Comparative proteomic analysis of thick-walled ray formation of *Haloxylon ammodendron* in the Gurbantunggut Desert, China

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Research article

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Abstract

Background

The thick-walled ray cells have been reported in *Haloxylon ammodendron* for the first time. This study measured the wall thickness of ray cells and performed a proteomic analysis of ray cell wall formation in the xylem of *H. ammodendron* using isobaric tags for relative and absolute quantitation.

Results

The wall thickness of ray cells in Jinghe (2.85 ± 0.42 μm) was significantly lower than that in Shihezi (3.08 ± 0.44 μm) ($P < 0.01$). In Shihezi, which has a thicker wall of ray cells than that in Jinghe, 795 differentially expressed proteins were upregulated. Phenylpropanoid biosynthesis, photosynthesis, glycolysis/gluconeogenesis, carbon metabolism, starch and sucrose metabolism, metabolic pathways, etc. promote ray cell wall biosynthesis of the xylem of *H. ammodendron* by providing substrates or energy. During the process of cell wall biosynthesis in the xylem of *H. ammodendron*, the nonspecific lipid-transfer protein and beta expansin EXPB2.1 (*Mirabilis jalapa*] first loosens the cell wall, followed by extension and expansion, and the xyloglucan endotransglycosylase/hydrolase 1 cleaves and links the xyloglucan chains. Then, photosystem I P700 apoprotein A1, reversibly glycosylated polypeptide 1 and GDP-mannose-3', 5'-epimerase, etc., are involved in cellulose, hemicellulose and pectin biosynthesis of the cell wall by providing components or energy. Finally, the proteins in phenylpropanoid biosynthesis promote the lignification of the ray cell wall and complete the biosynthetic process of the cell wall.

Conclusions

Phenylpropanoid biosynthesis, photosynthesis, glycolysis/gluconeogenesis, carbon metabolism, starch and sucrose metabolism, metabolic pathways, etc. promote ray cell wall biosynthesis of the xylem of *H. ammodendron* by providing substrates or energy. The results are important for improving the wood mechanical properties of timber plantations.

Background

Haloxylon ammodendron is an important afforestation species in the arid desert region both in Asia and Africa. Previous studies have found that the cell wall of ray cells was obviously thickened in the xylem of *H. ammodendron*, and the wall thickness of the ray cell can be up to 2.85 μm ~ 3.08 μm, which is 3 ~ 6 times the thickness of axial parenchyma and is slightly higher than that of fibre (2.64 μm ~ 2.97 μm) (Zhou and Gong 2017). Generally, ray tissue is composed of parenchyma cells, and the wall thickness of ray cells is much thinner than that of fibre cells in most species (Plavcová and Jansen 2015). However, in parenchyma, thick-walled ray cells have been reported in some species, such as *Melia azedarach* and *Symbolanthus macranthus* (Carlquist and Grant 2005). The mechanical properties will be enhanced when

tissues have thickened walls (Alves and Angyalossy-Alfonso 2002). Therefore, research on the regulatory proteins (genes) involved in the cell wall formation process will be important for both the environmental adaptability of the characteristic of xylem and improvement of wood quality in plantations.

The cell wall in higher plants consists mainly of cellulose, hemicellulose and lignin (Mellerowicz et al. 2001). The cell wall is mainly composed of polysaccharides. Both the primary wall and secondary wall contain cellulose and hemicellulose. The primary wall also contains pectin, enzymes and structural proteins; the secondary wall contains a small amount of proteins or pectin but generally contains lignin (Carpita and McCann 2000). The cell wall not only provides mechanical support and defence against pathogen invasion and nutrition stress but is also related to the physiological function of plant cells, such as material transport (Dhugga 2005). Moreover, photosynthates stored in plant secondary cell walls are important sources of fibre materials and raw materials of biomass energy (Huang and Li 2016), which play an important role in human survival and development.

Recently, with the development of genomics and molecular genetics, research on plant cell wall formation has made good progress. Research reports have mainly focused on *Arabidopsis thaliana* (Taylor et al. 2003), *Populus trichocarpa* (Suzuki et al. 2006), *Picea sitchensis* (Bong) Carr (Fernandes et al. 2011), and *Gossypium hirsutum* (Pear et al. 1996). Many achievements have been made in the regulatory mechanisms of cellulose, hemicellulose, lignin and pectin biosynthesis. Studies have shown that glucan chains in cellulose are synthesized by the cellulose synthase complex (CSC) on the plasma membrane and secreted into the extracellular space. The CSC is composed of three different types of cellulose synthase (CESA) catalytic subunits (Gonneau et al. 2014). The main chains of hemicellulose are synthesized by cellulose synthase-like (CSL), except for the xylan chain, and *Trigonella foenum-graecum* galactosyltransferase (TfGalT) was shown to be responsible for the synthesis of galactosyl side chains (Scheller and Ulvskov 2010). The main synthesis sites of hemicellulose and pectin are the Golgi apparatus, and the nucleotide sugar, which is the substrate of polysaccharose, is catalysed by CSL protein and glycosyltransferase and is synthesized mainly in the cytoplasm (Bar-Peled and O'neill 2011). The precursors needed for lignin biosynthesis are synthesized by the phenylpropanoid pathway, and deamination of phenylalanine to cinnamic acid initiates this process. All of these processes have been extensively studied (Mellerowicz et al. 2001). Although much work has been done in cell wall biosynthesis, the content and structure of cell wall components vary with species and tissues, which leads to diversity and complexity in cell wall composition (Burton et al. 2010). Therefore, the biosynthetic pathway of the cell wall in specific tissues of different species still needs to be studied.

Thus, the proteomic characteristics of ray cell wall formation in *H. ammodendron* were studied. GO annotation combined with KEGG pathway enrichment and other bioinformatics methods was used to explore the differentially expressed proteins and metabolic pathways related to the ray cell wall formation of this plant.

Methods

Sample location and sampling

The climate and growth characteristics of *H. ammodendron* plantation in the sample area were described by Zhou and Gong (2017). At the end of June 2017, samples were taken in Jinghe (82°53'35"E,44°36'10"N) and Shihezi (86°14'44"E，45°00'34"N) Desert Research and Experimental Station, Shihezi University, in the Gurbantunggut Desert, Xinjiang, China. The identification of *H. ammodendron* was performed according to the morphological characteristics in website (http://www.iplant.cn/info/Haloxylon%20ammodendron) built by Institute of Botany, Chinese Academy of Sciences. Perennial branches of *H. ammodendron* with a diameter of approximately 1 cm were collected. The bark, phloem and cambium were scraped from the branches, and a blade sterilized with anhydrous ethanol was used to scrape the xylem. The samples were wrapped in aluminium foil, placed in liquid nitrogen and quickly cooled, brought back to the laboratory and stored in a -80 ℃ refrigerator.

Measurement of indexes

Observation and measurement of the wall thickness of ray cells

Observations under a light microscope and measurements of the wall thickness of ray cells in *H. ammodendron* were performed as described in Zhou and Gong (2017).

Scanning electron microscopy (SEM): The xylem of *H. ammodendron* was cut into blocks of 1 cm x 1 cm x 1 cm, air dried and polished to a smooth surface. The samples were dehydrated using graded ethanol and dried using the liquid CO_2 critical point method. The sample was soaked in 98% H_2SO_4 for 5 min. The sample was attached to conductive tape, metal spraying was performed for 200 s, and then, the sample was observed by SEM.

Proteomic analysis

Protein Extraction: Samples (1 ~ 2 g) with 10% PVPP were ground into powder in liquid nitrogen and then sonicated on ice for 5 min in lysis buffer 3 (8 M urea and 40 mM Tris-HCl containing 1 mM PMSF, 2 mM EDTA and 10 mM DTT, pH 8.5) with 5-fold volumes of samples. After centrifugation at 25,000 rpm at 4 ℃ for 20 min, the supernatant was treated by adding 5-fold volumes of 10% TCA/acetone with 10 mM DTT to precipitate the proteins at -20 ℃ for 2 h/overnight. The precipitation step was repeated with acetone alone until there was no colour in the supernatant. The proteins were air dried and resuspended in lysis buffer 3 (8 M urea and 40 mM Tris-HCl containing 10 mM DTT, 1 mM PMSF and 2 mM EDTA, pH 8.5). Ultrasonication on ice for 5 min (2 sec/3 sec) was used to improve the protein dissolution. After centrifugation, the supernatant was incubated at 56 ℃ for 1 h for reduction and alkylated by 55 mM iodoacetamide (IAM) in the dark for 45 min. Fivefold volumes of acetone to samples were used to precipitate the proteins at -20 ℃ for 2 h/overnight. Lysis buffer 3 was used to dissolve the proteins with sonication on ice for 5 min (2 sec/3 sec).

QC of Protein Extraction: We separately added 0, 2, 4, 6, 8, 10, 12, 14, 16 and 18 μL of BSA (0.2 μg/μL) solution to a 96-well plate and then added 20, 18, 16, 14, 12, 10, 8, 6, 4 and 2 μL of pure water to the corresponding wells. We also prepared serial dilutions (20 μL each well) of the unknown sample to be measured. Then, 180 μL of Coomassie Blue was added to each well and mixed. The absorbance of each standard and sample well was read at 595 nm. Each sample had at least two duplicates. The absorbance of the standards vs. their concentrations was plotted. The extinction coefficient and the concentrations of the unknown samples were calculated.

We mixed 15 ~ 30 μg proteins with loading buffer in a centrifuge tube and heated them at 95 ℃ for 5 min. Then, the samples were centrifuged at 25000 rpm for 5 min, and the supernatant was added to the sample wells in a 12% polyacrylamide gel. SDS-PAGE was performed at constant voltage at 120 V for 120 min. Then, the gel was stained with Coomassie Blue for 2 h, destaining solution (40% ethanol and 10% acetic acid) was added and the gel was placed on a shaker (exchange destaining solution 3 ~ 5 times, 30 min a time).

Protein Digestion: The protein solution (100 μg) with 8 M urea was diluted 4 times with 100 mM TEAB. Trypsin Gold (Promega, Madison, WI, USA) was used to digest the proteins at a ratio of protein:trypsin = 40:1 at 37 °C overnight. After trypsin digestion, the peptides were desalted with a Strata X C18 column (Phenomenex) and vacuum-dried according to the manufacturer's protocol.

Peptide Labelling: The peptides were dissolved in 30 μL of 0.5 M TEAB with vortexing. After the iTRAQ labelling reagents were at ambient temperature, they were transferred and mixed with the proper samples. Peptide labelling was performed using the iTRAQ Reagent 8-plex Kit according to the manufacturer's protocol. The labelled peptides with different reagents were combined and desalted with a Strata X C18 column (Phenomenex) and vacuum-dried according to the manufacturer's protocol.

Peptide Fractionation: The peptides were separated on a Shimadzu LC-20AB HPLC Pump system coupled with a high pH RP column. The peptides were reconstituted with buffer A (5% ACN, pH 9.8) to 2 mL and loaded onto a column containing 5 μm particles (Phenomenex). The peptides were separated at a flow rate of 1 mL/min with a gradient of 5% buffer B (95% ACN, pH 9.8) for 10 min, 5% ~ 35% buffer B for 40 min, and 35% ~ 95% buffer B for 1 min. The system was then maintained in 95% buffer B for 3 min and decreased to 5% within 1 min before equilibrating with 5% buffer B for 10 min. Elution was monitored by measuring absorbance at 214 nm, and fractions were collected every 1 min. The eluted peptides were pooled as 20 fractions and vacuum dried.

HPLC: Each fraction was resuspended in buffer A (2% ACN and 0.1% FA in water) and centrifuged at 20,000 rpm for 10 min. The supernatant was loaded onto a C18 trap column at 5 μL/min for 8 min using an LC-20AD nano-HPLC instrument (Shimadzu, Kyoto, Japan) by the autosampler. Then, the peptides were eluted from the trap column and separated by an analytical C18 column (inner diameter 75 μm) packed in-house. The gradient was run at 300 nL/min starting from 8–35% of buffer B (2% H_2O and 0.1%

FA in ACN) in 35 min, increasing to 60% in 5 min, maintaining at 80% B for 5 min, and finally returning to 5% in 0.1 min, followed by equilibration for 10 min.

Mass Spectrometry Analysis: The peptides separated from nano-HPLC were subjected to tandem mass spectrometry Q EXACTIVE (Thermo Fisher Scientific, San Jose, CA) for DDA (data-dependent acquisition) detection by nanoelectrospray ionization. The parameters for MS analysis are listed as follows: electrospray voltage: 1.6 kV; precursor scan range: 350 m/z ~ 1600 m/z at a resolution of 70,000 in Orbitrap; MS/MS fragment scan range: >100 m/z at a resolution of 17,500 in HCD mode; normalized collision energy setting: 27%; dynamic exclusion time: 15 sec; automatic gain control (AGC) for full MS target and MS2 target: 3E6 and 1E5, respectively; the number of MS/MS scans following one MS scan: 20 most abundant precursor ions above a threshold ion count of 20000.

Bioinformatics: For peptide data analysis, raw mass data were processed using Mascot 2.3.02 (Matrix Science, London, UK) against a database (I-AZaGb004, 144985 sequences). The search parameters are shown in Table 1. Blast2GO software was used for the Gene Ontology (GO) analysis of differentially expressed proteins, and the protein functional categories were determined according to biological process, molecular function and cell component. An online database (http: www.genome.jp/kegg/) was used for the enrichment analysis of KEGG pathways for the differentially expressed proteins and detected the most significant pathways.

Table 1
The search parameters of Mascot

Item	Value
Type of search	MS/MS ion search
Enzyme	Trypsin
Fragment mass tolerance	0.05 Da
Mass values	Monoisotopic
Variable modifications	Oxidation (M), iTRAQ8plex (Y)
Fixed modifications	Carbamidomethyl (C), iTRAQ8plex (N-term), iTRAQ8plex (K)
Peptide mass Tolerance	20 ppm
False discovery rate (FDR)	< 0.01
Fold change	> 1.2
P value of significance test	< 0.05

Results

Wall thickness of ray cells in the xylem of *H. ammodendron*

The ray cell wall of the xylem in *H. ammodendron* showed an obviously thick wall structure (Fig. 1), and the wall thickness of *H. ammodendron* ray cells in Jinghe (2.85 ± 0.42 μm) was significantly lower than that in Shihezi (3.08 ± 0.44 μm) ($P < 0.01$).

Differentially expressed proteins

In total, 6767 peptides and 3076 proteins were identified with 1% FDR. Finally, repeat experiments defined differentially expressed proteins with a 1.2-fold change ($P < 0.05$). A total of 795 and 421 proteins were identified as upregulated and downregulated in Shihezi, respectively.

GO annotation for the identified proteins

The identified differentially expressed proteins were enriched in biological processes, cellular components and molecular functions using Gene Ontology. A total of 6,810 differentially expressed proteins functionally related to biological processes. The majority of functions belonged to the categories of metabolic process (24.92%), cellular process (20.73%), and single-organism process (18.43%) (Fig. 2). There were 6,685 proteins associated with cellular component function. The largest numbers of differentially expressed proteins were found in cells (23.08%), cell parts (23.08%) and organelles (16.92%) (Fig. 3). A total of 3317 differentially expressed proteins correlated with molecular function. The largest numbers of differentially expressed proteins were related to catalytic activity (47.45%), binding (39.16%) and structural molecule activity (3.65%) (Fig. 4).

Pathway annotation for the identified proteins

To better understand the pathways of differentially expressed proteins during the formation of ray cell walls in the xylem of *H. ammodendron*, we performed pathway annotation analysis for differentially expressed proteins based on the KEGG database. The results showed that a total of 9 pathways were related to ray cell wall synthesis in the xylem of *H. ammodendron*, such as phenylpropanoid biosynthesis, photosynthesis, glycolysis/gluconeogenesis, carbon metabolism, starch and sucrose metabolism, and metabolic pathways (Table 2).

Table 2
Significantly enriched pathways related to cell wall formation in H. ammodendron xylem

Number	Pathway	Different proteins with pathway annotation (1041)	Pathway ID
1	Photosynthesis	25 (2.4%)	ko00195
2	Starch and sucrose metabolism	36 (3.46%)	ko00500
3	Phenylpropanoid biosynthesis	34 (3.27%)	ko00940
4	Metabolic pathways	378 (36.31%)	ko01100
5	Amino sugar and nucleotide sugar metabolism	29 (2.79%)	ko00520
6	Glycolysis / Gluconeogenesis	39 (3.75%)	ko00010
7	Plant hormone signal transduction	7 (0.67%)	ko04075
8	Cysteine and methionine metabolism	18 (1.73%)	ko00270
9	Carbon metabolism	71 (6.82%)	ko01200

Discussion

The proteome related to ray cell wall formation in the xylem of *H. ammodendron* from the Gurbantunggut Desert was studied. A total of 3,076 proteins were identified, of which 795 proteins were significantly upregulated and 421 proteins were significantly downregulated in Shihezi, where the wall thickness of *H. ammodendron* ray cells was higher than that in Jinghe. Among the metabolic pathways involving differentially expressed proteins, phenylpropanoid biosynthesis, photosynthesis, glycolysis/gluconeogenesis, carbon metabolism, starch and sucrose metabolism, metabolic pathways, plant hormone signal transduction, cysteine and methionine metabolism as well as amino sugar and nucleotide sugar metabolism are related to ray cell wall synthesis (Table 2).

Metabolic pathways related to ray cell wall biosynthesis

Studies have shown that in poplar, more than 1,600 genes encode carbohydrate-active enzymes, and more than 34 genes are involved in phenylpropanoid biosynthesis and lignin biosynthesis. These results indicate the complexity of both cell wall biosynthesis and secondary xylem development. Phenylpropanoid biosynthesis and lignin biosynthesis are the key pathways in cell wall biosynthesis (Geisler-Lee et al. 2006). Studies utilizing reverse genetics and *Arabidopsis* mutants showed that phenylpropanoid biosynthesis is involved in cell wall biosynthesis and that carbohydrate-active enzymes are involved in polysaccharide biosynthesis (Brown et al. 2009). The biosynthesis of phenylpropanoid and lignin monomers has been well studied in poplar and *Arabidopsis* (Boerjan et al. 2003; Vanholme et al. 2008). The phenylpropanoid biosynthesis pathway provided the precursors required for lignin

biosynthesis, starting with phenylalanine for deamination to cinnamic acid. Lignin is a heterogeneous phenolic polymer found mainly in secondary thickened cell walls that plays a role in support and defending against diseases and insect pests. Due to its hydrophobicity, lignin provides impermeability for tubular molecules, allowing water and solutes to be transported in microtubule systems (Mellerowicz et al. 2001). In this paper, a total of 34 differentially expressed proteins were involved in phenylpropanoid biosynthesis (Table 2). These differentially expressed proteins catalyse the biosynthesis of p-hydroxy-phenyl lignin, guaiacyl lignin, 5-hydroxy-guaiacyl lignin and syringyl lignin (Fig. 5). The results suggested that phenylpropanoid biosynthesis may be involved in the ray cell wall biosynthesis of the xylem in *H. ammodendron*.

Some identified metabolic pathways, such as glycolysis/gluconeogenesis, carbon metabolism and photosynthesis, provide substrates or energy for cell wall biosynthesis. In starch and sucrose metabolism, sucrose degradation and metabolism provide high levels of energy and substrates during wood formation and participate in the biosynthetic process of both phenolic compounds and lignin during differentiation of xylem cambium (Hauch and Magel 1998). In this paper, a total of 36 differentially expressed proteins were involved in starch and sucrose metabolism (Table 2). In metabolic pathways, glucose-1-phosphate was catalysed, and UDP-glucose was synthesized. In plants, the glycogen units used in polysaccharide synthesis of the cell wall are derived from UDP-glucose or GDP-mannose (Gibeaut 2000). In this study, a total of 378 differentially expressed proteins were involved in metabolic pathways. Carbon metabolism provides energy and intermediates for secondary wall synthesis. Glucose is used in glycolysis to provide energy for cell wall synthesis (Yang et al. 2007). A total of 71 and 39 differentially expressed proteins were involved in carbon metabolism and glycolysis/gluconeogenesis, respectively. During photosynthesis, photosystem □ corresponds to $NADP^+$ reduction and the photophosphorylation cycle and consists of at least 8 peptides, and the main components are P700 chlorophyll α A1 and A2 apoproteins (Meng et al. 1988). PS I can also drive both cyclic and pseudocyclic electron transport (Brettel 1997). Cyclic and pseudocyclic electron transport provides the first stable product of photosynthesis in plants, ATP NADPH. These compounds provide the cells with all the reduction activities and energy required for biosynthesis and energy consumption (Fork and Herbert 1993). In this paper, photosystem I P700 apoprotein A1 was shown to be involved in the process of electron transport in photosynthesis and was significantly upregulated in the ray cell wall of *H. ammodendron* in Shihezi with a higher wall thickness of ray cells.

Upregulated proteins related to ray cell wall biosynthesis

Among the upregulated differentially expressed proteins, a total of 55 proteins, including beta expansin EXPB2.1 (*Mirabilis jalapa*), glucan endo-1,3-beta-D-glucosidase (*Beta vulgaris* subsp. *vulgaris*), hypothetical protein JCGZ_24101 (*Jatropha curcas*), pectin acetylesterase family protein (*Theobroma cacao*), polyphenol oxidase (*Spinacia oleracea*), etc. are related to ray cell wall formation in the xylem of *H. ammodendron*. Among them, five differentially expressed proteins were associated with cell wall loosening, the biosynthesis of cellulose and hemicellulose involved 46 differentially expressed proteins, and 4 differentially expressed proteins were involved in pectin biosynthesis (Table 3).

Table 3
Selected differentially expressed proteins for cell wall biosynthesis in H. ammodendron xylem

Protein_ID	Mass	Mean_RatioS	NCBInr Description
Loosening			
XP_021750963.1	18797.12	3.11	PREDICTED: non-specific lipid-transfer protein-like protein At5g64080-like [*Vitis vinifera*]
XP_021763617.1	10272.31	1.86	PREDICTED: probable non-specific lipid-transfer protein AKCS9-like [*Citrus sinensis*]
AAT40137.1	24799.11	1.25	putative xyloglucan endotransglycosylase, partial [*Bassia scoparia*]
XP_021838893.1	31624.29	1.36	xyloglucan endotransglycosylase/hydrolase 1 [*Neolamarckia cadamba*]
KMT01282.1	27706.02	1.27	beta expansin EXPB2.1 [*Mirabilis jalapa*]
Cellulose/hemicellulose			
CAA58475.1	49217.1	1.42	ADP-glucose pyrophosphorylase [*Spinacia oleracea*]
XP_021723825.1	104869.3	1.28	alpha-glucosidase, putative [*Ricinus communis*]
KMT12482.1	53853.96	1.49	beta-1,4-glucanase [*Atriplex lentiformis*]
XP_021757763.1	54480.48	1.53	beta-1,4-glucanase [*Atriplex lentiformis*]
XP_021742247.1	43121.16	1.29	GDP-D-mannose-3',5'-epimerase [*Malpighia glabra*]
CAA01679.1	36252.64	2.02	glucan endo-1,3-beta-D-glucosidase [*Beta vulgaris* subsp. *vulgaris*]
XP_021771858.1	37511.6	1.34	glucan endo-1,3-beta-D-glucosidase [*Beta vulgaris* subsp. *vulgaris*]
Protein_ID	Mass	Mean_RatioS	NCBInr Description
XP_021751492.1	22453.36	2.97	glucan endo-1,3-beta-D-glucosidase [*Beta vulgaris* subsp. *vulgaris*]
XP_021740932.1	53483.55	1.64	glycoside hydrolase family 28 family protein [*Populus trichocarpa*]
XP_021762462.1	111655.5	1.48	Glycosyl hydrolase family 38 protein [*Theobroma cacao*]
XP_021850714.1	25318.7	2.5	hypothetical protein CICLE_v10014737mg [*Citrus clementina*]

Protein_ID	Mass	Mean_RatioS	NCBInr Description
XP_021714131.1	229316.1	1.21	hypothetical protein JCGZ_24101 [*Jatropha curcas*]
XP_021755346.1	13203.1	2.05	hypothetical protein POPTR_0018s10730g [*Populus trichocarpa*]
XP_021757585.1	61555.73	2.32	hypothetical protein VITISV_032639 [*Vitis vinifera*]
KMT05369.1	60141.11	1.7	laccase, putative [*Ricinus communis*]
XP_021737467.1	57755.07	1.21	phosphoesterase family protein [*Populus trichocarpa*]
AGZ13351.1	83259.62	1.26	photosystem I P700 apoprotein A1 [*Beta vulgaris* subsp. *vulgaris*]
XP_021724027.1	41026.12	1.5	polygalacturonase inhibitor [*Actinidia deliciosa*]
KNA16501.1	37905.65	1.64	polygalacturonase inhibitor [*Actinidia deliciosa*]
KMT08955.1	69184.42	1.64	polyphenol oxidase [*Spinacia oleracea*]
KMT18466.1	134825.5	1.22	PREDICTED: alpha-mannosidase 2x [*Vitis vinifera*]
KMT11222.1	63161.73	1.28	PREDICTED: beta-glucosidase 24-like [*Fragaria vesca* subsp. *vesca*]
KNA15821.1	47701.31	1.36	PREDICTED: dolichyl-diphosphooligosaccharide–protein glycosyltransferase 48 kDa
Protein_ID	Mass	Mean_RatioS	NCBInr Description
KNA21582.1	43239.15	1.41	PREDICTED: dolichyl-diphosphooligosaccharide–protein glycosyltransferase 48 kDa subunit [*Vitis vinifera*]
KMS99901.1	75753.97	1.26	PREDICTED: dolichyl-diphosphooligosaccharide–protein glycosyltransferase subunit 2
			[*Cucumis melo*]
XP_021775930.1	60099.14	1.22	PREDICTED: LOW QUALITY PROTEIN: beta-glucosidase 12-like [*Brachypodium distachyon*]
KMT06847.1	114981.9	1.66	PREDICTED: lysosomal alpha-mannosidase isoform X1 [*Prunus mume*]

Protein_ID	Mass	Mean_RatioS	NCBInr Description
XP_021762464.1	115290.7	1.62	PREDICTED: lysosomal alpha-mannosidase isoform X1 [*Prunus mume*]
KMT06844.1	113997.4	1.28	PREDICTED: lysosomal alpha-mannosidase-like isoform X1 [*Citrus sinensis*]
KMT15365.1	37933.56	6.47	PREDICTED: polygalacturonase inhibitor-like [*Nicotiana tomentosiformis*]
XP_021738124.1	39376.37	1.49	PREDICTED: probable glycosyltransferase At5g11130 [*Pyrus x bretschneideri*]
XP_021765032.1	53270.9	1.3	PREDICTED: probable polygalacturonase [*Malus domestica*]
KMT16179.1	54521.81	1.45	PREDICTED: probable polygalacturonase [*Malus domestica*]
KMT16522.1	49622.32	1.21	PREDICTED: probable polygalacturonase [*Prunus mume*]
XP_021756977.1	39147.7	1.22	PREDICTED: pto-interacting protein 1-like [*Nicotiana sylvestris*]
XP_021764994.1	52495.78	2.14	PREDICTED: UDP-glycosyltransferase 88A1-like [*Vitis vinifera*]
Protein_ID	Mass	Mean_RatioS	NCBInr Description
KMS97535.1	47417.66	1.47	PREDICTED: UDP-N-acetylglucosamine–N-acetylmuramyl-(pentapeptide) pyrophosphoryl- undecaprenol N-acetylglucosamine transferase-like [*Vitis vinifera*]
KNA20972.1	59551.27	1.68	PREDICTED: uncharacterized protein LOC104597307 [*Nelumbo nucifera*]
KNA22897.1	52365.75	1.22	putative polygalacturonase [*Morus notabilis*]
KMT12043.1	52289.86	1.36	putative polygalacturonase [*Morus notabilis*]
XP_021765002.1	100318.7	1.25	Alpha-glucosidase; Flags: Precursor [*Spinacia oleracea*]
XP_021738992.1	54200.36	1.35	UDP-sulfoquinovose synthase, chloroplastic; Flags: Precursor [*Spinacia oleracea*]
KMT17940.1	54059.45	1.27	UDP-sulfoquinovose synthase, chloroplastic; Flags: Precursor [*Spinacia oleracea*]
KMT18678.1	41084.46	1.28	Reversibly glycosylated polypeptide 1 [*Theobroma cacao*]
XP_021757776.1	67481.2	1.41	UDP-Glycosyltransferase superfamily protein isoform 1 [*Theobroma cacao*]

Protein_ID	Mass	Mean_RatioS	NCBInr Description
KNA19339.1	47278.77	1.7	unnamed protein product [*Vitis vinifera*]
Pectin			
XP_021758812.1	62293.71	1.73	pectin methylesterase PME2.1 [*Nicotiana tabacum*]
XP_021734573.1	47139.74	1.36	Pectinacetylesterase family protein [*Theobroma cacao*]
KNA06798.1	63453.33	1.72	Pectinesterase-3 precursor, putative [*Ricinus communis*]
XP_021770654.1	65909.19	1.53	PREDICTED: putative pectinesterase/pectinesterase inhibitor 28 [*Nelumbo nucifera*]

Lipid transfer proteins can transfer lipids between membranes and may be involved in cell wall relaxation (Yeats and Rose 2008). In this manuscript, a protein (protein ID is XP_021763617.1, probable nonspecific lipid-transfer protein AKCS9-like [*Citrus sinensis*]) was upregulated in the xylem ray cell walls of *H. ammodendron* in Shihezi (with higher wall thickness of ray cells, Table 3), which may be involved in the process of cell wall loosening.

Expansin is a protein that loosens the cell walls of plants and participates in the process of cell wall expansion (Zhang et al. 2018). Expansin consists of four subfamilies: α-Expansin (EXPA), β-Expansin (EXPB), Expansin-like A (EXLA) and Expansin-like B (EXLB) (Kende et al. 2004). In our research, the β-Expansin EXPB2.1 was upregulated in the ray cell walls of *H. ammodendron* in Shihezi (Table 3), indicating that the protein was involved in the loosening process of the ray cell wall in the xylem of *H. ammodendron*.

Cell wall expansion depends on the ability of xyloglucan degradation or xyloglucan-cellulose hydrolysation, and xyloglucan endotransglycosylase (XET) plays an important role in this process (Darley et al. 2001). XETs were first identified and described by Fry et al. (1992) and Nishitani and Tominaga (1992). The main activity of XETs is transglycosidase activity, which catalyses the cleavage of the xyloglucan chain and moves the semi-chain to the non-reducing terminal of the second xyloglucan chain (Sulova et al. 1998). The cleavage (degradation) of cell wall xyloglucan promotes cell wall loosening, growth and cellulose accumulation (Park et al. 2004). Previous studies have shown the potential transformation and relaxation ability of XET in cell walls, and the expression and activity of XET are significantly correlated with the expansion area of the cell (Fry et al. 1992; Xu et al. 1996). Xyloglucan endotransglycosylase/hydrolase 1, which belongs to the pathway of plant hormone signal transduction, is upregulated in the xylem ray cell wall of *H. ammodendron* in Shihezi (Table 3). The xyloglucan endotransglycosylase/hydrolase 1, somatic embryogenesis receptor kinase 1-like precursor and kinase protein with tetratricopeptide repeat domain isoform 1 catalyse biochemical processes from brassinosteroid biosynthesis to cell elongation (Fig. 6, Table 4).

Table 4
Different proteins in pathway of plant hormone signal transduction

EC number	Protein_ID	Mass	Mean_Ratio	NCBInr Description
BAK1	KMT14924.1	24371.44	0.78	somatic embryogenesis receptor kinase 1-like precursor [*Glycine max*]
BSK	KMT01268.1	56957.73	0.7	PREDICTED: probable serine/threonine-protein kinase At4g35230 [*Prunus mume*]
	XP_010695172.1	54916.24	0.87	Kinase protein with tetratricopeptide repeat domain isoform 1 [*Theobroma cacao*]
	XP_021754164.1	54688.02	1.11	Kinase protein with tetratricopeptide repeat domain isoform 1 [*Theobroma cacao*]
	XP_021774662.1	57370.1	1.02	PREDICTED: probable serine/threonine-protein kinase At4g35230 [*Prunus mume*]
TCH4	KMT19882.1	31419.18	0.98	hypothetical protein VITISV_036640 [*Vitis vinifera*]
	XP_021838893.1	31624.29	1.36	xyloglucan endotransglycosylase/hydrolase 1 [*Neolamarckia cadamba*]

Reversibly glycosylated polypeptide 1 is involved in the transport of sugar into the Golgi cavity and the biosynthesis of non-cellulose polysaccharides (Saxena and Brown 1999). In this study, reversibly glycosylated polypeptide 1 was involved in amino sugar and nucleotide sugar metabolism and was significantly upregulated in Shihezi (ratio 1.28, Table 3).

GDP-mannose-3',5'-epimerase (GME) catalyses the conversion of GDP-D-mannose to GDP-L- galactose (Ma et al. 2014), which is a structural component of agar and cell wall polysaccharides (Siow et al. 2013). GME is involved in ascorbic acid biosynthesis in the Smirnoff-Wheeler pathway (Smirnoff and Wheeler 2000), and the first step is GME catalysing the formation of GDP-D-mannose (Wolucka and Van Montagu 2003). Ascorbic acid, an antioxidant and cofactor of enzymes, plays an important role in the photosynthesis and biosynthesis of cell wall components (Conklin and Barth 2004). In this paper, GME was shown to be involved in metabolic pathways and was upregulated in the ray cell walls of *H. ammodendron* in Shihezi (Table 3).

Beta-1,4-glucanase, encoded by the KORRIGAN gene, is involved in cellulose biosynthesis (Szyjanowicz et al. 2004). However, little is known about the functions of KORRIGAN EGase (KOR). In this experiment, beta-1,4-glucanase participated in starch and sucrose metabolism and was upregulated in the ray cell wall of *H. ammodendron* in Shihezi (Table 3).

In conclusion, during the process of ray cell wall biosynthesis in the xylem of *H. ammodendron*, it is assumed that the proteins (including predicted: nonspecific lipid-transfer protein-like protein At5g64080-like [*Vitis vinifera*], predicted: probable nonspecific lipid-transfer protein AKCS9-like [*C. sinensis*] and beta expansin EXPB2.1 [*M. jalapa*]) first cause cell wall loosening, extension and expansion, and the xyloglucan endotransglycosylase/hydrolase 1 cleaves and links xyloglucan chains. Then, photosystem I P700 apoprotein A1, reversibly glycosylated polypeptide 1 and GDP-mannose-3', 5'-epimerase, etc., are involved in cellulose, hemicellulose and pectin biosynthesis of the cell wall by providing components or energy. Finally, the proteins in phenylpropanoid biosynthesis promote the lignification of ray cell walls and complete the biosynthetic process of cell walls.

In this paper, it is notable that the upregulated proteins corresponded to significantly increased wall thickness of ray cells. The regulatory proteins or genes related to wall thickening of ray cells in *H. ammodendron* can be further explored to determine their functions, and the genes can be applied to the improvement of timber plantations, which is important for both identifying the effect of specific xylem on the environment and improving the wood mechanical properties of timber plantations.

Conclusions

(1) The wall thicknesses of *H. ammodendron* ray cells in Jinghe and Shihezi were 2.85 ± 0.42 μm and 3.08 ± 0.44 μm, respectively ($P < 0.01$).

(2) A total of 795 upregulated proteins and 421 downregulated proteins were found in the ray cell wall of *H. ammodendron*. Phenylpropanoid biosynthesis, photosynthesis, glycolysis/gluconeogenesis, carbon metabolism, starch and sucrose metabolism, metabolic pathways, plant hormone signal transduction, cysteine and methionine metabolism as well as amino sugar and nucleotide sugar metabolism may be related to the formation of the ray cell wall of *H. ammodendron*.

(3) During the process of ray cell wall biosynthesis in the xylem of *H. ammodendron*, it is assumed that nonspecific lipid-transfer protein-like proteins and beta expansin EXPB2.1 (*M. jalapa*) first loosen the cell wall loosening, followed by extension as well as expansion, and the xyloglucan endotransglycosylase/hydrolase 1 cleaves and links the xyloglucan chains. Then, photosystem I P700 apoprotein A1, reversibly glycosylated polypeptide 1 and GDP-mannose-3',5'-epimerase, etc. are involved in cellulose, hemicellulose and pectin biosynthesis of the cell wall by providing components or energy. Finally, the proteins involved in phenylpropanoid biosynthesis promote the lignification and complete the biosynthetic process of ray cell walls.

Abbreviations

ACN
Acetonitrile
DTT

Dithiothreitol
EDTA
Ethylene diaminetetraacetic acid
FA
Free folate
IAM
Iodoacetamide
iTRAQ
Isobaric tag for relative and absolute quantitation
PMSF
Phenylmethyl sulfonyl fluoride
PVPP
Polyvinylpolypyrrolidone
SDS
Sodium dodecyl sulfate
TCA
Trichloroacetic acid
TEAB
Triethylammonium bicarbonate

Declarations

Ethics approval and consent to participate:

not applicable

Consent to publish:

not applicable

Availability of data and materials:

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests:

the authors declare that they have no conflict of interest.

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Authors' Contributions:

Chaobin Zhou performed the material preparation and proteomic analysis, data analysis and written the manuscript. Junjie Ding and Xiaojing Hu performed the observation and measurement of wall thickness of ray cell. Wei Gong designed the experiment. All authors read and approved the final manuscript.

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Figures

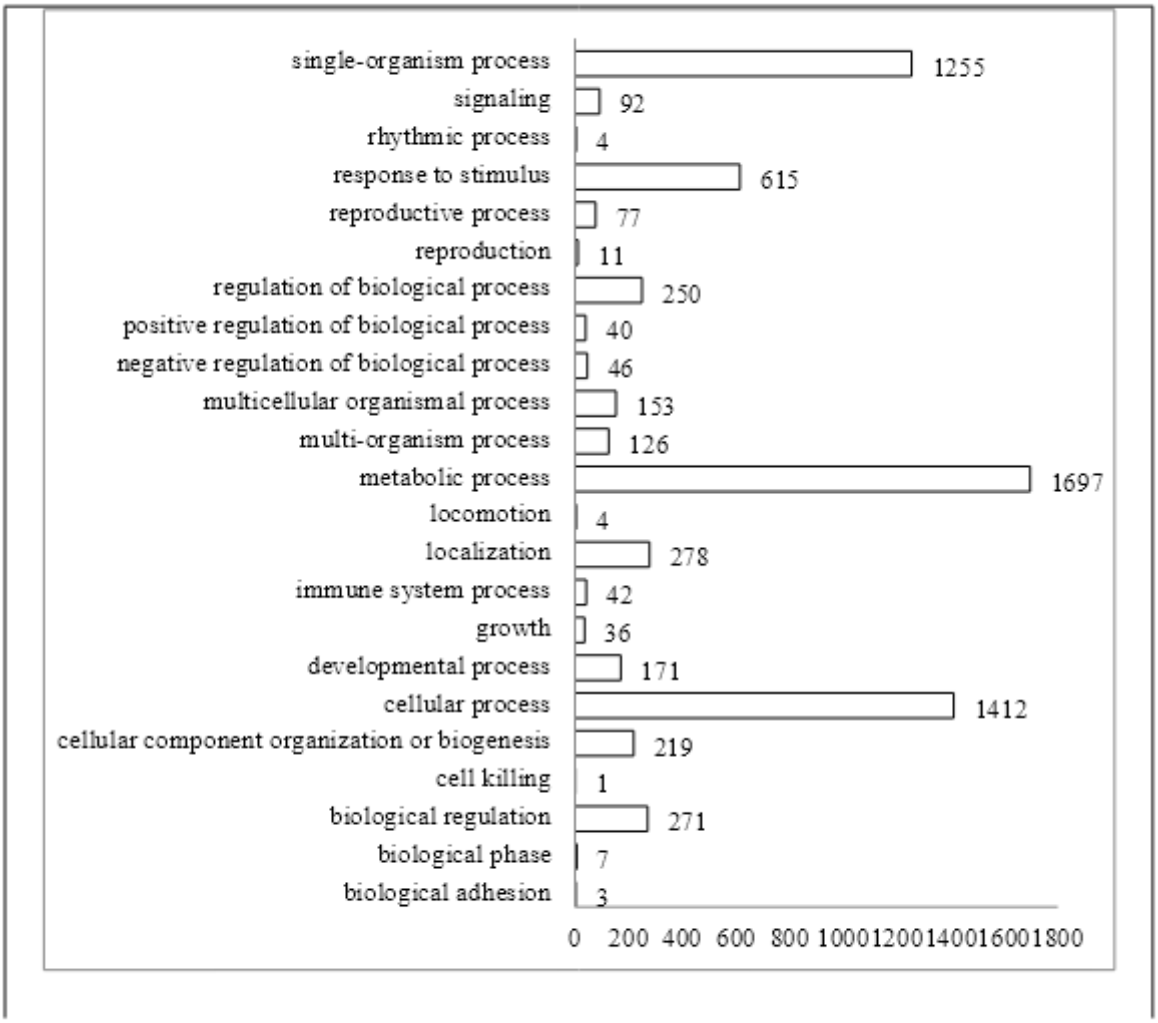

Fig. 2 Functional categorization of differentially expressed proteins based on

Figure 1

Functional categorization of differentially expressed proteins based on "biological process"

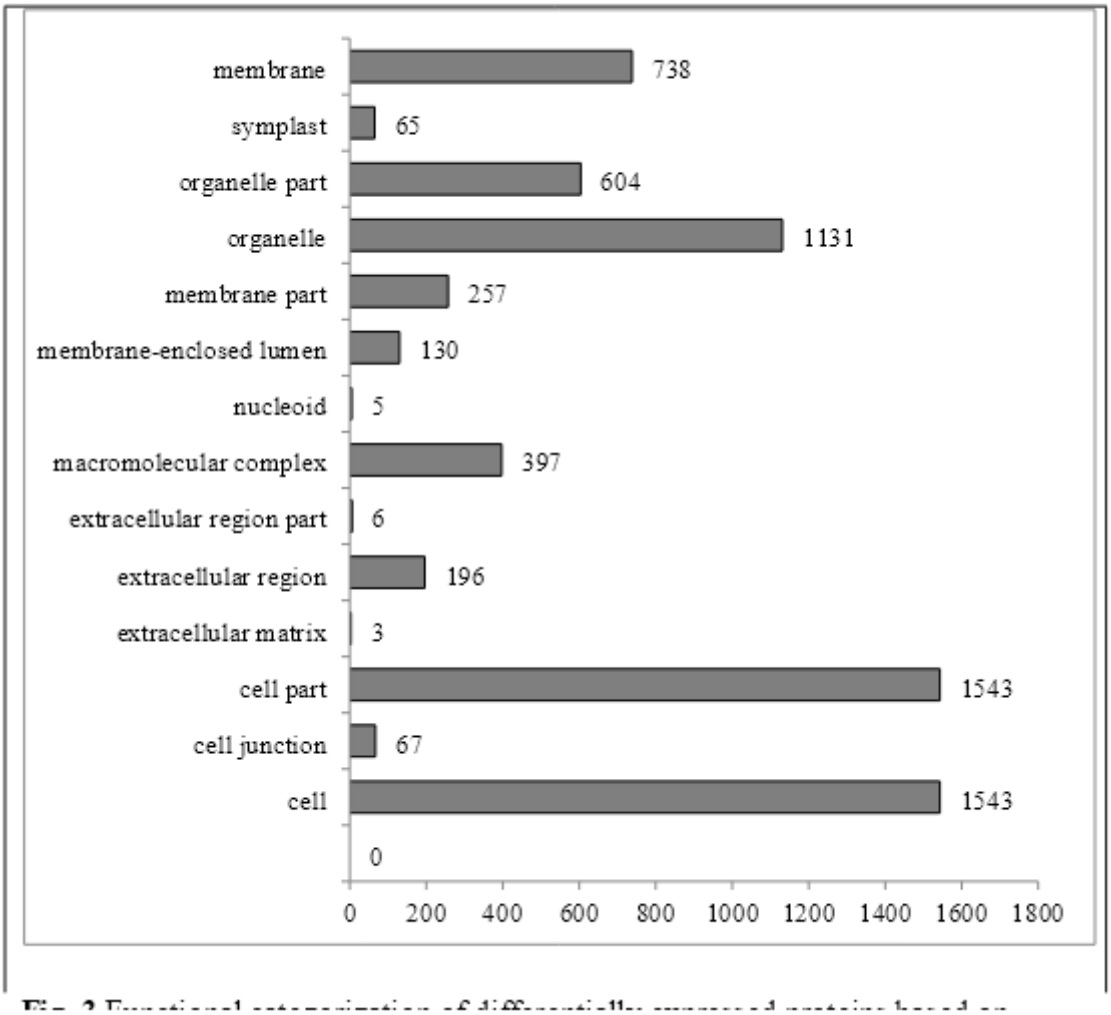

Figure 2

Functional categorization of differentially expressed proteins based on "cellular component"

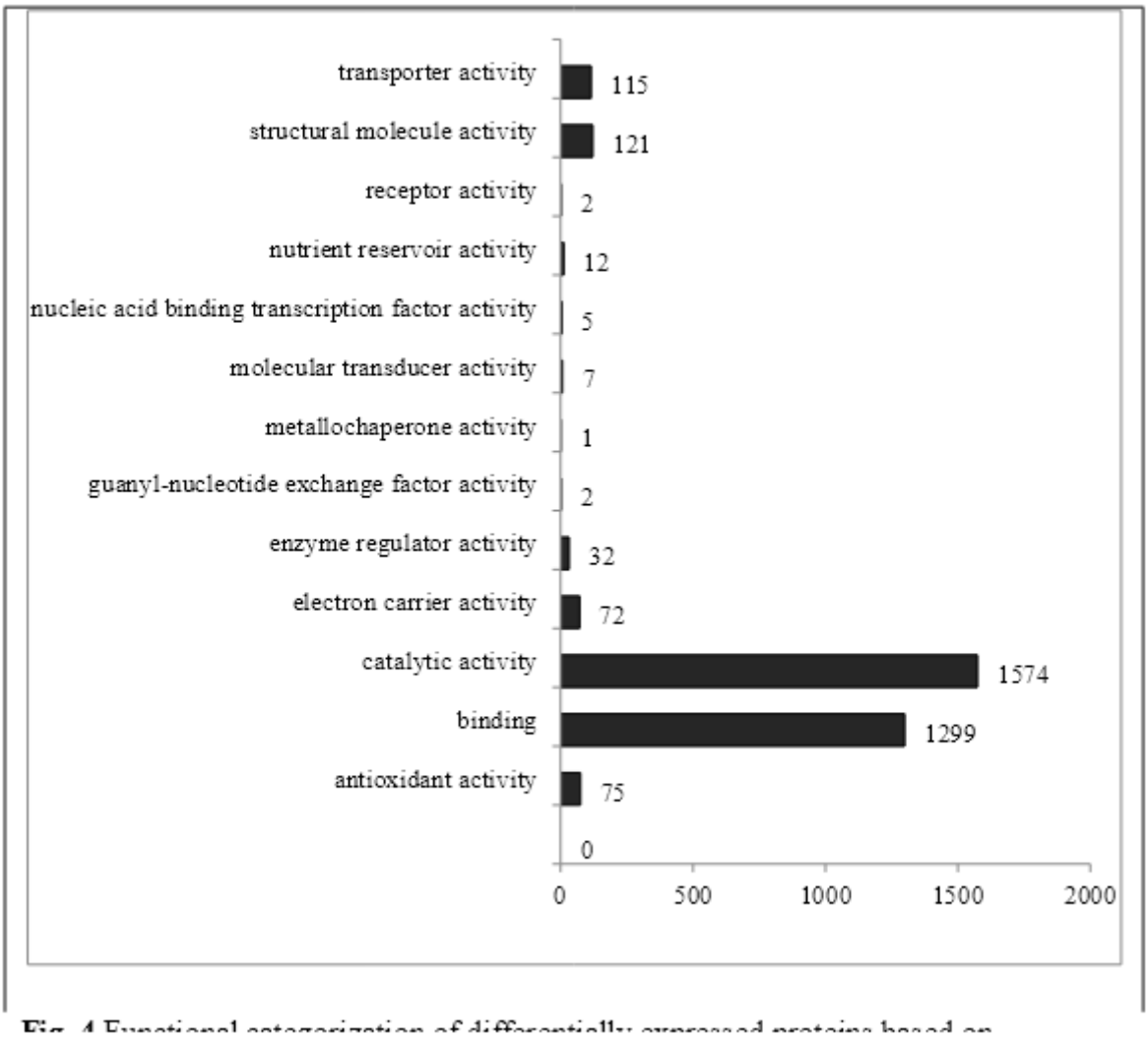

Figure 3

Functional categorization of differentially expressed proteins based on "molecular function"

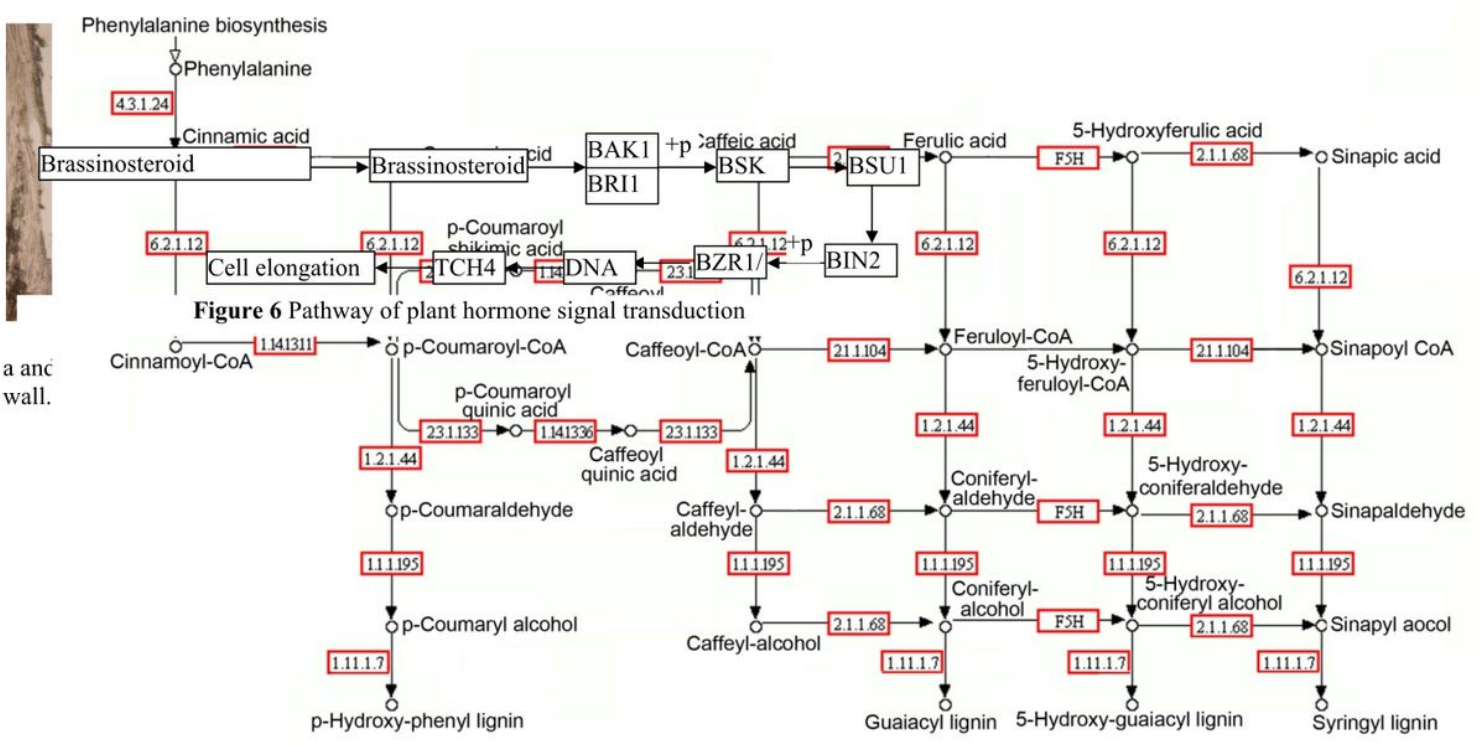

Figure 4

Pathway of phenylpropanoid biosynthesis

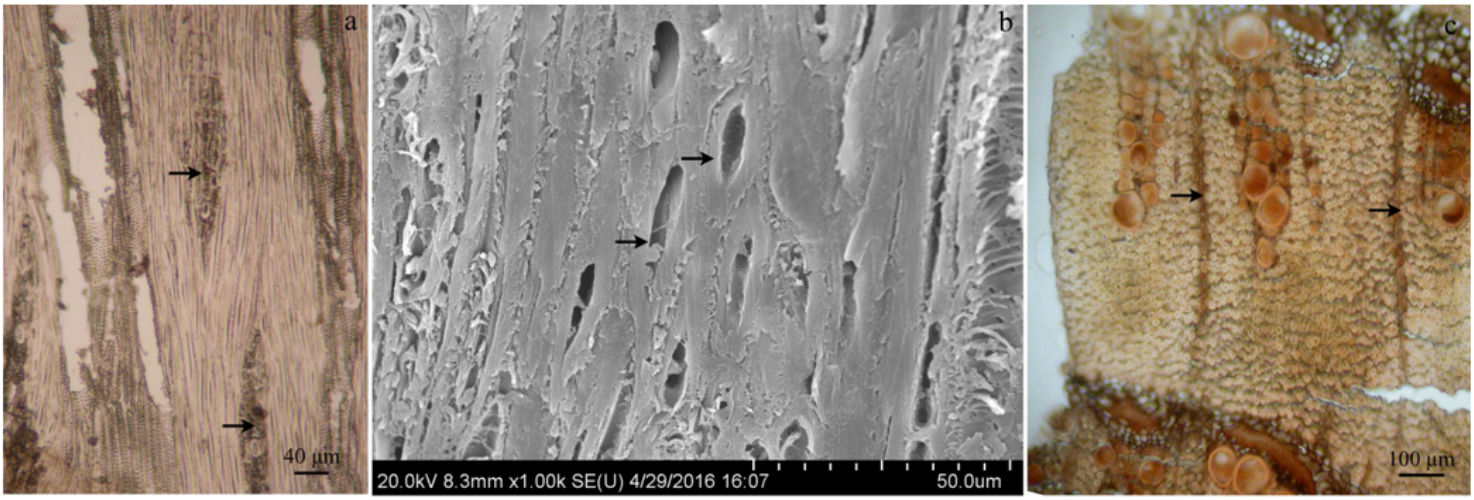

Figure 5

Anatomical characteristics of H. ammodendron rays a and b (SEM), Tangential section; c, Cross section. The arrows show ray cell with thick-wall.

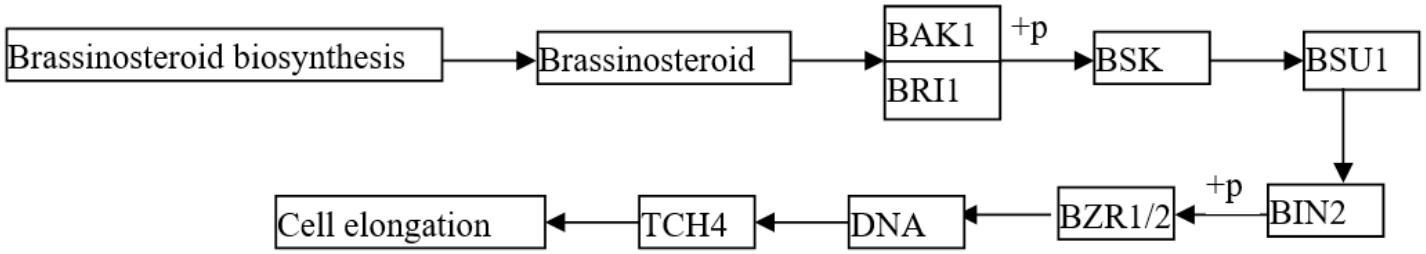

Figure 6

Pathway of plant hormone signal transduction

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