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Functional identification of bHLH transcription factor MdSAT1 in the ammonium response

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Abstract

Plants mainly uptake inorganic nitrogen from soil as ammonium and nitrate. Less energy is required to assimilate ammonium compared to nitrates, and plants prefer to take up ammonium when the external nitrogen concentration is low. Investigating the patterns and mechanisms of ammonium absorption can help improve crop nitrogen utilization. In this study, we isolated *MdSAT1*, a gene of apple encoding an ammonium-responsive bHLH transcription factor. MdSAT1 promoted the growth and development of lateral roots and root hairs. Overexpression of *MdSAT1* increased the transcript levels of genes related to ammonium uptake, indicating that MdSAT1 can regulate ammonium uptake and utilization at the transcriptional level. MdSAT1 also can modulate reactive oxygen species (ROS) accumulation to ultimately regulate plant growth. Taken together, these findings provide insight for us to further study the mechanisms by which *MdSAT1* controls ammonium utilization as well as plant growth and development.

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INTRODUCTION

Nitrogen is both the basis of metabolism and the primary determinant of growth and yield[1]. Nitrogen is a major component of nucleic acids, proteins, chlorophyll, and other substances, and is involved in photosynthesis, carbohydrate allocation, and root formation[2,3]. Plant nitrogen metabolism can also regulate the antioxidant system[4]. Thus, nitrogen is clearly an essential nutrient for plant growth.

Soils include inorganic nitrogen in the form of ammonium and nitrate, and organic nitrogen as amino acids, peptides, and proteins, with inorganic nitrogen are more readily absorbed by plants[5,6]. Ammonium is a major inorganic nitrogen source in most soils, its assimilation by plants requires less energy than nitrate, and plants prefer to take up ammonium when the external nitrogen concentration is low[7,8].

Ammonium transporters (AMTs) belong to the Ammonium transporter/Methylammonium permease/Rhesus (AMT/MEP/Rh) gene family, members of which have been identified in plants, microorganisms, and animals, indicating that ammonium transporter proteins are widely distributed in living organisms[9]. Two major groups of ammonium transporter proteins have been identified in plants: the AMT1 and AMT2 subfamilies[10]. The plant AMT2 subfamily is distantly related to the plant AMT1 subfamily[11], and in Arabidopsis, AtAMT2 is likely to play a significant role in moving ammonium[12]. Additional members of these families have also been characterized in Arabidopsis. AtAMT1;3, AtAMT1;4 and AtAMT1;5 exhibit high affinity for ammonium[13,14], and AtAMT1;2 exhibits a relatively low affinity for ammonium[15]. A plasma membrane NH_4^+ channel Ammonium Facilitator 1 (AMF1) has also been found to regulate plasma membrane permeability to NH_4^+ and NH_4^+ uptake indirectly through AMT/MEP/Rh[16].

The transcriptional regulation of ammonium uptake and utilization is driven by a series of transcription factors. In rice, transcription factor Indeterminate domain 10 (OsIDD10) binds to a *cis*-element motif present in the promoter region of *OsAMT1;2* to specifically activate expression. In *Arabidipsis*, transcription factor Long Hypocotyles 5 (HY5) negatively regulates the expression of *AtAMT1;2*, an orthologous gene of *OsAMT1;2*[17]. Another group of plant-specific transcription factors, DNA binding with one finger (OsDOF) transcription factors, positively regulate ammonium uptake, assimilation, and significantly increase amino acid content by regulating the transcript abundance of *OsAMTs*[18–21]. OsMYB55, a member of the R2R3-MYB gene family, plays a positive role in amino acid metabolism by promoting the expression of *OsGS1;2* and related genes[22].

A membrane-localized basic helix-loop-helix (bHLH) transcriptional factor, Glycine max Symbiotic Ammonium Transporter 1 (GmSAT1), encodes a novel regulatory gene involved in ammonium uptake during soybean root tumor development[23]. *GmSAT1* is involved in the regulation of nitrogen signaling regulatory networks related to nitrogen transport and metabolism[24]. GmSAT1 activates the transcription of plasma membrane NH_4^+ channel *ScAMF1*, which indirectly enhances NH_4^+ permeability and finally promotes ammonium uptake[23,16].

The growth and yield of plants are highly dependent on environmental nutrient factors, including nitrogen. However, in

pursuit of unilateral high yield, excessive input of nitrogen fertilizer has led to reduced nitrogen efficiency and decreased fruit quality, leading to lower agricultural production efficiency[25]. The over application of ammonium fertilizer presents a significant burden to both soil and plants[26], therefore, investigating the mechanism of ammonium utilization is an important goal in plant production[27]. Additionally, study of the tight regulation of transcription factors on nitrogen uptake can enable genetic engineering strategies to improve nutrient uptake regulation in plants[28]. In this study, we identified an ammonium-responsive *MdSAT1* gene in apple and found that MdSAT1 regulates the expression of genes related to ammonium uptake. MdSAT1 can also affect root conformation and root hair development, to ultimately promote nitrogen uptake. Overall, these findings provide insight into the mechanisms by which MdSAT1 controls ammonium uptake as well as plant growth and development.

RESULTS

Phylogenetic relationships, multiple sequence alignment, and protein structure analysis of *MdSAT1*

The *MdSAT1* (MD10G1115500) gene was identified from the NCBI website according to the *GmSAT1* sequence of soybean (*Glycine max*). A phylogenetic tree was constructed, and apple MdSAT1 was most closely related to pear PbSAT1 (Rosaceae) (Fig. 1a), indicating that these genes diverged recently in evolution. We compared the SAT1 protein sequences of apple with those of other plant species, and the results showed that all 13 proteins had high sequence similarity and belonged to the plant bHLH transcription factor superfamily, members of which contain a bHLH domain and an H-E-R DNA binding region (Fig. 1c; Supplemental Fig. S1). The bHLH structure domain of the MdSAT1 protein was predicted by the homology model, and the results indicated that the secondary and tertiary structures of bHLH structural domain match those of the core conserved domain (Fig. 1b, c).

MdSAT1 is an ammonium-responsive gene

MdSAT1 is homologous to GmSAT1, which is involved in ammonium uptake[23], and RT-PCR was used to detect the expression of *MdSAT1* in response to different nitrogen forms (KCl, as a control, represents 0 N; KNO_3, represents nitrate; and NH_4Cl, represents ammonium). The expression of *MdSAT1* was significantly induced by NH_4Cl both in shoots and roots, however, the transcript level of *MdSAT1* showed little change in response to nitrate (Fig. 2a, b), suggesting that *MdSAT1* was specifically responsive to ammonium.

The *ProMdSAT1*::*GUS* transgenic *Arabidopsis* seedlings-that can be used to localize the expression location and expression intensity of *MdSAT1*-were treated with different forms of nitrogen. We found that GUS staining results suggested that the highest GUS activity was observed under NH_4Cl treatment (Supplemental Fig. S2). With increasing time of different treatments, the expression activity of *ProMdSAT1*::*GUS* was specifically induced by NH_4Cl (Supplemental Fig. S3). Taken together, these results suggest that *MdSAT1* is specifically responsive to ammonium.

Overexpression of *MdSAT1* regulates ammonium uptake

Given that *MdSAT1* is an ammonium-responsive gene, we next treated *MdSAT1-OE* and wild type (Col) in a modified Hoagland's nutrient solution containing 0.5 mM NH_4Cl (Low NH_4^+) or 5 mM NH_4Cl (High NH_4^+) for four weeks and then assessed the effects on plant growth and ammonium content. Under low NH_4^+ conditions, ectopic expression of *MdSAT1* promoted seedling growth compared with Col, and *MdSAT1-OE* showed greater fresh weight and increased ammonium content (Fig 3a, c). In contrast, under high NH_4^+ conditions, ectopic expression of *MdSAT1* reduced fresh weight and accumulated higher ammonium (Fig. 3a–c). These results indicate that *MdSAT1* promotes ammonium uptake to regulate plant growth.

To further evaluate the role of MdSAT1 in ammonium uptake, the effects of MdSAT1 on the expression of genes related to ammonium uptake were analyzed. The result showed that

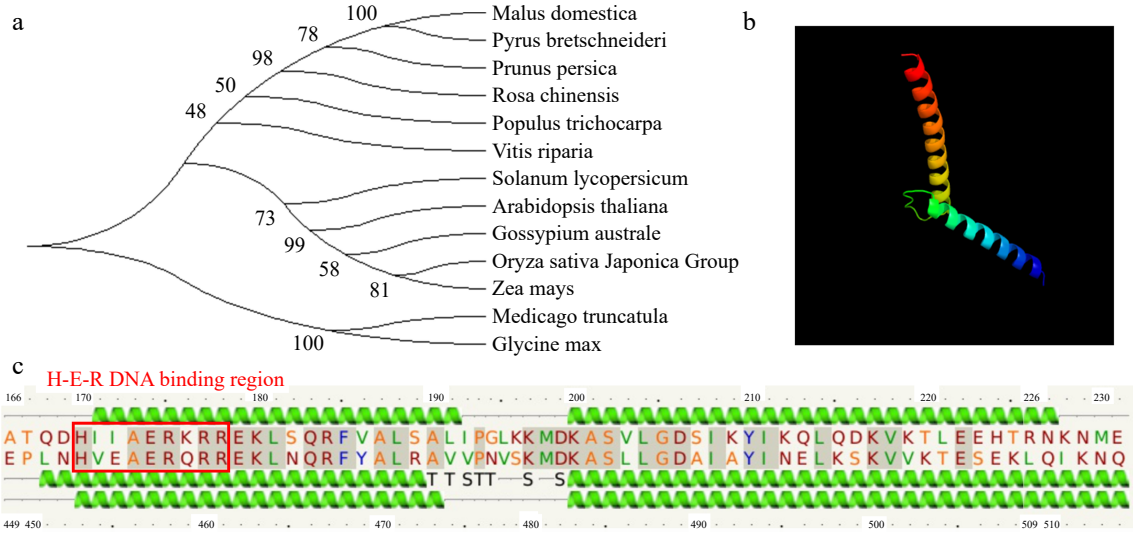

Fig. 1 Phylogenetic relationships, multiple sequence alignment, and protein structure of *MdSAT1*. (a) Phylogenetic tree of SAT1 sequences; the number on each branch represents the genetic distance. (b) Predicted bHLH 3D structure of MdSAT1 protein. (c) Predicted protein secondary of MdSAT1 bHLH structural domain.

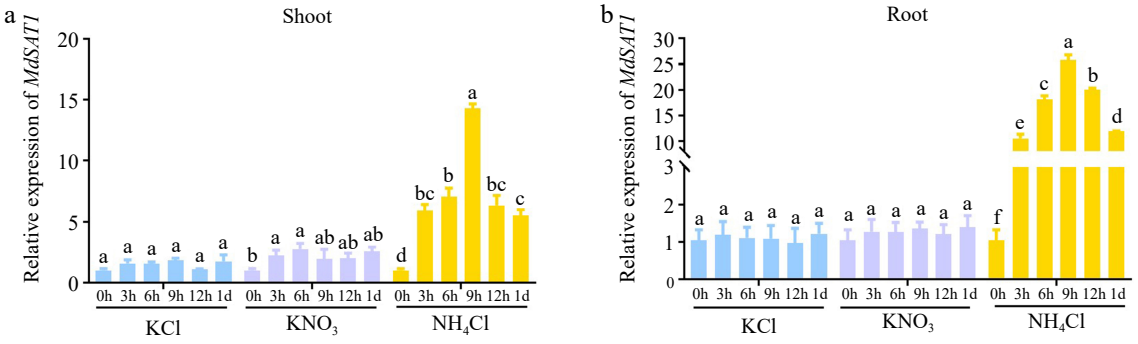

Fig. 2 Nitrogen response of *MdSAT1*. (a) Shoot and (b) root response of *MdSAT1* to KCl (represents 0 N), KNO_3 (represents nitrate), and NH_4Cl (represents ammonium). Error bars represent standard deviation (n = 3). Different letters above the bars indicate significantly different values ($P < 0.05$).

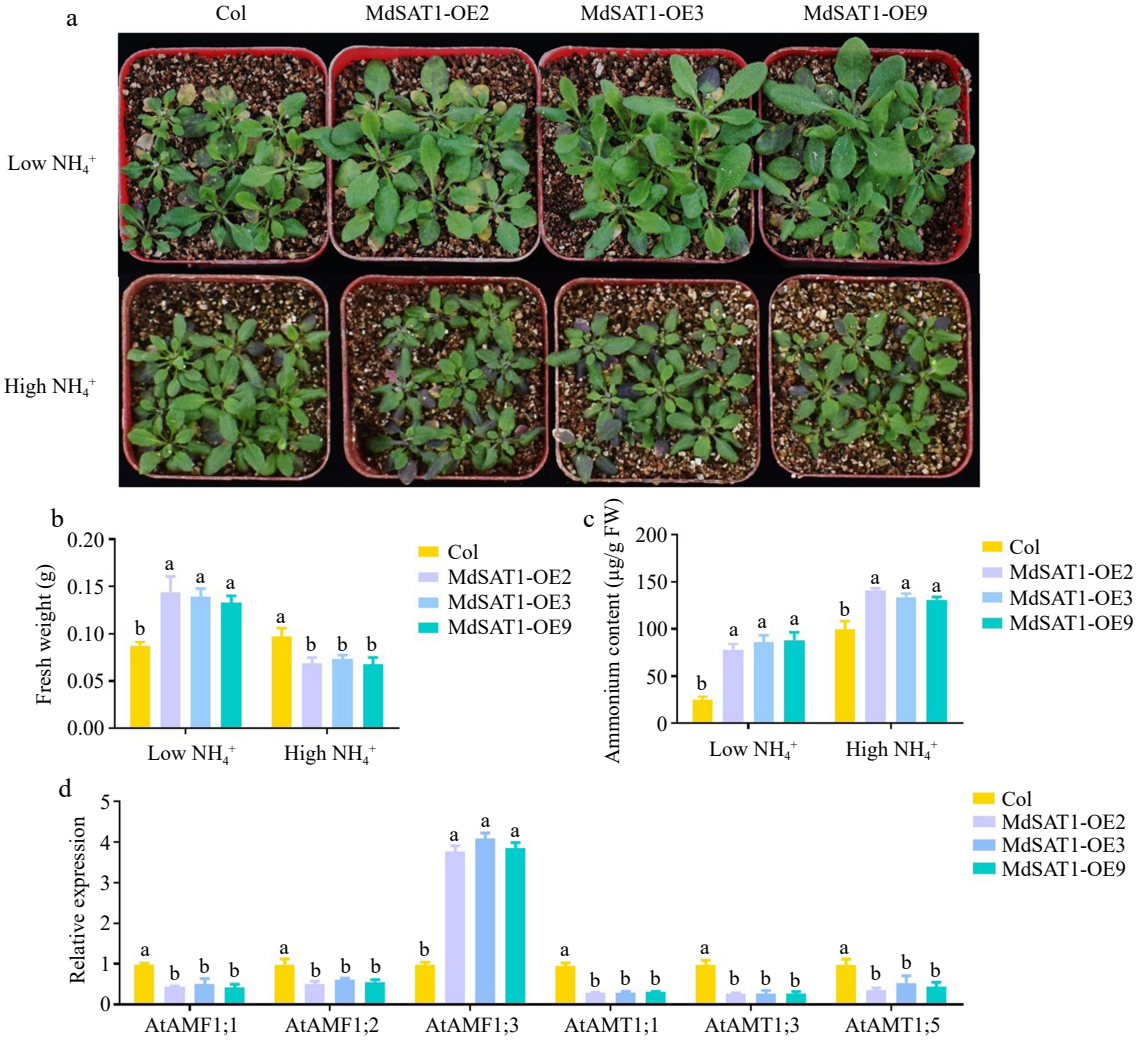

Fig. 3 *MdSAT1* regulates ammonium uptake and plant growth *MdSAT1-OE* and Col plants grown for four weeks under low NH_4^+ (0.5 mM NH_4Cl) or high NH_4^+ (5 mM NH_4Cl) conditions. (a) Morphological changes, (b) fresh weight, and (c) ammonium content are presented. (d) RT-PCR analysis of *AtAMF1;1*, *AtAMF1;2*, *AtAMF1;3*, *AtAMT1;1*, *AtAMT1;3* and *AtAMT1;5* expression in *MdSAT1-OE* and Col *Arabidopsis*. Error bars represent the standard deviation (n = 3). Different letters above the bars indicate significantly different values ($P < 0.05$).

transcript levels of *AtAMTs* were not increased in *MdSAT1-OE* lines, however, expression of *AtAMF1;3* was significantly induced in the *MdSAT1-OE* lines (Fig. 3d). AMF proteins promote NH_4^+ permeable transport[23] so these results indicated that *MdSAT1* promoted ammonium uptake by increasing the expression levels of genes related to ammonium uptake.

Overexpression of *MdSAT1* promotes lateral root development

Ammonium in the soil is actively taken up by the roots, mainly by ammonium ion transporters[29]. The tissue-specific localization of *MdSAT1* was detected using *ProMdSAT1*::*GUS* transgenic *Arabidopsis*. GUS staining results showed that

MdSAT1 was differentially expressed during lateral root growth, with the highest expression observed at the time of lateral root primordium genesis (Supplemental Fig. S4). There was no significant difference of primary root length, but the lateral root numbers were significantly increased in the *MdSAT1-OE* lines compared with those of Col (Fig. 4a–d). These results suggest that *MdSAT1* overexpression promotes lateral root growth and development.

Overexpression of *MdSAT1* regulates root hair growth and development

Root hairs play an important role in nutrient uptake[30], we next observed the phenotypes of root hairs in *MdSAT1-OE* and Col. *MdSAT1* significantly increased the number and length of root hairs under low NH_4^+ treatment (Fig. 5a, c, d), and the number and length of root hairs were inhibited under high NH_4^+ treatment (Fig. 5b–d). The expression levels of genes related to root hair development were also detected. The results showed significantly down-regulated transcript levels of *AtEGL3*, *AtGL3*, and *AtTTG1* genes that inhibit root hair development under low NH_4^+ treatment (Fig. 6a–c)[31,32] and significantly up-regulated levels of *AtSCM*, a positive regulator in root hair development (Fig. 6d)[33]; the expression patterns were reversed under high NH_4^+ treatment (Fig. 6a–d). These results suggest that *MdSAT1* promotes the growth and development of root hairs by regulating the transcript levels of root hair development-related genes.

MdSAT1 promotes ROS accumulation

ROS plays a role in root hair development[34], so we next measured ROS content by NBT staining. The results showed that overexpression of *MdSAT1* increased ROS accumulation of leaves compared with the level in Col (Fig. 7a). OFR content was promoted in the *MdSAT1-OE* lines, both in high and low NH_4^+

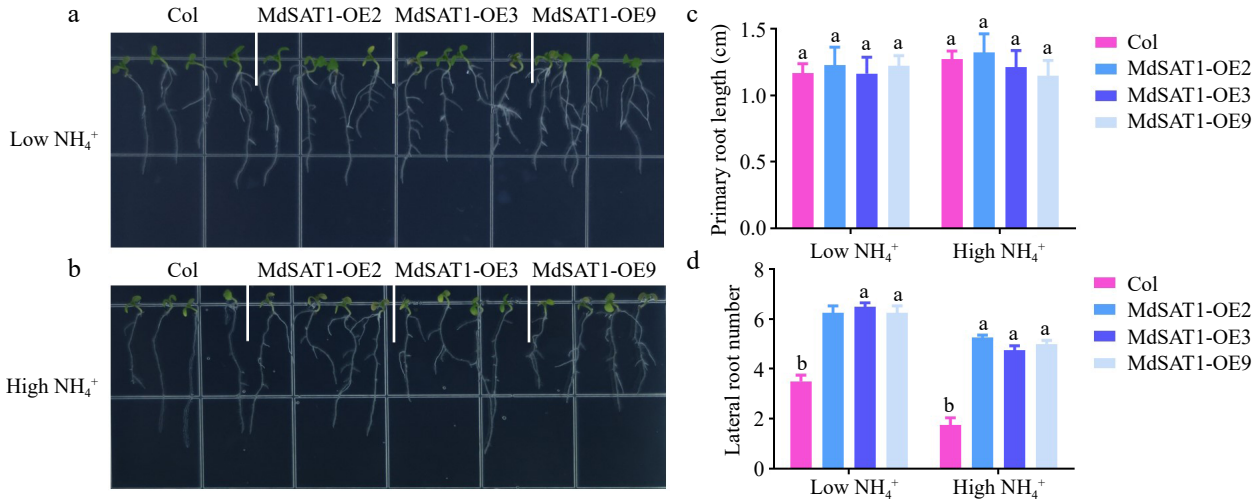

Fig. 4 *MdSAT1* regulates root system conformation *MdSAT1-OE* and Col plants grown for seven days under low NH_4^+ (0.5 mM NH_4Cl) or high NH_4^+ (1.5 mM NH_4Cl) conditions. (a), (b) Morphological changes, (c) primary root length and (d) lateral root number are presented. Error bars represent the standard deviation (n = 3). Different letters above the bars indicate significantly different values ($P < 0.05$).

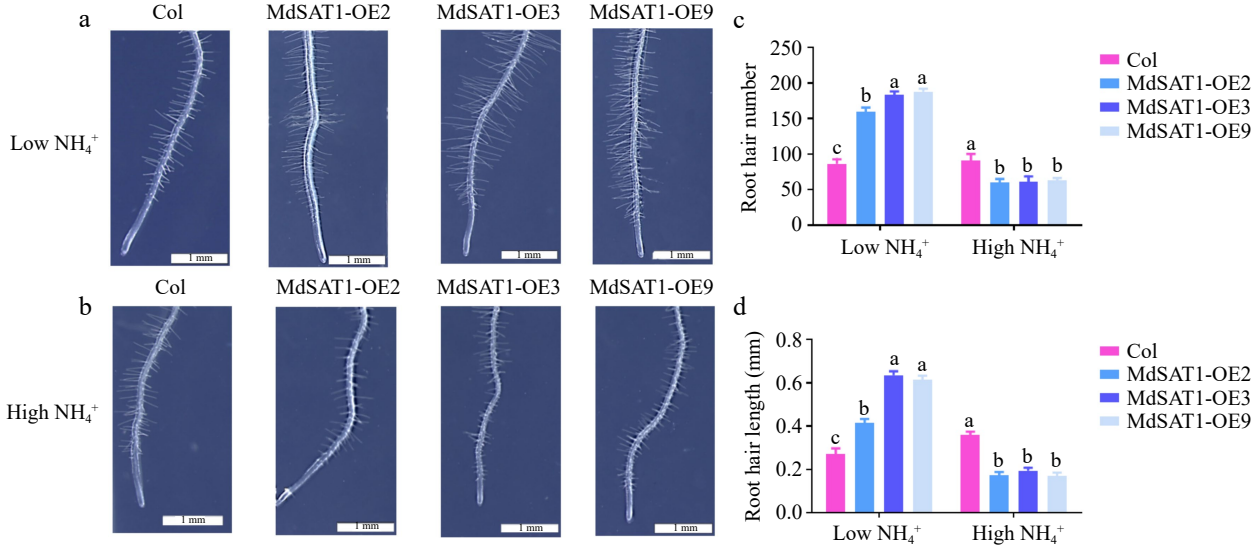

Fig. 5 *MdSAT1* regulates root hair growth and development *MdSAT1-OE* and Col plants grown for three days under low NH_4^+ (0.5 mM NH_4Cl) or high NH_4^+ (1.5 mM NH_4Cl) conditions. (a), (b) Morphological changes, (c) root hair number and (d) root hair length are presented. Error bars represent the standard deviation (n = 3). Different letters above the bars indicate significantly different values ($P < 0.05$).

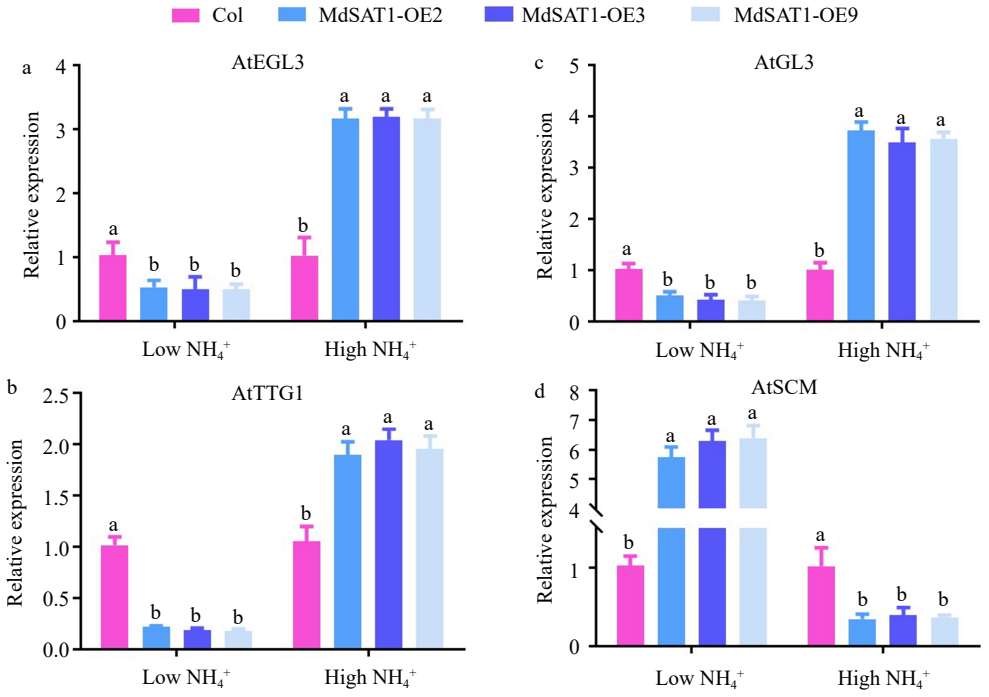

Fig. 6 *MdSAT1* regulates the expression of genes related to root hair development Analysis of root hair development-related gene expression by RT-PCR under low NH_4^+ (0.5 mM NH_4Cl) or high NH_4^+ (1.5 mM NH_4Cl) conditions: (a) *AtEGL3*, (b) *AtTTG1*, (c) *AtGL3*, and (d) *AtSCM* are presented. Error bars represent the standard deviation (n = 3). Different letters above the bars indicate significantly different values ($P < 0.05$).

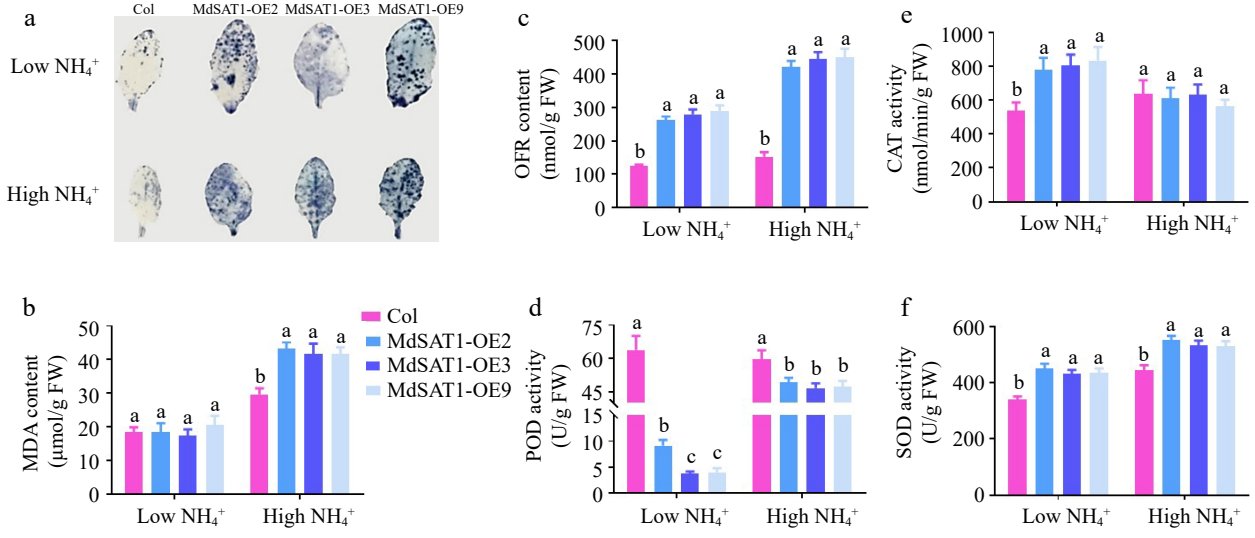

Fig. 7 *MdSAT1* regulates ROS accumulation Col and *MdSAT1-OE* plants grown for four weeks under low NH_4^+ (0.5 mM NH_4Cl) or high NH_4^+ (5 mM NH_4Cl) conditions. (a) NBT staining, (b) MDA content, (c) OFR concent, (d) POD activity, (e) CAT activity, and (f) SOD activity are presented. Error bars represent the standard deviation (n = 3). Different letters above the bars indicate significantly different values ($P < 0.05$).

treatment (Fig. 7c), while the MDA content was only promoted in high NH_4^+ treatment (Fig. 7b). CAT, SOD, and POD are important enzymes for ROS deconstruction[35,36] so the activities of these enzymes were measured. Under low NH_4^+ treatment, overexpression of *MdSAT1* resulted in higher CAT and SOD activities but significantly lower POD activity compared to Col (Fig. 7d–f). Overall, these results suggest that overexpression of *MdSAT1* promotes OFR production by affecting the activity of ROS-deconstruction-related enzymes.

DISCUSSION

When the soil nitrogen concentration is low, plants are more likely to take up ammonium[37]. However, the absorption and utilization efficiency of nitrogen by wild-type plants (Col) is not ideal when only ammonium is applied as nitrogen fertilizer. Moreover, the growth of plants is significantly inhibited at low concentration of single ammonium fertilizer, and the use of a higher concentration of single ammonium fertilizer will cause ammonium toxicity to plants. These trends will guide the appli-

cation of single ammonium as nitrogen fertilizer in agricultural production. Determination of the appropriate intermediate concentration of single ammonium for use as nitrogen fertilizer is essential to ensure the effective utilization of plants and also avoid the waste of resources and environmental pollution caused by excessive fertilization. Alternatively, combined application with nitrate nitrogen may be more appropriate. Therefore, it is important to study the law and mechanism of ammonium absorption by crops to optimize nitrogen utilization. GmSAT1 was functionally identified in soybean root nodule development[24]. In this study, phylogenetic and conserved domains analysis indicated that the MdSAT1 protein may be similar in function to GmSAT1 (Fig. 1; Supplemental Fig. S1). The function of MdSAT1 was characterized and the results showed that it is highly expressed, mainly in nutrient organs (Supplemental Fig. S5), and plays a key role in ammonium uptake and assimilation (Figs 3 & 4). MdSAT1 also regulates the accumulation of ROS and ultimately plant growth (Fig. 3a–c; Figs 4–7).

Several studies have shown that GmSAT1 is important for the symbiosis of soybean rhizobia and acts in NH_4^+ uptake during soybean rhizome development[23,24]. The Gmsat1 mutant negatively regulates nitrogen deficiency-induced genes to reduce nitrogen uptake[24]. Given that *MdSAT1* is an ammonium-responsive gene that is induced by NH_4^+ expression (Fig. 2; Supplemental Figs S2 & S3), we evaluated the role of *MdSAT1* in ammonium uptake and found that ectopic expression of *MdSAT1* promoted seedling growth under low NH_4^+ conditions compared with the wild type (Fig. 3a–c). A previous study showed that *SAT1* can regulate the nitrogen starvation response and coordinates related signaling regulatory networks[24]. SAT1 transcriptionally activates a unique plasma membrane NH_4^+ channel AMF1, indirectly enhancing NH_4^+ permeability, which also affects the regulation of MEP by SAT1[16]. Interestingly, SAT1 was unable to enhance the expression of MEP3 in the absence of AMF1, and SAT1 affects NH_4^+ uptake by indirect regulation of the AMT/MEP/Rh family through activation of AMF1[16]. Given this, we analyzed the expression of genes related to ammonium uptake and found that overexpression of *MdSAT1* significantly promoted the expression of *AtAMF1;3* (Fig. 3d), suggesting that *MdSAT1* may promote ammonium uptake by affecting the expression levels of *AMF1;3*.

NH_4^+ in soil is actively taken up by the root system mainly through ammonium ion transporters[38]. NH_4^+ can affect root system conformation, including primary roots, lateral roots, and root hairs. Previous work found that the primary root length, lateral root length, and root surface area gradually decreased with increasing NH_4^+ concentration[39] and addition of 0.1–10 mM of NH_4^+ could promote the number and elongation of lateral roots[40]. In this study, we found that the primary root lengths of *Arabidopsis* seedlings overexpressing *MdSAT1* were not significantly different from those of Col (Fig. 4c), but the number of lateral roots increased significantly compared with Col (Fig. 4d). We also found that GUS activity of *ProMdSAT1::GUS* transgenic *Arabidopsis* seedlings was expressed at the highest level at the beginning of lateral root primordia (Supplemental Figs S4). The above results suggest that *MdSAT1* is involved in the process of lateral root genesis and growth. A treatment of 0.1 mM NH_4^+ promotes the increase of root hair density and root hair number, but at NH_4^+ concentrations higher than 1 mM, the root hair density and root hair number gradually decrease with increasing NH_4^+ concentration[40]. Our results showed that under low NH_4^+ treatment, *MdSAT1* significantly promoted root hair development (Fig. 5a, c, d), but under high NH_4^+ treatment, it showed the opposite inhibitory effect (Fig. 5b–d). *AtEGL3*, *AtGL3*, and *AtTTG1*, which inhibit root hair development, were significantly down-regulated in *MdSAT1-OE Arabidopsis* under low NH_4^+ treatment (Fig. 6a–c), but the *AtSCM* genes were significantly up-regulated (Fig. 6d). These results suggest that *MdSAT1* regulates root development by regulating the transcript levels of root hair-related genes in an ammonium dosage-dependent manner. A role for ROS in root hair growth and formation mechanisms was previously reported[34]. To ask if *MdSAT1* regulates root hair development through the ROS pathway, we performed NBT staining and measured MDA and OFR content. The results suggested that overexpression of *MdSAT1* accumulated more OFR (Fig. 7a–c). Therefore, we speculate that OFR may regulate root hair development by ROS, a process that requires the involvement of the ammonium-responsive gene *MdSAT1*.

In conclusion, the results of this study showed that overexpression of *MdSAT1* promotes plant growth and biomass accumulation. These findings provide theoretical guidance to resolve the mechanisms by which MdSAT1 regulates ammonium uptake and plant growth and provide a reference for future selection of superior germplasm with more efficient nitrogen uptake.

MATERIALS AND METHODS

Plant materials and growth conditions

Apple seedlings (*Malus domestica* 'Royal Gala') were cultured in a plant growth chamber under 25/22 °C, 14/8 h temperature, and photoperiod. Apple group culture seedlings were grown in Murashige & Skoog (MS) medium (pH = 6.0) containing 6-Benzylamino Purine (6-BA, 0.5 mg L^{-1}), Naphthaleneacetic Acid (NAA, 0.1 mg L^{-1}), and Gibberellin (GA, 0.5 mg L^{-1}) for succession every 30 d. For the nitrogen treatment experiment, 1 month-old apple seedlings were selected for rooting in ½ MS rooting medium containing 1 mg L^{-1} 3-Indoleacetic acid (IAA). When rooting was completed, the seedlings were transferred to a nutrient bowl and cultured for about 30 d. Seedlings of uniform growth were selected and pre-treated in hydroponic conditions with ddH_2O for 1 week. The seedlings were then treated with 2 mM KCl (represents 0 N), KNO_3 (represents nitrate), or NH_4Cl (represents ammonium), and sampled after 0, 3, 6, 9, 12, and 24 h of treatment.

Arabidopsis seeds were disinfected with 75% ethanol and 3% sodium hypochlorite, and then sown on ½ MS medium solid culture plates (15 g L^{-1} sucrose and 8.0 g L^{-1} agar powder, pH adjusted to 5.9 with 1.0 M sodium hydroxide). The plates were incubated at 4 °C with dark vernalization for 4 d. Seeds were germinated and grown at 22 °C with a 16 h/8 h light/dark cycle.

Different types of *Arabidopsis* (*MdSAT1-OE*, Col) seedlings used for gene expression analysis by RT-PCR were germinated on ½ MS medium solid culture plates for 7 d before being transplanted to vermiculite, irrigated with tap water, and then watered weekly with a modified Hoagland's nutrient solution with either low NH_4^+ (0.5 mM NH_4Cl) or high NH_4^+ (5 mM NH_4Cl). The basic nutrient solution contained 1.0 mM $CaCl_2$, 1.0 mM NaH_2PO_4, 1.0 mM $MgSO_4$, 0.1 mM $FeNa_2EDTA$, 50 μM

$MnSO_4·H_2O$, 50 μM H_3BO_3, 0.05 μM $CuSO_4·5H_2O$, 0.5 μM $Na_2MoO_4·2H_2O$, 15 μM $ZnSO_4·7H_2O$, 2.5 μM KI, and 0.05 μM $CoCl·6H_2O$ with low or high concentration of NH_4Cl, and the pH was adjusted to 5.9 with 1.0 M sodium hydroxide. The final K^+ concentration was adjusted to 2,500 μM in both solutions by addition of K_2SO_4. After growing for four weeks, the *Arabidopsis* (*MdSAT1-OE*, Col) seedlings were subjected to phenotype observation and physiological analysis.

One or two days after germination, different types of *Arabidopsis* (*MdSAT1-OE*, Col) seedlings were transplanted and grown on low NH_4^+ (0.5 mM NH_4Cl) or high NH_4^+ (1.5 mM NH_4Cl) modified solid medium containing the above modified nutrient solution, plus inositol matter (2 μM $C_6H_{12}O_6·2H_2O$, 0.02 μM NC_5H_4COOH, 0.001 μM $C_{12}H_{17}ClN_4OS·HCl$, 0.01 μM $C_8H_{11}O_3N·HCl$, 0.1 μM $NH_2CN_2·COOH$), 30 g L^{-1} sucrose, and 8.0 g L^{-1} agar powder, with the pH adjusted to 5.9 with addition of 1.0 M sodium hydroxide. After three days, the root hairs of the seedlings grown on the above treatment medium were counted, and after seven days, the primary and lateral roots were counted.

Transgenic materials

MdSAT1-OE (MD10G1115500) and *ProMdSAT1::GUS Arabidopsis* seeds were obtained as described[41]. We identified the transgenic plant material using M5 Exceed Lightspeed PCR mix (Mei5Bio, Beijing, China) and Omni Plant RNA Kit (tDNase I) (Tiangen, Beijing, China) for DNA and RNA extraction, respectively (Supplemental Fig. S6)[41].

Bioinformatics analysis

The protein sequences of SAT1 from different species were obtained using blastp at the NCBI website (https://blast.ncbi.nlm.nih.gov/). The obtained sequences were used to construct a neighbor-joining phylogenetic tree with 1000 bootstrap replicates in MEGA-X[42] using the built-in ClustalW algorithm, Poisson model, and parameter settings for partial deletion (95%). The conserved domains of the SAT1 protein were predicted using Phyre^2 (www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index)[43].

Extraction of plant genomic RNA

Omni Plant RNA Kit (tDNase I) was used to extract plant RNA (Tiangen, Beijing, China). The extracted RNA was stored in tissue RNA preservation solution (RNAfollow M6100 New Cell & Molecular Biotech) for protection and stored in an ultra-low temperature refrigerator for gene expression analysis.

Real-time quantitative RT-PCR analysis of gene expression

From the extracted RNA, cDNA required for quantitative PCR was synthesized using the PrimeScript First Chain cDNA Synthesis Kit (Takara, Dalian, China). Reaction conditions: The RT-PCR experiments were conducted on ABI QuantStudio 3 a 96 Real-Time PCR system (Thermo Fisher, USA) using ChamQ™ Universal SYBR qPCR Master Mix (Vazyme, Q711-02, Nanjing, China), using a 20-μL reaction solution.This solution was made of 10-μL of 2× ChamQ Universal SYBR qPCR Master Mix, 0.4-μL 10 uM of each primer, 2-μL 10-folddilutions of cDNA template, and DEPC-water. All the qPCR protocols were performed in triplicate on 96-well plates, along with blank (template-absent) control. The PCR program is: 95 °C, 30 s; 95 °C, 10 s, 60 °C, 30 s, 40 cycles. Apple 18S rRNA and Arabidopsis actin rRNA genes were used as controls. PCR analysis was performed using specific primer sequences designed using Primer3Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi)[44] and listed in Supplemental Table S1. The RT-PCR analysis performed in triplicate, and relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Physiological measurements

Determination of substance content

Ammonium, Oxygen-derived free radicals (OFR), and Malondialdehyde (MDA) levels were measured by UV spectrophotometry as described below.

Ammonium interacts with hypochlorite and phenol in a strong alkaline medium to produce the water-soluble dye indophenol blue. Indophenol blue has a characteristic absorption peak at 625 nm and the absorbance value is proportional to the ammonium nitrogen content.

OFR react with hydroxylamine hydrochloride to form NO_2^-, which in the presence of p-aminobenzenesulfonic acid and α-naphthylamine produces a red azo compound with a characteristic absorption peak at 530 nm. The content of OFR in the sample can be calculated by measuring the change in absorbance at 530 nm.

MDA condenses with thiobarbituric acid (TBA) to produce a red product with a maximum absorption peak at 532 nm that can be used to estimate the amount of lipid peroxide in the sample. The absorbance at 600 nm was also measured, and the difference between the absorbance at 532 nm and 600 nm was used to calculate the amount of MDA.

Determination of enzymatic activities

Using 0.1g of plant material, Peroxidase (POD), Catalase (CAT), and Superoxide dismutase (SOD) activities were measured using activity assay kits (Comin, Suzhou, China) based on the below principles.

POD catalyzes the oxidation of specific substrates by H_2O_2 and exhibits characteristic light absorption at 470 nm.

CAT catalyzes the decomposition of H_2O_2 by CAT with characteristic light absorption at 405 nm.

SOD can scavenge OFR, and OFR can reduce azotetrazolium to produce blue methanamine, which exhibits absorption at 560 nm.

Nitroblue tetrazolium staining

Nitroblue tetrazolium (NBT) staining was performed according to existing methods[45].

GUS staining and enzyme activity assay

Transgenic *Arabidopsis* (*ProMdSAT1::GUS*) seedlings were immersed in GUS staining buffer consisting of 1 mM 5-bromo-4-chloro-3-indolyl-β-glutamic acid, 100 mM sodium phosphate (pH 7.0), 0.1 mM EDTA, 0.5 mM ferricyanide, and 0.1% (v/v) Triton X-100 37 °C for 1 h in the dark. To quantify GUS activity, proteins were extracted from the seedlings with 1 mL of extraction buffer (50 mM Na_2HPO_4/NaH_2PO_4 [pH 7.0]), 10 mM β-mercaptoethanol, 10 mM Na_2-EDTA, 0.1% (v/v) Triton X-100), and 1 mL of RIPA lysis buffer. A protein assay kit (Bio-Rad) was used to determine the total protein concentration. To measure GUS, 100 μL of the protein extract was added to 900 μL of GUS reaction buffer containing 1 mm 4-methylumbelliferone glucuronide and the mixture was incubated at 37 °C for 0, 5, 10, 15, 30, and 60 min. Then, 100 μL of the reaction mixture was added to 900 μL of the termination solution (1 M sodium carbonate). Fluorescence values were measured using a VersaFluor Spec-

trofluorometer (Bio-Rad) at an excitation wavelength of 365 nm and an emission wavelength of 455 nm. *Arabidopsis* (*ProMdSAT1*::*GUS*) seedlings (seven days-old) were pre-treated with ddH_2O for two days, treated with 1 mM KCl, KNO_3, or NH_4Cl for different time periods, immersed in the GUS staining solution, and photographed.

Root system analysis

Arabidopsis taproots were observed and photographed under a body view microscope. Digimizer software was used to measure and calculate the number and length of root hairs in a 4-mm area starting 2 mm from the root tip.

Data analysis

All experiments were repeated independently three times, unless otherwise indicated. The data are expressed as mean and standard deviation. Data were analyzed by one-way analysis of variance, and means were compared using Duncan's multiple range test. Different letters indicate significant differences at the $P < 0.05$ level.

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Conflict of interest

The authors declare that they have no conflict of interest.

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