Transcriptomic Analysis Reveals LncRNAs Associated with Flowering of Angelica sinensis during Vernalization

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Abstract: Angelica sinensis is a “low-temperature and long-day” perennial plant that produces bioactive compounds such as phthalides, organic acids, and polysaccharides for various types of clinical agents, including those with cardio-cerebrovascular, hepatoprotective, and immunomodulatory effects. To date, the regulatory mechanism of flowering under the photoperiod has been revealed, while the regulatory network of flowering genes during vernalization, especially in the role of lncRNAs, has yet to be identified. Here, lncRNAs associated with flowering were identified based on the full-length transcriptomic analysis of A. sinensis at vernalization and freezing temperatures, and the coexpressed mRNAs of lncRNAs were validated by qRT-PCR. We obtained a total of 2327 lncRNAs after assessing the protein-coding potential of coexpressed mRNAs, with 607 lncRNAs aligned against the TAIR database of model plant Arabidopsis, 345 lncRNAs identified, and 272 lncRNAs characterized on the SwissProt database. Based on the biological functions of coexpressed mRNAs, the 272 lncRNAs were divided into six categories: (1) chromatin, DNA/RNA and protein modification; (2) flowering; (3) stress response; (4) metabolism; (5) bio-signaling; and (6) energy and transport. The differential expression levels of representatively coexpressed mRNAs were almost consistent with the flowering of A. sinensis. It can be concluded that the flowering of A. sinensis is positively or negatively regulated by lncRNAs, which provides new insights into the regulation mechanism of the flowering of A. sinensis.

Keywords: Angelica sinensis; flowering; lncRNA; vernalization; transcriptomic analysis

1. Introduction

Angelica sinensis (Oliv.) Diels is a “low-temperature and long-day” perennial plant that is native to Gansu Province, Northwest China [1,2]. The roots have been used as a traditional Chinese medicine for over 2000 years [3,4]. Currently, the roots are also applied in cardio-cerebrovascular, hepatoprotective, and immunomodulatory clinical agents, largely relying on bioactive compounds such as phthalides, organic acids, and polysaccharides [5–7].

Recently, the planting area of A. sinensis has exceeded 40,000 ha to satisfy the increasing demand; however, a higher rate (>40%) of early bolting and flowering (EBF) in commercial cultivation increases the lignified rate of roots and decreases the yield accordingly [8,9]. In order to inhibit the EBF, effective measures that have been taken include selecting excellent germplasm resources with a lower rate of EBF [8], controlling the seedling size (0.4 to 0.6 cm) to delay the transition from vegetative to reproductive growth [10], storing seedlings below freezing temperatures (−3 to −10 °C) to avoid vernalization (0 to 10 °C) [2,11,12], and shading the plants with sunshade nets (40% to 60%) to avoid the long-day conditions during the adult stages [13].

Regarding the regulatory mechanism of flowering in A. sinensis, key genes and the regulatory network during the photoperiod have been identified and mapped based on transcriptomic analysis. Specifically, 13 genes associated with the photoperiod, vernalization,
sucrose, and GA pathways were identified from plants at the vegetative stage compared with the EBF stage [14]; 38 genes associated with the photoperiod, carbohydrates, hormone signaling, and floral development were identified from different development stages [15]; and 40 genes associated with the photoperiod, sucrose, GA, and floral development were identified from the EBF compared with Un-EBF [16]. In summary, key genes such as FLC, SOC1, FT, PHYA, API, and GA2OX1 were identified during the transition from the vegetative to the reproductive stage [14–16]. To date, physiological changes in the levels of carbohydrates, proteins, and hormones during vernalization have been investigated [17,18], while the regulatory network of flowering has yet to be identified.

Generally, ncRNAs include two categories, housekeeping and regulatory ncRNAs, and the latter can be further divided into sRNAs (i.e., miRNA, siRNA, and piRNA) and lncRNAs (>200 nucleotides) [19]. Both miRNAs and lncRNAs can influence plant developmental processes and stress responses [20], with the former being negative regulators functioning as specificity determinants, or guides, within complexes that promote the degradation of mRNA targets, and the latter acting either as precursors of miRNAs or endogenous target mimics (TMs), which mimic the real targets of miRNAs, thus rendering the corresponding miRNAs ineffective [21]. For example, previous studies on resistance against leaf rust in wheat found that 50 miRNAs and 1178 lncRNAs were identified and 49 lncRNAs were found to be the targets for miRNAs, with 1 lncRNA acting as a precursor of 2 miRNAs, and 3 lncRNAs acting as TMs [22].

Extensive investigations have demonstrated that lncRNAs regulate their downstream targets’ expression through the changing of epigenetic modification at the level of transcription and post-transcription by interacting with DNA, RNA, and proteins; thus, they are involved in various biological processes [23–25]. In this study, lncRNAs associated with flowering were identified based on the transcriptomic analysis of A. sinensis seedlings treated at vernalization and freezing temperatures (avoiding vernalization). We found that 272 lncRNAs directly or indirectly participate in regulating the flowering of A. sinensis and stress responses.

2. Materials and Methods

2.1. Plant Material

The seedlings (root–shoulder diameter 0.4–0.5 cm; Figure S1) of Angelica sinensis (cultivar Mingui 1) were stored at 0 (vernalization temperature) and −3 °C (freezing temperature), respectively. After storage at 0 °C for 14 (T1) and 60 days (T2), as well as at −3 °C for 125 days (T3), the shoot apical meristem (SAM) was cut from the root shoulder of the seedlings for transcriptomic analysis and qRT-PCR validation. Three biological replicates were performed for each treatment of T1, T2, and T3. Herein, the treatment of T1, T2, and T3 represents uncompleted, completed, and avoided vernalization, respectively, based on the EBF rate (Figure S2) when the stored seedlings were cultivated and grown in a long-day condition.

2.2. Full-Length Isoform Sequencing and Analysis

Total RNA of the SAM samples was extracted using Trizol reagent (Omega Bio-Tek, Norcross, GA, USA). The integrity of the RNA was determined using an Agilent 2100 Bioanalyzer (Agilent Technol., California, CA, USA) and agarose gel electrophoresis, and the purity and concentration of the RNA were determined using a microspectrophotometer (NanoDrop Technol., Wilmington, DE, USA). The high-quality RNAs were sequenced on a Pacific Biosciences Sequel platform (Gene Denovo Biotechnology Co., Ltd., Guangzhou, China). Raw reads of cDNA library were analyzed using a SMRT Link (V8.0.0) [26]. Briefly, high-quality CCS were extracted from the subreads BAM file; the integrity of transcripts (full-length sequences) was judged based on whether CCS reads contained primers (5′ and 3′) and polyAs; then, FLNC reads were generated by removing primers, barcodes, and polyAs; finally, FLNC reads were assembled to obtain the entire isoform [27].
2.3. Analysis of Long Noncoding RNAs (lncRNAs)

Isoforms that were not annotated against the four databases—NR, Swiss-Prot, Kyoto KEGG, and KOG—were used for the analysis of lncRNAs. The isoform that was assessed as a noncoding transcript by both CNCI and CPC software was finally confirmed as a lncRNA [28,29].

2.4. Characterization of lncRNAs

To date, the genome of *A. sinensis* has not been sequenced. Thus, the lncRNA analysis of *A. sinensis* was performed via a BLAST search with an E-value cut-off of ≤1 × 10^{-5} against the known lncRNAs from the TAIR database (https://www.arabidopsis.org accessed on 30 March 2022) [30]. The function of lncRNAs was annotated based on their coexpression mRNAs [31–33]. Herein, the biological functions of the coexpressed mRNAs were searched on the UniProt database (https://www.uniprot.org accessed on 30 March 2022).

2.5. qRT-PCR Validation

Based on the coding sequences (CDS) of coexpressed mRNA of lncRNA, 49 primer sequences of representatively coexpressed mRNAs (Table 1) were designed using the NCBI primer-blast tool. First-strand cDNA synthesis and qRT-PCR reaction were carried out using SuperRealPreMix Plus (FP205; Tiangen Biotech., Beijing, China) according to the manufacturer’s instructions; specifically, the cDNA was synthesized successively with one cycle (95 °C, 15 min) and 40 cycles (95 °C, 10 s; 60 °C, 20 s; and 72 °C, 30 s), and the qRT-PCR reaction was incubated successively at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 1 s. The *Actin* (*ACT*) gene was used as a reference control gene with forward: TGGTATGTGCTGGATTCTGGT and reverse: TGAGATCACCACCAGCAAGG (amplicon size 109 bp) [34]. Herein, the cycle threshold (Ct) values and standard curves of the ACT gene at different volumes (0.25, 0.5, 1.0, 1.5, 2.0, and 3.0 µL) was built to correct the gene expression level (Figure S3 and Figure S4), and the expression levels of the 49 candidate genes and their standard deviations for every variant were added to the Supplementary Materials (Table S1). The REL of coexpressed mRNAs was calculated using the $2^{-\Delta\Delta Ct}$ method [35] according to the following formula.

\[
\begin{align*}
\Delta Ct_{\text{Test gene}} &= Ct_{\text{Test gene}} - Ct_{\text{Reference gene}} \\
\Delta Ct_{\text{Target gene}} &= Ct_{\text{Target gene}} - Ct_{\text{Reference gene}} \\
-\Delta\Delta Ct(T2 vs. T1) &= -(\Delta Ct_{T2} - \Delta Ct_{T1}) \\
-\Delta\Delta Ct(T3 vs. T1) &= -(\Delta Ct_{T3} - \Delta Ct_{T1}) \\
\text{Relative expression level (REL)} &= 2^{-\Delta\Delta Ct}.
\end{align*}
\]

Table 1. Primer sequences used in qRT-PCR validation.

| lncRNA ID | Coexpressed mRNAs | mRNA ID | Primer Sequences (5' to 3') | Amplicon Size (bp) |
|-----------|--------------------|---------|-----------------------------|--------------------|
| Isoform0062250 | HMGB2 | NM_001035997.1 | Forward: CAAAGCTGCTGCTAAGGAC  
Reverse: GGACTTCCACTTGTCTCTACG | 155     |
| Isoform0061796 | HMGB3 | NM_001035998.1 | Forward: CTTCCGTTGCTGGCTTCCG  
Reverse: CTCAACCGTGGCTTCTCAG | 174     |
| Isoform0001498 | At1g05910 | NM_100472.2 | Forward: AGACCTCTCACCTGCCTTCC | 109     |
| Isoform0062769 | RID2 | NM_125110.6 | Forward: GCAGGGTCTAGCTGCTGTCTTCC | 100     |
| lncRNA ID | Coexpressed mRNAs | mRNA ID   | Primer Sequences (5’ to 3’) Amplicon Size (bp) |
|-----------|--------------------|-----------|------------------------------------------------|
| Isoform0061049 | At4g26600 | NM_001341820.1 | Forward: TTCCGATTGGTGCAACTCCT 118 |
|           |         | Reverse: GCCATGTCACAAACTGTC | |
| Isoform0034756 | H2AV     | NM_001339683.1 | Forward: CAGTTGGACGAATTCAGGC 176 |
|           |         | Reverse: CAGATGCCTGGGTGATC | |
| Isoform0050517 | At2g28720 | NM_128433.4 | Forward: GCAAGAAGCTCAAAATTCGC 107 |
|           |         | Reverse: TGCTTAGCAAGTTCACCAGG | |
| Isoform0062503 | HTR2     | NM_113651.2 | Forward: CACCCAGAGGAGKGAAGAC 189 |
|           |         | Reverse: TCTTGAAGAGCTGACTGC | |
| Isoform0027210 | HOS15    | NM_126132.4 | Forward: TACAGGCGCAAGACCTATGG 164 |
|           |         | Reverse: CTGTTGACCATCACCAGC | |
| Isoform0062048 | REF6     | NM_148863.4 | Forward: AGGGAACACAGCTTGCTGTG 124 |
|           |         | Reverse: TTCCCCAAATGGAGGCTG | |
| Isoform0062818 | SKP1A    | NM_106245.5 | Forward: GTGCTGTACCTGCAAGTAC 181 |
|           |         | Reverse: GTGCGGAAGCTTCTCGTGT | |
| Isoform0061474 | ASK21    | NM_001125404.1 | Forward: CCTGATGACCTTACTGAGAG 178 |
|           |         | Reverse: CAGGGTCACATCCCTGAACG | |
| Isoform0061497 | SRK2G    | NM_120946.5 | Forward: ACATCGAGAGAGGTCGCAAG 110 |
|           |         | Reverse: AGGTTGACATCACCAGC | |
| Isoform0062575 | SRK2H    | NM_125760.2 | Forward: TGGTCCTGTTGTTGACTCT 164 |
|           |         | Reverse: GAGAGAAAGTGTCTCGAC | |
| Isoform0062220 | SRR1     | NM_125348.4 | Forward: ATGCATTTTGTTGGGAACAGC 117 |
|           |         | Reverse: AGCAAACTCGCTGTGACTCT | |
| Isoform0061284 | PHL      | NM_001334526.1 | Forward: CAAAGTCTCCTTGTTGCG 104 |
|           |         | Reverse: GCAACTGCTCCATAGTGGG | |
| Isoform0057927 | PHYA     | NM_001123784.1 | Forward: GTGCGATATGCTGATCGCTG 149 |
|           |         | Reverse: CCTGAGGTGGAACCTACCT | |
| Isoform0041956 | AGL62    | NM_125437.5 | Forward: CTCTCAACCAACACAACAC 197 |
|           |         | Reverse: AAGCAGATTTACCAACCAAC | |
| Isoform0045502 | AGL79    | NM_113925.3 | Forward: AATCACCCATGAGCTTGC 107 |
|           |         | Reverse: TAGGGTCCGGCACTCATT | |
| Isoform0063170 | ATJ3     | NM_114279.4 | Forward: GAATACGCTACGAGGTTGC 135 |
|           |         | Reverse: GCATCCCACTTGGCTCTTCT | |
| Isoform0062470 | ACBP6    | NM_102916.4 | Forward: AAATCCACCATGAGCTTGC 107 |
|           |         | Reverse: TACGGGTTCGGGACGCTTACTT | |
| Isoform0061783 | ENO2     | NM_129209.4 | Forward: CACTTGAGTGTGGAACCGAGG 190 |
|           |         | Reverse: GGTCACTACCTCCCCAACCCTG | |
| Isoform0063049 | ADH1     | NM_106362.3 | Forward: TGATGACCAGTGTTGCAACC 123 |
|           |         | Reverse: TGAATCAGTGGCTACCAGC | |
| Isoform0062198 | CSP2     | NM_120029.3 | Forward: GATGTGGAGGAGTGAATCAGG 115 |
|           |         | Reverse: CAGTCTCTCGGCCATGTC | |
| lncRNA ID      | Coexpressed mRNAs | mRNA ID      | Primer Sequences (5’ to 3’)                        | Amplicon Size (bp) |
|---------------|-------------------|--------------|----------------------------------------------------|-------------------|
| Isoform0062617 | HSP17.8           | NM_100614.3  | Forward: AACATCGCGGATAACGAACG                      | 154               |
|               |                   |              | Reverse: CTCCACGTGCTCTCCAC                         |                   |
| Isoform0009507 | HSP70-3           | NM_001202918.1 | Forward: CGACTGAGGAGACACTCAT                      | 144               |
|               |                   |              | Reverse: TCTCACAGGCGGTTCTCAAC                      |                   |
| Isoform0034676 | HSP70-10          | NM_120996.4  | Forward: CGTGTCAGAAGGTGTCG                        | 167               |
|               |                   |              | Reverse: CCGACGATAGAGGTGAC                        |                   |
| Isoform0042993 | HSP90-3           | NM_124983.4  | Forward: AAACAGGGAAGTACGCTG                       | 193               |
|               |                   |              | Reverse: AGACACGACGACATAGG                        |                   |
| Isoform0061974 | CSY4              | NM_001337082.1 | Forward: GATGCAGAGCTCTACCGC                      | 197               |
|               |                   |              | Reverse: CTCCTCCGGGCTCAGAAT                      |                   |
| Isoform0062375 | GAPCP2            | NM_101496.3  | Forward: CATTTCTCAGCCTGTGGCG                      | 198               |
|               |                   |              | Reverse: TCTCAGTGGTGGTGTCG                        |                   |
| Isoform0062268 | At3g52990         | NM_115159.5  | Forward: GACAAACTTGCGACCATCG                      | 101               |
|               |                   |              | Reverse: AATCCAGGGGCGCTCAG                        |                   |
| Isoform0062585 | PGM2              | NM_001160993.2 | Forward: TGAACTGGATCAGAGAGAG                     | 158               |
|               |                   |              | Reverse: TCGGTCAGTACATGGAGAC                      |                   |
| Isoform0062370 | BAM1              | NM_113297.3  | Forward: AACTCTGCGTCTGTTCG                        | 165               |
|               |                   |              | Reverse: GGAAAGAAGGTGTCAGAAT                      |                   |
| Isoform0062586 | ARF1              | NM_001337250.1 | Forward: GTGACCGGTGTGTGAGCC                      | 148               |
|               |                   |              | Reverse: TGAAGCGAAAGGTGACG                        |                   |
| Isoform0061377 | CUL1              | NM_001036498.3 | Forward: GTGGCGTGGATGCTGAGAG                     | 153               |
|               |                   |              | Reverse: TCTCCGGGTGTGGAGAAG                      |                   |
| Isoform0057235 | SOFL4             | NM_123240.2  | Forward: AGGTCGATGAAAGACTAC                      | 144               |
|               |                   |              | Reverse: GAACCGCTGATAATGGGCC                     |                   |
| Isoform0043114 | SOFL5             | NM_001342234.1 | Forward: TGGGAGTCAGGAGCAGCTCT                    | 193               |
|               |                   |              | Reverse: TCCTGGACGAAGAGCAT                       |                   |
| Isoform0062152 | GRF2              | NM_106479.3  | Forward: AACTCCGGGAAAACTCGG                      | 192               |
|               |                   |              | Reverse: GAGCAAGATTGATTACGGCC                     |                   |
| Isoform0062828 | GRF11             | NM_001084180.2 | Forward: GTGGCTAGGAGCAGCACTGTG                   | 198               |
|               |                   |              | Reverse: GACGGTGAGATCTCCGGAA                     |                   |
| Isoform0061395 | ERF3              | NM_103946.3  | Forward: ATCTTTAGGCGGACAGAC                      | 101               |
|               |                   |              | Reverse: CGCAAATGCTGTGACCAAATCC                 |                   |
| Isoform0022533 | SF1               | NM_001036978.2 | Forward: GGCTTAGGCTCAACTCCGAC                    | 164               |
|               |                   |              | Reverse: CCAGTCACAGGTCCCTGAT                     |                   |
| Isoform0044730 | PURU1             | NM_124115.4  | Forward: ACGTCTCTTACTCCCGAAG                     | 125               |
|               |                   |              | Reverse: AGGCACACGAAACTGAA                      |                   |
| Isoform0047216 | MES16             | NM_117770.5  | Forward: CCATCCCTTCTCCGCACTTT                    | 195               |
|               |                   |              | Reverse: TCATAGGAGAGCCAC                       |                   |
| Isoform0063248 | GPT1              | NM_124861.5  | Forward: CGCTGTTTCTGTGATGAC                     | 193               |
|               |                   |              | Reverse: AAACGGCGGTTCACCAGCCT                   |                   |
### Table 1. Cont.

| IncRNA ID     | Coexpressed mRNAs | mRNA ID      | Primer Sequences (5’ to 3’) | Amplicon Size (bp) |
|---------------|--------------------|--------------|-----------------------------|--------------------|
| Isoform0062571| ABCF5              | NM_125882.3  | Forward: TGCTGATAGGCTTGTTGGT | 103                |
|               |                    |              | Reverse: CGGCTCATCAAGTAGCGAGCA |                    |
| Isoform0008194| SECA2              | NM_001198130.1 | Forward: ACTGAGGCCCATTGTCTG  | 117                |
|               |                    |              | Reverse: CTCACGACAGCATGGTATA |                    |
| Isoform0062373| VPS26A             | NM_124733.4  | Forward: TGTTCCGCTTCCTCCAATCAA | 196                |
|               |                    |              | Reverse: TGCTCCAGTTGATCTCGCC  |                    |
| Isoform0062449| CML19              | NM_119864.5  | Forward: CGAGCTCAACGTTGCTAGAG | 160                |
|               |                    |              | Reverse: GTCTATGGGATCTCGTCGCC  |                    |
| Isoform0061307| NHX6               | NM_106609.4  | Forward: GGCATTTGCTCTTGCTGC   | 112                |
|               |                    |              | Reverse: TCCTCCAATCACGTACCG    |                    |

### 2.6. Statistical Analysis

In order to obtain the precise estimation of PCR efficiency, each experiment for qRT-PCR validation was performed with three biological replicates, along with three technical replicates [36]. A t-test in SPSS 22.0 was performed for independent experiments, with \( p < 0.05 \) as the basis for statistical differences.

### 3. Results

#### 3.1. LncRNAs Analysis

In total, 2327 lncRNAs were obtained after assessing the protein-coding potential of coexpressed mRNAs based on the two software programs, CNCI and CPC (Figure 1A), with 607 genes aligned against the known lncRNAs from the TAIR database of model plant *Arabidopsis* (Figure 1B), and 345 lncRNAs with coexpressed mRNAs of *A. sinensis* identified (Figure 1C) based on the SwissProt database. Based on the biological functions, the 272 characterized lncRNAs (Figure 1D) were divided into six categories: chromatin, DNA/RNA and protein modification (29); flowering (36); stress response (24); metabolism (117); biosignaling (23), and energy and transport (43) (Figure 1E). The base sequences of the 272 lncRNAs are shown in Table S2.

#### 3.2. LncRNAs Linked with Chromatin, DNA/RNA and Protein Modification, as well as Expression Levels of Their Coexpressed mRNAs

Based on the biological functions of coexpressed mRNAs, 29 lncRNAs were linked with chromatin (HMGB2 and HMGB3), DNA/RNA (At1g05910, RID2 and At4g26600) and protein modification (H2AV, At2g28720, HTR2, HOS15, REF6, SKP1A, ASK21, SRK2G, SRK2H, DET1, BOPAt4g295601, At1g45180, At1g50840, At2g36630, At1g47890, UBP7, DER2.1, GRP3, MDH9.13, At3g24715, At3g16560, At3g62260, ESMD1, and At1g27930) (Table 2). The expression levels of 14 representative coexpressed mRNAs were confirmed by qRT-PCR, with 3 mRNAs (HMGB2, HMGB3 and At1g05910) showing down-regulation at T2 versus (vs.) T1, and 11 mRNAs showing lower levels at T2 vs. T1 than T3 vs. T1, with the exception of 2 mRNAs (H2AV and ASK21) (Figure 2).
**Table 2.** Twenty-nine lncRNAs linked with chromatin, DNA/RNA, and protein modification.

| lncRNA ID              | Coexpressed mRNAs                      | mRNA ID           | Proteins Encoded by Coexpressed mRNAs                                      |
|------------------------|----------------------------------------|-------------------|--------------------------------------------------------------------------------|
| **Chromatin modification (2)** | HMG B2 | AT1G20693.1 | High mobility group B protein 2                                               |
| Isoform0062250         | HMGB3 | AT1G20696.1 | High mobility group B protein 3                                               |
| **DNA/RNA modification (3)** | At1g05910 | AT1G05910.1 | ATPase family AAA domain-containing protein At1g05910                        |
| Isoform001498         | RID2  | AT5G57280.1 | 18S rRNA (guanine-N(7))-methyltransferase RID2 e S-adenosyl-L-methionine-dependent methyltransferases superfamily protein |
| Isoform0062769        |      | AT5G57280.1 | 18S rRNA (guanine-N(7))-methyltransferase RID2 e S-adenosyl-L-methionine-dependent methyltransferases superfamily protein |
| Isoform0061497        |      | AT4g26600.8 | ATPase family AAA domain-containing protein At1g05910                        |
| **Protein modification (24)** | H2AV  | AT3G54560.1 | Histone H2A variant 1                                                        |
| Isoform0034756         | HTR2  | AT1G09200.1 | Histone H2B.3                                                                |
| Isoform005017          | HOS15 | AT5G67320.1 | WD40 repeat-containing protein HOS15                                           |
| Isoform0062818         | REF6  | AT3G48401.0 | Lysine-specific demethylase REF6                                               |
| Isoform0062048         | SKP1A | AT1G75950.1 | SKP1-like protein 1A                                                          |
| Isoform0061474         | ASK21 | AT3G61415.1 | SKP1-like protein 21                                                          |
| Isoform0061497         | SRK2G | AT5G08590.1 | Serine/threonine-protein kinase SRK2G                                          |
| Isoform0062575         | SRK2H | AT5G63650.1 | Serine/threonine-protein kinase SRK2H                                          |
| Isoform0053138         | DET1  | AT4G10180.1 | Light-mediated development protein DET1                                        |
| Isoform0030135         | BOPAt4g295601 | AT3G57130.2 | Ankyrin repeat family protein/ BTB/POZ domain-containing protein              |
| Isoform0063715         | At1g45180 | AT1G45180.1 | Phototropic-responsive NPH3 family protein                                    |
| Isoform0044146         | At3g50840 | AT3G50840.3 | Sulfite exporter TauE/SafE family protein 4                                  |
| Isoform0034163         | At2g36630 | AT2G36630.1 | Ubiquitin carboxyl-terminal hydrolase-related protein                         |
| Isoform0058941         | At3g47890 | AT3G47890.2 | Ubiquitin carboxyl-terminal hydrolase-related protein                         |
| Isoform0007659         | UBP7  | A0A199LL79 | Ubiquitin carboxyl-terminal hydrolase-related protein                         |

**Figure 1.** Distribution and classification of lncRNAs in *Angelica sinensis* during vernalization, based on the biological functions of coexpressed mRNAs. Abbreviations: CNCI, coding–noncoding index; CPC, coding potential calculator. Images (A), (B), (C), (D) and (E) represent total, aligned, identified, characterized and classified lncRNAs, respectively.

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### References

CPC, coding potential calculator. Images (A), (B), (C), (D) and (E) represent total, aligned, identified, characterized and classified lncRNAs, respectively.
Table 2. Cont.

| IncRNA ID | Coexpressed mRNAs | mRNA ID | Proteins Encoded by Coexpressed mRNAs |
|-----------|--------------------|---------|---------------------------------------|
| Isoform0045576 | DER2.1 | AT3G21280.2 | Derlin-2.1 |
| Isoform0062510 | GRP3 | AT2G05520.1 | Glycine-rich protein 3 |
| Isoform0062575 | MDH9.13 | AT5G42440.1 | Kinase superfamily with octicosapeptide/Phox/Bem1p domain-containing protein |
| Isoform0051826 | AT3g24715 | AT3G24715.1 | |
| Isoform0061548 | AT3g16560 | AT3G16560.4 | Probable protein phosphatase 2C 40 |
| Isoform0049189 | AT3g2260 | AT3G2260.1 | Probable protein phosphatase 2C 49 |
| Isoform0060667 | ESMD1 | AT2G01480.1 | Protein ESMERALDA 1 |
| Isoform0027210 | At1g27930 | AT1G27930.1 | Probable methyltransferase At1g27930 |

Figure 2. The expression levels of coexpressed mRNAs of IncRNAs linked with chromatin, DNA/RNA and protein modification in A. sinensis at T2 vs. T1 and T3 vs. T1, as determined by qRT-PCR. T1, T2, and T3 represent uncompleted, completed, and avoided vernalization, respectively. The "*" represents a significant difference at $p < 0.05$ level between T2 vs. T1 and T3 vs. T2 for the same gene.

3.3. LncRNAs Linked with Flowering and Expression Levels of Their Coexpressed mRNAs

In total, 36 IncRNAs were linked with flowering based on the biological functions of their coexpressed mRNAs, with 12 IncRNAs directly associated with flowering, namely SRR1, PHL, PHYA, AGL62, AGL79, ATJ3, BBX29, CLE13, CLE44, MXC17.10, At1g06515, and BHLH30 (Table 3), and 24 IncRNAs indirectly associated with flowering, such as cell division, embryo development, and cell wall organization (Table S3). The expression levels of six representative coexpressed mRNAs (SRR1, PHL, PHYA, AGL62, AGL79, and ATJ3) were confirmed by qRT-PCR, with all six mRNAs showing down-regulation at T2 vs. T1 and T3 vs. T1, and five mRNAs showing lower levels at T2 vs. T1 than T3 vs. T1, with the exception of the gene AGL62 (Figure 3).

3.4. LncRNAs Linked with Stress Response and Expression Levels of Their Coexpressed mRNAs

In total, 24 IncRNAs were linked with the stress response based on the biological functions of their coexpressed mRNAs, with 14 IncRNAs associated with the temperature response, namely ACBP6, ENO2, ADH1, CSP2, RH20, RH52, RH53, RAB18, XERO1, MED14, HSP17.8, HSP70-3, HSP70-10, and HSP90-3 (Table 4), and 10 IncRNAs associated with other stresses responses, such as water, salt, and oxidative stress (Table S4). The expression levels of eight representative coexpressed mRNAs involved in the temperature response were confirmed by qRT-PCR, with four mRNAs (ACBP6, ENO2, CSP2, and HSP90-3) showing up-regulation at T3 vs. T1, and three mRNAs (ACBP6, ENO2, and CSP2) showing lower levels at T2 vs. T1 than T3 vs. T1 (Figure 4).
Table 3. Twelve lncRNAs directly linked with flowering.

| lncRNA ID          | Coexpressed mRNAs | mRNA ID                  | Proteins Encoded by Coexpressed mRNAs                                      |
|--------------------|-------------------|--------------------------|-----------------------------------------------------------------------------|
| Isoform0062220     | SRR1              | AT5G59560.2              | Protein SENSITIVITY TO RED LIGHT REDUCED 1                                 |
| Isoform0061284     | PHL               | AT1G72390.1              | Protein PHYTOCHROME-DEPENDENT LATE-FLOWERING                                 |
| Isoform0057927     | PHYA              | AT1G09570.6              | Phytochrome A                                                               |
| Isoform0041956     | AGL62             | AT5G60440.1              | Agamous-like MADS-box protein AGL62                                         |
| Isoform0045502     | AGL79             | AT3G30260.1              | AGAMOUS-like 79                                                             |
| Isoform0063170     | ATJ3              | AT3G44110.1              | Chaperone protein dnaJ 3                                                   |
| Isoform0028325     | BBX29             | AT5G54470.1              | At5g54470                                                                  |
| Isoform0054894     | CLE13             | AT1G73965.1              | CLAVATA3/ESR (CLE)-related protein 13                                      |
| Isoform0061298     | CLE44             | AT4G13195.1              | CLAVATA3/ESR (CLE)-related protein 44                                      |
| Isoform0056751     | MXC17.10          | AT5G24710.1              | Transducin/WD40 repeatlike superfamily protein                             |
| Isoform0062524     | At1g06515         | AT1G06515.2              | Membrane protein, putative (DUF3317)                                        |
| Isoform0061573     | BHLH30            | AT1G68810.1              | Transcription factor bHLH30                                                |

Figure 3. The expression levels of coexpressed mRNAs of lncRNAs directly linked with flowering in A. sinensis at T2 vs. T1 and T3 vs. T1, as determined by qRT-PCR. The “*” represents a significant difference at p < 0.05 level between T2 vs. T1 and T3 vs. T2 for the same gene.

Table 4. Fourteen lncRNAs directly linked with temperature response.

| lncRNA ID          | Coexpressed mRNAs | mRNA ID                  | Proteins Encoded by Coexpressed mRNAs                                      |
|--------------------|-------------------|--------------------------|-----------------------------------------------------------------------------|
| Isoform0062470     | ACBP6             | AT1G31812.1              | Acyl-CoA-binding domain-containing protein 6                                |
| Isoform0061783     | ENO2              | AT2G36530.1              | Bifunctional enolase 2/transcriptional activator                            |
| Isoform0063049     | ADH1              | AT1G77120.1              | Alcohol dehydrogenase class-P                                              |
| Isoform0062198     | CSP2              | AT4G38680.1              | Cold shock protein 2                                                        |
| Isoform0035932     | RH20              | AT1G35150.2              | DEA(D/H)-box RNA helicase family protein                                    |
| Isoform0019851     | RH52              | AT3G386570.1             | DEAD-box ATP-dependent RNA helicase 52                                     |
| Isoform0062484     | RH53              | AT3G22330.1              | DEAD-box ATP-dependent RNA helicase 53, mitochondrial                      |
| Isoform0058095     | RAB18             | AT5G6400.1               | Dehydrin Rab18                                                             |
| Isoform0061968     | XERO1             | AT3G30980.1              | Dehydrin Xero 1                                                            |
| Isoform0020919     | MED14             | AT3G04740.1              | Mediator of RNA polymerase II transcription subunit 14                    |
| Isoform0062617     | HSP17.8           | AT1G07400.1              | 17.8 kDa class I heat shock protein                                         |
| Isoform009507      | HSP70-3           | AT3G09440.2              | Heat shock 70 kDa protein                                                   |
| Isoform0034676     | HSP70-10          | AT3G0590.1               | Heat shock 70 kDa protein 10, mitochondrial                                 |
| Isoform0042993     | HSP90-3           | AT3G6010.1               | Heat shock protein 90-3                                                   |
3.5. LncRNAs Linked with Metabolism and Expression Levels of Their Coexpressed mRNAs

In total, 117 lncRNAs were linked with metabolism based on the biological functions of their coexpressed mRNAs, with 19 lncRNAs associated with carbohydrate metabolism, namely CSY4, FBA3, GAPC1, GAPCP2, At3g52990, PGM2, USP, GLCNAC1PUT2, UXS2, Xyla, GALS1, OFUT31, At1g59950, At1g26850, and BAM1 (Table 5), and 98 lncRNAs associated with other types of metabolism, such as nucleotide, protein, and lipid metabolism (Table S5). The expression levels of five representative coexpressed mRNAs (CSY4, GAPCP2, At1g52990, PGM2, and BAM1) involved in carbohydrate metabolism were confirmed by qRT-PCR, with all five mRNAs showing down-regulation at T3 vs. T1, and three mRNAs showing higher levels at T2 vs. T1 than T3 vs. T1, with the exception of the two genes GAPCP2 and BAM1 (Figure 5).

Table 5. Nineteen lncRNAs directly linked with carbohydrate metabolism.

| lncRNA ID     | Coexpressed mRNAs | mRNA ID       | Proteins Encoded by Coexpressed mRNAs                                      |
|---------------|--------------------|---------------|------------------------------------------------------------------------------|
| Isoform0061974| CSY4               | AT2G44350.2   | Citrate synthase 4, mitochondrial                                            |
| Isoform0062251| FBA3               | AT2G01140.1   | Fructose-bisphosphate aldolase 3, chloroplastic                              |
| Isoform0062131| GAPC1              | AT3G04120.1   | Glyceraldehyde-3-phosphate dehydrogenase GAPC1, cytosolic                   |
| Isoform0062375| GAPCP2             | AT1G16300.1   | Glyceraldehyde-3-phosphate dehydrogenase GAPCP2, chloroplastic              |
| Isoform0062268| AT3g52990          | AT3G52990.1   | Pyruvate kinase                                                              |
| Isoform0062585| PGM2               | AT1G70730.3   | Phosphoglucomutase                                                           |
| Isoform0063034| USP                | AT5G52560.1   | (alpha-D-glucose-1,6-bisphosphate-dependent)                                 |
| Isoform0042435| GLCNAC1PUT2        | AT2G35020.1   | UDP-sugar pyrophosphorylase                                                  |
| Isoform0062018| UXS2               | AT3G62830.1   | UDP-glucuron acid decarboxylase 2                                            |
| Isoform0062750| Xyla               | AT5G57655.2   | Xylose isomerase                                                             |
| Isoform0060700| GALS1              | AT2G33570.1   | Galactan beta-1,4-galactosyltransferase GALS1                               |
| Isoform0062032| OFUT31             | AT4G24530.1   | O-fucosyltransferase 31                                                     |
| Isoform0039872| A1g567460          | AT5G67460.1   | Glucan endo-1,3-beta-D-glucosidase                                           |
| Isoform0045893| RSW3               | AT5G63840.1   | Glycosyl hydrolases family 31 protein                                       |
| Isoform0036200| A1g59950           | AT1G9950.1    | Aldo/keto reductase                                                          |
| Isoform0042053| A1g525970          | AT5G25970.2   | beta-1,6-N-acetylglucosaminyl-transferase family protein                     |
| Isoform0059181| UGT76E7            | AT5G38040.1   | UDP-glucosyltransferase 76E7                                                 |
| Isoform0037698| A1g26850           | AT1G26850.2   | Probable methyltransferase PMT2                                              |
| Isoform0062370| BAM1               | AT3G23920.1   | Beta-amylase 1, chloroplastic                                                |

Figure 4. The expression levels of coexpressed mRNAs of lncRNAs linked with temperature response in A. sinensis at T2 vs. T1 and T3 vs. T1, as determined by qRT-PCR. The “*” represents a significant difference at p < 0.05 level between T2 vs. T1 and T3 vs. T2 for the same gene.
3.6. LncRNAs Linked with Biosignaling and Expression Levels of Their Coexpressed mRNAs

In total, 23 lncRNAs were linked with metabolism based on the biological functions of their coexpressed mRNAs, with 13 lncRNAs associated with hormone signaling, namely ARF1, CUL1, T4L20.330, SOFL4, SOFL5, GRF2, GRF11, ERF3, CIPK20, SF1, AGD9, TIFY4B, and At2g34810 (Table 6), and 10 lncRNAs associated with other types of signaling, such as protein kinase, phosphatidylinositol-mediated, and cell surface receptor signaling (Table S6). The expression levels of eight representative coexpressed mRNAs associated with hormone signaling were confirmed by qRT-PCR, with three mRNAs (ARF1, CUL1, and GRF11) showing up-regulation at T2 vs. T1 and T3 vs. T1, and four mRNAs (ARF1, SOFL4, GRF2, and ERF3) showing lower levels at T2 vs. T1 than T3 vs. T1 (Figure 6).

Table 6. Thirteen lncRNAs directly linked with hormone signaling.

| lncRNA ID       | Coexpressed mRNAs | mRNA ID               | Proteins Encoded by Coexpressed mRNAs                      |
|-----------------|--------------------|-----------------------|------------------------------------------------------------|
| lsoform0062586  | ARF1               | AT2G47170.1           | ADP-ribosylation factor 1                                  |
| lsoform0061377  | CUL1               | AT4G02570.1           | Cullin-1                                                  |
| lsoform0015752  | T4L20.330          | AT4G34750.1           | SAUR-like auxin-responsive protein family                  |
| lsoform0057235  | SOFL4              | AT5G38790.1           | Protein SOB FIVE-LIKE 4                                   |
| lsoform0043114  | SOFL5              | AT4G33800.2           | Protein SOB FIVE-LIKE 5                                   |
| lsoform0062152  | GRF2               | AT1G78300.1           | 14-3-3-like protein GF14 omega                              |
| lsoform0062828  | GRF11              | AT1G34760.1           | 14-3-3-like protein GF14 omicron                          |
| lsoform0061395  | ERF3               | AT1G50640.1           | Ethylene-responsive transcription factor 3                 |
| lsoform0007735  | CIPK20             | AT5G45820.1           | CBL-interacting serine/threonine-protein kinase 20        |
| lsoform0022533  | SF1                | AT5G51300.2           | Splicing factor-like protein 1                            |
| lsoform0046311  | AGD9               | AT5G46750.1           | Probable ADP-ribosylation factor GTPase-activating protein AGD9 |
| lsoform0057859  | TIFY4B             | AT4G14720.1           | Protein TIFY 4B                                           |
| lsoform0062437  | At2g34810          | AT2G34810.1           | Berberine bridge enzyme-like 16                           |
Forty-three lncRNAs linked with energy and transport.

Table 6. Thirteen lncRNAs directly linked with hormone signaling.

| lncRNA ID   | Coexpressed mRNAs | mRNA ID                                             | Proteins Encoded by Coexpressed mRNAs                  |
|------------|--------------------|-----------------------------------------------------|-------------------------------------------------------|
| Energy (5) |                    |                                                     |                                                       |
| Isoform0044730 | PURU1          | AT5G47435.2                                         | Formyltetrahydrofolate deformylase 1, mitochondrial   |
| Isoform0031698 | ndbhB           | ATCG01250.1                                         | NAD(P)H-quinone oxidoreductase subunit 2 A, chloroplastic |
| Isoform0029144 | NDB1            | AT4G28220.2                                         | NADH:ubiquinone reductase (nonelectrogenic)           |
| Isoform0046995 | WNK9            | AT5G28080.3                                         | Nonspecific serine/threonine protein kinase           |
| Isoform0047216 | SECA2           | AT4G16690.1                                         | pFDCC methylesterase MES16                            |
| Transport (38) |              |                                                     |                                                       |
| Isoform0047603 | At5g11230       | AT5G11230.1                                         | Probable sugar phosphate/phosphate translocator At5g11230 |
| Isoform0063248 | GPT1            | AT5G54800.1                                         | Glucose-6-phosphate/phosphate translocator 1, chloroplastic |
| Isoform0053163 | SMXL5           | AT5G57130.1                                         | Protein SMXL1-LIKE 5                                 |
| Isoform0062571 | ABCF5           | AT5G64840.1                                         | ABC transporter F family member 5                     |
| Isoform0062182 | SFH8            | AT2G21520.2                                         | Phosphatidylinositol/phosphatidylycholine transfer protein SFH8 |
| Isoform0022607 | BASS6           | AT4G22840.1                                         | Probable sodium/metalobite cotransporter BASS6, chloroplastic |
| Isoform0008194 | SECA2           | AT1G21650.3                                         | Protein translocase subunit SECA2, chloroplastic      |
| Isoform0014041 | At4g14160       | AT4G14160.1                                         | Protein transport protein SEC23                       |
| Isoform0062660 | Ycf2-A          | ATCG01280.1                                         | Protein Ycf2                                         |
| Isoform0036773 | At4g22990       | AT4G22990.1                                         | SPX domain-containing membrane protein At4g22990     |
| Isoform0032676 | At3g49350       | AT3G49350.1                                         | At3g49350                                           |
| Isoform0062443 | TMT2            | AT4G35300.5                                         | Tonoplast monosaccharide transporter2                  |
| Isoform0003274 | VPS24-1         | AT5G22950.1                                         | Vacuolar protein sorting-associated protein 24 homolog 1 |
| Isoform0062373 | VPS26A          | AT5G53530.1                                         | Vacuolar protein sorting-associated protein 26A       |
| Isoform0028575 | VPS52           | AT1G71207.1                                         | Vacuolar protein sorting-associated protein 52 A      |
| Isoform0038654 | VP560-2         | AT5G04850.1                                         | Vacuolar protein sorting-associated protein 60.2      |

3.7. LncRNAs Linked with Energy and Transport

In all, 43 lncRNAs were found to be linked with energy and transport based on the biological functions of their coexpressed mRNAs, with 5 lncRNAs associated with energy, such as PURU1, ndbhB1, and NDB1, and 38 lncRNAs associated with transport, such as At5g11230, GPT1 and SMXL5 (Table 7). The expression levels of eight representative coexpressed mRNAs (PURU1, MES16, GPT1, ABCF5, SECA2, VPS26A, CML19, and NHX6) were confirmed by qRT-PCR, with three mRNAs (PURU, GPT1, and VPS26A) showing up-regulation at T2 vs. T1 and T3 vs. T1, and six mRNAs (PURU1, GPT1, ABCF5, SECA2, VPS26A, and CML19) showing higher levels at T2 vs. T1 than T3 vs. T1, with the exception of the two genes MES16 and NHX6 (Figure 7).

Table 7. Forty-three lncRNAs linked with energy and transport.
Table 7. Cont.

| IncRNA ID   | Coexpressed mRNAs          | mRNA ID          | Proteins Encoded by Coexpressed mRNAs                                                                 |
|-------------|-----------------------------|------------------|-------------------------------------------------------------------------------------------------------|
| Isoform0062996 | At5g19500                   | AT5G19500.1      | At5g19500                                                                                             |
| Isoform0062800 | PIP1-5                      | AT4G23400.1      | Probable aquaporin PIP1-5                                                                            |
| Isoform0061992 | SULTR2;2                    | AT1G77990.1      | Sulfate transporter 2.2                                                                               |
| Isoform0062606 | MT2A                        | AT3G09390.2      | Metallothionein-like protein 2A                                                                       |
| Isoform0057136 | At3g52300                   | AT3G52300.1      | ATP synthase subunit d, mitochondrial                                                                |
| Isoform0061450 | ABCB23                      | AT4G28630.1      | ABC transporter B family member 23, mitochondrial                                                      |
| Isoform0062449 | CML19                       | AT4G37010.1      | Calcium-binding protein CML19                                                                        |
| Isoform0061763 | JJJ1                        | AT1G74250.1      | DNAJ protein JJJ1 homolog                                                                            |
| Isoform0061955 | PAP1                        | AT1G13750.1      | Probable inactive purple acid phosphatase 1                                                           |
| Isoform0062074 | HIPP04                      | AT1G29000.2      | Heavy metal-associated isoprenylated plant protein 4                                                  |
| Isoform0062972 | HIPP26                      | AT4G38580.1      | Heavy metal-associated isoprenylated plant protein 26                                                 |
| Isoform0014712 | KINLA                       | AT1G12430.1      | Kinesin-like protein KIN-UA                                                                           |
| Isoform0063051 | IQD30                       | AT1G18840.2      | Protein IQ-DOMAIN 30                                                                                 |
| Isoform0016777 | At3g18430                   | AT3G18430.1      | Calcineurin b subunit (Protein phosphatase 2b regulatory subunit)-like protein                        |
| Isoform0063921 | CBL10                       | AT4G33000.6      | Calcineurin B-like protein 10                                                                         |
| Isoform0061307 | NHX6                        | AT1G79610.1      | Sodium/hydrogen exchanger 6                                                                           |
| Isoform0024682 | CNGC15                      | AT2G28260.2      | Cyclic nucleotide-gated channel 15                                                                    |
| Isoform0034638 | 2-Oct                       | AT1G79360.1      | Organic cation/carotidine transporter 2                                                                |
| Isoform0033810 | CNGC13                      | AT4G01010.2      | Putative cyclic nucleotide-gated ion channel 13                                                       |
| Isoform0051075 | MTG13.4                    | AT5G16680.1      | RING/FYVE/PHD zinc finger superfAMILY protein                                                          |
| Isoform0051563 | SUP4                        | AT1G30970.3      | Zinc finger (C2H2 type) family protein                                                                |
| Isoform0037483 | AHA4                        | AT3G47950.2      | ATPase 4, plasma membranetype                                                                         |

Figure 7. The expression levels of coexpressed mRNAs of IncRNAs linked with energy and transport in *A. sinensis* at T2 vs. T1 and T3 vs. T1, as determined by qRT-PCR. The “*” represents a significant difference at p < 0.05 level between T2 vs. T1 and T3 vs. T2 for the same gene.

4. Discussion

Vernalization is a process considered to be an epigenetic switch whereby flowering is promoted by prolonged exposure to cold (0 to 10 °C); meanwhile, it can be lost at high temperatures or avoided below freezing temperatures [37,38]. Epigenetic regulation involves diverse molecular mechanisms including chromatin remodeling, DNA methylation, histone modification, histone variants, and ncRNAs [39]. Studies on *Brassica rapa* found that 127 differentially expressed IncRNAs were coexpressed with 128 differentially expressed genes, indicating that IncRNAs played an important role during vernalization [40]. In this study, 272 characterized IncRNAs were identified from *A. sinensis* and divided into six categories, namely (1) chromatin, DNA/RNA, and protein modification; (2) flowering;
(3) stress response; (4) metabolism; (5) biosignaling; and (6) energy and transport, based on their coexpressed mRNAs.

FLC is a MADS-box transcriptional regulator that acts as a potent repressor of flowering [38]. In Arabidopsis, the epigenetic silencing of the floral repressor gene FLC is a well-characterized key event of vernalization [41]. In this study, 29 lncRNAs linked with chromatin, DNA/RNA, and protein modification were identified in A. sinensis during vernalization. For the chromatin modification, two coexpressed mRNAs, HMGB2 and HMGB3, are involved in binding preferentially double-stranded DNA and up-regulated in response to cold stress [42]. For the DNA/RNA modification, At1g05910 is involved in DNA demethylation and the negative regulation of chromatin silencing [43]; R2D2 is involved in rRNA methyltransferase activity [44]; and At4g26600 is involved in RNA methylation [45]. For protein modification, 24 lncRNAs were involved and the roles of nine coexpressed mRNAs were represented as follows. H2AV plays a central role in regulating transcription, repairing DNA, replicating DNA, and stabilizing the nucleus chromosome [46]; At2g28720 and HTR2 are involved in compacting DNA into chromatin [47,48]; HOS15 promotes the deacetylation of histone H4 [49]; REF6 is involved in demethylating ‘Lys-27’ of histone H3, regulating flowering time by repressing FLC expression and interacting with the NF-Y complex to regulate SOC1 [50,51]; SKP1A and ASK21 are involved in ubiquitination and form an SCF E3 ubiquitin ligase complex together with CUL1, RBX1, and an F-box protein [52,53]; and SRK2G and SRK2H are involved in protein phosphorylation [54,55]. In previous studies, HMGB2 and HMGB3 were up-regulated in response to cold stress but down-regulated in response to drought and salt stresses [42]; H2AV, At2g28720 and HTR2 exhibited a high level in response to osmotic and drought stresses [56]; HOS15 was found to act as a repressor of cold stress-regulated gene expression and played a role in gene regulation for plant acclimation and tolerance to cold stress [49]; SKP1A and ASK21 were overexpressed in the host stress response [57]; and SRK2G and SRK2H were found to be positive regulators in stress responses such as drought, salt, and cold [54,55,58]. Currently, although most of the lncRNAs have been reported to be involved in the stress response in many plants, their roles in the regulation of flowering time have been studied in model plants [23]. Previous studies have found that FLC in Arabidopsis is epigenetically regulated by lncRNAs COOLAIR and COLDAIR [59–61]. In this study, a lower expression level was noted for almost all coexpressed mRNAs of lncRNAs involved in chromatin, DNA/RNA, and protein modification at vernalization (T2, 0 °C) compared with freezing temperature (T3, −3 °C), as well as down-regulation at T2, indicating that these lncRNAs participate in epigenetic silencing by transferring euchromatin to heterochromatin and confer early bolting and flowering of A. sinensis. Representatively, the down-regulation of mRNAs HMGB2 and HMGB3 during vernalization transfers the heterochromatin to the euchromatin [42], which generates the ability of flowering; in contrast, their up-regulation at freezing temperatures inhibits flowering by keeping the heterochromatin, which indicates that their coexpressed lncRNAs play positive roles in regulating the flowering of A. sinensis. The down-regulation of mRNA REF6 below 0 °C weakens the demethylation of histone H3 and delays flowering time by inducing FLC expression [50,51]; meanwhile, there is an increased expression level with decreased temperatures, which indicates that this coexpressed lncRNA plays a negative role in regulating the flowering of A. sinensis.

Flower formation occurs at the SAM and is a complex morphological event that is required not only for the circadian clock to measure the passage of time but also the regulation of meristem identity genes [42]. For the 6 representative coexpressed mRNAs of the 12 lncRNAs directly linked with flowering, SRR1 is involved in a circadian clock input pathway and regulation of the expression of clock-regulated genes such as CCA1 and TOCI [62]; PHL is involved in the circadian rhythm and the regulation of the timing of transition [63]; PHYA is involved in the regulation of flowering time and expression of its own gene as negative feedback [64]; AGL62 is required for promoting the nuclear proliferation of early endosperm [65]; AGL79 is involved in positively regulating the transition of the meristem from the vegetative to reproductive phase [66]; and ATJ3 plays a...
continuous role in plant development, such as in photoperiodism, flowering, and positive regulation of flower development [67]. Previous studies found that mRNAs (e.g., miR156, miR169 and miR172) play a crucial role in developmental processes in rice, wheat, and maize, especially in the formation of the floral meristem, with miR172 controlling AP2-like genes [23,68,69]. Studies on the flowering of Chenopodium quinoa found that pivotal flowering homologs, including photoreceptor genes PHYA and CRY1, as well as genes associated with florigen-encoding genes (FT and TWIN SISTER of FT) and circadian clock-related genes (ELF3, LHY, and HY5), were specifically affected by night-break and competed with the positive- and negative-flowering lncRNAs [70]. In this study, down-regulation of all the coexpressed mRNAs involved in circadian clock and meristem identity genes was observed, which can be considered acceptable and reasonable, because these mRNAs are often highly expressed at the plant development stage (at photoperiod), while their expression levels were examined during vernalization. In addition, increased expression with decreased temperatures indicates that their coexpressed lncRNAs play negative roles in regulating the flowering of *A. sinensis*.

The expression of numerous lncRNAs has been demonstrated to be significantly affected by various stresses [23,30]. During vernalization, plants have to face and adapt to low temperature [38]. To date, extensive studies have reported that lncRNAs participate in defense responses associated with plant immunity and adaptation to the environment [22]. Heat-responsive lncRNAs have been found to be differentially expressed in *Brassica juncea*, and cold-responsive lncRNAs have been identified in grape and *Arabidopsis* [71–73]. lncRNAs could regulate HSP family genes (HSP82 and HSP83) in response to heat stress in *Populus x canadensis* Moench, and HSP18.1 in response to Cd stress *Betula platyphylla* [74,75]. For the eight representative coexpressed mRNAs of the 14 lncRNAs linked with the temperature response, ACBP6 confers resistance to cold and freezing [76]; ENO2 acts as a positive regulator in response to cold stress [77]; ADH1 is required for survival and acclimation in response to abiotic stresses (e.g., cold, salt, and drought) [78,79]; CSP2 contributes to the enhancement of cold and freezing tolerance [80]; and HSP17.8, HSP70-3, HSP70-10, and HSP90-3 play vital roles in adapting to biotic and abiotic stresses [81,82]. In this study, increased expression for cold-tolerated mRNAs (ACBP6, ENO2, ADH1, and CSP2), and decreased expression for heat-tolerated mRNAs (HSP17.8, HSP70-3, HSP70-10, and HSP90-3), were observed with decreased temperatures, which indicates that their coexpressed lncRNAs play positive roles in adapting to low temperatures.

For vernalization to occur, sources of energy (sugars) and carbohydrate metabolism are required [37]. In recent years, the roles of lncRNAs in regulating metabolism in cancer, insulin, and chicken have been reported [83–85], while, in plants, studies are still limited. For the five representative coexpressed mRNAs of the 19 lncRNAs linked with carbohydrate metabolism, CSY4 is involved in the synthesis of scisic acid can either inhibit or promote flowering [37]. In previous studies, a pre-miRNA of miR93 was identified in *Brassica rapa* during vernalization, and the overexpression of an miR93-resistant form of TIR1 (mTIR1) could enhance auxin sensitivity, thus leading to pleiotropic effects on plant development [92]. For the 8 representative coexpressed mRNAs of the 13 lncRNAs linked with hormone signaling, ARF1 is involved in the recruitment
of COPI and GDAP1 to membranes and various auxin-dependent developmental processes [93]; CUL1 participates in forming a SCF complex together with SKP1, RBX1, and a F-box protein and is involved in floral organ development, the auxin signaling pathway and ethylene signaling [94]; SOFL4 and SOFL5 are involved in cytokinin-mediated development [95]; GRF2 and GRF11 participate in the brassinosteroid (BR)-mediated signaling pathway [96,97]; ERF3 is found to be differentially expressed in response to stresses and also regulates other ERFs [98]; and SF1 is required for development and is involved in the alternative splicing of FLM pre-mRNA [99]. In this study, the differential expression of these mRNAs involved in hormone signaling also played certain roles in regulating the flower-bud differentiation of seedlings and cold tolerance during vernalization. In previous studies, GRF2 and ERF3 were found to be down-regulated, while GRF11 was up-regulated in response to cold stress [100,101]; here, contrary findings for mRNAs GRF2 and GRF11 were observed with temperatures decreased, which indicate that their coexpressed IncRNAs may play negative roles in hormone signaling. The interaction between SF1 and FLM pre-mRNA controls flowering time in response to temperature [102]; here, decreased expression of mRNA SF1 was observed with decreased temperatures, which indicates that this coexpressed IncRNA may play a positive role in hormone signaling.

Energy generation and the transport of energy, nutrients and metabolites play essential roles in growth and development and stress tolerance [103]. For the 8 representative coexpressed mRNAs of the 43 IncRNAs linked with energy and transport, PURU1 is involved in photorespiration and participates in preventing the excessive accumulation of 5-formyl tetrahydrofolate [104]; MES16 is involved in chlorophyll breakdown by its demethylation [105]; GPT1 is involved in NADPH generation through a series of processes including Glc6P transport, starch biosynthesis, fatty acid biosynthesis, and oxidative pentose phosphate [106]; ABCF5 belongs to the ABC transporter superfamily and is involved in protein transport [107]; SECA2 is involved in protein export coupling ATP hydrolysis [108]; VPS26A plays a role in vesicular protein sorting and is essential in endosome-to-Golgi retrograde transport [109]; CML19 is a potential calcium sensor that binds calcium and is involved in the early response to stress [110]; and NHX6 is involved in trafficking to the vacuole and exchanging the low-affinity electroneutral Na(+) or K(+)/H(+) [111]. In this study, the differential expression of these coexpressed mRNAs associated with energy and transport provided energy, nutrients, and metabolites for A. sinensis seedlings to obtain the capacity for vernalization and, meanwhile, to adapt to low temperatures.

Based on the above functional analysis of IncRNAs identified in this study, flowering pathways proposed in the previous literature [14–16] and a general model of stress-responsive regulation by regulatory IncRNAs [23], a model of vernalization-induced bolting and flowering by regulatory IncRNAs in A. sinensis is proposed (Figure 8). Briefly, the vernalization of seedlings firstly triggers the differential expression of IncRNAs; then, the IncRNAs either act as a precursor of miRNAs or as a miRNA target mimic, which further binds their related targets; then, the binding of targets regulates the expression of their downstream mRNAs that are involved in various biological processes, including the temperature response, flowering pathways (i.e., epigenetic modification, flowering induction, carbohydrate metabolism, and hormone signaling), as well as energy and transport; finally, these biological processes promote the transition of the meristem from the vegetative phase to the bolting and flowering of A. sinensis.
Figure 8. A proposed model of vernalization-induced bolting and flowering by regulatory lncRNAs in *A. sinensis*.

5. Conclusions

From the above observations, we found that the lncRNAs positively or negatively regulated the expression of their downstream mRNAs through epigenetic changes at the level of transcription and post-transcription for the flowering of *A. sinensis* during vernalization. This coexpressed mRNA analysis of lncRNAs focused on five pathways, namely (1) chromatin, DNA/RNA, and protein modification; (2) floral development; (3) temperature response; (4) carbohydrate metabolism; and (5) hormone signaling. While several candidate lncRNAs were identified, their causative roles require further investigations.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cimb44050128/s1.

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Abbreviations

| Abbreviation | Description                                      |
|--------------|--------------------------------------------------|
| AP1          | APETALA 1                                        |
| CCS          | Circular consensus sequence                      |
| CNCI         | Coding-Noncoding Index                          |
| COLDAIR      | COLD-ASSISTED INTRONIC NON-CODING RNA            |
| COOLAIR      | COLD-INDUCED LONG ANTISENSE INTRAGENIC RNAs     |
| CPC          | Coding-Potential Calculator                     |
| FLC          | FLOWERING LOCUS C                                |
| FLML         | FLOWERING LOCUS M                                |
| FLNC         | Full-length nonchimeric                         |
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