Comparative transcriptomic analysis reveals the relationship between cold-acclimation and vernalization

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Abstract

Background:

The relationship between cold acclimation and vernalization has been a hanging debate. Cold-acclimation is a cold-responding process that enable plants adapt to cold temperature in a rapid time. While vernalization is that plants flower by quantitative accumulation of cold exposure. 4°C is both the acclimation temperature and vernalization temperature. We conducted transcriptomic analyses during 42 days 4°C vernalization process to explore the whole process of transcriptional dynamics during vernalization and responding situation of short-term cold exposure in Arabidopsis

Results:

Brassinosteroid was found as the most sensitive hormone in response to cold shock. Phosphorylation played an important role in long-term temperature drop by time-course analysis. Independency between cold-acclimation and vernalization was confirmed based on time-lag initiation of these two pathways. Cold-acclimation suppress flowering after vernalization initiation by continuously blocking photoperiod and gibberellin (GA) pathway. *FLM* inhibited flowering in a *FLM-β*-upregulation and *FLM-δ*-independent way. Autonomous and aging pathway positively responded but did little contribution to flowering. Alternative splicing mechanism mediated cold responding through the whole vernalization process.

Conclusion

Transcriptomic analysis revealed that Cold-acclimation and vernalization are independent. Cold as winter signal either indirectly suppress flowering through cold-acclimation involved photoperiod and GA pathways or directly suppress flowering through thermosensory pathway. Additionally, alternative splicing mediation worked through the whole vernalization process.

Background

Plants are sessile organisms, passively sensing environmental signals, one of which is temperature. The process that plants flower after exposing in prolonged cold termed vernalization, in which winter-annual accessions of Arabidopsis require 30-40 day's low temperature before flowering. Another type of accession named rapid-flowering, having no requirement of vernalization. Difference between two accessions is determined by a dominant allele of *FRI* (*FRIGIDA*) [1, 2]. *FRI* encodes a scaffold protein and acts with *FRL1* (*FRI-LIKE 1*), *FES1* (*FRI ESSENTIAL 1*), *SUF4* (*SUPPRESSOR OF FRI 4*), *FLX* (*FLC EXPRESSOR*) as a complex to recruit other transcription factors and chromatin modifiers, thus activating *FLC* (*FLOWERING LOCUS C*) expression [3]. *FLC* encodes a MADS-BOX transcription factor that can suppress flowering. *FLC* forms a complex with another MADS-box protein *SVP* (*SHORT VEGETATIVE*

PHASE), binding with promoter of floral integrator FT and SOC1 (SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1) [4].

Before vernalization, *FLC* chromatin is in an active-transcription state during vegetative growth, on the grounds that *FLC* chromatin is enriched of series of active marks including H3K4me3, H3K36me3 and acetylation [5]. ATX1 and ATX2 can directly bind the active *FLC* locus and methylate K4 of histone H3, positively regulating *FLC* expression. While ATX1 trimethylates it, ATX2 dimethylates it [6,7]. Upon cold exposure, suppression of *FLC* is initiated with up-regulation of noncoding *FLC* antisense transcripts *COOLAIR* [8]. Subsequent continuing suppression is maintained by Trimethylating lysine 27 of histone H3 via PRC2 (Polycomb repressive complex 2). PRC2 is conservatory in animals and plants. Homologs of the Drosophila H3K27 methyltransferase E(z): CLF (CURLY LEAF) and SWN (SWINGER) together with structure subunit VRN2 (VERNALIZATION 2) and FIE (FERTILIZATION INDEPENDENT ENDOSPERM) and MSI1 compose PRC2 core components [9, 10]. VIN3, encoding a chromatin-remodeling plant homeodomain (PHD) finger protein, form a heterodimer with a paralogue VIN3-like 1 (VIL1)/VERNALIZATION 5 (VRN5), thus joining in core PRC2 serving as cold specific PHD (VIN3)-PRC2. During cold exposure, PRC2 dynamically remove the active marks replacing by H3K27me3 [7]. Recent study revealed that Polycomb partners VAL1 and VAL2 serving as epigenome readers to recognize a cis-regulatory element at the *FLC* locus recruit histone deacetylase HDA9 and PRC2. The former catalyzes the deacetylation of H3K27ac to H3K27, and the latter catalyzes the trimethylation of H3K27 to H3K27me3, synergistically inhibiting the expression of *FLC* to promote flowering [11]. VAL1 also recruits the histone deacetylase HDA19 to reduced transcription level of *FLC* [12]. After recovering to 22°C, LIKE HETEROCHROMATIN PROTEIN 1 (LHP1) and VERNALIZATION (VRN2) recognize H3K27me3, thus stably maintaining *FLC* repression. Multiple genes participate in *FLC* inhibition by altering chromatin structure in *FLC* locus [13, 14]. Autonomous genes also partially participate in *FLC* suppression, including LD (LUMINIDEPENDENS), FCA, FY, FPA, FLD (FLOWERING LOCUS D), FVE, FLK (FLOWERING LOCUS KHDOMAN) and REF6 (RELATIVE OF EARLY FLOWERING 6) [15]. FLD, FVE and REF6 involve in chromatin remodeling of *FLC* [16, 17, 18]. While FCA, FLK and FY encode RNA-binding proteins, which inhibit *FLC* in RNA processing [19]. LD has a positive effect to the *LFY* promoter thus facilitating flowering, also suppress *FLC* by interacting with *SUF4* [20, 21].

Another cold responding process termed cold-acclimation, which is a relative short adaptation practice in cold exposure. A common belief of cold signal transduction is that it is initiated by membrane rigidification followed by an influx of Ca²⁺ into the cytosol, and subsequently cause the production of IP3 [22, 23, 24, 25]. There are two types of cold-signaling pathway: CBF-dependent pathway and CBF-independent pathway. Current research focus more on ICE1-CBF-COR. *COR* genes are rapidly induced by CBF in range of few minutes to several hours [26]. *KIN1*, *KIN2* are members of *COR* family, which expression is induced by cold and abscisic acid (ABA) [27]. LTI (LOW TEMPERATURE INDUCED) and RAB (RESPONSIVE TO ABSCISIC ACID) participate in freezing tolerance and osmotic stress [28]. Protein kinase OST1 (OPEN STOMATA 1) enhances freezing tolerance by regulating ICE1 phosphorylation [29, 30]. BES1 (BRI1-EMS-SUPPRESSOR 1) played an essential role in BR signal transduction, positively

regulating CBF expression [31]. When temperature is reaching non-freezing cold, ICE1 (INDUCER OF CBF EXPRESSION1) directly binds to promoter of *CBF*, activating its expression, then results in a further promotion of *COR* expression downstream, thus enhancing cold tolerance of plants [32, 33, 34].

One notion revolving around distinction between cold-acclimation and vernalization is time lag, which is approximately 10 days long [35]. In other words, the initiation of vernalization is 10 days later than that of cold acclimation. Thus, the debate that whether the cold responding pathway and vernalization are independent and what role cold acclimation play after vernalization initiation were born. For the purpose of investigating this mystery, we conducted a transcriptome analysis during the whole cold-acclimation and accompanying vernalization processes.

Results

Transcriptional dynamics of vernalization in *Arabidopsis thaliana*

Cold-acclimation is a cold-responding process that enable plants adapt to cold temperature in a rapid time. While vernalization is that plants flower by quantitative accumulation of cold exposure. At issue is whether overlapping and interactions exist between two pathways. With the aim of profiling the whole picture of transcriptional dynamics during vernalization process and responding situation of cold shock in *Arabidopsis* Col-0 with a functional *FRI* allele. We set up 8 time points of samples, 4 of which (0 d, 14 d, 28 d, 42 d) are designed based on time sequence of whole cold responding process, while 4 of which (0.5 h, 1 d, 29 d, 30 d) are designed for sake of exploring cold stress genes. Notably, among them 29 d samples experienced one day 22°C recovery, 30 d samples were exposed 1 d cold following the recovery, which were used to examine the validation of short-term responding gene's expression. BGISEQ-500 was applied to detecting differential expression genes (DEG). There are total of 31744 DEGs (including Non-coding RNA) identified. The differential genes of short-term response 0 h VS 1 d, 28 d VS 29 d, 29 d VS 30 d and long-term response 0 hVS14 d, 0VS28 d, 0VS42 d were respectively plotted by Wayne diagram. There are 693 common genes among both groups (Figure 1B). KEGG enrichment showed that they mainly enriched in plant hormone signal transduction, especially brassinosteroid (BR). This result suggests BR may play a most significant role in hormone responding of stress response, which is well-characterized in 00905 pathway map (Figure 1E) (Table S1). There are 5651 common genes in the long-term combination (Figure 1C). KEGG enrichment mainly focused on photosynthesis, metabolic and circadian rhythm (Figure 1D). This suggests that long-term responding changed the whole metabolism process and disrupted the photosynthesis, which is characterized by map 00195 (Figure 1F) (Table S2). Enrichment of long-term response and short-term response showed the difference between plant stress response and long-term acclimation. At the initiation stage of cold exposure, only 2709 genes had an expression change within 0.5 hour, which is almost 1/4 of 1 d samples (Figure 1A). Also, the greatest number of DEGs are detected in 1 d samples than any other points, which means one day is the most drastic responding timing of cold-shock. While genes from 30 d samples exhibited no such significant difference though they also experienced one day cold exposure after recovery. Acclimation maybe the explanation since plants had already exposed cold for 28 days. Point 14 d, 28 d and 42 d had similar

number of differential expression genes, suggesting that plants maintain high responding level to continuing cold exposure (Figure 1A).

Time-course analysis was conducted by clustering all genes from different time points in purpose of investigating expression dynamics (Figure 2). Genes in cluster 3 have a quick response to cold within 0.5 h (Figure 2C). Gene ontology (GO) indicates that these genes are sensitive to stress environment, which can be well characterized by enrichment of wounding response, defense response and ethylene-activated signaling pathway (Figure 3C). Genes in Cluster 5 and cluster 7 had similar expression pattern (Figure 2E, 2G). Both of them responded immediately to changing temperature at point 0.5 h and 29 d, and have a relative longer adaptation period than cluster 3 (Figure 2E). GO analysis showed that up-regulated genes in these two clusters were enriched in DNA repair and RNA modification, the reason of which maybe is that such lasting response can cause DNA impair and replication increase (Figure 2E, 2G). Genes in cluster 2 and cluster 8 were downregulated during cold exposure and are sensitive to raising temperature (Figure 2B, 2H). GO results reveals that cluster 2 were enriched in reductive pentose-phosphate cycle, oxidation-reduction process, response to cytokinin and cluster 8 were enriched in cell division and cell cycle. This indicates that life cycle, energy consumption and oxidative activity are active in relative high temperature and decrease in low temperature (Figure 2B). Genes in cluster1 maintained high expression after one-day cold exposure (Figure 2A). These genes are mainly enriched in phosphorylation-related processes including MKK, CIPK9 and intracellular protein transport (Figure 2A). This suggests that phosphorylation have an important role in long-term temperature change. Genes enriched in cold respond and cold-acclimation were from cluster 4 (Figure 2D). Compared to cluster 6 referring to regulation of flower development, the former expression level reached the peak at 14 d then dropped smoothly afterwards, the latter counterpart reached the peak at 42 d (Figure 2F). Also, genes in cluster 4 behaved sensitively to temperature change, but genes in cluster 6 had no activities like that. These results can preliminarily show the connections and differences between short-term and long-term response to cold (Figure 2F, 2G) (Table S3).

Cold-acclimation and vernalization are independent

Short-term response and long-term response as above-mentioned are well characterized by cold-acclimation and vernalization, respectively. These two processes are deemed to initiate with a time difference for about 10 days. To explore the overlapping and interactions between cold-acclimation and vernalization, we focused on genes involved in the relative pathways. Pivotal gene of cold-responding pathway CBF was induced to peak within 0.5 h, and had a smooth and steady expression pattern afterwards (Figure 3A). Other genes well known in respond to cold also showed a typical uptrend of expression. *ICE1*, *COR*, *LTI*, *RAB*, *KIN*, *OST1* and *BES1* all significantly ascended during response process within one day (Figure 3C). While *FLC*, central gene of vernalization process, was originally suppressed at around 14 days together with the induction of *VIN3*, which is considered as first gene in vernalization pathway. (Figure 3A). NTL8 was recently found to upregulate *VIN3* under long-term cold [36], which expression pattern was similar to *VIN3*. *PRC2* genes behaved a normal rising trend. *VAL 1* showed an obvious uptrend of expression during vernalization, while *VAL2* expression level fall to normal level after

a slight increase. Notably, almost all genes in vernalization pathway changed their expression level to regulate *FLC* expression after 14 d (Figure 3B). There is an overlapping gene *HOS1* between cold-acclimation and vernalization (Figure 3C). The instant rise of *FLC* and decrease of *ICE1* within one day are attributed to it, which promotes *FLC* transcription through chromatin remodeling and negatively regulates the *CBF* genes by mediates the ubiquitination and proteasomal degradation of *ICE1* [37, 38]. This may cause antagonistic responding between two pathways. However, this uptrend of *FLC* and downtrend of *ICE1* did not maintain too long. After one-day cold exposure, expression of these two genes went into a conserve expression trend, which means such interactive activity would not affect the initiation timing of two pathways. This indicates that cold-acclimation plays a predominant role in cold responding process. Consisting with the time-lag notion, rapid responding genes did not participate in quantitative responding process. On an objective note, cold-acclimation and vernalization are independent in overlapping-gene regulatory perspective (Table S4).

Cold acclimation suppress flowering after vernalization initiation

The significance of vernalization is to allow plants to get through the cold winter and flower in the warm spring. Thus, cold is supposed to recognized as winter signal to inhibiting other flowering pathways. The impacts of cold on flowering can be divided into two types. One is the direct effect on flowering that plants perceive the environmental signal thus exhibiting early flowering in warmer temperature and late flowering in lower temperature [39]. The other is the indirect effect on flowering by cold acclimation process in order to obtain cold tolerance. Genes in cold-acclimation are induced within a short period of time and keep stable in a high expression level afterwards. So, what role does cold-acclimation play after plants initiating vernalization response. It has been reported that mutant *shi1* (*Col* background), which is insensitive to cold, can flower after 95 days of 4°C treatment, but *Col* was delayed extremely longer than 95 days [40]. Interestingly, our analysis showed that many flowering genes upregulated during the cold exposure (Figure 4). Indirect effect involving cold acclimation is associated with photoperiod and gibberellin pathway. The genes related to circadian rhythm had a quick response to cold (Figure 4A). Almost all genes involved in circadian clock had an expression increase at 1 d and decrease at 29 d. Among them, *CCA1/LHY*, core genes of feedback loop, had similar expression pattern [41]. They were induced within one day and had a perfect high-regulating response to cold. *PRR7/9*, *TOC1* as the part of the morning loop and evening loop respectively also were up-regulated [42]. While TCP transcription factor *CHE* (*CCA1* HIKING EXPEDITION) participating in an additional module by which *CCA1* and *CHE* reciprocally repress each other showed a severe down-regulation [41]. Phytochromes *PHYA* and *PHYB* were upregulated during cold exposure, *PHYB*'s expression rose a little bit slower than *PHYA*. Another type of photo-receptor Cryptochrome, with ability of sensing blue light and UV-A also showed a coincident up-regulation. BBX proteins containing one or two B-box domains in N-terminal regions, provides a key link between the circadian clock and photoperiod [43]. Almost all members in BBX family had an expression increment. However, the expression level of central gene of photoperiod *CO* was found no distinct difference after cold treatment, which capable to activate *FT* expression [44]. This may be because *CCA1/LHY* contributed to cold tolerance by promoting *CBF* while facilitated expression of *CDF* at the same time [45, 46]. Besides this, *G1* showed the same expression level at 42 d as 0 d after ascending the

peak at 14 d. Also, *FKF1*, which can interact with CO to stabilize it [47, 48], gradually decreased after a quick induction at 0.5 h. Therefore, all increase shown in photoperiodic pathway actually had done nothing to flowering. These results suggest that when temperature falling to non-freezing cold, circadian rhythm gives preference to initiate cold acclimation, despite upregulation of certain genes may suppress flowering (Table S4).

Another pathway associates to both cold acclimation pathway and flowering is gibberellin (GA). *GA20ox* and *GA3ox* which play an important role in GA activation merely had a slight increase. While key gene for GA deactivation *GA2ox6* was distinctly upregulated. Negative regulator of GA signaling *SPY* also had an expression increment [49]. Other genes participating in GA signaling barely changed. These results indicate that in order to accumulate DELLA proteins to acquire cold tolerance, GA pathway was also inhibited during vernalization by reducing active GA form. Cold acclimation suppressed flowering by continuously block photoperiod and gibberellin pathway after vernalization initiation (figure 4g) (Table 4).

Low temperature suppress flowering through thermosensory pathway

Direct effect on flowering mainly revolves around thermosensory pathway. There are 5 homologous of *FLC*, *FLM* (FLOWERING LOCUS M)/*MAF1* (MADS AFFECTING FLOWERING 1) [50], *MAF2*, *MAF3*, *MAF4*, *MAF5* in Arabidopsis, which all serve as floral repressor in vernalization [50]. However low temperature did not inhibit their expression (Figure 4C). As a matter of fact, all of these homologous have an uptrend of expression under 4°C environments. This maybe because *MAF1* also participates in thermosensory pathway. *FLM-β* as a function form of *FLM* interact with *SVP* to suppress *SOC1* expression [51]. As temperature rising to 27°C, the ratio of two transcripts *FLM-β/δ* is reduced, thus decreasing the repression effect of *SOC1*. Recent research found that *MAF2* regulates flowering time in the same way [52]. Interestingly, under 4°C *FLM-β* was the only significantly increasing transcript, and *FLM-δ* had a relative stable expression pattern (Figure 4C), which means *FLM* repress flowering in a *FLM-β* upregulation and *FLM-δ*-independent way. *MAF2/MAF3* also regulate flowering by changing expression of sole transcript. Additionally, their interacting protein *SVP* were also upregulated distinctly to cooperate with them to suppress flowering. This indicates that *MAFs* served in thermosensory pathway to inhibit flowering rather than vernalization. However, there does existing upregulated genes that can promote flowering. *SPLs* from age pathway were up-regulated in juvenile period, with the result that *SOC1* expression was induced (Figure 4D) (Table 16). At the same time, expression level of genes in autonomous pathway like *FCA*, *FLD*, *FLK*, *LD* and *FY* had been increased during the cold treatment, which partially repressed *FLC* expression (Figure 4E) (Table 17). However, these pathways failed to activate the downstream flowering genes such as *AP1*, *LFY*. The reason of this maybe is that current expression increase of *SOC1* and decrease of *FLC* are not sufficient to initiate flowering transition. These results suggest that cold significantly suppress flowering by directly upregulating flowering repressor *FLM* in thermosensory pathway and continuously blocking photoperiod and GA pathway. It strikes the right note that, plants choose to acquire adaptation ability of temperature drop firstly then continue reproduction (Table 4).

Alternative splicing mediation during vernalization

Alternative splicing is a ubiquitous post-transcriptional RNA processing by which multiple transcripts can be generated by a single gene. It has been reported that temperature is closely associated with AS process. Several kinds of mechanisms of alternative splicing have been reported, including skipped exon (SE) (particular exon is excluded from mature mRNA), mutually exclusive exons (MXE) (choice between two constitutive exons), alternative 3'/5'splicing site (A3SS/A5SS) (distinct 3' or 5' splicing sites are generated in resulting isoforms) and retained intron (RI) [53]. IR is a predominant form of alternative splicing in plants, which main function is to generate transcripts with premature termination codons (PTCs), thus leading to nonsense mRNA decay (NMD) [54]. Total of 1540 differentially alternative splicing genes (DAS gene) were identified, which made up 4.85% of all DEG. In the grand scheme of things, the proportion of IR is decreasing during cold exposure, while MXE came to appearance from 1-day treatment, which is the AS form of *FLM*. MXE plays a role in generating variety kinds of protein without changing structure of them, by which adjust their functions. A3SS form also showed a significant increase (Figure 5A). Another reported alternative splicing regulation in flowering is *CCA1*, which respond to cold by increasing functional *CCA1-α* [55]. The mechanism of it is A5SS. These results suggest that plants attempt to improve production of proteins to deal with changing environment cues more efficiently (Figure 5B).

When view the perspective of particular responding pathways, it was found that AS mechanism enormously affect the whole cold exposure process. Activation of calcium channel is incidental to initiation of cold responding. Cyclic nucleotide-gated channels (CNGCs) are Ca^{2+} -permeable channels [56]. Full-length transcript of both *CNGC5* and *CNGC6* barely worked, *CNGC6* was even undetectable (Figure 5b). Line chart shows that *CNGC5* responded to cold by ratio variation of *NM_203224.1* and *NM_125179.5*, while three transcripts of *CNGC6*—*NM_127960.3*, *NM_001335885.1* and *NM_001335886.1* all had an expression increase, which means they all had a role in responding process. Phosphorylation functioned through whole cold treatment. CIPK3 (CBL-interacting protein kinase3) is crucial for Ca^{2+} signal transduction among different abiotic stresses [57]. Full-length transcript *NM_179761.2* was found no effect during responding process. However, *NM_179763.4* isoform showed a significant expression increase, which may be the functional form of *CIPK3*. With temperature signal transducing in vivo, upstream gene of cold-acclimation *ICE1* was induced [58]. *ICE1* worked by changing the ratio of *NM_001035699.2* and *NM_001035697.2*, thus leaving them reaching the same expression level. *COR15A* and *COR27* are activated by CBF. Both transcripts of *COR15A*—*NM_180040.3* and *NM_129815.5* up-regulated to enhance cold tolerance. While *COR27A* only functioned in full-length form. Hormones are main types of temperature responders. Transcription factor BES1 plays an important role in BR regulation. Full-length of *BES1* was also less contributory than splice variants. It was shown that *NM_202135.3* is the main responding transcript and *NM_202134.1* may react to temperature rise. EIN4 is one of the ethylene receptors [59]. At the early stage of the cold treatment, transcription level of *NM_202489.2* and *NM_111329.4* are almost the same. However, after long-term treatment when view against *NM_202489.2*, *NM_111329.4* increased drastically. ABA responding protein *RAB8* showed a

similar regulating pattern as *EIN4*. Expression level of *NM_115221.4* keep going up during the cold exposure, while *NM_180365.3* reach the bottom at 42 d.

Circadian rhythm plays a significant role in cold-acclimation. *PHYA* is a putative signal receptor in this process. Transcripts of *PHYA* all contributed to responding process since they all showed an uptrend of expression. While, splice variant *NM_001335612.1* of *PHYB* showed a distinct induction rather than main transcript. Blue light receptor *CRY2* responded to cold in a transcripts ratio change-dependent way. Full length of evening loop genes *PRR7*, *PRR9* had a similar rising expression trend. While splice variants of *PRR9* may participate more in responding process.

Vernalization process initiates as *FLC* suppression. *VRN5* showed a curious down-regulation during vernalization (Figure 3B). However, the reason of this is that splice variant *NM_001338682.1* decreased to ensure full transcript *NM_113351.3* working effectively. *VAL2* was also found having no expression level change (Figure 3B), the reason of which revolves around the ratio change of *NM_001342124.1* and *NM_119353.4* to respond cold. *ATX1* and *ATX2* which both have positive effect on *FLC* expression exhibited converse expression pattern. Low temperature causes the reduction of *ATX1* immediately at 0.5 h and keeps decreasing afterwards. However, after short-term stress response in 0.5 h, 1 d, 29 d, 30 d, *ATX2* always maintained a high expression level in long-term cold exposure in 14 d, 28 d and 42 d (Figure 3B). It was found that *ATX1*, *ATX2* experienced a different way of alternative splicing mediation. *ATX1* had a significant ratio change of two transcripts, while AS did not affect full length transcript of *ATX2*.

We classed these analyzed genes in three types: one is ratio change of different transcripts, the next is functional transcript induction, and last one is synergistic work of all transcripts (Figure 5B). Among them, *ICE1*, *EIN4*, *CRY2*, *RAB8*, *VRN5*, *VAL2*, *ATX1* belong to type one, which had a proportion change of two transcripts. This type of genes may have antagonistic functions between two transcripts. *CNGC5*, *CIPK3*, *COR27*, *PRR7*, *PRR9*, *ATX2* belong to type two, whose certain transcript showed a distinct expression rise. This type of genes which nonfunctional transcripts may involve in nonsense mRNA decay, thus affecting the potency of functional transcript. *CNGC6*, *BES1*, *PHYA*, *CPR15A*, *FCA* belong to type three, which transcripts all had response abilities. This type of genes may generate multiple proteins to respond temperature more efficiently. Therefore, alternative splicing responds to temperature change in a delicate regulatory way.

To further verify the genes transcript profiles obtained from RNA-Seq results, we selected six of DEGs: *FLC*, *VIN3*, *CBF2*, *CBF2*, *MAF1*, *SOC1* to confirm the validation by qRT-PCR assay. The outcome in each case was consistent with the RNA-Seq assay (Figure 6) (Table S5).

Discussion

Association between cold-acclimation and vernalization

The relationship between cold acclimation and vernalization has been a hanging mystery. How come two successive pathways have no interactions under same cold treatment. The heart of the contradiction is

the initial response time. Cold-acclimation is a rapid responding process in order to adapt the changing temperature as soon as possible, key requirement of which is quickness. Vernalization is a quantitative process in order to overwinter and flower in a warm spring, key requirement of which is accumulation. This led to the impossibility of overlapping regulation between two pathways and the priority of cold-acclimation in responding to cold. Time difference for about 10 days of two pathways' initiation verified this hypothesis (Figure 3A). Besides recent study found that NTL8 being upstream regulator of VIN3 does not function to cold stress genes like COR47 [36]. This further confirmed that vernalization is independent with cold acclimation process. Thus, another question is presented. What role does cold-acclimation play after initiation of vernalization. Photoperiod and gibberellin (GA) as crosstalk between cold acclimation and flowering characterized this interplay. Circadian rhythm oscillated instantly in response to cold. *CCA1/LHY* as core oscillators of circadian clock contributed to both *CBF* and *CDF* induction, upregulation of which mains this oscillation did not contribute to flowering, merely for the sake of promoting cold acclimation. External application of GA can enhance the activity of the *LFY* promoter and directly initiate the floral switch [60]. DELLA proteins acting as negative regulators in gibberellin (GA) signal transduction [49], was found accumulating under low temperature, thus contributing to CBF-mediated cold acclimation and late flowering [61]. Activation genes of GA did not exhibit obvious fluctuation while deactivation genes showed a distinct expression increase, which means GA pathway was also occupied by cold acclimation thus inhibiting the flowering. These results suggest that cold acclimation suppress flowering after vernalization initiation.

low temperature can also directly affect flowering. Cold did not suppress homologues of *FLC*. It was found that they all worked in AS-mediated thermosensory pathway. Functional transcript *FLM-β* was induced to interact with SVP which also had an uptrend expression level, thus repressing flowering (Figure 7). However, there are some expression change in flowering pathways actually contributory to floral transition. Endogenous autonomous and aging pathways did have a positive effect on flowering during cold exposure. Autonomous genes all had an expression increment, which partially participate in repressing *FLC* expression. Added to this, almost all *SPL* members were upregulated, which can promote *SOC1* expression. Nevertheless, it is the lonely voice, not sufficient to initiate floral switch. It was reported that Col flower after 200 days cold exposure under long days [40]. This suggest that plants would accumulate these flowering promoting genes in a relative slower pace under non-freezing cold. After thoroughly adapting to temperature change, they will flower despite the 22°C recovery after vernalization process (Figure 7).

We focused more on cold exposure process at different stages rather than 22°C recovery period of vernalization. It was found that WDR5a and ATX1 both facilitate FLC expression by depositing H3K4me3 in its locus, and both of them were downregulated during the vernalization (Figure 3B). While recently Xi et al (2020) explored and reported the full dynamic of vernalization. Continuous activity of H3K4me3 was shown during vernalization which is not consistent with this [62]. Interestingly, *ATX1* experienced alternative splicing mediation during cold exposure. Downregulation of it is due to expression decrease of a splicing variant to ensure a full function of trimethylated. And this indicates that ATX1 may be the main type of methyltransferase during vernalization.

Cold sensors of cold-acclimation and vernalization

What is the cold sensor in plants remains an open question. To explore this, we set a criterion that sensor should respond faster than CBF (0.5 h) and respond to temperature change in 29 d, 30 d quickly and accurately. Some potential cold sensors are proposed recently [63]. First is CNGCs (cyclic nucleotide-gated channels), which are Ca^{2+} -permeable channels mostly localizing at plasma membrane [64]. It was found that *CNGC5*, *CNGC6* differentially expressed during cold exposure and both were mediated by alternative splicing. However, functional transcript of *CNGC5* and *CNGC6* both start responding after 1d, which is later than CBF responding time (Figure 5b). Secondly, phytochromes served as photoreceptors in plants regulate the photomorphogenesis [65]. PhyB was recently reported as thermosensor in Arabidopsis [65]. However, *PHYB* also barely responded before 1d and showed no apparent expression fluctuation at 29d, 30d. While *PHYA* upregulated rapidly to cold at 0.5 h, and have a quick response to 29d, 30d temperature change. In tomato FR and R perceived by phyA and phyB have antagonistic effect to cold tolerance, and phyA plays a predominant role [66, 67], the mechanism of which may function in the same way in Arabidopsis. But whether phyA is the cold sensor remains lack of evidence.

Whether sensors of cold-acclimation and vernalization are the same is also in debate. Helliwell et al proposed that the initial response to a temperature change is physical in vernalization, by which chromosomes loops are gradually disrupted during cold treatment. Also, transcription activity would fall into lowest level within one week [68]. However, FLC did have a short-term elevation in response to cold at 1d in a HOS1-dependent way (Figure 2A) [37]. Added to this, it was shown that FLC expression maintained high expression at 10-day cold in another transcriptomic analysis recently [64], which means this postulation is questionable. Additionally, overexpression of CBFs in Col background increases the expression level of FLC in a vernalization-independent way [69]. Therefore, FLC may share the same cold sensor for downstream of CBF in response to short-term cold and for the sensor of vernalization initiation. As for cold sensor of vernalization process, recent research reported that growth condition can serve as thermo sensor. Temperature falling leads to slower growth and slowing growth leads to reduced NTL8 dilution, thus inducing VIN3 expression [36]. Therefore, there may be no specific sensor serving as vernalization inducer.

Alternative splicing mediation during cold exposure

Temperature has always been considered a key factor in regulating alternative splicing. Recent research found that plants would occur a rapid and dynamic AS mediation in response to short-term cold [70]. With the aim of exploring AS regulation under long-term cold, we explored the overall trend of change and the regulation of individual genes during 42 days cold treatment. It is found that the AS had been responding through the entire cold exposure. From an overall perspective, proportion of five mechanisms changed during the cold exposure in order to generate more functional transcripts, thus enhancing the cold tolerance more efficiently. From the perspective of particular genes, some of them which total expression has not changed may be greatly up-regulated in a single transcript. In general, genes in cold-acclimation and vernalization respond to cold mainly by three ways: ratio change of different transcripts,

functional transcript induction, and synergistic work of all transcripts, which enable plants addressing environmental signal more delicately and efficiently.

Conclusions

Transcriptomic analysis revealed that Cold-acclimation and vernalization are independent. Cold as winter signal either indirectly suppress flowering through cold-acclimation involved photoperiod and GA pathways or directly suppress flowering through thermosensory pathway. Additionally, BR was found as the most sensitive hormone to cold shock and phosphorylation played an important role in long-term cold shock. Alternative splicing mechanism regulated multiple genes' expression including *CNGC5*, *CNGC6*, *CIPK3*, *ICE1*, *COR15A*, *COR27*, *BES1*, *EIN3*, *RAB8*, *PHYA*, *PHYB*, *CRY2*, *PRR7*, *PRR9*, *VRN5*, *VAL2*, *ATX1*, *ATX2* during the whole vernalization process.

Methods

Plant materials and growth conditions

A functional *FRI* locus from the Sf2 line (CS6209) was introgressed into Col to construct *FRI*-Col by the R. Amasino lab [71], and the *FRI*-Col seeds were provided by Dr. Yuehui He (Shanghai Center for Plant Stress Biology, Chinese Academy of Sciences). Seeds were surface-sterilized by 75% ethyl alcohol for 1 min, 10% sodium hypochlorite for 15mins, washed 6 times by sterile water, and stratified at 4°C for 2 days before being sown on 1/2MS medium. Seedlings were grown in growth chambers at 22 °C under 16/8 h day/light periods for about 2 weeks (two true leaves grew out) and then harvested as T0h. Rest of them transferred to 8/16 h day/light period under 4°C condition for 30 mins (T0.5h), 1 day (T1d), 14 days (T14d), 28 days (T28d) then harvested. 29 d samples experienced one day 22°C recovery (T29d), 30 d samples was exposed 1 d cold following the recovery (T30d). 42 d samples experienced additional 14 days 4°C treatment under short-days condition then harvested.

RNA extraction and RNA-Seq library construction

Total RNA was extracted using RNAiso Plus (TaKaRa). Total RNA is processed by mRNA enrichment method or rRNA removal method. mRNA enrichment: Enrich the mRNA with polyA tail using magnetic beads with OligodT. Add appropriate amount of interruption reagent to the obtained mRNA to fragment it under high temperature conditions, use the interrupted mRNA as a template to synthesize the first-strand cDNA, and then configure the two-strand synthesis reaction system to synthesize the second-strand cDNA, and use the kit to purify the recovered End repair, add a base "A" to the 3'end of the cDNA and connect the linker, then select the size of the fragment, and finally perform PCR amplification; the quality of the constructed library is checked and sequenced after passing.

Different expression genes and different alternative splicing genes analysis

High-throughput sequencing was performed using the BGISEQ-500 platform. After several data processing steps (including removal of adaptor sequences, null reads, and low-quality reads), pure reads were obtained from the original sequence. After getting clean reads, we use HISAT to align clean reads to the reference genome sequence (GCF_000001735.4_TAIR10.1). Bowtie2 [73] was used to align Clean reads to the reference gene sequence, and then use RSEM [74] calculate the gene expression level of each sample.

rMATS was used to detect differential splicing genes between different samples and splicing events of the samples themselves. rMATS is a software for differential alternative splicing analysis of RNA-Seq data. It uses the rMATS statistical model to quantify the expression of alternative splicing events for different samples, and then calculates P value with a likelihood-ratio test to indicate that the two groups of samples are in IncLevel (Inclusion Level) The difference in level, IncLevel and using Benjamini Hochberg algorithm to correct the p value to obtain the FDR value.

qRT-PCR of DEGs

Reliability of DE genes or transcripts identified through RNA-seq was evaluated through qRT-PCR analysis of FLC, VIN3, CBF1, CBF2, MAF1, SOC1. The Eppendorf Mastercycler Ep Realplex 2S device (Hamburg, Germany) fluorescence quantifier was applied with 2 × SYBR Green qPCR Master Mix (Bimake) operating instructions. The specific system is: SYBR® Premix Ex Taq™ II 10.0μl, Forward primer (10μM) 1.0 μl, Reverse primer (10μM) 1.0μl, cDNA template 5.0 μl, ddH₂O 3.0μl. The specific reaction procedure is: 95°C 10 min, 95°C 15 s, 55°C 15 s, 72°C 20 s, for 40 cycles; finally insert the dissolution curve program.

Abbreviations

Not applicable

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated during the current study are supposed to submit to the NCBI repository.

(We are sorry that we failed to upload the clean data because of the server connection, we will try to acquire the accession number as soon as possible.)

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors have read and approved the manuscript. F. L., Q.H. and J.J designed the experiments. Q.H. and J.J prepared the samples for RNA-seq. F. L. analyzed the data. F. L. and J.J wrote the manuscript. F.C and J.J revised the manuscript.

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Figures

Figure 1

Comparison between short-term and long-term of cold responding (A) Bar graph showing total numbers of differentially upregulated (orange) and downregulated (green) genes of T0h VS 0.5h, 1d, 14d, 28d, 29d, 30d, 42d. (B) Venn diagram showing common genes and unique genes in group T0h Vs T0.5h, T0h Vs T1d, T28d Vs T29d, T29 Vs T30d and group T0h Vs T14d, T0h Vs T28d, T0h Vs T42d. (C-D) KEGG terms of short-term cold responding and long-term cold responding (E-F) KEGG pathway maps of short-term cold responding and long-term cold responding

Figure 2

Time-course analysis of dynamic expression change during vernalization (A-H) Clustering was performed using the time course analysis software Mfuzz [72]. Shown on the left were heat maps that expression level of genes in cluster1-8 were normalized to Log2, red and blue colors represent up- and down-regulated genes in heat map, respectively. Shown on the right were Gene ontology (GO) terms of each cluster.

Figure 3

Different expression pattern of *FLC*, *VIN3*, *CBF1* and heatmap of genes in vernalization and cold-acclimation pathway. (A) Line chart showing expression pattern of *FLC*, *VIN3*, *CBF1* in whole vernalization process as in Fragments Per Kilobase per Million (FPKM). Data were normalized to $\text{Log}_2(\text{Value}+1)$. (B, C) Data for gene expression level were normalized to Log_2 , Red and blue colors represent up- and down-regulated genes, respectively.

Figure 4

Heatmap of genes in flowering pathways. (A-F) Data for gene expression level were normalized to Log₂, Red and blue colors represent up- and down- regulated genes, respectively.

Figure 5

Alternative splicing of some DEG during cold exposure (A) Bar graph showing total numbers of differentially alternative splicing genes of five types of mechanisms (skipped exon, mutually exclusive exons, alternative 3'/5'splicing site and retained intron in 0.5h, 1d, 14d, 28d, 29d, 30d, 42d. (B) Line charts showing expression pattern of different transcripts of selected genes.

Figure 6

RT-qPCR validation of FLC, VIN3, CBF1, CBF2, MAF1, SOC1 at different time points ($P < 0.05$).

Figure 7

A working model that how cold-acclimation affect vernalization

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