Study on miRNAs-Mediated Seed and Stone-Hardening Regulatory Networks and the Mechanism of miRNAs' Manipulating Gibberellin-Induced Seedless Berries in Grapevine (Vitis vinifera L.)

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Abstract

A significant body of evidence supports the important roles of miRNAs in grape berry developments. However, their specific molecular functions during stone-hardening stage development remain unclear. Here, a total of 161 conserved and 85 species-specific miRNAs/miRNAs*(precursor) were identified in grape stone hardening stage berries using Solexa sequencing. Out of them, 30 VvmiRNAs were tissue-specific and identified as stone-hardening related to VvmiRNAs, whereas 52 exhibited differential expression profiles during berry development, potentially participating in modulation of development, and qRT-PCR analysis verified their expression patterns. Interestingly, high SNP variations in VvmiRNA sequences might result into generation of new VvmiRNA family members like VvmiR168, VvmiR479, VvmiR3636 families and so on. Through GO and KEGG pathway analyses, we revealed that 13 VvmiRNAs involved in the regulation of embryo development, 11 in lignin and cellulose biosynthesis, and 28 in the modulation of hormone signaling, sugar and proline metabolism. Furthermore, the target genes for novel VvmiRNAs related to berry development were validated using RLM-RACE and PPM-RACE methods, and it was revealed their cleavage sites mainly happened at the 9^{th}-11^{th} sites from the 5' ends of miRNAs at their binding regions. The potential roles of these VvmiRNAs in gibberellins repressing grape stone-hardening and embryo development by potentially inducing the expression of VvmiR31-3p and VvmiR8-5p to increase the cleavage product accumulation levels of corresponding target genes like lignin biosynthesis genes, *CAFFEOYL COENZYME A-3-O-METHYLTRANSFERASE* (*VvCCoAOMT*) and *DDB1-CUL4 ASSOCIATED FACTOR1* (*VvDCAF1*), as a potential key molecular mechanism involved in GA-induced grape seedless berry development. Based on the characterization of stone-hardening stage related to VvmiRNAs, a schematic model of miRNA-mediated grape seed and stone-hardening development was proposed in this work. This is the first report about the regulatory role of VvmiRNAs in the regulation of stone-hardening stage of grape berries, which provides valuable genetic information for the breeding of seedless grape varieties.

Key Messages

- Characterization of stage-specific VvmiRNA-mediated stone-hardening stage development in grape.
- High SNP variation in VvmiRNA families resulted into generation of new VvmiRNA members.
- GA might repress the stone-hardening and embryo development, at some extent, by inducing the expression of *VvmiR31-3p* and *VvmiR8-5p* to increase cleavage product accumulation levels of lignin biosynthesis enzyme genes *VvCCoAOMT* and *VvDCAF1*, as a potential key development.
- An assumed schematic model of miRNA-mediated stone-hardening development was proposed in this work.

Introduction

Grape (*Vitis vinifera* L.) is a soft pulpy berry with a thin edible outer skin (exocarp) and fleshy edible inner layers (mesocarp and endocarp) of storage tissues that contained seeds. During grape stone-hardening stage, the coats surrounding the seed gradually become hard, forming a lignified seed coat (stone). Stone-hardening is an essential strategy for seed protection and dispersal in different plant species, including cherry (*Prunuscerasus* and *P. avium*), peach (*P. persica*), plum (*P. salicina*) and grape (Callahan et al. 2009, Crisosto et al. 2012, Kritzinger et al. 2017). Lignin deposition plays a critical role in seed stone-hardening formation, and several transcriptome and proteome studies demonstrated that flavonoid and lignin biosynthesis pathways are highly involved in the lignification of seed coat structures (Dardick et al. 2010, Nashima et al. 2013, Reuscher et al. 2016, Xue et al. 2018). However, seed coat development might affect the expansion and ripening process of fruits, resulting in low-fruit quality (Xue et al. 2018). In contrast, seed abortion and seed coat degradation during seed development stage could lead to seedless berry, which is a favorable trait for consumer (Ren et al. 2014, Tang et al. 2017, Acheampong et al. 2017). Therefore, in-depth understanding of the regulatory mechanisms and molecular basis underlying seed stone-hardening formation during grape berry development is critical for the production of high-quality fruit.

With the recent development of high-throughput sequencing technologies, many miRNAs from various plant species have been released on the miRBase21.0 database (http://www.mirbase.org). MiRNAs play important roles in the regulation of fruit development and ripening process through miRNA-guided cleavage of target mRNAs and/or translational repression of ripening-related transcription factors (TFs) in different plant species, including grapevine (Zeng et al. 2015, Wang et al. 2018; Zhang et al. 2019). For example, Chinese pear (*Pyrus bretschneideri*) PbrmiR397a regulates the fruit stone cell lignification by inhibiting the expression of three *LACCASE* (*LAC*) genes involved in lignin biosynthesis, resulting in a decrease in lignin content and stone cell number in Chinese pear fruit (Xue et al. 2018). *Arabidopsis* AUXIN RESPONSE FACTOR8 (AtARF8) and FRUITFULL (AtFUL) MADS-domains act together to directly activate the expression of *MIR172C*, a valve-specific AtmiR172-encoding gene, leading to repression of the flower-patterning gene *APETALA2* (*AtAP2*) and promotion of fruit valve growth (Ripoll et al. 2015). Similarly, tomato (*Solanum lycopersicum*) SlmiR156/157 and SlmiR172 have been reported as important regulators in the ripening process of tomato fruits by inhibiting the expression of ripening regulatory genes *COLORLESS NON-RIPENING* (*CNR*) and *SlAP2a* (Karlova et al. 2013). Besides these, our research group identified and characterized many known and specific grape miRNAs (VvmiRNAs) during grape berry development, implying that a large number of VvmiRNAs might be involved in the modulation of berry development and seed formation (Wang et al. 2011b, Wang et al. 2014, Wang et al. 2018, Zhang et al. 2019). Moreover, we found that VvmiR058 negatively regulates the expression of *POLYPHENOL OXIDASE* (*VvPPO*) gene, involved in the synthesis of lignin in peel and seeds during berry development, suggesting that VvmiRNA might play regulatory roles in modulation of grape seed and stone-hardening development (Ren et al. 2014). Despites the large number of miRNAs involved in fruit and seed development have been identified in model plants, the regulatory networks and transcriptome dynamics of VvmiRNAs during the seed and stone-hardening development in grape still imperative.

Gibberellin (GA) is a well-known phytohormone involved in diverse biological processes of grape berry development, leading to the improvement of berry size, weight and seedless berry formation (Serrano et al. 2017, Zhang et al. 2019). Many VvmiRNAs are differentially expressed during the different stages of grape berry development in response to GA application (Han et al. 2014, Wang et al. 2018), and thus the identification of seed stone-hardening stage-specific VvmiRNAs responsive to GA signal is crucial for the molecular breeding of seedless grape production. Hence, in the present study, we attempted to identify

and characterize the expression of seed stone-hardening stage-specific VvmiRNAs and their target genes using Solexa sequencing technology. Moreover, GO and KEGG pathway analyses were investigated to explore the regulatory networks of VvmiRNA-mediated seed stone-hardening development stage in grape berries. In addition, transcriptome dynamics and functional annotations of several important miRNA downstream target genes in response to GA application across various stages of grape berry development were carried out. Our results provide novel source of genetic information for the improvement of grape breeding programs to advance the production of novel seedless grape varieties.

Results

Overview of sRNA sequencing at the stone-hardening stage of grape berries

The sRNA library from the 'Wink' cultivar grape stone-hardening berries (SB) at 45-days after flowering (45DAF) was constructed and sequenced in depth by Solexa technology to explore the role of sRNA in the grape stone-hardening process (Fig. 1A). After trimming and filtering the adaptor and low-quality tag sequences, a total of 11,456,656 redundant and 3,591,857 unique clean reads were obtained, respectively (Supplementary Table S1). The high-quality sRNA clean reads were mapped into the Rfam (https://rfam.xfam.org) to filter the rRNA, tRNA, snRNA and snoRNA etc. The filtered reads were then compared against known plant miRNA database in the miRBase 21.0 (http://www.mirbase.org/) with BLASTn. After searching against Rfam database and miRBase, the remaining reads were further mapped to grape reference genome (http://genomes.cribi.unipd.it/DATA/V2/), all about 18.40 and 8.79% of the redundant and unique reads, respectively, were annotated, of which 5.54 and 1.99% of the redundant and unique reads, respectively, were successfully mapped into the non-coding RNA of rRNAs, snRNAs, snoRNAs and tRNAs, while 5.71 and 0.04% of the redundant and unique reads, respectively, were identified to be putative known miRNAs, on the other hand, the majority of the redundant (81.6%) and unique (91.21%) reads, were mapped to un-annotated regions in the grape genome (Fig. 1B).

The length distribution of sRNAs in SB library are uneven, and most sRNA reads were 21 and 24 nucleotide (nt) in length, which are the characteristic lengths of miRNAs and siRNAs, respectively, and consistence with the expected size range generated by Dicer (Henderson et al. 2006). In contrast with other two sRNA libraries of 5 DAF berry (YB) and 90 DAF berry (MB) (Data not shown), the sRNA distribution at 21nt and 24nt at SB is more similar to that at MB, than that at YB (Fig. 1C). The comparative expression level using normalized counts per million (CPM) of miRNA (21 nt) and siRNA (24 nt) at grape YB, SB and MB stages indicated a gradual increased in the known miRNA (21 nt) reads towards the MB stage, whereas the siRNA (24 nt) reads exhibited a reverse trend with the highest level observed at the early YB stage followed by a gradual decrease towards the MB stage (Fig. 1C). The above result suggested that known miRNA (21 nt) reads are highly abundant during the later stage (MB) of grape berry ripening compared with siRNA (24 nt) reads.

Identification and characterization of known VvmiRNAs in the stone-hardening stage of grape berries

A total of 143 known VvmiRNAs and 18 corresponding precursors namely VvmiRNAs* belonging to 48 VvmiRNA families were identified in the stone-hardening stage of grape berries (Supplementary Table S2). Although the number of known VvmiRNA members within each family varied from 1 to 24 during grape berry development, they had the most variation at SB stage compared to other two stages (YB and MB) (Fig. 1D, E). Among total 48 VvmiRNA families, VvmiR169 family was highly represented during all three stages with 22-24 members, followed by VvmiR395 family with 13, VvmiR156 family with 8-9 and VvmiR166 family with 8 members, while the remaining miRNA families consisted of 1-7 members (Fig. 1D). This result indicated a diversification in the functions of these VvmiRNA families during grape development and ripening process. Interestingly, from the percent numbers of VvmiRNA members at different grape berry developments, we found that VvmiR3628 family was sequenced only at mature berries (90DAF), while VvmiR393 family was only detected at young berries (5DAF) (Fig. 1E), indicating the spatio-temporal specificity of VvmiRNAs' expressions.

Furthermore, we also observed that the characterization of most known VvmiRNA families with high expression abundances at the stone-hardening stage of grape berries. For instance, among the 143 known VvmiRNAs, about 68% exhibited high copy read number, including 42 known VvmiRNAs with read number > 1,000, while 55 known VvmiRNAs with read numbers ranging between 100 and 1,000 (Supplementary Table S2). Specifically, VvmiR166, VvmiR168, VvmiR479, VvmiR156 and VvmiR3636 families (not including their VvmiRNA* sequences) possessed more than 10,000 copy reads (Supplementary Table S2). On the other hand, 83.3% of known VvmiRNA* family members exhibited lower copy number than their corresponding VvmiRNAs (Fig. 1F, G; Supplementary Table S2), which might be derived from the fact VvmiRNAs* are easier to degrade than their corresponding VvmiRNAs, and thus, they usually have less copy number, similar to the previous report (Wang et al. 2012). Despite of this, it was revealed that two VvmiRNA* families, including VvmiR3623* and VvmiR2950*, showed higher copy number (14830 and 3029, respectively) than their corresponding VvmiR3623 and VvmiR2950 (4405 and 1773, respectively) (Fig. 1G; Supplementary Table S2). This result implied that both VvmiR3623* and VvmiR2950* might play potential roles during development and ripening stages of grape berries, similar to VvmiRNAs (Jagadeeswaran et al. 2010, Wang et al. 2014).

Screening of novel VvmiRNAs at grape stone-hardening stage and their validation using miR-RACE and qRT-PCR

On the basis of the annotation criteria of novel miRNAs (Bi et al. 2015), all un-annotated sRNAs were used to explore the stem-loop structures of their precursors for the prediction of novel miRNAs. In this work, a total of 90,352 reads were identified as novel VvmiRNAs, including 72 novel VvmiRNAs and 12 novel VvmiRNAs*, in the grape stone-hardening stage (Table 1). These novel precursors were folded into stable hairpin structures, and their negative minimal folding free energy (MFE) ranged from -108.2 to -20.88 kal mol^{-1} (Table 1), which was in line with the criteria of novel VvmiRNAs (MFE< −20.0 kal mol^{-1}) as previously reported (Bi et al. 2015). The novel VvmiRNAs and VvmiRNAs* were mainly 21 nt in length, accounting for 84.14% (69/82), and the first base with uracil (U) at the 5'-end of their mature sequences was up to 56.0%, confirming that these are novel VvmiRNAs (Fig. 2A). From our datasets, the novel VvmiRNAs were un-conserved, species-specific, and low abundance, usually exhibited lower accumulation level than conserved ones, which was in agreement with the results of previous reports (Pantaleo et al. 2010; Mica et al. 2010; Wang et al. 2012). Interestingly, it was also found a few novel VvmiRNAs with high abundance with read number >1000, such as VvmiR10, VvmiR13, VvmiR29, VvmiR30, VvmiR34, VvmiR37, VvmiR43 and VvmiR71

(Table 1). Among them, VvmiR37, VvmiR13 and VvmiR30 showed higher copy numbers (37985, 13748 and 10199, respectively) compared with others (Table 1). Moreover, their corresponding VvmiR37* and VvmiR13*also showed high copy numbers with 2588 and 1332, respectively, suggesting that VvmiR37/VvmiR37* and VvmiR13/VvmiR13* might play significant roles during the stone-hardening stage of grape berries. The location of novel VvmiRNAs in their precursors showed that 40 novel VvmiRNAs were located in the 5'-arm of their precursors, while 32 novel VvmiRNAs were located in the 3'-arm of their precursors (Table 1). Similarly, seven novel VvmiRNAs* were located in the 5'-arm of their precursors, whereas, five novel VvmiRNAs* were located in the 3'-arm of their precursors (Table 1). The above results indicated that the 5'-arm of miRNA precursors might be more efficient in generating miRNAs and miRNA* than the 3'-arm. However, further research is necessary to confirm this mechanism. The identified novel VvmiRNAs exhibited three types of VvmiRNA-3P or VvmiRNA-5P or the first both sequences (Table 1). The sequences marked 3p indicated only the 3' arm of miRNA precursor produced one miRNA sequences, and those marked 5p denoted just 5' arm of miRNA precursors yielded ones, while both sequences represented the both arms of miRNA precursors generated two sequences of miRNA and miRNA* (Table 1). The distribution of all these novel VvmiRNAs varied between the 19 grape Chrs and one unknown Chr (Fig. 2B). Chr19 possessed the highest number (10) of novel VvmiRNAs, followed by Chr8 with nine novel VvmiRNAs and the unknown Chr with eight novel VvmiRNAs (Fig. 2B), while Chr3, Chr4 and Chr11 did not harbor any novel miRNAs (Fig. 2B).

On the basis of the functional annotations of predicted target genes for miRNAs, six novel VvmiRNAs related to berry development, such as VvmiR8, VvmiR16, VvmiR31, VvmiR38-5p, VvmiR44-3p and VvmiR53-3p, were validated by miR-RACE and qRT-PCR. The precise sequences of the six novel VvmiRNAs were obtained by miR-RACE (Table 2), and these sequences were consistent with those obtained from high-throughput sequencing dataset (Table 1; Supplementary Table S2), confirming that these novel VvmiRNA sequences were true in grape berries. The qRT-PCR expression profiles of these six novel VvmiRNAs during grape berry development showed differential expression patterns similar to the high-throughput sequencing dataset (Fig. 2C). Therefore, our miR-RACE and qRT-PCR results confirmed the reliability and expression modes of VvmiRNA involved in the modulation of grape berry development.

Identification of grape stone-hardening stage-specific VvmiRNAs

The identification of grape stone-hardening stage-specific VvmiRNAs is essential for gaining insights into the modulation of grape berry development. Compared with our other two sRNA libraries from 5 DAF and 90DAF in our other work (data not shown), 35 VvmiRNAs/VvmiRNAs* were identified only at the stone-hardening stage of grape berries, including 28 VvmiRNAs (1 known and 27 novel ones) and 7 VvmiRNAs* (2 known and 5 novel ones) (Supplementary Tables S3 and S4; Fig. 2D). In addition, the high number of novel VvmiRNAs in this stage indicated that novel miRNAs might play the significant roles in the stone-hardening stage of grape berry development.

To identify the differential expression VvmiRNAs during grape berry development, the fold changes log2 (YB/SB) or log2 (MB/SB) >1 cut offs were applied, and the filtered VvmiRNA/VvmiRNA* possessed significant expression difference across diverse development stages of grape berries. Here, we discovered that 52 VvmiRNAs/VvmiRNAs* exhibited significant differences in their expression levels during grape berry development and ripening process, which comprised of 44 VvmiRNAs (34 known and 10 novel ones) and 8 VvmiRNAs* (5 known and 3 novel ones) (Supplementary Tables S3 and S4; Fig. 2D), implying that they might possess dynamic regulatory roles of grape berry development, and suggesting that known VvmiRNAs might have more dynamic variation in their regulatory roles than novel ones.

SNPs and their Edit types of known VvmiRNAs/VvmiRNAs* from grape berries during grape stone-hardening stage

Lots of SNP variations of known VvmiRNAs/VvmiRNAs* and their Edit types were detected in our datasets (Fig. 3), which were consistent with our previous work in 'Amur' grape (Wang et al. 2012). Identification of VvmiRNA SNPs' traits will contribute to the recognition of the evolution of VvmiRNAs and their comprehensive roles in the stone-hardening stage of grape berries. Among 161 VvmiRNAs/VvmiRNAs*, the SNPs and corresponding Edit types of 71 VvmiRNAs were identified, while the remaining 90 VvmiRNAs had no variation in their mature sequences (Table 3; Supplementary Table S5). Moreover, several VvmiRNA families exhibited high SNPs amongst their members. For example, VvmiR166, VvmiR156 and VvmiR167 families exhibited the much high SNPs amongst their members. In contrast, VvmiR169 family possessed the far low SNP in only eight members (Table 3). This finding suggests the divergence of conservation in the sequences among various VvmiRNA families (Table 3; Fig. 3).

Interestingly, it was observed that diverse VvmiRNA families had various SNP variations in their Edit types and numbers (Fig. 3). To depict this phenomenon clearly, all VvmiRNA families with SNPs were further classified into several groups (Fig. 3). Group I, most members of each VvmiRNA family had SNP, and each member with SNP possessed multiple Edit types of SNP. For example, the VvmiR166 family (VvmiR166s) has 8 members and the 282 Edit types of SNV (8, 282), followed by VvmiR156s (9, 118), VvmiR167s (5, 40), VvmiR164s (4, 17) and VvmiR535s (3, 9). From these VvmiRNA families, diverse members with sequence variations obviously exhibited divergent Edit types of SNP. Although the diverse members of one miRNA family with various precursors possessed same mature sequences (such as VvmiR166b/c/d/e/f/g/h, VvmiR156b/c/d, VvmiR156f/g/i, VvmiR167b/c/d/e, VvmiR164a/c/d and VvmiR535a/b/c), they had various Edit types. For instance, VvmiR166b and VvmiR166c/d/e/f/g/h had the same mature sequences, but they possessed 35 and 43 Edit types (35, 43), respectively, resembling VvmiR156b, VvmiR156c and VvmiR156d (10, 10 and 11); VvmiR167b, VvmiR167c, VvmiR167d and VvmiR167e (12, 9, 5 and 12) and VvmiR164a, VvmiR164b, VvmiR164c and VvmiR164d (5, 6 and 5) (Fig. 3). These findings suggested the diversification of the assorted VvmiRNA families in the evolution of the sequences. Group II, another VvmiRNA families only had one member, but it possessed multiple Edit types, such as VvmiR168 (34), VvmiR479 (31), VvmiR3636 (25), VvmiR3623* (19), VvmiR3624 (4), VvmiR3633a (4), VvmiR162 (3), VvmiR3623 (3) and VvmiR2950* (3), implying that the VvmiRNA families with single member exhibited the drastic divergence, and thus might be active factors during VvmiRNA sequence evolution. Group III, some VvmiRNA families, such as VvmiR169b/c/g/h/i/l/r/u, VvmiR160a/b/c/d, VvmiR399a/b/c/d and VvmiR3629 were also revealed only one edit type even though they had multiple members with SNPs, indicating that they might possess relatively high conservation during VvmiRNA sequence evolution process. Final group, the remaining VvmiRNA families had less members and Edit types (Fig. 3). All these results confirmed the diversification of VvmiRNA families in the evolution of their mature sequences.

In addition, we observed the total read number of VvmiRNAs with SNP was up to 77,141,827, and diverse VvmiRNA families and their various members had conspicuous divergence in the number of sequences with SNV, of which VvmiR166 and VvmiR156 families had considerably more reads with SNV compared with other families (Table 3). Generally, the number of SNPs in VvmiRNA families was less than that of the normal sequences. However, the SNP sequences of 21 VvmiRNAs had more read numbers than miRNAs themselves, including VvmiR156a, VvmiR156e, VvmiR156h, VvmiR160a, VvmiR160b, VvmiR169b, VvmiR169f, VvmiR169g, VvmiR169h, VvmiR169r, VvmiR169u, VvmiR171e, VvmiR3629a, VvmiR3629b, VvmiR3629c, VvmiR3631b*, VvmiR396b, VvmiR396c, VvmiR396d, VvmiR399b and VvmiR399c, suggesting that these VvmiRNAs had the stronger evolution than others. Interestingly, compared with homologous VvmiRNAs from grape cv. 'Pinot Noir' in miRBase 21.0 (http://www.mirbase.org/summary.shtml?org=vvi), some VvmiRNAs could not be identified in this work, but their SNP sequences were found, such as VvmiR164b, VvmiR169i and VvmiR828b (Supplementary Table S5; words with bold and italic), implying that SNPs might be one of the reasons for the generation of new members of VvmiRNA family in miRNA evolution.

Functional annotation of VvmiRNA targets during grape stone-hardening stage

To better recognize the roles of VvmiRNA during grape stone-hardening stage, PsRNA Target software (http://plantgrn.noble.org/psRNATarget/result?sessionid=1503987414486479) was utilized to predict the potential miRNA target genes on the basis of our previous RNA-seq data (GEO Accession: GSE77218) using mature miRNA sequences as queries. A total of 2,124 targets for known VvmiRNAs/VvmiRNAs* and 885 targets for novel VvmiRNAs/VvmiRNAs* were predicted in this work. BLAST analysis and GO term annotation were performed using these predicted target gene sequences to improve our understanding of their functions in grape stone-hardening stage (Supplementary Table S6). GO term enrichment analysis annotated approximately 38,530 sequences and classified them into three main categories, namely, biological process, molecular function and cellular component, using Blast2GO. As shown in Fig. 4, metabolic process (GO: 0008152; $P < 0.0011$), cellular process (GO: 0009987; $P < 0.001$), single organism process (GO:0044699; $P < 0.0001$), biological regulation (GO:0065007; $P < 0.0101$), response to stimulus (GO:0050896; $P < 0.0001$), developmental process (GO:0032502; $P < 0.001$) and reproductive process (GO:0022414) were among the top enriched terms in biological process category. In molecular function category, building and catalytic process (GO: 0005488; $P < 0.0101$) were the most enriched terms. With respect to cellular component, cell (GO: 0005623; $P < 0.0001$), cell part (GO:0044464; $P < 0.001$), organelle (GO:0043226; $P < 0.0001$), membrane (GO:0016020; $P < 0.001$) and membrane part (GO:0043227; $P < 0.001$) were most enriched terms (Fig. 4). We generated an overview of the metabolic pathways of the predicted VvmiRNA target genes using KEGG pathway analysis (http://www.genome.jp/kegg/). A total of 73 pathways were identified by 228 targets for VvmiRNAs (Supplementary Table S6), of which plant pathogen interaction (ko04626; $P < 0.0001$), pyrimidine metabolism (ko00240; $P < 0.000$), purine metabolism (ko00230; $P < 0.001$), RNA degradation (ko03018; $P < 0.101$) and plant hormone signal transduction (ko04075; $P < 0.965$) were the most enriched pathways (Fig. 4), indicating their significant roles during grape berry and seed development.

Verification of target genes for novel VvmiRNAs related to berry development at the stone-hardening stage of grape berries

Based on the expression profiles of novel VvmiRNAs shown in Fig. 2C, together with their potential functional annotation, of which 4 novel VvmiRNAs related to berry development of VvmiR8-5p, VvmiR31-3p, VvmiR38-5p and VvmiR53-3p, and their corresponding target genes involved in embryo and seed stone development [VIT_204s0008g03060, DDB1- and CUL4- ASSOCIATED FACTOR HOMOLOG 1 (*VvDCAF1*)], GA signaling [VIT_217s0000g10300, *GIBERELLIN INSENSITIVE* (*VvGAI*)], lignification [VIT_212s0028g03110, *CAFFEOYL-CoA O-METHYLTRANSFERASE* (*VvCCoAOMT*)] and cell wall expansion [VIT_218s0041g02160 (*ESTERASELLIPASE*, *VvGDSL*)] were further selected to verify their roles at the stone-hardening stage of grape berries. Here, the cleavage interactions of these 4 ones on their target genes above at the berries of grape stone-hardening stage were verified by our modified RLM-RACE and developed PPM-RACE procedures (Sun et al. 2012; Wang et al. 2013).

First, from RLM-RACE, the sequencing of the amplified 3'-end products confirmed VvmiRNAs' cleavage sites on their target genes were at the 9^{th}-11^{th}, of which the 10^{th} was their main cleavage site with the most cleavage frequency (Fig. 5), indicating the specificity of cleavage sites of miRNAs, which is consistent with previous studies (Sun et al. 2012; Wang et al. 2013). For example, although both VvmiR8 and VvmiR31 had the two cleavage sites on their corresponding targets VvDCAF1, VvCCoAOMT, the cleavage sites of the former were 9^{th} and 10^{th} (the higher frequency 18/20), those of the latter were 10^{th} (the higher frequency 10/16) and 11^{th}; similarly, VvmiR38-5p on VvGAI1 possessed three cleavage sites of 9^{th}-11^{th}, of them 10^{th} had the most frequency 18/22, whereas VvmiR53-3p on VvGDSL possessed only one cleavage site at 10^{th} with the same high frequency 18/22. Next, our developed PPM-RACE was employed to further confirm the target genes of VvmiR8-5p, VvmiR31-3p, VvmiR38-5p and VvmiR53-3p and their cleavage sites. The sequencing of the amplified 5'-end products identified the same cleavage sites as those of the 3'-end sequencing in the RLM-RACE experiment, but all their 5'- end cleavage frequency detected in PPM-RACE were the lower than their corresponding 3'-end cleavage frequency examined in RLM-RACE (Fig. 5), which might be derived from the fact that the 5'-end cleavage products were more easily degraded than 3'-end cleavage products, similar to the previous reports (Sun et al. 2012; Wang et al. 2013). The consistent results of both the RLM-RACE and PPM-RACE experiments demonstrated that *VvDCAF1*, *VvCCoAOMT*, *VvGAI* and *VvGDSL* are the downstream target genes of VvmiR8-5p, VvmiR31-3p, VvmiR38-5p and VvmiR53-3p, and verified their cleavage interaction mode in the stone-hardening stage of grape berries.

Spatio- temporal expression modes of VvmiRNAs and corresponding target genes during GA-induced grape seedless berry development process

To gain insight into the seed development during grape stone-hardening stage, and GA_3-induced parthenocarpy process, a comparative phenotyping of the berry and seed morphology of GA_3-treated and untreated control (CK) 'Wink' grape cultivar at 5, 20, 45 and 90 days after flowering (DAF) was carried out (Fig. 6A-D). Of them, the berries at 45 days after flowering (DAF) in untreated controls had the full seeds and hardening seed coats (Fig. 1A). GA_3-treated plants exhibited more distinct increase in vertical diameter than horizontal diameter of berry grains compared with untreated CK plants (Fig. 6B). In addition, GA_3-treated plants exhibited 99.6% seedless rate formation relative to untreated CK plants (Fig. 6D). These results confirmed the profound effects of GA_3-induced 'Wink' grape parthenocarpy, and suggested that the complicated regulatory networks might exist during GA-mediated grape berry and seed development.

To further determine the long-term roles of VvmiRNAs and their target genes validated above during GA-induced grape seedless berry development process, the relative expression levels of *VvMIR8-5p*, *VvMIR31-3p*, *VvMIR38-5p* and *VvMIR53-3p* and their corresponding target genes *VvDCAF1*, *VvGAI*, *VvCCoAOMT* and *VvGDSL* were examined in berries at 5DAF, 20DAF, 45DAF and 90DAF, respectively(Fig. 6E). Results showed that except for *VvMIR53-3p* and its target gene, the remaining three *VvMIRNAs* and their target genes exhibited significant expression differences in response to GA treatments relative to untreated control plants (Fig. 6E); while *VvMIR53-3p* and its target gene *VvGDSL* hardly any difference in response to GA treatment than control (Fig. 6E). Notably, the former three miRNAs *VvmiR8-5p*, *VvmiR31-3p* and *VvmiR38-5p* expression were strongly up-regulated by GA treatment (Fig. 6E). Specifically, *VvmiR8-5p* and *VvmiR31-3p* displayed the highest expression level at the grape stone-hardening stage (45DAF) in response to GA treatment relative to other stages (Fig. 6E), indicating that these two miRNAs might play significant roles through responding to GA during the stone-hardening stage of grape berries. In contrast, their corresponding target genes *VvDCAF1*, *VvCCoAOMT* and *VvGAI* expression exhibited strong down-regulation in response to GA treatment (Fig. 6E). And VvmiRNAs and their corresponding target genes above displayed the opposite expression trends during GA-induced grape seedless berry development, supporting that these VvmiRNAs negatively modulated their target gene expression during this process (Fig. 6E).

Interestingly, the expression analysis of *VvGAI*, a key DELLA protein negative interaction factor in GA signal pathway, was significantly down-regulated by GA_3 treatment (Fig. 6E). Similarly, *VvCCoAOMT* and *VvDCFA1*, key genes involved in lignin biosynthesis and embryo development, respectively, were also obviously down-regulated by GA_3 treatment at the grape stone-hardening stage (Fig. 6E). The above results demonstrated that GA might repress grape stone-hardening and embryo development by inducing the expression of *VvmiR31-3p* and *VvmiR8-5p* to negatively regulate the expression levels of *VvCCoAOMT* and *VvDCAF1* as a key molecular mechanism involved in the modulation of GA-induced grape seedless berry development. However, GA exhibited no effect on the expression levels of *VvmiR53-3p* and *VvGDSL* target genes compared with the untreated control (Fig. 6E); while *VvGDSL,* a gene related to cell wall development, exhibited the highest expression level at 20DAF (berry expanding stage), implying that it might participate in the regulation of the cell wall expansion development of young berries.

Dynamic accumulation of cleavage products of target genes for four VvmiRNAs above during GA-induced grape berry development process

Monitoring the accumulation patterns of the cleavage products of these four VvmiRNAs and target genes during GA-induced grape seedless berry development could contribute to determining the spatial-temporal variation of their cleavage roles. Here, the 3'- and 5'-end cleavage products were examined by RLM-RACE and PPM-RACE, respectively (Fig. 7). Results showed similar dynamic accumulation modes of both end cleavage products during different grape berry development (Fig. 7), confirming the dynamic variation of cleavage roles of these VvmiRNAs on their target genes during GA-induced grape seedless berry development process. Also, the accumulation modes of cleavage products resembled the expression modes of the corresponding VvmiRNAs, indicating that miRNAs might be the main factors in their interactions. Interestingly, we revealed that GA evidently promoted the cleavage roles of VvmiR8-5p, VvmiR31-3p and VvmiR38-5p on their corresponding targets, of which except for VvmiR53-3p/VvGDSL pair with nearly no change under GA treatment, the remaining VvmiRNAs were obviously up-regulated accumulation levels of corresponding target cleavage products by GA. Especially, the cleavage products of VvmiR31 and VvmiR8 on their corresponding targets *VvCCoAOMT* and *VvDCFA1* were promoted at the most level at the 45DAF (grape stone hardening stage), which might be due to the fact these two targets' potential functions are correlated to the embryo and stone development. Unlike these, those of VvmiR38-5p on *VvGAI1* were boosted drastically by GA at all stages of GA-induced seedless berries used in this work. These results indicated GA might involve in manipulating grape seedless berry development mainly through boosting the cleavage role of VvmiR38-5p on *VvGAI* at all stages here, while GA might repress grape stone hardening and embryo development by inducing the expression of VvmiR31-3p and VvmiR8-5p to negatively regulate expressions of lignin biosynthesis genes VvCCoAOMT and VvDCAF1, as a key molecular mechanism involve in modulation of GA-induced grape seedless berry development.

Discussion

Grape stone-hardening stage is a critical stage for seed embryo development (Ren et al. 2014), which plays significant roles in seed protection and dispersal. However, when grape inflorescences are treated with GA at ten days before anthesis, the seed embryo development and seed coat formation inhibited, leading to seedless berry formation (Wang et al. 2018). Therefore, GA is recognized as a key phytohormone regulator for seed embryo development and stone-hardening in grape. Previous studies showed that several VvmiRNAs were differentially expressed during grape berry development in response to GA application (Ren et al. 2014, Han et al. 2014, Wang et al. 2017, Wang et al. 2018). However, stone-hardening stage-specific VvmiRNAs and their regulatory networks during grape berry development remain imperative. Therefore, in the present work, we identified and characterized stone-hardening stage-specific miRNAs and their mRNA target genes in response to GA, to gain in-depth insights into the molecular basis of seedless berry development for the molecular breeding of novel seedless grape varieties.

VvmiRNA-mediated TFs and methylation-/acylation-related genes in grape during seed coat-hardening stage

Based on our RNA-seq data, many stone-hardening stage-specific VvmiRNAs and their target genes were predicted (Fig. 8). Among these VvmiRNAs, several VvmiRNA-mediated target genes were belong to different TF families, including SPBs, SCLs, GRAS, HB-Zips, MADS-boxes, AP2, MYBs, ARFs, NACs, ERFs, bZIPs, bHLHs, GATA, PHD, RFPs, WER, TCPs and WRKYs (Fig. 8). These TFs are well-known important regulators for plant growth and development (Zhang et al. 2010). For example, tomato AP2, bHLHs, HB-zips, ARFs and SPBs were involved in the fruit set development, starting from the fruit set initiation to burst color stage (Zhang et al. 2010). Similarly MADS-box, MYB, SPL, HB-zip, ERF, MYB, NAC, WRKYs, TCP, bZIP and GRAS TFs were also reported to play important roles in tomato fruit development and ripening process (Zhang et al. 2010). In a recent study by Xue et al. (2017), *WRKY16*, *WRKY56*, *WRKY31*, *MYB30* and *MYC* TFs exhibited differential expression pattern during the development and ripening process of hard-seed pomegranate 'Sanbai' (*Punica granatum*) and soft-seed pomegranate 'Tunisia', indicating their potential role in seed development. Several bZIP TFs have been also shown to play prominent roles during seed development. For instance, ABI5 (bZIPs) affects ABA sensitivity and controls the expression of some *LEA* genes in seeds,

whereas bZIP10 and bZIP25, which are homologous to maize OPAQUE2, also play signficiant roles during seed maturation (Santos-Mendoza et al. 2008). BpMADS12 promotes lignin accumulation through the up-regulation of key enzymes, namely, BpDWF4, BpDWF5, BpBR6OX, BpROT3 and BpDET2, in response to brassinosteroid signaling in *Betula platyphylla* (Li et al. 2016). All these findings supported the view that these VvmiRNA-mediated TFs might participate in the regulatory network of seed development and seed coat-hardening formation in grape.

In addition to the regulation of transcription levels, miRNA-mediated methylation/acylation-related genes might also participate in the modulation of grape berry development. We observed that VvmiRNAs targeted some genes related to methylation/acylation during seed coat-hardening stage in grape berries (Fig. 8), indicating that VvmiRNAs-induced methylation/acylation is important process for the development of seed coat-hardening in grape. As shown in Fig. 8, VvmiR28, VvmiR31, VvmiR40, VvmiR56, VvmiR166, VvmiR2950, VvmiR394a/b/c, VvmiR3633a/b, VvmiR396c/d and VvmiR399e predicted the target genes related to methylation/acylation, such as *VvASMDMT*, *VvDNMT*, *VvEHMT*, *VvMT*, *VvCCoAOMT*, *VvPAT*, *VvAcyl* and *VvLSD*. Interestingly, previous studies also revealed that DNA methylation plays important roles in modulating fruit development and ripening process (Xu et al. 2015, Gallusci et al. 2016). However, there are limited information about miRNA-mediated methylation/acylation; and hence, the present work points to a novel direction for gaining in-depth insights into fruit development and ripening process.

VvmiRNA-targeted genes involved in hormone signal pathways in grape berry at stone-hardening stage

GA and AUX play essential roles in grape berry growth and seed development (Ren et al. 2014, Silva et al. 2017, Wang et al. 2018). In the present study, we revealed that among our identified VvmiRNAs in grape berries at the stone-hardening stage, several VvmiRNA-mediated target genes were involved in hormone metabolism and signal transduction on the basis of functional annotations (Fig.8). For instance, *VvGA3ox* is the potential target of VvmiR3633, which is involved in the GA biosynthesis pathway, while *VvGID*, the receptor of GA signal, was identified as the predicted target for VvmiR396, which plays an important role in the GA signal transduction pathway (Fig. 8). Likewise, *VvDELLA* was identified as the potential target gene for VvmiR477, whereas *VvGAMYB* is the potential target gene for VvmiR159 and VvmiR319, and both *VvDELLA* and *VvGAMYB* genes are key interaction factors of the GA signal transduction pathway. GA treatment induced the expression level of miR159, leading to a reduction in the expression of *GAMYB* level, which resulted in delayed flowering, perturbed anther development and promoted parthenocarpy, and subsequently seedless fruit formation in *Arabidopsis*, tomato and grape plants (Liu et al. 2009, Wang et al. 2018). Likewise, GA treatment down-regulated the expression levels of miR319 and miR166 during the modulation of *Arabidopsis* and tomato plant development (Liu et al. 2009, Wu et al. 2006). In addition, our findings indicated that *VvARFs* are potential target genes for VvmiR160s, suggesting that VvmiRNAs might mediate auxin signal transduction to regulate grape berry and seed development at the stonet-hardening stage (Zhang et al. 2019). Our results are in line with previous studies reported that both miR160 and miR167 have important regulatory roles in female and male reproduction and parthenocarpy process of *Arabidopsis* plants (Liu et al. 2009, Wu et al. 2006). Besides this, *VvAP2* and *VvERF*, ethylene signaling-related genes, were identified as target genes for VvmiR172 and VvmiR3629 (Fig. 8). Also, our study also identified several VvmiRNAs that mediate the cleavage of ACC oxidase, a key enzyme involved in ethylene biosynthesis. Based on the above findings, we concluded that VvmiRNA-mediated phytohormone signaling as essential steps during early seed development in grape. Our conclusion was also supported by Curbara et al. (2012), who reported that ARFs and TIR1, an auxin receptors, were negatively regulated by miR160, miR167, miR390 and miR393; whereas ABI3, an ABA insensitive gene, was repressed by miR516 during the early seed development of barley. Similar reports were also found in the fruit development of pear, tomato, malus, banana and other plants (Moxon et al. 2008, Bi et al. 2015, Han et al. 2014, Wu et al. 2014, Zeng et al. 2015). All these findings implied that VvmiRNAs might negatively regulate several hormone signals-related genes during the modulation of grape berry and seed development at stone-hardening stage.

VvmiRNA-mediated regulatory networks in berry and seed development at the grape stone-hardening stage

The characterization of the potential target genes of VvmiRNAs at the grape stone-hardening stage is the key step for elucidating the miRNA-mediated regulatory networks associated with grape berry and seed development. Recently, several miRNAs and corresponding target genes have been reported during fruit developments in tomato (Pilcher et al. 2007), eggplant (Din et al. 2014), pear (Wu et al. 2014), grape (Carra et al. 2009, Wang et al. 2012), citrus (Song et al. 2009, Xu et al. 2010), banana (Bi et al. 2014), melon (Gonzalez-Ibeas et al. 2011), apple (Xia et al. 2012) and strawberry (Xu et al. 2013, Ge et al. 2012). In this study, lots of key berry development-related genes involved in sugar, acid, pigmentation and hormone metabolism, lignin synthesis and methylation/acylation process in grape berries and seeds were identified as the target genes for some known and novel VvmiRNAs (Fig. 8). On the basis of the identification and characterization of VvmiRNAs and their corresponding target genes in grape berries at the stone-hardening stage, a putative schematic mode of VvmiRNA-mediated berry and seed development was proposed (Fig. 8). From Fig. 8, it was revealed that VvmiR8, VvmiR27-3p, VvmiR28, VvmiR55, VvmiR56, VvmiR64, VvmiR156a, VvmiR166a-h, VvmiR171a/c/d, VvmiR319e, VvmiR3633a, VvmiR390, and VvmiR396c/d might be involved in the development of grape seeds and embryos by targeting their downstream target genes. Moreover, other 19 VvmiRNAs, including VvmiR53-5p, VvmiR156b, VvmiR164a/c, VvmiR166a-h, VvmiR3633b, VvmiR396c/d, VvmiR397a and VvmiR399b/c, might participate in modulation of lignin biosynthesis of grape stone. For instance, VvmiR397a, might negatively regulate five *LAC* genes involved in lignin biosynthesis. Also, 12 VvmiRNAs identified in this study, including VvmiR19 and VvmiR44-3p, might participated in seed and stone development, by targeting the genes related to proline metabolism (Fig. 8). Likewise, 10 VvmiRNAs, including VvmiR13-5p, VvmiR27-5p, VvmiR37-3p, VvmiR56, VvmiR63, VvmiR2950, VvmiR396c and d and VvmiR477b were found to be involved in the modulation of sugar metabolism. Our findings are in line with previous reports, demonstrating that miRNA modulate several target genes involved in lignin, sugars and hormonal signaling pathway in different plants during seed development, in which Zeng (Zeng et al. 2015) reported miR156 and miR164 regulated sugar metabolism in *Lycium barbarum* during the fruit development; While miR397a, miR1132, miR5077 and miR396b were involved in lignin, sugar and acid pathways during pear fruit development, especially, 27 *LAC* target genes of miR397a have been reported to be participated in the lignin synthesis of pear fruits . Whereas, in *Arabidopsis* (Abdel-Ghany and Pilon, 2008, Yamasaki et al. 2009) and poplar plants (Lu et al. 2013), 3 and 29 *LAC* genes, respectively were targeted by corresponding miR397. In tomatoes, miR156, miR166, miR167, miR168, miR393, miR172 and miR396 were preferentially/highly expressed during embryo development, whilst miR164 was mainly expressed in seeds (Bai et al. 2017). The outcomes of this study

provided insights into the miRNA-mediated seed and stone regulatory networks in grapes (Fig. 8), which could contribute to understand the molecular regulatory mechanism during grape berry development at the global transcriptome-wide level.

Regulatory modes of VvmiRNAs-mediated seedless berries during GA-induced grape parthnocarpy process

GA is one of key hormones inducing parthenocarpy to produce seedless fruit, and the change of the GA signaling pathway could induce parthenocarpy fruit set and fruit development, while DELLA protein is the key repressor of GA signal transduction, and the reduction of DELLA protein activity could lead to release GA signal and to appear the corresponding phenotypes like parthenocarpy (Joldersma and Liu, 2018). Nowadays, exogenous GA was widely used in inducing grape parthnocarpy, and recent studies showed that GA signaling induced the expression level of *miR159* and *miR160* to regulate grape parthnocarpy producing seedless berries (Wang et al. 2018; Zhang et al. 2019). With the development of research, more and more miRNAs were identified to involve in modulation of GA-mediated parthenocarpy, however, it is unclear what the regulatory network involved in miRNAs -GA signaling Here, we revealed that GA might negatively regulate expressions of lignin biosynthesis enzyme gene *VvCCoAOMT* and embryo developmental gene *VvDCAF1* to produce seedless berries through up-regulating of VvmiR31-3p and VvmiR8-5p, which provides further gaining insight into the regulatory mechanism of GA-induced grape seedless berries. But we also observed an odd phenomenon that GA obviously induced the expressions of *VvmiR38-5p* to strongly repress those of *VvGAI* (DELLA family member) at the transcription levels during GA-induced grape parthenocarpy, which contradicted the previous study that GA usually up-regulates *DELLA* transcription level, but decreases its protein activity after GA treatment (Chen et al. 2014). This contradiction might derive from the reason that in this work, we detected the expression of *VvGAI* at the berries of 5, 20, 45 and 90 DAF, that is 12, 27, 52 and 97 days after GA treatments (Fig. 6A), which might reflect the long or cascaded effects of GA in the expression of *VvGAI*, while the previous study examined the short effect of GA on *DELLA* genes within the short time after GA treatment.

Material And Methods

Plant materials and GA treatment

Five-year-old ‘Wink’ grape cultivar grown under common field conditions of conventional fertilization, irrigation and regular pruning management at Jiangpu Farm of Nanjing Agricultural University, Nanjing City, Jiangsu Province, China, was used in this study in 2019. First, the grape berries at 45DAF stage were collected to use in high throughput sequencing. Second, based on our preliminary trails, the total 18 inflorescence clusters with similar growth status from 6 grape plants were selected as materials, of which the 9 clusters were dipped into 50 mg/L GA_3 for 30s at 10 days before flowering to induce grape seedless berries. The other remaining 9 clusters were treated with water and used as control set. In the early morning (9 to10 AM), 3-4 grains from the middle of each cluster of GA_3-treated and water-treated control plants at different time points [5 days after flowering (5DAF), 20DAF, 45DAF and 90DAF] were collected and immediately frozen in liquid nitrogen and stored at −80°C until use. Each type of samples consisted of three biological replicates.

Small RNA (sRNA) library construction and sequencing

Total RNA was extracted from 200ug tissues of grape berries at 45DAF stage using our modified CTAB method for construction of small RNA library used in high throughput sequencing (Wang et al. 2011a). Library was prepared with 1 ug total RNA for each sample. Total RNA was purified by electrophoretic separation on a 15% urea denaturing polyacrylamide gel electrophoresis (PAGE) gel and small RNA regions corresponding to the 18-30 nt bands in the marker lane (14-30 ssRNA Ladder Marker, TAKARA) were excised and recovered. Then the 18-30 nt small RNAs were ligated to adenylated 3’ adapters annealed to unique molecular identifiers (UMI), followed by the ligation of 5’ adapters. The adapter-ligated small RNAs were subsequently transcribed into cDNA by SuperScript II Reverse Transcriptase (Invitrogen, USA) and then several rounds of PCR amplification with PCR Primer Cocktail and PCR Mix were performed to enrich the cDNA fragments. The PCR products were selected by agarose gel electrophoresis with target fragments 110~130 bp, and then purified by QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA). The library was quality and quantitated in two methods: check the distribution of the fragments size using the Agilent 2100 bioanalyzer, and quantify the library using real-time quantitative PCR (qPCR) (TaqMan Probe). The final ligation PCR products were sequenced using the BGISEQ-500 platform (BGI-Shenzhen, China).

Bioinformatics analysis and identification of VvmiRNA and VvmiRNA SNPs

The clean reads were screened from raw data by filtering out the corrupted adapter sequences, poly-A tails and sequences with ≤18 nt and ≥30 nt. The clean read sequences were mapped into the Rfam (https://rfam.xfam.org) to filter the rRNA, tRNA, snRNA and snoRNA etc. The filtered reads were then compared against known plant miRNA database existing in the miRBase 21.0 with BLASTn. Only matching (0-3 mismatches) sequences in their sequences’ ends were considered as known VvmiRNAs, while other sequences have one base variation with the known VvmiRNAs in the middle sites of their sequences and thus can be considered as miRNA SNV. On the other hand, the identification criteria of novel miRNA as follow: 1) Except for the identified known VvmiRNAs and VvmiRNA SNVs, the remaining sequences were mapped to the grape reference genome (http://genomes.cribi.unipd.it/DATA/V2/) and mRNA sequence. The reads mapped to genome but mRNA were used to predict the potential miRNA precursor with mireap, and then these reads werre processed by miRCat (http://srna-tools.cmp.uea.ac.uk/; Moxon et al. 2008; Wang et al. 2012) using default parameters to generate the secondary structures; 2) The negative free energy of folding structure were less than -20kj; 3) The both arms of stem-loop structures contained the bubbles with less than 6 mismatched bases; 4) The first base of miRNAs possessed the “U” preference; 5) The length of miRNA is usually in the range of 19 -24nt. In addition, as to the depth coverage and frequency filters for reliable calling of SNVs on miRNAs, here the depth coverage was required to be more than 4 (>4), and the frequency filters was more than 0.05 (>0.05).

Identification of precise sequences of VvmiRNAs by miR-RACE

The cDNA was amplified with miR-Racer 5’ primer, 3’ primer and their corresponding gene-specific primers to generate 5’- and 3’-miR-RACE products, respectively, for the identification of precise sequences of miRNAs (Wang et al. 2011b). The clone products of 5’- and 3’-miR-RACE were approximately 56 and 87 bp, respectively. 3’-miR-RACE was performed using Common primer 1(CP1) (ATTCTAGAGGCCGAGGCGGCCGACATG) and miRNA specific primer 1(MGSP1), while 5’-miR-RACE was performed using CP2 (GGAGCACGAGGACACTGACATGGACT) and MGSP2. The design procedure of MGSP1 and MGSP2 primers as follow: MGSP1primer consists of 10 bp adaptor sequence (GGAGTAGAAA) add 17 bp sequence intercepted from 5’end of miRNA, while MGSP2 primer includes 10bp Poly(T) and 17 bp complementary sequence cut off from 3’end of miRNA (Wang et al.2011a). Here, the 17 nucleotides complimentary to the miRNA were sufficient for the accurate and efficient PCR amplification of the opposite ends. The primer specificity was validated by inspecting the specific band of PCR product. All specific primers are listed in Supplementary Table S7.

Validation of potential target genes for VvmiRNAs with RLM-RACE and PPM-RACE

The mRNA library was ligated with 5’-adapter or 3’-PolyA tail (Wang et al. 2013) and then reverse transcribed as cDNA. RLM-RACE and PPM-RACE were performed with their corresponding cDNA and primers, respectively (Supplementary Table S6; Sun et al. 2012). The products of RLM- or PPM-RACE were mapped into the target genes for validation of potential target genes and identification of cleavage sites and frequency. RLM-RACE was carried out using the common primer 1 (GGAGCACGAGGACACTGACATGGACT) and the specific primers (P1); and PPM-RACE was performed with the common primer 2 (ATTCTAGAGGCCGAGGCGGCCGACATG) and the specific primers (P2). P1 is the reverse primer at the downstream of the predicted cleavage site in target gene, while P2 is the forward primer at the upstream of corresponding cleavage site in target gene. The primer specificity was validated by inspecting the specific band of PCR product. And P1 and P2 were listed in Supplementary Table S8.

qRT-PCR analysis of VvmiRNAs and their target gene*s*

For qRT-PCR expression analysis of VvmiRNAs and their target genes, the cDNA libraries for mRNA and miRNA from control and GA treatment at diverse stages were constructed using our developed methods (Zhang et al. 2019), and then these cDNAs were used as templates to detect their corresponding expression levels by their primers with three replicates (Table 2; Wang et al. 2011b), and the expression levels were normalized using 5.8S rRNA. The relative expression levels of the miRNAs and their targets were calculated by using the $2^{-\Delta\Delta CT}$ method (Wang et al. 2013). miRNA qRT-PCR was amplified using common primer (qP1:ATTCTAGAGGCCGAGGCGGCCGACATG) and specific primer(qP2: miRNA sequence). The primer specificity was validated by inspecting the specific band of PCR product. The U6 gene was used the reference gene for the nomalization of all miRNAs’ relative expression values, and the Actin gene was used as the referenced one for that of all target genes’ relative expression values. All primers listed in Supplementary Table S9.

Declarations

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Author contributions CW conceived the entire of this research. MA and SJ designed the rearch. ZT, PG and XW performed the experiments and analyzed date.WW and ZS drafted the manuscript. HJ and JF revised the paper. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflict of interest.

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Tables

Table 1 Novel miRNAs identified in grape berries of stone hardening stage

Id	location	mfe	Id(5p)	Id(3p)	count(5p)	count(3p)	seq(5p)
VvmiR1	chr10:4660933:4661043:+	-49.4	-	▲	-	15	-
VvmiR2	chr10:16154669:16154760:+	-54	▲	-	58	-	UCAGCGGCUGAGAUAAGCAAA
VvmiR3	chr12:836885:836996:-	-29.4	-	▲	-	6	-
VvmiR4	chr12:1137532:1137662:-	-32.3	-	▲	-	5	-
VvmiR5	chr12:17324634:17324749:-	-27.7	-	▲	-	5	-
VvmiR6	chr12:20076549:20076639:-	-26.3	-	▲	-	20	-
VvmiR7	chr13:20001280:20001472:+	-47.7	-	▲	-	10	-
VvmiR8	chr13:20934176:20934434:-	-64.1	▲	-	8	-	UCCAAGGAUGGAAAAGGCUUC
VvmiR9	chr13_random:2451486:2451802:-	-78.6	-	▲	-	7	-
VvmiR10	chr14:10959011:10959186:+	-20.55	▲	-	1447	-	CUAGAGAUUGUGGAUUAGGCU
VvmiR11*/VvmiR11	chr14:22335449:22335595:+	-70	△	▲	1	12	UUCUCAGCUACUAAUAUCAAG
VvmiR12	chr14:1713283:1713430:-	-46.7	▲	-	47	-	CACGGAAGUGGAGCGGGCGGGCG
VvmiR13/VvmiR13*	chr14:19755471:19755583:-	-57.9	▲	▲	13748	1332	GGAAUGGGCUGAUUGGGAUA
VvmiR14	chr14:22697840:22697962:-	-37.7	▲	-	23	-	UCAGCUGGGUUGGCAUCUGAA
VvmiR15/VvmiR15*	chr14:24560621:24560731:-	-54.7	▲	△	13	3	UCUGAACUCUCUCCCUCAUGGC
VvmiR16	chr15:6122847:6123043:-	-50.53	▲	-	7	-	UCUUUUCUUGAUAGAAGGCCU
VvmiR17	chr16:2126040:2126238:-	-38.7	▲	-	77	-	AUACCAUGUGGAAAAGAGGAAUC
VvmiR18	chr16:3111476:3111566:-	-51.7	-	▲	-	5	-
VvmiR19	chr16:17808410:17808741:-	-79	▲	-	41	-	UGCGGGUGGAAGAGAAGGAAG
VvmiR20	chr16:19208159:19208370:-	-67.7	-	▲	-	11	-
VvmiR21	chr17:4716715:4716853:+	-44.23	-	▲	-	10	-
VvmiR22	chr17:9575775:9575974:+	-57.7	▲	-	5	-	CGACGGCAAGGACACUUUCGU
VvmiR23	chr17:7265156:7265271:-	-43.9	-	▲	-	19	-
VvmiR24*/VvmiR24	chr18:4079210:4079312:+	-71.9	△	▲	2	93	GACAAGUUACAUACAUCCAAG
VvmiR25	chr18:29129189:29129421:+	-68.2	▲	-	17	-	UCCUUCGGCGUCGGCAAAUCC
VvmiR26	chr18_random:4558402:4558602:+	-51.3	-	▲	-	9	-
VvmiR27/VvmiR27*	chr19:607159:607251:+	-21.75	▲	△	22	1	UUUGAUCAGAUAUUGGAUUGC
VvmiR28	chr19:5046231:5046495:+	-67.97	▲	-	17	-	CAGGACUGGCAGUGAUGGUUA
VvmiR29*/VvmiR29	chr19:13510105:13510195:+	-44.7	△	▲	1	8174	UCCCUCAAAGGCUUCCAAUUU
VvmiR30	chr19:18678400:18678570:+	-51.9	▲	-	10199	-	GUUGGAAGUCGGUGGGGGAAC
VvmiR31	chr19:21910338:21910598:+	-54.19	-	▲	-	7	-
VvmiR32	chr19:580958:581064:-	-21	-	▲	-	28	-
VvmiR33	chr19:5446765:5447061:-	-68.9	-	▲	-	8	-
VvmiR34	chr19:18872600:18872761:-	-50	▲	-	1615	-	GUUGGAAGUCGGUGGGGGACC
VvmiR35	chr19:20383158:20383366:-	-66.7	▲	-	10	-	UGCUGAGUCAGUGAUGGUAGG
VvmiR36	chr19:22103176:22103318:-	-35.7	▲	-	11	-	UGGGCUUGUGGAGAAGAAAGUGA
VvmiR37/VvmiR37*	chr1:3865565:3865681:+	-46.2	▲	△	37985	2588	CAUGGGCGGUUUGGUAAGAGG
VvmiR38*/VvmiR38	chr2:1237534:1237664:+	-64.9	△	▲	17	629	ACUCUCCCUCAAGGGCUUCUG
VvmiR39	chr5:6017515:6017763:-	-98.3	▲	-	10	-	CAGCAGUUGCUAUUGUGGUUG
VvmiR40	chr5:19124470:19124728:-	-65.6	-	▲	-	15	-
VvmiR41	chr5:22090345:22090434:-	-37.9	-	▲	-	9	-
VvmiR42	chr5:23402211:23402278:-	-27.1	▲	-	10	-	CUGAACAGAACUGAGGACAGU

VvmiR43*/VvmiR43	chr5:24742118:24742235:-	-45.5	△	▲	13	2793	UUUUGUUGCUGGUCAUCUAGUC
VvmiR44/VvnuR44*	chr6:17896119:17896283:+	-75.22	△	▲	61	38	UGCAUUUGCACCUGCACCUUA
VvmiR45	chr6:777459:777636:-	-30.5	-	▲	-	61	-
VvmiR46	chr6:6489357:6489602:-	-103.01	▲	-	22	-	CACUCCCUCGAGCUCGUCGGC
VvmiR47	chr7:19450050:19450214:+	-49.6	-	▲	-	10	-
VvmiR48	chr7:2818487:2818617:-	-40.23	-	▲	-	6	-
VvmiR49	chr7:3130346:3130460:-	-26.9	-	▲	-	15	-
VvmiR50	chr7:3926329:3926600:-	-92.68	-	▲	-	5	-
VvmiR51	chr7:11137979:11138169:-	-42.6	▲	-	12	-	UCUGACGUAUAUGCUGAUGGA
VvmiR52	chr7_random:1422270:1422383:-	-52.2	▲	-	45	-	UGACAAAGAGAGAGAGCACAC
VvmiR53*/VvmiR53	chr8:2139178:2139403:+	-108.2	△	▲	3	23	GGGUAGUAUGCUGCUGUCUU
VvmiR54	chr8:22308229:22308469:+	-68.25	▲	-	14	-	AUGUAUUUGAGGGAAAGCAAA
VvmiR55	chr8:14593879:14594080:-	-98.9	▲	-	87	-	CCGAGGGAGAGAGCGAGAGGA
VvmiR56	chr8:16138745:16139039:-	-84.2	-	▲	-	8	-
VvmiR57	chr8:18999725:18999889:-	-57.1	▲	-	24	-	UCUGCAUUUGCACCUGCACCU
VvmiR58	chr8:19904186:19904332:-	-42.5	▲	-	11	-	CACAUAUAAUUUUUUCCCGUCA
VvmiR59	chr8:19949160:19949307:-	-45.7	▲	-	11	-	CACAUAUAAUUUUUUCCCGUCA
VvmiR60	chr8:20492988:20493218:-	-60.2	-	▲	-	10	-
VvmiR61	chr8:21905817:21906095:-	-100	▲	-	159	-	CAUCGUCCGAGGCUAUGGCGG
VvmiR62	chr9:406257:406420:+	-73.5	▲	-	9	-	UCUGUUGGGACGUCAUUUGUU
VvmiR63	chr9:1040473:1040614:+	-48.5	▲	-	22	-	CCUUGGCUGUUGGAGAGGAUA
VvmiR64	chr9:11237291:11237395:-	-28.8	▲	-	25	-	UGAUAGAUUAAGGUACCUCAA
VvmiR65	chrUn:11785723:11785836:+	-51.3	▲	-	45	-	UGACAAAGAGAGAGAGCACAC
VvmiR66	chrUn:11820795:11820908:+	-50.1	▲	-	45	-	UGACAAAGAGAGAGAGCACAC
VvmiR67	chrUn:20798908:20799202:+	-69.53	-	▲	-	8	-
VvmiR68	chrUn:10241355:10241468:-	-52.2	▲	-	45	-	UGACAAAGAGAGAGAGCACAC
VvmiR69	chrUn:10268710:10268823:-	-52.2	▲	-	45	-	UGACAAAGAGAGAGAGCACAC
VvmiR70	chrUn:10281947:10282060:-	-51	▲	-	45	-	UGACAAAGAGAGAGAGCACAC
VvmiR71*/VvmiR71	chrUn:16672978:16673068:-	-45.5	△	▲	1	8174	UCCCUCAAAGGCUUCCAAUUU
VvmiR72	chrUn:25396495:25396608:-	-52.2	▲	-	45	-	UGACAAAGAGAGAGAGCACAC

Notes: ▲denotes VvmiRNA; △ represents VvmiRNA*; - indicates no.

Table 2 Comparison of VvmiRNAs by miR-RACE and High throughput sequencing

ID	miR-RACE	High throughput seqencing	Consistence
VvmiR8	UCCAAGGAUGGAAAAGGCUUC	UCCAAGGAUGGAAAAGGCUUC	Yes
VvmiR16	UCUUUUCUUGAUAGAAGGCCU	UCUUUUCUUGAUAGAAGGCCU	Yes
VvmiR31	UUUCUUAGCAACCAAACAGAG	UUUCUUAGCAACCAAACAGAG	Yes
VvmiR38-5p	ACUCUCCCUCAAGGGCUUCUG	ACUCUCCCUCAAGGGCUUCUG	Yes
VvmiR44-3p	AGGUGCAGGUGAAGGUGCAGA	AGGUGCAGGUGAAGGUGCAGA	Yes
VvmiR53-3p	GGCAGCAGCAUACUACUUUG	GGCAGCAGCAUACUACUUUG	Yes

Table 3 List of VvmiRNAs Normal and SNP

miRNA_Name	SNP_Count	Normal_Count	SNP Rate	miRNA_Name	SNP_Count	Normal_Count	SNP Rate
VvmiR156a	10847	65	99.40%	VvmiR169g	27	7	79.41%
VvmiR156b	150	11417	1.30%	VvmiR169h	292	49	85.63%
VvmiR156c	145	10753	1.33%	***VvmiR169i***	292	0	100.00%
VvmiR156d	157	11570	1.34%	VvmiR169l	66	292	18.44%
VvmiR156e	9056	25	99.72%	VvmiR169r	269	57	82.52%
VvmiR156f	359	29990	1.18%	VvmiR169u	291	17	94.48%
VvmiR156g	359	29952	1.18%	VvmiR171e	116	1	99.15%
VvmiR156h	32	1	96.97%	VvmiR172d	14	2747	0.51%
VvmiR156i	359	29952	1.18%	VvmiR2950	8	1773	0.45%
VvmiR159c	27	1335	1.98%	VvmiR2950*	18	3029	0.59%
VvmiR160a	20	7	74.07%	VvmiR319e	63	100	38.65%
VvmiR160b	20	7	74.07%	VvmiR3623	18	4405	0.41%
VvmiR160c	7	17	29.17%	VvmiR3623*	201	14830	1.34%
VvmiR160d	7	21	25.00%	VvmiR3624	313	2652	10.56%
VvmiR160e	7	21	25.00%	VvmiR3629a	36	27	57.14%
VvmiR162	19	4100	0.46%	VvmiR3629b	34	27	55.74%
VvmiR164a	59	3790	1.53%	VvmiR3629c	36	27	57.14%
VvmiR164b	74	0	100.00%	VvmiR3631b*	103	28	78.63%
VvmiR164c	65	3809	1.68%	VvmiR3633a	26	6164	0.42%
VvmiR164d	59	3791	1.53%	VvmiR3635	5	3021	0.17%
VvmiR166a	270	17456	1.52%	VvmiR3636	195	12946	1.48%
VvmiR166b	1683	101565	1.63%	VvmiR3639	9	493	1.79%
VvmiR166c	6036	287126	2.06%	VvmiR396a	708	753	48.46%
VvmiR166d	6040	287317	2.06%	VvmiR396b	1163	1008	53.57%
VvmiR166e	6036	287126	2.06%	VvmiR396c	614	257	70.49%
VvmiR166f	6055	287902	2.06%	VvmiR396d	614	262	70.09%
VvmiR166g	6056	288001	2.06%	VvmiR399a	5	10	33.33%
VvmiR166h	6324	301916	2.05%	VvmiR399b	10	5	66.67%
VvmiR167a	19	341	5.28%	VvmiR399c	10	5	66.67%
VvmiR167b	120	9550	1.24%	VvmiR399h	5	10	33.33%
VvmiR167c	75	6468	1.15%	VvmiR479	465	34737	1.32%
VvmiR167d	56	6285	0.88%	VvmiR535a	17	3427	0.49%
VvmiR167e	120	9501	1.25%	VvmiR535b	17	3427	0.49%
VvmiR168	1070	55816	1.88%	VvmiR535c	17	3427	0.49%
VvmiR169b	292	50	85.38%	***VvmiR828b***	7	0	100.00%
VvmiR169f	27	7	79.41%				

Notes: Words with bold and italic denote the new generated VvmiRNAs

Figures

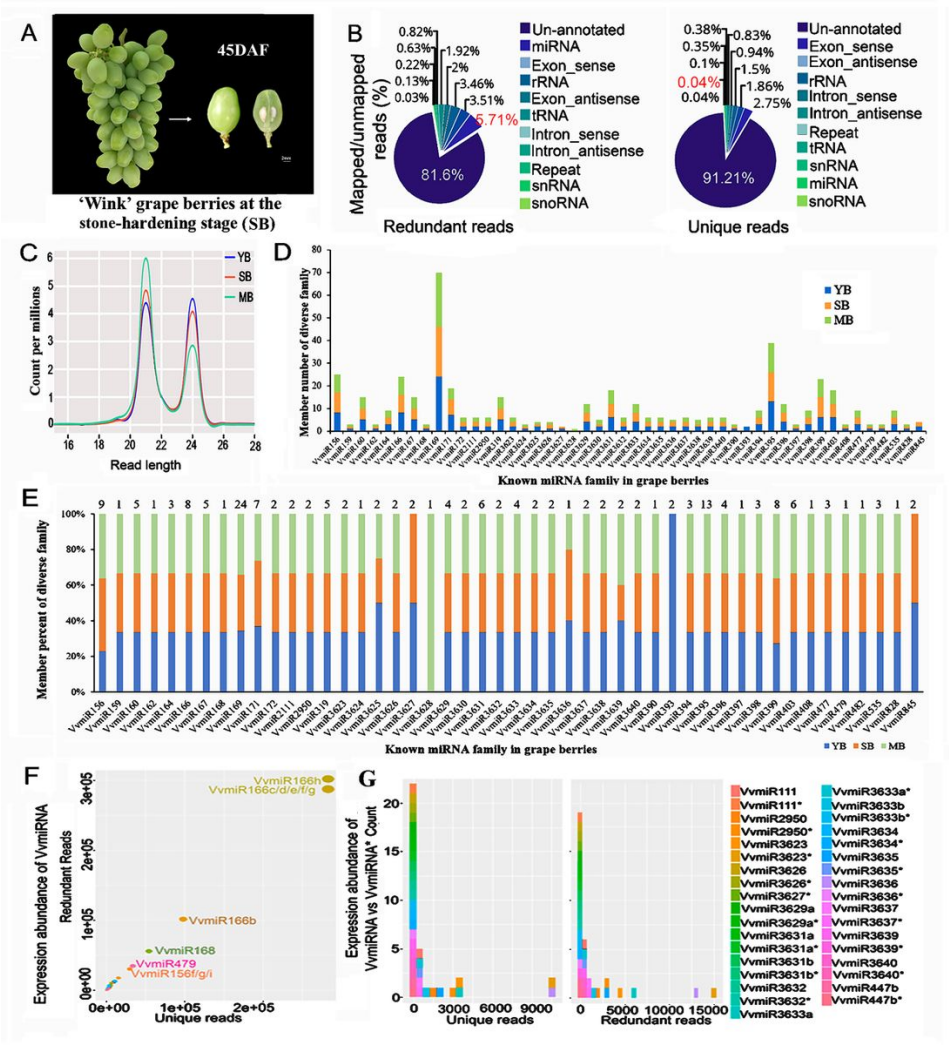

Figure 1

The sRNA dataset of grape berries at 45DAF stage. (A) Morphology of grape berries at the stone-hardening stage (SB). (B) Pie charts of diverse types of sRNAs derived from grape berry library at seed coat-hardening stage based on the redundant and unique reads, respectively. (C) Expression level of miRNA (21 nt) and siRNA (24 nt) reads at different berry developmental stages. (D) Percentage number of VvmiRNA family members at various developmental stages. (E) Number of diverse VvmiRNA family members at different berry developmental stages. (F-G) Expression abundance of (F) redundant vs unique reads in VvmiRNA and (G) VvmiRNA versus VvmiRNA* precursor. Young berries, YB [5 days after anthesis (5DAF)]; seed coat-hardening stage, SB (45DAF); ripening/mature berries, MB (90DAF).

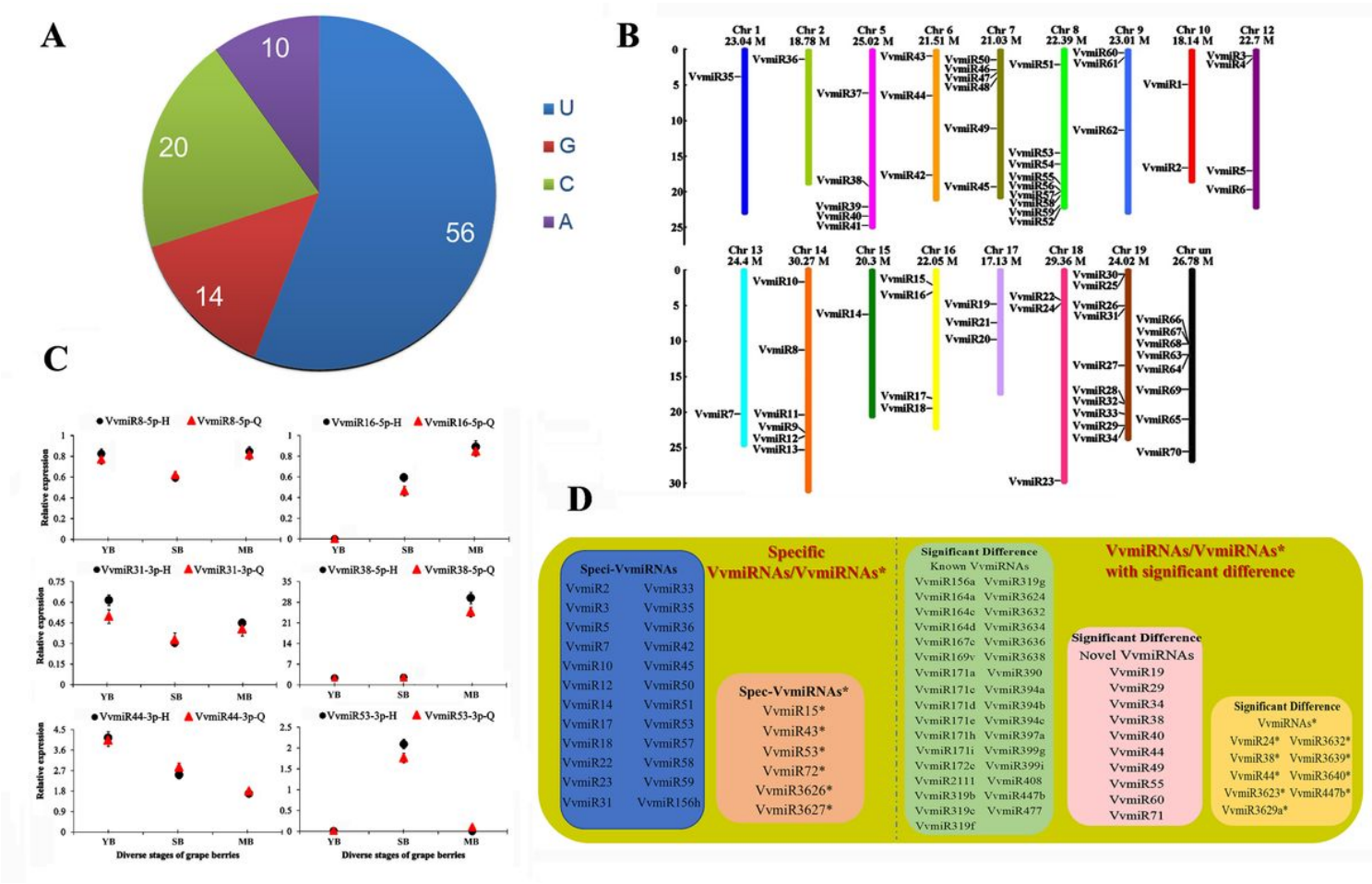

Figure 2

First base preference (A) and distribution in grape chromosomes (B) of VvmiRNAs and comparison of specific and significant VvmiRNAs in grape sRNA library (C).Validation of VvmiRNAs from high throughput sequencing data by qRT-PCR at different grape berry developmental time points [5 days after flowering (5DAF), 45DAF, 90DAF]. Each bar indicated the mean±SE of triplicate assays. H: high throughput sequencing; Q: qRT-PCR; each assay was conducted by using three repeats. There is no significant difference between high throughput sequencing and qRT-PCR results in $P < 0.05$ by Student's t-test. (D). VvmiRNAs with specific expressions at the seed coat-hardening stage, Spec-VvmiRNAs: VvmiRNAs with the absolute log2Fold (YB/SB) or log2Fold (MB/SB) > 1, Significant difference VvmiRNAs.

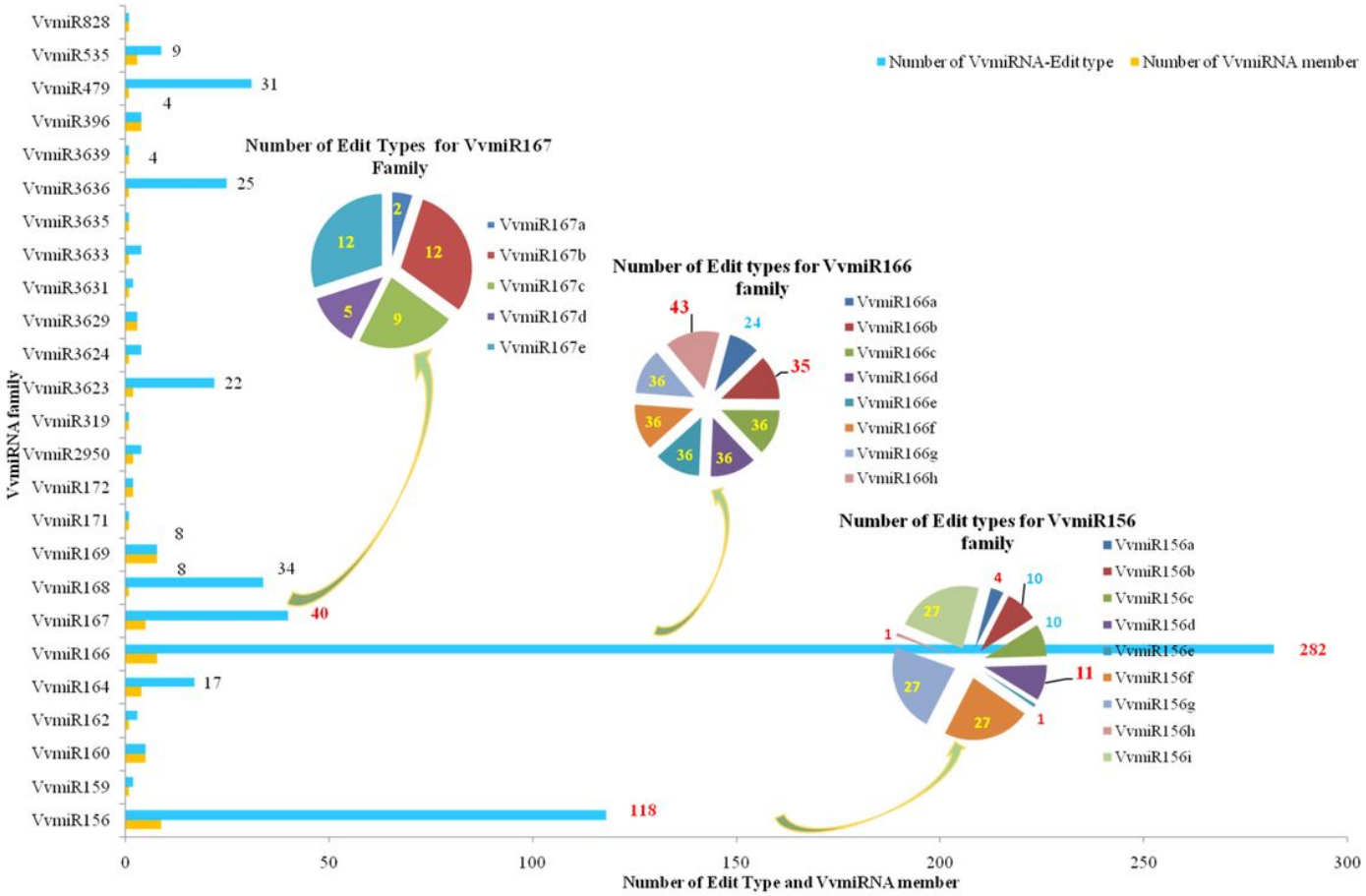

Figure 3

Number of SNP and their Edit types of various members of VvmiRNA families. Number in Pie denotes the number of different SNP edit types of VvmiRNA families.

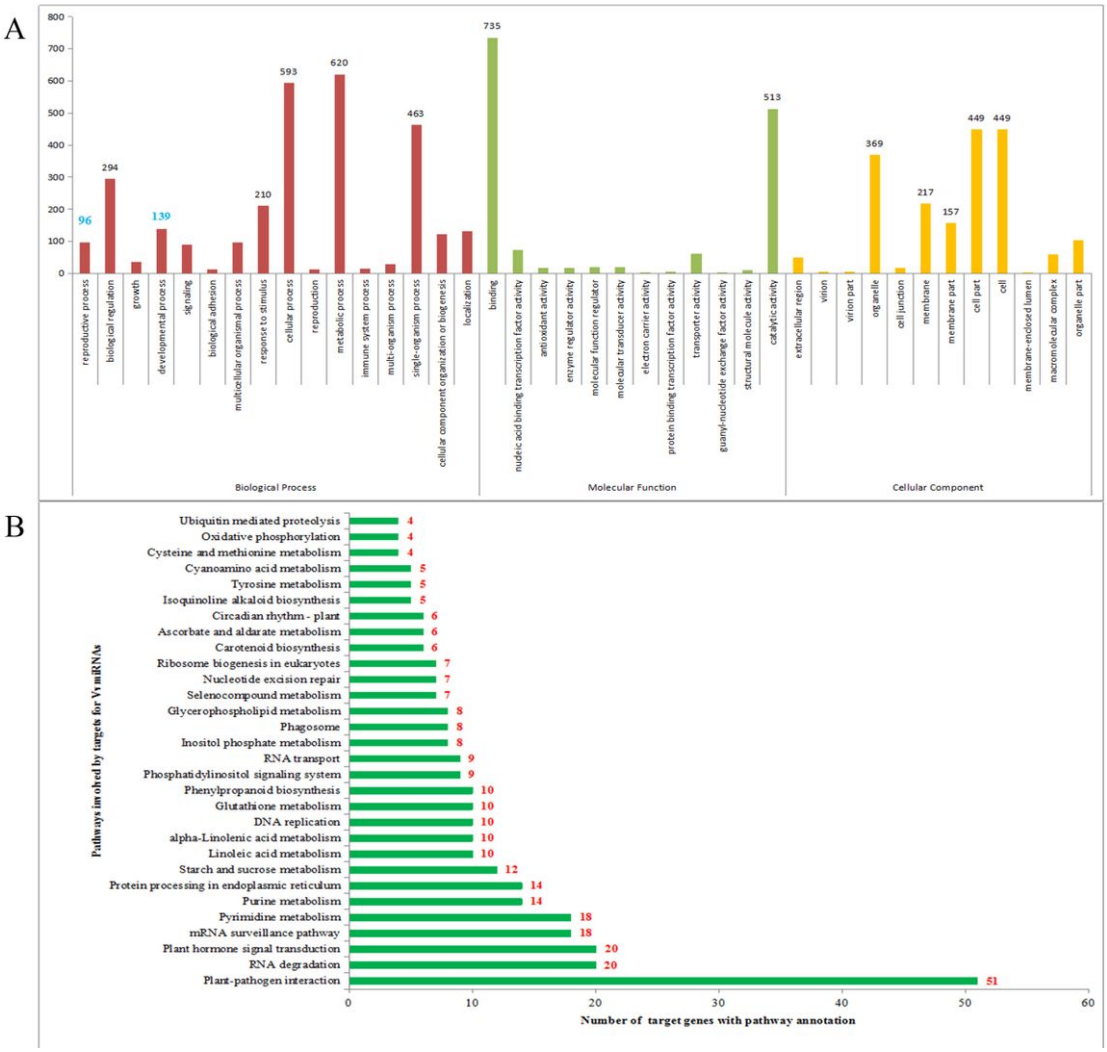

Figure 4

Number of target genes for VvmiRNAs involved in diverse Gene Ontology (GO) and KEGG pathway. (A) Number of VvmiRNAs target genes enriched in biological process, molecular function and cellular component. (B) Top 30 KEGG pathways highly enriched in identified miRNAs target genes.

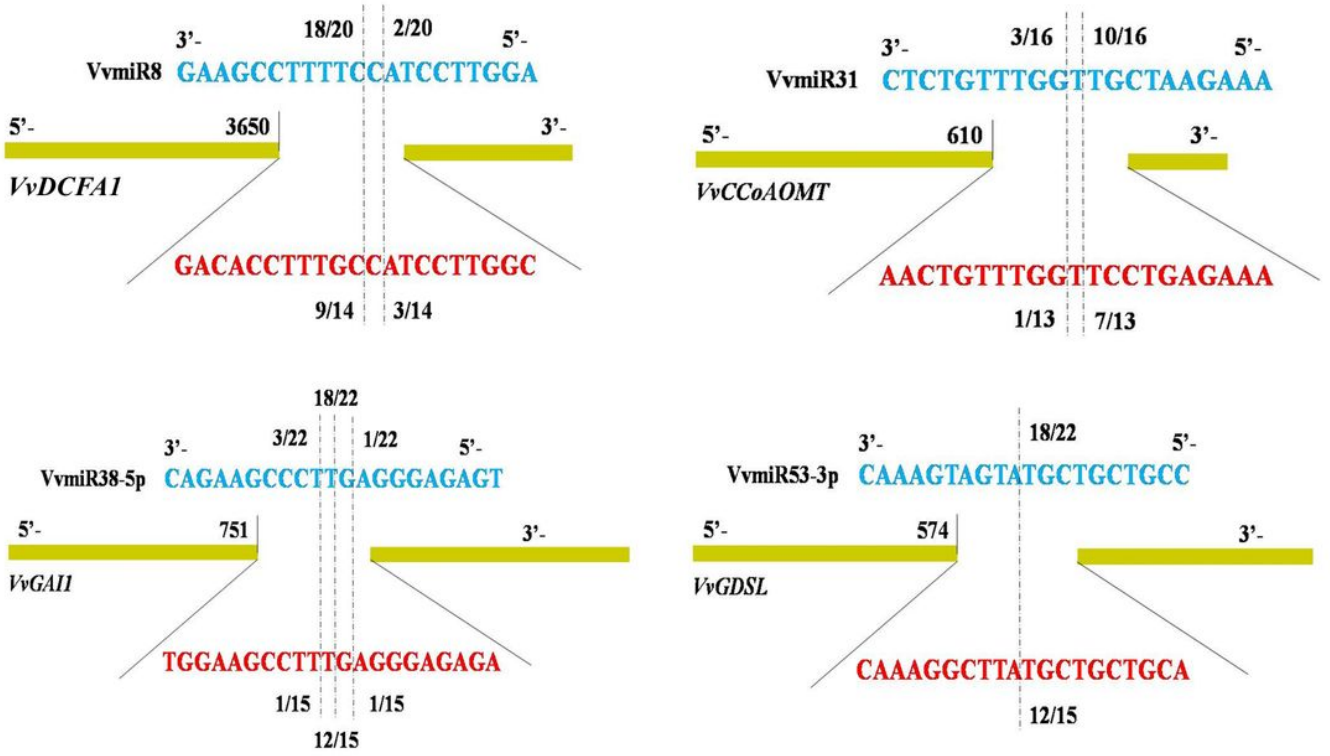

Figure 5

Validation of target genes cleaved by VvmiRNAs using RLM-RACE and PPM-RACE. The gray dotted lines indicate the cleavage sites of VvmiRNAs on target genes identified by 5' and 3'-ends of mRNA fragment using PPM-RACE and RLM-RACE, respectively. The blue and red regions denote the sequences of VvmiRNAs and complementary sequences of VvmiRNAs on their target genes. Number above the blue regions denote the cleavage counts by RLM-RACE, while the number below the red regions indicate the cleavage counts by PPM-RACE. Each assay was repeated the three times.

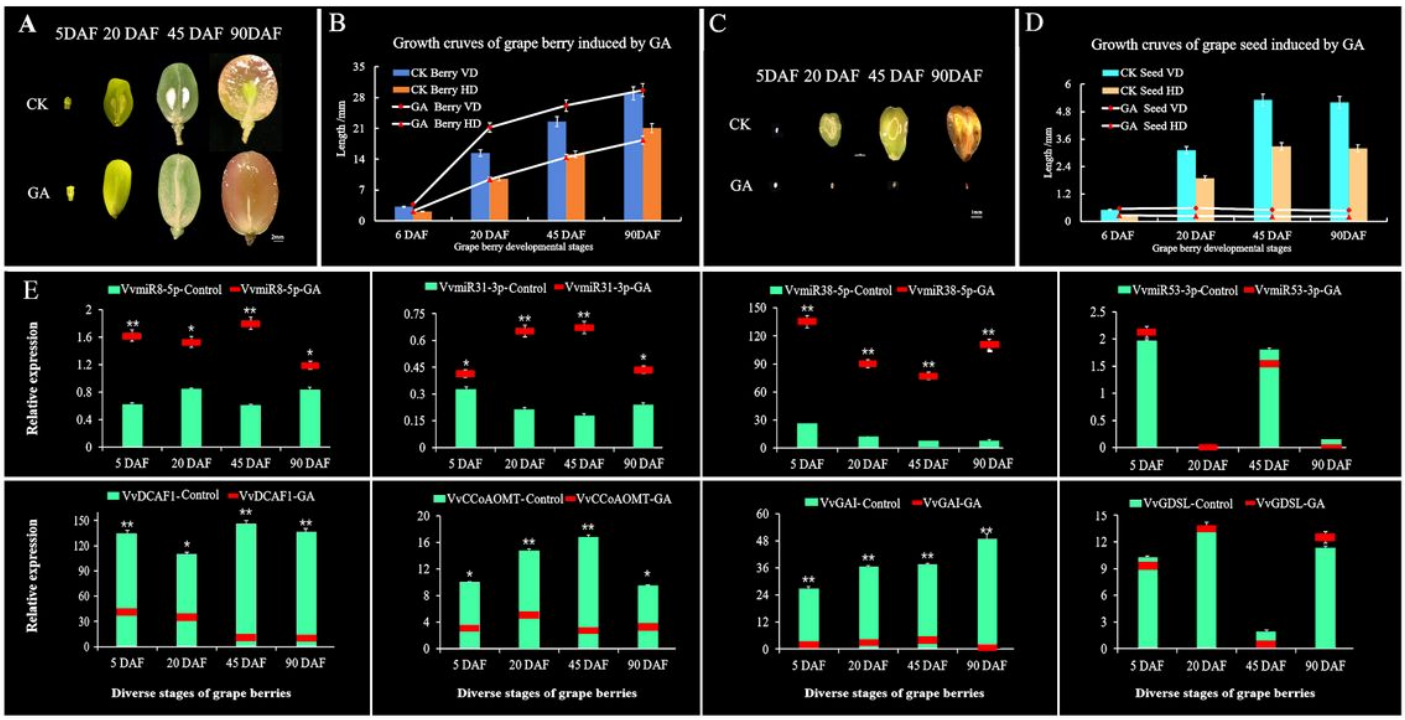

Figure 6

Characterization of grape berries at the seed coat-hardening stage and Gibberellin (GA3)-induced grape parthenocarpic berries. (A-B) Variation in morphologogy and growth curve of grape berries derived from GA3-treated and untreated control (CK) plants. (C-D) Variation in morphologogy and growth curve of seeds derived from GA3-treated and CK control plants. Vertical diameter, VD; horizontal diameter, HD. (E) Expression levels of VvmiRNAs responsive to GA3-treatment during grape berry development [5 days after flowing (5DAF), 20DAF, 45DAF, 90DAF]. The mean and SD values were obtained from three biological samples. ANOVA test was used to identify significant differences, Asterisks indicated statistically significant differences at (*P < 0.05; **P < 0.01) as determined by Student's t-test.

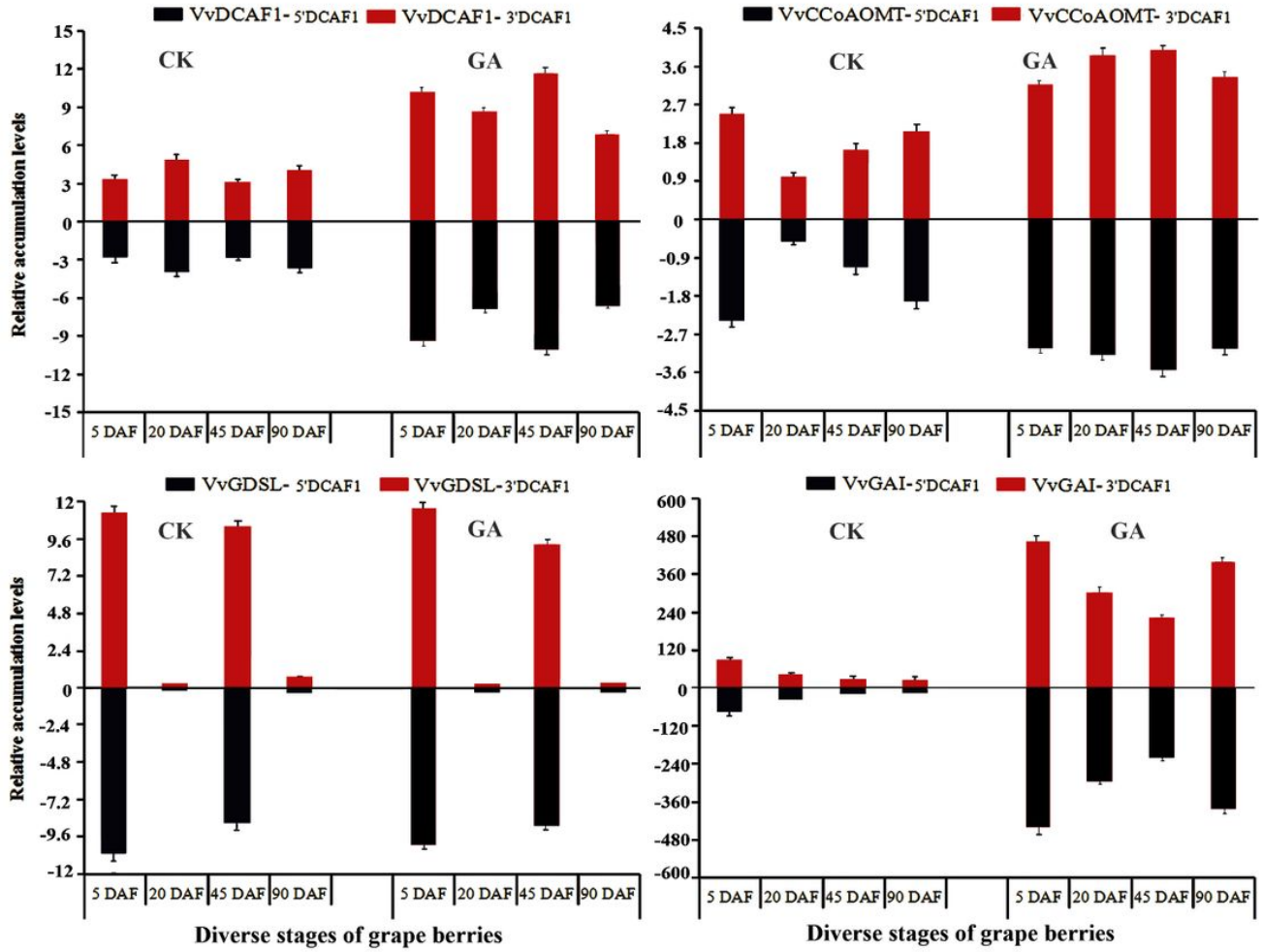

Figure 7

Accumulation levels of 3'-/5'- end cleavage products of VvmiRNAs on their target genes in GA-treated and untreated control (CK) plants at different stages of grape berry development by RLM-RACE and PPM-RACE. 3' DCAF1 denotes 3'- end cleavage products of target genes by VvmiRNAs, wheras 5'DCAF1 indicate 5'-end cleavage products of target genes by VvmiRNAs. Each experiment was repeated three times. ANOVA test was used to identify significant differences, Asterisks indicated statistically significant differences at (*P < 0.05; **P < 0.01) as determined by Student's t-test.

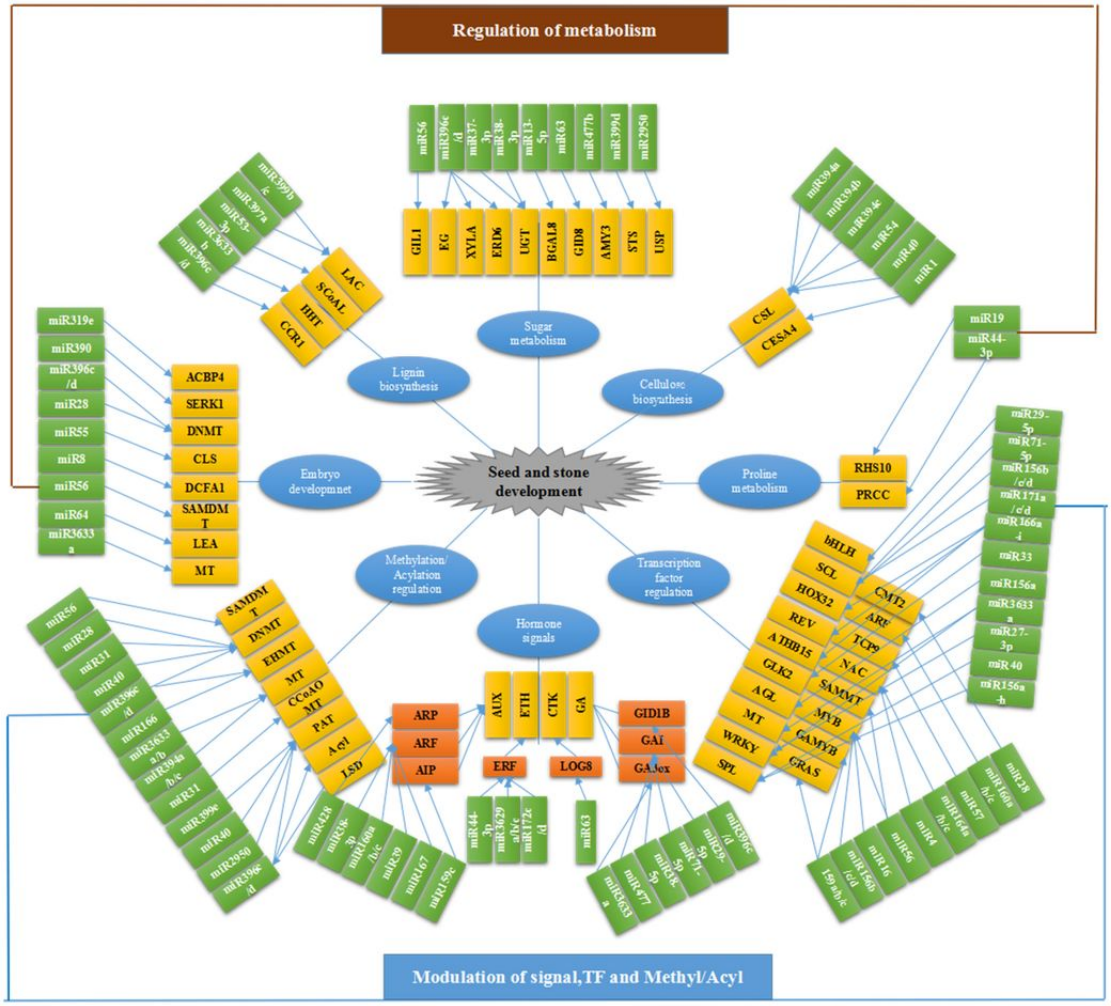

Figure 8

A mode chart of miRNA-mediated regulatory network related to grape berry seed and stone development. Green rectangle represents diverse VvmiRNAs which might regulate seed and seed coat development, whereas yellow rectangle indicates their corresponding target genes. The blue ellipse donates the metabolism pathways and specific tissues of VvmiRNAs and their target gene mediated in grape seed and seed coat development.

Supplementary Files

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

- Supplementarymaterials.docx