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Physiological and transcriptomic analysis of *Pinus massoniana* seedling response to osmotic stress

H. XU[1], X. GAO[1], and C. YU[2,*]

[1] *College of Eco-Environmental Engineering, Guizhou Minzu University, Guiyang 550025, P.R. China*
[2] *College of Forestry, Guizhou University, Huaxi district, Guiyang 550025, P.R. China*

[*]Corresponding author: E-mail: chifengyucun@163.com

Abstract

Masson pine (*Pinus massoniana* Lamb.) is an important tree species of high economic value in southern China, but osmotic stress threatens its growth and development. In this study, physiological measurements and RNA-Seq analysis were used to clarify the physiological and molecular responses of *P. massoniana* under osmotic stress. Osmotic treatment caused cell membrane damage and reactive oxygen species (ROS) accumulation in the tree seedlings, but it also increased their antioxidant enzyme (superoxide dismutase, peroxidase, and catalase) activities and osmotic substances (soluble sugars, proline, and trehalose) content so as to adjust to osmotic stress conditions. A total of 1 789 differentially expressed genes (DEGs) were identified by transcriptome sequencing, of which 962 were up-regulated and 827 genes down-regulated. A series of stress-induced genes associated with signal transduction, ROS-scavenging, osmotic regulation, late embryogenesis abundant (LEA) protein, pentatricopeptide repeat-containing protein, and transcription factors' regulation were distinguishable. This detailed investigation of the stress-responsive genes and pathways provides new insight into molecular mechanism of abiotic stress response in *P. massoniana*. Further, this study's data can contribute to genetic engineering or molecular breeding efforts to enhance osmotic resistance in *P. massoniana* stands.

Keywords: osmotic stress, physiological analysis, *Pinus massoniana*, plant molecular response, ROS-scavenging, transcription factors.

Introduction

Plants suffer from various biotic and abiotic stresses that greatly affect their growth and development, which causes major yield losses in agriculture and forestry globally (Hirt *et al.* 2010). Among these stressors, that of osmotic stress, which results in a cellular water deficit, is one of the most limiting factors to plant growth, reproduction, distribution, and general crop productivity (Rabbani *et al.* 2003). Drought, salt injury, chilling, and freezing all can induce osmotic stress in plants. Polyethylene glycol (PEG) is a non-ionic, inert molecule that can have a range of molecular masses and it is widely used to induce water stress without associated other environmental factors that usually complicate field experiments (Almansouri *et al.* 2001, Landjeva *et al.* 2008).

Osmotic stress can cause cell damage, nutrient imbalance, and the accumulation of reactive oxygen species (ROS) in plant. For instance, osmotic stress elicites high ROS content, mainly hydrogen peroxide (H_2O_2) and superoxide radicals ($O_2^{\cdot-}$), and increases membrane lipid peroxidation in cucumber plants (Wang *et al.* 2018). In other work, the content of malondialdehyde (MDA), $O_2^{\cdot-}$ and H_2O_2 in the leaves of wild-type *Arabidopsis* plants increases significantly under osmotic stress (Zang *et al.* 2016), and PEG treatments elevate *Brassica napus* MDA

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Abbreviations: APX - ascorbate peroxidase; CAT - catalase; DEGs - differentially expressed genes; LEA - late embryogenesis abundant protein; MDA - malondialdehyde; $O_2^{\cdot-}$ - superoxide radicals; PEG - polyethylene glycol; POD - peroxidase; PPR - pentatricopeptide repeat-containing proteins; ROS - reactive oxygen species; SOD - superoxide dismutase; TFs - transcription factors.
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content, a product of lipid peroxidation (Mirzaee *et al.* 2013). In response to osmotic stress, plants are engaged in a series of coordinated physiological and biochemical changes, and may exhibit an "osmotic tolerant" phenotype *via* increasing ROS scavenging capacity, osmotic adjustment, and stomatal movements (Thalmann *et al.* 2016, Liu *et al.* 2019). For instance, Mundada *et al*. (2020) found that the content of free proline and antioxidative enzyme activities are increased under osmotic stress in *Eleusine coracana*. Therefore, we expect that various types of osmotic stress can lead to transcriptional changes in plant species and induce expression of stress-responsive genes. The proteins encoded by these genes are known to mainly comprise proteins involved in signal transduction, transcription factors (TFs), osmolyte synthesis, and antioxidant proteins (Qiu *et al.* 2017, Wani *et al.* 2018).

Masson pine (*Pinus massoniana*) is one of the most economically valuable coniferous trees grown in southern China. It is commonly used for timber, pulp, and resin production industries (Liu *et al.* 2015). Guizhou is part of China's main *P. massoniana*-producing area, but soil drought and cold temperature caused a water deficit in plants, which is preventing the optimal development of *P. massoniana* stands in this region. Therefore, it would be prudent and timely to investigate the osmotic stress-induced mechanism(s) of *P. massoniana* trees for breeding osmotic-resistant cultivars. To date, most studies of this tree have focused on its morphology, physiology, and molecular response mechanisms (He *et al.* 2013, Fan *et al.* 2014, Quan and Ding 2017, Zhang *et al.* 2017, Du *et al.* 2018, Pan and Hu 2020). A comprehensive study on osmotic stress-induced mechanism in *P. massoniana* that combines physiological and transcriptomic responses has yet to be conducted.

In this study, the physiological parameters were measured in *P. massoniana* after osmotic stress treatment. Additionally, the transcriptomic responses of osmotic stress were detected using RNA-Seq method. The aims of this paper were: *1*) to improve our understanding of molecular mechanism of *P. massoniana* adaption to osmotic stress and *2*) to identify those stress-responsive genes that may serve as promising candidates for plant molecular breeding for osmotic resistance traits of *P. massoniana*.

Materials and methods

Plants and treatments: *Pinus massoniana* Lamb. seedlings were grown under day/night room temperatures of approximately 20/15 °C, a relative humidity of 70 %, an irradiance of 400 μmol m^{-2} s^{-1}, and a 14-h photoperiod. Three two-month-old healthy seedlings were grown in plastic pots (90 cm diameter × 75 cm depth) filled with nursey soil (Duyun City, Guizhou province, China). The well-watered seedlings were irrigated with 20 % (m/v) polyethylene glycol (PEG) solution for 2, 4, 6, 8, or 10 d. Plants treated with fresh water served as the control. At this time, the seedlings were divided evenly into 6 groups (one control and five PEG treatments with different times), with 3 seedlings per pot and 30 pots per group. The seedlings were sampled for physiological parameter measurements and all plants treated at different times were harvested at the same time. For the transcriptomic analysis, uniformly sized *P. massoniana* seedlings were treated with 20 % (m/v) PEG for 4 d, and the stem apex needles of the seedlings were selected for RNA extraction; plants treated with fresh water served as the control.

Observation of seedling phenotype and determination of chlorophyll content: Two-month-old *P. massoniana* seedlings grown in soil were treated with 20 % (m/v) PEG for 2, 4, 6, 8, or 10 d and the phenotypes of each group of *P. massoniana* seedlings carefully observed. Some representative individuals among them were selected and photographed. The fresh mass (f.m.) of each whole plant in the sample was recorded and chlorophyll was extracted from leaves with 80 % (v/v) acetone, and measured as described by Porra *et al* (1989).

Detection of cell death and ROS content: For cell death determination, the leaves were detached and incubated in Evans blue solution and stained following Kim *et al.* (2003). Malondialdehyde (MDA) content was measured to evaluate the level of membrane lipid peroxidation. MDA content in leaves was determined *via* the thiobarbituric acid (TBA) reaction method according to Campo *et al.* (2014). Electrolyte leakage was determined to monitor the membrane damage, and it was performed as described by Fan *et al.* (1997). To detect the hydrogen peroxide (H_2O_2) and superoxide anions ($O_2^{\cdot-}$) content by histochemical staining analysis, the detached leaves were infiltrated with nitroblue tetrazolium (NBT) or 3,3'-diaminobenzidine (DAB) solutions, respectively, as already described by Zhang *et al.* (2011). The H_2O_2 content was determined by following Velikova *et al.* (2000) and the $O_2^{\cdot-}$ content by using the protocol given by Elstner and Heupel (1976).

Antioxidative enzyme activities and content of proline and trehalose: Phosphate buffer (2 cm^3) was added to Masson pine tissues' powder (0.1 g per sample) and incubated at 4 °C for 30 min. After centrifugation at 6000 g for 10 min, the ensuing supernatant was used to measure the superoxide dismutase (SOD) and peroxidase (POD) activity according to the methodology of Han *et al.* (2008). Catalase (CAT) activity was quantified as a decrease in absorbance at 260 nm for 1 min following the decomposition of H_2O_2, carried out as described by Aebi (1984). Ascorbate peroxidase (APX) activity was determined according to Nakano and Asada (1981). For this, the assay mixture consisted of 0.5 mM of ascorbic acid, 0.1 mM of H_2O_2, 0.1 mM of EDTA, 50 mM of sodium phosphate buffer (pH 7.0), and 0.15 cm^3 of enzyme extract.

Masson pine tissues' powder (0.1 g) was dissolved in 2 cm^3 of water and incubated at 100 °C for 30 min, then centrifuged at 6000 g for 10 min. The supernatant was diluted with water to 10 cm^3, and the soluble sugar content was measured using an anthrone sulfuric acid reagent by following the method of Zhang and Huang (2013). Proline content was determined using the ninhydrin reagent according to Bates *et al.* (1973). The trehalose content in

the sample was determined according to the manufacturer's protocol using the kit (*Shuzhou Kemin Biotechnology Company*, Shuzhou, China). Three independent biological replicates (one pot indicates one biological replicate) were performed.

RNA-Seq analysis: The RNA was extracted from both treatment groups: seedlings treated with 20 % PEG for 4 d and seedlings treated with water as the control. Six libraries (control and PEG, three replicates each) were prepared and sequenced by *OE Biotech Company* (Shanghai, China) using *Illumina HiSeq X Ten* system. After raw read filtering, de novo transcriptome assembly was conducted using the short reads assembling program *Trinity* (Grabherr *et al.* 2011). Then, the unigenes were clustered among the six transcriptome libraries, by using *CD-HIT* software (Li *et al.* 2001).

Gene function was annotated by searching seven databases: *NR* (NCBI non-redundant protein), *Swissprot*, *KEGG* (Kyoto encyclopedia of genes and genomes), *KOG* (eukaryotic orthologous groups), *eggNOG* (evolutionary genealogy of genes: non-supervised orthologous groups), *GO* (gene ontology), and *Pfam*. Unigene expression was calculated and normalized by the FPKM (fragments per kb per million reads) method (Roberts *et al.* 2011). The DEGs were designated when the following criteria were satisfied: the | log2 Fold Change | > 1, and the corrected *P*-value < 0.05. DEG functions were explored through *GO* and *KEGG* pathway analyses, for which terms having a Q-value ≤ 0.05 were defined as being significantly enriched.

Reverse transcription-qPCR: For the verification of the RNA-Seq data, 10 DEGs related to osmotic stress response were selected for RT-qPCR analyses. *Ubiquitin-conjugating enzyme-like protein (UBC)* gene served as an internal control (Fan *et al.* 2014). All primers used are listed in Table 1 Suppl. For the RT-qPCR, the reaction system (20 mm^3) included 10 mm^3 of *SYBR Green Realtime PCR Master Mix*, 10 mM each of the forward or reverse primer, and 2 mm^3 of cDNA dilution products. The PCR was run at following conditions: 95 °C for 2 min; 45 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 40 s; and 79 °C for 1 s for the plate reading. The relative expression of a given gene was calculated by using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Three independent biological replicates were performed.

Statistical analyses: We used the *SPSS (v. 19.0)* software package to perform the statistical analysis of physiological data. Statistical differences were compared using one-way analysis of variance (*ANOVA*), followed by the comparison of mean values using Dunnett's test at $P < 0.05$.

Results

Under osmotic stress, the *P. massoniana* seedlings showed continuous phenotypic changes over time (Fig. 1*A*). After 6 d of osmotic stress, some needles began turning yellow and wilted. On day 10 of the osmotic treatment, the seedlings displayed severe damage, in that time all their needles had wilted and yellowed. Compared with normal conditions, there was no difference in terms of plant fresh mass and chlorophyll content of seedlings stressed for 2 d; but significant reductions were evident after 4 - 10 d of osmotic treatment ($P < 0.05$, Fig. 1*B,C*). These results indicated that osmotic stress caused continuous injury to *P. massoniana* seedlings.

According to Evans blue staining, seedlings displayed continuous damage under osmotic treatment from 2 to 10 d (Fig. 2*A*). Electrolyte leakage increased significantly in leaves after osmotic stress ($P < 0.05$), and enhanced obviously in roots on day 4 (Fig. 2*B*). The MDA content in leaves and roots significantly increased during osmotic stress ($P < 0.05$, Fig. 2*C*). The results suggested that osmotic stress caused cell membrane damage in *P. massoniana* seedlings.

As shown in Fig. 3*A* and *B*, a deeper blue or brown color was observed in leaves, revealing that the ROS content increased continuously during osmotic stress. In terms of the content of O_2^- and H_2O_2, seedlings displayed substantial increase in both leaves and roots when the treatment lasted 4 to 10 d (Fig. 3*C,D*). Next, we investigated ROS-scavenging capability by measuring POD, SOD, APX, and CAT activities. The SOD activity gradually rose to peak on day 8 in leaves or day 6 in roots (Fig. 3*E*). The POD activity was markedly increased in leaves treated from 8 to 10 d ($P < 0.05$), and in roots it increased significantly from 2 to 10 d of osmotic exposure ($P < 0.05$, Fig. 3*F*). The APX activity in roots was higher than in leaves, there was no obvious difference in APX activity after osmotic stress in leaves or roots (Fig. 3*G*). Seedlings had significantly increased CAT activity in roots when under osmotic stress for 2 to 8 d ($P < 0.05$, Fig. 3*H*). These results indicated that Masson pine seedlings could elevate antioxidant enzymes' activity to scavenge ROS when facing osmotic stress.

Soluble sugars, proline, and trehalose are important osmotic adjustment substances and they also figure prominently in ROS scavenging in plants. As Fig. 4 shows, the content of all three was greatly increased during osmotic stress, both in leaves and roots. The content of proline in leaves exceeded that in roots from 6 to 10 d of osmotic exposure. These results demonstrated that Masson pine seedlings can increase the content of their osmolytes in response to osmotic stress.

Six libraries were constructed, having an average of 49 228 582 raw reads and 47 783 731 clean reads, with a Q30 (Proportion of bases with Ophred > 30) ≥ 93.74 % and GC between 45.25 and 45.52 % (Table 2 Suppl.). After trimming the adapters, filtering out low-quality reads, and conducting the *de novo* assembly, a total of 40 931 unigenes were obtained, with a mean length of 1 114 bp and N50 (by ordering all sequences, then adding the length from longest to shortest until the summed length exceeded 50 % of the total length of all sequences) of 1 733 bp (Fig. 1 Suppl.).

Next, the 40 931 unigenes were annotated successfully, of which 24 571 unigenes (60.03 %) displayed significant similarity to the *NR* database; 17 964 unigenes (43.89 %)

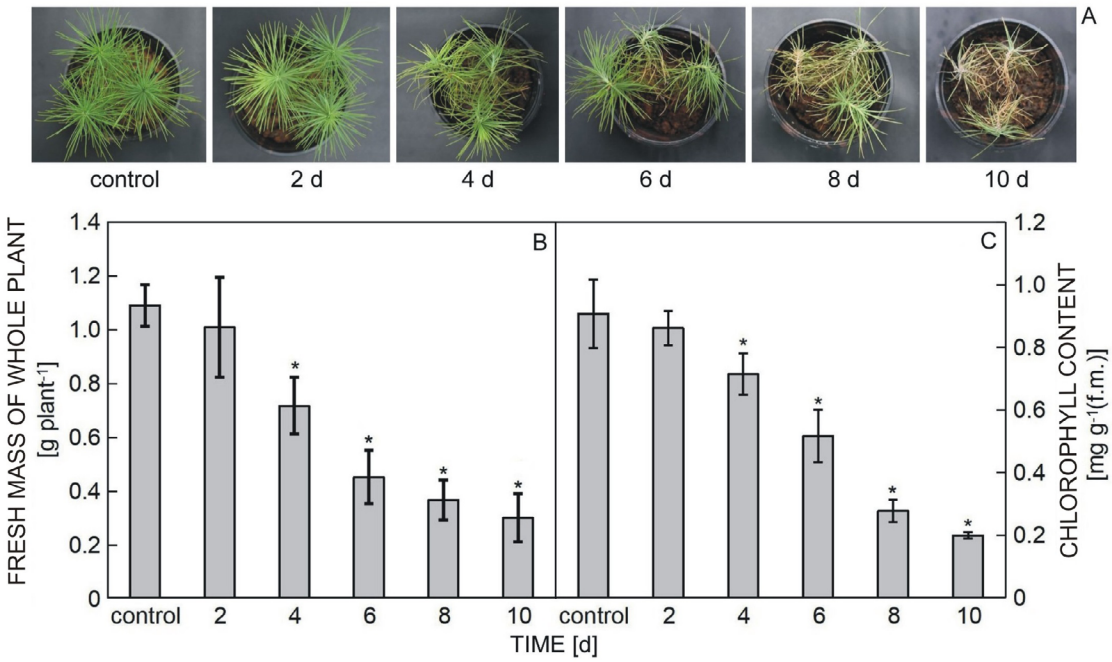

Fig. 1. Effects of osmotic treatment on the growth of Masson pine seedlings. *A* - Phenotypic changes of seedlings on 2, 4, 6, 8, and 10 d after 20 % PEG treatment; *B* - Measurement of fresh mass; *C* - Determination of chlorophyll content. Means ± SEs, $n = 3$ independent biological replicates, * indicates a significant differences at $P < 0.05$ compared with the control.

were annotated in *Swiss-Prot*; and likewise, from the other databases, 8 554 unigenes (20.90 %) in *KEGG*; 13 664 unigenes (33.38 %) in *KOG*; 21 655 unigenes (52.91 %) in *eggNOG*; 15 625 unigenes (38.17 %) in *GO* database; but just 24 unigenes (0.06 %) in *Pfam*.

Volcano plots show that a total of 1 789 DEGs were expressed under osmotic stress, of which 962 genes were up-regulated and 827 genes down-regulated (Fig. 2 Suppl.). The expression patterns of all DEGs were further analyzed by the hierarchical clustering between control and osmotic treatment (Fig. 3 Suppl.). Compared with the control, many genes were found differentially expressed under osmotic treatment.

To determine the reliability of the RNA-Seq results, RT-qPCR was performed. 10 DEGs from RNA-Seq were randomly selected for study. These results showed that the expressions of all these DEGs were similar to the RT-qPCR results (Fig. 4 Suppl.). Additionally, a linear regression analysis between the quantitative real-time PCR and the transcriptome data exhibited a significantly positive correlation ($R^2 = 0.9037$, $P < 0.05$), thus indicating the RNA-Seq results were reliable.

The *GO* classification of the above DEGs is shown in Fig. 5 Suppl. We used hypergeometric testing to determine the results of up- and down-regulation of significantly enriched *GO* categories compared with the genomic background ($P \leq 0.05$, after applying a Bonferroni correction) by the three ontology categories: biological processes, cellular components, and molecular function. The largest sub-groups distinguished under biological processes were “biological regulation”, “cellular process”, “metabolic process”, “regulation biological process”, and “response to stimulus”. The top five ranked *GO* terms for cellular components were identified as “cell”, “cell part”, “organelle part”, “membrane”, and “organelle”. Under the molecular function category, the largest sub-groups found were “binding”, “catalytic activity” and “transporter activity” (Fig. 5*A* Suppl.). To understand the functional classification of DEGs, the top 30 enriched *GO* terms in that the number of DEGs was greater than two were analyzed in detail. This revealed many categories associated with osmotic stress, such as biological processes of “galactose metabolic process”, “defense response”, “ethylene-activated signaling pathway”, “signal transduction”; cellular components of “peroxisomal membrane” and “plant-type vacuole membrane”; in addition to molecular function of “inositol 3-alpha-galactosyltransferase activity” and “chitinase activity” (Fig. 5*B* Suppl.).

To further understand the biological functioning and interactions of those genes, a *KEGG* annotation was performed. A total of 113 pathways (86 up-regulated and 63 down-regulated) were obtained. The DEGs were mainly involved in “carbohydrate metabolism”, “signal transduction”, “lipid metabolism”, and “amino acid metabolism” (Fig. 6*A* Suppl.). The top 20 most enriched pathways in that the number of DEGs was greater than two are shown in Fig. 6*B*,*C* Suppl. Most of the up-regulated DEGs were enriched primarily in the pathways of “plant hormone signal transduction”, “amino sugar and nucleotide sugar metabolism”, “galactose metabolism”, and “starch and sucrose metabolism”. Most of the down-regulated DEGs were involved in “carbon metabolism” and “flavonoid biosynthesis”.

Based on the transcriptomic data from the osmotic exposure experiment, a few representative pathways were involved in the osmotic-stress response of *P. massoniana*.

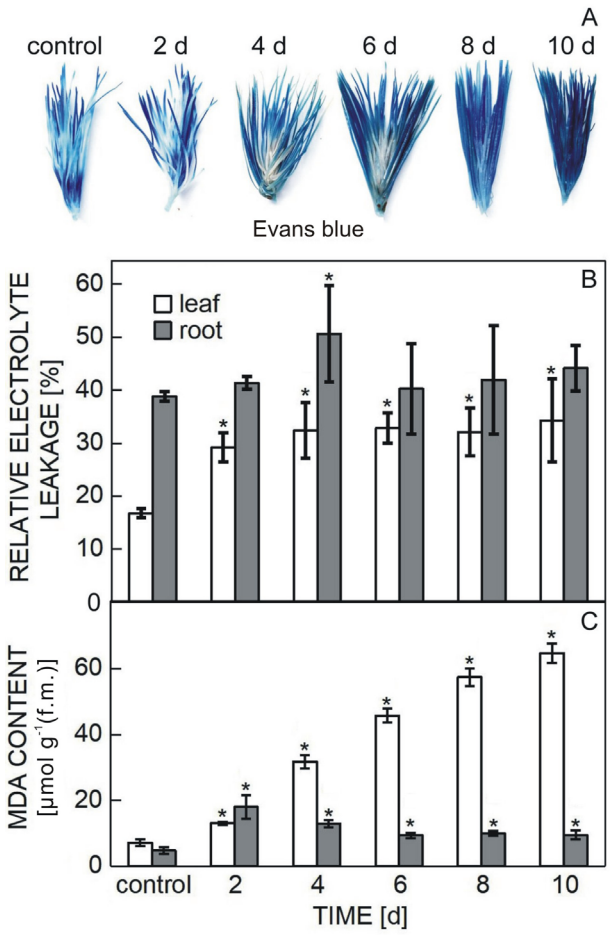

Fig. 2. Detection of cell death. *A* - Evans blue staining to detect cell death in Masson pine needles; *B* - Relative electrolyte leakage rate analysis; *C* - Determination of MDA content. Means ± SDs, $n = 3$ independent biological replicates, * indicates a significant differences at $P < 0.05$ compared with the control. Treatment time was used as a variable, leaf or root was analyzed separately by one-way *ANOVA*.

In all, 29 DEGs were closely associated with signal transduction, consisting of 16 genes participating in plant hormone signal transduction, 8 genes in calcium signaling pathway, and 5 genes in MAPK signaling pathway (Table 3 Suppl.). Five genes encoding antioxidant proteins were significantly up-regulated after osmotic stress, including one *glutathione peroxidase* (*GPX*) gene, one *glutathione S-transferase* (*GST*) gene, and three *peroxidase* (*PER*) genes (Table 4 Suppl.). There are 7 DEGs related to osmotic stress in *P. massoniana*, including one down-regulated *proline dehydrogenase* (*PRODH*) gene involved in proline degradation; conversely, the six up-regulated ones comprised one *trehalose-phosphate phosphatase D* (*TPPD*) gene associated in trehalose metabolism, one *sucrose synthase* (*SUS1*) gene, one *beta-amylase 1* (*BAM1*), and one *glucan endo-1,3-beta-glucosidase 2* (*E132*) gene involved in starch and sucrose metabolism, and two genes encoding aquaporins (Table 5 Suppl.). Further, seven of the significantly up-regulated genes encoded late embryogenesis abundant (LEA) proteins (Table 6 Suppl.), and seven genes encoding pentatricopeptide repeat-containing proteins were found down-regulated under osmotic stress (Table 7 Suppl.).

Transcription factors (TFs) play key roles in plant responses to abiotic stresses by regulating specific downstream genes. In this study, 492 TFs were found differentially expressed under osmotic stress, of which 300 genes were up-regulated and 192 genes were down-regulated. The ERF, WRKY, MYB, NAC, and bHLH were the main TF families involved, accounting for nearly 50 % of the total TFs identified (Fig. 7 Suppl). These results showed that ERF, WRKY, MYB, NAC, and bHLH play leading roles in *P. massoniana* response to osmotic stress.

Discussion

Previous experimental studies have shown that insufficient field moisture can significantly inhibit the growth of one-year-old *P. massoniana* seedlings, whose wilting became increasingly severe under a more pronounced drought stress condition (Du *et al*. 2018). Our results are similar to general finding, and we found that continuous osmotic stress caused wilting and cell death to seedlings. Nevertheless, when contending with abiotic stress, seedlings can regulate stress-related genes expressions to stimulate a wide range of metabolic and physiological responses (Qiu *et al*. 2017). Our study is the first to combine tree physiology and transcriptomic data to study the mechanisms of osmotic tolerance in *P. massoniana* seedlings.

Signal transduction under osmotic stress: Plant hormones, such as salicylic acid (SA), abscisic acid (ABA), and auxin in particular, reportedly play major roles in plant defense systems (Davies 1995). During the response to abiotic stresses, ABA is a key plant stress-signaling hormone that operates as the key signaling molecule regulating stress-responsive gene expression as part of ABA-dependent signaling pathways (Zhu 2002, Yoshida *et al.* 2014). We found that the expression of an ABA synthesis related-gene (9-*cis*-epoxycarotenoid dioxygenase, *NCED3*) was significantly up-regulated under osmotic stress, a result indicating it plays an important role in regulating ABA biosynthesis and accumulation in *P. massoniana* seedlings facing abiotic stress. Research has shown that when plants are exposed to abiotic stress, the increased ABA content results in its binding to pyrabactin resistance (PYR/PYL), to interact with the negative regulator type 2 C protein phosphatase (PP2C) to form a temporary complex (ABA-PYR/PYL-PP2C) (Sah *et al*. 2016). In response to abiotic stress, ABA appears to trigger the activation of Sucrose nonfermenting 1 related protein kinase 2 (SNRK2) *via* the ABA receptor complex (ABA-PYR/PYL-PP2C) (Sah *et al.* 2016); then, SNRK2 positively regulates downstream genes such as LEA protein genes, *via* the operation of transcription factors (TFs) (Yasunari *et al*. 2009). In this study, a gene encoding PYL was significantly up-regulated and three genes encoding PP2C were notably repressed; meanwhile,

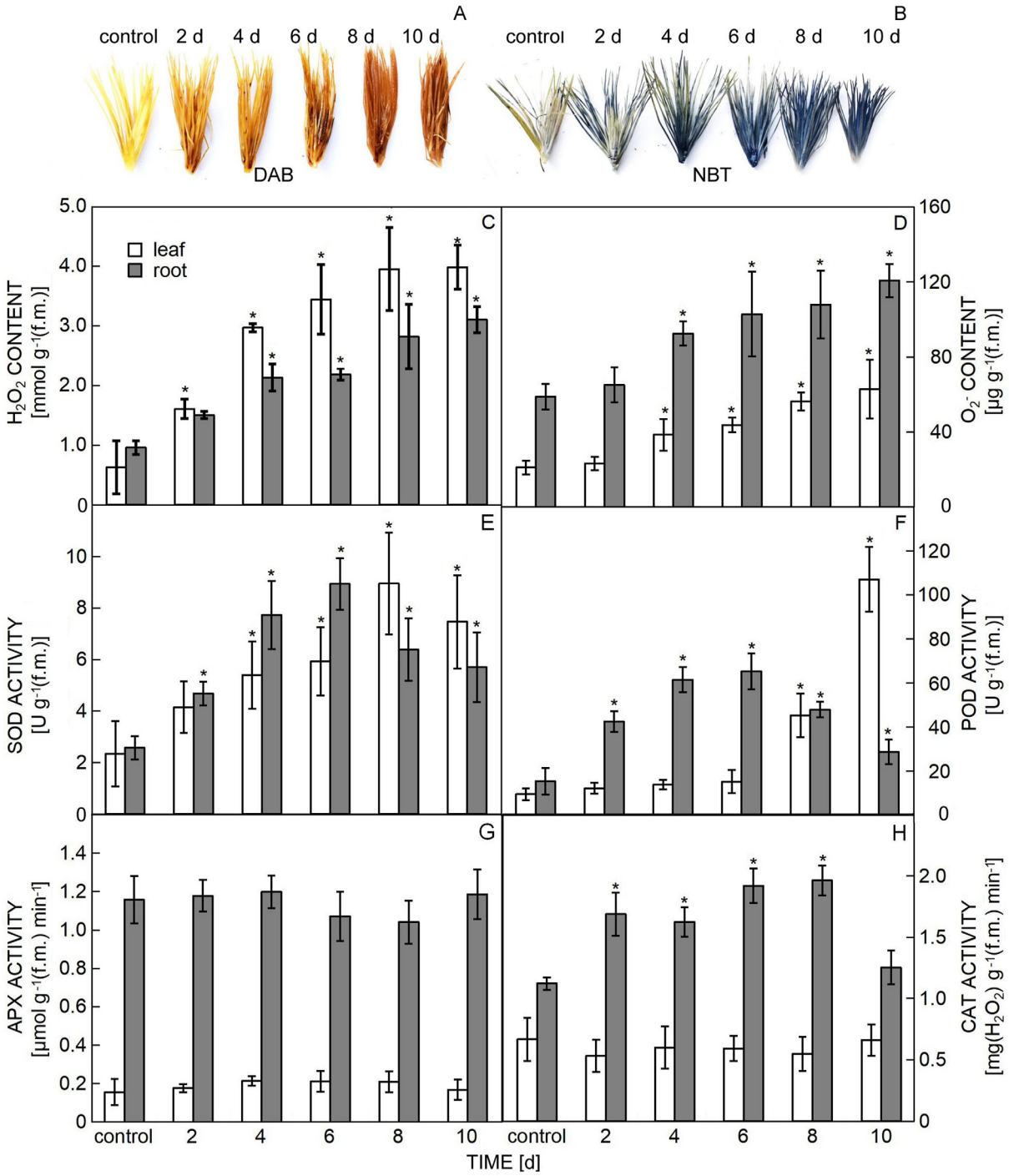

Fig. 3. Analysis of ROS content and ROS scavenging capability. *A*, *B* - DAB and NBT staining to detect of H_2O_2 (*A*) and $O_2^{\cdot-}$ (*B*), respectively; *C*, *D* - Measurement of H_2O_2 (*C*) and $O_2^{\cdot-}$ (*D*) content, respectively; *E-H* - Determination of antioxidant enzyme activity, SOD activity (*E*), POD activity (*F*), APX activity (*G*), CAT activity (*H*). Means ± SEs, $n = 3$ independent biological replicates, * indicates significant differences at $P < 0.05$ compared with the control. Treatment time was used as a variable, leaf or root was analyzed separately by one-way *ANOVA*.

a gene encoding SNRK2 was obviously up-regulated. These results are similar to previous findings (Du *et al.* 2018, Ye *et al.* 2018). These works suggested that the NCED3 in *P. massoniana* seedlings could regulate ABA biosynthesis to induce the dual expression of *PYL* and *PP2C* thereby activating SNRK2 activity to regulate the downstream osmotic stress-related genes, which stimulated the physiological changes needed for seedlings to survive under osmotic stress.

In this study, 54 ethylene signaling pathway-related genes, namely *ERF* (*ethylene-responsive transcription factor*) genes, were significantly up-regulated. ERFs are

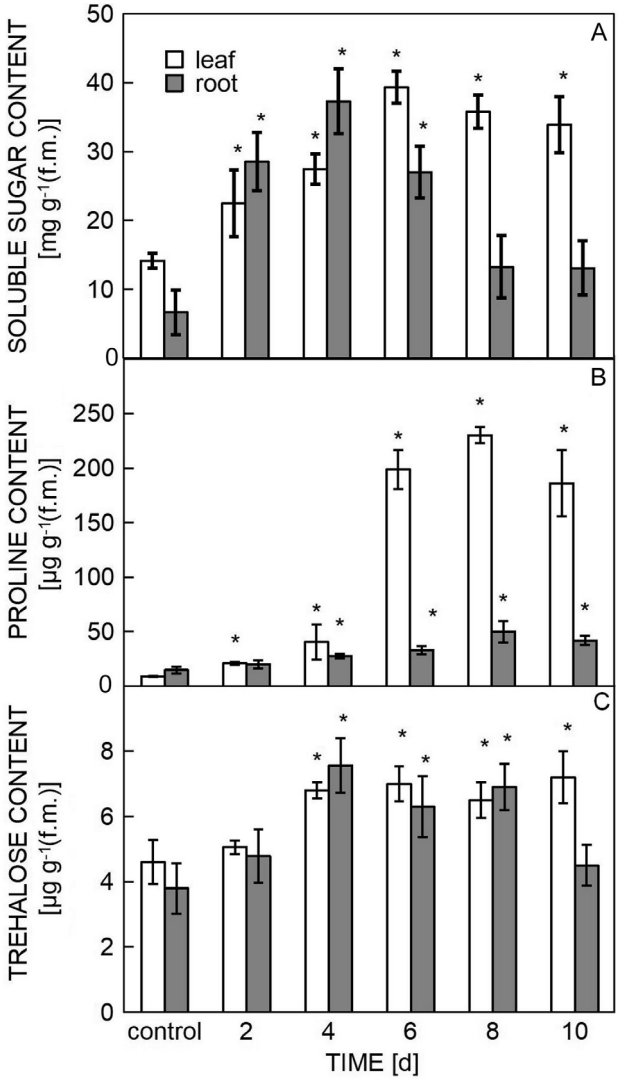

Fig. 4. Analysis of soluble osmolytes in Masson pine seedlings under osmotic stress conditions. *A* - Measurements of soluble sugar content; *B* - Proline content analysis; *C* - Determination of the trehalose content. Means ± SEs, $n = 3$ independent biological replicates, * indicates a significant differences at $P < 0.05$ compared with the control. Treatment time was used as a variable, leaf or root was analyzed separately by one-way *ANOVA*.

plant-specific transcription factors. ERFs are downstream regulatory factors of ethylene-signaling pathway, which influence ethylene and stress-responses. Most findings on the ERFs functions were from the studies of plant responses to various abiotic stresses, such as drought and salinity (Klay *et al*. 2018). Previous study found that ERFs could specifically bind to the GCC-box and/or dehydration-responsive element/C-repeat (DRE/CRT) *cis*-acting elements to regulate the downstream gene expression, such as ethylene-inducible pathogenesis-related (*PR*) genes and abiotic stresses-inducible genes (Allen *et al*. 1998). The results showed that ethylene-signaling pathway plays important roles in osmotic stress resistance of *P. massoniana*. In plants, crosstalk between ethylene and other phytohormones occurs coordinately and regulates various processes (Swarup *et al*. 2002). Meanwhile, research has found that ABA could interact with other hormones to improve the abiotic stress of plants. Rowe *et al*. (2016) found that ABA regulates root growth under osmotic stress conditions *via* an interacting hormonal network with cytokinin, ethylene, and auxin. In this study, we also found that a range of jasmonate-related, cytokinin-related, and auxin-related genes, namely *JAZ* (*jasmonate ZIM domain-containing protein*), *CKK* (*cytokinin dehydrogenase*), and *AUX1* (*auxin influx carrier*) were also significantly up-regulated. Further studies are needed to verify whether there is a cooperation among different plant hormone signaling pathways to improve osmotic resistance in *P. massoniana* seedlings.

Ca^{2+} is one of the major secondary messengers involved in many different signaling pathways (Dodd *et al*. 2010). Changes to the intracellular Ca^{2+} content are well recognized to act as cell signals that link various environmental stimuli to appropriate ecophysiological responses in plants. Calmodulin-like proteins (CMLs), calmodulin (CaM), and calcineurin B-like proteins

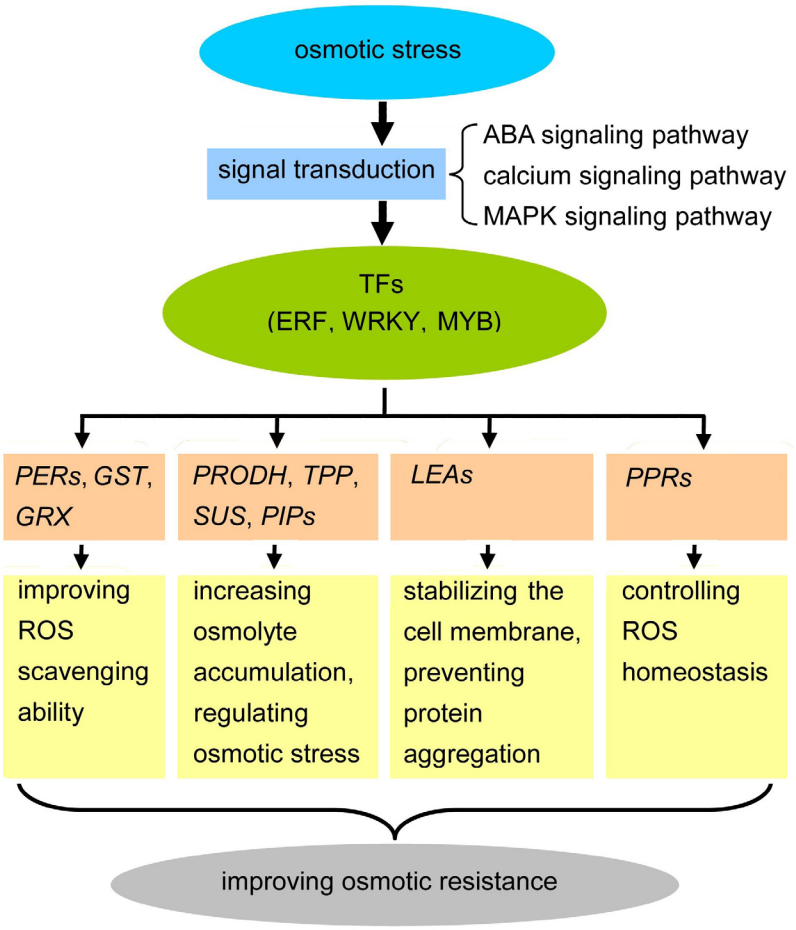

Fig. 5. A proposed model of the osmotic-stress response in *P. massoniana.*

(CBLs), act as Ca^{2+} sensors in plants and are known to be involved in various stress reactions. In this study, two *CaM,* one *CML2*, and one *CBL2* were up-regulated after the imposed osmotic stress, suggesting that those gene products could contribute to a coordinated osmotic stress response in *P. massoniana* seedlings. These results are consistent with previous studies. For instance, Xu *et al.* (2011) found that the expression of *OsMSR2* (*Oryza sativa multi-stress-responsive gene 2*), a novel calmodulin-like protein gene, enhances tolerance to high salt and drought conditions in *Arabidopsis* plants. Similarly, *Arabidopsis CBL5*-overexpressing plants display enhanced tolerance to high salt or drought stress (Cheong *et al.* 2010), and Wan *et al.* (2012) found the *CBP60g* overexpression lines show hypersensitivity to ABA and enhanced tolerance to drought stress.

Mitogen-activated protein kinase (MAPK) cascades are highly conserved signaling modules in plants, in which they play central roles in both developmental and environmental signal transduction (Singh and Jwa 2013). According to our results, two *MPKs* genes were significantly induced by osmotic stress, a result also agrees with previous research in other plant species. For instance, the overexpression of tomato *SpMPK3* in *Arabidopsis* increases its tolerance to osmotic stresses (Li *et al.* 2014). The ectopic expression of *GhMPK6a* in *Nicotiana benthamiana* reduces osmotic tolerance, with elevated malondialdehyde content, higher reactive oxygen species content, and lower abscisic acid content than in wild-type plants (Li *et al.* 2013). Considered together, these results suggested that the MAPK signaling pathway is instrument in the plant defense response to osmotic stress.

ROS-scavenging under osmotic stress: Under one or more various abiotic stresses, plants rapidly accumulate ROS, such as H_2O_2 and $O_2^{\cdot-}$. An excess of ROS can lead to oxidative damage and cause lesions and mutations and impair cellular components, resulting in metabolic dysfunction and finally cell death (Das and Roychoudhury 2014). Here, we found that ROS evidently accumulated and led to cell damage in the osmotic-treated *P. massoniana* seedlings. As a survival mechanism, an effective ROS scavenging capability is essential for plants to quickly adjust to temporary osmotic stress. Previous studies have demonstrated that POD, SOD, CAT, APX and glutathione transferase (GST), the main ROS-scavenging enzymes, could be highly induced in plants under abiotic stresses (Mittova *et al.* 2002, Rios-Gonzalez *et al.* 2002). In our study, POD activity was clearly enhanced in *P. massoniana* seedlings, meanwhile, *PER53* and *PER1* genes encoding

peroxidase were induced significantly by the osmotic stress, suggesting that these genes might make main contributions to the increased POD activity. SOD and CAT activities were improved in *P. massoniana*. However, no related *SOD* and *CAT* genes was highly expressed in this transcriptome data on 4th day of osmotic stress treatment, maybe more genes associated with antioxidant enzyme activities need to be discovered. Meanwhile, one *GST* gene and one *GPX* was obviously up-regulated, the result suggested that glutathione S-transferase and glutathione peroxidase activity might also play a part in ROS scavenging in *P. massoniana.* Further works need to verify if other antioxidant enzyme activities except for SOD, POD, and CAT enzymes, may contribute to osmotic stress resistance in *P. massoniana* tree seedlings.

Osmolyte accumulation and aquaporin gene expressions under osmotic stress: Plants are able to tolerate abiotic stresses by accumulating high-affinity osmotic substances, such as proline, sucrose, betaine, and trehalose (Yasar *et al.* 2006, Ben Ahmed *et al.* 2009, Wang *et al.* 2012). In our study, several genes associated with osmolyte synthesis were found differentially expressed. These results are in line with previous findings. For instance, *PRODH* is significantly down-regulated when *Betula platyphylla* plants are exposed to salt and osmotic stress (Zhang *et al.* 2016). Mijiti *et al.* (2017) reported that *TPP* (trehalose-6-phosphate phosphatase) genes related to trehalose biosynthesis are strongly induced by imposed salt stress. Sucrose synthase genes involved in sucrose metabolism are up-regulated by salt stress in Chinese cabbage (Qiu *et al.* 2017). Furthermore, the content of soluble sugar, proline, and trehalose were all significantly increased after osmotic stress. The gene expression results were consistent with the physiological responses recorded in our experiment with tree seedlings. Hence, it may be that DEGs involved in osmolyte metabolism drive osmolyte accumulation to enhance the osmotic potential of plant cells, thereby improving osmotic stress resistance in *P. massoniana*.

Besides osmotic regulation, the aquaporins (AQPs), membrane-bound pore-forming proteins, play key roles in water balance of plants (Moshelion *et al.* 2015). Differential expression of AQP proteins has been observed and reported on, and modulating AQP expression in transgenic plants can enhance their resistance to various abiotic stresses (Liu *et al.* 2013, Ayadi *et al.* 2019). For instance, overexpression of a wheat aquaporin gene, *TdPIP2;1*, increases salt and drought tolerance *via* a reduction in transpiration rates achieved through stomatal closure (Ayadi *et al.* 2019). Under salinity stress, the expression of *OsPIP1;1*, a rice aquaporin gene, increases in leaves but it is reduced in roots (Liu *et al.* 2013). In this study, two genes encoding AQPs, one up-regulated and one down-regulated, were differentially expressed under osmotic stress treatment. However, we need further studies done to understand the specific functions of AQPs in *P. massoniana* in its response to osmotic stress.

Late embryogenesis abundant (LEA) genes responsive to osmotic stress: Late embryogenesis abundant (LEA) proteins, which are important for abiotic stress tolerance, stabilize the cell membrane and serve as molecular chaperones or a shield, to prevent irreversible protein aggregation caused by abiotic stress, thereby protecting the plant from damage and injury (Serrano *et al.* 2003). Overexpression of *TaLEA3* increases drought resistance by inducing rapid stomatal closure (Yang *et al.* 2018), and overexpressing the *LEA Rab28* gene increases water stress tolerance of transgenic maize plants (Amara *et al.* 2013). In our study, seven DEGs encoding LEA proteins were significantly up-regulated, suggesting that the high expression of these LEA genes may contribute to improving osmotic stress resistance in *P. massoniana* seedlings.

Pentatricopeptide repeat-containing protein (PPR) is responsive to osmotic stress: The pentatricopeptide repeat (PPR) proteins are involved in various RNA processing in these organelles, such as RNA splicing, editing, 5′/3′ end processing, stability, cleavage, and translation (Meierhoff *et al.* 2003, Williams and Barkan 2003, Lurin *et al.* 2004). The PPR proteins also play diverse and critical roles in plants' developmental processes and their responses to environmental stresses. For example, the rosette leaves of *svr7-4*, a new mutant allele of *SVR7* encoding PPR proteins, accumulate more ROS and exhibit lower photo-oxidative stress tolerance (Lv *et al.* 2014). In other work, a cytosol-nucleus dual-localized PPR protein, SOAR1, likely regulates plant stress responses at least partly by integrating ABA-dependent and independent signaling pathways (Jiang *et al.* 2015). The *wsl* mutants (*WSL* encodes a PPR protein in rice plants) display high sensitivity to ABA, salinity, and sugars, and such individuals accumulate more H_2O_2 than wild-type counterparts (Tana *et al.* 2014). Inactivation of *PGN* results in susceptibility to ABA and loss-of-*PGN* functioning dramatically enhances ROS accumulation in seedlings in response to salt stress (Laluk *et al.* 2011). By examining previous studies, we found that PPR proteins are closely linked to ROS metabolism and ABA signaling in plant responses to abiotic stresses. In our study, we uncovered eight genes encoding PPR proteins that were differentially expressed, of which seven were down-regulated and just one up-regulated. We therefore speculate these *PPR* genes may function in the regulation of ROS homeostasis and participate in ABA signaling network during *P. massoniana* stress responses. Yet further studies are required to verify this in the future.

Transcription factors induced by osmotic treatment: TFs are the proteins that bind to specific DNA sequences in promoters to regulate the expression of downstream genes contributing to specific physiological processes and phenotypes. The ERF, WRKY, MYB, NAC, and bHLH protein families, as the main TFs in plants, play vital roles in their response to abiotic stress. These TFs can regulate many stress-responsive genes independently or dependently on ABA signaling. For example, OsERF71 in upland rice regulates the expression of several ABA-

responsive and proline biosynthesis genes under drought stress, resulting in enhanced sensitivity to an exogenous ABA treatment and proline accumulation (Li *et al.* 2018). ThCRF1 from *Tamarix hispida* could bind to the GCC-box and DRE and TTG motifs to regulate the expressions of stress-responsive genes, such as *P5CS*, *TPP*, *SODs* (encoding superoxide dismutase) and *PODs* (encoding peroxidase), which generally lead to elevated proline and trehalose content and enhanced SOD and POD activities to better adjust the osmotic stress (Qin *et al.* 2017). Overexpression of the *MuWRKY3* TF gene lead to higher free proline and total soluble sugar content, and it elicites a greater activity of antioxidant enzymes when compared with wild-type plants under drought stress. Moreover, a series of stress-related *LEA*, *HSP*, *MIPS*, *APX*, *SOD*, and *CAT* genes are found up-regulated in transgenic groundnut plants (Kiranmai *et al.* 2018). *AtDIV2,* an R-R-type MYB TF, plays negative roles in salt stress and is required for ABA signaling in *Arabidopsis* (Fang *et al.* 2018). In *Arabidopsis thaliana*, the *bHLH122* gene is highly induced by drought, as well as by NaCl and osmotic stresses, but not by an ABA treatment, and it could bind directly to the G-box or E-box *cis*-elements to regulate abiotic stress-responsive genes (Liu *et al.* 2014). Expression of *OsNAC5* in rice is induced by abiotic stresses such as drought, cold, high salinity, and also by ABA, and it improves the stress tolerance of rice by up-regulating the expression of stress-inducible rice genes such as *OsLEA3* (Takasaki *et al.* 2010). Here, we identified 300 differentially expressed TFs based on transcriptome data, and most of them were enriched in ERF, WRKY, and MYB families. Importantly, by combining the transcriptome and physiological data, we identified several signal transduction genes and stress-responsive genes associated with ROS scavenging and osmolytes synthesis. Accordingly, we speculate these TFs' involvement may be crucial for regulating these stress-responsive genes to cause plant physiological changes that improve the osmotic resistance of *P. massoniana* tree seedlings.

Conclusions

Based on our data and suite of previous results, a proposed model for osmotic resistance in *P. massoniana* is depicted in Fig. 5. Osmotic stress, as a key environmental stress factor, activates the signal transduction pathway, leading to transcriptional activation of TFs. These activated TF proteins regulate a serial of genes involved in abiotic stress tolerance, such as *PERs*, *GST*, *GRX*, *PRODH*, *TPP*, *SUS*, *PIPs*, *LEAs*, and *PPRs*. The altered expression of these genes is responsible for physiological changes, including an improved ROS-scavenging ability and osmolyte accumulation, increasing the expression of PPR genes involved in regulating ROS homeostasis and enhancing the expression of LEA genes, which may play a role in stabilizing the cell membrane and serving as molecular chaperones to prevent protein aggregation caused by stress. These physiological changes could then enhance osmotic stress resistance in this tree.

Data Availability: The raw sequencing data are available at NCBI:PRJNA648715 (https://dataview.ncbi.nlm.nih.gov/object/PRJNA648715?reviewer=fvm8c1pf4clpn3ukbcmp82ut1g)

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