miRNAs associated with auxin signaling, stress response, and cellular activities mediate adventitious root formation in apple rootstocks

Ke Li, Zhen Liu, Libo Xing, Yanhong Wei, Jiangping Mao, Yuan Meng, Lu Bao, Mingyu Han, Caiping Zhao**, Dong Zhang*

Department of Horticulture College, Northwest Agriculture & Forestry University, Yangling, 712100, China

ABSTRACT

Adventitious root (AR) formation is essential for the vegetative propagation of apple rootstocks. miRNAs play a significant role in regulating AR development, however, large-scale transcriptomic data on miRNA mediated AR formation in apple rootstocks is lacking. Therefore, in order to identify the molecular mechanisms underlying AR formation in ‘M9-T337’ apple rootstocks, transcriptomic changes occurring during key time points of AR formation (0, 3, and 16 days) were analyzed using high-throughput sequencing with a focus on miRNAs. A total of 84 known miRNAs and 56 novel miRNAs have differentially expressed were identified. Additionally, a total of 88 target genes of known miRNAs and 76 target genes of novel miRNAs were identified by degradome sequencing. The expression levels of the miRNAs and target genes were quantified by RT-qPCR. Results indicate that miRNAs and their target genes are associated with auxin signal-related (miR160 and miR390), stress response-related (miR398, miR395 and miR408), cell fate transformation-, proliferation- and enlargement-related (miR171, miR156, miR166, miR319 and miR396). These all involve pathways that participate in AR formation in ‘M9-T337’ apple rootstock. In addition, hormones (AUX, CTK, GA3, BR, JA, and ABA) are also involved in regulating AR formation. The candidate genes belonging to pathways associated with AR formation exhibited significantly higher expression levels, providing evidence that they may be involved in the regulation of AR development. The collective results of the present study indicate that the developmental process associated with AR formation in apple rootstock is extremely complex. The known and novel miRNAs and target genes that were identified by high-throughput and degradome sequencing, respectively, provide a framework for the future analysis of miRNAs associated with AR development in apple rootstocks, and provide new information that can be used to better understand AR development in woody plants.

1. Introduction

Apple (Malus x domestica Borkh.) is a widely consumed fruit crop worldwide, and China is the leading apple-producing country. There has been a major breakthrough in the development of dwarf and compact planting techniques for the modern apple industry in recent decades. The ‘M9-T337’ dwarfing apple rootstock is widely used and confers early fruiting and high yields. In order to maintain the genetic uniformity of rootstocks, the vegetative propagated method of mound layering and cutting propagation are widely used and the ability of AR formation plays a key role in that process. Breeding of dwarf rootstocks appropriate for the needs of the apple industry in China, however, has been a bottleneck. Therefore, the study of the molecular mechanisms of AR formation has both important theoretical and practical value for apple rootstock breeding and improvement.

AR formation is a complex process that emerges from stems, leaves or hypocotyls, being strategic for clonal propagation, and ARs may develop spontaneously, upon environmental stress or hormonal treatment; auxins strongly influence AR (Da Costa et al., 2018). A synthetic form of auxin, NAA and 2, 4-D also are widely used to induce AR formation in cuttings plants (Da Costa et al., 2018). In addition, stress signals are also important factors in AR formation. For example, a series of stress signal induced by injury results in the accumulation of endogenous auxin, triggering a series of cell cycle reactions (Zhou et al.,...
2018). However, the mechanism of AR formation is unclear, and still need further study.

MiRNAs play an important role in regulating plant development (Yan et al., 2018). Individual miRNAs, however, cannot regulate root development but rather must be associated with their target genes. For example, miR160 functions as a key controller of root cap cell formation, and also targets ARF17 to regulate AR initiation (Gutierrez et al., 2009). miR390, TAS3-derived trans-acting short-interfering RNAs (ta-siRNAs), and ARFs form an auxin-responsive regulatory network that controls lateral root initiation and growth (Marín et al., 2010). In addition, the interaction of miR393 with TIR1 (transport inhibitor response protein 1) modulates auxin sensitivity and affects primary root growth and lateral root emergence (Chen et al., 2011). Recently, a novel regulatory pathway involving bidirectional cell signaling mediated by miR165/miR166 and the transcription factors SHR (SHORT ROOT)/SCR (SCARECROW) has been identified as determining root cell fate (Carlsbecker et al., 2010). Elevated levels of miR156 promote adventitious root formation in maize, tomato, and tobacco (Feng et al., 2016). Overall, development of the root is clearly a dynamic process that requires the integration of plant hormones, transcriptional regulators, and small RNAs to produce the correct developmental outcome.

2. Material and methods

2.1. Plant material

Micro-propagated ‘M9-T337’ apple rootstock plantlets were grown in tissue culture at the Northwest Agriculture and Forestry University, Yangling, China. The tissue culture cuttings were maintained under a 16 h light at 25 ± 1 °C, followed by 8 h dark at 15 ± 1 °C. Stems of the microcuttings were treated with indole-3-butyric acid (IBA), which is widely used to promote adventitious rooting. The rooting medium was composed of 1/2 MS, 1 mg.L-1 IBA, 20 g.L-1 sugar and 8 g.L-1 agar, pH 5.8. The medium without IBA was used to determine root cell fate (Carlsbecker et al., 2010). Elevated levels of miR156 promote adventitious root formation in maize, tomato, and tobacco (Feng et al., 2016). Overall, development of the root is clearly a dynamic process that requires the integration of plant hormones, transcriptional regulators, and small RNAs to produce the correct developmental outcome (Meng et al., 2010). While the mentioned studies reveal the initial mechanism of AR initiation by some specific miRNAs, studies on the genome-wide identification of miRNAs regulating AR development in apple rootstocks is still lacking.

The main objective of the current study was to provide a framework of miRNAs associated with AR development in apple rootstocks at a whole genome level. High-throughput sequencing and a degradome library were used to identify miRNAs and their target genes, respectively, which were associated with AR formation in ‘M9-T337’ apple rootstocks. Hormone levels were also monitored. The combined analyses were used to provide the first global monitoring of changes occurring in the expression levels of miRNAs during AR formation in ‘M9-T337’ apple rootstocks. These findings contribute to the basic understanding of the molecular events underlying AR formation in apple rootstocks and provide direction for future research on AR formation in woody species.

2.2. Determination of hormone levels

Indole-3-acetic acid (IAA), zeatin riboside (ZR), Gibberellic acid 3 (GA3), Brassinolide (BR), Jasmonic acid (JA), and Abscisic acid (ABA) contents between stem cuttings harvested from IBA-treatment and control. Approximately 0.6 g fresh weight of stems collected during the AR formation and levels were determined using an indirect ELISA technique which was conducted at the Center of Plant Growth Regulator, China Agricultural University as described by Zuo et al. (2018). Detailed descriptions of the extraction and quantification methods are provided in our previous research (Fan et al., 2016). Three biological replicates were used for each hormone analysis (200 mg per replicate).

2.3. RNA library construction and high-throughput sequencing

RNA library construction followed the methods described in Xing et al. (2014). Total RNA was extracted at the three key time points of AR formation (0, 3, and 16 days). RNA extracts were obtained from three biological replicates (60 cuttings per replicate, totalling 180 cuttings) at each of the time points using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The RNA from each of the pooled extracts was used for small RNA library construction. The small RNAs with adapters were transcribed into cDNA using Super-Script II Reverse Transcriptase (Invitrogen, Shanghai, China). Lastly, the cDNA products were amplified by PCR and the purified PCR products were then sequenced by the Biomarker Biotechnology Corporation (Beijing, China) using an Illumina Genome Analyzer HiSeq2500. Total RNA from each of the pooled samples at the three time points was also used for degradome library construction and sequencing.

2.4. Profiling and differential expression analysis of known and novel miRNAs

The criteria for the sequence quality control was based on the studies of Xing et al. (2014) and included: 1) The removal of low-quality reads (base mass below 30 in more than 20% of the sequences); 2) removal of reads containing unknown bases (N) greater than or equal to 10% of the sequence; 3) removal of the 3’ end connector and barcode sequence; and, 4) removal of sequences shorter than 18 nt or longer than 30 nt. Sequence alignment and subsequent analysis were performed using the Malus x domestica Whole Genome v1.0p Assembly (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF_000148765.1_MalDomGD1.0/GCF_000148765.1 MalDomGD1.0_genomic.fna.gz) as a reference genome. mirDeep2 (Friedlander et al., 2012) software was used to compare unannotated reads with the reference genome to obtain position information within the reference genome, in order to map the unannotated reads.

DESeq (http://precedings.nature.com/documents/4282/version/2) was used to differentially expressed miRNAs. The criteria for defining differential expression of miRNAs were |log2 (FC)| ≥ 1 (Fold Change, FC) and FDR ≤ 0.01 (False Discovery Rate, FDR). Fold Change (FC) represents the ratio of the expression between two samples (groups). In addition, the accepted Benjamini-Hochberg correction method was used to correct the p value (p-value) of the original hypothesis test, and the false discovery rate (FDR) was used as the key index for initially identifying differentially expressed miRNAs.

2.5. Prediction, identification, and annotation of targets of miRNA

TargetFinder (http://carringtonlab.org/resources/targetfinder) software was used to predict the potential targets of both the known and novel miRNAs by matching the miRNA sequences to the apple genome (Malus x domestica Borkh.). In addition, Some targets of known and novel miRNAs were identified by degradome sequencing, which the degraded fragments were matched to the apple genome (Malus x domestica Borkh) after the removal of mRNAs and polyN fragments in order to reduce interference (Xing et al., 2014). Shear site detection was performed using CleaveLand software (Addoquaye et al., 2009). Set condition P-value < 0.05. Sequence alignment of target genes with NR,
Swiss-Prot, GO, KEGG, COG databases using BLAST software, obtain annotation information for the target genes.

2.6. RT-qPCR analysis of the expression levels of putative miRNA and target genes

The expression patterns of candidate DEGs, including auxin-, stress response-, and cell fate-, and AR formation-related genes, were validated by RT-qPCR. The sequences of the designed primer pairs were selected based on apple data (Malus × domestica) that were published in GenBank using Primer 6.0 software. RT-qPCR was conducted as described in previous research (Xing et al., 2014). An apple ACTIN gene was used for normalization. Each of the analyzed samples consisted of three biological and technical replicates. The 2^ΔΔCt method was used to calculate the relative expression of the analyzed genes (Livak and Schmittgen, 2001). The sequences of the utilized gene-specific primers are listed in Table S1.

2.7. Statistical analysis

Differences among means were evaluated with the Statistical Program for Social Science 19 (SPSS19, Chicago, IL, USA) using a two-tailed t-test at the 5% level. Statistical processing of plant phenotype data, hormone content, and RT-qPCR data was performed in Microsoft Excel (2010). Diagrams were generated in Microsoft Excel (2010) and OriginPro 7.5 (OriginLab Software, Inc.).

3. Results

3.1. IBA-induced changes in morphology and hormone levels during AR formation

The outline of ARs could be observed at the stem base during the elongation phase when the callus tissue expanded into a spheroid shape. Callus formation was observed at S1 (Fig. 1A). Indole-3-acetic acid (IAA) and zeatin riboside (ZR), an endogenous auxin and cytokinin, respectively, were the most abundant hormones measured. IAA and ZR levels were relatively higher at S1 and S2 than at S0 (Fig. 1B). GA content was highest at S1, while no significant difference between S0 and S2 (Fig. 1B). ABA, a stress-response hormone, increased significantly from S0 to S1 but JA content significantly decreased over the same period of time (Fig. 1B). The ratio of IAA/ZR at S2, however, was significantly lower than at S0 (Fig. 1C). The ratio of IAA to ABA exhibited a similar trend observed in the IAA/ZR ratio, while the ABA/GA ratio steadily during the entire AR formation sampling period (Fig. 1C). The different levels of hormones and changes in the ratios reflect their various functions during specific phases of AR development.

3.2. Construction and sequencing of the small RNA libraries

A total of 446,150,025 raw reads were obtained via high-throughput sequencing. The average numbers of raw reads at each time point were 53,703,123 at S0, 48,113,652 at S1, and 46,899,901 at S2 (Table S2). After removal of low quality reads, a total of 289,363,684 (64.86%) clean reads were selected from nine libraries (Table S2). A degradome library derived from mixed total RNA from the three key time points (S0, S1 and S2) was constructed and a total of 13,439,101 raw tags were generated, resulting in 13,410,565 (99.79%) clean tags and 4,783,127 (35.59%) unique tags (Table S3). The clean reads were categorized into rRNA, scRNA, snRNA, snoRNA, tRNA, Repbase, mapped reads and unmapped reads. Detailed information on the libraries is presented in Table S4.

3.3. Identification of known miRNAs and their expression patterns

A total of 190 known miRNAs belonging to 39 miRNA families were identified (Fig. S1). The mature sequences of the identified known miRNAs are listed in the database. The number of known miRNAs in different families varied significantly, from 1 (mdm-miR1511, 391, 535, 7125, 827 and 858) to 31 (mdm-miR156) (Fig. S1).

The expression levels of known miRNAs in the nine libraries were determined by the number of their read counts. The miRNAs were classed into one of six categories based on their reads counts (Fig. S2). Moderate expression (100–999 reads) contained the largest number of miRNAs at S0 but the low expression (10–99 reads) category was the largest at S1 and S2 (Fig. S2). After normalization, the identified known miRNAs were hierarchical clustered based on the TPM values to display their expression abundance (Fig. 2A). Some miRNAs had very high expression levels, with more than 10,000 read counts, including mdm-miR398b/c, miR408s and miR319a/b, (Fig. S2 and Fig. 2A), while others had very low expression levels, with less than 10 reads, including, miR398a, miR319c, miR7125; as well as some of the mdm-miR156, miR399, and miR171 family members (Fig. S2 and Fig. 2A).

3.4. The identification of known miRNAs that are differentially expressed during AR formation

A total of 84 known miRNAs, were identified. Among these miRNAs, 74 were down-regulated (including mdm-miR399a, miR156aa, and miR160a etc); and 10 miRNAs were up-regulated (including mdm-miR390a, miR396a, and miR3627a-c etc) (Figs. 2B and 3). The values of log_{2}FC of the known miRNAs were in the range of ~5.36 to 4.00, with miR390 exhibiting the largest increase; while miR398a exhibited the smallest decrease (Fig. 3). The Venn diagram illustrates the number of known miRNAs showed that a total of 65 miRNAs were differentially expressed in the initiation phase (S0 vs S1), 11 in the elongation phase (S1 vs S2), and 69 over the whole time course of AR formation (S0 vs S2) (Fig. S3A). More specifically, 12 miRNAs were only up-regulated, 66 miRNAs were only down-regulated, and 9 miRNAs were both up- and down-regulated over the course of AR formation (Fig. S3B).

3.5. Identification of putative novel miRNAs, that were differentially expressed during AR formation

The novel miRNAs had relatively lower levels of expression. The largest percentage of novel miRNAs fell into the very low category (1–9 reads) and the percentages varied from 35.08% to 40.74% in the different libraries. This was followed by those that fell into the low category (10–99 reads) and the percentages in each library varied from 23.52% to 35.49%. There were few novel miRNAs (< 4.123%) that exhibited more than 1000 reads (Fig. S4).

The Venn diagram illustrates that 56 novel miRNAs were differentially (Fig. S5). Among all of the novel miRNAs, 28 miRNAs were differentially expressed in S0 vs S1, and 4 within S1 vs S2. In addition, 25 miRNAs were only down-regulated, and 31 miRNAs were only up-regulated. None of the novel miRNAs exhibited both up- and down-regulated over the course of AR formation (Fig. S5B). The relative expression level of each novel miRNA is indicated by the blue-white-red gradient colors. The hierarchical clustering of the 56 novel miRNAs showed three major clusters (Fig. 4).

3.6. Degradome sequencing identification of target genes of the known and novel miRNAs

The results of targets of known and novel miRNAs analyzed by degradome sequencing were showed in Tables 1 and 2. After merging the miRNAs with their corresponding mature sequence, a total of 91 miRNAs with 144 target genes and 203 cleavage sites were found in the
degradome library (P-value < 0.05), and examples of the specific cleavage sites in the genes could be observed (Fig. S6). Most of the targets in the GO annotation were placed in the binding category of molecular function and the cellular category of biological process. A total of 82 known miRNA members belonging to 20 miRNA families (including miR156, 160, 166, 171 and 396) and 88 targets were identified (Table 1). The majority of the known miRNAs identified in the current study could potentially regulate multiple target genes. For example, mdm-miR156 regulates SPL6, SPL13B, and ACOS5, mdm-miR319 regulates TCP2 and TCP4, and mdm-miR396 regulates GNF1, GNF2, and GNF5. Mdm-miR160 regulates ARF16 and ARF17 (Table 1), and the cleavage site information for miR160 with its targets is presented in Fig. S7.

A total of 56 novel miRNAs and 76 targets were identified. After filtering out the miRNAs that fell into the no expression (0 read) category and the very low (1–9 reads) category (Fig. S4), and then the novel miRNAs with higher category were analyzed in Table 2. The targets of the novel miRNAs included genes (encoding regulatory proteins) and transcription factors (TFs), such as TCPs and the cytochrome P450 superfamily (Table 2). The targets of novel miRNAs have multiple functional types. Some of the targets were associated with phytohormone pathways, such as BSK2 (target of chr1_1772328) in BR signaling, IAA9 (target of chr5_2661918) in auxin signaling (Table 2). There were also some targets of that respond to stress signals, including CYP94B3 (chr3_2294129) in wounding signals, and DREB2C (chr1_1772328) in drought stress, etc (Table 2).
3.7. RT-qPCR analysis of differentially expressed miRNAs and targets during AR formation that are associated with auxin signaling

The expression levels of miRNAs (miR160 and miR390) and their targets, which were associated with auxin signaling, were examined by RT-qPCR (Fig. 5). The targets of miR160 and miR390 are AUXIN RESPONSE FACTORS (ARFs). The expression level of miR160a exhibits a significant decrease from S0 to S1, followed by an increase from S1 to S2. In contrast, the target gene, ARF16, displays its highest expression level at S1 (Fig. 5A). MiR390a increased by 8-folds from S0 to S2. However, the target gene of miR390a, APS1, reached its maximum at S1 (Fig. 6A). The target gene of miR398a and miR398b (CSD2, PYL4 and SPI1) gradually increased in expression from S0 to S2 (Fig. 6B C). The expression level of miR408a decreased by approximately 80% from S0 to S1, and stayed relatively low level at S2. DegP9 and ARPN, the target genes of miR408a, however, exhibited variable expression patterns during AR formation (Fig. 6D). Although the expression levels of miRNAs at several data points varied between RNA-seq and qRT-PCR, the differential expression trends detected by the two approaches were largely consistent (Fig. 6).

3.8. RT-qPCR analysis of differentially expressed miRNAs and their targets during AR formation that are associated with stress response and signal transduction

The expression level during AR formation of four miRNAs and their target genes, which are associated with stress response and signal transduction, were examined by RT-qPCR (Fig. 6). The expression profiles of miR398a, miR398b and miR408a exhibited a down-regulation pattern from S0 to S2 (Fig. 6B-D). However, the expression level of miR395a increased from S0 to S2 (Fig. 6A). The target gene of miR395a, APS1, reached its maximum at S1 (Fig. 6A). The target gene of miR398a and miR398b (CSD2, PYL4 and SPI1) gradually increased in expression from S0 to S2 (Fig. 6B C). The expression level of miR408a decreased by approximately 80% from S0 to S1, and stayed relatively low level at S2. DegP9 and ARPN, the target genes of miR408a, however, exhibited variable expression patterns during AR formation (Fig. 6D). Although the expression levels of miRNAs at several data points varied between RNA-seq and qRT-PCR, the differential expression trends detected by the two approaches were largely consistent (Fig. 6).
3.9. RT-qPCR analysis of differentially expressed miRNAs and their targets during AR formation that are associated with cell fate transformation, proliferation, and enlargement

The expression levels of miRNAs and target genes that associated with cell fate transformation, proliferation and enlargement were examined by qRT-PCR. RT-qPCR analysis indicated that the expression of miR171a and miR319a exhibited its lowest expression level at S1, while its target genes (HAM3, TCP2 and TCP4) exhibited its highest expression level at S1 (Fig. 7A-D). The expression level of mdm-miR156ab was down-regulated from S0 to S2, while the target genes (SPL6 and SPL13B) exhibited an opposite trend to mdm-miR156ab in expression (Fig. 7B). The expression level of miR166a significantly decreased from S0 to S2, while the target gene, PHB and REV, reached its maximum at S1 (Fig. 7C). The expression profiles of miR396a and miR396f were different from each other. MiR396a was up-regulated from S0 to S1 and decreased from S1 to S2. However, the relative expression level of miR396f was significantly down-regulated from S0 to S2 (Fig. 7E). Their target gene, GRF1, significantly increased from S0 to S1 but did not exhibit any significant difference in expression between S0 and S2 (Fig. 7E). In general, the RT-qPCR results were largely consistent with the data obtained from miRNAs sequencing TPM (Fig. 7).

3.10. RT-qPCR analysis of differentially expressed genes associated with pathways related to AR formation

The expression profiles of several genes that are specifically associated with AR formation were analyzed by RT-qPCR. The selected genes could be classified as auxin-related, cytokinin-related, cell cycle-related, root development-related, etc (Fig. 8). Among all of the selected genes, IAA3 exhibited its highest expression level at S1. The expression profiles of PIN1, PLT and RR12 showed significantly decreased from S0 to S1 and no significant difference between S1 and S2 (Fig. 8A). The expression level of IPT1-2, CYCD1;1 and CYCP4;1 exhibited its lowest expression level at S1 and no significant difference between S0 and S2 (Fig. 8). ACO1 and JAZ12 are stress-signal response genes and their expression levels were significantly up-regulated (from S0 to S2) (Fig. 8A). The expression level of WOX5, LRP1, and SCR1 were up-regulated during AR formation (Fig. 8B). The expression profile of RHS19, however, was opposite to SCR1 (Fig. 8B).

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Fig. 3. Fold-Change of differentially expressed known miRNAs. The red bars refer to log2FC of up-regulated miRNAs, and green ones to the down-regulated miRNAs. Vertical dotted lines refer to the screening criteria: |log2(FC)| = 1. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
4. Discussion

4.1. Hormone levels

Hormones play a crucial role in plant development and the interaction networks determine the capacity, position, and efficiency of AR formation (Negishi et al., 2014). The accumulation of auxin in the stem above the point of cutting is absolutely required for AR induction, but inhibits AR elongation (Sukumar et al., 2013) (Fig. 1B). The expression profiles of auxin-associated genes are approximately in accordance with the accumulation of auxin during the AR induction phase. These factors may be the reason why species with difficult to rooting are treated with exogenous auxin during the induction phase of AR formation in production.

In contrast to auxin, cytokinins (CTK) play a suppressive role during AR induction phase but promote cell division and differentiation during the extension phase of AR formation (Dello Ioio et al., 2012). A critical factor regulating AR development is the ratio of auxin to cytokinin. The cuttings used in this study did not have any primary roots (organs that favor an accumulation of CTK) which promoted a higher IAA/CTK ratio and thus also promoted the initiation and growth of AR (Rani and Taketa, 2005). The interaction and balance between auxin and cytokinin mediates AR formation. In the current study, the levels of JA, GA, ABA and BR also have changed during AR formation in apple rootstock. Collectively, the data on hormone patterns indicate that AR development is a complex process affected by various types of hormonal regulation. However, the mechanisms underlying and associated with their interaction, remain to be further experimentally demonstrated.

4.2. MiRNAs and their targets that are associated with auxin signaling pathways are involved in regulating AR formation in apple rootstocks

Auxin-related miRNAs were identified in the present study, including miR160 and miR390. The target gene of miR160 is ARF, which regulates the expression of auxin-responsive genes by binding to auxin response elements (AuxREs) in the promoters. MiR160 inhibits callus initiation from pericycle-like cells, while ARF10 promotes it (Liu et al., 2016). In addition, over-expression of miR160c resulted in a phenotype characterized by shorter and more gravitropic roots with an enlarged tumor-like apex (Wang et al., 2005). In the present study, the expression profiles of miR160a and ARF6 are consistent with these previous reports. Therefore, the involvement of miR160/ARFs in auxin signal response is readily apparent and may play a critical role in AR formation, although this will need to be confirmed by further studies.

MiR390 was also identified from the DEG library. In Arabidopsis, it has been reported that miR390 was specifically expressed at the sites of lateral root initiation where it triggers the biogenesis of tasiRNAs, followed by ARF-regulated lateral root growth (Marin et al., 2010). RT-qPCR results revealed that miR390a may be a positive regulator of AR development, but that ARF3 had dual target genes (Fig. 5B). The results also indicated that miR390 has regulatory capacity over ARFs, but may act indirectly in that it is capable of cleaving the non-coding TAS3 precursor RNA to produce tasiRNAs, which can then cleave the transcripts of ARF2, ARF3, and ARF4. This may be the reason of miR390-ARF cleavage sites could not be detected in the degradome library. In addition, ARF genes also display overlapping expression domains, interact genetically and constitute a complex regulatory network, including feedback regulation (Gutierrez et al., 2009). In the current study, miR160 was confirmed to have a cleavage relationship with ARF16 and ARF17, which might explain why miR390 and miR160 exhibited opposite expression patterns, even though they have similar targets (Fig. 5). Overall, the results indicate that miR160 and miR390 play a crucial role in AR formation and that the target genes of ARFs also act as key regulators of auxin signal transduction during AR formation in apple rootstocks. However, the mechanisms underlying and associated with their interaction remain to be further experimentally demonstrated.

4.3. MiRNAs and their targets that are associated with stress-response pathways are involved in regulating AR formation in apple rootstocks

Indeed, stress-response-related miRNAs were also identified in the present study, including miR398, miR395 and miR408.

Wounding is a primary stimulus for the induction of AR formation in the vegetative propagation of apple cuttings. The target gene of miR398 is CSD2, which belongs to subfamily of super oxide dismutase (SODs) (Fridovich, 1995). CSD2 is also induced in response to wound response; and function to serve as the initial signal for AR formation (Steffens and Rasmussen, 2016). Therefore, we consider MiR398a to be a rapid response miRNA in wounding-induced AR formation, which plays a primary role during the initial signal transmission.

In addition, miR395 targets APS1 (ATP sulfurylase gene) which regulates sulfate accumulation and allocation in Arabidopsis (Liang et al., 2010). In Arabidopsis, sulfur nutrient availability has also been
Detected targets of known miRNAs, some of their GO annotations and cleavage sites in 'M9-T337' apple rootstock AR formation.

<table>
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<tr>
<th>Known miRNA</th>
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<th>Description</th>
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<th>GO ID</th>
<th>Alignment Range</th>
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<td>MDP000023749</td>
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### Table 2
Detected targets of possible novel miRNAs and their reads counts in each stage of 'M9-T337' apple rootstock AR formation.

<table>
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<tr>
<th>novel miRNA</th>
<th>Mature sequence</th>
<th>Targets Description</th>
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<th>Reads counts</th>
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<tr>
<td>chr12_337666</td>
<td>ACAGGGCCGGGAAAUCGAUGAU</td>
<td>Protein kinase superfamily protein (AT1G54610.1)</td>
<td>MDP0000742986</td>
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<tr>
<td>chr15_1258354</td>
<td>UUGAGCAGUAAGGAGAGGCCC</td>
<td>TEOSSINTE BRANCHED 1, cyclidea and PCF transcription factor 2 (AT4G18390.1, TCP2)</td>
<td>MDP000027553, MDP0000287069, MDP0000763497, MDP000092127, MDP0000927314</td>
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<tr>
<td>chr3_2294129</td>
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<td>TCP family transcription factor 4 (AT3G15030.1, MEE35, TCP4)</td>
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<td>chr5_2661918</td>
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shown to regulate root stem cell niche maintenance and effect on root elongation (Zhao et al., 2014). Furthermore, sulfate is also associated with hormone metabolism, brassicales plants, auxin-dependent regulation of root growth and branching involves the glucosinolate pathway, which is regulated by S depletion (Honsel et al., 2012). In the current study, AR formation by targeting miR395 may take part in AR formation by targeting miR395 may be one of the induction factors of AR formation in apple rootstock.

In Arabidopsis, pyr/pyl mutant exhibited reduced sensitivity to ABA and reduced root hydrotropic response, and the expression of PYL4 was demonstrated initiated a reprogramming of cellular metabolism (Antoni et al., 2013). A member of the miR398 family, miR398b, was identified in the degradome library that targets PYL4 (PCAR10) (Table 1), which encodes proteins that function as ABA sensors and mediate ABA-dependent regulation of protein phosphate 2Cs (Pizzio et al., 2013). The expression patterns of miR398a and miR398b, however, are opposite of each other. Therefore, the results suggest that the miR398 family (miR398a and miR398b) may balance stress and regulate AR formation.

The degradome sequencing conducted in the present study revealed that the post-transcriptional regulated target of miR408a was Dep9. Dep9 can mediate the degradation of ARR4 and regulate the cross-talk between CTK and light-signaling pathways (Chi et al., 2016). Furthermore, type-A ARRs alter the pattern of cell division and differentiation in the stem cell niche of the root apical meristem by altering PIN levels, which intersects with cytokinin and auxin regulatory pathways (Zhang et al., 2011). These reports and the results obtained in the present study also infer that miR408 plays an important role in AR formation.

4.4. MiRNAs and their targets that are associated with cell fate determination, proliferation, and enlargement are involved in regulating AR formation

Five major differentially expressed miRNAs, miR171, miR156, miR166, miR319 and miR396, which are associated with cellular events such as cell fate determination, proliferation and enlargement, were identified within the degradome library. MiR171 is a relatively conserved miRNA present within different plant species that has been associated with root organ development. For example, miR171 up-regulated and enriched in Solanum tuberosum roots responding to drought (Hwang et al., 2011). GRAS TFs (DELLA, HAM, SCR, and others) are involved in regulating root growth, as well as the mediation of cell fate (Helariutta et al., 2000). In addition, miR171c regulated shoot apical meristem (SAM) development by HAM with WOX4 in a WUS-CLV feedback loop (Fan et al., 2015). In the current study, HAM3 (also named LOM3, SCL6-IV, and as a member of the GRAS protein family) was determined to be specifically targeted by miR171 (Table 1). Overall, the data suggest that miR171/HAM may interact with WOX to participate in cell fate determination during AR formation in apple rootstock.

In Malus xiaojinensis, auxin-induced AR formation relies on a relatively high expression of miR156 (Xu et al., 2017). However, miR156 expression in Eucalyptus grandis does not regulate AR induction (Levy et al., 2014). Therefore, miR156 regulated root development may be species specific. Alternatively, miR156 may be necessary but not a determining factor for AR induction in woody plants. In the present study, the target genes of miR166 have been verified to encode class III homeodomain zipper (HD-ZIP) TFs (including PHB and REV), which were also identified in the degradome library (Table 1). In Arabidopsis, PHB contributes to root vascular patterning and the differentiation of xylem cell types by directly binding to promoters of MP/ARF5 and IAA20 (Muller et al., 2016). PHB directly activates IPT7 (CTK biosynthesis gene), and then CTI feeds back to repress PHB and microRNA, thus balancing the ratio of cell division with differentiation during root development (Dello Ioio et al., 2012). Collectively, all of the evidences and results also strongly suggest that miR166/HD-ZIP plays a crucial role in vascular reconstitution during AR formation.

The data obtained from the degradome library in the present study indicates that the targets of miR319 are genes encoding TCP, specifically TCP2 and TCP4. TCP affects the morphogenesis of lateral organs, JA biosynthesis, and cell proliferation (Zhao et al., 2015). MiR396 regulates cell proliferation in meristems in Arabidopsis and roots of Medicago truncatula by targeting the growth-regulatory factors, GRF1, GRF2, and GRF5 (Bazin et al., 2013). It has been demonstrated that miR396a regulates both the conversion of stem cells and root growth in the elongation zone of Arabidopsis roots instead of the meristem (Bao et al., 2014), suggest that miR396/GRF plays vital roles in both cell proliferation and enlargement. In addition, miR319/TCP also had different regulatory mechanisms in tomato roots vs. leaves (Ori et al., 2007). Thus, it appears that the regulatory networks of miR319/TCPs interacting with miR396/GRFs have species and organ specificity. Overall, miR319a and miR396 established a complex and diverse regulatory network for fine-tune cell proliferation of AR formation in apple rootstock (Fig. 9). However, additional in-depth research is warranted and necessary to completely elucidate the specific mechanisms regulating this response.

4.5. Differential expression of genes mediates AR formation

In the current study, auxin-related, cytokinin-related, stress signal response-related, cell cycle-related, and root development-related genes were identified within the sequencing database. The types of genes indicate that AR formation involves multiple biological pathways. In order to confirm the reliability of the sequencing results, some representative genes related with AR formation were examined by RT-
Root development is a dynamic process which requires the integration of plant hormones, transcriptional regulators, and small RNAs (Meng et al., 2010). For example, miR160 acts as a key controller of root cap cell formation through repression of the AUXIN RESPONSE FACTOR (ARF) genes, ARF10 and ARF16, and also targets ARF17 to regulate adventitious root initiation (Gutierrez et al., 2009). The transcription factors SHR (SHORT ROOT) and SCR (SCARECROW) have been identified as determining root cell fate (Carlsbecker et al., 2010). In the current study, results of RT-qPCR analyses determined that the expression level of genes and miRNAs were induced during AR formation. In addition, hormone levels also changed over the course of AR formation. These data suggest that the aforementioned genes and miRNAs may interact with hormones to regulate AR induction, elongation, and development.

5. Conclusion

Base on the high-throughput sequencing and the degradome library, iconic miRNAs that are known to be auxin-related, stress response-related, and cell fate-related were identified within the database generated in present study. The range of differentially expressed miRNAs, and the range of target genes that are regulated by the miRNAs, clearly indicated that AR development in apple rootstocks is a complex biological process. We have used the collective data on AR development to construct a model of the interacting response mechanisms regulating AR formation in ‘M9-T337’ apple rootstocks (Fig. 9). Many auxin-, stress response-, and cell fate-related miRNAs and genes interact with hormones signaling to ultimately control AR formation (Fig. 9). Our study provides an overview of the transcriptomic changes that occur during AR formation in ‘M9-T337’ apple rootstocks. It will be a foundation for the further exploration of candidate miRNAs and genes, along with their associated pathways, that regulate AR formation. However, owing to technical limitations, post-transcriptional or (post)-translational evidence for AR formation still needs further analysis and experimentally demonstrated.

List of abbreviations

- AUX: auxin
- CTK: cytokinin
- IAA: Indole-3-acetic acid
- ZR: zeatin riboside
- GA: gibberellin
- BR: brassinolide
- JA: jasmonic acid
- ABA: abscisic acid
- AR: adventitious root

Author contributions

DZ, LX, CZ and KL designed and interpreted of all experiments. KL, DZ, ZL, LX and LB participated in the experimental design and data analysis. KL, ZL, LX, YW and JM performed the hormone and measurements. KL, DZ, ZL, LX, JM, YM and YW performed material sampling and the laboratory data measurements. KL, DZ, ZL and KL participated in the preparation of the manuscript. All authors have read and approved the manuscript.

Conflicts of interest

The authors declare that they have no competing interests.
Fig. 7. Identification by qRT-PCR of differentially expressed miRNAs and their targets associated with cell fate transformation, proliferation and enlargement in AR formation. (A) Expression level of miR171a and its target genes. (B) Expression level of miR156ab and its target genes. (C) Expression level of miR166a and its target genes. (D) Expression level of miR319a and its target genes. (E) Expression level of miR396a and miR396f and their target gene. Blue bars refer to the TPM values of miRNA, the star-lines refer to the miRNA expression level identified by qRT-PCR, and the gray bars refer to targets expression levels identified by qRT-PCR. Bars show SD with three biological replicates. Values represent means ± SE (n = 3). The statistical analysis was performed by Duncan’s multiple range test at level p ≤ 0.05. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 8. Identification by qRT-PCR of downstream key genes associated with related pathways in AR formation. (A) Expression level of genes related with phytohormone pathways. (B) Expression level of genes related with cellular events. Bars show SD with three biological replicates. Values represent means ± SE (n = 3). The statistical analysis was performed by Duncan’s multiple range test at level p ≤ 0.05.
Fig. 9. Hypothetical model of miRNAs regulating AR formation through the pathways of auxin, stress and cellular events in stem cuttings of ‘M9-T337’ apple rootstock.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.03.006.

References


