PacBio and Illumina RNA sequencing identify alternative splicing events in response to cold stress in two poplar species

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Running head: Alternative splicing events in poplar

Abstract

In eukaryotes, alternative splicing (AS) is a crucial regulatory mechanism that modulates mRNA diversity and stability. The contribution of AS to stress are known in many species related to stress. But the post-transcriptional mechanism in poplar under cold stress is still unclear. Recent studies have utilized the advantages of Single Molecular Real Time (SMRT) sequencing technology from Pacific Bioscience (PacBio) to identify full-length transcripts. We, therefore, used a combination of single-molecule long-read sequencing and Illumina RNA sequencing (RNA-Seq) for a global analysis of AS in two poplar species (*Populus trichocarpa* and *P. ussuriensis*) under cold stress. We further identified 1261 AS events in *P. trichocarpa* and 2101 in *P. ussuriensis*, among which intron retention, with a frequency of more than 30%, was

the most prominent type under cold stress. RNA-Seq data analysis and annotation revealed the importance of calcium, abscisic acid, and reactive oxygen species signaling in cold stress response. Besides, the low temperature rapidly induced multiple splicing factors, transcription factors, and differentially expressed genes through AS. In *P. ussuriensis*, there was a rapid occurrence of AS events. This study provides new insight into the complexity and regulation of AS during cold stress response in two poplar species.

KEYWORDS

Populus trichocarpa, *Populus ussuriensis*, alternative splicing, cold stress, PacBio, RNA-Seq

1. INTRODUCTION

In eukaryotes, precursor messenger RNAs (pre-mRNAs) with multiple introns undergo alternative splicing (AS) to generate two or more mature mRNA isoforms encoding structurally and functionally different proteins (Palusa, Ali, & Reddy, 2007). AS is a crucial regulatory mechanism that contributes to cellular and functional complexity and has been extensively studied in animals and plants (Wang et al., 2018; Li et al., 2019). Genome-wide investigation of AS has been performed on development or in response to stresses in multiple plants. Studies have revealed that an estimated 60% of Arabidopsis, 50% of soybean, 40% of cotton, and 40% of maize intron-containing genes undergo AS (Syed, Kalyna, Marquez, Barta, & Brown, 2012;

Marquez, Brown, Simpson, Barta, & Kalyna, 2012; Li et al., 2019). Five major types of AS events are recognized, namely exon skipping (ES), intron retention (IR), mutually exclusive exon (MXE), alternative 5' splice site (A5SS), and alternative 3' splice site (A3SS), of which IR is the most common event in plants (Reddy, Marquez, Kalyna, & Barta, 2013). Evidence suggests that plants employ AS to achieve phenotypic plasticity in response to different abiotic stresses, such as heat, drought, salinity, and cold (Zhao, Sun, Xu, Zhang, & Li, 2014; Liu et al., 2018; Zhu, Li, Zhang, & Guo, 2018).

Several studies have identified and reported the complexity of AS in plant species, such as cotton (Wang et al., 2018), cassava (Li et al., 2019), *Phyllostachys edulis* (Wang et al., 2017), *Populus trichocarpa* (Bao et al. 2013), Arabidopsis (Marquez et al., 2012), and rice (Zhang et al., 2019). Different from the constitutively spliced isoforms, alternatively spliced ones always show cell-, tissue-, or condition-specific expression patterns, and the extent of AS in plants depends on the complexity of tissues (Kalsotra & Cooper, 2011). Gan et al. (2011) identified multiple varied AS events among the different genotypes of *Arabidopsis thaliana*. Zhou, Moshgabadi, & Adams (2011) discovered a total of 45 AS events in *Brassica oleracea* in two organs and under two environmental conditions (heat and cold). While the splice sites in the pre-mRNA are recognized by splicing factors (SFs), which recruit the spliceosome for intron removal, to generate different alternatively spliced isoforms ((Fu & Ares, 2014; Lee & Rio, 2015). Besides, SFs are essential for plant growth and development processes, including control of flowering time, regulation of the circadian rhythms,

66 and response to abiotic stresses (Staiger & Brown, 2013; Schlaen et al., 2015). These
67 studies indicate that the regulated AS of downstream targets is essential for plants.
68 A recent survey by Calixto et al. (2018) has reported a massive and rapid AS response
69 under cold stress that involved changes in hundreds of cold-responsive transcription
70 factors (TFs) and SF RNA-binding proteins in *Arabidopsis*. Moreover, misexpression
71 of SFs altered cold sensitivity or tolerance in plants (Reddy et al., 2013; Staiger &
72 Brown, 2013). In Arabidopsis, low temperature regulated the expression and splicing
73 patterns of the serine/arginine-rich SFs (Palusa et al., 2007). These studies strongly
74 suggest the central role of SFs and the importance of AS in plant response to cold
75 stress.
76 Studies have widely used short-read RNA sequencing (RNA-Seq) technology to
77 detect AS events; however, it is challenging to identify full-length splicing isoforms
78 accurately by this method. By contrast, the SMRT developed by Pacific Biosciences
79 (PacBio, California, USA), offers an improvement in read length over the previous
80 technologies. PacBio technology is valuable for the annotation of newly sequenced
81 genes and the analysis of AS (Eid et al., 2009; Sharon, Tilgner, Grubert, & Snyder,
82 2013). Researchers have started using PacBio sequencing technology to characterize
83 the complexity of transcriptome in various plant species.
84 Low temperature is one of the most common stresses that negatively affect plant
85 growth and crop production (Calixto et al., 2018). Numerous studies have
86 demonstrated changes in AS events in response to external cold/chilling stimuli,
87 however, the regulation of AS in poplar species under cold stress is not clear at the

whole transcriptomic level. In this study, it was better cold tolerance in *P. ussuriensis* than in *P. trichocarpa.* we aim to investigate the reason for this. Our data reveal that cold intensely affects gene expression via alternative splicing regulated by SFs in *P. ussuriensis* to resistant cold stress and advances our understanding of the high complexity and specificity of species-specific AS regulation in response to cold.

2. MATERIALS AND METHODS

2.1. Plant materials and growth conditions

Populus trichocarpa 'Nisqually-1' and *P. ussuriensis* Kom. were used in this study. Stem segments (3 cm long) of these poplar species were cultured in ½ MS medium for one month. These clonally cultured seedlings were placed at 25 ± 2 °C under 16 h/ 8 h (light/dark) photoperiod. Two-week-old of both plants were transferred to a chamber for cold treatment at 3°C and -3°C. The young leaves and shoot apices were collected after 3 h of cold treatment, immediately frozen in liquid nitrogen, and stored at -80°C for RNA extraction. The nine RNAs samples from each poplar species (three biological replicates per time point) were subjected to 150 bp paired-end sequencing using the Illumina HiSeq platform. The nine RNAs samples from each poplar species were mixed in equal volume and sequenced on the PacBio RS II platform, respectively (Figure 1a).

2.2. RNA extraction and the SMRT sequencing library construction

Total RNA was extracted from the leaves and shoot apices of *P. trichocarpa* and *P.*

110 *ussuriensis* maintained under different temperatures (25°C, 3°C and -3°C) using the
111 CTAB method (Jaakola, Pirttila, Halonen, & Hohtola, 2001). The total RNA was
112 assessed using an Agilent Bioanalyzer 2100 (Agilent, https://www.agilent.com). RNA
113 purity was checked using the kaiaoK5500® Spectrophotometer (Kaiao, Beijing,
114 China). RNA integrity and concentration were assessed using the Bioanalyzer 2100
115 RNA Nano 6000 Assay Kit (Agilent Technologies, CA, USA). Total RNA of the nine
116 samples from each species were pooled in equal amounts, and 1 μg of the pooled
117 RNA was used for cDNA synthesis and SMRTbell library construction. The purified
118 RNA was reversely transcribed into cDNA using the SMARTer PCR cDNA Synthesis
119 Kit (Clontech Laboratories, Inc. Mountain View, CA, USA). The cDNA was
120 amplified using the Kapa HiFi PCR Kit (Kapa Biosystems, Wilmington, MA, USA).
121 Size selection was carried out on a BluePippin (Sage Science, Beverly, MA, USA),
122 and 1–2 kb, 2–3 kb, 3–6 kb, and 5–10 kb fractions were collected. These collected
123 cDNA fractions were treated with a DNA damage repair mix, followed by end repair
124 and ligation of SMRT adapters using the PacBio SMRTbell Template Prep Kit
125 (Pacific Biosciences, Menlo Park, CA, USA) to create SMRT sequencing libraries.
126
127 **2.3. PacBio data analysis**
128 Raw data generated by the PacBio RSII platform was processed following Iso-seq
129 method. The PacBio raw reads were processed into error-corrected ‘reads of inserts’
130 (ROIs) using ToFu (version 2.3.0) with the following parameters: minimum full pass
131 > 1, minimum ROI length > 200 nucleotides, and prediction accuracy > 0.8. The ROIs

were classified into circular consensus sequences (CCS) and non-CCS subreads based on the presence or absence of sequencing adapters. CCS subreads were classified into full-length non-chimeric reads (FLNC) or non-FLNC based on the presence of both the primer sequences (the 5′ and 3′ sequences) and the polyA tail signal.

Next, an isoform-level clustering algorithm ICE (Iterative Clustering for Error Correction) was applied to all the full-length (FL) transcripts to obtain the consensus transcripts based on the sequence similarity and generate a consensus sequence for each cluster. Quiver was used for error correction to obtain high-quality (HQ) and low-quality (LQ) isoforms (accuracy ≥ 99%). Finally, high-quality FL transcripts were combined to obtain all high-quality FL transcripts of each sample.

2.4. Library preparation and Illumina sequencing

Illumina sequencing was performed to generate data that can be used to validate and quantify the PacBio transcripts. Approximately 2 μg of RNA per sample was used as input material for the Illumina sequencing. The clustering of the index-coded samples was performed on a cBot cluster generation system (Illumina HiSeq PE Cluster Kit v4-cBot-HS) following the manufacturer's instructions. After cluster generation, the libraries were sequenced on an Illumina platform, and 150 bp paired-end reads were generated. In order to guarantee the data quality, the original data (Raw Data) was filtered. The read counts for each gene in each sample were generated using HTSeq v0.6.0, and FPKM (Fragments Per Kilobase Millon Mapped Reads) values were then calculated to estimate the expression level of the genes in each sample. Gene

annotation was based on the following databases: Nr (NCBI non-redundant protein sequences), Nt (NCBI non-redundant nucleotide sequences), Swiss-Prot (amanually annotated and reviewed protein sequence database), Pfam (Protein family), COG (Clusters of Orthologous Groups of proteins), GO (Gene Ontology), KEGG (Kyoto Encyclopedia of Genes and Genomes) orthology database (Kanehisa et al. 2008), GO enrichment analysis of the differentially expressed genes (DEGs) was implemented by the GOseq (Young, Wakefield, Smyth, & Oshlack, 2000).

2.5. Identification of differentially spliced events

The tool ASprofile (version b-1.0.4) was employed to classify the AS events using the raw.gtf files assembled from the Illumina RNA-Seq and SMRT-Seq data of *P. ussuriensis* compared to *P. trichocarpa*. The AS events, including IR, ES, MXE, A5SS, and A3SS were extracted and counted. The differential AS (DAS) events from two poplar comparison at different temperature were identified by rMATS.3.2.2 (Shen et al., 2014). The AS events with a false discovery rate (FDR) less than 0.05 were defined as DAS events. GO enrichment analyses with DAS were conducted using AgriGO (Tian et al., 2017).

2.6. Calcium and physiological indicators determination

After cold stress, the leaves of 25℃, 3℃, -3℃ for *P. trichocarpa* and *P. ussuriensis* was collected and dried in a 70 ℃ in the oven. Approximately 0.5mg (dry weight) of

175 samples were weighted and analyzed using inductively coupled plasma (ICP)
176 emission spectrometer (ICP-OES 5110 VDV, Agilent, USA).
177 For MDA content measurement, about 50 mg of leaves were ground and
178 homogenized in 1mL of 0.1% (w/v TCA for 10 min and centrifuged at 10,000g for 15
179 min at 4°C. The supernatant was reacted with 20% (w/v) TCA containing 0.5% (w/v)
180 thiobarbituric acid. After boiled and cooled, it was centrifuged at 10,000g for 5 min at
181 4°C. The detailed method was performed as described by Metwally, Finkemeier,
182 Georg, & Dietz (2003).
183 The H_2O_2 was measured using an Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit
184 (Invitrogen, Carlsbad, CA, USA) as described in Xing, Jia, & Zhang (2008). Leaves of
185 poplars were frozen in N_2 and ground. The phosphate buffer (20 mM K_2HPO_4, pH6.5) was
186 added to 50 mg of ground frozen tissue. After centrifugation, the supernatant was incubated
187 with 0.2 U ml^{-1} horseradish peroxidase and 100 μM Amplex Red reagent (10-acetyl-3,7-
188 dihydrophenoxazine) at room temperature for 30 min in darkness. The fluorescence was
189 quantified using FLUOStar Optima (excitation at 560 nm and emission at 590 nm).
190

191 **2.7. Validation of alternative splicing transcripts**

192 Total RNA was extracted from the leaves and shoot apices of *P. ussuriensis* as
193 described above. The TransScript One-Step gDNA Removal and cDNA Synthesis
194 SuperMix Kit (TransGen Biotech, China) was used for simultaneous genomic DNA
195 removal and cDNA synthesis (20 μL) following the manufacturer's instructions. The
196 AS transcripts were validated by reverse transcription-polymerase chain reaction (RT-

PCR) using specific primers listed in Supporting Information Table S1.

2.8. Validation of differentially expressed genes (DEGs) by quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA extraction and cDNA synthesis were performed as described above. qRT–PCR was performed to validate DEGs. The *PuActin* gene was used as the internal control. The relative gene expression values in *P. ussuriensis* were analyzed using the $2^{-\Delta\Delta Ct}$ method compared to *P. trichocarpa*. All reactions were performed using three biological replicates for each sample. The primers are listed in Supporting Information Table S2.

3. RESULTS

3.1. Phenotypic changes in two poplar species in response to cold stress

Low temperature is one of the key environmental stresses, which negatively impairs plant growth and development (Li, Li, Zhang, Zhang, Jiang, Yu, & Hou, 2017). To evaluate the tolerance of cold, we compared the phenotypic changes of two poplar species *P. trichocarpa* and *P. ussuriensis*, which are tolerant and sensitive to cold stress, respectively. About 5 cm height of seedlings were used for physiological and transcriptome analyses at 3°C for 3 h and -3°C for 3 h. The seedlings cultured at room temperature (Figure 1a) were used as control. Treatment-related changes in the phenotype of *P. trichocarpa* were observed after 4 h at -3°C (Figure 1d), the leaves

were damaged and wilted. While, the *P. ussuriensis* plants were not damaged (Figure 1d). In comparison, no difference was observed after 3 h at 3°C (Figure 1b) and -3°C (Figure 1c) between the two species.

3.2. Calcium, MDA, POD and H_2O_2 determination

At room temperature, the content of calcium, MDA, POD and H_2O_2 in *P. ussuriensis* was not different from them in *P. trichocarpa* (Figure 2). As the temperature fell, the calcium (Figure 2a), MDA (Figure 5b), POD (Figure 2c) and H_2O_2 (Figure 2d) content in both poplars was increased. But the calcium (Figure 2a), and POD (Figure 2c) content in *P. ussuriensis* increased more than that in *P. trichocarpa.* While, the MDA (Figure 2b) and H_2O_2 (Figure 2d) content in *P. ussuriensis* was decreased than that in *P. trichocarpa* at 3℃ and -3℃。

3.3. Overview of PacBio Iso-Seq data of two poplar species

We used *P. ussuriensis*, the widely cultivated species in northeast China, to compared with *P. trichocarpa* for this study. We performed PacBio sequencing and analyzed based on *P. trichocarpa* genome. From the phenotypic point of view, *P. ussuriensis* was more tolerant to lower temperatures than *P. trichocarpa.* To better understand the full-length splice variants, novel genes, and alternative polyadenylation (APA) sites of the two species in response to cold stress, the PacBio Iso-Seq method was used to sequence the transcriptomes. A total of two SMRT cells (r64053_20191003_023517_4_H01 for *P. trichocarpa*;

r64053_20191003_023517_4_H01 for *P. ussuriensis*) mixed with nine samples were constructed to eliminate instrumental bias towards short fragments respectively. A dataset with more than 20 Gb of clean reads was obtained after filtering using SMRTLink (6.0). Refer to *P. trichocarpa* genome, a total of 51,153 and 58,082 consensus isoforms were obtained from the two libraries (*P. trichocarpa* and *P. ussuriensis*), respectively (Table 1). The mean length of consensus isoforms in the two libraries was between 1683 and 1910 bp, respectively (Table 1). A total of 50,910 and 57,872 reads of HQ isoforms were produced, respectively. HQ isoforms with a mean length of 1678 and 1905 bp were identified from the two libraries, respectively (Table 1). Among which, a total of 228 and 188 HQ isoforms with a mean length of 1157 and 1344 bp were obtained, respectively (Table 1). Mapping on to the reference genome of *P. trichocarpa* revealed that a total of 97.66% and 97.51% of the isoforms detected by PacBio SMRT-Seq were mapped reads in *P. trichocarpa* (Figure 3a) and *P. ussuriensis* (Figure 3b) respectively; 1.8% and 1.02% were multi-mapped to the genome in *P. trichocarpa* (Figure 3a) and *P. ussuriensis* (Figure 3b) respectively. The mapped isoforms (density) were spread across different chromosomes. Our analysis indicates that the reference genome of *P. trichocarpa* genome was enriched with the PacBio SMRT-Seq results and used for further analysis.

Furthermore, we compared the genome-wide genetic variants, including chromosome distribution, gene density, novel gene, novel long non-coding RNA (lncRNA), APA site, and single-nucleotide polymorphism (SNP), between *P. trichocarpa* and *P. ussuriensis* (Figure 3) using the Circos program. A total of 852 (Figure 3c) and 1093

(Figure 3d) novel genes and 25 (Figure 3c) and 42 (Figure 3d) APA sites were detected in *P. trichocarpa* and *P. ussuriensis*, respectively. A total of 235,900 (Figure 3c) and 49,781 (Figure 3d) SNPs were detected in *P. trichocarpa* and *P. ussuriensis*, respectively. LncRNAs, which are widespread in eukaryotic genomes and play crucial roles in various biological processes (Shafiq, Li, & Sun, 2016; Chekanova, 2015).

3.3. Identification of known and novel alternative splicing (AS) events

To investigate the role of AS in response to cold stress, we surveyed the transcript isoforms in the two poplar species. We examined the five major types of AS events (IR, ES, MXE, A5SS, and A3SS) in the isoforms using SMRT-Seq (Figure 4a). We compared the frequency of AS types from the two poplar species at 25°C, -3°C and 3°C respectively. Based on the reference genome (*P. trichocarpa*), 35.7% of the AS events were IR, 30.3% were A5SS, 24.7% were A3SS, 9.0% were SE, and 0.3% were MXE in *P. trichocarpa* (Figure 4b) while 34.7% of the AS events were IR, 32.7% were A5SS, 24.5% were A3SS, 7.9% were SE, and 0.2% were MXE in *P. ussuriensis* (Figure 4c). The number of AS events in *P. trichocarpa* under cold stress was more than that in *P. ussuriensis* (Figure 4d). A total of 1261 AS events were identified from 834 genes in *P. trichocarpa* and 2101 AS events from 1257 genes in *P. ussuriensis* (Figure 4d). Among the AS types, IR, with a frequency of more than 30%, was the most abundant type under cold stress (Figure 4e). Interestingly, the percentage of different AS types changed with a decrease in temperature in both poplar species, especially IR dramatically increased and SE decreased at -3°C compared with that at

283 3°C both in *P. trichocarpa* (Figure 4f) and *P. ussuriensis* (Figure 4g).

284

285 **3.4. Analysis of DEGs, differential alternative splicing (DAS) events, and**
286 **corresponding cold-responsive genes**

287 RNA-Seq helps understand the network via which AS influence gene expression and
288 transcriptome reprogramming. To improve the accuracy of AS analysis, we
289 investigated the transcriptional changes and compared the the two poplar species in
290 response to cold stress by RNA-seq. Compared to *P. trichocarpa*, 6060 (Data S1),

291 5208 (Data S2), and 5867 (Data S3) DEGs ($P < 0.05$; $|\log2(FC)|>1$) were identified at
292 25°C, 3°C, and -3°C, respectively, in *P. ussuriensis*. We further analyzed the most
293 significantly enriched GO terms for DEGs (Figure 5, a-i) and DAS (Figure 5, j-l)
294 genes. The most significantly enriched GO terms for DEGs were "response to
295 stimulus and stress" in biological process (BP, Figures 5a–c), "intrinsic component of
296 membrane" in cellular component (CC, Figures 5d–f), and "catalytic activity" in
297 molecular function (MF, Figures 5g–i). The most significantly enriched GO terms for
298 DAS genes are shown in Figure 5j–l. As the temperature decreased, the "response to
299 abiotic stimulus" item of BP increased significantly, which is consistent with the GO
300 terms of DEGs.

301 From the RNA-Seq data, U1–U9 samples were classified into six subclasses of genes
302 that were upregulated or downregulated in *P. ussuriensis* compared with *P.*
303 *trichocarpa* (P1–P9 samples) in response to cold stress with different patterns of

304 expression (Figure S1). For example, genes of subclass 3 in *P. ussuriensis* were
305 upregulated compared with that in *P. trichocarpa* (Figure 6a, Supporting Information
306 Data 4). These upregulated genes included genes involved in calcium signaling, such
307 as calmodulin (CAM), calcium-dependent protein kinase (CPK), and CBL-interacting
308 protein kinase (CIPK) (Table 2), and many mitogen-activated protein kinase (MAPK)
309 cascades (Data S4). Additionally, a 9-*cis*-epoxycarotenoid dioxygenase (*NCED*) gene
310 encoding the key enzyme of abscisic acid (ABA) biosynthesis was upregulated under
311 cold stress (Table 2). Many peroxidase (POD) genes were also upregulated in *P.*
312 *ussuriensis* (Table 2). In total, 2785, 3613, and 3408 DAS genes were identified at
313 25°C, 3°C, and -3°C, respectively, in *P. ussuriensis* compared with that in *P.*
314 *trichocarpa* by RNA-seq (Figure 6b). MATS identified the DAS events in the two
315 species under different temperature conditions. A model pattern example from IGV
316 wiew of Potri.001G016200 alternative splicing (SE) type is shown in Figure 6c. Venn
317 analysis revealed a substantial overlap among the DEGs, DAS genes, and the cold-
318 responsive DEGs identified in *P. ussuriensis* compared to *P. trichocarpa* (Figures 6d–
319 f). About half of the DEGs were DAS genes (Figures 6d–f), of which 18.1%, 28.2%,
320 and 35.1% DEGs at 25°C, 3°C, and -3°C, respectively, in response to cold stress were
321 DAS genes (Figures 6d–f); these genes with significant expression ($P < 0.05$;
322 |log2(FC)|>1) are shown in Table 3 based on GO. We found that several DEGs related
323 to cold stress, such as cold-regulated inner membrane protein 1 (CORIMP1,
324 Potri.001G353500) at 25°C, low-temperature- and salt-responsive proteins (LTI6A,

Potri.013G001600 and CORIMP1 Potri.001G353500) at 3°C, and late elongated hypocotyl (LHY, Potri.014G106800), low-temperature-induced integral membrane protein (LTI6A, Potri.013G001600), and calcium-binding protein (CML, Potri.002G182500, Potri.014G108200) at -3°C in *P. ussuriensis* (Table 3) had more isoforms based on *P. trichocarpa* genome. RT-PCR validation revealed more than one isoform for these genes (Figure 7). We also randomly selected some DEGs using qRT-PCR to validate the reliability of the RNA-seq data. The result showed that the qRT-PCR was consistent with RNA-seq data (Figure 8).

3.5. Cold-induced changes in splicing factors (SFs) and transcription factors (TFs)

The splice site of pre-mRNA was recognized by SFs that recruit the spliceosome to remove the introns away (Chen & Moore, 2015). A number of SFs was reported that functions by diverse environmental conditions (Laloum, Martín, & Duque, 2018). We focused on the SFs that probably regulated the AS of downstream genes by different spliceosome complexes under cold condition. The AS events occurred in multiple SFs including small nuclear ribonucleoproteins (snRNPs), such as spliceosome-associated proteins (DEAH box helicase 1, YT521-B, LSM, AAR2, SART-1, and Prp series; Table 4, $P < 0.05$; $|\log2(FC)|>1$). At 25°C, only two SFs were differentially expressed; five SFs, including the two SFs at 25°C, were differentially expressed at

3°C. Many SFs, including SFs of the two temperatures (25°C and 3°C), were also responsive at -3°C (Table 4). Most importantly, all the SFs were upregulated. In short, more pre-mRNA SFs responded in *P. ussuriensis* than that in *P. trichocarpa* as the temperature decreased.

Analysis based on the reference genome revealed AS of numerous TFs. Table 2 shows significantly induced TFs. Under normal conditions, AS events of these TFs were different between the two poplar species and identified, such as WRKY, MYB, B3, and CAMTA. More and more AS events happened as the temperature lowered (Table 5).

4. DISCUSSION

Plants cope with adverse environmental conditions by reprogramming the gene expression and metabolism in a strict manner (Li et al., 2019). Although the expression of genes in some plant species under cold stress has been studied extensively, pre-mRNA splicing during the transcriptional changes in response to cold is still not clear in poplar species. In the current study, the result showed that the *P. ussuriensis* was more cold tolerance than *P. trichocarpa,* probably two poplar species have different abilities to deal with cold condition. We used SMRT sequencing and RNA-Seq (Illumina) for the first time for a global survey of AS in two poplar species under cold stress. The PacBio Iso-Seq data revealed that 97.66% and 97.51% of the isoforms detected were mapped reads based on the reference genome of *P. trichocarpa*, and 1.8% and 1.02% were multi-mapped to the genome. These findings

367 indicate that PacBio Iso-Seq data has higher transcript density than the reference
368 genome and the complexity of the response.
369 Plant cells sense cold stress through membrane rigidification (Chinnusamy, Zhu, &
370 Zhu, 2007). The rigidification subsequently activates mechanosensitive or ligand-
371 activated calcium channels that lead to transient accumulation of Ca^{2+} in the cytosol.
372 Studies have reported the higher levels of calcium, which lead to signal amplification,
373 act as the initial signals for cold stress response in plants (Vergnolle et al., 2005;
374 Chinnusamy et al., 2007). Ca^{2+} and MAPK signaling cascades play significant roles in
375 the cold response pathway of Arabidopsis (Li et al., 2017; Zhao et al., 2017). In our
376 study, *P. ussuriensis* exhibited more cold tolerance than *P. trichocarpa*. We found that
377 calcium signaling-related genes, such as CAM, CPK, and CIPK, and many MAPK
378 cascades were upregulated in *P. ussuriensis* at 25°C, 3°C and -3°C compared to *P.*
379 *trichocarpa*. Meanwhile, the Ca^{2+} content in *P. ussuriensis* increased more than that in
380 *P. trichocarpa* under cold stress in a short time (3 h) from 25℃ to -3℃ (Figure 5a),
381 which indicates that the cold response pathway is calcium-dependent in poplar
382 species.
383 Reactive oxygen species (ROS) also activate signal transduction pathways in response
384 to biotic and abiotic stresses (Miller, Suzuki, Ciftci-Yilmaz, & Mittler, 2010). As the
385 temperature lowed, the H_2O_2 content was increased, and the increased level was
386 greater in *P. trichocarpa* compared with in *P. ussuriensis*. In our study, low
387 temperature upregulated many POD genes in *P. ussuriensis*, which indicate the

388 influence of ROS on cold stress regulation of gene expression. Besides, ROS can
389 simulate Ca^{2+} accumulation that affects cold tolerance in plants (Chinnusamy et al.,
390 2007). Studies have also reported the role of ABA in abiotic stresses, such as drought,
391 low temperature, and salinity. ABA accumulates in plants to regulate stress-responsive
392 genes (Lv et al., 2018). ABA can also act as a secondary signal to change Ca^{2+} levels that
393 finally influence cold signaling (Boudsocq & Sheen, 2013). In this study, *NCED* gene
394 encoding the key enzyme involved in ABA biosynthesis at low temperature were
395 upregulated. Thus, we conclude that Ca^{2+} signaling, ROS, and ABA might have led to
396 cold tolerance in a short time.
397 Abiotic stress triggers AS events in plants (Pajoro, Severing, Angenent, & Immink,
398 2017; Laloum et al. 2018). These events regulate proteome diversity and gene
399 expression to adept adverse environment (Thatcher et al., 2016). Therefore, the DAS
400 events identified in our study under cold stress were not accidental. The extensive AS
401 information identified here demonstrates the complexity of cold stress response.
402 Studies based on the analysis of DEGs alone have significantly underestimated this
403 regulatory mechanism. We also compared the AS events in two poplar species in
404 response to cold stress. The comparison of the different AS types identified IR events
405 in a large proportion, followed by A3SS in both types of poplars. Studies have
406 reported IR as the most common splicing event in plant species, such as Arabidopsis
407 (Marquez et al., 2012; Calixto et al., 2018) and cassava (Li et al., 2019). Calixto et al.
408 (2018) reported differential splicing events very early under cold stress in
409 Arabidopsis. The AS exhibited significant changes within only 40–60 minutes under

cold stress (Calixto et al. 2018). Researchers have identified the cold-responsive ICE-CBF pathway, which was critical for the regulation of cold-responsive transcriptome in plants. One such cold-responsive pathway involved the binding of ICE1 (Inducer of CBF expression 1) to the promoter of CBFs (C-repeat binding factors) inducing expression; ICE1 enhances the expression of *COR* (Cold-regulated) genes also during cold acclimation (Ritonga, & Chen, 2020). However, the expression of these cold-responsive genes did not change in the two poplar species. Therefore, we speculated that AS happened probably before the differential expression of cold-responsive genes was induced. To identify DAS, we investigated the transcriptional changes in two poplar species in response to cold stress after 3 h by MATS. We identified few DAS events in *P. ussuriensis* when compared with *P. trichocarpa*. Many DAS genes regulated by both transcription and AS were differentially expressed in *P. ussuriensis* compared with *P. trichocarpa*. Accelerated cell death 6 (*ACD6,* Potri.013G133900) was downregulated in *P. ussuriensis* and subjected to IR under different low-temperature conditions. *ACD6* encodes a transmembrane protein with intracellular ankyrin repeats, which positively controls cell death and defense (Lu, Rat, Song, & Greenberg, 2003; Yao & Greenberg, 2006). Yao and Greenberg reported that *ACD* can prevent plant chlorophyll breakdown that induce programmed cell death (PCD) (Yao & Greenberg, 2006). In this study, *ACD6* was downregulated. This might have happened because PCD was not activated in *P. ussuriensis* at any temperature conditions, and thus, *P. ussuriensis* exhibited higher cold tolerance than *P. trichocarpa*. LHY is a protein that functions in floral growth (Yon et al., 2016) and

stress response (Adams et al., 2018). Studies have reported the influence of temperature changes on *LHY* transcript in Arabidopsis; moreover, AS in *LHY* was temperature-dependent (James, Sullivan, & Nimmo, 2018a; James et al., 2018b). In the present study, *LHY* (Potri.014G106800) was upregulated, and five types isoforms (A3SS, A5SS, MXE, RI, and SE) were identified at -3°C, which indicates the role of *LHY* in cold tolerance in poplar. Together, our results suggest that AS and DEGs play critical roles in the cold response. We have proposed a network (Figure 9) that reflects the rapid changes in DEGs and DAS genes during the cold response.

Differential expression of SFs is also a significant factor that determines the changes in stress-induced AS (Punzo et al., 2020). In Arabidopsis, DNA-DAMAGE REPAIR/ TOLERATION PROTEIN111 (DRT111)/SPLICING FACTOR FOR PHYTOCHROME SIGNALING (DRT111/SFPS) SF controlled ABA-sensitivity during seed germination (Punzo et al., 2020). In the present study, all the SFs identified via GO enrichment analysis were upregulated in *P. ussuriensis* compared with *P. trichocarpa*. Among these upregulated SFs, SC35, an arginine-serine rich protein, is known to regulate plant development by modulating splicing and transcription of a subset of genes was identified (Yan et al., 2017). Scarecrow-like (*SCL*) genes are involved in plant information transmission via signaling networks (Liu et al., 2017) and in the regulation of plant abiotic stresses, such as drought or salt stress (Golldack, Li, Mohan, & Probst, 2013; Zhou, Zhang, & Wang, 2013) were identified. The SF RS40, also an arginine-serine rich protein, participates in miRNA biogenesis (Chen & Moore, 2015). Therefore, the role of SFs in cold-induced AS

changes needs further investigation. Additionally, studies have reported numerous TFs that are subjected to AS and subsequently contribute to the regulation of gene expression (Zhang, Chernomoretz, & Yanovsky, 2015). In this study, at room temperature, few TFs had isoforms in *P. ussuriensis* compared with *P. trichocarpa*. As the temperature decreased, the amount of AS in TFs increased in *P. ussuriensis*. It will be interesting to investigate the functions of the novel and already known cold-responsive TFs regulated by AS under cold stress.

5. CONCLUSIONS

In summary, SMRT-Seq and Illumina RNA-Seq revealed that poplar trees rapidly responded at the pre-mRNA alternative splicing (AS) stage and AS regulated the transcript abundance to adjust to cold stress. Isoform abundance and rapid response in *P. ussuriensis*, indicate that the changes in AS transcripts might was the most element that tolerant to cold stress compared with *P. trichocarpa* species. Splicing factors and transcription factors are likely important for the regulation of DEGs by AS events. Our findings will support further research on the functions of AS and the coordination between transcriptional and AS responses to confer cold tolerance.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT

Illumina HiSeq data have been submitted to GEO under the accession numbers

PRJNA656151 for *Populus trichocarpa* and PRJNA656152 for *Populus ussuriensis*. PacBio SMRT sequencing data have been submitted to the Sequence Read Archive (SRA) of NCBI under the accession numbers PRJNA656159 for *P. trichocarpa* and PRJNA650237 for *P. ussuriensis*.

AUTHOR CONTRIBUTIONS

J.Y. and C.L. conceived and planned the experiments; J.Y. and W.L. performed the experiments; J.L. collected the materials; X.Z. analyzed the data; and J.Y. wrote the manuscript with support from all the authors.

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REFERENCES

Adams, S., Grundy, J., Veflingstad, S. R., Dyer, N. P., Hannah, M. A., Ott, S., …

500 Carré, I. A. (2018). Circadian control of abscisic acid biosynthesis and signaling
501 pathways revealed by genome-wide analysis of LHY binding targets. *New*
502 *Phytologiat,* 220(3):893–907. https://doi.org/10.1111/nph.15415.
503 Bao, H., Li, E., Mansfield, S. D., Cronk, Q. C. B., EI-Kassaby, Y.A., & Douglas, C.J.
504 (2013). The developing xylem transcriptome and genome-wide analysis of
505 alternative splicing in *Populus trichocarpa* (black cottonwood) populations.
506 *BMC Genomics,* 14:359. https://doi.org/10.1186/1471-2164-14-359.
507 Boudsocq, M., & Sheen, J. (2013). CDPKs in immune and stress signaling. *Trends in*
508 *Plant Science,* 18: 30–40. https://doi.org/10.1016/j.tplants.2012.08.008.
509 Calixto, C. P. G., Guo, W., James, A. B., Tzioutziou, N. A., Entizne, J. C., Panter, P.
510 E., … Browna, J. W. S. (2018). Rapid and Dynamic Alternative Splicing Impacts
511 the Arabidopsis Cold Response Transcriptome. *The Plant Cell,* 30:1424–1444.
512 https://doi.org/10.1105/tpc.18.00177.
513 Chekanova, J. A. (2015). Long non-coding RNAs and their functions in
514 plants. C*urrent Opinion in Plant Biology,* 27:207–216. https://doi.org/
515 10.1016/j.pbi.2015.08.003.
516 Chen, W., & Moore, M. J. (2015). Spliceosomes. *Current Biology,* 25:181–183.
517 https://doi.org/10.1016/j.cub.2014.11.059.
518 Chinnusamy, V., Zhu, J., & Zhu, J. (2007). Cold stress regulation of gene expression
519 in plants. *Trends in Plant Science,* 12(10): 444-451. https://doi.
520 org/10.1016/j.tplants.2007.07.002.
521 Eid, J., Fehr, A., Gray, J., Luong, K., Lyle, J., Otto, G., … Turner, S. (2009). Real-time

522 DNA sequencing from single polymerase molecules. *Science,* 323: 133–138.
523 https://doi. org/ 10.1126/science.1162986.
524 Fu, X.D., & Ares, M., Jr. (2014). Context-dependent control of alternative splicing by
525 RNA-binding proteins. *Nature Reviews Genetics,* 15: 689–701.
526 https://doi.org/10.1038/nrg3778.
527 Gan, X., Stegle, O., Behr, J., Steffen, J. G., Drewe, P., Hildebrand, K.L., … (2011).
528 Multiple reference genomes and transcriptomes for *Arabidopsis thaliana*. *Nature,*
529 477: 419–423. https://doi.org/10.1038/nature10414.
530 Golldack, D., Li, C., Mohan H., & Probst N. (2013) Gibberellins and abscisic acid
531 signal crosstalk: living and developing under unfavorable conditions. *Plant Cell*
532 *Reports,* 32:1007–1016. https://doi.org/10.1007/s00299-013-1409-2
533 Jaakola, L., Pirttila, A. M., Halonen, M., & Hohtola, A. (2001). Isolation of high
534 quality RNA from bilberry (*Vaccinium myrtillus* L.) fruit. *Molecular*
535 *Biotechnology,* 19: 201-203. https://doi.org/10.1385/MB:19:2:201.
536 James, A. B., Sullivan, S., & Nimmo, H. G. (2018a). Global spatial analysis of
537 Arabidopsis natural variants implicates 5′UTR splicing of LATE ELONGATED
538 HYPOCOTYL in responses to temperature. *Plant Cell and Environment,*
539 41(7):1524-1538. https://doi.org/10.1111/pce.13188.
540 James AB, Calixto CPG, Tzioutziou NA, Guo W, Zhang R, Simpson CG, … Nimmo,
541 H. G. (2018b). How does temperature affect splicing events? Isoform switching
542 of splicing factors regulates splicing of LATE ELONGATED
543 HYPOCOTYL (LHY). *Plant Cell and Environment,* 41(7):1539-1550.

https://doi.org/10.1111/pce.13193

Kalsotra, A., & Cooper, T. A. (2011). Functional consequences of developmentally regulated alternative splicing. *Nature Review Genetics,* 12: 715–729. https://doi.org/10.1038/nrg3052.

Kanehisa, M., Araki, M., Goto, S., Hattori, M., Hirakawa, M., Itoh, M., …, Yamanishi, Y. (2008). KEGG for linking genomes to life and the environment. *Nucleic Acids Research*, 36:480-484. https://doi.org/10.1093/nar/gkm882.

Laloum, T., Martín, G., and Duque, P. (2018). Alternative splicing control of abiotic stress responses. *Trends in Plant Science,* 23: 140–150. https://doi.org/10.1016/j.tplants.2017.09.019.

Lee, Y., & Rio, D.C. (2015). Mechanisms and regulation of alternative pre-mRNA splicing. *Annual Review of Biochemistry,* 84: 291–323. https://doi.org/10.1146/annurev-biochem-060614-034316.

Li, H., Ding, Y., Shi, Y., Zhang, X., Zhang, S., Gong, Z., … Yang, S. (2017). MPK3- and MPK6-mediated ICE1 phosphorylation negatively regulates ICE1 stability and freezing tolerance in Arabidopsis. Developmental Cell, 43: 630–642.e4. https://doi.org/10.1016/j.devcel.2017.09.025.

Li, P., Li, Y.J., Zhang, F.J. Zhang, G.Z., Jiang, X.Y., Yu, H.M., … Hou, B.K. (2017). The Arabidopsis UDP-glycosyltransferases UGT79B2 and UGT79B3, contribute to cold, salt and drought stress tolerance via modulating anthocyanin accumulation. *The Plant Journal,* 89: 85–103. https:// doi.org/10.1111/tpj.13324.

Li, S., Yu, X., Cheng, Z., Zeng, C., Li, W., Zhang, L., … Peng, M. (2019). Large-scale

566 analysis of the cassava transcriptome reveals the cold stress on alternative
567 splicing. *Journal of Experimental Botany*, 71(1):422-434.
568 https://doi.org/10.1093/jxb/erz444.
569 Liu, H., Qin, J., Fan, H., Cheng, J., Li, L., & Liu, Z. (2017). Genome-wide
570 identification, phylogeny and expression analyses of SCARECROW-LIKE(SCL)
571 genes in millet (*Setaria italica*). *Physiology and Molecular Biology of Plants,*
572 23(3): 629–640. https://doi.org/10.1007/s12298-017-0455-6.
573 Liu, Z., Qin, J., Tian, X., Xu, S., Wang, Yu., Li, H., … Sun, Q. (2018). Global
574 profiling of alternative splicing landscape responsive to drought, heat and their
575 combination in wheat (*Triticum aestivum* L.). *Plant Biotechnology Journal,* 16:
576 714–726. https://doi.org/10.1111/pbi.12822.
577 Lu, H., Rate, D. N., Song, J. T., Greenberg, J. T. (2003). ACD6, a novel ankyrin
578 protein, is a regulator and an effector of salicylic acid signaling in the
579 Arabidopsis defense response. *The Plant Cell,* 15: 2408–2420.
580 https://doi.org/10.1105/tpc.015412.
581 Lv, X., Li, H., Chen, X., Xiang, X., Guo, Z., Yu, J., … Zhou, Y. (2018). The role of
582 calcium-dependent protein kinase in hydrogen peroxide, nitric oxide and ABA-
583 dependent cold acclimation. *Journal of Experimental Botany,* 69:4127–4139.
584 https://doi.org/10.1093/jxb/ery212.
585 Marquez, Y., Brown, J. W., Simpson, C., Barta, A., & Kalyna, M. (2012).
586 Transcriptome survey reveals increased complexity of the alternative splicing
587 landscape in Arabidopsis. *Genome Research,* 22: 1184–1195.

https://doi.org/10.1101/gr.134106.111.

Metwally, A., Finkemeier, I., Georgi, M., Dietz, K.J. (2003). Salicylic acid alleviates the cadmium toxicity in barley seedlings. *Plant Physiology,* 132:272–281.

Miller, G., Suzuki, N., Ciftci-Yilmaz, S., & Mittler, R. (2010). Reactive oxygen species homeostasis and signalling during drought and salinity stresses. *Plant Cell Environment,* 33: 453–467. https://doi.org/10.1111/j.1365-3040.2009.02041.x.

Nurhasanah, R.F., & Chen, Su. (2020). Physiological and Molecular Mechanism involved in cold stress tolerance in plants. Plants, 9(5): 560. https://doi.org/10.3390/plants9050560.

Pajoro, A., Severing, E., Angenent, G.C., & Immink, R.G.H. (2017). Histone H3 lysine 36 methylation affects temperature-induced alternative splicing and flowering in plants. *Genome Biology,* 18: 102. https://doi.org/10.1186/s13059-017-1235-x.

Palusa, S. G., Ali, G. S., & Reddy, A. S. (2007). Alternative splicing of pre-mRNAs of Arabidopsis serine/arginine-rich proteins: regulation by hormones and stresses. *The Plant Journal,* 49: 1091–1107. https://doi.org/10.1111/j.1365-313X.2006.03020.x.

Punzo, P., Ruggiero, A., Possenti, M., Perrella, G., Nurcato, R., Costa, A., … Batelli, G. (2020). DRT111/SFPS Splicing Factor Controls Abscisic Acid Sensitivity during *Seed Development and Germination,* 183(2):793-807. *https://doi*.org/10.1104/pp.20.00037.

610 Reddy, A. S., Marquez, Y., Kalyna, M., & Barta, A. (2013). Complexity of the
611 alternative splicing landscape in plants. *The Plant Cell*, 25: 3657–3683.
612 https://doi.org/ 10.1105/tpc.113.117523.
613 Schlaen, R.G., Mancini, E., Sanchez, S. E., Perez-Santángelo, S., Rugnone, M.
614 L., Simpson, C. G., …Yanovsky, M. J. (2015). The spliceosome assembly factor
615 GEMIN2 attenuates the effects of temperature on alternative splicing and
616 circadian rhythms. *Proceeding of the National Academy of Sciences of the United*
617 *States of America,* 112: 9382–9387. https://doi.org/ 10.1073/pnas.1504541112.
618 Shafiq, S., Li, J., & Sun, Q. (2016). Functions of plants long non-coding RNAs. *BBA -*
619 *Gene Regulatory Mechanisms,* 1:1859:155–
620 162. https://doi.org/10.1016/j.bbagrm.2015.06.009.
621 Sharon, D., Tilgner, H., Grubert, F. & Snyder, M. (2013) A single-molecule long-read
622 survey of the human transcriptome. *Nature Biotechnology,* 31: 1009–1014.
623 https://doi.org/10.1038/nbt.2705.
624 Shen, S., Park, J. W., Lu, Z., Lin, L., Henry, M. D., … Xing, Y. (2014). rMATS:
625 robust and flexible detection of differential alternative splicing from replicate RN
626 A-Seq data. *Proceedings of the National Academy of Sciences of the United*
627 *States of America,* 111(51): 5593-5601.
628 https://doi.org/10.1073/pnas.1419161111.
629 Staiger, D., & Brown, J.W. (2013). Alternative splicing at the intersection of
630 biological timing, development, and stress responses. *The Plant Cell,* 25: 3640–
631 3656. https://doi.org/10.1105/tpc.113.113803.

632 Syed, N. H., Kalyna, M., Marquez, Y., Barta, A., & Brown, J. W. (2012). Alternative
633 splicing in plants: coming of age. *Trends in Plant Science,* 17: 616–623.
634 https://doi.org/ 10.1016/j.tplants.2012.06.001.
635 Thatcher, S. R., Danilevskaya, O. N., Meng, X., Beatty, M., Zastrow-Hayes, G.
636 Harris, H., … Li, B. (2016). Genome-wide analysis of alternative splicing during
637 development and drought stress in maize. *Plant Physiology,* 170: 586–599.
638 https://doi.org/10.1104/pp.15.01267.
639 Tian, T., Liu, Y., Yan, H., You, Q., Yi, X, Du, Z., …, Su, Z. (2017). agriGO v2.0: a GO
640 analysis toolkit for the agricultural community, 2017 update. *Nucleic Acids*
641 *Research*, 45: 122–129. https://doi.org/10.1093/nar/gkx382.
642 Vergnolle, C., Vaultier, M.N., Taconnat, L., Renou, J.P., Kader, J.C., Zachowski, A. …
643 Ruelland, E. (2005). The cold-induced early activation of phospholipase C and D
644 pathways determines the response of two distinct clusters of genes in
645 Arabidopsis cell suspensions. *Plant Physiology,* 139(3):1217-33. https://doi.org/
646 10.1104/pp.105.068171.
647 Wang, M., Wang, P., Liang, F., Ye, Z., Li, J., Shen, C., … Zhang, X. (2018). A global
648 survey of alternative splicing in allpolyploid cotton: landscape, complexity and
649 regulation. *New Phytologist*, 217:163-178. https://doi.org/ 10.1111/nph.14762.
650 Wang, T., Wang, H., Cai, D., Gao, Y., Zhang, H., Wang, Y., … Gu, L. (2017).
651 Comprehensive profiling of rhizome-associated alternative splicing and
652 alternative polyadenylation in moso bamboo (*Phyllostachys edulis*). The Plant
653 Journal 91:684-699. https://doi.org/10.1111/tpj.13597.

654 Williams, M.E., Torabinejad, J., Cohick, E., Parker, K., Drake, E.J., Thompson, J.E.,
655 … Dewald, D.B. (2005). Mutations in the Arabidopsis phosphoinositide
656 phosphatase gene SAC9 lead to overaccumulation of PtdIns(4,5)P2 and
657 constitutive expression of the stress-response pathway. *Plant Physiology,*
658 138(2):686-700. doi: 10.1104/pp.105.061317.
659 Xing, Y., Jia, W.S., Zhang, J.H. (2008). AtMKK1 mediates ABA-inducedCAT1
660 expression and H_2O_2 produced via AtMPK6-coupled signaling in Arabidopsis.
661 *The Plant Journal,* 54: 440–451. doi:10.1093/jxb/erq125.
662 Yan, Q., Xia, X., Sun, Z., & Fang, Y. (2017). Depletion of Arabidopsis SC35 and
663 SC35-like serine/arginine-rich proteins affects the transcription and splicing of a
664 subset of genes. *PLoS Genetic,* 13(3): e1006663.
665 https://doi.org/10.1371/journal.pgen.1006663.
666 Yao, N., & Greenberg, J. T. (2006). Arabidopsis accelerated cell death2 modulates
667 programmed cell death. *The Plant Cell,* 18: 397-411.
668 https://doi.org/10.1105/tpc.105.036251.
669 Yon, F., Joo, Y., Cortés Llorca, L., Rothe, E., Baldwin, I. T., Kim, S. G. (2016).
670 Silencing Nicotiana attenuata LHY and ZTL alters circadian rhythms in flowers.
671 *New Phytologist,* 209(3):1058–66. https://doi.org/10.1111/nph.13681.
672 Young, M.D., Wakefield, M.J., Smyth, G.K., Oshlack, A. (2010). Gene ontology
673 analysis for RNA-seq: Accounting for selection bias. *Genome Biololy,* 11:R14.
674 https://doi.org/10.1186/gb-2010-11-2-r14.
675 Zhang, G., Sun, M., Wang, J., Lei, M., Li, C., Zhao, D., … Zhang, B. (2019). PacBio

full-length cDNA sequencing integrated with RNA-seq reads drastically improves the discovery of splicing transcripts in rice. *The Plant Journal,* 97:296–305. https://doi.org/10.1111/tpj.14120.

Zhao, C., Wang, P., Si, T., Hsu, C.C., Wang, L., Zayed, O., … Zhu, J. K. (2017). MAP kinase cascades regulate the cold response by modulating ICE1 protein stability. *Developmental Cell,* 43: 618–629. https://doi.org/10.1016/j.devcel.2017.09.024.

Zhao, Y., Sun, J., Xu, P., Zhang, R., & Li, L. (2014). Intron-mediated alternative splicing of WOOD-ASSOCIATED NAC TRANSCRIPTION FACTOR1B regulate cell wall thickening during fiber development in *Populus* Species. *Plant Physiology,* 164:765-776. https://doi.org/10.1104/pp.113.231134.

Zhou, R., Moshgabadi, N., & Adams, K. L. (2011). Extensive changes to alternative splicing patterns following allopolyploidy in natural and resynthesized polyploids. *Proceeding of the National Academy of Sciences of the United States of America,* 108: 16122–16127. https://doi.org/10.1073/pnas.1109551108.

Zhu, G., Li, W., Zhang, F., & Guo, W. (2018). RNA-seq analysis reveals alternative splicing under salt stress in cotton, Gossypium davidsonii. *BMC Genomics,* 19: 73. https://doi.org/10.1186/s12864-018-4449-8.

FIGURE LEGENDS

Figure 1 Phenotypic changes in *Populus trichocarpa* and *Populus ussuriensis* in response to cold stress. *P. trichocarpa* and *P. ussuriensis* grown at 25°C for 3h (a); 3°C for 3 h (b); -3°C for 3 h (c); and -3°C for 4 h (d).

Figure 2 Circos visualization of PacBio Isoseq data in *Populus trichocarpa* and *Populus ussuriensis* compared using SMRT sequencing. (a-b) Distribution of full-length reads density in *P. trichocarpa*. and *P. ussuriensis* respectively on reference chromosome respectively. (c-d) The distribution of genetic variants in *P. trichocarpa*. and *P. ussuriensis* respectively.

Figure 3 Comparison of alternative splicing (AS) events in response to cold stress in *Populus trichocarpa* and *Populus ussuriensis* identified by PacBio sequencing. (a) AS types. (b) The proportion of different AS types in response to cold stress in *P. trichocarpa.* (c) The proportion of different AS types in response to cold stress in *P. ussuriensis.* (d) Statistical analysis of the AS types in response to cold stress in *P. trichocarpa* and *P. ussuriensis*. (e) Radar plot showing the percentage of AS types in response to cold stress in *P. trichocarpa* and *P. ussuriensis*. (f) Comparison of the AS types at different cold temperatures in *P. trichocarpa*. (g) Comparison of the AS types at different cold temperatures in *P. ussuriensis*.

Figure 4 Most significantly enriched gene ontology (GO) terms for differentially expressed genes (DEGs) and differential alternative splicing (DAS) genes identified by RNA sequencing analysis of two poplar species under cold conditions. (a–c) GO terms for DEGs between *Populus ussuriensis* and *Populus trichocarpa* at 25°C. (d–f)

715 GO terms for DEGs between *P. ussuriensis* and *P. trichocarpa* at 3°C. (g–i) GO terms
716 for DEGs between *P. ussuriensis* and *P. trichocarpa* at -3°C. (j–l) GO terms for DAS
717 genes between *P. ussuriensis* and *P. trichocarpa* at 25°C, 3°C, and -3°C.
718 **Figure 5** Calcium and physiological indicators measurement in *P. trichocarpa* and *P.*
719 *ussuriensis*. (a) Calcium content measurement using inductively coupled plasma (ICP)
720 emission spectrometer (ICP-OES 5110 VDV, Agilent, USA). (b) MDA, (c) POD and
721 (d) H_2O_2 content measurement.
722 **Figure 6** Changes in differentially expressed genes (DEGs) and differential
723 alternative splicing (DAS) genes in response to cold stress.
724 **Figure 7** Characteristic of randomly selected alternative splicing (AS) events and
725 validation of randomly selected alternative splicing events detected by RNA
726 sequencing using reverse transcription-polymerase chain reaction (RT-PCR). The
727 arrow on the left side indicates the position of PCR primers used for RT-PCR. The
728 electropherogram on the right side shows the alternatively spliced product bands.
729 **Figure 8** Validation of randomly selected differentially expressed genes (DEGs) using
730 quantitative real-time polymerase chain reaction (qRT-PCR).
731 **Figure 9** Model for cold signaling pathway and gene regulation in poplar.
732 **SUPPORTING INFORMATION**
733 **Data S1** Differentially expressed genes (DEGs) between *Populus ussuriensis* and
734 *Populus trichocarpa* identified by RNA sequencing analysis.
735 **Data S2** Differentially expressed genes (DEGs) between *Populus ussuriensis* and
736 *Populus trichocarpa* at 3°C identified by RNA sequencing analysis.
737 **Data S3** Differentially expressed genes (DEGs) between *Populus ussuriensis* and

738 *Populus trichocarpa* at -3°C identified by RNA sequencing analysis.

739 **Data S4** Differentially expressed genes (DEGs) of cluster 3 in *Populus ussuriensis*

740 compared to *Populus trichocarpa* identified by RNA sequencing analysis.

741 **Figure S1** Clustering of differentially expressed genes (DEGs) in *Populus ussuriensis*

742 compared to *Populus trichocarpa* in response to cold stress identified by RNA

743 sequencing analysis.

744 **Table S1** Primers used for reverse transcription-polymerase chain reaction (RT-PCR).

745 **Table S2** Primers used for quantitative real-time polymerase chain reaction (qRT-

746 PCR).

Tables

Table 1 Difference in genes as annotated in two poplar species compared to *P. trichocarpa* genome by the PacBio sequencing data respectively.

	P. trichocarpa	*P. ussurensis*
Cell_ID	r64053_20191003_023517_4_H01	r64053_20191003_023517_4_H01
Number of consensus isoforms	51,153	58,082
Mean length of consensus isoforms	1683.17	1910.19
Number of high-quality isoforms	50,910	57,872
Mean length of high-quality isoforms	1678.8	1905.13
Number of low-quality isoforms	228	188
Mean length of low-quality isoforms	1157.82	1344.64

750 Table 2 Partial DEGs response to cold in *Populus ussuriensis* compared with *Populus trichocarpa*.

25 ℃			+3 ℃			–3 ℃		
Gene ID	annotation	Log2FoldChange	Gene ID	annotation	Log2FoldChange	Gene ID	annotation	Log2FoldChange
Potri.001G138000	*CAM*	3.2		*CAM*	1.5	Potri.T124900	*CAM3*	1.5
Potri.007G042900	*CAM1*	2.0	Potri.T124900	*CAM3*	1.3	Potri.002G047300	*CAM*	1.4
Potri.005G052800	*CAM8*	1.8				Potri.001G138000	*CAM*	1.4
Potri.T124900	*CAM3*	1.7						
Potri.006G065900	*CAM3*	1.5						
Potri.009G168700	*CPK*	1.6	Potri.009G168700	*CPK*	3.6	Potri.009G168700	*CPK*	2.7
Potri.016G06570	*CPK*	1.2	Potri.004G015500	*CPK*	1.4	Potri.004G015500	*CPK*	1.3
Potri.004G015500	*CPK*	1.2				Potri.016G065700	*CPK*	1.2
Potri.018G122300	*CIPK*	1.4	Potri.018G108500	*CIPK*	1.3	Potri.016G133900	*CIPK*	1.4
Potri.018G108500	*CIPK*	1.2	Potri.018G122300	*CIPK*	1.3	Potri.017G118000	*CIPK*	1.2
Potri.009G152200	*NCED*	-1.8	Potri.001G393800	*NCED*	2.1	Potri.001G393800	*NCED*	1.3
			Potri.011G112400	*NCED*	1.5	Potri.011G112400	*NCED*	2.2
			Potri.019G093400	*NCED*	1.1			
Potri.010G156400	*CAB*	6.1	Potri.010G156400	*CAB*	9.1	Potri.010G156400	*CAB*	8.0
Potri.002G222800	*CAB*	4.5	Potri.002G222800	*CAB*	3.6	Potri.002G222800	*CAB*	2.8
Potri.008G198900	*CAB*	1.1	Potri.016G011300	*CAB*	3.3	Potri.016G011300	*CAB*	1.7
Potri.010G155400	*CAB*	-1.9	Potri.014G162100	*CAB*	2.6	Potri.010G155400	*CAB*	-1.9
			Potri.T137800	*CAB*	-1.4			
			Potri.010G155400	*CAB*	-2.1			
Potri.013G154400	*POD*	7.2	Potri.012G000300	*POD*	7.7	Potri.012G000300	*POD*	7.7
Potri.007G122100	*POD3*	5.5	Potri.007G122100	*POD*	4.6	Potri.017G038000	*POD3*	5.5
Potri.017G064100	*POD73*	5.4	Potri.017G038000	*POD3*	4.3	Potri.008G099900	*POD*	4.7

Potri.017G038000	*POD3*	4.3	Potri.011G062300	*POD19*	3.2	Potri.007G122100	*POD3*	4.3
Potri.T163100	*POD*	4.3	Potri.001G145800	*POD20*	3.1	Potri.008G022700	*POD*	3.7
Potri.013G083600	*POD4*	3.7	Potri.001G145800	*POD18*	3.1	Potri.013G154400	*POD*	3.4
Potri.012G006800	*POD5*	3.0	Potri.002G018000	*POD64*	2.8	Potri.016G125000	*POD*	3.4
Potri.010G036100	*POD6*	2.8	Potri.013G154400	*POD*	2.3	Potri.011G062300	*POD19*	2.9
Potri.016G132900	*POD*	2.6	Potri.003G214900	*POD15*	2.0	Potri.017G064100	*POD73*	2.8
Potri.018G015500	*POD18*	2.6	Potri.010G036100	*POD6*	1.9	Potri.018G015500	*POD18*	2.7
Potri.001G145800	*POD20*	2.6	Potri.003G214800	*POD15*	1.7	Potri.010G175100	*POD7*	2.6
Potri.001G013000	*POD*	2.1	Potri.001G013000	*POD*	1.6	Potri.001G145800	*POD20*	2.6
Potri.003G214900	*POD15*	2.1	Potri.017G064100	*POD73*	1.6	Potri.010G036100	*POD6*	2.2
Potri.015G003500	*POD5*	2.0	Potri.004G052100	*POD15*	1.5	Potri.003G214800	*POD15*	2.0
Potri.001G011500	*POD15*	1.9	Potri.003G214700	*POD15*	1.2	Potri.001G013000	*POD*	2.0
Potri.011G062300	*POD*	1.9				Potri.013G083600	*POD4*	1.6
Potri.002G018000	*POD64*	1.8				Potri.002G065300	*POD12*	1.6
Potri.003G214800	*POD15*	1.8				Potri.003G214700	*POD15*	1.5
Potri.006G267400	*POD18*	1.3				Potri.006G267400	*POD18*	1.2
Potri.007G053400	*POD73*	1.2						

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760 Table 3 DAS events in response to cold in *Populus ussurensis* compared with *Populus trichocarpa*.

25 ℃				+3 ℃				–3 ℃			
Gene ID	AS type	annotation	Log2Fold Change	Gene ID	AS type	annotation	Log2Fold Change	Gene ID	AS type	annotation	Log2Fold Change
Potri.013G133900	RI	*ACD6*	-2.9	Potri.003G017500	SE	*RECQ*	1.8	Potri.013G133900	RI	*ACD6*	-3.5
Potri.001G353500	SE, RI	*COR413IM1*	-1.7	Potri.013G001600	RI, A5SS, A3SS	*LTI6A*	-1.4	Potri.009G078500	SE	HVA22A	-1.9
Potri.013G014400	RI	*SWEET16*	-1.6	Potri.008G158200	RI	*ELIP*	-2.4	Potri.014G106800	A3SS, A5SS, MXE, RI, SE	*LHY*	2.2
				Potri.005G070900	SE	DGK	-2.6	Potri.013G001600	A3SS, A5SS, SE	*LTI6A*	-2.9
				Potri.008G161600	SE	EO	1.3	Potri.018G004200	SE	HIBCH	-6.0
				Potri.005G246000	SE, A5SS	PLD	-1.4	Potri.013G014400	A3SS, RI, SE	SWEET16	-1.9
				Potri.002G230000	SE	GLR6	1.5	Potri.004G230100	A3SS, SE	VRN1	-1.6
				Potri.017G041700	A3SS	ISPS	1.4	Potri.001G024900	A3SS, SE	ANN4	1.5

				Potri.013G133900	RI,	ACD 6	-3.3	Potri.002G182500	A3SS, RI, SE	CML	1.8
				Potri.002G182500	A3SS	CML	1.6	Potri.014G108200	A3SS, SE	CML	1.4
				Potri.001G353500	A3SS, MXE, RI, SE	CORIM1	-1.4	Potri.005G070900	SE	DAG	-1.6
								Potri.008G158200	RI	ELIP1	-1.5
								Potri.005G196700	A5SS, SE	GI	-2.1

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777 Table 4 Expression changed of splicing-associated factors in *P. ussurensis* compared to *P. trichocarpa* at different temperature.

25 ℃				+3 ℃				−3 ℃			
Gene ID	AS type	annotation	Log2Fold Change	Gene ID	AS type	annotation	Log2Fold Change	Gene ID	AS type	annotation	Log2Fold Change
Potri.015G112800		DEAH1	5.6	Potri.016G020200		PRO8	2.4	Potri.015G112800		DEAH1	8.6
Potri.016G020200		PRO8	1.7	Potri.015G112800		DEAH1	2	Potri.016G020200		PRO8	3.5
				Potri.001G147100		RES	1.6	Potri.009G041300		RBM22	2.3
				Potri.010G093100	RI	CEF-1	1.2	Potri.010G093100	RI	CEF1	1.9
								Potri.016G062500	A3SS	SCL30A	1.8
								Potri.009G041700	A5SS	SR45a	1.8
								Potri.006G197000		SCL30A	1.7
								Potri.012G012800		SF3A3	1.6
								Potri.007G070700	A3SS; RI	SF3A1	1.6
								Potri.001G147100		RES	1.5
								Potri.007G045900	A5SS; RI	Prp18	1.4
								Potri.004G076600		CTNNBL	1.4
								Potri.012G064100		SLU7-A	1.4
								Potri.015G048200	A3SS	SLU7-A	1.3
								Potri.012G133600	SE	RS40	1.3
								Potri.009G149600		SFU2af	1.3

								Potri.017G060200		ISY1	1.3
								Potri.004G039000	SE	SF3B2	1.3
								Potri.004G052700	SE	DEAH9	1.3
								Potri.002G153600		RS40	1.3
								Potri.003G087200		RES	1.3
								Potri.005G138200		SFRS16	1.3
								Potri.T134200	SE	SFRS1	1.2
								Potri.011G098100		RBM17	1.2
								Potri.006G000200		cwf25	1.2
								Potri.010G213300		PRP3	1.2
								Potri.005G024600	A3SS	SFRS1	1.2
								Potri.T125700		SC35	1.2
								Potri.005G210600		Prp18	1.2
								Potri.013G135300		PRP1	1.2
								Potri.008G168000	A5SS, MXE; SE	SF3B5	1.2
								Potri.005G258000		SFU2af	1.2
								Potri.019G039900		Prp19	1.2
								Potri.005G105700	A5SS; RI; SE	SR45a	1.2
								Potri.002G175000	A3SS; SE	RSP31	1.2
								Potri.014G101800	A5SS; SE	RS31	1.2

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781 Table 5 Comparison of AS events in TFs of two poplar species by the PacBio sequencing data respectively.

25 ℃				+3 ℃				−3 ℃			
Gene ID	AS type	annotation	Log2Fold Change	Gene ID	AS type	annotation	Log2Fold Change	Gene ID	AS type	annotation	Log2Fold Change
Potri.001G063000	A5SS	bHLH	4.3	Potri.001G283300	A3SS	ERF	5.8	Potri.005G184400	A5SS	B3	3.1
Potri.014G193800	A3SS	MYB	3.4	Potri.002G090600	A3SS, RI	bHLH	4.3	Potri.004G155800	MXE	CAMTA	4.0
Potri.012G016000	A3SS	NAC	2.1	Potri.001G395000	SE	MYB	5.3	Potri.014G106800	MXE	MYB	2.2
Potri.012G118300	A3SS	ERF	3.9	Potri.001G035500	A3SS, SE	WRKY	9.4	Potri.006G024000	RI	MYB	3.8
Potri.006G072200	A5SS	WRKY	2.6	Potri.001G010700	A3SS	WRKY	10.6	Potri.002G148000	RI	NAC	2.7
Potri.016G102100	A5SS	HD-ZIP	3.5	Potri.001G259100	A3SS, MXE, SE	NAC	5.9	Potri.001G184200	RI	WRKY	3.8

Potri.005G184400	A5SS	B3	2.4	Potri.001G056700	A3SS, SE	B3	8.8	Potri.002G102100	RI	ERF	2.6
Potri.004G155800	SE	CAMTA	3.4	Potri.002G002200	A3SS, A5SS	MYB	4.6	Potri.012G118300	SE	ERF	3.1
				Potri.001G063000	A5SS	WRKY	8.3	Potri.004G155800	SE	CAMTA	4.0
				Potri.002G225600	A5SS	HSF	3.8	Potri.007G119700	SE	bHLH	2.5
				Potri.001G075600	A5SS	MYB	8.0	Potri.T152100	SE	MYB	6.0
				Potri.001G290100	A5SS	WRKY	5.8	Potri.001G291700	SE	WRKY	2.9
				P otri.001G159400	MXE, SE	HSF	6.8	Potri.011G150100	SE	B3	2.8
				Potri.002G182500	RI	ERF	4.0				
				Potri.001G017700	RI	bHLH	9.6				
				Potri.001G436200	RI	B3	4.8				
				Potri.001G217700	RI	WRKY	6.2				
				Potri.001G056700	RI	B3	8.8				
				Potri.001G114000	RI	bHLH	7.5				

				Potri.001G255100	RI	bZIP	6.0				
				Potri.001G385300	SE	NAC	5.3				
				Potri.001G133500	SE	HSF	7.4				
				Potri.001G240300	SE	MYB	6.0				