Integrative investigation of root-related mRNAs, lncRNAs and circRNAs of 'Muscat Hamburg' (*Vitis vinifera* L.) grapevine in response to root restriction through transcriptomic analyses

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Abstract

Background

Root restriction is a physical and ecological cultivation mode which restricts plant roots into a limited container to regulate vegetative and reproduction growth by reshaping root architecture. However, little is known about related molecular mechanisms. To uncover the root-related regulatory network of endogenous RNAs under root restriction, transcriptome-wide analyses of mRNAs, long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs) involved in root development was performed.

Results

In this study, a total of 26588 mRNAs, 1971 lncRNAs and 2615 circRNAs were identified in root of annual 'Muscat Hamburg' grapevine by the next generation sequencing, respectively. The expression profile of mRNAs, lncRNAs and circRNA were further confirmed by the quantitative real-time PCR (qRT-PCR). Gene ontology enrichment analysis showed that a majority of the differentially expressed mRNAs, lncRNAs and circRNAs were annotated to the categories of cellular process, metabolic process, cell part, binding and catalytic activity. In addition, the regulatory network of endogenous RNAs was then constructed by the prediction of lncRNA-miRNA-mRNA and circRNA-miRNA-mRNA, implying that these RNAs play significant regulatory roles for root architecture shaping in response to root restriction.

Conclusion

Our results, for the first time, integrated the regulatory network of competitive endogenous RNAs (ceRNAs) functions of lncRNA and circRNA, and provided a basis for studying the potential functions of non-coding RNAs during root development of grapevine.

Background

'Muscat Hamburg' (*Vitis vinifera* L.) is a middle and late ripening variety of European subspecies with strong rose fragrance, which is popular among consumers. It is widely planted worldwide and can be used as a variety for production of table grape, juice and wine. To be inspired by potted practice, from the 1990s, agronomists turned to explore the cultivation ways of limiting roots of horticultural crops, named the root restriction (RR)^[1]. RR refers to limit the roots into a certain space, adjusts the aboveground and underground part growth to coordinate the relationship between vegetative and reproduction growth^[2]. It is supposed that root restriction effectively improve the yield and fruit quality by reduce the redundant growth. In grapevine, RR positively regulates sugar metabolism and anthocyanin related gene expression, results in up-regulated accumulation of sugar and anthocyanin in fruits^[3]. In general, the root architecture is obviously reshaped during RR which mainly significantly increases the number of lateral roots and improves the root regeneration rate^[4]. In addition, RR can also accelerates the grapevine ripening^[5],

increases stem sucrose content^[8], inhibits shoot growth^[6] and root growth^[9], reduces plant height, leaf area and dry plant mass^[7].

Transcriptomic analysis has shown that more than 90% DNA are transcribed and that most of the transcripts are non-coding RNAs (ncRNAs) in plant genome^[10–11]. ncRNAs refer to RNAs that are lack of the ability to encode proteins, which are initially regarded as inessential transcription ‘noises’. However, numerous researches have demonstrated that ncRNAs have significant effect on various biological processes^[12]. Generally, ncRNAs mainly consist of microRNAs (miRNAs), long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs). Studies have shown that some circRNAs and lncRNAs, harboring multiple binding sites for miRNAs, could regulate the activity of miRNAs by sponging miRNAs^[13–15]. It is supposed that ncRNAs act as competitive endogenous RNAs (ceRNAs) that sequester and suppress miRNA activity^[16–17]. and play vital roles in the development of plants and animals^[18–20]. However, the sponge effect of lncRNAs and circRNAs for miRNAs have not been identified and verified in plant.

The ncRNAs with length over 200 nucleotides are defined as long non coding RNAs (lncRNAs), which are always expressed at low levels and have short conserved sequences^[21]. lncRNAs are found to be involved in post-transcriptional gene regulation and chromatin modifications^[22–23]. With the rapid development of sequencing techniques, thousands of lncRNAs have been identified in several model plants, including Arabidopsis^[24], maize^[25], rice^[26], poplar^[27] and cotton^[28]. CircRNAs are a novel type of ncRNAs formed by the reverse splicing of one or more exons. CircRNAs occur widely in eukaryotic cells with tissue- and developmental-stage-specific expression^[29]. CircRNAs are widely distributed in plants and have been identified in several plants such as Arabidopsis^[30], tomato^[31], soybean^[32] and rice^[33], using deep RNA-seq and bioinformatic tools. In addition, a small number of circRNAs can be translated directly in mammals^[34]. Since circRNAs plays an important regulatory role at the transcriptional or post-transcriptional level, and it is important to study the expression of circRNAs for a transcriptional regulatory network. However, it is unclear whether lncRNAs and circRNAs plays a specific physiological role in plants. Although some researches have focused on the roles of lncRNAs and circRNAs in plants, lncRNAs and circRNAs involved in root development or root architecture reconstruction during RR have not been identified in grapevine.

In this study, ceRNA networks of lncRNAs, circRNAs and mRNAs were integrated based on the datas from high-throughput sequencing. For the first time, the potential ceRNA functions of lncRNAs and circRNAs involved in root growth and development have been investigated, which provides a basis for deciphering the regulatory networks of ncRNAs related to root architecture reshaping during root restricted cultivation.

Results

Root phenotype under conventional and root restricted cultivation

To observe the root phenotype differences under conventional cultivation (referred to NR) and root restricted cultivation (or root restriction, referred to RR) (Figure 1 and Figure 2). One-year-old self-rooted

grapevine *cv.* 'Muscat Hamburg' was planted by two different ways, respectively. The entire root system was sampled and photographed to record root phenotype during different developmental stages. A total of twelve sampling time points were conducted (at one development stage, root systems were simultaneously collected from two different cultivation methods). The phenotype of root samples from twelve different times of NR and RR were marked as NR1-12 (Figure 1) and RR1-12 (Figure 2), respectively. The two cultivations results showed that similar root formation orders including new adventitious roots, absorbing roots and secondary lateral roots. Finally, the old roots degenerated, and young roots developed into the main root system. However, from the the seventh sample (NR7 and RR7), the root morphology was significantly changed between NR and RR cultivation during the developmental process. Before the seventh sampling, new adventitious roots were generated and elongated continuously under NR and RR. After the seventh sampling, the old roots gradually aged and disappeared from bottom to top. The adventitious roots of NR became coarse and gradually lignified to brown, while the adventitious roots under RR further elongated to brown, differentiated more secondary lateral roots and produced a great deal of adventitious roots continuously.

In order to further detail the root architecture variation of *cv.* 'Muscat Hamburg' in response to root restriction. The weight of young roots, old roots and total roots were further counted (Figure 3). The weight of all three kinds of roots increased during developmental process. In the later stage of development, the total weight of roots under RR8-12 was significantly higher than that under NR8-12. However, there was no obvious difference in the weight of old roots during the whole development stage. Consistent with the total weight of root system, the weight of young roots, largely derived from the lateral root morphogenesis, significantly increased in the later stage of development under RR8-12. It shows that the difference in total roots weight were caused by enhancing the induction of young lateral roots, which were in keeping with phenotype of root system (Figure 1 and Figure 2). Combining the above results, The results shows that the root weight increasing and architecture remodeling of grapevine, to a great extent, contributes to the lateral root morphogenesis on the condition of RR.

Sequencing statistics in different roots samples

To systematically investigate the potential regulatory roles of genes and ncRNAs involved in root development in *cv.* 'Muscat Hamburg', we first collected the roots derived from two different developmental atages (NR7 and NR12) in NR and roots derived from the stage with abundant lateral root in RR (RR12) and performed integrative transcriptomic analyses. As a result, 59.62 G CleanReads from a total of 9 samples (NR7_1, NR7_2, NR7_3, NR12_1, NR12_2, NR12_3, RR12_1, RR12_2 and RR12_3) were obtained. The number of CleanBases ranged from 5.35 to 8.69 G in each samples, the Q30 base distribution ranged from 93.56% to 94.28%, and the average GC content was 47.07% (Table 1). The statistical assessment confirmed that the datasets were highly reliable.

Based on the expression pattern of mRNAs, lncRNAs and circRNAs, correlation and principal component analysis (PCA) of the 9 samples were conducted. The results indicated that the three independent biological triplicates were reliably repeatability (Figure 3). Meanwhile, The 9 samples were divided into

three clusters (NR7, NR12 and RR12), indicating that the closer the clustering distance or PCA distance of the samples, the more similar the samples were (Figure 4).

Identification and characterization of lncRNA and circRNAs

There were different distributions of lncRNAs and circRNAs on each chromosome, circRNA distributed on chromosome 18 was the most abundant and lncRNA located on chromosome 8 was the most (Figure 5a). The length of lncRNAs and circRNAs in grapevine were different, and the distribution of most lncRNAs and circRNAs were regular (Figure 5b). The length of 612 circRNAs (23.40 %) and 149 lncRNAs (7.56 %) were greater than 2000 nt, and 350 circRNAs were less than or equal to 200 nt. And the number of lncRNAs and circRNAs in the range of 201-2000 nt decreased with their sequence length (Figure 5b). Based on the position relationship between lncRNA and known protein coding transcripts, FEELnc software was used to count lncRNA types according to three levels, including direction, type and location. There are 657 antisense lncRNAs and 1308 sense lncRNAs were identified (Figure 5c). Bedtools software was used to classify the position relationship between circRNA and the coding genes, and five types of circRNAs were found. The largest number of circRNA of the sense-overlapping type containing 1878 circRNAs (72%). Intronic circRNAs was the lowest proportion containing 35 circRNAs (1.34%). Moreover, the percentage of exonic-derived circRNAs, antisense-derived circRNAs and intergenic-derived circRNAs accounted for 15.64% (409 circRNAs), 7.88% (206 circRNAs), and 3.33% (87 circRNAs), respectively (Figure 5d).

Differential expression analyses of mRNA, lncRNAs and circRNAs

There were three sample groups (NR12_vs_NR7, RR12_vs_NR7, RR12_vs_NR12) were used to analyze the differential expression of mRNA, lncRNAs and circRNAs. A total of 26588 mRNAs were detected in all groups. The numbers of differentially expressed genes (DEGs) were 2320, 1864 and 2440, respectively. For ncRNAs prediction, 2615 circRNAs were also characterized. However, a few circRNAs were differentially accumulated in all sample groups, which were 16, 17 and 9, respectively. Additionally, 1971 lncRNAs were identified in all sample pools, and 176, 173 and 137 were differential expression, respectively (Table 2).

We found 170 mRNAs, 5 lncRNAs, and 0 circRNA that were shared by three various groups (Figure 6a-c). There were 2320 mRNAs (1329 up-regulated and 991 down-regulated), 176 lncRNAs (110 up-regulated and 66 down-regulated), and 16 circRNAs (9 up-regulated and 7 down-regulated) that were differentially expressed in NR12_vs_NR7. 2440 mRNAs (1237 up-regulated and 1203 down-regulated), 173 lncRNAs (84 up- and 89 down-regulated), and 17 circRNAs (9 up-regulated and 8 down-regulated) that were differentially expressed in RR12_vs_NR12. Moreover, there were 1864 mRNAs (1222 up-regulated and 642 down-regulated), 137 lncRNAs (79 up-regulated and 58 down-regulated), and 9 circRNAs (including 7 up-regulated and 2 down-regulated) that were differentially expressed in RR12_vs_NR7 (Figure 6d-f) .

Functional enrichment analyses

To explore the functions of the mRNAs, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed. The GO terms for DEGs mainly related to biological regulation, metabolic process, cellular process, cell, cell part, binding, catalytic activity and transporter activity in three differential groups (NR12_vs_NR7, RR12_vs_NR7, RR12_vs_NR12). KEGG pathway analysis was also performed to further explore the functions of mRNAs. The results showed that biological functions of mRNAs were significantly enriched in three differential groups, including lipid metabolism, carbohydrate metabolism, biosynthesis of other secondary metabolites and signal transduction (Figure 7).

To investigate the potential function implication of lncRNAs, GO and KEGG enrichment analyses of lncRNAs and their total differentially expressed lncRNAs were also performed. The results showed that these lncRNAs were enriched in the cellular process, metabolic process, cell, cell part, binding and catalytic activity in three differential groups. The KEGG enrichment analysis showed that these lncRNAs were clustered into nucleotide metabolism, carbohydrate metabolism and signal transduction in three differential groups (Figure 8).

To further understand the potential functions of circRNAs, GO and KEGG analyses of the host genes of circRNAs were performed. Based on GO annotation, most the host genes of circRNAs in the root were annotated to the cellular process, metabolic process, cell, cell part, binding and catalytic activity in three differential groups. The parent genes of circRNAs were involved in 5 KEGG pathways and significantly enriched in metabolism of other amino acids, lipid metabolism, global and overview maps, energy metabolism and amino acid metabolism (Figure 9).

Experimental validation of the circRNA candidates

In the present study, we experimentally tested and verified the circRNA predictions in the grapevine. Since the majority of circRNA were derived from back-splicing of mRNA transcripts, convergent primers and divergent primers were designed and used to amplify the linear transcript and backsplice sites using cDNA and genomic DNA (gDNA) as template, respectively. Unlike convergent primers, divergent primers could amplified the back-splicing junctions in cDNAs synthesised by the random primers but did not in gDNAs. Additionally, We successfully validated the circRNA - circRNA_2377 associated with root development. The PCR amplification products were further analyzed by agarose gel electrophoresis and Sanger sequencing to confirm the occurrence of back-splicing (Figure 10).

Quantitative real-time PCR (qRT-PCR)

To verify the reliability of the sequencing data profiles, qRT-PCR was used to analyze the candidate genes related to root development (Figure 11). The expression pattern were generally consistent with the results of high-throughput sequencing, indicating that the research method used in this experiment is reliable. However, circRNA_2377 in NR12_vs_NR7 was not consistent with the high-throughput sequencing, which might be due to the expression level of circRNA itself is low (Figure 11).

CeRNA network analyses

It was supposed that lncRNA and circRNA generally contain one or multiple miRNA binding sites, and the method of miRNA target gene prediction can be used to identify lncRNA and circRNA which bound to miRNA. The functions of lncRNA and circRNA were clarified according to the functional annotations of miRNA target genes. In the present study, all the candidate ceRNA pairs (circRNA sequesters miRNA and miRNA targets mRNA) were first identified. Top 5 miRNA-lncRNA-circRNA interaction combinations were extracted based on minimum free energy according to website prediction. Furthermore, 3 miRNA-mRNA network with a high degree of confidence were also predicted and extracted. Finally, the lncRNA/circRNA-miRNA-mRNA target interaction network diagram was obtained and plotted by Cytoscape software, and constructed a hub prognostic ceRNA network consisting of 21 mRNAs, 14 miRNAs, 2 lncRNAs, and 1 circRNA (Figure 12).

As showed in Figure 12, a large proportion of mRNAs communicated with individual lncRNA and circRNA. lncRNA and circRNA acted as ceRNAs to communicate with multiple mRNAs by competing for specific miRNAs. These results suggested that the expression of lncRNA and circRNA potentially regulated gene expression through miRNA-mediated lncRNA/circRNA-mRNA ceRNA interactions, implying that ceRNA might important for lncRNA and circRNA function during root development.

Discussion

Root restriction which through limits the roots into a certain space to regulate the relationship between vegetative and reproductive growth, has been widely practised in fruit production^[1]. In this study, one-year old *cv.* 'Muscat Hamburg' was used as material to explore the regulatory mechanism involved in root architecture construction during conventional and root restricted cultivation. We firstly observed the root phenotype during the developmental stage on the condition of two distinct cultivation ways. Compared with NR, roots at the same stage of development emitted more lateral roots under the RR (Fig. 1 and Fig. 2). According to previous researches, RR accelerates root updatation^[4], which is consistent with this study. The weight of roots was positively affected under RR, and the young roots were significantly higher than it under NR during root development (Fig. 3). Therefore, these results indicated that root architecture reconstruction was attributed to the vigorous induction of lateral roots in response to RR condition.

The ceRNA hypothesis has been widely accepted since it was first reported in several years ago^[18]. CeRNA theory has been well applied to understand the mechanism of human disease^[35], however, only a few researches have been carried out in plants. In the ceRNA network, miRNA act as mediator which connect mRNAs and ncRNAs such as lncRNAs and circRNAs. In *Arabidopsis*, *IPS1*, as a non coding RNA containing a motif with defective complementarity to miR399 at the expected cleavage site, sequestered and inhibited miR399 activity by the target mimicry strategy^[36]. The mRNAs can be directly transcribed into proteins, while lncRNAs and circRNAs can indirectly influence mRNAs expression by competitive binding to common miRNAs^[18]. To investigate the potential regulatory functions of lncRNAs and circRNAs

during root development in grapevine, mRNAs, lncRNAs and circRNAs were analyzed by high-throughput sequencing.

In this study, a total of 26588 mRNAs, 1971 lncRNAs and 2615 circRNAs were identified through transcriptomic analyses in grapevine root, respectively. Compared with lncRNAs, the chromosome distribution of circRNAs were more uniform (Fig. 5). Through the GO analysis, differentially expressed mRNAs, lncRNAs and circRNAs were shown to be involved in the regulation of various biological processes, including cellular process, cell, cell part, binding, catalytic activity, etc.. In KEGG pathway, mRNAs, lncRNAs and circRNAs were mainly enriched in nucleotide metabolism, carbohydrate metabolism, signal transduction, lipid metabolism, energy metabolism and amino acid metabolism (Fig. 7–9). These key pathway, lncRNAs and circRNAs, form ceRNA networks by targeting miRNAs, and these networks may provide new evidence of the regulatory mechanisms during root development. To verify the reliability of the sequencing data profiles, the candidate genes were analyzed by qRT-PCR (Fig. 11), and the results were generally consistent with high-throughput sequencing.

A ceRNA hypothesis has been proposed that the lncRNAs, circRNAs and mRNAs can act as ceRNAs that competitively bind to miRNAs, thereby regulating a wide range of biological and developmental processes^[37–42]. MiRNAs act as the key regulatory factors for important root development. MiR160 has been reported to be involved in root cap and root elongation formation^[43]. MiRNAs target auxin response factors to influence the development of adventitious root^[44]. MiRNAs are likely to be an RNA bridge between non-coding RNAs and mRNAs^[45]. In addition, circRNAs possibly act as another type of ceRNA to sequester miRNAs and suppress their activity. CircRNAs have been shown to contain multiple binding sites for microRNAs in animals. One of the well studied examples is Cdr1as/ciRS-7, which can bind miR-7 as a miRNA sponge and affect miR-7 target gene expression^[46]. Based on the ceRNA hypothesis and the ceRNA network constructed in this study, a model of action for lncRNAs, circRNAs, miRNAs, mRNAs in response to the root development (Fig. 12).

Interestingly, miRNAs have been reported that involved in the response to root development^[47–52]. For example, *SPL 10*, one of the SPL family genes that represses lateral root growth in *Arabidopsis thaliana*, was significantly down-regulated in miR156 overexpressed plants, suggesting that it play an important role in plant growth and development^[47]. MiR166 reduced the number of lateral roots and symbiotic nodules, and induced ectopic development of vascular bundles in transgenic roots^[48]. Research has shown that the miR169defg isoform promote primary root growth and inhibit lateral root initiation^[49]. MiR2111 is not only the critical shoot-to-root factor that positively regulates root nodule development, but also shapes root system architecture^[50]. In addition, the transcription factor IREH1 may be involved in root hair elongation^[51], and was targeted by miR3623-3P. NAC1 has been reported to promote lateral root development and activates the expression of two downstream auxin-responsive genes, *DBP* and *AIR3*^[52]. Based on the ceRNA hypothesis, we speculate that lncRNAs and circRNAs binding miRNAs might play a critical role in the regulation of root growth, there is still a lot of work to be done to clarify how it plays the regulatory role.

Conclusions

Root restriction altered the expression profiles of mRNA, lncRNA and circRNAs in grapevine. We totally identified one mRNA, two lncRNA and one circRNA involved in the regulation of root development. They might act as miRNA sponges, trapping miRNAs from its target genes via the ceRNA network, thus affecting root development. To our knowledge, this is the first report of integrating the regulatory network of ceRNAs functions of lncRNA and circRNA, and provided a basis for studying the potential functions of non-coding RNAs during root development of grapevine.

Materials And Methods

Plant Material and Treatments

The grapevine materials used in this study were *cv.* 'Muscat Hamburg' (*Vitis vinifera* L.) which were planted in greenhouse of the Fruit Tree Laboratory in Shanghai Jiao Tong University. In this study, the materials were divided into conventional cultivation (referred to NR) and root restricted cultivation (referred to RR). In root restricted cultivation, 100 cuttings were planted in a container with diameter of 30 cm and height of 30 cm in the root zone (with holes around it) which were separated from the ground by a tray. The planting substrate was a 1:1:1 mixture of soil, organic fertilizer and perlite. In the control culture, 100 plants with consistent physiological state were cultivated on the same substrate with a planting distance of 60 cm × 60 cm. Sampling was performed in a zigzag pattern to ensure that there was sufficient space for the root system to develop. The treatment of the above-ground management were the same, and the growth of them was maintained without topping. The secondary shoots were trimmed and watered every 7 days. Moreover, the roots began to sample on March 10, 2021. The root samples of different cultivation models were taken every 10 days intervals during the first 7 time points and at 15-day intervals at the last 5 time point. Finally, grapevine root samples from two different cultivation models at 12 time points (53, 63, 73, 83, 93, 103, 113, 128, 143, 158, 173, and 188 days after planting) were collected. At each sampling time point, 4-6 trees were selected as biological replicates. All root samples were collected from control and treated plants, frozen in liquid nitrogen and stored at -80°C.

Library construction and Illumina sequencing

According to the root phenotype, samples from the seventh and twelfth under NR and the twelfth under RR were selected for sequencing, respectively. Three biological replicates were performed for each sample. Total RNA was extracted by the mirVana miRNA Isolation Kit (Ambion) following the manufacturer's protocol. RNA integrity was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The samples with RNA Integrity Number (RIN) ≥ 7 were subjected to the subsequent analysis. The libraries were constructed using TruSeq Stranded Total RNA with Ribo-Zero Gold according to the manufacturer's instructions. Then these libraries were sequenced on the Illumina sequencing platform (HiSeqTM 2500 or other platform) and 150 bp/125bp paired-end reads were generated. Three biological replicates were analyzed. The samples were named as NR7_1, NR7_2, NR7_3; NR12_1, NR12_2, NR12_3; RR12_1, RR12_2, RR12_3, respectively.

Data preprocessing and genomic alignment

Raw reads generated during high-throughput sequencing were fastq format sequences. In order to get high-quality reads that could be used for later analysis, raw reads needed to be further quality filtered. Trimmomatic^[53] software was first used for adapter removing, And then low-quality bases and N-bases or low-quality reads were filtered out. Finally, we got high-quality clean reads. Using hisat2^[54] to align clean reads to the reference genome of the experimental specie, the sample should be assessed by genomic and gene alignment.

Transcript splicing, lncRNA prediction and Gene quantification

The result of alignment with the reference genome was stored in a binary file, called a bam file. Using the Stringtie^[55] software to assemble the reads, the new transcript was spliced. Then the candidate lncRNA transcripts were selected by comparing the gene annotation information of the reference sequence produced by Cuffcompare^[56] software. Finally, transcripts with coding potential were screened out by CPC^[57], Pfam^[58] and PLEK^[59] to obtain lncRNA predicted sequences. Aligning the sequencing reads of each sample with the sequence of mRNA transcript sequences, known lncRNA sequences and lncRNA prediction sequences by bowtie2, and using express to make gene quantitative analysis, the FPKM value and counts value (the number of reads for each gene in each sample) were obtained.

Differential screening analysis and Functional Analysis

Using the estimateSizeFactors function of the DESeq^[60] R package to normalize the counts, and using nbinomTest function to calculate pvalue and foldchange values for the difference comparison. Selecting differential transcripts with p-values ≤ 0.05 and foldchange ≥ 2 , and analysing differential mRNA GO and KEGG enrichment by Hypergeometric Distribution Test.

CircRNA prediction, Expression analysis and Interaction research

In order to generate SAM file, we used BWA^[61] software to align the sequencing reads of each sample with reference genome. Then CIRI^[62] software was used to scan for PCC signals (paired chiastic clipping signals), and circRNA sequences were predicted based on junction reads and GT-AG cleavage signals. Using the RPM algorithm to quantify the circRNAs, and normalizing the number of junction reads counts and foldchange by DESeq. Enrichment of differentially expressed circRNAs was analysed through the annotation information of circRNA source transcripts. CircRNAs can serve as miRNA target molecules. psRNATarget^[63] software was used to predict lncRNA/ circRNA- miRNA- mRNA interactions.

Validation of circRNA in Grape

To confirm the grapevine circRNA_2377 that were predicted by the software, The convergent and divergent primers was designed using the Primer-Blast tools in NCBI (www.ncbi.nlm.nih.gov) (Table S1). Genomic

DNA (gDNA) and total RNA were used as templates for the PCR validation of circRNAs. The 2×Taq Master Mix (Vazyme, Nanjing) was used for cDNA and gDNA amplification with PCR to detect the circRNA templates. The PCR procedure was as follows: 94°C for 3 min; 39 cycles at 94°C for 30 s, 55°C for 15 s, and 72°C for 30 s; and then 1 cycle at 72°C for 5 min. The PCR products were separated using agarose gel electrophoresis and then purified with a Vazyme FastPure® Gel DNA Extraction Kit (v4.0). Sanger sequencing of the direct PCR products was performed to further verify the existence of the backsplice junction of the circRNAs predicted in grapevine.

qRT-PCR analysis

The qRT-PCR was performed on a CFX Connect Real-Time Detection System (Bio Rad, USA). The program settings are as follows: 95 °C for 150 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The relative expression levels were analyzed by the $2^{-\Delta\Delta Ct}$ method^[64]. *Actin* was used as an internal control for the mRNA, lncRNA and circRNA (Table S2). All qRT-PCRs were performed in triplicate.

Abbreviations

- RR
- root restriction
- NR
- conventional cultivation
- ncRNAs
- non-coding RNAs
- miRNAs
- microRNAs
- lncRNAs
- long non-coding RNAs
- circRNAs
- circular RNAs
- gDNA
- genomic DNA
- ceRNAs
- competitive endogenous RNAs
- DEGs
- differentially expressed genes
- qRT-PCR
- quantitative real-time PCR
- PCA
- principal component analysis
- GO
- Gene Ontology

Declarations

Ethics approval and consent to participate

The current study complies with relevant institutional, national, and international guidelines and legislation for experimental research and field studies on plants (either cultivated or wild), including the collection of plant material.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

The databases used in this study as follows,

NCBI database: <https://www.ncbi.nlm.nih.gov/sra/PRJNA808789>

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Authors' contributions

CM and HL conceptualized, designed, and performed the experiments; JH, WX, YR and HL supervised the experiments; JL, LZ and YS collected the sample; JL performed the experiments and drafted the manuscript. All authors have read and agreed to the published version of the manuscript.

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Tables

Table 1. The statistical qualification of high throughput sequencing

Sample	RawReads	RawBases	CleanReads	CleanBases	ValidBases	Q30	GC
NR12_1	50.65M	7.60G	50.23M	7.27G	95.70%	93.66%	47.69%
NR12_2	39.34M	5.90G	39.03M	5.67G	96.12%	93.64%	47.31%
NR12_3	39.31M	5.90G	39.00M	5.66G	96.07%	93.74%	46.50%
NR7_1	50.00M	7.50G	49.58M	7.12G	95.00%	94.25%	46.90%
NR7_2	48.08M	7.21G	47.72M	6.89G	95.54%	94.28%	46.57%
NR7_3	37.01M	5.55G	36.74M	5.35G	96.38%	93.62%	46.85%
RR12_1	42.49M	6.37G	42.13M	6.13G	96.22%	93.56%	46.97%
RR12_2	60.20M	9.03G	59.79M	8.69G	96.20%	93.85%	47.20%
RR12_3	47.77M	7.16G	47.37M	6.84G	95.40%	93.76%	47.64%

Table 2. Statistical table of sequence prediction information

Term	All	NR12_vs_NR7-diff	RR12_vs_NR7-diff	RR12_vs_NR12-diff
mRNA	26588	2320	1864	2440
lncRNA	1971	176	173	137
circRNA	2615	16	17	9

Figures

Figure 1

The developmental root system morphology of one-years-old self-rooted grapevine cv. 'Muscat Hamburg' under conventional cultivation (NR). Twelve different sampling points were recorded as NR1-NR12, respectively. Scale bar = 1 cm.

Figure 2

The developmental root system morphology of one-years-old self-rooted grapevine cv. 'Muscat Hamburg' under root restriction (RR). Twelve different sampling points were recorded as RR1-RR12, respectively. Scale bar = 1 cm.

Figure 3

Root weight variations of one-year-old self-rooted grapevine cv. 'Muscat Hamburg' in conventional (NR) and root restricted cultivation (RR). The weight of young roots (a), old roots (b) and total roots (c) was counted in NR and RR cultivation.

Figure 4

Correlation and PCA analyses of different samples. The Sample-to-Sample correlation analyses in mRNA (a), lncRNA (b) and circRNA (c). The horizontal axis and the vertical axis represent the corresponding sample names, and the color represents the size of the correlation coefficient. The PCA analyses among mRNA (d), lncRNA (e) and circRNA (f). The closer the clustering distance or PCA distance of the samples, the more reliable of the repeatability of the samples were.

Figure 5

Comparison of the features of lncRNAs and circRNAs in the grapevine root. Chromosomes distribution (a), Length distribution (b) and classification of lncRNAs (c) and circRNAs (d).

Figure 6

Comparative analysis of mRNAs, lncRNAs, and circRNAs. The specific mRNAs (a), lncRNAs (b), and circRNAs (c) shared by three differential groups (NR12_vs_NR7, RR12_vs_NR7, RR12_vs_NR12). The mRNAs (d), lncRNAs (e) and circRNAs (f) differentially expressed in various sample groups.

Figure 7

The functional analysis results of mRNAs. GO enrichment and KEGG pathway in NR12_vs_NR7 (a-b), RR12_vs_NR7 (c-d), RR12_vs_NR12 (e-f).

Figure 8

The functional analysis results of lncRNAs. GO enrichment and KEGG pathway in NR12_vs_NR7 (a-b), RR12_vs_NR7 (c-d), RR12_vs_NR12 (e-f).

Figure 9

The functional analysis results of circRNAs. GO enrichment and KEGG pathway in NR12_vs_NR7 (**a-b**), RR12_vs_NR7 (**c-d**), RR12_vs_NR12 (**e-f**).

Figure 10

PCR test was conducted to validate the stable expression of grape circRNA_2377. **(a)** According to the genomic loci, circRNA_2377 derived from GSVIVT01022505001 gene. **(b)** Sanger sequencing further confirmed head-to-tail backsplice site (blue line). **(c)** The agarose gel electrophoresis image showed the expected size of PCR product by divergent primers in cDNA. ◀▶ represents convergent primers, ▶▶ represents divergent primers.

Figure 11

Relative expression analyses of mRNAs, lncRNAs, and circRNAs in the roots of three differential groups. NR12_vs_NR7 (**a-d**), RR12_vs_NR7 (**e-h**), RR12_vs_NR12 (**i-l**). Significant difference at $p < 0.05$ is indicated by different letters above the columns.

Figure 12

Prediction of interaction network among lncRNAs, circRNAs, miRNAs and target genes. Yellow, green, red and blue represent lncRNA, circRNA, miRNA and target genes, respectively.

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