



## Article

# Development, Identification and Validation of a Novel SSR Molecular Marker for Heat Resistance of Grapes Based on miRNA

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**Abstract:** The adverse effects of high temperatures on fruit quality and yield have been reported in many studies of grapevines. MiRNAs play crucial roles in plant growth and development and also fulfill functions in regulating the high-temperature response. In this research, miRNA-based SSR (simple sequence repeat) polymorphisms were analyzed according to the grape genome sequence and then combined with polymerase chain reaction (PCR) amplification and Sanger sequencing methods to analyze the miRNA-SSR diversity of different heat-resistant grape varieties. A statistical analysis of SSR sequences showed that 391 and 12 SSRs were specific to pri-miRNA and pre-miRNA, respectively. In pri-miRNA containing SSR, hexa-nucleotide repeats were the most abundant (52.69%), followed by tetra-nucleotide (13.04%) and minimum penta-nucleotide (4.09%), which were also observed in pre-miRNA sequences. On the other hand, differences in heat resistance among grape varieties were observed. Based on the results of leaf Fv/Fm images, values and phenotypic changes under high-temperature stress, 20 heat-resistant (e.g., Niagara Rosada and Grand Noir) and 20 heat-sensitive (e.g., Shine Muscat and Jumeigui) grape varieties were identified. Further, PCR-Sanger sequencing was used to screen SSR differences in four thermos-tolerant and four thermos-sensitive grape varieties, and finally, eight SSR differential primers were found to be able to distinguish these varieties. Of these, four pairs of primers were selected for validation in 40 grape germplasm resources (20 thermo-tolerance and 20 thermo-sensitivity). The VMIRSSR167c3 highlights that the ratio of SSR (CT) repeat values greater than 17 in heat-resistant varieties was 90%. In conclusion, the VMIRSSR167c3 marker can accurately distinguish between heat-resistant and heat-sensitive grape varieties. The results provide a novel molecular marker for the genetic improvement of grape germplasm resources and will be beneficial to the breeding of heat-resistant varieties in the future.

**Keywords:** miRNA; SSR; molecular markers; heat stress; grape; germplasm



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## 1. Introduction

Grapevine (*Vitis vinifera* L.) is a vital crop with a high economic value and is widely cultivated around the world [1,2]. Heat stress is one of the main abiotic stresses that affect this crop [3]. Hence, a better understanding of the response of grapes to heat tolerance is crucial to improve their heat resistance [4]. In short, heat tolerance is a complex function that includes strength, duration and rate of temperature rise [5]. In high-temperature spaces, a

catastrophic collapse of cell tissue may occur within minutes, leading to severe cell damage and cell death [6]. These damages directly result in a reduction in the net photosynthetic rate, the inactivation of photosystem II (PS II) activity and the excessive accumulation of reactive oxygen species (ROS) [7]. In addition, studies have shown that Fv/Fm (maximum photosynthetic efficiency of photosystem II) and Malondialdehyde (MDA) contents could be used as a standard to evaluate heat tolerance [8]. Heat resistance is a variety of physiological and biochemical changes controlled by multiple genes, which makes heat resistance responses difficult to study [9]. Hence, traditional breeding methods have been unable to meet today's demand for heat-resistant varieties since complex selection schemes and time-consuming phenotypic evaluation rounds are needed [10]. Thus, basic research to understand the relationships between important traits that regulate horticultural crops can rapidly increase the selection of novel genotypes.

In plants, miRNAs are transcribed by RNA polymerase II into primary miRNAs (pri-miRNA). There are pri-miRNAs that may include sequences encoding multiple miRNAs of ~70 nucleotides in length by a Dicer-like 1 (DCL1) enzyme. These pri-miRNAs were cleaved to stem-loop structured pre-miRNAs (precursor miRNAs). Then, the pre-miRNA was processed into miRNA and miRNA \* double-stranded (ds). The ds-miRNA was finally processed into single-stranded mature miRNAs [11]. MiRNA into the RNA-induced silencing complex (RISC) was assembled and regulated gene expression by cleaving target gene (mRNA) at the transcriptional level [12]. Studies have shown that miRNAs play a crucial role in regulating leaf and root development, such as miR156, miR164 and miR167 [13–16]. MiR156, miR159, and miR160 participated in the stages of vegetative growth to reproductive growth and development in plants [17–19]. In apples, miR156 and miR828 regulated anthocyanin metabolic pathways by targeting to MYBs [20]. An increasing number of studies have reported that miRNAs were key regulators in response to heat stress in plants. Under high-temperature stress, miR398 enhanced heat resistance by decreasing the expression of target genes (*COPPER/ZINC SUPEROXIDE DISMUTASE 1* (*CSD1*), *CSD2*, and *COPPER CHAPERONE FOR SOD1* (*CCS*)) in *Arabidopsis*. The transgenic *Arabidopsis thaliana* that further overexpressed *CSD1*, *CSD2* and *CCS* transcripts was more sensitive to heat stress than the wild type [21]. The overexpression of *MIR398b* and *MIR398c* activates *NAT398b* and *NAT398c*, thereby inhibiting the biogenesis of miR398 and reducing the heat tolerance of plants [22]. In addition, the production of tocopherol (vitamin E) under heat stress could promote the accumulation of miR398, thus improving heat resistance [23]. The results highlight the significance of miR398 expression in plant thermotolerance. Other microRNAs, such as miR156, regulate memory in response to heat stress by targeting *SQUAMOSA-PROMOTER BINDING-LIKE* (*SPL*) transcription factors [24]. The miR160-ARFs (*AUXIN RESPONSE FACTOR*) gene module mediates thermostability [25].

Recent studies have shown that miR167 positively regulates heat resistance in grapes [8]. There have been an increasing number of reports on the involvement of miRNA in grape development and response to stress [26–28]. However, the large-scale development of molecular markers based on miRNA-SSR is still lacking in grapes. In recent years, with the convenience of computer software analysis, an increasing number of SSR sequences in pri-miRNAs and pre-miRNAs have been reported in plants [29,30]. In pri-miRNAs and pre-miRNAs, microsatellites were reported in many places and showed no obvious location preference. This suggests that SSRs are vital for pri-miRNAs and pre-miRNAs. It was suggested that a potential role for SNP or SSR molecular markers located in miRNAs could affect the expression of quantitative traits [31]. Polymorphisms include SSRs or SNPs across the entire pri-miRNA sequence, and these results may influence the interaction between miRNA and target genes [32]. In pre-miRNAs, further study indicates that they play a crucial role in SSRs [33]. SSRs could respond to environmental changes to produce mature RNA isomers by influencing alternative splicing.

As we all know, a variety of miRNA families affect stress response and development in plants [34]. For example, in the rice genome, studies have found salt-responsive miRNA-

SSRs, associating them with the expression of genes and phenotype [31]. Patil [35] found miRNA-SSRs associated with traits in the miRNA genome of pomegranate. Thus, there is a need for biological stress and the development of DNA markers related to miRNAs in grapes. This could promote the improvement of traits affected by miRNA [36]. Studies based on complete genome sequences of grapes have important potential for developing novel functional molecular markers. In this study, a large number of pri-miRNAs and pre-miRNAs SSR loci were analyzed in the grape genome. Then, combined with RT-qPCR, the differential genes related to heat resistance and carried-out SSR design were screened. The development of miRNA-SSRs in grapes, which were validated in combination with PCR-Sanger sequencing. In conclusion, a miRNA-SSR molecular marker (VSSRMIRNA) was developed, combining the comparison results of the heat resistance of different varieties, which will greatly strengthen the molecular marker breeding research of grapes. This will lay a certain foundation for the breeding of heat-resistant and high-quality fruit varieties in the future.

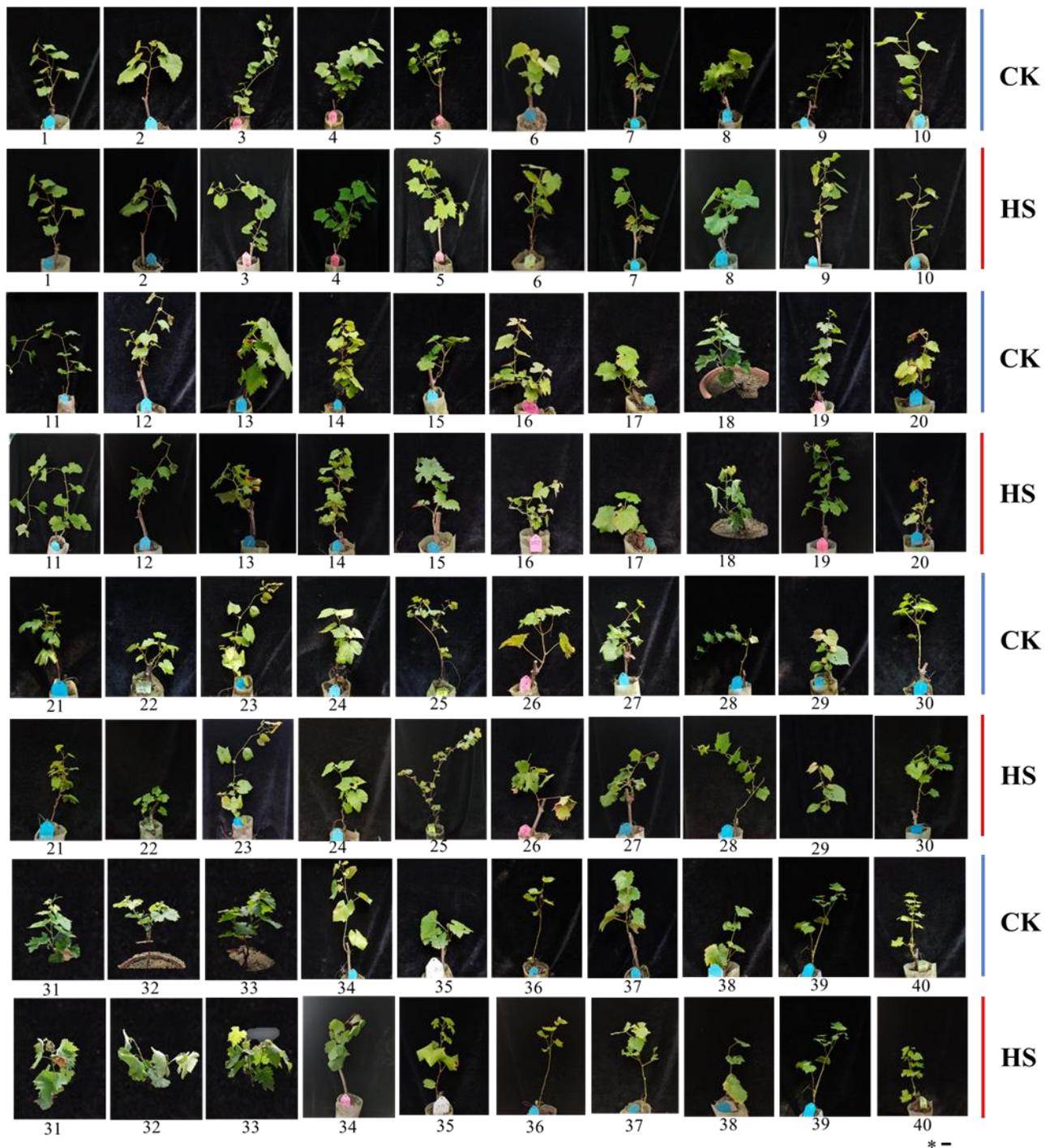
## 2. Materials and Methods

### 2.1. Plant Materials and Heat Treatments

In this study, forty grape genotypes were used during leaf growth and development in 2021, which were maintained at the School of Agriculture and Biology of Shanghai Jiao Tong University (Shanghai, China). There were 20 cuttings of grape per variety, totaling 800 plants. The experimental materials were selected from 100 days of growing cuttings of grapes with 7–10 leaves that could meet the needs of the experiment. They received standard pest control, disease control and horticultural practices. These grapes can be divided into table grapes and wine grapes by their economic value. The genotype names of all these varieties can be classified as VL: *V. vinifera* × *V. labrusca*; VI: *V. vinifera* L.; VA: *V. vinifera* × *V. amurensis*; AM: American wild grapes; AW: Asian wild grapes. For some detailed information on the notes and growing status of these grape varieties, refer to Supplementary Table S1 and Figure 1. The grape plants were acclimated for 7 days at 25 °C and were divided into four groups of each variety. On days 8 and 9, one group of 40 varieties of grapes at space 25 °C in the controlled environment room was kept. The other three groups were treated with high temperatures of 45 °C and 9:00–17:00, respectively. Fv/Fm (the maximum photochemical efficiency) was tested at 0, 2, 4 and 8 h. Handy PEA was used to measure chlorophyll fluorescence parameters Fv/Fm, and imaging photos were taken by imaging-PAM software. For all treatments, functional leaves for all treatments were collected at 0, 2, 4 and 8 h and stored in liquid nitrogen. The remaining parts were frozen in liquid nitrogen and then stored at −80 °C.

### 2.2. DNA, RNA Extraction and cDNA Synthesis

Genomic DNA was extracted using an improved CTAB method [37]. Total RNA was measured using a modified CTAB method [38] and treated with DNase I (Invitrogen, Sacramento, CA, USA) to remove DNA contamination. DNA and RNA integrity were verified on a 1% agar gel. DNA was used for PCR analysis and Sanger sequencing analysis of miRNA-SSR. Approximately 1–2 µg of RNA as template was used for first-strand cDNA synthesis using SuperScript reverse transcriptase (state for USA and Canada) according to the manufacturer's instructions (state for USA and Canada) for analyzing the abundance of pri-miRNAs. Total RNA from each of the three biological replicates in the RT-qPCR analysis was independently used.



**Figure 1.** Phenotypic change of forty grape varieties during high-temperature stress. Phenotypic photographs of control group (CK, samples at 25 °C) and heat treatment stress (HS, samples at 45 °C) were collected in different time points (0 h, 8 h), respectively. \* Bar = 3 cm.

### 2.3. Mining of SSRs from Pri-miRNAs and Pre-miRNAs of the Grapevine Genome

We downloaded pri-miRNAs and pre-miRNAs from the grape genome in silico dataset (<https://mirbase.org/> (accessed on 8 October 2022); <https://www.pmiren.com/> (accessed on 8 October 2022); <https://www.ncbi.nlm.nih.gov/> (accessed on 8 October 2022)). Pri-miRNA sequence was 500 bp before and after the pre-miRNA. Mining of microsatellites with 2–6 bp repeats, which were specific to pre-miRNA and pri-miRNA sequences, was performed by the MISA tool (<https://webblast.ipk-gatersleben.de/misa/> (accessed on

8 October 2022)). SSR motifs at genome-wide scale were surveyed by considering a minimum repeat length of 12 bp by defining 2, 3, 3, 4, 6 and 12 for hexa-, penta-, tetra-, tri-, di-, and mono-nucleotides, respectively. SSR types were denoted as P6–P1. Compounding SSRs were defined as two SSRs interrupted within 100 bases. SSR types were denoted as C. The MISA statistics in Microsoft Excel were analyzed further to draw frequency distribution graphs. For designing flanking primers for the SSRs present in pri- and pre-miRNA sequences, the PCR amplification primer designing software tool Batch Primer5 v1.0 was used. Primer pairs were designed to amplify products of 100–800 bp with the following parameters: primer length (bp) 18–28 bp; GC content (%) 40–60; T<sub>m</sub> (°C) 58–65. Other parameters used were those of default program values.

#### 2.4. RT-qPCR, PCR, and PCR-Sanger Sequencing Analysis

The PCR reaction system in 50 µL uses the Taq (*Thermus aquaticus*) enzyme (TaKaRa, Tokyo, Japan). According to the PCR product results, 30 µL were used for PCR-Sanger sequencing analysis. RT-qPCR in 10 µL reactions was conducted by SYBR Green Super-mix (TaKaRa, Tokyo, Japan) to analyze the expression of *vvi*-MIRNAs. The primers used for these reactions are listed in Supplementary Table S2. The relative abundance of *vvi*-MIRNAs was calculated by the  $2^{-\Delta\Delta CT}$  method and normalized by  $\beta$ -actin as references [39,40].

#### 2.5. About the Naming of miRNA-SSR Molecular Marker Sub

Referring to the SSR database of other cognate species, miRNA-based molecular markers were developed by combining the molecular characteristics of miRNA-SSR. For naming information, refer to VMIRSSR167c3. V represents *Vitis*; MIR represents *vvi*-pri-miRNA; SSR represents SSR diversity existed in the *vvi*-pri-miRNA; 167c represents specific *vvi*-pri-miRNA; 3 represents the sequence from 5' to 3' of the pri-miR167c sequence and the third SSR polymorphism site that meets the screening conditions.

#### 2.6. Prediction of Target Genes for miRNAs and Pathway Analyses

Target prediction of miRNAs by psRNATarget tools (<https://www.zhaolab.org/psRNATarget/> (accessed on 8 October 2022)) was performed, which involved loading miRNA reads into a FASTA file format to search for known targets in the grapevine (*Vitis vinifera*) transcript database. Predicted target genes for further validation were selected and functionally analyzed by BLASTN (<http://blast.ncbi.nlm.nih.gov> (accessed on 8 October 2022)). TBtools software (<https://github.com/CJ-Chen/TBtools/releases> (accessed on 8 October 2022)) was used to map the regulatory network pathway of miRNA-mRNA.

#### 2.7. Statistical Analysis

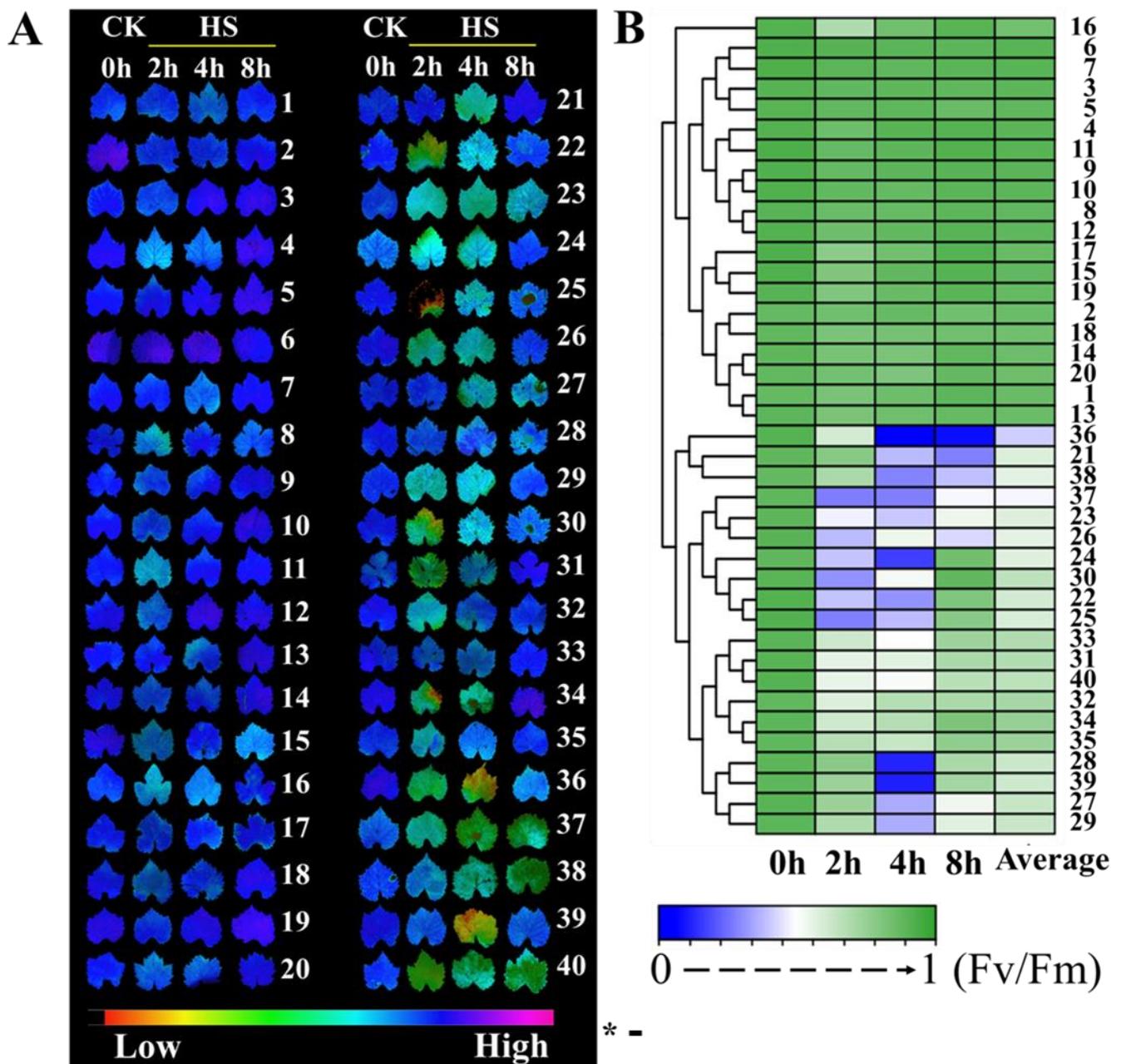
The plant phenotype data and physiological indexes were statistically analyzed by Microsoft Excel (2016). We used TBtools software to make diagrams. The statistical of Social Science software package IBM SPSS Statistics (Version 20.0) for Window was used.

### 3. Results

#### 3.1. Heat Injury in 40 Grape Varieties' Leaves Exposed to High-Temperature Stress

Pulse amplitude modulated (PAM) imaging of chlorophyll fluorescence measurements has been used as a high-efficiency method for the assessment of photosynthesis [41]. Photosynthesis is a biological process that is sensitive to high-temperature stress in plants [42]. The parameter F<sub>v</sub>/F<sub>m</sub> has been thought to be suitable for evaluating heat injury in grape leaves [43]. In this study, before high-temperature treatments (CK, 0 h), the F<sub>v</sub>/F<sub>m</sub> values of grape leaves ranged from 0.75 to 0.85 (Figure 2A). Chlorophyll fluorescence images of the leaves appeared dark blue, which showed that these grape leaves were healthy. After heat stress for 2 h and 4 h, the F<sub>v</sub>/F<sub>m</sub> value began to decrease (Figure 2B), suggesting that the leaves of the grapes had been obviously damaged at that time. The entire leaves with chlorophyll fluorescence were imaged, which allowed us to observe the changes in grape response to heat stress and assess the differences in photosynthetic performance

among different varieties (Figure 2). The maximum quantum yield efficiency (Fv/Fm) of PSII decreased during high temperatures, which was different for different heat-resistant grape varieties. However, the degree of photosynthetic inhibition in different varieties was different. According to chlorophyll fluorescence images and Fv/Fm values under heat stress, the varieties were divided into heat-sensitive (20 varieties) and heat-resistant (20 varieties). This was consistent with the phenotype of grapes responding to heat stress (Figure 1), where heat stress reduces photosynthesis in grapes. Heat-resistant varieties mainly include rootstock varieties such as *Vitis pseudoreticulata*, *Vitis rupestris*, and *Vitis bryoniifolia* and heat-sensitive varieties mainly include the table grape Jumeigui, Shine Muscat and Muscat Hamburg (Figure 2). In addition, the chlorophyll fluorescence parameters of grapes showed a recovery state after 8 h of heat treatment (Figure 2). It is possible that the grapes adapted to high-temperature stress and enhances their tolerance.



**Figure 2.** Representative pictures of chlorophyll fluorescence parameters. (A) Chlorophyll fluorescence imaging with Fv/Fm of grape leaves during heat stress. (B) Representative specific values of Fv/Fm. \* Bar = 1 cm.

### 3.2. RT-qPCR Analysis of the Pri-miRNA Response to Heat Stress

Previous work identified miRNAs involved in the heat stress of grapes in order to further explore the high-temperature response of pri-miRNA, the primary transcript of miRNA. In this study, Thompson seedless grape experimental materials that were treated with high temperatures for 1 h (Con) and 0 h (CK) at an early stage were selected [8]. RT-qPCR was used to further verify whether the primary transcripts with differentially expressed miRNAs participated in the heat stress response mechanism. The analysis results showed that among the 16 miRNA genes, the relative content of *vvi-MIR167d* was not significantly different in the CK and Con groups, and the *vvi-MIRNA* genes were significantly different (Supplementary Figure S1). Among the 16 *vvi-pri-miRNA* with SSR loci involved in the mechanism of grape high-temperature response, 4 *vvi-pri-miRNA* (*vvi-pri-miR319e*, *vvi-pri-miR159f*, *vvi-pri-miR156c* and *vvi-pri-miR169d*) were up-regulated. Eight *vvi-pri-miRNAs* with down-regulated expressions were *vvi-pri-miR828a*, *vvi-pri-miR398b/c* and *vvi-pri-miR164c* et al. These results suggest that the presence of SSR loci on *vvi-pri-miRNA* may change the expression abundance of the hyperthermia response in grape cells. The formation of miRNA was altered under heat stress, and the heat stress response of grapes was further regulated by cutting target genes or inhibiting the translation of target genes.

### 3.3. MiRNA and Target Genes Mediated Regulatory Networks

To further explore the relationship between the miRNAs and their targets, miRNA-mRNA-mediated regulatory networks were constructed (Supplementary Figure S2). Ten independent networks for miRNA families indicated multiplicity behavior for the majority of families; in other words, one miRNA can target more than one gene. Exploring these results, *vvi-miR156* has revealed the maximum number of targets (23 genes), followed by *vvi-miR159* (16 genes), *vvi-miR169* (11 genes), *vvi-miR157* (11 genes), *vvi-miR164* (8 genes), *vvi-miR828* (7 genes) and *vvi-miR160* (6 genes). The lowest targets were observed for *vvi-miR398* (5 genes), *vvi-miR167* (5 genes) and *vvi-miR171* (3 genes). The *vvi-miR156* (18 genes) was the largest regulatory network and shared maximum genes with *vvi-miR157* (11) for *vvSPL10-like* (*SQUAMOSA PROMOTER-BINDING-LIKE PROTEIN 10-RELATED*), *vvSPL4-like*, *vvSPL15-like*, *vvSPL3*, *vvSPL13-like*, *vv-POE1* (*POLLEN OLE E 1 ALLERGEN AND EXTENSIN FAMILY PROTEIN*), *vv-CYP2* (*Cytochrome P450 CYP2 subfamily*), *vv-GRAS* (*GRAS domain family*), *vv-BTB* (*BTB/POZ DOMAIN-CONTAINING PROTEIN NPY1*) and *vv-APRT* (*adenine phosphor ribosyl transferase*). Similarly, the *vvi-miR159* regulatory network shared common targets with *vvi-miR164*. Further, a network analysis for the individual miRNA families based on the alignment score revealed that *vvi-miR156* showed the strongest interaction with *SQUAMOSA PROMOTER-BINDING-LIKE PROTEIN 4-RELATED* (*vvSPL4-like*), *vvi-miR164* with *NAC DOMAIN-CONTAINING PROTEIN 100-RELATED* (*vv-NAC100-like*), *vvi-miR160* with *vv-ARFs* (*AUXIN RESPONSE FACTOR 17*, *AUXIN RESPONSE FACTOR 10-RELATED* and *AUXIN RESPONSE FACTOR 10*), *vvi-miR828* (*MYB-like DNA-binding domain*), *vvi-miR171* with *SCLs* (*SCARECROW-LIKE PROTEIN 15* and *SCARECROW-LIKE PROTEIN 22-RELATED*), *vvi-miR159* with *MYBs* (*MYB TRANSCRIPTION FACTOR-RELATED*) and many genes for *vvi-miR156* (*SQUAMOSA PROMOTER-BINDING-LIKE PROTEIN* family) (Supplementary Figure S2).

### 3.4. Identification and Frequency Distribution of miRNA-SSRs in the Grape Genome

We found the known 192 pri-miRNAs' and 192 pre-miRNAs' sequences from the miRbase and the PmiREN: Plant microRNA Encyclopedia. All the pre- and pri-miRNA sequences for the presence of SSRs were surveyed. There were approximately 18.99 kb of pre-miRNA and 209.61 kilo base (kb) of pri-miRNA sequences in the grapevine genome. The MISA analysis tool discovered a total of 12 SSRs from 192 (6.25%) pre-miRNAs and 391 SSR motifs from 154 (80.21%) pri-miRNAs. The distribution frequency of one SSR locus per 1.73 kb and 1.36 kb was observed for pre-miRNAs and pri-miRNAs, respectively. We found 1 (9.1%) pre-miRNA and 107 (55.73%) pri-miRNA sequences with more than



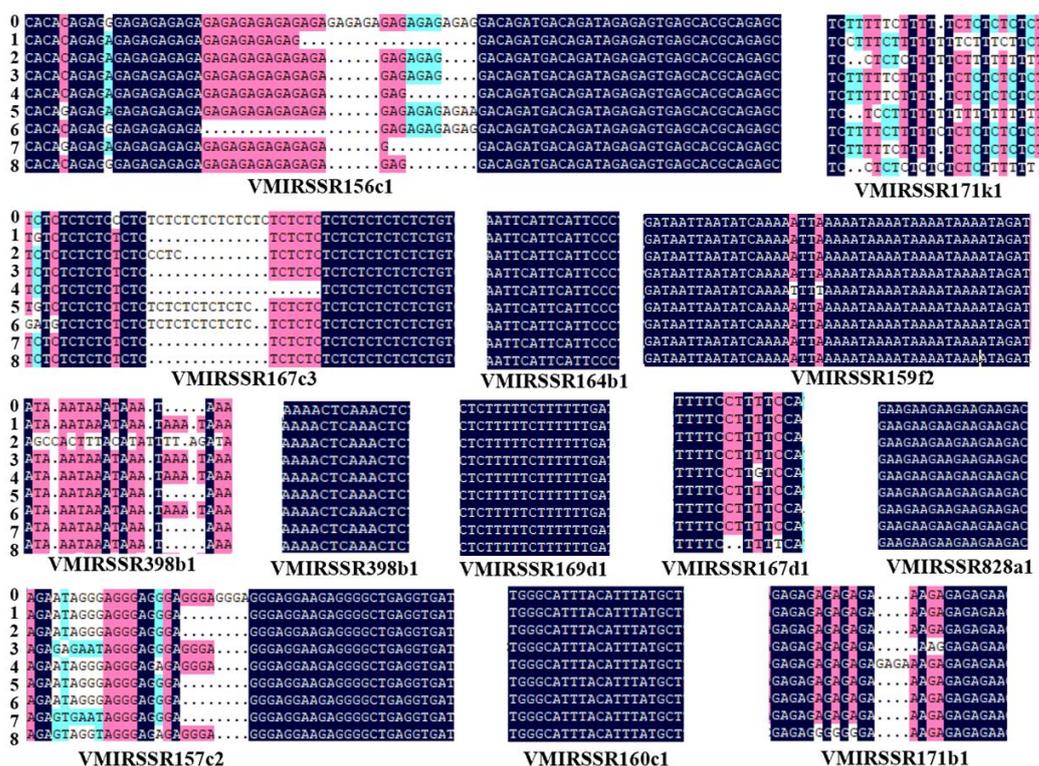


### 3.6. The Diversity of 13 MIRNA-SSR Loci Was Verified in 8 Grape Germplasm Samples

Based on the distribution of SSR loci in chromosome locations, RT-PCR verification and analysis of different SSR types. Thirteen different SSR loci were selected (Supplementary Table S4), and primers were designed (Supplementary Table S1). SSR polymorphisms in different genotypes were preliminarily screened using eight grape varieties (four heat-tolerant and four heat-sensitive) (Supplementary Table S5). The accuracy of PCR sequence amplification was verified by agarose gel electrophoresis (Supplementary Table S4). The PCR product sequencing results showed that SSR types in eight grape varieties showed different repeats, such as VMIRSSR156c1 (P2) and VMIRSSR398b1 (P4). The genotyping of multiple loci was obtained. The analysis of SSR polymorphism at the same locus found that there were different SSR repeats at one locus, such as VMIRSSR171b1 (GA), VMIRSSR398b1 (ATAA), VMIRSSR167d1 (TTTTCC) and VMIRSSR157c2 (AGGG). Combined with the differences in heat tolerance between varieties, this could provide more alternative markers for the identification of heat tolerance between grape varieties. Based on the distribution of SSR logic in chromosome location, RT-qPCR verification and the analysis of different SSR types, thirteen different SSR locals were selected, and primers were designed. SSR polymorphisms in different genotypes were preliminarily screened using eight grape varieties (four heat-tolerant and four heat-sensitive). The accuracy of PCR sequence amplification was verified by agarose gel electrophoresis. The PCR product sequencing results showed that SSR types in eight grape varieties showed different repeats, such as VMIRSSR156c1 (P2) and VMIRSSR398b1 (P4). The genotyping of multiple loci was obtained. The analysis of SSR polymorphism at the same locus found that there were different SSR repeats at one locus, such as VMIRSSR171b1 (GA), VMIRSSR398b1 (ATAA), VMIRSSR167d1 (TTTTCC) and VMIRSSR157c2 (AGGG) (Figure 5). Combined with the differences in heat tolerance between varieties, this could provide more alternative markers for the identification of heat tolerance between grape varieties.

### 3.7. Application of VMIRSSRNA Markers in Heat-Resistant and Heat-Sensitive Grape Varieties

Four pairs of SSR primer sequences were selected in grapes through the preliminary screening of miRNA and SSR polymorphisms in response to heat stress. Using 40 grape varieties as experimental materials, PCR products were amplified, sequenced and biological statistics were conducted to analyze the repeat times of SSR polymorphisms in many varieties. It was found that VMIRSSR167c3 (CT) had the largest difference in repetition times, and VMIRSSR167d1 (TTTTCC) had the smallest difference in grapes. The results of the identification of VMIRSSR167d1 indicated that the polymorphism of SSR showed only a SNP variation of T/G and C/T in a few varieties. Similarly, SNP variants also exist in VMIRSSR157c2 (AGGG) and VMIRSSR398b1 (ATAA) (Tables 1 and 2). The SSR polymorphism of VMIRSSR167c3 (CT)<sub>n</sub> was the most significant among 40 grape varieties. VMIRSSR167c3 (CT)<sub>n</sub> as a molecular marker to distinguish heat-resistant and heat-sensitive varieties was used. That is, if the CT repeat value was greater than or equal to 18, it was defined as a heat-resistant grape variety, whereas if it was less than 18, it was defined as a heat-sensitive grape variety. A biological statistical analysis showed that the differentiation rates of heat-resistant and heat-sensitive genotypes reached 90% (18/20) and 80% (16/20), respectively (Figure 6B). The establishment of miRNA-SSR molecular markers for VMIRSSR167c3 (CT)<sub>n</sub> not only has the advantages of simplicity and quickness but also provides alternative molecular markers to further improve the breeding of heat-resistant grape varieties.



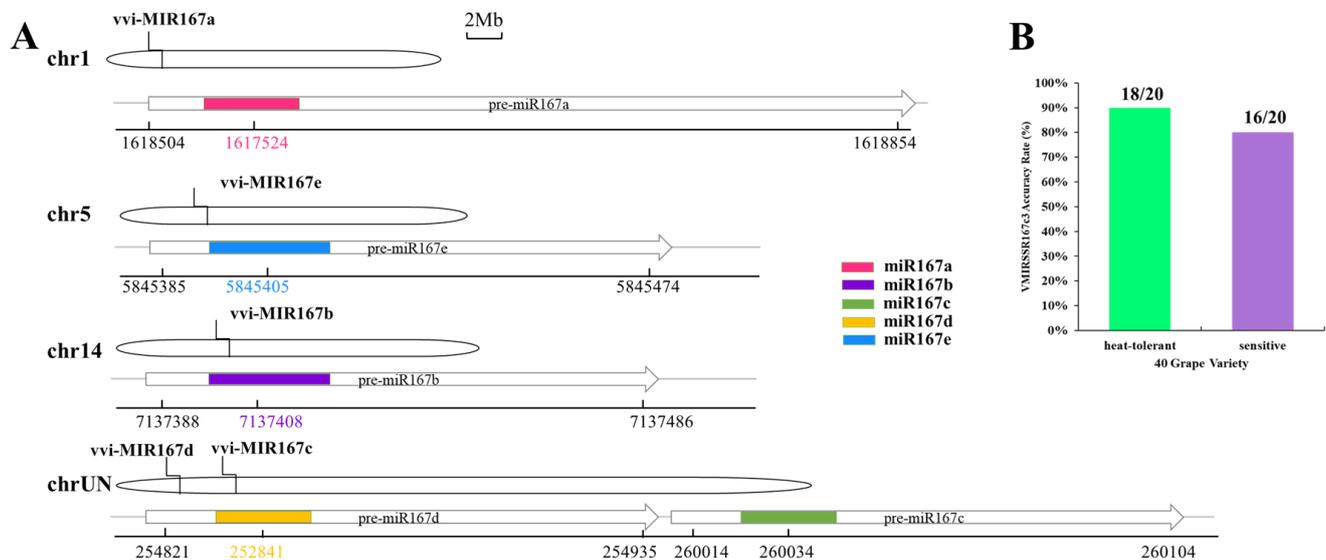
**Figure 5.** PCR-Sanger sequencing shows allelic variations as revealed by miRNA-SSR markers. Assaying thirteen grape genotypes with the markers VMIRSSR156c1, VMIRSSR171k1, VMIRSSR167c3, VMIRSSR164b1, VMIRSSR159f2, VMIRSSR398b1, VMIRSSR169d1, VMIRSSR167d1, VMIRSSR828a1, VMIRSSR157c2, VMIRSSR160c1 and VMIRSSR171b1.

**Table 1.** Identification of VMIRSSR167c3, VMIRSSR167d1, VMIRSSR157c2 and VMIRSSR398b1 in 20 heat-resistant grape varieties.

NO.	Varieties Thermostability	VMIRSSRNA-Marker			
		VMIRSSR167c3 (CT)n	VMIRSSR167d1 (TTTTCC)n	VMIRSSR157c2 (AGGG)n	VMIRSSR398b1 (ATAA)n
1	Niagara Rosada	(CT)20	(TTTTCC)2	(AGGG)4	(ATAA)5
2	Benzuiho	(CT)19	(TTTTCC)2	(AGGG)3, 1(AGAG)	(ATAA)5
3	<i>Vitis × Champion</i>	(CT)18, 1(CC)	(TTTTCC)2	(AGGG)4	(ATAA)5
4	Cuihong	(CT)17, 1(CC)	(TTTTCC)2	(AGGG)4, 1(AGAG)	(ATAA)5
5	Seibel Blanc	(CT)19	(TTTTCC)2	(AGGG)4	0
6	Lot Sand	(CT)20, 1(CC)	(TTTTCC)2	(AGGG)4	(ATAA)5
7	Damubo	(CT)23	(TTTTCC)2	(AGGG)4	(ATAA)5
8	Aromatic Rachachi	(CT)24	(TTTTCC)2	(AGGG)3, 1(AGAG)	(ATAA)5
9	Pollux	(CT)22, 1(CC)	(TTTTCC)2	(AGGG)2	(ATAA)5
10	Anisky	(CT)18, 1(CC)	(TTTTCC)2	(AGGG)4	(ATAA)5
11	Green beads	(CT)19	(TTTTCC)2	(AGGG)4	(ATAA)5
12	Volgarton	(CT)16	(TTTTCC)2	(AGGG)3,1(AGAG)	(ATAA)5
13	Augusta	(CT)17, 1(CC)	(TTTTCC)2	(AGGG)4	(ATAA)5
14	Jifeng	(CT)18, 1(CC)	(TTTTCC)2	(AGGG)3, 1(AGAG)	(ATAA)5
15	Grand Noir	(CT)23, 1(CC)	(TTTTCC)2	(AGGG)3, 1(AGAG)	0
16	Shenmei	(CT)18, 1(CC)	(TTTTCC)2	(AGGG)4	0
17	Jiangsu v. azuLene	(CT)24, 1(CC)	(TTTTCC)2	(AGGG)4	(ATAA)5
18	Shenyue	(CT)18, 1(CC)	(TTTTCC)2	(AGGG)4	(ATAA)5
19	Cabernet Sauvignon	(CT)17	(TTTTCC)2	(AGGG)3, 1(GAAT)	(ATAA)4
20	Shenhua	(CT)18, 1(CC)	TTTTCTTGTC	(AGGG)4, 1(AGAG)	(ATAA)5

**Table 2.** Identification of VMIRSSR167c3, VMIRSSR167d1, VMIRSSR157c2 and VMIRSSR398b1 in 20 heat-sensitive grape varieties.

NO.	Varieties Heat-Sensitive	VMIRSSRNA-Marker			
		VMIRSSR167c3 (CT)	VMIRSSR167d1 (TTTTCC)	VMIRSSR157c2 (AGGG)	VMIRSSR398b1 (ATAA)
21	Ruiduxiangyu	(CT)24, 1(CC)	(TTTTCC)2	(GA)3, 1(TA)	(ATAA)4
22	Shenbao	(CT)19	(TTTTCC)2	(AGGG)4	(ATAA)4
23	Hakuho	(CT)16, 1(GT)	(TTTTCC)2	(AGGG)4	(ATAA)5
24	Xinya	(CT)17	(TTTTCC)2	(AGGG)4	(ATAA)5
25	Beta	(CT)21	(TTTTCC)2	(AGGG)4	(ATAA)4
26	Beichun	(CT)13, 1(CC)	(TTTTCC)2	(AGGG)4	(ATAA)4
27	Molixiang	(CT)16, 1(CC)	TTTTCCTTTTTC	(AGGG)4	(ATAA)5
28	Yan 74	(CT)12, 1(CC)	(TTTTCC)2	(AGGG)4	(ATAA)5
29	Mgni Blanc	(CT)19	(TTTTCC)2	(AGGG)4, 1(AGAG)	(ATAA)5
30	Shine Muscat	(CT)14	(TTTTCC)2	(AGGG)4, 1(AGAG)	(ATAA)5
31	Fujiminori	(CT)14	(TTTTCC)2	(AGGG)3, 1(AGAG)	(ATAA)5
32	Italy grape	(CT)17	(TTTTCC)2	(AGGG)3, 1(AGAG)	(ATAA)5
33	SO4	(CT)14	(TTTTCC)2	(AGGG)4	3(ATAA), 1(ATTA)
34	Wuhecuibao	(CT)14	(TTTTCC)2	(AGGG)3, 1(AGAG)	(ATAA)5
35	Jumeigui	(CT)14	(TTTTCC)2	(AGGG)4	(ATAA)5
36	Kyoho	(CT)17	(TTTTCC)2	(AGGG)4	(ATAA)5
37	MuscatHamburgh	(CT)17	(TTTTCC)2	(AGGG)3, 1(AGAG)	(ATAA)5
38	Thompson Seedless	(CT)17	(TTTTCC)2	(AGGG)3, 1(AGAG)	(ATAA)4
39	Shuanghong	(CT)15	TTTTCCTTGTC	(AGGG)4, 1(AGAG)	(ATAA)5
40	1103P	(CT)14	(TTTTCC)2	(AGGG)4	3(ATAA), 1(ATTA)

**Figure 6.** (A) Chromosomal locations of *vvi-pri-miR167* family members in grapes. The numbers on the left side of chromosome represent the starting position of the *vvi-MIR167s* on that chromosome of grape. (B) Identification of VMIRSSR167c3 molecular marker accuracy rate (%) in heat-tolerant and heat-sensitive 40 grape germplasm resources.

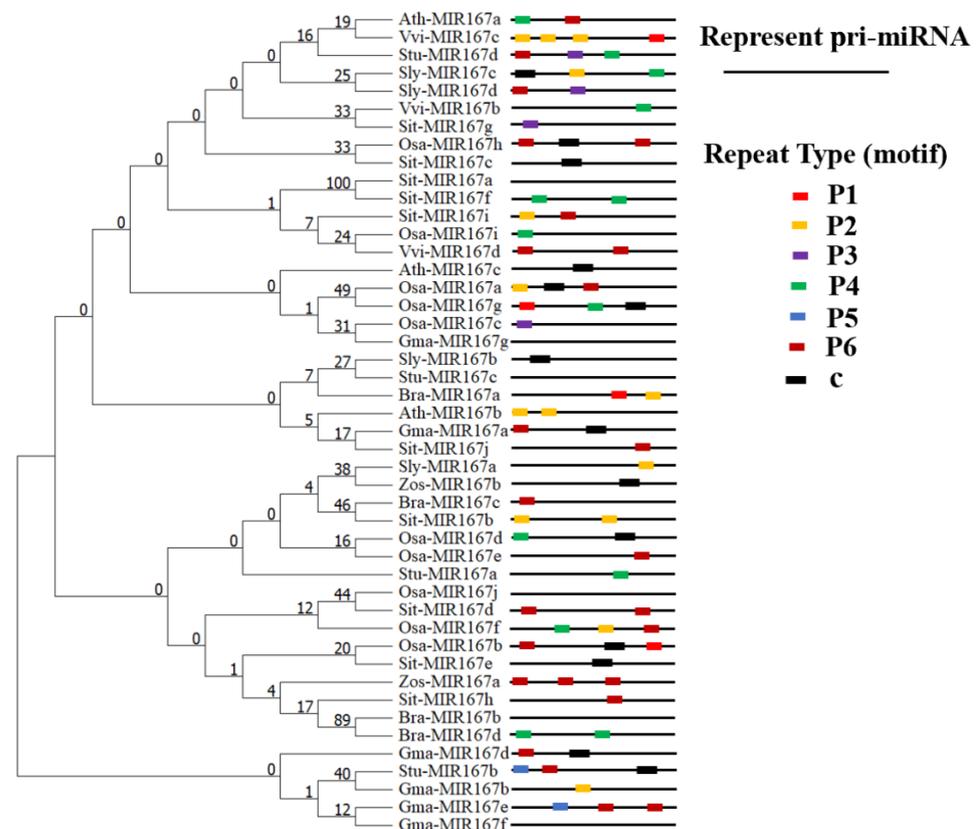
### 3.8. Chromosome Distribution of *vvi-miR167* Family Members in Grape

To determine the chromosomal location of *vvi-miR167* family members, the precursor sequences of *vvi-miR167s* (*vvi-MIR167s*) were aligned with the grape reference genome. The location of miRNA precursor sequences on individual chromosomes and the position of mature miRNAs on the precursors were shown in the upper and lower parts, respectively (Figure 6A). The results showed that five members of the *vvi-miR167* family were distributed on chromosomes chr1, chr5, chr14 and chrUN of the grape genome, *vvi-miR167c* and *vvi-miR167d* were located on unknown chromosomes and the rest were distributed

separately on different chromosomes (Figure 6A). The distance between vvi-miR167c and vvi-miR167d was 5193 bp.

### 3.9. Phylogenetic Development of the vvi-MIR167s Gene Family of Representative Species

MEGA X software was used to construct the phylogenetic tree of neighbor-joining (NJ) based on pri-miRNA sequences of miR167 family members of nine species (Figure 7). As can be seen from the results, the whole evolutionary tree has five major branches. On the whole, the evolutionary relationship of the miR167 family was related to the genetic relationship of species, just like *Oryza sativa* and *Setaria italica*, which were grass plants. Most members of the miR167 family cluster together in the evolutionary tree, such as *Osa-MIR167h* and *Sit-MIR167c*, *Osa-MIR167d* and *Osa-MIR167e*, *Osa-MIR167j/f* and *Sit-MIR167d*, *Osa-MIR167b* and *Sit-MIR167e*. Only some were dispersed in the whole evolutionary tree, and some were clustered with some members of soybean and grape; for example, *Sit-MIR167g* of *Setaria italica* clustered with *vvi-MIR167b* of grape, and *Osa-MIR167i* and *vv-MIR167d* of *Oryza sativa* clustered together. The vvi-miR167 family members of grapes were not clustered in one branch but dispersed in three branches. *Vv-MIR167c* and *Ath-MIR167a* were distributed in one branch, and the other two were clustered in rice and setaria, respectively. An SSR analysis on each sequence showed that 40 (87%) of all the sequences had SSR, and 6 (13%) sequences did not contain SSR. Further analysis revealed that *vv-MIR167c* had the largest number of SSR, including three P2 repeat types and one P1 repeat type, respectively (Supplementary Table S6). The number of sequences containing one SSR amounted to up to 16 (35%). A phylogenetic analysis of all repeat types showed that the evolutionary relationship of miRNA was different from that of species, and they might have their own special evolutionary mode. In addition, SSR was found in most *MIRNA* sequences of different species, indicating that SSR plays a very important role in the evolution of species and can be used as a molecular marker for certain traits.



**Figure 7.** Phylogenetic trees of Neighbor-joining (NJ) (Bootstrap = 1000) based on pri-miRNA sequences of miR167 family members of nine species. The number of SSR occurrences and different SSR types in pri-miRNA sequences.

## 4. Discussion

### 4.1. Functional Analysis for miRNAs and Their Regulatory Networks

As an important part of complex gene regulation, miRNAs can not only control plant development patterns but also play an important role in responding to stress. Previous studies highlight that miRNA was involved in the genesis of grape roots and that the ectopic expression of vvi-miR164 improves lateral root development [16]. Through RNA-seq, a comparative analysis of miRNA revealed that the function of vvi-miR828 under root domain restriction significantly reduced the anthocyanin content in transgenic *Arabidopsis thaliana* lines, indicating that this gene has the function of regulating fruit coloring [45]. In addition, Chen [46] found for the first time that the small peptide vvi-miPEP171d1 encoded by *vv-MIR171d* in grapes could specifically promote the expression of *vvi-MIR171d* and increase the number of grape adventitious roots. MiRNAs play an important role in the stress resistance of plants by targeting mRNA. In *Arabidopsis*, miR408 was implicated in abiotic stress responses, highlighting the core function of miR408 in plant survival [26]. Zma-miR167 targets auxin response factor 3 (*ZmARF3*) and *ZmARF30*, thereby regulating resistance to MCMV. The researchers then discovered the role of the Zma-miR167-*ZmARF3/30* modules in limiting MCMV infection by modulating *ZmPAO1* expression, while MCMV uses p31 to counteract this defense [47]. MiR156 inhibited the expression of the target gene SPL, promoted the stable expression of HSPs and HsfA2, improved *Arabidopsis* memory at high temperatures and enhanced heat resistance [24]. It was high temperature that up-regulated the expression of the transcription factor *vvi-MiR156c* (Supplementary Figure S1). The identification of grapevine heat-stress-responsive miRNAs revealed the positive regulatory function of vvi-miR167 in thermostability. In this study, the RT-qPCR results show that the expression of *vv-MIR167c/d* under heat stress was higher than that of CK (Supplementary Figure S1). To explore the relationship between the miRNAs and mRNAs, regulatory networks showed that vvi-miR156 has the maximum number of targets (23 genes) (Supplementary Figure S2). This may be because the miR156 family was the most involved in heat stress regulation. The overexpression of miR398 significantly inhibited the expression of *CSD1*, *CSD2* and *CCS*, induced the expression of HSPs and HSFs and enhanced the high temperature tolerance of *Arabidopsis* [21]. The results showed that the *vv-MIR398b/c* genes were significantly involved in the response to thermostability. In addition, the study revealed that the miR165/miR166-*PHABULOSA* (*PHB*) module regulates *HSFA1* at the transcriptional and translation levels, enhancing the thermal stress response of plants [48]. These studies suggest that miRNA regulatory networks play an important role in plant responses to heat stress.

### 4.2. Development and Application of miRNA-SSR Markers

SSRs (Simple sequence repeats) were one of the most vital genetic markers and widely exist in grape genomes. The comparative analysis and development of molecular markers of SSR could help us reveal the genetic variation underlying a variety of biologically functional genes in grape germplasm resources. SSR marker technology has become the main technology for grape germplasm resource identification due to its advantages of high polymorphism, strong stability, simple operation and low requirements on DNA quality [49]. In the early stages of SSR marker development, foreign researchers developed universal grape variety identification markers VVS2, VVMD5, VVMD7, VVMD25, VVMD27, VVMD28, VVMD32, VrZAG79 and VrZAG62 around the world [50–52]. However, due to the inability to satisfy the diverse identification of grape germplasm resources and the mixed use of markers in production, researchers have carried out a lot of phylogenetic studies on SSR molecular markers based on the grape genome. For example, Stavarakaki and Biniari [53] used SSR molecular markers and ampelographic description to analyze the genetic variation of the Eurasian grape. Dunlevy [54] analyzed the genotyping and sodium exclusion phenotype of grape hybrid populations (“K51-40 “×” Schwarzmänn”) based on SSR characteristics in the genome. Zhong [55] identified sequence variation and developed SSR markers across the whole genome of the *Munake Grape*

*Cultivar*. Although RNA-seq technology has made great progress in recent years, the large-scale development of specific SSR markers for miRNA in grapes has not been reported. MiRNA-SSRs were of potential utility for identifying master miRNAs that regulate various genes for growth and development. Given this, a group of miRNA-SSR markers was assayed on grape genotypes in order to indicate their immediate utility for genetic analysis (Figure 3; Supplementary Table S6). In the present study, the entire grape genome and 12 SSR motifs from 192 (6.25%) pre-miRNAs and 391 SSR motifs from 154 (80.21%) pri-miRNAs were analyzed. The total sequence of pre-miRNA was about 18.99 kb, and that of pri-miRNA was about 209.61 kb (Supplementary Table S3). This was similar to the analysis of miRNA-SSRs in other crops such as rice [31], Arabidopsis [36], and pomegranate [35]. In addition, the analysis found that hexa-nucleotide repeats were the most abundant class, followed by tetra-nucleotide repeats in comparison to penta-, tri-, di- and mono-nucleotide repeats (Figures 3 and 7). There was a difference from previous studies on the genome analysis of pomegranate and *Arabidopsis thaliana* [35,36]. These markers have many applications in grape breeding, such as linkage mapping, fine mapping of resistance genes and the selection of resistant varieties through genomic selection. There have been many reports on the research on SSRs in response to high-temperature stress. For example, Sun [56] analyzed the correlation between heat stress function and different tall fescue germplasm resources using SSR markers and identified marker alleles related to function. Simple sequence repeats and agricultural physiological traits were used to assess the heat stress resistance and genetic diversity of exotic and Indian wheat genotypes [57]. Based on SSR markers, the population structure of traits related to high-temperature stress was analyzed in chickpea, and it was found that some traits were significantly correlated with SSR markers [58]. SSRs have been very popular in heat-tolerant breeding. In this paper, 20 germplasm resources with sensitivity and heat tolerance were selected based on the results of the heat tolerance classification of preliminary physiological data (Fv/Fm) (Figure 2). Based on this, we further screened miRNA-SSR molecular markers related to heat resistance. In addition, the prediction analysis of miRNA-SSR (Supplementary Table S6) and the verification of PCR-Sanger sequencing results showed that VMIRSSR156c1, VMIRSSR398b1, VMIRSSR167c3 and VmirssR167d1 had SSR polymorphisms in different grape varieties (Figure 5). Therefore, we speculated that SSR loci on *vv-MIRNA* might alter miRNA expression. It further indicated that miRNA-SSR was closely related to the regulation of grape heat resistance.

Around the globe, grape breeding has been proactively adjusted to traditional targets to adapt to the environmental changes caused by global warming predictions. From this point of view, the development of new high-temperature resistant, high-yield varieties seems to be becoming more and more meaningful. In addition to quickly identifying grape heat resistance molecular markers, we can directly obtain the required target more quickly. In this study, we developed a molecular marker associated with miRNA based on PCR amplification and Sanger sequencing. This method can easily and directly determine the traits of varieties and can be better applied to production.

## 5. Conclusions

A total of 192 *vvi*-pri-miRNAs and 192 *vvi*-pre-miRNAs sequences were analyzed in the grape genome. A total of 391 SSR markers were identified in *vvi*-pri-miRNA, and 12 SSR markers were found in *vvi*-pre-miRNA. In this study, the accuracy of the presence of miRNA-SSR was verified by PCR amplification combined with Sanger sequencing, and a molecular marker VMIRSSR167c3 was developed to distinguish heat-resistant and heat-sensitive types. Notably, this was the genome-wide identification of miRNA-SSRs in grapes. A novel approach to developing molecular markers has been proposed and validated, which provides a reference for identifying more sequences from the genome's non-coding library. The development of heat-resistant molecular markers was beneficial to the genetic improvement of grape germplasm resources and the identification of heat-resistant, high-quality varieties.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae9080931/s1>. Supplemental Table S1: Forty grape germplasm resources; Supplemental Table S2: Sequence of primers used for the SSR and expression validation; Supplemental Table S3: Identification of SSR in Pri- and Pre-miRNAs sequences; Supplemental Table S4: Diversity analysis of grape heat resistance using 13 Pri-miRNA-SSR Markers; Supplemental Table S5: 8 grape germplasm resources; Supplemental Table S6: Frequency distribution of SSR; Supplemental Figure S1: Pri-miRNAs mediate the grape response to high-temperature stress in grape tissue culture plantlets. CK (control group), representing the 0 h; Con, representing the high-temperature stress treatment time (1 h). The line chart shows that SD has three biological replicates. Values represent means  $\pm$  SE (n = 3). Significant differences were determined by a one-tailed Student's *t*-test (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ); Supplemental Figure S2: A comprehensive miRNA-target gene regulatory network was identified in grape for 10 miRNA families (green lines for each family indicate stronger interactions for miRNAs with their target genes based on minimum free energy ratio of hybridization). Green represents the alignment score. The smaller the shape, the better the miRNA matches the target gene. Dark blue represents miRNAs. Red represents the miRNA target genes; Supplemental Figure S3: Gel images were shown, as revealed by miRNA-SSR markers. The markers miRNA-SSR (VMIRSSRNAs) on 1% metaphor gel, L, DNA mass ladder. Lanes 1–8, eight sets of grape genotypes.

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**Conflicts of Interest:** The authors have no competing interest.

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