Transcriptome analysis of miRNAs expression reveals novel insights into root formation under Root restriction cultivation in grapevine (*Vitis vinifera* L.)

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Research article

Keywords: MicroRNA, Grapevine, Root restriction cultivation, Root architecture, RNA sequencing

Posted Date: February 13th, 2020

DOI: https://doi.org/10.21203/rs.2.23429/v1

Abstract

Background: Root restriction cultivation not only promotes maturation and quality of fruit, but optimizes the architecture of root, especially in enhancing the occurrence of adventitious and lateral roots. While the molecular mechanism of this phenotype is still unknown.

Results: In this study, the development of roots was observed at 12 different time points under Root restriction and normal cultivations (control). Root phenotype showed a significantly different feature start from the seventh sampling, mainly in the increasing of adventitious roots numbers, degeneration of root tip and subsequent mass of lateral roots development. The 7th and 12th sampling of two different cultivations, named nR7, nR12, RR7, and RR12, were selected for small RNA sequencing. 214,439,588 raw reads were obtained and 168,741,687 clean reads were remained after the quality control steps, and finally got a total of 153 known miRNAs, and 119 predicted novel miRNAs. The predicted novel miRNAs blasted with the miRVIT and miRBase databases simultaneously. 96 new family members of grapevine miRNA and 23 grapevine-specific novel miRNAs were obtained. Differentially expressed miRNAs (DEMs) analysis showed that 26 and 33 miRNAs were differentially expressed in two different cultivation models (RR7 vs nR7; RR12 vs nR12), while 26 and 32 DEMs were obtained in different root development stages (nR12 vs nR7; RR12 vs RR7). MiRNA expression levels analysis found that conserved miRNA s were in higher expression level than novel miRNAs. The predicted target genes of DEMs were annotated on a variety of biological processes, and 24 participated in root development. An analysis of vvi-miR160 family members revealed that vvi-miR160c was highly expressed in grapevine root, indicating the potential role of miR160c in root development, which in accordance with the previous report in Arabidopsis .

Conclusions: Multiple miRNAs were jointly to regulate root development on Root restriction condition, mainly in lateral root development. And the specific expression of vvi-miR160c in the apex may be the main cause of apical degradation. Moreover, there were multiple miRNAs related to biotic or abiotic stresses, which indicated that multiple minor stresses exist in root development after Root restriction cultivation.

Background

In the early 1990s, researchers were inspired by the practice of garden plant potting, and began to explore the cultivation method of limiting the root system of plant, that was "Root Restriction" (RR) cultivation. RR cultivation refers to a cultivation technique that used physical or ecological materials to control the root system of a plant within a certain volume, and controlled the growth space of the root system to regulate vegetative and reproductive growth of above ground [1]. Some studies have reported that RR cultivation improved fruiting efficiency and fruit quality including total soluble solid and fruit color index, while reduced canopy volume and height in fruit trees, which was effective for dwarf cultivation of fruit trees and simplified production management[2–4].

Grapevine, as an economic fruit tree, fruit quality improvement has always been the ultimate goal of cultivation. RR cultivation could significantly inhibit the shoot perimeter, branch length and leaf area, while improving fruit coloring and increasing pigments content such as anthocyanin and carotenoid, and increasing the soluble sugar content in the pulp and reducing the tartaric acid content in grape berries[5, 6]. This may due to RR cultivation affected the absorption of nitrogen and phosphorus in grapevine[7, 8]. Further research reported that the amount of sugar phloem unloading in berries under RR cultivation were higher than control[9], which was related to the structural changes of the conducting tissues and activities of invertase[10]. RR cultivation also increased anthocyanin species and content, and up-regulated the expression of key genes for anthocyanin synthesis in grapevine peel[11].

Roots, as an important organ of perennial fruit trees, not only had a mechanical support function for the growth of tree, but played a key role in the process of absorbing water and mineral nutrients from soil. The development of the fruit tree root system directly affected its vegetative and reproductive growth[4]. Previous studies had shown that after RR cultivation, the developmental morphology of grapevine roots could be clearly distinguished from control cultivation. The dry weight of secondary roots (0.5 cm < thickness < 0.8 cm) and tertiary roots (0.3 cm < thickness < 0.5 cm) increased significantly due to the large number of roots occurrence, especially in the difference in fiber roots (< 0.2 cm) numbers between RR and control cultivation. These morphological observations revealed the root structure has been significantly altered under RR cultivation. However, these studies limited in observation of final root structure, which lacked a systematic observation of the whole process of root development and the reasons of such differences' formation. Moreover, the study of the molecular mechanism of grapevine root development and the RR cultivation will contribute to the fruit quality promotion and cultivation technical innovation.

Non-coding RNA (ncRNA) is a type of RNA that is widely present in many organisms and does not encode proteins[12, 13], which plays an important role in regulating plant growth and development. Among them, microRNA (miRNA) is a class of endogenous small non-coding RNA with a length of 19–25 nucleotides[14]. MiRNA was widely reported in various plants and animals, which played a crucial role in post-transcriptional regulation or transcriptional suppression of genes[15]. Plant miRNA not only affected plant resistance to biotic and abiotic stresses, but directly participated in plant growth and development. There were many miRNAs also reported to influence root development[16, 17]. For example, overexpression ath-miR164 also reduced lateral roots number in Arabidopsis[18]. And miR160 maintains proper auxin homeostasis and root tip development by regulateARF17 (Auxin Response Factor 17) in Arabidopsis[19, 20]. miR390 influenced lateral root development by regulating the expression of ARF2, ARF3, and ARF4 through the secondary siRNA produced by the TAS3(trans-acting siRNA 3) gene[21]. Except these conserved miRNAs, it was reported that some novel miRNA also participated in root development. For example, miR2111 regulated root nod development by long distance transportation[22].

In recent years, high throughput sequencing was wildly used to analyze the miRNA functions in biotic stress such as aster yellows phytoplasma infection[23] and abiotic stress such as cold stress[24] in grapevine. Moreover, a microRNA expression atlas of grapevine was analyzed including 70 small RNA libraries containing many tissues[25, 26]. In addition, Chitarra et al. integrated a novel miRNA database

“miRVIT” inferred from published small RNA libraries which was not uploaded in miRBase database[27]. However, the miRNA derived from grapevine root system wasless in the statistical sequencing data. This study will provide us the expression profile of miRNAs during grapevine root development under both normal and Root restriction cultivations.

Results

Phenotype variations of grapevine cv. Muscat Hamburg after Root restriction cultivation

The shoot length of one-year-old self-rooted seedlings of Vitis vinifera L. cv. Muscat Hamburg were measured from 60 to 125 days after planting (DAP) under two different cultivations. The results showed that the new shoot length both reached to 2 meters in 60 DAP. Root restriction (referred to RR) cultivation had a rapid growth and longer than control (non-restriction, referred to nR) cultivation on 100 DAP. From 100 to 125 DAP, the shoots of nR cultivation grew faster and reached about 5.5 meters, one meter longer than that of RR cultivation (Figure S1A). The base diameters of the new shoots in nR cultivation was always higher than that of the RR cultivation (Figure S1B). Since the secondary shoots were trimmed every 7–10 days, the growth of secondary shoots was measured between two pruning times. The results showed that the number of secondary shoots in nR cultivation was 18–20 per tree, which was almost 2 folds to RR’s 8–11 per tree (Figure S1E). According to the length of the secondary shoots, RR and nR cultivations both were divided into four grades, denoted as I-IV (Figure S1C, D). It was found that the length of the secondary shoots in nR cultivation was significantly higher than that of the RR cultivation, and the length difference from grades I-IV became larger and larger. Moreover, nR cultivation was almost twice as longer as the RR cultivationin grade IV (Figure S1F). The diameter of the secondary shoots was not significantly different in grade I, but nR cultivation was significantly higher than that of RR cultivationin grade II-IV (Figure S1G).

A total of 12 comparative phenotype photographs of the root system were taken every 10 or 15 days from April 24 to August 18, and the nR and RR cultivations were recorded as nR1-12 (Figure S2) and RR1-12 (Figure S3). Two cultivations showed a similar root formation orders including absorbing roots, secondary lateral roots and new adventitious roots. Finally, the old roots degenerate and new adventitious roots developed into the main root system. However, after the 7th sampling (70 DAP), the root morphology of the two cultivations changed significantly. The main manifestations were as follows:

(1) Compared whit the nR cultivation, a large number of new adventitious roots with thinner diameters emerged in RR cultivation. Adventitious roots occurred in clusters in both cultivations (Fig. 1A, B). The number and diameter of adventitious roots in the cluster were analyzed at the 7th sampling. The results showed that the number increased significantly after RR cultivation, which was 8 per cluster and about twice than that of 4 per cluster in the nR cultivation (Fig. 1C). While the diameter of adventitious roots in RR cultivation was about 0.15 cm, which was significantly lower than the nR (about 0.22 cm) (Fig. 1D).
(2) The number of lateral roots increased after RR cultivation. Comparing the roots morphology of the

same position in the two cultivations of the 12th sampling (125 DAP), the lateral roots mainly distributed in the upper part of roots in nR cultivation (Fig. 1E), but in RR cultivation lateral roots densely distributed on the whole roots (Fig. 1F). (3) Growth defect ofroot tips led to a large number of clustered roots emerged inRR cultivation. Clustered roots emerged from the degenerated root tips (Fig. 1G, I), and compared with the nR cultivation, almost all root tips were degraded after the RR cultivation. The secondary roots and tertiary lateral roots were appearing (Fig. 1H, J); (4) Root regeneration was accelerated after RR cultivation. Along with the lower new lateral roots occurred, the upper lateral roots became brown and disappeared, and the overall browning rate was faster than nR cultivation (Figure S4).

Sequencing Statistics In Different Grapevine Samples

The continuous phenotypic observation of grapevine root system revealed that significant differences were occurred from the 7th to 12th samplings in RR and nR cultivations. Then the 7th and 12th sampling points were selected for small RNA sequencing. These four root samples were named as nR7, nR12, RR7, and RR12, and each sample had three replicates, recorded as A, B, and C, respectively. A total of 214,439,588 raw reads were obtained and finally got 168,741,687 clean reads after the quality control steps. The clean reads of each library were between 11.29 and 15.60 M (Table 1). The copy number of clean reads uniq ranged from one to ten were more than 96.5%, among them, single copy and two copies accounted for 69.72% and 15.24%, respectively, totally reached 85% (Figure S5).

Identification Of Known And Novel Grapevine miRNAs

After a series of miRNA prediction analysis, a total of 153 grapevine known miRNAs, and 119 novel miRNAs (named by chromosome random number) were obtained. The length distribution results showed that the known miRNAs were distributed between 19 and 24 nt (nucleotide), of which more than 60% were 21 nt miRNAs (Fig. 2A). The length of novel miRNA ranges from 18 to 25 nt, of which the first peak was 23 nt, which accounts for more than 35%, and the proportion was slightly higher in RR cultivation; the second length peak is 21 nt, which accounts for about 25% (Fig. 2B). The miRBase database recorded a total of 48 known miRNA families in grapevine, and 45 of them were detected in this study except miR828, miR2950, and miR3628 families. And only 30 miRNA members from 13 known grapevine miRNA families were not detected. The predicted novel miRNAs were used to blast miRVIT database, and 18 of them was perfected matched. Among them, nine were similar to known miRNA families in grapevine, including Un_39994, 6_13658, 19_26046, 19_26048 and 19_25033 were similar to vvi-miR477a, 1_21167 were similar to vvi-miR482, 14_36566 and 17_1792 were similar to vvi-miR3627-5p, and 14_37516 were similar to vvi-miR3633b-3p,respectively. (Table S1).

To obtain the detail in formation of the predicted grape novel miRNA, they were aligned with mature miRNAs database of miRBase by default parameters.14 conserved grapevine miRNA family members were obtained (Fig. 3). Seven of the14 conserved miRNA also detected in the miRVIT database, but that was much better matched in miRBase than miRVIT database. Among them, five novel miRNAs annotated

in miRVIT that similar to vvi-miR477a matched better in miRBase database to ppt-miR477f (Physcomitrella patens).Meanwhile, 1_21167 and 14_37516 matched better with mtr-miR482-3p (Medicago truncatula) and gma-miR482a -3p (Glycine max), respectively. The remaining seven novel miRNA were6_12672, 17_2431,11_7793, 9_19848, 15_8904, 2_4979, and 9_20339, which were similar to csi-miR159b-5p (Citrus sinensis), mes-miR159a-5p (Manihot esculenta), vvi-miR396b, csi-miR156f-3p, ath-miR162a-3p (Arabidopsis thaliana), seu-miR319 (Salicornia europaea), and osa-miR396e- 3p (Oryza sativa), respectively (Table 2).In addition, there were 82 novel miRNAs aligned to miRNA families that had been reported in other species but not in grapevine (Table S3). 5_32700 and ghr-miR827a (Gossypium hirsutum), 14_36566 and vca-miR391-5p (Vrieseacarinata), 14_37655 and mes-miR1446 had an alignment score of more than 90 points, and mature sequences had high sequence homology with other species (Table S2). The remaining 23 novel miRNAs that did not match either databases were considered as grapevine-specific novel miRNAs (Table S3).

Differentially Expressed miRNAs (DEMs) Analysis

Principal component analysis (PCA) showed that the distribution was relatively concentrated among biological replicates. Moreover, nR7 and RR7 samples were close and some replicates could not be completely separated, and nR12 and RR12 could be completely separated (Figure S6). There were 26, 33, 26 and 32 differentially expressed miRNAs (DEMs) were identified in different cultivations (RR7 vs nR7; RR12 vs nR12) and different cultivation stages (nR12 vs nR7; RR12 vs RR7). Among them, both the know miRNA and novel miRNA showed up ordown regulation (Table S4). Hierarchical clustering heat map analysis showedthat different replicates of the same sample clustered together. In different cultivations, both vvi-miR3627-3p and 11_random_23 were up-regulated, while vvi-miR166a, vvi-miR482, vvi-miR2111-5p, Un_39994*, and 19_26046 showeda down-regulated expression (Fig. 4A). In different cultivation stages, 5_32700* and 18_33385 were up-regulated, and the down-regulated expressions miRNAs were vvi-miR3633a-3p, 17_2431*, and 2_4979 (Fig. 4B). In addition, in the later development stage of the nR cultivation (nR12 vs nR7), vvi-miR398a, vvi-miR3623-3p, and miR3634-3p were down-regulated, and vvi-miR167b, vvi-miR319g, 6_13658, and 14_37516 were up-regulated, which showed an opposite expression trend in RR cultivation (RR12 vs nR12) (Fig. 4B)

MiRNA expression levels analysis found that know miRNA were in higher TPM (transcript per million) values. 17 of know miRNA had TPM values higher than 30 and the highest reached to 3000 (Fig. 5A). TPM values above 10 in novel miRNA were only 11 (Fig. 5B). The highest five TPM of know miRNAs were vvi-miR3634-3p, vvi-miR166c, vvi-miR159c, vvi-miR482, and vvi-miR398b, and of novel miRNAs were 1_21167, 1_21167*, 14_37516, Un_39994*, and 11_7793.

Analysis Of vvi-miRNA Mediated Grapevine Root Formation

The number of predicted target genes in DEMs was 344, 738, 402, and 486 in different cultivations (RR7 vs nR7; RR12 vs nR12) and different cultivation stages (nR12 vs nR7; RR12 vs RR7), respectively. GO

annotation analysis revealed that the predicted target genes participated in a variety of biological processes, and both included regulation of transcription, oxidation-reduction process, serine family amino acid metabolic process and defense response. In different cultivation models, the target genes were predicted function on lignin catabolic process, electron transport, and response to water deprivation (Fig. 6A). In addition, response to abscisic acid stimulus, response to salt stress, regulation of meristem growth and polarity specification of adaxial/abaxial axis were in top 10 ranks in different cultivation stages (Fig. 6B). The firstcategory in cellular components classification was the nucleus, while the protein binding and ATP binding categories were the most abundant categories in molecular functions classification. Gene function annotation found a total of 24 target genes related to root development, which corresponding to 17 vvi-miRNAs. Target genes of vvi-miR156, vvi-miR166, vvi-miR2111-5p, and vvi-miR3624-3p participated in root hair development, as well as, vvi-miR164 and vvi-miR482 affected lateral root and root cap development; target genes of vvi-miR396 annotated in root development. In target genes of novel miRNAs were functioned on more different root developments, such as the target genes of 4_24249 and 17_2431 affected primary root development while 15_8868 and 15_8867 participated in root morphogenesis. KEGG metabolic pathways analysis was conducted and some target genes had corresponding metabolic pathway annotations. Among them, miR2111-5p participated in vasopressin-regulated water reabsorption, corresponding to its GO annotation in root hair development (Table 3).

Vvi-miR160 Family Contributes To Grapevine Root Development

Root tip degradation was one of the most obvious root phenotypes after RR cultivation. MiR160 had been reported to play an important role in root tip development[28]. Five members of the vvi-miR160 family named vvi-miR160a, b, c, d and e were obtained by miRbase search. Among them, the mature sequence lengths of vvi-miR160a and vvi-miR160b were 23 bp, and of vvi-miR160c, d and e were 21 nt. There was one base difference between the overlapping of vvi-miR160 mature sequences. MiR160 precursor sequence alignment result showed that flanking sequence was variable but the mature sequence was similar. Moreover, the mature sequence of vvi-miR160c, d, and e were 21nt, it’s the same with ath-miR160 (Fig. 7A). Phylogenetic analysis of miR160 precursors revealed that vvi-miR160a and vvi-miR160b were clustered into one branch, while vvi-miR160c, d, and e were clustered into another branch, and vvi-miR160c closed to ath-miR160c (Fig. 7B). And the RNAfold software was used to predict the stem-loop secondary structures of vvi-miR160 family members according to their precursors, and the vvi-miR160c got the highest free energy(Fig. 7C). Small RNA sequencing detected vvi-miR160 at a moderate expression level in different sequencing samples, but there was no differential expression among samples (Fig. 7D). Quantitative analysis of the relative expression of vvi-miR160 precursors found that vvi-miR160c was the highest expression number, followed by vvi-miR160b, and the expression of vvi-miR160a precursor was not detected (Fig. 7E).

Discussions

There were 186 grapevine miRNAs recorded in miRBase database, and in this work, 153 of them were detected in grapevine root, with a high detection rate of 82.3%, indicating that the crucial role of miRNA in grapevine root initialization and development (Fig. 3).Principal component analysis showed the distance of nR7 and RR7 were close and distinguished from nR12 and RR12(Figure S6), which was consistent with that the root phenotypic difference in the 12th sampling was more obvious. MiR828 had been reported to play a role in the anthocyanin metabolism pathway and affected fruit coloring[29]. The absence of vvi-miR828 in grapevine root was reasonable for no anthocyanin accumulation in the root. MiRNA length was considered to be 19–24 nt and usually showed a typical 21 nt and 24 nt 2-peak distribution in small RNA sequencing results. In previous research reported that the 24 nt length small RNA was most abundant in grapevine flowers and flower organs (carpels and stamens), and there was no significant peak in miRNA length distribution in seeds[25]. However, the remaining tissues, including roots, had the highest proportion of 21 nt sequences. In this study, 21 ntsequence was the largest content in known miRNAs(Fig. 2A), while novel miRNAs had two peaks, 21 nt and 23 nt in length, the proportion of 24 nt was relatively small(Fig. 2B),which was different from previous studies.

Compared with the abstract observation of the root system after Root restriction cultivation in previous studies, we summarized the characteristics of root development under different cultivation models through continuous observation in grapevine cv. Muscat Hamburg. Finally, the differences between the two root systems could be concluded to two basic phenotypes: degradation of the root tip and the occurrence of a large number of lateral roots, which accelerated the renewal rate of the root system(Fig. 1). The root system explores the soil for nutrients. In this exploration process, due to space limitation, the relative soil amount of the root system decreased. Therefore, in the case of insufficient soil, the root system may supplement the deficiency of root tip degradation by issuing more lateral roots, which increased the root surface area and helped to seek more soil and nutrients for growth. With the continuous occurrence of the root system, the roots were thinner. This was consistent with the phenotypes that the secondary roots, tertiary roots, and fibrous roots of the root system were promoted after RR cultivation.

Root tip includes root cap, root meristem and root distal, which showed complex behavioral patterns such as decision-making, and played an important role in the plant gravitropism. MiR160 had been reported in relation to root elongation and root cap formation[28]. Overexpression of ath-miR160c displayed uncontrolled cell division in root distal region and loss of gravity-sensing. Moreover, the root length of the seedling was reduced and the lateral root number was increased. Phylogenetic analysis revealed that vvi-miR160c was in the same clade with ath-miR160c(Fig. 7), which indicated vvi-miR160c may influence root tip development in grapevine. Although vvi-miR160c showed no difference expression in the root development after RR cultivation, the role of vvi-miR160 was still worthy of further study. In addition, multiple miRNAs have been reported to participate in lateral root development in plants(Fig. 8). For example, miR156 targeted Spls[30], miR164 targeted NAC[18], miR167 targeted ARF6 and ARF8[31], miR396 targeted bHLH74[32], and miR171 targeted GRAS[33] to regulate lateral root development. These miRNAs involved in lateral root development were differentially expressed in at least one of the roots develop stages in grapevine. Among them, miR167 was reported to negatively regulate the numbers of

lateral roots. In this study, miR167a was up-regulated while miR167b was down-regulated after RR cultivation, indicating function difference exist among miRNA family members. And the down-regulated expression of miR167b was in line with the characteristics of promoting lateral root development, which deserved further study.

Some differentially expressed miRNAs after root-restricted cultivation were rarely reported to participate in root development, but played a role in biotic or abiotic stresses(Fig. 8). Among them, miR482 was a miRNA related to disease resistance[34, 35], which was a highly conserved miRNA and only found in some species. MiR2111 was detected in the phloem sap under phosphorus-limited conditions, and the abundance of miR2111 in the phloem sap of Brassica napus strongly depended on the concentration of P or N, suggesting that it was effective at low phosphorus in plant roots[36]. In addition, miR167 targeted IAR3[37], miR169 targeted NFYAs[38], miR398 targeted CSDS[39], and miR408[40] were involved in drought stress. All these results indicate that the RR cultivation was s a combination of multiple stress processes, and the effect of drought was obvious. Meanwhile, several newly discovered miRNA family members miR3623-3p, miR3627-3p, miR3632-3p, miR3633a-3p, and miR3634-3p were also detected, which also high and differentially expressed, but the functions were still unclear and needed further study.

Conclusions

Grapevine root architecture had been changed in Root restriction cultivation after planting for 70 days, which was mainly manifested as root tip degradation, subsequently caused a large number of lateral roots, and also enhanced the rate of root regeneration. Small RNA sequencing was performed on the seventh and twelfth sampling time points of the Root restriction cultivation and control. A total of 153 known miRNAs and 119 predicted novel miRNAs were obtained. Annotations of the novel miRNAs by miRVIT and miRbase database obtained14 known new miRNA members and 23 grapevine-specific miRNAs. Differentially expressed miRNAs analysis found that multiple miRNAs were reported to be involved in root system development, and biotic and abiotic stresses, indicating that Root restriction cultivation was jointly regulated by multiple miRNAs, and multiple minor stresses exist in root development on root restriction condition. In addition, the specific expression of vvi-miR160c in the apex may be the main cause of apical degradation, which leads to the phenotype of root restriction cultivation.

Methods

Plant materials

200 one-year-old self-rooted seedlings Vitis vinifera L. cv. Muscat Hamburg were planted in the greenhouse of the Fruit Tree Laboratory in Shanghai Jiao Tong University (31°11'N, 121°29'W). The grapevine materials used in this study were cuttings.Two different cultivation models including Root restriction cultivation (referred to RR) and control cultivation (referred to nR) were used in this study. In Root restriction cultivation, 100 plants were cultivated in the root zone container with a diameter of 30 cm and a height of 30 cm (with holes around it) and separated from the ground by a tray. The planting

substrate was soil, organic fertilizer and perlite with 1: 1: 1 mixed. In control cultivation, 100 plants were planted on the ground with a height of 40 cm in the same substrate. The initial planting distance was 70 cm * 70 cm. Sampling was performed in a zigzag pattern to ensure that there was sufficient space for root development. The above-ground management were the same, and all of them maintain single-vine growth with no topping. The secondary shoots were trimmed every 7–10 days. Moreover, the experimental materials were equipped with unified control irrigation measures. The roots firstly sampled on April 24 when above-ground started to sprout, and then sampled every 10 or 15 days. Finally, 24 grapevine root samples from two different cultivation models at 12 developmental stages were collected. At each sampling time point, 6 trees were selected as biological replicates.

Small RNA Libraries Construction And Illumina Sequencing

According to root morphology observation, small RNA sequencing was performed on four root samples from the 7th (70 days after planting, simplified DAP) and 12th (125 DAP) sampling time points under two cultivation conditions. Total RNA was extracted from collected root samples using modified CTAB method. RNA concentration and quality were detected using both NanoDrop 2000 (Thermo Scientific, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, USA). 12 small RNA Libraries (four samples each with three biological replicates) were constructed according to the TruSeq Small RNA Sample Preparation Guide kit (Illumina, USA) and sequenced using Illumina Hiseq 2000. Briefly, 1 μg of total RNA was ligated to the 3'- and 5'-sequencing adapters by T4 RNA ligase and reversed into cDNA by Super Script III reverse transcriptase (Invitrogen, USA). The obtained cDNA template was PCR amplified using adaptor primers for 15 cycles. The product was separated and purified by 6% Novex TBE polyacrylamide gel electrophoresis (Invitrogen, USA), and RNA fragments in the range of 147–157 nt were excised and recovered. The length and quality of the library were determined by Agilent 2100 bioanalyzer.

Identification Of Known And Novel vvi-miRNAs

The criteria for the raw data quality control included: (1) remove the adapter sequence by cutadapt[41] software, and filter sequences less than 15 bp and greater than 41 bp in length; (2) use fastx_toolkit[42] software to perform Q20 quality control and retain sequences with Q20 above 80%; (3) filter reads containing N bases by NGSQCToolkit[43] and get clean reads; (4) remove redundant sequence and obtained clean reads uniq.

The process to obtain known and novel vvi-miRNAs contained the following steps: (1) Clean reads were mapped to the reference V. vinifera L. cv. Pinot Noir (PN40024) genomes to remove unmapped reads. (2) compared the filtered reads with Rfam[44] (version 10.0) database (http://www.sanger.ac.uk/Software/Rfam) by blastn[45], extracted the results with E-value ≤ 0.01, annotates and removed the sequences such as rRNA, snRNA, snoRNA, tRNA; (3) remove sequences perfect matched with the transcript and longer than 26 bp and less than 15 bp by bowtie[46] software; (4) remove redundant sequence by RepeatMasker[47] software. After the filtration and removal steps, the

remaining sequences were perfect matched with mature miRNAs database in the miRBase[48] (http://www.mirbase.org/), Sequences with perfect matches were considered as known miRNAs in V.vinifera. The unannotated sRNAs were performed secondary structure prediction by RNAfold[49] database, and sequences with miRNA hairpins was considered as novel miRNAs.

Moreover, the predicted novel miRNAs were searched in miRVIT[27] database to annotate novel miRNAs had reported in grapevine before. In addition, the novel miRNA also aligned with mature miRNAs database in the miRBase 21 by default Parameter (Evalue cutoff ≤ 10, Mismatch penalty = -4, Match score ≥ 60). Aligned novel miRNAs were considered as non-conserved miRNAs, while unaligned ones were considered as grapevine specific novel miRNAs.

Differentially expressed miRNAs analysis and annotation of the target genes

MiRNA expression quantification was normalized according to the expression of transcript per million (TPM)[50] and calculated as: TPM = Reads count of per miRNA/ Reads count of per miRNA × 10^6. The DESeq[51] (v1.18.0) software in the R package was used for miRNA differential expression analysis, the p value was calculated, and miRNAs with a P value < 0.05 were selected. At the same time, Hierarchical clustering analysis using the Euclidean distance measurement with the MeV software was performed on differentially expressedmiRNAs between different samples. Target genes were predicted using targetfinder[52] software, and GO functional annotation and KEGG Pathway metabolic pathway analysis were performed.

Structure Analysis Of vvi-miR160 Family

The miR160 family members and sequence information of grapevine and Arabidopsis were retrieved by NCBI (www.ncbi.nlm.nih.gov), and the obtained sequences were aligned and analyzed by BioEdit[53] software. In addition, vvi-miR160 was blast in NCBI to find miR160 sequences with high homology to other species. The MEGA 6 [54] software was used for phylogenetic analysis of miR160 in different species. The RNAfold[49] was used to predict the secondary structure of different vvi-miR160 members. Gene accession number listed in Table S5

RT-qPCR Analysis Of The Expression Levels Of vvi-miR160 Family

RNA extraction of grapevine root was performed according to the instructions of TianGenRNAprep Pure Plant Kit (TIANGEN, Beijing, China, DP441). RNA quality was detected by 1.2% agarose gel electrophoresis and NanoDrop 2000 (Thermo Scientific, Waltham, Massachusetts, USA) to ensure RNA OD 260/280 values ranged from 1.8 to 2.0. first-strand cDNA was generated according to the steps of

PrimeScript™ RT Master Mix (TaKaRa, Dalian, China, RR036A). Primer Premier 5 software was used for gene-specific primer design (TableS5). The RT-qPCR was performed on a CFX Connect Real-Time Detection System (Bio Rad, Hercules, USA). A 10-μl reaction volume included 5 μl of TB Green Premix Ex Taq II (TAKARA, Dalian, China), 0.5 μl of each primer (10 μM), 1 μl of cDNA template (diluted 10-fold), and 3 μl of double-distilled water. Two-step RT-qPCR was performed using the following conditions: initial denaturation at 95 °C for 150 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. The VvGAPDH (XM_002263109.3) was used as internal control[55].The relative expression of the genes was calculated using the $2^{-\Delta\Delta Ct}$ method. Each experiment was repeated three times.

Abbreviations

ARF: Auxin response factor; bHLH: basic helix-loop-helix; DAP: Days after planting; DEMs: Differentially expressed miRNAs; miRNA: microRNA; ncRNA: Non-coding RNA; PCA: Principal component analysis; RR: Root restriction; Spl: Squamosa-promoter binding protein-like; TAS: Trans-acting siRNA genes; TPM: Transcript per million.

Declarations

-Ethics approval and consent to participate

Not applicable.

-Consent to publish

Not applicable.

-Availability of data and materials

The miRNA-Sequencing raw data have been deposited to the National Centre for Biotechnology Information (NCBI) BioProject database under accession number PRJNA601829 (www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA601829).

-Competing interests

The authors declared that they have no conflicts of interest to this work.

-Funding

This work was supported by National Natural Science Foundation of China (31701888, 31972383), the Yunnan Natural Science Foundation (Grant No. 2018FB072) and the Special Funds of Modern Industrial Technology System for Agriculture (CARS-29-zp-7). The funders had no role in study design, data collection and analysis, or preparation of the manuscript.

-Authors' contributions

CZ, SW and CM conceived the study and designed the research. HL, ZG and DL performed most of the experiments. HL, LZ and SS analyzed the data. HL and MZ wrote the manuscript. LW, WX, and HJ proposed revision to the manuscript. All authors read and approved the final version of the manuscript.

-Acknowledgments

Not applicable.

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Tables

Due to technical limitations, Tables 1-3 are provided in the Supplementary Files section.

Supplementary Figure Captions

Figure S1. Aboveground phenotype variations of grapevine cv. Muscat Hamburg after root restriction cultivation. the length (A) and diameter (B) of shoots in two cultivation models. The grading of secondary shoots in Root restriction cultivation (C) and control (D) according to their length. (E) The number of secondary shoots per tree between two pruning. (F) and (G) represent the length and diameter of secondary shoots in different length grades.

Figure S2. The root morphology of grapevine cv. Muscat Hamburg in control cultivation at 12 sampling time points, recorded as nR1-12.

Figure S3. The root morphology of grapevine cv. Muscat Hamburg inRoot restriction cultivation at 12 sampling time points, recorded as RR1-12.

Figure S4. Root regeneration was accelerated after Root restriction cultivation.

Figure S5. The copy number of read counts.

Figure S6. Principal component analysis in four sequence samples with biological replicates.

Figures

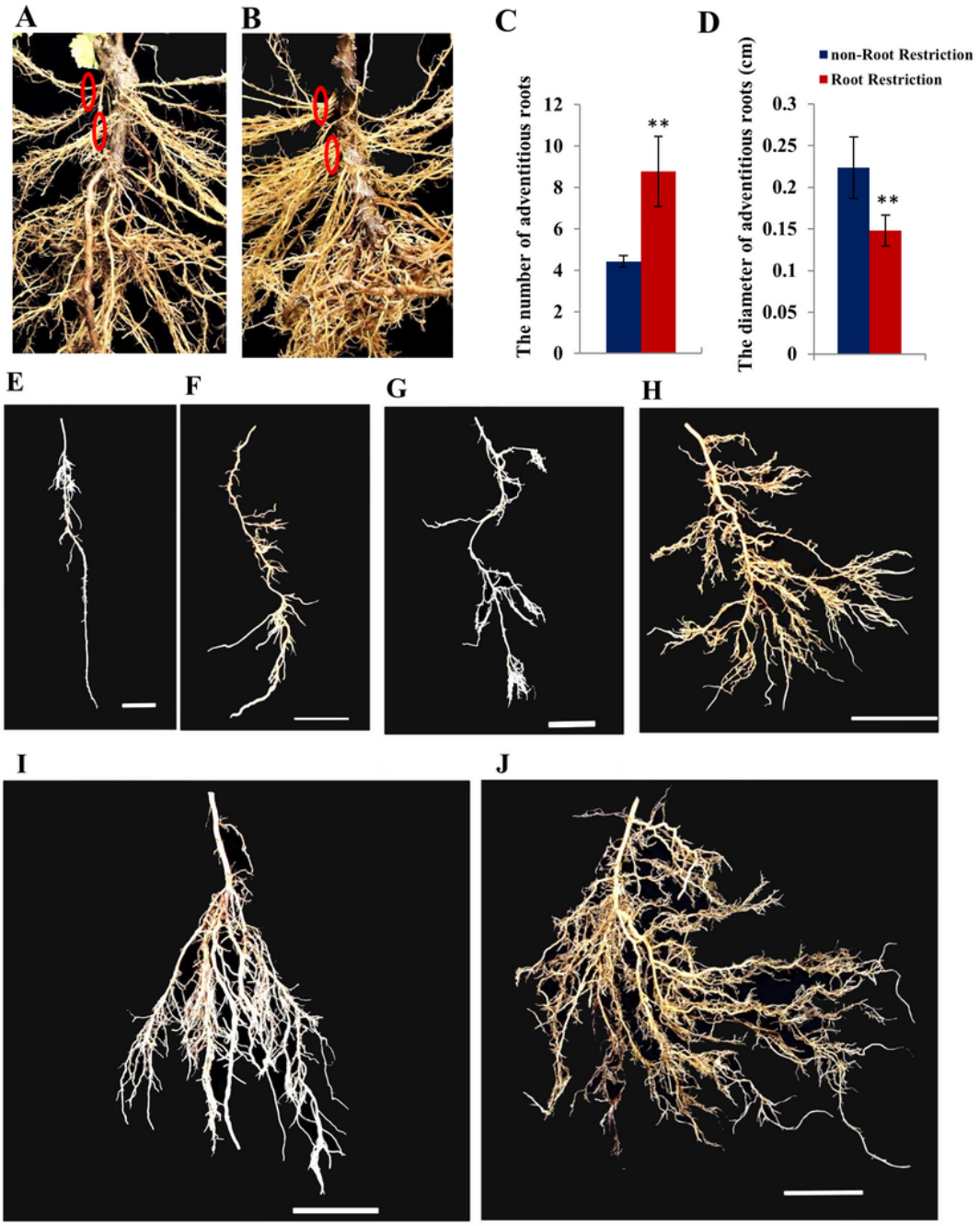

Figure 1

Root phenotype variations of grapevine cv. Muscat Hamburg after root restriction cultivation. The new adventitious roots developmentin control (A) and root restriction cultivation(B), red circles represent adventitious roots occurred in clusters. (C) and (D) represent the number and diameter of adventitious roots in two cultivation models. (E), (G) and (I) were the independent different root morphology in

controlcultivation, while (F), (H) and (J) were the same root morphology in root restrictioncultivation. Scale bar = 5 cm.

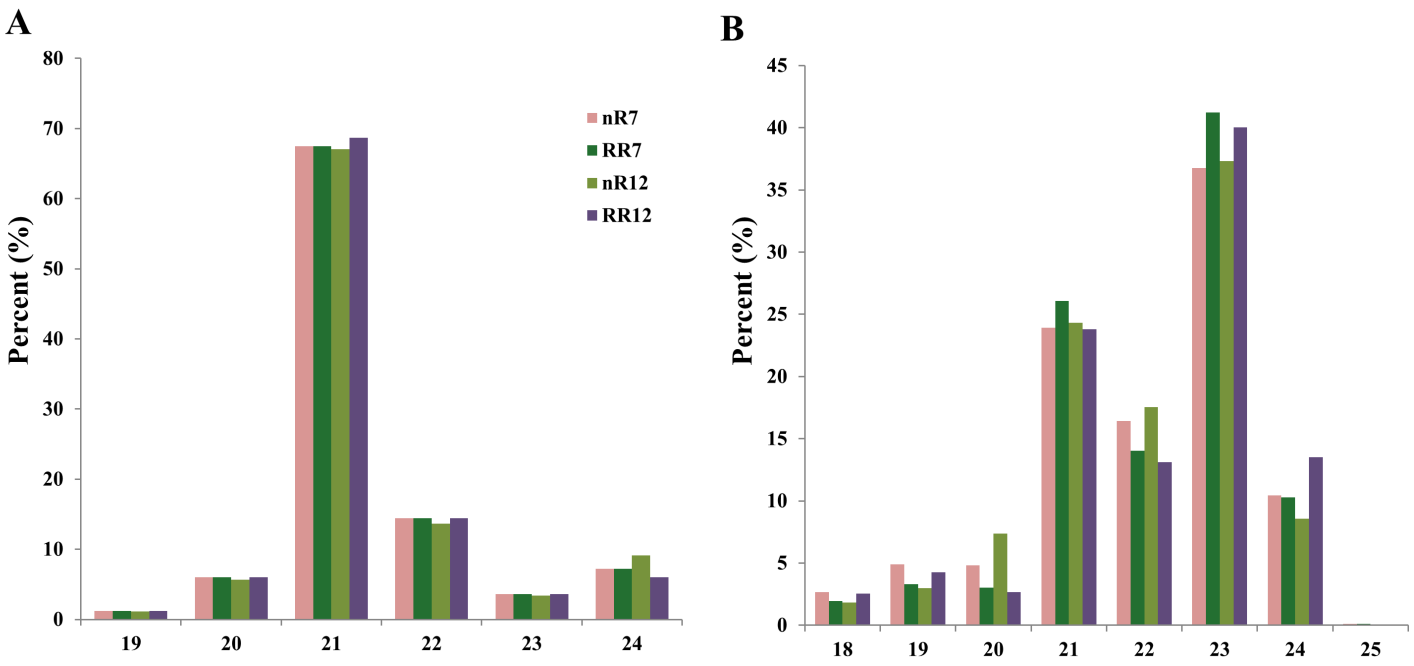

Figure 2

The length distribution percentage of the known (A) and novel (B) miRNAs in four root sequencing samples. nR7, RR7, nR12 and RR12 represented the 7th (70 days after planting, simplified DAP) and 12th (125 DAP) sampling time points under control and root restriction cultivation.

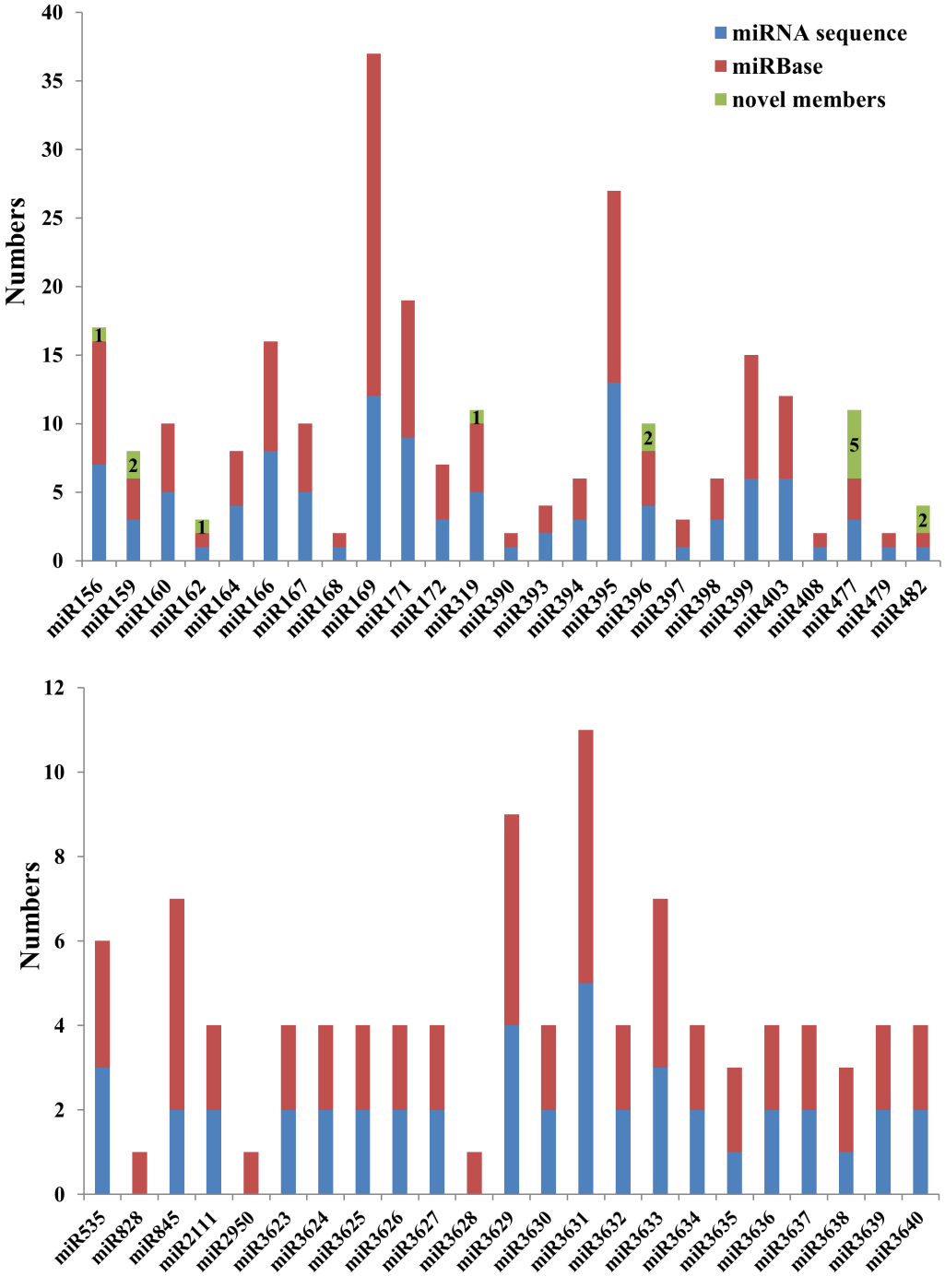

Figure 3

The number of known miRNA family members in grapevine obtained from miRNA sequence, predicted novel miRNA and miRbase database.

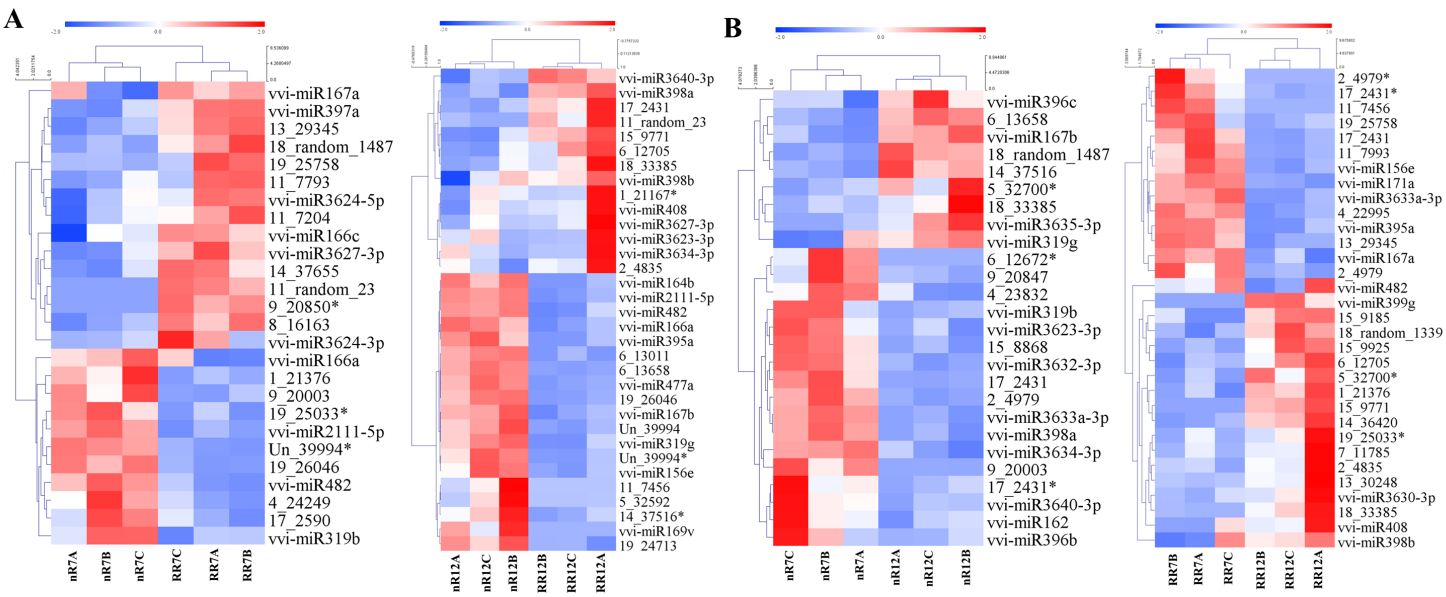

Figure 4

Hierarchical clustering heat map analysis of differentially expressed miRNAs between different cultivation (A) and different cultivation stages (B) in four root sequencing samples. nR7, RR7, nR12 and RR12represented the 7th (70 days after planting, simplified DAP) and 12th (125 DAP) sampling time points under control and root restriction cultivation, A, B, and C representthree replicates.

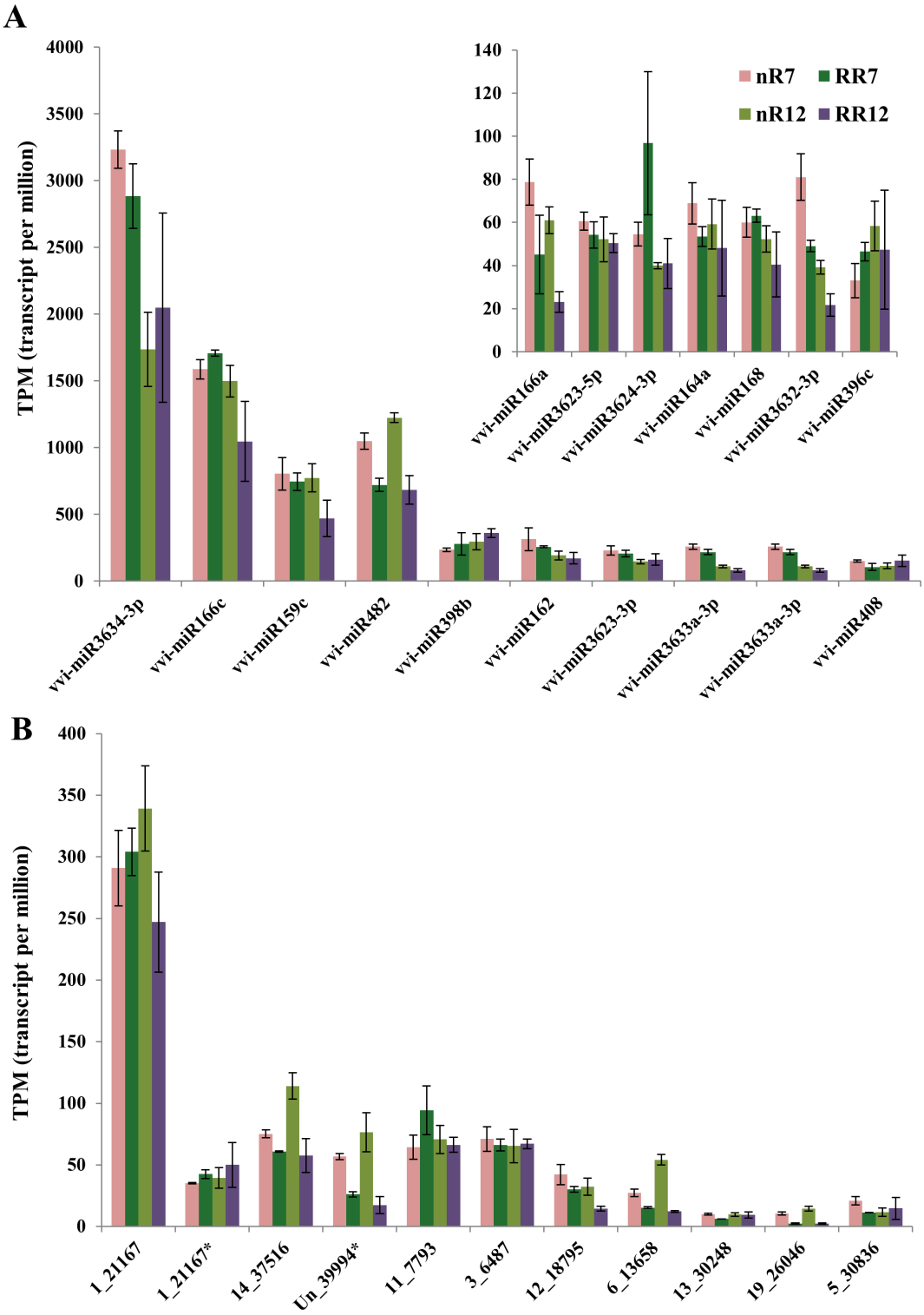

Figure 5

High TPM (transcript per million) values of know miRNA (A) and novel miRNA (B) in four root sequencing samples. nR7, RR7, nR12 and RR12 represented the 7th (70 days after planting, simplified DAP) and 12th (125 DAP) sampling time points under control and root restriction cultivation.

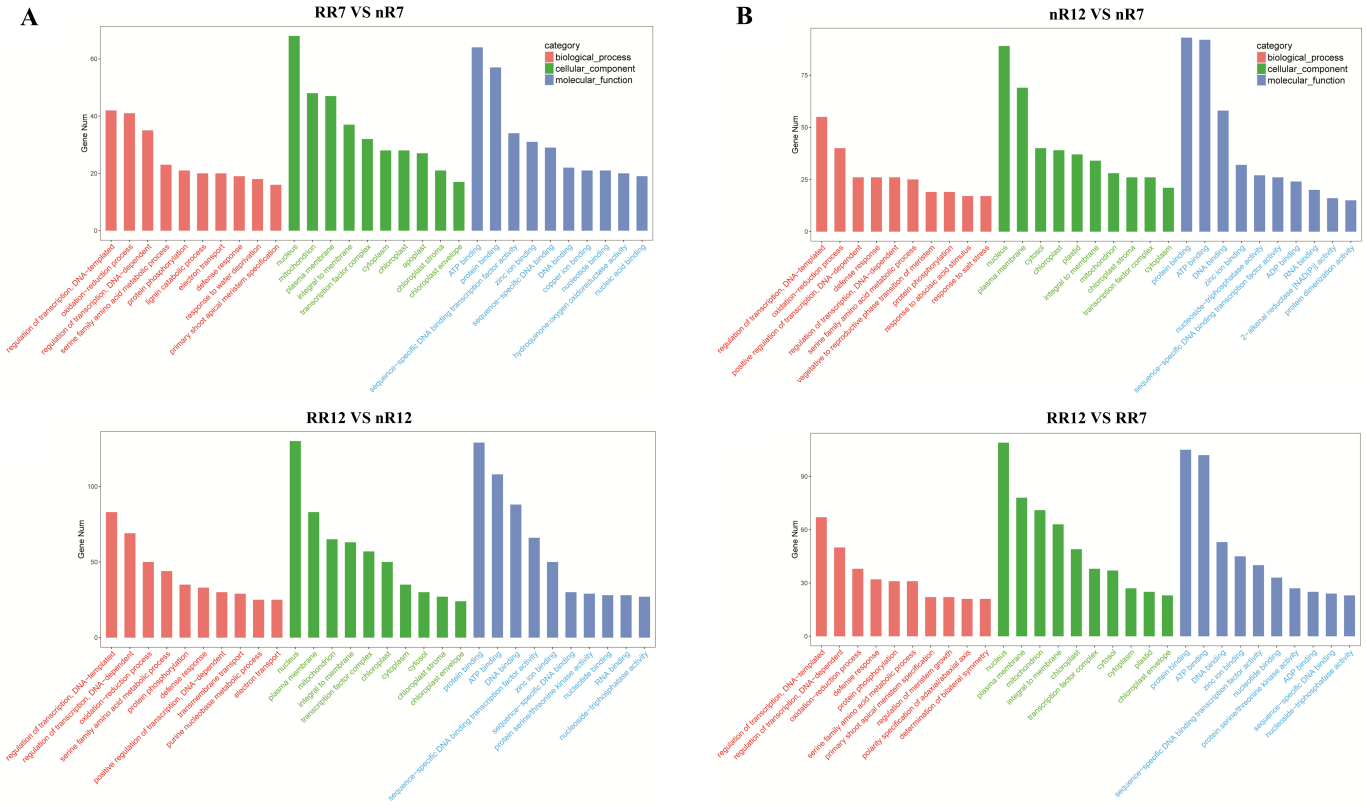

Figure 6

Gene ontology analysis of the predicted target genes of the differentially expressed miRNAs between different cultivation (A) and different cultivation stages (B) in four root sequencing samples. nR7, RR7, nR12 and RR12 represented the 7th (70 days after planting, simplified DAP) and 12th (125 DAP) sampling time points under control and root restriction cultivation.

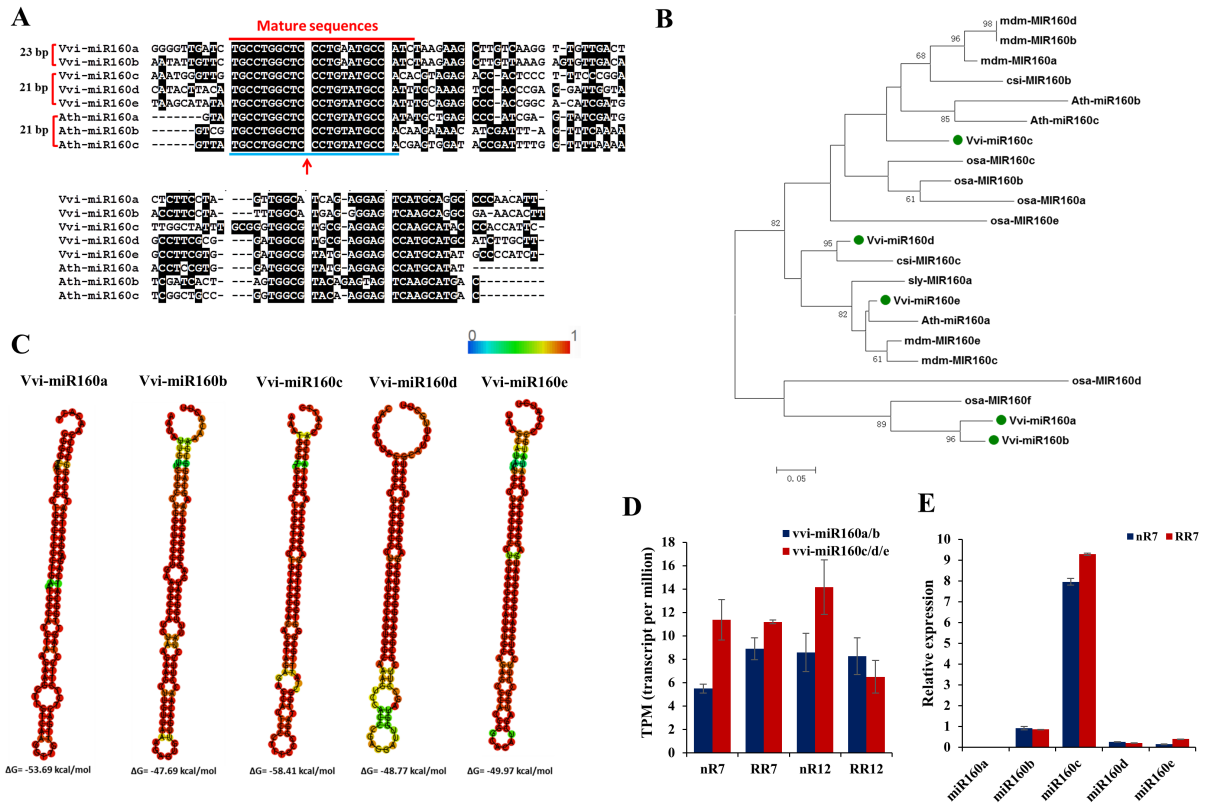

Figure 7

Structure analysis of vvi-miR160 family. (A) Sequence alignment analysis ofmiRNA precursor between vvi-miR160 and ath-miR160. (B) Phylogenetic analysis of miR160 in different species. (C) The secondary structure of different vvi-miR160 members.(D)TPM (transcript per million) values ofvvi-miR160members in four root sequencing samples. (E) The relative expression of the precursorof vvi-miR160members incontrol and root restriction cultivation at 7th sampling time points.

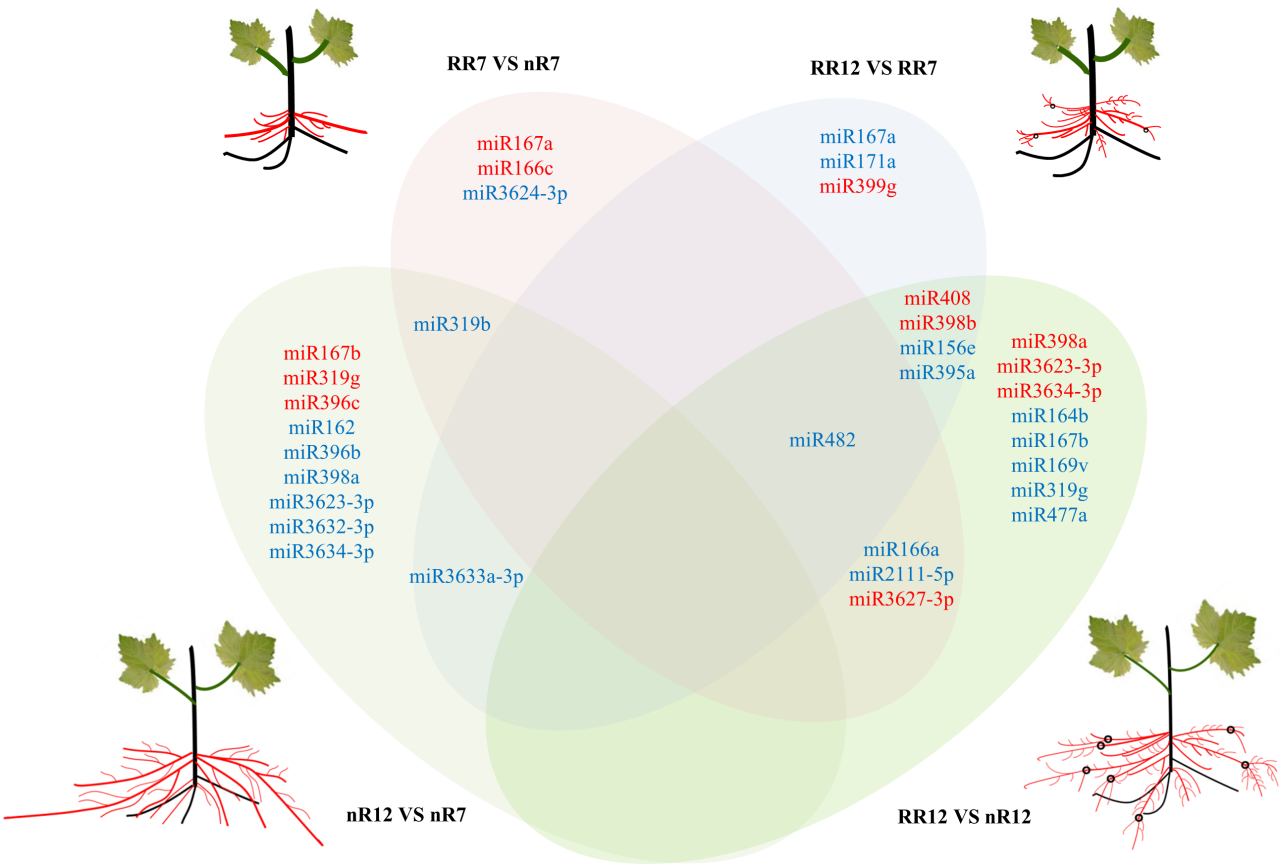

Figure 8

Hypothetical model of miRNAs regulating root formation in grapevine.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS5.xls
- FigureS3.tif
- FigureS2.tif
- FigureS4.tif
- FigureS6.tif
- FigureS5.tif
- FigureS1.tif

- TableS2.xlsx
- TableS4.xlsx
- TableS1.xlsx
- TableS3.xlsx