Osmotic stress-responsive promoter upstream transcripts (PROMPTs) act as carriers of MYB transcription factors to induce the expression of target genes in Populus simonii

Yu peng Song1,2,3, Anran Xuan1,2,3, Chen hao Bu1,2,3, Dong Ci1,2,3, Min Tian1,2,3 and Deqiang Zhang1,2,3* 1

1Beijing Advanced Innovation Center for Tree Breeding by Molecular Design, Beijing Forestry University, Beijing, China
2National Engineering Laboratory for Tree Breeding, College of Biological Sciences and Technology, Beijing Forestry University, Beijing, China
3Key Laboratory of Genetics and Breeding in Forest Trees and Ornamental Plants, College of Biological Sciences and Technology, Beijing Forestry University, Beijing, China

Summary
Complex RNA transcription and processing produces a diverse range catalog of long noncoding RNAs (lncRNAs), important biological regulators that have been implicated in osmotic stress responses in plants. Promoter upstream transcript (PROMPT) lncRNAs share some regulatory elements with the promoters of their neighbouring protein-coding genes. However, their function remains unknown. Here, using strand-specific RNA sequencing, we identified 209 differentially regulated osmotic-responsive PROMPTs in poplar (Populus simonii). PROMPTs are transcribed bidirectionally and are more stable than other lncRNAs. Co-expression analysis of PROMPTs and protein-coding genes divided the regulatory network into five independent subnetworks including 27 network modules. Significantly enriched PROMPTs in the network were selected to validate their regulatory roles. We used delaminated layered double hydroxide lactate nanosheets (LDH-lactate-NS) to transport synthetic nucleic acids into live tissues to mimic expression of specific PROMPTs and protein-coding genes. The altered expression of PROMPT_1281 induced the expression of its cis and trans targets, and this interaction was governed by its secondary structure rather than just its primary sequence. Based on this example, we proposed a model that a concentration gradient of PROMPT_1281 is established, which increases the probability of its interaction with targets near its transcription site that shares common motifs. Our results firstly demonstrated that PROMPT_1281 act as carriers of MYB transcription factors to induce the expression of target genes under osmotic stress. In sum, our study identified and validated a set of poplar PROMPTs that likely have regulatory functions in osmotic responses.

Introduction
Large-scale RNA sequencing analysis has indicated that more than 90% of eukaryotic genomes are actively transcribed to yield a highly complex network of protein-coding transcripts and non-coding RNAs (Djebali et al., 2012; Hangauer et al., 2013). Protein-coding genes make up only 1%–2% of all transcripts, indicating the widespread occurrence of noncoding RNAs in eukaryotic genomes (Hangauer et al., 2013; Kim and Sung, 2012). Functional noncoding RNAs are divided into housekeeping and regulatory RNAs (Chen and Carmichael, 2010; Shuai et al., 2014). Based on their extraordinary differences in transcript lengths and biogenesis, classification of regulatory noncoding RNAs remains difficult. Long noncoding RNAs (lncRNAs) are usually classified as RNAs greater than 200 nucleotides (nt) that lack significant protein-coding capacity (Ulitsky and Bartel, 2013). Depending on their orientation and/or proximity to protein-coding genes, ncRNAs are annotated as promoter upstream transcripts (PROMPTs), enhancer RNAs (eRNAs), long intervening/intergenic ncRNAs (lincRNAs) and natural antisense transcripts (NATs). Additionally, many lncRNAs are annotated as small nucleolar RNA-ended lncRNAs (sno-lncRNAs), 5’-snoRNA-ended and 3’-polyadenylated lncRNAs (SPAs), circular RNAs (circRNAs) and circular intronic RNAs (ciRNAs) depending on their RNA processing pathways (Wu et al., 2017). LncRNAs are key regulators of gene expression at both the transcriptional and the post-transcriptional levels in diverse cellular contexts and biological processes (Chen, 2016; Quinn and Chang, 2016). LncRNAs can regulate gene expression in cis- or trans-acting. Cis-acting lncRNAs function near the site of their synthesis and act directly on one or several contiguous genes on the same strand or chromosome. Thus, we speculated that the orientation and/or proximity of lncRNAs to protein-coding genes might be the main factor for determining whether they act in cis. The eRNAs have enhancer-like functions and can control promoter and enhancer interactions (Li et al., 2013; Melo et al., 2013). COOLAIR, a NAT transcribed from the FLOWERING LOCUS C (FLC) gene, mediates the formation of a stable RNA–DNA triplex and an R-loop (Sun et al., 2013a,b; Wahba and Koshland, 2013). The R-loop recruits a transcription repressor, which results in repression of FLC. By contrast, trans-acting lncRNAs diffuse from the site of their synthesis and can act directly on many genes at great distances, even genes on other chromosomes (Lee, 2012). These lncRNA-mediated interactions might be affected by the structure of the lncRNA. Considering the diversity in biogenesis and biological functions of lncRNAs, the biological function of...
each IncRNA should be validated, depending on its classification. Up to now, except lincRNAs, NATs and circRNAs, majority of IncRNAs regulatory function is still unclear that might hide some especial transcriptional mechanism of plants.

In mammals, PROMPTs are transcribed in the antisense orientation and from a distance of approximately 0.5–2.5 kb from the transcription start sites (TSSs) of protein-coding genes (Balbin et al., 2015; Preker et al., 2008). PROMPTs contain 5’-cap structures and 3’ adenosine tails and are diversified in length, ranging from 200 to 600 nt (Preker et al., 2011). PROMPTs also form complexes with RNA Polymerase II (Pol II) to act on protein-coding genes (Preker et al., 2011). PROMPTs are usually retained in the nucleus and undergo rapid degradation by the RNA nuclear exosome-targeting complex (Lubas et al., 2015; Preker et al., 2011). The expression of PROMPTs is cued by environment signals, and their accumulation influences the binding of transcription factors to promoters and is associated with the choice of promoter directionality (Ntini et al., 2013). This suggests that, although PROMPTs are short-lived, they may have important regulatory functions (Lloret-Llinares et al., 2016).

Unlike other IncRNAs, PROMPTs are transcribed from upstream of protein-coding genes and share many single-strand cis elements with the promoter regions of the neighbouring protein-coding genes. CREB-binding protein/ET1A-binding protein p300, a transcription co-activator, has a unique regulatory motif in which the RNA-binding region is bound by eRNAs to stimulate histone acetyltransferase activity (Bose et al., 2017). These findings suggest that these common cis elements might provide potential binding sites for PROMPTs, implying that PROMPTs may be co-activators for the expression of genes with common transcription factor interaction motifs. However, it is unknown if PROMPTs can induce gene expression in a trans-acting manner.

Higher-order structures govern most of the functions of IncRNAs, including interactions with proteins, small-molecule ligands, multicomponent complexes and other RNAs (Dethoff et al., 2012; Sharp, 2009). Plants, especially perennial and dioecious plants, have high levels of heterozygosity in their genomes. Abundant single nucleotide polymorphisms (SNPs), insertions/deletions (InDels) and simple sequence repeats in IncRNAs can affect their biological function by changing their secondary structure, altering their stability or interfering with RNA–protein interactions (Ding et al., 2012). It is worth noting whether these genetic variants will affect biological function of IncRNA alleles through changing their secondary structure. Differential expression between different IncRNA alleles is highly dependent on cell type or environment stimulus (Bell and Beck, 2009). Even small differences in the level of expression between alleles can strongly affect important physiological processes in mammals, but less is known about the molecular basis of differential expression between alleles in terms of adaptation to distinct developmental processes or different environmental signals.

Here, we systematically identified and characterized osmotic stress-responsive PROMPTs in poplar at a genomewide scale. We identified SNPs in the PROMPTs, detected their linkage disequilibrium (LD) and dissected the structural variation between the different PROMPT alleles. Then, we analysed the cis- and trans-acting regulatory functions of the PROMPT alleles. We developed a new procedure that uses layered double hydroxides (LDHs), sheet-like nanoparticles that can transport negatively charged biomolecules into intact plant cells, to deliver RNA molecules to mimic gene overexpression or gene silencing and used this method to validate the functions of a candidate PROMPT. In summary, the results of this study increased our understanding of osmotic stress-responsive PROMPTs in a perennial plant and provided a new layer for further research on the transcriptional mechanisms of IncRNAs.

Results

Identification and characterization of osmotic-responsive PROMPTs

To identify PROMPTs that are differentially expressed in response to osmotic stress, we conducted genomewide RNA sequencing on control and osmotic-treated poplar leaves. We obtained approximately 144 and 148 million clean reads from the control and osmotic-treated groups, respectively. Mapping showed that 78.6% of the reads from the control group and 88.4% from the osmotic-treated group mapped to the Populus trichocarpa genome (Table S1). In total, we obtained 17 603 IncRNAs between both libraries (Figure 1a, Table S2). The lengths of the IncRNAs ranged from 203 to 3002 bp, and most were in the range of 751–1346 bp (Figure 1b). We also obtained 4993 putative PROMPTs between both libraries (Table S3). The lengths of the PROMPTs ranged from 203 to 2902 bp, and most were in the range of 801–1697 bp, which were longer than the average length of the total IncRNAs (Figure 1b). The GC content of the antisense IncRNA loci ranged from 38.4% to 50.3%, which tended to be higher than antisense PROMPTs loci (Figure 1e). The minimum free energy (MFE) of the PROMPTs significantly decreased with increasing length (Figure 1c), and the MFE per bp of the PROMPTs was significantly lower than that of the other IncRNAs (Figure 1d). These results indicated that the structures of the osmotic-responsive PROMPTs are more stable than those of the other IncRNAs. The distribution of the PROMPTs in the Populus chromosomes was examined. In chromosome 10, there were 124.1 PROMPTs within every one Mb, which was the highest density of PROMPTs among all of the chromosomes (Figure 1f). Chromosome 19 had the lowest density, with 46.6 PROMPTs per one Mb (Figure 1f). We also calculated the expression levels of the PROMPTs in fragments per kilobase of transcript per million mapped reads (FPKM), which ranged from 2.9E-06 to 1121.9 FPKM (average 33.94 FPKM), which were significantly higher than the expression levels of the other IncRNAs (average 1.64 FPKM) (Table S4).

Expression of osmotic stress-responsive PROMPTs

To identify the potential transcriptional regulatory functions of the PROMPTs, we analysed the transcript abundance of the osmotic stress-responsive protein-coding genes and PROMPTs. In total, we identified 2598 IncRNAs that were differently expressed under osmotic stress, including 2133 that were down-regulated and 465 that were up-regulated (Figure 1a). The expression levels of the up-regulated IncRNAs in the osmotic and control groups averaged 1220 and 380 FPKM (average fold change 9.6), respectively (Figure 2a). The expression levels of the down-regulated IncRNAs in the osmotic and control groups averaged 38 and 141 FPKM (average fold change 0.13), respectively (Figure 2a). We also identified 209 PROMPTs that were differently expressed under osmotic stress (fold change >2 or <0.5, P < 0.005, FDR <0.05), including 113 that were down-regulated and 96 that were up-regulated (Table S5). The expression levels of the up-regulated PROMPTs in the osmotic and control groups averaged 69 and 15 FPKM (average fold change 13.9),
The expression levels of the downregulated PROMPTs in the osmotic and control groups averaged 4.3 and 113 FPKM (average fold change 0.03), respectively. The range of the fold changes of the osmotic stress-responsive PROMPTs was larger than that of the osmotic stress-responsive IncRNAs, suggesting that PROMPTs are more responsive to osmotic stress (Figure 2a,b). Protein-coding genes with osmotic stress-responsive PROMPTs in their upstream region also had larger fold changes than the other differentially expressed genes (Figure 2c), and the transcript abundance of these genes was positively correlated with the transcript abundance of the PROMPTs in the control and osmotic stress-stress groups ($r = 0.65, P < 10^{-5}$) (Figure 2d).

To explore the putative cis-regulatory functions of the osmotic stress-responsive PROMPTs, we compared the transcript abundance between the osmotic stress-responsive PROMPTs and their neighbouring genes. The expression of the PROMPTs transcribed from sense and antisense orientations was significantly higher than the expression of downstream protein-coding genes (Figure S1a,b). The expression of sense/sense pairs and antisense/antisense pairs of PROMPTs and downstream protein-coding genes was also consistent with the above tendency (Figure S1c,d). Downstream protein-coding genes were expressed significantly higher than the PROMPTs only when the PROMPTs were transcribed from the opposite direction (Figure S2).

**Motif prediction from the primary sequences of osmotic stress-responsive PROMPTs**

To identify the enriched regulatory elements in the PROMPTs, the motifs present in the promoters of the osmotic stress-responsive IncRNAs were used as the background. Six motifs, ERF, GATA, Dof, WRKY, MYB/SANT and AT-Hook, were specifically enriched in the osmotic stress-responsive PROMPTs and were present in 66.3%–82.6% of the osmotic stress-responsive PROMPTs (Figure 2e). In addition, bZIP, RAV and C2H2 elements were significantly reduced in the osmotic stress-responsive PROMPTs. Among these, all motifs bind transcriptional activators, except...
RAV1AAT, which bind transcriptional repressor. Two ethylene-responsive elements (RAV1AAT and ERF) were over-represented in the osmotic stress-responsive lncRNAs and PROMPTs, respectively, suggesting that osmotic stress-responsive PROMPTs might participate in an independent ethylene-responsive regulatory pathway. One abscisic acid (ABA)-responsive element (ATHBSAT-CORE), which acts as a positive regulator of ABA-responsive genes, was enriched in the PROMPTs. Examination of the distribution of these motifs revealed that MYB/SANT, RAV1AAT and Dehydrin were most enriched in the loop regions of the secondary structure of the PROMPTs (Figure 2f). Scanning of the sequences revealed that 43.8% of the osmotic stress-responsive PROMPTs contain multiple copies of these motifs. A MYB-related gene, Potri.001G219100, duplicated homeodomain-like superfamily protein. We found that 26% of the osmotic stress-responsive PROMPTs contain multiple copies of the MYB/SANT element and potentially interact with Potri.001G219100, implying that these PROMPTs might contain more potential interaction sites for transcription factors (Table S6).

**Co-expression of osmotic stress-responsive PROMPTs**

To identify potential novel regulators of osmotic stress-responsive PROMPTs, we constructed a co-expression network that included 209 osmotic stress-responsive PROMPTs and 2598 differentially expressed genes. The network connected pairs of genes with high normalized co-expression (Z-score > 5). PROMPTs were then ranked according to the number of co-expressed genes in their network cluster. The whole co-expression network consisted of five independent subnetworks (Figure S3). The two main subnetworks included 96 up-regulated PROMPTs and 113 down-regulated PROMPTs. The three other subnetworks contained only one osmotic stress-responsive PROMPT each. Notably, several of the osmotic stress-responsive PROMPTs were highly central in the co-expression network, indicating that they may serve important functional roles in response to osmotic stress in poplar.

To categorize the biological processes transcriptionally regulated by the osmotic stress-induced PROMPTs, we utilized the co-expression network to identify representative network modules (NMs) containing nonoverlapping sets of genes that were highly co-expressed with the most central genes in the network (Figure 3). Using Gene Ontology (GO) enrichment analyses, we assigned putative biological functions to the 27 main NMs (including 15 up-regulated and 12 down-regulated osmotic stress-responsive PROMPTs) containing at least 51 co-expressed genes (Figure 3 and Table 1). For the down-regulated osmotic stress-responsive PROMPTs, each node had an average of 135.9 co-expressed genes; for the up-regulated osmotic stress-responsive PROMPTs, the average was 469.6 co-expressed genes, which was significantly higher. We observed co-expressed genes significantly enriched within NMs. NM1 (PROMPT_1281, protein amino acid phosphorylation, $P < 5.65 \times 10^{-21}$; cell recognition, $P < 2.57 \times 10^{-7}$) contained 838 genes associated with protein phosphorylation and cell signalling, including seven homologs of WALL-ASSOCIATED KINASE (WAK1) and three homologs of MYB. This module was also enriched in phytohormone-related genes, including homologs of ETHYLENE RESPONSE FACTOR 1 (ERF1), CYTOKININ OXIDASE 6 (CKX6) and auxin-induced proteins, which are involved in the

---

**Figure 2** Transcript abundance and motif prediction of the osmotic stress-responsive lncRNAs and PROMPTs. (a) Accumulation frequency of transcript abundance of lncRNAs and PROMPTs. (b) Accumulation frequency of fold changes of lncRNAs and PROMPTs. (c) Expression patterns of PROMPTs and downstream genes. (d) Correlation of the expression of PROMPTs and downstream genes under osmotic stress. (e) Motif prediction of osmotic stress-responsive lncRNAs and PROMPTs. (f) Ratio of predicted motifs in the secondary structure of osmotic stress-responsive PROMPTs.
phytohormone-activated signalling pathway. Genes in this module were significantly down-regulated upon osmotic stress, suggesting negative roles in response to osmotic stress (Figure 3d). NM25 (PROMPT_3649, photosynthesis, \( P < 1.87^{-16} \)) was enriched in photosynthesis-related genes and included PHOTOSYSTEM I SUBUNIT F (PSAF), LIGHT HARVESTING COMPLEX PHOTOSYSTEM II SUBUNIT 6 (LHC6), PROTOCOLHROLPHILIDE OXIDOREDUCTASE A (PORA) and PHOTOSYSTEM II REACTION CENTER W (PSBW), which have a demonstrated role in light reactions and photosynthesis (Figure S3, Table S7). This module also included MYB4 and CTL genes. MYB4 gene is a well-known negative regulator of transcript (Zhao et al., 2007). CTL which encodes an endochitinase-like protein is essential for tolerance to heat, salt and osmotic stresses (Hong et al., 2003).

Effect of nucleotide variations in the secondary structure of osmotic stress-responsive PROMPTs

To investigate the function of the osmotic stress-responsive PROMPTs, we chose PROMPT_1281 (NM1) for further study, as the NM1 PROMPT_1281 node had the most connection with the other nodes in the co-expression network. Eight SNPs were found in the genomic regions of PROMPT_1281, including three SNPs in two exons and five SNPs in an intron (Figure 4). To examine the effects of the SNPs on the secondary structure of the RNA, we predicted the secondary structures of the different PROMPT_1281 variants and calculated the MFE. The average energy change due to the SNPs was 0.8 kcal/mol and ranged from 0 to 2 kcal/mol, which correlated the MFE. The average energy change due to the SNPs was 0.8 kcal/mol and ranged from 0 to 2 kcal/mol, which indicated that SNPs in osmotic stress-responsive lncRNAs undergo natural selection.

Allelic expression pattern of PROMPT_1281

To profile the allelic expression pattern of PROMPT_1281, we used seven tissue samples, four abiotic stress-treated samples and three phytohormone-treated samples for quantitative PCR (qPCR) analysis. We cloned full-length cDNAs of PROMPT_1281. Sequence analysis revealed several alleles, PROMPT_1281-Hap1 and PROMPT_1281-Hap2. As shown in Figure 5, the candidate genes exhibited different expression patterns. Transcript abundances of PROMPT_1281-Hap1 were significantly higher than those of PROMPT_1281-Hap2, indicating imbalanced expression of the different PROMPT_1281 alleles. The expression specificity value (tau score) of PROMPT_1281-Hap1 was 0.93, which was significantly higher than that of PROMPT_1281-Hap2. Among the nine tissues, both alleles of PROMPT_1281 were expressed except in cambium and mature xylem. The expression of PROMPT_1281-Hap1 was significantly higher than that of PROMPT_1281-Hap2 in leaves, male flowers and roots. The expression of PROMPT_1281-Hap2 was higher than that of PROMPT_1281-Hap1 only in immature xylem. For the different abiotic stress treatments, the two alleles were significantly up-regulated under salt stress and significantly down-regulated under osmotic and heat stress. Neither of the alleles was expressed under cold stress. For the phytohormone treatments, neither of the alleles was expressed under the gibberellin (GA) and auxin (IAA) treatments, but both were significantly down-regulated under cytokinin (6-BA) treatment.
the secondary structure. All five secondary structure mutant sequences were synthesized for subsequent lncRNA experiments. PROMPT_1281 mutants lacking loop 3 and loop 4 were not able to significantly induce the expression of the two alleles of its cis-target Ps/WAK2, suggesting that loops 3 and 4 are required for its cis-regulatory function.

To detect the interacting regions of the PROMPTs and candidate promoters, we used several criteria to scan the candidate regions. First, the length of the interacting regions must be sufficient to bind specific sequences. Second, unwinding of the interacting regions should not change the whole secondary structure of the PROMPTs. Based on these two criteria, we found

*Figure 3* Functional annotation of the osmotic stress-responsive PROMPTs in the co-expression network. (a and b) Co-expression network of osmotic stress-responsive PROMPTs and protein-coding genes (correlation coefficient >0.9999, \( P < 0.0001 \)). Blue nodes represent down-regulated osmotic stress-responsive PROMPTs. Red nodes represent up-regulated osmotic stress-responsive PROMPTs. (c) Functional annotation of the core node of the down-regulated co-expression network. Red nodes represent over 400 protein-coding genes co-expressed with PROMPTs. Orange nodes represent over 300 protein-coding genes co-expressed with PROMPTs. Yellow nodes represent over 200 protein-coding genes co-expressed with PROMPTs. Grey nodes represent PROMPTs with no significant GO enrichment. (d) Functional annotation of the core node of the up-regulated co-expression network. Red nodes represent over 800 protein-coding genes co-expressed with PROMPTs. Orange nodes represent over 700 protein-coding genes co-expressed with PROMPTs. Yellow nodes represent over 600 protein-coding genes co-expressed with PROMPTs. Grey nodes represent PROMPTs with no significant GO enrichment.
We identified six genes that are co-expressed with activates its co-expressed genes not interact with the promoter of its mutant lacking IR1. This result implied that the six co-expressed genes were significantly up-regulated under osmotic stress, and the transcript abundance of the co-expressed genes under overexpression of PROMPT_1281-Hap1 was significantly higher than that under overexpression of PROMPT_1281-Hap2. Therefore, we speculated that PROMPT_1281 might also function as an activator of its trans targets, and PROMPT_1281-Hap1 has stronger activation activity than PROMPT_1281-Hap2.

To test the regulatory functions of PROMPT_1281 via its secondary structure, we also deleted the five loop sequences from PROMPT_1281 one by one (Figure 6). All five secondary structure mutant sequences were synthesized for subsequent lncRNAe experiments. The results revealed that, without loop 4, neither of the PROMPT_1281 alleles could significantly induce the expression of the co-expressed genes, suggesting that only loop 4 is required for the trans-regulation function of PROMPT_1281. Additionally, the MYB1AT motif was found in loop 4, implying that the trans-regulation function of PROMPT_1281 is dependent on this MYB recognition site. To further validate the role of loop 4 in transcriptional regulation, we created mutants by changing three nucleotides in loop 4 to further validate the role of loop 4 in transcriptional regulation, we created mutants by changing three nucleotides in loop 4 to change the secondary structure to a stem (Figure S7). LncRNAe analysis showed that the transcript abundance of the co-expressed genes was not significantly changed after interaction with the PROMPT_1281 mutant lacking loop 4 (Figure S8).

Table 1 Annotation of the co-expression network modules

| Network module | PROMPT | Expression pattern | Enrichment GO term | Annotation |
|----------------|--------|--------------------|--------------------|------------|
| NM1            | PROMPT_1281 | Up-regulated | GO:0006468 | Protein amino acid phosphorylation |
| NM2            | PROMPT_3076 | Up-regulated | GO:0006888 | ER to Golgi vesicle-mediated transport |
| NM3            | PROMPT_4986 | Up-regulated | GO:0007264 | Small GTPase-mediated signal transduction |
| NM4            | PROMPT_1280 | Up-regulated | GO:0007165 | Signal transduction |
| NM5            | PROMPT_0386 | Up-regulated | GO:0006468 | Protein amino acid phosphorylation |
| NM6            | PROMPT_2924 | Up-regulated | GO:0006468 | Protein amino acid phosphorylation |
| NM7            | PROMPT_2536 | Up-regulated | GO:0006468 | Protein amino acid phosphorylation |
| NM8            | PROMPT_1393 | Up-regulated | GO:0007165 | Signal transduction |
| NM9            | PROMPT_0270 | Up-regulated | GO:0008037 | Cell recognition |
| NM10           | PROMPT_4201 | Up-regulated | GO:0043687 | Post-translational protein modification |
| NM11           | PROMPT_0293 | Up-regulated | GO:0007165 | Signal transduction |
| NM12           | PROMPT_1714 | Up-regulated | GO:0006468 | Protein amino acid phosphorylation |
| NM13           | PROMPT_4695 | Up-regulated | GO:0043687 | Post-translational protein modification |
| NM14           | PROMPT_4653 | Up-regulated | GO:0006511 | Ubiquitin-dependent protein catabolic process |
| NM15           | PROMPT_0982 | Down-regulated | GO:0034641 | Cellular nitrogen compound biosynthetic process |
| NM16           | PROMPT_1524 | Down-regulated | GO:006091 | Generation of precursor metabolites and energy |
| NM17           | PROMPT_4428 | Down-regulated | GO:0006457 | Protein folding |
| NM18           | PROMPT_0317 | Down-regulated | GO:0045454 | Cell redox homeostasis |
| NM19           | PROMPT_2181 | Down-regulated | GO:0006457 | Protein folding |
| NM20           | PROMPT_1259 | Down-regulated | GO:0043234 | Protein complex |
| NM21           | PROMPT_1588 | Down-regulated | GO:0034641 | Cellular nitrogen compound biosynthetic process |
| NM22           | PROMPT_3520 | Down-regulated | GO:0005840 | Ribosome |
| NM23           | PROMPT_0258 | Down-regulated | GO:0006412 | Translation |
| NM24           | PROMPT_1220 | Down-regulated | GO:0006520 | Cellular amino acid metabolic process |
| NM25           | PROMPT_3649 | Up-regulated | GO:0015979 | Photosynthesis |
| NM26           | PROMPT_2774 | Down-regulated | GO:0006091 | Generation of precursor metabolites and energy |
| NM27           | PROMPT_3662 | Down-regulated | None | None |

© 2018 The Authors. Plant Biotechnology Journal published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd., 17, 164-177
result suggested that both the secondary structure and sequence motifs are responsible for the transcriptional regulation functions of PROMPT_1281.

Discussion

PROMPTs are more stable than lncRNAs

In mammals, PROMPTs are transcribed in the antisense orientation (Balbin et al., 2015). Our results showed that approximately 4.5% of PROMPTs are transcribed in the sense orientation in poplar, indicating that PROMPTs are transcribed bidirectionally in plants. The distance of PROMPTs upstream of the active TSSs of most protein-coding genes ranges from 0.5 to 2.5 kb in mammals (Preker et al., 2008). In our study, PROMPTs were found approximately 0.4–0.8 kb upstream of the TSSs of protein-coding genes, implying that PROMPTs might share a common promoter with downstream genes, resulting in robust cis-mediated transcriptional regulation. Generally, PROMPTs are heterologous in length (about 200–600 nt) in mammals (Preker et al., 2011). By contrast, the length of PROMPTs ranged from 0.2 to 2.7 kb in poplar, indicating that the length of PROMPTs might be longer in plants.

Higher GC content confers higher thermostability in DNA and RNA. PROMPTs have higher GC content than other lncRNAs, indicating that PROMPTs might be unique among lncRNAs. CpG islands are regions with a high frequency of CpG sites that are always associated with the TSS of genes (Hartl and Jones, 2005). Because of the proximity of PROMPTs to TSSs, they overlap with CpG islands, which might be the main reason for their higher GC content. PROMPTs were found to be enriched in different chromosomes from other lncRNAs, suggesting that they might be involved in different transcriptional events. A low MFE means that the RNA has a more stable secondary structure (Mathews et al., 2004). Our results showed that the MFE of PROMPTs is significantly lower than that of other lncRNAs, suggesting that PROMPTs are more stable than other kinds of lncRNAs. Taken together, our results suggest that PROMPTs have bidirectional transcription in plants, and compared to other lncRNAs, they are longer in length, have a higher GC content and greater stability and are closer in proximity to protein-coding genes.
Osmotic stress-responsive PROMPTs are more sensitive to osmotic stress than other lncRNAs

Previous study reported that lncRNAs are expressed at significantly lower levels in plants and animals (Cabili et al., 2011; Shuai et al., 2014). In this study, our results showed that PROMPTs were expressed at about twofold higher levels, on average, than other lncRNAs. Osmotic-responsive PROMPTs were expressed about fourfold higher, on average, than other lncRNAs, and the average fold change of PROMPTs was also higher than that of other lncRNAs, suggesting that PROMPTs are more responsive than other lncRNAs to osmotic stress. In our study, the transcript abundance and fold changes of the genes located downstream of PROMPTs were positively correlated with the expression of the PROMPTs in the control and osmotic stress group, suggesting that PROMPTs might be positive transcriptional regulators of their cis targets. When PROMPTs and their gene targets were located in the same transcriptional orientation (sense/sense pairs and antisense/antisense pairs of PROMPTs/downstream protein-coding genes), the expression level of the PROMPTs was higher than when in the reverse orientation (sense/antisense pairs), implying that their orientation might also play an important role in their regulatory functions.

Sharing of regulatory elements in divergently transcribed genes is a primary factor for their co-expression (Williams and Bowles, 2004). In our study, the expression of PROMPTs was positively correlated with the expression of neighbouring genes that shared similar motifs, implying that these common motifs might be important regulation sites for the expression of PROMPT-mRNA pairs. Cis-regulatory elements in the promoter regions of sense and antisense transcripts play an important role in expression patterns (Williams and Bowles, 2004). A large percentage of lncRNAs physically interact with various chromatin regulatory proteins, including PRC2, WDR5 and other proteins involved in chromatin modifications (Guttman et al., 2011; Quinodoz and Guttman, 2014). These examples highlight how lncRNAs interact with proteins using their single-strand sequence. Our results showed that ERF, MYB, CBF/DREB and other motifs are enriched in PROMPTs and might provide potential target sites for transcriptional regulation factors.
The secondary structure of PROMPTs is more conserved than that of other lncRNAs

With the exception of the transcription factor binding site, the secondary structure of lncRNAs has critical roles in diversified processes including ligand sensing to the regulation of translation, polyadenylation and splicing (Cruz and Westhof, 2009). We predicted and characterized the conserved secondary structure of osmotic stress-responsive lncRNAs within the same NMs. All five PROMPTs in NM1, which was the largest NM among the co-expression network, had one conserved secondary structure that was annotated as being related to the regulation of transcription. The loop sequence of this conserved secondary structure contained a transcription factor binding site (a MYB1AT motif). This suggested that the expression pattern of the PROMPTs in NM1 might be mediated by the MYB1AT motif within their conserved secondary structure.

Single nucleotide polymorphisms are thought to be the most widespread factor affecting the structural variations, stability and transcript abundance of RNA (Ding et al., 2012; Gong et al., 2015). Our results showed that the density of SNPs in PROMPTs was lower than that of other lncRNAs. These SNPs may influence the stability, expression and functions of PROMPTs via changes in their secondary structure. Our analysis of the MFE of the PROMPTs showed that the average energy changes conferred by SNPs in poplar were 2.61 kcal/mol, which is higher than that in other lncRNAs in poplar and mammals, suggesting that SNPs might have more significant effects on the secondary structure of PROMPTs in plants. Linkage disequilibrium analysis showed that the number of PROMPT-SNPs in LD was significantly larger than that of lncRNA-SNPs, and most of the LD regions had conserved secondary structures. This suggests that the secondary structure of PROMPTs is more conserved than that in other lncRNAs, which might help maintain stable regulatory functions.

Figure 6 Allelic expression pattern of PROMPT_1281 and its trans targets. (a) Allelic expression pattern of PROMPT_1281 and its trans targets under osmotic stress and IncRNAe treatment. Relative transcript levels were calculated by qPCR with ACTIN as the standard. Data are mean ± SE of three separate measurements. Error bars represent standard error. (b) Allelic expression pattern of trans targets under overexpression of the PROMPT_1281 secondary structure mutant. Δ1–Δ5 represents deletions of loops 1–5. (c) Schematic diagram of how PROMPTs regulate their trans targets’ transcript abundance via a concentration gradient. The concentration of a PROMPT will be highest (red—inner circle) near its site of transcription and will decrease (pink—outer circles) the further the distance from its site of transcription, creating a concentration gradient. This concentration gradient establishes a nuclear domain with a high concentration of the PROMPT, where it can interact with site-specific targets.

© 2018 The Authors. Plant Biotechnology Journal published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd., 17, 164–177
To better understand the functionality of IncRNAs, we investigated whether IncRNAs exhibit signatures of purifying selection. ARs present in the last common ancestor appear to have evolved neutrally (Lunter et al., 2006). Their evolutionary rates provide appropriate proxies for the mutational rates in selectively neutral sequences (Ponjavic et al., 2007). Under neutrality, the length of intergap segments distributed similarly to distribution predicted for the distance between successive indels. Our Blast Z (compared the estimated rate of nucleotide substitutions the geometric distribution (Song, unpublished data). Thus, we evaluated whether lncRNAs exhibit signatures of purifying selection or lower mutation rates. The median that regulate transcription networks. Additionally, might not interact with DNA for transcriptional regulation of downstream genes. Interestingly, the concentration of PROMPT_1281 decreased with increasing distance from its site of transcription, creating a concentration gradient. PROMPT_1281 has a MYB transcription factor binding site (MYB1AT motif) that binds the MYB1 transcription factor (Yadav et al., 2017). In cotton, MYB1 regulates a specialized subcomponent of the secondary cell wall involving secondary metabolite synthesis and stress hormone signalling-related gene networks. Our results showed that PROMPT_1281 might be a carrier for MYB1 and other regulatory factors that regulate transcription networks. Additionally, PROMPT_1281 might undergo rapid degradation by the RNA nuclear exosome-targeting complex (Taft et al., 2009). Therefore, the concentration gradient of PROMPT_1281 might rapidly increase the probability of an interaction of its cis and trans targets with the MYB transcription factor.

**Experimental procedures**

**Plant materials and treatments**

One-year-old *Populus simoni* 'QL9' clones were grown in pots with inner size of 10 cm in height and 15 cm in diameter, containing a potting mix of a commercial medium and perlite at a ratio of 3 : 1. Those clones were maintained under natural light (1250 µmol/m²/s of photosynthetically active radiation), 25 ± 1 °C, 50% ± 1 relative humidity and a 12/12-h day/night regime in an air-conditioned greenhouse. Relative leaf water content was measured as Schonfeld's described (Schonfeld et al., 1988). Relative leaf water content was significantly decreased at 6-h osmotic stress (Figure S9), implying there might be a substantial change in gene expression at this time point. Therefore, we choose 6-h osmotic stress treatment for transcriptome analysis.

One-year-old clones of the same size (50 cm in height) were used for abiotic stress treatment. These groups were exposed to 150 mM NaCl, 30% polyethylene glycol (PEG) 6000, 42 and 4 °C for 6 h treated for salinity, osmotic, heat and cold stress treatments, respectively. Clones not exposed to abiotic stress...
were used as the control group (Song et al., 2014). For phytohormone treatment, 100 μM of GA3, IAA and 6-BA (Sigma-Aldrich, St. Louis, MO, USA) were, respectively, sprayed on clone leaves until drops of liquid dripped down. The control plants were treated with water in the same manner. Considering the effect of the developmental stages of leaves on gene expression, mature leaves with developmental stages which from the same position of control and treated plants were collected at 6 h after treatment. Three biological replicates were used in each treatment, including the control group. Fresh leaves were collected from all these groups, immediately frozen in liquid nitrogen and stored at −80 °C until analysed. For tissue-specific gene expression analysis, cambium, immature xylem, mature xylem, root, stem and bud were also collected from 1-year-old clones. Male and female flower were collected from 30-year-old male and female poplar, respectively. All these tissues also immediately frozen in liquid nitrogen and stored at −80 °C until analysed.

**Sequencing of IncRNA**

After the osmotic stress treatment, total RNA was isolated from fresh leaves by a modified CTAB method and was used for small RNA library construction. For IncRNA sequencing, a strand-specific cDNA library was constructed using the SMART library construction method (Levin et al., 2010). The detailed library construction process is presented in the supplemental data. LncRNAs were sequenced using an Illumina HiSeq 2000 at the Shanghai Bio Institute (Shanghai, China). The total number of reads and mapping results is shown in Table S1. The gene expression data reported here are available from NCBI with the SRA database accession numbers SRR5127346.

**Prediction of PROMPTs from the cDNA sequences**

Clean reads were obtained after filtering out low-quality reads and trimming the adaptor sequences. The P. trichocarpa (version 3.0) genome was used as a reference for clean reads mapping using TopHat (version: 2.0.9) (Trapnell et al., 2009). Mismatches of three bases or less and multihits of no more than one base were allowed in the alignment. We used three filter processes to identify the osmotic stress-responsive IncRNA candidates. First, the length of transcriptional units (TUs) had to be longer than 200 bp. Second, the longest open reading frame (ORF) of the TU had to be smaller than 300 bp (the longest ORF was predicted by OrfPredictor; http://proteomics.ysu.edu/tools/OrfPredictor.html) (Min et al., 2005). Sense and antisense strands of the TUs were used for prediction. The Coding Potential Calculator (CPC) and Coding--Non-Coding Index (CNCI) were used to assess the protein-coding potential of a transcript based on two criteria: a CPC score < 0 and a CNCI < 0 (Kong et al., 2007; Sun et al., 2013a,b). Finally, the IncRNA candidate sequences were mapped to the genome and full-length IncRNAs located in promoter regions were annotated as PROMPTs.

**Sequence and structural motif search**

Conserved sequence motif searches in a group of IncRNAs were carried out by MEME (http://meme-suite.org/tools/meme) (Bailey et al., 2009). For the IncRNAs selected by the target's GO term, we predicted the conserved structural motifs in grouped IncRNAs using RNApromo (https://gene.weizmann.ac.il/pubs/rnamotifs08/rnamotifs08_predict.html) (Rabani et al., 2008). The details of sequence and structural motif analysis are presented in the supplemental data.

**Quantitative PCR analysis**

To validate the expression patterns of PROMPTs acquired by high-throughput sequencing, we performed qPCR for 40 osmotic stress-responsive PROMPTs with different expression patterns (Table S10). We found a significant correlation between transcript abundance measured by qPCR and RNA-seq (r = 0.71, P < 0.001), indicating the reliability of the RNA-seq data (Figure S10). All primers used for candidate genes and PROMPTs are listed in Table S11. qPCR was performed using an ABI StepOne Plus instrument, and the results were subjected to the following calculations: sample cycle threshold (CT) values were determined and standardized relative to the endogenous control gene (ACTIN), and the 2^(ΔACT)-ΔCT method was used to calculate the relative changes in gene expression based on the qPCR data (Livak and Schmittgen, 2001). A melting curve was used to check the specificity of each amplified fragment. For all reactions, triplicate technical and biological repetitions were performed for each individual. After amplification, the PCR products were sequenced to check the specificity of the primer sets.

**Differential expression and co-expression analyses**

Cuffdiff was used to calculate fragments per kilobase per million fragments mapped (FPKMs). The FPKMs of IncRNAs and genes were computed by summing the FPKMs of transcripts in each transcript group. Cuffdiff provides statistical routines for determining differential expression in transcript data using a model based on the negative binomial distribution (Trapnell et al., 2010). Differential expression analysis of two conditions or groups was performed using the DESeq R package (1.8.3) (Heidelberg, Germany). The P-values were adjusted using the Benjamini & Hochberg method. Differences of mRNA, IncRNA and miRNA levels were considered statistically significant at a fold change >2 or <0.5 and P < 0.01.

Gene co-expression network analysis has been increasingly used to identify the biological functions of IncRNAs and their potential subnetworks for trans targets (Liao et al., 2011). One important end product of co-expression networks is the construction of gene modules composed of highly interconnected genes. To identify gene co-expression modules from 15 the RNA sequencing data (accession numbers SRR5127346, SRP095225, SRP073689 and SRP060593), the WGCNA package for R was used to calculate the correlation coefficient (Langfelder and Horvath, 2008). Normalized IncRNAs and mRNA expression values were used for co-expression analysis. One-step network construction with unsigned correlations type and consensus module detection was used for co-expression network construction. All other WGCNA parameters remained at their default settings. Assessment of module quality was assisted by examining trend plots of Z-score normalized expression values for all genes in a given module. The mRNA co-expression modules were used for GO enrichment analysis of the IncRNAs. Statistical significance for enrichment of genes was assessed using the hypergeometric distribution.

**Treatment with LDH--IncRNA conjugates**

Bulk Mg-Al-lactate LDH was synthesized using a coprecipitation method and delaminated in water into nano-scale sheets. The delaminated LDH-lactate is denoted as LDH-lactate-NS with a final concentration of 1 mg/mL. Candidate IncRNAs were artifically synthesized (Table S8). These IncRNAs were dissolved in distilled water to a concentration of 1 mg/mL (Bao et al., 2016).
The LDH-lactate-NS colloid in MS was added dropwise to the IncRNAs at a ratio of 3 : 1 (v:v) followed by gentle mixing. RNase inhibitor was added to a final concentration of 0.4 U/µL. The mixture was incubated for 1 h to form the LDH-lactate-NS–IncRNA conjugate. Then, 100 µL of LDH-lactate-NS–IncRNA conjugate was added to liquid MS medium for transport of the RNA into plant roots (Figure S6). Poplar seedlings were cultured in MS medium. For LDH–IncRNA conjugates treatment, poplar seedling lateral roots were dipped in liquid MS medium containing 30% PEG6000, the poplar seedling lateral roots with LDH-lactate-NS–IncRNA conjugate were dipped in liquid MS medium containing 30% PEG6000 (Figure S11). After incubation at room temperature for 3 h, the roots were rinsed several times with a standard growth medium and then stored at −80 °C for expression analysis. For the osmotic treatment with 30% PEG6000, the poplar seedling lateral roots with LDH-lactate-NS–IncRNA conjugate were dipped in liquid MS medium containing 30% PEG6000 (Figure S11). After incubation at room temperature for 1 h, the roots were stored at −80 °C for expression analysis.

Acknowledgements

This work was supported by the grants of the State ‘13.5’ Key Research Program of China (No. 2016YFD0600102) and the Project of the National Natural Science Foundation of China (no. 31400553 and 31770707). The authors would like to thank Professor Yinglang Wan for providing the LDH nanosheets. We are grateful for the sequence information produced by the U.S. Department of Energy Joint Genome Institute (http://www.jgi.doe.gov).

Conflict of interest

The authors declare no conflict of interest.

References

Abe, H., Urao, T., Ito, T., Seki, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2003) Arabidopsis AtMYC2 (AtHLS1 and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Plant Cell, 15, 63–78.
Bailey, T.L., Boden, M., Buske, F.A., Frith, M., Grant, C.E. and Clementi, L. (2009) MEME SUITE: tools for motif discovery and searching. Nucleic Acids Res. 37, W202–W208.
Balbin, O.A., Malik, R., Dhanasekaran, S.M., Prensner, J.R., Cao, X., Wu, Y.M., Robinson, D. et al. (2015) The landscape of antisense gene expression in human cancers. Genome Res. 25, 1068–1079.
Bao, W., Wang, J., Wang, Q., O’Hare, D. and Wan, Y. (2016) Layered double hydroxide nanotransporter for molecule delivery to intact plant cells. Sci. Rep. 6, 26738.
Bell, C.G. and Beck, S. (2009) Advances in the identification and analysis of allele-specific expression. Genome Med. 1, 56.
Bose, D.A., Donahue, G., Reinberg, D., Shiekhattar, R., Bonasio, R. and Berger, J.L. (2011) Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. Genes Dev. 25, 1915–1927.
Chen, L.L. (2016) Linking long noncoding RNA localization and function. Trends Biochem. Sci. 41, 761–772.
Chen, L.L. and Carmichael, G.G. (2010) Decoding the function of nuclear long non-coding RNAs. Curr. Opin. Cell Biol. 22, 357–364.
Cheung, V.G., Conlin, L.K., Weber, T.M., Arcaro, M., Jen, K.Y., Morley, M. and Spiesel, R.S. (2003) Natural variation in human gene expression assessed in lymphoblastoid cells. Nat. Genet. 33, 422–425.
Cruz, J.A. and Westhof, E. (2009) The dynamic landscapes of RNA architecture. Cell, 136, 604–609.
Dethoff, E.A., Chugh, J., Mustoe, A.M. and Al-Hashimi, H.M. (2012) Functional complexity and regulation through RNA dynamics. Nature, 482, 322–330.
Ding, J., Lu, Q., Ouyang, Y., Mao, H., Zhang, P., Yao, J., Xu, C. et al. (2012) A long noncoding RNA regulates photoperiod-sensitive male sterility, an essential component of hybrid rice. Proc. Natl Acad. Sci. USA, 109, 2654–2659.
Djebali, S., Davis, C.A., Merkel, A., Dobin, A., Lassmann, T., Mortazavi, A., Tanzer, A. et al. (2012) Landscape of transcription in human cells. Nature, 489, 101–108.
Enard, W., Khaitovich, P., Klose, J., Zöllner, S., Heissig, F., Giavalisco, P., Nieselt-Struwe, K. et al. (2002) Intra- and interspecific variation in primate gene expression patterns. Science, 296, 340–343.
Gong, J., Liu, W., Zhang, J.Y., Mao, X.P. and Guo, A.Y. (2015) IncRNAsNP: a database of SNPs in IncRNAs and their potential functions in human and mouse. Nucleic Acids Res. 43, D181–D186.
Gutmann, M., Donaghey, J., Carey, B.W., Garber, M., Grenier, J.K., Munson, G., Young, G. et al. (2011) IncRNAs act in the circuitry controlling pluripotency and differentiation. Nature, 477, 295–300.
Hauguer, M.J., Vaughn, I.W. and McManus, M.T. (2013) Pervasive transcription of the human genome produces thousands of previously unidentified long inter-genic noncoding RNAs. PLoS Genet, 9, e1003569.
Hartl, D.L. and Jones, E.W. (2005) Genetics: Analysis of Genes and Genomes, 6th ed. Mississauga, ON: Jones & Bartlett. 477 pp. ISBN 0-7637-1511-5.
Hong, S.W., Lee, U. and Vierling, E. (2003) Arabidopsis hot mutants define multiple functions required for acclimation to high temperatures. Plant Physiol. 132, 757–767.
Kim, E.D. and Sung, S. (2012) Long noncoding RNA: unveiling hidden layer of gene regulatory networks. Trends Plant Sci. 17, 16–21.
Knight, J.C. (2004) Allele-specific gene expression uncovered. Trends Genet. 20, 113–116.
Kong, L., Zhang, Y., Ye, Z.Q., Liu, X.Q., Zhao, S.Q., Wei, L. and Gao, G. (2007) CPC: assess the protein-coding potential of transcripts using sequence features and support vector machine. Nucleic Acids Res. 35, W345–W349.
Lai, F., Orom, U.A., Cesaroni, M., Beringer, M., Taatjes, D.J., Blobel, G.A. and Shiekhattar, R. (2013) Activating RNAs associate with Mediator to enhance chromatin architecture and transcription. Nature, 494, 497–501.
Langfelder, P. and Horvath, S. (2008) WGCNA: an R package for weighted correlation network analysis. BMC Bioinformatics, 9, 559.
Lee, J.T. (2012) Epigenetic regulation by long noncoding RNAs. Science, 338, 1435–1439.
Levin, I.Z., Yassour, M., Adiconis, X., Nusbaum, C., Thompson, D.A., Friedman, N., Gnirose, A. et al. (2010) Comparative comprehensive analysis of strand-specific RNA sequencing methods. Nat. Methods, 7, 709–715.
Li, W., Notani, D., Ma, Q., Tanasa, B., Nunez, E., Chen, A.Y., Merkurjev, D. et al. (2013) Functional roles of enhancer RNAs associate with Mediator to enhance chromatin architecture and transcription. Nature, 498, 516–520.
Liao, Q., Liu, C., Yuan, X., Kang, S., Mao, R., Xiao, H., Zhao, G. et al. (2011) Large-scale prediction of long non-coding RNA functions in a coding–non-coding gene co-expression network. Nucleic Acids Res. 39, 3864–3878.
Liska, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(−ΔΔCT) Method. Methods, 25, 402–408.
Lloret-Llinares, M., Mapedano, C.K., Martlew, L.H., Lykke-Andersen, S. and Jensen, T.H. (2016) Relationships between PROMPT and gene expression. RNA 13, 6–14.
Lubas, M., Andersen, P.R., Schein, A., Dziembowski, A., Kudla, G. and Jensen, T.H. (2015) The human nuclear exosome targeting complex is loaded onto newly synthesized RNA to direct early ribonucleolysis. Cell Rep. 10, 178–192.
Lunter, G., Ponting, C.P. and Hein, J. (2006) Genome-wide identification of human intergenic functional RNA using a neutral indel model. PLoS Comput. Biol. 2, e5.
Mathews, D.H., Disney, M.D., Childs, J.L., Schroeder, S.J., Zucker, M. and Turner, D.H. (2004) Incorporating chemical modification constraints into a dynamic programming algorithm for prediction of RNA secondary structure. Proc. Natl Acad. Sci. USA, 101, 7287–7292.
Melo, C.A., Drost, J., Wijchers, P.J., van der Werken, H., de Wit, E., Oude Vrielink, J.A., Elkon, R. et al. (2013) eRNAs are required for p53-dependent enhancer activity and gene transcription. Mol. Cell, 49, 524–535.

© 2018 The Authors. Plant Biotechnology Journal published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd., 17, 164–177.
Osmotic-responsive PROMPTs regulate gene expression

Williams, E.J. and Boxleis, D.J. (2004) Coexpression of neighboring genes in the genome of Arabidopsis thaliana. Genome Res. 14, 1060–1067.

Willingham, A.T., Orth, A.P., Batalov, S., Peters, E.C., Wen, B.G., Aza-Blanc, P., Hogenesch, J.B. et al. (2005) A strategy for probing the function of noncoding RNAs finds a repressor of NFAT. Science, 309, 1570–1573.

Wu, H., Yang, L. and Chen, L.L. (2017) The diversity of long noncoding RNAs and their generation. Trends Genet. 33, 540–552.

Yadav, V.K., Yadav, V.K., Pant, P., Singh, S.P., Maurya, R., Sable, A. and Sawant, S.V. (2017) GhMYB1 regulates SCW stage-specific expression of the GhGDSL promoter in the fibres of Gossypium hirsutum L. Plant Biotechnol. J. 15, 1163–1174.

Zhai, J., Zhang, W., Zhao, Y., Gong, X., Guo, L., Zhu, G., Wang, X. et al. (2007) SAD2, an importin-like protein, is required for UV-response in Arabidopsis by mediating MYB4 nuclear trafficking. Plant Cell, 19, 3805–3818.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Figure S1 Cis-regulatory functions of the osmotic stress-responsive PROMPTs.

Figure S2 Transcript abundance of PROMPTs and downstream protein-coding genes under osmotic stress.

Figure S3 Co-expression network of PROMPTs and mRNA.

Figure S4 Secondary structure of PROMPT_1281-Hap1 and PROMPT_1281-Hap2.

Figure S5 Nucleotide substitution rates are suppressed within PROMPT transcripts.

Figure S6 Schematic diagram of lncRNA interference and lncRNA enhance.

Figure S7 Secondary structure of PROMPT_1281-Hap1 and PROMPT_1281-Hap2 with mutated loop 4.

Figure S8 The secondary structure and spatial effect of PROMPTs regulate transcript of targets.

Figure S9 Correlation of qPCR and RNA-seq data.

Figure S10 Microscopic images of intact poplar root cells under FITC-PROMPT-LDH treatment.

Figure S11 Microscopic images of intact poplar root cells under the FITC-PROMPT-LDH treatment.

Table S1 Mapping results statistics.

Table S2 Information on the osmotic stress-responsive lncRNAs in poplar.

Table S3 Information on the PROMPTs and downstream genes in poplar.

Table S4 Differentially expressed lncRNAs of poplar under osmotic stress.

Table S5 Differentially expressed PROMPTs of poplar under osmotic stress.

Table S6 Annotation of the motifs of osmotic stress-responsive PROMPTs.

Table S7 Co-expression of osmotic stress-responsive PROMPTs and genes.

Table S8 Sequences of PROMPTs used for lncRNAe and lncRNAi analysis.

Table S9 Go term enrichment of NM1.

Table S10 qPCR primer sequences.

Table S11 Primer list of PROMPT_1281 and their targets.