Auxin response factor MdARF18 regulates MdNRT1.1 to affect nitrogen utilization in apple

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Abstract

Nitrate is the main source of nitrogen for plant growth and development under aerobic conditions, which serves as an important nutrient and signaling molecule, understanding the nitrate signaling pathway is important for agricultural production. Auxin response factors (ARFs) are associated with nitrate signaling, but their underlying mechanism is less known in apples. In this study, we found that MdARF18 can be expressed as a transcription factor activated by nitrate, and inhibited the uptake of nitrate in apples. Then, MdARF18 was found to bind directly to the TGTCCCT site of the MdNRT1.1 promoter and significantly reduced its expression. In conclusion, MdARF18 regulates nitrate uptake in plants by mediating the expression of MdNRT1.1, providing insight into the mechanism by which MdARF18 regulates nitrate uptake and utilization in apples.


Introduction

Crops require a variety of nutrients for growth, and nitrogen is particularly important. Nitrogen is the primary factor limiting plant growth and yield formation, and it also plays a significant role in improving product quality[1−4]. Nitrogen accounts for 1-3% of the dry weight of plants and is a component of many compounds. For example, it is an important part of proteins, a component of nucleic acids, the skeleton of cell membranes, and a constituent of chlorophyll[5,6]. When the plant is deficient in nitrogen, the synthesis process of nitrogen-containing substances such as proteins decreases significantly, cell division and elongation are restricted, and chlorophyll content decreases, and this leads to short and thin plants, small leaves, and pale color[7,8]. If nitrogen in the plant is in excess, a large number of carbohydrates will be used for the synthesis of proteins, chlorophyll and other substances, so that cells are large and thin-walled, and easy to be attacked by pests and diseases. At the same time, the mechanical tissues in stem are not well developed and are prone to collapse[3,8,9]. Therefore, the development of new crop varieties with both high yields and improved nitrogen use efficiency (NUE) is an urgently needed goal for a more sustainable agriculture with minimal nitrogen demand.

Plants obtain inorganic nitrogen from the soil, mainly in the form of NH₄⁺ and nitrate (NO₃⁻)[10−13]. Nitrate uptake by plants occurs primarily in aerobic environments[14]. Transmembrane proteins are required for nitrate uptake from the external environment as well as for transport and translocation between cells, tissues, and organs. NITRATE TRANSPORTER PROTEIN 1 (NRT1) / PEPTIDE TRANSPORTER (PTR) family (NPF), NRT2, CHLORIDE CHANNEL (CLC) family, and SLOW ACTIVATING ANION CHANNEL are four protein families involved in nitrate transport[14]. One of the most studied of these is NRT1.1, which has multiple functions[14,15]. NRT1.1 has been shown to be a major nitrate sensor, regulating many aspects of nitrate physiology and developmental responses, including regulating the expression levels of nitrate-related genes, modulating root architecture, and alleviating seed dormancy[15−18].

There is mounting evidence that plant growth and development are influenced by interactions across numerous phytohormone signaling pathways, including abscisic acid, gibberellins, growth hormones, and cytokinins[19,20]. To increase the effectiveness of plant nitrogen fertilizer application, it may be possible to tweak the signaling mediators or vary the content of certain phytohormones. Since the 1930s, research on the interplay between growth factors and N metabolism has also been conducted[21]. The Indole acetic acid (IAA) level of plant shoots is shown to decrease in early studies due to N shortage, although roots exhibit the reverse tendency[22,23]. In particular, low NO₃⁻ levels caused IAA buildup in the roots of Arabidopsis, Glycine max, Triticum aestivum, and Zea mays, indicating that IAA is crucial for conveying the effectiveness of exogenous nitrogen to the root growth response[20,22,23].
Plant materials and cultivation methods

Studies have shown that two families are required to control the expression of auxin-responsive genes: one is the Auxin Response Factor (ARF) and the other is the Aux/IAA repressor family\[24-26\]. As the transcription factor, ARF protein regulates the expression of auxin response genes by specifically binding to the TGTCCN auxin response element (AuxRE) in promoters of primary or early auxin response genes\[27\]. Among them, rice OsARF18, as a class of transcriptional repressor, has been involved in the field of nitrogen utilization and yield\[23,28\]. In rice (Oryza sativa), mutations in *rice salt tolerant 1* (rst1), encoding the OsARF18 gene, lead to the loss of its transcriptional repressor activity and up-regulation of OsAS1 expression, which accelerates the assimilation of NH₄⁺ to Asn and thus increases N utilization\[28\]. In addition, dao mutant plants deterred the conversion of IAA to OxlAA, thus high levels of IAA strongly activates OsARF18, which subsequently represses expression of OSARF2 and OsSUT1 by directly binding to the AuxRE and SuRE promoter motifs, resulting in the inhibition of carbohydrate partitioning\[23\]. As a result, rice carrying the dao has low yields.

Apples (*Malus domestica*) are used as a commercially important crop because of their high ecological adaptability, high nutritional value, and annual availability of fruit\[29\]. To ensure high apple yields, growers promote rapid early fruit yield growth by applying nitrogen. However, the over-application of nitrogen fertilizer to apples during cultivation also produces common diseases, and the over-application of nitrogen fertilizer is not only a waste of resources but also harmful to the environment\[29\]. Therefore, it is of great significance to explore efficient nitrogen-regulated genes to understand the uptake and regulation of nitrogen fertilizer in apples, and to provide reasonable guidance for nitrogen application during apple production\[30\]. In this study, we identify MdARF18, a key transcription factor involves in nitrate uptake and transport in apples, and *MdARF18* reduces NO₃⁻ uptake and assimilation. Further analysis suggests that *MdARF18* may inhibit the transcriptional level of *MdNRT1.1* promoter by directly binding to its TGTCTT target, thus affecting normal plant growth.

**Methods**

**Bioinformatics Analysis of the ARF18 Gene**

The protein sequence of apple MdARF18 (MD07G1152100) was obtained from The Apple Genome (https://iris.angers.inra.fr/gddh13/). Mutant of *arf18* (GABI_699809) sequence numbers were obtained from the official TAIR website (https://www.arabidopsis.org/). The protein sequences of ARF18 from different species were obtained from the protein sequence of apple MdARF18 on the NCBI website. Using these data, a phylogenetic tree with reasonably close associations were constructed\[31\].

Protein structural domain prediction of ARF18 was performed on the SMART website (https://smart.embl.de/). Motif analysis of ARF18 was performed by MEME (https://meme-suite.org/meme/tools/meme). Clustal was used to do multiple sequence comparisons. The first step was accessing the EBI web server through the Clustal Omega channel. The visualization of the results was altered using Jalview, which may be downloaded from https://www.jalview.org/download/\[32\].

**Extracting Plant Genomic DNA and RNA**

Plant DNA and RNA were obtained using the Genomic DNA Kit and the Omni Plant RNA Kit (tDNase I) (Tiangen, Beijing, China)\[37\].
We synthesized cDNA for qPCR by using the PrimeScript First Strand cDNA Synthesis Kit (Takara, Dalian, China). The cDNA for qPCR was synthesized by using the PrimeScript First Strand cDNA Synthesis Kit (Takara, Dalian, China). Quantitative real-time fluorescence analysis was performed by using the Ultra-SYBR Mixture (Low Rox) kit (ComWin Biotech Co. Ltd., Beijing, China). qRT-PCR experiments were performed using the 2^−ΔΔCT method for data analysis. The data were analyzed by the 2^−ΔΔCT method.

GUS Staining

GUS staining buffer contained 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, 0.01 mM EDTA, 0.5 mM hydrogen peroxide, 100 mM sodium phosphate (pH 7.0), and 0.1% (v/v) Triton X-100 and was maintained at 37 °C in the dark. The pMdNRT1.1::GUS construct was transiently introduced into apple calli. In order to confirm whether MdNRT1.1 is activated or inhibited by MdARF18, we co-transformed 35S::MdARF18 into pMDNRT1.1::GUS. The activity of transgenic calli was assessed using GUS labeling and activity assays.

Determination of Nitrate content, Nitrate Reductase activity

The specimens were crushed into fine particles, combined with 1 mL of ddH2O, and thereafter subjected to a temperature of 100 °C for a duration of 30 minutes. The supernatant was collected in a flow cell after centrifugation at 12,000 revolutions per minute for 10 minutes. The AutoAnalyzer 3 continuous flow analyzer was utilized to measure nitrate concentrations. The gas chromatographic (SEAL analytical, Mequon, WI, USA). Nitrate reductase (NR) activity was characterized by the corresponding kits (Solarbio Life Science, Beijing, China) using a spectrophotometric method.

Yeast one-hybrid (Y1H) assay

Y1H assays were performed as described by (Liu, et al. 2021). The coding sequence of MdARF18 was integrated into the pGAD7 expression vector, whereas the reporter region of MdNRT1.1 was included into the pHSIS reporter vector. Subsequently, the constitutive vectors were co-transformed into the yeast monohybrid strain Y187. The individual transformants were assessed on a medium lacking tryptophan, leucine, and histidine (SDT/-L/-H). Subsequently, the positive yeast cells were assessed on a medium lacking tryptophan, leucine, and histidine (SDT/-L/-H). Subsequently, the positive yeast cells were identified using polymerase chain reaction (PCR). The yeast strain cells were diluted at dilution factors of 10, 100, 1000, and 10,000. 10 μL of various doses were added to selective medium (SD-T/-L/-H) containing 120 mM 3-aminotriazole (3-AT) and incubated at 28 °C for 2−3 days.

Dual luciferase assays

Dual luciferase assays were performed as described previously. Full-length MdARF18 was cloned into pGreenII 62-SK to produce MdARF18-62-SK. The promoter fragment of MdNRT1.1 was cloned into pGentree 6800-LUC to produce pMdNRT1.1-LUC. Different combinations were transformed into Agrobacterium tumefaciens LBA4404 and the Agrobacterium solution was injected onto the underside of the leaves of tobacco (Nicotiana benthamiana) leaves abaxially. The Dual Luciferase Reporter Kit (Promega, https://www.promega.com) was used to detect fluorescence activity.

Protein degradation assays in vitro

Total protein was extracted from wild-type and transgenic apple calli with or without 100 μM MG132 treatment. The purified MdARF18-HIS fusion protein was incubated with total protein. Samples were collected at the indicated times (0, 1, 3, 5, and 7 h).

Protein gel blots were analyzed using GST antibody. ACTIN antibody was used as an internal reference. All antibodies used in this study were provided by Abmart (http://www.abmart.com)

Data Analysis

Unless otherwise noted, every experiment was carried out independently in triplicate. In this paper, values were provided as means standard deviation (SD). A one-way analysis of variance (ANOVA) was used to establish the statistical significance of all data, and Duncan’s test was used to compare results at the p < 0.05 level.

Results

Auxin regulates nitrogen uptake and utilization in apple

In order to investigate whether auxin affects the effective uptake of nitrate in apple, we first externally applied IAA under normal N (5 mM NO3−) environment, and this result showed that the growth of Gala apple seedlings in the IAA-treated group were better than control, and their fresh weights were heavier than control group (Fig. 1a, 1d). The N-related physiological indexes of apple seedlings also showed that the nitrate content and NR activity of the root part of the IAA-treated group were significantly higher than control group, while the nitrate content and NR activity of the shoot part were lower than control group (Fig. 1b−1c). These results demonstrate that auxin could promote the uptake of nitrate and thus promotes growth of plants.

To test whether auxin affects the expression of genes related to nitrogen uptake and metabolism. For root, the expression levels of MdNRT1.1, MdNRT2.1, MdNIA1, MdNIA2, and MdNIR were higher than control group (Supplemental Fig. S1a, S1f, S1h−j), while the expression levels of MdNRT1.2, MdNRT1.6 and MdNRT2.5 were lower than control group significantly (Supplemental Fig. S1b, S1d, S1g). For shoot, the expression of MdNRT1.1, MdNRT1.5, MdNRT1.6, MdNRT1.7, MdNRT2.1, MdNRT2.5, MdNIA1, MdNIA2 and MdNIR genes were significantly down-regulated (Supplemental Fig. S1a, S1c−s1i). This result infers that the application of auxin could mediate nitrate uptake in plants by affecting the expression levels of relevant nitrate uptake and assimilation genes.

Transcript levels of MdARFs are induced by nitrate

Since the auxin signaling pathway requires the regulation of the auxin response factors (ARFs), we investigate whether members of ARFs genes were nitrate responsive. Firstly, qPCR quantitative analysis showed that the five subfamily genes of MdARFs (MdARF9, MdARF2, MdARF12, MdARF3, and MdARF18) were expressed at different levels in various organs of the plant (Supplemental Fig. S2). Afterward, the expression levels of five ARF genes were analyzed under different concentrations of nitrate treatment (Fig. 2), and it was concluded that these genes represented by each subfamily responded in different degrees, but the expression level of MdARF18 was up-regulated regardless of low or high nitrogen (Fig. 2i−2j), and the expression level of MdARF18 showed a trend of stable up-regulation under IAA treatment (Supplemental Fig. S3). The result

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demonstrates that MdARFs could affect the uptake of external nitrate by plants, and MdARF18 may play an important role in the regulation of nitrate uptake by plants.

**Phylogenetic relationships and multiple sequence alignment of MdARF18**

MdARF18 (MD07G1152100) was predicted through The Apple Genome website (https://iris.angers.inra.fr/gddh13/) and it had high fitness with AtARF18 (AT3G61830). The homologs of ARF18 from 15 species were then identified in NCBI (https://www.ncbi.nlm.nih.gov/) and then constructed an evolutionary tree (Supplemental Fig. S4). The data indicates that MdARF18 was most closely genetically related to MbARF18 (Malus baccata), indicating that they diverged recently in evolution (Supplemental Fig. S4). Conserved structural domain analyses indicated that all 15 ARF18 proteins had highly similar conserved structural domains (Supplemental Fig. S5). In addition, multiple sequence alignment analysis showed that all 15 ARF18 genes have B3-type DNA-binding domains (Supplemental Fig. S6), which is in accordance with the previous reports on ARF18 protein structure[26].

**Overexpression of MdARF18 affects root development**

In order to explore whether MdARF18 could affect the development of the plant’s root system. Firstly, MdARF18 was heterologously expressed into Arabidopsis, and an arf18 mutant (GABI_699B09) Arabidopsis was also obtained (Supplemental Fig. S8). Seven-day-old MdARF18 transgenic Arabidopsis and arf18 mutants were treated in a medium with different nitrate concentrations for 10 days (Fig. 3a−3b). After observing results, it was found that under the environment of high nitrate concentration, the primary root of MdARF18 was shorter than arf18 and wild type (Fig. 3c), and the primary root length of arf18 is the longest (Fig. 3c), while there was no significant difference in the lateral root (Fig. 3d). For low nitrate concentration, there was no significant difference in the length of the primary root, and the number of lateral roots of MdARF18 was slightly more than wild type and arf18 mutant. These results suggest that MdARF18 affects root development in plants. However, in general, low nitrate concentrations could promote the transport of IAA by NRT1.1 and thus inhibit lateral root production[3], so it might be hypothesized that MdARF18 would have some effects on MdNRT1.1 thus leading to disruption of lateral root development.

**Overexpression of MdARF18 inhibits nitrate uptake in plants**

To investigate whether MdARF18 affects the growth of individual plants under different concentrations of nitrate, 7-day-old overexpression MdARF18, and arf18 mutants were planted in the soil and incubated for 20 days. It was found that arf18 had the best growth of shoot, while MdARF18 had the weakest shoot growth at any nitrate concentration (Fig. 4a). MdARF18 had the lightest fresh weight and the arf18 mutant had the heaviest fresh weight (Fig. 4b). N-related physiological indexes revealed that the nitrate content and NR activity of arf18 were significantly higher than wild type, whereas MdARF18 materials were lower than wild type (Fig. 4c−4d). More detail, MdARF18 had the lightest fresh weight under low and normal nitrate, while the arf18 mutant had the heaviest fresh weight, and the fresh weight of arf18 under high nitrate concentration did not differ much from the wild type (Fig. 4b). Nitrogen-related physiological indexes showed that the nitrate content of arf18 was significantly higher than wild type, while MdARF18 was lower than wild type. The NR activity of arf18 under high nitrate did not differ much from the wild type, but the NR activity of MdARF18 was the lowest in any treatment (Fig. 4c−4d). These
results indicate that \textit{MdARF18} significantly inhibits plant growth by inhibiting plants to absorb nitrate, and is particularly pronounced at high nitrate concentrations.

In addition, to further validate this conclusion, we obtained \textit{MdARF18} overexpression calli and treated them with different concentrations of nitrate. The result shows that the growth of overexpressed \textit{MdARF18} was weaker than wild type in both treatments (Supplemental Fig. S11a). The fresh weight of \textit{MdARF18} was significantly lighter than wild type (Supplemental Fig. S11b), and its nitrate and NR activity were lower than

\textbf{Fig. 2} Relative expression analysis of \textit{MdARF}s subfamilies in response to different concentrations of nitrate. Expression analysis of representative genes from five subfamilies of \textit{MdARF} transcription factors. Bars represent the mean ± SD (n = 3). Different letters above the bars indicate significant differences using the LSD test (p < 0.05).
wild type (Supplemental Fig. S11c−S11d), which was consistent with the above results (Fig. 4). This result further confirms that MdARF18 could inhibited the development of individual plants by inhibiting the uptake of nitrate.

**MdARF18 targets genes related to nitrogen uptake and utilization**

Nitrate acts as a signaling molecule that takes up nitrate by activating the NRT family as well as NIA1 and NIR[3,34]. To further investigate the pathway by which MdARF18 inhibits plant growth and reduces nitrate content, qRT-PCR was performed on the above plant materials treated with different concentrations of nitrate (Fig. 5). The result shows that the expression levels of AtNRT1.1, AtNIA1, AtNIA2 and AtNIR were all down-regulated in overexpression of MdARF18, and up-regulated in the arf18 mutant (Fig. 5a, 5h−5j). There was no significant change in AtNRT1.2 at normal nitrate levels, but AtNRT1.2 expression levels were down-regulated in MdARF18 and up-regulated in arf18 at both high and low nitrate levels (Fig. 5b). This trend in the expression levels of these genes might consist with the fact that MdARF18 inhibits the expression of nitrogen-related genes and restricts plant growth. The trend in the expression levels of these genes is consistent with MdARF18 restricting plant growth by inhibiting the expression of nitrogen-related genes. However, AtNRT1.5, AtNRT1.6, AtNRT1.7, AtNRT2.1, and AtNRT2.5 did not show suppressed expression levels in MdARF18 (Fig. 5c−5g). These results suggest that MdARF18 inhibits nitrate uptake and plant growth by repressing some of the genes for nitrate uptake or assimilation.

In addition, to text whether different concentrations of nitrate affect the protein stability of MdARF18. However, it was found that there was no significant difference in the protein stability of MdARF18 at different concentrations of nitrate (Supplemental Fig. S12). This result suggests that nitrate do not affect the degradation of MdARF18 protein.

**MdARF18 binds to the promoter of MdNRT1.1 to restrain its expression**

To further verify whether MdARF18 can directly bind N-related genes, firstly we found that the MdNRT1.1 promoter contains binding sites to ARF factors (Fig. 6a). The yeast one-hybrid research demonstrated an interaction between MdARF18 and the MdNRT1.1 promoter, as shown in Figure 6b. Yeast cells that were simultaneously transformed with MdNRT1.1-pHis and pGADT7 were unable to grow in selected SD medium. However, cells that were transformed with MdNRT1.1-pHis and MdARF18-pGADT7 grew successfully in the selective medium. The result therefore hypothesizes that MdARF18 could bind specifically to MdNRT1.1 to regulate nitrate uptake in plants.
To identify the inhibition or activation of MdNRT1.1 by MdARF18, we analyzed their connections by Dual luciferase assays (Fig. 6c), and also analyzed the fluorescence intensity (Supplemental Fig. S9). It was concluded that the fluorescence signals of cells carrying 35Spro and MdNRT1.1pro::LUC were stronger, but the mixture of 35Spro::MdARF18 and MdNRT1.1pro::LUC injected with fluorescence signal intensity was significantly weakened. Next, we transiently transformed the 35S::MdARF18 into pMdNRT1.1::GUS transgenic calli (Fig. 7). GUS results firstly showed that the color depth of pMdNRT1.1::GUS and 35S::MdARF18 were significantly lighter than pMdNRT1.1::GUS alone (Fig. 7a). GUS enzyme activity, as well as GUS expression, also indicated that the calli containing pMdNRT1.1::GUS alone had a stronger GUS activity (Fig. 7b−7d). In addition, the GUS activity of calli containing both pMdNRT1.1::GUS and 35S::MdARF18 were further attenuated under both high and low nitrate concentrations (Fig. 7a). These results suggest that MdARF18 represses MdNRT1.1 expression by directly binding to the MdNRT1.1 promoter region.

Discussion

Plants replenish their nutrients by absorbing nitrates from the soil[41,42]. Previous studies have shown that some of the plant hormones such as IAA, GA, and ABA interact with nitrate[35,43−45]. The effect of nitrate on the content and transport of IAA has been reported in previous studies, e.g., nitrate supply reduced IAA content in Arabidopsis, wheat, and maize roots and inhibited the transport of IAA from shoot to root[20,21]. In this study, it was found that auxin treatment promoted individual fresh weight gain and growth (Fig. 1a−1b). Nitrate content and NR activity were also significantly higher in their root parts (Fig. 1c−1d) and also affected the transcript expression levels of related nitrate uptake and assimilation genes (Supplemental Fig. S1). Possibly because IAA can affect plant growth by influencing the uptake of external nitrates by the plant.

ARFs are key transcription factors to regulate auxin signaling[46−50]. We identified five representative genes of the apple MdARFs subfamily and they all had different expression patterns (Supplemental Fig. S2). The transcript levels of each gene were found to be affected to different degrees under different concentrations of nitrate, but the expression level of MdARF18 was up-regulated under both low and high nitrate conditions (Fig. 2). The transcript level of MdARF18 was also activated under IAA treatment (Supplemental Fig. S3), so MdARF18 began to be used in the study of the mechanism of nitrate uptake in plants. In this study, we successfully cloned an Arabidopsis AtARF18 homolog and named it MdARF18 (Supplemental Fig. S4, S5). It contains a B3-type DNA-binding structural domain consistent with previous studies of ARFs (Supplemental Fig. S6), and arf18 mutants were also obtained and their transcript levels were examined (Supplemental Fig. S8).
Fig. 5 qPCR-RT analysis of N-related genes. Expression analysis of N-related genes in MdARF18 transgenic Arabidopsis at different nitrate concentrations. Bars represent the mean ± SD (n = 3). Different letters above the bars indicate significant differences using the LSD test (p < 0.05).
Plants rely on rapid modification of the root system to efficiently access effective nitrogen resources in the soil for growth and survival. The plasticity of root development is an effective strategy for accessing nitrate, and appropriate concentrations of IAA can promote the development of lateral roots\(^\text{[7,43]}\). Our study found that the length of the primary root was shortened.

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**Fig. 6** MdARF18 binds directly to the promoter of \(MdNRT1.1\). A. Schematic representation of \(MdNRT1.1\) promoter. B. Y1H assay of MdARF18 bound to the \(MdNRT1.1\) promoter in vitro. \(10^{-1}, 10^{-2}, 10^{-3}\), and \(10^{-4}\) indicate that the yeast concentration was diluted 10, 100, 1000, and 10000 times, respectively. 3-AT stands for 3-Amino-1,2,4-triazole. C. Dual luciferase assays demonstrate MdARF18 and \(MdNRT1.1\) binding. The horizontal bar on the left side of the right indicates the captured signal intensity. Empty LUC and 35S vectors were used as controls. Representative images of three independent experiments are shown here.

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**Fig. 7** MdARF18 inhibits the expression of \(MdNRT1.1\). A. GUS staining experiment of \(pMdNRT1.1\):GUS transgenic calli and transgenic calli containing both \(pMdNRT1.1\):GUS and \(35S::MdARF18\) with different nitrate treatments. B. GUS activity assays in MdARF18-overexpressing calli with different nitrate treatments. C. GUS expression level in MdARF18-overexpressing calli with different nitrate treatments. Bars represent the mean ± SD (n = 3). Different numbers of asterisk above the bars indicate significant differences using the LSD test (*p < 0.05 and **p < 0.01).
and the number of lateral roots did increase in IAA-treated Gl3 apple seedlings (Supplemental Fig. S7). Generally, an environment with low concentrations of nitrate promotes the transport of IAA by AtNRT1.1, which inhibits the growth of lateral roots[14]. However, in the research of MdARF18 transgenic Arabidopsis, it was found that the lateral roots of MdARF18-OX increased under low concentrations of nitrate, but there was no significant change in the mutant arf18 (Fig. 3). Therefore, it was hypothesized that MdARF18 might repress the expression of the MdNRT1.1 gene or other related genes that can regulate root plasticity, thereby affecting nitrate uptake in plants.

In rice, several researches has been demonstrated that OsARF18 significantly regulates nitrogen utilization. Loss of function of the Rice Salt Tolerant 1 (RST) gene (encoding OsARF18) removes its ability to transcriptionally repress OsAS1, accelerating the assimilation of NH₄⁺ to Asn and thereby increasing nitrogen utilization[28]. During soil incubation of MdARF18-OX Arabidopsis, it was found that leaving aside the effect of differences in nitrate concentration, the arf18 mutant grew significantly better than MdARF18-OX and had higher levels of nitrate and NR activity in arf18 than in MdARF18-OX. This demonstrates that MdARF18 may act as a repressor of nitrate uptake and assimilation, thereby inhibiting normal plant development (Fig. 4). Interestingly, an adequate nitrogen environment promotes plant growth, but MdARF18-OX Arabidopsis growth and all physiological indexes were poorer under high nitrate concentration than MdARF18-OX at other concentrations. We hypothesize that MdARF18 may be activated more intensively at high nitrate concentrations, or that MdARF18 suppresses the expression levels of genes for nitrate uptake or assimilation (genes that may play a stronger role at high nitrate concentrations), thereby inhibiting plant growth. In addition, we obtained MdARF18 transgenic calli (Supplemental Fig. S10) and subjected them to high and low concentrations of nitrate, and also found that MdARF18 inhibited the growth of individuals at both concentrations (Supplemental Fig. S11). This further confirms that MdARF18 inhibits nitrate uptake in individuals.

ARF family transcription factors play a key role in transmitting auxin signals to alter plant growth and development, e.g. osarf1 and osarf24 mutants have reduced levels of OsNRT1.1, OsNRT2.3a and OsNIA2 transcripts[22]. Therefore, further studies are needed to determine whether MdARF18 activates nitrate uptake through different molecular mechanisms. The result revealed that the transcript levels of AtNRT1.1, AtNIA1, AtNIA2, and AtNIR in MdARF18-OX were consistent with the developmental pattern of impaired plant growth (Fig. 5). Unfortunately, we attempted to explore whether variability in nitrate concentration affects MdARF18 to differ at the protein level, but the two did not appear to differ significantly (Supplemental Fig. S12).

ARF transcription factors act as trans-activators/repressors of N metabolism-related genes by directly binding to TGTCTT/NGGACA-containing fragments in the promoter regions of downstream target genes[57,58]. The NRT family plays important roles in nitrate uptake, transport, and storage, and NRT1.1 is an important dual-affinity nitrate transporter protein[57,58], and nitrogen utilization is very important for apple growth[59,60]. We identified binding sites in the promoters of these N-related genes that are compatible with ARF factors, and MdARF18 was found to bind to MdNRT1.1 by yeast one-hybrid technique (Fig. 6a-6b). It was also verified by Dual luciferase assays that MdARF18 could act as a transcriptional repressor that inhibited the expression of the downstream gene MdNRT1.1 (Fig. 6c), which inhibited the uptake of nitrate in plants. In addition, the GUS assay was synchronized to verify that transiently expressed pMdNRT1.1::GUS calli with 3SS::MdARF18 showed a lighter staining depth and a significant decrease in GUS transcript level and enzyme activity (Fig. 7). This phenomenon was particularly pronounced at high concentrations of nitrate. These results suggest that MdARF18 may directly bind to MdNRT1.1 promoter and inhibit its expression, thereby suppressing NO₃⁻ metabolism and decreasing the efficiency of nitrate uptake more significantly under high nitrate concentrations.

Conclusion

In conclusion, in this study, we found that MdARF18 responds to nitrate and could directly bind to the TGTCTT site of the MdNRT1.1 promoter to repress its expression. Our findings provide new insights into the molecular mechanisms by which MdARF18 regulates nitrate transport in apple.

Author Contributions

The authors confirm contribution to the paper as follows: study conception and design: Liu G; data collection:Liu G, Rui L, Liu R; analysis and interpretation of results: Liu G, Li H, An X; draft manuscript preparation: Liu G; supervision: Zhang S, Zhang Z; funding acquisition: You C, Wang X; All authors reviewed the results and approved the final version of the manuscript.

Data Availability Statement

Not applicable.

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Conflict of interest

The authors declare that they have no conflict of interest.

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