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Comparative transcriptome discovery and elucidation of the mechanism of long noncoding RNAs during vernalization in *Brassica rapa*

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Abstract

Flowering time is an important agronomic trait in *Brassica rapa* (*B. rapa*). However, our current understanding of the role of long noncoding RNAs (lncRNAs) in flowering time responded to vernalization is limited. The rapid development of the omics sequencing technology has facilitated the identification of thousands of lncRNAs in various plant species. Here, we used comparative transcriptome analysis between control and vernalized *B. rapa* to identify differentially expressed genes (DEGs) and lncRNAs (DELs). A total of 300 DEGs and 254 DELs were identified. Co-localization networks consisting of 128 DEGs and 127 DELs were established, followed by analyses of hierarchical categories, functional annotations, and correlation from mRNA-to-lncRNA. We found that the *BraZF-HD21* (*Bra026812)* gene which responds to photoperiods and vernalization is correlated with lncRNA TCONS_00035129. The correlated genes that were mapped to the plant hormone signal transduction pathway and increased gibberellin A3 (GA$_3$) content demonstrated that vernalization influences plant hormone levels. These findings suggest that vernalization alters the process of hormone biosynthesis, which in turn regulates flowering. This study provides an approach to elucidation of the regulatory mechanism of lncRNAs during vernalization in *B. rapa*.

Keywords Comparative transcriptome · *Brassica rapa* · Vernalization · LncRNA · Conetworks · Hormones

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Introduction

In flowering plants, the transition to flowering is an important developmental process linking vegetative growth to reproductive growth, which is sensitive to various seasonal

climatic signals (Koornneef et al. 2003). Some plants need to be exposed to winter cold to be competent to initiate the floral transition the following spring. Unlike photoperiods, winter cold does not immediately induce flowering. Rather, winter cold renders plants to be competent to flower under inductive photoperiods through a process known as vernalization (Bernier and Henichart 1981; Chouard 2003). The control of this process is particularly important in various crop plants to ensure high agricultural productivity. Our current understanding of vernalization's role in controlling flowering originated from the molecular genetic analysis of *Arabidopsis* (Henderson et al. 2003). The pathways conferring the requirement for, and the ability to respond to, vernalization converge on the regulation of the floral gene, *FLOWERING LOCUS C* (*FLC*) (Michaels and Amasino 1999; Sheldon et al. 1999). During vernalization, *FLC*, which encodes a MADS domain protein that acts as a repressor of flowering, is repressed, and the stable repression of *FLC* allows the activation of downstream floral activators to initiate flowering (Michaels and Amasino 2001; Helliwell et al. 2006; Searle et al. 2006). In *Arabidopsis*, the progressive repression and stable silencing of *FLC* is central to the vernalization mechanism (Michaels and Amasino 1999; Sheldon et al. 1999). In other plant species, *FLC* orthologs or other target genes are central to the developmental release that enables flowering (Sung et al. 2006; Oliver et al. 2009; Wang et al. 2009; Aikawa et al. 2010; Pin et al. 2010). *VERNALIZATION1* (*VRN1*) and *VERNALIZATION2* (*VRN2*) are required for the maintenance of *FLC* repression during subsequent development, following prolonged cold exposure (Gendall et al. 2001; Levy and Dean 2002). During cold, a unique, cold-specific Polycomb component known as *VERNALIZATION INSENSITIVE 3* (*VIN3*) is induced; VIN3 is a protein only produced during winter cold, which is necessary for Polycomb-mediated *FLC* silencing. The induction kinetics of *VIN3* by cold is correlated to the degree of the vernalization response (Sung and Amasino 2004; Wood et al. 2006; De et al. 2008). The Polycomb complex adds methyl groups to histone 3 (H3) at lysine 27 (K27) residues to form trimethylated H3 (H3K27me3), and increased H3K27me3 levels in the *FLC* chromatin appears to be one of the first chromatin changes accompanying *FLC* silencing (Bastow et al. 2004; Sung and Amasino 2004; Finnegan and Dennis 2007; Angel et al. 2011). Vernalization-mediated *FLC* silencing is also associated with increased lysine 9 (K9) trimethylation at H3 (Sung et al. 2006), and H3K9me3 appears to be required for the memory of winter at *FLC*.

The *Brassica* genus, comprising a large and diverse group of important oil, vegetable, fodder, and condiment crops, is closely related to *Arabidopsis thaliana*, which is a member of the Brassicaceae family. Flowering time, which is generally regulated by temperature and/or day length, is a very important developmental trait, and wide variation exists among *B. rapa* accessions. *B. rapa*, in contrast to *B. oleracea* (Lin et al. 2005), can be vernalized at the germinated seed stage, which can shorten generation time and thus speed up genetic research. However, the vernalization response varies among different cultivar groups. In general, oil types and several *B. rapa* cultivars flower very early, even under non-vernalized conditions. The very late flowering types are mainly Chinese cabbages and turnips, which need long-term vernalization to accelerate flowering. Currently, several *FLC* paralogs in *B. rapa* (*BrFLC1, BrFLC2, BrFLC3*, and *BrFLC5*) have been cloned and physically and genetically mapped (Yang et al. 2006; Kim et al. 2007). The study of *BrFLCs* expression in Chinese cabbage (Kim et al. 2007) and *B. napus* (Tadege et al. 2002) indicates that the *Brassica FLC* genes act similarly to *Arabidopsis FLC* gene.

In a recent study, long noncoding RNAs (lncRNAs) were found to have an important function in regulating Polycomb complexes (Kaneko et al. 2010, 2013; Zhao et al. 2010). In mammals, the polycomb repression complex 2 (PRC2)-associated lncRNAs and promoter-derived lncRNAs were identified, including promoter-derived lncRNAs (Kaneko et al. 2010; Negishi et al. 2014). During vernalization in *Arabidopsis*, an lncRNA called *COLDAIR* (the Polycomb complex often binds to lncRNAs) expresses, which directly associates with PRC2 to increase occupancy of PRC2 on *FLC* chromatin (Heo and Sung 2011). An additional Polycomb-binding lncRNA (*COLDWRAP*), derived from the repressed promoter of *FLC*, was identified. *COLDWRAP* is necessary for the establishment of stably repressed *FLC* by vernalization (Heo and Sung 2011). These results suggest that lncRNA plays an important role during vernalization. However, our understanding of the mechanism underlying the regulation of flowering time by lncRNAs during vernalization is limited.

In this study, using Illumina sequencing technology, we sequenced the whole transcriptome of control and vernalized *B. rapa*. Using RNA-seq, we identified vernalization lncRNA and mRNA clusters and characterized changes in their expression patterns. Through different transcription factors (TFs) and kyoto encyclopedia of genes and genomes (KEGG) pathway analysis, we found that some mRNAs were associated with plant hormone signal transduction. In addition, we measured the GA_3 content, which is closely related to flowering. Furthermore, we identified some vernalization lncRNA-mRNA modules, indicating that lncRNAs and mRNAs cooperate functionally during vernalization. We also predicted the functions of lncRNAs based on the cooperation between lncRNAs and mRNAs during vernalization, and additional vernalization might have impacted plant hormone levels (Fig. 1).
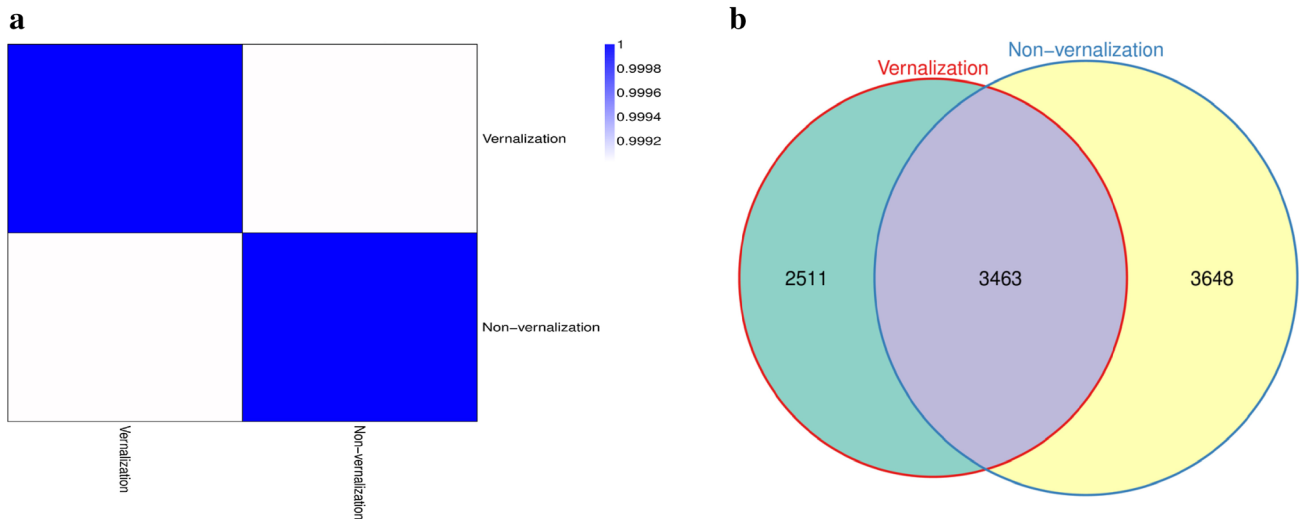
a

b

Fig. 1 Pearson correlation coefficient analysis and Venn chart of co-expressed genes. **a** Pearson correlation coefficient analysis of two libraries. The PCCs were calculated using Log_2(FPKM). **b** Co-expressed genes between non-vernalization and vernalization treatment. (Color figure online)

Results

Global expression of lncRNAs in *B. rapa*

For global transcriptome analysis of *B. rapa* under vernalization treatment, we constructed and sequenced two RNA-Seq libraries using the Illumina HiSeq™ 2000 platform. We obtained 110,985,906 and 107,687,374 clean reads in the control and vernalization libraries, respectively. The clean reads of the two cDNA libraries account for > 95% of the raw reads (Fig. S1, Table S1). However, the low mapping rate results from different cultivated varieties and polluted by the sequence of chloroplast. Transcript coverage was 40–50%, accounting for 36.42 and 31.66% of the control and vernalization libraries, respectively (Figs. S2 and S3). The reads were evenly distributed on the reference gene, indicating the randomness of the profiling data. We used TopHat to align paired-end reads to the *B. rapa* reference genome (*B. rapa* genome sequence, v1.5, downloaded from the BRAD database http://brassicadb.org/) and used Cufflinks to assemble the transcriptome. We identified 20,666 mRNAs and 2040 novel lncRNAs (> 200 nt and without coding potential) in the control and vernalization libraries (Tables S2 and S3). LncRNA and mRNA expression in the control and vernalization libraries were visualized for each sample (http://nhccdata.njau.edu.cn/). A total of 1961 lncRNAs and 23,289 mRNAs were detected, which showed differential expression during vernalization (Fig. 2).

Subsequently, we calculated the Pearson correlation coefficients (PCCs) of the transcriptome between the control and vernalization groups (Fig. 1a) based on fragments per kilobase of exon model per million mapped (FRKM). A total of

23,585 transcripts were detected, and relative information, including expression value (FPKM), coverage, the length of amino acid, as well as differential expression level between control and vernalized *B. rapa*, was respectively listed (Table S4). Approximately 3463 genes were selected for co-expression screening between the control and vernalized *B. rapa*. To further assess the expressed genes under vernalization, we constructed a Venn diagram, which showed that 2511 genes were exclusively expressed during vernalization, and 3648 genes only expressed in the control (Fig. 1b).

Classification of annotated lncRNAs

LncRNAs are a group of endogenous RNAs that are involved in the regulation of gene expression and have important functional roles during development and other physiological processes. Some lncRNAs are longer than 200 bp, and the short RNAs produced by lncRNAs act as primary transcripts. LncRNAs can be classified based on their position relative to the reference gene, and different strategies for function prediction were used in this study. For antisense lncRNAs, free energy calculations were conducted to identify possible hybridization sites for lncRNAs and mRNAs, which could be RNA–RNA interaction. A total of 400 lncRNAs were detected in the treatment. Because lncRNA are processed to yield small RNAs, small RNA precursor prediction was performed. We found 12 pri-miRNAs that included 3 known pri-miRNAs and 9 novel pri-miRNAs (Table S5). Further investigation determined that the identified pri-miRNAs have at least two stem-loop structures that were attained by self-folding (Fig. 3 and S4, Table S5), suggesting the existence of protein- or chromosome-binding
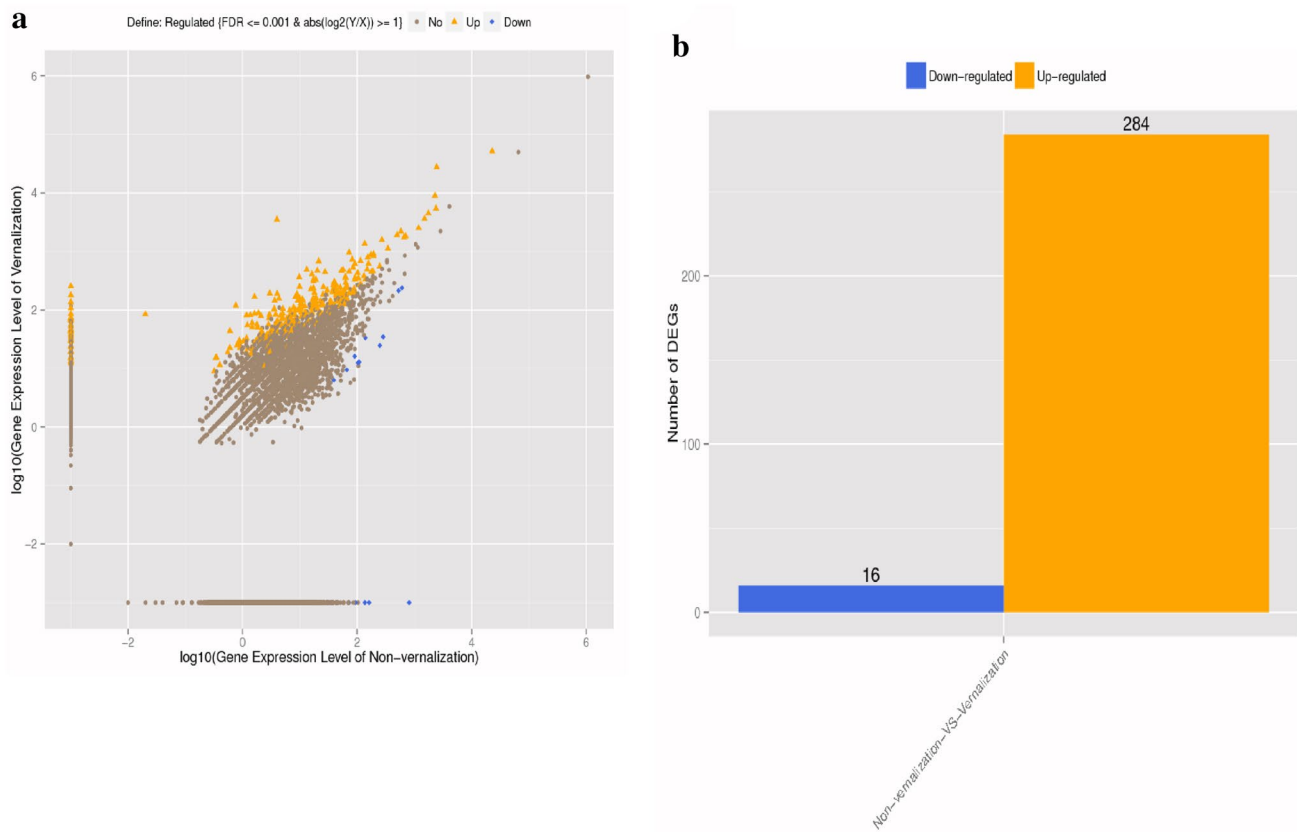
Fig. 2 Screening of differentially expressed genes (DEGs) **a** scatter chart of all expressed genes of each pair. **b** Stat chart of differentially expressed gene of each pair. (Color figure online)

sites. The selected lncRNAs may be involved in the complex chromatin–modifying complexes or in the regulation of gene transcription. We then screened for lncRNAs that were located within the flanking regions of genes. The results showed that 767 lncRNAs were situated upstream or downstream of genes. We also classified six lncRNAs into five families based on their RNA structure and sequence similarities, namely, LOC285194, TUG1_3, DLEU1_2, and JPX_1 WT1, which may also provide hints on their potential functions (Table S6).

Vernalization-dependent lncRNAs and mRNAs in *B. rapa* identified by RNA-Seq

FPKM was used to estimate the expression levels of the lncRNA transcripts. We identified 254 lncRNA transcripts that were differentially expressed during vernalization treatment (Table S7), which showed a higher number of upregulated lncRNAs than downregulated lncRNAs. The expression patterns of the differentially expressed lncRNAs were measured by systematic cluster analysis to explore the similarities and compare the relationship between the two libraries (Fig. 4). These lncRNA clusters exhibiting

vernalization-specific expression patterns may play distinct functional roles during vernalization. Moreover, some mRNA clusters showed vernalization-specific expression patterns similar to lncRNAs, indicating cooperation between lncRNAs and mRNAs during development.

To identify vernalization-responsive genes, the mRNA profiles of the control and vernalized plants were compared. Based on FPKM, 9621 genes were identified as differentially regulated gene pairs (Fig. 4). Subsequently, 300 genes were identified as DEGs that included 284 upregulated and 16 downregulated genes (Table S8).

Functional analysis of differentially expressed lncRNAs and mRNAs

To analyze the functions of lncRNAs and mRNAs, we clustered differentially expressed lncRNAs and mRNAs. Compared to mRNAs, there were 37 upregulated and 8 downregulated genes in the lncRNAs. For each comparison, the number of upregulated genes exceeded that of downregulated genes. To gain more insights into the functions of the DEGs, we searched for markedly enriched gene ontology (GO) terms and KEGG pathway enrichment by using the
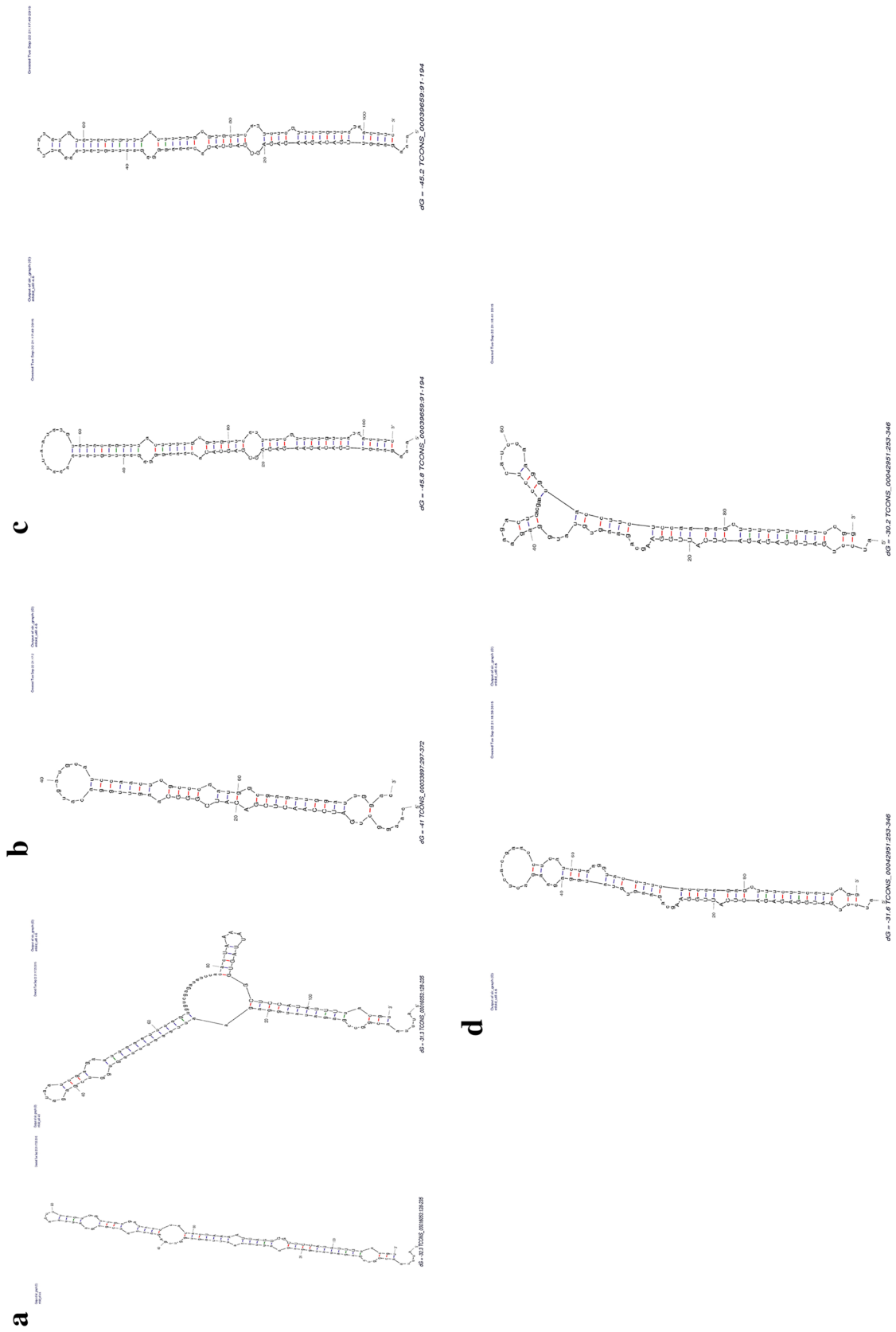
Fig. 3 Secondary structure of novel pre-miRNA. **a–d** Secondary structure of novel lncRNA TCONS_00016053_128-235, TCONS_00033897_297-372, TCONS_00039659_91-194 and TCONS_00042951_253-346
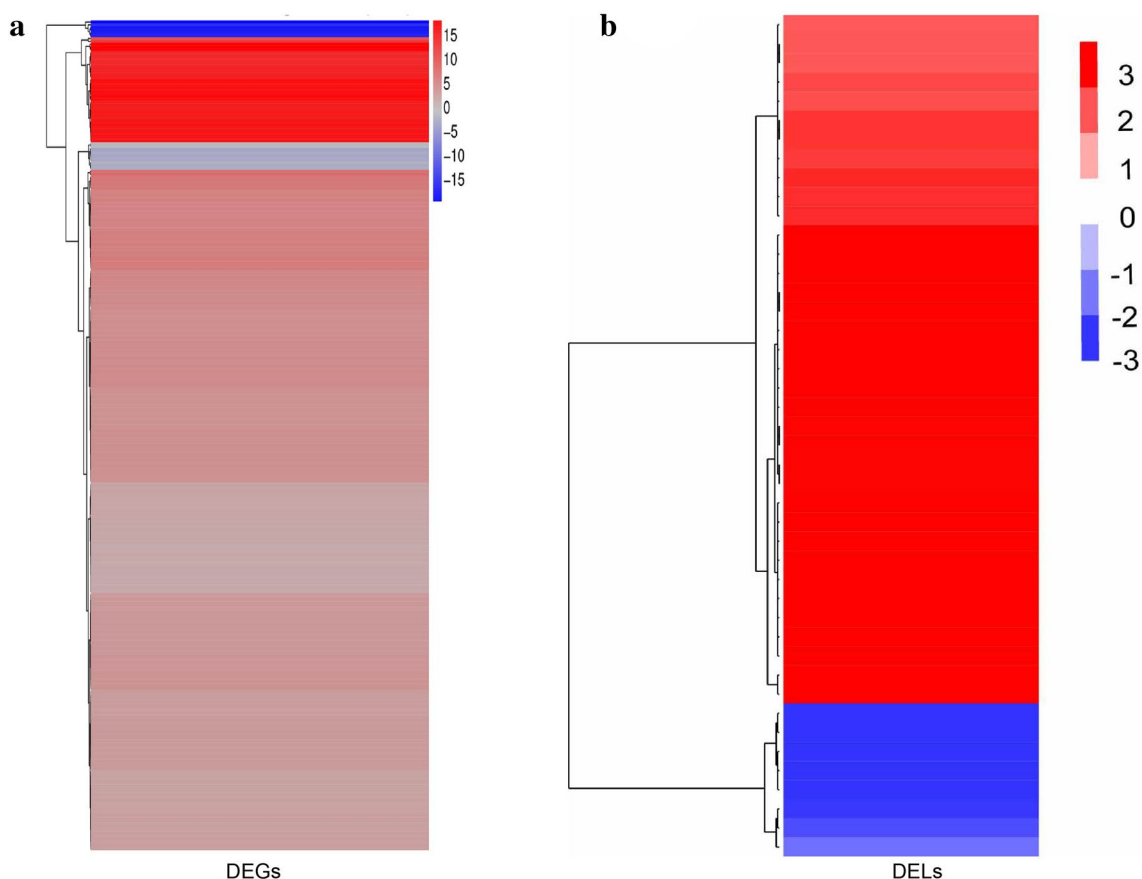
Fig. 4 Landscape of DEGs and DELs for *B.rapa* RNA-Seq transcriptome. **a** Cluster analysis of DEGs. **b** Cluster analysis of DELs. (Color figure online)

WEGO software and KEGG database (http://www.genome.jp/kegg/). GO analysis indicated that these DEGs could be classified into 9 molcular functions, 17 biological processes, and 9 cellular components (Fig. 5). Binding and catalytic activity was the largest subcategory under the molecular function category, metabolic process was the largest for the biological process category that included 122 DEGs, and the subcategories cell and cell part predominated the cellular component category, which comprised 100 DEGs (Table S9).

In addition, 164 (61%) significantly differentially expressed transcripts could be mapped to the reference canonical pathways in KEGG for functional categorization and annotation, thereby identifying 63 pathways. The top 20 enriched pathway terms are presented in Fig. 6 and Table S10. The KEGG enrichment scatterplot shows that significant DEGs were mostly enriched in two pathways that were related to metabolism (46 DEGs, 28.05%) and the biosynthesis of secondary metabolites (28 DEGs, 17.07%; Fig. 6). Additionally, KEGG enrichment analysis indicated that a total of 22,127 DEGs could be mapped to 123 pathways, of which 53 members exhibited similar profiles and

belonging to 43 pathways (Table S10). Moreover, we identified 14 DEGs (8.54%) that were enriched in the plant hormone signaling pathway. Finally, we selected 19 DEGs and 12 DELs that were used in qRT-PCR verification, which indicated that our results were indeed consistent and reliable (Fig. S7).

Identification of vernalization-related transcription factors (TFs)

Because TFs play an important role in the network of vernalization genes, we then sought to identify vernalization-inducible TFs. We observed that Trihelix, MYB, ZF-HD, GRAS, bHLH, WRKY, C2C2-Dof, AP2-EREBP, and HSF were differentially expressed after vernalization (Table S11). We also explored the functions of these differentially expressed TFs. ZF-HD TFs mainly harbored the ZF-HD_dimer domain, and all members were expressed in floral tissues. Furthermore, the *Bra026812* belongs to ZF-HD, which was differentially expressed during vernalization. RNAplex analysis predicts that the lncRNA TCONS_00035129 interacts with *Bra026812* (Fig. 7a). We previously reported that
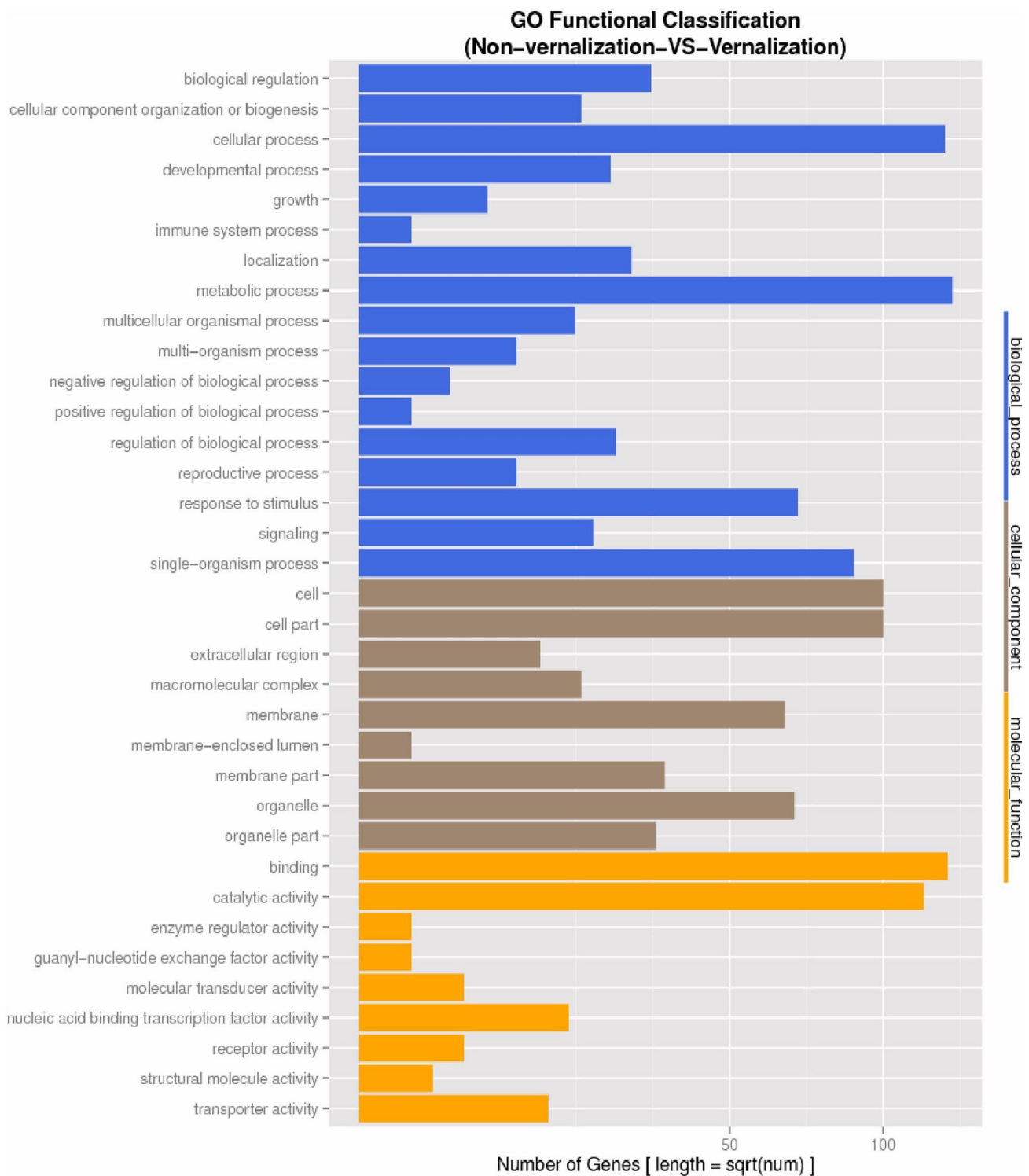
GO Functional Classification
(Non–vernalization–VS–Vernalization)

Fig. 5 Histogram of level GO term assignment of *B.rapa* re-annotated gene. (Color figure online)

the *BraZF-HD21* (*Bra026812*) gene responds to photo-periods and vernalization (Wang et al. 2016). Additionally, C2C2-Dof is involved in flower induction in higher plants. In present study, we determined that *Bra004396* (C2C2-Dof)

interacts with TCONS_0002673 (lncRNA) (Fig. 7c). qPCR analysis indicated an increase in both genes (*Bra026812* and *Bra004396*) and lncRNAs (TCONS_00035129 and TCONS_0002673) expression after vernalization, thereby
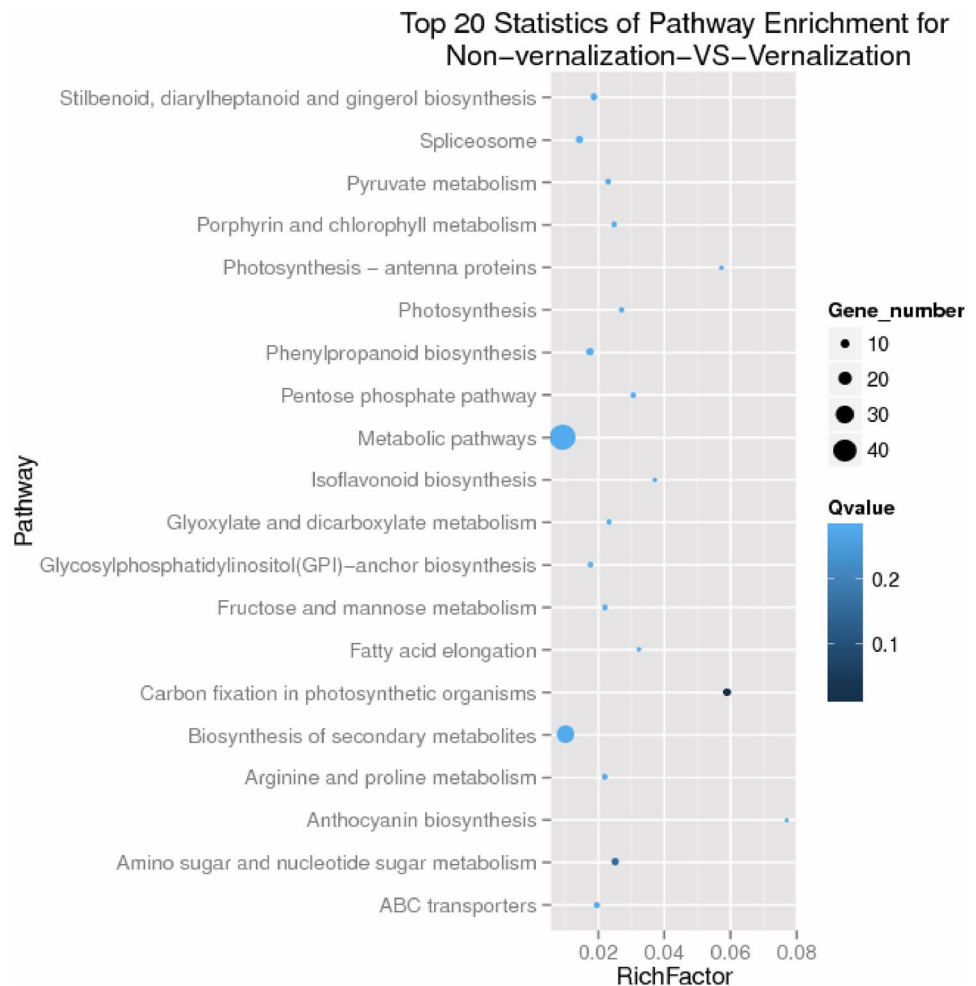
revealing the positive role of lncRNAs in genes regulation (Fig. 7e, f). These phenomena indicate that these lncRNAs and TFs respond to vernalization.

Some differentially expressed TFs also responded to temperature. Further investigation using expression profiling determined that the bHLH gene (*Bra037414*), a homolog of *AT4G01460* in *Arabidopsis* that may be involved in carbohydrate and amino acid biosynthesis, may also play a role in cold acclimation (Oono et al. 2006). Heat transcription factors (HSFs) mediate the activation of genes responsive to heat stress and various physiological stresses. However, some HSFs are involved in cold-responsive gene regulation such as *AT3G24520*. The homolog of *BrHsf-23* is upregulated by heat treatment and downregulated by cold treatment (Song et al. 2014). A number of TFs related to the plant hormones were also identified in this study.

Changes in hormone contents in vernalized plants

Significantly upregulated transcripts in *B. rapa* during vernalization were mapped to 14 genes belonging to different hormone signal transduction response pathways (Fig. S5,

Tables S2 and S10). These DEGs were hormone-related and involved in the cytokinin, abscisic acid, gibberellins, and brassinosteroids pathways (Fig. S5). In the gibberellins metabolism and signaling pathway, one upregulated DEG was identified. Similar results were observed in the cytokinin, abscisic acid, and brassinosteroid biosynthesis pathways. Because the gibberellin signaling pathway, which can be induced by GA, is an important pathway that triggers flowering, we measured the content of GA_3 in the control and vernalized plants. Increased GA_3 levels were observed after vernalization (Fig. 8, Table S13).

In addition, we also found that four AP2-EREBP genes (*Bra031371, Bra021200, Bra032724*, and *Bra030919*), which were associated with the ethylene signaling pathway, were differentially expressed after vernalization treatment (Table S11). A previous study has shown change in ethylene production in vernalized plants (Suge 1977). We also identified a pre-miRNA of miR393 (Table S12). The overexpression of an miR393-resistant form of TIR1 (mTIR1) enhances auxin sensitivity, leading to pleiotropic effects on plant development, including delayed flowering
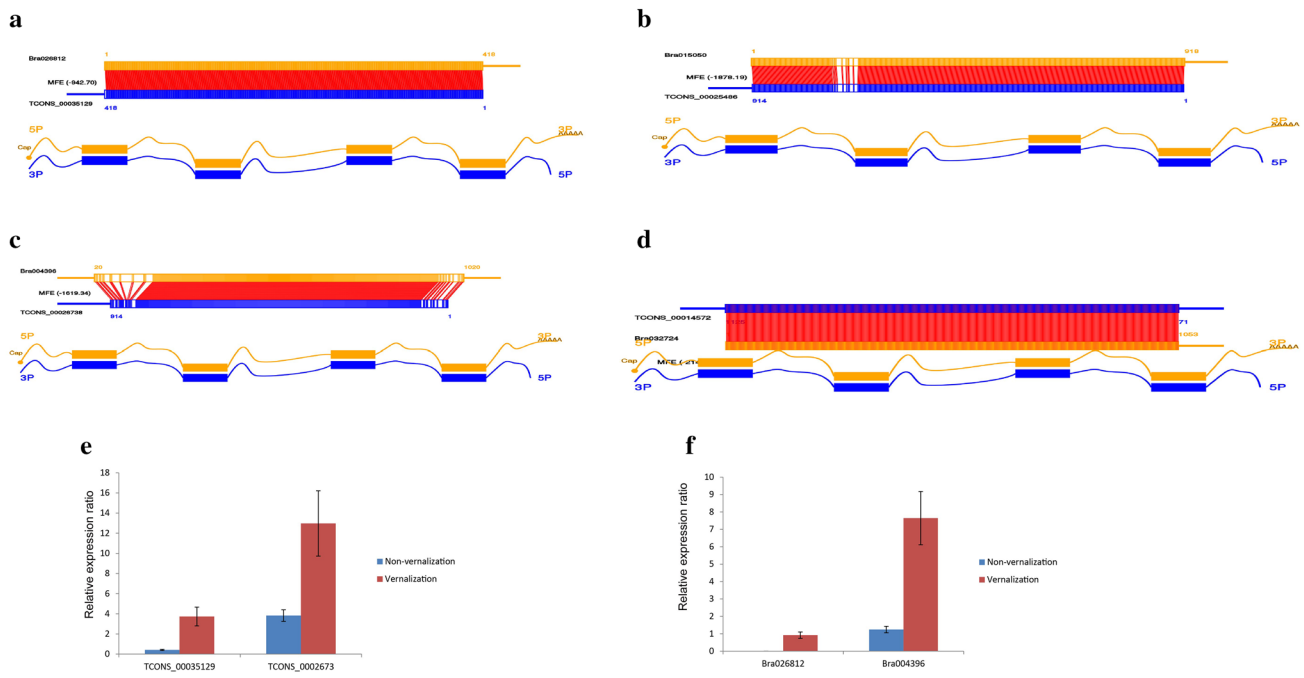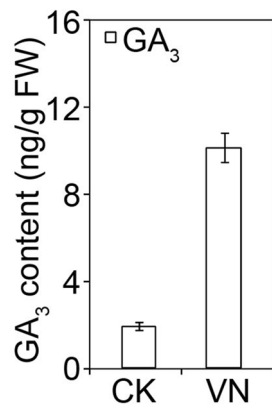
Fig. 7 Interaction analysis of complementary lncRNA-mRNA. **a–d** The lncRNA-mRNA of TCONS_00035129_Bra026812, TCONS_00025486-Bra015050, TCONS_00026738- Bra004396, TCONS_00014572-Bra032724. **e** and **f** The relative expression of TCONS_00035129, TCONS_00026738, Bra026812 and Bra004396. (Color figure online)

Fig. 8 The content of GA_3 in control and vernalized plants

(Oono et al. 2006). These results suggest that hormone levels in *B. rapa* are affected by vernalization.

Discussion

Various lncRNAs are involved in the control of gene expression in eukaryotes (Zhao et al. 2008; Rinn and Chang 2012; Mercer and Mattick 2013; Vance and Ponting 2014). However, most previous investigations have focused on the control of target genes expression by lncRNAs. LncRNAs have so far been reported to regulate various processes by coordinating gene expression, particularly during plant

development (Grob et al. 2013). During the process of vernalization, the lncRNA *COLDAIR* associates with the Polycomb protein, this in turn regulates the silencing of the floral repressor. The establishment of the repressed state of *FLC* by vernalization requires *COLDWRAP*, which is derived from the repressed promoter of *FLC* (Heo and Sung 2011). Both are required to form a repressive intragenic chromatin loop at the *FLC* locus during vernalization. Therefore, we aimed to explore whether other lncRNAs in *B. rapa* participated in vernalization.

In this study, we investigated the lncRNA and mRNA expression profiles of *B. rapa* during vernalization. A total of 1961 lncRNAs and 23,289 protein-coding transcripts were detected during vernalization. Among the lncRNAs, 254 were determined to be differentially expressed between the control and vernalization groups. mRNAs exhibited higher expression levels than lncRNAs, which is in agreement with previous findings (Grabherr et al. 2011). Recent studies have shown that lncRNAs with low expression levels regulate the expression of genes in complex tissues (Derrien et al. 2011; Rinn and Chang 2012). Therefore, these vernalization-induced lncRNAs may play an important role in development, including flowering.

To investigate the function of *B. rapa* lncRNAs, an lncRNA-mRNA co-expression network was constructed using RNAplex, and the functions of 400 lncRNAs-mRNAs were inferred. We observed that some lncRNAs

are not necessarily co-expressed with mRNAs. Subsequent localization of the lncRNAs and mRNAs indicated that most lncRNA-mRNA pairs were distantly situated. This finding suggested that these mRNAs were regulated *in trans* or indirectly regulated by their corresponding lncRNAs. However, chromosome looping may induce cooperation among these spatiotemporal-specific lncRNAs and mRNAs, thereby mediating the removal of mRNAs by lncRNAs (Guil and Esteller 2012).

TF and KEGG pathway analyses indicated that some mRNAs were associated with plant hormone signal pathways, including auxins, GAs, ethylene, cytokinins, and ABA. GAs promote flowering in *Arabidopsis* by activating genes that encode the floral integrator *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) in inflorescences, floral meristems, and leaves (Mutasagöttgens and Hedden 2009). Flowering may be inhibited by auxins during devernalization and when auxins are in excess in *Chrysanthemum* (Schwabe 1987). The role of ABA is apparently linked to the effect of GAs, which can be counteracted, whereas ethylene effects are directly or indirectly related to auxin effects (Schwabe 1987). The role of cytokinin remains elusive (Michalczuk et al. 1992). Here, we identified DEGs that are involved in hormone biosynthesis, deactivation, and signaling pathways, indicating that vernalization affects plant hormone levels. GAs is hormones that promote flowering. To check whether vernalization affects the levels of GAs hormones, we measured GA_3 levels before and after vernalization in *B. rapa*. As expected, GA_3 levels increased in the vernalized plants. These results reveal that vernalization not only downregulates *FLC* expression but also changes hormone levels in vernalized plants.

In summary, the present study conducted RNA-Seq transcriptome profiling to provide insights into the regulatory mechanism underlying vernalization in *B. rapa*. A total of 300 DEGs were detected between the control and vernalized libraries; of these, 284 DEGs were upregulated, and 16 were downregulated. Enrichment analysis demonstrated that vernalization affected hormone biosynthesis, as indicated by changes in GA_3 content. Approximately 2040 novel lncRNAs were identified in the *B. rapa* transcriptome dataset. Interaction analyses indicated that the expression of protein-coding transcripts was higher than that of pre-miRNAs and lncRNAs. *C2C2-Dof* (*Bra004396*) and *BraZF-HD21* (*Bra026812*) target genes were regulated by lncRNAs during vernalization. Finally, our study provides a comprehensive analysis of *B. rapa* lncRNAs and identifies changes in hormone content in vernalized plants. Our findings suggest that vernalization regulates flowering in two ways; namely, by downregulating *FLC* expression and by altering the biosynthesis of various hormones.

Materials and methods

Plant material and growth conditions

Brassica rapa late-flowering variety 'NHCC004' was used for the experiments. Plants were grown in pots containing a soil: vermiculite mixture (3:1) in the greenhouse and controlled-environment growth chamber programmed for cycles of 8 h of dark at 18 °C and 16 h of light (approximately 300 μmol photons $m^{-2} s^{-1}$) at 23 °C. For vernalization treatment, 40-days-old plants were transferred for an additional 4 weeks growth in a 4 °C cabinet under a 16-h day-length at 150 μmol photons $m^{-2} s^{-1}$, then transferred back to chamber for 1 week of recovery. For the control (CK) treatment, 40-days-old plants were transferred to a low light (150 μmol photons $m^{-2} s^{-1}$) chamber for 3 days at 4 °C, and then transferred back to normal chamber for 1 week of recovery. After treatment, the leaf from the top of the plant was collected, frozen in liquid nitrogen, and stored at − 80 °C for further analysis.

RNA isolation and qRT-PCR analyses

Total RNA was isolated from leaves using Trizol (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. To check the primer specificity, we used BLAST against the Brassica genome (Table S14). The qRT-PCR assays were performed with three biological and technical replicates. The reactions were performed using a StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Relative fold expression changes were calculated using the comparative Ct value method.

RNA extraction and mRNA-seq library construction

After extracting the total RNA from samples, mRNA and non-coding RNAs are enriched by removing rRNA from the total RNA with kit, and customers also can choose to remove mRNA by removing polyA RNAs, since a part of long noncoding RNA also with a polyA tail (Yang et al. 2011). By using the fragmentation buffer, the mRNAs and non-coding RNAs are fragmented into short fragments (about 200–500 nt), then the first-strand cDNA is synthesized by random hexamer-primer using the fragments as templates, and dTTP is substituted by dUTP during the synthesis of the second strand. Short fragments are purified and resolved with EB buffer for end reparation and single nucleotide A (adenine) addition. After that, the short fragments are connected with adapters, then the second strand is degraded using UNG (Uracil-*N*-Glycosylase) finally (Parkhomchuk et al. 2009). After agarose gel

electrophoresis, the suitable fragments are selected for the PCR amplification as templates. During the QC steps, Agilent 2100 Bioanaylzer and ABI StepOnePlus Real-Time PCR System are used in quantification and qualification of the sample library. At last, the library could be sequenced using Illumina HiSeqTM 2000 or other sequencer when necessary.

For the results of RNA-Seq, raw sequencing data was filtered by using the Trimmomatic program (v 0.33, Bolger et al. 2014). The clean reads were obtained by removing the reads with characteristics as followings: containing adaptor sequence, N10% "N" ratio and/or low-quality reads (that is, the base number of quality value $Q \leq 5$ accounts for N50% of the entire read). The filtered clean reads were further mapped to the reference genome (*B. rapa* genome, v1.5, downloaded from the BRAD database (http://brassicadb.org/)) by using Short Oligonucleotide Analysis Package tool TopHat (v.2.0.10) according to the following parameters: $N = 2$, $i = 50$, $I = 8000$ (Li et al. 2009), and two mismatches were allowed. We calculated the transcript expression level with the reads that could be uniquely mapped to a transcript. Moreover, the transcript expression level was normalized by per kilobase of exon region per million mappable reads (RPKM, Zheng et al. 2012), which were calculated by Cufflinks and then merged into a comprehensive final set of transcripts using Cuffmerge software K (v.2.1.1) (Mortazavi et al. 2008). Additionally, the Pearson correlation coefficient (PCC) between replicates was calculated using Log_2 transform of FPKM to evaluate the reliability of data among the biology repeats for each group.

Identification of novel lncRNAs

Primary sequencing data that produced by Illumina HiseqTM 2000, called as raw reads, which will be filtered into clean reads by remove adaptor contained and low quality reads. Due to the instability of the ribosomal RNA (rRNA) removement efficiency, it is also necessary to remove rRNA contained reads by alignment. To obtain lncRNAs, we removed transcripts containing no more than one exon, which may be considered transcriptional noise and DNA fragments in the library. Transcripts with length < 200 nt were excluded. After that, the retained transcripts were filtered by ORF length and only those with ORF < 300 nt were considered as lncRNA candidate. We utilize reference annotation based assembly method to reconstruct the transcripts, while background noise would be reduced by using FPKM and coverage threshold. Compared to the reference we would detect the novel transcripts, and then calculate the coding potential of these transcripts to identify the novel lncRNAs (Fig. S6, Table S15, S16, and S17).

Interaction analysis of complementary lncRNA-mRNA

To further reveal the potential antisense lncRNA-mRNA interaction, we searched all antisense lncRNA-mRNA duplex of complementary base pairing using RNAplex (Carrieri et al. 2012), a tool especially created to rapidly search for short interactions between two long RNAs. The program integrated ViennaRNA package, which predict best base–base pairing by using minimum free energy algorithm according to its thermodynamic features. Result shows the best lncRNA-mRNA base pairing sites and the minimum free energy of antisense lncRNA and its correspond mRNA.

Functional annotation and classification of the transcripts

All assembled transcripts were annotated with the publicly available protein databases, including Nr (http://www.ncbi.nlm.nih.gov), GO (http://www.geneontology.org) and KEGG (http://www.genome.jp/kegg) databases using BLAST (2.2.25) (E-value < 10^{-5}). Then, the best alignments were used to decide sequence direction and to predict coding regions of the unigenes. WEGO software was used to conduct GO classification for understanding the distribution of gene function. The unigenes were also aligned to COG database to predict and classify possible functions. GO enrichment was adopted to further investigate the DEGs (significant difference at the 0.05 and 0.01 level). Relative RPKM was log_2 (RPKM + 1) transformed to perform cluster analysis, and a heat map was then generated from log_2 'relative RPKM' using average linkage analysis. In addition, KEGG was used to annotate the pathway. GO terms and KEGG pathways with FDR-corrected P < 0.01 were considered statistically significant.

Measurements of hormone contents

The endogenous hormone contents in leaves were measured before and after vernalization. Each sample was prepared by three replicate samples. Hormone identification and quantification was performed by high-performance liquid chromatography-mass spectrometry (AB 5500, Beijing, China) following the protocol described previously (Pin et al. 2010).

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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