Melatonin enhances the hemiparasite *Santalum album* Linn. tolerance to low nitrogen stress via accelerated N metabolism and haustoria development

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Abstract

Santalum album is a hemiparasitic plant that obtains some of its water and nutritional requirements by parasitizing the roots of neighboring plants. Nitrogen (N) is a critical nutritional resource for all plants and nitrogen deficiency is typically detrimental to plant growth and development. As a hemiparasite, *S. album* can obtain organic N from parasitizing host roots as well as inorganic N by absorption thru its roots. In this current study, we evaluated changes in the physiology, transcriptional profiles and chromatin accessibility in *S. album* seedlings exposed to low N conditions with and without supplemental melatonin. We employed two complementary assays, global transcriptome analysis (RNA-seq) and assay for transposase-accessible chromatin with high throughput sequencing (ATAC-seq) to identify genes and genic regions differentially regulated in *S. album* roots under these conditions. Low N conditions disturbed the homeostasis of N metabolism, reducing both N uptake and assimilation. The inclusion of melatonin enabled *S. album* seedlings in low N conditions to achieve significantly higher levels of N uptake and assimilation compared to plants without melatonin. Interestingly, melatonin treatment also enhanced haustorium development through a mechanism associated with auxin accumulation. These results suggest that the application of supplemental melatonin may accelerate N metabolism and haustorium formation of *S. album* in low N conditions.

KEYWORDS

Santalum album Linn.; hemiparasitic plant; ATAC-seq; RNA-seq; melatonin; haustorium formation; N uptake and metabolism

1 | INTRODUTION

Plants have evolved various strategies to facilitate the acquisition of fundamental resources, including symbioses with mycorrhizal fungi and/or nitrogen-fixing bacteria (Tesitel et al., 2018). Approximately 1% of all plant species (*c.* 4500 species) have developed parasitic strategies for obtaining nutrients by parasitizing neighboring plants (Tesitel et al., 2015). Depending on their degree of host dependence parasitic plants are functionally classified as hemiparasites or holoparasites. Holoparasites are fully heterotrophic and dependent on host resources for a supply of fixed carbon. In constrat, hemiparasites, which constitute about 90% of parasitic plant species, retain the capacity for photosynthesis and in some cases can complete their life cycles without attaching to a host (Tesitel et al., 2018). Hemiparasites can acquire water and mineral resources either through absorption through their roots or through parasitism of a host (Tesitel et al., 2018). This mixotrophic strategy provides competitiveness over non-parasitic and holoparasitic plants in the same ecosystem and hemiparasites are often successful in nutrient-poor environments (Quested, 2008; Selosse et al., 2017).

Santalum album (Indian sandalwood) is a slow-growing, evergreen forest species with significant commercial value for its oil and hardwood (Mahesh et al., 2018). *S. album* is a hemiparasitic plant whose roots can invade those of other plants through invasive haustoria, parasite specific structures that penetrate host roots and provide luminal continuity between host and parasite xylem vessels. *S. album* has functional chloroplasts that perform photosynthesis and regular root system while just partially relying on host plants to absorb water and nutrients, especially nitrogenous compounds, through haustoria (Bell and Adams, 2011; Lu et al., 2013, 2014). The high commercial value of *S. album* has resulted in its exploitation and illegal trading which have decimated natural populations of this species. To meet commercial demands, large-scale plantations have been established in China and elsewhere but little is known about the biology of nutrient acquisition in parasitic trees.

N is a critical nutrient for plant growth and hemiparasites can obtain N either

through root absorption or by haustorial connections with a host. The uptake of NH_4^+ and NO_3^- into hemiparasite roots is similar to that of autotrophic plants. NH_4^+ can be directly assimilated into amino acids whereas NO_3^- needs to be first reduced to NH_4^+ by nitrate reductase (NR) and nitrite reductase (NiR) before being incorporated into amino acids (Xu et al., 2012). After either direct uptake or enzymatic reduction from NO_3^-, NH_4^+ is assimilated to glutamine and glutamate via pathways catalyzed by glutamate synthase (GOGAT), glutamine synthetase (GS) and glutamate dehydrogenase (GDH) (Dluzniewska et al., 2007; Mcallister et al., 2012). Glutamine and glutamate are subsequently converted into other nitrogenous compounds by various deamination and transamination reactions (Rennenberg et al., 2010).

Nitrogen uptake and assimilation are strongly regulated by N supply; for example, plants often decrease N uptake and reduce N metabolism in low N environments (Anne et al., 2011; Zhang et al., 2018). Plant responses to nutrient levels are often mediated through phytohormones, notably cytokinin and gibberellin (Nagar et al., 2015; Yang et al., 2013). Plant responses to N availability have not been well studied in *S. album*. In pilot experiments (data not shown), we evaluated the combined effects of N and hormones/biostimulating molecules on the growth of *S. album* and found that melatonin had positive effects on growth performance. Melatonin is an animal hormone that modulates sleep, mood, sexual behavior, reproductive physiology, and circadian rhythms while also acting as an antioxidant (Tan et al. 2012; Li et al. 2016; Galano et al, 2011). This hormone is ubiquitous and highly conserved in plants and animal kingdoms (Li et al. 2016). In plants, melatonin regulates root development, seed germination, stress resistance and circadian rhythms (Annia and J, 2010; Li et al., 2020; Wei et al., 2020). For instance, melatonin acts as a growth-stimulating compound and promotes lateral root regeneration and seed germination in lupin (*Lupinus albus* L.), cucumber (*Cucumis sativus* L.) and mustard (*Brassica juncea* L.) (Arnao and Josefa, 2010; Na et al., 2012; Qian et al., 2009). Endogenous melatonin deficiency aggravates high temperature-induced oxidative stress in *Solanum lycopersicum* L (Jalal et al., 2018). Melatonin also modulates the expression of potassium channel protein genes in *Malus rockii* Rehd and promotes the absorption of

K^+ in potassium deficient conditions (Li et al., 2016). Increasing evidence suggests that the auxin signaling is likely associated with melatonin mediated growth regulation (Ishida et al., 2018; Liang et al., 2017). While these results indicate that melatonin may play multiple roles in plant growth, nothing is known about the possible role of melatonin in acclimation to N deficiency in hemiparasites.

We used RNA-seq and ATAC-seq analyses to identify the genes differentially regulated in *S. album* roots in response to melatonin exposure in low N conditions. RNA-seq is used to quantify mRNA transcripts that are differentially abundant in the sampled tissue before and after treatment (Zhang et al., 2018), in this case with melatonin. ATAC-seq is a method for identifying regions of the genome that have open chromatin and are accessible to Tn5 transposase; these are highly enriched for expressed genes and their regulatory regions (Buenrostro et al., 2013; D'Ippolito et al., 2018; Maher et al., 2017; Rizzardi et al., 2019). Both of these assays are best served by a high quality reference genome that we describe in this manuscript. The following questions were specifically addressed: (i) to what degree does melatonin influence the growth of *S. album* under low N conditions, and (ii) what are the mechanisms and key pathways through which melatonin effects the acclimation to N deficiency in *S. album*?

2 | MATERIALS AND METHODS

2.1 | Plant growth conditions and treatments

Seeds of *S. album* were surface-sterilized with 3% sodium hypochlorite for 5 min and then germinated and grown in pots (vermiculite: perlite, 2: 1, v/v, pH 6.8). The plants were irrigated weekly with 100 mL of modified Hoagland's nutrient solution (Meng et al., 2019) and cultivated in a greenhouse (natural light; temperature: 20-25°C; relative humidity: 75%) at the Research Institute of Tropical Forestry, Guangzhou, China (23°11'N, 113°23'E). After 4 weeks, uniform plants that showed similar growth (10 cm in height) were selected for exposure to modified Hoagland's nutrient solution (10 μM EDTA·FeNa, 0.5 μM H_2MoO_4, 30 μM H_3BO_3, 1 μM $CuSO_4·5H_2O$, 1 μM $ZnSO_4·7H_2O$, 5 μM $MnSO_4·H_2O$, 1 mM $MgSO_4·7H_2O$, 1 mM KH_2PO_4, 1 mM

Na_2SO_4, 1 mM $CaCl_2$) containing: (i) high N, 1 mM NH_4NO_3 (HN) or (ii) low N, 0.01 mM NH_4NO_3 (LN). Melatonin treatment was applied by adding 1 μM melatonin to the nutrient solution and compared to seedlings grown in the nutrient solution without melatonin (HNM and LNM). For auxin treatment, the plants mentioned above were grown in the modified Hoagland's nutrient solution supplemented with 5 μM IAA, 10 μM L-Kynurenine (L-Kyn, an auxin biosynthesis inhibitor) and *N*-1-naphthylphthalamic acid (NPA, an polar auxin transport inhibitor). One seedling was transplanted into a 10 cm diameter round pot and each treatment had 12 biological replicates. All plants were randomly rotated every week to prevent possible edge effects and fertilized every 3 days. The treatments lasted for 2 months, from October to December 2018, before harvest. Six plants from each treatment were used for gas exchange determination and root morphological measurments and remaining 6 plants of each treatment were used for subsequent physiological and molecular analysis.

2.2 | Measurement of growth parameters

The net photosynthetic rate (*A*), stomatal conductance (*gs*) and transpiration rate (*E*) of three mature leaves (leaf plastochron index = 3-5) were measured with a LI-6400 portable photosynthesis system (Li-Cor, Inc., Lincoln, NE, USA). The CO_2 concentration in the greenhouse was 400 ppm, and light was provided by an attached LED light source (1000 μmol photon m^{-2} s^{-1}). The chlorophyll content of the chosen plants was determined with a portable meter (Minolta SPAD 502 Meter, Osaka, Japan).

After gas exchange determination, the roots (c. 2 g) of each plant were harvested and analyzed using a WinRHIZO root analyzer system (WinRHIZO version 2007b, Regent Instruments Canada, Montreal, Canada). The fresh weights of shoots and roots were recorded.

2.3 | Reference genome for *S. album*

To obtain a reference genome for our ATAC-Seq and RNA-seq studies, we made a new genome assembly and annotation for *S. album* based on the raw sequence data (Accession ID, PRJNA411901). Augustus (RRID:SCR_008417) was used to predict

coding genes in *de novo* prediction. The gene structure was formed using Cufflinks (RRID:SCR_014597). Finally, 30,445 consensus protein-coding genes were predicted in the *S. album* genome by integrating all gene models by MAKER. Gene functions of protein-coding genes were annotated by searching functional motifs, domains, and the possible biological process of genes to known databases such as TrEMBL, InterPro, NR database, SwissProt with a maximal e- value of 1e-5, GO and KEGG by using the software Blast2GO.

2.4 | Transcriptome sequencing (RNA-Seq) and data processing

Total RNA was extracted and purified from roots of 3 month-old seedlings using the Omega reagent (R6827, Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's instructions. RNA quality was verified using gel electrophoresis, a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Sequencing libraries were sequenced using standard methods on an Illumina HiSeq 2500 platform at Frasergen (Frasergen Bioinformatics Co. Ltd, Wuhan, China).

We mapped reads to the *S. album* reference genome (Supplementary Table S1 and S2). Reads were aligned, and low-quality alignments were discarded using TopHat version 2.0.6 (Daehwan et al., 2015). We calculated gene expression levels using the fragments per kilobase of transcript per million mapped reads (FPKM) method in Cufflinks version 2.2.1 (Cole et al., 2010). By comparing the FPKM values of samples from different treatments, differentially expressed genes were then identified using the DESeq2 package in R (www. r-project. org). Kruskal's nonmetric multidimensional scaling was applied to the fold change matrix, and the genes whose absolute fold change was greater than 2 were considered to be differentially expressed genes (DEGs). Functional category and pathway analyses were performed using MapMan (Thimm et al., 2010).

2.5 | Transposase-accessible chromatin with sequencing assay (ATAC-seq)

Intact nuclei were isolated and purified from ground roots (two biological plant

replicates for each treatment) following a standard nuclear isolation protocol (Maher et al., 2017; Wilkins et al., 2016). Briefly, the tissue was suspended in nuclear isolation buffer (20 mM MOPS [pH 7], 40 mM NaCl, 90 mM KCl, 2 mM EDTA, 0.5 mM EGTA, 0.5 mM spermidine, 0.2 mM spermine, and 1× Roche Complete protease inhibitors). This mixture was then filtered through a 40-μM cell strainer. After centrifugation (500 g, 10 min, 4°C), the pellet was suspended in nuclear isolation buffer in an ice bath. The mixture was then centrifuged at 500 g for 10 min at 4°C and washed in nuclear wash buffer I (0.25 M sucrose, 10 mM Tris [pH 8], 10 mM $MgCl_2$, 1% Triton X-100, and 1× Roche Complete protease inhibitors). The nuclei were again pelleted at 500 g for 10 min at 4°C and resuspended in nuclear wash buffer II (1.7 M sucrose, 10 mM Tris [pH 8], 2 mM $MgCl_2$, 0.15% Triton X-100, and 1× Roche Complete protease inhibitors).

Approximately 50,000 purified nuclei were used for the transposase integration reaction at 37°C (Nextera, Illumina, FC-121-1030). The digested DNA fragments were purified using a MinElute PCR Purification Kit (28704, Qiagen, Germany) and then amplified using High Fidelity PCR Mix (R050Q, Takara, China). These amplified libraries were purified using Qiagen MinElute columns and analyzed on a Bioanalyzer High Sensitivity DNA Chip (Agilent 2100) prior to sequencing. Library sequencing was carried out using an Illumina HiSeq PE150 at Frasergen (Frasergen Bioinformatics Co. Ltd, Wuhan, China).

Adapters and low quality (phred quality < 10) bases were removed from raw sequencing reads with Trimmomatic (version 0.38), and trimmed reads were aligned to the reference genome with bowtie2 (version 2.4.3). High-quality properly paired alignments (mapping quality >= 30) were extracted with samtools (version 1.9). PCR duplications were removed with Picard tools, and then organelle contacts were excluded from downstream analysis. The distribution of insertion size was plotted with the R program (version 3.5.1) in order to evaluate chromatin integrity. Transcription Start Sites (TSS) enrichment scores were calculated as the ENCODE project suggested. In brief, read counts around TSS (3 kb) were summed per bin (400 bp) after shifting 75 bp toward the 5-prime end of each read and then extending to 150

bp uniformly, and then the average read counts of all transcripts in each bin were calculated. The number of bins that overlapped with TSS was taken as the TSS enrichment score. MACS2 was used for peak calling with the following parameters: '--nomodel --shift -75 --extsize 150' after converting alignments from BAM to BED format. For peak reproducibility evaluation, half of the reads were randomly selected as one pseudo replicate if replicates were not available, and then peaks from two datasets were evaluated with the method IDR (irreproducible discovery rate). Peaks that passed a cutoff of 0.05 were chosen as being reliable.

2.6 | *In situ* measurements of net fluxes of NH_4^+ and NO_3^-

Three fine roots (c. 1.0 mm in diameter, 50 mm in length) were randomly selected from each treatment to determine net fluxes of NH_4^+ and NO_3^- along the root. Measurements of NH_4^+ and NO_3^- fluxes along the roots were performed noninvasively using Noninvasive Micro-test Technology (NMT, system BIO-IM; Younger USA, LLC., Amherst, MA, USA). The NMT system and its application in net ion flux detection were previously described in detail (Meng et al. 2016a). Briefly, silanized glass micropipettes filled with a backfilling solution (100 mM NH_4Cl for the NH_4^+ electrode; 10 mM KNO_3 for the NO_3^- electrode) were front-filled with 30-μm columns of selective liquid ion-exchange cocktails (NH_4^+ LIX, #09879, Sigma; NO_3^- LIX, #72549, Sigma; reference electrode YG003-Y05, Younger USA). Electrodes with Nernst slopes greater than 55 mV per tenfold concentration difference were used after calibration. The measuring solution contained 0.1 mM KCl and 0.1 mM $CaCl_2$, as well as 10 or 1000 μM NH_4NO_3 depending on treatment. By moving the microelectrode perpendicular to the root axis, voltage gradients near the root surface (approximately 5 μm above the root surface) were measured. Fluxes were measured in steps of either 300 μm (approximately 0-2 mm) or 8 mm (approximately 2-30 mm) from the root tip. The voltage gradients were transformed to ion fluxes using the MageFlux software attached to the NMT system.

2.7 | *Ex situ* N uptake using the ^{15}N tracer method

Roots (three replicates per treatment) were excised and rinsed in 1 mM $CaSO_4$ for 20 min to preserve membrane integrity followed by incubation for 20 min in ^{15}N labeling solution (0.5 mM $CaCl_2$, 0.01 M sucrose, 0.01 mM or 1 mM NH_4NO_3 (either $^{15}NH_4NO_3$ or $NH_4{}^{15}NO_3$), and finally washed in 1 mM $CaSO_4$ solution to remove ^{15}N tracer solution adsorbed onto the outer surfaces of roots (Templer and Dawson, 2004; Finzi and Berthrong 2005; Socci and Templer 2011). The samples were dried for 72 h at 72 °C in an oven until the dry weight was steady and ground into powder. Roots without ^{15}N-labeled N served as the control. The ^{15}N abundances and total N were analyzed using a Thermo Electron Flash EA 1112 elemental analyzer coupled to a Thermo Electron Delta V isotope ratio mass spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The ^{15}N abundance was calculated as follows:

$$\delta^{15}N = [(R_{sample}/ R_{standard}) - 1] \times 1{,}000,$$

where R_{sample} is the isotope ratio of the sample and $R_{standard}$ is the isotope ratio of atmospheric N_2. The ^{15}N uptake was determined as ^{15}N uptake = DW× N% × (atom%$_{root}$ –atom%$_{control}$), where DW is the dry weight.

2.8 | Determination of activities of enzymes involved in N assimilation

The activities of enzymes involved in N assimilation (NR, EC 1.7.99.4; NiR, EC 1.7.2.1; GS, EC 6.3.1.2; GOGAT, EC 1.4.7.1 and GDH, EC 1.4.1.2) in roots of *S. album* were determined using kits following the manufacturer's instructions (Solarbio LIFE SCIENCES, BC0080; BC1540; BC0080; BC0070; BC1460, Beijing, China).

2.9 | Immunocytochemistry and determination of IAA

Immunolocalization of IAA in the haustoria of *S. album* was conducted based on the method of Escandon et al. (2016). Haustoria (c. 0.8 cm in diameter) before penetrating into the host root were isolated and immediately fixed for 24 h at 4 °C in 3% (w/v) paraformaldehyde containing 0.1% (v/v) Triton X-100 (Sigma-Aldrich Co., St Louis, MO, USA) to which was added 4% (w/v) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma Aldrich Co.) to immobilize the IAA by covalent binding to proteins. Sections of 50 μm were cut with a sliding cryotome CM1510S (Leica

Microsystems, Wetzlar, Germany) and immersed for 5 min in an ascending and descending 25, 50, 75 and 100% ethanol series, then washed for 30 min in PBS (137 mM NaCl, 2.7 mM KCl, 7.9 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, at pH 7.3) containing 0.1% (v/v) Tween 20, and finally washed for 5 min in PBS. Samples were incubated for 10 min in PBS containing 5% (w/v) bovine serum albumin followed by incubation for 12 h in IAA primary antibody (Ref.: AS09 421 AGRISERA, Vannas, Sweden) and then washed three times for 10 min in PBS containing 0.1% (v/v) Tween 20. After incubation for 1 h in darkness in the secondary antibody Alexa 488 (Molecular Probes, Gottingen, Germany), sections were washed again in PBS containing 0.1% (v/v) Tween 20. Finally, samples were counterstained with DAPI (4',6-diamidino-2-phenylindole), and fluorescence was visualized using a confocal microscope (Leica TCS SP2 AOBS) and then imaged with Fiji Software.

The levels of IAA were determined as described previously (You et al., 2016). Haustoria (c. 0.5 g) were ground into powder in an ice bath and the powder was extracted in 4 ml of isopropanol/hydrochloric acid. The extract was shaken for 30 min at 4°C and then 10 mL dichloromethane was added. The mixture was shaken for 30 min at 4°C again. After centrifugation (13,000 rpm, 5 min, 4°C), the lower, organic phase were dried under N_2 and dissolved in 150 μL of methanol (0.1% methane acid) and fifiltered with a 0.22 μm fifilter membrane. High-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) was then performed using a ZORBAX SB-C18 (Agilent Technologies) column (2.1 mm × 150 mm; 3.5 mm). MS conditions were as follows: the spray voltage was 4500 V; the pressure of the air curtain, nebulizer, and aux gas were 15, 65, and 70 psi, respectively; and the atomizing temperature was 400°C.

2.10 | Statistical analysis

All statistical tests were performed using IBM SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) and graphs were plotted with origin 8.0. We analyzed all variables by one-way ANOVA, and differences between means were determined on the basis of Duncan's multiple range tests ($P < 0.05$).

3 | RESULTS

3.1 | Growth parameters

After 2 months of melatonin treatment and different levels of N supply, *S. album* plants exhibited different morphological and photosynthetic characteristics after being treated with 1 μM melatonin in low N conditions. Low N conditions significantly decreased plant growth, resulting in higher root-shoot ratio and inhibited root development compared with plants in 1 mM NH_4NO_3 (Figure 1 and Table 1). Chlorophyll content, net photosynthetic rate (*A*), stomatal conductance (*gs*) and transpiration rate (*E*) were also significantly reduced in LN conditions (Table 1).

Melatonin significantly increased total root length and root surface area in LN conditions while this effect was not significant in HN conditions (Table 1). Similarly, almost all photosynthetic characteristics were higher in melatonin-treated plants (Table 1). Compared to the nonmelatonin treatment plants, melatonin treated plants grew more haustoria in roots under low N supply (Figure 1c).

3.2 | RNA-seq and ATAC-seqinterrogation of differentially expressed genes and enriched pathways induced by melatonin treatment and low N stress

An average of 3.5 GB clean reads of the RNA-libraries were obtained and mapped to the *S. album* genome (Table S1 and S2). A total of 1467 DEGs (adjusted $P < 0.05$) were identified by pairwise sample time and treatment level comparisons (Table S3). The number of high-quality reads generated from each of the ATAC-libraries and biological replicates varied from 19.10 to 35.06 million (File S1). Approximately 6.31–16.56 million clean reads per library were mapped to the reference genome of *S. album,* and the open regions were distributed throughout the genome in genic and intergenic regions (File S1). Additionally, the enriched regions (transposase hypersensitive sites, THS) distribution showed a sharp peak approximately 50-150 bp before the transcription start sites (TSS) (Figure 2a). More than 90% of the clean data were reproduced across the biological repeats, which showed a high degree of correlation within replicates (Figure 2b). Directional RNA-seq libraries were

constructed from the same samples used for ATAC-seq to assess the relationship between chromatin accessibility and gene expression. Generally, chromatin accessibility showed a positive correlation with the transcript abundance of the downstream gene (Figure 2c). The relative abundance of corresponding mRNAs was generally correlated with the levels of ATAC-seq signals (Figure 2d).

We combined chromatin accessibility and gene expression data from *S. album* roots exposed to 1 mM or 0.01 mM N with and without 1 μM melatonin to profile gene regulatory events. To identify the genes that might be impacted by melatonin, we derived a list of 2,214 melatonin-induced differentially accessible regions (mDARs) in LN conditions and 2,684 mDARs in HN conditions (Figure 3a and File S2). We then asked whether the melatonin-induced DARs were also associated with differentially expressed genes (DEGs). Of the 2, 214 mDARs in LN conditions, 119 genes were associated with melatonin-induced DEGs (Figure 3a). Among the 2,684 mDARs in HN conditions, 127 genes were overlap with melatonin-induced DEGs (Figure 3a). In total, 89 and 149 genes differentially expressed in response to N supply were also identified under melatonin and nonmelatonin treatment respectively (Figure 3b). These DARs and DEGs were classified according to their different expression patterns and those enriched in specific functional categories after different treatments, including melatonin and/or low N stress, were identified.

To elucidate the biological processes that were involved in the response to melatonin in *S. album*, overrepresentation analyses on the DARs and DEGs were performed. The DARs and DEGs in response to melatonin in LN conditions showed considerable enrichment in terms related to “amino acid biosynthesis,” “amino/nucleotide sugar metabolism” and “flavonoid biosynthesis” (Figure 3c). In HN conditions, the DARs and DEGs were strongly enriched in “hormone signal transduction,” “MAPK signaling pathway” and “phenylpropanoid biosynthesis” (Figure 3d). Strikingly, N metabolism and hormone signal transduction (auxin) were significantly altered (Figure 3c, d) and these pathways were investigated thereinafter more in detail.

3.3 | Effects of melatonin treatment and low N stress on nitrogen metabolism

Net fluxes of NH_4^+ and NO_3^- were different at different positions along the roots of *S. album* with maximal net influxes of NH_4^+ (Figure 4a) and NO_3^- (Figure 4b) occurred approximately 10 and 15 mm from the root tip respectively. At this position, both NH_4^+ and NO_3^- influxes displayed little fluctuation in 10 min (Figure 4c, d). Net fluxes of NH_4^+ and NO_3^- at these two positions were investigated to detect the uptake of NH_4^+ and NO_3^-.

LN treatment significantly decreased net uptake of NH_4^+ (Figure 5a) and NO_3^- (Figure 5b) in both melatonin-treated and untreated plants. However, melatonin treatment plants maintained significantly higher NH_4^+ and NO_3^- uptake rates (except in the case of NH_4^+ uptake under high N) compared with plants that received no additional melatonin (Figure 5a, b). Total N concentrations in the roots and leaves were dramatically decreased in response to LN treatment, and melatonin had no effects on these concentrations in roots and leaves (Figure 5c, d). An *ex situ* N uptake experiment using ^{15}N tracer showed that low N conditions decreased ammonium uptake but had no influence on nitrate uptake (Figure 5e, f). The roots of *S. album* supplied with melatonin had significantly higher ^{15}N-labeled ammonium uptake under low N conditions (Figure 5e) and higher nitrate uptake in response to high N supply (Figure 5f).

3.4 | Differentially expressed genes and activities of enzymes involved in N uptake and assimilation

The transcript levels of most genes predicted to encode key *NRTs* and *AMTs* greatly varied in response to low N stress and melatonin treatment in roots of *S. album*. The expression of predicted *NRT1;1* and *NRT2;4* genes was upregulated by low N stress, whereas *AMT1;1* and *AMT3;1* were downregulated by low N stress (Figure 6a and Table S4). However, melatonin decreased the abundances of *NRT1;1* and *NRT2;4* transcripts but increased the expression levels of *AMT1;1* and *AMT3;1* (Figure 6a). Almost all genes encoding N assimilation enzymes (*NR1*, *NR2*, *NiR*, *GS1;3*, *GS2* and *GDH*) were significantly downregulated in response to LN stress (only *GOGAT*

expression was increased), while melatonin treatment induced higher transcript abundances of these genes under both low and high N supply (Figure 6a). Enzyme activities of both NR (Figure 6b) and GS (Figure 6d) significantly decreased in fine roots in LN conditions which was alleviated with 1 μM melatonin. Melatonin increased NR activities in LN conditions but had no significant effects on them under high N conditions (Figure 6b). However, GS activities were increased by melatonin under both LN and HN conditions (Figure 6d). Neither low N nor melatonin had any significant effects on NiR and GOGAT activities in the roots of *S. album* (Figure 6c, e).

Transcript levels of genes involved in the synthesis of most amino acids were decreased in response to LN supply (Figure 7 and Table S3). For example, in LN conditions, the expression of the genes encoding anthranilate synthase (*AS*) and Tryptophan aminotransferase (*TAR*) was decreased, whereas some genes related to N metabolism and amino acid degradation (*GDH*, *PGDH*, *PSAT*, *GAD*, *MGL* and *ProDH*) were clearly upregulated (Figure 7). Melatonin treatment increased the transcript levels of most genes involved in amino acid metabolism under conditions of low and/or high N supply (Figure 7).

3.5 | Alteration of IAA metabolism by melatonin treatment and low N stress

IAA immunolocalization analysis (transversal haustorium section) showed different intensities and distributions across the haustorium in response to melatonin addition and N supply levels (Figure 8a). Under the LN and HN treatments, IAA signals were weak (Figure 8a). However, melatonin treatment induced significantly increased IAA signals under both low N and high N supply, and the magnitudes of the increment under high N supply were greater than those under low N stress (Figure 8a, b).

To further investigate the roles of IAA in haustorium formation, we monitored haustorium development in the presence of exogenous IAA, L-Kyn (the auxin synthesis inhibitor) and NPA (the polar auxin transport inhibitor). In general exposure of plants to IAA resulted in more haustorium formation while treatment with the L-Kyn and NPA significantly decreased the level of haustorium formation (Figure 8c).

4 | DISCUSSION

N deficiency is a major limitation to plant growth. Plants have developed various strategies, including altering the architecture of the root system and improving nutrient acquisition and remobilization, to overcome N deprivation, by activating ion transport (Jie et al., 2015; Meng et al., 2016b). Our results showed *S. album* alters root architecture and nutrient acquisition under low N conditions (Table 1). The inhibition of *S. album* growth may result from a lower net photosynthetic rate under low N conditions, as photosynthesis is a major source of building blocks and energy for biomass production and maintenance. The observed stunted growth, higher root-shoot ratio and decreased photosynthesis may facilitate plant survival via the redistribution of limited resources.

To identify major changes in *S. album* under low N and/or melatonin treatments, global gene transcription was analyzed by high-throughput sequencing. RNA-seq is a method that quantifies steady state levels of mRNA transcripts under different conditions. ATAC-seq, delineates open chromatin regions and potentially transcription factor binding sites across the genome using a hyperactive Tn5 transposase that cleaves DNA and inserts sequencing adapters as probes (Buenrostro et al., 2013; Corces et al., 2018; Maher et al., 2017; Rizzardi et al., 2019). To map the DEGs and DARs from these two analyses, we made and annotated a *de novo* assembly genome of *S. album* for the mapping of DARs (Table S1 and S2). DEGs and DARs were coupled on the assumption that most genes are regulated by the nearest regulatory elements. Complementary RNA-seq and ATAC-seq identified major changes in N metabolism and plant hormone signal transduction in *S. album* under melatonin treatment (Figure 3). It was clear that the nitrogen uptake and metabolism pathways in *S. album* were significantly influenced by N availability (Figure 3). In plants, NH_4^+ and NO_3^- are first incorporated into glutamine and glutamate and then used to synthesize other amino acids by transamination (Castro-Rodriguez et al., 2011; Rennenberg et al., 2010). Growing *S. album* under low N conditions resulted in low N concentration in tissues (Figure 5). Previous studies showed that plants decreased N

uptake and assimilation in low N conditions (Anne et al., 2011; Zhang et al., 2018). Indeed, the roots of *S. album* under low N conditions had lower NH_4^+/ NO_3^- influx and ^{15}N-labeled NH_4^+ uptake than those in high N (Figure 5). This is consistent with results from poplar (*Populus*), Douglas fir (*Pseudotsuga menziesii*) and soybean (*Glycine max*) (Hawkins and Robbins, 2010; Luo et al., 2013). Additionally, the roots of *S. album* under lLN treatment had significantly higher ^{15}N-labeled NO_3^- uptake but lower ^{15}N-labeled NH_4^+ uptake than those of the HN treatment (Figure 5), suggesting that nitrate may play a key role in maintaining plant survival (Xu et al., 2012).

Nitrogen uptake from soils is mediated mainly by the products of *AMTs* and *NRTs*, transcription of which is sensitive to N availability. We measured the expression of the genes encoding the key transporters *NRT1;1*, *NRT2;4*, *AMT1;1* and *AMT3;1* and found that the transcription levels of *NRT* genes were significantly increased under low N conditions, while *AMT1;1* and *AMT3;1* were downregulated. *NRT1;1* is not only a nitrate transporter but also functions as a major nitrate sensor that is sensitive to nitrogen deficiency in plants (Wang et al., 2018). *NRT2;4* is a key high-affinity transporter that is quickly and highly induced by low nitrate (Xuan et al., 2017). These results indicate that plants attempt to maintain regular N metabolism while adapting to low N stress at the molecular level. In agreement with the decreased NH_4^+/ NO_3^- uptake, NR, the critical rate-limiting enzyme in nitrate assimilation, was significantly inhibited by low N supply in *S. album* (Figure 6b). These findings suggest that the processes of N uptake and assimilation slowed in acclimation to low N stress. Indeed, most of the genes involved in amino acid synthesis were downregulated under low N treatment; this may be associated with the substrates derived from inorganic N. However, a few genes, e.g., *PGDH* and *PSAT*, two key enzymes involved in Ser synthesis (Ros et al., 2014), showed higher transcription levels in response to N deficiency (Figure 7). *PGDH* and *PSAT* are active in meristems, and it has recently been suggested that serine biosynthesis acts as a signal controlling cell proliferation (Ros et al., 2014), suggesting that root cell renewal was active in replacing senescent cells under low N stress. Consistent with these findings, key genes involved in GABA synthesis (*GADs*), which are related to cell elongation

510 (Hausler et al., 2014), were also upregulated under low N stress (Figure 7).

511 Melatonin application stimulates the growth of roots and photosynthesis under LN
512 conditions and our experiments are focused on understanding of how melatonin
513 regulates N metabolism. The application of melatonin enabled plants to maintain
514 significantly higher nitrogen uptake and assimilation rates compared to plants not
515 exposed to melatonin. Previous studies show that melatonin modulates nutrient
516 metabolism and promotes lateral root formation in rice (Chao et al., 2016; Liang et al.,
517 2017; Zhao et al., 2015). Based on the results of high-throughput sequencing analysis,
518 auxin signaling may be associated with the melatonin responses in *S. album* (Figure
519 3d). In this study, supplemental melatonin upregulated N transporters at low N levels
520 (Figure 5). The addition of melatonin also increased the activities of the enzymes NR,
521 GS and GOGAT (Figure 6), suggesting that melatonin has a function in controlling
522 NH_4^+/ NO_3^- transporters and assimilation enzymes. However, the processes of N
523 assimilation (downstream processes after N uptake) is influenced by N-containing
524 precursors (Luo et al., 2013). Thus, in the present study, higher activities of N
525 assimilation enzymes under melatonin treatment may also be an indirect effect of
526 increased N uptake. These results indicate that low N stress disturbs nitrogen
527 metabolism homeostasis and that melatonin can play an important role in regulating
528 these processes.

529 Movement of nutrients from host to parasite across the haustorium provides another
530 important N source for *S. album*. In parasitic plants, endogenous auxin synthesis and
531 accumulation are key factors associated with haustorium development (Ishida et al.,
532 2016; Zhang et al., 2015). For example, the application of both the auxin efflux
533 inhibitor (2,3,5-triiodobenzoic acid) and an inhibitor of auxin activity
534 (*p*-chlorophenoxyisobutyric acid) resulted in a significant reduction in haustorium
535 number (Tomilov et al., 2005). In *Arabidopsis*, ZAT6 is involved in melatonin-
536 mediated auxin signaling through forming an interacting complex of auxin signaling
537 pathway (Shi et al., 2018). A positive response to 2,6-dimethoxy-p-benzoquinone
538 (DMBQ, commonly recognized as a haustorium-inducing factor) by an
539 auxin-inducible promoter-reporter construct further confirmed that auxin accumulates

during haustorium development (Tomilov et al., 2005). Similarly, earlier studies demonstrated that the expression of *YUC3* and auxin biosynthesis in roots play a pivotal role in haustorium formation in the root parasitic plant *P. japonicum*, which is consistent with the results of our study (Figure 8) (Ishida et al., 2016). However, in the present study, haustorium development in *S. album* was promoted by IAA and inhibited by the auxin biosynthesis inhibitor L-Kyn and the polar auxin transport inhibitor NPA (Figure 8c). Thus, haustorium development in *S. album* may be enhanced by auxin both synthesized in the root and transported from shoots. Melatonin had positive effects on growth performance of *S. album* under low nitrogen condition. However, this effect will be confused if the *S. album* plants were co-cultivate with host plants because the host plants may also benefit from the melatonin treatment. We cannot distinguish direct roles of melatonin or a secondary effect of host plants on growth performance of *S. album*. However, in some degree, more haustoria means greater water and nitrogen uptake capacity. Thus, we hypothesize that melatonin may improve both the growth performance of the hemiparasite *S. album* as well as its N acquisition via auxin-mediated haustorium development and this will be confirmed in further study.

Taken together, the findings of our study indicate that melatonin has positive effects on the growth performance of *S. album*, especially under LN stress. An integrated analysis of ATAC- and RNA-seq data in response to melatonin application and/or low N has provided a comprehensive view of the major processes underlying the N metabolism of hemiparasites in low N environments. Our results revealed that accelerated N uptake and assimilation together with stimulation of auxin-mediated haustorium formation may play key roles in increasing LN stress toleration in melatonin-treated *S. album* hemiparasites.

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AUTHOR CONTRIBUTIONS

SM and XLW conceived the ideas and designed the experiment. ZB, ZSL and FCY carried out the experiments and generated the data. SM, JIY and JKL analyzed the data and wrote the manuscript.

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TABLE 1 Growth and photosynthetic characteristics of *S. album* as affected by nitrogen nutrition and melatonin treatment. Data indicate means ± SE (n = 6). Bars labeled with different letters indicate statistically significant differences between treatments ($p < 0.05$). HN, 1 mM NH_4NO_3; HNM, 1 mM NH_4NO_3 and 1 μM melatonin; LN, 0.01 mM NH_4NO_3; LNM, 0.01 mM NH_4NO_3 and 1 μM melatonin.

Treatment	Root biomass (mg DW)	Shoot biomass (mg DW)	Root: Shoot ratio	Total root length (cm)	Total root surface area (cm^2)	Total root volume (cm^3)	Chlorophyll content	*A* (mmol CO_2 m^{-2} s^{-1})	g_s (mol H_2O m^{-2} s^{-1})	*E* (mmol H_2O m^{-2} s^{-1})
HN	0.29±0.11a	3.20±0.11a	0.09±0.00c	156±9.70a	15.1±0.58ab	4.29±0.79a	31.5±1.02a	7.57±0.33a	0.17±0.01a	2.50±0.07a
HNM	0.27±0.18a	3.36±0.13a	0.08±0.00c	173±6.81a	16.8±0.86a	5.75±0.25a	28.4±0.60a	7.88±0.21a	0.16±0.01a	2.36±0.07a
LN	0.21±0.01c	0.95±0.11c	0.23±0.02a	74.6±3.19c	7.55±0.40c	1.03±0.10b	14.9±0.93c	3.07±0.20c	0.08±0.01b	1.19±0.06c
LNM	0.25±0.02bc	1.56±0.06b	0.16±0.01b	102±3.07b	13.1±0.94b	2.47±0.17b	23.1±0.97b	4.91±0.24b	0.11±0.01b	1.67±0.06b

SUPPORTING INFORMATION

Table S1 *S. album* genome data splicing and annotation.

Table S2 Predicted coding sequences of *S. album.*

Table S3 Differentially expressed genes (DEGs) among treatments in *S. album*.

Table S4 Accession number and expression data of genes in nitrogen uptake, assimilation and amino acid metabolism pathways among treatments in *S. album*.

File S1 peak calling among treatments in *S. album*.

File S2 Differentially accessible regions (DARs) among treatments in *S. album*.

FIGURE LEGENDS

FIGURE 1 Growth performance of *S. album* as affected by nitrogen nutrition and melatonin treatment. (a) and (b) Plants after 2 months of exposure to different N supply levels and melatonin treatment. (c),Visual examination of haustorium formation under different N supply levels and melatonin treatment. HN, 1 mM NH_4NO_3; HNM, 1 mM NH_4NO_3 and 1 μM melatonin; LN, 0.01 mM NH_4NO_3; LNM, 0.01 mM NH_4NO_3 and 1 μM melatonin.

FIGURE 2 Landscape of ATAC-Seq detecting chromatin accessibility in *S. album* as affected by nitrogen nutrition and melatonin treatment. (a), Distribution of open chromatin around THSs. Peaks were assigned to the closest downstream gene (c. 21273 genes), and the distance between the ATAC peak summit and the TSS or ATG was calculated. The line depicts the smoothed distribution of distances (kernel density estimate). (b), Pearson correlation coefficients of RNA abundance for the downstream gene and relative chromatin accessibility, demonstrating a high degree of reproducibility. (c), Genome browser screenshot (locus = NXEK01000034.1:3984487- NXEK01000034.1: 4000908) of ATAC-seq chromatin accessibility profiles (top blue) and corresponding RNA-seq profiles (bottom red). (d), Heatmap depicting relative chromatin accessibility and mRNA abundance profiles of the downstream gene. Relative accessibility in ATAC-seq peaks and the relative transcript abundances of the downstream located genes were plotted in the same order.

HN, 1 mM NH_4NO_3; HNM, 1 mM NH_4NO_3 and 1 μM melatonin; LN, 0.01 mM NH_4NO_3; LNM, 0.01 mM NH_4NO_3 and 1 μM melatonin.

FIGURE 3 Main gene expression patterns in *S. album* and functional category enrichment as affected by nitrogen nutrition and melatonin treatment. (a), Venn diagram of overlaps between differentially expressed genes (DEGs) and differentially accessible regions (DARs) following treatment with melatonin. (b), Venn diagram of overlaps between DEGs and DARs following treatment with N conditions. (c), Functional category enrichment of DARs and DEGs in response to melatonin treatment under low N conditions. (d), Functional category enrichment of the DARs and DEGs in response to melatonin under high N conditions. The green boxes show three representative pathways regulated by melatonin. HN, 1 mM NH_4NO_3; HNM, 1 mM NH_4NO_3 and 1 μM melatonin; LN, 0.01 mM NH_4NO_3; LNM, 0.01 mM NH_4NO_3 and 1 μM melatonin.

FIGURE 4 Net NH_4^+ (a) and NO_3^- (b) (pmol cm^{-2} s^{-1}) fluxes along the root of *S. album*. Net NH_4^+ (c) and NO_3^- (d) (pmol cm^{-2} s^{-1}) fluxes at specified distances (for NH_4^+, maximal influx position at 10 mm; for NO_3^-, maximal influx position at 15 mm) from the tips in 10 min. Net influxes correspond to positive values, and net effluxes are indicated by negative values. Data indicate means ± SE (n = 6).

FIGURE 5 Nitrogen uptake and total N concentration in roots of *S. album* as affected by nitrogen and melatonin. (a), *In situ* net NH_4^+ uptake; (b), *In situ* net NO_3^- uptake; (c), Total N concentration in roots; (d), Total N concentration in leaves; (e), *Ex situ* ^{15}N- NH_4^+ uptake; (f), *Ex situ* ^{15}N- NO_3^- uptake. Data indicate means ± SE (n = 6). Bars labeled with different letters indicate statistically significant differences between treatments ($p < 0.05$).HN, 1 mM NH_4NO_3; HNM, 1 mM NH_4NO_3 and 1 μM melatonin; LN, 0.01 mM NH_4NO_3; LNM, 0.01 mM NH_4NO_3 and 1 μM melatonin.

FIGURE 6 (a) Pathway viewer illustration of nitrogen uptake and assimilation in *S.*

album as affected by nitrogen nutrition and melatonin treatment. The expression in three nutrient treatments (HNM, LN, LNM) relative to HN are represented by 3-box strings. Heat maps were drawn using *log2*- transformed FPKM values. (b-e), Bar graphs represent the means ± SE (n = 6) of enzyme activities. Bars labeled with different letters indicate statistically significant differences between treatments ($p < 0.05$). (b), Nitrate reductase (μM NO_3^- h^{-1} (g FW)$^{-1}$); (c), Nitrite reductase (mmol NO_2^- h^{-1} (mg protein) $^{-1}$); (d), Glutamine synthetase (nkat (mg protein) $^{-1}$); (e), Glutamate synthase (nkat (g protein) $^{-1}$). Detailed accession number and expression data are provided in Table S4 available as Supplementary Data. *AMT*, ammonium transporter; *NRT*, nitrate transporter; *NR*, nitrate reductase; *NiR*, nitrite reductase; *GS*, glutamine synthetase; *GOGAT*, glutamate synthase; *GDH*, glutamate dehydrogenase). HN, 1 mM NH_4NO_3; HNM, 1 mM NH_4NO_3 and 1 μM melatonin; LN, 0.01 mM NH_4NO_3; LNM, 0.01 mM NH_4NO_3 and 1 μM melatonin.

FIGURE 7 Amino acid metabolism pathway in *S. album* as affected by nitrogen nutrition and melatonin treatment. Progression of gene expression in response to different treatment conditions (HNM, LN, LNM) compared with the control (HN) are indicated in 3-box strings. Detailed accession number and expression data are provided in Table S4 available as Supplementary Data. HN, 1 mM NH_4NO_3; HNM, 1 mM NH_4NO_3 and 1 μM melatonin; LN, 0.01 mM NH_4NO_3; LNM, 0.01 mM NH_4NO_3 and 1 μM melatonin. *TAR*, Tryptophan aminotransferase; *AS*, anthranilate synthase; *CM*, chorismate mutase; *ADT*, arogenate dehydratase; *DHA/SDH*, 3-dehydroquinate dehydratase/shikimate 5-dehydrogenase; *TDC*, tyrosine decarboxylase; *TAT*, tyrosine aminotransferase; *PGDH*, phosphoglycerate dehydrogenase; *PSAT*, phosphoserine aminotransferase; *SAT*, serine O-acetyltransferase; *CYSC*, cysteine synthase; *GAT*, glyoxylate aminotransferase; *BCAT*, branched-chain amino acid aminotransferase; *MCC*, methylcrotonyl-CoAcarboxylase; *GS*, glutamine synthetase; *GOGAT*, glutamate synthase; *GDH*, glutamate dehydrogenase; *P5CS*, pyrroline-5-carboxylate synthase; *GAD*, glutamate decarboxylase; *ProDG*, proline dehydrogenase; *ADC*, arginine decarboxylase; *AspAT*, aspartate aminotransferase; *ANS*, asparaginase; *ASN*,

asparagine synthase; *AK*, aspartate kinase; *LKR/SDH*, lysine ketoglutarate reductase-saccaropine dehydrogenase; *HMT*, homocysteine S-methyltransferase; S*AMS*, S-adenosylmethionine synthase; *MGL*, methionine gamma-lyase; *TA*, threonine aldolase; *MCC*, methylcrotonyl-CoA carboxylase.

FIGURE 8 Auxin signals in haustorium and effects of IAA on haustorium number. (a), IAA immunolocalization across the haustorium in response to melatonin addition and N supply levels. HN, 1 mM NH_4NO_3; HNM, 1 mM NH_4NO_3 and 1 μM melatonin; LN, 0.01 mM NH_4NO_3; LNM, 0.01 mM NH_4NO_3 and 1 μM melatonin. Immunolocalization of DAPI (blue signal) and IAA (Green signal) in transversal haustorium section. (b), IAA concentration (ng g^{-1}) in haustorium in response to melatonin addition and N supply levels. (c), Haustorium number in the presence of exogenous IAA, L-Kyn and NPA under different treatments. Data indicate means ± SE (n = 6). Bars labeled with different letters indicate statistically significant differences between treatments ($p < 0.05$).