Melatonin biosynthesis gene MdASMT9 confers tolerance to nitrogen deficiency in an MdHY5-dependent manner in apple plants

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Abstract

Nitrogen (N) is a vital nutrient for crop growth and development which influences both yield and quality. Melatonin (MT), a known enhancer of abiotic stress tolerance, has been extensively studies; however, its relationship with nutrient stress, particularly N, and the underlying regulatory mechanisms of MT on N uptake remain unclear. In this study, exogenous MT treatment was found to improve the tolerance of apple plants to N deficiency. Apple plants overexpressing the MT biosynthetic gene N-acetylserotonin methyltransferase 9 (*MdASMT9*) was used to further investigated the effects of endogenous MT on low-N stress. The overexpression of *MdASMT9* improved the light harvesting and heat transfer capability of apple plants, thereby mitigating the detrimental effects of N deficiency on the photosynthetic system. Proteomic and physiological data analyses indicated that *MdASMT9* overexpression enhanced the trichloroacetic acid (TCA) cycle and positively modulated amino acid metabolism to counteract N-deficiency stress. Additionally, both exogenous and endogenous MT promoted the transcription of *MdHY5*, which in turn bound to the *MdNRT2.1* and *MdNRT2.4* promoters and activated their expression. Notably, MT-mediated promotion of *MdNRT2.1* and *MdNRT2.4* expression in an *MdHY5*-dependent manner, ultimately enhancing N absorption. Taken together, these results may provide useful insights into the relationship between *MdASMT9*-mediated MT biosynthesis and N uptake under N-deficiency conditions in apple plants.

Title

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Abstract

Nitrogen (N) is a vital nutrient for crop growth and development which influences both yield and quality. Melatonin (MT), a known enhancer of abiotic stress tolerance, has been extensively studies; however, its relationship with nutrient stress, particularly N, and the underlying regulatory mechanisms of MT on N uptake remain unclear. In this study, exogenous MT treatment was found to improve the tolerance of apple plants to N deficiency. Apple plants overexpressing the MT biosynthetic gene N-acetylserotonin methyltransferase 9 (*MdASMT9*) was used to further investigated the effects of endogenous MT on low-N stress. The overexpression of *MdASMT9* improved the light harvesting and heat transfer capability of apple plants, thereby mitigating the detrimental effects of N deficiency on the photosynthetic system. Proteomic and physiological data analyses indicated that *MdASMT9* overexpression enhanced the trichloroacetic acid (TCA) cycle and positively modulated amino acid metabolism to counteract N-deficiency stress. Additionally, both exogenous and endogenous MT promoted the transcription of*MdHY5* , which in turn bound to the *MdNRT2.1* and*MdNRT2.4* promoters and activated their expression. Notably, MT-mediated promotion of *MdNRT2.1* and *MdNRT2.4* expression in an *MdHY5* -dependent manner, ultimately enhancing N absorption. Taken together, these results may provide useful insights into the relationship between *MdASMT9* -mediated MT biosynthesis and N uptake under N-deficiency conditions in apple plants.

Keywords: melatonin, *MdASMT9* , nitrogen deficiency,*MdHY5* , nitrate transporters proteins

INTRODUCTION

Nitrogen (N) is an essential macronutrient for plant growth and development, serving as a main component of the nucleotides and proteins. Its deficiency hinders the synthesis of nucleic acids, hormones, chlorophyll, and other metabolites required for primary metabolism (Yang et al. , 2015; Zrenner et al. , 2006). As a result, plant productivity is largely dependent on N fertilization, which is a limiting factor in agricultural production (Kaur et al. , 2017). However, excessive N application leads to increased costs, environmental pollution, and imperiled ecological conditions (Yu et al. , 2019; Zhang et al. , 2015). Accordingly, enhancing the N use efficiency of plants is of utmost importance for sustainable crop production and effective agricultural research.

For plants, nitrate is one of the main forms of N available (Crawford and Glass, 1998). In response to varying nitrate conditions, plants have evolved the high-affinity nitrate transport system (HATS) and low-affinity nitrate transport system (LATS) (Crawford and Glass, 1998; Glass et al. , 1992). HATS operates at very low external nitrate concentrations (below 1 mM) while LATS is engaged when the external nitrate concentration exceeds 1 mM (Behl et al. , 1988; Siddiqi et al. , 1990). Nitrate transporters proteins (NRTs) can be categorized as NRT1 or NRT2 (Krapp et al. , 2014; Léran et al. , 2014). Among the 53 NRT1 genes in *Arabidopsis* , NRT1.1 functions as a dual-affinity transporter, while the remaining members are all low-affinity transporters (Liu et al. , 1999; Tsay et al. , 2007). NRT2 proteins play a key role in response to low nitrate conditions, with NRT2.1 being a major contributor to HATS (Okamoto et al. , 2003; You et al. , 2022). However, NRT2.4 also exhibits a very high-affinity for nitrate and exerts dual effects on plant shoots and roots under N-deficiency conditions (Kiba et al. , 2012). In *nrt2.4* mutants, reduced nitrate uptake and nitrate content has been observed in shoot phloem exudates under low external supply (Kiba et al. , 2012). Furthermore, AtNRT2.5 is primarily expressed in nitrate-deprived plants roots as a complex with AtNAR2.1 (Kotur and Glass, 2015).

Transcription factors (TFs) sever as regulators in plant signaling networks and have been shown to regulate N uptake and transport. Dof1 promotes plant growth during N deficiency by regulating the expression of genes related to carbon-skeleton production and by enhancing N assimilation (Yanagisawa et al. , 2004). ANR1, a member of MADS box, participates in the NRT1.1 signaling pathway and regulates lateral root elongation in *Arabidopsis* (Remans et al. , 2006; Zhang and Forde, 1998). In *Arabidopsis* , ELONGATED HYPOCOTYL5 (HY5), a member of basic leucine zipper (bZIP) family, promotes nitrate absorption by activating *NRT2.1* as a shoot-to-root mobile signal (Chen et al. , 2016). The overexpression of *MdHY5* in apple calli enhances the expression of *MdNRT2.1* , *MdNRT2.4* , and*MdNRT2.7* , potentially aiding in the coordination of carbon (C) and N acquisition (An et al. , 2017; Chen et al. , 2016).

Melatonin (MT, N-acetyl-5-methoxytryptamine) is a pleiotropic molecule in living organisms responsible for a variety of effects. In plants, MT is recognized as an antioxidant that confers tolerance to various types of abiotic stress caused by drought (Liang et al. , 2018), salinity (Li et al. , 2019; Yu et al. , 2018), cold (Li et al. , 2018), and heavy metals (Yan et al. , 2019). Recent studies have also revealed the positive influence of MT on N uptake. Application of 1 μM MT has been shown to significantly enhance N absorption and assimilation by increasing the activities of glutamine synthetase and nitrate reductase under N-deficiency conditions (Qiao et al. , 2019). Liang et al. (2018) found that MT application increased ^{15}N uptake, utilization, and accumulation by upregulating genes involved in N absorption and metabolism during water deficiency.

N-acetylserotonin methyltransferase (ASMT) is considered a key enzyme in MT biosynthesis and is extremely important in regulating MT levels in plants (Kang et al. , 2011; Liu et al. , 2017; Park et al. , 2013). In our previous study, we characterized*MdASMT9* in apple plants, and demonstrated that its overexpression increased MT levels and improved water-use efficiency in apple plants (Zhou, Li, et al. , 2022). However, the specific functions of endogenous MT in apple plants under low N conditions remain unclear. In the present study, we used *MdASMT9* -overexpressing (OE) apple lines to reveal the function of endogenous MT in response to N deficiency. Our findings indicate that the overexpression of*MdASMT9* enables plants to maintain higher photosynthetic capacity and plays a positive role in the tolerance to N deficiency. Proteomic and physiological data analyses indicate that *MdASMT9*overexpression enhances the TCA cycle and positively modulates amino acid metabolism to alleviate N-deficiency stress. Furthermore, MT promotes *MdNRT2.1* and *MdNRT2.4* expression in an*MdHY5* -dependent manner, thereby improving N absorption.

MATERIALS AND METHODS

2.1 Plant materials and growth conditions

In vitro shoot cultures of *Malus domestica* 'GL-3' ('Royal Gala') wild type (WT) and *MdASMT9* -OE apple lines (OE-3 and OE-4) were cultivated as described previously (Zhou, Li, et al. , 2022). After the tissue-cultured seedlings developed roots, the WT and*MdASMT9* -OE lines were transplanted into pots filled with a loam-perlite mixture in a 1:1 ratio (by volume). The plants were grown in a greenhouse with a light/dark cycle of 16 h/8 h at 24 °C temperature for a duration of eight weeks.

2.2 Vector construction and genetic transformation

To generate *MdHY5* overexpression constructs, the coding sequence (CDS) of *MdHY5* was cloned into the pGWB415 vector containing a CaMV 35S promoter and 3xHA tag. For *MdHY5* RNA interference (RNAi), a specific 200-bp fragment of *MdHY5* was cloned into the RNAi vector pK7GWIWG2D (a GFP tag). *Agrobacterium tumefaciens* -mediated transgenic apple calli were obtained as described by Xie et al. (2012).

The root transformation of *MdHY5* was carried out using an*A. rhizogenes* (K599)-mediated system with tissue-cultured*M. domestica* (cv. Gala) as the explant, following the protocol used by Meng et al. (2019) with slightly modifications. Tissue-cultured plants with 3–4 leaves were used for the root transformation. In a clean bench, the base of each tissue-cultured plant was obliquely cut before immersing it directly into a glass beaker containing 100 ml of K599 *A. rhizogenes* suspension. The treated seedlings were vacuumed for 15 min using a vacuum pump set to 0.08 MPa. The explants were blot-dried and transferred to the MS media supplemented with 25 mg/L kanamycin for the selection and further culture.

2.3 Low-nitrate treatment

A hydroponic system was utilized to evaluate the tolerance of apple plants to N deficiency using. Initially, healthy and consistently growing plants were selected for hydroponic culture using half-strength Hoagland's nutrient solution. An air pump was used to supply oxygen to the plant roots and maintain the dissolved oxygen concentration in the solution at 8.0-8.5 mg L^{-1}. After 15 days of pre-cultivation, both WT and *MdASMT9* -OE plants were divided into half-strength Hoagland's nutrient solution with 6 mM NO_3^- (control) and with 0.2 mM NO_3^- (low-N conditions). After 35 d of treatment, leaves and roots were harvested.

Before sampling, the nutrient solution was replaced with a new solution containing^{15}N-KNO_3 (98% atom, Sigma Aldrich, USA) as the sole N source for 3 h.

M. hupehensis were used to screen the concentration of MT in the hydroponic culture. After 10 d of pre-cultivation, the plants were designated to receive half-strength nutrient solutions containing 0, 0.05, 0.5, or 2.5 μM MT, respectively. After 10 d of MT treatment, a nutrient deficiency (0.2 mM NO_3^-) was induced. The deficiency trials spanned 30 d. The transgenic apple plants mediated by *A. rhizogenes* were pre-cultured with or without MT and subjected to low-nitrate treatment according to the method described above.

2.4 RT-qPCR analysis

Total RNA was extracted using a Foregene Plant RNA Isolation Kit (Foregenes, Chengdu, China). The first-strand cDNA was synthesized via a Thermo Scientific Kit (Thermo Scientific, Carlsbad, CA, USA). RT-qPCR was conducted with a Roche LightCycler® 96 Real-Time PCR System (Roche, Indianapolis, IN, USA). The *malate dehydrogenase* (*MdMDH*) gene in *M. domestica* was used to normalize the expression of different genes. The primers used are listed in Table S1.

2.5 Photosynthetic pigment content, photosynthesis, and chlorophyll fluorescence

Photosynthetic pigments were extracted with 80% acetone for 48 h and observed by spectrophotometry based on Arnon (1949). Net photosynthesis (P_n) and stomatal conductance (G_S) were assessed with a CIRAS-3 portable photosynthesis system (CIRAS, Amesbury, MA, USA).

The apple plants were kept in darkness for 30 min before measuring their chlorophyll fluorescence. The F_V/Fm ratio was measured by placing fully expanded leaves on an Imaging-Pam Chl fluorimeter (Walz, Effeltrich, Germany), then determining chlorophyll fluorescence parameters with Imaging WinGegE software.

2.6 Measurements of melatonin, soluble sugars, and amino acids

First, 0.3 g of the sample was ground with liquid N and placed into 2 mL of pre-cooled extraction solution (concentrated hydrochloric acid:water: isopropanol, v:v:v=0.002:1:2), then vortexed for 1 min. After 24 h at -20°C in the dark, the sample was centrifuged at 4 °C and 4000 rpm for 10 min and the supernatant was transferred to a 10 mL centrifuge tube. After adding 1 mL of dichloromethane, samples were left at -20 °C for 0.5 h and centrifuged again at 4000 rpm for 10 min. After the supernatant was dried with N gas, 200 μL of chromatographic grade methanol was added, mixed well, and filtered through a 0.22 μm organic membrane before injection. Detection was performed using LC-MS (SCIEX, QTRAP5500) with an injection volume of 2 μL.

Soluble sugars content was measured according to Gao et al. (2020) and amino acids levels were assessed as outlined by Huo et al. (2020) described.

2.7 Measurement of total N content and^{15}N-NO_3^- absorption

Total N content was measured as described by Makino et al. (1997). NO_3^- quantitation in plant tissue was measured with high performance liquid chromatography (Agilent Technologies, USA) (Patterson et al. , 2010).^{15}N-NO_3^- uptake activity was determined on an isotope-ratio mass spectrometry system (Thermo Scientific, USA) and^{15}N-NO_3^- influx rate was calculated based on the amount of ^{15}N taken up per unit weight of the sample per unit time (Zhang et al. , 2021).

2.8 Electrophoretic mobility shift assay (EMSA)

Biotin-labeled, unlabeled, and mutant probes of promoters of*MdNRT2.1* and *MdNRT2.* 4 (Sangon Biotech, China) and MdHY5-His protein were prepared. The corresponding probes were incubated with MdHY5-His protein or without protein in binding buffer (5 mM $MgCl_2$, 2.5% glycerol, 10 mM EDTA, and 50 mM KCl) for 20 min at 23 °C. EMSA was then conducted with an EMSA kit (Thermo Scientific, Waltham, MA, USA) following the manufacturer's instructions.

2.9 Dual-luciferase reporter assay

MdNRT2.1 and *MdNRT2.* 4 promoters were cloned into pGreenII 0800-LUC vectors and generated the reporter construct*MdNRT2.1* pro:LUC and *MdNRT2.4* pro:LUC. The CDS of the*MdHY5* was cloned into the pGreenII 62-SK vector and generated the effector 35Spro:*MdHY5* . LUC/REN activity was evaluated using a Dual-Luciferase® reporter gene assay kit (Yeasen Biotechnology, Shanghai).

2.10 Proteomics sequencing analysis

Quantitative proteomics analysis was performed by PTM BioLab, Inc. (Hangzhou, China) as a customer service. Approximately 0.1 g of each sample was pulverized in liquid N and mixed with four volumes of acetone containing 10% trichloroacetic acid. The samples were stored at- 20 for 4 h. The supernatant was discarded and the precipitate was washed three times with ice-cold acetone. The precipitate was air-dried and extracted with SDS/phenol method. Following trypsinization, peptides were reconstituted in 0.5 M TEAB and labeled with a TMTsixplex kit (ThermoFisher Scientifc).

The peptides were fractionated and the resulting peptides were subjected to an NSI source for analyzed by tandem mass spectrometry (MS/MS) in Q Exactive^{TM} (Thermo Scientific, US) coupled online to the UPLC-MS/MS. The acquired data were analyzed using the MaxQuant search engine (v.1.5.2.8). Proteins with a fold change (FC) of >1.2 or <0.833, plus a P -value <0.05, were set as thresholds for significant up- and down- regulation, respectively. The differentially expressed proteins (DEPs) were annotated using sub-cellular localization predictions (WolF Psort), Clusters of Orthologous Groups (COG/KOG) (https://www.ncbi.nlm.nih.gov/research/cog-project/), and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways (http://www.genome.jp/kegg/kaas/) (Galperin et al. , 2021; Horton et al. , 2007; Moriya et al. , 2007).

2.11 Statistical analysis

All data in this study were analyzed using SPSS software (Version 20) and are presented as means +- standard deviation (SD). Significant differences were assessed with one-way ANOVA and Tukey's tests (*P < 0.05, **P < 0.01, and ***P < 0.001).

RESULTS

3.1 Application of exogenous MT improved the low-nitrate tolerance of apple plants

To characterize the roles of MT in response to low-N stress, the effects of different concentrations of MT (0, 0.05, 0.5 and 2.5 μM) on plant height and biomass were examined in apple plants under low-N (0.1 mM) conditions. Among all MT concentrations, plants treated with 0.5 μM MT showed the highest plant height, shoot and root fresh weight (Figure 1a-c). Thus, MT treatment improved the low-nitrate tolerance, with 0.5 μM MT was determined as the optimal concentration.

3.2 Overexpression of *MdASMT9* enhanced low-N tolerance in apple plants

The expression of *MdASMT9* , which encodes the enzyme involved in MT synthesis, was measured in apple plants under low-N stress. The results showed that *MdASMT9* expression was significantly upregulated under N-deficiency treatment, first increasing on the 8th day and peaking on the 16th day (Figure 1d).

To further explore the function of endogenous MT in low-N responses, transgenic 'Royal Gala' apple seedlings overexpressing *MdASMT9*(*MdASMT9* -OE) were generated and analyzed. LC-MS analysis revealed a significant increase in MT content in the leaves and roots of*MdASMT9* -OE plants compared to WT plants. Moreover, endogenous MT levels in both leaves and roots increased under low-N stress (Figure 1f). Under control conditions, there was no significant difference in phenotype between WT and *MdASMT9* -OE plants (Figure 1e). However, after 35 d of low-N treatment, the WT plants exhibited reduced plant height while the damage suffered by OE lines was less severe (Figure 1g). The biomass of the transgenic plants was also less affected by N deficiency compared to WT plants (Figure 1h). These findings suggest that apple plants overexpressing *MdASMT9* have enhanced low-N tolerance.

3.3 Overexpression of *MdASMT9* improved photosynthetic capacity in apple plants

The significant difference in plant biomass between WT and*MdASMT9* -OE plants under N-deficiency conditions indicates potential variations in the photosynthetic capacity of different genotypes. There was no conspicuous difference in $P_n$between the WT and *MdASMT9* -OE plants under control conditions (Figure 2a). However, when exposed to N-deficiency stress, the decrease in P_n was much less pronounced in the *MdASMT9* -OE plants. Under control and low-N conditions, OE-4 lines exhibited higher G_S than WT plants (Figure 2b). We also assessed the total chlorophyll and carotenoid contents (Figure 2c,d) to find that after low-N treatment, OE-3 and OE-4 lines showed higher total chlorophyll and carotenoid contents than WT.

Chlorophyll fluorescence (ChlF) parameters are closely related to photosynthesis under environmental stress (Gao et al., 2020). Hence, we further examined the ChlF characteristics of WT and *MdASMT9* -OE lines with disparate tolerance to low N levels (Figure 2e,f). After low-N treatment, F_0 and $F_m$values were lower whereas the F_V/Fm ratio was higher in *MdASMT9* transgenic plants compared to WT plants.*MdASMT9* -OE lines exhibited higher YII than WT as well. Under normal N supply and low-N conditions, OE lines showed lower Y(NO) and higher NPQ/4. After low-N treatment, OE-4 lines showed higher Y(NPQ) and qN than WT. These results altogether suggest that the photosynthetic capacity in *MdASMT9* -OE plants was less compromised under N-deficiency stress.

3.4 Identification of DEPs by proteomic analysis

We conducted proteomic analysis based on tandem mass tag (TMT) quantification to identify DEPs between WT and OE-4 line treated with low N for 35 d (Figure 3). Principal component analysis (PCA) analyses confirmed the reliability of the replicated samples data (Figure 3a). By comparing the protein expression levels and applying a threshold value of FC > 1.2 and p-value < 0.05, we identified 253 downregulated proteins and 440 upregulated proteins were identified in the WT and OE-4 comparisons (Figure 3b,c). To gain a deeper understanding of DEPs in the sequencing data, the locations, characteristics, and functions of these proteins were annotated. Subcellular localization prediction indicated that 38.67% of DEPs were localized in the chloroplast, followed by the cytoplasm (26.12%), nucleus (18.76%), plasma membrane (8.08%), mitochondria (2.6%), other locations (5.77%) (Figure 3d).

The COG/KOG database was used to categorize the 502 DEPs into 22 groups based on their primary cellular functions (Figure 4a; Table S2). In the metabolism category, numerous DEPs were involved in carbohydrate, lipid, and amino acid transport and metabolism (G, I, and E), energy production and conversion (C), and secondary metabolites biosynthesis, transport, and catabolism. KEGG pathways analysis was also performed to predict potentially important pathways. We observed that the upregulated proteins were significantly enriched in pathways such as 'alanine, aspartate and glutamate metabolism' (map00250), 'stilbenoid, diarylheptanoid and gingerol biosynthesis' (map00945), 'photosynthesis' (map00196), 'butanoate metabolism' (man0060), and 'flavonoid biosynthesis' (map00941) (Figure 4b; Table S3). These findings suggest that DEPs between WT and OE-4 lines are involved in some processes associated with N deficiency such as photosynthesis, carbohydrate and amino acid metabolism, and energy production and conversion.

3.5 Overexpression of *MdASMT9* promoted TCA cycle and positively modulated amino acid metabolism

In the proteomic data, several upregulated DEPs (HK, FRK, GAD, GABA-T, and SSADH) were identified as involved in carbohydrate and N metabolism, potentially promoting TCA cycle (Figure 4c). To investigate this further, the contents of soluble sugars in WT and *MdASMT9* -OE plants were measured. The results revealed that *MdASMT9*overexpression promoted the accumulation of sucrose, glucose and fructose under N-deficiency stress. As the TCA cycle accompanies amino acids synthesis and metabolism, the contents of nine amino acids in WT and *MdASMT9* -OE lines were determined under normal N supply and N-deficiency conditions. After 35 d of N-deficiency treatment, the levels of most measured amino acids in all genotypes significantly decreased. However, compared to WT, the reduction in *MdASMT9* -OE lines was less pronounced. The levels of six aromatic amino acids (ARG, GLU, HIS, LYS, THR, and ASP) were significantly higher in*MdASMT9* -OE lines than in WT during N deficiency. These results indicate that the

overexpression of *MdASMT9* enhanced the TCA cycle and positively modulated amino acid metabolism to resist N-deficiency conditions.

3.6 MT promoted nitrate accumulation and transcription of*MdHY5*

Previous studies have shown that exogenous MT treatment enhances N uptake during N deficiency (Du et al. , 2022; Qiao et al. , 2019). To investigate the function of *MdASMT9* in N absorption, the ^{15}N-NO_3^- influx rate in WT and *MdASMT9* -OE plants were measured. We found that under N-deficiency conditions, the^{15}N-NO_3^- influx rate of *MdASMT9* -OE plants was substantially higher compared to WT plants (Figure 5b). Under normal conditions, the NO_3^- concentration in the roots of OE lines were similar to those of WT plants (Figure 5a). However,*MdASMT9* -OE plants exhibited more NO_3^- in their roots under N-deficiency conditions. NRT1.1 and NRT2 proteins also play key roles in NO_3^- absorption in response to the low NO_3^- conditions (Okamoto et al. , 2003; You et al. , 2022), so we analyzed the expression of *MdNRT1.1* , *MdNRT2.1* , *MdNRT2.2* ,*MdNRT2.3* , *MdNRT2.4* , and *MdNRT2.5* in the roots of WT and *MdASMT9* transgenic plants under low-N conditions as well (Figure 5c,d; Figure S1). The results showed that *MdASMT9* OE plants exhibited higher expression of *MdNRT2.1* and*MdNRT2.4* in roots than that of WT plants during low-N treatment (Figure 5c,d).

Previous studies have reported that HY5 positively regulates root nitrate absorption by activating *NRT2.1* (Chen et al. , 2016; Huang et al. , 2015). Yao et al. (2021) found that exogenous MT and altered MT biosynthesis in *Arabidopsis* enhance the expression of HY5 to protect the plant from oxidative stress. Therefore, we examined whether MT increased the expression of *MdHY5* under N-deficiency conditions (Figure 6a). *MdHY5* expression was detected in apple plants treated with 0.5 μM melatonin (WT+MT) and overexpressed with *MdASMT9* under N-deficiency conditions. The expression of *MdHY5* in the roots of WT, WT+MT, OE-3, and OE-4 plants first increased and then decreased, reaching the maximum value at 16 d of low-N stress. Starting from the fourth day of low-N treatment, WT+MT, OE-3, and OE-4 plants had higher expression of *MdHY5* than WT. Exogenous MT treatment and overexpression of *MdASMT9* in apple plants thus appeared to enhance the expression of *MdHY5* during low-N treatment.

3.7 *MdHY5* plays a positive role in N-deficiency tolerance

Previous studies have shown that shoot-derived HY5 moves to roots promotes NO_3^- uptake by activating*NRT2.1* (Chen et al. , 2016). To investigate whether MdHY5 binds to the promoters of *MdNRT2.1* and *MdNRT2.4* , their promoters were analyzed (Table S3). The results showed that the*MdNRT2.1* promoter contains MdHY5 binding elements P1 and P2 (CACGTC), while the *MdNRT2.4* promoter contains MdHY5 binding elements P3 (TACGTA) and P4 (CACGTA) (Figure 6b,c). EMSA results indicated that MdHY5 directly and specifically binds to the promoters of the *MdNRT2.1* and *MdNRT2.4* . Further, a transient expression assay showed that LUC were activated by the co-expression of*MdHY5* with *MdNRT2.1* or *MdNRT2.4* reporters significantly beyond the control (Figure 6d,e). These findings demonstrated that MdHY5 protein can directly and specifically bind to*MdNRT2.1* and *MdNRT2.4* promoters, thereby activating their expression.

To examine the function of MdHY5 in low-N stress responses, transgenic calli overexpressing *MdHY5* (*MdHY5* -OE) and interfering with*MdHY5* (*MdHY5* -RNAi) were generated (Figure 7a; Figure S2). After low-N treatment, the *MdHY5* -OE apple callus had higher fresh weight (FW) and total N content than WT, while the FW and total N content of the *MdHY5* -RNAi apple callus were significantly lower than WT (Figure 7a-c). In addition, under low-N conditions,*MdHY5* -OE apple calli showed higher expression of *MdNRT2.1*and *MdNRT2.4* than WT and significantly lower the expression of*MdNRT2.1* and *MdNRT2.4* in *MdHY5* -RNAi apple callus than WT (Figure 7d,e). These results indicated that *MdHY5* enhanced the expression of*MdNRT2.1* and *MdNRT2.4* , thereby improving the tolerance to low-N stress.

3.8 MT promoted NO_3^- uptake in an *MdHY5*-dependent manner

In this study, MT increased the expression of *MdHY5* ,*MdNRT2.1* , and *MdNRT2.4* . MdHY5 activated the transcription of *MdNRT2.1* and *MdNRT2.4* . Accordingly, we further investigated whether MT promotes

MdNRT2.1 and *MdNRT2.4* expression in an *MdHY5* -dependent manner. Transgenic apple plants interfering with *MdHY5* (Ri-1 and Ri-2) were generated for hydroponic N-deficiency experiments (Figure 8 a; Figure S3). The results showed that under N-deficiency conditions, the FW and $^{15}N\text{-}NO_3^-$ influx rates of WT with exogenous MT application were significantly higher than those of WT without MT application, but there was no significant difference between the FW and $^{15}N\text{-}NO_3^-$ influx rate of *MdHY5* RNAi plants with versus without exogenous MT application (Figure 8 b,c).

Additionally, RT-qPCR results indicated that the expression of *MdHY5* , *MdNRT2.1* , and *MdNRT2.4* of WT with exogenous MT application was significantly higher than that of WT without MT application (Figure 8 d-f), but there was no significant difference between *MdHY5* RNAi plants with versus without exogenous MT application. These results showed that MT-mediated promotion of *MdNRT2.1* and *MdNRT2.4* expression in an *MdHY5* -dependent manner, ultimately enhancing N absorption.

DISCUSSION

MT, as an emerging biomolecule, has been shown to regulate plant growth and increase resistance to abiotic and biotic stresses (Gao et al. , 2022). Its content is closely related to the key genes involved in its biosynthesis (Zhang et al. , 2022). ASMT encodes an enzyme that catalyzes the synthesis of MT from N-acetylserotonin, which is the final step in MT biosynthesis. The upregulation of *MzASMT1* in the apple plants is consistent with MT production in the 24-h dark/light cycle (Zuo et al. , 2014). Previous research revealed that *MdASMT9* transgenic apple plants have elevated MT accumulation (Zhou, Li, et al. , 2022). In this study, we also observed a positive correlation between *MdASMT9* gene expression and endogenous MT levels as-induced by N-deficiency stress. To confirm the function of *MdASMT9* in regulating low-N resistance, we analyzed *MdASMT9*- OE apple plants under low-N stress. We found that overexpressing *MdASMT9* led to increased plant height, plant biomass, and enhanced low-N tolerance. These findings indicate that *MdASMT9* is involved in enhancing resistance to low-N treatment in apple plants by regulating MT production.

Leaf N primarily contributes to the allocation of the photosynthetic apparatus, which in turn influences photosynthetic capacity (Bassi et al. , 2018). In this study, N-deficiency treatment affected the N concentration in leaves, which then decreased photosynthesis (Hiratsuka et al. , 2015; Wu et al. , 2019). Key functional attributes of photosynthesis, such as G_S, play an important role in regulating P_n during plant growth (Tantray et al. , 2020). Previous studies have shown that N-deficiency conditions impair the photosynthetic apparatus and decrease G_S (Wu et al. , 2019), ultimately affecting the production of photosynthates and leading to a decrease in P_n. P_n and G_S decreased after low-N treatment in our tests, however, *MdASMT9* -OE plants exhibited higher P_n and G_S levels compared to WT plants.

ChlF is closely linked to photosynthesis processes and serves as an intuitive, efficient indicator of environmental stress (Wu et al. , 2019). Previous studies have shown that N-deficiency conditions lead to photoinhibition, which impairs PSII photochemical efficiency and reduces the activity of reaction centers, ultimately damaging the photosynthetic structure (Jin et al. , 2015; Zhao et al. , 2017). A decrease in F_V/Fm indicates that the reaction center of PSII has induced photoinhibition, decreasing light energy use efficiency (Abbasi et al. , 2015). Y(II) reflects the proportion of absorbed light that is used in PSII photochemistry and is considered an important indicator of plant stress (Murchie and Lawson, 2013). Our results demonstrated a decrease in F_V/Fm and Y(II) after N-deficiency treatment, while *MdASMT9* -OE plants exhibited higher F_V/Fm and Y(II). An increase in F_0 and Y(NO) indicates progressive damage to the photosynthetic system, ultimately unbalancing the electron transport chain in the chloroplasts, accelerating reactive oxygen species production, and causing oxidative damage (Affenzeller et al. , 2009; Demmig-Adams and Adams, 1992; Fahnenstich et al. , 2008). Conversely, an increase of NPQ and qN protects the photosynthetic system by dissipating excess energy in the form of heat, thereby reducing oxidative damage (Jia et al. , 2019). Here, *MdASMT9* -OE lines showed lower F_0 and Y(NO) and higher NPQ during N deficiency treatment. These results indicated that overexpressing *MdASMT9* helps maintain higher light harvesting and heat transfer capacity, sustains PSII utilization, and improves photosynthetic capacity under N-deficiency conditions.

NO_3^- is the one of the main available N sources for plants (Crawford and Glass, 1998). In the present study,*MdASMT9* -OE plants accumulated more NO_3^- in leaves and roots after low-N stress than WT. Previous studies have shown that NRT1.1 and NRT2 protein play crucial roles in response to the low NO_3^- conditions (Okamoto et al. , 2003; You et al. , 2022). *AtNRT2.1* and*AtNRT2.4* are primarily expressed in roots and are important for NO_3^- influx (Okamoto et al. , 2003; Orsel et al. , 2002; Wirth et al. , 2007). In apple plants, there are five main NRT2 proteins: MdNRT2.1, MdNRT2.2, MdNRT2.3, MdNRT2.4, and MdNRT2.5 (Tahir 2020). We detected the expression of*MdNRT1.1* , *MdNRT2.1* , *MdNRT2.2* , *MdNRT2.3* ,*MdNRT2* .4, and *MdNRT2.5* to find that application of MT and overexpression of *MdASMT9* enhanced the expression of*MdNRT2.1* and *MdNRT2.4* in apple plants.

HY5, a bZIP TF, regulates root development and NO_3^- absorption (Gangappa and Botto, 2016). Recently studies have revealed that HY5 moves to roots and activates its own expression, which in turn activates *NRT2.1*expression to promote N uptake while promoting C assimilation and translocation in shoots (Chen et al. , 2016). EMSA and LUC/REN activity provided evidence here that *MdHY5* can bind to the*MdNRT2.1* and *MdNRT2.4* promoters to activate their expression. In apple calli, overexpression of *MdHY5* increased*MdNRT2.1* and *MdNRT2.4* expression while silencing of*MdHY5* reduced *MdNRT2.1* and *MdNRT2.4* expression. Previous research showed that exogenous MT and altered biosynthesis of endogenous MT enhance the expression of HY5 to protect plants from oxidative stress (Yao et al. , 2021). Here, *MdASMT9*overexpression and application of MT enhanced the expression of*MdHY5* under N-deficiency conditions. However, exogenous application of MT had no effect on the accumulation of N or the expression of *MdNRT2.1* and *MdNRT2.4* in *MdHY5* RNAi plants under N-deficiency conditions. These results indicate that MT promotes N absorption in an *MdHY5* -dependent manner.

C and N are two of the most important essential nutrients in all living organisms. Their metabolism is crucial to biological systems (Zhang et al. , 2018). C metabolism includes the TCA cycle, glycolysis, fatty acid, sugar, and organic acid metabolism; nucleotide and amino acid metabolism are related to N metabolism (Zhang et al. , 2018). N deficiency impairs nitrate reduction and amino acid assimilation, thereby affecting C and N metabolism (Schlüter et al. , 2012). It is necessary to balance C and N metabolism for plants to avoid metabolic inefficiencies in response to low-N stress (Santos-Filho et al. , 2014). The TCA cycle is a recognized source of C skeletons, supplying biosynthetic precursors and energy (Niehaus, 2021). It also plays a vital role in N assimilation by providing reducing power and the C-skeleton α-ketoglutarate (KG) and oxaloacetate (Oxa) (Zhou, Hu, et al. , 2022). The photosynthetic apparatus also relies on the availability of N to synthesize Chl, other metabolites, and cellular components (Kaachra et al. , 2018; Nunes-Nesi et al. , 2010). Thus, higher photosynthetic capacity and N utilization efficiency activate the TCA cycle and enhance the TCA cycle activity, which provide the thus energy necessary to withstand adverse environmental conditions.

The TCA cycle is closely linked with amino acids synthesis and metabolism. Amino acid metabolism is central to N management, involving the efficiency of N absorption, assimilation, and reactivation. It is finely regulated under N-deficiency treatment (Dellero, 2020). Previous studies have shown that N use efficiency is closely related to amino acid metabolism, and the level of free amino acids is significantly decreased when N supply is insufficient (Dellero, 2020; Sung et al. , 2015). Enhanced NO_3^-uptake contributes to maintenance of the amino acid pool under N deficiency (Jia et al. , 2020). N-deficiency-tolerant wild soybean can enhance amino acids synthesis and the TCA cycle to improve the low-N tolerance (Liu et al. , 2020). MT has also been proven to be related to amino acid metabolism in N deficiency conditions (Wang et al. , 2022). Wang et al. (2021) showed that MT can promote the expression of glutamate synthase (GOGAT) and amino acid transporter genes, highlighting its importance in N use efficiency. MT enhances the activities of glutamate dehydrogenase (GDH) and GOGAT, leading to enhanced amino acids synthesis under N-deficiency conditions (Wang et al. , 2022). In this study, *MdASMT9* -OE plants showed higher levels of certain amino acids (Glu, Asp, Arg, His, Lys, and Thr) compared to WT under low-N stress. This relatively higher and more stable amino acid pool may explain why *MdASMT9* transgenic apple lines maintained better growth performance compared to WT during N-deficiency treatment.

In summary, a working model of the manner in which *MdASMT9*mediates MT biosynthesis to enhance low N tolerance was developed. We found that N-deficiency conditions induce the expression of*MdASMT9* and the accumulation of MT. Overexpression of*MdASMT9* enabled apple plants to maintain higher light harvesting and heat transfer capability while alleviating the damage of low-N stress to the photosynthetic system. *MdASMT9* overexpression also promoted the^{15}N-NO_3^- influx rate and N accumulation. MT promoted the expression of *MdNRT2.1* and*MdNRT2.4* in an *MdHY5* -dependent manner, thereby improving N absorption. Furthermore, an increase in photosynthetic capacity and N uptake was found to enhance the TCA cycle, which positively modulated amino acid metabolism to resist N-deficiency stress.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data in this study are available in the Supporting Information of this article.

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Figure LEGENDS

Figure 1. Exogenous MT treatment and overexpression of*MdASMT9* improves low-N tolerance of apple plants. (a) The phenotype, (b) plant height, and (c) plant biomass of apple plants pretreated with 0 mM, 0.05 mM, 0.5 mM, or 2.5 mM MT were measured after 30 d of low-N stress. (d) Relative expression patterns of *MdASMT9*under N-deficiency conditions. (e) Phenotypes of WT and*MdASMT9* -OE apple lines under normal N supply and N-deficiency conditions (scale bar = 3 cm). Determination of MT contents in leaves and roots (f), plant height, (g) and plant biomass (h) of WT and*MdASMT9* -OE apple plants. Values are shown as the means +- SD. Asterisks indicate significant differences (*P < 0.05, **P < 0.01, and ***P < 0.001).

Figure 2. *MdASMT9* transgenic plants exhibit higher photosynthetic capacity and chlorophyll contents under N-deficiency conditions. Changes in the net photosynthesis rate (P_n) (a), stomatal conductance (Gs) (b), total chlorophyll content (c), and carotenoid content (d) of WT and *MdASMT9* -OE apple plants after 35 d of N-deficiency conditions. Chlorophyll fluorescence images (e) and quantitative measurements of F_0 , Fm ,F_V/Fm , Y(II), Y(NO), NPQ/4, Y(NPQ), and qN (f). Values are shown as the means +- SD. Asterisks indicate significant differences (*P < 0.05, **P < 0.01, and ***P < 0.001).

Figure 3. Proteomic analysis of WT and OE-4 apple plants under N-deficiency conditions. (a) PCA diagram of all quantifiable proteins. (b) Quantitative volcano diagram of differentially expressed proteins (DEPs). (c) Statistical diagram of the number of DEPs. (d) Subcellular structure locations of DEPs.

Figure 4. COG/KOG and KEGG pathway analysis of DEPs. (a) COG/KOG annotation of DEPs, and the ontology covers four domains: Cellular process and signaling, information storage and processing, metabolism, and poorly characterization. (b) KEGG pathway analysis of DEPs (Q1, Q2, Q3, and Q4 respectively represent DEPs of FC<0.667, 0.667<FC<0.769, 1.3<FC<1.5 and FC>1.5). (c) Specific DEPs involved in C and N cycle with determination of related amino acid contents. Heatmap shows the levels of sucrose, glucose, fructose, lysine (Lys), methionine (Met), threonine (Thr), isoleucine (Ile), aspartate (Asp), proline (Pro), arginine (Arg), histidine (His) and glutamate (Glu) in WT and transgenic apple plants. Color key normalizing the amino acid content to (-2, 2) is in the upper right corner. Redder color indicates higher the amino acid content while bluer color indicates lower amino acid content.

Figure 5. *MdASMT9* transgenic plants maintain higher nitrate uptake under N-deficiency conditions. (a) The content of NO_3^- in roots of WT and*MdASMT9* -OE plants. (b)$^{15}NO_3^-$ influx rate in WT and *MdASMT9* -OE lines. Overexpression of *MdASMT9* and exogenous MT treatment affect the expression of *MdNRT2.1* (c) and*MdNRT2.4* (d) in apple plants during N deficiency conditions. Values are shown as the means +- SD. Asterisks indicate significant differences (*P < 0.05, **P < 0.01, and ***P < 0.001).

Figure 6. MT promotes the transcription of *MdHY5* under low N stress (a). (b-c) EMSA assay of MdHY5 protein binding to*MdNRT2.1* and *MdNRT2.4* promoters. The recombinant protein was incubated with biotin-labelled or mutant oligos P1, P2, P3, and P4. (d-e) Transient expression assay of *MdHY5* interacting with*MdNRT2.1* and *MdNRT2.4* promoters and quantitative analysis of luminescence intensity. The value for Luc+Empty vector was set to 1. Asterisks indicate significant differences (***P<0.001).

Figure 7. *MdHY5* transgenic apple calli maintained higher N content and higher expression of *MdNRT2.1* and *MdNRT2.4* . (a) Phenotypes of WT and transgenic apple calli after N-deficiency treatment (*HY5* -OE: *HY5* -overexpressing apple calli;*HY5* -RNAi: *HY5* -silenced apple calli). (b) Fresh weights (FW) and (c) total N contents of WT and transgenic apple calli under low-N treatment. (d-e) Relative expression level of *MdNRT2.1* and*MdNRT2.4* in WT and transgenic apple calli under low-N treatment.

Values are shown as the means +- SD. Asterisks indicate significant differences (*P < 0.05, **P < 0.01, and ***P < 0.001).

Figure 8. Effects of exogenous MT treatment on WT and*MdHY5* interfered lines (Ri-1 and Ri-2) under N-deficiency conditions. Phenotypes (a) and total FW (b) of WT, Ri-1, and Ri-2 with or without MT treatment under low N treatment. (c)$^{15}NO_3^-$ influx rates of WT and *MdHY5* interfered lines with or

without MT treatment under low-N conditions. (d-f) Effects of exogenous MT on the expression of *MdHY5* , *MdNRT2.1* , and *MdNRT2.4* in the roots of WT and *MdHY5* interfered lines under low-N stress. Values are shown as the means +- SD. Asterisks indicate significant differences (*P $<$ 0.05, **P $<$ 0.01, and ***P $<$ 0.001).

Figure 9. Proposed model for the response of*MdASMT9* -mediated biosynthesis of MT to low-N stress in apple plants. Solid arrows refer activation, while dashed arrows refer indirect activation.

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