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Authors

Hori, Chiaki

Yu, Xiang

Mortimer, Jenny C

et al.

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Impact of abiotic stress on the regulation of cell wall biosynthesis in *Populus trichocarpa*

Chiaki Hori[1,2,†], Xiang Yu[1,†], Jenny C. Mortimer[1,3], Ryosuke Sano[4], Tomoko Matsumoto[1], Jun Kikuchi[1,5], Taku Demura[1,4,*], Misato Ohtani[1,4,6,**]

[1] RIKEN Center for Sustainable Resource Science, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan; [2] Research Faculty of Engineering, Hokkaido University, North 13, West 8, Sapporo, Hokkaido 060-8628, Japan; [3] Joint BioEnergy Institute, Environmental Genomics and Systems Biology Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA; [4] Division of Biological Science, Graduate School of Science and Technology, Nara Institute of Science and Technology, 8916-5 Takayama-cho, Ikoma, Nara 630-0192, Japan; [5] Graduate School of Medical Life Science, Yokohama City University, 1-7-29 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan; [6] Department of Integrated Biosciences, Graduate School of Frontier Sciences, The University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8562, Japan

* E-mail: demura@bs.naist.jp Tel: +81-743-72-5460 Fax: +81-743-72-5469

** E-mail: misato@edu.k.u-tokyo.ac.jp Tel: +81-4-7136-3673 Fax: +81-4-7136-3674

Abstract Growth of biomass for lignocellulosic biofuels and biomaterials may take place on land unsuitable for foods, meaning the biomass plants are exposed to increased abiotic stresses. Thus, the understanding how this affects biomass composition and quality is important for downstream bioprocessing. Here, we analyzed the effect of drought and salt stress on cell wall biosynthesis in young shoots and xylem tissues of *Populus trichocarpa* using transcriptomic and biochemical methods. Following exposure to abiotic stress, stem tissues reduced vessel sizes, and young shoots increased xylem formation. Compositional analyses revealed a reduction in the total amount of cell wall polysaccharides. In contrast, the total lignin amount was unchanged, while the ratio of S/G lignin was significantly decreased in young shoots. Consistent with these observations, transcriptome analyses show that the expression of a subset of cell wall-related genes is tightly regulated by drought and salt stresses. In particular, the expression of a part of genes encoding key enzymes for S-lignin biosynthesis, caffeic acid *O*-methyltransferase and ferulate 5-hydroxylase, was decreased, suggesting the lower S/G ratio could be partly attributed to the down-regulation of these genes. Together, our data identifies a transcriptional abiotic stress response strategy in poplar, which results in adaptive changes to the plant cell wall.

Introduction

Woody biomass is the most abundant terrestrial biomass, and it is used industrially for construction, paper making, energy and the manufacture of many other materials and chemicals. Recently woody biomass has been identified as a key feedstock for sustainable biofuel production (Himmel et al. 2007; U.S. Department of Energy 2016), but increased demands for woody biomass raises concerns about the effect of the environment upon wood productivity and quality (Plomion et al. 2001). In addition, information about how woody plants respond to the environment will give us hints about forest breeding under global climate changes and/or the potential for increased forestry on degraded land. Abiotic stresses, such as drought and salinity, are major drivers of global agricultural losses, leading to plant morphological, anatomical and chemical changes (Shao et al. 2008). Generally, drought and salt stresses result in a water deficit that causes physiological alterations

Abbreviations: 4CL, 4-coumarate: CoA ligase; ABA, abscisic acid; C3H, *p*-coumarate 3-hydroxylase; C4H, cinnamate-4-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; CESA, CELLULOSE SYNTHASE A; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; COMT, caffeic acid *O*-methyltransferase; DMSO, dimethyl sulfoxide; F5H, ferulate 5-hydroxylase; GO, Gene ontology; HCT, *p*-hydroxycinnamoyl CoA: quinate/shikimate *p*-hydroxycinnamoyl transferase; HSQC, heteronuclear single-quantum coherence; IRX, IRREGULAR XYLEM; NST3, NAC SECONDARY WALL THICKENING PROMOTING FACTOR 3; NMR, nuclear magnetic resonance; PAL, phenylalanine ammonium-lyase; PFP, percentage of false-positives; SND1, SECONDARY WALL-ASSOCIATED NAC DOMAIN 1; VNS, VND, NST/SND, SMB-related; XTH, xyloglucan endotransglucosylase/hydrolase.

† These authors contributed equally to this work.

in higher plants including loss of turgor, osmotic adjustment and water potential. Many studies have intensively investigated the genes involved in molecular networks, such as the identification of signaling cascades in response to abiotic stresses (Hirayama and Shinozaki 2010; Shinozaki and Yamaguchi-Shinozaki 2006).

Woody biomass is comprised of cell wall polymers accumulated in the secondary cell wall in xylems. Recent progress in plant molecular biological research has identified many enzymes involved in the biosynthesis of cell wall polysaccharides (Dhugga 2012; Hussey et al. 2013; Pauly et al. 2013), and the cell wall response to drought and salt stresses have been extensively investigated (Le Gall et al. 2015; Moura-Sobczak et al. 2011; Tenhaken 2015; Wang et al. 2016). For example, cellulose content was reduced during osmotic stress in many angiosperms including tobacco suspension cells, grape leaves, wheat roots and Arabidopsis (*Arabidopsis thaliana*) shoots, roots and leaves (Bray 2004; Iraki et al. 1988; Piro et al. 2003; Sweet et al. 1990). Loss of functional copies of the secondary cell wall-type cellulose synthase genes, *AtCesA7* and *AtCesA8*, improved osmotic stress tolerance of Arabidopsis plants by controlling water loss (Chen et al. 2005; Liang et al. 2010). In contrast, *CesA* expression is induced by salt stress in alfalfa (*Medicago sativa*) stems (Guerriero et al. 2014). Moreover, the total amount of xyloglucan increased in response to abiotic stresses (Yang et al. 2006) and overexpression of a pepper xyloglucan endotransglucosylase/hydrolase (XTH) gene enhanced abiotic stress tolerance in Arabidopsis and tomato (Cho et al. 2006; Choi et al. 2011).

In addition to cell wall polysaccharide biosynthesis, phenolic polymer lignin biosynthesis is crucial to determine cell wall quality (Hussey et al. 2013; Vanholme et al. 2010). Lignin mainly consists of three different monolignols, *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S), which are randomly cross-linked together. Lignin is considered to be important for survival under abiotic stress, since lignin content usually increases under abiotic stresses in roots and leaves, with the exception of Eucalyptus leaves (Fan et al. 2006; Lee et al. 2007; Moura-Sobczak et al. 2011; Terzi et al. 2013). Expression of the lignin biosynthetic genes encoding phenylalanine ammonium lyase (PAL), caffeoyl-CoA *O*-methyltransferase (CCoAOMT) and cinnamoyl-CoA reductase (CCR), were elevated in various plants under abiotic stresses (Fan et al. 2006; Pandey et al. 2010; Terzi et al. 2013; Yoshimura et al. 2007).

However, most of these studies focused on the leaves and roots of herbaceous plants. Previous works on woody plants showed the transcriptional, anatomical and compositional changes to poplar (a model for woody biomass) stems in response to osmotic stress, e.g., reduced stem diameter and decreased vessel lumen diameter, and increased ratio of lignin:carbohydrate (Chen and Polle 2010; Janz et al. 2012; Junghans et al. 2006; Polle et al. 2019). Transcriptome analysis on poplar species also indicated that specific set of cell wall-related genes can be significantly changed by salt stress treatment (Janz et al. 2012; Wildhagen et al. 2018), suggesting an active transcriptional regulation of cell wall biosynthesis in response to abiotic stress in trees.

In this study, we further investigated the response of poplar cell walls to abiotic stress. The cell walls of poplar trees grown under drought or high-salt conditions were investigated by analytical chemistry methods, including 2D-NMR, as well as transcriptomic analyses. We identified specific changes to the cell wall in response to these stresses, and identified the responsible genes. This work provides new insights into the impacts of abiotic stresses on woody biomass quantity and quality, which is important for future utilization of woody biomass as a sustainable bioresource.

Materials and methods

Plant materials

Populus trichocarpa (poplar) plantlets were propagated and grown in jars containing Murashige and Skoog (MS) medium (pH 5.6, Duchefa), under long-day (16-h light/8-h dark) conditions at 23°C (Ohtani et al. 2011). For soil cultured poplar, 1-month-old plants grown on MS medium were transferred to the soil, and cultivated in the greenhouse for 3 months under long-day conditions (16-h light/8-h dark) at 23°C.

Abiotic stresses and ABA treatment

Soil-grown poplar plantlets were used for the abiotic stress treatments. They were grown in water-sufficient (control), salt (NaCl) and water-deficient (drought) conditions for 1 month. In water-sufficient condition, they were well watered with 250 ml tap water (3 times/week). For salt treatments, they were irrigated with 200 ml of 50, 75, or 100 mM NaCl solutions (3 times/week). For drought treatment, the water supply was reduced to 150 ml, once per week. The height and stem diameter of each treated poplars were measured every week, and the growth rate of stem diameter was calculated as "[(Diameter at each time point)−(Diameter before stress treatment)] / (Diameter before stress treatment)". Following 4-weeks of treatment, the plant material was harvested and stored at −80°C for anatomical observation, cell wall monosaccharide analysis and NMR analysis.

For gene expression analysis, the soil-grown poplar plantlets were treated with stresses as below; for the drought stress treatment, the water supply was suspended. For salt stress treatment, they were irrigated with 100 mM NaCl solution 3 times/week. According to poplar stem growth, there was not a significant change between control and after one-week of 50 mM NaCl treatment, which was used for four weeks treatments for the anatomical and biochemical observations. Therefore, 100 mM NaCl treatment was used

for gene expression to observe gene regulation by salt stress. After one week, young shoot tissues (top 3 cm) were harvested, immediately frozen in liquid nitrogen, and stored at −80°C.

For ABA treatment, platntlets grown on MS medium for 1 month were used. Three platntlets were transferred to liquid medium containing 50 μM ABA, and sampled after 24 h of treatment. The samples were immediately frozen in liquid nitrogen, and stored at −80°C.

Anatomical observation of poplar stem tissues

Young shoot and mature stem were sampled from 3rd internode from the top of the stem, and the 4th internode from the base part of the stem, respectively. The samples were fixed in 10% acetic acid in ethanol and the fresh cross sections were stained with toluidine blue and observed by optical microscopy. To measure vessel frequency and size, vessel cells were counted in a 350 μm square area of the section, and then the average diameter of all the detected vessels was measured from two directions. Data is presented normalized to a 100 μm square, $n=3$.

Chemical analysis of poplar stem tissues

Young shoots (Ys) and debarked mature stem (Xylem, Xy) were sampled from 10 to 25 cm from the top or the ground of the stem, respectively. The frozen stem organs were milled by automill (TK-AM7, Tokken Inc., Japan). The samples were then washed twice in 60°C distilled water, and washed with 60°C methanol 3 times. The washed samples were dried in an evaporator for 3 h. To destarch the samples, 20 mg was incubated at 25°C in an α-amylase solution (400 μl 0.1 M KH_2PO_4 [pH 6.0], 4 mg/ml α-amylase 40 μl and 0.2% sodium-azaide 20 μl) for 48 h. Samples were washed with distilled water, then methanol respectively, and then dried at 45°C for 1 h.

To hydrolyze the cell wall polysaccharides, 10 mg of destarched sample was added to 200 μl 72% sulphuric acid and incubated at 60°C for 1 h, and then the sulphuric acid was diluted to 3% with distilled water for hydrolysis at 121°C for 1 h. After cooling to room temperature, the supernatant was collected, erythritol was added as an external standard, and then neutralized with barium carbonate. The neutral sugars in the supernatant were separated using an SP0810 column (Shodex) on a UHPLC system (Ultimate™ 3000, Thermo Fisher Scientific) equipped with corona CAD detector. The amount of detected sugars was calculated based on standard curves of glucose, xylose, mannose, galactose and arabinose. The total lignin quantity of the destarched plant powder samples was quantified by using thioglycolic acid method according to the previous report (Suzuki et al. 2009).

NMR analysis of poplar stem tissues

The sampling and treatments of stem organs were performed according to the procedure described above until washing with methanol and distilled water. The obtained samples were further grounded in Pulverisette 5 ball mill (Fritsch GmbH, Idar-Oberstein, Germany) for 12 h. The ball-milleded powder was dissolved in dimethyl sulfoxide (DMSO)-d_6: pyridine-d_5 (4:1) and heated at 50°C for 30 min with shaking at 14,000 rpm in a Thermomixer Comfort (Eppendorf AG, Hamburg, Germany). After centrifugation at 15,000 rpm for 5 min, the supernatant was transfered to NMR tubes for analysis. NMR spectra were collected on an Avance II HD-700 instrument (Bruker, Billerica, MA, USA) with a 5-mm cryoTCI probe. For ^{1}H and ^{13}C analysis, 700.130 and 176.06 MHz resonance frequencies were used respectively. The temperature for all NMR samples was kept at 318 K. Chemical shifts were referenced to the methyl group of DMSO-d_6 at ^{1}H=2.49 ppm and ^{13}C=39.5 ppm. The echo/antiecho gradient selection were used for collection of 2D ^{1}H-^{13}C HSQC-NMR spectra. Fifty-one regions of interest (ROI) were identified based on previously assigned chemical shifts (Komatsu and Kikuchi 2013; Mori et al. 2015; Watanabe et al. 2014). HSQC quantification used in this study was essentially same protocols with the previous reports (Mansfield et al. 2012; Tsuji et al. 2015). The peak intensity of pyridine-d_5 used for normalization.

Expression analysis by microarray and qPCR

Total RNA was isolated from sampled organs using Plant RNA Isolation Reagent (Invitrogen), and then purified using an RNeasy Mini Kit (QIAGEN). For complementary DNA synthesis, 2 μg total RNA was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen) with oligo(dT)12–16 primer, after DNase I treatment. Aliquots of the reaction solution (0.5 μl for each gene) were used as templates for subsequent PCR amplifications. Quantitative PCR analysis was performed with a LightCycler 480 System II (Roche) using the LightCycler 480 SYBR Green I Master reagent (Roche), following the previous method (Yu et al. 2017a, b) *PtELF4A* was used as an internal control.

Microarray analysis was performed using the GeneChip Poplar Genome Array (Affymetrix) on three independent biological replicates from each organ of stress-treated and non-treated plants grown in soil, as previously described (Ohtani et al. 2011). The data was deposited in GEO database (accession number, GSE124496). Quality control (RMA normalization), statistical analysis, and filtering were then carried out using the R packages affy (Gautier et al. 2004) and RankProd (Breitling et al. 2004; Hong et al. 2006), and the percentage of false-positives (PFP), the statistic used in the Rank products method (Breitling et al. 2004; Hong et al. 2006), was used to control the false positives; differentially expressed genes with a cutoff of PFP<0.05 were selected.

Results

Growth of poplar stem under salt and drought stresses

To investigate the phenotypic response of poplar stem to abiotic stresses, four-month-old poplar plantlets (45 cm in height) were grown in water-sufficient (control), water-deficient (drought), and NaCl-treated (salt)

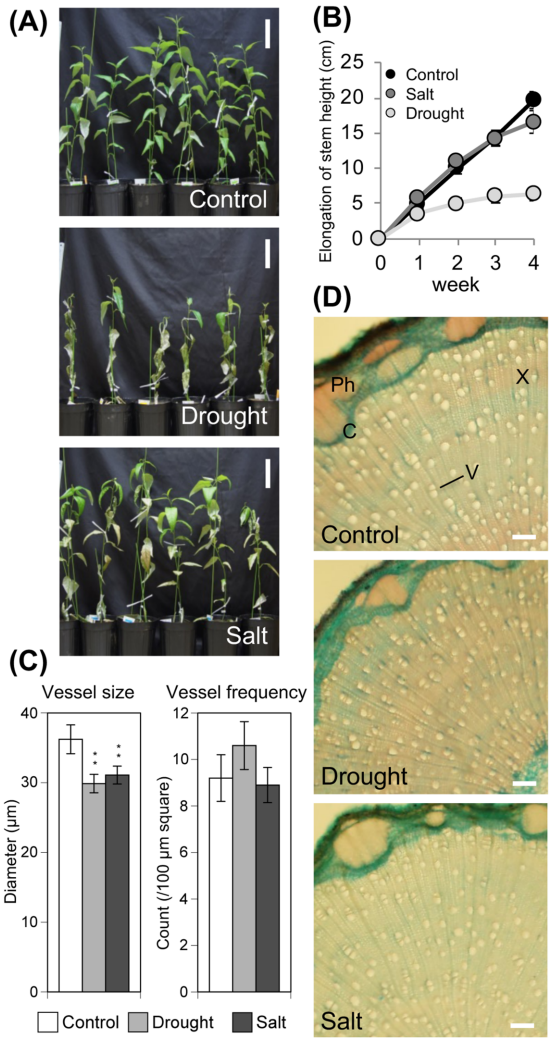


Figure 1. Phenotypic response of poplar to salt (50 mM NaCl) or drought (water deficiency) stress. (A) Overview of poplar growth after four weeks of treatment. Bars=10 cm. (B) Growth data of poplar during exposure to drought (water deficiency) and salt (50 mM NaCl) for 4 weeks (means±SD, n=10). (C) Vessel size and frequency in the internode 4th from the base of the stem (as shown in Supplementary Figure S1C) were quantified (means±SE, n=3). (t-test; ** p<0.01). (D) Anatomical views of poplar stem 4th internode from basal part during salt and drought. Bars=100 μm. V, Vessels; C, Cambium; Ph, Phloem; X, Xylem; Pi, Pith.

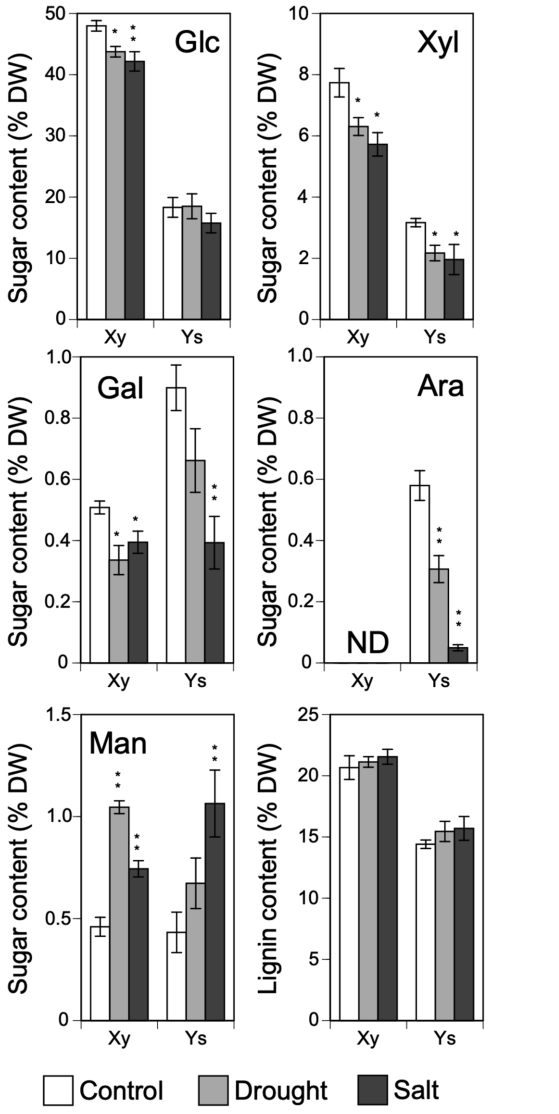
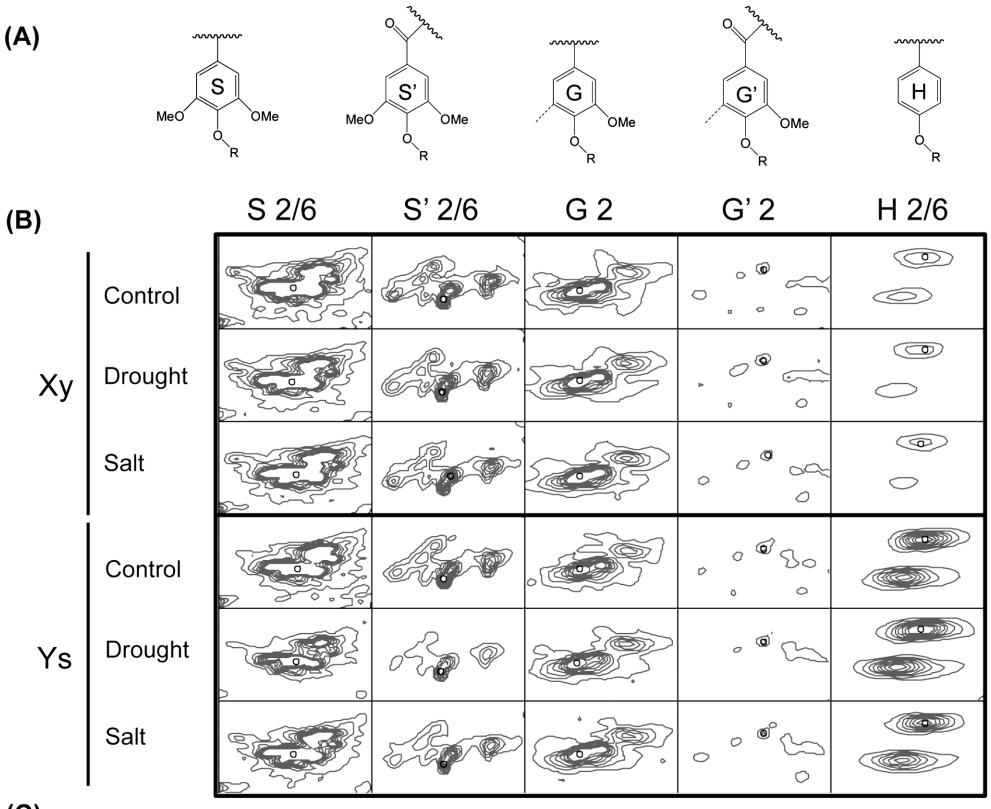


Figure 2. Chemical composition of poplar stem under control, salt and drought conditions. Monosaccharide sugars and lignin content of AIR. Error bars represent SE (n=5). Asterisks represent statistically significant difference between control condition and stress conditions (t-test; * p<0.05, ** p<0.01). Glc, glucose; Xyl, Xylose; Gal, Galactose; Ara, Arabinose; Man, Mannose; ND, Not Detected.

conditions for 4 weeks. Plantlets under drought stress showed serious leaf damage and stunted stem growth (Figure 1A, B). For the salt treatment, we tested irrigation regimes using 50 mM, 75 mM, or 100 mM NaCl; the poplar plantlets which were irrigated with 50 mM NaCl showed leaf damage (Figure 1A), and decreased growth in the third week (Figure 1B). However, plantlets irrigated with 75 mM and 100 mM NaCl solution were completely wilted within three weeks (Supplementary Figure S1A). Thus, we used 50 mM NaCl treatment for all subsequent salt-stress experiments except for gene expression analyses.

Analysis of the basal stem anatomy revealed that drought stresses decreased the diameter of stem (Supplementary Figure S1B). In addition, the drought and salt stresses significantly decreased the size of vessel cells, and drought stress tended to increase the frequency of vessel cells within xylems (Figure 1C, D), as previously reported in salt sensitive poplar (Janz et al. 2012; Junghans et al. 2006; Wildhagen et al. 2018). In the young shoot stems, which are newly generated during abiotic stress, the secondary xylem formation was enhanced as compared with the control plantlets (Supplementary Figure S1C). These results indicate that different poplar stem tissues (young shoot and mature stem) have phenotypic responses to both osmotic stresses, and xylem formation was affected during drought or salt stress.



(C)

		S%	S'%	G%	G'%	H%	(S+S')/(G+G')	(S+S')/(G+G')/H
Xy	Control	53.4	11.9	31.0	2.7	1.0	1.9	65/34/1
	Drought	52.2	11.9	32.2	2.9	0.9	1.8	64/35/1
	Salt	52.9	11.4	32.3	2.5	0.9	1.9	64/35/1
Ys	Control	49.8	12.1	29.0	2.8	6.3	2.0	62/32/6
	Drought	45.7	7.4**	37.3**	2.7	7.0	1.3 **	53/40/7
	Salt	48.1	9.1**	34.1**	2.6	6.1	1.6 *	57/37/6

Figure 3. HSQC NMR analysis of lignin components in poplar stem tissues under control, salt and drought conditions. (A, B) Representative structures and signal peaks of S-lignin, G-lignin and H-lignin. Contour threshold=5.2. (C) The compositional ratios of the three major lignin components. Asterisks represent statistically significant difference between control condition and stress conditions (*t*-test; * $p<0.05$, ** $p<0.01$, $n=5$).

Cell wall monosaccharide composition of poplar shoots and stems under abiotic stresses

Next, young shoots (Ys) and debarked mature stems (Xylem, Xy) were sampled to analyze changes in cell wall monosaccharide composition under abiotic stresses. The majority of monosaccharides showed a decrease in amount during salt and drought stresses (Figure 2). For example, glucose, the most abundant monosaccharide in the cell wall, was significantly decreased under abiotic stresses compared to the control in Xy but not in Ys (Figure 2). Xylose and galactose contents were decreased significantly in both tissues by the stress treatment (Figure 2). These results indicate that the total amount of cellulose, glucuronoxylan, and pectin tends to be reduced in stem tissue during abiotic stresses. On the other hand, mannose, which is a trace component in poplar cell walls, showed a significant increase in Xy and Ys during abiotic stress (Figure 2A). Unlike the sugar content, the total amount of lignin was not changed in Xy or Ys by the stress treatment (Figure 2B). This suggests that the response of Xy or Ys to drought and salt stresses is basically similar, and these abiotic stresses would influence cell wall polysaccharides, but not the total lignin content.

Structural changes in lignin polymer during abiotic stresses

To examine in detail any changes of lignin structures in poplar cell wall, solution state NMR spectroscopy (Lu et al. 2013; Mansfield et al. 2012) was performed on the same sets of tissues (Young shoots, Ys; Xylem, Xy). All three lignin subunits (Syringyl, S; Guaiacyl, G;

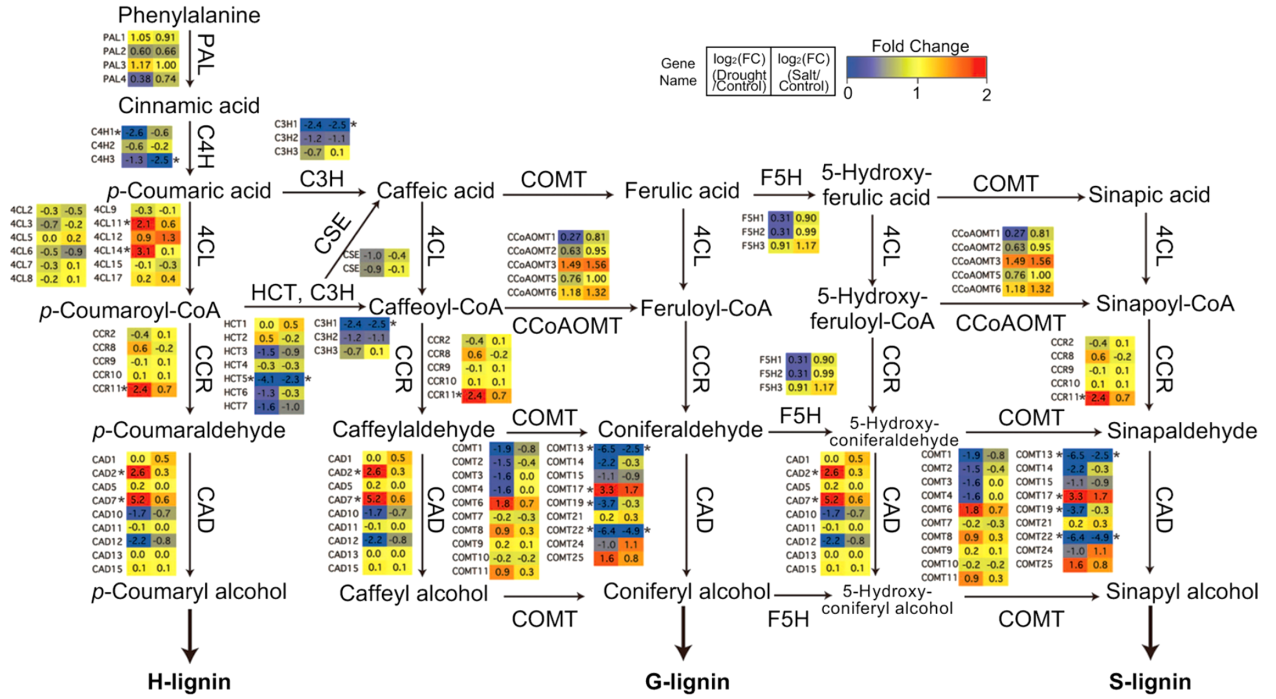


Figure 4. Transcriptome of poplar young shoot in lignin biosynthesis pathways response to abiotic stresses. PAL, phenylalanine ammonia-lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumarate: CoA ligase; HCT, *p*-hydroxycinnamoyl CoA: quinate/shikimate *p*-hydroxycinnamoyl transferase; C3H, coumarate 3-hydroxylase; CSE, caffeoyl shikimate esterase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; F5H, ferulate 5-hydroxylase; COMT, caffeic acid *O*-methyltransferase; CAD, cinnamyl alcohol dehydrogenase; SAD, sinapyl alcohol dehydrogenase. Enzyme gene highlighted with red is up-regulated, while enzyme gene highlighted with blue is downregulated by abiotic stress. Black arrows represent the principal pathway of monolignol synthesis pathway in poplars.

hydroxyphenyl, H) as shown in Figure 3A were clearly resolved in the 2D heteronuclear single-quantum coherence (HSQC) spectra of all samples (Figure 3B). Under the control conditions, S (S′)-lignin and G (G′)-lignin contents were similar between Xy and Ys, and small amounts of H-lignin were detected in both Ys and Xy (Figure 3C). Under abiotic stress conditions, the lignin composition of Ys was dramatically altered, although that of Xy was not changed. In the Ys samples, the S′-lignin and G-lignin contents were significantly reduced and increased, respectively, under both salt and drought stress conditions, whereas the H-lignin content was not affected (Figure 3B, C). These differences resulted in the decrease of S/G ratio by the stress treatment in Ys (Figure 3C).

To further examine the detailed changes in lignin structure, we examined several peaks of *β*-*O*-4 linkage, which is the dominant bond between monolignol units in the lignin polymer structure (Supplementary Figure S2A). Consistent with the results of Figure 3, the signal of A-H/G structure derived from the bond included G- and/or H-lignin units (Supplementary Figure S2A), was significantly increased only in Ys but not in Xy, under the drought stress condition (Supplementary Figure S2B). These data revealed that the lignin structures were changed in Ys under abiotic stresses, despite the overall amount of lignin was constant.

Transcriptional responses of cell wall biosynthesis to abiotic stresses

To explore the mechanism of cell wall alteration in response to abiotic stresses, we performed microarray analysis for young shoot (Ys) tissue after the treatment of drought and salt stresses. Considering no significant difference in growth between the control and 50 mM NaCl-treated plants after 1 week (Figure 1A,B), we treated the plants with 100 mM NaCl for transcript analyses, to observe early response of transcriptome to salt stress.

We identified 1251 and 1509 significantly up- and down-regulated genes, respectively, by drought-treatment (PFP$<$0.05) (Supplementary Table S1). In contrast, in response to salt stress, only 133 and 309 genes were significantly up- and down-regulated, respectively (PFP$<$0.05) (Supplementary Table S1). Of these, 55% of the up-regulated and 75% of the down-regulated genes were overlapped between drought and salt stress treatment (Supplementary Table S1). Gene ontology (GO) term enrichment analysis (Supplementary Figure S3) indicated that the down-regulated genes by drought and salt treatment contained similar molecular function; interestingly, the genes annotated to be related with "cell wall" were enriched in both cases (Supplementary Figure S3A, C), suggesting that the gene for cell wall metabolism would be actively regulated by abiotic stresses.

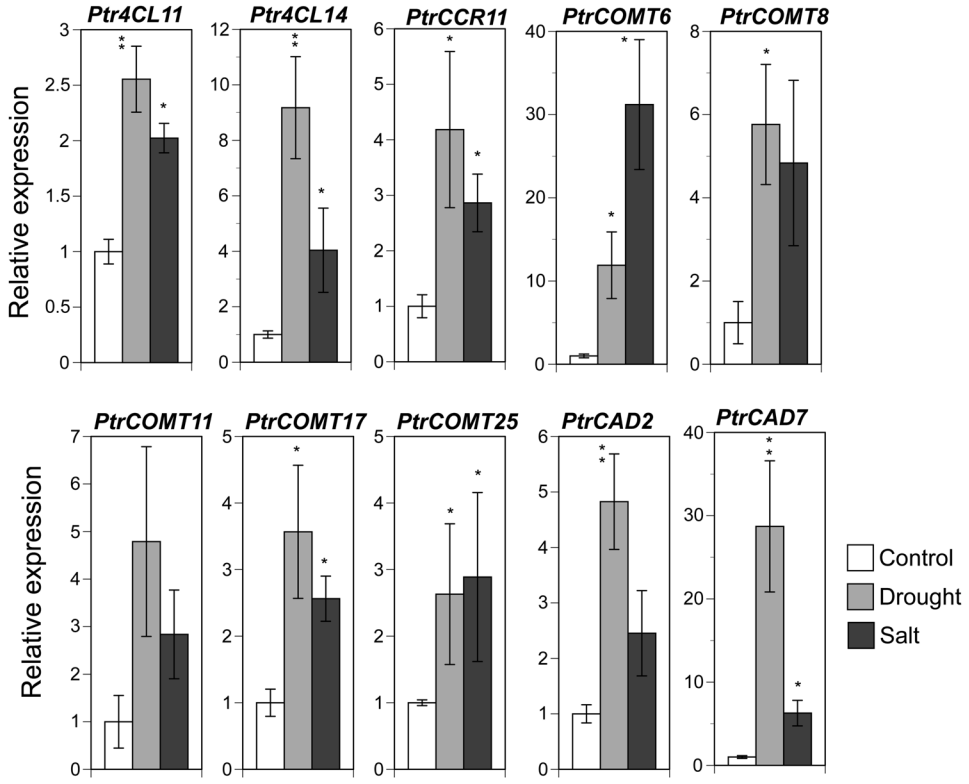


Figure 5. Quantitative RT-PCR analysis of putative lignin biosynthesis genes (up-regulated in young shoot) in poplar under control, salt and drought conditions. Relative expression was normalized based on average of control expression=1. Error bars represent SE (n=4). Asterisks represent statistically significant difference between control condition and stress conditions (t-test; * $p<0.05$, ** $p<0.01$).

For detailed analysis of the genes involved in cell wall metabolism, we identified orthologs of genes associated with cellulose, hemicellulose and lignin biosynthesis (Dhugga 2012; Hussey et al. 2013; Pauly et al. 2013; Shi et al. 2010; Tsai et al. 2006; Vanholme et al. 2010) and extracted the transcriptomics data for these genes (Figure 4, Supplementary Figure S4). The putative poplar genes involved in cellulose and hemicellulose biosynthesis, such as *CELLULOSE SYNTHASE* (*CESA*) and *IRREGULAR XYLEM* (*IRX*) genes, were strongly repressed by the drought and salt stresses (Supplementary Figure S4), with the exception of *GATL9* genes, putative pectin synthase genes, which were up-regulated by the drought stress (Supplementary Figure S4). The overall reduction tendency of polysaccharide biosynthetic genes would be consistent with the decrease in monosaccharide (Figure 2).

In the case of lignin biosynthesis, the expression of genes encoding phenylalanine ammonium-lyase (PAL) and cinnamate-4-hydroxylase (C4H), which collectively convert phenylalanine to 4-hydroxylcinnamic acid (a precursor of all monolignols), was tended to be repressed (Figure 4, Supplementary Table S2). In addition, the genes for *p*-hydroxycinnamoyl CoA: quinate/shikimate *p*-hydroxycinnamoyl transferase (HCT) and *p*-coumarate 3-hydroxylase (C3H) were significantly down-regulated (PFP$<$0.05). In contrast, the part of genes encoding 4-coumarate: CoA ligase (4CL), cinnamoyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) were significantly up-regulated (PFP $<$0.05). In the case of caffeic acid *O*-methyltransferase (COMT), 3 and 1 genes were significantly down- and up-regulated significantly, respectively (PFP $<$0.05). The expression of genes for caffeoyl-CoA *O*-methyltransferase (CCoAOMT) and ferulate 5-hydroxylase (F5H) showed the downward trends in the microarray data (Figure 4, Supplementary Table S2).

Such changes in the gene expression shown in microarray data (Figure 4, Supplementary Table S2) were validated by quantitative RT-PCR analysis (Figure 5, 6). The results were basically consistent with the microarray analysis; we found that the part of *Ptr4CL*, *PtrCCR*, *PtrCOMT*, and *PtrCAD* genes were up-regulated by the treatment of abiotic stresses (Figure 5). In contrast, the part of *PtrPAL*, *PtrC4H*, *PtrHCT*, *PtrCOMT*, *PtrCSE*, *PtrCCoAOMT*, and *PtrF5H* genes were downregulated by the treatment of abiotic stresses, especially by the drought stress (Figure 6). It has been reported that *F5H* and *COMT* are key genes for S-lignin biosynthesis (Jouanin et al. 2000; Stewart et al. 2009; Weng and Chapple 2010). Our data showed that the expression of specific set of *COMT* and *F5H* genes was decreased by the stress treatment (Figure 4, 6), suggesting that the decreased S/G ratio by the abiotic stresses (Figure 3C)

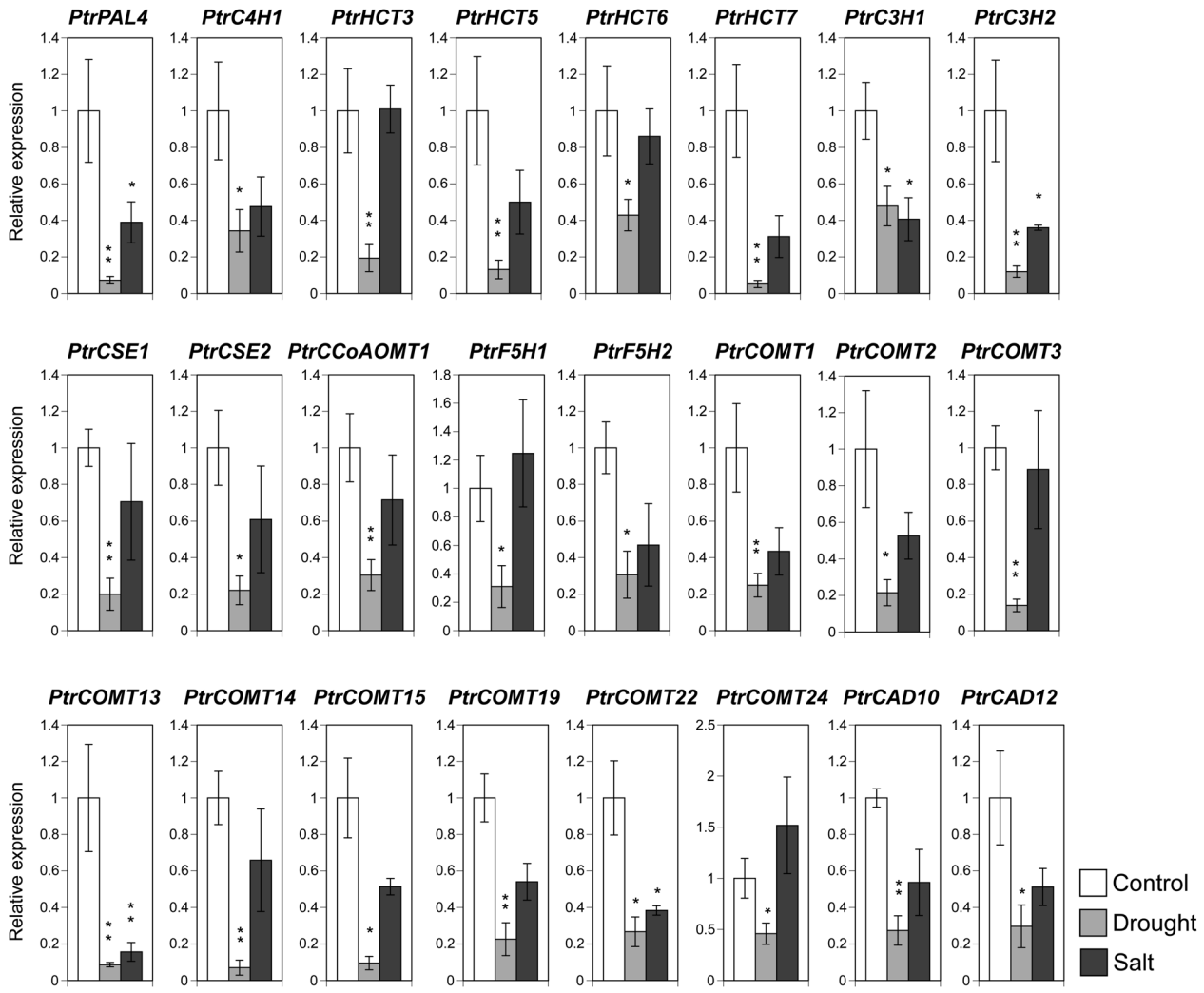


Figure 6. Quantitative RT-PCR analysis of putative lignin biosynthesis genes (down-regulated in young shoot) in poplar under control, salt and drought conditions. Relative expression was normalized based on average of control expression=1. Error bars represent SE (n=4). Asterisks represent statistically significant difference between control condition and stress conditions (t-test; * $p<0.05$, ** $p<0.01$).

would be caused by the down-regulation of *COMT* and *F5H* genes.

Upregulation of lignin biosynthesis genes is partly mediated by ABA signaling

Abscisic acid (ABA) is a key mediator of stress response (Popko et al. 2010), and the overexpression of ABA signaling regulators can enhance salt tolerance in poplar (Song et al. 2016; Yu et al. 2017a, b). Therefore, we tested the responsiveness of monolignol synthesis genes to ABA treatment by quantitative RT-PCR analyses (Supplementary Figure S5). All tested genes, which were up-regulated under drought and salt stress condition (Figure 5), were also induced by the ABA treatment in the young shoots (Supplementary Figure S5). However, the *PtrCOMT8*, *PtrCOMT17*, and *PtrCOMT25* genes were not significantly up-regulated by the ABA treatment ($p>0.05$) (Supplementary Figure S5). These results suggest that the up-regulation of monolignol synthesis genes is mainly mediated by ABA signaling, but ABA-independent factors can be also involved in the regulation for cell wall biosynthesis-related genes.

Discussion

In this study, we observed the changes of poplar cell wall in response to two abiotic stress conditions (drought and salt stresses), in two different tissues (young shoots and mature stem xylem). Under both conditions, the similar anatomical changes were observed in newly generated stem tissues, i.e. the decreased size of vessels in xylem (Figure 1C, D). Differences in toluidine blue dye staining in young shoots (Supplementary Figure S1C) also suggested that the secondary xylem formation in young shoot, which newly generated during abiotic stress, was altered in response to the stress treatments. Indeed, the chemical analysis revealed that the total production of structural polysaccharides such as cellulose and xylan was

significantly reduced (Figure 2). Interestingly, our data indicated that total lignin content was not changed by the stress treatment (Figure 2). However, the NMR analysis clearly demonstrated that the lignin structure including the S/G ratio was significantly altered under the stress conditions, in young shoots (Figure 3, Supplementary Figure S2). These results indicate that the developing tissues will actively respond to abiotic stresses by altering cell wall biosynthesis, while the established xylem tissues can respond to abiotic stresses only through changing vessel cell differentiation. Therefore, it is possible that woody plants would possess different strategies to conquer abiotic stresses by tissues and/or organs.

Transcriptomic analysis demonstrated the drastic changes in expression of cell wall-related genes in young shoots under the abiotic conditions (Figure 4, Supplementary Figure S3, S4). Especially, genes involved in cellulose and hemicellulose biosynthesis were significantly decreased (Supplementary Figure S4), which is consistent with the chemical components results (Figure 2). Notably, the expression of the core lignin biosynthesis pathway genes, *F5H* and *COMT*, were significantly decreased by the drought and salt treatment (Figure 6), likely leading to the observed decrease in the lignin S/G ratio (Figure 3C). Similar down-regulation of *F5H* and *COMT* was reported in rice under salt and osmotic stress conditions (Xu et al. 2009), suggesting that the down-regulation of *F5H* and *COMT* might be a common abiotic stress response in plants. Altering the lignin S/G composition will change the hydrophobicity and rigidity of the cell wall (Le Gall et al. 2015; Moura et al. 2010; Tenhaken 2015), e.g. the enriched G-lignin in vessels is considered to be related to the activity of water transportation (Abreu et al. 2009; Li et al. 2001; Saito et al. 2012). Together, we propose that the lower S/G ratio could be a strategy to decrease cell wall damage as well as to increase the hydrophobicity of cells when plants are exposed to drought and salt stresses.

It is notable that *F5H* is directly regulated by the NAC-type transcription factor SECONDARY WALL-ASSOCIATED NAC DOMAIN 1 (SND1)/NAC SECONDARY WALL THICKENING PROMOTING 3 (NST3), one of the VNS (VND, NST/SND, SMB-related) family proteins that are master regulators of secondary cell wall formation in woody cells (Nakano et al. 2015; Ohtani et al. 2017; Ohtani and Demura 2019), whereas other lignin biosynthesis genes are regulated by MYB transcription factors through a MYB-specific *cis*-element (Zhong and Ye 2009). Our data also demonstrated that *PtVNS09*, which is homologous to the poplar *SND1*/*NST3* (Ohtani et al. 2011) was significantly down-regulated (PFP$<$0.05) by the drought stress (Supplementary Table S3). Other *NST*-related genes, *PtVNS10*, *PtVNS11*, and *PtVNS12*, also showed a tendency towards decreased expression under drought (PFP $<$0.1) (Supplementary Table S3). However, interestingly, the expression of secondary cell wall-related *MYB* genes, such as *PtMYB020* and *PtMYB021*, was unchanged by the stress treatment (Supplementary Table S3). Thus, the down-regulation of *F5H* would be the result of decreased level of *PtVNS* family gene expression. Recently, Arabidopsis *SND1*/*NST3* was reported to be a possible modulator of ABA biosynthesis and lignin biosynthesis, striking a balance between plant growth and stress responses (Jeong et al. 2018). Taken together, the NST-related *PtVNS* gene can be one of promising molecular targets of future engineering, to change the lignin S/G ratio, without decreasing the lignin quantity, for high tolerant to abiotic stresses.

Collectively, in the present work, we identified a molecular strategy by which poplar responds to abiotic stresses by altering gene expression and adapting cell wall composition. Both the size of vessel cells within the xylem and the lignin S/G/H ratio are important factors determining the quantity and quality of woody plant biomass, including saccharification efficiencies (Li et al. 2010; Studer et al. 2011; Van Acker et al. 2013). These effects on woody biomass should be the things to pay attention to, when planning their usage. We also demonstrated the candidate genes for engineering of cell wall adaptation under abiotic stress, i.e. the *F5H* and *COMT* genes. Further studies will support the development of a molecular breeding strategy for trees (Ohtani and Demura 2019), for the high quality biomass even when plants are exposed to severe abiotic stress.

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