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# Identification and Analysis of the Expression of microRNAs during the Low-Temperature Dormancy Release of *Tulipa thianschanica* Seeds

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**Abstract:** With versatile biological functions, microRNAs (miRNAs) participate in the regulation of post-transcriptional gene expression in plants. *Tulipa thianschanica* Regel is a key wild tulip resource of the Liliaceae; however, it reproduces poorly under natural conditions, and the spreading and expansion of its population rely on the release of its seeds. In this study, *T. thianschanica* seeds were subjected to stratification at 4 °C for three different durations: 1 d (physiological dormancy), 20 d (dormancy release), and 40 d (non-dormancy). Nine samples were selected, and miRNA databases were established and annotated. This research revealed information on 34 miRNA families, including 14 newly discovered families and 20 families that were previously known. *TpmiRNA167* and *TpmiRNA395* were observed to be upregulated. Conversely, *TpmiRNA166*, which targets the key enzymes involved in antioxidant reactions, was downregulated. Notably, *PC-5p-84014* and *TpmiRNA159* were found to target *TpPYL* and *TpDELLA*, respectively, which influenced the abscisic acid/gibberellin (ABA/GA) ratio. Additionally, *TpmiRNA160* and *TpmiRNA164* were found to participate in the indole-3-acetic acid signal transduction pathway and interact with ABA, thereby contributing to the release of dormancy. In conclusion, this study provides a comprehensive understanding of the mechanism used to release dormancy in *T. thianschanica* seeds at the molecular level.

**Keywords:** *Tulipa thianschanica*; dormancy; low temperature; microRNAs



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## 1. Introduction

*Tulipa thianschanica* Regel is distributed in western Xinjiang and central China and showcases its striking colors, unique flower types, and strong resistance. *T. thianschanica* reproduces through bulbs or seeds in the wild [1,2]. Bulb propagation is advantageous because it expedites the time of cultivation, which ensures earlier returns on investment. However, this method has the following drawbacks: (1) it diminishes the population of bulbs in natural habitats and harms the environment and (2) the coefficient of bulb propagation is low because a 4 to 5 year old bulb yields only approximately 1–3 cormels. Consequently, seed propagation has become the preferable choice to artificially cultivate *T. thianschanica* on a large scale [3]. Unfortunately, *T. thianschanica* seeds display low rates of germination under natural conditions. Seeds released from mature capsules require an extended period to fully break morphological and physiological dormancy before germination [3]. Investigating the mechanism of germination of tulip seeds holds significant implications to accelerate the germination and breeding of wild tulip seeds.

MicroRNAs (miRNAs), a class of biosynthesized small RNAs that are involved in degrading target gene mRNAs or inhibiting mRNA translation [4,5], are widely studied and relatively conserved in plants. Seed development is a complex biological process

that encompasses the development of embryos, endosperms, and seed coats and the accumulation of storage substances [6]. This process undergoes intricate genetic regulation at multiple levels with miRNAs playing a crucial role as a mode of genetic regulation. miRNAs govern the development, size, vitality, and activity of seeds, while also influencing their physicochemical properties and nutritional composition [7,8]. Studies have demonstrated the regulatory roles of various miRNAs, including miR160, miR159, miR417, miR395, miR402, miR165/166, miR164, miR167, miR156, miR172, and miR158, in the germination and dormancy of seeds [9–11]. An increase in the levels of miR156 in mature embryos leads to a decrease in the levels of SQUAMOSA Promoter-Binding Protein-Like (SPLs) and miR172, which effectively reduces the speed of the seed developmental process and maintains dormancy [9]. During seed imbibition, 12 miRNA families, namely, miR156, miR159, miR164, miR166, miR167, miR168, miR169, miR172, miR319, miR393, miR394, and miR397, have been reported to be down regulated to varying degrees, while four miRNA families (miR398, miR408, miR528, and miR529) are upregulated during seed germination [12]. miR156 and miR172 have also been associated with the transition from the vegetative to the reproductive period [13], which highlights their diverse functions. The identification of two abscisic acid (ABA)-sensitive mutants, absg1 and absg2, revealed that there is complex regulation between indole-3-acetic acid (IAA) and the small RNAs, with absg1 and absg2 identified as alleles of dcl1 and hen1, respectively; these mutants positively regulate the expression of ABA-responsive genes [14,15]. miR159 plays a crucial role in the regulation of seed germination by modulating gibberellic acid (GA) and ABA signals [11]. miR159 regulates GAMYB mRNAs during flower development, fertility, and seed germination [5]. The GAMYB protein acts as a positive regulator, while DELLA serves as a negative regulator in the GA signaling cascade [16,17]. Additionally, miR159 also regulates the transcription factors (TFs) MYB33 and MYB101, which act as positive regulators of ABA signals during seed germination and dormancy [5,11]. miR159 was upregulated in the rdr2, dcl2, dcl3, and dcl4 triple mutants. RDR2, DCL2, DCL3, and DCL4 are critical factors that are involved in the generation of small interfering RNAs (siRNAs), particularly during the biosynthetic pathway of heterochromatin siRNAs [18,19]. This indicates that in addition to miRNAs, different types of small RNAs also play a significant role in seed germination and dormancy.

Drawing on the characteristic stem-ring structure of pre-miRNAs, many researchers have developed multiple software programs to predict pre-miRNAs and mature miRNAs in plants. These studies primarily utilize genomic data combined with the sequencing information for small RNAs. The comprehensive collection of miRNA data is available in miRbase V23 [20], which is a database that encompasses miRNAs from both plant and animal species. Recently, a plant-specific miRNA database called the Plant MicroRNA Encyclopedia (PmiREN) has been introduced, which incorporates information on the miRNA of 88 plant species and more than 16,000 predicted miRNA loci (http://www.pmiren.com/, accessed on 16 December 2022). While high-throughput sequencing has been used to identify numerous miRNAs from various plant species that have been cataloged in miRBase22.1 (http://www.mirbase.org/, accessed on 14 March 2021), there are no reported miRNAs specific to *T. thianschanica*.

In this study, we used high-throughput sequencing to analyze nine small RNA sequencing libraries. Our objective was to classify miRNAs and their corresponding target genes during the release of *T. thianschanica* seeds from dormancy under low-temperature conditions. The aim was to predict the regulatory role of miRNAs in seed dormancy and advance our understanding of the mechanisms that underlie the seed dormancy of *T. thianschanica*. Furthermore, molecular methods were utilized to modulate germination and accelerate the breeding of tulip seeds.

## 2. Materials and Methods

### *2.1. Collection and Pre-Treatment of Seeds*

The materials used in this experiment were obtained from the National Tulip Germplasm Bank, Liaoning Academy of Agricultural Sciences (Shenyang, China) (N 41°49′41.40″, E 123°32′39.66″). Mature *T. thianschanica* seeds were dried and stored at room temperature. *T. thianschanica* seeds (Figure 1) were subjected to stratification (according to Zhang, 2020) at 4 °C for 1 day (physiological dormancy [PD]), 20 days (dormancy release [DR]), and 40 days (non-dormancy [ND]) and then stored at −80 °C.

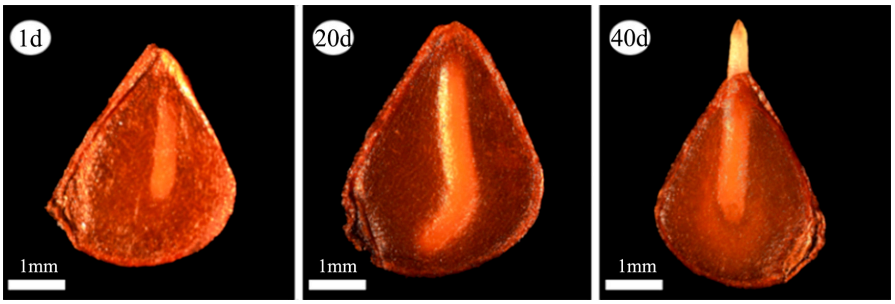


**Figure 1.** Seeds of *Tulipa thianschanica* used in deep sequencing.

### *2.2. Total RNA Extraction and Establishment of the miRNA Library*

The total RNA was extracted from *T. thianschanica* seeds using the CTAB method. The degree of RNA degradation and contamination was assessed using 1.0% agarose gel electrophoresis. The concentration and purity of RNA ($A_{260/280}$ and $A_{260/230}$, respectively) were determined using a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). MiRNA sequencing libraries were established for nine samples, including PD-1, PD-2, PD-3, DR-1, DR-2, DR-3, ND-1, ND-2, and ND-3, using TruSeq Small RNA Sample Prep Kits (Illumina, San Diego, CA, USA). Subsequently, sequencing was performed on an Illumina HiSeq 2500 platform with a 50 bp/read length. The resulting high-quality data were compared with the GtRNAdb, Silva, Rfam, and Repbase databases. Non-coding RNAs, such as rRNA, tRNA, snRNA, and snoRNA, as well as repeat sequences, were filtered out. miRDP2-v1.1.4 software (San Diego, CA, USA) was utilized to compare the sequenced reads with the reference genome sequences of closely related species to obtain mapped reads. PD was the control group, and DR and ND were the treatment groups.

### *2.3. Identification and Functional Annotation of the Target Genes of miRNAs*

BLAST was utilized to compare the predicted miRNA target genes with the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases to obtain functional annotations. Low-quality and spliced tags were removed to obtain high-quality clean tags and the corresponding clustering data. These data were then compared with the Rfam database to annotate non-coding RNAs, and degradation sites were identified using a Cleveland analysis with a significance threshold set at $p < 0.05$.

### *2.4. Analysis of the Differential Expression of miRNA*

To identify differentially expressed miRNAs, the levels of expression of miRNA were normalized as tags per million (TPM), and differential expression was detected using IDEG6 with the criteria of $|\log2(FC)| \geq 1$; FDR $\leq 0.01$. The fold change (FC) represents the ratio of the expression between two samples. The $p$ value of the original hypothesis represents the probability of expressing indifference, which was corrected by Benjamini–Hochberg, and FDR was eventually selected as the key indicator to screen differentially expressed genes.

### *2.5. Verification of the Target Genes of miRNAs by qRT-PCR*

To explore the correlation between miRNAs and their predicted target genes, six pairs of miRNA–mRNA were randomly selected from the sequencing database for a real-time

quantitative reverse transcription PCR (qRT-PCR) analysis of the target genes using tulip *Actin* as the internal reference. Reference and candidate genes were measured simultaneously with each sample repeated three times, and gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Specific primer sequences are shown in Table S1. The method of extracting the total RNA was the same as that described above. The RNA from *T. thianschanica* seeds was extracted after stratification for 1 day, 20 days, and 40 days to quantitatively measure the gene. The qRT-PCR reaction system, program, and reactants are shown in Tables S2 and S3.

### *2.6. Statistical Analysis*

SPSS 24.0 (IBM, Inc., Armonk, NY, USA) was utilized for statistical analyses. The data were expressed as the mean ± standard deviation, and Duncan's method was used to assess the significance and perform inter-group comparisons. A difference was considered significant when $p < 0.05$. GraphPad Prism 8.0.1 (GraphPad Software, La Jolla, CA, USA) was used to visualize the data.

## 3. Results and Analysis

### *3.1. miRNA Sequencing of Seeds at Different Stages of Low-Temperature Stratification*

To investigate the regulatory mechanism of miRNAs during the germination of *T. thianschanica* seeds at different stages of stratification, the total RNA was collected and subjected to sequencing. Illumina sequencing technology was utilized, which resulted in the generation of 46,145,598 raw reads across nine samples (three for each of PD, DR, and ND). After the adapter sequences were removed along with low-quality reads and sequences outside the range of 18–30 nt, more than 60% of the original reads remained, which yielded 73,534,151 valid reads from the nine samples tested (Table 1).

**Table 1.** Results of miRNA sequencing of the *Tulipa thianschanica* seed libraries.

| Sample | Total Reads (%) | 3ADT and Length Filter (%) | Junk Reads (%) | Rfam (%) | Repeats (%) | Valid Reads (%) |
|---|---|---|---|---|---|---|
| PD-1 | 17,035,681 (100) | 3,287,565 (19.30) | 53,503 (0.31) | 1,425,279 (8.37) | 8550 (0.05) | 12,266,027 (72.00) |
| PD-2 | 13,530,443 (100) | 3,063,073 (22.64) | 38,381 (0.28) | 1,346,965 (9.96) | 7442 (0.06) | 9,079,240 (67.10) |
| PD-3 | 15,579,474 (100) | 3,112,538 (19.98) | 49,475 (0.32) | 1,286,262 (8.26) | 8658 (0.06) | 11,127,570 (71.42) |
| DR-1 | 14,669,850 (100) | 5,914,655 (40.32) | 18,357 (0.13) | 2,270,576 (15.48) | 45,245 (0.31) | 6,456,516 (44.01) |
| DR-2 | 11,288,606 (100) | 3,695,920 (32.74) | 27,623 (0.24) | 1,439,851 (12.75) | 16,100 (0.14) | 6,120,230 (54.22) |
| DR-3 | 13,881,039 (100) | 4,534,585 (32.67) | 36,418 (0.26) | 1,700,172 (12.25) | 15,765 (0.11) | 7,604,768 (54.79) |
| ND-1 | 14,222,034 (100) | 4,408,012 (30.99) | 27,732 (0.19) | 1,718,145 (12.08) | 21,017 (0.15) | 8,060,083 (56.67) |
| ND-2 | 12,884,793 (100) | 4,592,940 (35.65) | 18,327 (0.14) | 1,563,487 (12.13) | 26,889 (0.21) | 6,701,697 (52.01) |
| ND-3 | 12,143,228 (100) | 4,863,545 (40.05) | 25,445 (0.21) | 1,131,736 (9.32) | 11,006 (0.09) | 6,118,020 (50.38) |

Note: Rfam, number of non-miRNA sequences annotated in the Rfam database; repeats, the number of non-miRNA sequences annotated in the Repbase database.

As shown in Figure 2, small RNA sequences that ranged from 18 to 25 nt in length were subjected to a statistical analysis. The most abundant small RNA sequence was 24 nt, followed by 20 nt and 22 nt. The frequency share of 24 nt decreased from PD to DR and then to ND, and the frequency share of 19 nt, 21 nt, and 22 nt increased from PD to ND. There was a lower proportion of 18 nt sequences than those for the other small RNA sequences, and their summed frequency share accounted for <40%. These patterns of length distribution closely resembled the typical distribution of small RNAs in angiosperms.

The tested miRNAs were simultaneously compared with ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nuclear RNAs (snRNAs), and nucleolar RNAs (snoRNAs). The results revealed that only a cumulative small fraction of 8.5–16.0% of the miRNAs detected could be identified. During the release of *T. thianschanica* seeds from dormancy, more than 80% of miRNAs with unknown functions were not annotated, as shown in Table 2.

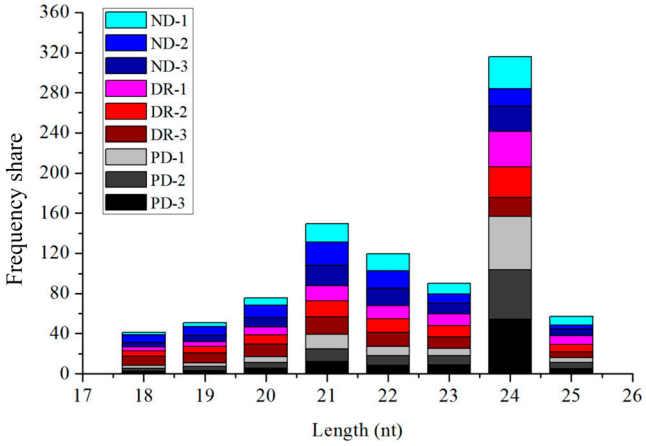


**Figure 2.** Distribution of miRNA lengths in *Tulipa thianschanica* seeds. DR, dormancy release; ND, non-dormancy; PD, physiological dormancy.

**Table 2.** Numbers of reads classified according to the types of miRNAs identified.

| Sample | rRNA | tRNA | snoRNA | snRNA | Rfam RNA |
|---|---|---|---|---|---|
| PD-1 | 813,906 (4.78%) | 519,783 (3.05%) | 11,783 (0.07%) | 33,001 (0.19%) | 46,806 (0.27%) |
| PD-2 | 765,727 (5.66%) | 476,440 (3.52%) | 12,430 (0.09%) | 46,725 (0.35%) | 45,643 (0.34%) |
| PD-3 | 683,463 (4.39%) | 504,865 (3.24%) | 12,422 (0.08%) | 40,735 (0.26%) | 44,777 (0.29%) |
| DR-1 | 1,267,257 (8.64%) | 865,419 (5.90%) | 18,345 (0.13%) | 29,577 (0.20%) | 89,978 (0.61%) |
| DR-2 | 887,866 (7.87%) | 468,740 (4.15%) | 6274 (0.06%) | 28,979 (0.26%) | 47,992 (0.43%) |
| DR-3 | 1,091,435 (7.86%) | 501,606 (3.61%) | 7150 (0.05%) | 39,114 (0.28%) | 60,867 (0.44%) |
| ND-1 | 879,050 (6.82%) | 317,343 (2.61%) | 4989 (0.04%) | 26,628 (0.22%) | 48,241 (0.40%) |
| ND-2 | 734,535 (6.05%) | 571,427 (4.43%) | 9242 (0.07%) | 43,361 (0.34%) | 60,407 (0.47%) |
| ND-3 | 1,061,289 (7.46%) | 541,407 (3.81%) | 8030 (0.06%) | 41,243 (0.29%) | 66,176 (0.47%) |

Note: DR, dormancy release; ND, non-dormancy; PD, physiological dormancy; rRNA, ribosomal RNA; tRNA, transfer RNA; snRNA, small nuclear RNA; snoRNA, nucleolar RNA.

### *3.2. Identification of the Conserved miRNAs*

Numerous miRNAs are relatively conserved among plants. As illustrated in Figure 3, a consensus-based approach identified 54 conserved miRNAs, which belonged to 18 families. The most abundant family was miRNA167, which consisted of seven members, followed by miRNA159, miRNA166, and miRNA171. Furthermore, most families, such as miRNA156, miR169, miRNA160, miRNA164, and miRNA403, only contained one or two members.

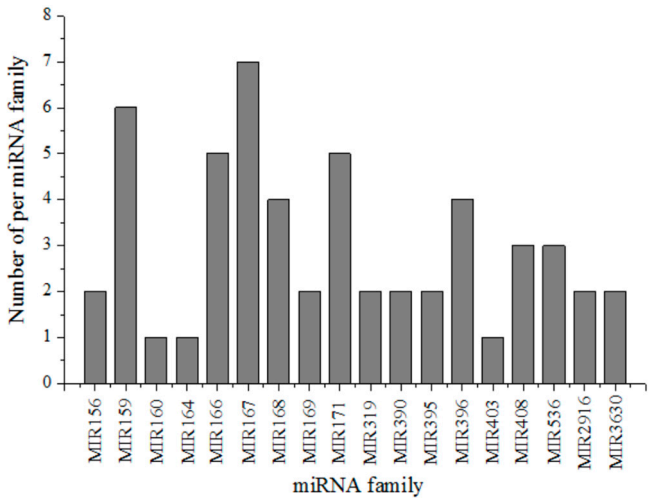


**Figure 3.** Numbers of conserved miRNAs in each miRNA family of *Tulipa thianschanica* seeds.

### *3.3. Identification of Novel miRNAs*

To identify novel miRNAs from unlabeled small RNA labels, miREvo and miRDeep2 were used to predict the secondary structure and Dicer cutting sites and to measure the minimum free energy. As shown in Table 3, 14 novel miRNAs were predicted from nine samples. The length of new mature miRNAs ranged from 18 to 24 nt, and approximately 64.3% of the miRNAs were 21 nt. The rest were 19 nt, 20 nt, and 24 nt. There was >35% CG in all the novel miRNAs. Except for PC-3p-190777_6, the expression abundance of other miRNAs was relatively high. There were fewer newly discovered miRNAs (14) than conserved miRNAs (67), and these novel miRNAs could represent novel miRNA families or correspond to unknown members of known miRNA families.

**Table 3.** Novel miRNAs identified in the *Tulipa thianschanica* seeds.

| miRNA | miRNA Sequence | Length (nt) | CG % | dG | Level of Expression |
|---|---|---|---|---|---|
| PC-5p-12886_384 | TGGACCAATCACAGCAAGAAT | 21 | 35.40 | −101.90 | high |
| PC-3p-2448_3032 | TATTCTTGCTGTGATTGGTCC | 21 | 35.40 | −101.90 | high |
| PC-5p-29011_123 | ACGGAACGCACCTCCGAACGCACC | 24 | 61.00 | −104.10 | high |
| PC-3p-190777_6 | TGCGTTTCGGAGGTGCGTTCC | 21 | 61.00 | −104.10 | low |
| PC-5p-26456_140 | ACCCCAAATCCCCCGAGAAGC | 21 | 54.00 | −48.10 | high |
| PC-3p-212_24745 | TTTTCGGGTGATTTGAGGTGG | 21 | 54.00 | −48.10 | high |
| PC-5p-20023_207 | GGAATGGTGTGATCGGTAAAT | 21 | 44.00 | −53.30 | high |
| PC-5p-84014_23 | GTTCCCTCCGGCACTTCACC | 20 | 54.40 | −51.70 | high |
| PC-5p-80803_25 | AAGCAAAGTGAACTAGAGC | 19 | 46.30 | −68.90 | high |
| PC-3p-73564_29 | TTGTATCTCACTTGTAGTCCT | 21 | 41.90 | −79.60 | high |
| PC-3p-104391_16 | CTCAATTGTAGACTTGACC | 19 | 37.80 | −28.70 | high |
| PC-3p-95380_19 | TTGTAGACCTAACCTTGTAGA | 21 | 43.60 | −101.30 | high |
| PC-5p-54971_47 | TGATAGTGATTGATGAAGCTC | 21 | 34.30 | −31.50 | high |
| PC-5p-46859_61 | ACCTCAAATCCCCCGAAAAGC | 21 | 55.10 | −66.30 | high |

Note: miRNA, microRNA.

### *3.4. Analysis of the Differentially Expressed miRNAs during the Release of Seed Dormancy*

Based on the findings shown in Figure 4, 60 differentially expressed miRNAs ($p < 0.05$) were determined from nine samples throughout three sequencing periods. A total of 21 miRNAs were significantly differentially expressed, while 71 miRNAs reached a differential level of expression of 0.1. A screening criterion of 0.05 was used to observe that during the ND vs. PD process, the highest number of differentially expressed miRNAs was 24 with 14 upregulated and 10 downregulated. During the early stages of low-temperature stratification (PD-DR), 13 miRNAs were downregulated, whereas eight miRNAs were upregulated. As the dormancy gradually dissipated at the later stages of stratification, the number of differentially expressed miRNAs decreased. Notably, there were five downregulated miRNAs, which was precisely half of the number of 10 upregulated miRNAs.

Considering the characteristics of the expression of miRNAs, 21 miRNAs that exhibited different patterns of co-expression were selected for cluster analysis. As shown in Figure 5, the 21 differentially expressed miRNAs could be categorized into two groups. The first group was highly expressed in the PD stage, and their levels of expression gradually decreased with the progression of stratification stages. In this group, eight miRNAs showed no significant changes from DR to ND but increased in PD, and three miRNAs changed from higher, lower, to higher. The second group exhibited an increasing pattern of expression as the time of stratification advanced. There were three miRNAs that also changed from higher, lower, to higher, and seven miRNAs showed a trend of continuous increasing. These phased changes in expression signified the critical regulatory role of miRNAs during the process of seed germination.

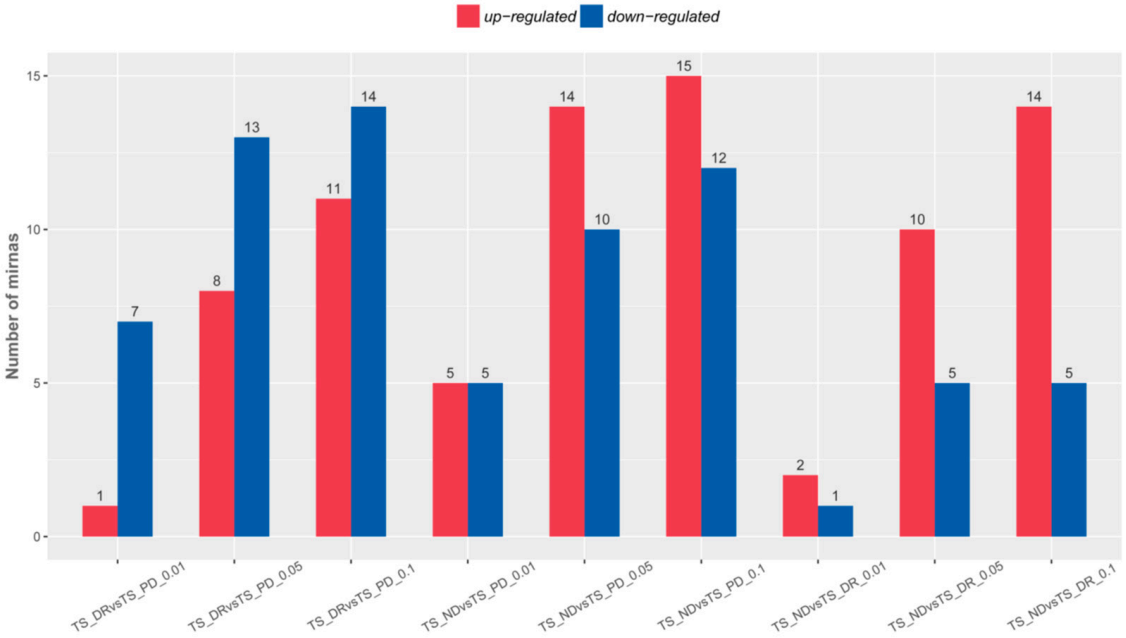


**Figure 4.** Number of differentially expressed miRNAs.

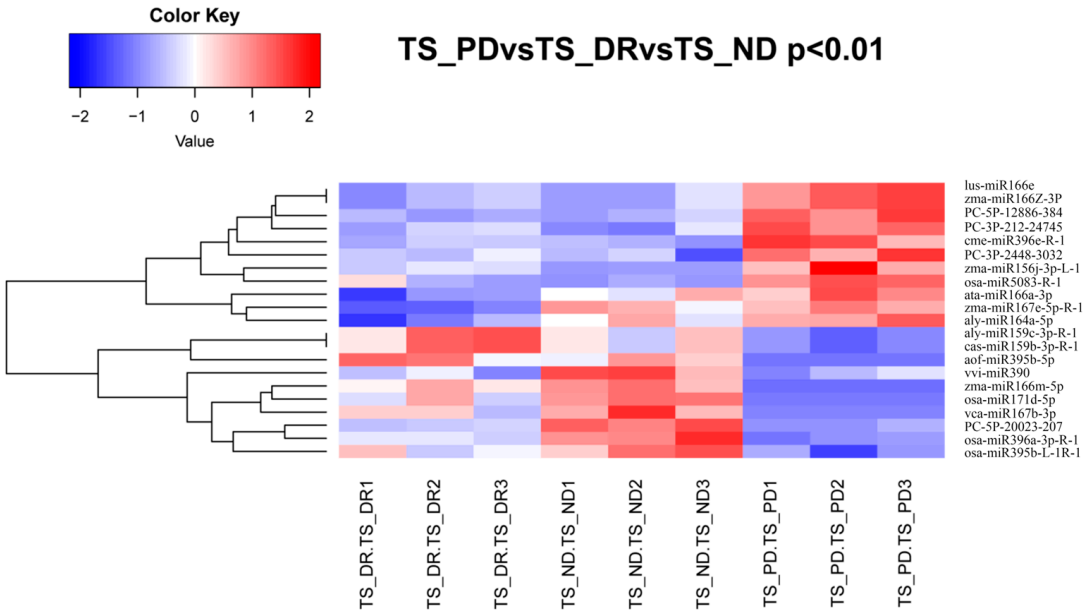


**Figure 5.** Heat map of differentially expressed miRNAs.

### *3.5. Prediction and Functional Annotation of the Target Genes of the miRNAs*

To further understand the important role of miRNAs in the release of *T. thianschanica* seeds from dormancy, the known sequenced miRNAs and their corresponding all target genes were analyzed by GO enrichment. As shown in Figure 6, 337 target genes were noted to be involved in biological processes (150, total 44.51%), cell components (101, total 29.97%), and molecular functional modules (86, total 25.52%) with significant differences in expression ($p < 0.05$).

As shown in Table 4, further attention was paid to subclasses that are related to seed dormancy, including regulation of transcription (DNA-templated) in the BP components, transcription (DNA-templated), primary shoot apical meristem specification, meristem initiation, regulation of RNA biosynthetic processes, cellular response to freezing, response to gibberellin, and auxin-activated signaling pathway. The CC components primarily included

the histone deacetylase complex, mitochondrion, nuclear chromosome, and peroxisome, which are involved in transport and aerobic metabolism. Of the MF components, the activities and transport functions of the enzymes related to energy metabolism have been largely annotated, including double-stranded DNA binding, ATP binding, carboxypeptidase activity, and acetyl-CoA C-acetyltransferase activity, among others, were annotated. There were some differences in the number of commented target genes in different subclasses with the proportion of significantly commented target genes ranging from 33.3 to 100%.

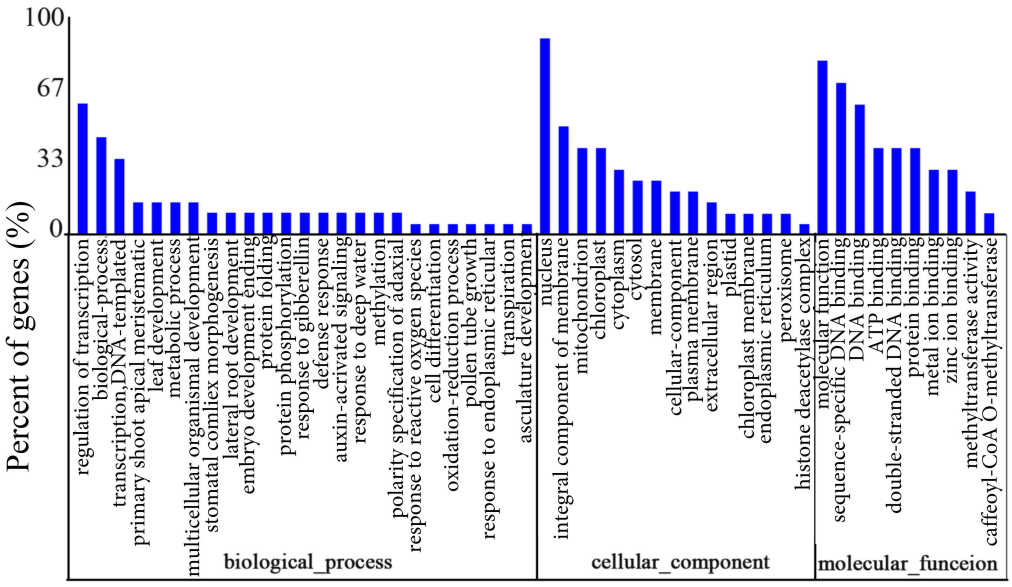


**Figure 6.** Enriched GO term analysis of target genes of the miRNAs in *Tulipa thianschanica* seeds.

**Table 4.** GO functional annotation of the target genes of differentially expressed miRNAs.

| GO Term | GO ID | Annotated | Significant | % |
|---|---|---|---|---|
| **Biological Process** | | | | |
| Primary shoot apical meristem specification | GO:0010072 | 5 | 3 | 60 |
| Regulation of transcription, DNA-templated | GO:0006355 | 15 | 12 | 80 |
| Response to deep water | GO:0030912 | 2 | 2 | 100 |
| Stomatal complex morphogenesis | GO:0010103 | 2 | 2 | 100 |
| Leaf development | GO:0048366 | 3 | 3 | 100 |
| Polarity specification of adaxial/abaxial axis | GO:0009944 | 4 | 2 | 50 |
| Lateral root development | GO:0048527 | 2 | 2 | 100 |
| Transcription, DNA-templated | GO:0006351 | 9 | 7 | 77.8 |
| Regulation of lignin biosynthetic process | GO:1901141 | 1 | 1 | 100 |
| Meristem initiation | GO:0010014 | 3 | 1 | 33.3 |
| Regulation of RNA biosynthetic process | GO:2001141 | 1 | 1 | 100 |
| Determination of bilateral symmetry | GO:0009855 | 3 | 1 | 33.3 |
| Cellular response to freezing | GO:0071497 | 1 | 1 | 100 |
| Integument development | GO:0080060 | 3 | 1 | 33.3 |
| Response to gibberellin | GO:0009739 | 2 | 2 | 100 |
| Auxin-activated signaling pathway | GO:0009734 | 2 | 2 | 100 |
| **Cellular component** | | | | |
| Histone deacetylase complex | GO:0000118 | 1 | 1 | 100 |
| Mitochondrion | GO:0005739 | 9 | 5 | 55.6 |
| Nuclear chromosome | GO:0000228 | 1 | 1 | 100 |
| Nucleus | GO:0005634 | 20 | 8 | 40 |
| Integral component of mitochondrial outer membrane | GO:0031307 | 1 | 1 | 100 |
| Peroxisome | GO:0005739 | 8 | 2 | 25 |

**Table 4.** *Cont.*

| GO Term | GO ID | Annotated | Significant | % |
|---|---|---|---|---|
| **Molecular function** | | | | |
| Double-stranded DNA binding | GO:0003690 | 4 | 4 | 100 |
| Sequence-specific DNA binding transcription factor activity | GO:0003700 | 9 | 7 | 77.8 |
| Sigma factor activity | GO:0016987 | 1 | 1 | 100 |
| Plastid sigma factor activity | GO:0001053 | 1 | 1 | 100 |
| Carboxypeptidase activity | GO:0004180 | 1 | 1 | 100 |
| Acetyl-CoA C-acetyltransferase activity | GO:0003985 | 1 | 1 | 100 |
| Chorismate mutase activity | GO:0004106 | 2 | 1 | 50 |
| L-ascorbate peroxidase activity | GO:0016688 | 2 | 1 | 50 |
| Methyltransferase activity | GO:0008168 | 2 | 2 | 100 |
| Calcium-transporting ATPase activity | GO:0005388 | 1 | 1 | 100 |
| Caffeoyl-CoA O-methyltransferase activity | GO:0042409 | 1 | 1 | 100 |
| DNA binding | GO:0003677 | 8 | 6 | 75 |
| Signal transducer activity | GO:0004871 | 2 | 1 | 50 |
| ATP binding | GO:0005524 | 4 | 2 | 50 |
| Oxidoreductase activity | GO:0016491 | 1 | 1 | 100 |

Note: GO, Gene Ontology; miRNA, microRNA.

### *3.6. Analysis of the Expression of miRNAs Related to Dormancy and Their Target Genes*

To further investigate the role of miRNAs in the seed dormancy of *T. thianschanica*, a comprehensive screening was conducted to identify 26 candidate miRNAs associated with the release of seed dormancy considering the annotation information of target genes (Table 5). Among these candidates, 20 were conserved miRNAs, whereas 6 were newly annotated miRNAs, which corresponded to a total of 37 and 9 target genes, respectively. Remarkably, it was observed that certain miRNAs can simultaneously regulate multiple target genes. For example, aly-miR164a-5p was found to target three genes, while cme-miR396e_R+1 regulated five target genes. Interestingly, more than half of the miRNAs targeted a single gene. Additionally, miRNAs from the same family exhibited diverse functions by regulating different target genes. For example, cas-miR159b-3p_R+1 and aly-miR159c-3p_R+1_1ss20CT targeted *CM1* (Chorismate mutase 1), and aof-miR159f targeted *DELLA*. Similarly, some miRNAs targeted the same gene, such as *lus-miR166e* and *zma-miR166l-3p*, suggesting that miRNAs from different families may share similar regulatory patterns or possibly represent functionally equivalent miRNAs. Similar findings were observed for the newly annotated miRNAs in this study, such as PC-3p-212_24745 and cme-miR396e_R+1, which both targeted ARF4.

To establish the correlation between the dynamic expression of miRNAs and their target genes, fluorescence quantitative experiments were conducted on six miRNAs that were related to hormone metabolism and their corresponding target genes. Figure 7 illustrates the patterns of expression of these miRNAs, which could be classified into two categories. The first category included *Tp-mi156* and PC-5p-84014, which were continuously upregulated or downregulated. The second category comprised the remaining four miRNAs, which exhibited phased changes in their expression. Notably, *Tp-mi156*, *Tp-mi159*, *Tp-mi164*, *Tp-mi4995*, and PC-5p-84014 negatively correlated with their respective target genes *TpGA20ox-like*, *TpDELLA8-like*, *TpNAC22-like*, *TpCYP707A1-like*, and *TpPYL1-like*. This observation was consistent with the expected function of miRNA-mediated cleavage. However, PC-3p-212-24745 and its corresponding target gene *TpARF4-like* displayed similar patterns of expression, indicating a positive correlation. These findings suggest the possibility of a feedback regulatory mechanism between the miRNA and its target gene (Figure 8).

**Table 5.** Target genes of the miRNAs that are related to the release of dormancy in *Tulipa thianschanica* seeds.

| miRNA Family | Target Genes | Annotation |
|---|---|---|
| zma-miR156j-3p_L-1_2ss14 | TRINITY_DN30618_c0 | Uncharacterized protein LOC108987072 |
| osa-miR156a | TRINITY_DN30823_c0 | Gibberellin 20 oxidase 1-D-like |
| cas-miR159b-3p_R+1 | TRINITY_DN42042_c1 | Chorismate mutase 3, chloroplastic |
| aly-miR159c-3p_R+1_1ss20CT | TRINITY_DN42042_c1 | Chorismate mutase 3, chloroplastic |
| aof- miR159f | TRINITY_DN46102_c0 | DELLA protein DWARF8-like |
| bdi-miR162 | TRINITY_DN41525_c2 | Plant UBX domain-containing protein 7 |
| aly-miR164a-5p | TRINITY_DN32775_c0 | NAC domain-containing protein 21/22 |
| | TRINITY_DN31804_c0 | NAC domain-containing protein 21/22-like |
| | TRINITY_DN35987_c1 | NAC domain-containing protein 100-like |
| zma-miR166m-5p | TRINITY_DN42896_c0 | Uncharacterized protein LOC103700952 |
| | TRINITY_DN42749_c0 | Probable calcium-binding protein CML21 |
| lus-miR166e | TRINITY_DN45178_c0 | Homeobox-leucine zipper protein HOX32 |
| | TRINITY_DN38326_c0 | NDR1/HIN1-like protein 12 |
| ata-miR166a-3p | TRINITY_DN45178_c0 | Homeobox-leucine zipper protein HOX32 |
| vca-miR167b-3p | TRINITY_DN27282_c0 | Non-specific lipid-transfer protein-like |
| vca-miR168a-5p | TRINITY_DN45301_c1 | Hypothetical protein POPTR_0012s03410g |
| osa-miR171d-5p | TRINITY_DN33637_c1 | -- |
| vvi-miR390 | TRINITY_DN43406_c0 | Serine/threonine-protein kinase BAM3 |
| | TRINITY_DN39224_c0 | LRR receptor-like serine/threonine-protein kinase |
| | TRINITY_DN46340_c0 | - |
| | TRINITY_DN37038_c0 | LRR receptor-like serine/threonine-protein kinase |
| osa-miR395b_L-1R+1 | TRINITY_DN43730_c0 | ATP sulfurylase 1, chloroplastic-like |
| | TRINITY_DN16935_c0 | - |
| | TRINITY_DN40931_c0 | Transcriptional corepressor LEUNIG isoform X4 |
| aof-MIR395b-p5_2ss11AG20 | TRINITY_DN46806_c1 | Calcium-transporting ATPase 8 |
| cme-miR396e_R+1 | TRINITY_DN36128_c0 | Growth-regulating factor 4-like |
| | TRINITY_DN34323_c1 | Growth-regulating factor 1 |
| | TRINITY_DN45792_c0 | Growth-regulating factor 10-like isoform X2 |
| | TRINITY_DN44678_c0 | CSC1-like protein ERD4 |
| | TRINITY_DN34323_c1 | Growth-regulating factor 1-like |
| zma-miR166l-3p | TRINITY_DN45178_c0 | Homeobox-leucine zipper protein HOX32 |
| | TRINITY_DN38326_c0 | NDR1/HIN1-like protein 12 |
| gma-miR4995 | TRINITY_DN37500_c0 | Abscisic acid 8′-hydroxylase 1-like |
| osa-miR5083_R-1 | TRINITY_DN41267_c1 | Uncharacterized protein LOC105045431 isoform |
| | TRINITY_DN39322_c1 | -- |
| | TRINITY_DN44382_c0 | -- |
| | TRINITY_DN42545_c0 | Uncharacterized protein LOC103973876 isoform |
| **Novel miRNA** | | |
| PC-3p-212_24745 | TRINITY_DN36128_c0 | Growth-regulating factor 4-like |
| PC-3p-2448_3032 | TRINITY_DN41146_c2 | -- |
| | TRINITY_DN37385_c0 | Uncharacterized protein LOC103721212 |
| | TRINITY_DN22436_c0 | -- |
| | TRINITY_DN31474_c0 | Transcription factor vib-1 |
| PC-5p-20023_207 | TRINITY_DN34607_c0 | Uncharacterized protein LOC103979330 isoform |
| PC-5p-12886_384 | TRINITY_DN41146_c2 | -- |
| PC-5p-29011_123 | TRINITY_DN39092_c1 | Ethylene-responsive transcription factor 4 |
| PC-5p-84014_23 | TRINITY_DN39500_c3 | Abscisic acid receptor PYR1-like |

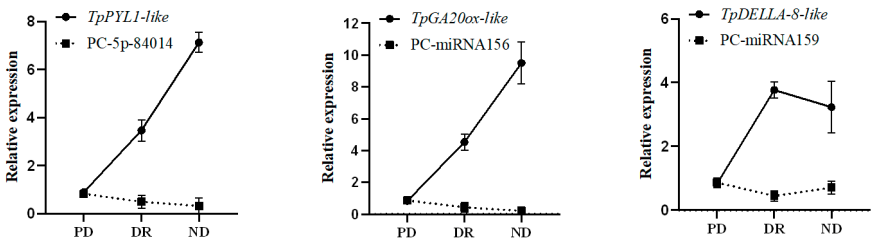


**Figure 7.** *Cont.*

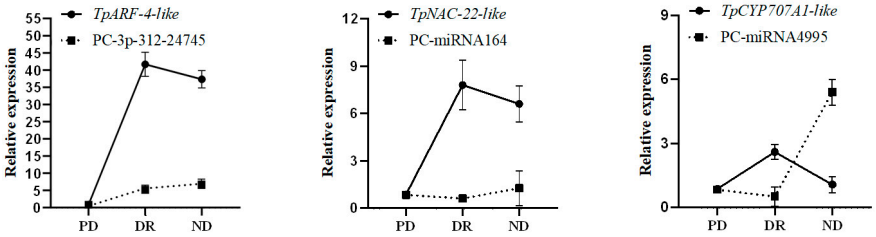


**Figure 7.** Verification of the target genes of miRNAs by qRT-PCR. DR, dormancy release; ND, non-dormancy; PD, physiological dormancy; qRT-PCR, real-time quantitative reverse transcriptase PCR. miRNAs, microRNAs.

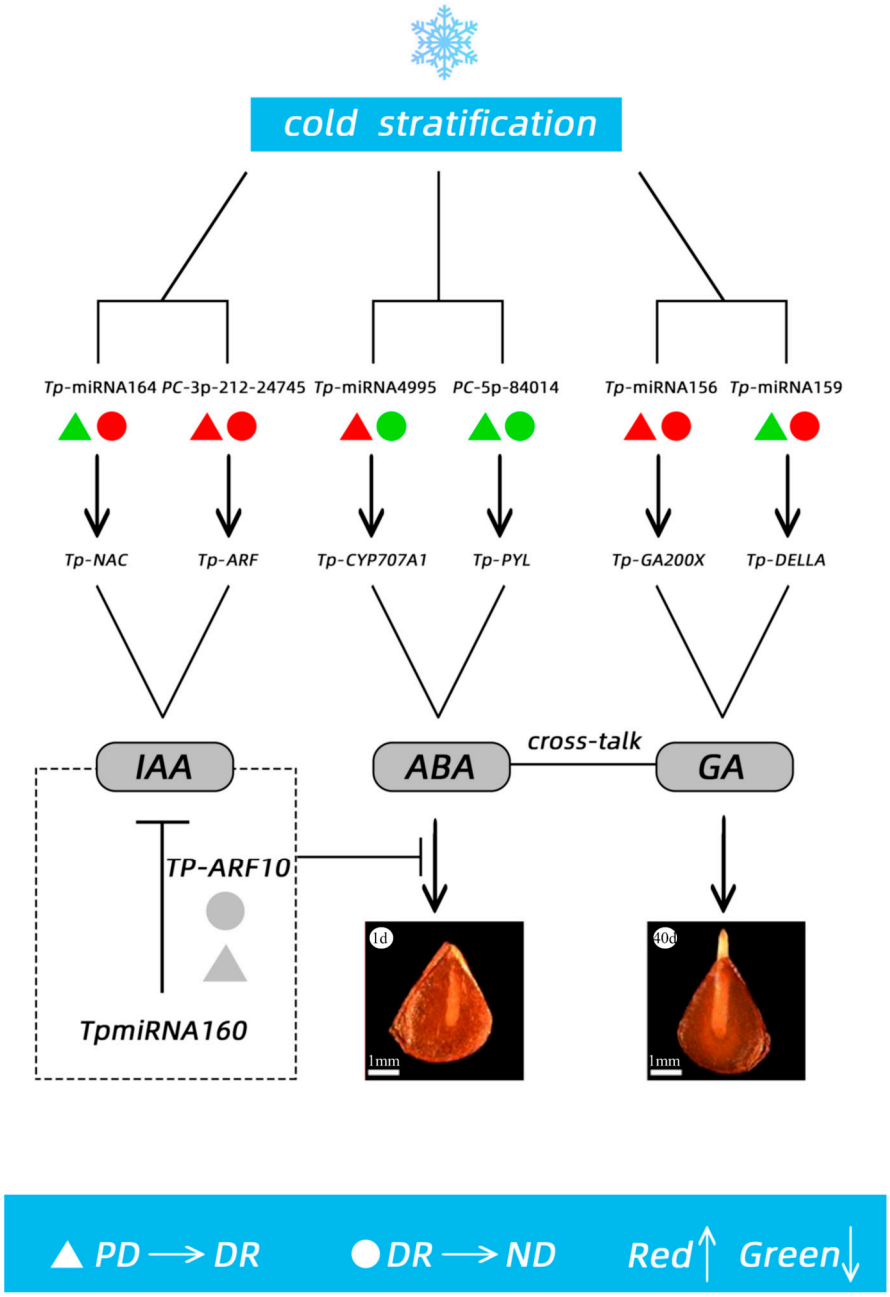


**Figure 8.** Hypothetical model of the miRNA regulatory network of dormancy release in *Tulipa thianschanica* seeds. ABA, abscisic acid; DR, dormancy release; GA, gibberellin; IAA, indole-3-acetic acid; miRNA, microRNA; ND, non-dormancy; PD, physiological dormancy.

## 4. Discussion

### *4.1. miRNA Dynamic Patterns of Expression during the Dormancy Release of T. thianschanica Seeds*

In most eukaryotic cells, miRNAs play a crucial role as a class of non-coding small RNAs involved in gene regulation. Extensive research has demonstrated their widespread involvement in various stress responses, such as drought, cold, and salt [21,22]. Over the past decade, significant progress has been made in understanding miRNAs in numerous plant species. However, the identification and mining of miRNAs using traditional methods, such as Sanger sequencing and bioinformatics predictions, are time-consuming, labor-intensive, and often unable to detect new miRNAs or those that are present at low levels. Advancements in high-throughput sequencing have revolutionized the field and enabled the exploration of novel or rare miRNAs and the analysis of small RNA clusters at different developmental stages under diverse natural environmental stress conditions [23,24]. High-throughput sequencing was first applied to the model species *Arabidopsis thaliana*, and it has since played a pivotal role in the discovery of miRNAs in various plant species, including *Magnolia sieboldii*, narrow-leafed lupin (*Lupinus angustifolius*), *Ginkgo biloba* L., and *Chlorophytum borivilianum* [25–28]. To date, 7474 hairpin sequences and 9168 mature miRNA sequences have been identified from 74 angiosperms. Despite the incorporation of these advances into miRBase 22.1, the sequence information for tulip miRNAs remains completely absent from the database.

Seed germination is initiated by the absorption of water, which causes the seeds to expand [29]. This results in the mobilization of nutrients within the seeds and subsequent cell elongation until the radicle breaks through the seed coat. The mobilization of stored substances involves a series of metabolic responses that can be modulated by alterations in gene expression and the utilization of different metabolic or signal transduction pathways under biological and abiotic stress conditions [30,31]. *T. thianschanica* seeds are physiologically dormant, and their germination requires exposure to low temperatures. This distinctive regulatory mechanism necessitates breaking dormancy under cold conditions before germination can be completed. In this study, nine small RNA libraries were constructed and analyzed to identify and predict the miRNAs and their target genes that are involved in the release of *T. thianschanica* seeds from dormancy under low-temperature conditions. A total of 67 conserved miRNAs were identified, which represented 34 families that encompassed the majority of miRNAs that have been reported in previous studies on other plant species. Additionally, 14 novel miRNAs were discovered. The predominant identified miRNA family was miRNA167, which was composed of seven members, followed by miRNA159, miRNA166, and miRNA171 with six, five, and five members, respectively. Several other families, including miRNA156, miRNA160, and miRNA164, were also identified. These families had mostly one or two validated members.

### *4.2. Regulatory Mechanism of the miRNA of T. thianschanica Seeds*

The characteristics of *T. thianschanica* seeds, particularly the presence of dormancy, limit the successful establishment of seedlings, which thereby impedes the utilization of this wild species. Numerous studies have confirmed the significant regulatory role of miRNAs in the processes of seed dormancy and germination. To obtain a better understanding of the miRNA–mRNA regulatory relationship, we performed an miRNA–mRNA correlation analysis using the expression data of small RNAs obtained in this study and previously acquired transcriptome data by predicting sequencing data. In this experiment, six pairs of miRNA–target gene interactions were validated, and five of them exhibited a negative correlation in their patterns of expression, which was consistent with the silencing function of miRNAs. Only one pair showed a positive correlation, which could be attributed to the presence of a feedback regulatory mechanism between the target gene and miRNA or the combined effect of multiple miRNAs and target genes. Zhou [32] also reported a similar positive correlation between miRNAs and patterns of target gene expression in her study

on the dormancy characteristics of a lily with fine leaves. Therefore, further research is required to confirm the intricacies of this regulatory mechanism.

The target genes ARF and miRNA396, which were identified in this study, have been extensively reported to be involved in auxin transduction [32]. Furthermore, the newly annotated miRNA PC-3p-212_24745 may have a similar functional role to that of miRNA396; furthermore, we will next verify this bold conjecture. Additionally, *CYP707A1*, a key gene that encodes ABA 8′-hydroxylase that is involved in the catabolism of ABA, could potentially be the target gene for miRNA4995 [33]. In *A. thaliana*, *CYP707A* that encodes ABA 8′-hydroxylase plays critical roles in various physiological processes [33]. Previous studies have demonstrated the importance of ABA 8′-hydroxylase genes, including *CYP707A1* and *CYP707A2* in *A. thaliana*, in regulating the endogenous levels of ABA during seed dormancy and germination [34,35]. In our study, seeds in the early stratification stage exhibited dormancy. Genes for the synthesis of ABA were upregulated along with miRNA4995, while *CYP707A1* was downregulated. However, as dormancy was released owing to the continuous catabolism of ABA, the level of expression of miRNA4995 decreased, while that of *CYP707A1* increased. Moreover, the prediction of this study identified PYL as the target gene for the newly predicted miRNA PC-5p-84014_23, which directly participates in ABA metabolism. Throughout the process, from dormancy to germination (PD to ND), PC-5p-84014_23 was consistently downregulated, whereas the corresponding target gene PYL was continuously upregulated. Previous studies have suggested that miRNA159 regulates the TFs that encode GA-MYB, which interacts with the GA response elements and influences the duration of flowering and anther development [36]. In this study, the expression of miRNA159 was inhibited in the absence of GA, indicating that the target gene DELLA directly interacts with miRNA159. Additionally, since DELLA is also targeted by GA-induced proteolysis, miRNA159 is closely associated with GA.

## 5. Conclusions

The miRNA databases for the PD, DR, and ND stages were constructed, and the corresponding raw reads were 46,145,598, 36,839,495, and 39,250,055, respectively. A total of 73,534,151 valid reads were obtained from the nine samples with 24 nt and 21 nt miRNAs identified as the predominant types. Furthermore, 34 miRNA families were identified and annotated. They encompassed functions that were related to seed germination, auxin signaling, and various aspects of signal transduction and endogenous metabolism. Among the miRNAs identified, *TpmiRNA159*, *TpmiRNA156*, *TpmiRNA4995*, and *PC-5p-84014* target *TpDELLA*, *TpGA20ox*, *TpCYP707A1*, and *TpPYL*, respectively, thereby influencing the GA/ABA ratio. In particular, *TpmiRNA4995* and *TpCYP707A1* exhibit direct negative regulation with the level of expression of *TpCYP707A1* increasing and the level of ABA decreasing during the late lamination period. Consequently, these molecular interactions contribute to the process of germination of *T. thianschanica* seeds.

**Supplementary Materials:** The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy13123067/s1. Table S1: Primer sequences for qRT-PCR analysis of target genes of miRNAs; Table S2: cDNA synthesis; Table S3: Reaction system and procedure of qRT-PCR.

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## References


1. Qu, L.W.; Xue, L.; Xing, G.M.; Zhang, Y.Q.; Chen, J.J.; Zhang, W.; Lei, J.J. Karyotype analysis of eight wild *Tulipa* species native to China and the interspecific hybridization with tulip cultivars. *Euphytica* **2018**, *214*, 2–12. [CrossRef]
2. Xing, G.M.; Qu, L.W.; Zhang, Y.Q.; Xue, L.; Su, J.W.; Lei, J.J. Collection and evaluation of wild tulip (*Tulipa* spp.) resources in China. *Genet. Resour. Crop Evol.* **2017**, *64*, 641–652. [CrossRef]
3. Zhang, W.; Zhao, J.; Xue, L.; Dai, H.P.; Lei, J.J. Seed Morphology and Germination of Native *Tulipa* Species. *Agriculture* **2023**, *13*, 466. [CrossRef]
4. Chen, X.M. MicroRNA biogenesis and function in plants. *FEBS Lett.* **2005**, *579*, 5923–5931. [CrossRef] [PubMed]
5. Cuperus, J.T.; Fahlgren, N.; Carrington, J.C. Evolution and functional diversification of miRNA genes. *Plant Cell* **2011**, *23*, 431–442. [CrossRef]
6. Bai, B.; Peviani, A.; van der Horst, S.; Gamm, M.; Snel, B.; Bentsink, L.; Hanson, J. Extensive translational regulation during seed germination revealed by polysomal profiling. *New Phytol.* **2017**, *214*, 233–244. [CrossRef]
7. Mallory, A.C.; Vaucheret, H. Functions of microRNAs and related small RNAs in plants. *Nat. Genet.* **2006**, *38*, 31–36. [CrossRef]
8. Ramya, R.; Herv, V.; Jerry, T.; Bartel, D.P. A diverse and evolutionarily fluid set of microRNAs in *Arabidopsis thaliana*. *Genes Dev.* **2006**, *20*, 3407–3425.
9. Huang, D.Q.; Koh, C.; Feurtado, J.A.; Tsang, E.W.T.; Cutler, A.J. MicroRNAs and their putative targets in *Brassica napus* seed maturation. *BMC Genom.* **2013**, *14*, 140. [CrossRef]
10. Kim, J.Y.; Lee, H.J.; Jung, H.J.; Maruyama, K.; Suzuki, N.; Kang, H. Over expression of microRNA395c or 395e affects differently the seed germination of *Arabidopsis thaliana* under stress conditions. *Planta* **2010**, *232*, 1447–1454. [CrossRef]
11. Martin, R.C.; Liu, P.P.; Goloviznina, N.A.; Nonogaki, H. MicroRNA, seeds, and Darwin: Diverse function of miRNA in seed biology and plant responses to stress. *J. Exp. Bot.* **2010**, *61*, 2229–2234. [CrossRef]
12. Li, J.Y.; Reichel, M.; Li, Y.J.; Millar, A.A. The functional scope of plant microRNA-mediated silencing. *Trends Plant Sci.* **2014**, *19*, 750–756. [CrossRef]
13. Wu, G.; Park, M.Y.; Conway, S.R.; Wang, J.W.; Weigel, D.; Poethig, R.S. The sequential action of mi156 and mi172 regulates developmental timing in *Arabidopsis*. *Cell* **2009**, *138*, 750–759. [CrossRef] [PubMed]
14. Liu, Y.; El-Kassaby, Y.A. Regulatory crosstalk between microRNAs and hormone signalling cascades controls the variation on seed dormancy phenotype at *Arabidopsis thaliana* seed set. *Plant Cell Rep.* **2017**, *36*, 705–717. [CrossRef] [PubMed]
15. Xue, X.F.; Jiao, F.C.; Xu, H.C.; Jiao, Q.Q.; Zhang, X.; Zhang, Y.; Du, S.Y.; Xi, M.H.; Wang, A.; Chen, J.T.; et al. The role of RNA-binding protein, microRNA and alternative splicing in seed germination: A field need to be discovered. *BMC Plant Biol.* **2021**, *21*, 194. [CrossRef]
16. Finkelstein, R.; Reeves, W.; Ariizumi, T.; Steber, C. Molecular aspects of seed dormancy. *Annu. Rev. Plant Biol.* **2008**, *59*, 387–415. [CrossRef] [PubMed]
17. Weitbrecht, K.; Muller, K.; Leubner-Metzger, G. First off the mark: Early seed germination. *J. Exp. Bot.* **2011**, *62*, 3289–3309. [CrossRef] [PubMed]
18. Allen, E.; Howell, M.D. miRNAs in the biogenesis of transacting siRNAs in higher plants. *Semin. Cell Dev. Biol.* **2010**, *21*, 798–804. [CrossRef] [PubMed]
19. Axtell, M.J.; Jan, C.; Rajagopalan, R.; Bartel, D.P. A two-hit trigger for siRNA biogenesis in plants. *Cell* **2006**, *127*, 565–577. [CrossRef]
20. Griffiths, J.; Murase, K.; Rieu, I.; Zentella, R.; Zhang, Z.L.; Powers, S.J.; Gong, F.; Phillips, A.L.; Hedden, P.; Sun, T.P. Genetic characterization and functional analysis of the GID1 gibberellin receptors in *Arabidopsis*. *Plant Cell* **2006**, *18*, 3399–3414. [CrossRef]
21. Wang, J.W.; Wang, L.; Mao, Y.B.; Cai, W.J.; Xue, H.W.; Chen, X.Y. Control of root cap formation by microRNA-targeted auxin response factors in *Arabidopsis*. *Plant Cell* **2005**, *17*, 2204–2216. [CrossRef] [PubMed]
22. Zhou, L.; Liu, Y.; Liu, Z.; Kong, D.; Duan, M.; Luo, L. Genome-wide identification and analysis of drought-responsive microRNAs in *Oryza sativa*. *J. Exp. Bot.* **2010**, *61*, 4157–4168. [CrossRef] [PubMed]
23. Wang, Y.; Zhao, Z.; Deng, M.; Liu, R.; Niu, S.; Fan, G. Identification and functional analysis of microRNAs and their targets in *Platanus acerifolia* under lead (Pb) stress. *Int. J. Mol. Sci.* **2015**, *16*, 7098–7111. [CrossRef] [PubMed]
24. Saminathan, T.; Bodunrin, A.; Singh, N.V.; Devarajan, R.; Nimmakayala, P.; Jeff, M.; Aradhya, M.; Reddy, U.K. Genome-wide identification of microRNAs in pomegranate (*Punica granatum* L.) by high throughput sequencing. *BMC Plant Biol.* **2016**, *16*, 122. [CrossRef] [PubMed]
25. Mei, M.; Wei, J.; Ai, W.F.; Zhang, L.J.; Lu, X.J. Integrated RNA and miRNA sequencing analysis reveals a complex regulatory network of *Magnolia sieboldii* seed germination. *Sci. Rep.* **2021**, *11*, 10842. [CrossRef]
26. DeBoer, K.; Melser, S.; Sperschneider, J.; Kamphuis, L.G.; Garg, G.; Gao, L.L.; Frick, K.; Singh, K.B. Identification and profiling of narrow-leafed lupin (*Lupinus angustifolius*) microRNAs during seed development. *BMC Genom.* **2019**, *20*, 135. [CrossRef] [PubMed]

27. Cui, J.W.; Zhao, J.G.; Zhao, J.Y.; Xu, H.M.; Wang, L.; Jin, B. Cytological and miRNA expression changes during the vascular cambial transition from the dormant stage to the active stage in *Ginkgo biloba* L. *Trees* **2016**, *30*, 2177–2188. [CrossRef]
28. Kajal, M.; Singh, K. Small RNA profiling for identification of miRNAs involved in regulation of saponins biosynthesis in *Chlorophytum borivilianum*. *BMC Plant Biol.* **2017**, *17*, 265. [CrossRef]
29. Zhang, L.; Xu, C.H.; Liu, H.N.; Tao, J.; Zhang, K.L. Seed dormancy and germination requirements of *Torilis scabra* (Apiaceae). *Agronomy* **2023**, *13*, 1250. [CrossRef]
30. Wan, Y.L.; Zhang, M.; Liu, A.Q.; Yuan, Q.P.; Liu, Y. Morphology and physiology response to stratification during seeds epicotyl dormancy breaking of *Paeonia emodi* Wall. Ex. *Agronomy* **2022**, *12*, 1745. [CrossRef]
31. Han, Z.P.; Jin, Y.Q.; Wang, B.; Guo, Y.Y. Multi-omics revealed the molecular mechanism of Maize (*Zea mays* L.) seed germination regulated by $GA_3$. *Agronomy* **2023**, *13*, 1929. [CrossRef]
32. Zhou, Y.W.; Wang, W.; Yang, L.H.; Su, X.X. Identification and expression analysis of microRNAs in response to dormancy release during cold storage of *Lilium pumilum* Bulbs. *J. Plant Growth Regul.* **2021**, *40*, 388–404. [CrossRef]
33. Okamoto, M.; Kuwahara, A.; Seo, M.; Kushiro, T.; Asami, T.; Hirai, N.; Kamiya, Y.; Koshiba, T.; Nambara, E. CYP707A1 and CYP707A2, which encode abscisic acid 8′-hydroxylases, are indispensable for proper control of seed dormancy and germination in *Arabidopsis*. *Plant Physiol.* **2006**, *141*, 97–107. [CrossRef] [PubMed]
34. Zhu, G.H.; Liu, Y.G.; Ye, N.H.; Liu, R.; Zhang, J.H. Involvement of the abscisic acid catabolic gene CYP707A2 in the glucose-induced delay in seed germination and post-germination growth of *Arabidopsis*. *Physiol. Plant.* **2011**, *143*, 375–384. [CrossRef]
35. Matakiadis, T.; Alboresi, A.; Jikumaru, Y.; Tatematsu, K.; Pichon, O.; Renou, J.P.; Kamiya, Y.; Nambara, E.; Truong, H.N. The *Arabidopsis* abscisic acid catabolic gene CYP707A2 plays a key role in nitrate control of seed dormancy. *Plant Physiol.* **2009**, *149*, 949–960. [CrossRef]
36. Patrick, A.; Alan, H.; Baulcombe, D.C.; Harberd, N.P. Modulation of floral development by a gibberellin-regulated microRNA. *Development* **2004**, *131*, 3357–3365.