Functional identification of bHLH transcription factor MdSAT1 in the ammonium response in apple

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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 of apple, we isolated *MdSAT1*, a gene 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 and assimilation and promoted the activities of ammonium assimilation-related enzymes, indicating that MdSAT1 can enhance ammonium uptake and utilization. *MdSAT1* also can modulate ROS accumulation to ultimately regulate plant growth. Taken together, these findings provide insight into the mechanisms by which *MdSAT1* controls ammoniun utilization as well as plant growth and development in apple.

Key Message

The bHLH transcription factor MdSAT1 can enhance ammonium uptake and utilization in apple.

1. Introduction

Nitrogen is both the basis of metabolism and the primary determinant of growth and yield (Lawlor, 2001). Nitrogen is a major component of nucleic acids, proteins, chlorophyll, and other substances, and is involved in many physiological and biological processes in plant growth and metabolism, including photosynthesis, carbohydrate allocation, and root formation (Ohyama, 2010, Viktor and Cramer, 2005). Plant nitrogen metabolism can also regulate the antioxidant system (Zhao, 2009). 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 more readily absorbed by plants (Jackson *et al.*, 2008, Patterson *et al.*, 2010). 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 (Bloom, 1997, Noctor *et al.*, 1998).

Inorganic nitrogen can be used for the metabolism of organic compounds in the form of ammonium, and non-ammonium nitrogen sources are generally converted to ammonium before amino synthesis (Xu *et al.*, 2012). Oxidative deamination of glutamate is catalyzed by glutamate dehydrogenase (GDH), and is generally involved in the oxidative decomposition of amino acids rather than their synthesis (Lopes *et al.*, 2015). Asparaginase (ASN) catalyzes the hydrolysis of asparagine to produce aspartate and ammonia, and participates in nitrogen fixation (Lopes et al., 2015, Atkins *et al.*, 1975). Glutaminase (GLS) catalyzes the formation of glutamate from glutamine as part of amino acid catabolism (Yang *et al.*, 2017). These

enzymes play important roles in the process of amino acid cycling in plants, indirectly facilitating the uptake and fixation of ammonium.

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 (Marini *et al.*, 1997). Two major groups of ammonium transporter proteins have been identified in plants: the AMT1 and AMT2 subfamilies (Couturier *et al.*, 2007). The plant AMT2 subfamily is more distantly related to the plant AMT1 subfamily (Guether *et al.*, 2009), and in Arabidopsis, AtAMT2 is likely to play a significant role in moving ammonium (Sohlenkamp *et al.*, 2002). Additional members of these families have also been characterized in Arabidopsis. AtAMT1;3, AtAMT1;4 and AtAMT1;5 exhibit high affinity for ammonium (Lopez-Pedrosa *et al.*, 2006, Yuan *et al.*, 2007), and AtAMT1;2 exhibits a relatively low affinity for ammonium (Neuhauser *et al.*, 2007). 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 (Mazurkiewicz, 2013).

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* (Huang *et al.*, 2015). 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* (Yanagisawa *et al.*, 2004, Santos *et al.*, 2012, Wu *et al.*, 2017, Yanagisawa, 2000). 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 (El-Kereamy *et al.*, 2012).

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 (Chiasson *et al.*, 2014). *GmSAT1* is involved in the regulation of nitrogen signaling regulatory networks related to nitrogen transport and metabolism (Dehcheshmeh, 2013). GmSAT1 activates the transcription of plasma membrane NH_4^+ channel *ScAMF1*, which indirectly enhances NH_4^+ permeability and finally promotes ammonium uptake (Chiasson et al., 2014, Mazurkiewicz, 2013).

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 (Miao *et al.*, 2011). The over application of ammonium fertilizer presents a significant burden to both soil and plants (Dawar *et al.*, 2021), therefore, investigating the mechanism of ammonium utilization is an

important goal in plant production (Rubio-Asensio and Bloom, 2017). Additionally, study of the tight regulation of transcription factors on nitrogen uptake can enable genetic engineering strategies to improve nutrient uptake regulation in plants (Wei *et al.*, 2019). 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 and the enzymatic activities of ammonium assimilation-related proteins. MdSAT1 also can 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 in apple.

2. Materials And Methods

2.1 Plant materials and growth conditions

Apple seedlings (*Malus domestica*) were cultured in a plant growth chamber under 25°C/22°C, 14 h/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), Naphthaleneacetic Acid (NAA, 0.1 mg/L), and Gibberellin (GA, 0.5 mg/L) for succession every 30 days. For the nitrogen treatment experiment, 1 month-old apple seedlings were selected for rooting in ½MS rooting medium containing 1 mg/L 3-Indoleacetic acid (IAA). When rooting was completed, the seedlings were transferred to a nutrient bowl and cultured for about 30 days. 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% alcohol 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 days 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 be the same 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 and biochemical 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 organic matter (2 μM $C_6H_{12}O_6{\cdot}2H_2O$, 0.02 μM NC_5H_4COOH, 0.001 μM $C_{12}H_{17}ClN_4OS{\cdot}HCl$, 0.01 μM $C_8H_{11}O_3N{\cdot}HCl$, 0.1 μM $NH_2CN_2{\cdot}COOH$), 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 observed, and after seven days, the primary and lateral roots were observed.

2.2 Transgenic materials

MdSAT1-OE and *ProMdSAT1::GUS Arabidopsis* seeds were obtained as described (Fig. S1) (Yang *et al.*, 2021).

2.3 Bioinformatics analysis

2.3.1 Multiple sequence alignment and phylogenetic tree construction

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 (Kumar *et al.*, 2018) using the built-in ClustalW algorithm, Poisson model, and parameter settings for partial deletion (95%).

2.3.2 Prediction of conserved domains

The conserved domains of the SAT1 protein were predicted using Phyre^2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi? id = index) (Kelley *et al.*, 2015).

2.4 Extraction of plant genomic DNA and RNA

Genomic DNA Reagent Kit and Omni Plant RNA Kit (tDNase I) were used to extract plant DNA and RNA (Tiangen, Beijing, China), respectively.

2.5 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). These synthesized products were used as templates for real-time quantitative RT-PCR to detect the expression levels of selected genes. 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) (Untergasser *et al.*, 2007) and listed in Supplemental Table S1. The qRT-PCR analysis performed in triplicate, and relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.6 Physiological measurements

2.6.1 Determination of substance content

Ammoninum, 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.

2.6.2 Determination of enzymatic activities

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

GDH catalyzes the formation of glutamate and NAD^+ from NH_4^+, α-ketoglutarate and NADH. This causes a decrease in absorbance at 340 nm, so GDH activity can be calculated by measuring the rate of decrease in absorbance at 340 nm.

ASN catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia, and its enzymatic activity can be calculated by detecting the rate of ammonia increase using Nessler's reagent.

GLS catalyzes the hydrolysis of glutamine to L-glutamate and ammonia, and the rate of increase of ammonia was measured by using Nessler's reagent to calculate the enzymatic activity.

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.

2.6.3 Nitroblue tetrazolium staining

Nitroblue tetrazolium (NBT) staining was performed according to existing methods (CORPAS, 2004).

2.7 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 Spectrofluorometer (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.

2.8 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.

2.9 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.

3. Results

3.1 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 closest 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. 1B-C). The high-level structure of the MdSAT1 protein was predicted by homology model, and the results indicated that the secondary and tertiary structures of MdSAT1 match those of the core conserved domain (Fig. 1C-D).

3.2 *MdSAT1* is an ammonium-responsive gene

MdSAT1 is homologous to GmSAT1, which is involved in ammonium uptake (Chiasson et al., 2014), and qRT-PCR was next used to detect the expression of *MdSAT1* in response to different nitrogen forms (KCl, KNO_3, and NH_4Cl). 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.

ProMdSAT1::GUS transgenic *Arabidopsis* seedlings were treated with different forms of nitrogen, and GUS staining results suggested that the highest GUS activity was observed under NH_4Cl treatment (Fig. 2C-D). With increasing time of different treatments, the expression activity of *ProMdSAT1::GUS* was specifically induced by NH_4Cl (Fig. S2). Taken together, these results suggest that *MdSAT1* is specifically responsive to ammonium.

3.3 Overexpression of *MdSAT1* regulates ammonium uptake

Given that *MdSAT1* is an ammonium-responsive gene, we next treated *MdSAT1-OE* and wild type (Col) in MS medium 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 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 (Chiasson et al., 2014), so these results indicated that *MdSAT1* promoted ammonium uptake by increasing the expression levels of genes related to ammonium uptake.

3.4 Overexpression of *MdSAT1* affects the enzymatic activities of ammonium assimilation-related proteins

After taken up by plant roots, NH_4^+ is then assimilated to amino acids or amides through the action of GDH, ASN, and GLS (Yang et al., 2017, Lea, 2006, Lopes et al., 2015, Atkins et al., 1975). Therefore, we measured the activities of these ammonium assimilation-related enzymes in *MdSAT1* transgenic

Arabidopsis. The results showed that overexpression of *MdSAT1* promoted GDH, ASN, and GLS activities *in vivo*, independent of ammonium treatment concentration (Fig. 4). The expression levels of ammonium assimilation-related genes showed the same trend (Fig. S3). Therefore, the results demonstrate that *MdSAT1* increases the activities of ammonium assimilation-related enzymes to influence ammonium assimilation.

3.5 Overexpression of *MdSAT1* promotes lateral root development

Ammonium in the soil is actively taken up by the roots mainly by ammonium ion transporters (von Wittgenstein *et al.*, 2014). 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 (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. 5A-D). These results suggest that *MdSAT1* overexpression promotes lateral root growth and development.

3.6 Overexpression of *MdSAT1* regulates root hair growth and development

Root hairs play an important role in nutrient uptake (Moon *et al.*, 2019), and 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. 6A, C-D), and the number and length of root hairs were inhibited under high NH_4^+ treatment (Fig. 6B-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. 7A-C) (Bernhardt *et al.*, 2003, Schiefelbein *et al.*, 2014) and significantly up-regulated levels of *AtSCM*, a positive regulator in root hair development (Fig. 7D) (Kwak and Schiefelbein, 2014); the expression patterns were reversed under high NH_4^+ treatment (Fig. 7A-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.

3.7 *MdSAT1* promotes the ROS accumulation

ROS plays a role in root hair development (Monshausen *et al.*, 2007), 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. 8A). OFR content was promoted in the *MdSAT1-OE* lines, both in high and low NH_4^+ treatment (Fig. 8C), while the MDA content was only promoted in high NH_4^+ treatment (Fig. 8B). CAT, SOD, and POD are important enzymes for ROS deconstruction (Waszczak *et al.*, 2018, Miller *et al.*, 2008) 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. 8D-F). Overall, these results suggest that overexpression of *MdSAT1* promotes OFR production by affecting the activity of ROS-deconstruction-related enzymes.

4. Discussion

When the soil nitrogen concentration is low, plants are more likely to take up ammonium (Bloom *et al.*, 1992). 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 application 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 (Dehcheshmeh, 2013). In this study, phylogenetic and conserved domains analysis indicated that the MdSAT1 protein may be similar in function to GmSAT1 (Fig. 1). The function of MdSAT1 was characterized and the results showed that it is highly expressed, mainly in nutrient organs (Fig. S5), and plays a key role in ammonium uptake and assimilation (Fig. 2; Fig. 3D; Fig. 4). MdSAT1 is phenotypically similar to GmSAT1, further confirming the genetic relationship of MdSAT1 with GmSAT1. MdSAT1 also regulates the accumulation of ROS and ultimately plant growth (Fig. 3A-C; Fig. 5–8).

Several studies have shown that GmSAT1 is important for the symbiosis of soybean rhizobia and acts in NH_4^+ uptake during soybean rhizome development (Chiasson et al., 2014, Dehcheshmeh, 2013). The Gmsat1 mutant negatively regulates nitrogen deficiency-induced genes to reduce nitrogen uptake (Dehcheshmeh, 2013). Given that *MdSAT1* is an ammonium-responsive gene that is induced by NH_4^+ expression (Fig. 2; Fig. S2), 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). Previous study showed that *SAT1* can regulate the nitrogen starvation response and coordinates related signaling regulatory networks (Dehcheshmeh, 2013). 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 (Mazurkiewicz, 2013). 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 (Mazurkiewicz, 2013). 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*.

Most of the NH_4^+ absorbed by plant roots is enzymatically assimilated to glutamate in the root system, and glutamate is then converted to other amino acids and subsequently transported to various parts of the plant organs and tissues via the xylem (Lea PJ, 2006, Comadira *et al.*, 2015). ASN is a amide hydrolase that participates in amino acid metabolism and GDH and GLS catabolize ammonia by deamination (Yang et al., 2017, Lopes et al., 2015, Atkins et al., 1975). Our experiments showed that independent of the external NH_4^+ concentration, overexpression of *MdSAT1* promoted the activities of GDH, ASN, and GLS, consistent with the observed changes in transcript levels of the corresponding genes (Fig. 4; Fig. S3). In this way, *MdSAT1* can help regulate the free ammonia level *in vivo* and promote the plant's own ammonia cycle.

NH_4^+ in soil is actively taken up by the root system mainly through ammonium ion transporters (Wang *et al.*, 2012). 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 (Li and Sun, 2007) and addition of 0.1–10 mM of NH_4^+ could promote the number and elongation of lateral roots (Yang, 2010). In this study, we found that the primary root lengths of *Arabidopsis* seedlings overexpressing *MdSAT1* were not significantly different from those of Col (Fig. 5C), but the number of lateral roots increased significantly compared with Col (Fig. 5D). 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 (Fig. 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 (Yang, 2010). Our results showed that under low NH_4^+ treatment, *MdSAT1* significantly promoted root hair development (Fig. 6A, C-D), but under high NH_4^+ treatment, it showed the opposite inhibitory effect (Fig. 6B-D). *AtEGL3*, *AtGL3*, and *AtTTG1*, which inhibit root hair development, were significantly down-regulated in *MdSAT1-OE Arabidopsis* under low NH_4^+ treatment (Fig. 7A-C), but the *AtSCM* genes were significantly up-regulated (Fig. 7D). 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 (Monshausen et al., 2007). 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. 8A-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.

Declarations

Author Contributions:

Xiao-Fei Wang and Wen-Sheng Gao designed the experiments. Tong Li, Zi-Quan Feng, Bai-Hui Zhu, Ming-Li Li and Guo-Dong Li performed the research. Tong Li, Zi-Quan Feng, Bai-Hui Zhu and Chun-Xiang You analyzed the data. Tong Li, Xiao-Fei Wang and Wen-Sheng Gao wrote the paper.

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Competing Interest:

The authors declare no conflict of interest.

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Figures

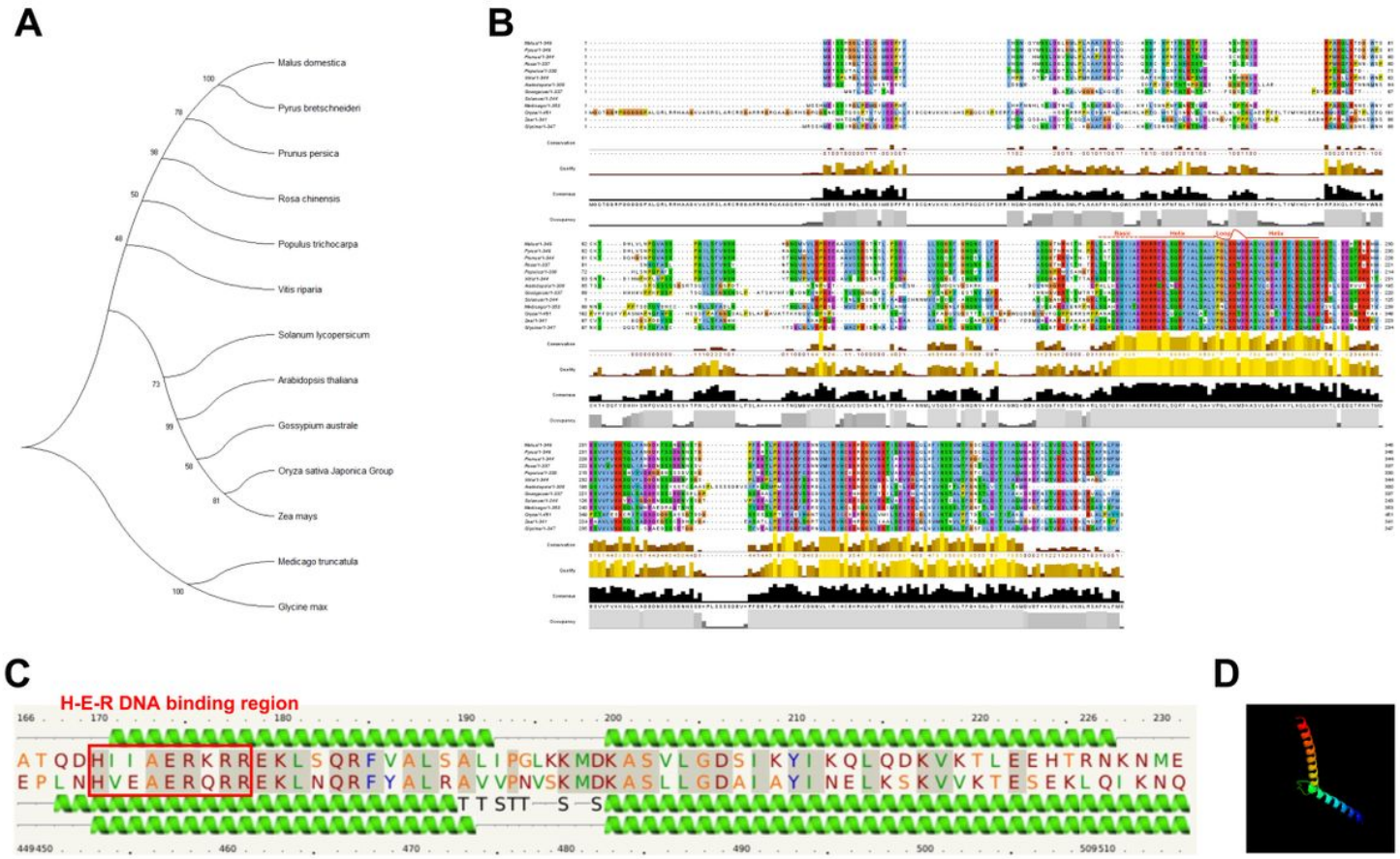

Figure 1

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) Multiple sequence alignment for above 13 proteins. (C) Predicted protein secondary of MdSAT1 structural domain. (D) Predicted protein 3D structure of MdSAT1.

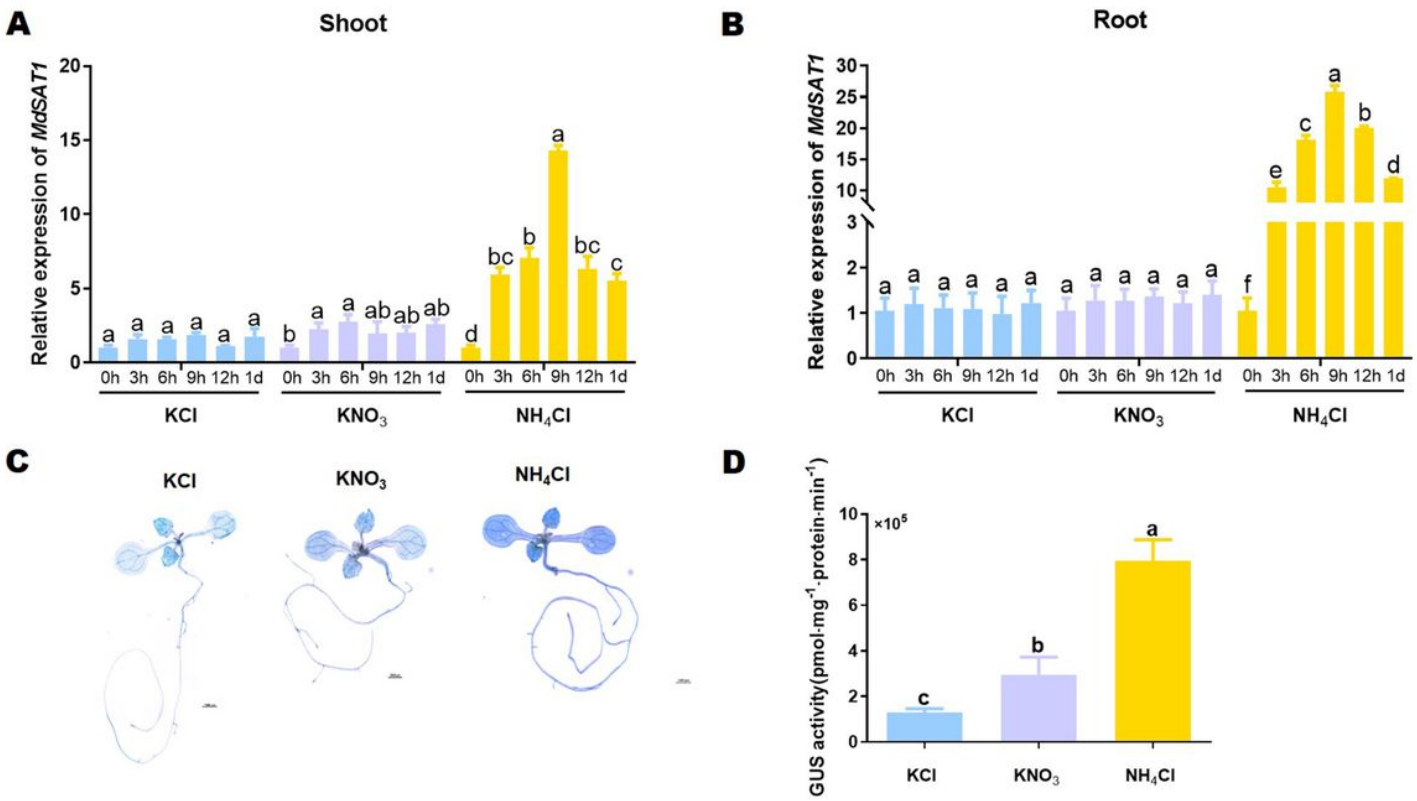

Figure 2

Nitrogen response of *MdSAT1*

Shoot (A) and root (B) response of *MdSAT1* to KCl (represents 0 N), KNO_3 (represents nitrate), and NH_4Cl (represents ammonium). (C) GUS staining of *ProMdSAT1::GUS* transgenic *Arabidopsis* under above treatments. (D) GUS activity of *MdSAT1* under above treatments. Error bars represent standard deviation ($n \geq 3$). Different letters above the bars indicate significantly different values ($P<0.05$).

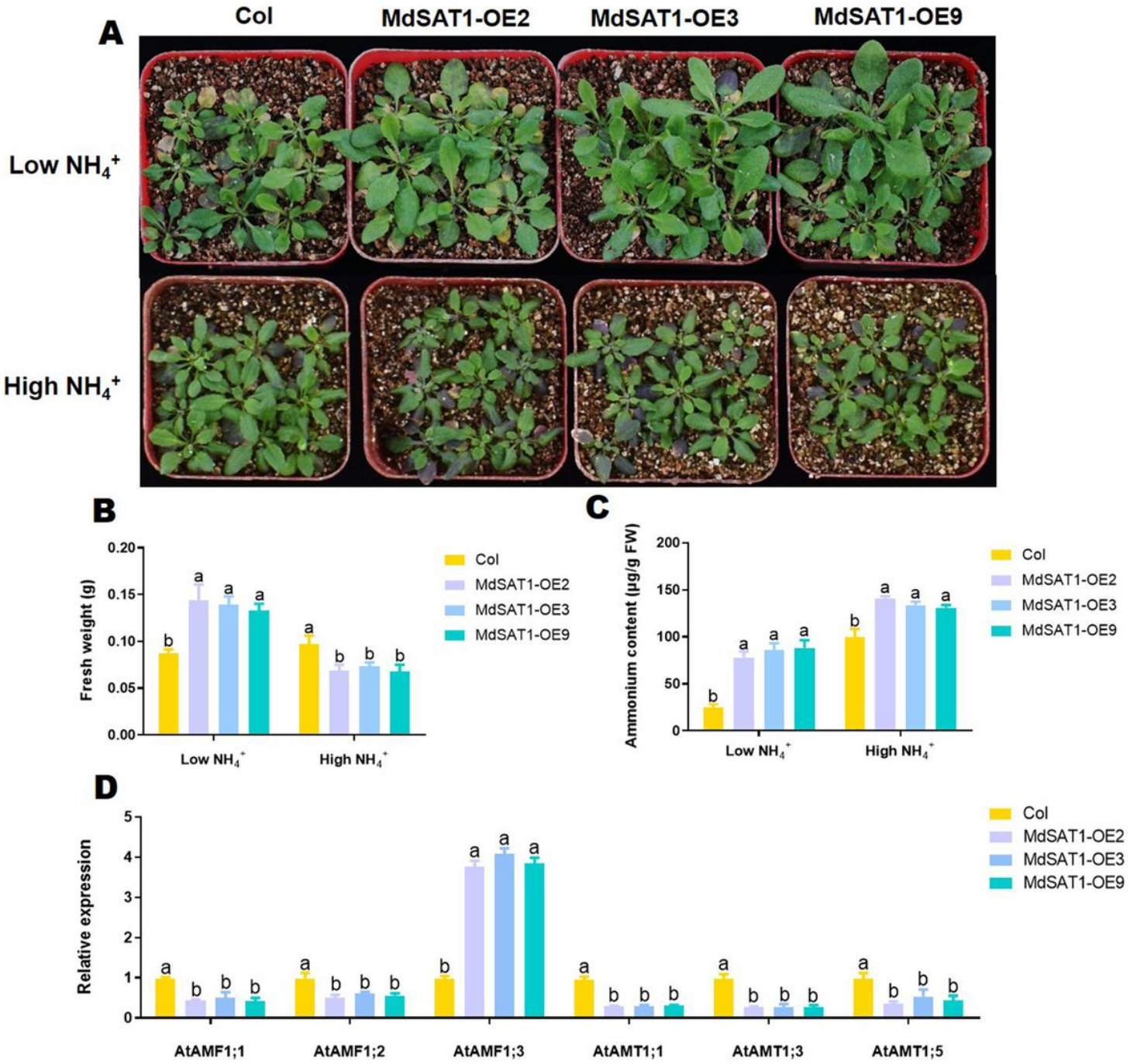

Figure 3

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. Morphological changes (A), Fresh weight (B), and Ammonium content (C) are presented. (D) qRT-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).

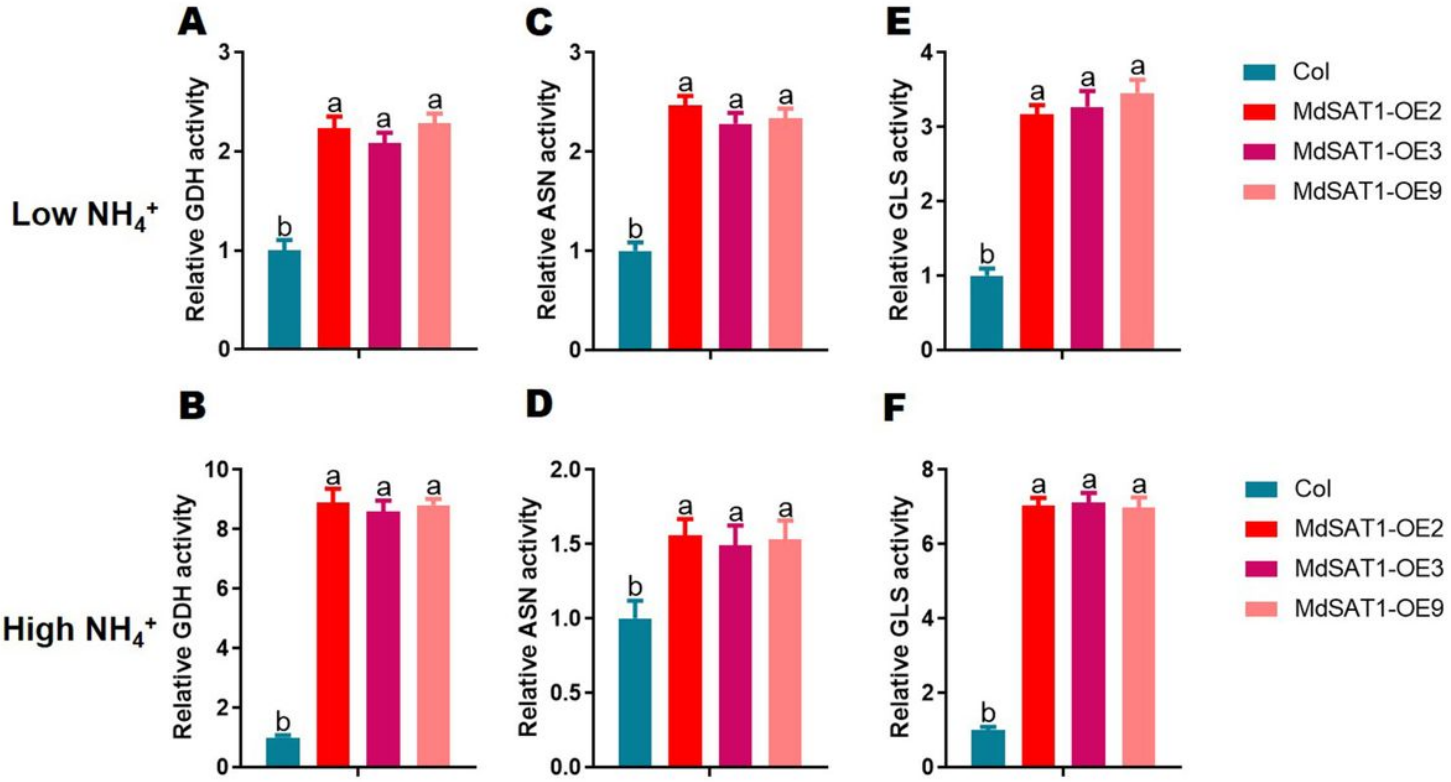

Figure 4

MdSAT1 affects ammonium uptake through enzymatic activity

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. Relative GDH activity (A and B), Relative ASN activity (C and D), and Relative GLS activity (E and F) are presented. Error bars represent the standard deviation ($n \geq 3$). Different letters above the bars indicate significantly different values ($P<0.05$).

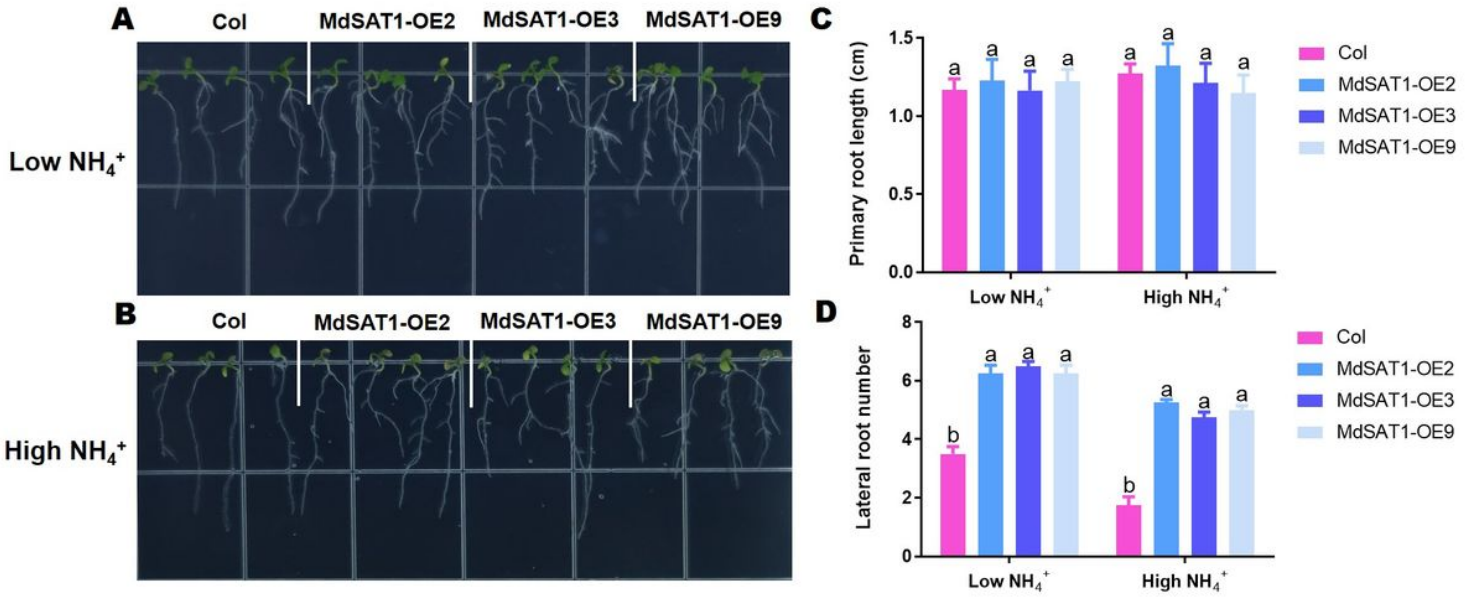

Figure 5

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. Morphological changes (A and B), Primary root length (C) and Lateral root number (D) are presented. Error bars represent the standard deviation (n≥3). Different letters above the bars indicate significantly different values (P<0.05).

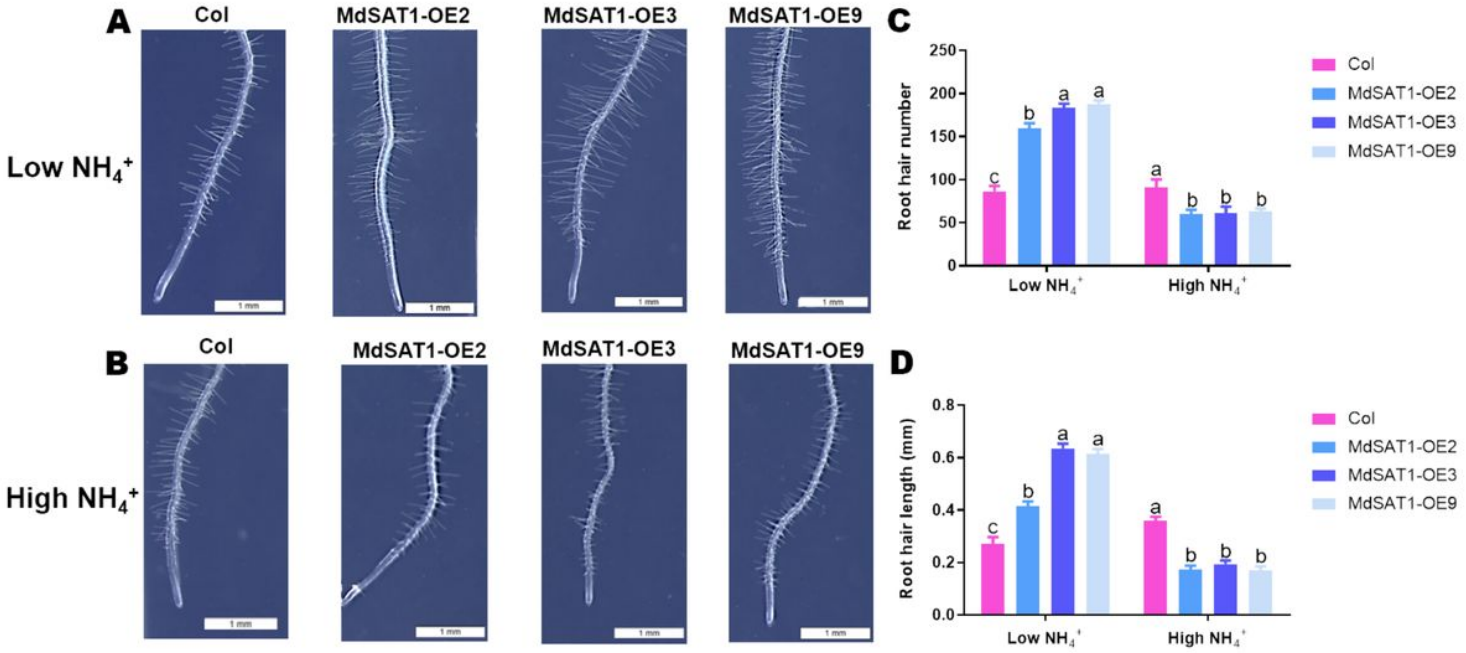

Figure 6

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. Morphological changes (A and B), Root hair number (C) and Root hair length (D) are presented. Error bars represent the standard deviation (n≥3). Different letters above the bars indicate significantly different values (P<0.05).

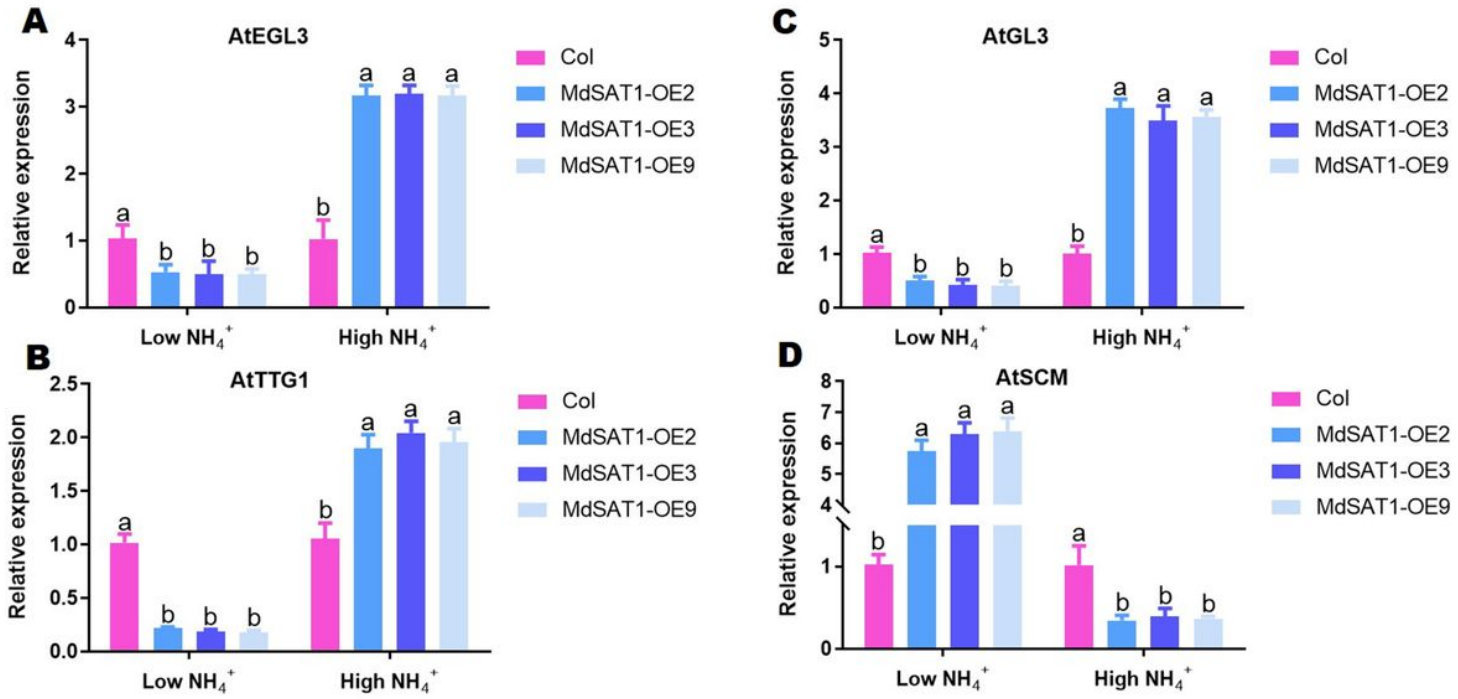

Figure 7

MdSAT1 regulates the expression of genes related to root hair development

Analysis of root hair development-related gene expression by qRT-PCR under Low NH_4^+ (0.5 mM NH_4Cl) or High NH_4^+ (1.5 mM NH_4Cl) conditions: *AtEGL3* (A), *AtTTG1* (B), *AtGL3* (C), and *AtSCM* (D) are presented. Error bars represent the standard deviation (n≥3). Different letters above the bars indicate significantly different values (P<0.05).

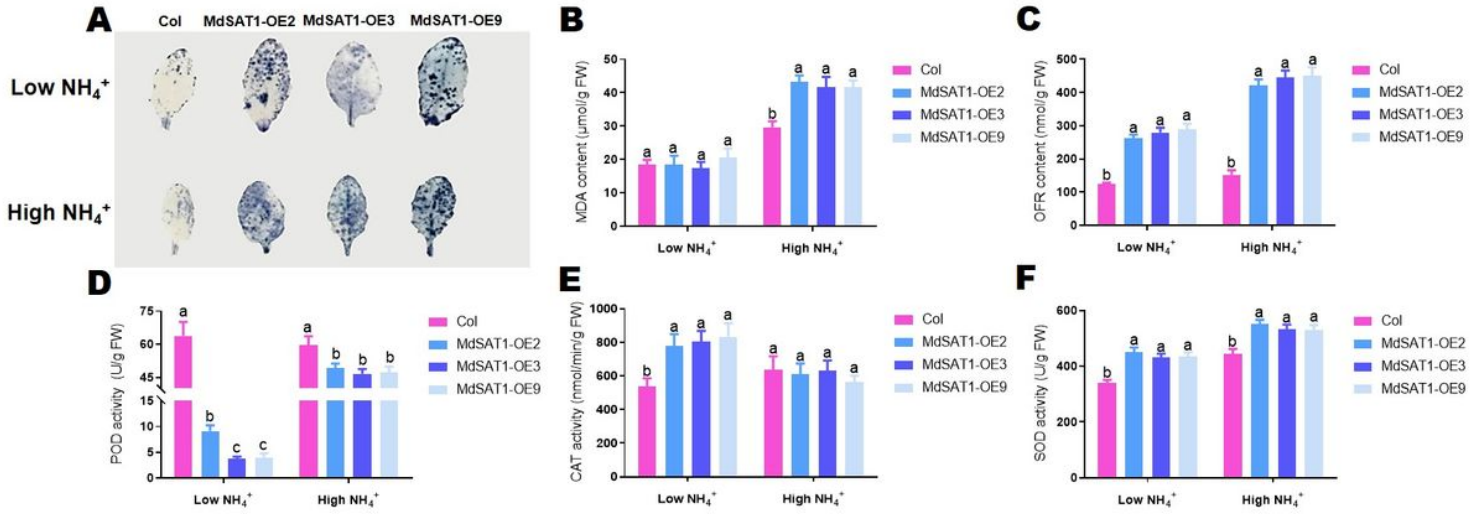

Figure 8

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. NBT staining (A), MDA content (B), OFR concent (C), POD activity (D), CAT activity (E), and SOD activity (F) are presented. Error bars represent the standard deviation ($n \geq 3$). Different letters above the bars indicate significantly different values ($P<0.05$).

Supplementary Files

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