MdASMT9 -mediated melatonin biosynthesis enhances basal thermotolerance in apple plants

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Abstract

High temperature negatively impacts the yield and quality of fruit crops. Exogenous melatonin (MT) application has shown the capability to enhance heat tolerance, but the response of endogenous MT to heat stress, particularly in perennial fruit trees, remains elusive. This study investigated the effects of high temperatures on transgenic apple plants overexpressing the MT biosynthetic gene N-acetylserotonin methyltransferase 9 (*MdASMT9*). Endogenous MT protected transgenic plants from heat stress, scavenging reactive oxygen species (ROS) and increasing soluble carbohydrates and amino acids levels. *MdASMT9*-overexpressing plants also maintained higher photosynthetic activity by protecting the chloroplasts from damage. Transcriptome sequencing indicates that *MdASMT9* overexpression promoting the expression of *HSFA1d*, *HSFA2-like*, and *HSFA9b*, and inhibiting the transcription of *HSFB1* and *HSFB2b*. Application of MT and overexpression of *MdASMT9* reduced abscisic acid (ABA) accumulation through promoting MdWRKY33-mediated transcriptional inhibition of *MdNCED1* and *MdNCED3*, thus promoting stomatal opening for better heat dissipation. Furthermore, melatonin enhanced autophagic activity through promoting MdWRKY33-mediated transcriptional enhancement of *MdATG18a* under heat stress . These findings provide new sight into the regulation of endogenous MT and its role in improving heat tolerance in perennial fruit trees.

Title

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Abstract

High temperature negatively impacts the yield and quality of fruit crops. Exogenous melatonin (MT) application has shown the capability to enhance heat tolerance, but the response of endogenous MT to heat stress, particularly in perennial fruit trees, remains elusive. This study investigated the effects of high temperatures

on transgenic apple plants overexpressing the MT biosynthetic gene N-acetylserotonin methyltransferase 9 (*MdASMT9*). Endogenous MT protected transgenic plants from heat stress, scavenging reactive oxygen species (ROS) and increasing soluble carbohydrates and amino acids levels.*MdASMT9* -overexpressing plants also maintained higher photosynthetic activity by protecting the chloroplasts from damage. Transcriptome sequencing indicates that *MdASMT9* overexpression promoting the expression of *HSFA1d* , *HSFA2-like* , and*HSFA9b* , and inhibiting the transcription of *HSFB1* and*HSFB2b* . Application of MT and overexpression of *MdASMT9*reduced abscisic acid (ABA) accumulation through promoting MdWRKY33-mediated transcriptional inhibition of *MdNCED1* and*MdNCED3* , thus promoting stomatal opening for better heat dissipation. Furthermore, melatonin enhanced autophagic activity through promoting MdWRKY33-mediated transcriptional enhancement of*MdATG18a* under heat stress. These findings provide new sight into the regulation of endogenous MT and its role in improving heat tolerance in perennial fruit trees.

Key words: *MdASMT9* , melatonin, high temperature,

MdWRKY33

INTRODUCTION

The sixth assessment of the Intergovernmental Panel on Climate Change (IPCC) stated that the global mean temperature is expected to rise by 2.8±0.7°C at the end of this century (IPCC, 2021). The frequency and amplitude of heat episodes are projected to increase as global warming intensifies (Ummenhofer and Meehl, 2017). These problems negatively effects fruit production capacity and quality. Consequently, plants have evolved complex and diverse responses to short or periodic exposure to extreme temperatures. These responses include acquired and basal thermotolerance. Acquired thermotolerance is the increase of thermotolerance after pretreatment at high temperature (Sung *et al.* , 2003), while basal thermotolerance is the ability to survive high temperature without preacclimation (Yeh *et al.* , 2012).

Heat stress triggers a series of metabolic alterations in plants. When subjected to high temperatures, the dynamic balance between the removal and production of reactive oxygen species (ROS) is disrupted, resulting in an excess of ROS (such as O_2^-, H_2O_2, -OH.,$^1O_2$) (Dong *et al.* , 2021). This excess could induce damage to DNA, proteins, lipids, and cell membranes in plants, ultimately leading to oxidative stress (Gill and Tuteja, 2010). In response, plants have developed efficient enzymatic (peroxidase, POD; catalase, CAT; superoxide dismutase, SOD) and non-enzymatic defense mechanisms to counteract the harmful effects of ROS (Gill and Tuteja, 2010; Hoffman *et al.* , 2012; Sheikh-Mohamadi*et al.* , 2018). Furthermore, plants regulate cell osmotic pressure by accumulating osmoprotectants, including soluble sugars, amino acids, soluble proteins, and lipids (Hare *et al.* , 1998; Hasanuzzaman*et al.* , 2013; Jia *et al.* , 2021).

Notably, plant photosynthesis, one of the most fundamental biochemical processes, is hypersensitive to heat stress at the subcellular level. In the early stage, high temperature leads to the stomata closure and reduces stomatal conductance (G_S), directly limiting the photosynthetic function (Wise *et al.* , 2004). Under strong heat stress, the thylakoid membranes integrity and chloroplasts ultrastructure are damaged, damaging the oxygen-producing complex of photosystem II (PSII) and impairing electron transfer within the reaction center of PSII (Kouřil *et al.* , 2004; Lichtenthaler*et al.* , 2005).

MT (N-acetyl-5-methoxytryptamine) is a versatile signaling molecule that responds to various abiotic stresses during plant development (Arnao and Hernández-Ruiz, 2015; Zhang *et al.* , 2015). Since its detection in plants, it has been shown to provide physiological protection against environmental stresses, including temperature stress (Ahammed *et al.* , 2018; Altaf *et al.* , 2021; Byeon and Back, 2014). Byeon and Back (2014) showed that endogenous MT levels increase in rice (*Oryza sativa*) seedlings when exposed to heat stress, through increased activities of N-acetylserotonin methyltransferase (ASMT) and serotonin N-acetyltransferase (SNAT), two enzymes involved in the MT synthesis in plants. Exogenous MT application in tomato (*Solanum lycopersicum*) plants enhance cellular protein protection by inducing autophagy and heat shock proteins, which help degrade or refold heat-denatured (Xu *et al.* , 2016). Additionally, overexpression of *SlSNAT* in tomato plants improves the maximum photochemical quantum yield of PSII (F_V/F_M) and increased the transcription of heat shock factors under high-temperature stress.

Previous research found that *MdASMT9* overexpression in apple increased endogenous MT levels and improved water use efficiency (WUE) (Zhou *et al.* , 2022). However, the beneficial roles of endogenous MT in apple thermotolerance remain unclear. Therefore, this study investigated *MdASMT9* -overexpressing (OE) apple plants under extreme heat stress. Results showed that *MdASMT9* overexpression positively impacted the apple plant's response to heat stress by improving thermotolerance through scavenging harmful ROS and maintaining higher photosynthetic capacity. *MdASMT9* overexpression also increased soluble sugar and amino acid levels under heat stress. Furthermore, *MdASMT9* overexpression increased stomatal aperture through promoting MdWRKY33-mediated transcriptional inhibition of*MdNCED1* and *MdNCED3* . Moreover, exogenous MT and overexpression of *MdASMT9* enhanced autophagic activity through promoting MdWRKY33-mediated transcriptional enhancement of*MdATG18a* under heat stress.

MATERIALS AND METHODS

2.1 Plant materials and high-temperature treatment

In vitro shoot cultures of *Malus domestica* 'GL-3' ('Royal Gala') and two *MdASMT9* -OE lines were cultivated as described previously (Zhou *et al.* , 2022). After culturing on rooting medium, WT and*MdASMT9* -OE plants were transplanted to pots containing a 1:1 (by volume) loam-perlite mixture and grown in a growth chamber with a 16h-light/8h-dark cycle at 24°C. For the heat treatment, 2-month-old apple plants were exposed to a 48°C for 8 h.

2.2 TRV-mediated VIGS (virus-induced gene silencing)

The specific fragments of *MdWRKY33* were inserted into pTRV2 vector in the antisense orientation (TRV-*MdWRKY33*). The antisense expression plasmids pTRV1 or pTRV2 derivatives were transformed into*Agrobacterium* GV3101 with a ratio of 1:1 (v/v). Rooted apple plants and plantlets of apple cultured in vitro were vacuum infiltrated. After five days, the injected apple materials were treated were exposed to 48°C.

2.3 RNA extraction and qRT-PCR

Total RNA was extracted using a Foregene Plant RNA Isolation Kit (Foregenes Co. Ltd., Chengdu, China). cDNA synthesis was performed using a Thermo Scientific Kit (Thermo Scientific, Carlsbad, CA, USA). qRT-PCR was then conducted with an SYBR® Premix Ex Taq II Kit (Takara, Kyoto, Japan) and a Roche LightCycler$^{(r)}$ 96 Real-Time PCR System (Roche, Indianapolis, IN, USA). Three biological replicates were used for each experiment. The *malate dehydrogenase*(*MdMDH*) gene was used to standardize gene expression (Table S1). Relative gene expression was determined using the 2^{-CT} method (Livak and Schmittgen, 2001).

2.4 Evaluation of REL and MDA

REL was analyzed using the Dionisio-Sese and Tobita method (1998). The MDA levels were measured using an MDA kit (Comin, Suzhou, China).

2.5 Antioxidant enzyme activities and histochemical staining

SOD, POD, and CAT activities, and ROS (H_2O_2 and O_2^-) levels were estimated using colorimetric assay kits (Comin, Suzhou, China). After 8h of heat exposure, the presence of H_2O_2 and O_2^- were stained with DAB and NBT, respectively.

2.6 Determination of photosynthetic parameters

The CIRAS-3 portable photosynthesis system (CIRAS, Amesbury, MA, USA) was used to determine the P_n and G_s. For each measurement, data were collected from six fully mature leaves of different seedlings. Before measuring chlorophyll fluorescence, the apple plants were kept in darkness for 30 minutes. To measure F_V/F_M ratio, fully expanded leaves were placed on a modulated chlorophyll fluorescence imaging system from Walz (Effeltrich, Germany).

2.7 Observations of leaf stomata, chloroplasts, and autophagosome

The fully expanded leaves were immediately cut into small pieces and fixed in a 4% glutaraldehyde solution, then kept at 4°C for 24 h. After three washing with 0.2 M phosphate buffered solution, the samples underwent dehydration with different ethanol concentrations (70%, 80%, and 90%). The samples were then coated with gold using isoamyl acetate and imaged using a JSM-6360LV scanning electron microscope (SEM, JEOL Ltd., Tokyo, Japan) for observation and photography. The width/length ratio was calculated using the ImageJ software. The chloroplast and autophagosome were observed using a JEOL-1230 transmission electron microscope (TEM, Hitachi, Japan).

2.8 RNA quality assessment, Illumina sequencing, and DEGs analysis

Advanced molecular biology techniques were applied to assess the concentration and quality of the RNA sample. The RNA that met the qualifications was t used for library construction. To ensure library quality, the cDNA concentration and insert size were assessed using Qubit 2.0 and Agilent 2100. The effective concentration of the library (>2 nM) was accurately measured through qPCR. Upon confirmation of the library's suitability, the pooling and sequencing were performed using the Illumina platform. The selection criteria for DEGs were established as FC [?] 2 and FDR < 0.01(Anders and Huber, 2010). The FDR refers to the adjusted p-value used to determine the significance of the difference.

2.9 Determination of ABA, soluble sugar, and amino acid

The ABA extraction was performed using an early described method (Huo*et al.* , 2020) and measured using a liquid chromatography-mass spectrometry (LC-MS) system. The soluble sugar levels were measured according to Li et al. (2016). The amino acid content was determined based on Huo *et al.* (2020). The supernatant was filtered through a 0.22 μm organic filter for high-performance LC-MS analysis using the QTRAP5500 system (AB, America).

2.10 Determination of MT contents

A 0.3 g sample was ground with liquid nitrogen and placed in 2 mL of pre-cooled extraction solution (isopropanol:water:concentrated hydrochloric acid, v:v:v=2:1:0.002), vortexed for 1 min, and left it in the dark at -20°C for 24 h. Then, the sample was centrifuged at 4°C and 4000 rpm for 10 min, and the supernatant transferred to a new 10 mL centrifuge tube. After adding 1 mL of dichloromethane, samples were left in the dark at -20°C for 30 minutes, and centrifuged at 4°C and 4000 rpm for 10 minutes. The supernatant was transferred to a new 10 mL centrifuge tube and dried with nitrogen gas. After drying, 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.

2.11 Electromobility shift assays (EMSA)

The MdWRKY33-His and biotin-labeled probes (*NCED1* -probe and*NCED3* -probe) were obtained from Sangon Biotech, China. The probes were mixed with MdWRKY33-His protein or a protein-free binding buffer for 25 min at 24 °C. EMSA was then conducted using an EMSA kit from Thermo Scientific (Waltham, MA, USA) following the manufacturer's instructions.

2.12 LUC reporter assay

The LUC reporter assay was conducted following a previously described protocol (An *et al.* , 2017). The *MdNCED1* and *MdNCED3*promoters were amplified, and then cloned into the pGreenII 0800-LUC vector resulting in the reporter constructs *MdNCED1* pro:LUC and*MdNCED3* pro:LUC. The *MdWRKY33* ORF was cloned into the pGreenII 62-SK vector to generate the effector (35Spro:*MdWRKY33*). The LUC reporter assay was conducted using the Dual-Luciferase® Reporter Gene Assay Kit from Yeasen Biotechnology (Shanghai, China), referring to the manufacturer's instructions.

RESULTS

3.1 Endogenous MT play a positive role in thermotolerance of apple plants

Endogenous MT content in apple plants increased significantly under high temperature stress (48°C) (Figure 1A). QRT-PCR was used to measure expression of four synthase genes following 8 h high temperature treatment: *MdTDC1* , *MdT5H4* , *MdAANAT2* and*MdASMT9* (Figure 1B-E). The gene expression levels of*MdTDC1* , *MdAANAT2* , and *MdASMT9* were altered by high temperature treatment. *MdASMT9* showed the greatest increase, with the expression level 120 times higher than the control after 1h of heat treatment. To further study the role of endogenous MT under heat stress, two previously generated *MdASMT9* -OE transgenic apple lines (OE-3 and OE-4) were subjected to high-temperature stress (Zhou *et al.* , 2022). OE-3 and OE-4 transgenic apples had significantly higher MT content than the wild-type (WT) under both normal and high-temperature conditions (Figure 1F). In addition, WT, OE-3, and OE-4 showed increased MT levels under high temperature compared to normal conditions. These findings indicate that endogenous MT may play a role in apple's response to heat stress.

The top leaves of WT plants were charred and shriveled, while those of the transgenic lines showed symptoms of slight dehydration after exposure to 48°C for 8 h (Figure 1G). The relative electrolyte leakage (REL) increased significantly because of heat stress damage. However, it was lower in OE lines compared to WT (Figure 1H). As shown in Figure 1I, OE lines and WT plants showed the same trend for the malondialdehyde (MDA) content. These results indicate that both transgenic lines suffered less physiological damage under heat stress, implying a positive role for endogenous MT in apple plants' response to high-temperature stress.

3.2 Overexpression of *MdASMT9* stimulated ROS scavenging under heat stress

High levels of ROS, such as H_2O_2 and O_2^-, can damage macromolecules and membrane lipid structures, leading to oxidative damage with deleterious effects on plants (Mittler *et al.* , 2011). Therefore, we stained the leaves with 3,3'-diaminobenzidine (DAB) and nitro blue tetrazolium (NBT) separately to detect H_2O_2 and O_2-, accumulation, respectively. Results showed that heat stress caused ROS accumulation in all genotypes; however,*MdASMT9* -OE lines exhibited fewer brown areas and blue spots on their leaves compared to the WT plants (Figure 2A). Quantitative measurements of H_2O_2 and O_2- were consistent with these observations, showing that after 8h of heat treatment, the *MdASMT9* -OE lines had lower ROS levels than the WT plants (Figure 2B and 2C). Moreover, activities of antioxidant enzymes (SOD, CAT, and POD) were quantified. Exposure to 48degC for 8h increased SOD, POD, and CAT activities, with OE-3 and OE-4 exhibiting a greater increase than WT plants (Figure 2D-F). These findings indicate that *MdASMT9* -OE apple plants showed less oxidative damage than WT plants when exposed to high-temperature stress.

3.3 Overexpression of *MdASMT9* enhanced photosynthetic capacity and increased stomatal aperture under heat stress

Heat stress has a particularly adverse impact on plant photosynthesis, one of the most vital biochemical processes. A significant decrease in net photosynthesis (P_n) and stomatal conductance (G_s) was determined in both WT and *MdASMT9* -OE lines after 8h of exposure to 48degC and 24 h of recovery. However,*MdASMT9* -OE lines exhibited higher P_n compared to the WT (Figure 3A). Consistent with the P_n value, G_s showed a similar pattern after the recovery period (Figure 3B). To assess the effect of heat stress on photosynthesis, chlorophyll fluorescence imaging was used to measure the maximum photochemical quantum yield of PSII as theF_V/F_M ratio (Figure 3C and 3D). Under normal conditions, both WT and *MdASMT9* -OE lines showed comparable fluorescence intensities, but after heat stress exposure, theF_V/F_M value was higher in the*MdASMT9* -OE plants compared to the WT.

Exposure to high temperatures can negatively impact the chloroplast ultrastructure, thereby reducing photosynthetic efficiency, as confirmed through TEM (Figure 3E). After exposure to 48degC for 3 h, the ultrastructure of chloroplasts in WT plants was damaged, characterized by an accumulation of larger starch grains. However, *MdASMT9* -OE lines exhibited less fragmented and better-organized chloroplast ultrastructure. These results collectively suggest that *MdASMT9*overexpression protected the photosynthetic apparatus from damage and enhanced photosynthetic capacity under heat stress.

Stomatal movement impacts leaf transpiration and heat dissipation, playing a crucial role in heat stress response. Thus, the stomatal morphology of *MdASMT9* -OE and WT plants was compared (Figure 3F).

Their stomatal aperture exhibited no significant difference under normal conditions, and both declined significantly when exposed to heat stress. However, OE-3 and OE-4 showed greater stomatal aperture than the WT line after exposure to heat stress (Figure 3F and 3G). ABA plays a crucial part in regulating stomatal movement, and we assessed the ABA levels in WT plants and *MdASMT9* -OE lines. Both WT and transgenic lines displayed significantly higher ABA contents under high stress (Figure 3H). However, the *MdASMT9* -OE plants had notably lower ABA levels than the WT. These results demonstrated that *MdASMT9*overexpression significantly reduced ABA synthesis and promoted stomatal opening, conducive to transpiration and heat dissipation in plants under heat stress.

3.4 Overexpression of *MdASMT9* increased soluble sugar and amino acid levels in apple leaves under heat stress

The tolerance of plants to heat stress is related to the accumulation of soluble carbohydrates and amino acids. Therefore, the soluble sugar and amino acid content in WT and *MdASMT9* -OE lines were also measured. The transgenic lines showed a great increase in the galactose, sorbitol, and sucrose contents compared to the WT (Figure 4A-C). Meanwhile, the contents of the measured amino acids (histidine, arginine, aspartate, proline, glutamine, and glycine) significantly increased in*MdASMT9* -OE lines, allowing them to better withstand heat stress (Figure 4D-I). For example, the OE-3 and OE-4 lines exhibited about 3.2 and 2.5 times greater histidine levels than the WT, respectively (Figure 4D). The arginine contents in *MdASMT9* -OE plants were approximately 2.3 times greater than those in the WT after exposure to 48degC for 8 h (Figure 4E).

3.5 Transcriptome sequencing analysis of WT and*MdASMT9*-OE lines under heat stress

To gain a deeper understanding of the impacts of overexpressing*MdASMT9* on the heat tolerance of apple plants, transcriptional sequencing was conducted on the leaves exposed to 48degC for 1h. Three cDNA libraries were constructed in triplicate, and after quality control, 95.56 Gb of clean data were obtained, with a minimum of 94.02% Q30 bases per sample. The clean data from each sample were separately mapped to the apple reference genome, resulting in mapping rates between 90.85% and 91.95%. The sequencing data were proved suitable for transcriptome analysis based on these results.

Analysis of differentially expressed genes (DEGs) between WT and transgenic plants was conducted based on fragments per kilobase per million mapped fragments (FPKM) with a fold change (FC) of at least 2 and a false discovery rate (FDR) of less than 0.01. A total of 370 DEGs were identified between WT and OE-3, with 205 being up-regulated and 165 being down-regulated. In the comparison between WT and OE-4, there were 453 DEGs identified in total, including 361 up-regulated genes and 92 down-regulated (Figure 5A and 5B). Among these DEGs, we identified 48 DEGs are expressed simultaneously in both OE-3 and OE-4 (Figure 5C and 5D; Table S2). There are 9 transcription factors (TFs), including 5 heat stress transcription factors (HSFs). The relative transcripts of 5 HSFs were induced by heat stress in both WT and OE lines; however,*HSFA1d* , *HSFA2-like* and *HSFA9b* abundance was higher in OE lines than in WT (Figure 5E). In contrast, *HSFB1* and*HSFB2b* abundance was lower in OE lines than in WT.

3.6 MdWRKY33 negatively regulates the transcription of*MdNCED1* and *MdNCED3*

Analysis of the transcriptome data demonstrate that the expression of 9-cis-epoxycarotenoid dioxygenase (*MdNCED1*) (MD10G1194200) and*MdNCED3* (MD05G1207300), involved in ABA biosynthesis, were down-regulated in OE-3 and OE-4 (Figure 5D). QRT-PCR results showed that after 1 h of heat stress, the *MdNCED1* and *MdNCED3*expressions in *MdASMT9* -OE lines were remarkably lower than in the WT (Figure 6A and 6B).

Among these differentially expressed TFs, *MdWRKY33* (MD04G1167700) was significantly upregulated in OE-3 and OE-4 lines, with $\log_2$FC values of 1.75 and 1.62, respectively (Figure 5D). Earlier studies have shown that WRKY33 acts on the*NCED3/NCED5* promoter, thereby inhibiting its expression and negatively impacting ABA biosynthesis in Arabidopsis (Liu *et al.,*2015). To investigate whether endogenous MT negatively regulated ABA biosynthesis through *MdWRKY33* , the *MdWRKY33* expression pattern in

WT and *MdASMT9* -OE lines under heat stress was determined. QRT-PCR showed that *MdWRKY33* expression increased then decreased, reaching its highest level after 2 h of heat treatment (Figure 6C). In addition, OE-3 and OE-4 lines had remarkably higher*MdWRKY33* expression than the WT during high-temperature stress. This confirms the *MdASMT9* -driven activation of *MdWRKY33*expression under heat stress.

Analysis of the *MdNCED1* and *MdNCED3* promoters showed that both contained the MdWRKY33 binding elements 'TTGAAT' (Figure 6D and 6E). To verify whether MdWRKY33 binds to *MdNCED1 and MdNCED3*promoters and assesses its effect on their expression, electromobility shift assays (EMSA) and dual-luciferase (LUC) reporter assays were conducted. MdWRKY33 could bind to *MdNCED1* promoter-probe P1 and *MdNCED3* promoter-probe P2. MdWRKY33 cannot bind to mutated probes. These findings suggest that MdWRKY33 specifically recognizes 'TTGAAT' elements in the *MdNCED1* and *MdNCED3* promoters in vitro. Transient expression assays were performed in tobacco (*Nicotiana benthamiana*) leaves transformed with*Agrobacterium tumefaciens* to verify transcriptional activity. The luminescence intensity of cells co-expressing 35S::*MdWRKY33* and*NCED1* pro::LUC was higher than in cells co-expressing empty vector and *NCED1* pro::LUC (Figure 6F and 6H). Similarly, cells co-expressing 35S::*MdWRKY33* and *NCED3* pro:: LUC had higher luminescence intensity than cells co-expressing empty vector and*NCED3* pro::LUC (Figure 6G and 6I). These results demonstrated that MdWRKY33 binds to *MdNCED1* and *MdNCED3* promoters and inhibits their expression. This result preliminarily indicated that MdWRKY33 negatively regulates the transcription of *MdNCED1* and*MdNCED3* .

3.7 MT increased stomatal aperture through promoting MdWRKY33-mediated transcriptional inhibition of *MdNCEDs*

Because MdWRKY33 negatively regulate ABA biosynthesis, and*MdASMT9* enhances *MdWRKY33* transcription, we speculated that melatonin inhibited ABA biosynthesis through an*MdWRKY33* -mediated pathway. Therefore, *MdWRKY33* was transiently suppressed in WT apple plants using a tobacco rattle virus (TRV) system (TRV-*MdWRKY33*), validated by detected expression level of *MdWRKY33* (Figure 7B). After exposure to 48degC for 8 h, plants infiltrated by empty TRV (EV) pretreated with 100 μM MT had less dehydration symptoms and lower REL than those without MT treatment (Figure 7A and 7D). TRV-*MdWRKY33* plants had higher degree of wilting and higher REL than EV plants with or without MT. The concentration of MT used in this experiment was determined through concentration gradient analysis (unpublished data).

After exposure to heat stress, EV plants pretreated with 100 μM MT had higher stomatal aperture than those without MT treatment, but there was no significant difference in stomatal aperture between TRV-*MdWRKY33* plants pretreated with MT and TRV-*MdWRKY33*plants not treated with MT (Figure 7E and 7F). As shown in Figure 7G,H,I, exogenous application of MT reduced the content of ABA and the expression of *MdNCED1 /3* in EV plants under heat stress. However, there was no significant difference in ABA contents and*MdNCED1 /3* expression levels between TRV-*MdWRKY33*plants pretreated with MT and TRV-*MdWRKY33* plants not treated with MT.

To further explore whether *MdWRKY33* plays an important role in endogenous MT-mediated stomatal movement under heat stress,*MdWRKY33* was transiently suppressed in WT and *MdASMT9* -OE plants before the plants underwent heat treatment (WT/*MdWRKY33*RNAi, OE-3/*MdWRKY33* RNAi, OE-4/*MdWRKY33* RNAi, respectively), validated by detected expression level of *MdWRKY33*(Figure 8A and 8B). Under high-temperature conditions, the REL of OE-3/EV and OE-4/EV was significantly lower than that of WT/EV, while the REL of OE-3/*MdWRKY33* and OE-4/*MdWRKY33* is not significantly different from that of WT/*MdWRKY33* (Figure 8C). After exposure to heat stress, OE-3/EV and OE-4/*MdWRKY33* had higher stomatal aperture than WT/EV, while the stomatal aperture of OE-3/*MdWRKY33* and OE-4/*MdWRKY33* is not significantly different from that of WT/*MdWRKY33* (Figure 8D and 8E). OE-3/EV and OE-4/*MdWRKY33* had lower ABA contents and*MdNCED1 /3* expression levels than WT/EV under heat treatment (Figure 8F-H). However, ABA contents and*MdNCED1 /3* expression levels of OE-3/*MdWRKY33* and OE-4/*MdWRKY33* is not significantly different from that of WT/*MdWRKY33* . The

above results indicate that MT increased stomatal aperture through promoting MdWRKY33-mediated transcriptional inhibition of *MdNCED1* and *MdNCED3* .

3.8 Melatonin enhances autophagic activity through promoting MdWRKY33-mediated transcriptional enhancement of *MdATG18a*

Among these 48 DEGs, *MdATG18a* (MD11G1303400) was significantly upregulated in OE-3 and OE-4 lines (Figure 5D). Huo *et al.* (2020) found that overexpressing *MdATG18a* in apple plants leads to stronger autophagy activity, thereby enhancing tolerance to heat stress (Huo *et al.* , 2020). Consequently, the *MdATG18a* expression pattern and autophagic activity in WT and *MdASMT9* -OE lines under heat stress was determined. qRT-PCR showed that OE-3 and OE-4 lines had remarkably higher *MdATG18a* expression than the WT after 2 h of heat treatment (Figure 9A). TEM was used to observe the formation of autophagosome in response to high temperature (Figure 9B and 9C). The results showed that there were fewer autophagosome in all plants under control conditions, but more autophagosome were accumulated in OE lines than in WT plants under heat stress.

Previous studies found that silencing of *WRKY33* reduced*ATGs* gene expression and autophagosome accumulation, and compromised tomato heat tolerance (Zhou *et al.* , 2014). In this study, the correlation analysis of *MdWRKY33* and *MdATG18a*expression levels found that the expression of *MdWRKY33* was significantly positively correlated with the expression of*MdATG18a* (R=0.78, p <0.001) (Figure 9D). After exposure to heat stress, EV plants pretreated with 100 μM MT had higher expression level of *MdATG18a* than those without MT treatment, but there was no significant difference in *MdATG18a* expression level between TRV-*MdWRKY33* plants pretreated with MT and TRV-*MdWRKY33* plants not treated with MT (Figure 9E). As shown in Figure 9F, OE-3/EV and OE-4/*MdWRKY33* had higher *MdATG18a*expression levels than WT/EV under heat treatment. However,*MdATG18a* expression levels of OE-3/*MdWRKY33* and OE-4/*MdWRKY33* is not significantly different from that of WT/*MdWRKY33* . The above results indicate that exogenous MT and overexpression of *MdASMT9* enhanced autophagic activity through promoting MdWRKY33-mediated transcriptional enhancement of*MdATG18a* .

DISCUSSION

MT, an essential antioxidant, plays multifarious roles in the stress tolerance of plants (Arnao and Hernández-Ruiz, 2015). Earlier research has demonstrated that MT helps plants alleviate the stress caused by high temperatures. Xu *et al.* (2016) suggested that heat stress could increase the endogenous MT content in tomato plants. MT pretreatment has alleviated heat-induced damage by promoting antioxidant defense mechanisms and regulate the biosynthesis of polyamine and nitric oxide (Jahan *et al.* , 2019). Exogenous MT application and*ASMT* overexpression enhance thermotolerance and protect cellular protein in tomato by inducing HSPs and autophagy (Xu *et al.* , 2016). The relationship between endogenous MT and heat tolerance has only been described in model plants, while the physiological mechanisms of how endogenous MT responds to heat stress, particularly in perennial fruit trees, remain elusive. Accordingly, exploring the functions of MT biosynthesis genes in non-model plants such as apple would help us better understand the multiple functions of MT, thus furthering our knowledge in this area.

In the present study, apple plants overexpressing *MdASMT9* were utilized to explore the role of *MdASMT9* in high-temperature response. The findings indicated that *MdASMT9* overexpression leads to greater heat tolerance in apple. *MdASMT9* -OE lines experienced less damage than the WT under heat stress, as manifested by lower ion leakage and reduced MDA and ROS levels. High-temperature stress causes an imbalance of the osmotic potential and excessive ROS production in plant tissues (Choudhury *et al.* , 2017; de Pinto*et al.* , 2015). High concentrations of ROS can be detrimental to plants, resulting in oxidative damage due to the oxidation of crucial cellular components such as DNA, proteins, and lipids (Farmer and Mueller, 2013). Previous studies have shown that an antioxidant system is necessary for plants to prevent oxidative damage under high temperatures (Dong *et al.* , 2021; Huo *et al.* , 2020). In this study, *MdASMT9* -OE apple lines maintained lower ROS accumulation under high temperatures than WT. The ROS regulatory system was also assessed, and it was discovered that the *MdASMT9* -OE lines had significantly high SOD, POD, and

CAT levels compared to WT under high-temperature stress.

Plants' heat stress tolerance is related to the accumulation of soluble carbohydrates and amino acids (Akter and Rafiqul Islam, 2017; El Habti*et al.* , 2020). Under environmental stress, plant soluble sugar accumulation can promote photosynthesis and delay senescence, and involve intracellular signal transduction and transcriptional regulation (Xalxo *et al.,* 2020). Amino acids are the basis of protein synthesis and play a critical role in cell stress response and signal transduction (Häusler *et al.* , 2014; Zeier, 2013). Under high temperatures, arginine increased the activities of antioxidant enzymes and reduced lipid peroxidation (Khalil et al., 2009). Spraying glutamic acid on leaves inhibited leaf senescence induced by high temperature by inhibiting chlorophyll degradation, promoting amino acid metabolism, and maintaining nitrogen balance (Rossi *et al.* , 2021). MT can regulate soluble carbohydrates and amino acids levels under high-temperature stress (Iqbal *et al.* , 2021; Li *et al.* , 2020). 100 μM MT increases the amount of soluble sugar in wheat (*Triticum aestivum* L.) and improves its resistance to heat stress (Iqbal *et al.* , 2021). Exogenous MT application also increased the levels of amino acid, polyphenol, and caffeine, regulated photosynthesis, and promoted the growth and development of tea (*Camellia sinensis* L.) plants (Li *et al.* , 2020). In the present study, *MdASMT9* overexpression in apple plants exposed to heat stress significantly increase the accumulation of sorbitol, galactose, sucrose, and all measured amino acids (histidine, arginine, aspartic acid, proline, glutamine, and glycine). However, many complex factors can influence carbohydrate and amino acid metabolism. The mechanism by which MT affects plant carbohydrate and amino acid accumulation under high-temperature stress requires further investigation.

Stomata can facilitate leaf cooling through transpiration (Gommers, 2020). Kostaki *et al.* (2020) showed that stomata rapidly opened once exposed to heat stress, resulting in a significant decrease in leaf surface temperature. Transgenic apple plants overexpressing*MdATG18a* showed greater stomatal pore aperture than WT plants and improved basal thermotolerance (Huo *et al.* , 2020). ABA is a key hormone for stomatal closure and plant tolerance to biotic and abiotic stress (Nambara and Marion-Poll, 2005). Li G *et al.* (2020) demonstrated that ABA negatively modulates heat tolerance by increasing leaf temperature and reducing transpiration. ABA-treated plants exhibited lower stomatal conductance than controls at 40°C (Feller, 2007). Exogenous MT application confers heat tolerance through increased cytokinin (CK) levels, whereas it decreases ABA levels in *Lolium perenne* (Zhang *et al.* , 2017). Jahan *et al.* (2021) also showed that MT inhibited leaf senescence induced by heat stress by inhibiting ABA biosynthesis and activating GA biosynthesis pathways in tomato. Furthermore, we found that *MdASMT9* overexpression was associated with decreased ABA content and increased stomatal aperture under heat stress, which may enable leaves to keep a steady rate of transpiration and maintain a suitable temperature.

ABA biosynthesis occurs mainly through several enzymatic steps requiring NCED, zeaxanthin epoxidase (ZEP), and aldehyde oxidase (AO) (Nambara and Marion-Poll, 2005). Among them, NCED is a critical rate-limiting enzyme in ABA biosynthesis. In soybean (*Glycine max* L.), the positive effects of MT treatment during heat stress were associated with the decrease in ABA levels and down-regulated *gmNCED3* expression (Imran *et al.* , 2021). In the present study, *MdASMT9* -OE lines showed significantly decreased expressions of *MdNCED1* and*MdNCED3* . In a previous study, WRKY33 acted on the *NCED3*and *NCED5* promoters, repressing their expression, thereby negatively regulating ABA biosynthesis (Liu *et al.* , 2015). Zhou*et al.* (2014) showed that *WRKY33* expression increased after heat treatment, and silencing *WRKY33* reduced the heat tolerance in tomato plants. *MdWRKY33* expression was significantly upregulated in transcriptome data of transgenic apple plants overexpressing *MdASMT9* . EMSA and LUC reporter assays provided evidence that *MdWRKY33* binds to *MdNCED1* and *MdNCED3*promoters suppresses their expression. These results suggested that*MdASMT9* overexpression promoted a WRKY33-mediated decrease in*MdNCED1* and *MdNCED3* expression.

Autophagy is an evolutionarily conserved protein degradation pathway, which helps plants alleviate heat stress (Huo *et al.* , 2020). Huo*et al.* (2020) found that overexpressing *MdATG18a* in apple plants leads to stronger autophagy activity, thereby enhancing tolerance to heat stress (Huo *et al.* , 2020). In previous studies, silencing of *WRKY33* reduced *ATGs* gene expression and autophagosome accumulation, and compromised

tomato heat tolerance (Zhou *et al.* , 2014). Lai *et al.* (2011) found that WRKY33 plays a positive role in autophagy activity, and in the *wrky33* mutant, the induction of autophagy and *ATG18a* by *Botrytis cinerea* was impaired. In this study, the correlation analysis found that the expression of*MdWRKY33* was significantly positively correlated with the expression of *MdATG18a* . Exogenous MT application and overexpression of *MdASMT9* significantly increased expression of*MdATG18a* and enhanced relative autophagic activity. However, but there was no significant difference in *MdATG18a* expression between TRV-*MdWRKY33* plants with versus without exogenous MT application. *MdATG18a* expression levels of OE-3/*MdWRKY33*and OE-4/*MdWRKY33* was not also significantly different from that of WT/*MdWRKY33* under heat stress. Therefore, melatonin enhances autophagic activity and *MdATG18a* expression through an MdWRKY33-mediated pathway.

HSFs act as key components of signal transduction in heat tolerance of plants, which are divided into three subfamilies (HSFA, HSFB, and HSFC) (Zeng *et al.* , 2021). A lot of HSFAs play active regulatory roles in response to heat stress in plants (Friedrich *et al.* , 2021; Mishra *et al.* , 2002; Zhang *et al.* , 2022). *HsfA1d*enhanced high-temperature resistance by regulating the expression of heat-stress-responsive genes in *Thellungiella salsuginea* (Higashi*et al.* , 2013). In kiwifruit plants, *AcHsfA2–1*overexpression upregulated transcripts of multiple genes and onferred enhanced heat tolerance. Overexpression of *MdHSFA9b* in Arabidopsis improves heat tolerance of plants (Zhang *et al.* , 2022). In this study, transcriptome data showed that *HSFA1d* ,*HSFA2-like* and *HSFA9b* expression levels was higher in*MdASMT9* -OE lines. In contrast with *HSFA* , the *HSFB*has been reported as a a negative regulator in responses to heat stress (Tan *et al.* , 2021; Xie *et al.* , 2023). Under higher temperature, overexpression of *MdASMT9* inhibited the expression of *HSFB1* and *HSFB2b* . These suggested that *MdASMT9*overexpression enhanced heat tolerance by promoting the expression of*HSFAs* (*HSFA1d* , *HSFA2-like* , *HSFA9b*) and inhibiting the transcription of *HSFBs* (*HSFB1* and*HSFB2b*), negative regulators in heat response.

In conclusion, we have determined the mechanism of*MdASMT9* -mediated MT biosynthesis in promoting thermotolerance (Figure 10). Heat stress induces the expression of *MdASMT9* and the accumulation of MT. Endogenous MT can eliminate excessive ROS by prompting activities of antioxidant enzymes (SOD, CAT, and POD) and improved soluble sugars and amino acids contents under heat stress. Transcriptome sequencing and qRT-PCR indicates that *MdASMT9*overexpression promoting the expression of *HSFA1d* ,*HSFA2-like* , and *HSFA9b* , and inhibiting the transcription of *HSFB1* and *HSFB2b* . Furthermore, application of MT and*MdASMT9* overexpression promotes the *MdWRKY33*transcription. *MdWRKY33* directly targeted and inhibit the expression of *MdNCED1* and *MdNCED3,* reducing ABA accumulation and promoting stomatal openings under heat stress to facilitate plant transpiration and dissipation. In addition, melatonin enhances autophagic activity by promoting the transcription of

MdWRKY33.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Tengteng Gao and Danni Zhang performed the experiments and wrote the manuscript. Chao Li and Fengwang Ma conceptualized and designed the experiments. Shuo Xu, Xumei Jia, Xiaomin Liu and Kexin Tan and conducted the experiments. Yi Zhou and Zhijun Zhang analyzed the data and provided the necessary reagents and materials.

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. Endogenous melatonin (MT) play a positive role in thermotolerance of apple plants. (A) MT contents of apple plants under high-temperature stress. Relative expression patterns of MT biosynthetic gene *MdTDC1* (B), *MdT5H4* (C), *MdAANAT2* (D) and*MdASMT9* (E) under heat stress. (F) MT contents in *MdASMT9*overexpressing (OE) apple plants under heat stress. (G) Phenotype, (H) relative electrolyte leakage (REL), and (I) malondialdehyde (MDA) content of WT and *MdASMT9* -OE apple plants after 8 h of exposure to 48°C. Asterisks indicated significant differences (*P<0.05, **P<0.01, ***P<0.001 according to Tukey's test).

Figure 2. Reactive oxygen species (ROS) levels and activities of antioxidant enzymes in WT and *MdASMT9* -OE apple plants. (A) Histochemical staining for H_2O_2 and O_2^-. The (B) H_2O_2 and (C) O_2^- contents and (D) SOD, (E) POD, and (F) CAT activities in apple leaves with and without heat stress. Asterisks indicated significant differences (*P<0.05, **P<0.01, ***P<0.001 according to Tukey's test).

Figure 3. The impact of high temperature on the photosynthetic capacity and stomatal aperture of *MdASMT9* -OE apple lines. Changes in (A) the net photosynthesis rate (P_n) and (B) stomatal conductance (G_S) after heat treatment. Chlorophyll fluorescence images (C) and F_V/F_Mratios (D) of WT and *MdASMT9* -OE plants with and without heat stress. The color in the images represents theF_V/F_M ratio, with black indicating 0 and red indicating 1. (E) Arrows indicate chloroplasts in mesophyll cells. (F) The scanning electron microscopy (SEM) images of stomata. (G) Stomatal apertures and (H) ABA content of WT and*MdASMT9* -OE apple plants under control and heat stress conditions. Asterisks indicated significant differences (*P<0.05 and **P<0.01 according to Tukey's test).

Figure 4. The levels of soluble sugars (A-C) and amino acids (D-I) in WT and *MdASMT9* -OE apple plants under heat stress. Changes in (A) galactose, (B) sorbitol, (C) sucrose, (D) histidine, (E) arginine, (F) aspartate, (G) proline, (H) glutamine, and (I) glycine contents after heat treatment. Asterisks indicated significant differences (*P<0.05, **P<0.01, ***P<0.001 according to Tukey's test).

Figure 5. Volcano plot showing differentially expressed genes (DEGs) between WT and OE-3 (A), and between WT and OE-4 (B) under heat treatment. (C) Venn diagram showed the number of DEGs in WT and*MdASMT9* -OE apple plants. (D) Heatmap showing expression levels of overlapping DEGs in both OE-3 and OE-4 plants (values are presented as $\log_2$ fold change). (E) The relative transcripts of heat stress

transcription factors (HSFs). Asterisks indicated significant differences (***P<0.001 according to Tukey's test).

Figure 6. The relative expression levels of (A) *MdNCED1* , (B) *MdNCED3* , and (C) *MdWRKY33* in WT and *MdASMT9* -OE apple plants under heat stress. MdWRKY33 binds to the *MdNCED1* and*MdNCED3* promoters (D-I). EMSA assay of MdWRKY33 binding to*MdNCED1* (D) and *MdNCED3* (E) promoters. The recombinant proteins were incubated with biotin-labeled P1;2 or mutant oligos P1;2-mut. (F-I) Transiently expressed *MdWRKY33* interacts with*MdNCED1* and *MdNCED3* promoters. The value for the Luc+Empty vector was set as one. Asterisks indicated significant differences (*P<0.05 and ***P<0.001 according to Tukey's test).

Figure 7. Exogenous MT increased stomatal aperture through promoting MdWRKY33-mediated transcriptional inhibition of *MdNCED1*and *MdNCED3* . (A) Phenotype, (B) *MdWRKY33* expression, (C) MT contents and (D) REL of TRV-*MdWRKY33* plants with or without MT treatment under heat treatment. (E-F) Stomatal apertures and (G) ABA content of TRV-*MdWRKY33* plants with or without MT treatment under control and heat stress conditions. Relative expression patterns of*MdNCED1* (H) and *MdNCED* 3 (I) under heat stress. Asterisks indicated significant differences (*P<0.05, **P<0.01, ***P<0.001 according to Tukey's test).

Figure 8. Overexpression of *MdASMT9* increased stomatal aperture through promoting MdWRKY33-mediated transcriptional inhibition of *MdNCED1* and *MdNCED3* . (A) Phenotype, (B) *MdWRKY33*expression, (C) REL of apple plants overexpressing *MdASMT9* and suppressing *MdWRKY33* under heat treatment. (D-E) Stomatal apertures and (F) ABA content of apple plants overexpressing*MdASMT9* and suppressing *MdWRKY33* . Relative expression patterns of *MdNCED1* (G) and *MdNCED* 3 (H) under heat stress. Asterisks indicated significant differences (*P<0.05, **P<0.01, ***P<0.001 according to Tukey's test).

Figure 9. Exogenous MT and overexpression of *MdASMT9*enhanced autophagic activity through promoting *MdWRKY33* -mediated transcriptional enhancement of *MdATG18a.* (A) Relative expression patterns of *MdATG18a* in *MdASMT9* -OE apple plants under heat stress. (B) TEM images of autophagic structures (arrows indicate autophagosomes in mesophyll cells). (C) Relative autophagic activity in WT and *MdASMT9* -OE apple plants. (D) The correlation analysis of*MdWRKY33* and *MdATG18a* expression levels. (E)*MdATG18a* expression of TRV-*MdWRKY33* plants with or without MT treatment under heat treatment. (F) *MdATG18a* expression of apple plants overexpressing *MdASMT9* and suppressing*MdWRKY33* . Asterisks indicated significant differences (**P<0.01 and ***P<0.001 according to Tukey's test).

Figure 10. Model of *MdASMT9* -mediated biosynthesis of MT in apple plants responding to heat stress. The arrows refer to activation, and the blocked arrows refer to inhibition. The dashed arrows refer to indirect effects or unknown effects.