Physiologia Plantarum
An International Journal for Plant Biology

Vernalization can regulate flowering time through microRNA mechanism in *Brassica rapa*

Feiyi Huang†, Xiaoting Wu†, Xilin Hou, Shuaixu Shao and Tongkun Liu*

State Key Laboratory of Crop Genetics and Germplasm Enhancement, College of Horticulture, Nanjing Agricultural University, Nanjing 210095, China

Correspondence
*Corresponding author,
e-mail: liutk@njau.edu.cn

Received 21 September 2017;
revised 8 January 2018

doi:10.1111/ppl.12692

Vernalization is an important process that regulates the floral transition in plants. MicroRNAs (miRNAs) are endogenous non-coding small RNA (sRNA) molecules that function in plant growth and development. Despite that miRNAs related to flowering have previously been characterized, their roles in response to vernalization in pak-choi (*Brassica rapa ssp. chinensis*) has never been studied. Here, two sRNA libraries from *B. rapa* leaves (vernalized and non-vernalized plants) were constructed and sequenced. Two hundred eight known and 535 novel miRNAs were obtained, of which 20 known and 66 new miRNAs were significantly differentially expressed and considered as vernalization-related miRNAs. The corresponding targets were predicted on the basic of sequence homology search. In addition, 11 miRNAs and eight targets were selected for real-time quantitative PCR to confirm their expression profiles. Functional annotation of targets using gene ontology and Kyoto encyclopedia of genes and genomes results suggested that most targets were significantly enriched in the hormone signaling pathway. Moreover, a decreased indole-3-acetic acid (IAA) and an increased GA_3 hormone were detected after vernalization, indicating that the IAA and GA_3 might response to vernalization. These results indicated that vernalization regulates flowering through microRNA mechanism by affecting endogenous hormone level in *B. rapa*. This study provides useful insights of promising miRNAs candidates involved in vernalization in *B. rapa*, and facilitates further investigation of the miRNA-mediated molecular mechanisms of vernalization in *B. rapa*.

Introduction

Plant development consists of two phases, vegetative and reproductive growth. Flowering is an important biological process that ensures reproductive success, which can be stimulated by various environmental stimuli and endogenous signals. This process is controlled by numerous genes, as well as some evolutionary conserved microRNAs (miRNAs) (Irish 2010, Pose et al. 2012). It has been determined that there are five major

Abbreviation – *AFB*, *AUXIN SIGNALING F-BOX*; *AP2*, *APETELA2*; COLDAIR, cold assisted intronic noncoding RNA; COOLAIR, cold induced long antisense intragenic RNA; *FT*, *FLOWERING LOCUS T*; *FLC*, *FLOWERING LOCUS C*; GA, gibberellin; GO, gene ontology; IAA, indole-3-acetic acid; KEGG, Kyoto encyclopedia of genes and genomes; *LFY*, *LEAFY*; lncRNA, long non-coding RNA; *MYB*, *MYB DOMAIN PROTEIN*; miRNA, microRNA; nt, nucleotides; pre-miRNA, miRNA precursor; pri-miRNA, primary miRNA; qPCR, real-time quantitative PCR; RISC, the RNA-induced silencing complex; SAM, shoot apical meristem; *SOC1*, *SUPPRESSOR OF OVEREXPRESSION OF CO 1*; *SPL*, *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE*; snRNA, small nuclear RNA; snoRNA, small nucleolar RNA; sRNA, small RNA; *TIR1*, *Toll/interleukin receptor 1*.

†These authors equally contributed to this work.

pathways regulating the floral transition in *Arabidopsis thaliana*, namely the photoperiod, vernalization, autonomous, gibberellins (GAs) and ageing pathways (Srikanth and Schmid 2011). The vernalization pathway mainly mediates the response to the appropriate environmental conditions. In temperate climates, flowering is usually coordinated with temperature and photoperiod. In Arabidopsis, the models of flowering-time regulation suggest that vernalization and photoperiod influence different signal genes. A series of 'integrator' genes, such as *FT*, *SOC1*, causes interactions between vernalization and photoperiod response signals (Samach et al. 2000). Plant hormones and internal regulatory factors also function in the floral transition process (Li et al. 2015). Recent studies reported that GAs function to induce floral transition and regulate floral development (Mutasa-Gottgens and Hedden 2009). In addition to GAs, indole-3-acetic acid (IAA) also play a vital role in the reproductive phase transition. GAs signaling triggers early flowering, while IAA signaling leads to late-flowering slightly in *Agapanthus praecox* ssp. *orientalis* (Zhang et al. 2014).

Increasing studies have been reporting that miRNAs play vital roles in controlling various plant developmental processes, included flower development (Zhao et al. 2007, Yant et al. 2010). MiRNAs are endogenous 18–25 nt single-stranded non-coding small RNAs (sRNAs), that functioned as post-transcriptional and transcriptional negative regulators (Voinnet 2009). They are first transcribed to long primary miRNAs (pri-miRNAs) by RNA polymerase II in the nucleus that fold to a secondary structure (stem-loop) with partly complementarity. The pri-miRNAs are processed into pre-miRNAs via Dicer-like one enzyme and further cleaved to small double-stranded RNA duplexes. The duplexes are then transported to the cytoplasm, and cleaved by Dicer, forming a miRNA (Bartel 2004, Kurihara and Watanabe 2004). The miRNA strand is loaded into RNA-induced silencing complex (RISC, Baumberger and Baulcombe 2005). The miRNA acts as a guide for RISC to degrade targets or inhibit their translation (Vazquez et al. 2010).

To date, nine conserved miRNA families have a verified function in flowering, such as miR156, miR159 and miR172 (Luo et al. 2013). MiR156 is required for flower transition by targeting *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*s) in Arabidopsis, maize and rice (Chuck et al. 2007, Wang et al. 2009, Jiao et al. 2010). MiR172 targets *AP2* to regulate organ identity and flowering time (Varkonyi-Gasic et al. 2012). MiR159 is essential for normal anther development through controlling *MYB*s expression (Tsuji et al. 2006). Generally, these reports revealed that miRNA-mediated gene regulation probably play crucial roles in plant flower development.

Pak-choi (*Brassica rapa ssp. chinensis*), a subspecies of *B. rapa*, is a major vegetable crop and widely cultivated in Asia (Tian et al. 2004). The cultivar 'Wuyueman', a cold-requiring *B. rapa* which flowers later than other *B. rapa* cultivars, was used in this study. Despite that numerous miRNAs have been identified in *B. rapa*, no systematic studies were available for the roles of miRNAs in the vernalization pathway. Herein, we constructed two sRNA libraries (vernalization and non-vernalization) and performed the high-throughput Solexa sequencing to analysis miRNAs and targets in *B. rapa*. The expression profiles of vernalization-related miRNAs and their targets were validated and analyzed by real-time quantitative PCR (qPCR). Then, we further analyzed *B. rapa* miRNAs targets functions on the basic of the information provided by a Blastx (Beijing, China) search against the Arabidopsis genome and gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) analysis. This work offers information to understand the regulatory roles and molecular mechanisms of miRNAs in *B. rapa* vernalization process.

Materials and methods

Plant materials

Young emerging leaves of *B. rapa* were used for sequencing. Seeds were sowed in pots in a greenhouse (22/18°C, 16/8 h light/dark). For vernalization treatment, 1-month-old seedlings were transferred to a growth chamber exposed to 4°C for 6 weeks and then transferred to the greenhouse (22/18°C, 16/8 h light/dark daily) for 1 week. Seedlings grown in the greenhouse without vernalization treatment were used as control. Leaves of 10-week-old vernalized and non-vernalized (control) seedlings grown in the greenhouse were harvested and frozen immediately in liquid nitrogen and stored at −80°C.

Construction of two small RNA library and bioinformatics analysis of sequencing data

Total RNA isolation and construction of two sRNA libraries (vernalized and control seedlings) were completed following reported procedures (Sunkar et al. 2008). Solexa technology was used to sequence sRNAs at BGI (Shenzhen, China). Small RNA reads were generated by an Illumina Genome Analyzer at BGI. After removing the undesired raw reads, the extracted clean reads of 18–30 nt were obtained for subsequent analysis. The retained miRNA reads were mapped to *B. rapa* genome using SOAP software under default parameters (Li et al. 2008). Only perfectly matched sequences were kept. The mappable sequences were search against

Rfam (http://www.sanger.ac.uk/software/Rfam) and the NCBI GenBank noncoding RNA (http://www.ncbi.nlm.nih.gov/) database, the small RNA sequences exactly matching rRNA, tRNA, small nuclear RNA (snRNA), small nucleolar RNA (snoRNA) together with sequences containing poly (A) tails were removed (Gardner et al. 2009). We then aligned the remainder of the unique small RNA sequences against miRBase 21.0 (http://www.mirbase.org/index.shtml) to identify *B. rapa* known miRNAs. The sequences that match miRBase with up to two mismatches were considered as known miRNAs. Novel miRNAs were predicted using the program Mireap (http://sourceforge.net/projects/mireap) with default parameters. The secondary structures of candidate novel miRNAs were confirmed by Mfold (Zuker 2003).

Differential expression analysis of miRNAs

To investigate significantly differentially expressed miRNAs between the two libraries (control and vernalization), the read counts of miRNAs were normalized as transcripts per million according to Bayesian methods (Audic and Claverie 1997). If the normalized read count is zero, the value was modified to 0.001. The fold-change and *P*-value were calculated by normalized expression according to previously reported methods (Li et al. 2011). When $P \leq 0.05$ and fold change ($\log_2$ (vernalization/control) >2 or <0.5, it was considered up- or down-regulated during the process of vernalization.

Prediction and annotation of potential targets

We used miRNA candidate targets as query sequences to blast against Chiifu Chinese cabbage database (http://brassicadb.org/brad/) by a custom Perl script, based on the published rules (Allen et al. 2005, Schwab et al. 2005). A BlastX search with the Arabidopsis genome was then applied to the predicted targets, considering they both belong to Brassicaceae. Moreover, to further study the biological functions of miRNA targets, we obtained GO annotations through Blast2GO program from UniGene database based on the BLAST searches against the Nr database in NCBI under an *E*-value threshold of less than 10^{-5} (Conesa et al. 2005).

QPCR analysis

We performed qPCR to confirm the quality of small RNA sequencing and expression patterns of miRNAs and their targets. Total RNA was extracted from the previously frozen plant tissues. The qPCR assay and relative expression calculation were carried out followed our previous report (Huang et al. 2016). The 5S rRNA and *B. rapa actin* gene was used as the reference gene. The primers used are showed in Table S9.

Measurements of hormone contents

Approximately 500 mg frozen samples of leaves before and after vernalization were used for endogenous phytohormone extraction. IAA and GA_3 contents were measured by the previously described methods (Takei et al. 2001, Dobrev and Vankova 2012). To link the RNA sequencing results to hormone contents, the examined leaves were to the same ones than for sequencing. The hormone was isolated based on the previously published protocol (Agar et al. 2006). High-performance liquid chromatography-mass spectrometry (AB 5500, Beijing, China) was used to detect and quantify hormone following the previously reported protocol (Pan et al. 2010). Both standard IAA and GA_3 sample were purchased from Sigma-Aldrich (St. Louis, MO). The results were analyzed using six replicates.

Results

Global analysis of sequences from small RNA libraries

To analysis the roles of miRNAs in the vernalization process, we constructed two sRNA libraries from leaves of control and vernalized plants. 30 677 485 clean reads in control and 20 504 643 clean reads in vernalization library were obtained from Solexa sequencing (Table S1). Among them, 17% total sRNAs were non-vernalization library-specific with 50.8% unique sRNAs, 10% total sRNAs were vernalization library-specific with 33% unique sRNAs and 73% total sRNAs were present in both with 16.2% unique sRNAs (Fig. 1; Table S2). Then, we annotated and filtered out undesired sRNAs, such as rRNAs, tRNAs and mRNA. The proportions and numbers for different categories of small RNA were summarized in Table S3.

The size distribution of reads displayed similar trends between the two libraries (Fig. 2). The majority of the sRNAs were 24 nt long, which accounted for 47.2 and 47.1% of total reads in control and vernalization library, respectively, followed by the 21 nt (23.5 and 19.2%). This was consistent with previous studies on *A. thaliana* (Rajagopalan et al. 2006), *Oryza sativa* (Zhu et al. 2008), *Medicago truncatula* (Szittya et al. 2008) and *Populus trichocarpa* (Puzey et al. 2012). However, a difference in the size distribution was noticed. The relative abundance of 21 nt sRNAs in vernalization library was significantly lower than those in control library, indicating that the 21 nt sRNAs might be repressed in *B. rapa* vernalization process.

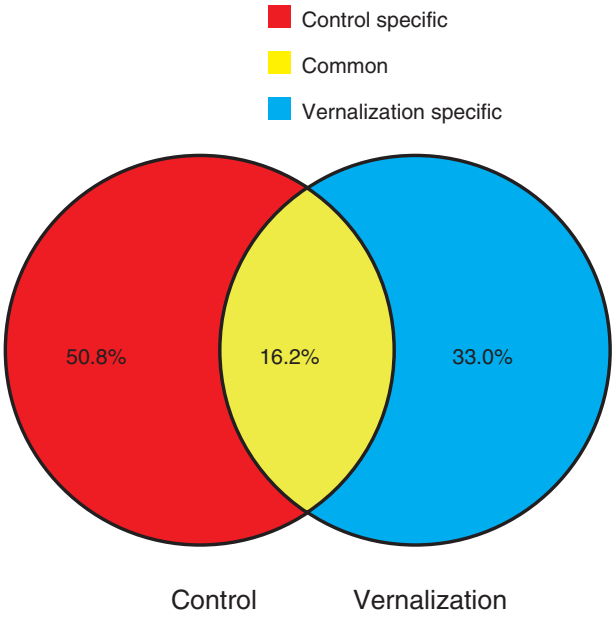

Fig. 1. Summary of the common and specific sequences of the unique sRNAs from control and vernalization library.

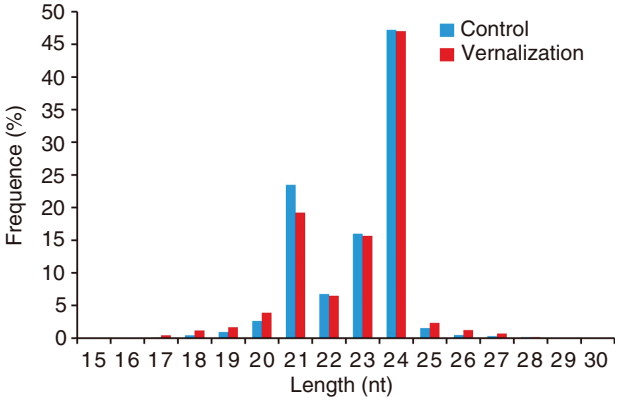

Fig. 2. Length distribution and frequency percent of sRNA sequences in control and vernalization libraries in *B. rapa*. Y-axis represents percentages of sRNAs identified in this study; x-axis represents the length of sRNAs. The two libraries are shown by different colors.

Identification of known and novel miRNAs

By sequencing, we obtained 43 389 (control) and 35 545 (vernalization) miRNA candidates. To isolate known miRNAs in *B. rapa*, the sequences of sRNAs were blasted against the known miRNAs of various plant species deposited in miRBase 21.0. After BLASTn and sequence analysis, 208 expressed known miRNAs were identified in both libraries. Detailed sequences and reads of the known miRNAs are shown in Table S4. Five hundred thirty-five potential novel miRNAs candidates in *B. rapa* were also identified and named as bra-miRn. Most of the novel miRNAs were control-specific or vernalization-specific, which accounted for 47.1 (252) and 28.4% (152), respectively. Only 131 novel miRNAs appeared in both control and vernalization libraries. Information about the novel miRNAs was listed in Table S5. We also found that conserved miRNAs level was higher than non-conserved and novel miRNAs level (Tables S4 and S5), which is in agreement with the previous findings that evolutionary conservation may have a correlation with the expression level (Chi et al. 2011).

Identification of vernalization-related miRNAs in *B. rapa*

After annotating known and novel miRNAs, we examined vernalization-responsive miRNAs based on the differential expression analysis. We used a threshold and absolute value of $\log_2$Ratio ≥ 1 and *P*-value ≤ 0.05 to search for miRNAs up- or down-regulated during the process of vernalization. Twenty known and 66 novel miRNAs were observed to be significantly differentially expressed (Table S6). Among these, 43 miRNAs (14 known and 29 novel ones) were upregulated, and 43 miRNAs (6 known and 37 novel ones) were downregulated. The changes in expression levels of known miRNAs were much lower than the novel miRNAs. Of these known miRNAs, bra-miR1063a was remarkably downregulated with more than fourfold change, while bra-miR5714, bra-miR157a and bra-miR5077 were sharply upregulated with more than twofold change. The majority of the novel vernalization-responsive miRNAs were only detected in the control or vernalization library, indicating that these novel miRNAs may have stage-specific functions. Of them, seven novel miRNAs (bra-miRn93, bra-miRn25 and bra-miRn345 were downregulated; bra-miRn426, bra-miRn458, bra-miRn503 and bra-miRn395 were upregulated) were significantly differentially expressed with an absolute value of $\log_2$ratio > 10. These results indicated that these identified vernalization-responsive miRNAs might play vital roles in *B. rapa* vernalization.

Prediction and annotation of vernalization-related miRNA targets

To investigate the biological roles of the predicted targets in *B. rapa*, we searched against EST and cDNA sequences in *B. rapa* genome annotation database (http://brassicadb.org/brad/index.php) and Arabidopsis genome database (https://www.arabidopsis.org/index.jsp). A total of 714 and 666 potential targets for 92 known and 213 new miRNAs were identified, with an average of 7.76 and

3.13 target genes per miRNA, respectively (Table S7). We viewed that the known miRNAs targeted more genes on average compared to novel miRNAs, and this finding suggested that the known miRNAs could function in a broader range of biological process than the novel miRNAs. In addition, no targets for 116 known and 322 novel miRNAs were found, possibly due to the targets expression levels below the detection level during the process of vernalization. Additionally, 114 targets related to vernalization were isolated as the targets of 33 vernalization-related miRNAs (11 known and 22 novel miRNAs). These targets were related to a wide variety of biological processes, and several targets were transcription factors. For instance, *MYBs* (targeted by bra-miR159a) and *SPLs* (targeted by bra-miR157a), which were in accord with the previous research in rice and Chinese cabbage (Sunkar et al. 2008, Wang et al. 2011). Additionally, many hypothetical and unknown function genes were targeted, indicating possible new functions for these miRNAs in *B. rapa*.

To functionally analyzed the annotation, the targets of vernalization-related miRNAs were subjected to GO enrichment analysis by Blast2 GO program with default parameters. GO analysis indicated that these predicted targets could be classified into five molecular functions, 16 biological processes and nine cellular components (Fig. 3). Most targets, which were classified as the binding category, encode transcription factors. Since transcription factors play an important role in gene expression, these targets are thought to be the major targets of miRNAs (Eldem et al. 2013). Interestingly, we found that most targets were enriched in hormone-mediated signaling pathway, including GAs-mediated signaling pathway (Fig. S1). To further investigate the biological pathways influenced by vernalization in *B. rapa*, KEGG pathway enrichment analysis was carried out. We found 106 pathways enriched with targets of significantly differentially expressed miRNAs, and each target was assigned to at least one KEGG annotation. Among these pathways, plant hormone signal transduction involved the most miRNA targets (*Bra032954*, *Bra003518*, *Bra007720*, *Bra026953* and *Bra016754*, all targeted by bra-miR393b) (Table S8). The functional annotations of these genes showed that they all acted in auxin signal pathway. The GO and KEGG results suggested that plant hormone (GA and auxin) signal transduction has vital influences on the process of *B. rapa* vernalization.

qPCR analysis of miRNAs and their targets

To confirm the deep sequencing data and quantify the expression patterns of both the identified miRNAs and their corresponding targets at different stages of vernalization (4°C for 0, 2, 4 and 6 weeks), 11 vernalization-related miRNAs (9 known and 2 novel miRNAs) and 8 targets were randomly selected for qPCR analysis. The expression profiles were displayed in Figs. 4 and 5, and the primers were listed in Table S9. As Fig. 4 shown, there exists a similar tendency between the qPCR and sRNA sequencing results of the selected miRNAs expressions, suggesting that the results of sRNA sequencing were reliable. Nine miRNAs (*bra-miR157*, *bra-miR159a*, *bra-miR393b*, *bra-miR5020b*, *bra-miR5054*, *bra-miR5072*, *bra-miR5077*, *bra-miR5368* and *bra-miRn429*) expressions were increased and two miRNAs (*bra-miR397a* and *bra-miRn93*) expressions were reduced. The results suggested that the levels of the tested miRNAs vary significantly during the process of vernalization. Furthermore, some miRNAs showed stage-specific expression, probably involved in vernalization.

Additionally, we also validated the expression patterns of eight corresponding targets. The qRCR results showed that different targets of one miRNA expressed varied during the process of vernalization and in different tissues in *B. rapa*, and that most targets expression were relatively higher at flower or leaf tissues (Fig. 5). For example, *NEDD1-Bra009155* exhibited highest expression level in flower, while *SAP130B-Bra034172* was highly expressed in leaf, and both of them are targeted by *bra-miR5077*. These results showed that different targets of the same vernalization-related miRNA may have different function. In addition, there are inverse correlations between the expression levels of the selected miRNAs and those of their targets, suggesting that these miRNAs might negatively regulate their targets. The relationships between miRNAs and their targets were shown in Supplementary Table S10. These findings suggested that miRNA-mediated silencing of their potential target genes occur during the process of vernalization in *B. rapa*.

Endogenous hormone measurements

As mentioned above, GO and KEGG results suggested that plant hormones may function in the process of *B. rapa* vernalization. To further investigate the roles of endogenous hormones in the vernalization process, the IAA and GA_3 contents were measured in *B. rapa*. Samples were collected from the leaves before and after vernalization. As Fig. 6 shown, the IAA level decreased and the GA_3 level increased after vernalization, indicating that the IAA and GA signaling might be involved in the process of *B. rapa* vernalization.

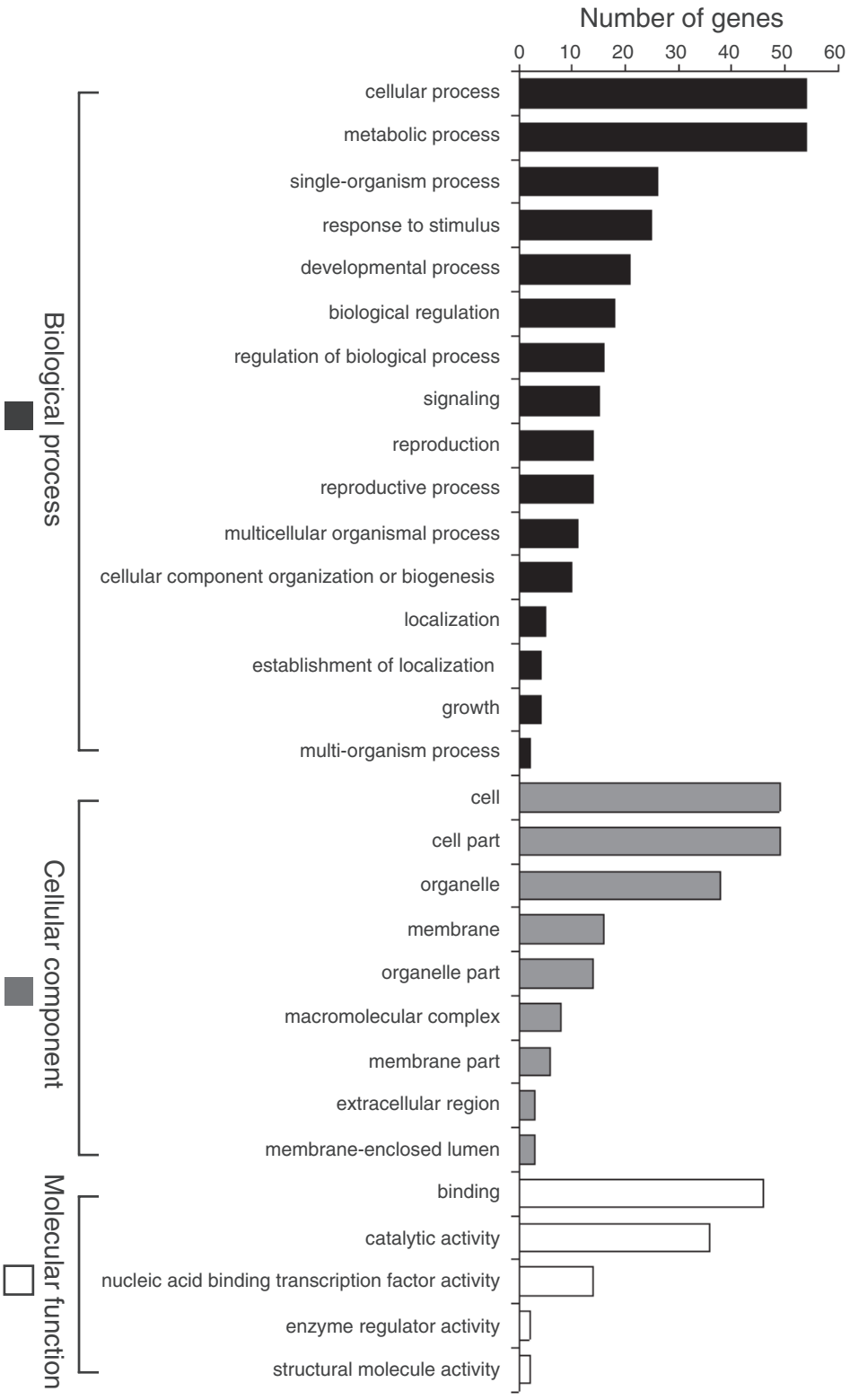

Fig. 3. GO annotation of targets of vernalization-associated miRNAs.

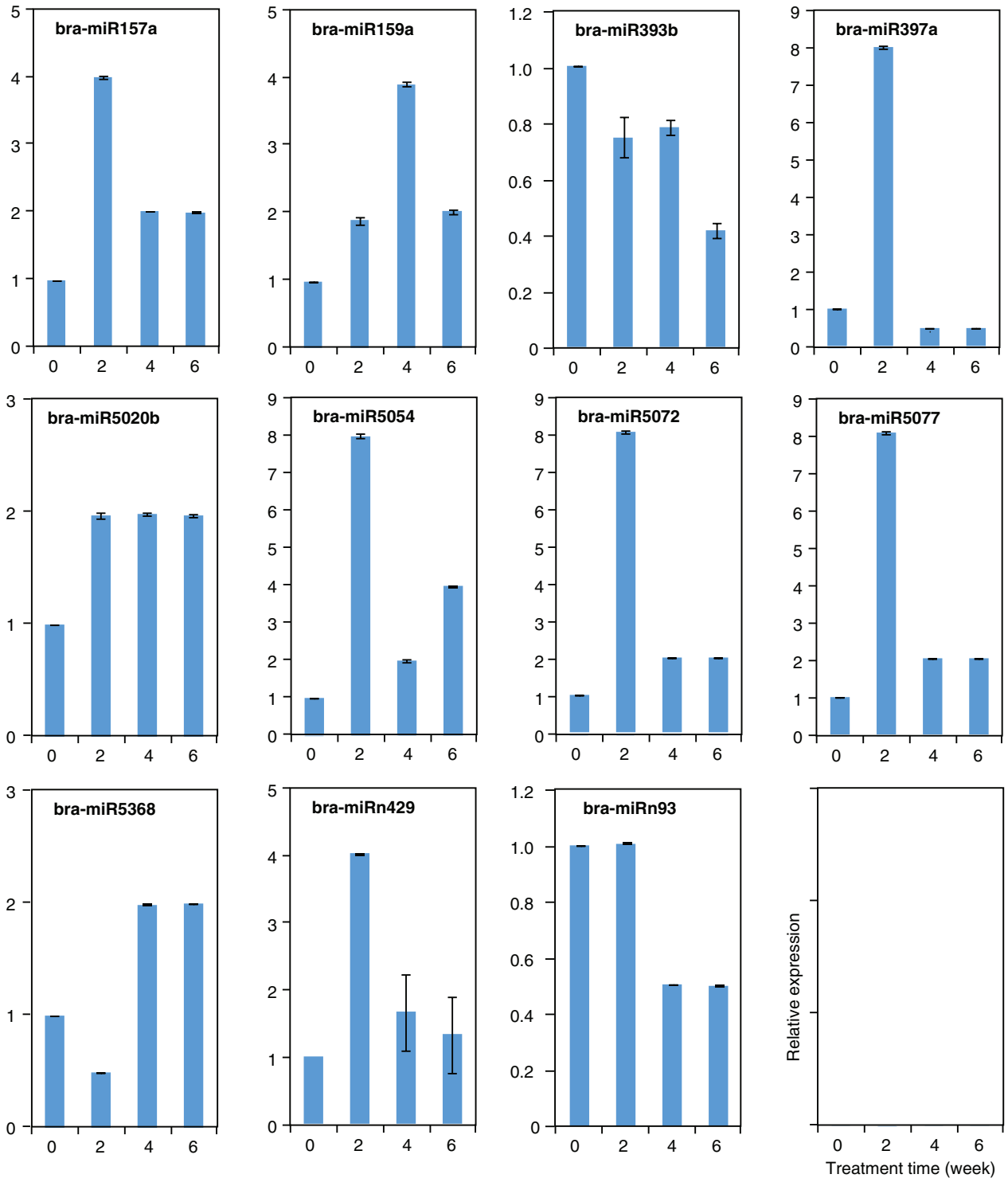

Fig. 4. The expression patterns of miRNAs during the process of vernalization by qRT-PCR. The transcript levels of miRNAs relative to the transcript levels of internal reference were quantified using $2^{-\Delta\Delta CT}$. The expression level in the untreated sample (0 h) was set to a value of 1. Each bar shows the mean ± SE of triplicate biological assays.

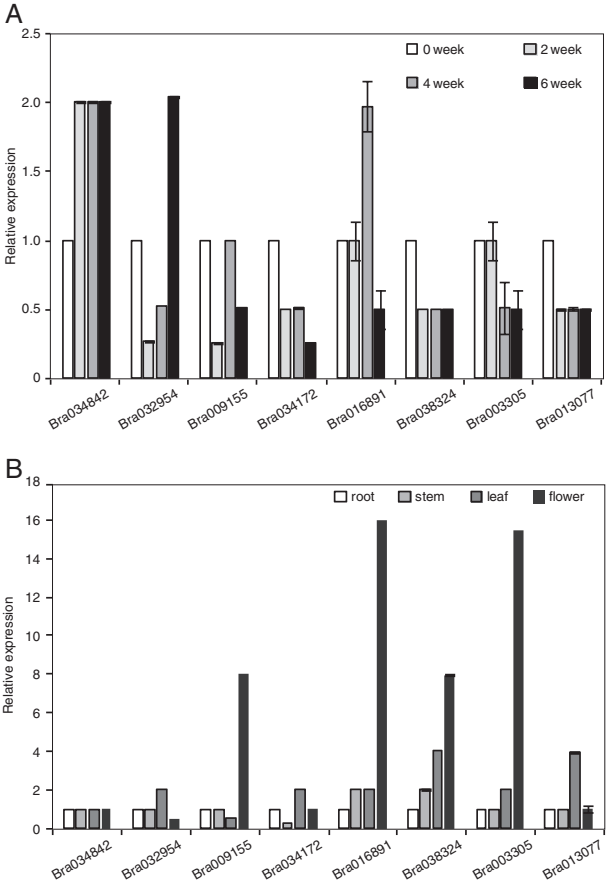

Fig. 5. The temporal and spatial expression patterns of targets during the process of vernalization by qPCR. (A) The relative expression levels of targets during different stages of vernalization. (B) The relative expression levels of targets in various tissues (root, stem, leaf and flower). Each bar shows the mean ± SE of triplicate assays. The targets detected in qPCR were *Bra034842* (*MYB65*), *Bra032954* (*AFB2*), *Bra026953* (*AFB3*), *Bra009155* (*NEDD1*), *Bra034172* (*SAP130B*), *Bra016891* (*SPL9*), *Bra038324* (*SPL6*), *Bra003305* (*SPL15*), *Bra013077* (*CHR35*).

Discussion

With genomic and biocomputing tools widely developed, high-throughput sequencing becomes a straightforward method to analyze small RNA profiling for plants in most cases. To date, 28 645 miRNAs (miRBase database, Release June 21, 2014) have been discovered from various kinds of multicellular eukaryotes, including humans, animals and plants. To analyze the roles of miRNAs during vernalization process in *B. rapa*, we constructed two sRNAs libraries from the leaves with or without vernalization treatment. With Solexa sequencing, we identified 25 million clean reads per library, more than in the previous study on *B. rapa* that generated about 9 million clean reads by deep sequencing (Wang et al. 2015). This suggest that we generated a relatively complete small RNA libraries that will discover more novel miRNAs, especially those specifically expressed in leaves after vernalization. In this work, we have obtained 208 known and 535 novel miRNAs involved in *B. rapa* vernalization and predicted their targets using bioinformatics approaches (Tables S5–S7). Most of the identified known miRNAs were evolutionarily conserved across diverse plant species, and 54 conserved miRNAs (belonging to 32 families) were detected. The expression patterns of several selected miRNAs and their targets were confirmed through qPCR analysis (Figs 4 and 5), validating the accuracy of the RNA-seq results in this study.

To date, nine flowering-related miRNA families were studied, which together with their target genes controlled the floral transition in Arabidopsis. Of these miRNAs, miR156/157 and miR159 were downregulated during the process of floral transition, whereas miR172 was upregulated (Luo et al. 2013). However, both sequencing and qPCR results showed that *bra-miR157a* was upregulated after vernalization, which targets 11 *SPL* genes (Table S10). Interestingly, previous studies reported that high levels of miR156 can prevent precocious flowering (Wang 2014). Vernalization just works as the first step to obtain the competence to flower. For most vernalization-requiring plants, like 'wuyueman', they are also LD plants, still needing long days after vernalization, which ensures that flowering does not begin during the decreasing day lengths of late fall and occurs in the spring. The results indicated that vernalization could affect flowering through microRNA mechanism in *B. rapa*.

GO and KEGG analyses revealed that the targets were significantly enriched in the hormone-mediated

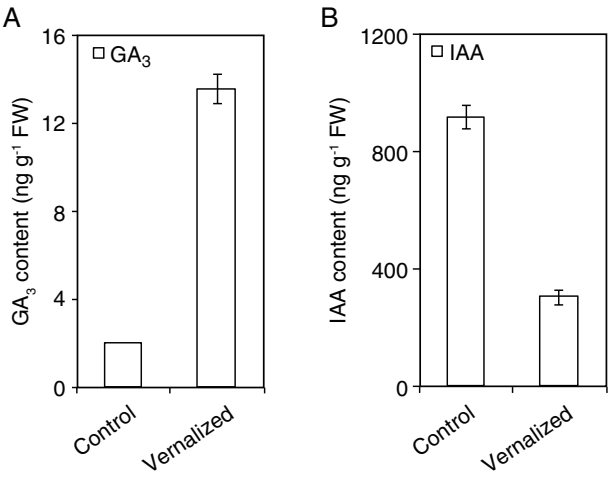

Fig. 6. Gibberellic acid 3 (A) and auxine IAA (B) contents in leaves before and after vernalization in *B. rapa*.

signaling pathway, and most of which were involved in GA and IAA mediated signaling pathway. We therefore measured the contents of IAA and GA_3 in *B. rapa* during the process of vernalization. The results revealed that the content of IAA was reduced and the content of GA_3 was increased after vernalization (Fig. 6), suggesting that vernalization could induce GA_3 and reduce IAA.

Plant hormones, components of the flowering time syndrome, have been reported in model plants (Davis 2009). However, the regulatory roles of hormones in *B. rapa* vernalization process are still unclear. Auxin functions in the initiation of floral primordia and flower development (Alabadi et al. 2009). In this work, we found five auxin-related genes were significantly changed, *Bra032954*, *Bra026953*, *Bra016754*, *Bra003518* and *Bra007720*, which were homologous genes of *AtAFB2*, *AtAFB3* and *AtTIR1* respectively. These five genes were all targeted by *bra-miR393b*. In Arabidopsis, miR393 is encoded by *miR393a* and *miR393b* (Jones-Rhoades and Bartel 2004). Exogenous IAA treatment could enhance *miR393b* transcription (Chen et al. 2011). In our sequencing and qPCR results, *bra-miR393b* was reduced after vernalization, which was consistent with the decrease in the contents of IAA after vernalization, indicating that the IAA signaling involved in flowering time regulation is regulated by miR393 (Figs. 4 and 6). MiR393 post-transcriptionally regulates *AtTIR1*, *AtAFB2* and *AtAFB3* to alter plant auxin responses (Xia et al. 2012). Auxin response factors (*ARFs*) constituted a small subset of F-box-containing auxin receptors and played key roles in bud growth and flower development (Nagpal et al. 2005, Zhao 2010). *TIR1* acted as auxin receptors and played a role in the degradation of the Aux/IAAs repressors, which promotes AFB mediating auxin-related specific genes (Dharmasiri et al. 2005, Sato and Yamamoto 2008). Furthermore, *osmiR393* overexpression resulted in early flowering in rice (Xia et al. 2012). These results suggested that vernalization could reduce *bra-miR393* expression by downregulation of IAA, and finally prevent premature flowering (Fig. 7).

In addition to auxin, GAs also plays important roles in flowering regulation. GAs could induce phase transitions of flowering and regulate floral development (Mandaokar and Browse 2009). Vernalization could regulate GAs biosynthesis in vegetative plants, and control regulatory signals which control the GAs responsive genes expressions (Oka et al. 2001). The *ga1-3* mutant Arabidopsis, which contains a deletion in an enzyme that acts on an early step of gibberellic acid biosynthesis, is responsive to vernalization (Michaels and Amasino 1999). Additionally, the endogenous level of GAs was upregulated during vernalization in

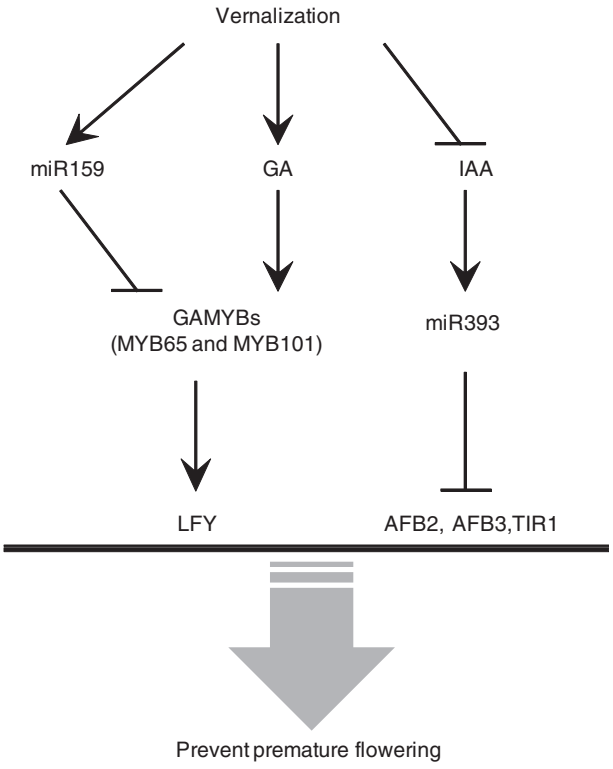

Fig. 7. Hypothetical model for flowering regulation by miRNAs association with IAA and GA after vernalization in *B. rapa*. The up- and down-regulation are marked red and green, respectively.

Brassica napus (Zanewich and Rood 1995). Interestingly, GA_3 level was also triggered by vernalization in *B. rapa* (Fig. 6). However, the increased GA_3 did not lead to early flowering, why?

In Arabidopsis, miR159 is involved in the floral transition through targeting *MYB*s in response to GA signaling (Simpson and Dean 2002). GAs treatment directly degrades DELLA proteins and finally result in an increase in *LFY* level (Yamaguchi and Abe 2012). In this study, we got seven targets of *bra-miR159a*, *MYB65* (*Bra034842* and *Bra002042*), *MYB81* (*Bra000531*), *MYB101* (*Bra022888* and *Bra021791*) and *MYB120* (*Bra035547* and *Bra002938*). *Bra034842* is the homologous gene of *AtMYB65,* which is a GA-specific transcriptional regulator regulating the floral initiation through activating *LFY* in the GA signaling pathway in Arabidopsis (Gocal et al. 2001). *AtmiR159* overexpression transgenic plants showed late flowering with decreasing *GAMYB*s (*MYB33*, *MYB65* and *MYB101*) and *LFY* expression levels (Achard et al. 2004), and a similar result have been shown here (Figs. 4 and 5; Table S10). *GAMYB*s, a downstream component of GA response, acts on binding to the promoter of *LFY* and then induces *LFY* expression (Gocal et al. 2001). So, upregulated

expression of *bra-miR159a* and downregulated expression of *MYB*s inhibited the promotion of *LFY* by GAs to prevent premature flowering (Fig. 7).

As we know, *FLC*, a key gene in vernalization pathway, is regulated by two lncRNAs, COLDAIR and COOLAIR (Swiezewski et al. 2009, Heo and Sung 2011). In this work, we did not find any vernalization-related miRNA targeting *FLC*. Our findings showed that miRNAs mainly act on interactions between vernalization and hormone pathways, rather than directly act on vernalization. We therefore speculated that vernalization can regulate flowering by modulating hormones level via miRNAs and by modulating *FLC* at the epigenetic level. These results help us understand the roles of miRNAs in vernalization pathway in *B. rapa*. Whether these effects are produced via miRNA-target modules requires further functional analysis.

Author contributions

Conception and design of the study: F.H. and T.L. Performed the experiments: F.H., S.S. and X.W. Wrote the paper: F.H. Manuscript revision and approval: F.H., T.L. and X.H. All authors red and approved the final manuscript.

Acknowledgements – This work was supported by grants from the Fundamental Research Funds for the Central Universities (Y0201700179), the Natural Science Foundation of Jiangsu province (BK20171374) and National Natural Science Foundation of China (Nos. 31330067, 31301782).

References

Achard P, Herr A, Baulcombe DC, Harberd NP (2004) Modulation of floral development by a gibberellin-regulated microRNA. Development 131: 3357–3365

Agar G, Turker M, Battal P, Emre EM (2006) Phytohormone levels in germinating seeds of *Zea mays* L. exposed to selenium and aflatoxines. Ecotoxicology 15: 443–450

Alabadi D, Blazquez MA, Carbonell J, Ferrandiz C, Perez-Amador MA (2009) Instructive roles for hormones in plant development. Int J Dev Biol 53: 1597–1608

Allen E, Xie Z, Gustafson AM, Carrington JC (2005) microRNA-directed phasing during trans-acting siRNA biogenesis in plants. Cell 121: 207–221

Audic S, Claverie JM (1997) The significance of digital gene expression profiles. Genom Res 7: 986–995

Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281–297

Baumberger N, Baulcombe D (2005) Arabidopsis ARGONAUTE1 is an RNA Slicer that selectively recruits microRNAs and short interfering RNAs. Proc Natl Acad Sci USA 102: 11928–11933

Chen ZH, Bao ML, Sun YZ, Yang YJ, Xu XH, Wang JH, Han N, Bian HW, Zhu MY (2011) Regulation of auxin response by miR393-targeted transport inhibitor response protein 1 is involved in normal development in Arabidopsis. Plant Mol Biol 77: 619–629

Chi X, Yang Q, Chen X, Wang J, Pan L, Chen M, Yang Z, He Y, Liang X, Yu S (2011) Identification and characterization of microRNAs from peanut (*Arachis hypogaea* L.) by high-throughput sequencing. PloS One 6: e27530

Chuck G, Cigan AM, Saeteurn K, Hake S (2007) The heterochronic maize mutant Corngrass1 results from overexpression of a tandem microRNA. Nat Genet 39: 544–549

Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, Robles M (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21: 3674–3676

Davis SJ (2009) Integrating hormones into the floral-transition pathway of *Arabidopsis thaliana*. Plant Cell Environ 32: 1201–1210

Dharmasiri N, Dharmasiri S, Weijers D, Lechner E, Yamada M, Hobbie L, Ehrismann JS, Jürgens G, Estelle M (2005) Plant development is regulated by a family of auxin receptor F box proteins. Dev Cell 9: 109–119

Dobrev PI, Vankova R (2012) Quantification of abscisic acid, cytokinin, and auxin content in salt-stressed plant tissues. Methods Mol Biol 913: 251–261

Eldem V, Okay S, Unver T (2013) Plant microRNAs: new players in functional genomics. Turk J Agric For 37: 1–21

Gardner PP, Daub J, Tate JG, Nawrocki EP, Kolbe DL, Lindgreen S, Wilkinson AC, Finn RD, Griffiths-Jones S, Eddy SR, Bateman A (2009) Rfam: updates to the RNA families database. Nucleic Acids Res 37: D136–D140

Gocal GF, Sheldon CC, Gubler F, Moritz T, Bagnall DJ, MacMillan CP, Li SF, Parish RW, Dennis ES, Weigel D, King RW (2001) GAMYB-like genes, flowering, and gibberellin signaling in Arabidopsis. Plant Physiol 127: 1682–1693

Heo JB, Sung S (2011) Vernalization-mediated epigenetic silencing by a long intronic noncoding RNA. Science 331: 76–79

Huang F, Tang J, Hou X (2016) Molecular cloning and characterization of BcCSP1, a Pak-choi (*Brassica rapa* Ssp. *chinensis*) cold shock protein gene highly co-expressed under ABA and cold stimulation. Acta Physiol Plant 38: 1–8

Irish VF (2010) The flowering of Arabidopsis flower development. Plant J 61: 1014–1028

Jiao Y, Wang Y, Xue D, Wang J, Yan M, Liu G, Dong G, Zeng D, Lu Z, Zhu X, Qian Q, Li J (2010) Regulation of OsSPL14 by OsmiR156 defines ideal plant architecture in rice. Nat Genet 42: 541–544

Jones-Rhoades MW, Bartel DP (2004) Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. Mol Cell 14: 787–799

Kurihara Y, Watanabe Y (2004) Arabidopsis Micro-RNA biogenesis through dicer-like 1 protein functions. Proc Natl Acad Sci USA 101: 12753–12758

Li D, Liu C, Shen L, Wu Y, Chen H, Robertson M, Helliwell CA, Ito T, Meyerowitz E, Yu H (2008) A repressor complex governs the integration of flowering signals in Arabidopsis. Dev Cell 15: 110–120

Li H, Dong Y, Yin H, Wang N, Yang J, Liu X, Wang Y, Wu J, Li X (2011) Characterization of the stress associated microRNAs in Glycine max by deep sequencing. BMC Plant Biol 11: 170

Li L, Zhang W, Zhang L, Li N, Peng J, Wang Y, Zhong C, Yang Y, Sun S, Liang S, Wang X (2015) Transcriptomic insights into antagonistic effects of gibberellin and abscisic acid on petal growth in *Gerbera hybrida*. Front Plant Sci 6: 168

Luo Y, Guo Z, Li L (2013) Evolutionary conservation of microRNA regulatory programs in plant flower development. Dev Biol 380: 133–144

Mandaokar A, Browse J (2009) MYB108 acts together with MYB24 to regulate jasmonate-mediated stamen maturation in Arabidopsis. Plant Physiol 149: 851–862

Michaels SD, Amasino RM (1999) The gibberellic acid biosynthesis mutant *ga1-3* of *Arabidopsis thaliana* is responsive to vernalization. Dev Genet 25: 194–198

Mutasa-Gottgens E, Hedden P (2009) Gibberellin as a factor in floral regulatory networks. J Exp Bot 60: 1979–1989

Nagpal P, Ellis CM, Weber H, Ploense SE, Barkawi LS, Guilfoyle TJ, Hagen G, Alonso JM, Cohen JD, Farmer EE, Ecker JR, Reed JW (2005) Auxin response factors ARF6 and ARF8 promote jasmonic acid production and flower maturation. Development 132: 4107–4118

Oka M, Tasaka Y, Iwabuchi M, Mino M (2001) Elevated sensitivity to gibberellin by vernalization in the vegetative rosette plants of *Eustoma grandiflorum* and *Arabidopsis thaliana*. Plant Sci 160: 1237–1245

Pan X, Welti R, Wang X (2010) Quantitative analysis of major plant hormones in crude plant extracts by high-performance liquid chromatography-mass spectrometry. Nat Protoc 5: 986–992

Pose D, Yant L, Schmid M (2012) The end of innocence: flowering networks explode in complexity. Curr Opin Plant Bio 15: 45–50

Puzey JR, Karger A, Axtell M, Kramer EM (2012) Deep annotation of *Populus trichocarpa* microRNAs from diverse tissue sets. PLoS One 7: e33034

Rajagopalan R, Vaucheret H, Trejo J, Bartel DP (2006) A diverse and evolutionarily fluid set of microRNAs in *Arabidopsis thaliana*. Gene Dev 20: 3407–3425

Samach A, Onouchi H, Gold SE, Ditta GS, Schwarz-Sommer Z, Yanofsky MF, Coupland G (2000) Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. Science 288: 1613–1616

Sato A, Yamamoto KT (2008) Overexpression of the non-canonical aux/IAA genes causes auxin-related aberrant phenotypes in Arabidopsis. Physiol Plant 133: 397–405

Schwab R, Palatnik JF, Riester M, Schommer C, Schmid M, Weigel D (2005) Specific effects of microRNAs on the plant transcriptome. Dev Cell 8: 517–527

Simpson GG, Dean C (2002) Arabidopsis, the Rosetta stone of flowering time? Science 296: 285–289

Srikanth A, Schmid M (2011) Regulation of flowering time: all roads lead to Rome. Cell Mol Life Sci 68: 2013–2037

Sunkar R, Zhou X, Zheng Y, Zhang W, Zhu JK (2008) Identification of novel and candidate miRNAs in rice by high throughput sequencing. BMC Plant Biol 8: 25

Swiezewski S, Liu F, Magusin A, Dean C (2009) Cold-induced silencing by long antisense transcripts of an Arabidopsis Polycomb target. Nature 462: 799–802

Szittya G, Moxon S, Santos DM, Jing R, Fevereiro MP, Moulton V, Dalmay T (2008) High-throughput sequencing of *Medicago truncatula* short RNAs identifies eight new miRNA families. BMC Genom 9: 593

Takei K, Sakakibara H, Taniguchi M, Sugiyama T (2001) Nitrogen-dependent accumulation of cytokinins in root and the translocation to leaf: implication of cytokinin species that induces gene expression of maize response regulator. Plant Cell Physiol 42: 85–93

Tian C, Wan P, Sun S, Li J, Chen M (2004) Genome-wide analysis of the GRAS gene family in rice and Arabidopsis. Plant Mol Biol 54: 519–532

Tsuji H, Aya K, Ueguchi-Tanaka M, Shimada Y, Nakazono M, Watanabe R, Nishizawa NK, Gomi K, Shimada A, Kitano H, Ashikari M, Matsuoka M (2006) GAMYB controls different sets of genes and is differentially regulated by microRNA in aleurone cells and anthers. Plant J 47: 427–444

Varkonyi-Gasic E, Lough RH, Moss SM, Wu R, Hellens RP (2012) Kiwifruit floral gene APETALA2 is alternatively spliced and accumulates in aberrant indeterminate flowers in the absence of miR172. Plant Mol Biol 78: 417–429

Vazquez F, Legrand S, Windels D (2010) The biosynthetic pathways and biological scopes of plant small RNAs. Trends Plant Sci 15: 337–345

Voinnet O (2009) Origin, biogenesis, and activity of plant microRNAs. Cell 136: 669–687

Wang JW (2014) Regulation of flowering time by the miR156-mediated age pathway. J Exp Bot 65: 4723–4730

Wang JW, Czech B, Weigel D (2009) miR156-regulated SPL transcription factors define an endogenous flowering pathway in *Arabidopsis thaliana*. Cell 138: 738–749

Wang J, Hou X, Yang X, Gustafson P (2011) Identification of conserved microRNAs and their targets in Chinese cabbage (*Brassica rapa* subsp. *pekinensis*). Genome 54: 1029–1040

Wang Z, Jiang D, Zhang C, Tan H, Li Y, Lv S, Hou X, Cui X (2015) Genome-wide identification of turnip mosaic virus-responsive microRNAs in non-heading Chinese cabbage by high-throughput sequencing. Gene 571: 178–187

Xia K, Wang R, Ou X, Fang Z, Tian C, Duan J, Wang Y, Zhang M (2012) OsTIR1 and OsAFB2 downregulation via OsmiR393 overexpression leads to more tillers, early flowering and less tolerance to salt and drought in rice. PLoS One 7: e30039

Yamaguchi A, Abe M (2012) Regulation of reproductive development by non-coding RNA in Arabidopsis: to flower or not to flower. J Plant Res 125: 693–704

Yant L, Mathieu J, Dinh TT, Ott F, Lanz C, Wollmann H, Chen X, Schmid M (2010) Orchestration of the floral transition and floral development in Arabidopsis by the bifunctional transcription factor APETALA2. Plant Cell 22: 2156–2170

Zanewich KP, Rood SB (1995) Vernalization and gibberellin physiology of winter canola (endogenous gibberellin (GA)) content and metabolism of [^{3}H]GA1 and [^{3}H]GA20. Plant Physiol 108: 615–621

Zhang D, Ren L, Yue JH, Wang L, Zhuo LH, Shen XH (2014) GA4 and IAA were involved in the morphogenesis and development of flowers in *Agapanthus praecox* ssp. *orientalis*. J Plant Physiol 171: 966–976

Zhao Y (2010) Auxin biosynthesis and its role in plant development. Annu Rev Plant Biol 61: 49–64

Zhao L, Kim Y, Dinh TT, Chen X (2007) miR172 regulates stem cell fate and defines the inner boundary of APETALA3 and PISTILLATA expression domain in Arabidopsis floral meristems. Plant J 51: 840–849

Zhu QH, Spriggs A, Matthew L, Fan L, Kennedy G, Gubler F, Helliwell C (2008) A diverse set of microRNAs and microRNA-like small RNAs in developing rice grains. Genome Res 18: 1456–1465

Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 31: 3406–3415

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. GO enrichment of targets of vernalization-associated miRNAs. (A) GO enrichment of biological process. (B) GO enrichment of molecular function.

Table S1. Summary of cleaning data from control and vernalization sRNA.

Table S2. Summary of common and specific sequences between control and vernalization sRNA libraries.

Table S3. Distribution of small RNAs among different categories in B. rapa.

Table S4. Known *B. rapa* miRNAs in the control and vernalization library.

Table S5. Novel *B. rapa* miRNAs in the control and vernalization library.

Table S6. Differentially expressed miRNAs between control and vernalization in *B. rapa*.

Table S7. Summary of target genes from control and vernalization libraries

Table S8. Top 10 KEGG Pathway annotations of genes targeted by differentially expressed miRNAs.

Table S9. Primers of miRNAs and targets in *B. rapa* used for qPCR.

Table S10. Putative targets of differentially expressed miRNAs identified in *B. rapa*.

Edited by S. Pelaz