Functional identification of bHLH transcription factor MdSAT1 in the ammonium response

Tong Li¹, Zi-Quan Feng¹, Bai-Hui Zhu¹, Ming-Li Li², Guo-Dong Li², Chun-Xiang You¹, Wen-Sheng Gao²*, and Xiao-Fei Wang¹*

¹ State Key Laboratory of Crop Biology, Shandong Green Fertilizer Technology Innovation Center, Collaborative Innovation Center of Fruit & Vegetable Quality and Efficient Production, Shandong Agricultural University, Ta‘an 271018, Shandong, China
² Shandong Agricultural Technology Extension Center, Jinan 250013, Shandong, China
*Corresponding authors, E-mail: gaowensheng@shandong.cn; xfwang2004@163.com

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.

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 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 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 NH4⁺ channel Ammonium Facilitator 1 (AMF1) has also been found to regulate plasma membrane permeability to NH4⁺ and NH3⁺ 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 Arabidopsis, transcription factor Long Hypocotyls 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, Glicine 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 NH4⁺ channel ScAMF1, which indirectly enhances NH4⁺ 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

© The Author(s)
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\textsuperscript{[29]}. The over application of ammonium fertilizer presents a significant burden to both soil and plants\textsuperscript{[28]}, therefore, investigating the mechanism of ammonium utilization is an important goal in plant production\textsuperscript{[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\textsuperscript{[28]}. In this study, we identified an ammonium-responsive \textit{MdSAT1} gene in apple and found that \textit{MdSAT1} regulates the expression of genes related to ammonium uptake. \textit{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 \textit{MdSAT1} controls ammonium uptake as well as plant growth and development.

RESULTS

Phylogenetic relationships, multiple sequence alignment, and protein structure analysis of \textit{MdSAT1}

The \textit{MdSAT1} (MD10G1115500) gene was identified from the NCBI website according to the \textit{GmSAT1} sequence of soybean (\textit{Glycine max}). A phylogenetic tree was constructed, and apple \textit{MdSAT1} was most closely related to pear \textit{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 \textit{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).

\textbf{MdSAT1 is an ammonium-responsive gene}

\textit{MdSAT1} is homologous to \textit{GmSAT1}, which is involved in ammonium uptake\textsuperscript{[23]}, and RT-PCR was used to detect the expression of \textit{MdSAT1} in response to different nitrogen forms (KCl, as a control, represents 0 N; KNO\textsubscript{3} represents nitrate; and NH\textsubscript{4}Cl, represents ammonium). The expression of \textit{MdSAT1} was significantly induced by NH\textsubscript{4}Cl both in shoots and roots, however, the transcript level of \textit{MdSAT1} showed little change in response to nitrate (Fig. 2a, b), suggesting that \textit{MdSAT1} was specifically responsive to ammonium.

The \textit{ProMdSAT1::GUS} transgenic \textit{Arabidopsis} seedlings-that can be used to localize the expression location and expression intensity of \textit{MdSAT1}-were treated with different forms of nitrogen. We found that GUS staining results suggested that the highest GUS activity was observed under NH\textsubscript{4}Cl treatment (Supplemental Fig. S2). With increasing time of different treatments, the expression activity of \textit{ProMdSAT1::GUS} was specifically induced by NH\textsubscript{4}Cl (Supplemental Fig. S3). Taken together, these results suggest that \textit{MdSAT1} is specifically responsive to ammonium.

Overexpression of \textit{MdSAT1} regulates ammonium uptake

Given that \textit{MdSAT1} is an ammonium-responsive gene, we next treated \textit{MdSAT1-OE} and wild type (Col) in a modified Hoagland’s nutrient solution containing 0.5 mM NH\textsubscript{4}Cl (Low NH\textsubscript{4}\textsuperscript{+}) or 5 mM NH\textsubscript{4}Cl (High NH\textsubscript{4}\textsuperscript{+}) for four weeks and then assessed the effects on plant growth and ammonium content. Under low NH\textsubscript{4}\textsuperscript{+} conditions, ectopic expression of \textit{MdSAT1} promoted seedling growth compared with Col, and \textit{MdSAT1-OE} showed greater fresh weight and increased ammonium content (Fig 3a, c). In contrast, under high NH\textsubscript{4}\textsuperscript{+} conditions, ectopic expression of \textit{MdSAT1} reduced fresh weight and accumulated higher ammonium (Fig. 3a–c). These results indicate that \textit{MdSAT1} promotes ammonium uptake to regulate plant growth.

To further evaluate the role of \textit{MdSAT1} in ammonium uptake, the effects of \textit{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$^{[23]}$ 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 [15], so 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. 5a–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$_4$**$^+$ 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. 4**  **MdSAT1** regulates root system conformation **MdSAT1-OE** and **Col** plants grown for seven days under low **NH$_4$**$^+$ (0.5 mM **NH$_4$Cl**) or high **NH$_4$**$^+$ (1.5 mM **NH$_4$Cl**) 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$_4$Cl**) or high **NH$_4$**$^+$ (1.5 mM **NH$_4$Cl**) 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).
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 AtITG1, 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-Benzylaminopurine (6-BA, 0.5 mg L$^{-1}$), Naphthaleneacetic Acid (NAA, 0.1 mg L$^{-1}$), and Gibberellin (GA, 0.5 mg L$^{-1}$) for success every 30 d. For the nitrogen treatment experiment, 1 month-old apple seedlings were selected for rooting in 1/2 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 hydronic conditions with ddH$_2$O for 1 week. The seedlings were then treated with 2 mM KCl (represents 0 N), KNO$_3$ (represents nitrate), or NH$_4$Cl (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 1/2 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 1/2 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$_4$Cl) or high NH$_4^+$ (5 mM NH$_4$Cl). The basic nutrient solution contained 1.0 mM CaCl$_2$, 1.0 mM NaH$_2$PO$_4$, 1.0 mM MgSO$_4$, 0.1 mM FeNa$_2$EDTA, 50 μM
MnSO₄·H₂O, 50 µM H₂BO₃, 0.05 µM CuSO₄·5H₂O, 0.5 µM Na₂MoO₄·2H₂O, 15 µM ZnSO₄·7H₂O, 2.5 µM KI, and 0.05 µM CoCl₂·6H₂O with low or high concentration of NH₄Cl, 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 K₂SO₄. After growing for four weeks, the Arabidopsis (MdSAT1-1OE, Col) seedlings were subjected to phenotype observation and physiological analysis.

One or two days after germination, different types of Arabidopsis (MdSAT1-1OE, Col) seedlings were transplanted and grown on low NH₄⁺ (0.5 mM NH₄Cl) or high NH₄⁺ (1.5 mM NH₄Cl) modified solid medium containing the above modified nutrient solution, plus inositol matter (2 µM C₆H₁₂O₆·2H₂O, 0.02 µM NC₆H₅COOH, 0.001 µM C₆H₇CaCl₂, 0.01 µM C₆H₇O₃·N·NH₄Cl, 0.1 µM NH₂CN₂·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
MdSAT1-1OE (MD10G1155000) and ProMdSAT1::GUS Arabidopsis seeds were obtained as described[41]. We identified the transgenic plant material using MS 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 Phyre² (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 µM of each primer, 2-µL 10-fold dilutions 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⁻ΔΔCT 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 NO₂⁻, which in the presence of p-aminobenzenesulfonic acid and 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 thioibarbituric 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 H₂O₂ and exhibits characteristic light absorption at 470 nm.

CAT catalyzes the decomposition of H₂O₂ 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-D-glucuronic acid, 100 mM sodium phosphate (pH 7.0), 0.1 mM EDTA, 0.5 mM ferricyanide, and 0.1% (w/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 Na₂HPO₄/NaH₂PO₄ [pH 7.0]), 10 mM β-mercaptoethanol, 10 mM Na₂EDTA, 0.1% (w/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 ddH2O for two days, treated with 1 mM KCl, KNO3, or NH4Cl 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.

**ACKNOWLEDGMENTS**

This work was supported by Shandong Province Key R&D Program, 2021CXGC010802 (Chun-Xiang You); Taishan scholar foundation, LNY202026 (Wen-Sheng Gao); National Natural Science Foundation of China, 31972378 (Xiao-Fei Wang) and China Agriculture Research System of MOF and MARA, CARS-27 (Xiao-Fei Wang). We sincerely thank our team leader Dr. Yu-Jin Hao, who will be remembered for his great achievement and for the support and help in our work.

**Conflict of interest**

The authors declare that they have no conflict of interest.

**Supplementary Information** accompanies this paper at (https://www.maxapress.com/article/doi/10.48130/FruRes-2022-0017)

**Dates**

Received 5 September 2022; Accepted 31 October 2022; Published online 22 November 2022

**REFERENCES**


Copyright: © 2022 by the author(s). Published by Maximum Academic Press, Fayetteville, GA. This article is an open access article distributed under Creative Commons Attribution License (CC BY 4.0), visit https://creativecommons.org/licenses/by/4.0/.