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ORIGINAL ARTICLE

Transcript Profiles Differentiate Cold Acclimation-Induced Processes in a Summer and Winter Biotype of Camelina

Hongxia Wang[1] · Münevver Doğramacı[1] · James V. Anderson[1] · David P. Horvath[1] · Wun S. Chao[1]

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Abstract
Camelina (*Camelina sativa* L. Crantz) is a short-season oilseed crop of the *Brassicaceae* family that consists of both summer and winter annual biotypes. Winter biotypes require non-freezing cold conditions for acquiring freezing tolerance (cold acclimation) and floral initiation (vernalization). Transcriptome profiles of a summer (CO46) biotype with poor freezing tolerance after acclimation and a winter (Joelle) biotype with excellent freezing tolerance after acclimation were compared prior to and after an 8-week cold treatment to identify key molecular pathways and genes responsive to cold acclimation and vernalization and potentially associated with freezing tolerance. Gene-set enrichment analyses identified AraCyc pathways involved in photosynthesis and lipid and hormone biosynthesis that were different between the two biotypes. Sub-network enrichment analyses identified hubs of molecular networks such as circadian clock, flowering, and hormone and stress responsive genes that were likely involved in vernalization but may also overlap with cold-induced freezing tolerance. A microRNA involved in floral initiation (MIR172A) was identified as a central hub for microRNA targets among upregulated genes for Joelle post-acclimation. Combined results are generally consistent with many previously identified molecular pathways and genes acting together to control vernalization, cold acclimation, and freezing tolerance. Our research provides new insights into the regulation of cold acclimation and molecular genetic mechanisms underlying cold tolerance and floral induction for the winter biotype Joelle.

Keywords Camelina · Cold acclimation · RNAseq · Vernalization

Introduction

Camelina (*Camelina sativa* L. Crantz) is a short-season oilseed crop in the *Brassicaceae* family that can grow under water-limited environments (Al-Shehbaz et al. 2006; Berti et al. 2016; Obour et al. 2018). Both summer- and winter-annual Camelina biotypes have been identified, and winter biotypes require vernalization for flower initiation (Mirek 1980; Anderson et al. 2018; Gesch et al. 2018; Wittenberg et al. 2019). Summer- and winter-annual biotypes are different in leaf and seed morphology (Wittenberg et al. 2019) and can be accurately (100%) distinguished using allele-specific markers for the floral development gene *FLC* (Chao et al. 2019). Winter biotypes are freezing tolerant and have been used in double or relay cropping systems in the Upper Midwestern United States (Gesch and Archer 2013). Camelina is also beneficial as a cover crop to ease soil erosion, suppress springtime weed growth, reduce nutrients (nitrogen and phosphorus) leaching and runoff, and enhance habitat for pollinators (Eberle et al. 2015; Berti et al. 2017).

Camelina is closely related to the model flowering plant Arabidopsis (*Arabidopsis thaliana*) (Al-Shehbaz et al. 2006; Berti et al. 2016). The reference genome of Camelina is similar to the Arabidopsis genome but is more complex due to its hexaploid genome (Kagale et al. 2014; Berti et al. 2016). Since

Key message
- Transcriptome profiles for winter and summer biotypes of Camelina provided evidence for induction of freezing tolerance and vernalization.
- Molecular pathways involved in photosynthesis; fatty acid and hormone biosynthesis; differentially regulated genes such as *VIN3*, *FLC*, *LHY*, and *LEA14*; and microRNA *MIR172A* have various roles in vernalization, cold acclimation, and potentially freezing tolerance.

✉ Wun S. Chao
wun.chao@usda.gov

[1] Edward T. Schafer Agricultural Research Center, Sunflower and Plant Biology Research Unit, USDA-Agricultural Research Service, 1616 Albrecht Blvd. N, Fargo, ND 58102-2765, USA

a high degree of synteny is observed between Camelina and Arabidopsis, the annotated Camelina genes are proposed to play similar roles as that of Arabidopsis (Kagale et al. 2014; Berti et al. 2016).

Winter biotypes of Camelina must cold acclimate to survive freezing temperatures during the winter (Horvath et al. 2019). Cold acclimation in plant species is partially mediated by the C-repeat binding factor (CBF) regulon, and the expression of *CBF* genes is regulated by low temperature, circadian clock, light quality, and photoperiod, among other signals (Liu et al. 2019). For example, low temperature quickly induces *CBF* genes during cold acclimation (Thomashow 1999). Circadian clock proteins CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) bind to *CBF* promoters directly and positively regulate *CBF* expression (Dong et al. 2011), whereas pseudo-response regulators (PRR5/7/9) repress *CBF* expression by inhibiting the expression of *CCA1* and *LHY* (Nakamichi et al. 2009, 2010). CBFs regulate the expression of cold responsive (*COR*) genes (Lee et al. 2005). Many COR proteins such as late embryogenesis abundant proteins, transcription factors, protein kinases, proteins for hormone responses, and chloroplast proteins play critical roles in regulating cold acclimation and subsequent freezing tolerance (Zhao et al. 2016; Liu et al. 2019). Besides the CBF-mediated pathway, the expression of *COR* genes is also regulated by several CBF-independent pathways including abscisic acid signaling in Arabidopsis to control cold acclimation and freezing tolerance (Chinnusamy et al. 2006; Park et al. 2015; Liu et al. 2019).

After acclimation, the winter biotype Joelle has significantly greater survival rates following exposure to freezing temperatures than the summer biotype CO46 (Horvath et al. 2019). Genetic analysis of freezing tolerance suggested that survival is regulated by as few as two dominant genes (Horvath et al. 2019). However, both biotypes show elevated *CBF* gene expression following acclimation (Horvath et al. 2019; Anderson et al. 2018). In this study, we compare the transcriptome profiles of CO46 and Joelle pre- and post-acclimation to identify key signal pathways and molecular components for cold acclimation and vernalization. Many key pathways and genes were differentially regulated between Joelle and CO46.

Materials and Methods

Plant Growth and Cold Acclimation

Seeds of a winter (Joelle) and a summer (CO46) biotype of Camelina were obtained from the USDA-ARS Laboratory in Morris, MN, USA, following several cycles of field production. The USDA-ARS in Morris originally obtained the seeds from the North Dakota State University Extension Center, Carrington, ND, in 2007 (Anderson et al. 2018). Seeds were grown to the 3–4 leaf stage and cold acclimated (4 °C with 8/16 h light:dark for 8 weeks) as previously described by Anderson et al. (2018). For the winter biotype "Joelle," previous reports indicated that 8 weeks of cold acclimation provided maximum flowering potential (Anderson et al. 2018) and freezing tolerance (Horvath et al. 2019). Plant tissues including meristem and young leaves were collected from plants exposed to 0 or 8 weeks of cold acclimation (pre- and post-acclimation, respectively), flash frozen in liquid nitrogen, and stored at −80 °C until RNA extraction.

RNAseq Analysis

RNA samples of Joelle and CO46 were used to prepare stranded RNAseq libraries as described for Illumina next-generation sequencing (Anderson et al. 2018). Briefly, twelve RNAseq libraries (i.e., 2 Camelina biotypes × 2 treatments × 3 replicate plants) were prepared, and sequencing was performed on the HiSeq2500 platform. Fastq files were generated and demultiplexed with the bcl2fastq v1.8.4 Conversion Software (Illumina). Trimmed and filtered RNAseq reads were mapped to the *C. sativa* (DH55; a doubled haploid line derived from *C. sativa* genotype SRS933) reference genome (Kagale et al. 2014). All sequences were deposited into NCBI BioProject ID = PRJNA292793, and the details of RNAseq read mapping, quantification, and differential gene expression analysis were described and published previously by Anderson et al. (2018). To validate RNAseq results, a heat map including 21 stably and differentially expressed genes was generated to compare RNAseq and RT-qPCR results (Additional Table S1). These genes were selected and previously characterized to determine reference genes for RT-qPCR in Camelina (Chao et al. 2019). The results confirmed that transcripts of stably expressed genes identified from RNAseq (primer numbers 1–19) were generally stably expressed for RT-qPCR as well; in contrast, transcripts of two differentially expressed genes (*FLC* and *SOC1*) exhibited similar differential expression patterns between RNAseq and RT-qPCR. This study was consistent with our earlier findings that transcript abundance generated by RNAseq and RT-qPCR was very similar (Chao et al. 2016; 2017).

Bioinformatics Analysis

A dendrogram of hierarchical grouping between pre- and post-acclimated sample replicates was produced using the Ward method (Murtagh and Legendre 2014). Pathway Studio software (http://www.ariadnegenomics.com) and AGI

designations for Arabidopsis genes were used for Gene Set Enrichment Analysis (GSEA) of AraCyc pathways (Mueller et al. 2003; Subramanian et al. 2005) and for Sub-Network Enrichment Analysis (SNEA) (Yuryev et al. 2006). GSEA is a statistical method to determine if predefined sets of genes are over-represented between treatments, and AraCyc pathways describe the metabolic pathways for Arabidopsis while putting genes and enzymes within their metabolic framework (Mueller et al. 2003 and http://pmn.plantcyc.org/ARA/class-instances?object=Pathways). GSEA identified significantly over-represented ($P < 0.05$) up- and downregulated AraCyc pathways in (1) Joelle vs. CO46 pre-acclimation (Jo-pre vs. CO-pre), (2) Joelle vs. CO46 post-acclimation (Jo-post vs. CO-post), (3) Joelle post-acclimation vs. Joelle pre-acclimation (Jo-post vs. Jo-pre), and (4) CO46 post-acclimation vs. CO46 pre-acclimation (CO-post vs. CO-pre) (Additional Table S2); Table 1 only includes pathways highlighted in the text of this paper. SNEA uses regulatory and interacting network relationships to assist in interpretation of experimental data and development of new hypotheses. The pathway studio program has a database of regulatory genes and microRNA and a database of targets for each regulator drawn from the literature. The program uses Fischer's exact test to determine if those targets are over-represented in the list of genes provided to the program (Yuryev et al. 2006; Daraselia et al. 2012). Thus, SNEA reveals hubs of signaling pathways over-represented in the above comparison datasets (Table 2; Additional Table S3).

Results

Heat Map and Cluster Analysis of Transcript Profiles for Pre- and Post-acclimated Leaf Samples in CO46 and Joelle

Transcript profiles for pre- and post-acclimated leaf samples in two biotypes of Camelina, CO46 and Joelle, identified 22,157 non-redundant genes with fragments per kilobase of transcript per million mapped reads (FPKM) values ≥ 5 in all replicates of at least one treatment (Additional Table S4; Anderson et al. 2018). Of those genes, 18,344 were considered "good" (FPKM ≥ 5 for all three replicates of any single treatment) and were used for cluster analysis (Fig. 1) to reveal similarities and differences of the molecular states among these samples. Cluster analysis grouped 3 replicates of Joelle and CO46 pre-acclimation together and likewise grouped post-acclimated samples together. Thus, although CO46 was flowering competent pre-acclimation but Joelle was not, and Joelle was considerably more freezing tolerant than CO46 post-acclimation, cluster analysis demonstrated that treatment effects were more prominent than biotype effects.

Gene Set and Sub-network Enrichment Analysis

Previously, transcriptome profiles of Joelle and CO46 were compared prior to and after an 8-week cold treatment, and the results of differential gene expression analysis

Table 1 Gene set enrichment analysis. Over-represented AraCyc pathways associated with acclimation and freezing tolerance were listed for comparisons of Jo-pre vs. CO-pre, Jo-post vs. CO-post, Jo-post vs. Jo-pre, and CO-post vs. CO-pre. Up and down arrows indicate the regulation level in the former part of the comparison (i.e., Jo-pre vs. CO-pre: up means up in Jo-pre)

AraCyc pathways	Jo-pre vs. CO-pre		Jo-post vs. CO-post		Jo-post vs. Jo-pre		CO-post vs. CO-pre	
Calvin cycle	↑							
Cholesterol biosynthesis					↑			
Choline biosynthesis I						↓		
Ethylene biosynthesis from methionine				↓				
Galactose degradation I	↑							
Galactose degradation II	↑							
Jasmonic acid biosynthesis				↓		↓		↓
Oleate biosynthesis I (plants)			↑					
Phosphatidylcholine biosynthesis I							↑	
Phosphatidylcholine biosynthesis IV				↓				
Phospholipases							↑	
Photosynthesis	↑							↓
Salicylic acid biosynthesis					↑		↑	
Sphingolipid biosynthesis (plants)			↑	↓	↑			
Starch degradation	↑		↑					
Sterol biosynthesis					↑			
Sucrose biosynthesis	↑				↑		↑	
Sucrose degradation					↑			
Superpathway of starch degradation to pyruvate	↑							
Superpathway of sucrose and starch metabolism II	↑							
UDP-sugars interconversion	↑							

Table 2 Sub-network enrichment analyses. Expression targets and miRNAs identified as central hubs for comparisons of Jo-pre vs. CO-pre, Jo-post vs. CO-post, Jo-post vs. Jo-pre, and CO-post vs. CO-pre

	Expression targets_up	Expression targets_down	MiRNA targets_up	MiRNA targets_down
Jo-pre vs. Co-pre	Calmodulin, DREB2C, Myb- factors, PHR1, DREB2A, MAX2, CBF, CCA1	EREBP, ZTL, PRR5, ELF4, PCL1, PRR9, PRR7, EIN2, ERF6, BRM, ERF-1, MPK4, photoreceptor, ELF3	MIR172A	MIR172B, MIR396A
Jo-post vs. Co-post	CBF1, PRR9, DREB2A, HOS1, XRN4, EREBP, MPK6, AP3, PI, EIN2	ACD6, ETR1, AP2, ERF6, WRKY70, BES1, AvrRpm1	MIR172A	MIR159A
Jo-post vs. Jo-pre	ZTL, ATAN11, CBF1, DREB2A, DREB1A, RPK1, CBF, basic-helix-loop-helix protein, histone H3, TOC1, PRR5, ELF4, PCL1, PLC, SKB1, CCA1	BRI1, SIZ1, COI1	MIR172A	ath-miR837
Co-post vs. Co-pre	CBF1, CBF, RPK1, DREB1A, DREB2A, histone H3, PRR5, ELF4, PCL1, ATAN11, ZTL, CBL1, basic-helix-loop-helix protein, PLC, ERF6, EREBP, WRKY70, ABA1, ERF4, ETR1, TOC1, SKB1, LHY, MEKK1, EIN2, MPK3, ELF3, CCA1	CCA1, BES1	MIR169B	

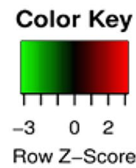
were reported (Anderson et al. 2018; Horvath et al. 2019). The data were used to study the impact of vernalization on abundance of key transcripts involved in floral regulation pathways (Anderson et al. 2018) and to analyze COR genes and the promoters of coordinately expressed gene clusters (Horvath et al. 2019). In this study, we specifically applied GSEA and SNEA to identify key signal pathways and molecular components for cold acclimation and vernalization in CO46 and Joelle pre- and post-acclimation. Of the 22,157 non-redundant genes (FPKM ≥ 5), 21,659 had TAIR ID; thus, 21,659 genes were used for GSEA and SNEA studies (Additional Table S4).

Joelle vs. CO46 Pre-acclimation Among genes upregulated in Joelle, 29 pathways were over-represented (Additional Table S2). Half of the pathways were biosynthetic pathways including ABA glucose ester, ajugose, ascorbate, coumarin, cytokinins, flavonoid, galactosylcyclitol, kaempferol, monolignol, pelargonidin, and quercetin glucoside, sucrose, UDP-glucose, and xylan biosynthesis. The rest of the pathways included Calvin cycle, galactose degradations I and II, lysine degradation II, photosynthesis, starch degradation, sucrose degradation to ethanol and lactate, starch degradation to pyruvate, sucrose and starch metabolism II, triacylglycerol degradation, and UDP-sugars interconversion. The biosynthetic pathways may imply that Joelle was more active in generating those biosynthetic products compared to CO46 prior to acclimation treatment.

Among genes downregulated in Joelle, 13 pathways were over-represented. Many of the biosynthetic pathways were involved in glucosinolate, myo-inositol, superpathway of pantothenate, coenzyme A, and polyamine biosynthesis. The rest of the pathways included nitrate assimilation pathway, phenylalanine degradation, sulfate reduction, and tyrosine degradation. One pathway, IAA degradation, was represented in both up- and downregulated gene sets.

SNEA of genes upregulated in Joelle showed over-representation of expression targets for central hubs including calmodulin, *C-REPEAT BINDING FACTOR* (*CBF*), *CIRCADIAN CLOCK-ASSOCIATED 1* (*CCA1*), *DROUGHT RESPONSE ELEMENT BINDING FACTOR 2A* and *2C* (*DREB2A* and *DREB2C*), *MORE AXILLARY GROWTH 2* (*MAX2*), *MYB* (*Myeloblastosis*)-factors, and *PHOSPHATE STARVATION RESPONSE 1* (*PHR1*). In addition, MIR172A was identified as a central hub for miRNA targets among upregulated genes (Table 2; Additional Table S3).

SNEA of downregulated genes in Joelle identified *BRAHMA* (*BRM*); *ETHYLENE INSENSITIVE2* (*EIN2*); *EARLY FLOWERING 3* and *4* (*ELF3* and *ELF4*); *ETHYLENE-RESPONSIVE ELEMENT-BINDING PROTEIN* (*EREBP*); *ETHYLENE RESPONSE FACTOR* 1 and 6 (*ERF-1* and *ERF6*); *MITOGEN-ACTIVATED PROTEIN KINASE 4* (*MPK4*); *PEROXY-CAGED LUCIFERIN 1* (*PCL1*); photoreceptor; *PSEUDO RESPONSE REGULATOR 5*, *7*, and *9* (*PRR5*, *7*, and *9*); and *ZEITLUPE* (*ZTL*) as central hubs for expression targets. MIR172B and MIR396A were identified as central hubs for miRNA targets among downregulated genes. Many of these hubs are associated with ethylene signaling/regulation and circadian clock in plant development and under stress conditions (Lewandowska-Sabat et al. 2012).



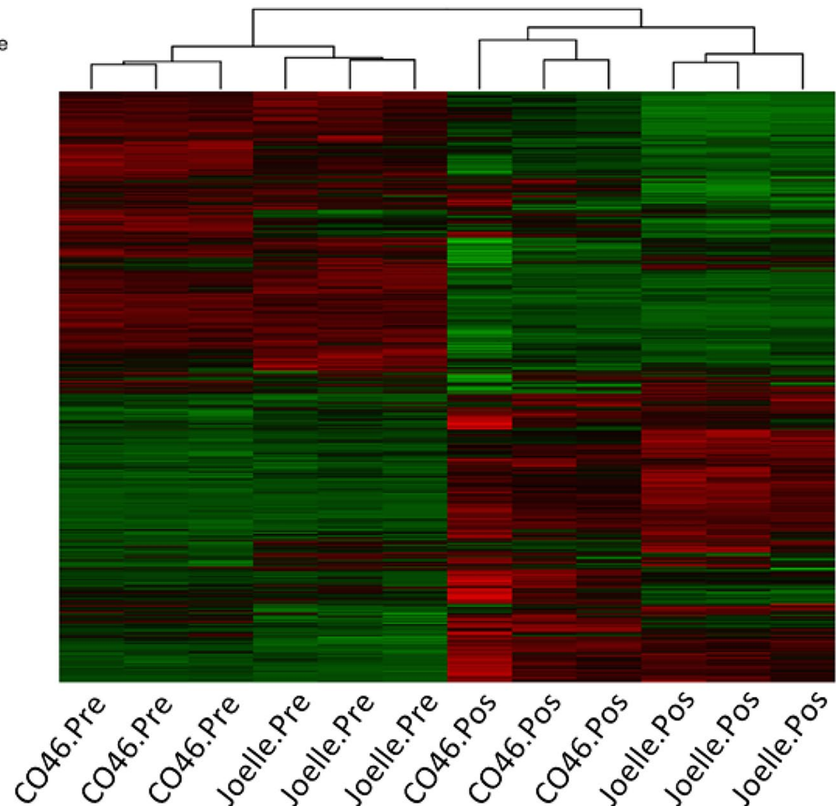


Fig. 1 Heat map and cluster analysis based on principal component analysis ($P < 0.05$) using 18,344 genes from RNAseq analysis with FPKM ≥ 5 in all replicates of at least one treatment. The standard score is indicated in the color key at the top left, where positive values are red, means are black, and negative values are green

Joelle vs. CO46 Post-acclimation and Vernalization Among genes up regulated in Joelle, 21 pathways were over-represented (Additional Table S2), of which only five were uniquely over-represented in this gene set. These pathways are glutathione redox reactions, oleate, and stachyose biosynthesis, starch degradation, and superpathway of citrulline metabolism. The oleate biosynthesis pathway may be involved in cold acclimation (Miquel et al. 1993; Martz et al. 2006).

Among genes downregulated in Joelle, 27 pathways were over-represented (Additional Table S2). Eleven of these pathways unique to this gene set are ammonia assimilation cycle, ethylene biosynthesis from methionine, homogalacturonan degradation, jasmonic acid biosynthesis, methionine salvage pathway, nitrate assimilation pathway, phosphatidylcholine biosynthesis, suberin biosynthesis, superpathway of phosphatidylcholine biosynthesis, tetrahydrofolate biosynthesis, and triacylglycerol degradation. The downregulation of ethylene biosynthesis from methionine correlated well with an increase in freezing tolerance for Joelle post-acclimation (Shi et al. 2012).

Sixteen pathways were over-represented among up- and downregulated gene sets. Twelve of the 16 pathways are involved in the following biosynthetic processes: abscisic acid glucose ester, ajugose, cellulose, cytokinins, galactosylcyclitol, kaempferol glucoside, monolignol glucoside, pelargonidin conjugates, quercetin glucosides, sphingolipid, anthocyanin, and xylan biosynthesis.

SNEA of genes upregulated in Joelle showed over-representation of expression targets for central hubs including *APETELA 3* (*AP3*), *CBF1*, *DREB2A*, *EIN2*, *EREBP*, *HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1* (*HOS1*), *MITOGEN ACTIVATED PROTEIN KINASE 6* (*MPK6*), *PISTILLATA* (*PI*), *PRR9*, and *EXORIBONUCLEASE 4* (*XRN4*). In addition, MIR172A was identified as a central hub for miRNA targets among upregulated genes (Table 2; Additional Table S3).

SNEA of downregulated genes identified *ACCELERATED CELL DEATH 6* (*ACD6*), *APETELA 2* (*AP2*), *AVIRULENCE RESISTANCE TO P. SYRINGAE PV MACULICOLA 1* (AvrRpm1), *BRASSINOSTERIOD INSENSITIVE1 EMS SUPPRESSOR 1* (*BES1*), *ERF6*, *ETHYLENE RESPONSE 1* (*ETR1*), and *WRKY70* as central hubs for expression targets. In addition, MIR159A was identified as a central hub for miRNA targets among downregulated genes. Many of these hubs are associated with hormone and stress signaling pathways.

Joelle Post- vs. Joelle Pre-acclimation Among genes up regulated in Joelle post-acclimation, 31 pathways were over-represented (Additional Table S2). Eleven of the 31 pathways are involved in the following biosynthetic processes: beta-alanine, cholesterol, epoxysqualene, homomethionine, IAA, salicylic acid, sphingolipid, sterol, sucrose, anthocyanin, and xylan biosynthesis. The rest of the pathways included fatty acid alpha- and omega- oxidation, glutathione redox reactions, oxidative ethanol degradation, and sucrose degradation. Among these pathways, sphingolipids and sterols are major membrane lipids and play important roles in regulating plant growth and development, as well as cold acclimation (Mishra et al. 2015; Valitova et al. 2016; Huby et al. 2020).

Many pathways over-represented among downregulated genes were also biosynthetic processes, and they were involved in cellulose, chlorophyllide a, choline, chorismate, coumarin, pyrimidine deoxyribonucleotide, glucosinolate, jasmonic acid, lysine, phenylpropanoid, threonine, methionine, proto- and siroheme, and UDP-glucose biosynthesis. The rest of the pathways included branched-chain alpha-keto acid dehydrogenase complex, cyanate degradation, homogalacturonan degradation, purine nucleotide metabolism, ribose degradation, superpathway of ribose and deoxyribose phosphate degradation, and triacylglycerol degradation pathways.

Pathways over-represented in both the up- and downregulated gene sets include abscisic acid glucose ester, ajugose, cytokinins, galactosylcyclitol, kaempferol glucoside, monolignol glucoside, pelargonidin conjugates, quercetin glucosides, suberin biosynthesis, and degradation pathways of IAA and phenylalanine.

SNEA of upregulated genes identified central hubs for expression targets (Table 2; Additional Table S3). These central hubs included *ANTHOCYANIN11* (*ATAN11*), basic helix-loop-helix protein, *CBF*, *CBF1*, *CCA1*, *DREB1A*, *DREB2A*, *ELF4*, histone H3, *PCL1*, *PHOSPHOLIPASE C* (*PLC*), *PRR5*, *RECEPTOR-LIKE PROTEIN KINASE1* (*RPK1*), *SHK1 KINASE-BINDING PROTEIN 1* (*SKB1*), *TIMING OF CAB 1* (*TOC1*), and *ZTL*. In addition, MIR172A was identified as a central hub for miRNA targets among upregulated genes.

SNEA of downregulated genes identified *BRASSINOSTEROID INSENSITIVE 1* (*BRI1*), *CORONATINE-INSENSITIVE 1* (*COI1*), and *SAP AND MIZ1 DOMAIN-CONTAINING LIGASE1* (*SIZ1*) as central hubs for expression targets. SIZ1 is involved in regulating SA accumulation and defense-related gene expression (Cheong et al. 2009; Miura and Ohta 2010). Ath-miR837 was identified as a central hub for miRNA targets among downregulated genes.

CO46 Post- vs. CO46 Pre-acclimation Thirty-five pathways were over-represented by genes upregulated in CO46 post-acclimation (Additional Table S2). These pathways include abscisic acid glucose ester, ajugose, aliphatic glucosinolate, beta-alanine, cytokinins, flavonoid, galactosylcyclitol, homomethionine, kaempferol glucoside, leucodelphinidin, monolignol glucoside, pelargonidin conjugate, phenylethanol, phosphatidylcholine, quercetin glucoside, salicylic acid, simple coumarin, suberin, sucrose, superpathway of anthocyanin, and xylan biosynthesis. Additional pathways included ethanol degradation, fatty acid alpha and omega oxidation, glutathione redox reactions, IAA degradation, oxidative ethanol degradation, phenylalanine degradation, phospholipases, pyruvate fermentation to ethanol, and sulfate activation for sulfonation.

Among genes downregulated in CO46 post-acclimation, over-represented pathways were involved in arginine, chlorophyllide a, chorismate, cutin, purine nucleotide, pyrimidine deoxyribonucleotide, pyrimidine ribonucleotide, uridine-5′-monophosphate, glucosinolate, heme, isoleucine, jasmonic acid, lysine, superpathway of isoleucine, leucine, and valine, superpathway of proto- and siroheme, tetrapyrrole, triacylglycerol, and valine biosynthesis. Additional pathways included branched-chain alpha-keto acid dehydrogenase complex, leucine degradation, photosynthesis, purine nucleotide metabolism, ribose degradation, superpathway of ribose and deoxyribose phosphate degradation, triacylglycerol degradation, tRNA charging pathway, and Urea cycle. Only one pathway (photosynthesis-light reaction) was over-represented in both up- and downregulated gene sets.

Interestingly, all 16 central hubs for expression targets identified in SNEA of upregulated genes in Joelle post- vs. pre-acclimation were also identified in SNEA of upregulated genes in CO46 post- vs. pre-acclimation. Besides these 16 hubs, 12 additional hubs including *ABA DEFICIENT 1* (*ABA1*), *CALCINEURIN B-LIKE 1* (*CBL1*), *EIN2*, *ELF3*, *EREBP*, *ERF4*, *ERF6*, *ETR1*, *LATE ELONGATED HYPOCOTYL (LHY)*, *MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 1* (*MEKK1*), *MITOGEN-ACTIVATED PROTEIN KINASE 3* (*MPK3*), and WRKY70 were identified. The only miRNA target among upregulated genes was MIR169B. SNEA of downregulated genes identified *BES1* and *CCA1* as central hubs for expression targets. No miRNA targets were identified among downregulated genes (Table 2; Additional Table S3).

Transcript Abundance of Some Key Regulatory Genes During Acclimation

Flowering, Circadian Clock, and CBF Genes Some key flowering and circadian genes (Table 3; Additional Table S5) including *EARLY FLOWERING 4* (*ELF4*), *FLC*, and *JUMONJI DOMAIN CONTAINING 5* (*JMJD5*) had decreased transcript abundance post-acclimation for both biotypes. In contrast, other floral regulating genes such as

SOC1, *SHORT VEGETATIVE PHASE* (*SVP*), and *VIN3* had increased transcript abundance post-acclimation in both biotypes, but their transcript abundance was considerably greater in Joelle than that of CO46. Interestingly, although two *SOC1* paralogous genes were upregulated dramatically post-acclimation for Joelle, the abundance of *SOC1* transcript was much greater in CO46 than that of Joelle pre-acclimation. Because the summer biotype CO46 flowers without a vernalizing cold treatment (Anderson et al. 2018; Chao et al. 2019), these results were consistent with SOC1's known function as a flowering activator (Richter et al. 2019).

In this study, transcript abundance of three key components of circadian clock *CCA1*, *LHY*, and *PRR9* increased dramatically post-acclimation for both biotypes. However, *CCA1* and PRR9 transcript abundance in Joelle was considerably

Table 3 Fold changes of transcription abundance for some flowering, circadian clock, and stress-responsive genes. Some of these genes are floral regulators, and their involvement in floral regulation pathways were discussed in Anderson et al. (2018). Here "Fold changes" are determined by dividing the FRKM value of one physiological condition with the FRKM value of another physiological condition and represented by positive or negative fold numbers (e.g., Jo-pre vs. CO-pre: the numerator is Jo-pre and the denominator is CO-pre for positive fold numbers, but the numerator is CO-pre and the denominator is Jo-pre for negative fold numbers)

Process	Gene	TAIR ID	Feature ID	Fold changes			
				Jo-pre vs. CO-pre	Jo-post vs. CO-post	Jo-post vs. Jo-pre	CO-post vs CO-pre.
Flowering and circadian clock	*ELF4*	AT2G40080	Csa04g054280.1	1.2	-1.5	-12.8	-7
			Csa05g012830.1	1.5	-1.2	-10.8	-5.8
			Csa06g044320.1	1.5	-1.4	-9.7	-4.5
	FLC	AT5G10140	Csa08g054450.1	-1.2	-2.7	-41.1	-17.8
			Csa20g015400.1	11.9	2.1	-127.7	-22.2
	JMJD5	AT3G20810	Csa01g023880.1	3.3	-0.9	-8.2	-2.3
			Csa15g031170.1	2.8	-0.9	-5.6	-1.9
			Csa19g028780.1	2.9	-1.3	-6.9	-1.9
	SOC1	AT2G45660	Csa04g063650.1	-1.4	-4.8	1.3	4.3
			Csa06g052060.1	-16.6	-1.7	31.6	3.2
			Csa08g054450.1	-80.6	-1.5	193.6	3.7
	SVP	AT2G22540	Csa07g052630.1	-2.2	-1.2	3.2	1.7
			Csa09g086860.1	-1.6	-1.1	2.4	1.7
			Csa16g044290.1	-1.4	1.2	3.6	2.2
	VIN3	AT5G57380	Csa02g061850.1	-2.1	1.1	1420.6	626
			Csa11g090940.1	-2.1	1.3	1373.9	507
	CCA1	AT2G46830	Csa04g065960.1	-3	-1.1	54.7	19.2
			Csa05g002330.1	-3.1	1.8	161.7	28.7
			Csa06g053340.1	-3.6	-1.2	97.6	31.8
	LHY	AT1G01060	Csa03g002180.1	-2.4	-1.4	241.7	142.6
			Csa14g002120.1	-1.2	-1.6	202.1	268
			Csa17g001540.1	-1.3	-1.7	102.1	133.2
	PRR1	AT1G32100	Csa14g042020.1	1.4	1.9	5.4	3.9
			Csa17g050430.1	-1.3	-3.9	-15.2	-4.9
	PRR3	AT5G60100	Csa02g066970.1	1.1	-1.1	-3.4	-2.8
			Csa02g065900.1	-1.3	-2.2	8.7	14.5
			Csa18g033380.1	-1	-1.5	-1.1	1.4
	PRR5	AT5G24470	Csa08g018960.1	-1.1	-1.3	1.9	2.3
			Csa13g029150.1	-1.4	-1.5	2.3	2.5
			Csa20g040910.1	-1.2	-1.4	2.6	3.2
	PRR7	AT5G02810	Csa08g061810.1	-1.4	-1.2	5.8	5
			Csa13g002660.1	-1.4	-1.1	5.6	4.2
			Csa20g002850.1	-1.3	-1	5.6	4.4
	PRR9	AT2G46790	Csa04g065920.1	-5.8	1.6	140.8	15
			Csa05g002370.1	-3.9	2.8	102.6	9.2
			Csa06g053300.1	-1.7	1.7	61.2	22.1
CBF pathway	*CBF1 (DREB1B)*	AT4G25490	Csa12g027680.1		1		309.2
	CBF2 (DREB1C)	AT4G25470	Csa11g019080.1	2.6	3.8	13.5	9.2
	CBF3 (DREB1A)	AT4G25480	Csa12g027690.1	1.2	2.8	6.2	2.6
Light	*PIF4*	AT2G43010	Csa04g060610.1	-1.5	-1.0	1.1	-1.3
			Csa05g007450.1	-1.4	1.2	-1.6	-2.6
			Csa06g049200.1	-1.7	1.5	-1.3	-3.2
	PIF5	AT3G59060	Csa04g046570.1	-2.5	1.0	6.6	2.6
			Csa05g089590.1	-1.6	-1.1	3.6	2.4
			Csa06g036750.1	-3.3	1.5	10.5	2.2
	PIF7	AT5G61270	Csa02g068210.1	-1.2	1.5	9.2	5.1
			Csa11g095090.1	-2.0	-1.1	12.0	6.4
			Csa18g034590.1	-2.0	1.2	23.3	9.6
Stress-related	*LEA14*	AT1G01470	Csa03g001760.1	-1.2	4.5	14.9	2.7
			Csa14g001670.1	-1.3	2.9	26.1	6.7
			Csa17g001940.1	-1.7	4.7	48.9	6.3
	ABR	AT3G02480	Csa15g002110.1	-5.9	447.1	1721.2	-1.5
			Csa19g003140.1		19.7		3.9
	WRKY70	AT3G56400	Csa04g042830.1	-1.2	-5.9	-4.1	1.2
			Csa06g031130.1	-1.2	-4.6	-3.7	-1
			Csa09g068700.1	1.1	-3.8	-3.9	1.1

greater relative to CO46 among different paralogues. Transcript abundance varied only slightly for the rest of *PRR*s (*PRR1*, 3, *5*, and *7*) compared with *PRR9* in both Joelle and CO46. The CBF cold response pathway is known to be positively regulated by the circadian clock components *CCA1* and *LHY* (Dong et al. 2011). The transcript abundance of three *CBF* genes, *CBF1*, *2*, and *3* (also known as *DREB1B*, *1C*, and *1A*), increased post-acclimation in both Joelle and CO46. The transcript abundance for *CBF1* was very low in CO46 and unidentifiable in Joelle pre-acclimation.

Light and Stress-Related Genes The transcript abundance of *PIF* genes was examined for Joelle and CO46 pre- and post-acclimation. *PIF5* and *PIF7* transcripts increased moderately in CO46 post-acclimation, while the transcript abundance of these two genes in Joelle increased considerably. *LATE EMBRYOGENESIS ABUNDANT* (*LEA*) *14* increased post-acclimation in both Joelle and CO46. However, transcript abundance for all three paralogues was considerably greater in Joelle compared with CO46 post-acclimation. A similar trend was observed for another *LEA* gene, *ABA RESPONSIVE PROTEIN* (*ABR*). In contrast, the transcript abundance of three stress-related *WRKY70* paralogues decreased specifically in Joelle post-acclimation but was virtually the same for CO46 post-acclimation (Table 3; Additional Table S5).

Discussion

In this study, analytical tools (cluster analysis, GSEA, and SNEA) provided new insights to the physiological states of summer and winter biotypes of Camelina at pre- and post-acclimation. Because the summer and winter biotypes have different freezing tolerance following cold acclimation, the uniquely expressed genes and differentially regulated pathways identified in this study help to differentiate the acclimation-induced molecular mechanisms impacting freezing tolerance in the winter biotype. Additionally, because vernalization occurs during the cold acclimation process, it is possible the vernalization process overlaps with genes and pathways involved in freezing tolerance of the winter biotype.

Photosynthesis and Photoperiod Involvement in Vernalization and Cold Acclimation

Photosynthesis is sensitive to changes in environmental stimuli and is important for balancing the light energy absorbed by the photosystems with the energy used by metabolic sinks such as reduction of CO_2 through the Calvin cycle to form carbohydrates in chloroplasts (Ensminger et al. 2006; Ding et al. 2016, 2017; Fürtauer et al. 2019). In the present study, Calvin cycle and photosynthesis pathways were over-represented among upregulated genes for Joelle in relation to CO46 pre-acclimation. Because increased activity of the Calvin cycle is positively correlated with cold tolerance in Arabidopsis (Koç et al. 2018), we propose that Joelle is predisposed to have a higher capacity for cold tolerance than that of CO46. In transgenic tomato plants exposed to cold treatment and over-expressing a Calvin cycle enzyme (sedoheptulose-1, 7-bisphosphatase), photosynthetic rates and tolerance to chilling-induced oxidative stress increased (Ding et al. 2017), which supports our hypothesis.

AraCyc pathways associated with carbon metabolism (e.g., starch and sugar biosynthesis and degradation) were also over-represented among upregulated genes for Joelle pre-acclimation and/or post-acclimation compared to CO46 (Table 1). The starch degradation pathways identified in this study correlated well with studies in leafy spurge (*Euphorbia esula* L.), where an inverse shift in starch and sucrose during the fall to winter was associated with underground buds acquiring freezing tolerance (Anderson et al. 2005). Similar results were observed by Nagler et al. (2015). These authors found that sucrose levels increased significantly in the leaves of cold tolerant Arabidopsis accession (Rschew) after cold acclimation than that of cold-sensitive accession (Cvi). Interestingly, starch levels were also increased in both accessions after cold acclimation; however, Cvi showed significantly higher starch levels than that of Rschew.

Moreover, photoperiod is involved in regulating cold acclimation and freezing tolerance through the activity of phytochromes and phytochrome interacting factors (PIFs) (Franklin and Quail 2010). PIFs belong to a family of light labile basic helix-loop-helix (bHLH) transcription factors that interact with the phytochromes and act primarily as negative regulators of photomorphogenesis (Pham et al. 2018). PIFs are known to negatively regulate plant freezing tolerance by inhibiting the CBF pathway (Leivar and Quail 2011; Lee and Thomashow 2012; Jiang et al. 2020). In this study, *PIF5* and *PIF7* transcript abundance increased post-acclimation in both Joelle and CO46 but transcript abundance was considerably greater in Joelle relative to CO46 post-acclimation. For example, the fold change for a *PIF7* paralogue (Csa18g034590.1) reached as high as 23-fold in Joelle relative to a tenfold increase in CO46 post-acclimation (Table 3; Additional Table S5). The increased expression of *PIF* genes observed post-acclimation is contradictory to the concurrent increased expression of CBFs (Table 3). However, because PIF4, 5, and 7 are also known to positively regulate flowering (Galvão et al. 2019), it is possible that PIFs have a functional role in vernalization (Jiang et al. 2020), and thus, their induction during extended cold could be a part of the induction of floral competency for Joelle.

Lipid Biosynthetic Pathways May Be Involved in Freezing Tolerance

Plants can improve cold tolerance by increasing the levels of unsaturated fatty acids and membrane lipid fluidity (Upchurch 2008; Nozawa 2011; Theocharis et al. 2012; Fürtauer et al. 2019). In this study, phytosterol biosynthesis pathways were different between Joelle and CO46; cholesterol and sterol biosynthesis were over-represented among upregulated genes specifically for Joelle post-acclimation (Table 1). Sterols are present in three forms, free sterols, steryl esters, and steryl glucosides. Free sterols are integral components of the membrane lipid bilayer and involved in the regulation of membrane fluidity and permeability. Mishra et al. (2015) found that higher content of free sterols and steryl glycosides correlated well with increased freezing tolerance in Arabidopsis after cold acclimation.

The oleate biosynthesis pathway was also over-represented among upregulated genes in Joelle compared with CO46 post acclimation (Table 1). Oleate is a salt or ester of oleic acid (18:1), and oleic acid was required for normal plant growth at low temperature in Arabidopsis *fad2* mutants, which were deficient in the activity of microsomal 18:1 desaturase (Miquel et al. 1993). In *Pinus resinosa* needles, the proportion of oleic acid increased in total glycerolipids extracted from the plasma membrane fraction during cold acclimation and the proportion decreased during deacclimation (Martz et al. 2006). Also, oleic acid increased from 30 to 47% of the total fatty acid pool during rapid cold hardening (8 h at 4 °C) for pharate flesh flies (*Sarcophaga crassipalpis*) (Michaud and Denlinger 2006).

Phosphatidylcholine (PC) biosynthesis I and phospholipases pathways, in contrast, were over-represented among upregulated genes specifically in cold-sensitive biotype CO46 post-acclimation. In addition, phosphatidylcholine biosynthesis IV was over-represented among downregulated genes for Joelle in relation to CO46 post-acclimation. PC is the major phospholipid in higher plants and is the most abundant lipid on the outer chloroplast envelope membrane (32%) (Block et al. 2007). Lipid analysis revealed higher conversions of PC to triacylglycerol (TAG) during cold acclimation in Arabidopsis, and the accumulation of free polyunsaturated fatty acids and TAG during cold acclimation is associated with freezing tolerance (Arisz et al. 2018). The observation that PC biosynthesis pathways were over-represented among upregulation genes post-acclimation in CO46 (not Joelle) appears contradictory to the above supposition that higher PC and TAG levels promote freezing tolerance. However, considering the multiple pathways plants use to acquire freezing tolerance, this result may not be completely unanticipated; the levels of PC and TAG may contribute partially to freezing tolerance. Alternatively, Rajashekar et al. (2006) found that suppression of a phospholipase Dα1 induces freezing tolerance in Arabidopsis. Phospholipase D is one of the four types of phospholipases, and it hydrolyzes PC to produce phosphatidic acid (a signal molecule) and choline (Jenkins and Frohman 2005). The over-representation of choline biosynthesis I pathway among downregulated genes in Joelle post-acclimation correlated well with the over-representation of phospholipase pathway among upregulated genes in CO46 post-acclimation (Table 1). This data suggests that conversion of PC to phosphatidic acid and choline does not correlate well to freezing tolerance in the winter biotype of Camelina.

Hormones and Genes Associated with Cold Acclimation

Plant hormones (phytohormones), including auxin (IAA), abscisic acid (ABA), ethylene, cytokinins, gibberellins, jasmonic acid, brassinosteroids, salicylic acid, nitric oxide, and strigolactone, are small molecule compounds produced within plants (Peleg and Blumwald 2011; Koç et al. 2018); they play important roles in assisting plant adaption to environmental stimuli and to mediate plant growth, development, and nutrient allocation (Santner and Estelle 2009; Peleg and Blumwald 2011; Khan et al. 2020). In this experiment, ethylene biosynthesis via methionine pathway was over-represented among downregulated genes in Joelle post-acclimation relative to CO46 post-acclimation (Table 1). Shi et al. (2012) showed that ethylene negatively regulates plant responses to freezing stress in Arabidopsis and inhibits the expression of *CBF* and type-A *ARABIDOPSIS RESPONSE REGULATOR* (*ARR*) genes, which correlate well with our observations that *CBF* genes were upregulated in Joelle post-acclimation relative to CO46 (Table 3). In addition, SNEA of upregulated genes identified a putative *CBF1* as a central hub in Joelle for expression targets of transcripts with increased abundance post-acclimation relative to CO46 (Table 2). These observations also agree with the assumption that ethylene inhibits the expression of *CBF* (Shi et al. 2012).

Salicylic acid (SA) biosynthesis pathways were over-represented among upregulated genes post-acclimation for both Joelle and CO46 (Table 1). It is known that cold temperatures stimulate the accumulation of free SA and glucosyl SA in wild-type Arabidopsis shoots (Scott et al. 2004) and leaves of winter wheat (*Triticum aestivum* L.) (Kosova et al. 2012). In addition, low concentrations of SA (0.1–0.5 mM) improved tolerance to chilling stress in bean, tomato, maize, cucumber, and rice (Senaratna et al. 2000; Kang and Saltveit 2002); however, high concentrations and the continual application of SA caused a decrease in the cold tolerance capacity in addition to growth damage (Senaratna et al. 2000; Horváth et al. 2007) due to the production of ROS (Scott et al. 2004). In this study, the over-representation of SA biosynthesis pathways

among upregulated genes post-acclimation for Joelle and CO46 may indicate that SA was involved in cold tolerance for both cultivars. Interesting, a gene *ACCELERATED CELL DEATH 6* (*ACD6*) that is involved in salicylic acid (SA) signaling pathway was identified as a central hub for expression targets in SNEA of downregulated genes in Joelle post-acclimation relative to CO46 (Table 2). Mutation of this gene increased endogenous SA levels in Arabidopsis, and this SA-accumulating mutant (*acd6*) was more sensitive to freezing temperatures than wildtype plants (Rate et al. 1999; Miura and Ohta 2010), whereas the introduction of a SA-degrading salicylate hydroxylase, nahG, into *acd6* suppressed freezing sensitivity (Miura and Ohta 2010). The identification of *ACD6* as a central hub in SNEA of downregulated genes in Joelle relative to CO46 may indicate that SA levels were higher in CO46 than that of Joelle post-acclimation, and the higher SA levels may trigger decreased capacity of cold tolerance in CO46.

Endogenous jasmonic acid (JA) biosynthesis is activated in response to cold exposure, and exogenous application of JA improves Arabidopsis freezing tolerance (Hu et al. 2017). In addition, JA positively regulates the CBF regulon (Hu et al. 2017). However, surprisingly, the JA biosynthesis pathway was over-represented among downregulated genes in Joelle post-acclimation relative to CO46, and this pathway was also over-represented among downregulated genes post-acclimation for both Joelle and CO46 relative to their pre-acclimated samples (Table 1). These results are unexpected and may suggest that the role of JA in freezing tolerance is minimal in Camelina.

MicroRNA and Cold Tolerance

A class of small non-coding RNA molecules microRNAs (miRNAs) plays key roles of gene expression in both plants and animals (Zhang et al. 2019). In this study, we identified a MIR172A as a central hub among upregulated genes in Joelle pre- and post-acclimation relative to CO46 (Table 2). MIR172A is known to improve salt tolerance in soybean (Pan et al. 2016) and play positive roles in flowering (Wang et al. 2016). MIR172A also acts as a negative regulator of specific AP2 transcription factors that negatively regulate FLOWERING LOCUS T (FT), as well as regulating floral organ identity in Arabidopsis (Aukerman and Sakai 2003). In addition, the transcript abundance of MIR172A increased under long-term cold treatment in *Populus ssp.* (Howe et al. 2015; Zhou et al. 2019). Because MIR172A was a central hub among upregulated genes in flowering-competent Joelle post-acclimation vs. non-vernalized Joelle pre-acclimation but not in CO46 post-acclimation vs. CO46 pre-acclimation which is flowering competent in both conditions, the Camelina MIR172A network may be specifically cold inducible in Joelle. In addition, the induction of MIR172A network in Joelle presumably highlights its role in the vernalization process in Camelina.

Other Floral and Stress-Related Genes Were Differentially Regulated Post-acclimation

Based on the result of gene expression (FPKM) in Joelle and CO46, the fold differences for floral and stress-related regulators between pre- and post-acclimation are presented in Table 3. In general, transcript abundance of most genes (and of their paralogues) in Table 3 is similar (fold differences between -2 and 2) for comparisons between the two biotypes (Jo-pre vs. CO-pre and Jo-post vs. CO-post); however, dramatic fold differences in transcript abundance were observed for comparisons between two treatments from the same biotype (Jo-post vs. Jo-pre and CO-post vs. CO-pre). In present study, the transcript abundance of two *FLC* paralogues located on chromosomes 8 (Csa08g054450.1) and 20 (Csa20g015400.1) decreased greatly post-acclimation, and *FLC* transcript abundance decreased to a greater level in Joelle than that of CO46. Similar results were also observed in Arabidopsis as the downregulation of *FLC* was quantitatively related to the duration of the cold treatment (Sheldon et al. 2000). It is known that prolonged cold exposure promotes flowering through epigenetic silencing of *FLC* and the expression of *VIN3* (Kim and Sung 2013). The 1000- and 500-fold increases in *VIN3* transcript abundance post-acclimation in Joelle and CO46, respectively, are consistent with the downregulation of *FLC* (Table 3). Although cold acclimation induces *VIN3* as expected in both summer and winter biotypes, previous studies (Anderson et al. 2018; Chao et al. 2019) have proposed that flowering in summer biotypes of Camelina without vernalization is due to a mutation in chromosome 20 *FLC* that produces a non-functional protein.

LATE EMBRYOGENESIS ABUNDANT (LEA) proteins such as COR15A, COR47, and WCS19 and their transcripts are induced by cold acclimation (Sasaki et al. 2014; Wang et al. 2014) and are known to play a role in freezing tolerance (Artus et al. 1996; NDong et al. 2002; Puhakainen et al. 2004; Sanghera et al. 2011). In this study, transcript abundance of a *LEA* gene, *LEA14*, was significantly increased post-acclimation in both Joelle and CO46, but transcript abundance was considerably greater in Joelle than that of CO46. Transcript abundances of three paralogous *LEA14* genes (Table 3) increased 14.9-, 26.1-, and 48.9-fold in Joelle vs. 2.7-, 6.7-, and 6.3-fold in CO46, respectively, post-acclimation. The Arabidopsis *LEA14* belongs to the LEA_2 subgroup (Singh et al. 2005), and over-expression of this gene in Arabidopsis and foxtail millet (*Setaria italica*) enhances salt stress tolerance (Jia et al. 2014; Wang et al. 2014). Two paralogues of another *LEA* gene *ABR* were also significantly increased post-acclimation in Joelle; one paralogous *ABR* gene (Csa15g002110.1) increased 1721-fold in Joelle relative to −1.5-fold in CO46 post-acclimation (Table 3). Giving the relatedness of salt and chilling stress tolerance for some *LEA* genes (Imai et al. 1996), the increased abundance of *LEA*s in winter biotypes of Camelina suggests they may play a role in

freezing tolerance. Another stress-related gene *WRKY70* was downregulated specifically in Joelle post-acclimation. WRKY70 is involved in SA signaling and acts as a negative regulator of the SA response pathways (Li et al. 2013). The downregulation of *WRKY70* gene in Joelle post-acclimation correlated well with the over-representation of SA biosynthesis pathway among upregulated genes post-acclimation.

CBFs regulate the expression of many *COR* genes such as the *LEA*s described above. However, CBFs themselves are gated by the circadian clock (Fowler et al. 2005), which regulates many physiological processes through endogenous 24-h oscillations in gene expression (Dong et al. 2011; Greenham and McClung 2015; Jones et al. 2019; Leone et al. 2019). Our studies showed that the transcript abundance of key components of circadian clock *CCA1*, *LHY*, and some *PSEUDO RESPONSE REGULATOR*s (*PRR5*, *7*, and *9*) increased post-acclimation for both biotypes. CCA1 and LHY are directly involved in the induction of *CBF* genes (Dong et al. 2011), but PRRs suppress *CBF* expression (Nakamichi et al. 2009). Although *CCA1*, *LHY*, and *PRR*s were all highly expressed post-acclimation in both biotypes, their transcript abundance was considerably greater in Joelle than that of CO46. The upregulation of *PRR9* in this study appears to contradict its role of inhibiting the expression of

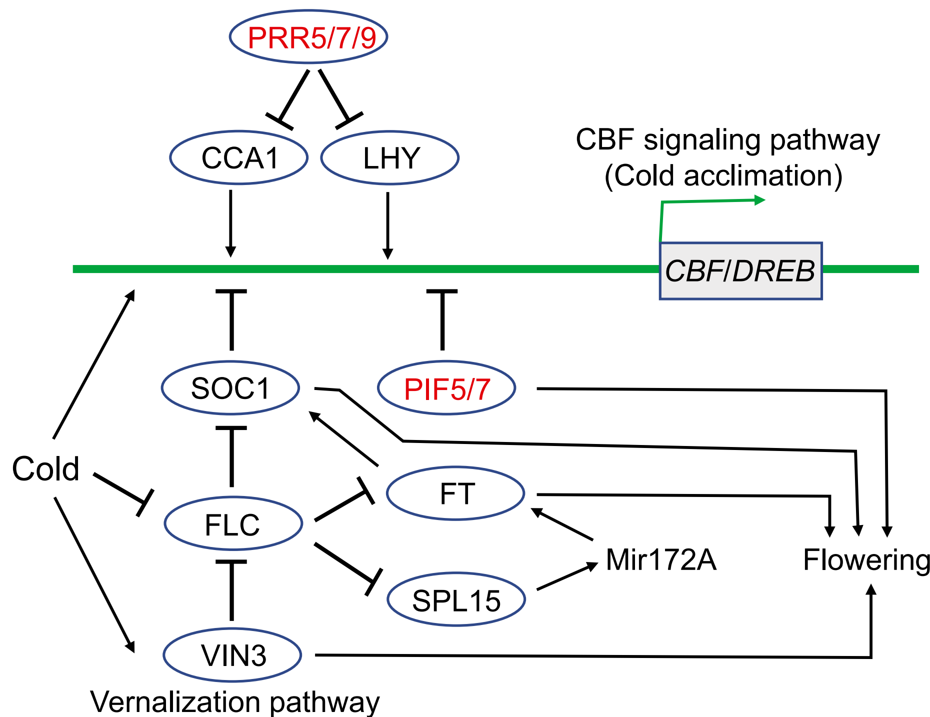


Fig. 2 Regulation of CBF signaling pathway. The CBF regulon plays an important role in cold acclimation of evolutionarily diverse plant species. CCA1 and LHY directly bind to the *CBF* promoters to upregulate their expression (Dong et al. 2011). Both SOC1 and PRRs (PRR5/7/9) are negative regulators of CBFs. The expression of *SOC1* is positively regulated by FT. SOC1 directly binds to the *CBF* promoters to repress their expression (Seo et al. 2009), whereas PRRs repress *CBF* expression by inhibiting the expression of *CCA1* and *LHY* (Nakamichi et al. 2009, 2010). However, although PRR proteins are known to play negative roles in regulating the cold stress response (Nakamichi et al. 2009), the transcript abundance of *PRR5*, *7*, and *9* was increased after vernalization in this study. PIFs are known to negatively regulate plant freezing tolerance by inhibiting the CBF pathway (Leivar and Quail 2011; Lee and Thomashow 2012; Jiang et al. 2020). However, the increased expression of *PIF5* and *PIF7* observed post-acclimation is contradictory to the concurrent increased expression of *CBF*s, and thus, these PIFs may have a functional role in vernalization. FLC is a master regulator of flowering; during vernalization, *FLC* expression is repressed possibly via *VIN3* (Wood et al. 2006). The repression of *FLC* facilitates the activation of downstream floral activators, FT and SPL15 (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE15), to initiate flowering. The transcript abundance of *FT* and *SPL15* was low (FPKM values <5) pre- and post-acclimation for both Joelle and CO46 in this study. FLC also directly represses the transcription of *SOC1* (Searle et al. 2006; Deng et al. 2011), which may reverse *CBF* repression. A microRNA, MIR172A, was identified as a central hub among upregulated genes in Joelle pre- and post-acclimation relative to CO46. MIR172A is known to play positive roles in flowering (Wang et al. 2016) possibly via the regulation of SPL15 (Wu et al. 2009) and through its target genes (such as TARGET OF EAT) to upregulate the expression of *FT* (Teotia and Tang 2015)

Table 4 Major differences between the winter biotype (Joelle) and the spring biotype (CO46)

Names	Functional roles	Differences between Joelle and CO46
AraCyc pathways		
Calvin cycle	Calvin cycle plays a role in reduction of CO2 to form carbohydrates in chloroplasts	Calvin cycle pathway was over-represented among upregulated genes for the winter biotype (Joelle) in relation to the spring biotype (CO46) pre-acclimation
Cholesterol biosynthesis	Plants can improve cold tolerance by increasing the levels of unsaturated fatty acids and membrane lipid fluidity	Cholesterol biosynthesis pathway was over-represented among upregulated genes specifically for Joelle post-acclimation
Ethylene biosynthesis from methionine	Ethylene negatively regulates plant responses to freezing stress in Arabidopsis and inhibits the expression of *CBF* and type-A *ARR* genes	Ethylene biosynthesis via methionine pathway was over- represented among downregulated genes in Joelle post-acclimation relative to CO46 post-acclimation
Oleate biosynthesis I (plants)	Oleic acid is required for normal plant growth at low temperature	Oleate (a salt or ester of oleic acid) biosynthesis pathway was over-represented among upregulated genes in Joelle compared with CO46 post-acclimation
Starch degradation	Starch degradation is associated with freezing tolerance	Starch degradation pathway was over-represented among upregulated genes for Joelle pre- and post-acclimation
Sterol biosynthesis	Plants can improve cold tolerance by increasing the levels of unsaturated fatty acids and membrane lipid fluidity	Sterol biosynthesis pathway was over-represented among upregulated genes specifically for Joelle post-acclimation
Central hubs to the subnetworks		
CBF1	CBFs are involved in cold acclimation	*CBF1* was identified as a central hub in Joelle for expression targets of transcripts with increased abundance post-acclimation relative to CO46
MIR172A	*MIR172A* is involved in the regulation of *FT*, which triggers flowering	*MIR172A* network in Joelle presumably highlights its role in the vernalization process in Camelina
Putative genes involvement in cold acclimation and vernalization		
CCA1	CCA1 is directly involved in the induction of *CBF* genes	Although three *CCA1* paralogues were all highly expressed post-acclimation in Joelle and CO46, *CCA1* transcript abundance was considerably greater in Joelle than that of CO46
FLC	Prolonged cold exposure promotes flowering through epigenetic silencing of FLC	The transcript abundance of the two *FLC* paralogues decreased post-acclimation in both Joelle and CO46; however, cold acclimation caused a greater decease in the transcript abundance of *FLC* in Joelle than in CO46
LEA14	Some LEA proteins are known to play a role in freezing tolerance	Three *LEA14* paralogues were significantly increased post-acclimation in both Joelle and CO46, but transcript abundance was considerably greater in Joelle than that of CO46
PIF5/7	PIFs are known to positively regulate flowering	*PIF5* and *PIF7* transcript abundance increased post-acclimation in both Joelle and CO46, but transcript abundance was considerably greater in Joelle relative to CO46 post-acclimation. Their induction during extended cold could be a part of the induction of floral competency for Joelle
VIN3	Prolonged cold exposure promotes flowering through the induction of *VIN3* transcript	The transcript abundance of two *VIN3* paralogues increased greatly post-acclimation in both Joelle and CO46; however, *VIN3* transcript abundance increased to a greater level in Joelle than that of CO46

CCA1 and *LHY* genes (Nakamichi et al. 2010). Assuming protein abundance of CCA1 correlated to the increase in transcript abundance of *CCA1* in the summer and winter biotype post-acclimation, these results would be in line with the abundance of *CBF* observed in this study.

It is noteworthy that *CBF1*, *2*, and *3* transcripts were induced within minutes of exposing Arabidopsis to low non-freezing temperatures and decreased to basal levels after a few days (Stockinger et al. 1997; Gilmour et al. 1998). However, the transcript abundance of three *CBF* genes increased dramatically after 8 weeks of acclimation for both Joelle and CO46. These results may indicate the species-specific expression of *CBF* genes, which are in accord with studies showing that natural variation in the CBF pathway has contributed to local adaptation in two different Arabidopsis ecotypes (Park et al. 2018). Figure 2 summarizes the regulation of CBF signaling pathway based on important differentially regulated genes identified in this study.

JUMONJI DOMAIN CONTAINING 5 (JMJD5) is a histone demethylase that is considered a positive regulator of *CCA1* and *LHY* (Jones et al. 2010). The downregulation in gene expression for *JMJD5* was inconsistent with the upregulation of *CCA1* and *LHY* in Joelle and CO46 post-acclimation. However, there is also evidence that *JMJD5* transcript is alternatively spliced in cold temperatures (Shen et al. 2016) and our analysis could have quantified both active and inactive expression products. Thus, we cannot rule out the possibility that the change in gene expression could have produced non-functional proteins that would not impact *LHY* and *CCA1* expression.

It is noteworthy that in addition to the unanticipated findings such as JA biosynthesis pathway and the expression of *PIF*s, *PRR9*, and *JMJD5* genes mentioned above, potentially contradictory results were also observed for other genes. For example, SNEA identified HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1 (HOS1) and MPK6 as central hubs of transcripts with increased abundance in Joelle post-acclimation relative to CO46 (Table 2). HOS1 mediates the ubiquitination of ICE1 to negatively regulate the expression of cold-responsive genes that contribute to cold acclimation (Dong et al. 2006). MPK6 mediates the phosphorylation of ICE1 and also negatively regulates ICE1 stability leading to the reduction of cold tolerance (Li et al. 2017). However, there is recent evidence that ICE may not play as significant a role in the CBF regulon as previously understood (Kidokoro et al. 2020). Thus, the results of this study suggest that freezing tolerance in the winter biotype of Camelina involves complex and potentially overlapping molecular mechanisms associated with cold acclimation and vernalization processes. A table (Table 4) was generated to show the most notable and essential differences in molecular pathways, central hubs, and individual genes during cold acclimation based on the overall results of RNAseq analysis.

Conclusions

Although previous genetic analysis indicated that freezing tolerance may be regulated by two dominant genes in the winter biotype Joelle, transcriptomics analysis indicated many downstream molecular pathways and genes are involved in cold acclimation–induced freezing tolerance. The over-represented molecular pathways (photosynthesis and carbon metabolism, fatty acid and hormone biosynthesis), significantly up- or downregulated genes (*VIN3*, *SOC1*, *FLC*, *LHY*, *LEA14*), and microRNA (MIR172A) appear to play prominent roles in vernalization and/or cold acclimation processes. Thus, regulation of cold acclimation in hexaploid Camelina relies on the collective effects of many molecular pathways and genes. Because vernalization occurs during the cold acclimation process in the winter biotype of Camelina, some molecular pathways and genes involved in the vernalization process may also be associated with freezing tolerance.

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Author Contribution JVA conceived and designed the study. JVA, MD, and DPH analyzed the RNAseq data; JVA, MD, WSC, and HW analyzed the Pathway Studio results; WSC and HW wrote the initial draft; and WSC, HW, JVA, MD, and DPH revised and approved the final draft.

Declarations

Conflict of Interest The authors declare no competing interests.

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