***NnLAC17*, a Gene Related to Lignin Synthesis, is Involved in Adventitious Root Formation in the Seedlings of Lotus (*Nelumbo Nucifera* Gaertn)**

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Abstract

Background

Adventitious roots (ARs), which are considered as an important member of root system, have an unmatched status in plant growth and metabolism due to the degeneration of primary roots in lotus. The regulation of AR formation was previously revealed and multiple factors were recognized to be involved in this biological process.

Results

In the present study, we sought to assess the effect of sucrose on AR formation. Based on our results, lignin metabolism, which is regulated by the sucrose signal transduction pathway, is involved in AR development. The lignification degree of the AR primordium was weaker in plants treated with 20 g/L sucrose than in control plants. However, based on the microstructural observation of the AR developmental process, 50 g/L sucrose promoted the lignification process. Lignin content, including monomer and polymer lignin, was determined in the present study. Compared with control plants, the monomer (containing 30%–45% S type and 55%–70% G type) and polymer lignin contents were lower in plants treated with 20 g/L sucrose and higher in plants treated with 50 g/L sucrose. The precursors of monomer lignin were identified in four libraries of differential developmental stages in seedlings using LC-MS/MS technique. The contents of four metabolites, including p-coumaric acid, caffeate, sinapinal aldehyde and ferulic acid for monomer lignin synthesis were lower in the GL50 library than in the GL20 library. Further analysis revealed that the gene expression of these four metabolites had no novel difference in the GL50/GL20 libraries. However, *NnLAC17*, a gene involved in polymer lignin synthesis, had a higher expression in the GL50 library than in the GL20 library.

Conclusions

Therefore, *NnLAC17* was cloned, and the overexpression of *NnLAC17* was found to directly result in a decrease in AR number in transgenic *Arabidopsis* plants. These findings suggest that *NnLAC17*, which is relevant to lignin synthesis, is involved in AR formation in lotus seedlings.

Background

The lotus is an aquatic plant that contains multiple nutrients. The lotus is believed to be an important vegetable and is widely cultivated in the Yangtze River and Yellow River basins owing to the suitable climate [1, 2]. In recent years, the lotus has become the largest cultivated area of aquatic vegetables. ARs are important member of root system that play an important role in water and nutrient uptake in plants, especially in lotus, which has underdeveloped primary roots. ARs are always located on the hypocotyl of seedlings in lotus, and approximately two weeks are required for ARs emergence [3]. There are three developmental stages in the entire period of ARs formation [4, 5]: the first stage is the induced period, where the normal cell is divided and developed into meristematic cells; the second stage is the

development stage, where the root primordium is formed from meristematic cells [6]; and the third stage, where the root primordium emerges from the epidermis of the stem or leaf [7, 8]. All biological processes of AR formation are strictly controlled by internal and external factors, such as light, plant hormones, damage, and sucrose.

The signaling transduction pathway of plant hormone is found to be involved in various metabolic processes of plant growth. In the past years, IAA has been reported to play an important role in root formation at the introduced, developmental, and emergence stages [9, 10]. Any factors that affect the synthesis, degradation, and transport of IAA can affect the development of AR. The formation of AR, an irreplaceable organ for water and nutrition uptake, is strictly controlled by ethylene [11]. Thus, the role of ethylene in AR formation is mainly carried out at the induction stage [12, 13, 14]. IAA and ethylene could also cooperatively regulate AR development. The metabolism and sensitivity of auxin are affected by ethylene. However, the role of ethylene is also regulated by IAA [15, 16, 17]. Sugar is involved in root development [18]. In fact, the role of sucrose in the regulation of root formation has been revealed to be similar to that of some types of plant hormone. Further, sucrose can promote the formation of root primordium and late development [10, 19]. In lotus, there is an interaction between sucrose and IAA during AR formation and the exogenous application of sucrose can affect IAA content. Exogenous IAA also changes endogenous sucrose content during AR formation [20]. Lignin is an important component of cells during plant growth and metabolism [21]. Normally, lignin, which is a polyphenolic polymer, contains three types of monomers, namely the S-type, H-type, and G-type [22].

According to recently reported data, a close relationship exists between lignin metabolism and AR formation. Cho et al. [23] reveals that IBA treatment promotes root primordial development but suppressed lignin synthesis. Peroxidase genes are responsible for lignin synthesis [24]. In fact, these peroxidase genes expression is found to be upregulated after IBA treatment [25]. Moreover, two different types of *Ebenus cretica* (rooting and non-rooting genotypes) are found to have different lignin and peroxidase activities [26]. In sweet potato, the formation of storage root is reduced and vascular lignification is promoted after the exogenous application of gibberellin [27]. A high expression of genes related to lignin synthesis occurs in wild species (not from storage roots) relative to cultivated species [28]. In mung bean seedlings, the genes responsible for lignin biosynthesis are downregulated after IBA treatment, which significantly promotes AR formation [29]. The development of rice roots is previously found to be inhibited while lignin deposition is increased in cell walls under conditions of stress [30].

Sucrose is an important signal molecular, which is involved in various biological process in plant. In the previous study, we found that sucrose metabolism was involved in AR development, and the expression of the gene, *NnLAC17* (which was related to lignin metabolism), was altered after plants were treated with 20 and 50 g/L sucrose. Fifty g/L sucrose (which inhibited AR formation in lotus seedlings) caused a greater transcription level of *NnLAC17* than 20 g/L sucrose (which promoted AR formation in lotus seedlings) [20]. Therefore, *NnLAC17* might play a critical role in lignin synthesis and regulate AR formation. In this study, the lignin content, a lignin metabolite, gene expression at the genomic level, and

the biological function of *NnLAC17* during AR formation were assessed to explore the AR mechanism regulated by sucrose in lotus seedlings.

Methods

Plant growth

The lotus seed species, Taikong36, was selected for all experiments in this study. The seeds used in this study were derived from the open field of Yangzhou University, and necessary permission was obtained to collect these samples. In spring (usually in April), the lotus was planted in the field for germination with moist soil. After the petiole broke through the soil surface, the water depth was maintained at 5-10 cm; a deeper water (20-40 cm) was required for the development of the plant and temperature was maintained between 20 and 30 °C during the entire growth season. The application of fertilizer and pest control was carried out in the same manner employed in the conventional management of the field. The seeds were harvested in November and placed in a container at normal temperature.

The role of sucrose in AR formation

The seed coat of lotus was punched to enable the uptake of available water (for approximately three days) and placed at 26 °C for approximately three days under dark conditions. Fifty germinated seedlings from each treatment were selected and placed into 0 g/L, 20 g/L, and 50 g/L sucrose solutions for two days. The germination rates and numbers were counted on days 0, 2, 4, and 6 after transfer into water for 2 d. All statistical data were expressed as the mean $\pm$ SE of three repetitions of experiments. The SPSS software ver. 14.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses.

Observation of the paraffin sections

The lotus seed coat was broken and immersed in water for approximately three days for germination at 26 °C in the dark. The germinated seeds were selected for treatment with various concentrations of sucrose (0, 20, and 50 mg/L) for two days. The seedling hypocotyls of plants subjected to the above treatments were selected from four time points (0, 2, 4, and 6 d) after treatment. The selected samples were cut into small pieces of 2.5 mm x 2.5 mm x 2 mm (length, width and height), and then placed in a container filled with FAA fixing fluid (solution amount was 20-fold that of samples).

The container with fixed samples was produced in a vacuum state using a syringe for 5 s. The lid of the container was opened for gas exchange after five min. This process was repeated in triplicate. The container was transferred to a clean bench at normal temperature for 24 h. Thereafter, 50%, 70%, 85%, 95%, and 100% ethanol were applied to dehydrate the samples for approximately 25-30 min. After dehydration with ethanol, the samples were treated with a mixed solution (one-half volume of ethanol and one-half volume of xylene and pure xylene) for approximately 25-30 min. Paraffin debris was

prepared and thawed on an electric stove and then poured into the container with the samples for 24 h. The tissues embedded in paraffin were cut into small blocks, and 10 µm of wax tape was prepared using a slicer. The wax tapes were placed on a glass slide, and then respectively transferred into pure xylene, mixed solution (one-half volume of pure xylene and one-half volume of absolute ethanol), and absolute ethanol for 10 min. The slide was dried at room temperature, and 2-3 drops of 0.75% crystal violet solution were placed on the samples for 5 min. The samples were washed with distilled water and then observed using an optical microscope.

Determination of monomer lignin and polymer lignin contents

The seed coat was broken for water absorption. The broken seeds were immersed in water (26 °C in the dark) for three days. After germination, two concentrations (20 mg/L and 50 mg/L) of sucrose were applied as treatments to the plants for two days, with water as a control. The hypocotyls of seedlings were used as materials to determine the monomer lignin and polymer lignin contents. Ten milligrams of dried sample was added to a mixture (2.5% boron trifluoride, 10% ethanethiol, 87.5% dioxane) for 4 h in a metal bath followed by 300 µL of 0.4 m sodium bicarbonate. The mixture was vibrated, and 2 ml water and 0.3 ml ethyl acetate were added. The mixture was centrifuged at 14000 rpm for 10 min at normal temperature, and the supernatant was retrieved. The supernatant was dried with nitrogen, and 150 µL pyridine (including an internal standard) and 50 µL trimethylsilane were allowed to react at 60 °C for 1 h. The mixture was centrifuged at 10000 rpm for 10 min at normal temperature, and the supernatant was retrieved for lignin identification. The lignin content was determined using a GC-MS instrument (7820A-5977B) from Agilent Technologies Inc. (CA, USA). The conditions for chromatography (mass spectrometry) and quantitative analysis lignin were based on a previous protocol [31]. Three biological replicates was carried out in this experiment. In addition, the roots of five- to six-leaf stage seedlings of transgenic *Arabidopsis* plants and none of the transgenic plants were collected for polymer lignin identification. The material treatment and identification method was the same as that mentioned above.

Metabolite analysis of lignin during AR formation

Lotus seedlings were treated with sucrose (20 g/L and 50 g/L) for two days, the detail progress were the same as above mentioned. The hypocotyls of control plants and plants treated with 20 g/L and 50 g/L were selected for metabolites analysis. All freeze-dried hypocotyls were crushed using a mixer mill (MM 400, Retsch) with a zirconia bead for 1.5 min at 30 Hz. Powder powder (100 mg) was weighed and extracted overnight at 4 °C with 1.2 ml 70% aqueous methanol. Following centrifugation at 10,000 g for 10 min at normal temperature, the extracts were absorbed (CNWBOND Carbon-GCB SPE Cartridge, 250 mg, 3 ml; ANPEL, Shanghai, China, www.anpel.com.cn/cnw) and filtered (SCAA-104, 0.22µm pore size; ANPEL, Shanghai, China, <http://www.anpel.com.cn/>) before UPLC-MS/MS analysis.

UPLC conditions

The sample extracts were analyzed using a UPLC-ESI-MS/MS system (UPLC, Shim-pack UFLC SHIMADZUCBM 30A system, www.shimadzu.com.cn/; MS, Applied Biosystems 6500QTRAP, www.appliedbiosystems.com.cn). The analytical conditions were as follows: column, Waters ACQUITYUPLCHSST3C18 (1.8 μm , 2.1 mm*100 mm); and mobile phase, solvent A- pure water with 0.04% acetic acid and solvent B- acetonitrile with 0.04% acetic acid. Sample measurements were performed with an Agilent program that employed the starting conditions of 95% A and 5% B. Within 10 min, a linear gradient to 5% A and 95% B was employed, and a composition of 5% A and 95% B was maintained for 1 min. Subsequently, a composition of 95% A and 5.0 % B was employed for 0.10 min and retained for 2.9 min. The column oven temperature was set to 40 °C, and the injection volume was 2 μL . The effluent was alternatively connected to an ESI-triple quadrupole-linear ion trap (QTRAP)-MS.

ESI-QTRAP-MS/MS

LIT and triple quadrupole (QQQ) scans were acquired on a triple quadrupole-linear ion trap mass spectrometer (Q TRAP), API 6500 Q TRAP UPLC/MS/MS System, equipped with an ESI Turbo Ion Spray interface, operating in positive and negative ion mode and controlled by Analyst 1.6.3 software (AB Sciex). The ESI source parameters were as follows: ion source, turbo spray; source temperature, 550 °C; ion spray voltage (IS), 5500 V (positive ion mode)/-4500 V (negative ion mode); ion source gas I (GSI), gas II (GSII), and curtain gas (CUR) 50, 60, and 30.0 psi, respectively; and collision gas (CAD), high. Instrument tuning and mass calibration were performed with 10 and 100 $\mu\text{mol/L}$ polypropylene glycol solutions in the QQQ and LIT modes, respectively. QQQ scans were acquired as MRM experiments with a collision gas (nitrogen) set at 5 psi. DP and CE for individual MRM transitions were performed with further optimization of DP and CE. A specific set of MRM transitions was monitored for each period, according to the metabolites eluted within this period.

RNA-seq analysis of the differentially expressed genes

For the analysis of differentially expressed genes, all genes related to monomer and polymer lignin synthesis were selected and their expression profiles were monitored after treatment with sucrose during ARs formation. The lotus seed coat was broken before germination and placed in 5 cm water at 28-30 °C for germination. After germination, the seedlings were treated with sucrose (20 mg/L and 50 mg/L) and IAA (10 μM and 50 μM) for two days, and then placed in water for continued growth. RNAs were then extracted, and eight libraries were constructed for gene expression analysis (low concentrations of sucrose and IAA were demonstrated to promote AR formation while high concentration inhibited AR development in lotus seedlings). In the previous study, four libraries treated with different concentration of IAA (CK library: germinating seeds without treatment; A library: initial AR stages: 2 days of seedlings with 10 μM IAA treatment; B library: 2 days of seedlings without IAA treatment; C library: 2 days of

seedlings with 50 μ M IAA treatment) and sucrose (C0 library: the samples were collected at day 0; CK1, GL20 and GL50 libraries: the samples were collected at day 1) were constructed, and the data has been published by Cheng et al. [32] and Cheng et al. [20] respectively. Gene expression related to the synthesis of lignin including monomer and polymer lignin was selected from above libraries for further analysis.

Cloning and sequence analysis of NnLAC17

The sequence of *NnLAC17* was derived from the NCBI database according to the gene expression profile under sucrose treatment. Lotus RNA was extracted from the hypocotyls of three old seedlings using a plant RNA extract mix (Tiangen, Beijing, China). DNase was applied to eliminate residual DNA before cDNA synthesis, which was performed according to the protocol of the mix kit (Promega, USA). A total of 20 μ L of PCR reaction mixture, which included 2.5 μ L dNTP, 2 μ L forward and reverse primers, 2.5 μ L $MgCl_2$, 0.5 μ L Taq polymerase, 2 μ L cDNA fragments, and 9.5 μ L dH_2O , was used. The PCR program included 35 cycles: 94 °C for 1 min, 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and 72 °C for 10 min. Forward primer: 5-ATGGGTTTCCTTTGTTCTTCC-3; Reverse primer: 5-AGGCGGTAGTTTCTGATTTG-3. The plasmid of DNA was sent to Sangon Biotechnology Co., Ltd (Shanghai, China) for sequencing. For sequence analysis, five LACs including lotus, vitis riparia, vitis vinifera, spatholobus suberectus, and trema orientale were used for alignment analysis by DNAMAN software. In addition, thirty-four LACs from different plants were selected, and MEGA-X software was used for phylogenetic tree construction.

Expression analysis of NnLAC17 in lotus

The seed of the lotus was placed in a glass container with a water depth of 5 cm to allow germination at 28-30 °C. The germinated seeds were treated with sucrose (20 mg/L and 50 mg/L) and IAA (10 μ M and 150 μ M) for 2 d and transferred into water to allow growth. The expression profile of *NnLAC17* was determined using qRT-PCR. The treatment of lotus seeds and the germination conditions were the same as those mentioned above. A total of 150 two-leaf seedlings which were divided into three groups, were treated with various concentrations of sucrose (20 g/L and 50 g/L) and IAA (10 μ M and 150 μ M) for 3 days respectively, and then transferred to water for continued growth. The hypocotyls of seedlings at 4 d after treatment were selected for transcription-level analysis. For the expression of *NnLAC17* in different organs, the leaf, stem, and hypocotyls of seedlings at 6 d (cultivation in water) were selected to monitor expression changes. Total plant RNA was derived from these samples, and DNase was used to eliminate residual DNA. The primer was designed based on the *NnLAC17* sequence using the primer 5.0 software. Forward primer was 5-GGGTTCCTTTGTTCTTCCA-3, and reverse primer was: 5-GCCAGTGCAAGGTGATATT-3. *NnActin* was used as the internal standard. The *NnActin* forward primer was 5'-ACGCGTATGAAGTCAGTTGT-3' and reverse primer, 5'-TTTATGGGGATCAGCTGGT-3'. A 25 μ L reaction mixture was prepared, which contained 12.5 μ L SYBR Premix Ex Taq II (Tli RNaseH Plus) (2 $\times$), 1 μ L of each of the forward and reverse primers, 2 μ L cDNA, and 8.5 μ L dH_2O . The PCR program consisted of 30 s at 94 °C, followed by 40 cycles of 95 °C for 5 s and 60 s at 60 °C. Three biological replicates were

carried out in this experiment. For data analysis, the $2^{-\Delta\Delta C_t}$ method was used to identify *NnLAC17* expression. The ΔC_t value was obtained according to the C_t (target) and C_t (actin) values in treated plants (ΔC_t (target)) and control (ΔC_t (normal)), and the $\Delta\Delta C_t$ value was calculated according to the data of ΔC_t (target) and ΔC_t (normal). Based on the $\Delta\Delta C_t$ value, the $2^{-\Delta\Delta C_t}$ value was determined.

Vector construction

NnLAC17 was cloned and inserted into a clone vector (pGEM-T). pGEM-T was transformed into *Escherichia coli* to expand reproduction. After identification, positive clones were cultured in LB culture medium. The plasmid was extracted from bacterial fluid and digested with *Bam*HI and *Kpn*I enzymes. Thereafter, the digestion product (sequence of *NnLAC17*) was inserted into the plant transformation vector with a CaMV 35S promoter. The pSN1301:: *NnLAC17* plasmid was inserted into the *Agrobacterium tumefaciens* strain, GV3101. The floral dip method [33] was employed to transform *NnLAC17* into the wild-type *Arabidopsis* plant. The transformed *Arabidopsis* was cultured in the greenhouse, and seeds were harvested after approximately three months of growth. Sterilized seeds of the T0 generation were tilled on MS medium containing $20 \mu\text{g}\cdot\text{g}^{-1}$ hygromycin B to select 'positive' plants. The temperature of the chamber was 22 °C with 12 h light and 12/dark light cycle. In addition, RT-PCR method was applied for further identification, and the primers was: Forward primer was 5-GGGTTCCTTTGTTCTTCCA-3, and reverse primer was: 5-GCCAGTGCAAGGTGATATT-3. Reaction mixture and program process were the same as gene expression. Base on the information of data, more than five lines of positive plants were selected for further study.

Identification of NnLAC17 function in Arabidopsis plants

The seeds of transgene (T2 generation) and wild-type plants were cultured in pots, and six-leaf-old transgenic seedlings and wild-type plants were selected for functional analysis. The development of all emerged roots including root length and number were determined. For root number analysis, all the root including ARs, primary root and lateral root were together counted (the hypocotyl of *Arabidopsis* was too short, so it was difficult to clearly distinguish ARs and primary root. Therefore, all the roots were identified together). For statistical analysis, all data are presented as the mean $\pm$ SE of triplicate independent treatments with approximately 10 seedlings per experiment, and about thirty seedlings was used for three biological replicates. The SPSS software ver. 14.0 (SPSS Inc., Chicago, IL, USA) was for data analysis.

Results

Effect of sucrose on AR formation

The lotus seedlings were treated with different concentrations (0, 20 g/L, and 50 g/L) of sucrose to determine the effect of sucrose on AR development. The AR of seedlings treated with 20 g/L sucrose

broke through the hypocotyl within 2 d. Further, the number of ARs in plants treated with 20 g/L sucrose was greater than that of control plants after transferal to water. Such findings indicate that 20 g/L sucrose significantly promoted the formation of ARs. The developmental process of ARs was markedly delayed in seedlings treated with 50 g/L sucrose. In fact, the number of ARs was lower in plants treated with 50 g/L sucrose than in control plants. Further analysis revealed that the seedlings treated with 20 g/L sucrose had higher rates of AR formation than those treated with 50 g/L sucrose. The rate of AR formation was only 50% at 6 d of growth in seedlings treated with 50 g/L sucrose. In contrast, the rate of AR formation was 100% at 5 d in the seedlings treated with 20 g/L sucrose (Fig. 1). In summary, we concluded that low concentrations of sucrose accelerated AR formation while high concentrations inhibited this process.

Observation of the Paraffin sections

The microstructure of hypocotyls was analyzed for AR development following treatment with 20 mg/L and 50 mg/L sucrose. The primordial of ARs were formed around the stomata in the hypocotyl. The induction and development of AR took approximately 2 d after treatment with 20 g/L sucrose; however, a longer time was taken following treatment with 50 g/L sucrose. In the last stage (expressed stage) of AR development, a greater number of ARs broke through the epidermis of hypocotyl following treatment with 20 mg/L sucrose than that at CK0 and after treatment with 50 g/L sucrose. Crystal violet staining was used to monitor the acclimation of lignin. Different degrees of staining were observed after treatment at CK0 and after treatment with 20 g/L and 50 g/L sucrose during AR development. A more intense crystal violet staining was observed after treatment with 50 g/L sucrose than at CK0 and after treatment with 20 g/L sucrose, indicating that the accumulation of lignin was higher following treatment with 50 g/L sucrose than at CK and with 20 g/L sucrose. Further, the lowest content of lignin was observed within 6 d of treatment with 20 g/L sucrose (Fig. 2).

Determination of monomer lignin and polymer lignin contents

The seedlings were treated with 20 mg/L and 50 mg/L sucrose for three days, and the contents of monomer lignin and polymer lignin were determined at two stages (CK: 0 d of treatment; CK1, three days after treatment (20 g/L and 50 g/L)). The lotus monomer lignin was found to be composed of two types of lignin (G and S types); thus, it did not contain the H type. Of the two lignin types, the content of the G-type lignin was greater than that of the S-type lignin. The lignin content of the G and S types was significantly higher at CK1 than CK0. At the same time, seedlings treated with 50 g/L sucrose had higher lignin content than at CK0, while a remarkable decrease was observed after treatment with 20 g/L sucrose (Fig. 3a). Further analysis revealed that more than 60% lignin was of the G-type lignin, and less than 40% lignin was of the S-type lignin at CK0 and CK1 (20 g/L and 50 g/L sucrose treatment) (Fig. 3b). The content of polymer lignin was also determined following the above treatments. The content of polymer lignin was found to increase at CK1 with 50 g/L sucrose and CK0. Further, more content was found at CK1 and 50 g/L sucrose than at CK0. However, total lignin was significantly decreased after treatment with 20 g/L sucrose (Fig. 3c).

The change in metabolites related to lignin biosynthesis owing to sucrose treatment

Four libraries, namely CK0, CK1, GL20, and GL50, were constructed to monitor the change in 14 metabolites (p-coumaric acid, p-coumaraldehyde, sinapic acid, sinapyl alcohol, p-coumaryl alcohol, caffeoyl alcohol, caffeoyl aldehyde, sinapinaldehyde, L-phenylalanine, coniferyl alcohol, caffeate, ferulic acid, 4-hydroxy-3-methoxycinnamaldehyde, and cinnamic acid), which was involved in synthesis of monomer lignin in lotus seedlings treated with different concentrations of sucrose. Seven metabolites (sinapinaldehyde and 4-hydroxy-3-methoxycinnamaldehyde was involved in S-lignin synthesis; coniferyl alcohol, caffeate and ferulic acid were involved in G-lignin and S-lignin synthesis; L-phenylalanine and cinnamic acid were primary product for H lignin, G-lignin and S-lignin synthasins), six (sinapinaldehyde and 4-hydroxy-3-methoxycinnamaldehyde were involved in S-lignin synthesis; coniferyl alcohol, ferulic acid and, and cinnamic acid were involved in G-lignin and S-lignin synthesis; L-phenylalanine was primary product for P lignin, G-lignin and S-lignin synthasins), and six metabolites (sinapinaldehyde and 4-hydroxy-3-methoxycinnamaldehyde were involved in S-lignin synthesis; coniferyl alcohol, ferulic acid and cinnamic acid were involved in G lignin and S-lignin synthesis; L-phenylalanine was primary product for P lignin, G-lignin and S-lignin synthasins) were found to increase, and only one metabolite (p-coumaric acid was involved in H-lignin synthesis) decreased in the CK1/CK0 libraries, GL20/CK0, and GL50/CK0 libraries, respectively. Based on the data obtained from these libraries, we further analyzed the altered metabolites in the GL50/GL20 libraries. The expression of four important metabolites, namely p-coumaric acid, caffeate, sinapinal aldehyde, and ferulic acid, were found to decrease (Table 1).

Table 1

Analysis of the metabolism of monomer lignin in lotus seedlings treated with 20 g/L and 50 g/L sucrose.

Compounds	Pvalue	Fold Change	Type
CK1/CK0 libraries			
p-Coumaric acid	0.0112	0.2743	Down-regulated
p-Coumaraldehyde	NA	NA	
Sinapic acid	NA	NA	
Sinapyl alcohol	NA	NA	
p-Coumaryl alcohol	NA	NA	
Caffeyl alcohol	NA	NA	
Caffeyl aldehyde	NA	NA	
Sinapinaldehyde	0.0015	3.4747	Up-regulated
L-Phenylalanine	0.00525	1.8344	Up-regulated
Coniferyl alcohol	0.0225	23.0678	Up-regulated
Caffeate	0.0305	1.4309	Up-regulated
Ferulic acid	0.0564	1.6682	Up-regulated
4-Hydroxy-3-methoxycinnamaldehyde	0.0019	10.5479	Up-regulated
Cinnamic acid	0.0036	2.5194	Up-regulated
GL20/CK0			
p-Coumaric acid	0.0407	0.1107	Down-regulated
p-Coumaraldehyde	NA	NA	
Sinapic acid	NA	NA	
Caffeate	0.0369	1.156	
Sinapyl alcohol	NA	NA	
p-Coumaryl alcohol	NA	NA	
Caffeyl alcohol	NA	NA	
Caffeyl aldehyde	NA	NA	
Sinapinaldehyde	0.0147	7.2121	Up-regulated
L-Phenylalanine	0.0497	1.6486	Up-regulated
Coniferyl alcohol	0.01762	19.5263	Up-regulated
Ferulic acid	0.0113	2.3898	Up-regulated

4-Hydroxy-3-methoxycinnamaldehyde	0.0415	16.7289	Up-regulated
Cinnamic acid	0.02470	2.1252	Up-regulated
GL60/CK0			
p-Coumaric acid	0.04766	0.079	Down-regulated
p-Coumaraldehyde	NA	NA	
Sinapic acid	NA	NA	
Caffeate	0.0325	0.899	
Sinapyl alcohol	NA	NA	
p-Coumaryl alcohol	NA	NA	
Caffeyl alcohol	NA	NA	
Caffeyl aldehyde	NA	NA	
Sinapinal dehyde	0.0057	5.2929	Up-regulated
L-Phenylalanine	0.0073	1.5573	Up-regulated
Coniferyl alcohol	0.0072	20.4520	Up-regulated
Ferulic acid	0.03586	1.9201	Up-regulated
4-Hydroxy-3-methoxycinnamaldehyde	0.0001	17.3515	Up-regulated
Cinnamic acid	0.0004	2.4017	Up-regulated
GL60/GL20			
p-Coumaric acid	0.0386	0.7140	Down-regulated
p-Coumaraldehyde	NA	NA	
Sinapic acid	NA	NA	
Caffeate	0.0339	0.7823	Down-regulated
Sinapyl alcohol	NA	NA	
p-Coumaryl alcohol	NA	NA	
Caffeyl alcohol	NA	NA	
Caffeyl aldehyde	NA	NA	
Sinapinal dehyde	0.0157	0.7338	Down-regulated
L-Phenylalanine	0.0014	——	
Coniferyl alcohol	0.0082	——	

Ferulic acid	0.0254	0.7034	Down-regulated
4-Hydroxy-3-methoxycinnamaldehyde	0.0497	——	
Cinnamic acid	0.0145	——	
NA: The metabolite was not checked; “——”: No difference was found between two libraries.			

Expression of genes related to monomer and polymer lignin contents

All the genes related to monomer and polymer lignin contents were selected from the libraries to further analyze their expression after sucrose and IAA treatment. The data of these libraries has been published in previous study [20, 32](Cheng et al. 2008, Cheng et al. 2020a). A total of eight genes related to monomer lignin was identified, and while expression of these genes did not change in the CK1/C0, GL20/C0, and GL50/C0 libraries after sucrose treatment (Fig. 4a), which indicated that sucrose had no effect on the content of monomer lignin at the transcription level. Genes involved in polymer lignin synthesis were also identified with these concentration of sucrose treatment. In fact, five genes were identified to be expressed at the transcriptional level. The mRNA levels of four genes, including *NnLAC4*, *NnLAC7*, *NnLAC11*, and *NnLAC17* were increased, and one gene (*NnLAC1*), were decreased after 20 g/L and 50 g/L sucrose treatment (Fig. 4b). For these altered genes including *NnLAC4*, *NnLAC7*, *NnLAC11*, no different change in expression was found between the CK1/CK0 libraries, GL20/C0 libraries, and GL50/C0 libraries. However *NnLAC17* was observed to have lower transcriptional level in GL20/C0 libraries than that of CK1/CK0 and GL50/C0 libraries (Fig. 4b), which indicated that 20 g/L sucrose decreased *NnLAC17* expression which was involved in polymer lignin synthesis.

For treatments with 10 μ M and 50 μ M IAA, *C4H*, *CCR*, *CAD*, and *F5H* related with monomer lignin synthesis altered their expression, and while *PAL* and *COMT* had no significant changes in transcriptional level. *CCR* and *CAD* had increased expression in the A1/C0 and C1/C0 libraries compared to the B1/C0 library, and *F5H* had more transcriptional level in the C1/C0 libraries than in the A1/C0 libraries and B1/C0 libraries (Fig. 4c). In addition, all the gene relevant to polymer lignin synthesis was chosen from these libraries. We found that the expression of two genes (*NnLAC4* and *NnLAC17*) involved in polymer lignin synthesis was identified, and *NnLAC17* was found to have increased transcription level in the C1/C0 libraries compared to the A1/C0 libraries and B1/C0 libraries (Fig. 4d).

Cloning and expression of NnLAC17

NnLAC17 was cloned from the lotus and sequenced by Sangon Biotech (Shanghai) Co., Ltd. This gene had a full-length sequence of 1,749 bp and encoded 583 amino acid residues. The deduced protein of this gene showed 84%, 84%, 83%, and 85% similarity with that of *Vitis riparia* (XP_034687366.1), *Vitis vinifera* (XP_002284473.1), *Spatholobus suberectus* (TKY66042.1), and *Trema orientale* (PON84167.1), respectively (additional Fig. 1). In addition, a phylogenetic analysis of *LAC17s* revealed that all *LAC17s*

were classified into seven groups, and most of these groups contained several sub-groups. *SiLAC17*, *CsLAC17*, *NnLAC17*, *PeLAC17*, *PtLAC17*, *PdLAC17* and *RcLAC17* were classified into a group, among which, *CsLAC17* and *NnLAC17* were classified into one sub-group, which indicated that *LAC17* from lotus was closely related to that of cucumber in gene sequence (Fig. 5a).

Different concentrations of IAA and sucrose were applied to lotus seedlings, and *NnLAC17* expression was analyzed after 2 days of treatment. No significant difference in *NnLAC17* was observed at the transcription level in control plants and plants treated with 20 g/L sucrose. However, 50 g/L sucrose was found to remarkably enhance *NnLAC17* gene expression (Fig. 5b). A similar change was observed in seedlings treated with IAA. The seedlings treated with 150 µg/L IAA had higher mRNA levels than the control plants and those treated with 10 µg/L IAA (Fig. 5c). Based on an analysis of the gene expression in different organs, a higher expression of *NnLAC17* was observed in roots than in stems and leaves (Fig. 5d).

Functional analysis of NnLAC17 in transgenic Arabidopsis thaliana

sn1301: *NnLAC17* was constructed and overexpressed in *Arabidopsis* plants under the control of a cauliflower mosaic virus 35S promoter to assess gene function. The “positive” plants were identified by hygromycin screen and the RT-PCR technique (Additional Fig. 2). The T2 generation of transgenic plants was planted in the base material, and root length and number were assessed with seedlings at 5–6 leaf age. Plant growth and the length of roots in transgenic plants and non-transgenic plants had no significant difference, while the number of roots in transgenic plants was greater than that in non-transgenic plants (Fig. 6a,b). Average twenty-six roots in each plant was observed in none transgenic plants at 5–6 leaf age, and approximately twenty roots were found in transgenic plants (Fig. 6c). Further analysis revealed that the content of polymer lignin was markedly increased in transgenic *Arabidopsis* plants after the overexpression of *NnLAC17* (Fig. 6d). Compared with non-transgenic plants, *NnLAC17* was found to regulate root formation by enhancing lignin content. Therefore, *NnLAC17* regulated by sucrose signaling in the lotus is involved in AR development.

Discussion

The ARs of lotus play an important role in water and nutrition uptake in the lotus. In this experiment, we observed that a high concentration (50 g/L) of sucrose inhibited AR formation while a low concentration (20 g/L) of sucrose promoted AR development (Fig. 1). The developmental process of AR, including the induction stage, which is the initial formation; the developmental stage of root primordium; and the expressed stage are found in lotus seedlings [6, 34]. These stages are regulated by multiple factors, including light, plant hormones, mechanical damage, and sucrose [20, 32, 35, 36]. IAA is involved in the early stages of root formation, and any incidence relevant to IAA synthesis and flux could affect root development [37, 38, 39]. Cheng et al. [32] reported that the exogenous application of low concentrations of IAA (10 µM) increased the AR number. Through further analysis, these researchers revealed an IAA

regulating role at the induction stage and developmental stages in lotus [20]. Another important regulator of AR development is sucrose, which has already been found in the plant kingdom [20]. The effect of sucrose, which is similar to that of hormones, occurs throughout the entire stage of AR development [36]. An interaction has been found to occur between sucrose and auxin in AR formation [20], which suggests that sucrose and auxin might simultaneously regulate AR development in lotus. To date, how sucrose and auxin regulate AR formation has not been revealed.

In this study, a microstructural observation of the development of AR was carried out. Treatment with 50 g/L sucrose resulted in deeper crystal violet staining in AR primordium than that observed at CK0 and after treatment with 20 g/L sucrose (Fig. 2). Such findings suggest that a high correlation may exist between AR formation and lignin deposition. This phenomenon has also been identified in carrot taproots. Lignin accumulation is decreased after the application of exogenous IBA, which results in an increase in root length [40]. Lignin metabolism originates from the phenylpropanoid pathway and participates in various biological processes during plant growth [41, 42]. Lignin depletion causes plant dwarfism, which is caused by the disruption of cell wall formation [43]. Silencing of *C4H* can markedly decrease lignin content and directly result in seedling lethality and an obstacle to leaf development [44]. Lignin deposition is found during root development in sweet potato, suggesting that lignin metabolism is closely correlated with root formation [45]. Further experiments shows that the decreased expression of *CH4* reduces the number of Arabidopsis lateral roots relative to AUX transport [46]. Lotus seedlings treated with 50 g/L sucrose were found to have a higher lignin content than at CK0. In addition, there was a significant decrease in lignin content following treatment with 20 g/L sucrose (Fig. 3), suggesting that sucrose affected AR formation derived from the regulation of lignin metabolism. Lignin deposition is related to the age of the plant. Durkovic et al. [47] reported that 3-year-old plants have higher concentrations of lignin than 1-year-old plants. Further, callus cells in roots have higher lignin content than meristem [48]. In summary, root development and lignin deposition have a negative correlation, and lignin accumulation negatively affects root development.

Lignin synthesis includes two steps: monomer lignin synthesis and polymer lignin synthesis, and many genes are involved in these processes [49, 50]. These biological processes are affected by many factors, including plant hormones, light, stress, temperature, and nitrate [51, 52, 53, 54]. For the synthesis of monomer lignin, *PAL*, *C4H*, *4CL*, *CCR*, *CAD*, *SAD*, *COMT*, and *F5H* catalyze the conversion of phenylalanine into p-coumaryl alcohol, sinapyl alcohol, and coniferyl alcohol, resulting in the formation of monomer lignin with the help of *POD* [24, 49, 55, 56, 57, 58, 59]. The decreased expression of *PALs* and *4CL* reduces lignin accumulation [24, 60], and the increased expression of *POD* can significantly improve lignin content in plants [61]. In this study, we found no difference in the expression of *PAL*, *C4H*, *4CL*, *CCR*, *CAD*, *SAD*, *COMT*, and *F5H* under various concentrations of sucrose (Fig. 4). However, the contents of p-coumaric acid, caffeate, sinapinal aldehyde, and ferulic acid were markedly decreased in the GL50/GL20 libraries (Table 1), which indicated that more lignin was used in the synthesis of polymer lignin after 50 mg/L sucrose treatment. A further analysis was carried out in libraries treated with different concentrations of sucrose and IAA, and all *NnLACs* were selected to monitor the changes in gene expression related to lignin synthesis. We found that only *NnLAC17* showed the same expression tendency; thus, the

involvement of *NnLAC17* in polymer lignin synthesis might be the main factor resulting in different lignin contents in lotus seedlings responding to various concentrations of sucrose and IAA.

LACs play an important role in the polymerization of lignin, which is involved in

cell wall formation. In addition, the polymer distribution of lignin is regulated by *LACs* in gymnosperm compression wood [62]. Recently, *LACs* have been found to be involved in various abiotic stress responses in plants [53]. The overexpression of *LAC2P* can improve water transport, which leads to enhanced plant drought adaptation [63]. H_2O_2 is reported to be an important signaling molecule. In fact, the exogenous application of H_2O_2 promotes AR formation in mung bean seedlings, and while the expression of *Laccase7*, which is related to lignin synthesis, was found to be significantly reduced [64], suggesting that the formation of AR and lignin content are opposing processes. Our data further revealed that increased lignin affected root development (i.e., a decrease in root number in transgenic plants) in transgenic plants of *Arabidopsis thaliana* after the overexpression of a lotus gene, *NnLAC17* (Fig. 6). Such finding also indicated that *LAC17* might be an important gene involved in lignin content, which regulated AR formation in lotus.

Conclusion

Based on the monomer and polymer lignin content, microstructural observation, and metabolite product analysis of the lignin metabolism pathway, a higher concentration of sucrose promotes lignin content than a lower sucrose concentration in lotus seedlings. Genes involved in monomer and polymer lignin were analyzed. However, no difference in expression profile was found in the libraries following treatment with different concentrations of sucrose. As a result, the difference in monomer lignin was found to be derived from the efficiency of polymer lignin synthesis. Only *NnLAC17*, which is related to polymer lignin synthesis, was found to display different expression levels when exposed to different concentrations of sucrose and IAA. *NnLAC17* had markedly enhanced expression in seedlings treated with 50 g/L sucrose and 150 μ M IAA. Further, its overexpression markedly reduced root number. Therefore, we believed that lignin synthesis affect AR formation, and *NnLAC17* might be an important factor in the regulation of AR formation in lotus seedlings.

Declarations

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Conflicts of interest/Competing interests.

The authors declare that they have no competing interests.

Ethics approval and consent to participate

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The materials of all the experiment was supported by aquatic vegetable Lab of Yangzhou University. The collection of seed complied with local and national guidelines and permissions of seed were obtained. The detail data derived from sucrose treatment has been deposited in NCBI database (Bioproject number). All the raw tags derived from IAA treatment were deposited in the National Center for Biotechnology Information (BioProject ID: PRJNA398315; accession number: SRR5944803–SRR5944814)

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Figures

Figure 1

The influence of 20 g/L and 50 g/L sucrose on AR formation in lotus. Some data of this experiment had been published in Cheng et al. [20].

Figure 2

The change in lignin metabolism based on an observation of the microstructure during AR formation in lotus seedlings treated with 20 g/L and 50 g/L sucrose.

Figure 3

Identification of the AR lignin in lotus seedlings treated with 20 g/L and 50 g/L sucrose. a. The lignin was analyzed by ESI-QTRAP-MS/MS. b. Determination of the monomer lignin content in the C0, C1, GL50, and GL20 libraries. c. Determination of the monomer lignin types in the C0, C1, GL50, and GL20 libraries. d. Analysis of polymer lignin content in the C0, C1, GL50, and GL20 libraries.

Figure 4

The expression of genes related to lignin synthesis during AR formation in lotus seedlings. a. The expression of genes related to monomer lignin synthesis in the GL20 and GL50 libraries. b. The expression of genes related to polymer lignin synthesis in the C0, C1, GL20, and GL50 libraries. c. The expression of genes related to monomer lignin synthesis in the C0, A1, B, and C1 libraries. d. The expression of genes related to polymer lignin synthesis in the C0, A1, B, and C1 libraries.

Figure 6

Functional analysis of NnLAC17 in transgenic *Arabidopsis thaliana*. a. Phenotypic investigation of NnLAC17 transgenic plants and wild-type plants at 6-7-leaf age. b. Comparative analysis of root length and number of transgenic plants and wild-type plants. c. Investigation of polymer lignin in NnLAC17 transgenic plants and wild-type plants. d. Determination of root length and number in NnLAC17 transgenic plants and wild-type plants.

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