Identification and functional prediction of cold-related long non-coding RNA (lncRNA) in grapevine

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Plant long non-coding RNA (lincRNA) undergoes dynamic regulation and acts in developmental and stress regulation. In this study, we surveyed the expression dynamics of lncRNAs in grapevine (Vitis vinifera L.) under cold stress using high-throughput sequencing. Two-hundred and three known lncRNAs were significantly up-regulated and 144 known lncRNAs were significantly down-regulated in cold-treated grapevine. In addition, 2 088 novel lncRNA transcripts were identified in this study, with 284 novel lncRNAs significantly up-regulated and 182 novel lncRNAs significantly down-regulated in cold-treated grapevine. Two-hundred and forty-two differentially expressed grapevine lncRNAs were predicted to target 326 protein-coding genes in a cis-regulatory relationship. Many differentially expressed grapevine lncRNAs targeted stress response-related genes, such as CBF4 transcription factor genes, late embryogenesis abundant protein genes, peroxisome biogenesis protein genes, and WRKY transcription factor genes. Sixty-two differentially expressed grapevine lncRNAs were predicted to target 100 protein-coding genes in a trans-regulatory relationship. The expression of overall target genes in both cis and trans-regulatory relationships were positively related to the expression of lncRNAs in grapevines under cold stress. We identified 31 known lncRNAs as 34 grapevine micro RNA (miRNA) precursors and some miRNAs may be derived from multiple lncRNAs. We found 212 lncRNAs acting as targets of miRNAs in grapevines, involving 150 miRNAs; additionally, 120 grapevine genes were predicted as targets of grapevine miRNAs and lncRNAs. We found one gene cluster that was up-regulated and showed the same expression trend. In this cluster, many genes may be involved in abiotic stress response such as WRKY, Hsf, and NAC transcription factor genes.

In eukaryotes, many transcripts are non-coding RNAs (ncRNAs)1-2. Long ncRNA (lincRNA) is a type of ncRNA that is generally >200 nt long and has no discernable coding potential3-4. Most lncRNAs can be broadly classified into three types based on their genomic positions: (1) lncRNAs transcribed from intergenic regions of lncRNAs are known as lincRNAs (long intergenic non-coding RNA); (2) lncRNAs transcribed from intronic regions are long intronic RNAs, which can be transcribed in any orientation relative to coding genes; and (3) long non-coding nature antisense transcripts (lncNAT) that overlap with protein-coding regions or ncRNAs on the opposite strand and antisense RNA5-7. In eukaryotes, different lncRNAs have been shown to be differentially expressed in different tissues or under different stress conditions. This indicates that lncRNAs undergo dynamic regulation and act in the regulation of development and stress response8. LncRNAs have been shown to be involved in gene silencing, the control of flowering time, photomorphogenesis in seedlings, organogenesis in roots, and reproduction in plants9-16. Some lncRNAs can also serve as precursors to small RNAs17-22. Some lncRNAs can regulate proteins or microRNAs (miRNA) by acting as decoys that mimic target DNA or RNA. For example, the Arabidopsis microRNA target mimics the IPS1 lncRNA and the decoy ASCO-lncRNA14,23. This illustrates the competing endogenous RNA (ceRNA) theory, which is well-supported and is now widely accepted17,24. The ceRNA theory states that mRNA, lncRNAs, pseudogenes, and other miRNA sponges share common miRNA binding sites because the amount of any given miRNA is limited25.

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Currently, growing evidence supports the view that non-coding RNAs, including lncRNAs, play important roles in regulating responses to a variety of abiotic and biotic stressors. A previous study has identified 318 lncRNAs responsive to cold and/or drought stress in cassava. In cotton, some lncRNAs were shown to possibly be involved in regulating plant hormone pathways in response to drought stress. Several stress-responsive lncRNAs have been functionally characterized in plant signaling pathways such as lncRNA npc4830, At4/IPS123, and npc53632. In addition, miRNA, another non-coding RNA, was shown to be involved in various abiotic stress responses such as cold stress (chilling or freezing) in plants33–36. LncRNAs compete with other miRNA sponges, such as target gene mRNA, to play important roles in eukaryotes30,37–40. Therefore, lncRNA may play important roles in various abiotic stress responses via the ceRNA mechanism.

Cold stress is an important environmental factor that negatively affects grapevine productivity and quality. However, in grapevine, the function of lncRNA and the relationship between grapevine lncRNA and cold stress or cold stress tolerance are unknown. Here, cold-inducible lncRNAs in grapevine were detected using RNA-sequencing and analysis. The potential function of these lncRNAs, their target genes, and the relationship between grapevine mRNAs, lncRNAs, and miRNAs were also predicted and analyzed. Our aims were to identify the cold-responsive lncRNAs and determine if or how cold stress response in grapevine is related to lncRNA regulation.

**Results**

**Data mining of transcriptome sequencing and identification of lncRNAs in grapevine.** To systematically identify lncRNAs related to cold stress in grapevine, we performed whole transcriptome RNA-seq of grapevine cv. Cabernet Sauvignon that had been submitted to a cold-stress treatment of 4 °C. We generated an average of 12.65 gigabases (Gb) of raw reads per sample from the six samples used for Illumina RNA-sequencing. The total number of raw reads per control (CK) sample (plants were kept under a 16-h light/8-h dark photoperiod at 26 °C) ranged from 220842362 to 274931726, and the number of clean reads in each CK sample ranged from 216561108 to 270342092. The total number of raw reads in each cold treatment sample ranged from 191766324 to 233777742, and the number of clean reads in each cold treatment sample ranged from 186259776 to 223345340. The average mapping rate to the grapevine genome is 63.77%. In total, we identified 56732 transcripts, including 44644 known mRNA transcripts, 2031 known lncRNA transcripts, 7969 novel mRNA transcripts, and 2088 novel lncRNA transcripts. The transcripts of novel lncRNAs predicted here are listed in Table S1, and the transcript names and the related lncRNA gene IDs are listed in Table S2. The transcripts of novel mRNAs predicted here are listed in
Table S3, and the transcript names and the related mRNA gene IDs are listed in Table S4. In all samples, we identified 212 novel lincRNAs, 1933 novel long intronic RNAs, and 688 novel lncNAT. We also found 1893 known lincRNAs, 511 known long intronic RNAs, and 803 known lncNAT in total samples. In addition, we found that it was the most common for the lncRNAs to contain only one exon; lncRNAs containing two exons were the next most common, followed by lncRNAs containing three exons and four exons (Fig. 1A). The lncRNAs less than 500 bp long were most common, followed by the 500–1000 bp long lncRNAs and 1000–1500 bp long lncRNAs (Fig. 1B). We also found that most lncRNAs were located on chromosome 1 (Fig. 1C).

Variation in lncRNA expression among cold stress. In grapevine, 17 known lncRNAs were expressed only in the CK library and 97 known lncRNAs were expressed only in the cold-treated library. The expression heatmaps of all known and novel grape lncRNAs in the CK and cold treatment based on the average FPKM value of each set of replicates. (B) The box plot of expression levels of grape lncRNAs under the control and cold treatment conditions. The y-axis represents the average log2 (FPKM) value of each set of replicates. (C) The violin map of expression levels of grape lncRNAs and mRNAs in the control. The y-axis represents the average log2 (FPKM) value of three replicates. T-test p-values < 0.05 are considered to be significantly different. (D) The violin map of expression levels of grape lncRNAs and mRNAs under cold treatment. The y-axis represents the average log2 (FPKM) value of three replicates. T-test p-values < 0.05 are considered to be significantly different.
treatment, followed by LXLOC_011153 and LXLOC_017876. These significantly up- and down-regulated lncR-NAs were considered the differentially expressed novel lncRNAs (Fig. 3B, Table S5).

**Prediction of target genes of cold-related lncRNA targets in cis-regulatory relationships.** To investigate the possible functions of grape lncRNAs, we predicted the potential targets of lncRNAs in cis-regulatory relationships. We searched for known protein-coding genes located within 10 kb downstream and upstream of all the identified grape lncRNAs. These genes were thought to be the targets of lncRNAs in cis-regulatory relationships if the Pearson and Spearman correlation coefficients between the expression levels of these genes were $\geq 0.6$ or $\leq -0.6$, and $P < 0.05^{26}$.

Our results predicted a total of 2 527 target genes in cis-regulatory relationships of 1 650 lncRNAs in grapevine. In our study, significantly up-regulated or down-regulated lncRNAs were thought to be differentially expressed lncRNAs. Specifically, we found that 242 differentially expressed grapevine lncRNAs were predicted to target 326 protein-coding genes in cis-regulatory relationships, and many differentially expressed grapevine lncRNAs targeted stress response-related genes such as CBF4 transcription factor genes, late embryogenesis abundant protein genes, peroxisome biogenesis protein genes, and WRKY transcription factor genes (Table S6). The expression levels of some target genes in cis-regulatory relationships were positively related to lncRNAs. For example, VIT_216s0100n00030, LXLOC_027751, LXLOC_010422, and VIT_202s0025n00100 were up-regulated under cold stress compared to the CK. Based on our RNA-seq data, their target genes VIT_216s0100g00380 (CBF4

**Figure 3.** Differentially expressed lncRNAs in grapevine treated with cold stress. (A) The Volcano map of differentially expressed known lncRNAs and (B) differentially expressed novel lncRNAs. The x-axis represents the log2 (FPKM) values of the differentially expressed lncRNAs, and the y-axis represents the $-\log_{10}(P\text{ value})$ values of the differentially expressed lncRNAs.
transcription factor), VIT_208s0058g00960 (transcription factor bHLH61), VIT_215s0046g02110 (late embryogenesis abundant protein Lea14-A), and VIT_202s0025g01280 (WRKY transcription factor 41) respectively, were also up-regulated under cold stress compared to the CK. The RNA-seq data was validated by the qRT-PCR results (Fig. 4). The expression levels of some target genes in cis-regulatory relationships were negatively related to lncRNAs. For example, compared to the CK, LXLOC_013001 was down-regulated under cold stress, but its target gene in the cis-regulatory relationship, VIT_217s0000g06350 (chlorophyll a-b binding protein 4), was up-regulated under cold stress when compared to the control. LXLOC_019156 was up-regulated under cold stress, but its target gene in the cis-regulatory relationship, VIT_202s0154g00610 (peroxisome biogenesis protein), was down-regulated under cold stress (Table S6, Fig. 5A). We calculated the correlation coefficient between the expression changes of lncRNAs and their target genes in cis-regulatory relationships under cold stress. As shown in Fig. 5B, the values of the x-axis are the log2fold change of lncRNAs (fold change = FPKM value of genes in the cold treatment/FPKM value of lncRNA genes in the control). The values along the y-axis are the log2fold change of their target genes in cis-regulatory relationships (fold change = FPKM value of genes in the cold treatment/FPKM value of genes in the control). The correlation coefficient was 0.53 (t-test, P < 0.05), indicating that the expression of overall target genes with a cis-regulatory relationship was positively related to the expression of related lncRNAs in grapevine under cold stress (Fig. 5B). The heatmap of expression of lncRNAs and their target genes in cis-regulatory relationships under cold stress based on the log2fold change value also showed that the expression of the overall target genes with cis-regulatory relationships were positively related to the expression of related lncRNAs in grapevine under cold stress (Fig. 5A).

Analysis of target genes of cold-related lncRNAs in trans-regulatory relationships. To investigate the possible functions of grapevine lncRNAs, we predicted the potential targets of lncRNAs in trans-regulatory relationships. RNAplex software was used to identify the lncRNA (parameters: > −30 binding energy) as described in a previous study. The Pearson and Spearman correlation coefficients between the expression of these genes identified using RNAplex and the expression of related lncRNAs must be ≥0.6 or ≤0.6 and P < 0.05, or will be filtered out.

In grapevine, we predicted a total of 574 target genes in trans-regulatory relationships with 422 lncRNAs (Table S6). The results showed that 62 differentially expressed grapevine lncRNAs were predicted to target 100 protein-coding genes in trans-regulatory relationships such as NADH dehydrogenase subunit genes, UDP-glycosyltransferase genes, calcium-transporting ATPase genes, disease resistance protein genes, and glutamate receptor genes. However, most target genes in trans-regulatory relationships were unknown protein coding genes (Table S6). The expression levels of some target genes in trans-regulatory relationships were positively related with lncRNAs. For example, VIT_200s0225n00020 was down-regulated under cold stress, and its target gene, VIT_200s0246g00150 (NADH dehydrogenase subunit 5), in the trans-regulatory relationship was up-regulated under cold stress. Some target genes with trans-regulatory relationships were negatively related to lncRNAs (Fig. 5C).

We calculated the correlation coefficient between the expression changes of lncRNAs and their target genes in trans-regulatory relationships in the cold stress treatment. As shown in Fig. 5D, the values along the x-axis are the log2fold change of lncRNAs (fold change = FPKM value of genes in the cold treatment/FPKM value of lncRNA genes in the CK). The values along the y-axis are the log2fold change of their target genes in trans-regulatory relationships (fold change = FPKM value of genes in the cold treatment/FPKM value of genes in the control). The correlation coefficient was 0.71 (t-test, P < 0.05), indicating that the expression levels of overall target genes

Figure 4. Expression level of cold inducible grapevine lncRNAs and their target genes validated by qRT-PCR. T-test p-values < 0.05 are considered to be significantly different, and "*" represents a p-value < 0.05.
with trans-regulatory relationships were positively related to IncRNAs in grapevine under cold stress (Fig. 5D). The heatmap of expression of IncRNAs and their target genes in trans-regulatory relationships under cold stress based on the log2fold change value also showed that the expression of overall target genes with a trans-regulatory relationship were positively related to the expression of IncRNAs in grapevine under cold stress (Fig. 5C).

**GO enrichment and KEGG pathway analyses for differentially expressed IncRNA targets.** The potential function of grapevine IncRNAs in response to cold stress was studied using gene ontology (GO) annotation and enrichment analysis. Targets of differentially expressed cultivated grapevine IncRNAs were classified into three categories, 438 in biological processes, 231 in molecular functions, and 455 in cellular components. Biological processes contained 16 sub-categories with 299 terms, including the regulation of jasmonic acid mediated signaling pathway (GO: 2000022), regulation of defense response (GO: 0031347), regulation of signal transduction (GO: 0009966), hormone metabolic process (GO: 0042445), regulation of hormone levels (GO: 0010817), transmembrane transport (GO: 0055085), lipid metabolic process (GO: 0006629), chloroplast organization (GO: 0009658), flavonoid biosynthetic process (GO: 0009813), and flavonoid metabolic process (GO: 0009812). Molecular functions contained nine sub-categories with 156 terms, including chlorophyll binding (GO: 0016168), transcription factor activity, sequence-specific DNA binding (GO: 0003700), signal transducer activity (GO: 0004871), transcription factor activity, and transcription factor binding (GO: 0000989). Cellular components contained 10 sub-categories with 85 terms, including chloroplast (GO: 0009507), photosystem (GO:.

**Figure 5.** Comparison of the expression changes of differentially expressed IncRNAs, related target genes, and the correlation between them. The heatmap was generated from the fold change values in the RNA-seq data and was used to visualize the IncRNAs and cis-regulated relation target expression changes (A) and the IncRNAs and trans-regulated relation expression changes (D). (B) The correlation between the expression changes of IncRNAs and cis-regulated relation target. The values along the x-axis are the log2fold change of IncRNAs (fold change = FPKM value of genes in the cold treatment/FPKM value of IncRNA genes in the control). The values along the y-axis are the log2fold change of their target genes in cis-regulatory relationships (fold change = FPKM value of genes in the cold treatment/FPKM value of genes in the control). (D) The correlation between the expression changes of IncRNAs and trans-regulated relation target. The values along the x-axis are the log2fold change of IncRNAs (fold change = FPKM value of genes in the cold treatment/FPKM value of IncRNA genes in the control). The values along the y-axis are the log2fold change of their target genes in trans-regulatory relationships (fold change = FPKM value of genes in the cold treatment/FPKM value of genes in the control).
In molecular functions, the significantly enriched (P < 0.05) GO term was calcium ion transmembrane transporter activity (GO: 0015085). In target genes of differentially expressed grapevine lncRNAs, 87 KEGG (The Kyoto Encyclopedia of Gene and Genome) pathways were obtained and significantly enriched (P < 0.05) KEGG pathways included plant-pathogen interaction (ko04626), anthocyanin biosynthesis (ko00942), homologous recombination (ko03440), and zeatin biosynthesis (ko00908) (Table 1, Fig. 6B).

**Validation of lncRNA expression using qRT-PCR.** We performed qRT-PCR analyses to validate the RNA-seq results from six randomly selected grapevine lncRNAs, VIT_201s0010n00070, VIT_209s0002n00020, VIT_200s0179n00030, VIT_207s0141n00070, VIT_208s0007n00270, and VIT_207s0005n00480. The primers for qRT-PCR are listed in Table S8. The expression results were similar to the deep sequencing data (Fig. 7). VIT_206s0179n00030, VIT_207s0141n00070, and VIT_207s0005n00480 were shown to be up-regulated by the qRT-PCR data, showing a positive correlation with the deep sequencing results. VIT_200s0179n00030, VIT_206s0179n00030, VIT_208s0007n00270 and VIT_209s0002n00020 were down-regulated in both the qRT-PCR and RNA-seq results (Fig. 7, Table S5).

**LncRNAs as potential miRNA precursors.** By aligning miRNA precursors to grapevine lncRNAs, we identified 31 known lncRNAs as 34 grapevine miRNA precursors, including vvi-MIR169h, vvi-MIR399a, vvi-MIR394b, vvi-MIR166a, and vvi-MIR156c (Table 2). We identified 25 novel lncRNA transcripts (19 IncRNA genes) as 22 grapevine miRNA precursors, including vvi-MIR162, vvi-MIR168, vvi-MIR535, vvi-MIR403a,
vvi-MIR3623, and vvi-MIR3630. Some miRNAs may be derived from multiple lncRNAs. For example, vvi-MIR396b may be derived from VIT_211s0016n00330 and LXLOC_003224 (Table 2).

The relationships between grape mRNAs, lncRNAs, and miRNAs. We predicted the lncRNAs as targets or target mimics of miRNAs. In some previous studies, lncRNAs were found as both targets and target mimics of miRNAs. As target mimics, lncRNAs could bind to miRNAs with a three-nucleotide bulge. In our study, we only found lncRNAs that paired with miRNAs without any bulges. These lncRNAs may be targets of miRNAs but not target mimics of miRNAs. Here, 212 lncRNAs as targets of miRNAs in grapevine were involved with 150 miRNAs (Table S9). Additionally, 120 predicted grapevine genes were both the target of grapevine miRNAs and lncRNAs (Table S10).

Gene clusters show the same trends. The Mufzz software was used to cluster grapevine genes into gene clusters showing similar expression trends based on the expression changes of genes in cold treated grapevines. Nine clusters were identified and the genes with the same expression trend were clustered together (Fig. 8). Cluster 5 only contained 19 lncRNAs, Cluster 1 contained one lncRNA, and Cluster 7 did not contain any lncRNAs. Clusters 2, 3, 4, 6, 8, and 9 contained more lncRNAs and their target genes than other clusters (Table S11). For example, Cluster 9 contained 137 lncRNAs and their 89 target genes. In cold treated grapevines, Cluster 9 showed an up-regulated expression pattern (Fig. 8), and in this cluster, 45 lncRNAs were significantly up-regulated under cold stress. In addition, 12 of their target genes were in Cluster 9, and the 12 target genes, which were significantly up-regulated under cold stress, contained LRR receptor-like serine/threonine-protein kinase, hydroxyacylglutathione hydrolase, prolyl 4-hydroxylase subunit alpha-1, calcium-transporting ATPase 2, and some unnamed proteins (Table 3). Cluster 9 contained many ethylene-responsive transcription factor genes, such as two ERF5s and four ABSCISIC ACID-INSENSITIVE 5-like protein genes. Cluster 9 also contained NAM/ATAF/CUC (NAC) trans-cription factor genes, such as NAC 68 and 94, as well as Hsf transcription factor genes, such as HsfA3, MYBA1, flavanone 7-O-glucoside 2’-O-beta-L-rhamnosyltransferase, isoflavone-7-O-methyltransferase 9, and WD repeat-containing protein (Table S11). Cluster 9 also contained 19 WRKY transcription factor genes including WRKY 3, 7, 11, 22, 28, 33, 40, 41, 46, 47, 48, and 50 (Table 4). RNA-seq data showed that 17 of these WRKY genes were significantly up-regulated (Fig. 9A), which was confirmed by the qRT-PCR (Fig. 9B). In cold-treated grapevines, Cluster 3 showed a down-regulated expression pattern (Fig. 8). Cluster 3 contained some ABSCISIC ACID-INSENSITIVE protein genes, auxin response factor genes, proline synthase co-transcribed bacterial homolog protein genes, NAC domain-containing protein genes, basic helix-loop-helix DNA-binding super family protein genes, cold-inducible RNA-binding protein genes, and WRKY transcription factor genes (Table S11).

Discussion
A previous study reported the existence of lncRNAs in plants. As next generation sequencing technology developed, it became possible to identify lncRNAs including those identified in Arabidopsis, rice, maize, cassava, and grapevine (http://genomes.cribi.unipd.it/DATA/V2/V2.1/IncRNA/). However, few studies have been conducted on the roles of lncRNAs involved in abiotic and biotic stress responses. In addition, there has been limited research conducted on the roles of lncRNA involved in abiotic stress response, such as response to cold stress, in grapevine. In this study, we detected the expression changes of lncRNAs in grapevine exposed to cold treatment.
and found 2,088 novel grapevine lncRNAs. Previous studies have also identified novel lncRNAs in other plant taxa including 6,500 novel lncRNAs in *Arabidopsis thaliana*\(^8\), 1,704 novel lncRNAs in maize\(^45\), and 682 novel lncRNAs in cassava\(^28\).

Here, we found that the average expression level of the total lncRNAs was lower than the average expression level of mRNAs in grapevine in both the control and cold treatment conditions (Fig. 2C,D). This indicates that the expression levels of total lncRNAs should be lower than mRNAs in grapevine. In *A. thaliana*, approximately 300 lncRNAs were evidenced to be differentially expressed under abiotic stressors\(^27,31\), and 318 cassava lncRNAs were differentially expressed under cold and drought conditions\(^28\). Here, we found 813 differentially expressed grapevine lncRNAs in the cold stress treatment, showing that more grapevine lncRNAs were differentially expressed under cold stress. We hypothesize that many grapevine lncRNAs may be related to cold stress and may play important roles in cold stress response. Though the expression levels in many lncRNAs changed in the cold treatment, the average expression levels of the total lncRNAs in the cold treatment were similar to the average expression levels of the total lncRNAs under control conditions (Fig. 2B).

We predicted the target genes of cold inducible grape lncRNAs, finding more target genes of cold inducible grapevine lncRNAs in cis-regulatory relationships than in trans-regulatory relationships. This indicated that the target genes in cis-regulatory relationships may be more related to cold stress response. We also analyzed the expression correlation between the total cold inducible grapevine lncRNAs and their target genes, and our results showed that the expression patterns were positively related.

The expression correlation between cold inducible grapevine lncRNAs and their target genes in trans-regulatory relationships were higher than in cis-regulatory relationships. However, some of the expression patterns of lncRNAs were negatively related to their target genes (Fig. 3). A previous study showed that lncRNAs could act as enhancers of gene expression\(^47\). In kiwifruit, the expression of both protein-coding genes and lncRNA

| miRNA and IncRNA as precursor | miRNA and IncRNA as precursor |
|-----------------------------|-----------------------------|
| vvi-MIR156c VIT_204d0088n00030 | vvi-MIR162 LXLOC_012888 |
| vvi-MIR159a VIT_215d0046n00070 | vvi-MIR162 LXLOC_012888 |
| vvi-MIR159b VIT_215d0046n00080 | vvi-MIR164c LXLOC_029337 |
| vvi-MIR160c VIT_210a0092n00020 | vvi-MIR167a LXLOC_00093 |
| vvi-MIR164b VIT_209d0002n00040 | vvi-MIR167b LXLOC_009023 |
| vvi-MIR166a VIT_208d0032n00030 | vvi-MIR167b LXLOC_007999 |
| vvi-MIR166b VIT_212d0034n00230 | vvi-MIR167b LXLOC_007999 |
| vvi-MIR166c VIT_215d0048n00320 | vvi-MIR168 LXLOC_019119 |
| vvi-MIR166d VIT_216a0098n00100 | vvi-MIR168 LXLOC_019119 |
| vvi-MIR166e VIT_202d0025n00230 | vvi-MIR169g LXLOC_028343 |
| vvi-MIR166f VIT_207d0031n00260 | vvi-MIR169r LXLOC_003511 |
| vvi-MIR167d VIT_208d0179n00030 | vvi-MIR169t LXLOC_003511 |
| vvi-MIR167e VIT_205d0020n00290 | vvi-MIR169u LXLOC_003511 |
| vvi-MIR169y VIT_201d0146m00060 | vvi-MIR396b LXLOC_003224 |
| vvi-MIR169z VIT_211d0103n00100 | vvi-MIR396d LXLOC_003867 |
| vvi-MIR169r VIT_211d0103n00110 | vvi-MIR398a LXLOC_000033 |
| vvi-MIR169t VIT_211d0103n00110 | vvi-MIR535a LXLOC_033356 |
| vvi-MIR169u VIT_211d0103n00110 | vvi-MIR535a LXLOC_033356 |
| vvi-MIR171a VIT_214d0068n00210 | vvi-MIR535a LXLOC_033356 |
| vvi-MIR171b VIT_212d0059n00020 | vvi-MIR535b LXLOC_033356 |
| vvi-MIR394b VIT_218d0011n00020 | vvi-MIR535b LXLOC_033356 |
| vvi-MIR394c VIT_218d0011n00020 | vvi-MIR535b LXLOC_033356 |
| vvi-MIR396b VIT_211d0166n00330 | vvi-MIR535c LXLOC_033356 |
| vvi-MIR396d VIT_211d0166n00340 | vvi-MIR535c LXLOC_033356 |
| vvi-MIR399a VIT_210d0033n00240 | vvi-MIR535c LXLOC_033356 |
| vvi-MIR399b VIT_216d0100n00020 | vvi-MIR399b LXLOC_022332 |
| vvi-MIR169h VIT_211d0103n00060 | vvi-MIR403a LXLOC_033356 |
| vvi-MIR169i VIT_211d0103n00070 | vvi-MIR477a LXLOC_033061 |
| vvi-MIR169l VIT_211d0103n00080 | vvi-MIR477a LXLOC_016789 |
| vvi-MIR169m VIT_211d0103n00100 | vvi-MIR3623 LXLOC_014879 |
| vvi-MIR169o VIT_211d0103n00090 | vvi-MIR3630 LXLOC_013003 |
| vvi-MIR319e VIT_211d0166n00290 | vvi-MIR3630 LXLOC_013632 |
| vvi-MIR394c VIT_218d0001n00020 | vvi-MIR3630 LXLOC_013003 |
| vvi-MIR828a VIT_216d0098n00140 | vvi-MIR3633a LXLOC_012920 |
| vvi-MIR3636 VIT_216d0131n00110 | vvi-MIR3633b LXLOC_012920 |

Table 2. grape miRNAs and the lncRNAs as their precursors.

and found 2,088 novel grapevine lncRNAs. Previous studies have also identified novel lncRNAs in other plant taxa including 6,500 novel lncRNAs in *Arabidopsis thaliana*\(^8\), 1,704 novel lncRNAs in maize\(^45\), and 682 novel lncRNAs in cassava\(^28\).

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genes tended to be more positively correlated than negatively correlated in trans-regulatory relationships. Here, we found that the expression of the overall target genes with a cis-regulatory relationship was also positively related to the expression of related lncRNAs in grapevine under cold stress.

Some target genes of cold inducible grapevine lncRNAs in cis-regulatory relationships may be involved in abiotic stress response such as VIT_216s0100g00380 (CBF4 transcription factor), VIT_215s0046g02110 (late embryogenesis abundant protein Lea14-A), and VIT_202s0025g01280 (WRKY transcription factor 41). These genes were also up-regulated in the cold stress treatment. Previous research has shown that CBF family genes...
hormones, such as abscisic acid (ABA), are related to abiotic responses in plants. Molecular function terms of genes that were related to the cold-related lncRNAs contained photosystem (GO: 0009521) and involved in cold response and the regulation of other downstream genes involved in cold response. Cellular component terms of genes were transcription factors or were related to transcription factors. These transcription factors may be binding (GO: 0003700), and transcription factor binding (GO: 0000989) (Fig. 6A, Table S7), indicating that many of genes that are related to cold stress lncRNAs contained transcription factor activity, sequence-specific DNA binding (GO: 0003700), and transcription factor binding (GO: 0000989) (Fig. 6A, Table S7), indicating that many target genes were transcription factors or were related to transcription factors. These transcription factors may be involved in cold response and the regulation of other downstream genes involved in cold response. Cellular component terms of genes that were related to the cold-related lncRNAs contained photosystem (GO: 0009521) and photosystem I (GO: 0009535) (Fig. 6A, Table S7), showing that many target genes may be related to photosystems.

The GO analysis showed that the biological process terms that are related to cold stress lncRNAs contained the regulation of jasmonic acid (JA) mediated signaling pathway (GO: 2000022), regulation of defense response (GO: 0031347), regulation of signal transduction (GO: 0009966), hormone metabolic process (GO: 0042445), and regulation of hormone levels (Fig. 6A, Table S7). Jasmonic acid is related to cold stress response in plants. Other hormones, such as abscisic acid (ABA), are related to abiotic responses in plants. Molecular function terms of genes that are related to cold stress lncRNAs contained transcription factor activity, sequence-specific DNA binding (GO: 0003700), and transcription factor binding (GO: 0000989) (Fig. 6A, Table S7), indicating that many target genes were transcription factors or were related to transcription factors. These transcription factors may be involved in cold response and the regulation of other downstream genes involved in cold response. Cellular component terms of genes that were related to the cold-related lncRNAs contained photosystem (GO: 0009521) and photosystem I (GO: 0009535) (Fig. 6A, Table S7), showing that many target genes may be related to photosystems. Under cold stress, the photosystems have been shown to be related to cold tolerance.

We identified 31 known lncRNAs as 34 grapevine miRNA precursors, including vvi-MIR169h, vvi-MIR399a, vvi-MIR394b, vvi-MIR166a, and vvi-MIR156c (Table 2). In cassava, 12 lncRNAs were identified as 11 known cassava miRNA precursors, including miR156g, miR160d, miR166d, miR167g, and miR169d. The lncRNAs that are precursors of miR156 and miR169 family members were identified in both grape and cassava, indicating that some lncRNAs from different species might have been derived from same ancestral genes.

A previous study has shown that the lncRNAs that acted as target mimics could bind to miRNAs with three-nucleotide bulges. However, our data did not predict similar target mimics that have been found in previous studies, but the data did predict some targets that could bind to miRNAs without three-nucleotide bulges. These lncRNA may be targets of miRNAs. Similarly, a previous report has shown that lncRNAs acting as target genes could bind to miRNAs without bulges. We found that lncRNAs and protein coding genes shared common miRNAs, which could target both lncRNAs and protein coding genes, and miRNAs and lncRNAs shared common target genes in grapevine. We hypothesize that the lncRNAs may regulate protein coding genes via complex pathways in grapevines.

The genes with the same expression trends were clustered together, and the genes in the same cluster may be involved in the same biological process. We identified one cluster (Cluster 9) that showed an up-regulated expression pattern under cold treatment (Fig. 8). In this cluster, many genes may be involved in abiotic stress response such as WRKY transcription factor genes, Hsf transcription factor genes, and NAC transcription factor genes. In Cluster 9, we also found 19 WRKY transcription factor genes, most of which were significantly up-regulated. Cluster 9 contained many lncRNAs and many protein coding genes that are the target genes of the lncRNAs in this cluster. Therefore, we suggest that the cluster may contain one or more pathways related to cold stress response and that lncRNAs may play important roles in cold stress response in this pathway. Because many WRKYs were found in Cluster 9, WRKY family members may play important roles in the key cold stress response pathway. Although none of the WRKY genes in Cluster 9 was a target gene of the lncRNAs, they may still be indirectly

| Genes ID      | Gene annotation | Expressed change       |
|---------------|-----------------|------------------------|
| MXLOC_026074  | WRKY47          | Up-regulated significantly |
| MXLOC_025409  | WRKY33          | Up-regulated significantly |
| VIT_202s0025g01280 | WRKY41    | Up-regulated significantly |
| VIT_204s0008g01470 | WRKY50    | Up-regulated significantly |
| VIT_204s0008g05760 | WRKY3    | Up-regulated significantly |
| VIT_205s0077g00730 | WRKY48    | Up-regulated significantly |
| VIT_206s0004g07500 | WRKY33    | Up-regulated significantly |
| VIT_207s0031g00080 | WRKY7     | No change              |
| VIT_208s0058g00690 | WRKY33    | Up-regulated significantly |
| VIT_209s0018g00240 | WRKY40    | Up-regulated significantly |
| VIT_210s0003g01600 | WRKY65    | Up-regulated significantly |
| VIT_211s0116g01200 | WRKY16    | Up-regulated significantly |
| VIT_211s0052g00450 | WRKY11    | Up-regulated significantly |
| VIT_212s0028g00270 | WRKY28    | No change              |
| VIT_213s0067g03140 | WRKY70    | Up-regulated significantly |
| VIT_215s0046g01140 | WRKY46    | Up-regulated significantly |
| VIT_215s0046g02190 | WRKY22    | Up-regulated significantly |
| VIT_218s0001g10030 | WRKY7    | Up-regulated significantly |

Table 4. WRKY genes in cluster 9.
regulated by lncRNAs or regulated by the expression of lncRNAs; however, this requires further study. Additionally, in this cluster, there are some genes related to anthocyanin or flavonoid biosynthesis such as VvMYBA1, flavanone 7-O-glucoside 2′-O-beta-L-rhamnosyltransferase, isoflavone-7-O-methyltransferase 9, and WD repeat-containing protein (Table 11)\(^57–59\). Previous studies have shown that abiotic stressors (such as cold or heat stress) may regulate anthocyanin or flavonoid biosynthesis-related genes\(^57,58\). Anthocyanins have been shown to be synthesized as protective compounds in response to cold stress \(^60\). Our cluster analysis showed that some key anthocyanin biosynthesis related genes may be located in pathways involved in cold stress response; therefore, lncRNAs in pathways involved in cold stress response are related to these anthocyanin biosynthesis related genes. Supporting our findings, previous studies have shown that biotic or abiotic stressors are related to the biosynthesis of anthocyanins or flavonols in grapevine \(^58,59,61\). Further studies should be conducted on the relationship between anthocyanin/ flavonoid biosynthesis pathways and cold stress as additional results will positively impact viticulture and breeding.

Materials and Methods

Plant materials. One-year-old self-rooted seedlings of the grapevine cv. Cabernet Sauvignon were grown and maintained in the greenhouse under a 16 h light/8 h dark photoperiod at 26 °C. For the cold stress treatment, plant materials under a 16-h light/8-h dark photoperiod were transferred to 4 °C for 4 hours. For the control (CK), plants were kept under a 16-h light/8-h dark photoperiod at 26 °C for 4 hours. The shoot apices with well-developed leaves from these plant materials were collected. Each treatment consisted of three independent replicates. RNA was isolated for the construction of RNA-seq libraries and real-time PCR analysis.

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**Figure 9.** Expression level of WRKY genes in Cluster 9. (A) The heatmap was generated from the FPKM value of WRKY genes in Cluster 9 in each set of replicates. (B) Expression levels of some WRKY genes in Cluster 9 validated by qRT-PCR. *T*-test *p*-values < 0.05 are considered to be significantly changed, and “*” represents a *p*-value < 0.05.
Transcriptome library construction and high-throughput sequencing. Extracted RNA was sent to BGI (Shenzhen, China) for transcriptome library construction. In this process, RNA was treated with a Ribo-Zero™ Magnetic Kit to degrade RNA. First-strand cDNA was generated by First Strand Master Mix and Super Script II reverse transcription (Invitrogen). High-throughput sequencing was performed using a HiSeq 2500 instrument. The clean reads generated by high-throughput sequencing were mapped on the grape genome (http://genomes.criibi.unipd.it/grape/) using the HISAT software (V2.0.4)62, and the reads mapped on the genome were assembled into transcripts using the stringTie software (V1.0.4)93.

Identification of lncRNA. To identify novel grapevine lncRNA transcripts, we first filtered out all mRNA transcripts, transcripts with a length < 200 nt, and known lncRNA transcripts predicted in data from the grape genome database (http://genomes.criibi.unipd.it/grape/)28. Then, we predicted the protein coding ability of the remaining transcripts using the CPC24, txCdsPredict, and CNCi software65. The transcripts without protein coding ability were subsequently employed in the remainder of the study. If transcripts without protein coding ability were not found in any known domain using the pfam database66, we considered them lncRNA transcripts. The known lncRNAs were annotated in the grape genome database (http://genomes.criibi.unipd.it/grape/). Finally, the transcripts with FPKM < 0.5 were removed28.

LncRNAs that were not found near any protein-coding locus (within < 10 kb) are considered lincRNAs56. LncRNAs transcribed from intronic regions are long intronic RNAs, which can be transcribed in any orientation relative to coding genes. LncNAT are those that overlap with protein-coding regions or ncRNAs on the opposite strand and antisense RNA5–7.

Analysis of differentially expressed lncRNAs and mRNAs. DEGseq67 was used to identify the differentially expressed lncRNAs and miRNAs based on an MA-plot68. The lncRNAs significantly up-regulated (fold change > 2, P < 0.05) and down-regulated (fold change < −2, P < 0.05) under cold stress were considered the differentially expressed known lncRNAs.

Quantitative real time PCR validation of lncRNA and protein-coding genes. Quantitative RT-PCR (qRT-PCR) was performed to analyze the expression of lncRNAs following the methods outlined by a previous study26. Primers used in all qRT-PCR experiments are listed in Table S8.

Prediction of lncRNAs as miRNA targets. Target genes (lncRNAs and protein-coding genes) of grape miRNAs were identified using psRobot software set to moderate parameters (penalty score threshold = 2, five-prime boundary of essential sequence = 2, three-prime boundary of essential sequence = 17, maximal number of permitted gaps = 1, and position after gaps permitted = 17)70. Grape miRNAs were downloaded from the mirBase database (http://www.mirbase.org/).

GO and KEGG pathway analysis. Target genes were annotated based on the GO database (http://www.geneontology.org/). Pathway analyses of target genes were performed using the KEGG database (http://www.genome.jp/kegg/kegg1.html)71.

Analysis of miRNAs derived from lncRNAs. The grape precursors of the miRNAs dataset from miRbase were downloaded. The miRNAs were mapped to lncRNAs using the STAR program. The miRNAs were thought to be derived from lncRNAs if the identification between precursors of the miRNAs could be mapped to lncNRas28.

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**Author Contributions**
P.W., Y.W. and F.R. designed the study, P.W. wrote the manuscript. P.W., J.A. and L.D. carried out most of the experiment, data analysis, and wrote the method section of the manuscript.

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