

Interfering small ubiquitin modifiers (SUMO) exhibits apple's enhanced tolerance to nitrogen deficiency

Zeyuan Liu[#], Ziqing Ma[#], Jia Li, Ningning Bian, Zitong Guo, Jianyan Guo, Shuang Zhao, Baohua Chu, Chundong Niu, Fengwang Ma, Qingmei Guan* and Xuewei Li*^{ORCID}

State Key Laboratory of Crop Stress Biology for Arid Areas/Shaanxi Key Laboratory of Apple, College of Horticulture, Northwest A&F University, Yangling, Shaanxi 712100, China

[#] These authors contributed equally: Zeyuan Liu, Ziqing Ma

* Corresponding authors, E-mail: qguan@nwfau.edu.cn; vivian@nwfau.edu.cn

Abstract

Nitrogen is one of the most critical macroelements required for apple biomass accumulation and quality formation. Thus, identification and characterization of candidate genes involved in nitrogen deficiency stress is of great importance to the sustainable development of the apple industry. SUMOylation is a kind of post-translational modification, which is widely involved in plant life processes, including responses to nitrogen (N) deficiency. Here, we identified apple small ubiquitin modifiers (SUMO) that participated in responding to nitrogen deficiency. Firstly, all isoforms of apple SUMOs were induced by N deficiency stress. Under N deficiency stress conditions, the *MdSUMO2* RNAi transgenic apple plants showed better growth potential than the wild type GL-3, including more new leaves, increased height, thicker stems, and heavier fresh weight. In addition, the *MdSUMO2* RNAi transgenic apple plants exhibited darker green leaves and greater root volume than GL-3 in response to N deficient conditions. Finally, the activated glutamine synthetase (GS) activity, nitrate reductase (NR) activity, as well as the induced expression of genes involved in nitrogen assimilation in the root might contribute to the tolerance of *MdSUMO2* RNAi transgenic apple plants in response to nitrogen deficiency. Taken together, our finding provides insight to further study the mechanisms underlying the essential roles of apple *MdSUMOs* in responding to N deficient conditions.

Citation: Liu Z, Ma Z, Li J, Bian N, Guo Z, et al. 2023. Interfering small ubiquitin modifiers (SUMO) exhibits apple's enhanced tolerance to nitrogen deficiency. *Fruit Research* 3:24 <https://doi.org/10.48130/FruRes-2023-0024>

Introduction

Nitrogen is a major component of chlorophyll, nucleic acids, proteins, vitamins, and several other secondary metabolites in plants, which plays an irreplaceable role in the regulation of plant photosynthesis, carbohydrate allocation, root formation, and resistance to abiotic stress^[1]. Previous studies have demonstrated that the application of an optimal amount of nitrogen (N) fertilizer can enhance the electron transfer capacity between photosystem I (PSI) and photosystem II (PSII) in wheat, which leads to improved photosynthetic efficiency, higher biomass production, and increased crop yield^[2]. However, N deficiency stress is a common nutrient stress in plants. Under conditions of mild nitrogen deficiency stress, plants usually exhibit slow growth and pale or yellow-green leaves, while severe nitrogen deficiency stress can inhibit plant photosynthesis and accelerate leaf senescence, ultimately affecting plant yield and quality. Given the potential negative impact of nitrogen deficiency stress on plant growth and development, understanding the molecular mechanisms underlying plant tolerance to this stress has become an urgent challenge in current research.

Apple (*Malus domestica* Borkh) is one of the most widely cultivated and important commercial crops worldwide. In 2020, the total output of apples reached 46 metric tons in China, which accounted for over 55% of global apple production (FAO STAT, 2022, <http://www.fao.org/faostat/en/#home>). However,

the quality and yield of apples are usually affected by unfavorable environmental conditions, such as N-deficiency. Studies have shown that in response to nitrogen deficiency stress, the color of apple leaves change from dark green to light yellow-green^[3]. There is also a significant increase in the levels of malondialdehyde (MDA), soluble protein, and proline content, while the chlorophyll content significantly decreases. These changes are adaptive responses of the plant to nitrogen deficiency stress^[3]. At the same time, the molecular regulation mechanism of apple trees in response to nitrogen deficiency stress conditions has also been discovered. Many genes involved in this process have been identified, including Apple *MdBT2* (BTB and TAZ DOMAIN PROTEIN 2), which was repressed by nitrate starvation. Overexpression of *MdBT2* negatively regulates apple response to tolerate N deficiency stress^[4]. *MdMYB10*, an important MYB (v-Myb avian myeloblastosis viral oncogene homolog) transcription factor in apples, was also involved in regulating nitrate uptake and usage. Overexpression of the *MdMYB10* gene can enhance the absorption of nitrate by apple roots, promote the transport and redistribution of nitrogen in the plant, thus increasing the nitrogen content and accumulation of anthocyanins in apple fruits^[5]. However, research on apple N deficiency stress response is still insufficient. Understanding the functions of the genes involved in this process will be crucial for developing strategies to improve the growth and productivity of apple trees under conditions of N deficiency.

SUMOylation is a crucial post-translation modification (PTM) that is the covalent attachment of small ubiquitin-associated modifier (SUMO) molecules to target proteins catalyzed by enzyme cascade, which regulates numerous cellular and physiological processes of plants, particularly in response to abiotic stress^[6,7]. Currently, SUMOylation has been proven to be involved in the physiological processes of many plants, including Arabidopsis, rice, soybean, maize, potato, tomato, peanut, apple, poplar, and so on^[6,8–10]. In Arabidopsis, the SUMO E3 ligase SIZ1 (SAP and MIZ1 protein interacting zinc finger protein 1) functions in abiotic stress responses, phytohormone signaling, nitrogen assimilation, development, and photomorphogenesis^[11]. Additionally, rice *OsSIZ1* is involved in the phosphate and nitrogen-dependent responses^[12]. Apple *MdSIZ1* is induced by the transcription factor MdMYB2, which has been found to regulate cold tolerance and anthocyanin accumulation^[13]. However, to date, how SUMOylation participates in the underlying N deficiency stress response in apple trees is largely unknown.

In this study, we found that interfering SUMOs expression could facilitate tolerance of the transgenic apple plants to N deficiency stress conditions. The *MdSUMO2* RNAi transgenic apple plants exhibited better growth phenotype under N deficient conditions compared to the wild type, as well as the developed root system. The activated nitrate reductase *NR* and nitrate transporters *NRT1.1/1.2/2.5* in the root system of *MdSUMO2* RNAi plants might partly contribute to the tolerance of *MdSUMO2* RNAi in response to N deficiency stress conditions. Our findings highlight the important role of apple *MdSUMOs* during N deficiency response and provide a foundation for improving the efficiency of nitrogen use in apple.

Results

Expression patterns analysis of *MdSUMO2s* in apple under N deficient conditions

It was reported that apple small ubiquitin modifiers (SUMO) - *MdSUMO2A*, *MdSUMO2B*, and *MdSUMO2C* were induced by various abiotic stress^[6,7]. To understand the expression pattern of apple *MdSUMO2s* in response to N deficient conditions, we examined the relative expression levels of *MdSUMO2A/B/C* by qRT-PCR (qRT-PCR). The results showed that *MdSUMO2A*, *MdSUMO2B*, and *MdSUMO2C* were all induced by N deficiency

stress conditions (Fig. 1), indicating that apple *MdSUMO2A/B/C* were N deficiency responsive genes.

MdSUMO2 RNAi transgenic apple plants exhibit enhanced tolerance to N deficiency

The induced expression levels of *MdSUMO2A*, *MdSUMO2B*, and *MdSUMO2C* suggested these genes might function in the N deficiency stress response. To examine this hypothesis, *MdSUMO2* RNAi transgenic apple seedlings which interfering all SUMOs expression and the wild type (GL-3) were used as experimental materials. Under normal hydroponic culture conditions, no significant difference was observed between the GL-3 and *MdSUMO2* RNAi plants. However, under N deficient treatment conditions, *MdSUMO2* RNAi transgenic apple plants exhibited a more vigorous phenotype than GL-3 (Fig. 2a). A significant reduction of new leaves was found in GL-3 under N deficient conditions, but the *MdSUMO2* RNAi transgenic apple plants were less affected (Fig. 2b). These results showed that GL-3 ceased to produce new leaves under N deficiency stress, while the growth of *MdSUMO2* RNAi transgenic apple seedlings was not significantly affected (Fig. 2c & e). Under nitrogen deficient conditions, the *MdSUMO2* RNAi apple plants exhibited significantly increased height and fresh weight in comparison to the GL-3 plants, which was the opposite of the control conditions (Fig. 2c & e). All apple seedlings displayed a noticeable reduction in stem diameter when exposed to nitrogen deficiency, suggesting a general impact on stem development under limited nitrogen availability (Fig. 2d). Interestingly, in contrast to the control conditions, the *MdSUMO2* RNAi plants demonstrated a strikingly thicker stem than the GL-3 plants under nitrogen deficiency (Fig. 2d). Together, these findings indicate that knock-down of the *MdSUMO2s* in apple enhanced the tolerance to N deficiency.

MdSUMO2 RNAi transgenic apple plants increased more chlorophyll content in response to N deficient conditions

Nitrogen deficient conditions typically result in yellowing of the leaves^[14]. Here, we found that the *MdSUMO2* RNAi transgenic apple plants tended to keep green leaves compared to GL-3 under N deficient conditions (Fig. 2a). Since the SPAD value has been commonly used as an indicator of nitrogen levels in plants^[15], we evaluated the leaves SPAD value of *MdSUMO2* RNAi transgenic apple plants and GL-3 in response

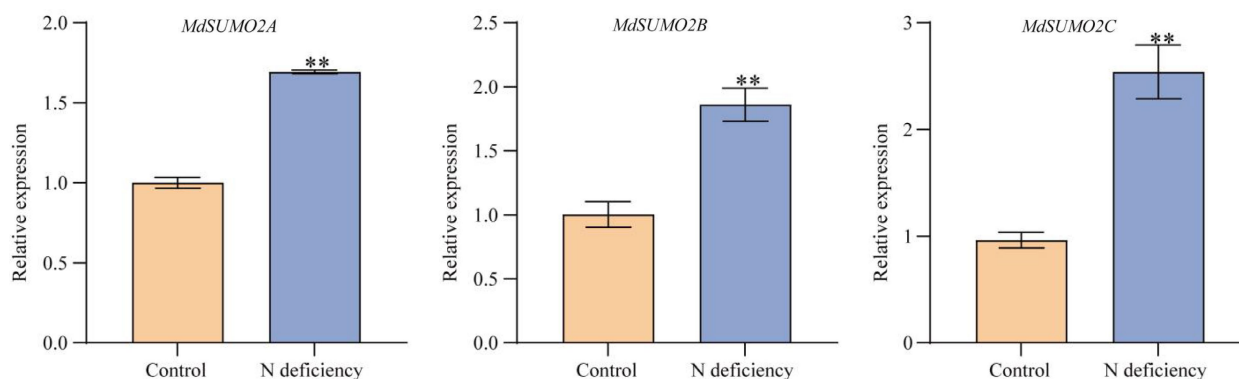


Fig. 1 Expression patterns of *MdSUMO2s* in apple under N deficient conditions. Hydroponically-cultured GL-3 were treated with normal (6 mM) or low nitrate (0.2 mM) for 4 d. Root samples were collected and used for gene expression analysis. Error bars indicate SD, n = 3. Asterisks indicate statistically significant differences. Student's t test was performed and statistically significant differences were indicated by * $p \leq 0.05$, ** $p \leq 0.01$.

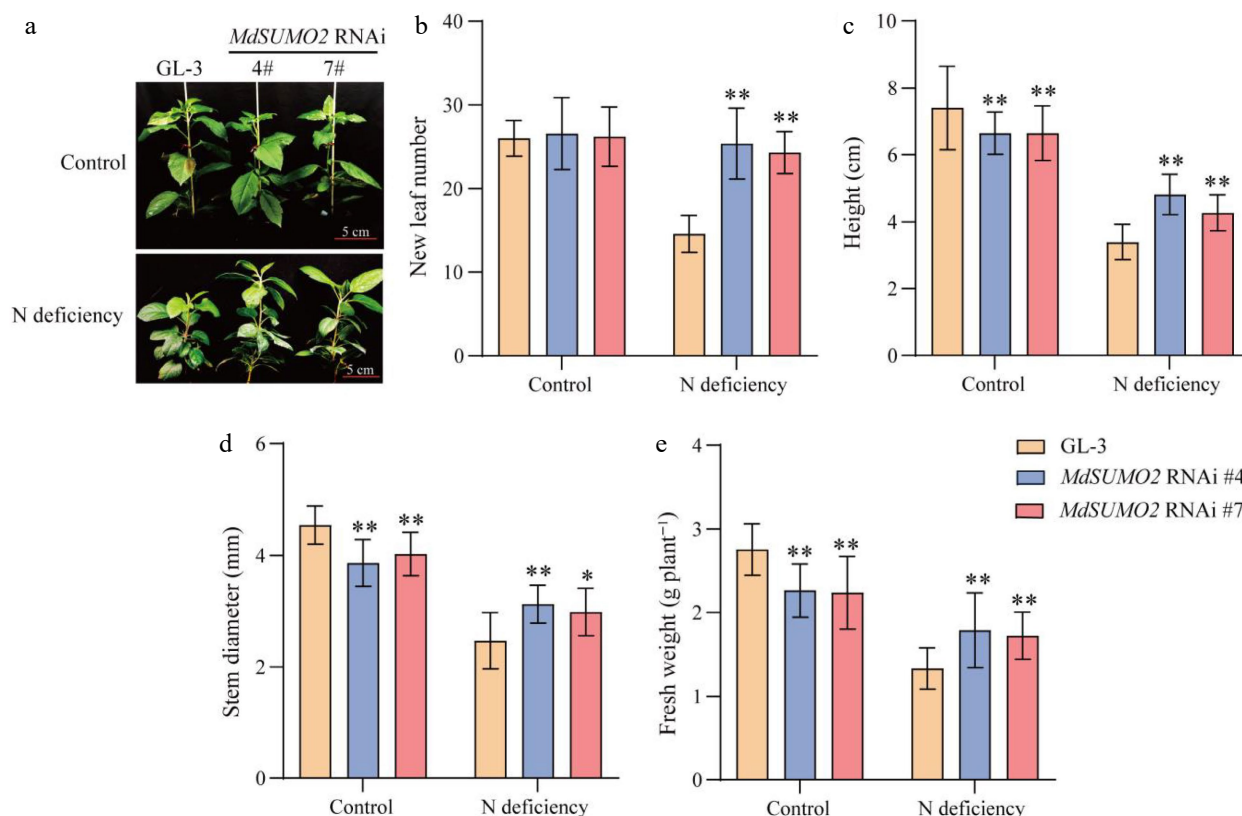


Fig. 2 The *MdSUMO2* RNAi transgenic apple plants are more tolerance than the wild type (GL-3) under N deficient conditions. (a) Morphology of three-month-old *MdSUMO2* RNAi transgenic apple plants and GL-3 treated with normal nitrogen (Control, 10 mM KNO₃) and low nitrogen (N deficient, 0.2 mM KNO₃) under hydroponic culture. Scale bars = 5 cm. (b) New leaf number, (c) height, (d) stem diameter, (e) fresh weight in (a). Error bars indicate SD, n = 12 in (b)–(e). Asterisks indicate significant differences between *MdSUMO2* RNAi transgenic apple plants and GL-3 plants in each group (control and N deficiency). Student's *t* test was performed and statistically significant differences were indicated by **p* ≤ 0.05, ***p* ≤ 0.01.

to N deficiency stress conditions. The results showed that the SPAD values of all the plants decreased significantly upon N deficient conditions, and the *MdSUMO2* RNAi plants kept higher SPAD than GL-3 after N deficiency stress treatment (Fig. 3a). We investigated the chlorophyll content of *MdSUMO2* RNAi plants and GL-3 in response to N deficiency stress. The results showed that the content of chlorophyll a, chlorophyll b, and total chlorophyll significantly decreased in leaves under N deficiency stress conditions (Fig. 3b, c & d). However, in the presence of N deficiency stress conditions, all the chlorophyll content in *MdSUMO2* RNAi transgenic apple plants were higher than in GL-3.

***MdSUMO2* regulates root system conformation in response to N deficient conditions**

The roots of apple trees serve as the primary organs for nutrient absorption, including nitrogen uptake^[16]. We observed the root systems of the *MdSUMO2* RNAi transgenic apple plants and GL-3. As can be seen from the root morphology results (Fig. 4a), N deficient conditions induced the root system development of both GL-3 and the *MdSUMO2* RNAi transgenic apple plants (Fig. 4b, c & d). And the *MdSUMO2* RNAi plants showed significantly greater average diameter and larger root volume than GL-3 in response to N deficient conditions (Fig. 4c & d). These results suggested that the improved root system of *MdSUMO2* RNAi transgenic apple plants may be responsible for their increased tolerance to nitrogen deficiency.

Activated glutamine synthetase and nitrate reductase contribute to the tolerance of *MdSUMO2* RNAi transgenic apple plants in response to N deficient conditions

According to previous studies, both glutamine synthetase (GS) and nitrate reductase (NR) are known as the principal enzymes involved in plant nitrogen conversion^[17]. To further explore whether *MdSUMO2* function under N deficiency stress conditions in apple, the GS and NR activity were determined in roots of *MdSUMO2* RNAi transgenic apple plants and GL-3 under control and N deficient conditions. Under control conditions, the *MdSUMO2* RNAi transgenic apple plants showed higher GS activity than GL-3 in the root system. The N deficiency treatment produced more significant results—the GS activity markedly increased more in *MdSUMO2* RNAi plants than GL-3 (Fig. 5a). With regards to the NR activity, *MdSUMO2* RNAi transgenic apple plants were higher than GL-3 under N deficient conditions. Nevertheless, the NR activity was not significantly affected by interfering *MdSUMO2* expression in the control (Fig. 5b).

The genes responsible for nitrogen assimilation play roles in the tolerance of *MdSUMO2* RNAi transgenic apple plants

Nitrate uptake and distribution is carried out by nitrate transporters (NRTs)^[18]. To determine the mechanism behind the increased tolerance of *MdSUMO2* RNAi transgenic apple plants

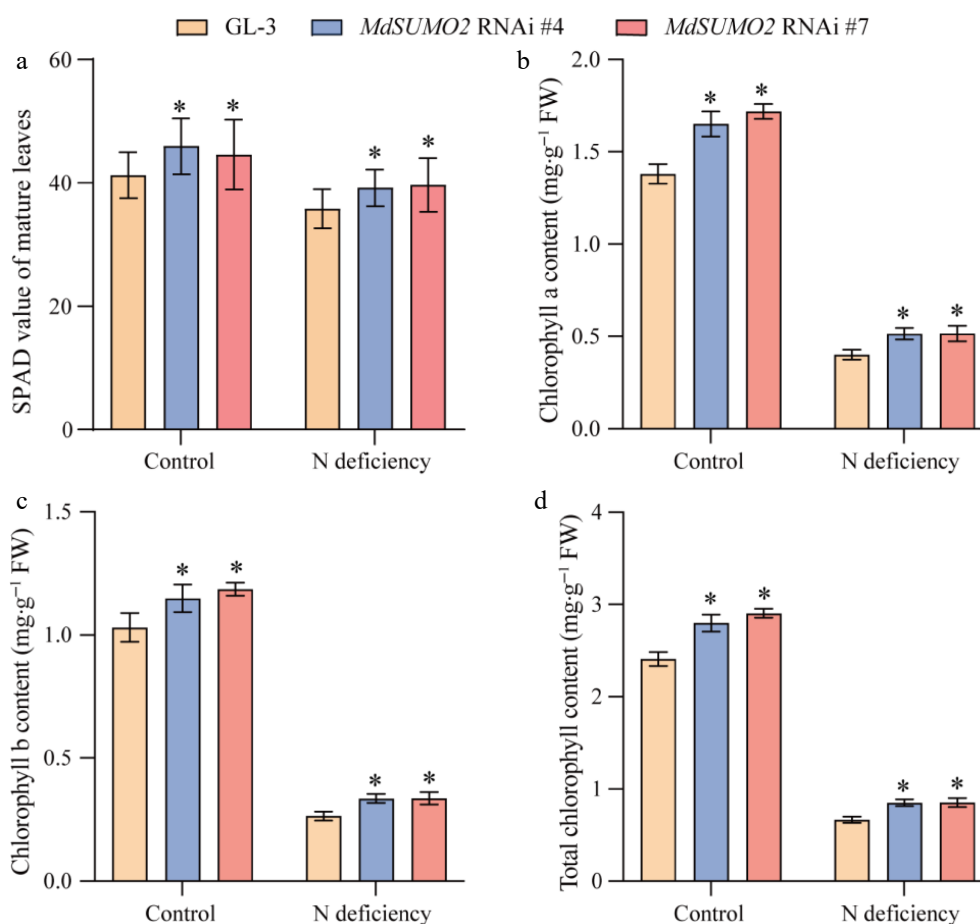


Fig. 3 The *MdSUMO2* RNAi transgenic apple plants displayed darker green leaves than the wild type (GL-3) under N deficient conditions. (a) The SPAD value of leaves in response to N deficiency in *MdSUMO2* RNAi transgenic apple plants and GL-3. (b) Chlorophyll a, (c) chlorophyll b, and (d) total chlorophyll content of leaves in *MdSUMO2* RNAi transgenic apple plants and GL-3 under N deficient conditions. Error bars indicate SD, n = 12 in (a)–(d). Asterisks indicate significant differences between *MdSUMO2* RNAi transgenic apple plants and GL-3 plants in each group (control and N deficiency). Student's *t* test was performed and statistically significant differences were indicated by * $p \leq 0.05$, ** $p \leq 0.01$.

under nitrogen deficient conditions, we further detected the expression levels of genes involved in nitrogen uptake. According to our results, *MdNRT1.1*, *MdNRT1.2* were inhibited in response to N deficiency stress, while *MdNRT2.5* is significantly induced and up-regulated. Additionally, the expression levels of *MdNRT1.1*, *MdNRT1.2* and *MdNRT2.5* were all higher in *MdSUMO2* RNAi transgenic plants than GL-3 under control and N deficient conditions (Fig. 6a–c). These results indicate that *MdSUMO2* RNAi transgenic plants have higher levels of *NRT* gene expression compared to GL-3.

Nitrate reductase genes (NRs) has been shown to play a pivotal role in nitrogen assimilation and stress tolerance in plants^[19]. Thus, the expression levels of the *MdNR* in *MdSUMO2* RNAi transgenic apple plants and GL-3 was examined using qRT-PCR experiment. The result showed that the expression of *MdNR* was significantly lower in *MdSUMO2* RNAi transgenic plants under control conditions compared to the GL-3 (Fig. 6d). N deficient conditions induced the expression of *MdNR* significantly, and much higher in *MdSUMO2* RNAi transgenic plants than GL-3. These results demonstrated that the induced expression of genes involved in nitrogen uptake and assimilation in *MdSUMO2* RNAi transgenic plants might contribute to its tolerance to N deficiency stress.

Discussion

The growth and development of plants require various nutrients^[20]. Among all necessary nutrients, nitrogen is the primary limiting factor for plant growth and yield formation on a global scale^[20]. The application of nitrogen fertilizer is crucial for improving agricultural yields and enhancing crop quality. However, the long-term excessive use of fertilizers has increasingly caused environmental consequences^[21]. How to improve the utilization efficiency of nitrogen fertilizer has always been a hot scientific issue in agricultural research. Understanding the molecular mechanisms related to plant perception and response to nitrogen availability can be utilized to improve nitrogen utilization efficiency. While the knowledge of the molecular mechanisms behind improving nitrogen utilization efficiency in model plants has greatly expanded, more extensive research is still needed in woody fruit trees. In this study, we identified that *MdSUMO2s* were up-regulated in response to N deficiency (Fig. 1), indicating that apple *MdSUMO2s* might function in the N deficiency stress response. Meanwhile, we found that *MdSUMO2* RNAi transgenic apple plants exhibited a more vigorous phenotype compared to GL-3 (Fig. 2a). Under low nitrogen conditions, plants adapt to the limitation by making physiological and morphological adjustments,

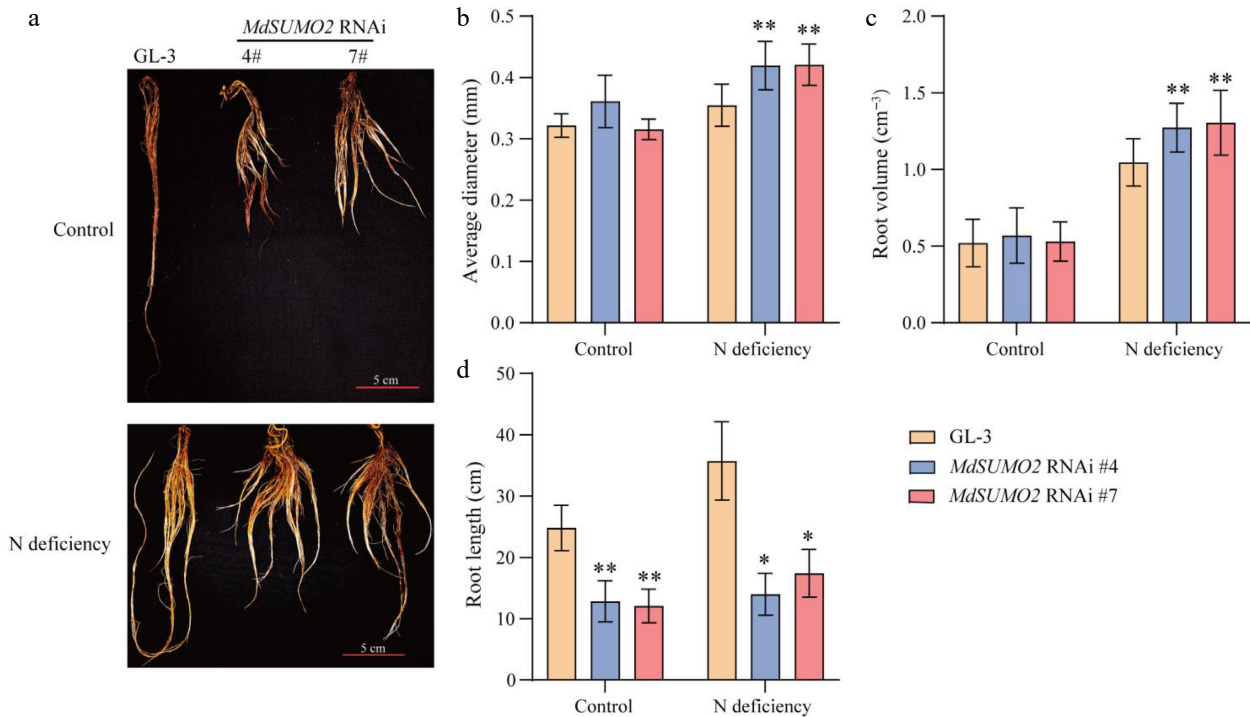


Fig. 4 *MdSUMO2* regulates root system in response to N deficient conditions. (a) Morphological characteristics of the root system in three-month-old *MdSUMO2* RNAi transgenic apple plants and GL-3 treated with normal nitrogen (Control, 10 mM KNO₃), and low nitrogen (N deficiency, 0.2 mM KNO₃) under hydroponic culture. Scale bars = 5 cm. (b) Average diameter, (c) root volume, and (d) root length in (a). Error bars indicate SD, n = 12 in (b)–(d). Asterisks indicate significant differences between *MdSUMO2* RNAi transgenic apple plants and GL-3 plants in each group (control and N deficiency). Student's *t* test was performed and statistically significant differences were indicated by **p* ≤ 0.05, ***p* ≤ 0.01.

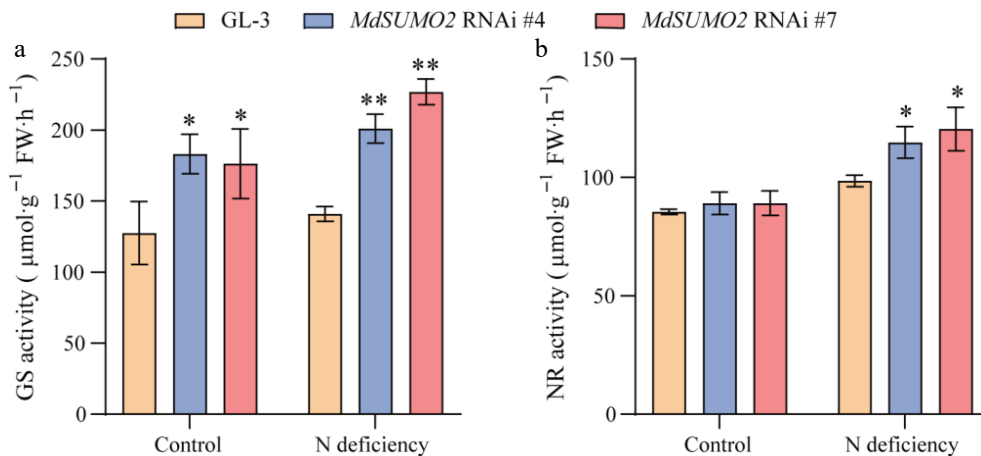


Fig. 5 Activated glutamine synthetase and nitrate reductase in root contribute to the tolerance of *MdSUMO2* RNAi transgenic apple plants in response to N deficiency. (a) Glutamine synthetase (GS) activity in *MdSUMO2* RNAi transgenic apple plants and GL-3 under control and N deficient conditions. (b) Nitrate reductase (NR) activity in *MdSUMO2* RNAi transgenic apple plants and GL-3 under control and N deficient conditions. Error bars indicate SD, n = 3 in (a) and (b). Asterisks indicate significant differences between *MdSUMO2* RNAi transgenic apple plants and GL-3 plants in each group (control and N deficiency). Student's *t* test was performed and statistically significant differences were indicated by **p* ≤ 0.05, ***p* ≤ 0.01.

including new leaf number, height, stem diameter and fresh weight^[22]. One such adjustment is increasing leaf production to enhance leaf area for improved nitrogen absorption and utilization efficiency^[23]. Due to their higher nitrogen use efficiency and effective utilization of limited nitrogen resources, plants under low nitrogen conditions exhibit a tendency to decelerate the growth of older leaves while allocating increased resources towards the growth of new leaves^[24]. By measuring

the number of new leaves, we can assess a plant's ability to adapt to low-nitrogen environments and its growth status. Additionally, measuring the number of new leaves provides information about plant growth rate and physiological condition^[25]. Changes in the number of new leaves under low-nitrogen treatment reflect both a plant's response level to nitrogen nutrition as well as the extent to which its growth is restricted. Three-month-old *MdSUMO2* RNAi transgenic apple plants

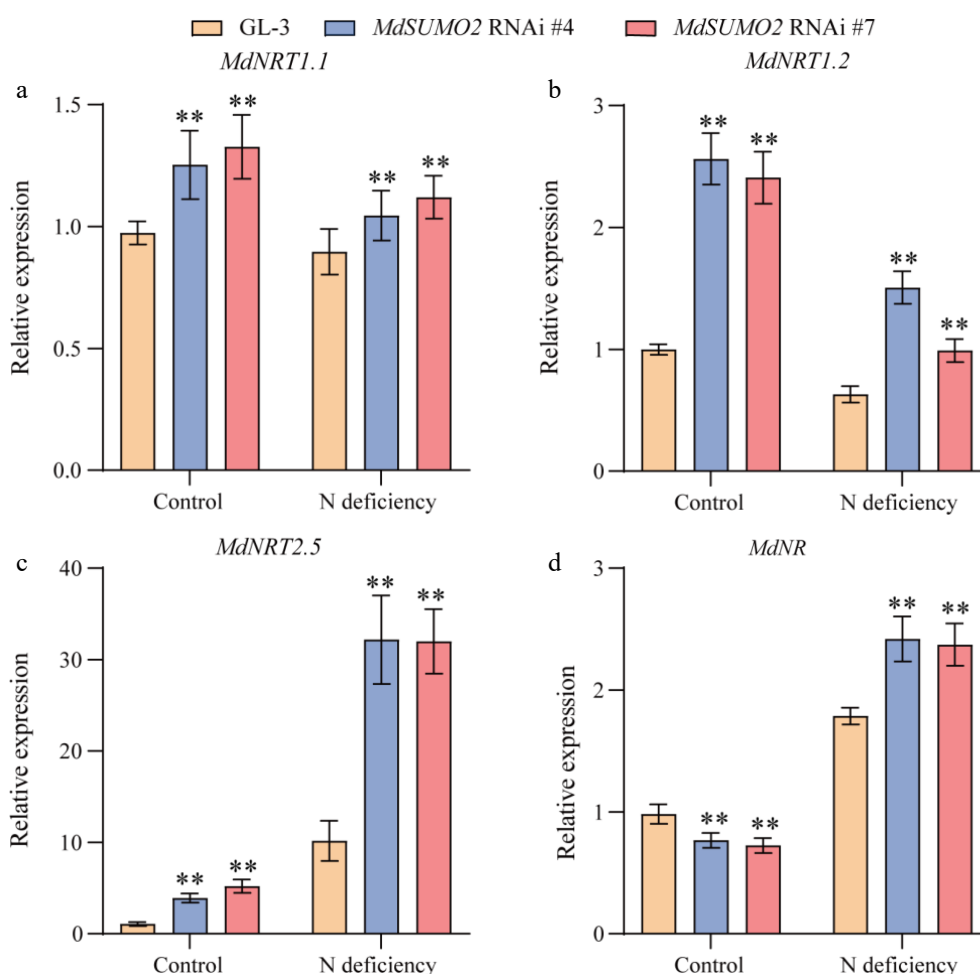


Fig. 6 Genes involved in nitrogen assimilation show a response in the *MdSUMO2* RNAi transgenic apple plants under N deficient conditions. Relative expression levels of (a) *MdNRT1.1*, (b) *MdNRT1.2*, (c) *MdNRT2.5* and (d) *MdNR* in the root under control and N deficient conditions. Error bars indicate SD, n = 3 in (a)–(d). Asterisks indicate significant differences between *MdSUMO2* RNAi transgenic apple plants and GL-3 plants in each group (control and N deficient). Student's *t* test was performed and statistically significant differences were indicated by * $p \leq 0.05$, ** $p \leq 0.01$.

produce more new leaves compared to GL-3 under N deficiency stress (Fig. 2b). Additionally, Under N deficient conditions, the *MdSUMO2* RNAi apple plants showed lower height, stem diameter and fresh weight compared to GL-3 plants (Fig. 2b–e). All these findings indicate that knock-down of the *MdSUMO2*s in apple enhances the N deficiency tolerance.

When crops are deficient in nitrogen, protein synthesis is inhibited, leading to a decrease in the amount of protein and enzymes in the crop^[26]. The structure of chloroplasts is also damaged due to nitrogen deficiency, and the synthesis of chlorophyll decreases, causing the leaves to turn yellow^[27]. These changes slow down the growth process of the plant, which in turn severely affects crop yield and quality. In this study, we investigated the color of apple leaves. The results showed that *MdSUMO2* RNAi transgenic apple plants exhibited higher chlorophyll content compared to GL-3 (Fig. 3b–d). Relevant studies have shown that SPAD can be used to accurately predict the chlorophyll content and nitrogen concentration of various crop leaves, and has been applied in the low-nitrogen tolerance evaluation of crops such as rice and corn^[15]. Our results showed higher SPAD value in *MdSUMO2* RNAi transgenic apple plants than GL-3 (Fig. 2a). So, we speculated the

darker green leaves of *MdSUMO2* RNAi transgenic apple plants may help assimilate more energy and make the plants more vigorous.

The morphology and physiological state of the root system are closely related to the growth status of the aboveground part, which is crucial for both the movement of nutrients within plants and the absorption of nutrients by plants^[28]. Plants can better absorb and store nitrogen when their root systems are properly arranged and distributed, and the availability of nitrogen also has an impact on how well plant roots grow^[29]. Therefore, we analyze the root system of *MdSUMO2* RNAi transgenic apple plants and GL-3. Although the GL-3 had significantly longer total root lengths compared to *MdSUMO2* RNAi transgenic apple plants, the average diameter and root volume were significantly higher in the *MdSUMO2* RNAi than in GL-3 plants under N deficiency stress (Fig. 4a–d). The root volume is crucial for nutrient absorption of plants, especially by influencing the root density and root length density to affect their nutrient uptake^[30]. So, the larger root volume of *MdSUMO2* RNAi might be an important factor in enabling them to perform better under N deficiency. Meanwhile, in plant roots, GS and NR play important roles in the uptake and assimilation of nitrogen from

MdSUMO2 RNAi exhibits tolerance to nitrogen deficiency

the soil^[20]. GS is mainly located in the root cortex, where it assimilates ammonium derived from nitrate reduction or directly absorbed by the roots^[26]. NR is mainly located in the root epidermis and cortex, where it reduces nitrate to nitrite^[31]. Our results showed higher GS activity and NR activity in *MdSUMO2* RNAi transgenic apple plants than GL-3 (Fig. 5a,b). In coincide, the expression of genes involved in nitrogen assimilation in the roots were higher in *MdSUMO2* RNAi transgenic apple plants than GL-3 (Fig. 6a–e). Therefore, we speculated that the root system of *MdSUMO2* RNAi transgenic apple plants had stronger nitrogen assimilation capabilities, which made their growth less affected under N deficient conditions.

In conclusion, the results of this study showed that interfering small ubiquitin modifiers (SUMO) promoted plant growth and biomass accumulation under N deficient conditions. Meanwhile, *MdSUMO2* RNAi transgenic apple plants produced darker green leaves and larger root volume than GL-3 in response to N deficiency stress. In addition, the GS activity, NR activity, and the expression of genes involved in nitrogen assimilation in the root were higher in *MdSUMO2* RNAi transgenic apple plants than GL-3. These findings provide theoretical guidance for revealing the mechanism by which MdSUMO2 regulates nitrate uptake and plant growth, as well as candidate genes for future apple breeding to improve nitrogen uptake efficiency.

Materials and methods

Plant material and growth conditions

The *MdSUMO2* RNAi transgenic apple plants were produced previously^[6]. The tissue-cultured apple seedlings were cultured in Murashige and Skoog (MS) medium (4.43 g/L MS salts, 30 g/L sucrose, 0.2 mg/L 6-Benzylaminopurine [6-BA], 0.2 mg/L 3-Indoleacetic acid [IAA] and 7.5 g/L agar, pH 5.8) under long-day conditions (14 h light [cool white, ~100 $\mu\text{mol}/\text{m}^2/\text{s}$, T5 LED batten]: 10 h dark) for 4 weeks at 25 °C^[32]. One month-old tissue-cultured apple seedlings were selected for rooting, and rooted on 1/2 MS medium containing 0.5 mg/L IAA and 0.5 mg/L indole butyric acid (IBA). When the tissue-cultured apple seedlings were rooted, the plants were transformed to a nutrient bowl containing substrate (Pindstrup, Denmark), and cultured in a growth chamber (16 h light: 8 h dark, 25 °C, ~55% relative humidity) for 30 d.

RNA extraction and qRT-PCR analysis

RNA extraction was performed as described previously^[33]. RNase-free DNase I (Thermo Scientific, USA) was used to remove genomic DNA. After extracting total RNA, it was detected through agarose gel electrophoresis and UV spectrophotometer (Thermo Nanodrop 2000) for concentration determination.

Then, 1 μg of RNA was reverse transcribed to synthesize cDNA by using reverse transcription kit HiScript II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme, R223-01, China). Quantitative reverse transcription PCR (qRT-PCR) analysis was carried out using a ChamQ Universal SYBR qPCR Master Mix (Vazyme, C601, China) on the CFX96 real-time system (Bio-Rad, USA). Primers used in this study are listed in Table 1.

N deficiency treatment

N deficiency treatment was performed as described previously^[4]. Hydroponically-cultured plants were cultured in 1/2 Hoagland nutrient solution (1.75 mM $\text{Ca}(\text{NO}_3)_2$, 2.5 mM KNO_3 , 0.5 mM KH_2PO_4 , 0.86 mM MgSO_4 , 100 μM EDTA-FeNa, 46.26 μM H_3PO_4 , 9.15 μM MnCl_2 , 0.77 μM ZnSO_4 , 0.32 μM CuSO_4 , 0.5 μM H_2MoO_4). Hydroponic culture was chosen for three-month-old GL-3 and *MdSUMO2* RNAi transgenic apple plants with identical growth conditions. After pre-culturing for about 10 d, the plants were in nitrogen starvation for 7 d and then divided into two groups with low and normal nitrogen solution (0.2 mM and 6 mM KNO_3) with the same base nutrient medium. The two groups continued to grow in the nutrient solution for 30 d and the solution was refreshed every 3 d. Hydroponically-cultured GL-3 (three-month-old) were treated with normal (6 mM) or low nitrate (0.2 mM) for 4 d. Root samples were used for gene expression analysis^[34].

Growth and root architecture measurements

Twelve plants were measured for each genotype. The physiological indicators of the above-ground part, including new leaf number, height, stem diameter, and fresh weight. To obtain an accurate assessment of the root systems, the roots were meticulously cleansed without causing any damage. After flattening and spreading the roots, their images were captured using a SNAPSCAN 310 scanner, which allowed for high-resolution imaging. Finally, WinRHIZO image analysis software (V4.1c; Regent Instruments, Quebec City, QC, Canada) was used to examine root system parameters.

Measurements of leaf SPAD

For measurements of leaf SPAD, we used a portable chlorophyll meter (SPAD-502; Minolta, Japan) according to the method of Markwell et al^[35]. The results obtained from the experiment were measured in SPAD values, which is a unit used to assess the chlorophyll content in plants. The SPAD values ranged from 0 to 100, with higher values indicating that the plants had more chlorophyll content.

Determination of chlorophyll content

Chlorophyll content was determined as described previously^[36]. To measure the concentration of chlorophyll, samples of leaves were taken from hydroponic experiment at

Table 1. Sequences of primers used in this study.

Gene name	Forward primer	Reverse primer	Purpose
<i>MdSUMO2C</i>	GCACAGAAGAAACCCCTGGAT	CACTTCATTACCATCCTGCCCT	qRT-PCR analysis
<i>MdSUMO2A</i>	GTGTTTGGGAAGTGGTGGTAGCTC	TCTTGTCTCCTCCTGGTTCG	qRT-PCR analysis
<i>MdSUMO2B</i>	CGACTCCGACGAAGAAGAG	CITCAGCTGAGTGCTTCGC	qRT-PCR analysis
<i>MdSUMO2-RNAi</i>	GGGGACAAGTTGTACAAAAAGCAGGCTTC TCAGGCGTCACGAACCAGG	GGGGACCACTTTGTACAAAGCTGGGTCCG TGGGTACTTCTTGATCCTGA	Plasmid construction of <i>MdSUMO2</i> RNAi
<i>MdNRT1.1</i>	ACGGGCACCACGCACTTG	GGTTGGAGGCTGGGGACTG	qRT-PCR analysis
<i>MdNRT1.2</i>	TCCTATCTTCCCGTACTCTTC	GGAGTTGGTCTGCTACACGCT	qRT-PCR analysis
<i>MdNRT2.5</i>	CGTCCACCTGTCTTGTTTTTC	CGATGTCGCTGGCGGTTAG	qRT-PCR analysis
<i>MdNR</i>	TGACGATAGCGTTGTCAGG	AGTGAGGCGGACCATTGAAG	qRT-PCR analysis

regular intervals throughout the study. The chlorophyll was extracted from the leaves using a solution of 80% ethanol, which was then analyzed using a multi-function enzyme marker meter (Victor Nivo, PerkinElmer, Waltham, MA, USA) at wavelengths of 664 and 647 nm.

Determination of nitrate reductase (NR) activity and glutamine synthetase (GS) activity

According to the method described earlier^[37], the activity of nitrate reductase was determined in roots. The definition of nitrate reductase activity is the decrease in the amount of NO₂⁻ in the reaction mixture^[38]. The activity of glutamine synthetase was analyzed by spectrophotometry^[39], whereas the activity of glutamate synthase was determined according to the method of Lin & Kao^[40].

Statistical analysis

For statistical analysis, Prism 9.4 software (GraphPad software, USA) was used with Student's *t*-test. Differences were considered significant if *p* < 0.05 or 0.01.

Acknowledgments

This work was supported by the National Key Research and Development Project (2022YFD1602107) and the Key S&T Special Projects of Shanxi Province, China (202201140601027-6). We thank Dr. Zhihong Zhang from Shenyang Agricultural University for providing tissue-cultured GL-3 plants.

Conflict of interest

The authors declare that they have no conflict of interest.

Dates

Received 25 June 2023; Accepted 9 August 2023; Published online 8 October 2023

References

- Leberecht M, Dannenmann M, Tejedor J, Simon J, Rennenberg H, et al. 2016. Segregation of nitrogen use between ammonium and nitrate of ectomycorrhizas and beech trees. *Plant, Cell & Environment* 39:2691–700
- Lawlor DW. 2002. Carbon and nitrogen assimilation in relation to yield: mechanisms are the key to understanding production systems. *Journal of Experimental Botany* 53:773–87
- Wen B, Gong X, Chen X, Tan Q, Li L, et al. 2022. Transcriptome analysis reveals candidate genes involved in nitrogen deficiency stress in apples. *Journal of Plant Physiology* 279:153822
- Zhang D, Yang K, Kan Z, Dang H, Feng S, et al. 2021. The regulatory module MdBT2-MdMYB88/MdMYB124-MdNRTs regulates nitrogen usage in apple. *Plant Physiology* 185:1924–42
- Liu X, Liu H, Li H, An X, Song L, et al. 2022. MdMYB10 affects nitrogen uptake and reallocation by regulating the nitrate transporter MdNRT2.4-1 in red-fleshed apple. *Horticulture Research* 9:uhac016
- Li X, Zhou S, Liu Z, Lu L, Dang H, et al. 2022. Fine-tuning of SUMOylation modulates drought tolerance of apple. *Plant Biotechnology Journal* 20:903–19
- Liu Z, Bian N, Guo J, Zhao S, Khan A, et al. 2023. Interfering small ubiquitin modifiers (SUMO) improves the thermotolerance of apple by facilitating the activity of MdDREB2A. *Stress Biology* 3:10
- Novatchkova M, Tomanov K, Hofmann K, Stuitable HP, Bachmair A. 2012. Update on sumoylation: defining core components of the plant SUMO conjugation system by phylogenetic comparison. *New Phytologist* 195:23–31

MdSUMO2 RNAi exhibits tolerance to nitrogen deficiency

- Liu Y, Zhu J, Sun S, Cui F, Han Y, et al. 2019. Defining the function of SUMO system in pod development and abiotic stresses in Peanut. *BMC Plant Biology* 19:593
- Ghimire S, Tang X, Zhang N, Liu W, Qi X, et al. 2020. Genomic analysis of the SUMO-conjugating enzyme and genes under abiotic stress in Potato (*Solanum tuberosum* L.). *International Journal of Genomics* 2020:9703638
- Lin X, Niu D, Hu Z, Kim DH, Jin Y, et al. 2016. An *Arabidopsis* SUMO E3 ligase, SIZ1, negatively regulates photomorphogenesis by promoting COP1 activity. *PLoS Genetics* 12:e1006016
- Wang H, Sun R, Cao Y, Pei W, Sun Y, et al. 2015. OsSIZ1, a SUMO E3 ligase gene, is involved in the regulation of the responses to phosphate and nitrogen in rice. *Plant and Cell Physiology* 56:2381–95
- Jiang H, Zhou L, Gao H, Wang X, Li Z, et al. 2022. The transcription factor MdMYB2 influences cold tolerance and anthocyanin accumulation by activating SUMO E3 ligase MdSIZ1 in apple. *Plant Physiology* 189:2044–60
- Kusaba M, Ito H, Morita R, Iida S, Sato Y, et al. 2007. Rice NON-YELLOW COLORING1 is involved in light-harvesting complex II and grana degradation during leaf senescence. *The Plant Cell* 19:1362–75
- Xiong D, Chen J, Yu T, Gao W, Ling X, et al. 2015. SPAD-based leaf nitrogen estimation is impacted by environmental factors and crop leaf characteristics. *Scientific Reports* 5:13389
- Smithwick EAH, Eissenstat DM, Lovett GM, Bowden RD, Rustad LE, et al. 2013. Root stress and nitrogen deposition: consequences and research priorities. *New Phytologist* 197:712–19
- Lam HM, Coschigano KT, Oliveira IC, Melo-Oliveira R, Coruzzi GM. 1996. The molecular-genetics of nitrogen assimilation into amino acids in higher plants. *Annual Review of Plant Physiology and Plant Molecular Biology* 47:569–93
- Orsel M, Krapp A, Daniel-Vedele F. 2002. Analysis of the NRT2 nitrate transporter family in *Arabidopsis*. Structure and gene expression. *Plant Physiology* 129:886–96
- Bardzik JM, Marsh HV Jr, Havis JR. 1971. Effects of water stress on the activities of three enzymes in maize seedlings. *Plant Physiology* 47:828–31
- Thomas W. 1927. Nitrogenous metabolism of *Pyrus Malus* L.: III the partition of nitrogen in the leaves, one and two year branch growth and non-bearing spurs throughout a year's cycle. *Plant Physiology* 2:109–37
- Erisman JW, Bleeker A, Galloway J, Sutton MS. 2007. Reduced nitrogen in ecology and the environment. *Environmental Pollution* 150:140–49
- Mu X, Chen Y. 2021. The physiological response of photosynthesis to nitrogen deficiency. *Plant Physiology And Biochemistry* 158:76–82
- Meng X, Wang X, Zhang Z, Xiong S, Wei Y, et al. 2021. Transcriptomic, proteomic, and physiological studies reveal key players in wheat nitrogen use efficiency under both high and low nitrogen supply. *Journal of Experimental Botany* 72:4435–56
- Chen KE, Chen HY, Tseng CS, Tsay YF. 2020. Improving nitrogen use efficiency by manipulating nitrate remobilization in plants. *Nature Plants* 6:1126–35
- Hirel B, Le Gouis J, Ney B, Gallais A. 2007. The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches. *Journal of Experimental Botany* 58:2369–87
- Xu Z, Zhou G. 2006. Nitrogen metabolism and photosynthesis in *Leymus chinensis* in response to long-term soil drought. *Journal of Plant Growth Regulation* 25:252–66
- Bassi D, Menossi M, Mattiello L. 2018. Nitrogen supply influences photosynthesis establishment along the sugarcane leaf. *Scientific Reports* 8:2327
- Kiba T, Krapp A. 2016. Plant nitrogen acquisition under low availability: regulation of uptake and root architecture. *Plant and Cell Physiology* 57:707–14

MdSUMO2 RNAi exhibits tolerance to nitrogen deficiency

29. Remans T, Nacry P, Pervent M, Girin T, Tillard P, et al. 2006. A central role for the nitrate transporter NRT2.1 in the integrated morphological and physiological responses of the root system to nitrogen limitation in *Arabidopsis*. *Plant Physiology* 140:909–21
30. Pan S, Liu H, Mo Z, Patterson B, Duan M, et al. 2016. Effects of nitrogen and shading on root morphologies, nutrient accumulation, and photosynthetic parameters in different rice genotypes. *Scientific Reports* 6:32148
31. Ruffy TW Jr, Thomas JF, Remmler JL, Campbell WH, Volk RJ. 1986. Intercellular localization of nitrate reductase in roots. *Plant Physiology* 82:675–80
32. Niu C, Jiang L, Cao F, Liu C, Guo J, et al. 2022. Methylation of a MITE insertion in the MdRFNR1-1 promoter is positively associated with its allelic expression in apple in response to drought stress. *The Plant Cell* 34:3983–4006
33. Xie Y, Chen P, Yan Y, Bao C, Li X, et al. 2018. An atypical R2R3 MYB transcription factor increases cold hardiness by CBF-dependent and CBF-independent pathways in apple. *New Phytologist* 218:201–18
34. Jia X, Wang Q, Ye Y, Li T, Sun X, et al. 2022. MdATG5a positively regulates nitrogen uptake under low nitrogen conditions by enhancing the accumulation of flavonoids and auxin in apple roots. *Environmental and Experimental Botany* 197:104840
35. Markwell J, Osterman JC, Mitchell JL. 1995. Calibration of the Minolta SPAD-502 leaf chlorophyll meter. *Photosynthesis Research* 46:467–72
36. Jiang L, Zhang D, Liu C, Shen W, He J, et al. 2022. MdGH3.6 is targeted by MdMYB94 and plays a negative role in apple water-deficit stress tolerance. *The Plant Journal* 109:1271–89
37. Högberg P, Granström A, Johansson T, Lundmark-Thelin A, Näsholm T. 1986. Plant nitrate reductase activity as an indicator of availability of nitrate in forest soils. *Canadian Journal of Forest Research* 16:1165–69
38. Seith B, Setzer B, Flaig H, Mohr H. 1994. Appearance of nitrate reductase, nitrite reductase and glutamine synthetase in different organs of the Scots pine (*Pinus sylvestris*) seedling as affected by light, nitrate and ammonium. *Physiologia Plantarum* 91:419–26
39. Wang L, Zhou Q, Ding L, Sun Y. 2008. Effect of cadmium toxicity on nitrogen metabolism in leaves of *Solanum nigrum* L. as a newly found cadmium hyperaccumulator. *Journal of Hazardous Materials* 154:818–25
40. Lin C, Kao C. 1996. Disturbed ammonium assimilation is associated with growth inhibition of roots in rice seedlings caused by NaCl. *Plant Growth Regulation* 18:233–38



Copyright: © 2023 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/>.