The **peu-miR160a–PeARF17.1/PeARF17.2** module participates in the adventitious root development of poplar

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**Summary**

Deep roots give rise to flourishing leaves, and the two complement each other. However, the genetic mechanisms underlying adventitious rooting for forest trees have remained elusive. In this study, we verified that pea-miR160a targets six poplar genes **AUXIN RESPONSE FACTORS** (ARFs), **PeARF10.1**, **PeARF16.1**, **PeARF16.2**, **PeARF16.3**, **PeARF17.1** and **PeARF17.2**, using 5’RLM-RACE. Interaction experiments with pea-miR160a and ARFs in poplar protoplasts further confirmed that pea-miR160a targets and negatively regulates the six ARFs. Peu-miR160a and its target genes exhibited obvious temporal expression in different stages of adventitious root development, and they could also be induced by IAA and abscisic acid. Peu-miR160a-overexpressing lines exhibited a significant shortening of adventitious root length, an increase in the number of lateral roots, severe dwarfing and shortened internodes. In addition, the overexpression of **PeARF17.1** or mPeARF17.2 (pea-miR160a-resistant version of **PeARF17.2**) significantly increased the number of adventitious roots. Furthermore, **PeARF17.1**-overexpressing lines had multiple branches with no visible trunk, although the adventitious root length of the **PeARF17.1**-overexpressing lines was significantly increased. Our findings reveal that the pea-miR160a — **PeARF17.1**/**PeARF17.2** module is an important regulator involved in the development of the adventitious roots of poplar.

**Introduction**

MiRNAs are a group of non-coding RNA molecules of approximately 21–24 nt, and they are common in eukaryotes (Debernardi et al., 2012; Rogers and Chen, 2013). MiRNA is complementary to its target gene sequence and forms an RNA-induced silencing complex, which cleaves or inhibits translation of the target gene to regulate post-transcriptional expression (Debernardi et al., 2012). In plants, miRNAs are highly conserved, exhibit spatiotemporal specificity and are involved in a series of important processes in the growth and development of plants, including regulation of leaf morphogenesis (Kidner, 2010; Palatnik et al., 2003), floral differentiation and development (Aukerman and Sakai, 2003), root formation and development (Guo et al., 2005), and transformation of plants from the juvenile phase to the reproductive phase (Schwab et al., 2005). In addition, miRNAs play important regulatory roles in the plant response to the external environment (e.g., drought stress [Chen et al., 2012] and viruses [Chellappan et al., 2005]). In previous studies, the miR390/TAS3/ARFs module was able to regulate the growth of the lateral roots of poplar under salt stress (He et al., 2018).

The miR160 family targets AUXIN RESPONSE FACTORS (ARFs) and involves auxin signalling pathways, which play an important regulatory role in plant growth and development. In **Arabidopsis thaliana**, miR160a, miR160b and miR160c share the same mature sequences; their target genes are **ARF10**, **ARF16** and **ARF17** (Rhoades et al., 2002). Overexpression of the miR160a-resistant version of **ARF10** (mARF10) revealed phenotypic variation in transgenic **Arabidopsis**, such as serrated leaves, curled stems, and abnormal flower and fruit development (Liu et al., 2007). Furthermore, mARF10 mutant seeds and plants were highly sensitive to abscisic acid (ABA), although the overexpression of miR160a produced the opposite result (Liu et al., 2007). MiR160a can negatively regulate the target genes **ARF16** and **ARF17** and can affect the embryonic development of **Arabidopsis** (Liu et al., 2010).

In addition, miR160 regulates the growth and development of roots, especially root tips and lateral root development. In **A. thaliana**, overexpressing miR160c or arf10 arf16 double mutants showed root tip defects, loss of gravity sensing, impaired differentiation of root cap cells and ectopic expansion of the stem cell niche (Wang et al., 2005). Overexpression of the miARF16 mutant in **Arabidopsis** significantly decreased the lateral root density, which was in contrast to the overexpression of miR160c (Khan et al., 2011; Wang et al., 2005). This effect suggests that miR160 may potentially regulate the formation of lateral roots, but it also may be an indirect consequence of the influence of miR160 on the development of primary root apical meristems (RAMs) (Khan et al., 2011; Wang et al., 2005). An overexpressing mPeARF17 (miR160a-resistant version of **PeARF17**) mutant showed a phenotype with shortened primary roots. MiR160 also positively regulated the formation of adventitious roots by degrading **ARF17** instead of **ARF10** and **ARF16** (Khan et al., 2011).
The functions of miR160 in Arabidopsis have been clearly studied, but its functions in poplar are still unclear. To explore the functions of peu-miR160a in poplar, we predicted and confirmed that peu-miR160a targets PeARF10.1, PeARF16.1, PeARF16.2, PeARF16.3, PeARF17.1 and PeARF17.2. Furthermore, the functions of peu-miR160a were investigated by overexpressing peu-miR160a and its targets in poplar. Our study showed that peu-miR160a and its target genes play crucial roles in the growth and development of adventitious and lateral roots of poplar.

Results

Identification and sequence analysis of miR160 in Populus

From previous small RNA sequencing, we obtained six differentially expressed miR160 family members (peu-miR160a, peu-miR160b-3p, peu-miR160e-5p, peu-miR160e-3p, peu-miR160g and peu-miR160h) from the adventitious root development of the hybrid poplar Nanlinb95 (Liu et al., 2019). A multiple sequence alignment analysis revealed that the miR160 mature sequences derived from the 5’ arm of the miR160 precursor were highly conserved among different plant species (Figure 1a), whereas the mature miR160 sequences derived from the 3’ arm of the miR160 precursor were less conserved in plants (Figure 1b). Among different plants, conservation between the mature sequences of miR160 derived from the 5’ arm and the mature sequences of miR160 derived from the 3’ arm was very low (Figure 1c). A phylogenetic analysis showed that miR160 derived from the 5’ arm and miR160 derived from the 3’ arm were apparently divided into two clades (Figure 1d). Based on a comparison of the precursor sequences of miR160a in different plants, we found that except for the mature sequences, the other sequences were not conserved (Figure S1a). The meu-miR160a precursor had a typical stem-loop structure, and the meu-miR160a mature sequence was derived from the 5’ arm of the stem-loop structure (Figure S1b). The minimum free energy of miRNA precursors was predicted using the classic algorithm of Zuker and Stiegler (1981). A MFE analysis revealed that the miR160 mature sequences derived from the 5’ arm of the stem-loop structure (Figure S1b). The precursor sequences of miR160a in different plants, we found that except for the mature sequences, the other sequences were not conserved (Figure S1a). The meu-miR160a precursor had a typical stem-loop structure, and the meu-miR160a mature sequence was derived from the 5’ arm of the stem-loop structure (Figure S1b). The minimum free energy (MFE) of miRNA precursors was predicted using the classic algorithm of Zuker and Stiegler (1981). A MFE analysis indicated that the stability of the meu-miR160a precursor was −56.30 kcal/mol. In summary, meu-miR160a is more conserved in different plants than the other five differentially expressed miRNAs; therefore, we selected peu-miR160a for subsequent functional studies.

Validation of cleavage sites of peu-miR160a

In previous studies, we obtained the full-length cDNAs of the six target genes using rapid amplification of cDNA ends (RACE) (Yang et al., 2014). The peu-miR160a sequence was highly complementary to its target genes, with two mismatched bases relative to PeARF16.1 and only one mismatched base relative to the remaining five target genes. Here, we validated the pequ-miR160a cleavage site using modified 5’RLM-RACE and showed that peu-miR160a cleaves its target genes. The cleavage sites for PeARF16.2 and PeARF16.3 were between the 9th and 10th nucleotides of peu-miR160a, and the cleavage sites for PeARF10.1, PeARF16.1 and PeARF17.1 were between the 10th and 11th nucleotides of peu-miR160a (Figure 2a). The 5’RLM-RACE assay revealed that PeARF17.2 had a cleavage site at the 12th and 13th nucleotides of peu-miR160a and a cleavage site outside the complementary sequences of peu-mir160a (Figure 2a). To further confirm the PeARF17.2 cleavage site, we analysed the degradome sequencing data (accession number PRJNA498391) and found that the PeARF17.2 cleavage site was at the 12th and 13th nucleotides of peu-miR160a.

Interaction of peu-miR160a with its targets

Previous studies have shown that synonymous substitutions in complementary sequences of miRNA can reduce the inhibitory effect of miRNA on target genes and do not alter the amino acid sequence of the target gene (Wang et al., 2005). Thus, peu-miR160a-resistant versions of PeARF16.1 (mPeARF16.1) and PeARF17.2 (mPeARF17.2) were generated by two rounds of synonymous substitutions using PCR that changed the complementary sequence of peu-miR160a (Figure 2b,c). psRNATarget (Dai and Zhao, 2011) prediction showed that compared with PeARF16.1 and PeARF17.2, mPeARF16.1 and mPeARF17.2 are not target genes of peu-miR160a (Figure S2). This result indicated that we successfully obtained peu-miR160a-resistant versions of PeARF16.1 (mPeARF16.1) and PeARF17.2 (mPeARF17.2).

To visualize the interaction of peu-miR160a and its targets, the constructed expression vectors for peu-miR160a and its target PeARF genes (and mPeARFs) were co-transfected into Populus protoplasts. The results showed that if only one target gene of peu-miR160a was transfected, then the target would emit intense green fluorescence in the nucleus (Figure 3a). However, when peu-miR160a was co-transfected with a target gene, the green fluorescence of the target gene was very weak or devoid of light (Figure 3a). When mPeARF16.1 or mPeARF17.2 was singly transfected, it produced strong GFP fluorescence (Figure 3a). Compared with peu-miR160a and PeARF16.1 or PeARF17.2 co-transfection, when peu-miR160a was co-transfected with mPeARF16.1 (or mPeARF17.2), mPeARF16.1 (or mPeARF17.2) still had stronger green fluorescence (Figure 3a). However, the green fluorescence of co-transfected peu-miR160a and mPeARF16.1 (or mPeARF17.2) was not as strong as the green fluorescence of single transfected mPeARF16.1 or mPeARF17.2 (Figure 3a).

To accurately detect the interaction between miR160a and its target genes, we collected protoplasts transfected with empty vectors (CK), protoplasts transfected with Pro35S::PeARFs and protoplasts co-transfected with Pro35S::mPR160a and Pro35S::mPeARFs for RNA isolation. Subsequently, the expression levels of meu-miR160a and its target genes were analysed by real-time quantitative PCR (qRT-PCR). When peu-miR160a interacted with a target gene, the expression level of the target gene was significantly reduced (Figure 3b–g). When peu-miR160a interacted with mPeARF16.1 or mPeARF17.2, the expression level of PeARF16.1 or PeARF17.2 increased, but it did not reach the expression observed when only PeARF16.1 or PeARF17.2 was transfected (Figure 3c,g). These qRT-PCR results were consistent with the green fluorescence experiments above, which indicated that peu-miR160a inhibited the expression of its target genes. At the same time, it was also shown that the peu-miR160a-resistant versions of PeARF16.1 and PeARF17.2 reduced the inhibitory effect of peu-miR160a.

Expression patterns of peu-miR160a and its targets

Peu-miR160a and its target genes exhibited significant temporal specificity in different developmental stages of poplar adventitious roots (Figure 4a). Peu-miR160a had the highest expression level in 30DR (30-day-old adventitious roots) and the lowest expression level in 20DR (20-day-old adventitious roots) (Figure 4a). Interestingly, the six target genes of peu-miR160a had the highest expression levels in 10DR (10-day-old adventitious roots).
miR160 regulates the development of poplar roots

In four developmental stages of adventitious roots (10DR–30DR), PeARF10.1 and PeARF16.2 showed consistent trends of decreased expression, and the expression of the remaining four target genes showed a trend of decreasing and then increasing (Figure 4a).

ABA response elements (ABREs) were found on the promoter of peu-miR160a; therefore, the expression levels of peu-miR160a and its target genes were detected at different times following ABA treatment. The results showed that the expression of peu-miR160a first increased and then decreased with increased treatment time with 10 µM ABA (Figure 4b). After 1 h of treatment, the expression reached a peak, which was almost twice the control (0 min) value, and the expression reached a minimum after 24 h of treatment (Figure 4b). The expression levels of the peu-miR160a targets were different (Figure 4b). The expression of PeARF16.2, PeARF17.1 and PeARF17.2 changed greatly after induction with ABA, but the expression of PeARF10.1, PeARF16.1 and PeARF17.2 changed slightly after induction with ABA (Figure 4b). An analysis of the expression trends of peu-miR160a targets indicated that those of PeARF16.3 and PeARF17.1 were equivalent and those of PeARF10.1 and PeARF17.2 were equivalent (Figure 4b).
Figure 2 5'RLM-RACE validation and synonymous nucleotide substitutions of target genes of peu-miR160a. (a) 5'RLM-RACE validation of target genes of peu-miR160a. The arrows indicate the cleavage sites. The numbers above the arrows refer to the number of clones that detected the cleavage site and the total number of clones in the 5'RLM-RACE assay. M indicates a DNA marker. (b) Synonymous nucleotide substitutions at the peu-miR160a binding sites of PeARF16.1 to create peu-miR160a-resistant PeARF16.1 (mPeARF16.1). (c) Synonymous nucleotide substitutions at the peu-miR160a binding sites of PeARF17.2 to create peu-miR160a-resistant PeARF17.2 (mPeARF17.2).
miR160 regulates the development of poplar roots

Because the six PeARFs belong to the auxin response factor family, their expression may be induced by auxin. Therefore, the expression levels of peu-miR160a and its target genes were detected at different indole-3-acetic acid (IAA) treatment times. The results showed that peu-miR160a was differentially expressed after 50 µM IAA treatment and reached its highest level after 30 min of IAA treatment (Figure 4c). After being induced by IAA, the expression levels of PeARF16.2 and PeARF10.1 were obviously different and 3.3 times higher than that of the control (0 min) after 1 h of IAA treatment (Figure 4c). PeARF10.1, PeARF17.1 and PeARF17.2 had the same expression patterns; they all had the lowest expression level after IAA treatment for 1 h and the highest expression level after IAA treatment for 12 h (Figure 4c). In addition, PeARF16.1 and PeARF16.2 had the same expression patterns, which first increased and then decreased, while the expression trend of PeARF16.3 first decreased, then increased and then decreased (Figure 4c).

Overexpression of seu-miR160a in Populus

To investigate the functions of miR160a, multiple Pro35S::miR160a transgenic lines were obtained and validated by PCR. Then, five transgenic lines (L2, L3, L5, L6 and L7) were used to detect the expression level of seu-miR160a using qRT-PCR. The qRT-PCR analysis results showed that the expression level of seu-miR160a in transgenic poplars was significantly higher than that of non-transgenic poplars (WT) (Figure 5a). In Pro35S::miR160a transgenic poplars, the expression levels of the six target genes of seu-miR160a decreased to varying degrees (Figure 5b). Of these six target genes, the expression of PeARF17.2 had the largest decrease.

To determine whether overexpression of seu-miR160a affected the growth and development of transgenic plants, we observed and examined the phenotypes of transgenic plants. After 50 days on Murashige and Skoog (MS) medium, transgenic poplars exhibited severe dwarfing, with shorter internode lengths (Figure 5c). In addition, the lateral roots near the root tips of the transgenic poplars increased significantly compared to those of the WT poplars (Figure 5c). The statistical results showed that the number of adventitious roots was significantly reduced in transgenic lines L2 and L5 compared to that of the WT poplars, whereas the decrease was not significant in other lines (Figure 5d). In addition, the length of the adventitious roots of the transgenic lines was significantly shortened, and the number of lateral roots increased significantly (Figure 5e,f).

Overexpression of PeARF17.1 in Populus

Figure 5b shows that only PeARF17.1 and PeARF17.2 were significantly down-regulated in all Pro35S::miR160a transgenic lines; therefore, we separately overexpressed these two genes in poplar to further understand the regulatory functions of seu-miR160a. After verifying the transgenic plants, we selected seven Pro35S::PeARF17.1 plants for subsequent experiments. The qRT-PCR results showed that the expression levels of PeARF17.1 were significantly higher in the transgenic lines than the WT poplars (Figure 6a). We observed and evaluated the phenotypes of transgenic plants Pro35S::PeARF17.1. Compared with the WT poplars, the Pro35S::PeARF17.1 plants had numerous stems, no obvious trunk, flat stem bases, an increased number of adventitious roots, and smaller and slightly curled leaves (Figure 6b). The number of adventitious roots of Pro35S::PeARF17.1 plants increased significantly (Figure 6c), and the adventitious root length of the four lines increased significantly, while the other three lines did not change significantly (Figure 6d). In addition, the number of branches of transgenic lines increased significantly, that is fourfold higher than that of the WT poplars (Figure 6e).

Overexpression of mPeARF17.2 in Populus

In the Pro35S::mPeARF17.2 transgenic lines, the expression level of PeARF17.2 was significantly higher than that of the WT poplars (Figure 7a). A significant change in the aerial parts of Pro35S::mPeARF17.2 plants was not observed compared to those of the WT poplars (Figure 7b). Similar to Pro35S::PeARF17.1 plants, the number of adventitious roots increased significantly in Pro35S::mPeARF17.2 plants (Figure 7c), but the lateral roots did not change significantly (Figure 7b). In addition, unlike Pro35S::PeARF17.1 plants, the adventitious root lengths of the Pro35S::mPeARF17.2 plants were significantly shortened (Figure 7d).

Discussion

Peu-miR160a targeted PeARFs

Degradome sequencing and 5'SRLM-RACE are common methods for verifying miRNA cleavage sites. Degradome sequencing is a high-throughput detection method that can effectively detect miRNA cleavage sites, and 5'SRLM-RACE can accurately identify miRNA cleavage sites (Shamimuzzaman and Vodkin, 2012; Wang et al., 2017; Zhang et al., 2018). Both methods have been applied to many plants, including Arabidopsis and Vitis vinifera (Adam et al., 2011; Addo-Quaye et al., 2008; Beauclair et al., 2010; German et al., 2008; Jiang et al., 2018; Wang et al., 2012). Previous studies in Arabidopsis and Dimocarpus longan revealed that miR160a targets ARF10, ARF16 and ARF17 (Lin et al., 2015; Wang et al., 2005). We predicted that peu-miR160a had six target genes (PeARF10.1, PeARF16.1, PeARF16.2, PeARF16.3, PeARF17.1 and PeARF17.2) in poplar, and this prediction was confirmed by a modified 5'SRLM-RACE assay and degradome sequencing. Similar to the results of studies in plants, such as A. thaliana, Solanum lycopersicum and Zea mays (Guo et al., 2005; Hendelman et al., 2012; Li et al., 2012; Wang et al., 2016), the five target genes for peu-miR160a had a cleavage site in the seed region (9th–11th nucleotides) of peu-miR160a. Furthermore, we observed interactions between miR160a and its target genes in poplar protoplasts and used qRT-PCR to quantitatively analyse the interactions between miR160a and the six target genes. This not only verified the authenticity of the miR160a target genes but also showed that miR160a had an inhibitory effect on the target genes.

Peu-miR160a and its target genes were induced by hormones

Auxin is a key regulator involved in almost all plant growth and development processes. ARFs are plant-specific transcription factors that regulate the expression of auxin-responsive genes (Tiwari et al., 2003). They bind specifically to the auxin response element (AuxRE) in the promoter regions of auxin-responsive genes, thereby activating or suppressing gene expression (Li et al., 2016; Roosjen et al., 2018; Vernoux and Robert, 2017). Recent studies have shown that auxin binds directly to some ARFs and regulates the interactions of ARFs with other transcription factors (Simonini et al., 2016; Vernoux and Robert, 2017). By promoting the expression of ARF10 and ARF16, which in turn inhibits WOX5,
auxin participates in the establishment of the root stem cell niche and columella cell differentiation of the RAM (Ding and Friml, 2010). In D. longa, miR160a and its target genes ARF10, ARF16 and ARF17 participate in transduction of the auxin signal (Lin et al., 2015). In Glycine max, miR160a equilibrates auxin and cytokinin in plants and regulates the formation of root nodules by inhibiting the expression of ARFs (Nizampatnam et al., 2015). Overexpression of miR160 increases soybean sensitivity to auxin and significantly reduces nodule formation (Marie et al., 2013). In our study, miR160a and its target genes were induced by IAA. Among them, miR160a, PeARF16.1 and PeARF16.2 responded rapidly to IAA and quickly reached expression peaks under IAA induction. In previous studies, miR160 was up-regulated under ABA induction in Arabidopsis and D. longa (Lin et al., 2015; Liu et al., 2007). After overexpression of miR160, Arabidopsis seeds showed reduced susceptibility to ABA during germination, whereas after overexpression of mARF10, Arabidopsis seeds and plants showed increased sensitivity to ABA (Liu et al., 2007). Here, we found that peu-miR160a responded more quickly to ABA induction than to the IAA treatment. The expression levels of PeARF16.2, PeARF17.1 and PeARF17.2 increased significantly under ABA induction.

Expression patterns and functions of peu-miR160a and its target genes

MiR160 plays crucial roles in seed germination (Liu et al., 2007; Liu et al., 2013), seedling development (Mallory et al., 2005), root cap formation and shoot regeneration in vitro (Qiao et al., 2012; Wang et al., 2005). In S. lycopersicum, miR160 regulates leaf development, fruit development, ovule patterning and floral organ abscission (Damodharan et al., 2016; Hendelman et al., 2012). Pri-miR160, ARF10, ARF16 and ARF17 were highly expressed in the roots of D. longa but were not detected in mature leaves (Lin et al., 2015). In the current study, peu-miR160a had a lower expression level in the early stage of adventitious root development (10DR) and had the highest expression level in the mature stage of adventitious root development (30DR) (Figure 4a). In contrast, its six target genes had the highest expression in the early stages of adventitious root development (Figure 4a). This finding suggested that peu-miR160a and its six target genes may play crucial roles in the development of adventitious roots of poplar.

MiR160 regulates the growth and geotropism of Arabidopsis roots (Chien et al., 2017; Khan et al., 2011; Wang et al., 2005). In Arabidopsis, after overexpressing miR160, the expression levels of ARF10 and ARF16 were reduced, root lengths were shortened, root cap development was abnormal, and cell division and RAM differentiation were out of control (Wang et al., 2005). The arf10arf16 double mutants exhibited the same phenotype as plants overexpressing miR160, but arf10 and arf16 single mutants did not show significant phenotypic changes, which indicated that the functions of ARF10 and ARF16 were redundant (Wang et al., 2005). To further understand the function of miR160a in poplar, Pro35S::miR160a was overexpressed in poplar. The expression levels of miR160a in transgenic poplars were significantly elevated, and the transcript levels of the six target genes were down-regulated, which indicated that mature miR160a was successfully released in poplars and the six target genes were cleaved to various degrees. Pro35S::miR160a transgenic poplars showed significant phenotypic variation compared to that of the WT poplars, such as dwarf plants, shortened internodes, increased lateral roots and shorter root lengths. Similarly, miR160 promoted the formation of lateral roots in Arabidopsis, and in Medicago truncatula, overexpression of miR160a also resulted in shortened root length (Bustos-Sanmamed et al., 2013; Mallory et al., 2005; Wang et al., 2005).

In Arabidopsis, only one ARF17 gene was identified, but we obtained two ARF17 genes (PeARF17.1 and PeARF17.2) in poplar (Yang et al., 2014). The protein sequences of PeARF17.1 and PeARF17.2 had high similarity (Figure S3), and their expression trends in the different developmental stages of adventitious roots of poplar were similar (Figure 4a). Understanding the functions of miRNA target genes is an important method for determining miRNA functions. Therefore, although we overexpressed peu-miR160a in poplar, we also overexpressed PeARF17.1 and PeARF17.2. In Arabidopsis, the overexpression of the miR160a-resistant version of ARF17 (SmARF17) led to shortened primary roots and hypocotyls, reduced lateral roots, defects in leaf shape, abnormal stamens and sterility (Mallory et al., 2005). The Pro35S::PeARF17.1 and Pro35S::mPeARF17.2 transgenic poplars showed an increase in the number of adventitious roots; in addition, the leaves of Pro35S::PeARF17.1 plants developed abnormally and the adventitious root lengths of Pro35S::mPeARF17.2 plants were shortened. Unlike Pro35S::mPeARF17.2, Pro35S::PeARF17.1 plants had multiple branches with no visible trunk, which may be due to abnormal shoot tip development and a loss of apical dominance. The results showed that the functions of PeARF17.1 and PeARF17.2 had some similarities and some differences. In addition, the Pro35S::PeARF17.1, Pro35S::mPeARF17.2 and Pro35S::miR160a transgenic poplars had both complementary and similar phenotypes, indicating that the miR160a regulatory network was more complex in poplar than in Arabidopsis.

ARF10, ARF16 and ARF17 showed distinct functional divergence in Arabidopsis, in which ARF10 and ARF16 have functional redundancy (Gutierrez et al., 2009; Wang et al., 2005). ARF10 and ARF16 were specifically expressed in the root cap, which was necessary for root cap formation and development, but they were not involved in adventitious root formation (Gutierrez et al., 2009; Wang et al., 2005). However, ARF17 was closely related to adventitious root formation and development, and

Figure 3 Interactions of miR160a and its target genes. (a) Verification of the interactions between miR160a and its target PeARF genes in poplar protoplasts. PeARFs: Pro35S::PeARFs were transfected into poplar protoplasts; miR160a + PeARFs: Pro35S::miR160a was co-transfected with Pro35S::PeARFs-GFP (miR160a-resistant version of PeARFs) GFP fluorescence (GFP), chlorophyll autofluorescence (Auto); bright images are shown. Scale bars: 20 μm. (b–g) qRT-PCR analysis of the interaction between miR160a and PeARFs. CK: protoplasts transfected with empty vectors; PeARFs: Pro35S::PeARFs was transfected into protoplasts; miR160a-PeARFs: Pro35S::miR160a was co-transfected with Pro35S::PeARFs into protoplasts; miR160a-mPeARFs: miR160a was co-transfected with Pro35S::mPeARFs (peu-miR160a-resistant version) into protoplasts. The error bars indicate the mean ± SE (n = 3). Asterisks indicate a significant difference as determined by a t-test (*P < 0.05, **P < 0.01).
overexpression of ARF17 resulted in a significant decrease in the number of adventitious roots (Gutierrez et al., 2009; Sorin et al., 2005; Wang et al., 2005). In Arabidopsis, miR160 positively regulated the formation of adventitious roots and lateral roots by degrading ARF17 (Gutierrez et al., 2009; Wang et al., 2005). Interestingly, we found that the functions of peu-miR160a and PeARF17.1/PeARF17.2 had similarities and differences in Arabidopsis and poplar. There was only one ARF17 in Arabidopsis but two homologous genes, PeARF17.1 and PeARF17.2, in poplar. PeARF17.1 and PeARF17.2 are also the targets of peu-miR160a in poplar. Moreover, we showed that both PeARF17.1- and PeARF17.2-overexpressing plants could promote adventitious root initiation, which is opposite to the phenotypes displayed by ARF17-overexpressing Arabidopsis plants. The protein sequences of PeARF17.1 and PeARF17.2 were highly similar, but there were some differences (Figure S3). The phylogenetic analysis found that PeARF17.1 and PeARF17.2 had distant evolutionary relationships with Arabidopsis AtARF17 while PeARF17.1 and PeARF17.2 did not belong to the same evolutionary branch (Figure S4). PeARF17.1 regulated the formation of the adventitious roots and lateral roots of poplar, while PeARF17.2 also promoted adventitious root formation but inhibited lateral root formation.

In Arabidopsis, overexpressing either miR160a or miRNA160c would promote both adventitious root initiation and elongation (Gutierrez et al., 2009). However, in poplar, our results showed that overexpressing miR160a could inhibit adventitious elongation significantly and initiation as well. The functional divergence of miR160 and its target genes in poplar and Arabidopsis may be due to the more complex mechanism of adventitious root formation in poplar, and the other regulatory molecules involved in the poplar miR160-ARFs module. We found that four long non-coding RNAs and one circular RNA were involved in the miR160a-PeARFs module (Liu et al., 2019). These results indicate that the adventitious root formation mechanism of poplar is complex.

In summary, we found that peu-miR160a targets six PeARF genes in poplar. We obtained peu-miR160a-resistant versions of PeARF16.1 (mPeARF16.1) and PeARF17.2 (mPeARF17.2) by the ‘megaprimer’ PCR method. By co-transfecting peu-miR160a and its targets PeARFs-GFP/mPeARFs-GFP in poplar protoplasts, we found that peu-miR160a inhibited the GFP fluorescence of the target gene and the expression level of the target gene. It was further verified by the Pro35S::miR160a transgenic poplars that peu-miR160a reduced the expression levels of its target genes. Observation of the phenotypes of Pro35S::miR160a, Pro35S::PeARF17.1 and Pro35S::mPeARF17.2 transgenic poplars revealed that peu-miR160a and its target genes PeARF17.1 and PeARF17.2 are involved in the adventitious root development of poplar. By investigating the functions of the peu-miR160a–PeARF17.1/PeARF17.2 module, new insights have been proposed to reveal...
Figure 5  Overexpression of miR160a inhibits adventitious root formation. (a) The expression levels of miR160a in Pro35S::miR160a transgenic lines. The error bars indicate the mean ± SE (n = 3). (b) The expression levels of target genes in Pro35S::miR160a transgenic lines. The error bars indicate the mean ± SE (n = 3). (c) Comparison of Pro35S::miR160a transgenic poplars with WT phenotypes. (d–f) Comparison of adventitious root number, length and lateral root number between WT and Pro35S::miR160a transgenic lines. The error bars indicate the mean ± SE (n = 7). L2: miR160a-L2; L3: miR160a-L3; L5: miR160a-L5; L6: miR160a-L6; L7: miR160a-L7. ARs: adventitious roots; LRs: lateral roots. Asterisks indicate a significant difference as determined by a t-test (*P < 0.05, **P < 0.01).
the key regulatory roles of this module in the adventitious root development of poplar.

Materials and methods

Plant materials

Tissue culture seedlings of the hybrid poplar ‘Nanlin895’ (Populus deltoides × Populus euramericana cv. ‘Nanlin895’) were grown in a 25 °C (day)/18 °C (night) greenhouse under a 16-h/8-h (light/ dark) photoperiod.

Gene cloning and 5’RLM-RACE

The miR160a precursor was cloned from Populus genomic DNA and analysed by RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) and DNAMAN (Woffelman, 2004). MEGA7 (Kumar et al., 2016) was used to construct the phylogenetic tree using the neighbour-joining (NJ) method. The six target genes (PeARF10.1, PeARF16.1, PeARF16.2, PeARF16.3, PeARF17.1 and PeARF17.2) of miR160a were predicted by psRNATarget (Dai and Zhao, 2011). Based on our previous study (Yang et al., 2014), the full-length cDNA sequences of the six target genes were cloned using RACE technology. To determine the internal cleavage sites of the miR160a target genes, a modified 5’RLM-RACE experiment was performed using the First Choice RLM-RACE kit (Ambion, Austin, TX). In brief, when the target gene was cleaved by miRNA, there was only one 5’ monophosphate without a 5’ cap; therefore, the alkaline phosphatase and tobacco acid pyrophosphatase treatments were not needed (Adam et al., 2011). The free hydroxyl groups were bound to 5’ monophosphate by RNA ligase and then subjected to nested PCR (Adam et al., 2011). The specific primers used for the above experiments were designed by Oligo 7 (Table S1).

Site-directed synonymous mutagenesis

Site-directed synonymous mutagenesis of PeARF16.1 and PeARF17.2 was performed using the single-tube ‘megaprimers’ PCR method (Ke and Madison, 1997). This experiment requires three primers for two rounds of PCR. The mutated miR160a-PeARF16.1 (or miR160a-PeARF17.2) binding site sequence was designed as a mutagenic primer. A megaprimer was synthesized in the first PCR using a mutagenic primer and a low-melting-temperature flanking primer. The second PCR was carried out using the megaprimer and a high-melting-temperature flanking primer.

Interaction of miR160a and its target genes

The plasmids used for transient overexpression of miR160a and its target genes in Populus protoplasts were constructed using Gateway technology (Invitrogen). The miR160a precursor and

![Image](figure6.png)

Figure 6 Overexpression of PeARF17.1 promotes adventitious root formation and increases the number of branches. (a) The expression levels of PeARF17.1 in Pro35S::PeARF17.1 transgenic lines. The error bars indicate the mean ± SE (n = 3). (b) Comparison of Pro35S::PeARF17.1 transgenic poplars with WT phenotypes. (c), (d) and (e) Comparison of adventitious root number, length and branch number between the WT and Pro35S::mPeARF17.1 transgenic lines. The error bars indicate the mean ± SE (n = 7). L10: PeARF17.1-L10; L11: PeARF17.1-L11; L14: PeARF17.1-L14; L15: PeARF17.1-L15; L18: PeARF17.1-L18; L20: PeARF17.1-L20; L21: PeARF17.1-L21. ARs: adventitious roots. Asterisks indicate a significant difference as determined by a t-test (*P < 0.05, **P < 0.01).
ORFs of the six target genes were cloned into the entry vector pCR8/GW/TOPO (Invitrogen, Carlsbad, CA). After verification by sequencing, the inserted fragments of the miR160a precursor and the six target genes in the entry vector were transferred to the destination vectors p2GWF7 and p2FGW7 by an LR reaction. The constructed vectors (Pro35S::miR160a, Pro35S::PeARF-GFP and Pro35S::mPeARF-GFP) were driven by a double CaMV 35S promoter. The Pro35S::miR160a and Pro35S::PeARF-GFP (Pro35S::mPeARF-GFP) plasmids were co-transfected into Populus protoplasts. GFP fluorescence was captured using a BX51 fluorescence microscope (Olympus, Tokyo, Japan).

To further accurately detect the interaction between miR160a and its target genes, we collected protoplasts transfected with empty vectors (CK) and protoplasts transfected with Pro35S::mir160a and Pro35S::PeARF-GFP (Pro35S::mPeARF-GFP) were driven by a double CaMV 35S promoter. The Pro35S::mir160a and Pro35S::PeARF-GFP (Pro35S::mPeARF-GFP) plasmids were co-transfected into Populus protoplasts. Populus protoplasts were isolated using 2% cellulase and 0.5% pectinase, and then, the vectors were transfected using the polyethylene glycol-mediated (PEG-mediated) method (Tan et al., 2013). GFP fluorescence was captured using a BX51 fluorescence microscope (Olympus, Tokyo, Japan).

qRT-PCR
qRT-PCR was performed on an ABI ViiA 7 Real-time PCR platform. PowerUp™ SYBR™ Green Master Mix was used for the qRT-PCR assays according to the manufacturer’s protocol. The relative expression of miR160a and its target genes was calculated by the delta-delta Ct method and normalized using the internal control gene 18S. The specific stem-loop reverse transcription primers of miR160a and qRT-PCR primers of all genes are listed in Table S1.

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Author contributions
M.X. conceived and designed the project. S.L. and M.X. analysed the data and wrote the manuscript. S.L. and C.Y. performed the experiments. L.W. and H.C. participated in the data analysis. H.L. provided helpful suggestions in the design of the project.

Conflicts of interest
The authors declare no conflicts of interest.

References
Adam, H., Marguerettaz, M., Qadri, R., Adroher, B., Richaud, F., Collin, M., Thuillet, A.C. et al. (2011) Divergent expression patterns of miR164 and miR160 regulates the development of poplar roots 467
CUP-SHAPED COTYLEDON genes in palms and other monocots: implication for the evolution of meristem function in angiosperms. Mol. Biol. Evol. 28, 1439–1454.

Addo-Quaye, C., Eshoo, T.W., Bartel, D.P. and Axtell, M.J. (2008) Endogenous siRNA and miRNA targets identified by sequencing of the Arabidopsis degradome. Curr. Biol. 18, 758–762.

Aukerman, M.J. and Sakai, H. (2003) Regulation of flowering time and floral organ identity by a microRNA and its APETALA2-like target genes. Plant Cell, 15, 2730.

Beaucle, L., Yu, A. and Bouche, N. (2010) microRNA-directed cleavage and translational repression of the copper chaperone for superoxide dismutase mRNA in Arabidopsis. Plant J. 62, 454–462.

Bustos-Sammamed, P., Mao, G., Deng, Y., Elouet, M., Khan, G.A., Bazin, J., Turner, M. et al. (2013) Overexpression of miR160 affects root growth and nitrogen-fixing nodule number in Medicago truncatula. Funct. Plant Biol. 40, 1208–1220.

Chellappan, P., Vanitharani, R. and Fauquet, C.M. (2005) MicroRNA-binding viral protein interferes with Arabidopsis development. Proc. Natl Acad. Sci. USA, 102, 10381.

Chen, H., Li, Z. and Xiong, L. (2012) A plant microRNA regulates the adaptation of roots to drought stress. FEBS Lett. 586, 1742–1747.

Chen, P.S., Chiang, C.B., Wang, Z. and Chiou, T.J. (2017) MicroRNA-mediated signaling and regulation of nutrient transport and utilization. Curr. Opin. Plant Biol. 39, 73–79.

Dai, X. and Zhao, P.X. (2011) priRNAtarget: a plant small RNA target analysis server. Nucleic Acids Res. 39, W155–W159.

Damedhara, S., Zhao, D. and Arazi, T. (2016) A common miR160/167-based mechanism regulates ovary patterning, floral organ abscission and lamina outgrowth in tomato. Plant J. 86, 458–471.

Debernardi, J.M., Rodriguez, R.E., Mecchia, M.A. and Palatnik, J.F. (2012) Functional specialization of the plant miR396 regulatory network through distinct microRNA-target interactions. PLoS One, 8, e1002419.

Ding, Z. and Friml, J. (2010) Auxin regulates distal stem cell differentiation in Arabidopsis roots. Proc. Natl Acad. Sci. USA, 107, 12046.

German, M.A., Pillay, M., Jeong, D.-H., Hetawal, A., Luo, S., Janardhanan, P., Kannan, V. et al. (2008) Global identification of microRNA–target RNA pairs by parallel analysis of RNA ends. Nat. Biotechnol. 26, 941.

Guo, H.-S., Xie, Q., Fei, J.-F. and Chua, N.-H. (2005) MicroRNA directs mRNA cleavage of the transcription factor NAC1 to downregulate auxin signals for Arabidopsis lateral root development. Plant Cell, 17, 1376.

Gutierrez, L., Bussell, J.D., Pacurar, D.I., Schwambach, J., Pacurar, M. and Bellini, C. (2009) Phenotypic plasticity of adventitious rooting in Arabidopsis is controlled by complex regulation of AUXIN RESPONSE FACTOR transcripts and microRNA abundance. Plant Cell, 21, 3119–3132.

He, F., Xu, C., Fu, X., Shen, Y., Guo, L., Leng, M. and Luo, K. (2018) The MicroRNA390/TRANS–ACTING SHORT INTERFERING RNA3 module mediates lateral root growth under salt stress via the auxin pathway. Plant Physiol. 177, 775–791.

Hendelman, A., Buxdorf, K., Stav, R., Kravchik, M. and Arazi, T. (2012) Inhibition of lamina outgrowth following Solanum lycopersicum AUXIN RESPONSE FACTOR 10 (SIA10) derepression. Plant Mol. Biol. 78, 561–576.

Jiang, N., Meng, J., Cui, J., Sun, G. and Luan, Y. (2018) Function identification of miR482b, a negative regulator during tomato resistance to Phytophthora infestans. Hortic Res. 5, 9.

Ke, S.-H. and Madison, E.L. (1997) Rapid and efficient site-directed mutagenesis by single-tube ‘megaprimer’ PCR method. Nucleic Acids Res. 25, 3371–3372.

Khan, G.A., Declerck, M., Sorin, C., Hartmann, C., Crespi, M. and Lelandais-Briere, C. (2011) MicroRNAs as regulators of root development and architecture. Plant Mol. Biol. 77, 47–58.

Kdimer, C.A. (2010) The Many roles of small RNAs in leaf development. J. Genet. Genomics, 37, 13–21.

Kumar, S., Stecher, G. and Tamura, K. (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol. Biol. Evol., 33, 1870–1874.

Li, J., Guo, G., Guo, W., Guo, G., Tong, D., Ni, Z., Sun, Q. et al. (2012) miRNA164-directed cleavage of ZmNAC1 confers lateral root development in maize (Zea mays L.). BMC Plant Biol. 12, 220.
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Wang, C., Han, J., Liu, C., Kibet, K.N., Kayesh, E., Shangguan, L., Li, X. et al. (2012) Identification of microRNAs from Amur grape (Vitis amurensis Rupr.) by deep sequencing and analysis of microRNA variations with bioinformatics. *BMC Genom.*, 13, 122.

Wang, L., Zhao, H., Chen, D., Li, L., Sun, H., Lou, Y. and Gao, Z. (2016) Characterization and primary functional analysis of a bamboo NAC gene targeted by miR164b. *Plant Cell Rep.* 35, 1371–1383.

Wang, M., Wu, H.-J., Fang, J., Chu, C. and Wang, X.-J. (2017) A long noncoding RNA involved in rice reproductive development by negatively regulating osa-miR160. *Sci. Bull.* 62, 470–475.

Woffelman, C. (2004) *DNAMAN for Windows*, Version 5.2.10. Leiden: Lynon Biosoft, Institute of Molecular Plant Sciences. Leiden University.

Xu, M., Chen, C., Cai, H. and Wu, L. (2018) Overexpression of PeHKT1;1 improves salt tolerance in Populus. *Genes* 9, 475.

Yang, C., Xu, M., Xuan, L., Jiang, X. and Huang, M. (2014) Identification and expression analysis of twenty ARF genes in Populus. *Gene*, 544, 134–144.

Zhang, J., Zhang, H., Srivastava, A.K., Pan, Y., Bai, J., Fang, J., Shi, H. et al. (2018) Knockdown of rice microRNA166 confers drought resistance by causing leaf rolling and altering stem xylem development. *Plant Physiol.* 176, 2082.

Zuker, M. and Stiegler, P. (1981) Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. *Nucleic Acids Res.* 9, 133–148.

**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1** Primer sequences of peu-miR160a and its target genes.

**Figure S1** Structural analysis of the miR160a precursor.

**Figure S2** Prediction of target sites for peu-miR160a by psRNATarget.

**Figure S3** Protein sequence alignment of PeARF17.1 and PeARF17.2.

**Figure S4** Phylogenetic tree analysis of ARF17 in different plants.