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Phylogenetic analysis and target gene prediction of miR477 gene family in grape

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Abstract: To understand the molecular characteristics of the miR477 gene family of grape (*Vvi*-miR477) and to predict its target genes, the *Vvi*-miR477 genes were identified from previous small RNA sequencing data, then phylogenetic analysis and prediction of target gene were conducted. The *Vvi*-miR477 family consists of two precursor sequences and three mature sequences. The miR477 family members were mostly 19-22nt in length. The sequence is relatively conservative. *Vvi*-MIR477a and *Vvi*-MIR477b are located on chromosomes 1 and 2, respectively. These precursor sequences can form the typical stable stem-loop structure. Their minimum folding free energy is −39.10 kcal/mol and −50.90 kcal/mol, respectively. The MIR477 family can be divided into three groups. The prediction of target genes showed that *Vvi*-miR477 targets 26S proteasome, DEAD-box, GRAS family protein, Protein Phosphatase 2C, etc. The GO function of target genes was mainly enriched to six categories. The catabolic process, carboxylic ester hydrolase activity is shown to be high. This study provided a theoretical basis for further exploration of the molecular mechanism of miR477 in grape berry ripening.

Introduction

MicroRNAs (miRNAs) are endogenous, non-coding single-stranded small RNA molecules with about 21–23 nucleotides, which are widely distributed in eukaryotes (Ye *et al.*, 2019). A large number of studies have shown that miRNAs play a crucial role in regulating various metabolic and biological processes, including growth (Ding *et al.*, 2020; Lelandais-Brière *et al.*, 2010), flowering time (Yang *et al.*, 2019), sex determination (Sun, 2012), and phytohormone signaling (Liu and Chen, 2009). For example, miR172 regulates the development of *Brassica napus* flower organs by targeting the *euAP2* gene (Wang *et al.*, 2019). Auxin response factors of miR167 plays an important role in flower development and fertility of rice (Li *et al.*, 2020b). The overexpression of miR319a reduced the transcription level of its targeted gene family TCP, significantly improved the trichome density of transgenic *Populus tomentosa* leaves, and thus relieved the harm of insect herbivores (Fan *et al.*, 2020). In strawberry, overexpression of miR399a could increase the content of soluble sugar, soluble solids, and vitamin C, indicating that the expression of miR399a is positively correlated with the content of sugar (Wang *et al.*, 2017). The regulation of anthocyanin biosynthesis by miR858 has been revealed in grape (Tirumalai *et al.*, 2019), tomato (Jia *et al.*, 2015) and kiwifruit (Li *et al.*, 2020a). By means of base complementary pairing, miRNA cleaves the mRNA or inhibits the translation of target genes to participate in the regulation of plant growth and development (Chipman and Pasquinelli, 2019).

The miR477 family is conserved and present in a number of plant species (Wang *et al.*, 2020). Studies have shown that miR477 plays an important role in the biological processes of plants (Wang *et al.*, 2020). For example, the downregulation of miR477 resulted in the accumulation of lipids and the transformation of nutrients during the fruit ripening process of *Camellia oleifera* (Liu *et al.*, 2019). In cotton, *ghr*-miR477 directly lyses the mRNA of *Gh*CBP60A during the post-transcriptional process. While miR477-CBP60A module participates in the resistance response of plants to *V. dahliae*. The silencing of *ghr*-miR477 and the knockout of *Gh*CBP60A increased the susceptibility and the resistance of plants to *V. dahlia*, respectively (Hu *et al.*, 2020). In addition, miR477 was differently expressed in the roots and leaves of bread wheat under drought stress (Lu *et al.*, 2008) and up-regulated under salt stress in maize (Ding *et al.*, 2009).

Grape is an important fruit crop in the world and has high nutritional value. In China, grapes are mainly used for

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natural consumption, and there are also many processed products, such as wine, raisins, grape juice, grape seeds, etc., Guo *et al.* (2020). Fruit development is a sequential process, which involves fruit set, fruit growth and fruit ripening (Osorio *et al.*, 2013). During fruit development, each step is strictly regulated by complex molecular mechanisms, post-transcriptional regulation by microRNAs is one of the important ways (Farinati *et al.*, 2020). At present, most of our knowledge about the genetic and molecular mechanisms of fruit ripening comes from the model plant, tomato (Gao *et al.*, 2015). The detailed information of grape berry ripening is relatively few.

Previous reports showed that Fengzao is an early-ripening bud mutant of Kyoho, which matures nearly 30 days earlier (Guo and Zhang, 2015). The whole transcriptome characteristics of Kyoho and Fengzao were determined by high-throughput sequencing, which demonstrated that *Vvi*-miR477b-3p was one of the major differentially expressed miRNAs between Kyoho and Fengzao and up-regulated in Fengzao (Guo *et al.*, 2018).

In this study, two precursor sequences and three mature sequences of *Vvi*-miR477 gene family were identified based on the high-throughput sequencing of small RNAs in the previous study (Guo *et al.*, 2018). Sequence alignment, phylogenetic analysis, prediction and GO enrichment analysis of the target genes were performed for miR477 family members. This study provided the bases for future research on the regulatory mechanism of miR477 in grape early ripening process.

Materials and Methods

Acquisition of precursor and mature sequences of miR477 family members

The family members, precursor and mature sequences of *Vvi*-miR477 were identified from previous small RNA sequencing data (Guo *et al.*, 2018) through homologs searches of blast using already known miRNAs in other species according to the method of Xie *et al.* (2010). The precursor and mature sequences of miR477 for the investigated plants were downloaded from the miRBase database (http://www.mirbase.org/index.shtml).

Analysis of the mature sequence and precursor sequence of the miR477 family

The miR477 mature sequences of *Vitis vinifera, Nicotiana tabacum, Malus domestica, Citrus sinensis, Manihot esculenta, Populus trichocarpa, Fragaria vesca, Physcomitrella patens, Lotus japonicus, Prunus persica, Solanum tuberosum, Aquilegia caerulea, Solanum lycopersicum, Asparagus officinalis, Gossypium raimondii, Amborella trichopoda, Carica papaya, Cucumis melo* were subjected to multiple sequence alignment using CLUSTALW online software with the default parameters (https://www.genome.jp/tools-bin/clustalw). Meanwhile, the grape miR477 precursor sequence (*Vvi*-MIR477) and the mature sequence (*Vvi*-miR477) were separately aligned. The phylogenetic tree of miR477 gene family was constructed using the Maximum Likelihood method of MEGA version 7.0.26. Bootstrap confidence values were obtained by applying 1000 replications. Kimura 2-parameter model was chosen as the substitution model. Other parameters were set at their default value. RNAfold (http://rna.tbi.univie.ac.at/) of the ViennaRNA package was used to analyze the secondary structure of the *Vvi*-MIR477. The default values of the software were used.

Gene location of Vvi-MIR477 family member

The localization of *Vvi*-MIR477a and *Vvi*-MIR477b on chromosomes was analyzed by local blast method. MapGene2Chrom V1.0 (http://mg2c.IASK.in/mg2C_v1.0/) online software was employed to map the chromosome location.

Target gene prediction of grape miR477 family members

PsRNATarget (http://plantgrn.noble.org/psRNATarget/) online software was used to predict the target genes of *Vvi*-miR477 family members. The grape transcript from University of Padua CRIBI Genomics V2.1 (http://genomes.cribi.unipd.it) was used as target prediction database. The maximum expectation value was set to 3 and the remaining parameters were as the default values.

Expression of miR477 and functional enrichment analysis of its target genes

A heatmap was drawn using the R pheatmap package to demonstrate the expression of *Vvi*-miR477 family members in Kyoho and Fengzao. The predicted miR477 target genes were then for Gene Ontology (GO) enrichment analysis with ClusterProfiler 3.8.0. GO terms that had a *P*-value less than 0.05 after Bonferroni correction were scored as significant.

Results

Analysis of plant miR477 family mature sequence

Seventy-four mature miR477 sequences belonging to 17 species from the miRBase database were obtained. The number of members of the miR477 family varies greatly among the species. For example, there are 11 miR477 mature sequences for *Manihot esculenta* which is the largest. *Citrus sinensis* and *Physcomitrella patens* have 10 miR477 mature sequences. There are 9 miR477 mature sequences in *Populus trichocarpa*, 7 in *Aquilegia caerulea*, 6 in *Prunus persica*, 4 in *Solanum tuberosum*. There are two miR477 mature sequences in the *Nicotiana tabacum, Malus domestica, Fragaria vesca, Lotus japonicus, Solanum lycopersicum, Asparagus officinalis, Cucumis melo*. There is only one miR477 mature sequence in *Gossypium raimondii*, *Amborella trichopoda*, and *Carica papaya*. Three mature miR477 sequences of *Vitis vinifera* were identified from small RNA sequencing data, including *Vvi*-miR477a, *Vvi*-miR477b-3p and *Vvi*-miR477b-5p (Guo *et al.*, 2018).

The mature sequences of miR477 from investigated plants were compared using CLUSTALW online software. The results showed that the length of most miR477 family members in different species was 20–21 nt, while few of them were 19 nt and 22 nt long (Fig. 1). Members of the same family from different species and different members of the same species have the different sequence length. For example, the sequences of miR477a-3p, miR477a-5p, miR477b-3p, miR477b-5p were different between

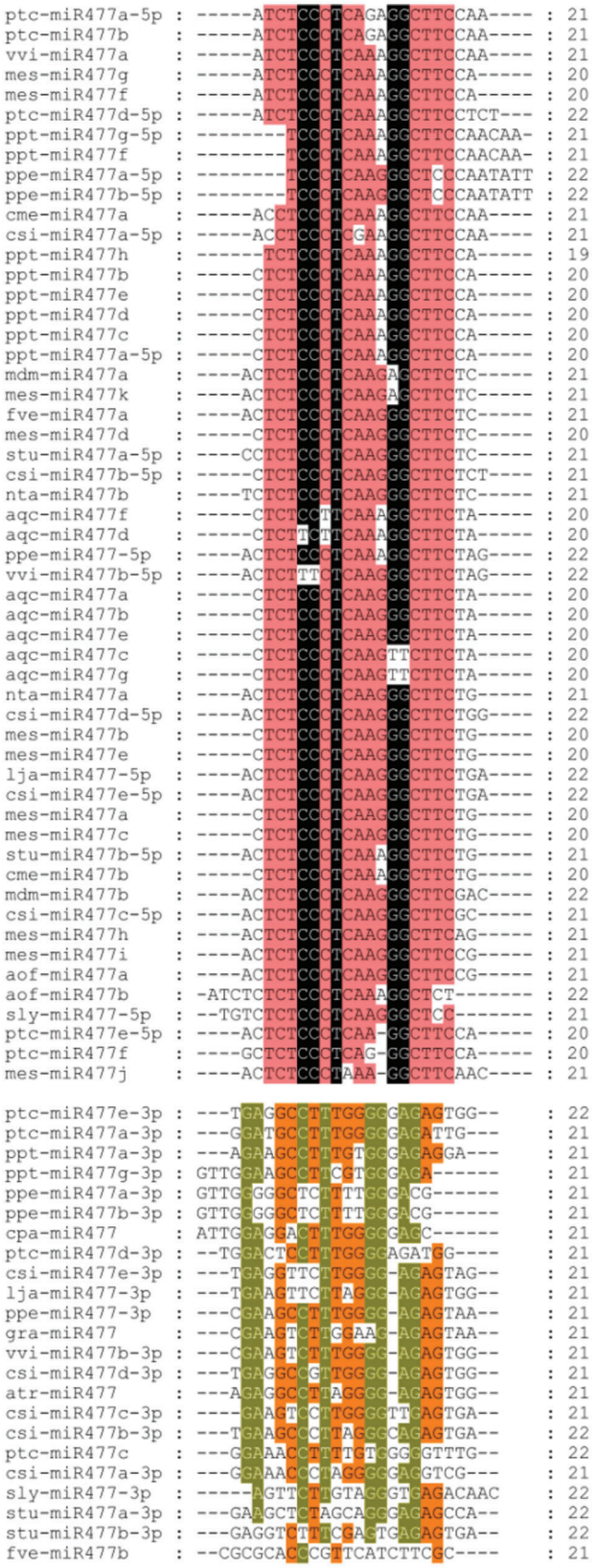

FIGURE 1. Sequence alignment of the mature miR477 from different plants.

Note: ptc, *Populus trichocarpa*; vvi, *Vitis vinifera*; mes, *Manihot esculenta*; ppt, *Physcomitrella patens*; ppe, *Prunus persica*; cme, *Cucumis melo*; csi, *Citrus sinensis*; mdm, *Malus domestica*; fve, *Fragaria vesca*; stu, *Solanum tuberosum*; nta, *Nicotiana tabacum*; aqc, *Aquilegia caerulea*; lja, *Lotus japonicus*; aof, *Asparagus officinalis*; sly, *Solanum lycopersicum*; gra, *Gossypium raimondii*; atr, *Amborella trichopoda*; cpa, *Carica papaya*.

Prunus persica and *Citrus sinensis*. The mature sequences of *fve*-miR477a and *fve*-miR477b were also different even if both of them are from *Fragaria vesca* miR477 family. The mature sequences of three members of the miR477 family of grapes are also not identical (Fig. 2). But their sequence is relatively conservative.

Localization of the Vvi-MIR477 gene family in the genome

The chromosome distribution of the *Vvi*-miR477 precursors is shown in Fig. 3. *Vvi*-MIR477 was unevenly distributed in the 19 chromosomes of grape, which was restricted to chromosomes 1 and 2. The *Vvi*-MIR477a was located on chromosome 1, and *Vvi*-MIR477b was located on chromosome 2.

Sequence analysis and secondary structure prediction of precursors of MIR477 family in grape

Previous studies have shown that the precursor miRNAs of miR477 family members in grape are *Vvi*-MIR477a and *Vvi*-MIR477b. Their lengths are 94 nt and 117 nt, respectively (Fig. 4). There is a 23-nt difference between these two precursors, and the sequences had a certain diversity. The RNAfold Server (http://rna.tbi.univie.ac.at/) in ViennaRNA Web Services is used to predict the secondary structure of the *Vvi*-MIR477 members. The minimum folding free energy (dG) of *Vvi*-MIR477a and *Vvi*-MIR477b is −39.10 kcal/mol, −50.90 kcal/mol, respectively (Fig. 5). It showed the high reliability forecast of miR477 family members. The red part indicated the mature sequence of miR477 (Fig. 5).

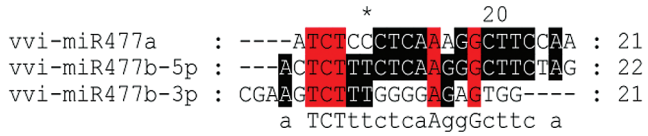

FIGURE 2. Sequence alignment of miR477 mature sequences in grape. Note: Red indicates the highest level of conservatism, followed by black.

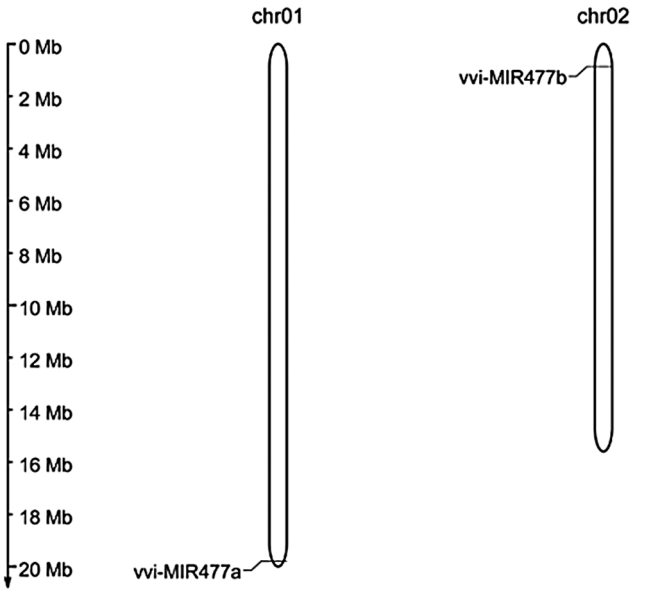

FIGURE 3. Localization of the *Vvi*-miR477 gene family in the grape genome.

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                    *         20          *         40          *
vvi-MIR477a : ----------------GAAGTTGTCAATCTCCCTCAAAGGCTTCCAATT :  33
vvi-MIR477b : GCCAACACGAGGCATTTCTATTGCTCACTCTTTCTCAAGGGCTTCTAGCA :  50
                            A T  TCA TCT  CTCAA GGCTTC A

                   60         *         80         *        100
vvi-MIR477a : TTCCTGG---CTGATTATCAAGGTTGGAAAGTTGGAAGACAGTGGGGGAC :  80
vvi-MIR477b : TTGCCAGTAACAAAATGGCAAGCTCGAAGTCTTTGGGGAGAGTGGACAAA : 100
              TT C  G   C  A T  CAAG T G A   TT G  GA AGTGG   A

                    *
vvi-MIR477a : CTTGGAAGCTTACC--- :  94
vvi-MIR477b : CGTTGTCTCGAATATGC : 117
              C T G   C  A
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FIGURE 4. Sequence alignment of miR477 precursor sequences in grape.
Note: Red indicates the highest level of conservatism, followed by black.

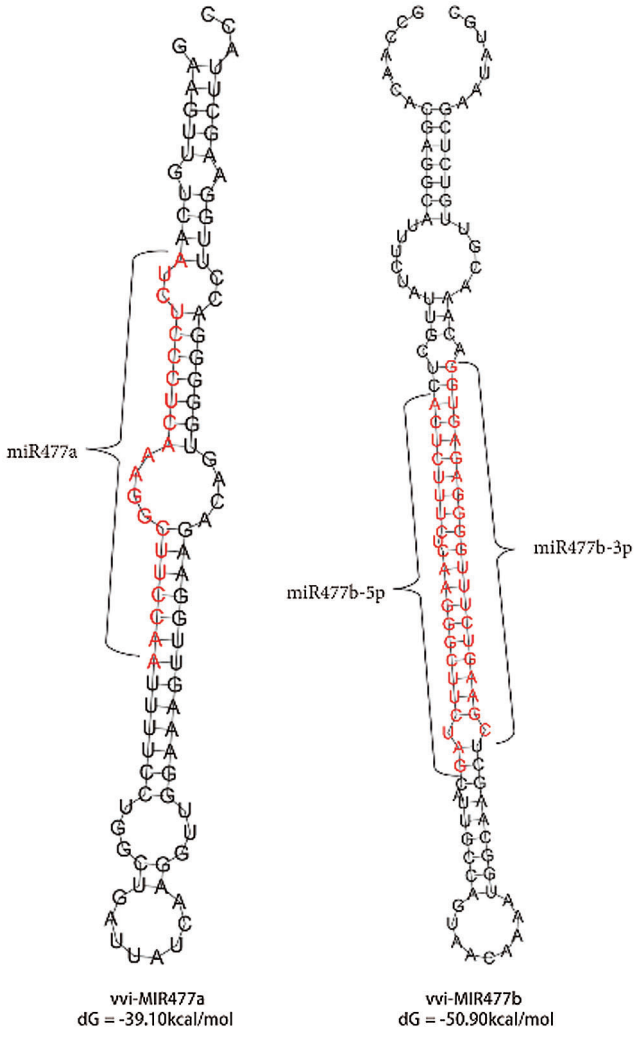

FIGURE 5. The stem-loop structure of *Vvi*-MIR477 genes.
Note: The mature miRNA sequences or putative exons encoding the precursors are in red. The junction of the red and black region are the canonical splice sites.

Phylogenetic tree of the miR477 family

To explore the phylogenetic relationships among members of the MIR477 family. The precursor sequences of MIR477 for the investigated plants from the miRBase database were downloaded. The maximum likelihood method in MEGA Version 7.0.26. Software was used to construct precursor evolutionary trees of MIR477. According to phylogenetic tree analysis, the MIR477 family can be divided into three major groups (Fig. 6). MIR477b from strawberry and MIR477h from cassava are clustered in individual group, and the rest of the precursors were clustered into another group. The MIR477 family of grape were clustered with apple and Populus trichocarpa MIR477. MIR477 members were found to be relatively dispersed among different species, indicating that the phylogenetic relationship of MIR477 family members is less related to species.

Different MIR477 members of the same species clustered into different branches, which also reflected the phylogenetic diversity of the plant MIR477 family. It is speculated that MIR477 genes may evolve at different rates and in different ways, indicating the complexity of plant miRNA precursor evolution.

Prediction of Vvi-miR477 target gene

The target genes of miR477 in grape were predicted with PsRNATarget software. The prediction results showed that different members of miR477 gene family in grape have the same target genes (Table 1). For example, both *Vvi*-miR477a and *Vvi*-miR477b-3p target 26S Proteasome components. Both *Vvi*-miR477a and *Vvi*-miR477b-5p target DEAD-box ATP-dependent RNA helicase. In addition, all *Vvi*-miR477 family members inhibit the transcription of target genes by cleavage. The target genes of different members of the miR477 family are not the same.

Expression of miR477 and functional enrichment analysis of its target genes

In order to specifically reflect the expression levels of *Vvi*-miR477 in Kyoho and Fengzao, a heat map of gene expression at different berry developmental stages were made from the previous data (Guo *et al.*, 2018). As can be seen from the diagram (Fig. 7a), the expression levels of miR477 family members are different at different developmental stages. *Vvi*-miR477b-5p and *Vvi*-miR477b-3p were mostly up-regulated in Fengzao, and miR477b-5p was the highest in FZ3 period.

To reflect a global overview of the regulatory functions of miRNAs, the GO terms of all target genes were analyzed through a GO annotation. Target prediction analysis showed that the target genes regulate a wide range of biological processes, cellular components, and molecular

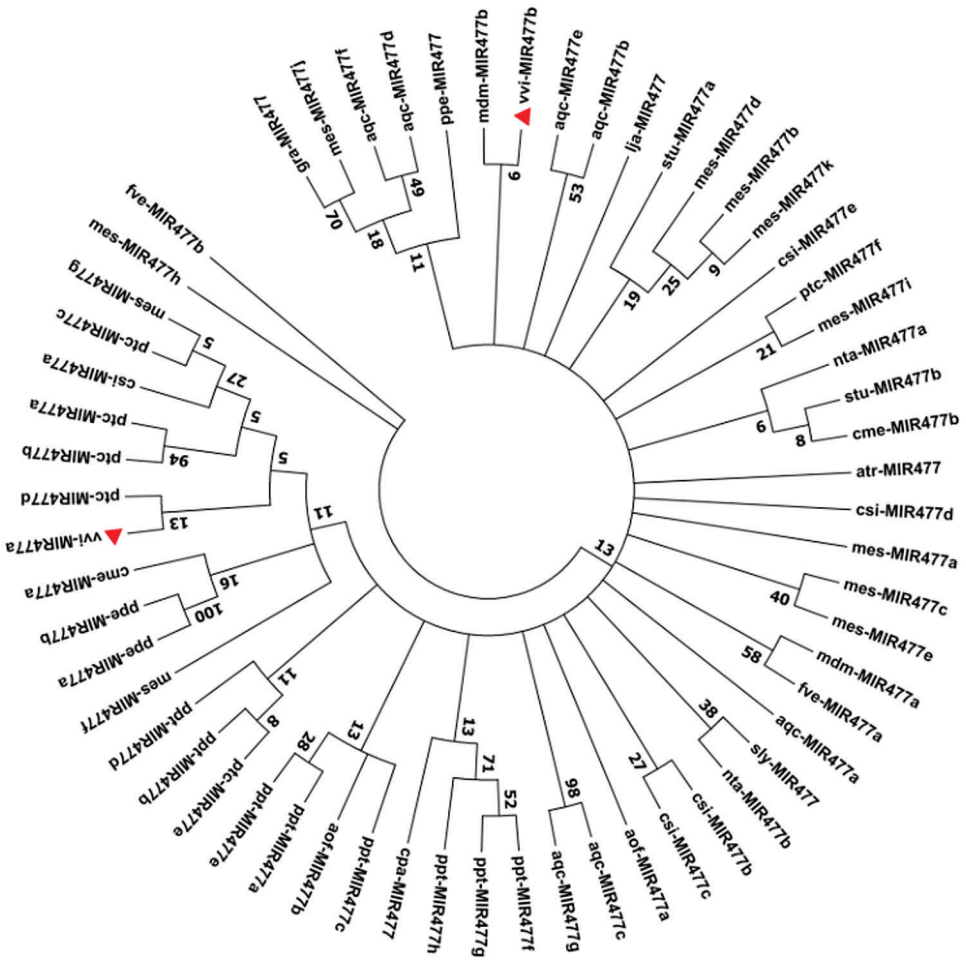

FIGURE 6. Phylogenetic tree of plants MIR477 precursor sequences based on the Maximum Likelihood method of MEGA version 7.0.26.
Note: ptc, *Populus trichocarpa*; Vvi, *Vitis vinifera*; mes, *Manihot esculenta*; ppt, *Physcomitrella patens*; ppe, *Prunus persica*; cme, *Cucumis melo*; csi, *Citrus sinensis*; mdm, *Malus domestica*; fve, *Fragaria vesca*; stu, *Solanum tuberosum*; nta, *Nicotiana tabacum*; aqc, *Aquilegia caerulea*; lja, *Lotus japonicus*; aof, *Asparagus officinalis*; sly, *Solanum lycopersicum*; gra, *Gossypium raimondii*; atr, *Amborella trichopoda*; cpa, *Carica papaya*.

TABLE 1

Target genes prediction of grape miR477 family

Name	Target_ID	NCBI_ID	Expectation	Target gene annotation	Inhibition
Vvi-miR477a	VIT_17s0000g10300	XP_002262921.1	1	26S proteasome non-ATPase regulatory subunit 6	Cleavage
	VIT_01s0010g02270	XP_002267538.2	1	GRAS family protein RAM1	Cleavage
	VIT_16s0022g02350	XP_002263260.1	2	uncharacterized protein LOC100245789	Cleavage
	VIT_16s0050g02000	XP_010662634.1	2.5	WD repeat-containing protein 91 homolog	Cleavage
	VIT_03s0017g01800	XP_002269136.1	2.5	Pentatricopeptide repeat-containing protein At1g71210, mitochondrial	Cleavage
	VIT_00s2364g00010	XP_003635287.2	2.5	U-box domain-containing protein 8	Cleavage
	VIT_09s0054g01830	XP_002270644.1	3	DEAD-box ATP-dependent RNA helicase 21	Cleavage
	VIT_12s0035g01130	XP_019079109.1	3	elongation factor 1-gamma	Cleavage
	VIT_04s0008g06260	XP_010648724.1	3	Exosome complex component RRP42	Cleavage
	VIT_12s0035g01120	XP_034701601.1	3	Prolycopene isomerase, chloroplastic	Cleavage
Vvi-miR477b-3p	VIT_18s0001g05870	XP_003634414.1	1	Probable RNA-binding protein ARP1	Cleavage
	VIT_06s0004g08220	XP_002276130.2	1.5	26S proteasome regulatory subunit 6B homolog	Cleavage
	VIT_00s0275g00060	XP_019073855.1	2.5	TMV resistance protein N	Cleavage
	VIT_00s0160g00090	XP_019074280.1	2.5	Disease resistance protein RPP2A	Cleavage
	VIT_12s0028g01140	XP_002278419.1	2.5	Uncharacterized oxidoreductase At1g06690, chloroplastic	Cleavage

(Continued)

Table 1 (continued).

Name	Target_ID	NCBI_ID	Expectation	Target gene annotation	Inhibition
	VIT_09s0002g03460	XP_002283628.2	3	LysM domain receptor-like kinase 3	Cleavage
	VIT_10s0116g00340	XP_010655347.1	3	Probable UDP-3-O-acylglucosamine N-acyltransferase 2, mitochondrial	Cleavage
	VIT_00s0551g00030	XP_010647191.1	3	Protein transport protein Sec61 subunit alpha	Cleavage
	VIT_04s0008g05410	XP_002285090.3	3	Probable carboxylesterase 17	Cleavage
	VIT_04s0008g05380	XP_002285085.1	3	Probable carboxylesterase 17	Cleavage
	VIT_04s0008g05390	NP_001268122.1	3	serine hydrolase-like	Cleavage
	VIT_04s0008g05350	XP_002285077.1	3	Probable carboxylesterase 17	Cleavage
	VIT_18s0001g14530	XP_002273785.1	3	Endoplasmin homolog	Cleavage
	VIT_01s0010g03820	XP_010656224.2	3	LRR receptor-like serine/threonine-protein kinase GSO1	Cleavage
	VIT_01s0010g00380	XP_002265191.2	3	LRR receptor-like serine/threonine-protein kinase GSO1	Cleavage
	VIT_05s0020g01420	XP_002273066.1	3	Flowering-promoting factor 1-like protein 1	Cleavage
	VIT_03s0038g03130	XP_002281225.1	3	Probable flavin-containing monooxygenase 1	Cleavage
	VIT_02s0154g00280	XP_002271619.1	3	14 kDa proline-rich protein DC2.15	Cleavage
Vvi-miR477b-5p	VIT_04s0008g06260	XP_002285435.1	2.5	Exosome complex component RRP42	Cleavage
	VIT_01s0010g02270	XP_002267538.2	2.5	GRAS family protein RAM1	Cleavage
	VIT_06s0004g04600	XP_010651025.1	2.5	Protein phosphatase 2C	Cleavage
	VIT_08s0040g02540	XP_002274849.2	2.5	Type I inositol polyphosphate 5-phosphatase 8	Cleavage
	VIT_10s0003g04030	XP_002279094.1	2.5	DEAD-box ATP-dependent RNA helicase 1	Cleavage
	VIT_14s0006g01530	XP_002273909.1	3	U-box domain-containing protein 4 isoform X2	Cleavage
	VIT_13s0067g02380	XP_002279029.1	3	LOB domain-containing protein 15	Cleavage
	VIT_14s0068g00420	XP_002279659.2	3	Putative methylesterase 11, chloroplastic	Cleavage
	VIT_16s0050g02460	XP_010662592.1	3	Probable inactive receptor kinase At4g23740	Cleavage
	VIT_11s0037g01230	XP_002270946.1	3	Very-long-chain aldehyde decarbonylase CER3	Cleavage
	VIT_18s0001g15290	XP_002266110.1	3	Transcription factor MYB52	Cleavage
	VIT_14s0030g01930	XP_002278422.1	3	peptide-N(4)-(N-acetyl-beta-glucosaminyl) asparagine amidase	Cleavage
	VIT_19s0014g03190	XP_002282052.1	3	WD repeat-containing protein 44	Cleavage
	VIT_17s0000g06710	XP_002281604.2	3	probable LRR receptor-like serine/threonine-protein kinase At1g74360	Cleavage
	VIT_18s0001g02570	XP_002285864.1	3	Protein disulfide-isomerase	Cleavage
	VIT_07s0031g02570	XP_010652932.1	3	Mediator of RNA polymerase II transcription subunit 15a	Cleavage
	VIT_06s0061g01020	XP_002272430.2	3	uncharacterized protein LOC100249926 isoform X1	Cleavage

Note: All predicted target genes with the lowest expectation scores (shown in parentheses) of ≤3 are listed; Cleavage indicates miR477 regulate target genes by cleaving the mRNA at complementary base pairs.

functions. The GO enrichment analysis showed that the predicted target genes are enriched in catabolic process; carboxylic ester hydrolase activity; water homeostasis; regulation of root morphogenesis; water transport; hydrolase activity; Cellular response to hypoxia; regulation of root development (Fig. 7b).

Discussion

Molecular characteristics of miR477 family members

Many studies have shown that miRNAs play the important role in fruit development and ripening (Gao *et al.*, 2015; Karlova *et al.*, 2013). In this study, the molecular characteristics of miR477 were analyzed. The miR477 mature sequences of the investigated plants were obtained from the miRBase database, and the results showed that miR477 is widely distributed in angiosperms and mosses (Wang *et al.*, 2020). Three mature miR477 members were revealed in grape. Sequence alignment analysis showed that miR477 had multiple conserved base regions, and MIR477 in grape could form a stable secondary structure. Wang *et al.* (2020) also indicated in tea plant (*Camellia sinensis*) that miR477 family members are conservative. The phylogenetic analysis showed that *Vvi*-miR477a and

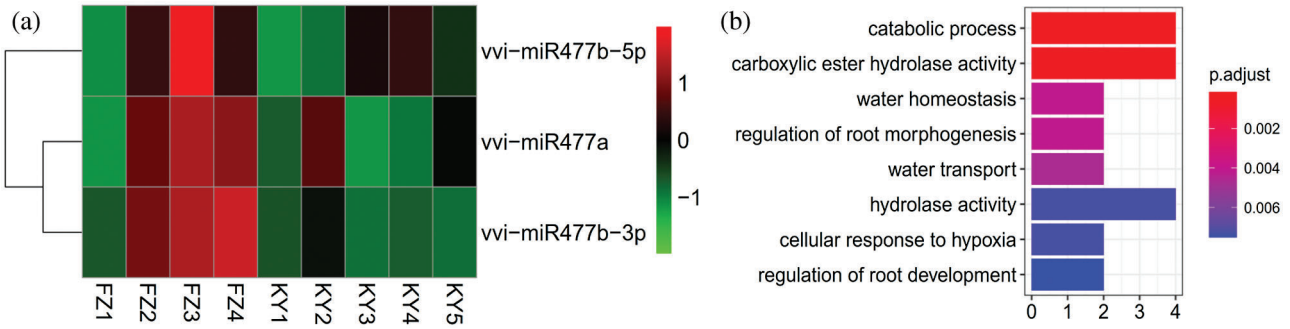

FIGURE 7. The expression level of V*vi*-miR477 at different berry developmental stages and the GO enrichment analysis of their target genes. (a) Expression analysis of miR477 family members at different stages of Kyoho and Fengzao. The abscissa represents the different developmental stages of Kyoho and Fengzao (data from the previous results (Guo *et al.*, 2018)). FZ represents Fengzao and KY represents Kyoho. Red represents up-regulated expression and green represents down-regulated expression. (b) Scatter gram of overrepresented GO terms ($P < 0.05$) in molecular function categories from GO enrichment analysis of miRNA targets gene of all the miRNAs identified in this study using ClusterProfiler. Enrichment term is represented by colored dots (red indicates high enrichment and blue indicates low enrichment).

Vvi-miR477b were clustered into different branches. It can also be seen from the larger branches that different members of the same family are distributed in different branches. The similar cases were documented in tomato and Korla fragrant Pear (Liu *et al.*, 2017; Ma *et al.*, 2020b). The conservation of miR477 was also reflected in the results of target gene prediction.

In plants, miRNA genes are mostly discrete, independent transcription units, and about 50% of miRNAs have multiple sites in the genome, similar to protein-coding genes (Liang *et al.*, 2012). Sequence alignment revealed that the length of the mature sequence of miR477 was inconsistent, with a difference of 2–3 nt. In addition, the secondary structure of MIR477 has a different minimum folding free energy (dG). The target genes of miR477 are not all the same. Tan *et al.* (2020) also discovered this by studying the function of miRNAs in plants. This is due to evolutionary processes which leads to the diversity of miRNA sequences with nucleotide substitutions, insertions, or deletions (Liang *et al.*, 2012). These results suggested that miR477 is conserved in different species but is also diverse. Similar results have been reported for members of the MIR156 family in Korla fragrant pears (Ma *et al.*, 2020b).

Target genes prediction of miR477 members

Fruit development is a highly coordinated and complex process. A range of physiological and biochemical changes influence the flavor, color, and texture of the berry (Ma *et al.*, 2020a). Fengzao is an early-ripening bud mutant of Kyoho, which matures nearly 30 days earlier (Guo and Zhang, 2015). In addition, previous studies have shown that miR477 is differentially expressed in Kyoho and Fengzao and upregulated in Fengzao (Guo *et al.*, 2018).

miRNAs exert their functions by regulating target genes (Alptekin *et al.*, 2017). The prediction results of target genes showed that miRNAs targeted 26S proteasome, GRAS family protein, Protein Phosphatase 2C, DEAD-box ATP-dependent RNA helicase and other target genes. A growing number of genetic analyses have shown that the Ub/26S proteasome pathway plays a role in plant physiological processes, such as embryonic development, hormonal response, flower homeosis, cell differentiation and senescence (Gu and Ma, 2018). GRAS family protein regulates plant growth and development (Hakoshima, 2018). The negative regulator of type 2C protein phosphatase ABI1 functioned in strawberry fruit ripening (Jia *et al.*, 2013). DEAD-box RNA helicase not only involved in plant normal growth and development, cell proliferation, differentiation, miRNA biological process, but also participating in plant abiotic stress responses (Wang *et al.*, 2020). Therefore, we speculate that the precocity of Fengzao may regulated by miR477 through the regulation of the above target genes. However, the authenticity of target gene prediction and the function of target gene still need to be verified by further experiments.

GO enrichment analysis showed that *Vvi*-miR477 was involved in Cellular response to hypoxia. Hypoxic conditions can lead to hypoxic respiration and accumulation of toxic end products of reactive oxygen species (ROS) (Yang, 2014). Hydrogen peroxide (H_2O_2) is a type of reactive oxygen species (ROS). Under oxygen-deficient conditions, production of both hydrogen peroxide (H_2O_2) is triggered by hypoxia signaling (Pucciariello *et al.*, 2012). Vergara *et al.* (2012) showed that hypoxia and respiratory inhibitors, such as potassium cyanide (KCN) and sodium nitroprusside (SNP), triggered the production of H_2O_2 in grape buds. While hydrogen peroxide (H_2O_2) treatment could promote the ripening of Kyoho grape (Guo *et al.*, 2020). In addition, Hydrogen peroxide is also involved in ripening tomatoes (Jimenez *et al.*, 2002) and pears (Chiriboga *et al.*, 2013). Therefore, miR477 may involve in the regulation of grape ripening.

Conclusions

In this study, three mature sequences and two precursor sequences of the miR477 family of grape were obtained by bioinformatic analyses, *Vvi*-miR477a, *Vvi*-miR477b-3p, *Vvi*-miR477b-5p, *Vvi*-MIR477a, *Vvi*-MIR477b. The phylogenetic analysis showed that the members of *Vvi*-miR477 were clustered in different clades. Multiple sequence alignment results showed that most miR477 family members were 20 nt and 21 nt in length. The secondary structure diagrams

of the two MIR477 members in grape are relatively stable. The results of target gene prediction showed that *Vvi*-miR477 targeted different genes, including 26S proteasome, DEAD-box ATP-dependent RNA helicase, Protein phosphatase 2C and GRAS family protein, etc. It showed the diversity of target genes of miR477. The GO function of target genes were enriched, mainly in six functions. The catabolic process, Carboxylic ester hydrolase activity is shown to be high. The expression levels, and the target gene functions of miR477, suggested that *Vvi*-miR477 may involve grape fruit ripening by regulating its target genes.

Availability of Data and Materials: The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request.

Author Contribution: D-LG conceived and designed the experiment. M-SP analyzed and interpreted the data. H-YJ wrote the manuscript. All authors read and approved the final manuscript.

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