



Nitrate Application Induces Adventitious Root Growth by Regulating Gene Expression Patterns in Apple Rootstocks

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Abstract

Adventitious root (AR) formation is an essential step in the vegetative propagation of apple rootstocks. Nitrate serves as an essential signaling molecule for regulating root architecture by inducing the expression of auxin-related genes. However, the underlying mechanisms of nitrate-mediated ARs remain to be explored in apple. In this study, stem cuttings of B9 apple rootstocks were treated with different nitrate treatments: T1 (9.4 mM/L), T2 (28.1 mM/L), and T3 (46.9 mM/L). The root morphological parameters indicated that T2 was the optimum nitrate level for AR formation and development in B9 apple rootstocks. Therefore, to identify the underlying molecular mechanism by which nitrate promotes AR formation, stem cuttings of B9 were grown on T2 and T3. Furthermore, morphological and anatomical observations of stem cuttings also revealed that the nitrate treatment (T2) promoted AR formation. The results indicated that nitrate perceptibly upregulated the relative expression of genes related to nitrate (*MdNRT1.1*, *MdNRT2.1*, *MdNIA1*, and *MdANR1*) and auxin biosynthesis (*MdIAA14* and *MdIAA23*) in T2 cuttings compared with T3 cuttings. This resulted in enhanced expression of AR development-related genes (*MdWOX11*, *MdARRO1*, and *MdSHR*), collectively resulting in elevated expression of the cell cycle-related genes (*MdCYCD1;1*, *MdCYCD3;1*, and *MdCYCP4;1*). Overall, this study established a foundation for applied research work and shed light on nitrate-mediated AR formation in B9 apple rootstock and other fruit rootstock cuttings.

Keywords Apple rootstock · Adventitious root (AR) · Nitrate · Gene expression

Introduction

Apple is a highly nutritious fruit and is widely cultivated worldwide. High-density planting is currently necessary in the modern apple industry, which is greatly dependent on the practice of dwarfing rootstocks to maintain and control the

growth and architecture of trees (Li et al. 2015a). Various apple rootstocks with appropriate features exhibit inadequate rooting ability when grown from stem cuttings. Therefore, increasing rooting capacity is important for the commercial use of apple rootstocks. The B9 apple rootstock is the most commonly used rootstock in the apple industry and has strong architecture; resistance to pests, diseases, and other environmental challenges; good fruiting ability, and high production; however, the formation of ARs is a barrier/limiting aspect for apple rootstock breeding programs. AR formation is a key step in vegetative propagation. It is a multifaceted process affected by several factors, including nutritional status, hormones, temperature, light conditions, wounding, waterlogging, phenolic compounds, and genetic characteristics (Li et al. 2009). The AR formation process consists of four successive stages (activation, induction, initiation, and emergence) that depend on certain physiological and metabolic markers (Legué et al. 2014; Atkinson et al. 2014). The molecular mechanisms underlying AR formation are well characterized in various crops, including rice (Jiang

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et al. 2017), poplar (Legué et al. 2014), and Arabidopsis (Hu and Xu 2016). However, they remain to be addressed in apple rootstocks. Subsequently, it is important to determine the problems related to ARs for a better understanding of and to address the complications pertaining to AR formation in apple rootstocks.

Nitrogen is considered an essential macronutrient, which is crucial for plant growth and development and higher yield (Wang et al. 2012). In the soil, nitrogen assimilation occurred in two ways: organic and inorganic (Masclaux-Daubresse et al. 2010). Nitrate is an indispensable source of nitrogen and serves as an essential signaling molecule for regulating root architecture, modulating flowering time, regulating leaf growth, and inducing the expression of auxin-related genes (Sheng et al. 2017; O'Brien et al. 2016). Moreover, nitrate ions control root initiation and their consequences as nutrients in nitrogen metabolism in auxin-related pathways (Forde and Lorenzo 2001). In addition, AR formation and nitrate nutrition are intimately related. Generally, exogenous nitrate triggers dual effects on root growth depending on its concentration, promoting root growth at low concentrations. In contrast, a systemic inhibiting effect of nitrate at high concentrations was detected in Arabidopsis (Zhang et al. 1999, 2021). Our previous work elucidated the physiological and molecular mechanisms behind the inhibition of ARs by high nitrate supply in B9 apple rootstock, where high nitrate prevented ARs formation and development by causing a hormonal imbalance within the plant. Furthermore, transcriptome analysis revealed that high nitrate activated most genes involved in nitrate and hormone metabolism, while repressing genes involved with AR growth and development, and cell cycle (Zhang et al. 2021). However, nitrate abundance is usually low in the soil due to its high solubility, leaching tendency, and rapid acquisition by fungi and bacteria (López-Bucio et al. 2003).

The low-affinity transport system (LATS) and high-affinity transport system (HATS) are nitrate transportation mechanisms that are primarily responsible for the absorption, distribution, and storage of nitrate in higher plants (Crawford and Glass 1998). According to previous work, LATS participates in nitrate uptake at high external nitrate concentrations in the soil (> 1 mM) and HATS uptakes nitrate when the external nitrate concentration is low in the soil (< 1 mM) (Crawford and Glass 1998). Nitrate is promptly and strongly detected by plant cells and then the nitrate signaling pathway adapts the expression of a large number of gene sets to adjust cell and organ metabolism and growth. *NRT1.1*, a dual affinity nitrate transporter, serves in the auxin-mediated nitrate signaling pathway and modulates root architecture (Mounier et al. 2014). *NRT2.1*, a HATS-type gene, plays a key role in regulating root

development at low nitrate concentrations and is repressed at a high level (Little et al. 2005; Li et al. 2009). After transport to leaves, nitrate is reduced to ammonium through nitrate reductase (NR, NIA1, and NIA2) and nitrite reductase (NiR) in the cytoplasm and then carried to the chloroplast. Glutamic acid and glutamine are then produced by assimilation via glutamine synthetase (GS) and glutamate synthase (GOGAT) (Maeda et al. 2014; Plett et al. 2016). *ANRI* is a well-characterized nitrate-inducible gene that shows high involvement in nitrate-stimulated root elongation (Gan et al. 2005). However, the molecular mechanism underlying nitrate-induced AR formation in apples needs to be elucidated.

Previous investigations have found a relationship between the expression of auxin-related genes and the formation of ARs, comprising auxin influx (*AUX1*) and efflux (*PINs*) carriers, which play a key role in the basipetal movement of auxin and permit the local accumulation of auxin at the base of the stem (Da Costa et al. 2013). The polar transport of the PIN protein supports the gradient of auxin concentration in root tips, consequently regulating root growth and development (Grieneisen et al. 2007). Therefore, *AUX1*, *PIN1*, and *PIN2* may also have essential roles in AR formation and development in apple. Concerning the auxin signaling pathway, the degradation of AUX/IAA proteins promoted auxin response factors (ARFs) which activate the expression of auxin-responsive genes (Gray et al. 2001; Orman-Ligeza et al. 2013). Furthermore, the ARF gene family is involved in various processes related to plant growth, where the *ARF7* and *ARF19* genes exhibit highly distinctive and/or vigorous trends of gene expression during seedling growth, embryo development, and root formation in Arabidopsis (Okushima et al. 2005; Wilmoth et al. 2005). Interestingly, recent reports have suggested that ARFs regulate the induction of root formation by positively regulating the expression of LATERAL ORGAN BOUNDARIES DOMAIN16 (*LBD16*) and *LBD29*, where the overexpression of *LBD16* and *LBD29* induces LR formation in Arabidopsis (Okushima et al. 2007). *SHORT ROOT* (*SHR*) is strongly involved in developing the LR stem cell niche and AR meristem (Tian et al. 2014). The *ARRO-1* gene also promotes the formation of apple ARs (Smolka et al. 2009).

In this work, the underlying molecular mechanism driving ARs began to unfold. Subsequently, the effects of different nitrate treatments on root morphology, anatomical observation, and gene expression related to nitrate, IAA, AR development, and the cell cycle were analyzed. Relying on our findings, we elucidated how nitrate regulates AR formation in B9 apple rootstock by altering gene expression. Finally, the results of the present work provided a scientific basis for understanding the regulation of ARs in apple.

Materials and Methods

Plant Material, Growth Conditions, and Nitrate Treatments

B9 stem cuttings derived from Northwest Agriculture and Forestry University, Yangling, China, were tested as a plant material. Morphologically homogeneous cuttings were cultured under sterile conditions on 1/2 strength MS medium under a 16/8 h light/dark cycle at 25 ± 1 °C, followed by 8 h at 15 ± 1 °C. The relative humidity was 70–80%. First, 270 four-week-old cuttings were cultured on 1/2 MS medium, 30 g/L sugar, 1.2 mg/L IBA, 7.5 g/L agar, and pH 5.8 with three different concentrations of KNO_3 for 30 days. The 1/2 MS medium in its general formulation includes 9.4 mM/L KNO_3 , so the total amounts of KNO_3 for each treatment were T1, 9.4 mM/L; T2, 28.1 mM/L; and T3, 46.9 mM/L. Then, 720 fresh B9 stem cuttings were cultured on 1/2 MS medium, 30 g/L sugar, 1.2 mg/L IBA, 7.5 g/L agar, pH 5.8, and supplemented with two treatments (T2 and T3) of KNO_3 . Samples were collected at four stages after treatment: 0 days (competent cells), 3 days (cell cycle reactivation), 7 days (activation of AR primordia), and 16 days (outgrowth of ARs). Generally, the basal part of the stems (0.5 cm) is considered the rooting zone, and it was harvested as a sample from each cutting (Mao et al. 2020b; Meng et al. 2020). Ninety stem cuttings of B9 were randomly sampled at each time point for each treatment. The harvested samples were immediately dipped in liquid nitrogen and then stored at -80 °C until future experiments.

Measurement of Morphological and Anatomical Parameters

An Expression 10000XL scanner (Epson, Sydney, Australia) was used to measure the morphological parameters, including the root length (cm), root surface area (cm^2), root volume (cm^3), root projection area (cm^2), number of root forks, number of root tips, number of root crossing, and root average diameter (mm). Then, a WinRHIZO-Reagent instrument (Inc., Quebec, Canada) was utilized to analyze the images (Mao et al. 2020a). The collected samples for fixation, paraffin embedding, and sectioning were processed by previously reported protocols (Xu et al. 2012; Shah et al. 2020).

RNA Extraction and cDNA Synthesis

The basal portion of stems (0.5 cm) was used to extract RNA at 0, 3, 7, and 16 days after the treatments. Total RNA was extracted by a CTAB-based method (Gambino et al. 2008). Total RNA integrity was confirmed by running samples on

2% agarose gels. cDNA was synthesized by a Prime Script RT Reagent Kit with gDNA Eraser (TaKaRa Bio, Shiga, Japan).

RT-qPCR Analysis

The relative gene expression levels of genes related to nitrate transportation and assimilation (*MdNRT1.1*, *MdNRT2.1*, *MdNRT2.4*, *MdNRT2.5*, *MdNPF3.3*, *MdNIA1*, *MdNIA2*, *MdANR1*, *MdANR2*, *MdNIR*, *MdGS*, *MdGOGAT*, *MdNR*, and *MdATG18a*); auxin synthesis, transport, and signal transduction (*MdPIN1*, *MdPIN2*, *MdPIN3*, *MdARF7*, *MdARF22*, *MdARF19*, *MdIAA3*, *MdIAA23*, *MdIAA14*, and *MdAUX1*); root development (*MdLBD16*, *MdLBD29*, *MdWOX5*, *MdWOX11*, *MdARRO1*, and *MdSHR*); and the cell cycle (*MdCYCP4;1*, *MdCYCD1;1*, and *MdCYCD3;1*) were analyzed using RT-qPCR. The pair of specific primers was designed using Primer 6.0 (Table S1). The RT-qPCR assay was executed as reported by Li et al. (2016). The apple ACTIN gene was used for normalization. Three biological and technical replicates were performed for each treatment. The expression levels of analyzed genes were measured using the $2^{-\Delta\Delta\text{Ct}}$ method (Livak and Schmittgen 2001).

Statistical Analysis

The obtained data were analyzed using analysis of variance (ANOVA), and differences among the means were analyzed with an LSD test at the 0.05% level using Statistics 8.1 software (Tallahassee FL 32317 USA). The graphs were drawn with GraphPad Prism version 7.00 for Windows [(Shah et al. 2019), GraphPad Software, San Diego California, USA, www.graphpad.com].

Results

Effect of Nitrate Treatments on the Formation and Development of AR in B9 Apple Rootstock

To identify the suitable amount of nitrate, B9 apple rootstock cuttings were grown under different nitrate treatments. The nitrate-treated B9 apple rootstock cuttings exhibited substantial divergence in the formation and development of ARs (Fig. 1). By carefully examining the data, it was found that the T2 cuttings had the highest rooting percentage, which was 28.5% and 127% higher than those of the T1 and T3 cuttings, respectively (Fig. 2a). A massive difference in the number of roots was observed in the T2 cuttings (120.7), which was 285.1% higher than that in the T3 cuttings (Fig. 2b). The highest root length, 241 cm, was measured in the T2 cuttings, and the lowest was 34.2 cm in the T3 cuttings (Fig. 2c). As expected, the data on root volume

Fig. 1 Effect of nitrate treatments on the phenotype of ARs, scale bar = 1. The B9 stem cuttings were cultured on 1/2 MS medium, 30 g/L sugar, 1.2 mg/L IBA, 7.5 g/L agar, and pH 5.8 supplemented with three nitrate treatments: T1 (9.4 mM/L), T2 (28.1 mM/L), and T3 (46.9 mM/L) for 30 days

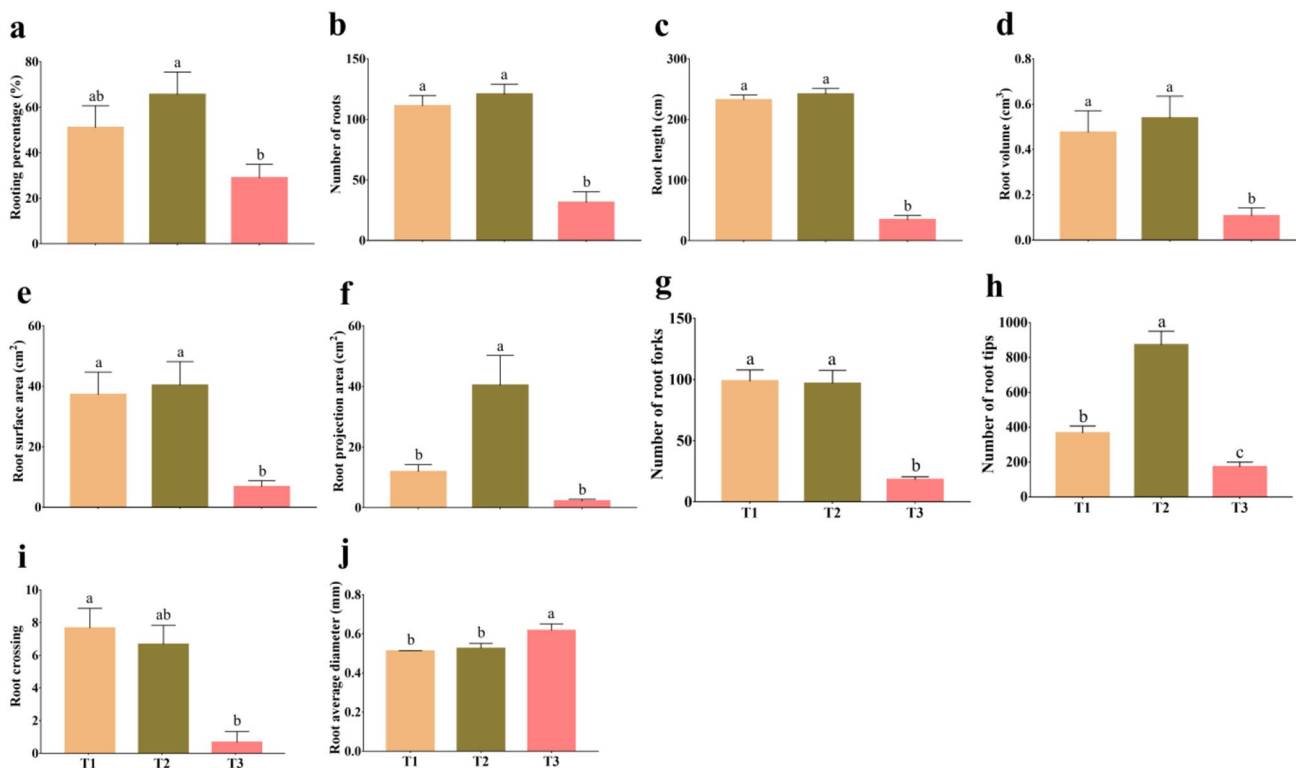
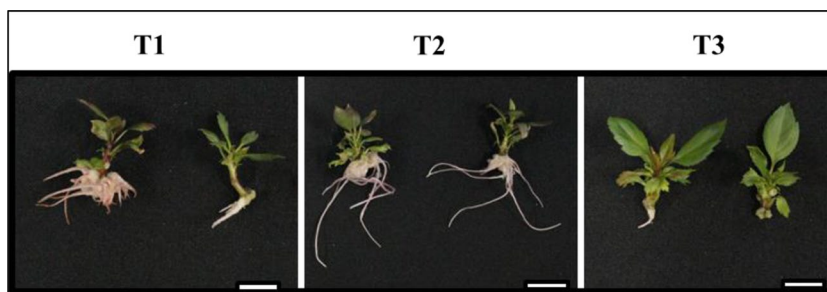


Fig. 2 Effect of nitrate treatments: T1 (9.4 mM/L), T2 (28.1 mM/L), and T3 (46.9 mM/L) on root morphological features: rooting percentage (a), number of roots (b), root length (cm) (c), root volume (cm³) (d), root surface area (cm²) (e), root projection area (cm²) (f), number of root forks (g), number of root tips (h), root crossing (i), and

root average diameter (mm) (j). The B9 stem cuttings were cultured for 30 days on nitrate treatments: T1 (9.4 mM/L), T2 (28.1 mM/L), and T3 (46.9 mM/L). Error bars refer to the average value \pm SD from three biological replicates. Different letters above columns indicate significant differences by LSD test at $P \leq 0.05$

(Fig. 2d), root surface area (Fig. 2e), and the number of root forks (Fig. 2g) were in line with the patterns observed for root length and root number (Fig. 2c, b). Moreover, the root projection area and the number of root tips had similar statistical results (Fig. 2f, h), where the maximum root projection, area 40.3 cm², and number of root tips, 871 were observed in T2, while the lowest were observed in T3 (2.2 cm² and 173, respectively). Root crossing was observed to be higher in the T1 and T2 cuttings than in the T3 cuttings (Fig. 2i). Unsurprisingly, the T3 cuttings had the highest root average diameter (0.67 mm) compared to other treatments (Fig. 2j). Based on the available data, T2 appeared to be a suitable

amount of nitrate for AR formation and development in the B9 apple rootstock.

Previously, it was proposed that root roughness is directly related to mineral and nutrient absorption abilities; based on the available data, the roots were categorized into three categories according to their respective diameters: 0–0.5 mm, 0.5–2 mm, and 2–5 mm (Nagata and Saitou 2009). The results of this classification are shown in Fig. 3. Our findings showed that the category 2–5 mm was generally lowest in all measured root parameters for all treatments. However, the root numbers and root length were found to be higher at 0.5–2 mm than in the other categories (Fig. 3). The root

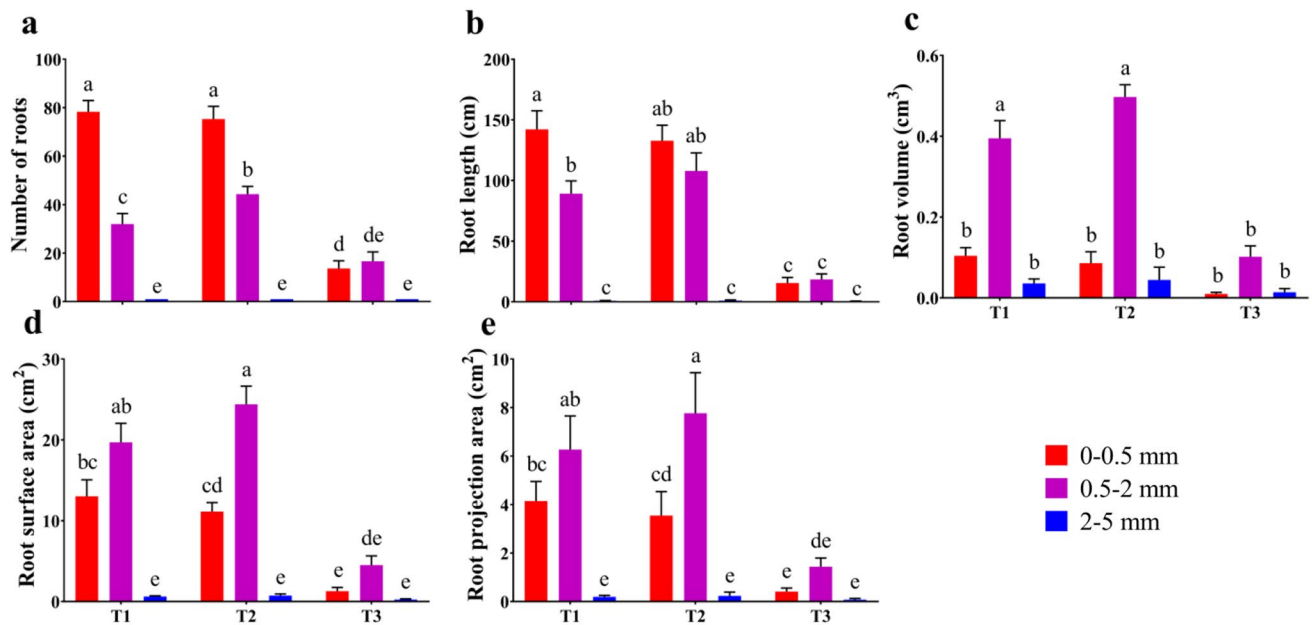


Fig. 3 Effect of nitrate treatments T1 (9.4 mM/L), T2 (28.1 mM/L), and T3 (46.9 mM/L) on the number of roots (a), root length (cm) (b), root volume (cm³) (c), root surface area (cm²) (d), and root projection area (cm²) (e). Roots were classified into three different size groups based on their diameter: 0–0.5 mm, 0.5–2 mm, and 2–5 mm. The B9

stem cuttings were cultured for 30 days on nitrate treatments. Error bars refer to the average value \pm SD from three biological replicates. Different letters above columns indicate significant differences by LSD test at $P \leq 0.05$

surface area and root projection area shared similar statistical values/patterns among all diameters in all treatments. It was quite astonishing to find that the root volumes in the categories between 0–0.5 mm and 0.5–2 mm have similar statistical patterns (Fig. 3). Taken together, these results suggest that the roots obtained from T2 were superior to the roots from the other treatments.

Morphological and Anatomical Observations of AR Formation in Stem Cuttings of B9 Apple Rootstock

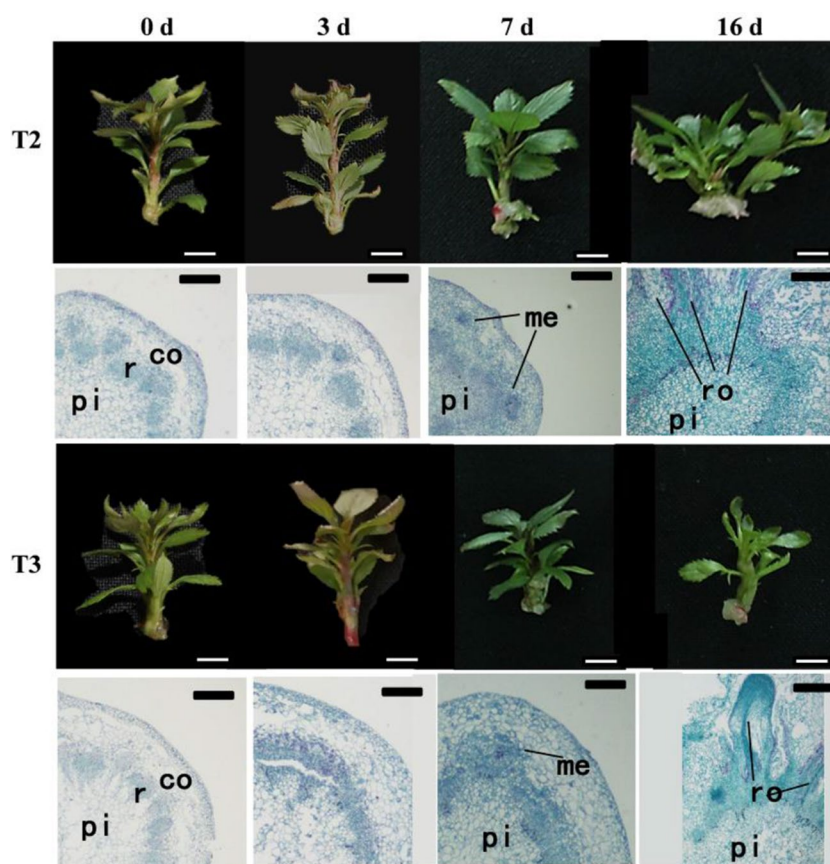
Morphological and anatomical observations were also carried out in this work. The results of this work were analyzed and shed light on the understanding of AR formation during the time course of this study (0 day, 3 days, 7 days, and 16 days). Within 3 days, no morphological variations were detected between the T2 and T3 cuttings. In comparison, on day 7, AR emergence at the stem base was visible in the T2 cuttings but was not observed in the T3 cuttings. Furthermore, at 16 day, it was not surprising to find that the T2 cuttings started to produce more ARs than the T3 cuttings (Fig. 4). Moreover, anatomical observations were performed with paraffin-embedded samples, and a light microscope (Nikon Eclipse E100) was utilized to examine stem fragments (Ahkami et al. 2009). On day 0, cross-sections of the samples revealed the presence of the cortex (co), pith parenchyma (pi), and ring of vessels (r). More first root meristems

(me) were observed in T2 cuttings than in the T3 cuttings at 7 days. Finally, on day 16, the T2 cuttings showed more root cortex (ro) relative to the T3 cuttings (Fig. 4). Our findings demonstrated that the application of the optimum level of nitrate was an effective method of increasing AR formation in B9 apple rootstock.

Effect of Nitrate Treatments on the Expression of Nitrate-Related Genes

This section determined the expression levels of nitrate transporters and nitrate assimilation-related genes by RT-qPCR under the nitrate treatments during the time course of the study (Fig. 5). After rigorous examination, it was found that the relative expression of *MdNRT1.1* and *MdNRT2.1* was higher in T2 cuttings than in T3 cuttings (Fig. 5). These results indicate that these genes play an essential role in nitrate transportation during AR formation and development in B9 apple rootstock; conversely, the expression of *MdNRT2.4* and *MdNRT2.5* was significantly higher at 3 days, and then decreased levels were detected at other time points in the T2 cuttings (Fig. 5). Furthermore, *MdNPF3.3*, *MdANR1*, and *MdNIR* shared parallel expression pattern levels with a significant increase at 16 days in T2 cuttings relative to T3 cuttings (Fig. 5). *MdNIA1* and *MdNR* established significantly higher expression levels at 3 days and 16 days, but no significant difference was observed at 7 days

Fig. 4 Morphological and anatomical observations of AR formation in stem cuttings of B9 apple rootstock at 0 day, 3 days, 7 days, and 16 days. Scale bars for morphological and anatomical photos are 1 cm and 100 μ m, respectively. The stem cuttings were cultured on half-strength MS medium with two nitrate treatments: T2 (28.1 mM/L) and T3 (46.9 mM/L). Cortex (co), pith parenchyma (pi), ring of vessels (r) root meristems (me), and root cortex (ro)



in T2 cuttings in comparison with T3 cuttings (Fig. 5). *MdGS* and *MdATG18a* demonstrated a range of induction responses in the T2 cuttings, with noticeable enrichment at 3 days (Fig. 5). However, significantly decreased or no variation in the expression levels of *MdNIA2*, *MdANR2*, and *MdGOGAT* was perceived in T2 cuttings at all time points, excluding *MdGOGAT* at 7 days in comparison with T3 cuttings (Fig. 5). This section offers conclusions and identifies avenues for further research work related to nitrate-regulated ARs.

Effect of Nitrate Treatments on the Expression of IAA-Related Genes

The effects of nitrate treatments on the expression of IAA synthesis-, transport-, and signal transduction-related genes were assessed by RT-qPCR (Fig. 6). This section describes the divergent expression pattern of IAA-related genes at different time points during this study. From our results, we found that *MdPIN1*, *MdPIN2*, and *MdPIN3* had notably higher expression levels at 3 days and 7 days in the T2 cuttings, whereas at 16 days, no significant difference was observed between the two treatments (except in *MdPIN2*, 3 expression) (Fig. 6). In addition, the transcript abundances of *MdARF7* and *MdARF19* were higher at 3 days and 7 days

and then significantly decreased at 16 days, while *MdARF22* expression was higher at 3 days and then gradually declined toward 7 days and 16 days in the T2 cuttings compared to the T3 cuttings (Fig. 6). *MdIAA14* and *MdIAA23* showed similar expression patterns at all time points; however, the expression of both genes increased in T2 cuttings at 3 days and 7 days and suddenly decreased at 16 days (Fig. 6). The expression of *MdIAA3* was higher at 3 days and lower at 7 days and 16 days in the T2 cuttings. In contrast, the relative transcript abundance of *MdAUX1* was apparently upregulated in the T2 cuttings at all time points in comparison with that in the T3 cuttings (Fig. 6). This portion of the study summarizes the findings related to the effects of the nitrate treatments on IAA-related genes that promote AR formation in B9 apple rootstock.

Effect of Nitrate Treatments on the Expression of AR Development- and Cell Cycle-Related Genes

Next, the relative expression levels of root development- and cell cycle-related genes associated with AR formation were also determined using RT-qPCR. The resulting data were assessed at multiple stages of AR formation (Fig. 7). From our findings, we established that the relative transcript abundance of *MdCYCP4;1* and *MdCYCD1;1* was

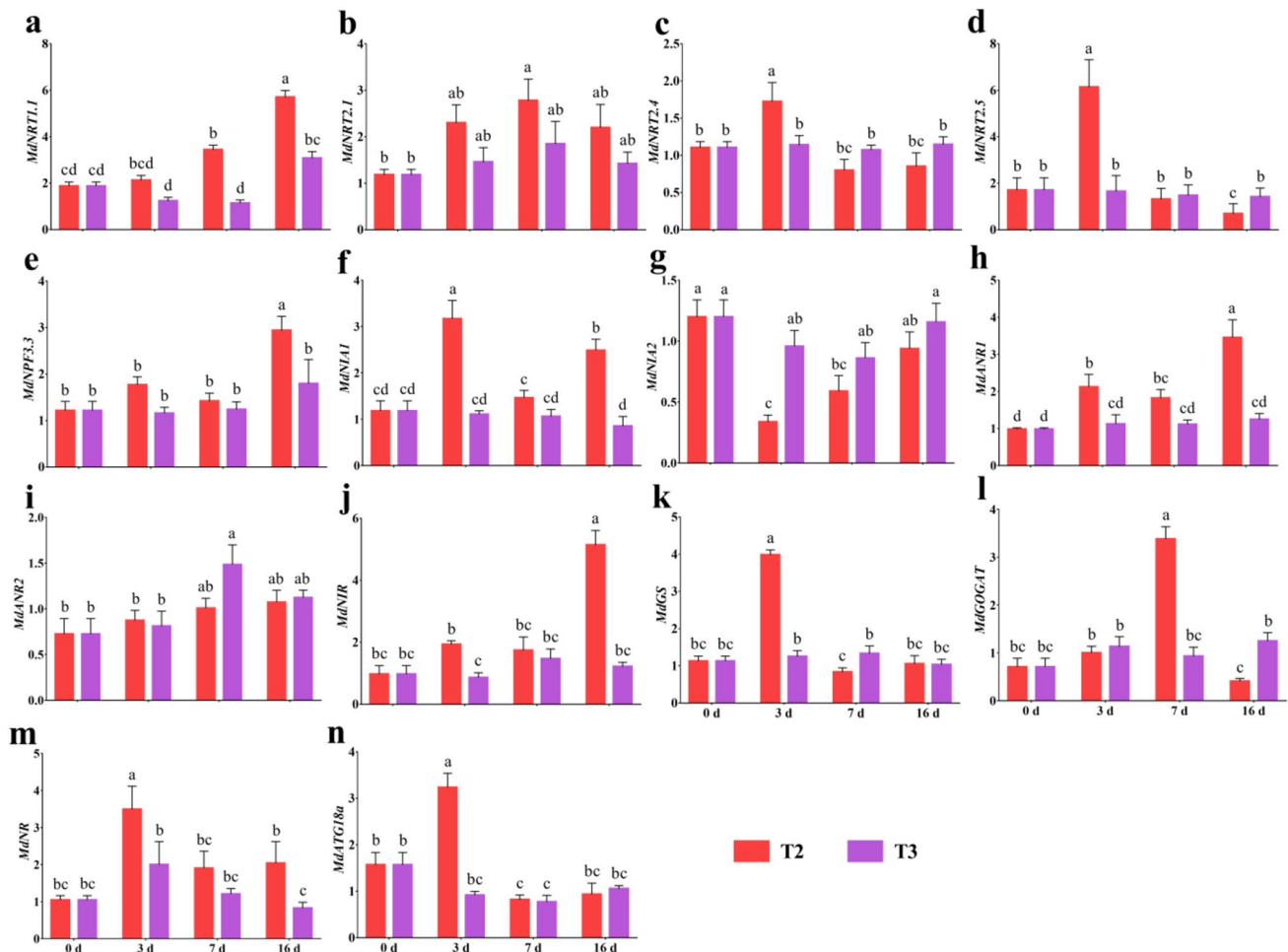


Fig. 5 Effect of nitrate treatments T2 (28.1 mM/L) and T3 (46.9 mM/L) on the relative expression of nitrate-related genes, including *MdNRT1.1* (a), *MdNRT2.1* (b), *MdNRT2.4* (c), *MdNRT2.5* (d), *MdNPF3.3* (e), *MdNIA1* (f), *MdNIA2* (g), *MdANR1* (h), *MdANR2* (i), *MdNIR* (j), *MdGS* (k), *MdGOGAT* (l), *MdNR* (m),

and *MdATG18a* (n) over the time course of the study (0 day, 3 days, 7 days, and 16 days) during AR formation. Error bars refer to the average value \pm SD from three biological replicates. Different letters above columns indicate significant differences by LSD test at $P \leq 0.05$

significantly upregulated in T2 cuttings at all sampling points, which indicates the strong influence of these genes on the AR induction, initiation, and development stages (Fig. 7). Moreover, the relative expression of *MdCYCD3;1*, *MdWOX11*, and *MdSHR* was also higher in T2 cuttings than in T3 cuttings at some time points (Fig. 7). The highest expression of *MdARRO1* was observed on day 3 in T2 cuttings, which gradually decreased at other time points. On the other hand, the expression levels of *MdLBD29* and *MdWOX5* were initially lower in T2 cuttings and then continuously increased in the expression of *MdLBD29* toward 16 days (Fig. 7). Furthermore, *MdLBD16* had significantly high expression at 7 days and lower expression at 16 days, but no difference was observed at 3 days in T2 cuttings. This section summarizes the discoveries related to and the influences of nitrate treatment on AR development- and

cell cycle-related genes for increasing AR formation and development in B9 apple rootstock.

Discussion

AR formation is crucial for the vegetative propagation of horticultural plants. It is a complicated biological process that involves variations in the expression of multiple sets of genes. The process of AR formation is typically divided into four independent phases, namely, 0–1 day (activation phase), 1–3 day (induction phase), 3–7 day (initiation phase), and after 7 days AR emergence phase (De Klerk et al. 1997). The AR induction stage represents an influential point for molecular reprogramming, while the AR initiation stage is important for AR formation, as the AR primordia are formed

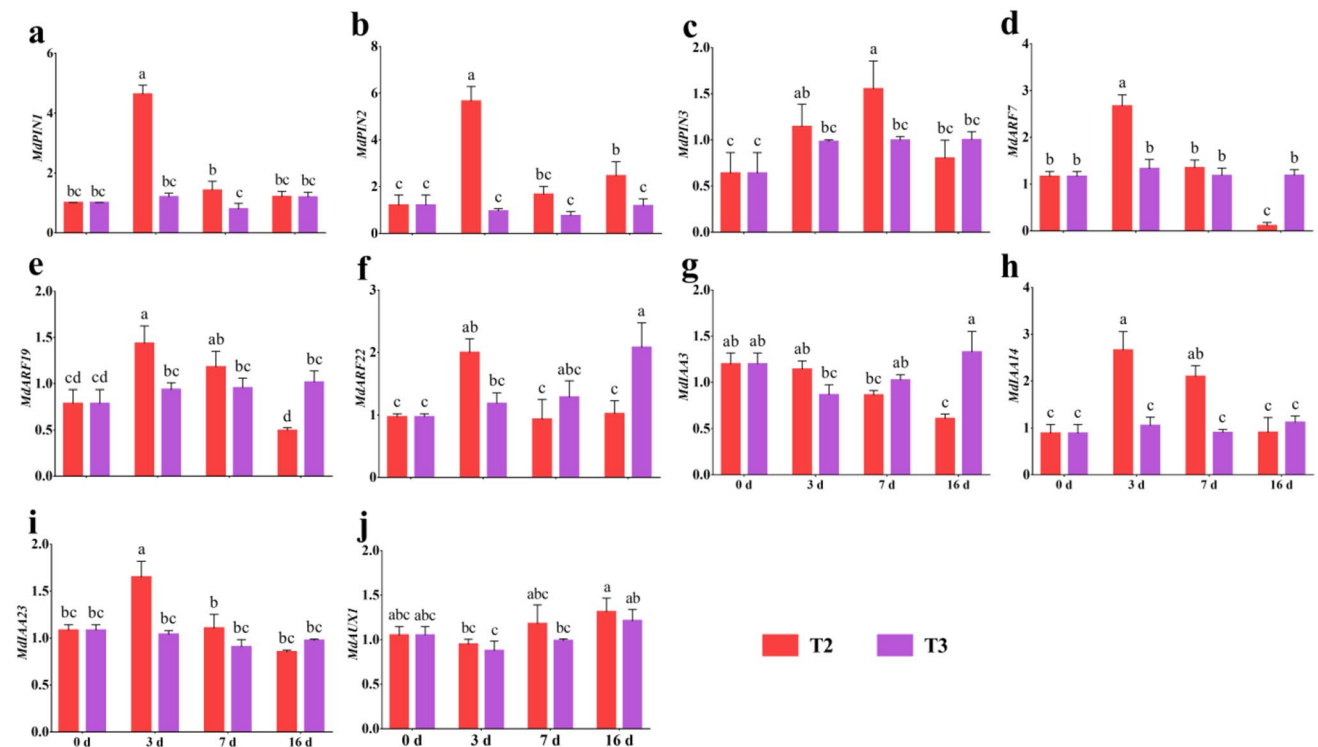


Fig. 6 Effect of nitrate treatments T2 (28.1 mM/L) and T3 (46.9 mM/L) on the relative expression of IAA-related genes, including *MdPIN1* (a), *MdPIN2* (b), *MdPIN3* (c), *MdARF7* (d), *MdARF19* (e), *MdARF22* (f), *MdIAA3* (g), *MdIAA14* (h), *MdIAA23* (i), and

MdAUX1 (j) over the time course of the study (0 day, 3 days, 7 days, and 16 days) during AR formation. Error bars refer to the average value \pm SD from three biological replicates. Different letters above columns indicate significant differences by LSD test at $P \leq 0.05$

at this phase. If the number of AR primordia is limited, it will reduce the number of ARs (Li et al. 2015b; Guan et al. 2015). Morphological and anatomical observations have been performed in apple, where the AR emergence stage and an increase in first root meristems appeared at 7 days in *Malus prunifolia* var. Ringo rootstock (Wang et al. 2020). Our results are in line with these observations, as the AR emergence stage was observed in T2 cuttings at 7 days, and at 16 days, the T2 cuttings started to produce more ARs than the T3 cuttings (Fig. 4). Additionally, anatomical observations revealed that an increase in first root meristems was detected at 7 days, and more root cortex appeared in the T2 cuttings at 16 days than in the T3 cuttings (Fig. 4). Our observations provide essential information pertaining to the AR formation process in B9 apple rootstock.

Previous study has proposed that low nitrate promotes ARs formation and high nitrate inhibits it by JA and CTK signaling in B9 apple rootstocks (Zhang et al. 2021). Furthermore, the regulation of root formation and development by nitrate is not exerted through the direct perception of external nitrate but relies on the magnitude of nitrate absorbed by the plant (Zhang and Forde 2000; Zhang et al. 1999). Moreover, the effect of exogenous nitrate on LR elongation is greatly restricted by the increase in the

meristematic activity of the mature LR tips (Remans et al. 2006b; Zhang and Forde 2000; Zhang et al. 1999). Schwambach et al. observed higher AR numbers under a nitrate treatment in *Eucalyptus globulus* microcuttings (Schwambach et al. 2015); consequently, a sufficient nitrate amount is also crucial for root elongation. Furthermore, the magnitude of the root surface area is directly associated with the area available for root absorption, which is essential indicator of root activity. Root diameter is also an important indicator for root activity; fine roots have a key function in the absorption of minerals and nutrients. The results presented here are in accordance with previous findings; the AR morphological characteristics were increased by the adequate (T2) provision of nitrate in comparison with other treatments (Figs. 1, 2, 3).

NRT genes have diverse biological functions during the uptake and distribution of nitrate within the plant body (Wang et al. 2012). Two distinct gene families, *NRT1* and *NRT2*, encode low- and high-affinity nitrate transporters, respectively. It has been reported that the expression of *NRT1.1* is induced by nitrate, and it has a main function in the nitrate signaling pathway, which leads to increased rates of root elongation in *Arabidopsis* (Remans et al. 2006b, 2006a). In addition, Krouk et al. found that *NRT1.1* provides

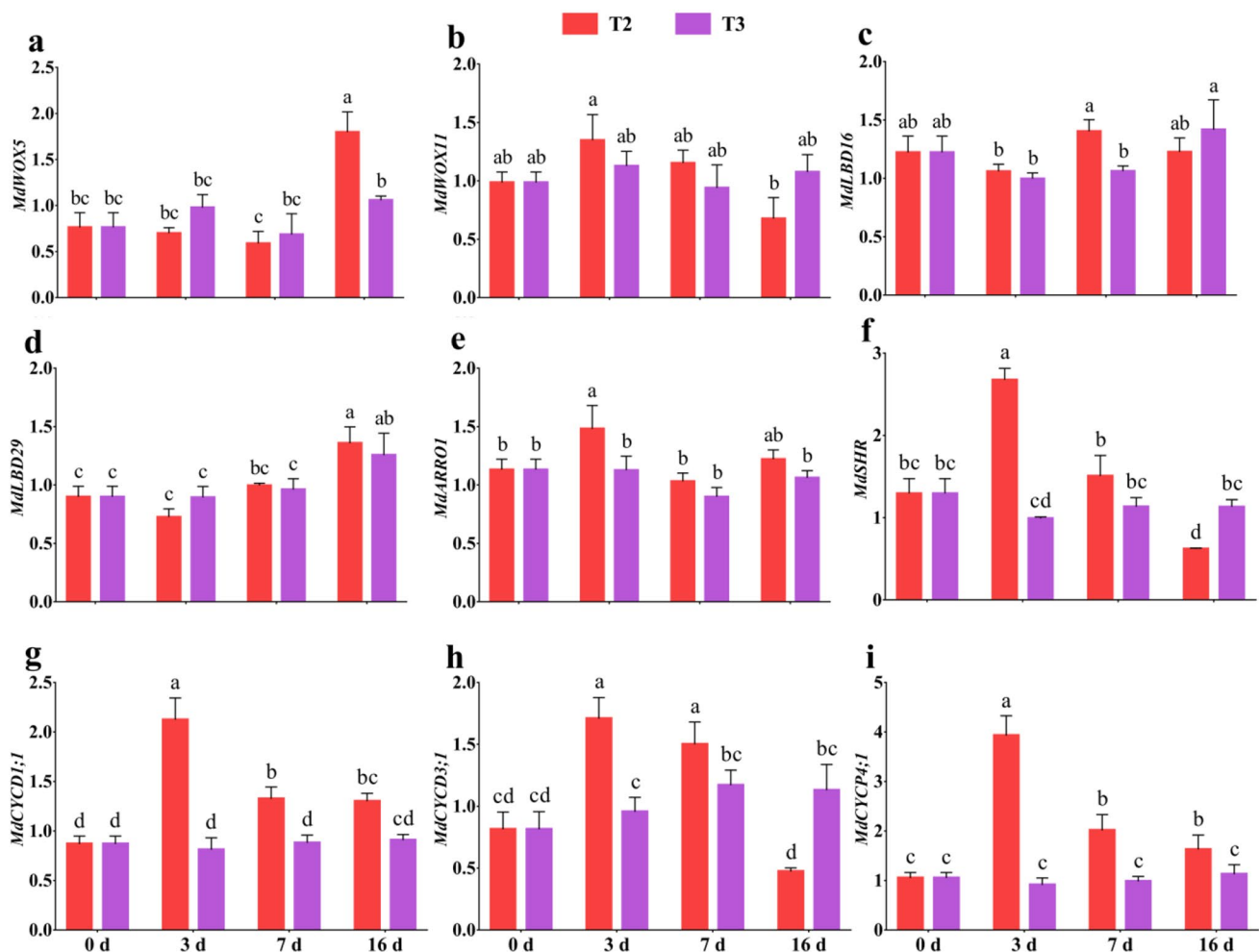


Fig. 7 Effect of nitrate treatments T2 (28.1 mM/L) and T3 (46.9 mM/L) on the relative expression of AR development- and cell cycle-related genes, including *MdWOX5* (a), *MdWOX11* (b), *MdLBD16* (c), *MdLBD29* (d), *MdARRO1* (e), *MdSHR* (f), *MdCYCD1;1* (g), *MdCYCD3;1* (h), and *MdCYCP4;1* (i) over the time

course of the study (0 day, 3 days, 7 days, and 16 days) during AR formation. Error bars refer to the average value \pm SD from three biological replicates. Different letters above columns indicate significant differences by LSD test at $P \leq 0.05$

support in auxin transport, suggesting that transduction of nitrate signals by *NRT1.1* is related to auxin transport (Krouk et al. 2010). Our present findings confirm this, as the expression of *MdNRT1.1* was higher in T2 cuttings at all time points, suggesting that *MdNRT1.1* has a key role in the nitrate signaling pathway, which turns to increase in root growth. We also observed that *MdNRT2.1* had a prominent effect on the regulation of ARs by a moderate nitrate supply (T2) in B9. Moreover, the relative transcript abundance was observed to be higher in response to T2 during the course of this study; however, the expression indicated first increasing trend at 3 days and 7 days, and decreased expression levels were observed toward 16 days in both treatments (Fig. 5), which was parallel with the *CsNRT2.1* expression profile (Li et al. 2018). *NRT2.1* expression and nitrate cellular contents under different nitrate application levels expose the

complicated regulation of nitrate homeostasis and nitrogen metabolism during plant growth (Fraisier et al. 2000). Additionally, the expression of *NRT2.1* relies on the N demand inside the plant body. In addition, for HATS regulation, the plant regulates the spatial condition of RSA to endure adaptable nitrate provision; in Arabidopsis, RSA is possibly regulated by both exogenous and endogenous concentrations of nitrate (Krapp et al. 2014). In higher plants, the *ANR1* gene was the first characterized regulatory factor involved in the modulation of root architecture by changes in external nitrate (Zhang and Forde 1998). From our results, *MdANR1* expression was higher at 3 days, 7 days, and 16 days during AR formation, which confirms its positive role in AR formation in B9 apple rootstock (Fig. 5). *MdATG18a*, an ATG gene from apple, is functionally involved in nitrate transport and assimilation by upregulating the expression

of high-affinity nitrate transporters (*MdNRT2.1/2.4/2.5*) and nitrate reductase (*MdNIA2*) (Sun et al. 2018). In the present study, we found that *MdATG18a* might positively control nitrate uptake and assimilation during AR formation by affecting the expression of the nitrate transporters *MdNRT2.1*, *MdNRT2.4*, and *MdNRT2.5*; conversely, it did not influence *MdNIA2* (Fig. 5). *NIR* and *NR* contribute to the process of reducing nitrate to ammonium in coupled regulation. *NR* is induced by nitrate, and its expression depends on external nitrate (Kovács et al. 2015). From our findings, *MdNR* and *MdNIR* shared similar expression patterns/trends in both treatments, and upregulated expression appeared in T2 cuttings compared to T3 cuttings (Fig. 5), which indicated that these genes may play a key role during AR formation in B9 apple rootstock. However, more research work is needed to clarify the functions of nitrate-related genes during AR formation.

PINs play essential roles in the regulation of root formation and development (Grieneisen et al. 2007), and the level of auxin can promote *AUX1* expression (Benková et al. 2003). Recently, Xu et al. observed in rice mutants that the gene expression of *OsPIN1* was involved in the transport of polar auxin and influenced the processes of AR emergence and tillering; they established that auxin allocation and concentration levels are essential in various plant organs (Xu et al. 2005). Moreover, members of the PIN family control the auxin efflux system and act as promoters of auxin efflux in the plasma membrane, which is vital for root growth. However, not every PIN mutation affects root growth; some PIN mutations result in major defects in root formation processes (Benková et al. 2003). Interestingly, this work showed that the transcript abundance of *MdPIN1*, *MdPIN2*, and *MdPIN3* was clearly upregulated during the induction and initiation stages in T2 cuttings, which indicated that these genes have a major role in the regulation of ARs in B9 (Fig. 6). The mutation of *AUX1* decreases IAA accumulation in the roots of young seedlings; it behaves as an auxin influx carrier and promotes LR growth through IAA distribution between shoots and roots (Marchant et al. 2002). In this study, the *AUX1* expression was apparently higher in T2 cuttings during AR induction, initiation, and emergence stages (Fig. 6). Previously, *arf7* and *arf19* single mutants decreased the LR and AR numbers, although double mutants of *arf7* and *arf19* showed very fewer LRs and ARs (Okushima et al. 2007; Wilmoth et al. 2005). In Arabidopsis, it was detected that *ARF7* and *ARF19* are directly involved in the activation of *LBD16* and *LBD29* to induce root formation (Okushima et al. 2007). Our work shows that the transcript abundance of *MdARF7* and *MdARF19* was higher at 3 days and 7 days and significantly lower at 16 days (Fig. 6), while *MdLBD16* and *MdLBD29* expression was upregulated at 7 days and 16 days (except *MdLBD16* at 16 days) in response to T2 cuttings (Fig. 7). Thus, *MdARF7*

and *MdARF19* regulated the expression of *MdLBD16* and *MdLBD29*, which in turn regulated the process of AR formation in B9 apple rootstock. Auxin induces *WOX11* at the first stage of cell fate transition, which leads to upregulate the relative expression of *LBD16* and *LBD29* during AR formation in Arabidopsis (Liu et al. 2014). The combined expression of these genes promoted LR growth by the upregulation of cell cycle-related genes (Mao et al. 2018). Similar gene expression patterns were also perceived in this study, where the relative expression of *WOX11* was upregulated at 3 days and 7 days, and the transcript abundance of *MdCYCD1;1*, *MdCYCD3;1*, and *MdCYCP4;1* was higher at all time points (except *MdCYCD3;1* at 16 days) in response to T2 cuttings than T3 cuttings (Fig. 7).

Conclusion

This study found that nitrate treatment triggers the process of AR formation in B9 apple rootstock by promoting the expression of nitrate- and auxin-related genes, which may lead to an increase in auxin levels. In addition, *MdARF7* and *MdARF19* regulated the transcript abundance of *MdLBD16* and *MdLBD29*, and the upregulation of *MdSHR* and *MdARRO1* together promoted AR formation. This phenomenon was related to nitrate-mediated pathways of interaction with auxin. *MdWOX11* may be induced by auxin, which leads to the regulation of the relative expression of *MdLBD16* and *MdLBD29*; the collective effects of these genes stimulate AR formation by upregulating cell cycle-related genes. Our work suggests the association of particular genes and pathways in nitrate-mediated AR formation. Some essential questions remain; the specific mechanism related to the crosstalk between nitrate and hormones needs research in future work and might be helpful in identifying the mechanism of AR formation.

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Author Contributions MMT is the main author. He collected all test data performed statistical analyses, interpreted the results, and drafted the manuscript. LZ, WC, KS, LS, and ZX gave help in the section of the results of the manuscript. MJ, LY, AS, and LK mainly helped in the paper discussion. BL and ZD helped in the paper's writing and are the leaders of our team.

Declarations

Conflict of interest The authors declare no conflict of interest.

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