Fruit Research

ARTICLE

Open Access

https://doi.org/10.48130/frures-0024-0021

Fruit Research **2024**, 4: e027

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

Guo-Dong Liu[1], Xiu-Hong An[2], Lin Rui[1], Ran-Xin Liu[1], Hong-Liang Li[1], Shuai Zhang[3], Zhen-Lu Zhang[1], Chun-Xiang You[1*] and Xiao-Fei Wang[1*]

[1] *Apple Technology Innovation Center of Shandong Province, Shandong Collaborative Innovation Center of Fruit & Vegetable Quality and Efficient Production, National Key Laboratory of Wheat Improvement, College of Horticulture Science and Engineering, Shandong Agricultural University, Tai-An 271018, Shandong, China*

[2] *National Engineering Research Center for Agriculture in Northern Mountainous Areas, Agricultural Technology Innovation Center in Mountainous Areas of Hebei Province, Hebei Agricultural University, Baoding 071001, Hebei, China*

[3] *Key Laboratory of Agricultural Film Application of Ministry of Agriculture and Rural Affairs, College of Chemistry and Material Science, Shandong Agricultural University, Tai'an 271018, Shandong, China*

* Corresponding authors, E-mail: youchunxiang@sdau.edu.cn; xfwang2004@163.com

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, it was 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 TGTCTT 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.

Citation: Liu GD, An XH, Rui L, Liu RX, Li HL, et al. 2024. Auxin response factor MdARF18 regulates *MdNRT1.1* to affect nitrogen utilization in apple. *Fruit Research* 4: e027 https://doi.org/10.48130/frures-0024-0021

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 decrease significantly, cell division and elongation are restricted, and chlorophyll content decreases, and this leads to short and thin plants, small leaves, and pale color[2,7,8]. 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 the 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 more sustainable agriculture with minimal nitrogen demand.

Plants obtain inorganic nitrogen from the soil, mainly in the form of NH_4^+ and nitrate (NO_3^-)[10–13]. Nitrate uptake by plants occurs primarily in aerobic environments[3]. 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]. NRT1.1 is 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[3,19,20]. 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[3]. 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[3,21]. In particular, low NO_3^- 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].

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, the ARF protein regulates the expression of auxin response genes by specifically binding to the TGTCNN 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 NH_4^+ to Asn and thus increases N utilization[28]. In addition, *dao* mutant plants deterred the conversion of IAA to OxIAA, thus high levels of IAA strongly activates *OsARF18*, which subsequently represses the 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, MdARF18 is identified which is a key transcription factor involved in nitrate uptake and transport in apples and *MdARF18* reduces NO_3^- uptake and assimilation. Further analysis suggests that MdRF18 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_699B09) sequence numbers were obtained from the official TAIR website (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 was 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 www.jalview.org/download.[32]

Plant materials and cultivation methods

The apple 'Orin' callus was transplanted on MS medium containing 1.5 mg·L^{-1} 6-benzylaminopurine (6-BA) and 0.5 mg·L^{-1} 2,4 dichlorophenoxyacetic acid (2,4-D) at 25 °C, in the dark, at 21-d intervals. 'Royal Gala' apple cultivars were cultured in vermiculite and transplanted at 25 °C every 30 d. The Arabidopsis plants used were of the Columbia (Col-0) wild-type variety. Sowing and germinating Arabidopsis seeds on MS nutrient medium, and Arabidopsis seeds were incubated and grown at 25 °C (light/dark cycle of 16 h/8 h)[33].

The nutrient solution in the base contained 1.0 mM $CaCl_2$, 1.0 mM KH_2PO_4, 1.0 mM $MgSO_4$, 0.1 mM $FeSO_4{\cdot}7H_2O$ 0.1 mM $Na_2EDTA{\cdot}2H_2O$, 50 μM $MnSO_4{\cdot}H_2O$, 50 μM H_3BO_3, 0.05 μM $CuSO_4{\cdot}5H_2O$, 0.5 μM $Na_2MoO_4{\cdot}2H_2O$, 15 μM $ZnSO_4{\cdot}7H_2O$, 2.5 μM KI, and 0.05 μM $CoCl{\cdot}6H_2O$, and 0.05 μM $CoCl{\cdot}6H_2O$, and 0.05 μM CoCl· $6H_2O$. $2H_2O$, 15 μM $ZnSO_4{\cdot}7H_2O$, 2.5 μM KI and 0.05 μM $CoCl{\cdot}6H_2O$, and 0.05 μM $CoCl{\cdot}6H_2O$, supplemented with 0.5 mM, 2 mM, and 10 mM KNO_3 as the sole nitrogen source, and added with the relevant concentrations of KCl to maintain the same K concentration[33,34].

For auxin treatment, 12 uniformly growing apple tissue-cultured seedlings (*Malus domestica* 'Royal Gala') were selected from each of the control and treatment groups, apple seedlings were incubated in a nutrient solution containing 1.5 mg·L^{-1} 6-BA, 0.2 mg·L^{-1} naphthalene acetic acid, and IAA (10 μM) for 50 d, and then the physiological data were determined. Apple seedlings were incubated and grown at 25 °C (light/dark cycle of 16 h/8 h).

For nitrate treatment, Arabidopsis seedlings were transferred into an MS medium (containing different concentrations of KNO_3) as soon as they germinated to test root development. Seven-day-old Arabidopsis were transplanted into vermiculite and then treated with a nutrient solution containing different concentrations of KNO_3 (0.5, 2, 10 mM) and watered at 10-d intervals. Apple calli were treated with medium containing 1.5 mg·L^{-1} 6-BA, 0.5 mg·L^{-1} 2,4-D, and varying doses of KNO_3 (0.5, 2, and 10 mM) for 25 d, and samples were examined for relevant physiological data. Apple calli were subjected to the same treatment for 1 d for GUS staining[35].

Plasmid construction and plant transformation

To obtain *MdARF18* overexpression materials, the open reading frame (ORF) of *MdARF18* was introduced into the pRI-101 vector. To obtain *pMdNRT1.1* material, the 2 kb segment located before the transcription start site of *MdNRT1.1* was inserted into the pCAMBIA1300 vector. The Agrobacterium tumefaciens LBA4404 strain was cultivated in lysozyme broth (LB) medium supplemented with 50 mg·L^{-1} kanamycin and 50 mg·L^{-1} rifampicin. The *MdARF18* overexpression vector and the *ProMdNRT1.1::GUS* vector were introduced into Arabidopsis and apple callus using the flower dip transformation procedure. The third-generation homozygous transgenic Arabidopsis (T3) and transgenic calli were obtained[36]. Information on the relevant primers designed is shown in Supplemental Table S1.

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].

Real-Time quantitative Polymerase Chain Reaction (qPCR)

cDNA was synthesized 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

UltraSYBR Mixture (Low Rox) kit (ComWin Biotech Co. Ltd., Beijing, China). qRT-PCR experiments were performed using the $2^{-\Delta\Delta CT}$ method for data analysis. The data were analyzed by the $2^{-\Delta\Delta CT}$ method[31].

GUS staining

GUS staining buffer contained 1 mM 5-bromo-4-chloro-3-indolyl-β-glutamic acid, 0.01 mM EDTA, 0.5 mM hydrogen ferrocyanide, 100 mM sodium phosphate (pH 7.0), and 0.1% (v/v) Triton X-100 was maintained at 37 °C in the dark. The *pMdNRT1.1::GUS* construct was transiently introduced into apple calli. To confirm whether *MdNRT1.1* is activated or inhibited by *MdARF18*, we co-transformed *35S::MdARF18* into *pMdNRT1.1::GUS* is calling. The activity of transgenic calli was assessed using GUS labeling and activity assays[33,38].

Determination of nitrate content, nitrate reductase activity

The specimens were crushed into fine particles, combined with 1 mL of ddH_2O, and thereafter subjected to a temperature of 100 °C for 30 min. The supernatant was collected in a flow cell after centrifugation at 12,000 revolutions per minute for 10 min. The AutoAnalyzer 3 continuous flow analyzer was utilized to measure nitrate concentrations. (SEAL analytical, Mequon, WI, USA). Nitrate reductase (NR) activity was characterized by the corresponding kits (Solarbio Life Science, Beijing, China) using a spectrophotometric method[31].

Yeast one-hybrid (Y1H) assay

Y1H assays were performed as previously described by Liu et al.[39]. The coding sequence of *MdARF18* was integrated into the pGADT7 expression vector, whereas the promoter region of *MdNRT1.1* was included in the pHIS2 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 identified using polymerase chain reaction (PCR). The yeast strain cells were diluted at dilution factors of 10, 100, 1,000, and 10,000. Ten 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 d[37].

Dual luciferase assays

Dual-luciferase assays were performed as described previously[40]. Full-length *MdARF18* was cloned into pGreenII 62-SK to produce MdARF18-62-SK. The promoter fragment of *MdNRT1.1* was cloned into pGreenII 0800-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, 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[41]. 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 (www.ab-mart.com).

Data analysis

Unless otherwise noted, every experiment was carried out independently in triplicate. 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[31].

Results

Auxin regulates nitrogen uptake and utilization in apple

To investigate whether auxin affects the effective uptake of nitrate in apple, we first externally applied IAA under normal N (5 mM NO_3^-) environment, and this result showed that the growth of Gala apple seedlings in the IAA-treated group were better than the control, and their fresh weights were heavier than the control group (Fig. 1a, d). 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 the control group, while the nitrate content and NR activity of the shoot part were lower than the control group (Fig. 1b, c). 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 the root, the expression levels of *MdNRT1.1*, *MdNRT2.1*, *MdNIA1*, *MdNIA2*, and *MdNIR* were higher than control group (Supplemental Fig. S1a, f, h–j), while the expression levels of *MdNRT1.2*, *MdNRT1.6* and *MdNRT2.5* were lower than control group significantly (Supplemental Fig. S1b, d, g). For the 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, c–j). 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)[25,27], it was investigated whether members of ARF 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, j), and the expression level of *MdARF18* showed a trend of stable up-regulation under IAA treatment (Supplemental Fig. S3). The result 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.

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

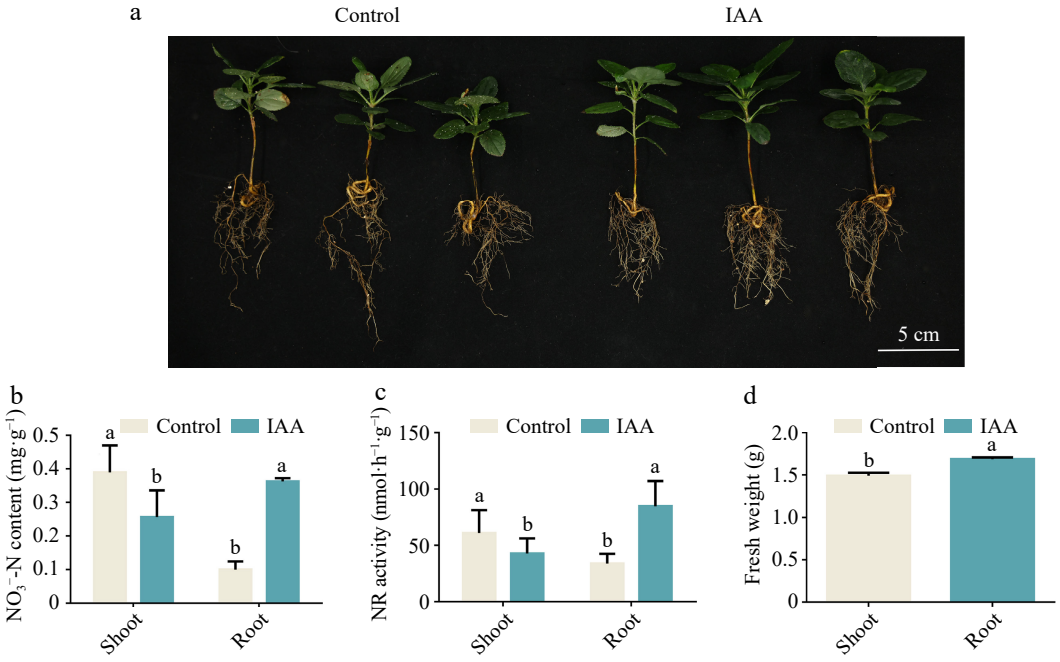

Fig. 1 Auxin enhances nitrate uptake of Gala seedlings. (a) Phenotypes of apple (*Malus domestica* 'Royal Gala') seedlings grown nutritionally for 50 d under IAA (10 μM) treatment. (b) Nitrate content of shoot and root apple (*Malus domestica* 'Royal Gala') seedlings treated with IAA. (c) NR activity in shoot and root of IAA treatment apple (*Malus domestica* 'Royal Gala') seedlings. (d) Seedling fresh weight under IAA treatment. Bars represent the mean ± SD (n = 3). Different letters above the bars indicate significant differences using the LSD test ($p < 0.05$).

ARF18 from 15 species were then identified in NCBI (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

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. S7). Seven-day-old *MdARF18* transgenic Arabidopsis and *arf18* mutants were treated in a medium with different nitrate concentrations for 10 d (Fig. 3a, b). 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 effect on *MdNRT1.1* thus leading to the 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 d. 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, d). 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, d). These results indicate that *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, *MdARF18* overexpression calli were obtained and treated with different concentrations of nitrate (Supplemental Fig. S8). The results show that the growth of overexpressed *MdARF18* was weaker than wild type in both treatments (Supplemental Fig. S9a). The fresh weight of *MdARF18* was significantly lighter than wild

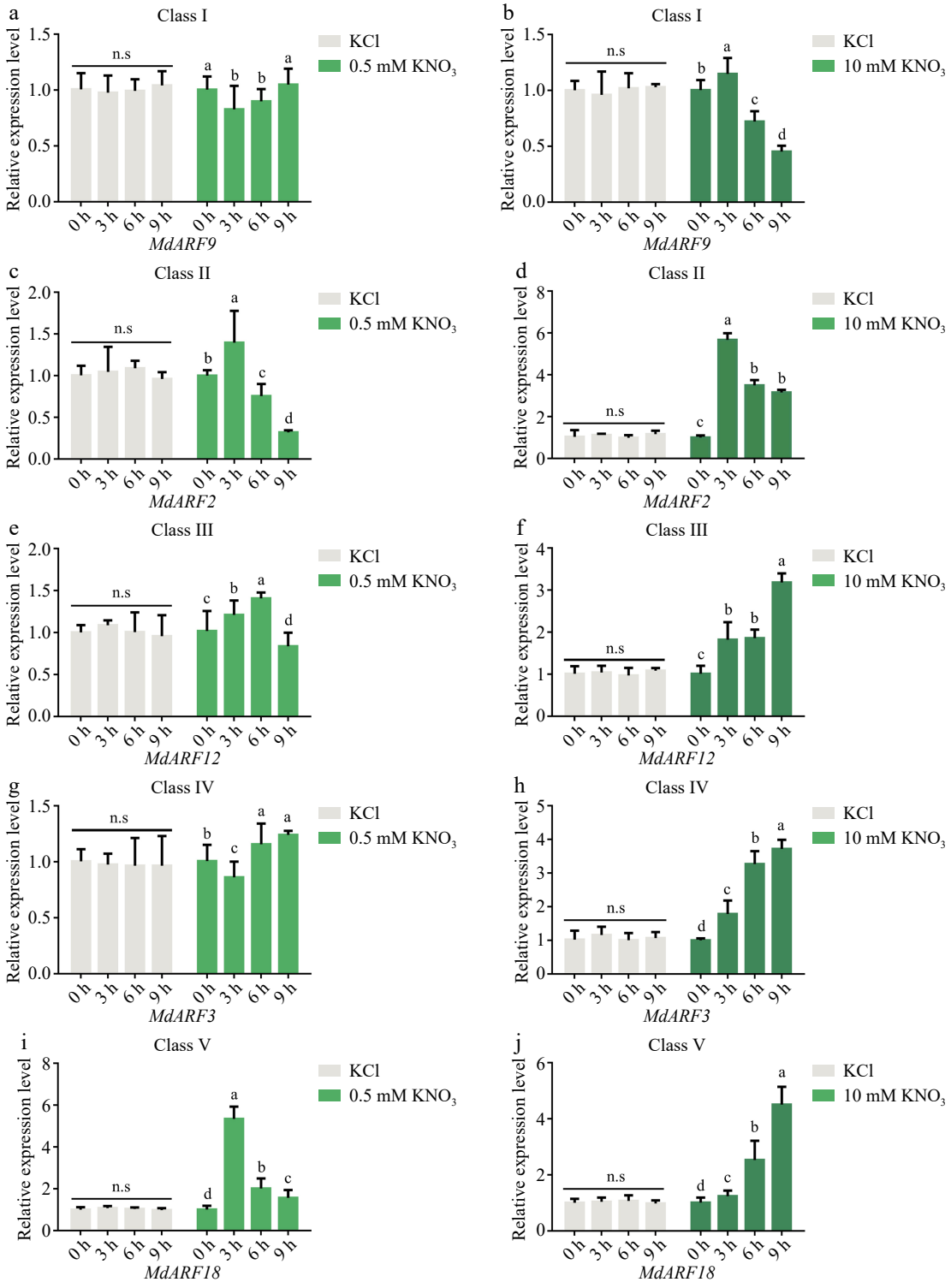

Fig. 2 Relative expression analysis of *MdARFs* subfamilies in response to different concentrations of nitrate. Expression analysis of representative genes from five subfamilies of MdARF transcription factors. Bars represent the mean ± SD (n = 3). Different letters above the bars indicate significant differences using the LSD test ($p < 0.05$).

type (Supplemental Fig. S9b), and its nitrate and NR activity were lower than wild type (Supplemental Fig. S9c, d), which was consistent with the above results (Fig. 4). This result further confirms that *MdARF18* could inhibit 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 NIAs and NIR[3,34]. To further investigate the pathway by which *MdARF18* inhibits plant

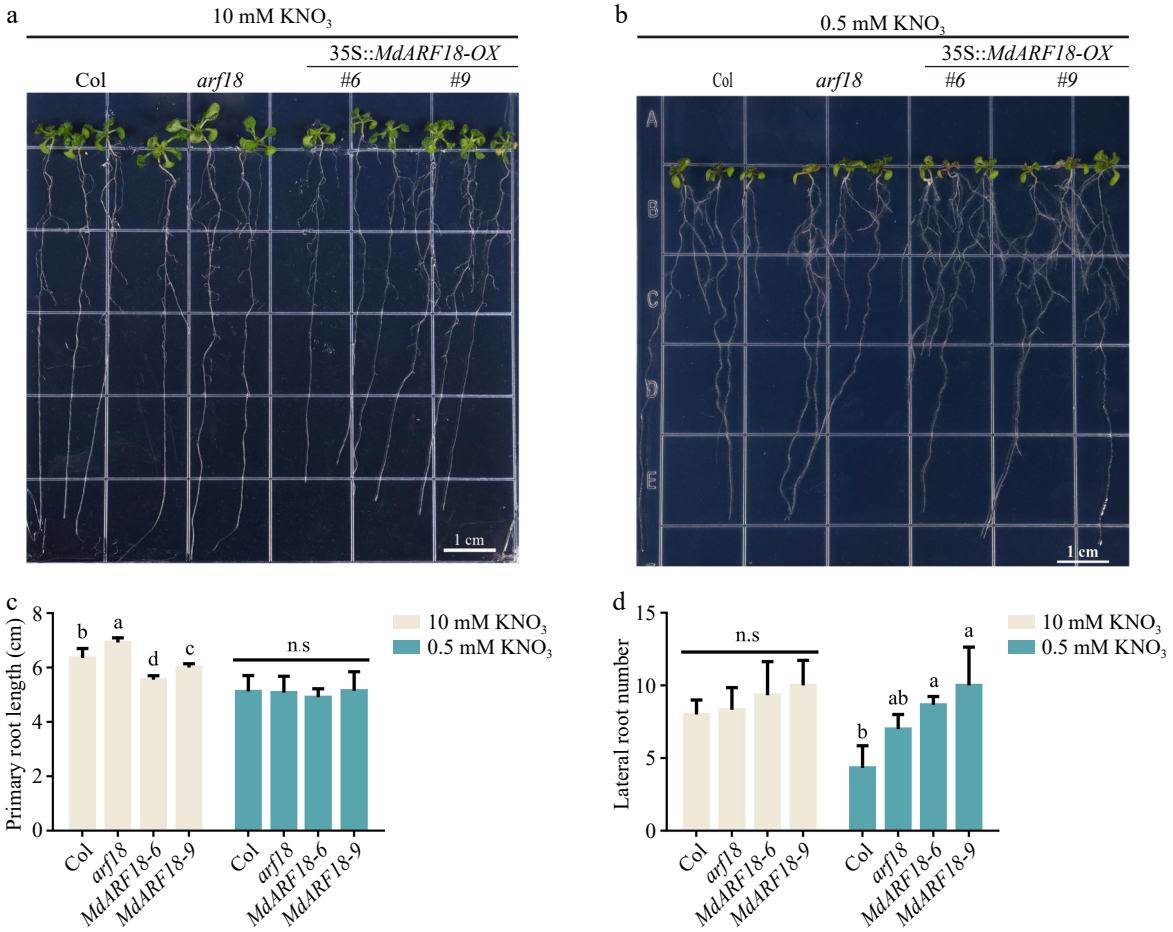

Fig. 3 MdARF18 inhibits root development. (a) MdARF18 inhibits root length at 10 mM nitrate concentration. (b) MdARF18 promotes lateral root growth at 0.5 mM nitrate concentration. (c) Primary root length statistics. (d) Lateral root number statistics. Bars represent the mean ± SD (n = 3). Different letters above the bars indicate significant differences using the LSD test ($p < 0.05$).

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, h–j). 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 be consistent 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–g). 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 test 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. S10). This result suggests that nitrate does 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 Fig. 6b. Yeast cells that were simultaneously transformed with *MdNRT1.1-P-pHIS* and *pGADT7* were unable to grow in selected SD medium. However, cells that were transformed with *MdNRT1.1-P-pHIS* and *MdARF18-pGADT7* grew successfully in the selective medium. The result therefore hypothesizes that MdARF18 could bind specifically to *MdNRT1.1* promoter 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. S11). 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 first showed that the color depth of *pMdNRT 1.1::GUS* and *35S::MdARF18* were significantly lighter than

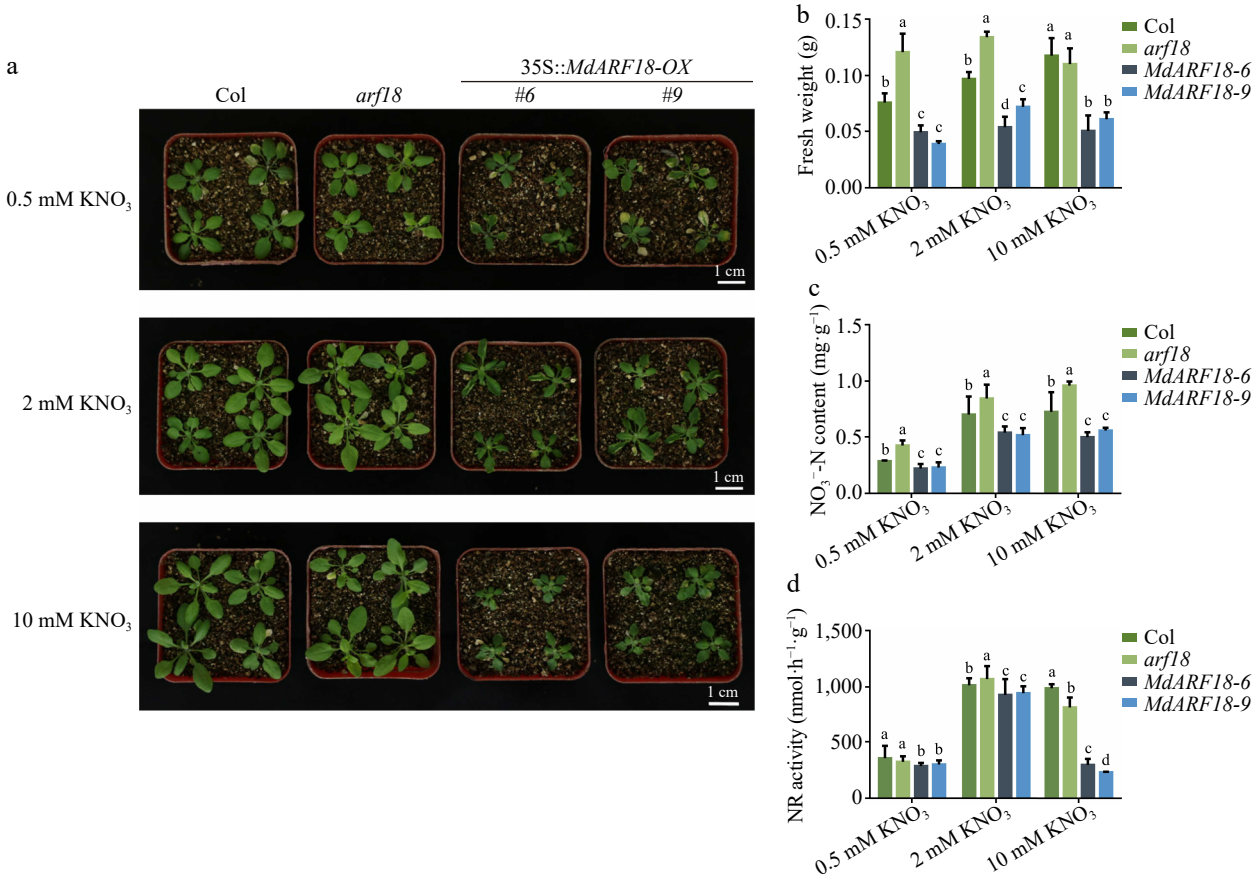

Fig. 4 Ectopic expression of *MdARF18* inhibits Arabidopsis growth. (a) Status of Arabidopsis growth after one month of incubation at different nitrate concentrations. (b) Fresh weight of Arabidopsis. (c) Nitrate content of Arabidopsis. (d) NR activity in Arabidopsis. Bars represent the mean ± SD (n = 3). Different letters above the bars indicate significant differences using the LSD test ($p < 0.05$).

pMdnNRT1.1::GUS alone (Fig. 7a). GUS enzyme activity, as well as GUS expression, also indicated that the calli containing *pMdnNRT1.1::GUS* alone had a stronger GUS activity (Fig. 7b, c). 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[42,43]. Previous studies have shown that some of the plant hormones such as IAA, GA, and ABA interact with nitrate[25,44–45]. 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, b). Nitrate content and NR activity were also significantly higher in their root parts (Fig. 1c, d) 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[46–49]. 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, an Arabidopsis *AtARF18* homolog was successfully cloned and named *MdARF18* (Supplemental Figs 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. S7).

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[7,44]. The present study found that the length of the primary root was shortened and the number of lateral roots did increase in IAA-treated Gl3 apple seedlings (Supplemental Fig. S12). 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

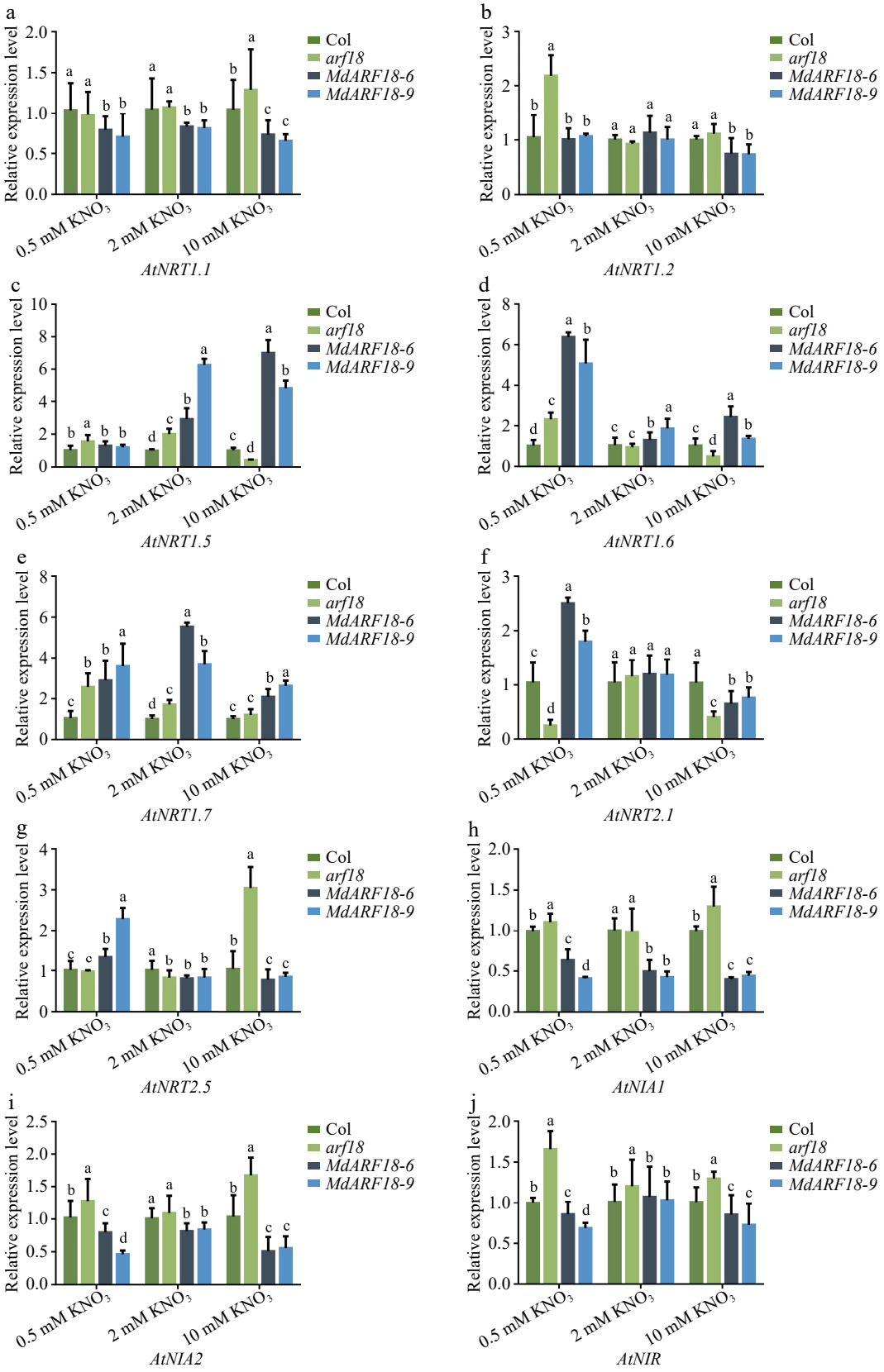

Fig. 5 qPCR-RT analysis of N-related genes. Expression analysis of N-related genes in MdARF18 transgenic Arabidopsis at different nitrate concentrations. Bars represent the mean ± SD (n = 3). Different letters above the bars indicate significant differences using the LSD test ($p < 0.05$).

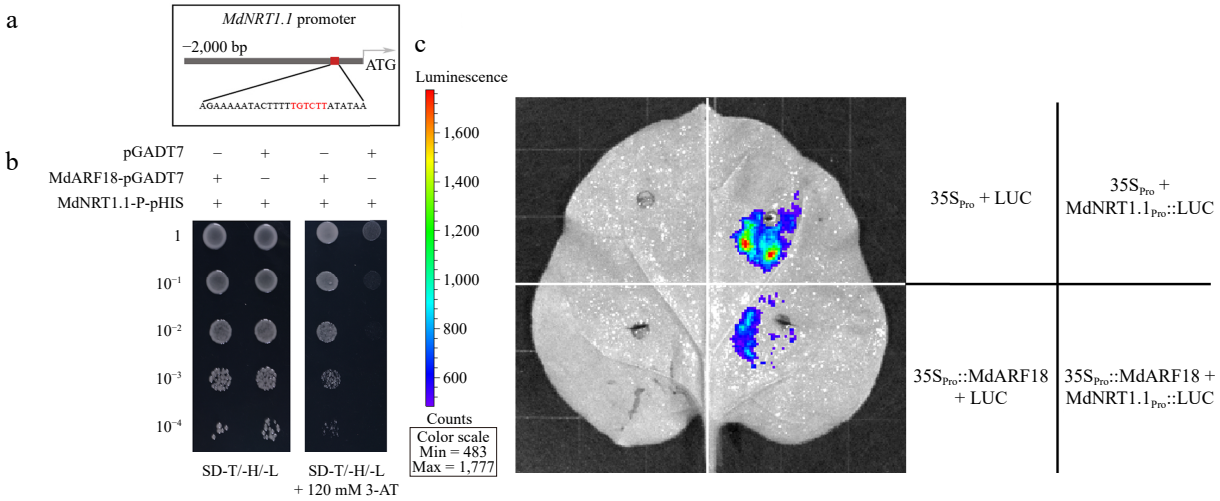

Fig. 6 MdARF18 binds directly to the promoter of *MdNRT1.1*. (a) Schematic representation of *MdNRT1.1* promoter. (b) Y1H assay of MdARF18 bound to the *MdNRT1.1* promoter *in vitro*. 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} indicate that the yeast concentration was diluted 10, 100, 1,000, and 10,000 times, respectively. 3-AT stands for 3-Amino-1,2,4-triazole. (c) Dual luciferase assays demonstrate the binding of MdARF18 with *MdNRT1.1* promoter. 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.

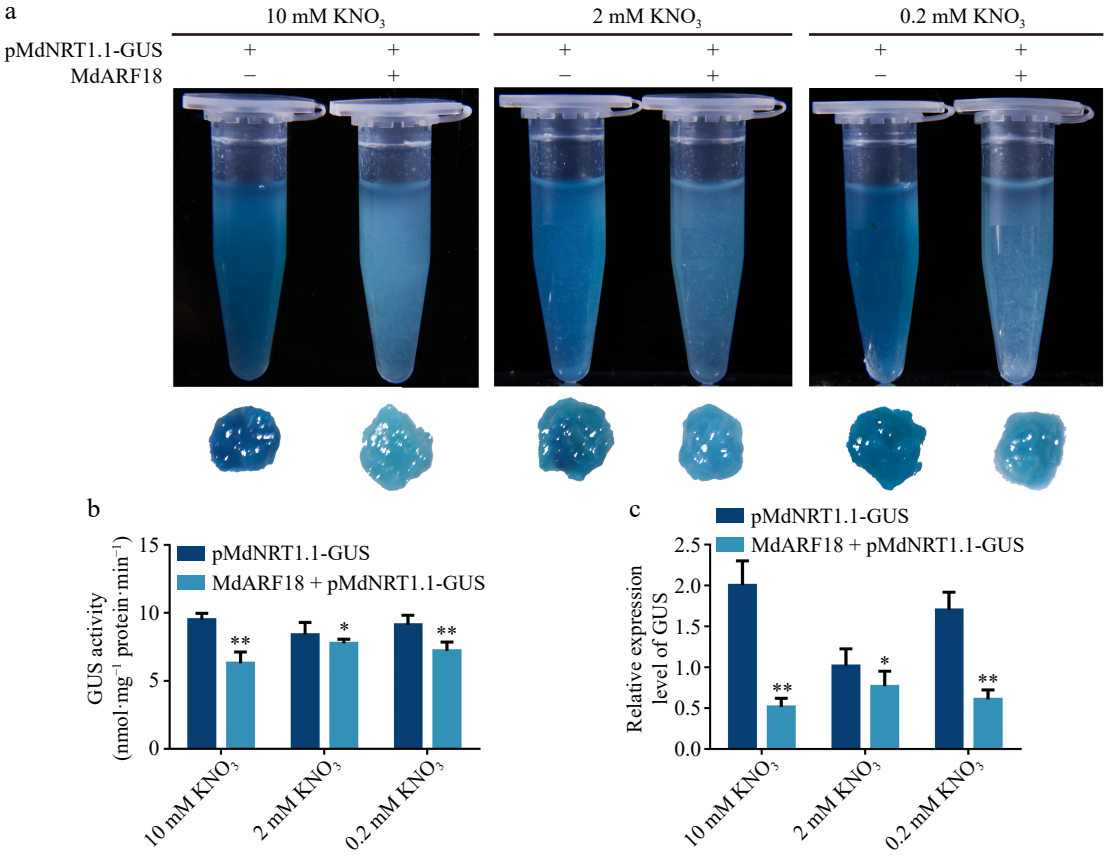

Fig. 7 MdARF18 inhibits the expression of *MdNRT1.1*. (a) GUS staining experiment of *pMdNRT1.1::GUS* transgenic calli and transgenic calli containing both *pMdNRT1.1::GUS* and *35S::MdARF18* with different nitrate treatments. (b) GUS activity assays in *MdARF18* overexpressing calli with different nitrate 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 **$p < 0.01$).

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 researchers have demonstrated that *OsARF18* significantly regulates nitrogen utilization. Loss of function of the *Rice Salt Tolerant 1* (*RST1*) gene (encoding OsARF18) removes its ability to transcriptionally repress *OsAS1*, accelerating the assimilation of NH_4^+ 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. S8) 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. S9). 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 *OsNRT1.1B*, *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. S10).

ARF transcription factors act as trans-activators/repressors of N metabolism-related genes by directly binding to TGTCNN/NNGACA-containing fragments in the promoter regions of downstream target genes[27,50]. The NRT family plays important roles in nitrate uptake, transport, and storage, and NRT1.1 is an important dual-affinity nitrate transporter protein[7,50–52], and nitrogen utilization is very important for apple growth[53,54]. 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* promoter by yeast one-hybrid technique (Fig. 6a, b). 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 *35S::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 the *MdNRT1.1* promoter and inhibit its expression, thereby suppressing NO_3^- metabolism and decreasing the efficiency of nitrate uptake more significantly under high nitrate concentrations.

Conclusions

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 GD; data collection: Liu GD, Rui L, Liu RX; analysis and interpretation of results: Liu GD, Li HL, An XH; draft manuscript preparation: Liu GD; supervision: Zhang S, Zhang ZL; funding acquisition: You CX, Wang XF; All authors reviewed the results and approved the final version of the manuscript.

Data availability

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (32272683), the Shandong Province Key R&D Program of China (2022TZXD008-02), the China Agriculture Research System of MOF and MARA (CARS-27), the National Key Research and Development Program of China (2022YFD1201700), and the National Natural Science Foundation of China (NSFC) (32172538).

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-0024-0021)

Dates

Received 6 May 2024; Revised 8 June 2024; Accepted 17 June 2024; Published online 5 August 2024

References

1. Castaings L, Marchive C, Meyer C, Krapp A. 2011. Nitrogen signalling in *Arabidopsis*: how to obtain insights into a complex signalling network. *Journal of Experimental Botany* 62:1391–97
2. Nacry P, Bouguyon E, Gojon A. 2013. Nitrogen acquisition by roots: physiological and developmental mechanisms ensuring plant adaptation to a fluctuating resource. *Plant and Soil* 370:1–29
3. Wang YY, Cheng YH, Chen KE, Tsay YF. 2018. Nitrate transport, signaling, and use efficiency. *Annual Review of Plant Biology* 63:85–122
4. Raytek LM, Dastmalchi M. 2022. Plant nutrition: an architect of nitrate-hunger cues. *Current Biology* 32:R1320–R1323

5. Ahmed M, Rauf M, Akhtar M, Mukhtar Z, Saeed NA. 2020. Hazards of nitrogen fertilizers and ways to reduce nitrate accumulation in crop plants. *Environmental Science and Pollution Research* 27:17661–70
6. Bi YM, Kant S, Clark J, Gidda S, Ming F, et al. 2009. Increased nitrogen-use efficiency in transgenic rice plants over-expressing a nitrogen-responsive early nodulin gene identified from rice expression profiling. *Plant, Cell & Environment* 32:1749–60
7. Bouguyon E, Perrine-Walker F, Pervent M, Rochette J, Cuesta C, et al. 2016. Nitrate controls root development through posttranscriptional regulation of the NRT1.1/NPF6.3 transporter/sensor. *Plant Physiology* 172:1237–48
8. Tahir MM, Lu Z, Wang C, Shah K, Li S, et al. 2022. Nitrate application induces adventitious root growth by regulating gene expression patterns in apple rootstocks. *Journal of Plant Growth Regulation* 41:3467–78
9. Tahir MM, Li S, Mao J, Liu Y, Li K, et al. 2021. High nitrate inhibited adventitious roots formation in apple rootstock by altering hormonal contents and miRNAs expression profiles. *Scientia Horticulturae* 286:110230
10. Ishikawa K, Ohmori T, Miyamoto H, Ito T, Kumagai Y, et al. 2013. Denitrification in soil amended with thermophile-fermented compost suppresses nitrate accumulation in plants. *Applied Microbiology and Biotechnology* 97:1349–59
11. Jian S, Liao Q, Song H, Liu Q, Lepo JE, et al. 2018. NRT1.1-related NH_4^+ toxicity is associated with a disturbed balance between NH_4^+ uptake and assimilation. *Plant Physiology* 178:1473–88
12. Rashid M, Bera S, Medvinsky AB, Sun GQ, Li BL, et al. 2018. Adaptive regulation of nitrate transceptor NRT1.1 in fluctuating soil nitrate conditions. *iScience* 2:41–50
13. Unkefer PJ, Knight TJ, Martinez RA. 2023. The intermediate in a nitrate-responsive ω-amidase pathway in plants may signal ammonium assimilation status. *Plant Physiology* 191:715–28
14. Fang X, Fang S, Ye Z, Liu D, Zhao K, et al. 2021. NRT1.1 dual-affinity nitrate transport/signalling and its roles in plant abiotic stress resistance. *Frontiers in Plant Science* 12:715694
15. Chai S, Li E, Zhang Y, Li S. 2020. NRT1.1-mediated nitrate suppression of root coiling relies on PIN2- and AUX1-mediated auxin transport. *Frontiers in Plant Science* 11:671
16. Chiba Y, Shimizu T, Miyakawa S, Kanno Y, Koshiba T, et al. 2015. Identification of *Arabidopsis thaliana* NRT1/PTR FAMILY (NPF) proteins capable of transporting plant hormones. *Journal of Plant Research* 128:679–86
17. Kiba T, Krapp A. 2016. Plant nitrogen acquisition under low availability: regulation of uptake and root architecture. *Plant and Cell Physiology* 57:707–14
18. Krapp A, David LC, Chardin C, Girin T, Marmagne A, et al. 2014. Nitrate transport and signalling in *Arabidopsis*. *Journal of Experimental Botany* 65:789–98
19. Su H, Wang T, Ju C, Deng J, Zhang T, et al. 2021. Abscisic acid signaling negatively regulates nitrate uptake via phosphorylation of NRT1.1 by SnRK2s in *Arabidopsis*. *Journal of Integrative Plant Biology* 63:597–610
20. Zhao Y. 2010. Auxin biosynthesis and its role in plant development. *Annual Review of Plant Biology* 61:49–64
21. Hu Q, Shu J, Li W, Wang G. 2021. Role of auxin and nitrate signaling in the development of root system architecture. *Frontiers in Plant Science* 12:690363
22. Zhang S, Zhu L, Shen C, Ji Z, Zhang H, et al. 2021. Natural allelic variation in a modulator of auxin homeostasis improves grain yield and nitrogen use efficiency in rice. *The Plant Cell* 33:566–80
23. Zhao Z, Wang C, Yu X, Tian Y, Wang W, et al. 2022. Auxin regulates source-sink carbohydrate partitioning and reproductive organ development in rice. *Proceedings of the National Academy of Sciences of the United States of America* 119:e2121671119
24. Ellis CM, Nagpal P, Young JC, Hagen G, Guilfoyle TJ, et al. 2005. *AUXIN RESPONSE FACTOR1* and *AUXIN RESPONSE FACTOR2* regulate senescence and floral organ abscission in *Arabidopsis thaliana*. *Development* 132:4563–74
25. Keller AH, Fallon MD. 2012. *Auxins : structure, biosynthesis and functions*. New York: Nova Science Publishers
26. Li S, Xie Z, Hu C, Zhang J. 2016. A review of *auxin response factors* (ARFs) in plants. *Frontiers in Plant Science* 7:47
27. Chandler JW. 2016. Auxin response factors. *Plant, Cell & Environment* 39:1014–28
28. Deng P, Jing W, Cao C, Sun M, Chi W, et al. 2022. Transcriptional repressor RST1 controls salt tolerance and grain yield in rice by regulating gene expression of asparagine synthetase. *Proceedings of the National Academy of Sciences of the United States of America* 119:e2210338119
29. Tan BZ, Close DC, Quin PR, Swarts ND. 2021. Nitrogen use efficiency, allocation, and remobilization in apple trees: uptake is optimized with pre-harvest N supply. *Frontiers in Plant Science* 12:657070
30. Kowalczyk W, Wrona D, Przybylko S. 2022. Effect of nitrogen fertilization of apple orchard on soil mineral nitrogen content, yielding of the apple trees and nutritional status of leaves and fruits. *Agriculture* 12:2169
31. Liu G, Rui L, Yang Y, Liu R, Li H, et al. 2023. Identification and functional characterization of *MdNRT1.1* in nitrogen utilization and abiotic stress tolerance in *Malus domestica*. *International Journal of Molecular Sciences* 24:9291
32. Zheng P, Wang X, Yang Y, You C, Zhang Z, et al. 2020. Identification of phytochrome-interacting factor family members and functional analysis of *MdPIF4* in *Malus domestica*. *International Journal of Molecular Sciences* 21:7350
33. Feng Z, Li T, Wang X, Sun W, Zhang T, et al. 2022. Identification and characterization of apple MdNLP7 transcription factor in the nitrate response. *Plant Science* 316:111158
34. Liu R, Li H, Rui L, Liu G, Wang T, et al. 2023. An apple *NITRATE REDUCTASE 2* gene positively regulates nitrogen utilization and abiotic stress tolerance in *Arabidopsis* and apple callus. *Plant Physiology and Biochemistry* 196:23–32
35. Wang D, Yang K, Wang X, Lin X, Rui L, et al. 2022. Overexpression of MdZAT5, an C2H2-type zinc finger protein, regulates anthocyanin accumulation and salt stress response in apple calli and *Arabidopsis*. *International Journal of Molecular Sciences* 23:1897
36. Rui L, Yang Y, Zheng P, Wang C, Wang X, et al. 2022. Genome-wide analysis of *MdABF* Subfamily and functional identification of *MdABF1* in drought tolerance in apple. *Environmental and Experimental Botany* 199:104904
37. Liu Y, Gao N, Ma Q, Zhang J, Wang X, et al. 2021. The MdABI5 transcription factor interacts with the *MdNRT1.5/MdNPF7.3* promoter to fine-tune nitrate transport from roots to shoots in apple. *Horticulture Research* 8:236
38. Yang Y, Zheng P, Ren Y, Yao Y, You C, et al. 2021. Apple *MdSAT1* encodes a bHLHm1 transcription factor involved in salinity and drought responses. *Planta* 253:46
39. Liu X, Liu H, Li H, An X, Song L, et al. 2022. MdMYB10 affects nitrogen uptake and reallocation by regulatingthe nitrate transporter MdNRT2.4-1 in red flesh apple. *Horticulture Research* 9:uhac016
40. An J, Zhang X, Liu Y, Wang X, You C, et al. 2021. ABI5 regulates ABA-induced anthocyanin biosynthesis by modulating the MYB1-bHLH3 complex in apple. *Journal of Experimental Botany* 72:1460–72
41. Ji X, Li H, Qiao Z, Zhang J, Sun W, et al. 2022. The BTB protein MdBT2 recruits auxin signaling components to regulate adventitious root formation in apple. *Plant Physiology* 189:1005–20
42. Cox KL Jr. 2021. Nodding on and off: transcription factor cis-elements that regulate nitrate-dependent gene expression for root nodule symbiosis. *The Plant Cell* 33:2101–03
43. Li S, Xiao F, Yang D, Lyu X, Ma C, et al. 2021. Nitrate transport and distribution in soybean plants with dual-root systems. *Frontiers in Plant Science* 12:661054

44. Krouk G, Crawford NM, Coruzzi GM, Tsay YF. 2010. Nitrate signaling: adaptation to fluctuating environments. *Current Opinion in Plant Biology* 13:265–72
45. Hu J, Israeli A, Ori N, Sun T. 2018. The interaction between DELLA and ARF/IAA mediates crosstalk between gibberellin and auxin signaling to control fruit initiation in tomato. *The Plant Cell* 30:1710–28
46. Jia Z, Giehl RFH, Hartmann A, Estevez JM, Bennett MJ, et al. 2023. A spatially concerted epidermal auxin signaling framework steers the root hair foraging response under low nitrogen. *Current Biology* 33:3926–3941.e5
47. Lin J, Ali A, Chu N, Fu H, Huang M, et al. 2023. Identification of ARF transcription factor gene family and its defense responses to bacterial infection and salicylic acid treatment in sugarcane. *Frontiers in Microbiology* 14:1257355
48. Mo Z, Zhang Y, Hu L, Zhai M, Xuan J. 2023. Genome-wide identification and expression analysis of *auxin response factor* (*ARF*) gene family in pecan indicates its possible roles during graft union formation. *Scientia Horticulturae* 322:112401
49. Chen X, Liu Y, Zhang X, Zheng B, Han Y, et al. 2023. PpARF6 acts as an integrator of auxin and ethylene signaling to promote fruit ripening in peach PpARF. *Horticulture Research* 10:uhad158
50. Wang Y, Dai M, Wu X, Zhang S, Shi Z, et al. 2022. An ARF1-binding factor triggering programmed cell death and periderm development in pear russet fruit skin. *Horticulture Research* 9:uhab061
51. Sun J, Zheng N. 2015. Molecular mechanism underlying the plant NRT1.1 dual-affinity nitrate transporter. *Frontiers in Physiology* 6:386
52. Ye J, Tian W, Zhou M, Zhu Q, Du W, et al. 2021. STOP1 activates NRT1.1-mediated nitrate uptake to create a favorable rhizospheric pH for plant adaptation to acidity. *The Plant Cell* 33:3658–74
53. 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
54. Guo T, Yang Z, Bao R, Fu X, Wang N, et al. 2023. The m^6A reader MhYTP2 regulates the stability of its target mRNAs contributing to low nitrogen tolerance in apple (*Malus domestica*). *Horticulture Research* 10:uhad094