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Transcriptional profiling analysis in *Populus yunnanensis* provides insights into molecular mechanisms of sexual differences in salinity tolerance

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Abstract

Physiological responses to abiotic stress in plants exhibit sexual differences. Females usually experience greater negative effects than males; however, little is known about the molecular mechanisms of sexual differences in abiotic stress responses. In the present study, transcriptional responses to salinity treatments were compared between male and female individuals of the poplar *Populus yunnanensis*. It was found that several functional groups of genes involved in important pathways were differentially expressed, including photosynthesis-related genes, which were mainly up-regulated in males but down-regulated in females. This gene expression pattern is consistent with physiological observations showing that salinity inhibited photosynthetic capacity more in females than in males. Furthermore, genes located in autosomes rather than in the female-specific region of the W chromosome are the major contributors to the sexual differences in the salinity tolerance of poplars. In conclusion, this study provided molecular evidence of sexual differences in the salinity tolerance of poplars. The identified sex-related genes in salinity tolerance and their functional groups will enhance our understanding of sexual differences in salinity stress at the transcription level.

Key words: Next-generation sequencing, physiological responses, *Populus*, salinity tolerance, sexual differences, transcriptional profiling.

Introduction

More than 800 Mha of land throughout the world are affected by salt (FAO, 2008). Among various salt types, sodium chloride is considered to be the most soluble and abundant salt. Although plants cannot escape from high salinity, they can trigger signals to alter their physiological characteristics and growth in order to survive (Zeller *et al.*, 2009). Indeed, salinity stress affects virtually every aspect of plant physiology and metabolism. Therefore, an improvement of salinity tolerance in plants is an urgent issue and has been the target of several previous studies (Hasegawa *et al.*, 2000; Zhu, 2000, 2001; Yokoi *et al.*, 2002; Bartels and Sunkar, 2005; Munns, 2005; Munns and Tester, 2008). Many salt-responsive genes have been identified (Kawasaki

et al., 2001; Seki *et al.*, 2002; Rabbani *et al.*, 2003; Dinneny *et al.*, 2008) and several salinity-related signalling pathways have been predicted and confirmed (Knight and Knight, 2001; Zhu, 2002). Comparative transcriptional profiles of salt-sensitive (*Arabidopsis thaliana*) and salt-tolerant species (*Thellungiella halophila*) revealed a common set of genes in response to salt (Taji *et al.*, 2004), and genes involved in photosynthesis, osmolyte production and transcription (Gong *et al.*, 2005) exhibited species-specific differences, with only a few genes differentially regulated in the salt-tolerant *T. halophila* plants (Wong *et al.*, 2006). Transcriptome studies also found that different functional categories of transcripts were differentially regulated at

different time points (Kawasaki *et al.*, 2001) and there is a cross-talk between signalling pathways in various stress conditions (Seki *et al.*, 2002; Rabbani *et al.*, 2003; Wong *et al.*, 2006). These transcriptome studies have provided new knowledge of how to improve the salinity tolerance of plants.

Dioecious plants are an important component of terrestrial ecosystems, representing nearly 6% (14 620 of 240 000) of angiosperm species (Renner and Ricklefs, 1995). They play a crucial role in maintaining the stability of the structure and function in terrestrial ecosystems. Many dominant woody species, such as *Populus*, are dioecious. Sexual differences have been reported in several dioecious plants in response to salinity and other environmental stresses (Li *et al.*, 2007; F. Chen *et al.*, 2010; Zhang *et al.*, 2011), and females usually show a lower tolerance capacity compared with males. For instance, *P. yunnanensis* males and females showed significant differences under salinity, drought, and, in particular, under a combination of both these stresses (L. Chen *et al.*, 2010). Furthermore, male *P. tremuloides* trees were more responsive to elevated CO₂ than females in terms of photosynthetic rates (Wang and Curtis, 2001). Sexual differences have also been observed in earlier studies on *P. cathayana* subjected to drought stress (Xu *et al.*, 2008a, b), UV-B radiation, and elevated CO₂ (Zhao *et al.*, 2009, 2011; Xu *et al.*, 2010).

Because there is considerable variation in physiological responses between males and females, it is hypothesized that molecular responses may parallel salinity stress. However, molecular mechanisms underlying sex-related differences are largely unknown. Here the Solexa/Illumina's digital gene expression (DGE) system (Blow, 2009; Morrissy *et al.*, 2009) is used to investigate differential gene expression in responses to salinity stress between males and females of the poplar *P. yunnanensis*, which is naturally distributed at altitudes of 1300–2700 m in southwest China. The aims of the study are (i) to assess whether males are more tolerant to salinity stress than females; and (ii) to elucidate sex-related molecular mechanisms for salinity tolerance, and their possible relationship to sex chromosomes.

Materials and methods

Plant materials and experimental design

Two male and female trees of *P. yunnanensis* collected from a natural population in Meigu, Sichuan Province, China (28°18'N and 103°06'E), were used for a controlled intraspecific cross. From the F₁ progeny, 10 male and 10 female individuals were vegetatively propagated to produce 50 male and 50 female cuttings, respectively. The cuttings were planted in 10 litre plastic pots filled with 8 kg of homogenized soil. After sprouting and growing for ~2 months, healthy plants were replanted (one plant per pot). After another 2 months (July 2009), healthy plants with a similar crown size and equal height were chosen for the experiments. The experimental layout was completely randomized with two factors (sex and salt application). Therefore, there were finally four treatments: (i) males without salt application (control); (ii) females without salt application (control); (iii) males with salt application; and (iv) females with salt application. Nine plants of

each sex were exposed to each treatment. Three replicates with three plants each were used to account for sampling errors. Salt-treated plants were firstly exposed to short-term low concentration salt pre-treatment (25 mM NaCl for 24 h) avoiding a large osmotic shock, and then to long-term salinity (50 mM NaCl for 7 d). Control plants were kept in well-watered conditions. After 8 d of salinity treatment, the fourth fully expanded and intact young leaves near the shoot apex of each plant were harvested and frozen immediately in liquid nitrogen, then stored at -80 °C for physiological monitoring and molecular analyses.

Measurements of gas exchange and chlorophyll fluorescence

Gas exchange and chlorophyll fluorescence traits were measured using the fourth fully expanded and intact leaves. The net photosynthesis rate (P_n), transpiration rate (E), and intercellular CO₂ concentration (C_i) were measured with a Li-Cor 6400, a portable photosynthesis measuring system (Li-Cor Inc., Lincoln, NE, USA). The gas exchange was measured in the morning (08:00–12:00 h) using the following conditions: leaf temperature, 25 °C; leaf-to-air vapour pressure deficit, 1.5 ± 0.5 kPa; photosynthetic photon flux (PPF), $1400 \mu\text{mol m}^{-2} \text{s}^{-1}$; relative air humidity, 50%; and ambient CO₂ concentration, $350 \pm 5 \mu\text{mol mol}^{-1}$. To measure chlorophyll fluorescence kinetic traits, the variable and maximum fluorescence (F_v/F_m) and qP (photochemical quenching coefficient) were determined using a PAM chlorophyll fluorometer (PAM 2100, Walz, Effeltrich, Germany) between 07:30 and 09:30 h (Kooten and Snel, 1990).

Determinations of chlorophyll, proline, and Cl⁻ contents

Leaf discs (0.3 g) were treated with 80% chilled acetone (v/v) at 4 °C for ~20 h until they changed to a white colour and were then quantified using a spectrometer (Unicam UV-330, Unicam, Cambridge, UK). Chlorophyll was extracted (Lichtenthaler, 1987) and the absorbances of chlorophyll *a* (Chl *a*) and Chl *b* were determined at 646 nm and 663 nm, respectively. Proline was measured by a spectrophotometric analysis at 515 nm using the ninhydrin reaction (Bates *et al.*, 1973).

Cl⁻ contents of leaves were analysed as described by Chen *et al.* (2001). Leaf samples were ground and passed through a 20 mesh screen after drying at 80 °C for 40 h. Dry powder (0.5 g) was extracted with 1 N HNO₃. Abundant AgNO₃ solution (0.025 N) was used to precipitate chloride from the aqueous extracts, and excess Ag⁺ was estimated by 0.02976 N NH₄SCN titration. NH₄Fe(SO₄)₂ was used as a colour indicator for determination of the isoionic point. The chloride concentration was calculated using the following formula: $\text{Cl}^- (\text{mmol g}^{-1} \text{DW}) = (\text{N}_{\text{AgNO}_3} \text{V}_1 - \text{N}_{\text{NH}_4\text{SCN}} \text{V}_2) / \text{DW}$, where DW (g) is the dry weight of plant tissue, V₁ (ml) represents the total volume of AgNO₃ solution in Cl⁻ extracts, and V₂ (ml) is the volume of NH₄SCN solution used for excess Ag⁺ precipitation.

Physiological data analysis

Physiological data were analysed using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Two-way analyses of variance were performed to evaluate the interaction effect of sex and salinity conditions. Sexual differences were analysed using a model with salinity and sex as fixed effects. Significant individual differences among means of different treatments were determined by Tukey's multiple range tests after conducting tests of homogeneity for variances. Differences were considered as statistically significant at the $P < 0.05$ level.

RNA isolation and DGE library preparation

Four DGE libraries representing four different treatments were prepared. Total RNA was extracted from three biological replicates using the E.Z.N.A.™ Plant RNA Kit (Omega, USA)

according to the manufacturer's instructions. Residual genomic DNA was removed with DNase I and the integrity of RNA was checked using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). RNA samples from nine individuals from each treatment were pooled with equal proportions. Double-stranded cDNA was synthesized using oligo(dT) beads. The cDNA was then digested with an anchoring restriction enzyme *Nla*III, which recognizes and cuts 3' CATG. The digested cDNA samples were ligated to the Illumina-specific adaptor A, containing a recognition site for the enzyme *Mme*I. Following *Mme*I digestion, the Illumina-specific adaptor B containing a 2 bp degenerated 3' overhang was ligated. The adaptor-ligated cDNA tag library was then enriched by PCR primers annealing to the adaptor ends. The PCR program was as follows: 30 s at 98 °C, followed by 15 cycles of 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 15 s, and then 72 °C for 5 min. After gel purification, cDNA was used for cluster generation on a separated flow cell lane. Sequencing by synthesis was performed using the Illumina Genome Analyzer according to the manufacturer's protocol. Image analysis, base calling, extraction of 17 bp tags, and tag counting were performed using the Illumina pipeline.

DGE data analysis

After data processing, raw sequences were transformed into clean tags. Sequences of 21 bp length (17 bp tag plus 4 bp *Nla*III recognition site) were aligned against the poplar genome (*Populus trichocarpa* v1.1) (Tuskan *et al.*, 2006) using the Short Oligonucleotide Alignment Program (SOAP) (Li *et al.*, 2008). No more than a 1 bp mismatch was taken into account for the differences between species. Clean tags mapped to reference sequences from multiple genes were filtered. The remaining clean tags were designated as the distinct tags for further analysis. The number of distinct tags for each gene was calculated and then normalized to the TPM (number of transcripts per million clean tags) (t Hoen *et al.*, 2008; Morrissy *et al.*, 2009).

To identify differentially expressed genes (DEGs) between two samples, a rigorous algorithm was developed by BGI (Shenzhen, China), similar to 'the significance of digital gene expression profiles' (Audic and Claverie, 1997). A brief description of the algorithm is as follows: the number of distinct tags from gene A is denoted as x , and as every gene's expression occupies only a small part of the library, the $p(x)$ follows the Poisson distribution.

$$p(x) = \frac{e^{-\lambda} \lambda^x}{x!}$$

where λ is the real transcript of a gene.

The total clean tag number of sample 1 is N_1 , and the total clean tag number of sample 2 is N_2 ; gene A holds x tags in sample 1 and y tags in sample 2. The probability of gene A being expressed equally in two samples can be calculated as follows:

$$2 \sum_{i=0}^{i=y} p(y|x) \text{ or } 2 \times \left(1 - \sum_{i=0}^{i=y} p(y|x)\right) \left(\text{If } \sum_{i=0}^{i=y} p(y|x) > 0.5\right)$$

$$p(y|x) = \left(\frac{N_2}{N_1}\right)^y \frac{(x+y)!}{x!y!(1 + \frac{N_2}{N_1})^{(x+y+1)}}$$

The P -value represents the significance of differential gene expression. The false discovery rate (FDR) determines the threshold of the P -value in multiple tests and analyses through manipulating the FDR value. If R differentially expressed genes were selected among which S genes really showed differential expression, then the other V genes are false positive. If the error ratio 'Q=V/R' must stay below a cut-off (e.g. 5%), then the FDR should be pre-set to a number not larger than 0.05 (Benjamini and Yekutieli, 2001). FDR < 0.001 and the absolute value of log-2 ratio ≥ 1 were the thresholds for the significance of a gene with differential expression. The full data set has been deposited in the

Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) and is accessible through accession number GSE35223.

For the analysis of DEGs between males and females in response to salinity, singular enrichment analysis (SEA) and parametric analysis of gene set enrichment (PAGE, <http://bioinfo.cau.edu.cn/agriGO/>) were performed using plant GO slim with the *P. trichocarpa* gene models as background, followed by multiple testing with Bonferroni (corrected P -value ≤ 0.05) (Du *et al.*, 2010). Functional annotation and gene ontology (GO) were also retrieved by querying with BLASTN against the *Arabidopsis* AGI transcripts database (<http://arabidopsis.org/>; TAIR10 release). Only BLASTN hits of e -value $\leq 1E-5$ were considered further. The AGI number of each corresponding poplar DEG was categorized into functional groups and mapped using the MapMan (version 3.5.1; <http://www.gabipd.de/projects/MapMan/>) according to the standard protocol (Thimm *et al.*, 2004; Usadel *et al.*, 2005).

qRT-PCR

Ten transcripts from DGE libraries were validated by quantitative reverse transcription-PCR (qRT-PCR; Supplementary Table S1 available at *JXB* online) using the same RNA samples as in the DGE library construction. Total RNA was treated with DNase I and used for cDNA synthesis with ReverTra Ace Moloney murine leukaemia virus reverse transcriptase (Toyobo, Osaka, Japan) and oligo(dT)₁₈ primer. Synthesized cDNAs were diluted to a final volume of 100 μ l, and 1 μ l was used as a template for qRT-PCRs. qRT-PCR was performed in triplicate on the Opticon Monitor 3 DNA engine (Bio-Rad). The detailed cycling conditions were described in the manufacturer's instructions of SYBR[®] Premix Ex Taq[™] II (TaKaRa, Dalian, China). Sequences of the gene-specific primers are presented in Supplementary Table S1. The gene encoding ubiquitin-conjugating enzyme (NCBI accession number: XM_002307243.1) with a similar intensity value across all DGE libraries was used as the reference gene for normalization. Gene expression was evaluated using the comparative cycle threshold method, and data were expressed as the mean \pm SE (Livak and Schmittgen, 2001).

Results

Sexual differences in physiological traits

Under salinity stress, the photosynthetic capacity, P_n , E , C_i , total chlorophyll content (TC), F_v/F_m , and qP significantly decreased; while the proline and Cl^- contents, and the Chl *alb* ratio increased in both males and females (Table 1). Significant sexual differences in these physiological traits were also observed, among which P_n , E , C_i , F_v/F_m , and qP decreased more in females than in males, whereas the Chl *alb* ratio increased less in males than in females. Furthermore, salinity stress significantly increased the proline content in males but it had less effect on females (Fig. 1a); while it significantly increased the Cl^- content in females compared with that in males (Fig. 1b). In addition, the interaction between sex and salinity stress was significant in P_n , E , C_i , F_v/F_m , qP, Chl *alb* ratio, and proline content (Fig. 1a and Table 1).

Transcriptional profiles

Four DGE libraries were sequenced using the Solexa technology. After data processing according to DGE system requirements, the total number of clean tags per library

Table 1. Sexual differences in photosynthetic responses to salinity stress in *P. yunnanensis*

	P_n ($\mu\text{mol m}^{-2}\text{s}^{-1}$)	E ($\text{mmol m}^{-2}\text{s}^{-1}$)	C_i ($\mu\text{mol mol}^{-1}$)	F_v/F_m	qP	TC (mg g^{-1})	Chl <i>a/b</i>
Control							
Male	16.17±0.38 a	4.22±0.13 a	256±2.65 a	0.82±0.009 a	0.88±0.02 a	1.23±0.01 a	2.10±0.05 c
Female	14.97±0.46 a	4.22±0.15 a	267±9.17 a	0.82±0.004 a	0.88±0.013 a	1.22±0.01 a	1.93±0.02 b
50 mM NaCl							
Male	12.33±0.09 b	3.49±0.11 b	236±5.69 ab	0.78±0.004 b	0.81±0.002 b	1.01±0.04 b	2.58±0.09 c
Female	9.44±0.09 c	2.89±0.10 c	206±8.66 c	0.69±0.008 c	0.69±0.018 c	0.93±0.15 c	5.05±0.40 a
P (sex)	0.000 ***	0.046 *	0.214 NS	0.000***	0.001**	0.065 NS	0.000 ***
P (salt)	0.000 ***	0.000 ***	0.000 ***	0.000***	0.000***	0.000 ***	0.000 ***
P (sex×salt)	0.025 *	0.042 *	0.020 *	0.000***	0.002**	0.174 ns	0.000 ***

Different letters represent statistical significance between treatments (means \pm SE, $n=3$) at $P < 0.05$ according to Tukey's multiple range tests. Significant values of the factorial analysis (ANOVA) for the effects of sex, salt, and sex×salt interaction are denoted as follows: NS, non-significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

P_n , net photosynthesis rate; E , transpiration rate; C_i , intercellular CO_2 concentration; F_v/F_m , variable and maximum fluorescence; qP, photochemical quenching coefficient; TC, total chlorophyll content; Chl *a/b*, the chlorophyll *a/b* ratio.

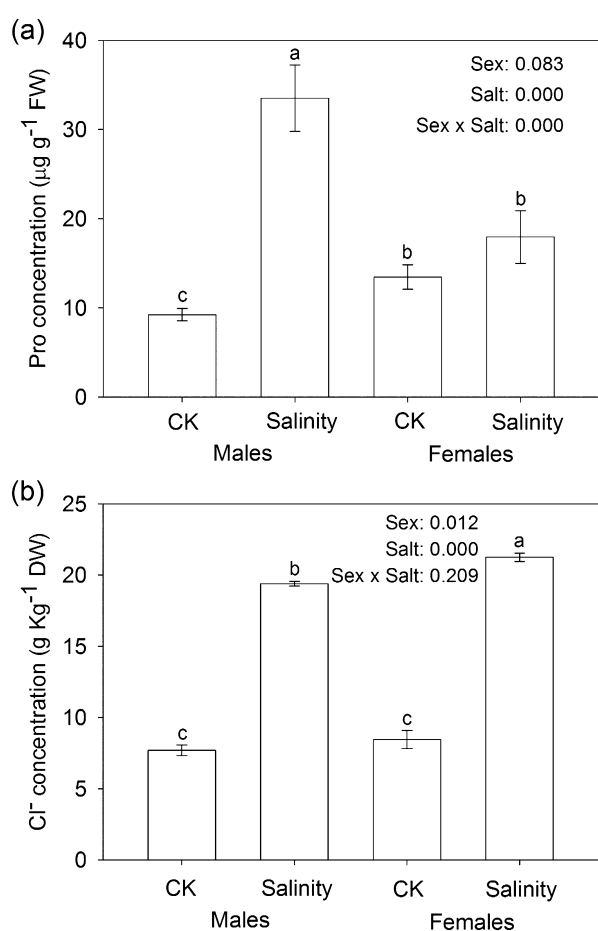


Fig. 1. Salt-induced accumulation of (a) proline and (b) Cl^- (mean \pm SE, $n=3$) in males and females. Different letters above the bars represent statistically significant differences between treatments at $P < 0.05$ according to Tukey's multiple range tests. Significance values of the factorial analysis (ANOVA) are denoted as follows: sex, sex effect; salt, salt effect; sex×salt, sex×salt interaction effects.

ranged from ~ 2.3 million to 3.4 million, and distinct tags representing unique nucleotide sequences varied from ~ 0.15 million to 0.22 million. Figure 2 shows the distribution

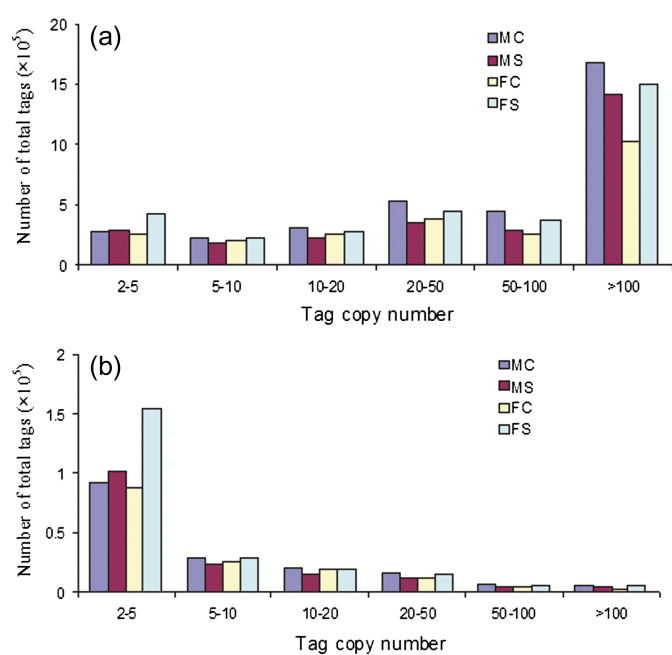


Fig. 2. Distribution of clean tag copy numbers in males and females. (a) Distribution of all clean tags. (b) Distribution of distinct clean tags, which represent unique nucleotide sequences. MC, males with 0 mM NaCl; MS, males with 50 mM NaCl; FC, females with 0 mM NaCl; FS, females with 50 mM NaCl. (This figure is available in colour at *JXB* online.)

profiles of the clean and distinct tags. Tags with an abundance of >100 copies in quantity and <5 copies in variety were dominant. A total of 4224 and 5980 DEGs were detected in males and females, respectively. These DEGs were involved in metabolism, regulation, and other aspects. A large proportion ($\sim 77\%$) of the DEGs were down-regulated in response to salinity stress. To confirm the DGE library data, 10 transcripts were selected for qRT-PCR analysis. The expression tendency of these transcripts was highly consistent when these two methods were compared (Supplementary Fig. S1 at *JXB* online).

Functional categories of DEGs

Two strategies were used to identify functional gene groups differentially expressed in the two sexes under salinity. First, an overview of the main results was obtained by PAGE, and significant GO terms were listed (Tables 2 and 3). It was found that the most enriched term in the biological process was ‘photosynthesis’ (GO: 0015979), which was up-regulated under salinity stress in males. In contrast, ‘cellular macromolecule metabolic process’ (GO: 0044260), ‘developmental process’ (GO: 0032502), and ‘anatomical structure development’ (GO: 0048856) were down-regulated in females. In the molecular function, ‘DNA binding’ (GO: 0003677), ‘nuclease activity’ (GO: 0004518), and ‘nucleic acid binding’ (GO: 0003676) were detected more frequently in males; while ‘motor activity’ (GO: 0003774), ‘chromatin binding’ (GO: 0003682), ‘pyrophosphatase activity’ (GO: 0016462), etc. were relatively abundant in females. The most enriched terms of the cellular component in males and females were ‘nuclear lumen’ (GO: 0031981) and ‘intracellular part’ (GO: 0044424), respectively. The second strategy was based on the gene expression levels in males and females. The DEGs were classified into two groups: the salinity-induced co-regulated DEGs in both males and females (Figs 3a, 4a) and the sexually different DEGs (Figs 3b, 4b). A total of 1420 and 609 DEGs were found to be down- and up-regulated, respectively, only in males, while 2944 and 841 DEGs were down- and up-regulated, respectively, only in females. Interestingly, 78 DEGs were up-regulated in males but down-regulated in females (UMDF), while 51 DEGs were regulated in the opposite way (UFDM).

To test whether the DEGs above are directly related to sexual differences in response to salinity stress, they were compared with the *Arabidopsis* genome, and the best matches and AGI numbers were obtained. Further MapMan analysis

indicated that these DEGs were widely involved in plant metabolic and regulatory pathways (Fig. 5; Supplementary Fig. S2 at *JXB* online), including DEGs related to metabolism, such as photosynthesis, major and minor carbohydrates, cell wall, lipids, and amino acids. DEGs related to plant regulation mostly concerned transcription factors, protein modification and degradation, and hormones. These results suggested that salinity stress has a great influence on poplars, which changed the transcript abundance of many genes to tackle salinity. These results also indicated that a set of common functions and pathways are induced in the salinity responses, regardless of the sex. Nevertheless, distinct differences between males and females were shown in some pathways, such as photosynthesis and transcription (see the later sections), highlighting sexual differences in salinity responses.

Sexual differences of co-regulated DEGs under salinity stress

Most co-regulated DEGs in both males and females were down-regulated under salinity stress. Due to different gene expression levels, these DEGs were divided into three groups. The first group consisted of 34 genes with similar expression patterns in both males and females, such as *SPL7*, *TOR*, *GI*, and *DEX1*. These genes were mostly involved in development. Genes in the second group represented a cluster with greater expression in males, including eight ribosomal protein genes (*RPS3C*, *RPS4D*, *RPS17C*, *RPS18C*, *RPS30B*, *RPL15A*, *RPL31B*, and *MEE49*) and genes with function in amino acid metabolism (such as *MAT3*, *MTO1*, and *CBL*), minor carbohydrates, signalling, redox, and DNA repair. The third group of genes up-regulated in females had functions in starch degradation, tetrapyrrole synthesis, post-transcriptional

Table 2. Overview of significant GO terms analysed by the parametric analysis of gene set enrichment (PAGE) in males

GO term	Onto	No.	Description	Z-score	Mean	FDR
GO: 0015979	P	63	Photosynthesis	5.6	0.74	1.6e-06
GO: 0006350	P	361	Transcription	-3.6	-2.8	0.023
GO: 0010467	P	703	Gene expression	-3.7	-2.6	0.018
GO: 0043170	P	1381	Macromolecule metabolic process	-3.8	-2.5	0.0094
GO: 0040029	P	42	Regulation of gene expression, epigenetic	-3.9	-4.5	0.0088
GO: 0006259	P	171	DNA metabolic process	-4.6	-3.5	0.00038
GO: 0006807	P	908	Nitrogen compound metabolic process	-4.7	-2.7	0.00016
GO: 0016043	P	600	Cellular component organization	-4.9	-2.9	8.8e-05
GO: 0006139	P	779	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	-5.6	-2.9	1.2e-06
GO: 0003677	F	361	DNA binding	-3.5	-2.8	0.014
GO: 0004518	F	46	Nuclease activity	-3.7	-4.2	0.0076
GO: 0003676	F	723	Nucleic acid binding	-3.8	-2.6	0.0038
GO: 0031981	C	199	Nuclear lumen	-3.5	-3.1	0.02
GO: 0005856	C	152	Cytoskeleton	-3.6	-3.2	0.018
GO: 0044428	C	311	Nuclear part	-5	-3.2	3.2e-05
GO: 0005634	C	806	Nucleus	-5	-2.8	2.3e-05

P, biological process; F, molecular function; C, cellular component. Mean, mean log₂ expression ratio, >0 represents up-regulation, <0 represents down-regulation.

Table 3. Overview of significant GO terms analysed by the parametric analysis of gene set enrichment (PAGE) in females

GO term	Onto	No.	Description	Z-score	Mean	FDR
GO: 0044260	P	1727	Cellular macromolecule metabolic process	-3.4	-2.7	0.049
GO: 0032502	P	1147	Developmental process	-3.5	-2.8	0.043
GO: 0048856	P	887	Anatomical structure development	-3.5	-2.9	0.037
GO: 0048869	P	368	Cellular developmental process	-3.5	-3.2	0.032
GO: 0043170	P	1960	Macromolecule metabolic process	-3.6	-2.7	0.027
GO: 0030154	P	299	Cell differentiation	-3.6	-3.3	0.025
GO: 0000003	P	609	Reproduction	-3.8	-3	0.011
GO: 0009653	P	412	Anatomical structure morphogenesis	-3.9	-3.2	0.0063
GO: 0009790	P	416	Embryonic development	-4.1	-3.2	0.0038
GO: 0006807	P	1290	Nitrogen compound metabolic process	-4.2	-2.9	0.0024
GO: 0006139	P	1094	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	-4.5	-2.9	0.00054
GO: 0016043	P	792	Cellular component organization	-4.9	-3.1	8.3e-05
GO: 0007049	P	309	Cell cycle	-5.3	-3.7	9.3e-06
GO: 0006259	P	230	DNA metabolic process	-5.7	-4	9e-07
GO: 0003774	F	53	Motor activity	-3.4	-4.5	0.019
GO: 0003682	F	55	Chromatin binding	-3.5	-4.5	0.015
GO: 0016462	F	357	Pyrophosphatase activity	-4.6	-3.4	0.00013
GO: 0016817	F	364	Hydrolase activity, acting on acid anhydrides	-4.7	-3.4	9.8e-05
GO: 0016818	F	361	Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	-4.7	-3.4	9.4e-05
GO: 0017111	F	349	Nucleoside-triphosphatase activity	-5	-3.5	2.1e-05
GO: 0000166	F	993	Nucleotide binding	-5.2	-3.1	4.9e-06
GO: 0044424	C	2977	Intracellular part	-3.5	-2.6	0.024
GO: 0005622	C	3057	Intracellular	-3.7	-2.6	0.013
GO: 0032991	C	856	Macromolecular complex	-3.8	-2.9	0.0079
GO: 0005856	C	199	Cytoskeleton	-3.9	-3.6	0.0043
GO: 0005730	C	113	Nucleolus	-4	-4	0.0036
GO: 0043227	C	2464	Membrane-bound organelle	-4	-2.7	0.0027
GO: 0043231	C	2461	Intracellular membrane-bound organelle	-4	-2.7	0.0027
GO: 0005634	C	1088	Nucleus	-4.1	-2.9	0.0025
GO: 0005654	C	162	Nucleoplasm	-4.1	-3.8	0.0019
GO: 0043229	C	2652	Intracellular organelle	-4.2	-2.7	0.0014
GO: 0043226	C	2654	Organelle	-4.2	-2.7	0.0014
GO: 0044446	C	1223	Intracellular organelle part	-4.4	-2.9	0.00051
GO: 0044422	C	1227	Organelle part	-4.4	-2.9	0.00048
GO: 0031974	C	343	Membrane-enclosed lumen	-4.8	-3.5	6.9e-05
GO: 0070013	C	340	Intracellular organelle lumen	-4.9	-3.5	5e-05
GO: 0043233	C	340	Organelle lumen	-4.9	-3.5	5e-05
GO: 0031981	C	262	Nuclear lumen	-5.7	-3.9	7.4e-07
GO: 0043232	C	571	Intracellular non-membrane-bound organelle	-6.1	-3.5	4.1e-08
GO: 0043228	C	571	Non-membrane-bound organelle	-6.1	-3.5	4.1e-08
GO: 0044428	C	406	Nuclear part	-7	-3.9	1.8e-10

P, biological process; F, molecular function; C, cellular component. Mean, mean log-2 expression ratio, >0 represents up-regulation, <0 represents down-regulation.

modification of proteins, light signalling, DNA synthesis, protein targeting, and transport.

Genes up-regulated only in males or in females

Under salinity stress, 609 genes were up-regulated only in males (Supplementary Table S2 at *JXB* online), such as genes encoding ketoacyl ACP synthase, enoyl ACP reductase, long chain fatty acid-CoA ligase, and acyl-CoA-binding protein, which are involved in fatty acid (FA) synthesis and elongation. Two genes, *GGT1* and *ALAAT2*, encoding aminotransferases involved in central amino acid metabolism and two genes encoding galactosyltransferase

family proteins were also detected only in males. More importantly, five genes encoding photosystem II (PSII) polypeptide subunits were exclusively up-regulated in males, providing possible reasons why males maintained relatively higher F_v/F_m than females under salinity (Table 1).

There were 841 genes up-regulated only in females (Supplementary Table S3 at *JXB* online), including genes related to lipid metabolism (nine genes), stress (four genes), miscellaneous enzyme families (22 genes), and other proteins (66 genes). This result suggested that females increase gene expression of regulatory pathways during salinity. The functional categories FA synthesis and FA elongation were also detected in females, but the identified genes in females

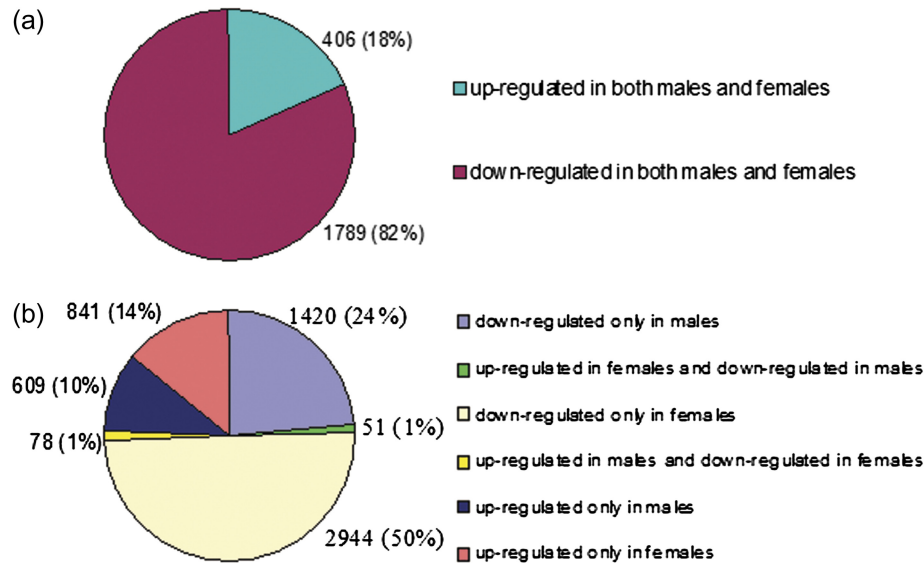


Fig. 3. Classifications of (a) co-regulated and (b) sexually different DEGs up- or down-regulated in males and females under salinity stress. (This figure is available in colour at *JXB* online.)

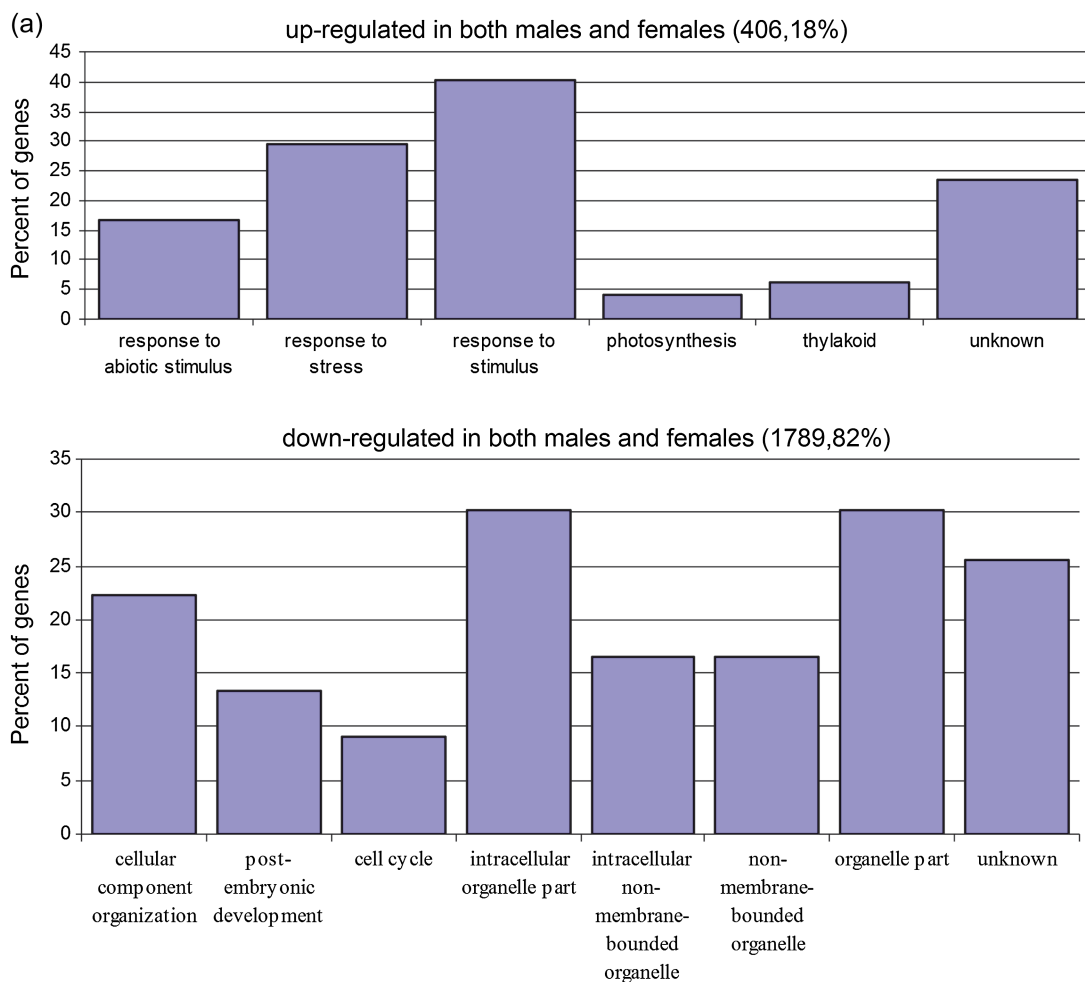


Fig. 4. SEA analysis implying significant GO terms of (a) co-regulated and (b) sexually different DEGs up- or down-regulated in males and females under salinity stress, excluding categories 'up-regulated only in females' (only one: vacuole) and 'up-regulated in females and down-regulated in males' (none). (This figure is available in colour at *JXB* online.)

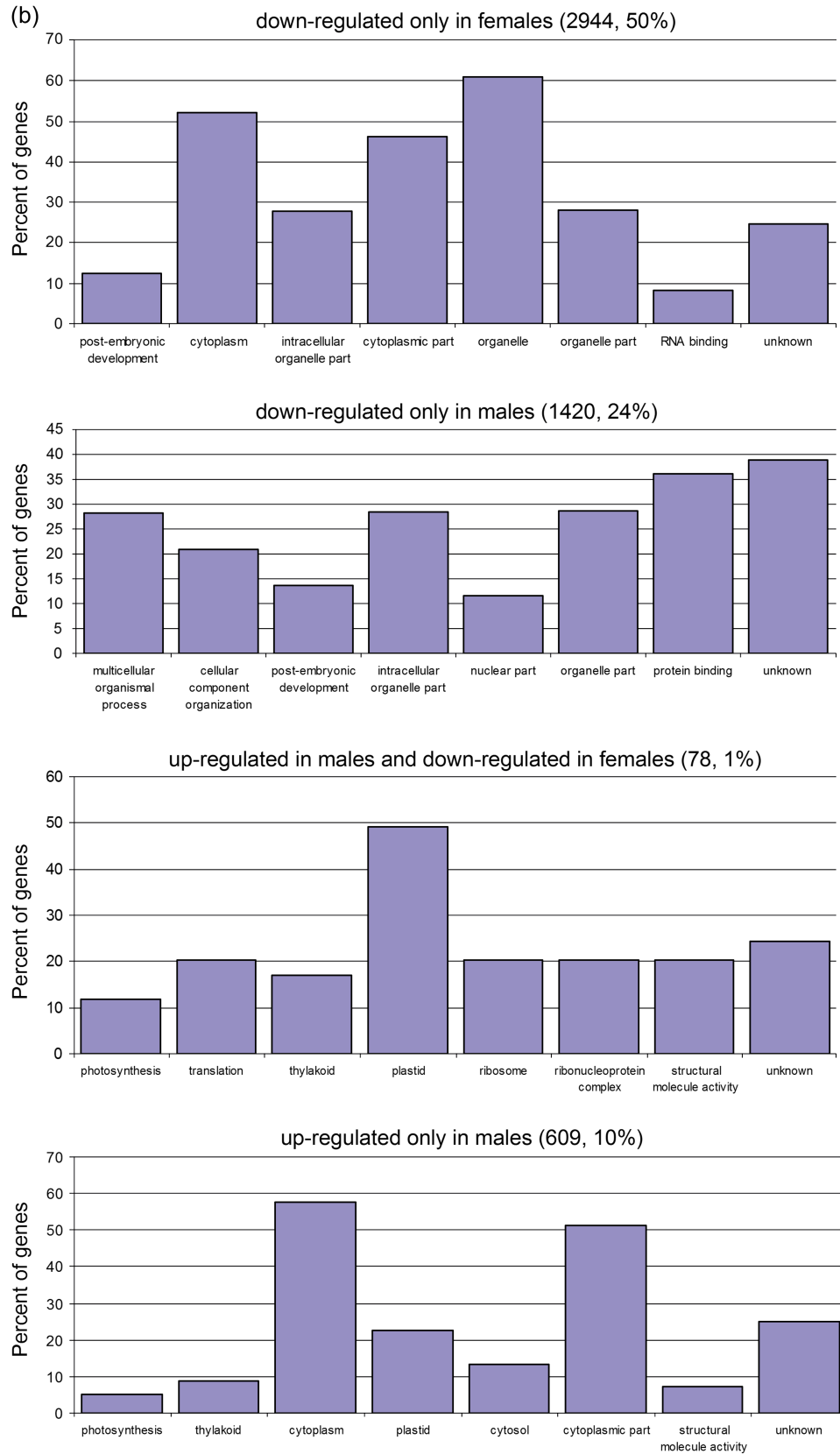


Fig. 4. Continued

were different from those in males. In females, genes involved in FA synthesis and FA elongation were *KCS11* and *MCD*, which encode β -ketoacyl-CoA synthase and

malonyl-CoA decarboxylase, respectively. The salinity stress-altered gene expression pattern involved miscellaneous enzyme families, including genes encoding a universal

stress protein family protein, pollen Ole e 1 allergen and extensin family protein, and germin-like protein 5. Interestingly, DEGs identified only in females included genes related to four functional groups of proteins: protein synthesis (12 genes, e.g. *RPS9C*, *RPS13A*, *RPS19C*, *RPS20A*, *RPS24A*, and *RPS29C*), targeting (four genes, e.g. *NTF2* and *ALB3*), post-translational modification (18 genes, e.g. *CKA2*, *CIPK25*, and *PP2C*), and degradation (32 genes, e.g. *EGY2*, *PUX3*, and *PADI*). This result suggested that genes encoding enzymes and other proteins could play key roles in the salinity tolerance of female poplars, and their different expression patterns may make a significant contribution to the different physiological responses in males and females under salinity stress.

Genes down-regulated only in males or in females

There were 1420 genes down-regulated only in males (Supplementary Table S4 at *JXB* online), including genes related to cell functions, such as hormone metabolism, transcription, and DNA synthesis. In hormone metabolism, the genes found mainly had functions in the synthesis and degradation of auxin, abscisic acid, brassinosteroid, and jasmonate, such as *ARG1*, *ABA3*, *XF1*, and *OPR2*. Two signal transduction genes, and genes encoding squalene monooxygenase and ethylene-responsive family protein, were also detected. This result suggested that male poplars changed their phytohormone levels to cope with salinity stress, and the genes detected only in males may further expand the sexually different adaptation. Three genes involved in lipid degradation (*IBR3*, *MFP2*, and *FAR4*), and 15 genes related to chromatin structure involving DNA synthesis, such as *DPB2*, *SLD5*, *CHR4*, *RHL2*, *NRP1*, *EMB2411*, and *MAA3*, were also down-regulated. This observation suggested that lipids and chromatin of male poplars could develop reorganization in response to salinity.

In contrast, 2944 genes were down-regulated only in females, including five major pathways: minor carbohydrate (16 genes), lipid (40 genes), hormone (24 genes), protein (39 genes), and cell (85 genes). In the pathway 'cell', 85 genes fall into four groups: organization (32 genes), division (23 genes), cycle (15 genes), and vesicle transport (15 genes). This result suggested that genes with a role in the cell were significantly depressed in female poplars when exposed to salinity stress. In addition, seven genes involved in metal handling, such as *FRO4*, *MTO3*, and *MT3*, and two genes, *GAUT8* and *GAUT1*, encoding polygalacturonate 4- α -galacturonosyltransferase and galacturonic acid transferase involved in pectin synthesis, were also down-regulated in females under salinity stress. Other genes which were down-regulated only in females are listed in Supplementary Table S5 at *JXB* online.

Genes with complex regulation patterns in males and females

A small number of genes exhibited opposite expression patterns in males and females under salinity stress. In the category UMDF, DEGs were mainly related to cell wall

formation, photosynthesis, and protein pathways (Table 4). Eight DEGs in photosynthesis (Fig. 4) were involved in light reactions (Chl *a/b*-binding protein 2, *CAB2*; light-harvesting complex II Chl *a/b*-binding protein 2, *LHCB2.2*; chlorophyll-binding protein D1, *PSBA*; photosystem II PsbY protein, *PSBY*; gamma subunits of chloroplast ATP synthase, *ATPCL1*; photosystem I subunit PsaN, *PSAN*; and leaf ferredoxin, *FED A*), the Calvin cycle (ribulose biphosphate carboxylase small chain 1A, *RBCS1A*), and photorespiration (*RBCS1A*).

Genes belonging to the UFDM category were regulated in the opposite way to those in UMDF. RNA and protein were two important pathways in this category (Table 5). Three DEGs involved in the pathway of RNA processing and transcription regulation were down-regulated in males but up-regulated in females (Table 5). Genes in this category included HNH endonuclease domain-containing protein, *MXM12.5*; TRAF-type zinc finger-related protein, *F21M12.31*; and A20/AN1-like zinc finger family protein, *SAP5*.

Genes located in the female-specific region of the W chromosome

In order to investigate further the molecular mechanisms underlying salinity-induced gene expression and different sexual responses, genes located in the female-specific region of the W chromosome (FSW) (Yin *et al.*, 2008) were analysed. Surprisingly, none of the identified differentially expressed genes in females was located in the FSW. Thus, it appears that the genes located in autosomes rather than in the FSW make a major contribution to sexual differences in the salinity tolerance of poplars.

Discussion

Sexual differences in responses to salinity can occur in osmotic and ionic phases and involve accumulation of Cl⁻ and proline

Previous studies have clearly demonstrated that salt tolerance of plants includes two main phases, the osmotic stress and the salt-specific effect (Munns, 2005). In *P. yunnanensis*, Cl⁻ rather than Na⁺ content exhibited a large increment in the two sexes, but more significantly in females than in males (Fig. 1b). This phenomenon was consistent with an earlier study (L. Chen *et al.*, 2010) suggesting that Cl⁻ is the more toxic ion, as also observed in grapevine and citrus (Storey and Walker, 1999). Na⁺ can be withheld effectively in woody roots and stems, while Cl⁻ continues to accumulate in leaves as the major toxic ion. Accordingly, increased transcription was observed of a gene encoding a chloride channel (CLC) family protein (*CLC-c*) with osmotic adjustment function under high salinity (Diédhiou and Gollmack, 2006; Li *et al.*, 2006), but only in females. This observation suggests that under the same salt conditions, specific signals triggered to cope with Cl⁻ influx accumulated more in

Table 4. Functional categories and gene expression patterns of 59 DEGs up-regulated in males but down-regulated in females under salinity stress

<i>Populus</i> gene model ID	AGI	AGI annotation and function description	Log-2 ratio	
			MS/MC	FS/FC
Cell wall				
estExt_Genewise1_v1.C_LG_XIV0850	AT2G45470	AGP8; fasciclin-like arabinogalactanprotein 8	1.09	-1.71
eugene3.00140737	AT3G62830	UXS2; UDP-glucuronate decarboxylase activity, catalytic activity, dTDP-glucose 4,6-dehydratase activity	1.66	-1.3
gw1.III.2269.1	AT5G47500	MNJ7.9; pectin lyase-like superfamily protein, pectinesterase activity	1.39	-2.27
Lipids				
fgenes4_pm.C_scaffold_44000016	AT3G15730	PLD alpha 1; phospholipase D activity	1.04	-1.15
estExt_fgenes4_pm.C_LG_VI0352	AT3G11170	FAD7; omega-3 fatty acid desaturase activity	1.3	-1.56
Redox				
estExt_fgenes4_pg.C_LG_IX1399	AT1G07890	Cytosolic ascorbate peroxidase APX1; L-ascorbate peroxidase activity	1.26	-1.4
TCA				
grail3.0010030801	AT3G17240	mtLPD2; dihydrolipoyl dehydrogenase activity	1.68	-1.12
Photosynthesis				
estExt_fgenes4_kg.C_LG_IV0017	AT1G67090	RBCS1A; ribulose-bisphosphate carboxylase activity	2.3	-1.3
eugene3.00110470	AT1G29920	CAB2; chlorophyll binding	2.55	-1.37
estExt_fgenes4_pm.C_LG_II0962	AT2G05070	LHCB2.2; chlorophyll binding	1.31	-2.47
gw1.XIII.2252.1	ATCG00020	PSBA; chlorophyll binding	1.21	-2.83
estExt_Genewise1_v1.C_LG_X5024	AT1G67740	PSBY; manganese ion binding	1.68	-1.63
estExt_Genewise1_v1.C_LG_V2453	AT5G64040	PSAN; calmodulin binding	1.9	-1.35
eugene3.00040033	AT4G04640	ATPC1; enzyme regulator activity	3.28	-1.45
estExt_fgenes4_kg.C_1630003	AT1G60950	FED A; electron carrier activity	1.17	-1.65
Amino acid				
eugene3.00090483	AT5G11880	DAPD; diaminopimelate decarboxylase activity	1.83	-3.09
eugene3.00050687	AT1G22410	DAHPI; 3-deoxy-7-phosphoheptulonate synthase activity	1.17	-2.41
C1-metabolism				
grail3.0066005802	AT3G03780	ATMS2; 5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase activity, methionine synthase activity	1.18	-1.54
grail3.0050014702	AT1G02500	SAM1; methionine adenosyltransferase activity	2.38	-1.65
eugene3.00012227	AT4G13930	SHM4; catalytic activity, glycine hydroxymethyltransferase activity	1.08	-1.51
Nucleotides				
fgenes4_pg.C_LG_XII000913	AT5G63310	NDPK2; ATP binding, protein binding, nucleoside diphosphate kinase activity	1.54	-1.85
Protein				
eugene3.00410149	AT1G35340	T9I1.11; ATP-dependent peptidase activity	2.29	-1.73
grail3.0028002001	AT5G60360	AALP; cysteine-type peptidase activity	2.02	-1.19
fgenes4_pm.C_LG_II000602	AT1G74970	RPS9; nuclear encoded component of the chloroplast ribosome	1.87	-3.16
gw1.XI.877.1	AT1G32990	PRPL11; mutant has decreased effective quantum yield of photosystem II	1.25	-2.38
gw1.XVI.542.1	AT3G54210	F24B22.170; structural constituent of ribosome	1.11	-1.61
eugene3.00060477	AT1G58380	XW6; structural constituent of ribosome	1.25	-3.37
estExt_fgenes4_pm.C_LG_IV0210	AT3G02560	RPS7B; structural constituent of ribosome	1.97	-1.91
estExt_fgenes4_pg.C_LG_V0222	AT3G49910	RPL26A; structural constituent of ribosome	1.58	-1.63
eugene3.00061117	AT1G70600	F24J13.17; structural constituent of ribosome	1.29	-1.57
estExt_fgenes4_pg.C_410046	AT4G22380	Ribosomal protein L7Ae/L30e/S12e/Gadd45 family protein; RNA binding	1.23	-2.09
estExt_Genewise1Plus.C_LG_VII0502	AT4G17300	NS1; asparagine-tRNA ligase activity	1.12	-2.03
grail3.0002059601	AT1G04940	TIC20; Tic20 is believed to function as a component of the protein-conducting channel at the inner envelope membrane	2.95	-1.9
estExt_fgenes4_pg.C_LG_IX1267	AT2G28800	ALB3; P-P-bond-hydrolysis-driven protein transmembrane transporter activity	2.09	-2.6
Hormones				

Table 4. Continued

Populus gene model ID	AGI	AGI annotation and function description	Log-2 ratio	
			MS/MC	FS/FC
e_gw1.XIX.2349.1 Signalling	AT4G19170	NCED4; similar to nine- <i>cis</i> -epoxycarotenoid dioxygenase	3.78	-2.34
estExt_Genewise1_v1.C_LG_XV2501 Stress	AT5G61790	CNX1; calcium ion binding, unfolded protein binding	1.38	-1.59
gw1.XIV.3121.1	AT1G05850	POM1; chitinase activity	1.49	-1.2
estExt_fgenes4_pg.C_1500058 Development	AT5G49910	HSC70-7; protein binding	1.98	-1.7
gw1.VI.1538.1 Not assigned	AT1G67440	EMB1688; GTPase activity	2.66	-2.03
gw1.XVIII.2260.1	AT2G26900	F12C20.6; bile acid:sodium symporter activity, transporter activity	1.05	-1.49
grail3.0061011301	AT2G31400	GUN1; DNA binding	1.31	-3.12
eugene3.00570020	AT4G35760	F4B14.2; NAD(P)H dehydrogenase (quinone) activity	1.2	-1.04
e_gw1.VI.585.1	AT2G23990	ENODL11; electron carrier activity	1.85	-4.67
eugene3.00012979	AT1G79090	YUP8H12R.29; molecular function unknown	3.97	-4.21
eugene3.00870040	AT1G65230	T23K8.14; molecular function unknown	1.2	-1.03
estExt_Genewise1_v1.C_LG_XVI3139	AT2G36885	Unknown	2.34	-1.72
estExt_fgenes4_pg.C_LG_II2402	AT3G07090	T1B9.26; PPPDE putative thiol peptidase family protein	1.68	-1.19
estExt_fgenes4_pg.C_LG_IX0791	AT3G49720	T16K5.70; molecular function unknown	1.03	-2.87
estExt_fgenes4_pm.C_LG_II0597	AT1G47740	T2E6.19; PPPDE putative thiol peptidase family protein	1.65	-1.76
estExt_Genewise1Plus.C_LG_III1018	AT1G32080	F3C3.12; membrane protein, putative	1.31	-1.39
fgenes4_pg.C_scaffold_137000002	AT5G55930	OPT1; oligopeptide transporter activity	2.57	-2.28
fgenes4_pg.C_LG_XIV000399	AT4G01030	F3I3.50; pentatricopeptide (PPR) repeat-containing protein	1.6	-3.11
eugene3.00012101	AT3G01780	TPLATE; a cytokinesis protein targeted to the cell plate, binding	1.67	-1.81
eugene3.00170125	AT5G65260	MQN23.21; RNA binding, nucleic acid binding, nucleotide binding	1.19	-1.67
estExt_Genewise1_v1.C_LG_IX2088	AT3G48690	CXE12; carboxylesterase activity	1.55	-3.46
estExt_fgenes4_pg.C_LG_II0348	AT1G06200	F9P14.6; peptidase S24/S26A/S26B/S26C family protein	1.41	-1.4
estExt_fgenes4_pg.C_LG_VII0502	AT2G22170	T26C19.17; lipase/lipooxygenase, PLAT/LH2 family protein	1.21	-1.14
estExt_fgenes4_pg.C_290237	AT4G17730	SYP23; SNAP receptor activity	1.78	-1.4
e_gw1.I.2717.1	AT3G01660	F4P13.20; methyltransferase activity	1.03	-1.05

MC, males with 0 mM NaCl; MS, males with 50 mM NaCl; FC, females with 0 mM NaCl; FS, females with 50 mM NaCl; NS, not statistically significant.

females than in males. In this way, males are more efficient in Cl^- management than females.

Interestingly, salinity significantly increased the proline content but only in males (Fig. 1a), and the interaction effect between sex and salinity stress was significant ($P=0.000$). Proline is a proteinogenic amino acid with an exceptional conformational rigidity. It accumulates in many plant species in response to environmental stress, and it plays a crucial role in osmotic protection (Yamada *et al.*, 2005; Schat *et al.*, 2006; Szabados and Saviouré, 2010). However, little is known about the detailed links between proline increment and Cl^- accumulation. Previous physiological studies revealed an interactive relationship between proline increment and Cl^- accumulation during the osmotic phase (F. Chen *et al.*, 2010; L. Chen *et al.*, 2010). The significantly lower Cl^- content in males is presumably partially due to the higher accumulation of proline in males than in females.

Transcriptional profiling analysis revealed a massive influence of salinity on proline metabolism, including induction of several key enzymes, such as pyrroline-5-carboxylate synthetase (P5CS) (Székely *et al.*, 2007). In

plants, proline is synthesized mainly from glutamate, which is reduced to glutamate-semialdehyde (GSA) by P5CS, and then converted to pyrroline-5-carboxylate (P5C) (Hu *et al.*, 1992; Saviouré *et al.*, 1995). P5C reductase (P5CR) further reduces the P5C intermediate to proline (Szoke *et al.*, 1992; Verbruggen *et al.*, 1993). Alternatively, plants can synthesize proline from ornithine, which is firstly transaminated by ornithine- δ -aminotransferase (OAT) to produce GSA and P5C (Roosens *et al.*, 1998). During osmotic stress, proline biosynthesis is controlled by *P5CS1* (Székely *et al.*, 2007). In the present study, the transcript of *P5CS1* was found more frequently in females while *OAT* was found more frequently in males, suggesting that males and females adopted distinct strategies that trigger different signals to synthesize more osmoprotectant proline to cope with the predicament. However, no obvious transcriptional changes were found in other key genes, such as *P5CS2* (Székely *et al.*, 2007), *P5CR* (Szoke *et al.*, 1992), *PDH1*, *PDH2* (Kiyosue *et al.*, 1996), and *P5CDH* (Deuschle *et al.*, 2001). Because males accumulate more proline than females, the OAT pathway could be important in the salinity tolerance of poplars.

Table 5. Functional categories and gene expression patterns of 28 DEGs down-regulated in males but up-regulated in females under salinity stress

Populus gene model ID	AGI	AGI annotation and function description	Log-2 ratio MS/MC	FS/FC
Cell wall				
fgenes4_pg.C_LG_XVI000539	AT3G52370	FLA15; fasciclin-like arabinogalactanprotein 15 precursor	-2.37	1.5
Lipids				
fgenes4_pm.C_LG_VIII000723	AT3G23510	MEE5.5; cyclopropane-fatty-acyl-phospholipid synthase activity	-2.69	1.01
Major CHO metabolism				
estExt_fgenes4_pg.C_LG_II1784	AT2G47470	UNE5; protein disulphide isomerase activity	-1.3	2.63
fgenes4_pm.C_LG_VI000400	AT4G26140	BGAL12; beta-galactosidase activity, catalytic activity, hydrolase activity, hydrolyzing O-glycosyl compounds	-1.57	1.24
fgenes4_pg.C_LG_IX001086	AT1G50460	HKL1; hexokinase activity	-2.94	2.01
RNA				
eugene3.00120596	AT5G07810	MXM12.5; endonuclease activity, helicase activity	-2.4	1.26
grail3.0003096203	AT1G09920	F21M12.31; TRAF-type zinc finger-related	-2	1.12
eugene3.00011778	AT3G12630	A20/AN1-like zinc finger family protein; DNA binding, zinc ion binding	-2.35	1.34
Protein				
estExt_fgenes4_pm.C_LG_XIII0008	AT1G11910	APA1; aspartic proteinase, endopeptidase activity	-1.3	2.29
estExt_Genewise1_v1.C_570194	AT3G51350	F26O13.3; aspartic-type endopeptidase activity	-1.77	2.11
estExt_Genewise1Plus.C_44690001	AT2G35780	SCPL26; serine-type carboxypeptidase activity	-2.22	2.98
estExt_fgenes4_pm.C_LG_XVIII0083	AT5G25760	PEX4; ubiquitin-protein ligase activity	-1.53	1.58
estExt_Genewise1_v1.C_LG_I8663	AT5G41700	UBC8; ubiquitin-protein ligase activity	-1.57	1.5
estExt_fgenes4_pm.C_280017	AT5G20570	RBX1; protein binding	-1.92	0.88
Signalling				
grail3.0033033502	AT4G11880	AGL14; DNA binding, sequence-specific DNA binding transcription factor activity	-1.49	1.69
estExt_Genewise1_v1.C_LG_V0143	AT1G43860	F28H19.11; sequence-specific DNA binding transcription factor activity	-1.22	1.77
eugene3.00131252	AT4G18700	CIPK12; kinase, protein kinase, protein serine/threonine kinase activity	-1.47	1.03
eugene3.00400111	AT1G02130	ARA5; GTP binding	-1.59	1.25
grail3.0001009101	AT4G35860	ATGB2; GTP binding	-1.26	1.41
Stress				
estExt_Genewise1_v1.C_LG_II2154	AT4G33300	ADR1-L1; ATP binding	-1.99	1.68
Large enzyme families				
eugene3.00141430	AT5G43940	HOT5; S-(hydroxymethyl) glutathione dehydrogenase activity, S-nitrosoglutathione reductase activity	-1.64	2.56
Not assigned				
gw1.i.9523.1	AT1G15520	PDR12; ATPase activity, coupled to transmembrane movement of substances	-1.46	1.68
eugene3.01470017	AT4G31480	F3L17.50; binding, clathrin binding, structural molecule activity	-1.18	1.08
estExt_fgenes4_pm.C_LG_I1158	AT5G55940	EMB2731; molecular function unknown	-3.85	3.27
gw1.VII.1749.1	AT4G39970	T5J17.140; catalytic activity, hydrolase activity	-2.3	1.25
estExt_fgenes4_pm.C_LG_VI0047	AT4G16480	INT4; carbohydrate transmembrane transporter activity, myo-inositol:hydrogen symporter activity, sugar:hydrogen symporter activity	-1.01	1.08
estExt_fgenes4_pg.C_LG_VIII0748	AT2G02990	RNS1; endoribonuclease activity, ribonuclease activity	-1.4	3.75
e_gw1.XVIII.760.1	AT5G11700	T22P22.90; similar to glycine-rich protein, putative	-2.55	1.72

MC, males with 0 mM NaCl; MS, males with 50 mM NaCl; FC, females with 0 mM NaCl; FS, females with 50 mM NaCl; NS, not statistically significant.

Sexual differences in photosynthetic capacity under salinity stress

Photosynthesis is closely related to the salinity tolerance of poplars (Wang *et al.*, 2007; F. Chen *et al.*, 2010; L. Chen *et al.*, 2010). The present results showed that salinity significantly inhibits gas exchange in both males and females because of lowered P_n , E , and C_i (Table 1). Similar observations have been reported for *P. cathayana* (F. Chen

et al., 2010) and *P. popularis* (Wang *et al.*, 2007). The mean values of P_n and E decreased by 37% and 31.5% in females, but by only 23.7% and 17.3% in males, respectively (Table 1). This observation suggested that the inhibitory effects of salinity were more significant in females than in males, and the result is consistent with an earlier study (L. Chen *et al.*, 2010). Chlorophyll fluorescence, as an indicator of the photochemical efficiency of PSII, can provide insights into both the ability and extent to which plants tolerate

environmental stresses (Maxwell and Johnson, 2000; Xu *et al.*, 2008a). It was observed that F_v/F_m and qP decreased more in females than in males, indicating less photodamage to PSII reaction centres in males. Chlorophyll degradation is an important symptom of leaf senescence. In *P. yunnanensis*, the total chlorophyll content significantly decreased in both males and females, but the Chl *alb* ratio significantly increased only in females. This result implies that salinity aggravates the senescence of leaves particularly in females, which will further affect photosynthesis and increase sexual differences in salinity responses.

Salinity significantly changed gene expression related to photosynthesis, a similar result to that detected in physiological responses. A total of 56 and 66 genes were detected to have altered transcription levels in response to salinity stress in males and females, respectively, among which 78.5% in males but only 19.7% in females were up-regulated (Supplementary Tables S6, S7 at *JXB* online). It was concluded that genes with high up-regulation may enable males to maintain higher photosynthetic capacity than females under salinity stress. In addition to co-regulated genes, sexually differently expressed genes were also mainly up-regulated in males (25 up- and one down-regulated) but down-regulated in females (34 down- and two up-regulated). This result suggests that expression of genes involved in photosynthesis differed between males and females, and males can up-regulate the gene expression level in order to cope with salinity stress. Furthermore, sex-related genes up-regulated only in males had functions in light-harvesting complex I (LHC-I), LHC-II, PSI, and PSII polypeptide subunits, ATP synthase, and so on, while sex-related DEGs detected only in females included genes with additional functions (cytochrome *b₆f*, ferredoxin reductase, plastocyanin, etc).

Of eight DEGs up-regulated in males but down-regulated in females in the category 'photosynthesis' (Table 4), seven DEGs had a role in light reactions (Supplementary Fig. S3 at *JXB* online). These genes may directly contribute to sexual differences in photosynthetic capacity. Salinity down-regulated *PSBA* in females, which encodes D1 protein involved in repairing the damaged PSII. A previous study on *Synechocystis* (Allakhverdiev *et al.*, 2002) showed up-regulation of *PSBA* in males, suggesting that *PSBA* can repair the damaged PSII. In contrast, down-regulation of *PSBA* to low levels aggravates the decline of PSII in females. *PSBY*, the single nuclear gene, is imported into chloroplasts, where it is processed into two integral membrane proteins with identical topology (*PSBY-1* and *PSBY-2*). The protein appears to bind manganese, but its role is not well understood. Meentemeyer *et al.* (1999) reported that the *PSBY* protein is not essential for oxygenic photosynthesis. However, sexual differences in *PSBY* expression patterns do exist between males and females.

CAB2 and *LHCB2.2* are included in the category of photosynthesis. *CAB2* encodes LHC proteins that constitute the antenna system of the photosynthetic apparatus. Both of them are closely related to chlorophyll binding, which suggests that transcription of these two genes to high levels in males may help to enhance their photosynthetic capacity

in response to salinity stress. In the redox chain, *ATPCI* encoding the gamma subunit of chloroplast ATP synthase was significantly up-regulated in males but down-regulated in females. The absence of *ATPCI* decreased ATP synthase activity, which restricted overall rates of leaf photosynthesis (Wu *et al.*, 2007). Thus, different gene expression patterns of *ATPCI* contribute to salinity-induced sexual differences in photosynthesis. *PSAN*, encoding the only subunit of PSI and located entirely in the thylakoid lumen, may be involved in the interaction between plastocyanin and the PSI complex (Haldrup *et al.*, 1999), which together with *FED A* is related to photosynthetic electron transport.

Sexual differences in amino acid metabolism and transcription under salinity stress

Metabolism of amino acids plays a key role in the salinity tolerance of plants (Sanchez *et al.*, 2008). In the present study, 59 and 81 salinity-responsive DEGs related to amino acid metabolism were identified in males and females, respectively (Supplementary Table S6 at *JXB* online). These DEGs are mainly involved in the alanine–glycine pathway, alanine–glutamate pathway, GABA (γ -aminobutyric acid) metabolism, N metabolism, homoserine synthesis, and sulphate assimilation. Nearly half of the identified DEGs (23 in males and 45 in females) showed obvious sexual differences in gene expression patterns. For instance, genes encoding glutamate decarboxylase (*GAD1* and *GAD5*) and glutamate dehydrogenase (*GDH1* and *GDH2*) involved in GABA metabolism were detected only in females. Three genes that function in homoserine dehydrogenase, kinase, and dehydrogenase were down-regulated only in females. Interestingly, two genes encoding DAHP (Dyer *et al.*, 1990) and DAPD (Less and Galili, 2008), respectively, were up-regulated in males but down-regulated in females, suggesting different regulation strategies in amino acid metabolism between males and females under salinity stress. DAHP is the first enzyme in a series of metabolic steps, such as the shikimate pathway, responsible for biosynthesis of some amino acid (Dyer *et al.*, 1990). Different expression patterns of DAHP may produce diverse amino acid components and contents, leading to sexual differences under salinity stress.

Previous studies have shown that a significant alternation of transcription-related gene expression was closely linked to plant stress tolerance (Luo *et al.*, 2009; Brinker *et al.*, 2010; Cohen *et al.*, 2010). The present results provided new insights into sexual differences in transcription related to the salinity tolerance of plants. During salinity stress, 324 and 416 DEGs related to transcription factors were differentially expressed in males and females, respectively, most of which were down-regulated, such as MYB domain, NAC domain, Aux/IAA, basic helix–loop–helix, and the C2H2 zinc finger. This result suggested that salinity suppressed the normal transcription of genes. In addition, the transcript abundances of several genes were altered only in males during salinity stress, including genes involved in chromatin assembly or disassembly (*CMT2*), brassinosteroid signalling transduction (*BEH3*), DNA-binding bromodomain (*F4II.24*), asymmetric cell

division (*HSFB4*), circadian rhythm (*ELF3*), regulation of transcription (*LUH*), and DNA-mediated transformation (*ASF1B*). More transcription factors were identified in females than in males, such as genes encoding WRKY domain, zinc finger (GATA type), and ABI3/VP1-related B3-domain-containing transcription factors. This observation implied that the influence of salinity stress on transcription was more severe or more extensive in the female transcriptome when compared with that of males.

Genes located in the female-specific region of the W chromosome are not the main cause of sexual differences in salinity responses

Sex chromosomes of plants, when present, are distinctive not only because of their gender-determining role (Charlesworth, 1985) but also due to genomic features (Ming and Moore, 2007). In the present study, numerous DEGs related to sexual differences in physiological responses were detected. The presence of ZW sex chromosomes in poplar (Yin *et al.*, 2008) provides an opportunity to ascertain whether the DEGs identified in this study are associated with sex chromosomes. Recombination suppression in male- and female-specific chromosomal regions is a hallmark of sex chromosomes (Ming *et al.*, 2011). The female-specific region of the poplar W chromosome (FSW) is a 706 kb region with no counterpart on the Z chromosome. Surprisingly, based on the chromosomal location of the identified DEGs, salinity-induced DEGs of females were mainly located in autosomes rather than within the FSW. This discovery suggested that molecular responses of *P. yunnanensis* under salinity stress mainly rely on genes located in autosomes, and gene regulation related to the FSW makes less of a contribution to sexual differences in the plant's salinity tolerance. Does this conclusion simply prove and supplement the established conclusion that the principal function of the sex chromosome is to reinforce dioecy? Dioecy in plants means that unisexual flowers are produced on two types of individuals, staminate flowers on males and pistillate flowers on females (Ming *et al.*, 2011). Genes controlling staminate or pistillate flower development are randomly distributed in the genome and scattered on every chromosome (Wellmer *et al.*, 2004). It is proposed here that more attention should be paid to genes located in autosomes rather than only on sex chromosomes when relating sexual differences and sex chromosomes.

In conclusion, the present study showed that *P. yunnanensis* males and females exhibit distinct sexual differences in response to salinity stress. A series of changes in physiological and biochemical traits were identified between the two sexes, clearly showing that salinity stress restrained normal metabolism and regulation, particularly in females. Transcriptional profiling analysis revealed many genes in response to salinity stress, most of which were down-regulated. Some genes with different expression patterns in males and females may cause the differences visible in growth responses, including genes involved in photosynthesis, amino acid metabolism, and transcription. Functional genes located in autosomes rather

than in the FSW could play a key role in poplar salinity tolerance. Identified DEGs with different expression patterns between males and females are excellent targets for further functional studies in order to understand more specific molecular mechanisms of sexual differences in plant salt tolerance.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. qRT-PCR validation of DGE library data

Figure S2. Overview of the DEGs assigned to 'regulation' by MapMan.

Figure S3. Overview of the DEGs assigned to 'photosynthesis' by MapMan.

Table S1. Primer sequences of candidate genes used for validation in qRT-PCR.

Table S2. Genes up-regulated only in males under salinity stress.

Table S3. Genes up-regulated only in females under salinity stress.

Table S4. Genes down-regulated only in males under salinity stress.

Table S5. Genes down-regulated only in females under salinity stress.

Table S6. Comparisons of three major pathways between males and females.

Table S7. Differentially regulated genes involved in photosynthesis under salinity stress.

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