, with 54 cis × trans and 88 compensatory regulated genes, compared with only 4 cis × trans and 9 compensatory regulated genes uniquely expressed in females (table 3). By comparing ASE of female and male F1 progeny individuals, genes with significant cis-regulation were significantly biased towards maternal parent (P1) in the shoot tip and internode transcriptome of females, as well as the shoot tip transcriptome of males, irrespective of the magnitude of ASE (table 4). On a per-site basis, antagonistic cis × trans and compensatory regulation in the F1 shoot tip transcriptome was driven by upregulation of the paternal (P2) allele, whereas the maternal (P1) allele was upregulated for...
either pure cis- or trans-regulation or cis + trans regulation (fig. 5B).

The absolute magnitude of trans-regulated genes was greater than that of cis-regulated genes for both males and females (fig. 5C). Although the magnitude of effect class sizes of genes that showed pure cis- or trans-regulation were not significantly different by sex, a significantly greater frequency of cis-effects were present at the tails (<0.5 and >2-fold) and a greater frequency of trans-effects between tails (>0.5 and <2-fold) (fig. 5D). By scaling cis- and trans-regulatory divergence by the total regulatory divergence, as in Coolon et al. (2013), we obtained a relative percent divergence due to cis- and trans-effects. With regard to females, the proportion of genes with evidence of cis-regulatory divergence was significantly greater than was observed in males. The proportion of genes showing significant trans-regulatory divergence was greater in females than in males, but with weaker significance than the cis-regulated genes (fig. 5E).

## Discussion

### Dominance and Regulatory Divergence in F1 and F2 S. purpurea

The majority of gene expression inheritance and ASE studies in both plants and animals have focused on F1 hybrids generated from crossing stable, inbred parents. Unlike what has been described in model crop plants, like maize (Stupar and Springer 2006) and rice (Song et al. 2013), we show that in S. purpurea, the greatest proportion of differentially expressed genes did not exhibit a primarily additive mode of inheritance, but rather showed strong patterns of dominance. Preference of uniparental expression in progeny is thought to be orchestrated by epistatic interactions, which primarily function to silence one of the parental alleles in a parent-of-origin manner (Chen and Pikaard 1997; Stupar et al. 2007). Here, maternal dominance represented the greater proportion of the nonadditive gene expression in both tissue transcriptomes of the F1 and F2 families. Other cases of expression-level dominance has been described in hybrids of intraspecific thistle (Bell et al. 2013), interspecific coffee (Combes et al. 2015), synthetic allotetraploid Arabidopsis (Shi et al. 2012). Reciprocal hybridization has become a useful technique to examine genomic imprinting by comparing common patterns of uniparental expression of alleles in reciprocal family progeny (Donoghue et al. 2014; Baldauf et al. 2016). In developing seeds of Arabidopsis, there is strong evidence that imprinting genes regulate early endosperm development and nutrient translocation and partitioning in the seed (McKeown et al. 2011). While epistasis may well contribute to the prodigious levels of dominant gene expression observed in F1 and F2 S. purpurea, since Salix spp. are dioecious, reciprocal crosses cannot be generated in the classical sense.

The inheritance and regulatory patterns described in this study were most similar to what was reported in hybrids derived from heterozygous parents that were collected from natural C. arvense populations (Bell et al. 2013). For instance, for both F1 S. purpurea and F1 C. arvense, more divergently expressed genes showed higher expression of the maternal allele than the paternal allele and that a significantly greater proportion of dominant cases had maternal expression patterns. Importantly, there were similar trends between S. purpurea and C. arvense in cis- and trans-divergence, such that trans-divergence was associated with higher expression of the paternal allele, whereas cis-divergence tended to increase expression of the maternal allele. However, there were differences in transgressive inheritance classes between the species. The frequency of overdominant gene expression in S. purpurea (shoot tip) was significantly less than underdominant expression, whereas the opposite was detected in C. arvense.

The transcription of two divergent parental alleles in the hybrid can be controlled by cis-regulatory elements as well as trans-acting factors, but parental expression differences may be reduced in the hybrid for genes subject to strong
trans-regulation (Xu et al. 2014). Allele-specific tests in hybrids of inbred maize (Stupar and Springer 2006) established that cis-acting regulatory variation accounted for the majority of the observed parental expression divergence and that pure cis-regulation correlated with additive expression patterns in the F1 hybrid. In synthetic allotetraploids of indica and japonica rice subspecies, parental expression differences were found to be intensified in the allotetraploid but showed a reduction of expression divergence in reciprocal F1 hybrids, owing to the effects of a common trans environment on divergent cis-factors (Xu et al. 2014). Using allotetraploids of Arabidopsis thaliana as well as resynthesized allotetraploids of A. arenosa, Shi et al. (2012) showed that the higher level of sequence divergence (and dominance) in A. arenosa promote flexibility of trans-factors for their binding to interacting factors and cis-elements of A. thaliana and A. arenosa alleles. In F1 and F2 S. purpurea, both cis- and trans-effects account for nonadditive or transgressive gene expression. These data also show significantly greater cis × trans and cis-trans-compensatory regulation in the F1 shoot tip transcriptome compared with the internode transcriptome, fitting the model in which greater transgressive inheritance tends to show greater proportions of cis × trans regulatory divergence (McManus et al. 2010). Further, for genes showing both cis- and trans-effects, antagonistic cis × trans and compensatory interactions were driven by the up-regulation of the paternal allele. The compatibility of novel trans-factors with their target binding sites could explain the high proportion of expression-level dominance in S. purpurea, which suggests that compensatory interactions and stabilizing selection may play an important role in maintaining parental gene expression levels.

The Fasciclin Gene Family Is Highly Expressed in the F1 Internode

Fasciclin-like AGPs were found to be over-represented and highly expressed in stem internode tissue, whereas genes encoding defense-related proteins were primarily upregulated in shoot tip tissue. Found in most angiosperms, AGPs are a class of Hyp-rich glycoproteins that are highly abundant in the cell wall and plasma membrane which are involved in various aspects of plant growth (Kitazawa et al. 2013) and primarily function in cell adhesion (Johnson et al. 2003). In Populus and Eucalyptus, FLAs were highly expressed during xylem differentiation (Hefer et al. 2015) and implicated in secondary cell wall thickening (Lafarguette et al. 2004). Salix FLA11 and FLA12 gene models are closely related to Arabidopsis FLA11

### Table 4

| Tissues       | Shoot Tip | Internode |
|---------------|-----------|-----------|
|               | cis       | trans     | cis       | trans     |
| Males         |           |           |           |           |
| All genes     | 40 16     | 37 34     | 66 51     | 68 54     |
| >1.5-fold     | 31 10     | 1 4       | 48 36     | 2 5       |
| >2-fold       | 21 7      | 1 1       | 34 29     | 0 4       |
| Females       |           |           |           |           |
| All genes     | 64 25     | 46 36     | 79 37     | 71 50     |
| >1.5-fold     | 47 10     | 1 2       | 59 23     | 3 1       |
| >2-fold       | 38 5      | 1 1       | 49 18     | 0 1       |

Note.—Numbers in boldface indicate significant (P < 0.01) deviations from a 1: 1 ratio according to a χ² test.
and FLA12 homologs and have been shown to contribute to stem tensile strength and the modulus of stem elasticity (MacMillan et al. 2010, 2015), which is consistent with FLA roles in cellulose deposition during secondary xylemogenesis. These data suggest that constitutive defense in the shoot tips of *S. purpurea* is coordinated with the rapid development of secondary xylem in stem internodes. Functional analysis of FLA homologs in *Salix* may prove to be useful in characterizing genes involved in the regulation of stem strength for the genetic improvement of shrub willow bioenergy crops.

**Sexually Dimorphic Gene Expression**

The effect of sex may explain a significant amount of the variation driving the evolution of gene expression in dioecious plants. While differential expression in the F1 family shoot tip transcriptome was found to be almost entirely nonadditive, the primary contributors of nonadditive expression were only discernable by barcoding and sequencing the F2 progeny individuals. This study provides evidence of sexually dimorphic expression in intraspecific F1 *S. purpurea*, where both cis- and trans-effects accounted for the observed differences in magnitude among regulatory patterns; however, the effect of sex on gene expression in the F1 was tissue-specific. Although allelic effects were comparable, Meiklejohn et al. (2014) described sexually dimorphic regulatory divergence among *Drosophilia simulans* and *D. mauritiana* introgression hybrids, proposing that pure-species genotypes carry modifier alleles that increase sexually dimorphic expression.

Although cis-effects accounted for more of the regulatory divergent expression in F1 females, compensatory regulation was enriched in F1 males. It may be the sex determining system itself can help explain sexually dimorphic ASE in *S. purpurea*. In females, the Z haplotype is paternally (ZZ) inherited and the W is maternally (ZW) inherited, but in males, both the maternal and paternal Z haplotypes are inherited equally. If there is substantial divergence between the Z and W, theoretically, cis-effects should outweigh trans-effects in females. In contrast, the two Z haplotypes present in males are considerably less polymorphic, such that parental expression differences on the Z would be explained by more antagonistic trans-regulatory interactions.

It is possible that a subset of the sexually dimorphic genes in F1 *S. purpurea* actually reflect unresolved conflicts between females and males and that sex-biased gene expression on autosomes or in pseudo-autosomal regions could be due to sexual antagonism (Alström-Rapaport et al. 1997; Nagamitsu and Futamura 2014; Su et al. 2016; Zhai et al. 2016). The accumulation of sexually antagonistic loci are predicted to occur in newly formed SDRs, where differential selection via tight linkage is beneficial to one sex and harmful to the other (Rice 1992). Over time, accumulation of sexually antagonistic genes within a population may eventually lead to a considerable conflict between sexes such that adaptation by each sex would be compromised. As female-benefit and male-detriment genes accumulated, Rice (1992) found that the sex ratios (m/f) of *Drosophilia* declined, suggesting that pseudo-autosomal regions (PARs) near the SDR can act as a hot spot for the accumulation of genes detrimental to the homogamic sex.

There are a number of studies which have reported consistent 2:1 (f: m) sex ratio-biases in natural willow populations (Ueno et al. 2007; Che-Castaldo et al. 2015). Disproportionate sex-ratios in natural populations of *Salix* spp. could promote evolutionary biases by sustaining the regulatory roles of the predominant sex. The higher proportion of genes with female-specific expression in *S. purpurea* may be a consequence of cyclic asexual reproduction leading to a relaxation of purifying selection on male-biased genes, whereby conflicting modes of inheritance and regulatory divergence patterns could lead to unequal investments in reproduction and reproductive strategies (Parsch and Ellegren 2013). However, sex-biased gene expression in *S. purpurea* may reflect resolved conflicts in favor of females.

**Sex-Biased Expression Localizes to the SDR of *S. purpurea* chr15**

The genus *Salix* exhibits a ZW sex determination system (Alström-Rapaport et al. 1997; Gunter et al. 2003; Semerikov et al. 2003; Liu et al. 2013), where the female is heterogametic (ZW) and the male is homogametic (ZZ), in contrast to the male heterogametic XY system of *P. trichocarpa* (Yin et al. 2008; Gerald et al. 2015). Although the genomes are rather collinear, is not entirely clear whether the SDR developed before or after the Salicoid duplication (Rogers-Melnick et al. 2012) and divergence of *Salix* from *Populus* (Hou et al. 2016). We have delimited the SDR of *S. purpurea* to a centromeric region on chr15 (Zhou et al. 2017) using a full-sib F2 *S. purpurea* mapping population (described here) and a diverse panel of *S. purpurea* naturalized North American genotypes. Zhou et al. (2017) show in *S. purpurea* that females are heterozygous (or hemizygous) and are males homozygous at polymorphic sites within the coding sequences of genes found in the SDR of *S. purpurea* chr15. As low recombination near the *Salix* SDR has prevented the identification of a single gene responsible for sex determination in experimental mapping populations (Pucholt et al. 2015; Chen et al. 2016; Pucholt et al. 2017), increasing the overall experimental scale is likely required to pinpoint the causative sex-determining genes.

Of the previous gene expression studies on sex determination in *Salix*, none have yet identified the gene(s) responsible. Even though a relatively small proportion of genes in the F1 shoot tip transcriptome of *S. purpurea* are sex-biased (<0.1%), there was no substantial evidence of sex-biased expression in the F1 internode transcriptome. At the time shoot tips were collected, it was likely that floral buds were
developing near the shoot apical meristem and it is possible this amalgam of cells included a subset of that were expressing genes involved in the determination of male or female flowers. Genes differentially expressed among the F1, male and female shoot tip transcriptome are not necessarily indicative of sex determination alone, but altogether represent a cascade of developmental gene expression involved in patterning, signaling, and organ suppression during the vegetative-to-reproductive transition leading to sexual dimorphism (Fairbairn and Roff 2006).

Tests for differential expression between mature catkins of female and male willows likely confound the search for sex determining genes because there are profound morphological and phenological differences between females and males at floral maturity. For instance, RNA-sequencing of male and female catkins of *S. suchowensis* (Liu et al. 2013) and *S. viminalis* (Pucholt et al. 2017) identified a plethora of differentially expressed genes, but failed to establish any biological links among those with significant associations. Yet, a link between sex determination and meristem fate is well-described in oil palm (Ho et al. 2016) and in maize via RNA-induced silencing of *TS1* and *AP2* by miRNA172 (Hartwig 2011). Genes related to DNA methylation, *MET1* and *DDM1*, as well as SAUR-like auxin responsive genes were implicated in sex determination in andromonecious *Populus tomentosa* (Song et al. 2013) and *P. trichocarpa* (McKown et al. 2017). In *Asparagus officinalis*, the MYB-like transcription factor, *MSE1*, is specifically expressed in males and has been shown to induce male sterility in knockouts of Arabidopsis (Murase et al. 2017). The identification of *MeGI*, an autosomal homeobox transcription factor in *Diospyros lotus*, has been shown to dominantly suppress male organ development can be suppressed by the small RNA OGI on the Y chromosome that targets *MeGI* for gene silencing (Akagi et al. 2014).

The most significant sex-biased gene highly expressed in males (log10(P) = 6.4) was most similar to the 40S ribosomal subunit, *RPS3* (SapurV1A.0582s0010), a positive regulator of apoptosis during UV irradiation in Arabidopsis (Lee et al. 2010). Conversely, the most significant upregulated gene in females (log10(P) = 42.6) was most similar to an inhibitor of apoptosis in Arabidopsis, a C3CH zinc finger homolog of *NUCLEAR INTERACTING PARTNER OF ALK* (*NIPA*) (SapurV1A.0934s0010). Developmental requirements controlling the vegetative-to-reproductive transition in meristematic tissues are likely to differ by sex. The Arabidopsis *ASSOCIATE OF C-MYC* (*AMY1*) homolog in *S. purpurea* (SapurV1A.1254s0040), was highly expressed in females and has been shown to interact with numerous chromatin modifiers and transcription factors in Arabidopsis (Taira et al. 1998) and implicated as a universal amplifier of gene expression, acting to increase output at all active promoters. Programmed cell death (PCD) in developing floral buds could lead to the specification of sex at the cost of structural reproductive components integral to the opposite sex. If PCD is a primary component in the determination of male or female flowers in *S. purpurea*, both the greater numbers and magnitude of differential gene expression in females could indicate that the role of PCD is more active in the development of female flowers.

**REPRODUCTIVE MERISTEM 1 (REM1)** (SapurV1A.0301s0070), AGAMOUS-LIKE 98 (*AGL98*) (SapurV1A.2504s0020), and *DOWN-REGULATOR OF TRANSCRIPTION 1 (DR1)* (SapurV1A.0301s0160) were highly expressed in F1 female shoot tip tissues and among the top differentially expressed genes that encode for known flowering-time genes in Arabidopsis (Pagnussat et al. 2005). The *REM1* gene aligns to a region within the SDR of *S. purpurea* chr15 and highly expressed in females, but showed complete null expression in males. In maturing inflorescences of Arabidopsis, *REM1* expression localized to only a few vegetative cells in the shoot apical meristem, but during the vegetative-to-reproductive transition, *REM1* was progressively restricted to the gynoeicum which gives rise to the stigma, style, and septum (Franco-Zorrilla et al. 2002). Although SapurV1A.1250s0040 is paralogous to SapurV1A.0301s0070, the former is highly expressed in males and is homologous to *A. thaliana* AG09. Implicated in the vegetative-to-reproductive transition during male gametogenesis in *A. thaliana*, the primary role of AG09 is to silence transposable elements (TEs) in the female gametophyte; thereby establishing the transgenerational epigenetic information required to control gametophytic fate (Hernandez-Lagana et al. 2016). Thus, it is conceivable that there is sRNA-induced silencing of W-specific genes via AGO loading in the RdDM pathway.

We have identified *TCP-1* (SapurV1A.1538s0020) as one of the few sex-biased genes that exhibited complete null expression in male shoot tip. TCP transcription factors play pivotal roles in the control of shoot morphogenesis by negatively regulating the expression of boundary-specific genes (Koyama et al. 2007; Koyama et al. 2010; Li et al. 2012), such as suppression of secondary wall thickening of the anther endothecium in Arabidopsis (Wang et al. 2015). Geraldes et al. (2015) identified two TCP-1 chaperonin family cpn-60 proteins associated with sex determination in the *TS2* *P. trichocarpa* association population, and this gene could play a major role in suppression of the anther endothecium in female *S. purpurea* catkins. Two sex-biased genes encoding the DExH-box ATP-dependent RNA helicase, *BRR2C*, are highly conserved components of the spliceosome and are required for efficient splicing of *FLC* introns, as well as regulation of *FT* and *SOC1* in Arabidopsis (Mahrez et al. 2016). Of the two sex-biased *Dr1/NF-Y* paralogs in *S. purpurea*, SapurV1A.0934s0060 was highly expressed in males, whereas SapurV1A.0301s0160 was highly expressed in females. *Dr1* represses RNAi transcription by binding to TBP to prevent the formation of an active transcription complex. Members of the heterotrimeric NF-Y transcription factor

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family in Arabidopsis initiate photoperiod-dependent flowering and also required for activation of the FT promoter by initiating downstream events leading to floral transition (Siriwardana et al. 2016).

We observed a number of genes with null expression in males that are highly expressed in females, yet we found no evidence for the converse. Rather, low levels of gene expression in females were always accompanied by low expression levels in males. Nearly 65% of all sex-biased genes were more highly expressed in females than in males, indicating disproportionate W-specific genes or Z-specific pseudogenes in the SDR. Given our findings on sexually dimorphic expression in S. purpurea, a reasonable hypothesis is that the transcription of genes involved in early floral meristem identity is differentially regulated by the relative abundance of RNAP II core subunits, whose promoter site specificity, initially guided by TBP components, are likely sex-specific.

We conclude that chr15 contributes to sexual dimorphism in S. purpurea, as genes with sex-biased expression were vastly over-represented within or near the SDR, compared with other autosomes. Although there was no apparent localization of sex-biased genes along chr19 as was found for chr15, over 12% of the sex-biased genes were most similar to P. trichocarpa chr19 gene models, many of which were highly expressed in males. In addition to mapping sex QTL on all three linkage maps to chr15 in F2 S. purpurea (family 317), Zhou et al. (2017) identified a secondary sex QTL on chr19; however, this QTL was only present in the male backcross map. It is not clear whether chr19 is epistatic to chr15, yet it seems likely that chr19 is the ancestral sex determining chromosome of Salix and Populus, and may very well continue to contribute to sex determination, sex ratio bias, and sex dimorphism in Salix.

Conclusions

This study provides the first detailed analysis of transcriptome-wide regulatory divergent expression in Salix. Expression-level dominance and sexual dimorphism are prevailing features of differential gene expression in S. purpurea. Expanding upon transcriptomic resources in Salix will not only contribute to our understanding of the evolution of dioecy in the Salicaceae, but also facilitate the functional characterization of genes underlying sex determination in dioecious species.

Supplementary Material

Supplementary data are available at Genome Biology and Evolution online.

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