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Comparative Study of Small-RNA and Degradome Sequencing Reveals Role of Novel stu-miR8006 in Regulating Root Development in *Solanum tuberosum* L.

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Abstract: MicroRNAs are a class of endogenous, non-coding, small-RNA molecules with important functions in plant development and stress response processes. Root systems are important because they allow plants to absorb nutrients and water from the soil and are fundamental for anchoring the plant and responding to environmental signals. However, the roles of miRNAs underlying root development remain poorly investigated in potato. In this research, small-RNA sequencing was performed to thoroughly detect underlying miRNAs and their roles in regulating root development between the early root (ER) stage and the mature root (MR) stage of potato roots. A total of 203 known and 137 novel miRNAs were obtained, and 64 differentially expressed miRNAs (DEMs) were identified between the ER and MR stages. The expression patterns of 12 DEMs were also determined via qRT-PCR. In addition, a mixed degradome library was constructed from the ER and the MR stages to identify the targets of the identified miRNAs, and 2400 target genes were verified to be the targets of 131 miRNAs. Based on target annotation, we identified that nine target genes of six DEMs were probably involved in potato root development, and eight targets of six DEMs were validated via 5′-RLM-RACE assays. These targets may participate in root development by regulating cell proliferation, root cultures (PGSC0003DMT400013837), root meristem growth (PGSC0003DMT400079970), root morphogenesis (PGSC0003DMT400040282), post-embryonic root development (PGSC0003DMT400021612), root hair elongation (PGSC0003DMT400034518), cell wall repair (PGSC0003DMT400074930), and auxin polar transport (PGSC0003DMT400079970), and by negatively regulating cell proliferation (PGSC0003DMT400009997) and cell growth (PGSC0003DMT400003464). The qRT-PCR analysis indicated that most miRNAs have opposing expression patterns to their targets. It is widely accepted that potato root development is regulated by miRNAs, among which stu-miR8006-p5-1ss9AT is substantially down-regulated during root development. We show here that the suppression of stu-miR8006-p5-1ss9AT led to an alteration in the potato root architecture and that it targeted auxin induction in the root culture protein 12-encoding gene that is potentially involved in the regulation of root development. In addition, the suppression of stu-miR8006-p5-1ss9AT led to a significant alteration in the potato root architecture. Altogether, our results might provide some useful insights into stu-miR8006-p5-1ss9AT and the crucial role that it plays in potato root development; they could also facilitate the molecular genetic breeding of potato.

Keywords: root development; microRNA; small-RNA sequencing; degradome sequencing; *Solanum tuberosum*

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1. Introduction

Roots are important organ systems of plants that are involved in the extraction of nutrients and water; anchorage; reproduction; the storage function, synthesis, and accumulation of secondary metabolites; and they are also the main site for the interaction of nitrogen-fixing organisms and various pests and pathogens [1–3]. Therefore, root biology has been studied in depth to enhance crop productivity and survival rates in adverse environmental conditions. In recent years, a large number of genes involved in root development have been identified through genetic methods, such as *AtSWP1/LDL1*, which has been reported to negatively regulate the elongation of lateral roots via the direct or indirect transcriptional inhibition of lateral root promoters (such as auxin response factors (ARFs) and GATA23) in *Arabidopsis thaliana* [4]. Kang et al. (2019) also found that *OsZFP350* had a positive impact on the root morphology of rice [5]. The rice *OsZFP350* gene was induced by osmotic stress, and *OsZFP350*-overexpressed lines produced a pleiotropic phenotype with improved root configuration, and the primary root length and the lateral root numbers were significantly increased [5]. Researchers have also made significant advances in the study of genes related to the architecture of maize roots. The regulation of crown roots, lateral roots, and root hairs is governed by genes such as *NAC*, *IRT1*, and *SLR1/2. RTCS* is also responsible for regulating the development of crown roots, and the RTH family (*RTH1*, *RTH2*, *RTH3*, *RTH5*, and *RTH6*) regulates root hair development. *SlHSP90.2* was found to regulate tomato lateral root formation, with the length and number of the lateral roots increasing by 35–38% in *SlHSP90.2*-overexpressed lines. Further, the increase in root biomass in overexpressed lines has been demonstrated to enhance tomato's tolerance to salinity and drought stress [6]. In wheat, numerous genes have been identified that regulate the development of roots, such as *WAK1*, *RSL2* and *RSL4*. Studies have also shown that the overexpression of *TaWRKY51-2B* can increase the number of lateral roots by 20–50% in wheat. The overexpression of *TaWRKY51-2b* in wheat can increase the lateral root numbers from 150% to 340%. Further investigation revealed that *TaWRKY51* regulates lateral root formation through the auxin signaling pathway [7–9].

Potato (*Solanum tuberosum* L.) is a typical shallow-root crop, and the root system is often characterized as shallow and inefficient, with weak penetration in the compact soil to extract water and nutrition; thus, various abiotic stresses at any stage of growth and development will frequently affect the yield and quality of potato tubers [10]. Therefore, it is of great significance to understand how the potato root system develops holding potential to increase potato yields and increase stress resistance. At present, the genetic mechanisms related to potato development domestically and abroad mainly focus on the leaves, flowers, tubers, and stress responses. It has been proven that miR160 targets *StARF10*/16/17 to regulate potato leaf development [11]. *StSP3D* has been identified as the flowering signal of potato, and silencing *StSP3D* causes a delay in potato flowering [12]. *StAN11* regulates the biosynthesis of potato anthocyanins by controlling the expression of *DFR*. The overexpression of *StAN11* can lead to the accumulation of anthocyanins and a much darker tuber color [13]. *StTCP15* regulates the dormancy and germination of potato tubers by affecting the dynamic balance between ABA and GA [14]. Compared to the model plant Arabidopsis and other graminaceous crops, few studies on the genes or miRNAs related to the potato root architecture have been reported so far [15–17].

MicroRNAs are small, non-coding, single-stranded RNAs with regulatory functions in eukaryotes and base lengths between 21 and 25 [18]. MicroRNAs usually target one or more mRNAs and regulate target genes by complementing and binding to the transcription products of the target mRNAs to degrade the target mRNAs, inhibit translation, or mediate DNA methylation [19]. MicroRNAs are one of the main components of molecular networks [20]. More and more plant miRNAs have been discovered as mass sequencing has progressed. Plant miRNAs have been shown to not only regulate various plant development processes, such as leaf development [21], stem development [22], flowering time [23], organ polarity [24], and signal transduction [25], but also to play a significant role in biotic and abiotic stress responses [26–29]. For example, miRNAs are involved

in the drought stress responses of cereals [30], rice [31,32], wheat [33], and other plants. Although many miRNA functions have been identified, most studies have focused on some model plants. However, since the functions of miRNAs in many non-model plants have not been determined, it is extremely important that the functions of miRNAs in potato root development be thoroughly investigated.

Herein, to explore the miRNAs involved in potato root development, small-RNA samples were extracted from the early root stage (ER) and mature root stage (MR) of potato. Based on small-RNA sequencing, a total of 203 known miRNAs and 137 novel miRNAs were obtained, and 64 differentially expressed miRNAs were identified between ER and MR. A total of 2400 target genes of 131 miRNAs were also obtained by degradome sequencing, and eight target genes of six DEMs were confirmed by 5′-RACE assays, which have identified that miRNAs play an important role in the regulation of plant root development. This study provides new insights into a potential regulatory mechanism for the development of potato roots.

2. Materials and Methods

2.1. Plant Materials

The potato cultivar "Desiree", used as test material, was cultivated in a standard light incubator with a 16 h light/8 h dark photoperiod. Early stage (ER) and mature stage (MR) were categorized as developmental stages of potato roots based on root length and adventitious root differentiation. For analyses of root development, potatoes were grown on MS medium and root tissues were collected at 10 days (ER) and 24 days (MR), snap-frozen in liquid nitrogen, and stored at −80 °C for RNA extraction.

2.2. Small-RNA Isolation and Sequencing

The total RNA was extracted from potato root samples using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Small RNA (sRNA) with a length of 15–30 nt was isolated through gel electrophoresis and linked with 5′ and 3′ adapters. The cDNA library was constructed and amplified by RT-PCR. The constructed potato libraries were sequenced using the high-throughput Illumina HiSeq™ 2500 platform (Illumina, San Diego, CA, USA) at Biomarker Technologies (Beijing, China).

2.3. Sequencing Data Analysis and Identification of Potato miRNAs

The sequencing data are divided into following processes:. First, clean reads were obtained from the original reads after removing the 3 'joints, and the sequences between 18 and 25 nt were retained. Then, the clean reads were used to blast with known RNA database sequences (excluding miRNA). Finally, the potential miRNAs were obtained by blasting miRbase 21.0 (https://www.mirbase.org/ftp.shtml; accessed on 16 April 2021). The mature miRNAs and precursor miRNAs that have been reported in plants were compared and the conserved miRNAs were identified. To identify potato novel miRNAs, the sequences (excluding conserved miRNAs) were analyzed by the miRDeep2 program and RNAfold web server (http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi; accessed on 16 April 2021), including dicer restriction sites identification, secondary structure prediction, and free energy calculation.

2.4. Differential Expression Analysis of Potato miRNAs

In order to identify differentially expressed miRNAs, we normalized the expression levels of miRNAs in ER and MR samples via TPM values; then, DEseq2 was used to calculate differentially expressed miRNAs. The miRNAs with $|\log2\ (\text{MR/ER fold change})| \geq 1$ and a significance threshold < 0.05 were identified as differentially expressed miRNAs.

2.5. Degradome Library Construction and Sequencing

A mixed degradome library was constructed from ER and MR samples to verify the pairing relationship between miRNAs and their predicted targets. Equal amounts of enriched

RNAs from both ER and MR samples were mixed. Poly-A molecules were ligated to the 5′ terminus of purified RNA samples. The ligation fragments were subjected to reverse transcription and PCR amplification. The amplified products were purified by gel electrophoresis and subsequently subjected to sequencing using the Illumina HiSeq™ 2500 platform (Illumina, San Diego, CA, USA). The degradome library was constructed and sequenced at Biomarker Technologies (Beijing, China).

2.6. Identification of miRNA Targets

The CleaveL and 3.0 pipeline (University Park, PA, USA) were used to identify targets of potato miRNAs in this study. Briefly, after the removal of adapter reads, polyN fragments and low-quality tags from the raw reads, and obtained clean reads, were used to blast with the Rfam database to remove ncRNAs. The remaining sequences were used for cleavage site analysis by CleaveL and 3.0 pipeline with a *p*-value of less than 0.05. Based on the relative abundance of degraded sequences at the 9th to 11th positions of a miRNA binding site, T-plots were built to show the distribution of the t-signatures and abundances on the target mRNAs. In addition, Gene Ontology (GO) was used to functionally annotate the target genes in various biological processes, molecular functions, and cellular component groups. The KEGG database was also used for pathway analyses of identified target genes.

2.7. RLM-5′-RACE Assay

The RLM-5′-RACE assay was used to validate 9 potential targets of 6 potato miRNAs (Table 1) by using the GeneRacer kit (Invitrogen). Briefly, total RNA was ligated with an RNA adapter and a reverse transcription was performed to obtain cDNA using oligo dT. The resulting cDNA was performed in nested PCR using the GeneRacer out primer and gene-specific out primer in the first round of amplification; then, the amplified PCR fragments served as a template for nested PCR using the GeneRacer inter primer and gene-specific inter primer in the second round. The amplified products were gel purified and sequenced to determine cleavage sites in the mRNAs of target genes.

Table 1. Identification of miRNAs involved in potato root development.

MiRNA	MiRNA Sequences	Target Gene	Transcript Annotation	GO_Term
PC-3p-230042_5	CAAGGCTAGGACAG ATGGAAAGAA	PGSC0003DMT400074930	uncharacterized protein LOC102590046 isoform X1	GO:0048364 (root development); GO: 0071433 (cell wall repair)
PC-5p119431_10	TCTTAGGCCTAATTCACACCC	PGSC0003DMT400003464	receptor-like protein kinase FERONIA isoform X2	GO:0030308 (negative regulation of cell growth); GO: 0048364 (root development)
stu-miR477b-5p	ACTCTCCCTCAAAGGCTTCTG	PGSC0003DMT400021612	serine/threonine-protein kinase CTR1-like isoform X1	GO:2000069 (regulation of post-embryonic root development)
stu-miR5303g	TTTTTGAAGAGTCTGGGCAAC	PGSC0003DMT400009997	growth-regulating factor 9-like	GO:0008285 (negative regulation of cell proliferation); GO: 0048364 (root development)
		PGSC0003DMT400079970	uncharacterized protein OsI_027940-like	GO:0010449 (root meristem growth); GO:0080037 (negative regulation of cytokinin-activated signaling pathway); GO:2000012 (regulation of auxin polar transport)
stu-miR5303j-p5	ATTCAAATTTCTGCCCTA	PGSC0003DMT400034518	tropinone reductase-like 3	GO:0048767 (root hair elongation)
stu-miR8006-p5-1ss9AT	CATACTTTTGGGACATTG	PGSC0003DMT400013837	auxin-induced in root cultures protein 12 isoform X1	Function unknown
		PGSC0003DMT400040282	protein NRT1/PTR FAMILY 1.2-like isoform X2	GO:0010015 (root morphogenesis);

2.8. qRT-PCR Analysis

The qRT-PCR analysis was employed to test the expression profiles of potato miRNAs and their targets between ER and MR samples. In the study, total RNA extraction, cDNA synthesis, and the reactions were performed by strictly following the manufacturer's instructions. *Stef1a* gene was used as the internal control, and three biological replicates in each reaction were performed. The gene-specific primers are given in Table 2.

Table 2. Oligonucleotides used for qRT-PCR analysis.

Gene	Sequence of Oligonucleotides Forward (F) and Reverse (R)	Length (bp)
stu-miR8020	R 5′-GGAATTTCATTGAGTATGTTGTTGTT-3′	-
stu-miR167d-3p	R 5′-CAGATCATGTGGTTGCTTCAC-3′	-
stu-miR477a-5p	R 5′-CCTCTCCCTCAAGGGCTTCT-3′	-
stu-miR408a-3p	R 5′-TGCACAGCCTCTTCCCTG-3′	-
stu-miR398a-3p	R 5′-TATGTTCTCAGGTCGCCCCT-3′	-
stu-MIR319-p5	R 5′-TGCTGCTGAATCATTGGTTC-3′	-
PC-3p-28523_59	R 5′-GCGTTATTTTCACCTGACAAGACT-3′	-
PC-5p-82220_15	R 5′-GCGTTCTAGGATATAAATTGCACTACT-3′	-
PC-5p-138631_8	R 5′-TCAACTATCCAGTCTTTCCTCAGA-3′	-
PC-5p-107179_11	R 5′-CGAGTTTCCTACCTGAACTATCACC-3′	-
PC-5p-656396_2	R 5′-ACTTCCTGATCACATGGCGA-3′	-
PC-3p-291253_4	R 5′-TAAGGTAGGGCGCTCTTCG-3′	-
PC-3p-230042_5	R 5′-CAAGGCTAGGACAGATGGAAAG-3′	-
PC-5p119431_10	R 5′-GTCTTAGGCCTAATTCACACCC-3′	-
stu-miR477b-5p	R 5′-ACTCTCCCTCAAAGGCTTCTG-3′	-
stu-miR5303g	R 5′-GTTTTTGAAGAGTCTGGGCAAC-3′	-
stu-MIR5303j-p5	R 5′-GCGATTCAAATTTCTGCCCTA-3′	-
stu-miR8006-p5-1ss9AT	R 5′-CGCATACTTTTGGGACATTG-3′	-
St18sRNA	R 5′-TTAGAGGAAGGAGAAGTCGTAACAA-3′	-
uncharacterized protein LOC102590046	F 5′-CAAGCATACCCACTTCCTCT-3′ R 5′-GTCCTAACTCCTGAGACAACG-3′	182
receptor-like protein kinase FERONIA	F 5′-CGCCGAGGATAGTTTACACAA-3′ R 5′-ACCCTGTCGTTCACTTCCAG-3′	150
serine/threonine-protein ki′-se CTR1-like	F 5′-TTGTGTTACCGACGCAGT-3′ R 5′-GCAGGTTGCTTCAGTAGAGAT-3′	187
growth-regulating factor 9-like	F 5′-CAGAGCCAAGAAGATGTAGGAG-3′ R 5′-TTTGAAGAGTCCGAACAACG-3′	168
uncharacterized protein OsI_027940	F 5′-TGACTTCTCGGACTGGCTGT-3′ R 5′-GCAGGATGTCTTCAACTCTGG-3′	182
tropinone reductase-like 3	F 5′-CTGAAAGGCTTGGTTTAGAAGG-3′ R 5′-GCCACGGTAGGATTTACAGC-3′	225
auxin-induced in root cultures protein	F 5′-CGTATTCCCTTCCATCCTATC-3′ R 5′-GGCTGAGAAAGTTTGAGAGG-3′	254
protein NRT1/PTR FAMILY 1.2	F 5′- CTGATTCTTACTGTGGTCGCT -3′ R 5′-CCTCTTGTTCTCGGGTTTC-3′	118
Stef1α	F 5′- ATTGGAAACGGATATGCTCCA-3′ R 5′- TCCTTACCTGAACGCCTGTCA-3′	101

2.9. Identification and Expressional Analysis of stu-miR8006-p5-1ss9AT

To identify stu-miR8006-p5-1ss9AT in potato, mature sequence and precursor sequence of stu-miR8006-p5-1ss9AT were obtained from small-RNA sequencing, and 215 nt precursor length was used for evaluating the secondary structure using the available online tool UNAFold. The qRT-PCR was used to analyze the expression levels of stu-miR8006-p5-1ss9AT in different tissues and examine its target genes expression levels during the potato root development; the method is the same as in Section 2.8. The gene-specific primers are also listed in Table 2.

2.10. Vector Construction and Potato Transformation

The STTM fragment of stu-miR8006-p5-1ss9AT was constructed to reduce endogenous stu-miR8006-p5-1ss9AT activity in this study. The STTM fragment was designed as described by Wu et al. (2013) [34], Briefly, the stu-miR8006-p5-1ss9AT imperfect binding site motif on its target gene was used as a mimic target site motif, which contains 3 additional nucleotides of insertion (CTA) in the mimic target site motif. A 96-nucleotide STTM fragment containing two copies of the mimic target sites motif was linked with a 48 nt fragment,

and the STTM fragment was cloned into the pBI121 vector and named PBI121-STTM8006. The potato transformation was performed using the previously reported method [35].

2.11. Root Architecture Analysis

In order to study the function of stu-miR8006-p5-1ss9AT in potato root development, the three potato transgenic lines (T2, T3, and T5) were used for root architecture analysis. Test-tube plantlets that were 21 days old were harvested for photographing to display the differences in root architecture between the CK and transgenic plants. The length, biomass of plant roots, and lateral root numbers were measured at full maturity.

2.12. Statistical Analysis

Statistical analysis of experimental data was performed with Microsoft Excel 2010. Asterisks indicate significant differences between transgenic lines and WT control plants based on Student's *t*-test (* $p < 0.05$ and ** $p < 0.01$).

3. Results

3.1. Overview of sRNA Sequencing

Two sRNA libraries were constructed from the early root stage (ER) and the mature root stage (MR) of potatoes to identify miRNAs involved in potato root development. We obtained 15,443,787 and 12,099,703 raw reads from the ER and MR libraries, respectively (Table 3). After removing the low-quality sequences, adaptor sequences, Junk reads, reads shorter than 18 nt or more than 30 nt, and producing 8,985,433 clean sequences in ER and 316,653 clean reads in MR (Table 3), clean reads were used to annotate the rRNAs, snRNAs, snoRNAs, tRNAs, and other ncRNAs (Figure 1); the unannotated reads were further searched against the potato genome sequence; and totals of 30,578,981 and 28,408,840 unique sequences accounting for 88.39% and 88.49% were obtained in ER and MR, respectively (Table 3). The length distribution analysis showed that most of the small RNAs distribute from 18 to 25 nt in length, and the 24 nt sequences had the highest sequence redundancy in all libraries (Figure 2), which was consistent with the typical size range generated by the dicer. In addition, nucleotide bias analysis showed the dominant bias for U at the first two positions in the mature small-RNA sequences (Figure 3).

Table 3. Date of sequencing reads from ER and MR sRNA libraries.

Term	ER	MR
Raw reads	15,443,787	12,099,703
3ADT and length filter	2,202,430 (54.26%)	6,595,079 (54.51%)
Junk reads	56,274 (0.36%)	25,332 (0.21%)
Rfam	1,693,395 (10.96%)	1,189,792 (9.83%)
Repeats	24,663 (0.16%)	24,286 (0.20%)
Valid reads	8,985,433 (5%)	3,166,534 (26.17%)
Mapped reads	46,382,473 (88.29%)	44,040,313 (88.39%)
Unique Mapped reads	30,578,981 (58.21%)	28,408,840 (57.02%)
Q20 (%)	99.73	99.72
Q30 (%)	98.10	98.08
GC content (%)	43	43.5

Notes: ER means early root stage; MR means mature root stage.

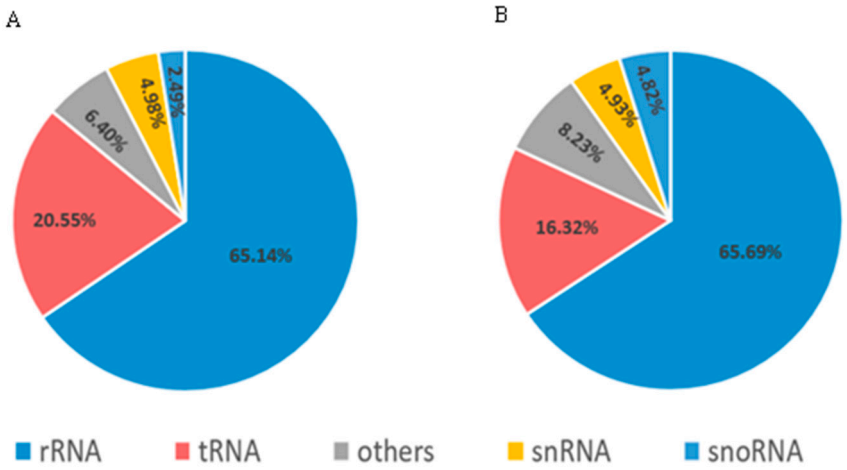

Figure 1. Percentage of annotated sRNAs in early root stage (**A**) and mature root stage (**B**).

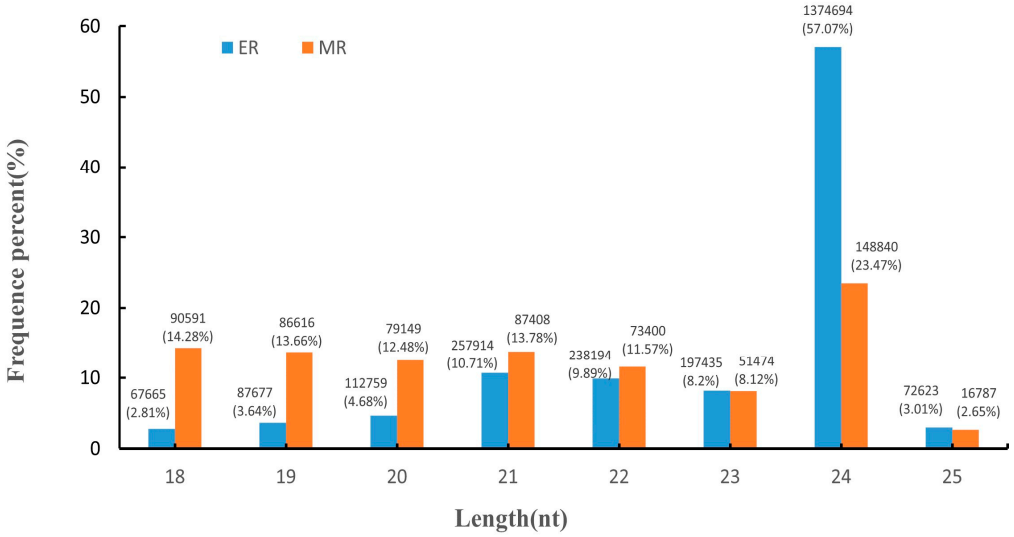

Figure 2. Length distribution of small RNAs in two libraries, ER means early root stage; MR means mature root stage.

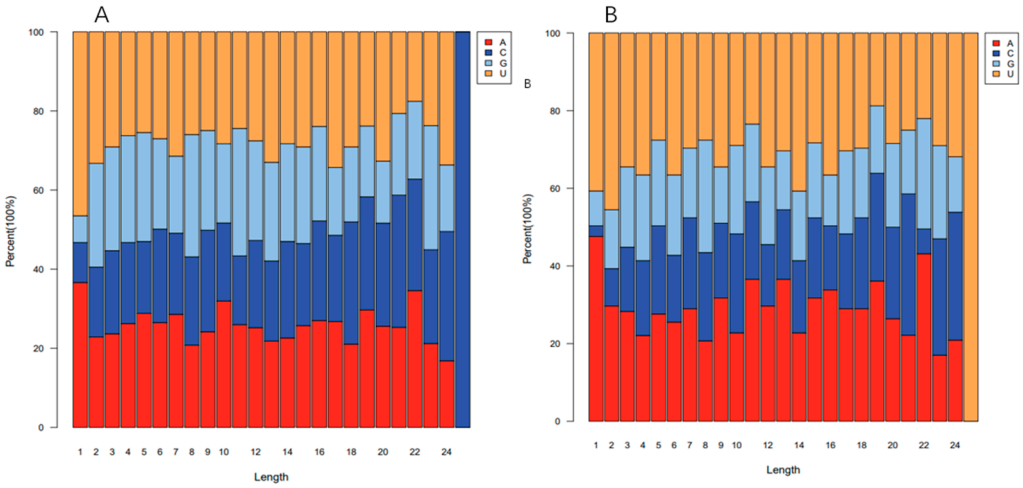

Figure 3. Nucleotide bias analysis. (**A**) The nucleotide distribution of potato known miRNAs. (**B**) The nucleotide distribution of potato novel miRNAs; the nucleotide numbering is from 5′ to 3′.

3.2. Identification of Known and Novel miRNAs in Potato

To identify known miRNAs in potato roots, the mapped reads were used to blast with mature sequences of known miRNAs in the miRBase database (release 21.0). A total of 203 known miRNAs were obtained, belonging to 82 miRNA families (Supplementary Materials S1). Among the 82 identified families, the MIR7984 family has the largest family, with 12 members; followed by the MIR5303 and MIR8006 families, with 10 family members; and there are 35 families with only one member (Supplementary Materials S1). miRDeep2 was used to identify novel miRNAs in potato roots with default plant parameters, and 137 novel miRNAs were obtained in potato roots (Supplementary Materials S2). The expression analyses showed that novel miRNAs have lower expression levels than known miRNAs. Significantly, only five novel miRNAs (PC-3p-158843_7, PC-5p-77773_16, PC-5p-223762_5, PC-5p-5426_338, and PC-3p-51799_27) had more than 100 reads in two libraries. Figure 4 shows the part of secondary structures of newly predicted miRNA precursors.

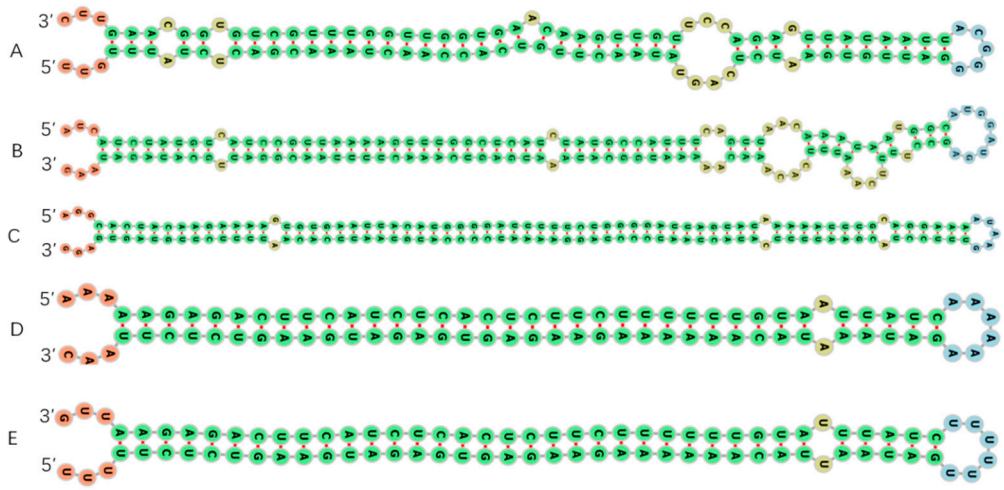

Figure 4. The secondary structure of some predicted miRNAs in potato: (**A**) PC-3p-158843_7; (**B**) PC-5p-77773_16; (**C**) PC-5p-223762_5; (**D**) PC-5p-5426_338; (**E**) PC-3p-51799_27.

3.3. Differential Expression Analysis of miRNAs between ER and MR

To identify miRNAs involved in potato root development, a T-test was performed to screen differentially expressed miRNAs between the ER and MR libraries. Using the ER libraries as the control, miRNAs with $|\log_2$ (Fold-change)(MR/ER)$| > 1$ and a significance threshold < 0.05 were considered as differentially expressed miRNAs. In total, 64 differentially expressed miRNAs (DEMs) were obtained, including 46 known DEMs and 18 novel DEMs. Moreover, the number of down-regulated miRNAs was much higher than that of up-regulated miRNAs, with 12 up-regulated miRNAs and 52 down-regulated miRNAs between the ER and MR samples (Supplementary Materials S3). To confirm the expression pattern of miRNAs from small-RNA sequencing data, qRT-qPCR was performed to verify the expression pattern of twelve differentially expressed miRNAs during potato root development, including six up-regulated miRNAs (stu-miR408a-3p, stu-miR398a-3p, stu-miR319-p5, PC-5p-107179_11, PC-5p-656396_2, and PC-3p-291253_4) and six down-regulated miRNAs (stu-miR8020, stu-miR167d-3p, stu-miR477a-5p, PC-3p-28523_59, PC-5p-82220_15, and PC-5p-138631_8). The results nearly agreed with the sRNA sequencing data. (Figure 5).

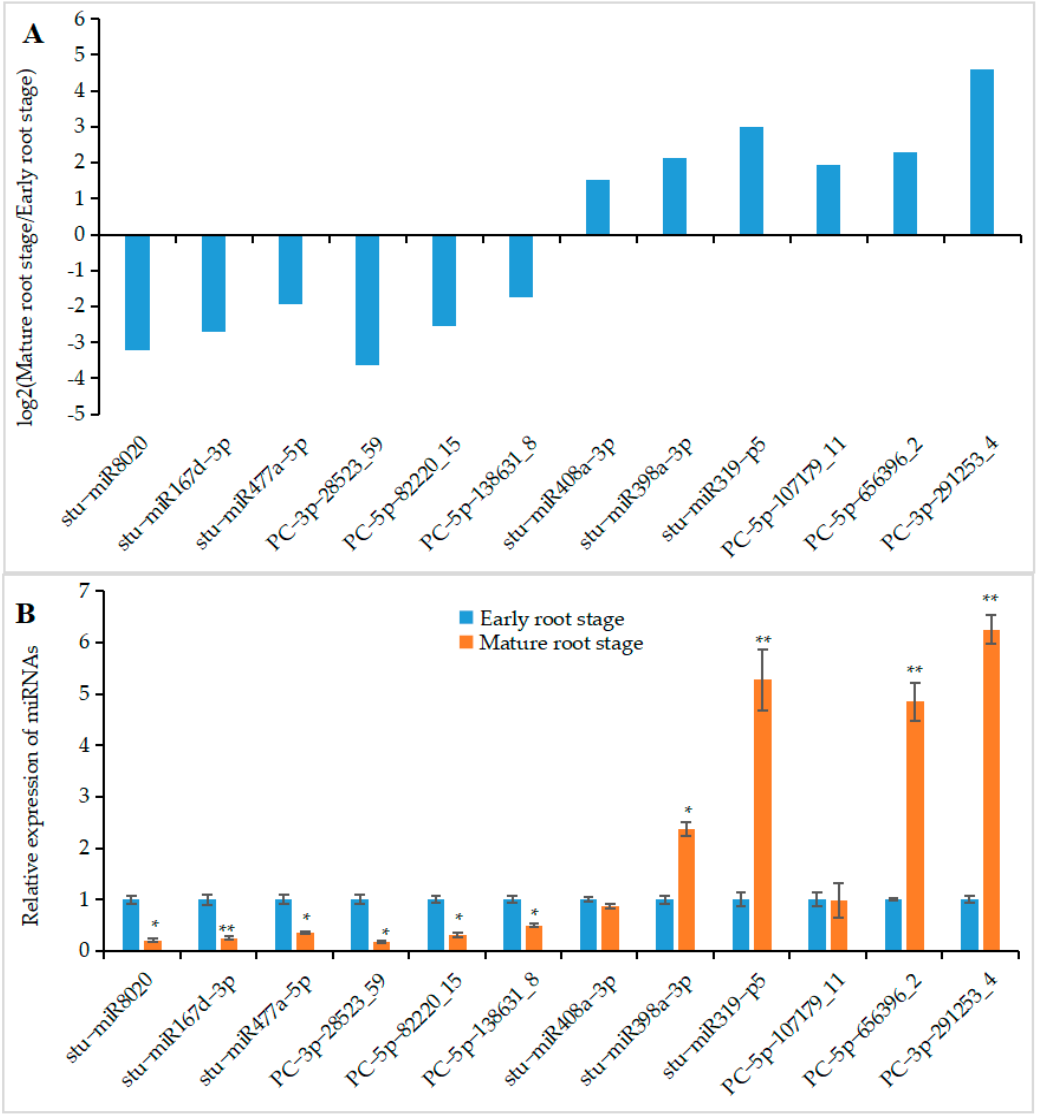

Figure 5. Validation of the differentially expressed miRNAs between the ER and MR libraries by qRT-PCR. (**A**) Fold change (log_2) of DEMs between the ER and MR libraries detected by small-RNA sequencing. (**B**) The relative expression level of DEMs between the ER and MR libraries measured by qRT-PCR. Asterisks indicate a significant difference between transgenic lines and WT control plants based on Student's *t*-test at $p < 0.05$ (*) and $p < 0.01$ (**).

3.4. Identification of miRNA Targets by Degradome Analysis

To investigate the functions of potato miRNAs involved in root development, TargetFinder was used to predict the target genes of potato miRNAs. To further validate the targets, a mixture degradome library was constructed from both ER and MR samples to identify transcriptome-wide miRNA-cleaved mRNAs. A total of 26,207,517 raw reads were obtained from the mixed degradome library. After removing the adapter, PolyN fragments, and low-quality sequences, 9,690,091 unique mappable reads were obtained; among them, 99.31% of unique sequences were mapped to the potato genome (Table 4). All mapped reads were subjected to further analysis using the CleaveL and 3.0 pipeline software. As a result, a total of 2400 targets for 131 potato miRNAs were identified in the mixed degradome library (Supplementary Materials S4). The T-plots are shown in Supplementary Materials S5; among those miRNA families, stu-miR5303g and stu-MIR7997c-p3 have the largest number of target genes.

Table 4. Summary of data from potato degradome sequencing.

Type	Number of Reads	Percentage (%)
Raw reads	26,207,517	100
Reads < 15 nt after removing 3 adapters	229,844	0.88
Mappable reads	25,977,673	99.12
Unique raw reads	9,690,091	100
Unique reads < 15 nt after removing 3 adapters	78,098	0.81
Unique mappable reads	9,611,993	99.19
Transcript mapped reads	18,191,670	69.41
Unique transcript mapped reads	5,637,124	58.17
Number of input transcript	56,218	100
Number of covered transcript	43,889	78.07

3.5. GO Enrichment and KEGG Pathway Analyses of Target Genes

Gene ontology (GO) was used to annotate identified target genes. The results showed that all targets were classified into three categories: biological process, cellular component, and molecular function (Figure 6). The greatest numbers of functional groups in biological processes are transcription and DNA templating; nuclear, cytoplasm, and organelle functional groups in cellular components; and DNA binding, transcription factor activity, and sequence-specific DNA binding functional groups in molecular functions. KEGG transduction analysis showed that miRNA targets are mainly involved in environmental adaptation, metabolism, folding, sorting and degradation, and the signal transduction pathway; notably, most of the targets are involved in the metabolism pathway and genetic information processing (Figure 7).

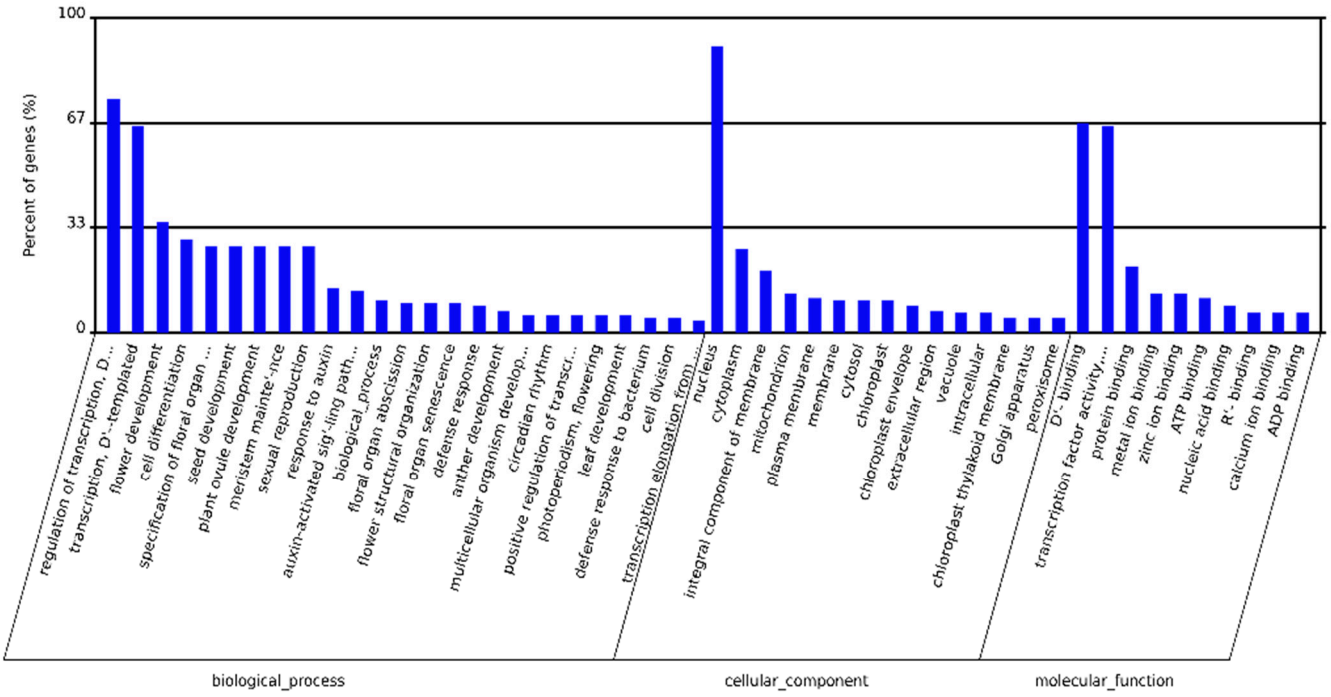

Figure 6. GO annotation of target genes for degradation sequencing.

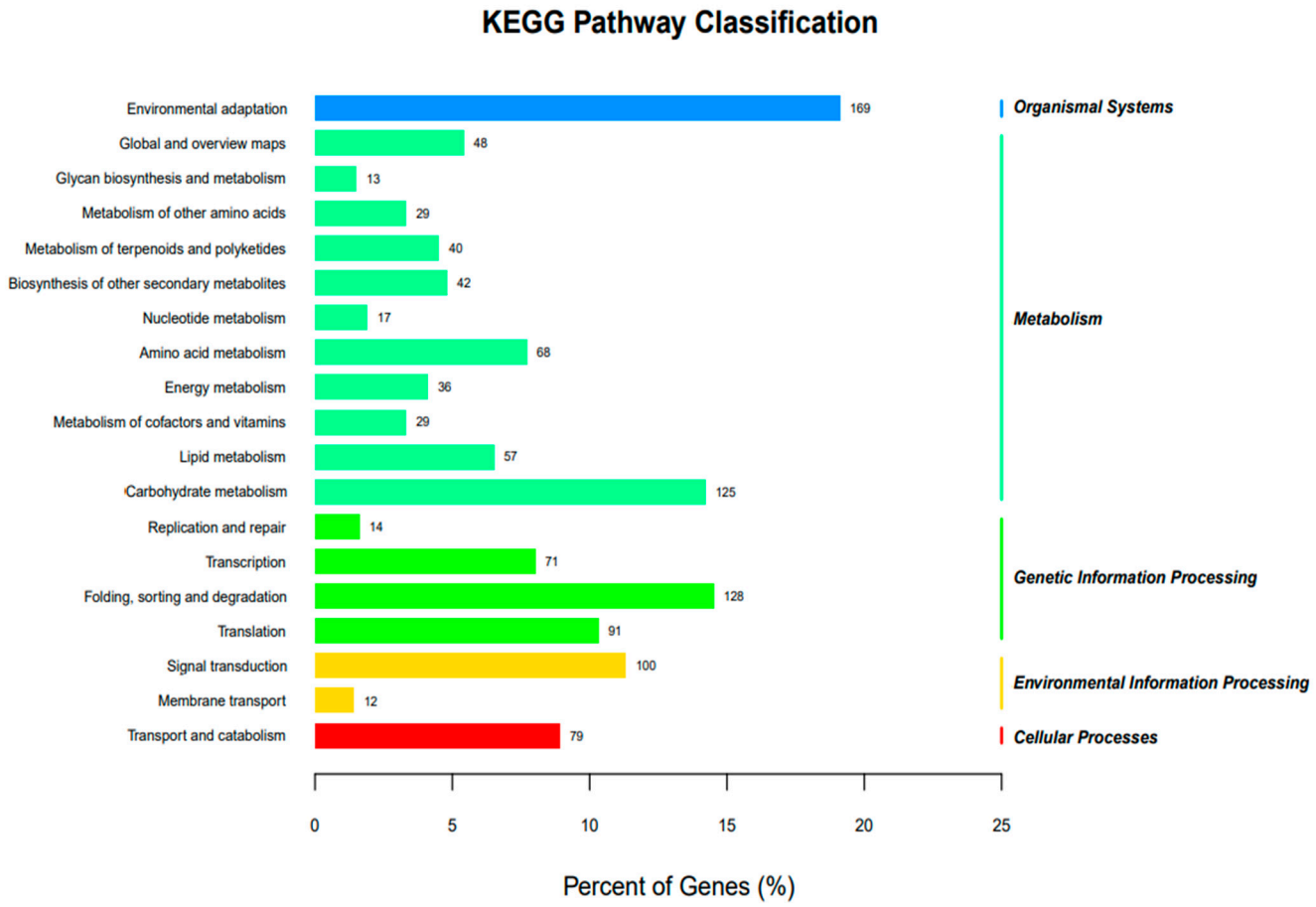

Figure 7. KEGG pathway analysis of the target genes.

3.6. Identification of miRNAs and Their Targets Involved in the Development of Potato Roots

To identify the roles of miRNA in potato root development, based on the gene function annotation and previous literatures, nine targets of six DEMs were identified as putative genes showing involvement in potato root development, including three potential targets (PGSC0003DMT400009997, PGSC0003DMT 400,079,970, and PGSC0003DMT400081457) of stu-miR5303g; among them, PGSC0003DMT400009997, encoding growth-regulating factor, was predicted to be involved in cell proliferation of the plant root; PGSC0003DMT400081457, encoding probable linoleate 9S-lipoxygese 5 genes, was predicted to regulate lateral root formation of plant; and PGSC0003DMT 400,079,970, encoding an uncharacterized protein OsI_027940-like, was predicted to play an important role in plant root meristem growth, cytokinin-activated signaling pathway, and auxin polar transport. Two potential target genes (PGSC0003DMT400013837 and PGSC0003DMT400040282) of stu-miR8006-p5-1ss9AT—PGSC0003DMT400013837, encoding auxin-induced in root cultures protein 12, which is involved in root development; and PGSC0003DMT400040282, encoding protein NRT1/PTR FAMILY 1.2-like isoform X2—were predicted to be involved in root morphogenesis. The potential target of stu-miR477b-5p is a serine/threonine-protein kinase CTR1-like gene (PGSC0003DMT400021612) that plays a vital role in the post-embryonic root development of plants. The potential target of stu-miR5303j-p5 is a tropinone reductase-like 3 (PGSC0003DMT400034518), which plays an important role in plant root hair elongation; the potential target of PC-5p119431_10 is uncharacterized protein LOC102590046, which is involved in plant root development and cell wall repair; and the potential target of PC-3p-230042_5 is receptor-like protein kinase FERONIA, which is involved in plant root development and cell growth.

3.7. Validation of miRNAs' Targets Involved in the Development of Potato Roots

To further validate the potential targets of miRNAs involved in potato root development from degradome sequencing, RLM-5′-RACE was used to confirm nine putative targets of six miRNAs. The results indicated that eight targets of six miRNAs were cleaved at the expected position of the target mRNA fragment and directed the cleavage (Figure 8).

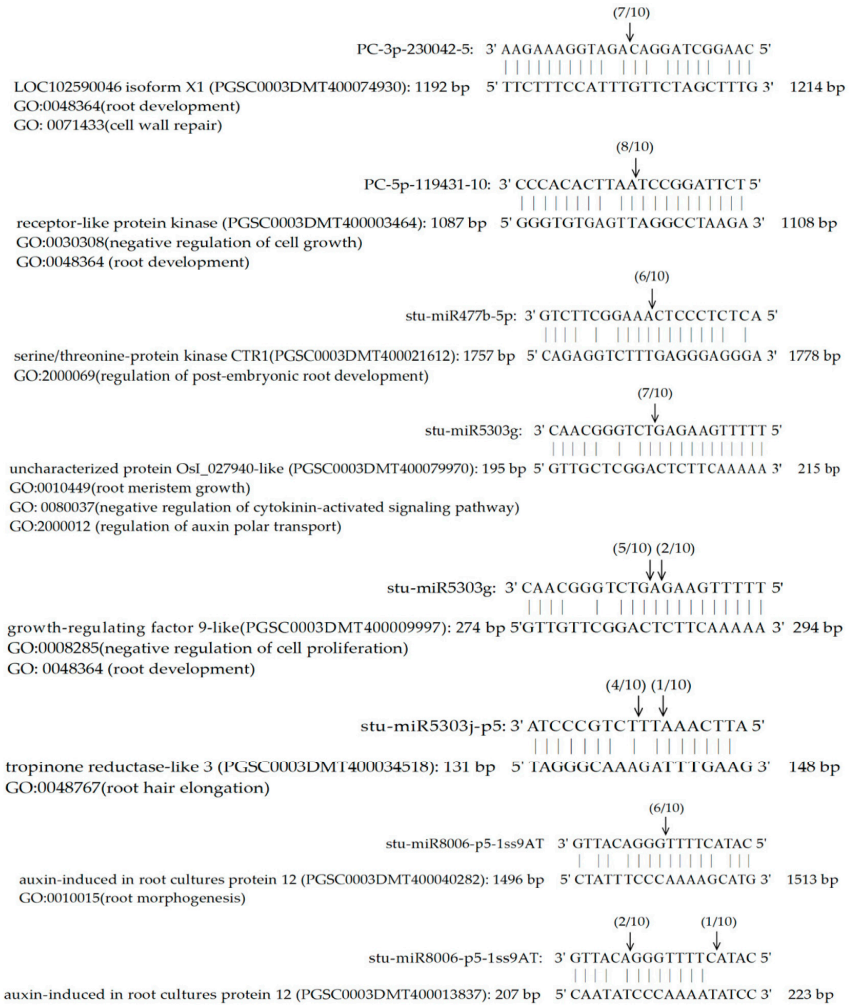

Figure 8. Verification of cleavage sites of miRNA target mRNA by RLM-5′-RACE.

3.8. Expression Analysis of Root-Development-Related miRNAs and Their Target Genes

To confirm the existence and expression patterns of root-development-related miRNAs, qRT-PCR was used to analyze the expression of six miRNAs (stu-miR477b-5p, stu-miR5303g, stu-miR5303j-p5, stu-miR8006-p5-1ss9AT, PC-3p-230042_5, and PC-5p119431_10). Experiment results revealed that all six miRNAs showed similar expression profiles to the results from sequencing data (Figure 9), including five miRNAs (PC-3p-230042_5, PC-5p119431_10, stu-miR477b-5p, stu-miR8006-p5-1ss9AT, and stu-miR5303g) that were down-regulated between the ER and MR samples and only stu-miR5303j-p5 being up-regulated. In addition, to determine the regulatory relationship of miRNAs and their target genes, qRT-PCR was also performed to analyze the expression patterns of miRNAs and their targets. The results showed that all six miRNAs had a negative expression pattern with their targets, including PC-3p-230042_5, PC-5p119431_10, stu-miR477b-5p, stu-miR5303g, and stu-miR8006-p5-1ss9AT, which were down-regulated during potato root development, whereas their target genes (uncharacterized protein LOC102590046 isoform X1, receptor-like protein kinase FERONIA isoform X2, serine/threonine-protein kinase CTR1-like isoform X1, growth-regulating factor 9-like and uncharacterized protein OsI_027940-like, auxin-induced in

root cultures protein 12 isoform X1, and protein NRT1/PTR FAMILY 1.2-like isoform X2) were up-regulated; tropinone reductase-like 3, targeted by stu-miR5303j-p5, was down-regulated, while stu-miR5303j-p5 was up-regulated. These results indicate that these miRNAs exhibit a negative expression pattern with their targets during the process of potato root development.

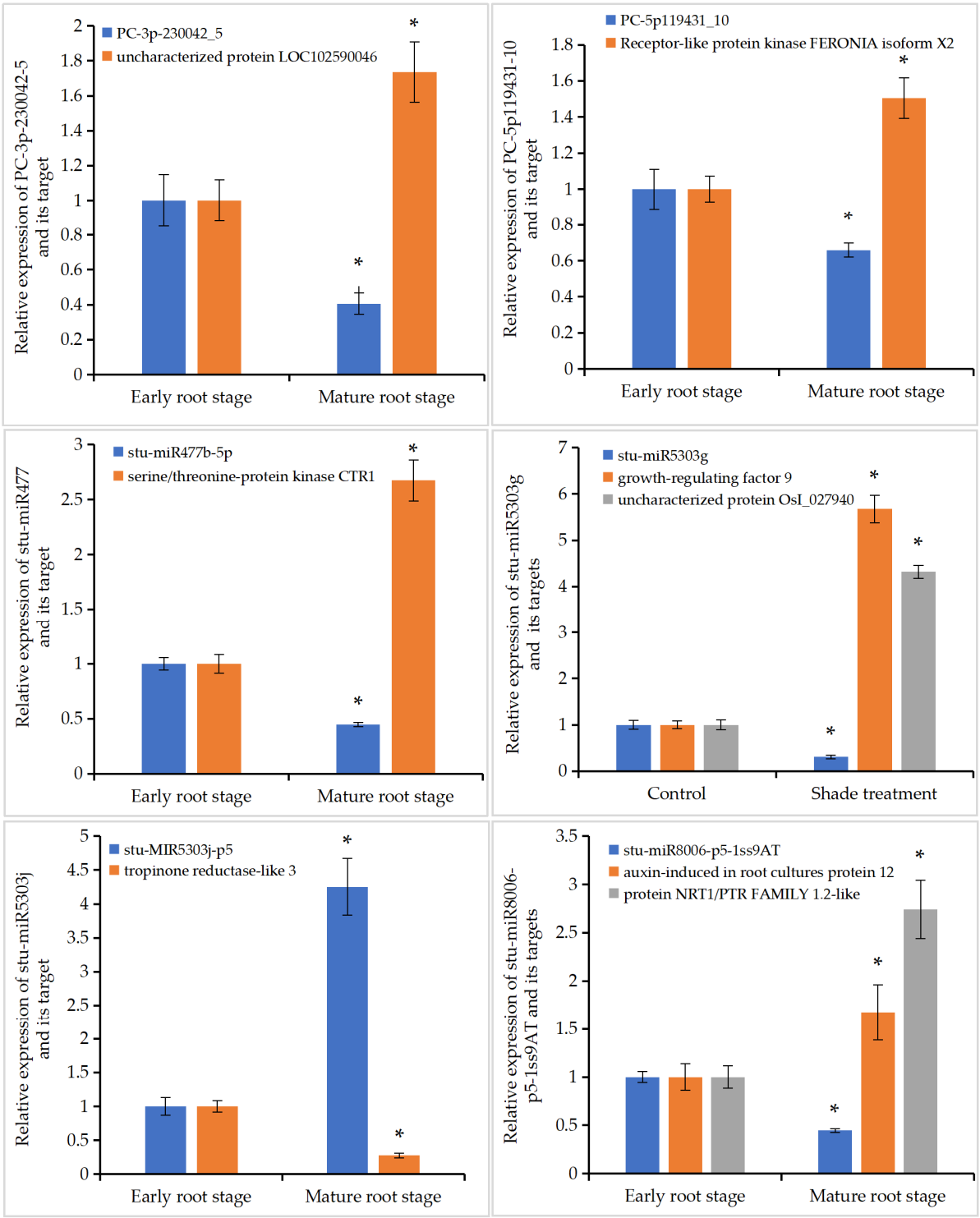

Figure 9. RT-qPCR analysis of the expression levels of miRNAs and their targets. Asterisks indicate a significant difference between transgenic lines and WT control plants based on Student's *t*-test at $p < 0.05$ (*).

3.9. Clone, Characterization, and Expressional Analysis of stu-miR8006-p5-1ss9AT in Potato

The stu-miR8006 was first reported in 2013 by small-RNA sequencing [36]. In this study, stu-miR8006-p5-1ss9AT was selected for further functional study in potato root development. qRT-PCR was used to analyze the expression levels of stu-miR8006-p5-1ss9AT and its target gene (auxin-induced in root cultures protein 12). The results showed that stu-miR8006-p5-1ss9AT has a lower level of expression in potato roots compared with stems or leaves (Figure 10A). Also, stu-miR8006-p5-1ss9AT was significantly down-regulated during the potato root development process, while the expression levels of its target (auxin-induced in root cultures protein 12) were greatly up-regulated (Figure 10B). These results demonstrate the potential role of stu-miR8006-p5-1ss9AT in potato root development.

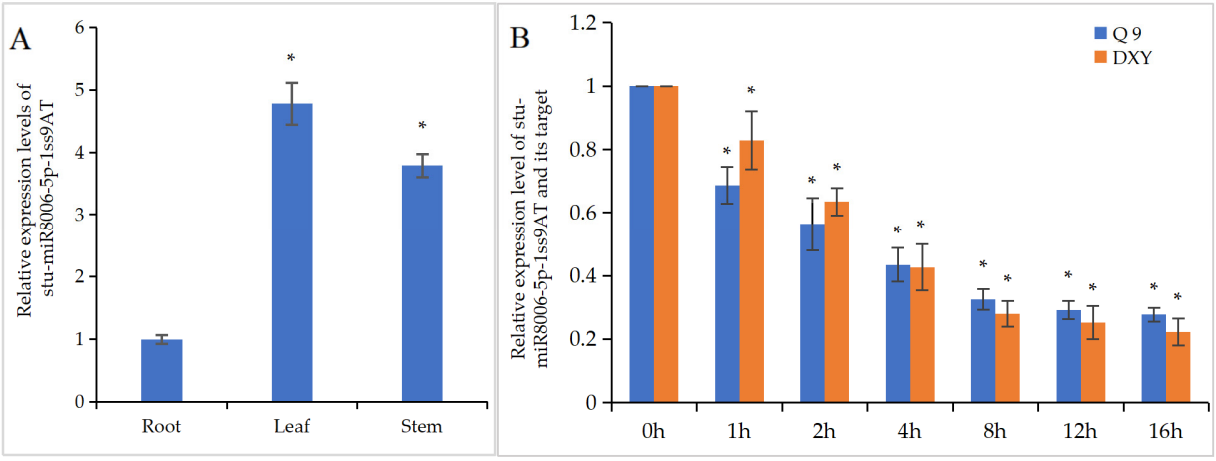

Figure 10. Expressional analysis of stu-miR8006-p5-1ss9AT. (**A**) Expression analysis of stu-miR8006-p5-1ss9AT in different potato tissues. (**B**) Expression analysis of stu-miR8006-p5-1ss9AT and its target gene auxin-induced in root cultures protein 12. Asterisks indicate significant differences between transgenic lines and WT control plants based on Student's *t*-test at $p < 0.05$ (*).

3.10. Suppression of stu-miR8006-p5-1ss9AT Resulted in Alteration in Potato Root Architecture

In order to reveal the roles of stu-miR8006-p5-1ss9AT in potato root development, we constructed the transgenic lines with overexpressing short tandem target mimic (STTM) of stu-miR8006-p5-1ss9AT under the CaMV35S promoter to block stu-miR8006-p5-1ss9AT from binding with its target. We detected the root architecture of transgenic potato lines. The results indicate that highly significant differences were observed between transgenic and WT lines for root phenotypic values of all characters (Figure 11A), the number of lateral roots in transgenic plants was increased (Figure 11B), the length of the main root was significantly shortened (Figure 11C), and the transgenic plant roots had no apparent variation in biomass (Figure 11D). Taken together, these results implied a crucial role of stu-miR8006-p5-1ss9AT in the regulation of potato root system architecture.

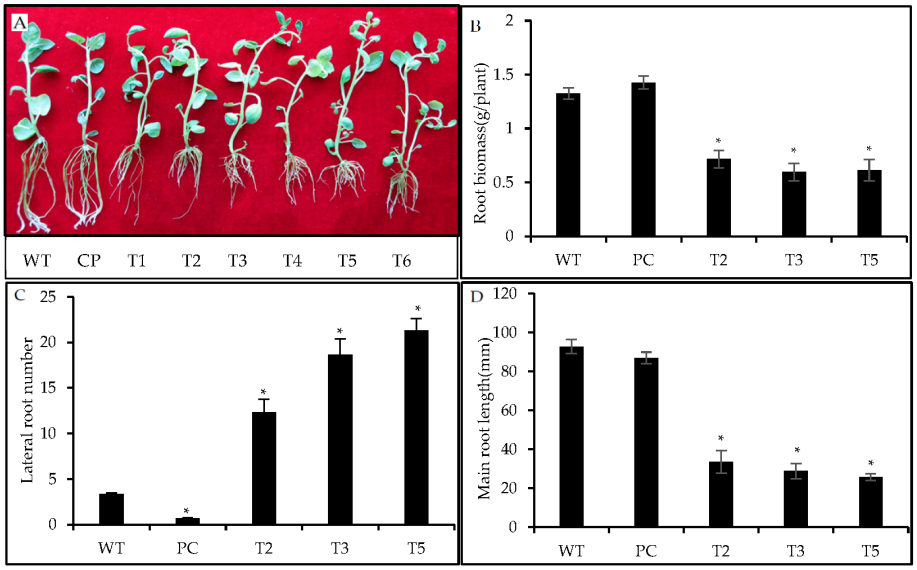

Figure 11. Effect of stu-miR8006-p5-1ss9AT on potato root architecture. (**A**) Root architecture of potato transformed and non-transformed lines. (**B**) The lateral root number of potato transformed and non-transformed lines. (**C**) The main root length of potato transformed and non-transformed lines. (**D**) The biomass changes of potato transformed and non-transformed lines. WT means wild-type plants; PC means transgenic plants with empty vectors; T2, T3, and T5 mean transgenic line2, line3, and line5, respectively. Asterisks indicate significant difference between transgenic lines and WT control plants based on Student's *t*-test at $p < 0.05$ (*).

4. Discussion

The plant root system plays a constructive role in anchoring the plant and extracting water and nutrients from the soil. However, root development is a highly ordered and systematic regulatory process that is affected by several genetic and genetic factors. Potato is one of the staple crops around the world, and the development of root systems seriously affects the yield and quality of potatoes. Previous studies have highlighted a large number of genes that contribute to the regulation of root development in potatoes [37,38]. However, the post-transcriptional regulative mechanisms of those genes remain incompletely understood. MicroRNA is one of the non-coding RNAs that negatively regulates the expression of targets at the post-transcriptional level, and the function of microRNA has been well determined to regulate gene silencing in plant development and stress response. However, the molecular mechanisms of miRNAs that regulate the development of potato roots have seldom been reported. Recently, small-RNA sequencing technologies have become an effective method to identify and evaluate the expression profiles of miRNAs in many plants [39,40]. In this research, small-RNA high-throughput sequencing was performed to investigate miRNAs in the early root stage and mature root stage of potatoes. Our results suggest that the small-RNA length distributed from 18 to 25nt in two libraries, and the 24 nt small RNAs took up the biggest share, which was consistent with the results in previous papers [41,42]. Previous work has shown that the 5′-terminal nucleotide sequence of miRNA is U, which is more conducive to binding with argonaute1 [43,44]. Our data also show that most of the 5′ ends of miRNA are U. In addition, the Q20 and Q30 values of the two libraries were 99% and 98%, and the GC contents were 43% and 43.5%, respectively, indicating that our dates could be used for further analysis [45,46]. In a previous study, 89 known and 112 novel miRNAs were identified in potatoes [47]. In our research, as many as 203 known and 137 novel miRNAs were certified in potato root, consistent with previous reports, and more potato miRNAs were certified, indicating that high-throughput sequencing is the most effective way to discover miRNAs in potato root.

MiRNAs involved in plant root development have been previously reported in many plants [48–54]. For example, miR162a regulates the length of initial roots and the number of

crown roots in rice [48]. Numerous studies claim that miR319 regulates the development of leaves, flowers, and roots by targeting TCP genes [49–51]. miR167 is involved in regulating root, stem, leaf, and flower development by targeting ARFs and IAR3. miR408 was found to regulate the formation of root hairs and crown roots [52]. miR482 was identified to regulate the density of lateral and length of tap roots [53]. miR6024 has also been identified in the transcriptomic profile of tobacco roots [54]. In our research, 64 DEMs were obtained between ER and MR, including 12 up-regulated miRNAs and 52 down-regulated miRNAs. GO annotation indicated that the targets of DEMs were mainly annotated to transcription regulation, transcription factor activity, and nucleus functional groups. KEGG enrichment analysis also suggested that targets of DEMs are involved in metabolic pathways and signaling transduction pathways, which was similar to previous reports in soybean, and sweet potato [55,56]. These results indicate that miRNAs play vital roles in the regulation of potato root development; however, the regulation mechanism remains unclear.

Based on the degradome analysis and RLM-5′-RACE assay, eight target genes of six DEMs were detected that are probably involved in potato root development, including lateral root formation, root meristem growth, auxin polar transport, root morphogenesis, post-embryonic root development, root hair elongation, cell proliferation, cell wall repair, and cell growth according to GO function analysis—including growth-regulating factor 9-like, the target of stu-miR5303g. It has shown that GRFs play important roles in regulating the reprogramming of root cells [57]. Auxin-induced in root cultures protein 12 isoform X1, the target of stu-miR8006-p5-1ss9AT. It was demonstrated that AIR plays a crucial role in the aberrant cell-file rotation of the root differentiation zone [58]. A serine/threonine-protein kinase CTR1-like isoform X1, as the target of stu-miR477b-5p, was demonstrated to be involved in plant root auxin homeostasis [59]. Tropinone reductase-like 3 was identified as the candidate target gene of stu-MIR5303j-p5; it was demonstrated to play a key role in the aberrant cell-file rotation of the root differentiation zone [60]. An uncharacterized protein LOC102590046 isoform X1 was identified as the candidate target gene of PC-3p-230042_5; GO function analysis showed that this protein is involved in root development and cell wall repair. A receptor-like protein kinase FERONIA isoform X2 was identified as the candidate target gene of PC-5p119431_10. Recent studies show that receptor kinases regulate many important processes of root formation and growth [61]. These miRNA targets may play vital roles in the morphogenesis of potato roots.

5. Conclusions

In the present study, we identified 48 known and 16 novel miRNAs that are differentially expressed during potato root development. Based upon the degradome sequencing and GO annotation, eight targets of six differentially expressed miRNAs were identified to be involved in potato root development. The RLM-5′-RACE analysis was successfully performed to verify the cleavage sites in eight target mRNAs. These miRNA target genes are involved in hormone-mediated signaling pathways, cell proliferation and differentiation, and developmental processes. These identified miRNAs and their targets provide valuable clues for further investigation of miRNAs involved in potato root development.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13122942/s1. Supplementary Materials S1: Number of known miRNAs in each miRNA family. Supplementary Materials S2: Number of potato novel miRNAs. Supplementary Materials S3: Statistics of differentially expressed miRNAs in potato. Supplementary Materials S4: Target genes of miRNAs from degradome sequencing. Supplementary Materials S5: The T-plots of miRNA targets.

Author Contributions: Conceived and designed the experiments: J.Y. and H.S.; performed the experiments: X.D. and J.Y.; analyzed the data: X.D. and J.Y., contributed reagents/materials/analysis tools: F.Z., N.Z., M.L., Y.H., Y.G. and J.Y.; wrote the paper: X.D., J.Y., F.Z and H.S. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All data are available in the manuscript and Supplementary Materials.

Conflicts of Interest: The authors declare no conflict of interest.

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