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Transcriptome profiling in *Salix matsudana* during refilling xylem vessels after embolism

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Abstract

The objective of this study is to understand the role of bark water uptake in refilling of xylem vessels after embolism in *Salix matsudana.* Further, we determined expression of genes closely related to xylem embolism repair. The isolated branch segments of *S. matsudana* were soaked in deionized water and after 2 h, the micro-computed tomography (CT) images, volume of xylem sap, osmotic potential of xylem sap, and the content of starch and soluble sugar were measured. At the same time, *Illumina* high-throughput sequencing (Hi Seq TM) was applied to obtain the xylem transcriptome profiling. The results showed that the soaked branches had 58.86 % fewer embolized vessels than branches before soaking. The volume of xylem sap was increased by 21.51 % in comparison with initial sample and the osmotic potential (Ψs) of xylem sap was decreased by 87.18 %. The content of ions and soluble sugars in the xylem sap significantly increased after soaking. Fourteen genes were clarified and found to have a significant correlation with osmotic potential of xylem sap. Our findings showed that water can enter the xylem vessels through the bark and can repair the xylem embolism in branches. The saccharides played an important role in osmotic potential difference formation and the genes *UXL1 (uridine diphosphate-xylose synthase 1), UGDH (uridine diphosphate-glucose 6-dehydrogenase), BGLU40 (β-glucosidase 40), PPE8B (pectinesterase/pectinesterase inhibitor), UG1 (uridine diphosphate-glucuronate 1)*, and *XYL4 (β -D-xylosidase 4)*) were the key genes to promote embolized vessels refilling.

Additional key words: bark water uptake, embolism repair, micro-computed tomography, osmotic potential, soluble sugars.

Introduction

Woody plants have a developed water transport system that can efficiently transport water from the soil to the leaves, which meets the leaf transpiration demand for water (Meinzer and McCulloh 2013). According to the cohesion-tension theory, the water is transported upward in xylem vessels in the form of a water column under negative pressure where the water is in a metastable state. When the negative pressure of the xylem exceeds a certain threshold, air seeds will be sucked in functional vessels. Afterward, embolisms form, which hinders xylem water transport (Tyree and Zimmermann 2002, Martorell *et al.* 2014). Embolism reduces the hydraulic conductivity of xylem, causes the stomata closure, blocks photosynthesis, decreases productivity yields, and even causes the death of the whole plant (Hacke and Sperry 2003, Urli *et al.* 2013).

For a long time, it was speculated that embolism repair is driven by root pressure, which is approximately 0.1 MPa. However, this only plays a role in a woody plant of no more than 10 m height. For the higher woody plants, the embolism *in situ* repair of distal branches is important (Zwieniecki and Holbrook 2009, Wheeler *et al.* 2013). The formation of root pressure requires a low transpiration rate and high available water in the soil. Additionally, it can be easily limited by the root activity. Numerous experiments

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Abbreviations: BGLU40 - hydroxyisourate hydrolase family protein; CT - computed tomography; DEGs - differently expressed genes; PLC - percentage of loss of conductivity; PPE8B - pectinesterase inhibitor PPE8B; UGDH - UDP-glucose dehydrogenase; UG1 - UDP-D-glucuronate 4-epimerase 6 family protein; UXL1- UDP-xylose synthase 4 family protein; XYL4 - β-glucosidase family protein; Ψs - osmotic potential.

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proved that embolization occurs frequently and it is not restricted by space-time factors (Tyree *et al.* 1999). In recent years, more and more experiments have proven that the establishment of different osmotic potential in and out of the embolism vessels is the main driving force for stimulating water flow toward embolism vessels. Therefore, synchronous repair *in situ* is very important for maintaining the continuity of water conduction and ensures the gas exchange of the leaf.

As pointed out by several studies, plants can take up water not only *via* the root system but also *via* the leaves and branches (McCulloh *et al.* 2010). In *Pinus contorta*, water uptake *via* branches during the winter leads to a recovery of embolism induced in winter (Limm *et al.* 2009). Furthermore, previous research found that the percentage loss of conductivity (PLC) is approximately 35 % in *Pinus albicaulis* and 25 % in *Larix lyallii* after snow fall in the winter, which were significantly lower than that of before snow fall. A significant decrease in PLC was also observed in branches of *Picea abies* trees in foggy weather (Mayr *et al.* 2002). Furthermore, transcriptome studies have shown that specific genes are expressed during the recovery of embolism (Secchi and Zwienieki 2010, Secchi and Zwieniecki 2011, Perrone *et al.* 2012, Brodersen *et al.* 2013). Therefore, embolism repair not only requires the supply of external water but also depends on the driving force of water transport to the embolized vessels. The driving force for water shifts may be created by the degradation of starch to osmotically active sugars. However, water transport efficiency, the ability to repair embolism, and the hydraulic mechanism may be different between coniferous and broadleaf species due to their different xylem water transport structures. After the leaves of deciduous trees fall off in winter, deciduous species can repair embolism by directly absorbing water from bark. It is essential to know the key genes that are involved in the radial water supply in the embolism repair process.

Salix matsudana is not only an important fast-growing timber, but it is also a tree used in farmland protection and soil and water conservation in northern China. It has a strong adaptability to drought, water flooding, salinity, and other environmental constrains. In this study, the segments excised from the branches with 50 % conductivity loss were taken and the ratio of xylem sap volume and the xylem sap osmotic potential before and after soaking were investigated. In order to explore the key genes involved in the embolism refilling *via* bark water uptake, the transcriptome profiling of xylem during the refilling process was revealed.

Materials and methods

Samples used in this study were collected from three *Salix matsudana* Koidz. trees (~4 m tall and ~0.1 m diameter) naturally growing in the experimental station of the Chinese Academy of Forestry. Small branches (~1 m in length and 5 to 10 mm in diameter) were randomly selected from low hanging limbs (~2.5 m above ground). The selected branches had over 30 cm long segments with no leaves, which ensured full coverage with bark. Branches were collected from limbs facing the same aspects around the trees. The branches were naturally dehydrated to P_{50} (water potential at 50 % loss of conductivity) on the experimental table (according to the preliminary experimental results, the P_{50} was -1.9 ± 0.05 MPa). After dehydration, we cut each branch into two segments (50 mm length) under water. The upper segment I was used as an initial control (pre-soaking), and measured immediately after excision from the branch. The lower segment II was quickly sealed at both ends with a siliconized acrylic caulk and placed in a container containing 5 dm^3 of deionized water (Limm *et al.* 2009). After 2 h of soaking, segment II was removed from the water bath and measured. Three independent biological replicates were analyzed for each treatment.

The xylem sap was extracted by centrifugation of segments after removing the bark at 12 000 *g* for 1 min. Then, ~3 mm off of the segment was cut, spun again, and this procedure was repeated until the entire segment was cut and spun (Earles *et al.* 2015).

Osmotic potential of the xylem sap was determined by *Osmomat 030* (*Gonotech*, Berlin, Germany). The osmotic potential was calculated as $\Psi s = -icRT$ where i refers to the dissociation coefficient of solute, c to the concentration of solutes, R to the gas constant, and T to the temperature.

For determination of xylem sap soluble sugar content, 0.05 cm^3 of the diluted extracted xylem sap (×25) was mixed with 0.15 cm^3 of 98 % (m/v) sulfuric acid with 0.1 % (m/v) anthrone and placed in a 96 micro-plate well on ice (< 4 °C) for 10 min. Then, the plates were heated at 100 °C for 20 min and allowed to equilibrate to room temperature for 20 min (*ca*. 20 °C). The sugar content was determined as glucose equivalent from the colorimetric reading (*Thermo Scientific Multiskan*, Waltham, USA) at absorbance of 620 nm (A_{620}) and calculated according to a standard curve.

Sections 30 µm thick were obtained from each segment, rinsed with distilled water, and then immersed in a Lugol solution (iodine-potassium iodide) for 1 min, which stained the starch dark blue. Sections were again rinsed with distilled water to remove excess staining and observed under a microscope (*Laborlux S-Leitz Esselte*, *Leitz*, Stuttgart, Germany) equipped with a digital camera (*Leica DC300F*, Leica, Solms, Germany).

To determine starch and soluble sugar content in the xylem cell, the xylem tissue (0.1 g) was scraped by a scalpel after the xylem was dried**.** The content of soluble sugar was measured by using the *Plant Soluble Assay* kit

(*Solarbio*, Beijing, China). The content of starch was measured by using the *Plant Starch Assay* kit (*Solarbio*).

For gene expression analysis in xylem cells, the bark of the segment was peeled and the cambium was scraped with a scalpel. The xylem was frozen with liquid nitrogen and sent to *Majorbio* (Shanghai, China) for RNA extraction and sequencing. Further, total RNA was isolated from xylem using an *RNeasy* plant mini kit (*Qiagen*, Berlin, Germany). The first-strand cDNA was synthesized using the *PrimeScriptTM RT* reagent kit (*TaKaRa*, Dalian, China) and real-time qPCR was performed in a *Roche Light Cycler 480* (*Roche Applied Science*, Penzberg, Germany) according to the protocol described by Wang *et al*. (2017). The relative expressions were determined using the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen 2001). The sequences of all primer used in this study are shown in Table 1 Suppl. The *UBQ-L* gene was used as a reference gene.

Three biological replicates per treatment and four technical replicates were analyzed. Statistical analysis of data was processed using analysis of variance (*one-way ANOVA*) and differences between treatments were assessed by the Tukey-test using *SPSS 19.0* statistics software (*SPSS*, Chicago, USA). Differences between treatments were considered significant if $P < 0.05$.

Results

Micro-computed tomography (micro-CT) analysis provided indirect evidence that water entered the bark and decreased the percentage of embolized vessels. The initial branch segment, which was not soaked prior to scanning, had more embolized vessels than that soaked in water. It showed that soaked branches had 58.86 % fewer embolized vessels than un-soaked branches. In order to demonstrate that water enters the xylem vessels, the volume of xylem sap was measured. The initial xylem sap volume of the segment was 0.13 ± 0.001 $mm^3 \cdot mm^{-3}$ and it was increased by 21.51 % after soaking for 2 h (Fig. 1). Thus, the bark can absorb water and some of this water was transported into the xylem vessels for repair of the embolized vessels. According to the theory of refilling, the consumption of saccharide storage in xylem parenchyma cells causes the radial movement of sugar and water through the xylem ray cells, which forms different osmotic potential inside and outside the embolized vessels. Our study found that the initial Ψs of xylem sap was 0.07 ± 0.05 MPa and it was decreased to -0.12 MPa after soaking for 2 h (Fig. 1). We could consider that establishing differential osmotic potential is the main driving force to move water flow to embolized vessels.

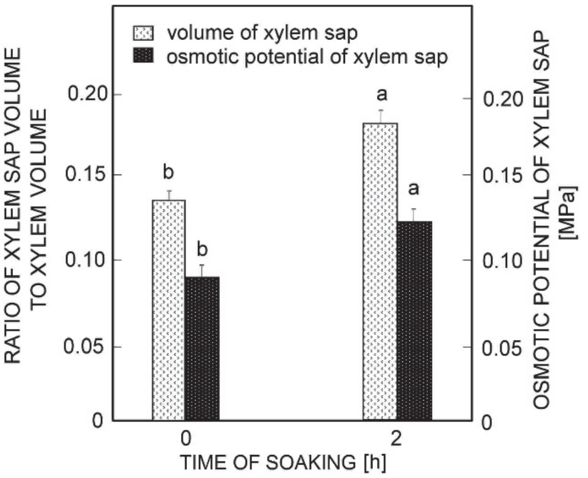

Fig. 1. Volume and osmotic potential of xylem sap. Means ± SEs, $n = 3$, different letters denote significant differences ($P < 0.05$).

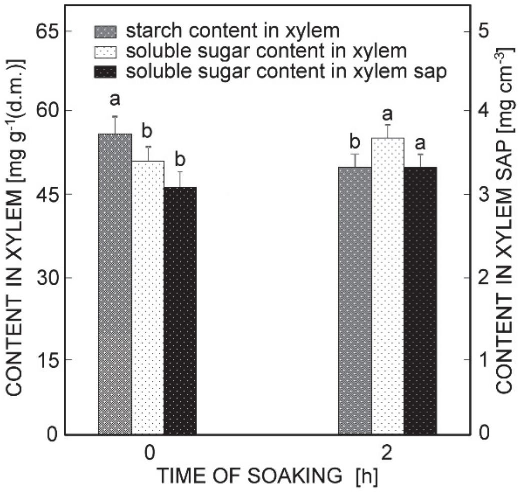

Fig 2. Content of starch and the soluble sugars in xylem and xylem sap. Means ± SEs, $n = 3$, different letters denote significant differences ($P < 0.05$).

Most of the starch granules were distributed around the vessels. We found that the content of soluble sugars in xylem sap was increased by 12.86 % after soaking for 2 h. The content of soluble sugars in xylem was increased by 15.52 % and the content of starch in xylem was decreased by 13.77 % after soaking. It is assumed that the changes in the osmotic potential of the embolized vessel might be derived from hydrolysis of the xylem starch.

The RNA from the initial samples and the samples after soaking was used to construct libraries for sequencing. The Q20 (the proportion of all bases with a mass value greater than 20) occupied 97.25 % of the overall bases and the GC content was approximately 46.05 % on average. Finally, a total of 55 077 uni-genes were obtained. Their length ranged from 201 to 16 872 bp and the average length was 937.86 bp (Table 1).

Table 1. Summary of the *S. matsudana* transcriptome (Q20 - the proportion of all bases with a mass value greater than 20).

Item	Number
Total number of unigenes	55077
Q20 [%]	97.25
GC content [%]	46.05
Largest length of unigenes [bp]	16872
Smallest length of unigenes [bp]	201
Average length of unigenes [bp]	937.86

A total of 55 077 uni-genes were obtained from the RNA-sequence. The differentially expressed genes (DEGs) were determined by applying the screening thresholds of false discovery rate (FDR) ≤ 0.05 and $|\log_2(2h/initial)| \geq 1$. Based on this analysis, a total of 1 986 significant DEGs were detected between initial and soaking samples. Among these DEGs, 1 293 genes were up-regulated and 693 genes were down-regulated. In the up-regulated genes, 879 genes satisfied the screening thresholds of FDR ≤ 0.01 and $|\log_2(2h/initial)| \geq 1$. Additionally, in the down-regulated genes, 362 genes satisfied the screening thresholds of FDR ≤ 0.01 and $|\log_2(2h/initial)| \geq 1$ (Fig. 1 Suppl.). Furthermore, we obtained GO functional annotation using the non-redundant (Nr) annotation. In total, we submitted 8 564 DEGs, which were categorized into 51 functional groups. The 51 functional groups were divided into three main categories including the biological process group (1 716, 20.04 %), the cellular component group (2 687, 31.38 %), and the molecular function group (4 161, 48.41 %) (Fig. 2 Suppl.).

We analyzed the saccharide metabolism path in the *KEGG* pathway (Fig. 3 Suppl.). We identified 20 significantly-expressed genes involved in saccharide metabolism during the process of refilling. There were 16 genes that showed significantly up-regulated expression and 4 genes showed significantly down-regulated expression after 2 h of soaking (Table 2). The up-regulated genes mainly encoding UDP-glucuronic acid decarboxylase, UDP-xylose synthase 4, UDP-glucos-6-dehydrogenase, sucrose synthase, *etc.* The down-regulated genes encoding β-amylase 1, UDP-D-glucuronate 4-epimerase 1, glycosyl transferases, and sucrose-phosphate synthase.

To test the validity of the data generated using the *Illumina* sequencing platform, qPCR analysis was performed. We analyzed the expression of six genes involved in saccharide metabolism upon embolized vessels refilling in xylem. The genes *UXS1*, *glgA*, *XYL4*, and *UGDH* were significantly up-regulated after 2 h of refilling. The genes *GAUT1* and *E2.4.1.13* were not significantly expressed in the process of refilling (Fig. 3). Therefore, the results of digital gene expression (DGE) analysis were overall reliable.

We analyzed the relationship between the 20 significantly-expressed genes involved in saccharide metabolism and the osmotic potential in the process of refilling. The results showed that there were 14 genes with a significant negative correlation with osmotic potential of xylem sap ($P < 0.05$) and seven genes that had a highly significant negative correlation with osmotic potential of xylem sap ($P < 0.01$) (Table 2 Suppl.). These genes mainly encoded UDP-glucuronic acid decarboxylase, UDP-xylose synthase 4, UDP-glucose-6-dehydrogenase, hydroxyisourate hydrolase, UDP-D-glucuronate 4-epimerase 6, β-glucosidase, and UDP-glucose dehydrogenase. We believe that the genes (*UXL1, UGDH, BGLU40, PPE8B, UG1,* and *XYL4*) were the key genes that promoted embolized vessel refilling through osmotic regulation. The up-regulation of these genes was important for transport the sugar to the embolized vessels and so for production of an osmotic gradient, which is used to drive the water into the embolized vessels.

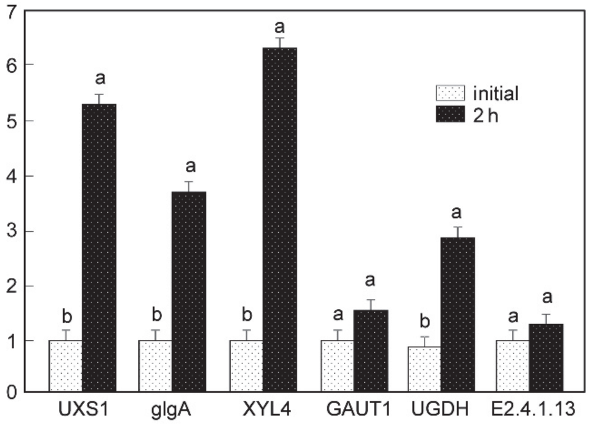

Fig. 3. Verification of digital gene expression results by qPCR. Means of quantitative gene expression data ± SEs, $n = 3$. The *UBQ-L* gene was used as reference gene (*UXL1- UDP-xylose synthase 4 family protein; glgA - glycogen synthase; XYL4- beta-glucosidase family protein; GAUT1- glycosyl transferase family 8;UGDH- UDP-glucose dehydrogenase;E2.4.1.13- sucrose synthase*).

Discussion

Embolism can cause the rapid decline in the hydraulic conductivity of xylem and hinder the normal growth of plants. The embolism will be repaired during rainy days or after irrigation. It is very important to study the repair mechanism of embolised vessels (Christman *et al.* 2012, Zwieniecki *et al.* 2013). Plants can directly absorb water

Table 2. Relative expression profile of genes involved in saccharide metabolism during embolized vessels refilling in xylem of branch segment of *Salix matsudana*. The fragments per kilobase million (FPKM) value of each unigene identified in the transcriptomes. The means of three independent biological samples. Dark grey/light grey refers to the up-regulated/down-regulated genes.

Gene name	Unigene ID	FPKM	Description
UXS1	c95333_g1	26.90	UDP-glucuronic acid decarboxylase
UXS1	c70619_g1	8.79	UDP-xylose synthase 4
UGDH	c55418_g1	9.66	UDP-glucose 6-dehydrogenase
BGLU40	c67131_g1	20.53	hydroxyisourate hydrolase
β-amylase	c69220_g1	0.03	β-amylase 1
E3.1.1.11	c68272_g1	7.03	pectinesterases
UG3	c62946_g1	0.17	UDP-D-glucuronate 4-epimerase 1
PPE8B	c66969_g1	20.91	pectinesterase inhibitor PPE8B
glgA	c65600_g1	3.96	glycogen synthase
UG1	c69293_g2	6.05	UDP-D-glucuronate 4-epimerase 6
PMEpcrA	c62377_g1	4.95	pectin methylesterase
XYL4	c70726_g1	8.20	β-glucosidase
sacA	c63969_g1	3.68	β-fructofuranosidase, insoluble isoenzyme CWINV1
HK	c70420_g1	3.18	hexokinase
E2.4.1.13	c41346_g1	72.10	sucrose synthase
GAUT1	c66251_g1	16.81	glycosyl transferase family 8
TPS	c95022_g1	0.27	glycosyl transferase family 20
E2.4.1.14	c70790_g1	0.37	sucrose-phosphate synthase
GAUT2	c66017_g1	3.07	galacturonosyltransferase 15
UGDH	c56919_g1	2.87	UDP-glucose dehydrogenase

from fog, snow, and rain which can promote the hydraulic recovery of leaves (Laur *et al*. 2013). The role of bark in the hydraulic recovery is only mentioned in some coniferous plants such as *Abies fabri* and *Picea asperata*, *e.g.*, the PLC of the branch of *Picea asperata* decreases from 86 to 29 % after soaking in water for five days (Mayr *et al*. 2014). The micro-CT is a non-destructive and non-invasive technique. Using this method, we found that the branches soaked in water for 2 h had 58.86 % fewer embolized vessels than un-soaked branches. To restore the xylem hydraulics function, the exogenous water was transported through the bark to enter the conduits to repair the xylem embolism. The future research should focus on clarification of the role of bark water uptake in the repair of plant embolism in a large range of time, because it is not clear whether prolonged immersion will affect the efficiency of embolization repair. The conduit structure and cellular anaerobic respiration may be affected by the soaking. Furthermore, we extracted xylem sap and found that the volume of xylem sap was increased by 21.51 % after soaking for 2 h. Previous studies also have shown that embolism can be recovered in a short time (Holtta *et al*. 2006).

The embolism repair is not spontaneous but it is a consequence of series of metabolic processes that promote water flow to embolized conduits. We found that the Ψs of xylem sap decreased after soaking. This suggests that the osmotic potential difference between embolized conduits and vessel associated cells provided the power for water refilling. Our study found that the content of starch slightly decreased in the xylem cell and the content of soluble sugars slightly increased in the xylem and xylem sap. This suggests that starch degradation in xylem cells might be the main reason of osmotic potential difference between the embolized vessels and their surroundings as previously suggested Salleo *et al*. (2006, 2009). Salleo *et al*. (2009) found that starch hydrolysis in parenchyma cells increases the swelling pressure of parenchyma cells and pushes water into the embolization conduits to promote the embolism repair. Bucci *et al*. (2003) found that plants with high soluble sugar content in petiole tend to have higher water conductivity and these soluble sugars are hydrolyzed from starch in parenchyma cells of the xylem vascular bundle. However, it is not clear what is the critical point initiating the conversion of starch to soluble sugar during this process. It is also unclear what kind of signal triggers the hydrolysis of starch. It has been found that acoustic vibrations caused by a bubble change are the main signal of embolism repair and the intensity of vibration can affect the conversion of starch into soluble sugars. However, the fate of these sugars is unknown. We cannot conclude whether they are used for respiration or they are further transported by xylem. As an important energy and carbon source in plants, soluble sugars are involved in many processes of metabolism. Drought stress hindered the normal distribution of saccharides and decreased the total carbon assimilation. Mild drought stress can increase the soluble sugar content of plants and improve the ability of water absorption and retention (Ameglio *et al*. 2004). At the same time, soluble sugar can maintain the specific structure and function of proteins.

The RNA-sequencing can provide reasonable data for comprehensive analysis of gene expression especially for species without complete genome sequencing. The *GO* analysis of *S. matsudana* confirmed that the expressions of genes associated with saccharide metabolism, DNA-dependent transcription factors, and cellular activity were increased during embolism repair. This aligns with findings of gene expression studies of water-stressed plants during the process of refilling (Maurel *et al*. 2008). From the *KEGG* analysis, we focused on 16 genes that showed significant up-regulation and 4 genes that showed significant down-regulation after 2 h of refilling. Secchi and Zwieniecki (2014) also found that the genes involved in carbon metabolism and aquaporin formation are upregulated during the refilling in the leaf petiole of the grape.

Conclusions

In this study, we showed the role of bark water uptake in promoting xylem embolism repair of *S. matsudana.* We studied the expression of genes in the process of refilling by RNA-sequencing. It was found that water can enter the xylem vessels through the bark and repair the embolized vessels. The increase of soluble sugar content in embolized vessels might create an osmotic gradient necessary for water transport and so refilling embolized vessels. During this process, most of the genes related to saccharide metabolism were up-regulated. Combined with correlation analysis, we conclude that the genes of *UXL1, UGDH, BGLU40, PPE8B, UG1*, and *XYL4* were the key genes to promote embolized vessel refilling.

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