The transition to flowering in winter rapeseed during vernalization

Sarah Matar[1], Avneesh Kumar[1], Daniela Holtgräwe[2], Bernd Weisshaar[2] and Siegbert Melzer[1].

[1]Plant Breeding Institute, Christian-Albrechts-University of Kiel, Olshausenstr. 40, D-24098 Kiel, Germany

[2] Center for Biotechnology – CeBiTec Universitätsstraße 25 33615 Bielefeld.

Correspondence to: Dr. Siegbert Melzer

email: s.melzer@plantbreeding.uni-kiel.de

Tel.: +49 431 880-256

Running head: Cold-driven floral transition in rapeseed

Abstract

Flowering time is a major determinant of adaptation, fitness and yield in the allopolyploid species rapeseed (*Brassica napus*). Despite being a close relative to *Arabidopsis thaliana*, little is known about the timing of floral transition and which genes govern this process. Winter, semi-winter and spring type rapeseed have important life history characteristics that differ in vernalization requirements for flowering and are important for growing rapeseed in different regions of the world. In this study, we investigated the timing of vernalization-driven floral transition in winter rapeseed and the effect of photoperiod and developmental age on flowering time and vernalization responsiveness. Microscopy and whole transcriptome analysis at the shoot apical meristems of plants grown under controlled conditions showed that floral transition is initiated within few weeks of vernalization. Certain *Bna.SOC1* and *Bna.SPL5* homeologs were among the induced genes, suggesting that they are regulating the timing of cold-induced floral transition. Moreover, the flowering response of plants with shorter pre-vernalization period correlated with a delayed expression of *Bna.SOC1* and *Bna.SPL5* genes. In essence, this study presents a detailed analysis of vernalization-driven floral transition and the aspects of juvenility and dormancy and their effect on flowering time in rapeseed.

Key words: Vernalization, floral transition, transcriptome, *Bna.SOC1*, *Bna.SPL5*, rapeseed

1 Introduction

The allopolyploid rapeseed (*Brassica napus*) is a major oil crop that was formed by spontaneous hybridization of the two diploid species *Brassica rapa* (AA, 2n=20) and *Brassica oleracea* (CC, 2n=18) and retained both genomes (Chalhoub et al*.,* 2014). Rapeseed is adapted to different environments by specific life history traits. Winter types are biennials, grown in temperate climates such as Northern Europe and have an obligate requirement of cold periods for flowering, a process known as vernalization (Chouard, 1960; Amasino, 2004). Semi-winter types, with milder or no cold requirements, are grown in regions with moderate winter temperatures, such as central China, whereas spring types are annuals that flower in the same year of sowing without any vernalization requirement (Leijten et al., 2018). Since flowering time has a profound impact on adaptation, life cycle and yield, understanding the molecular genetic basis of flowering time in rapeseed has been in the limelight of research for years.

The evolution of the control of flowering by vernalization is an important adaptive trait that ensures flowering in favorable conditions after winter (Bouché et al., 2017). In winter annuals of *Arabidopsis thaliana*, the floral repressor FLOWERING LOCUS C (FLC) is a central regulator of the vernalization response (Michaels & Amasino, 1999, Sheldon et al., 1999). *FLC* is expressed in shoot apical meristems (SAM) and vascular tissues of leaves. In leaves, FLC directly represses the expression of *FLOWERING LOCUS T* (*FT*), whereas in SAMs, it impairs the upregulation of *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) and *FLOWERING LOCUS D* (*FD*), which prevents flowering (Searle et al., 2006). Vernalization leads to a rapid decrease in *FLC* transcript abundance, and continuous cold exposure results in its epigenetic silencing creating a winter memory state (Costa & Dean, 2019), therefore, liberating *FT* and *SOC1* and other flowering time genes, conferring a seasonal competence to flowering (He et al., 2020).

In winter annual Brassicaceae, sensitivity to vernalization depends on the plant's age. Certain plants, like *A. thaliana* and *B. rapa,* respond to vernalization in metabolically active seeds or at the seedling stage, whereas other species need a longer pre-vernalization growth phase to become vernalization-responsive (Friend, 1985). The role of pre-vernalization growth was most clearly demonstrated in the Brassicaceae *Arabis alpina* and *Cardamine flexuosa*, which responded to vernalization only after the levels of microRNA156 (miR156) declined below a certain threshold (Bergonzi et al., 2013; Zhou et al., 2013). In *A. alpina,* the downregulation of miR156, through ageing, caused an increased expression of SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) with miR156 target sites in the respective mRNAs (Bergonzi et al., 2013). The first *SPL* genes were identified in *Antirrhinum majus* (Klein et al., 1996) as *AmSBP1* and *AmSBP2* for *SQUAMOSA PROMOTER BINDING PROTEIN* gene 1 and 2. The encoded proteins share a conserved SBP-box that represents the DNA-binding domain of SPL transcription factors. In *A. thaliana*, out of 16 *SPL* genes 10 are targeted by miR156 and are involved in flowering time control (Preston & Hileman, 2013). In *A. alpina*, in addition to being a target of miR156, *AaSPL15* is a downstream target of the *FLC* ortholog, *PERPETUAL FLOWERING 1 (PEP1*) (Hyun et al., 2019). The downregulation of *PEP1*, by cold, further releases the repression of *AaSPL15*, which in turn initiates floral transition (Hyun et al., 2019). While *AaSPL15* is indispensable for floral transition to take place in *A. alpina*, *A. thaliana* plants are not dependent on *SPL15*, because the cold induced downregulation of *FLC* promotes flowering through the photoperiodic pathway (Hyun et al., 2019).

Numerous studies have addressed the genetic basis of flowering time in rapeseed. Thereby, many quantitative trait loci (QTL) that control time to flowering were mapped and several flowering time gene orthologs were found to be aligned with these QTLs or associated with them (Long et al., 2007; Raman et al., 2013; Wang et al., 2016). Orthologs of the key regulator of the vernalization response in *A. thaliana*, *FLC*, and the main flowering promoter in the photoperiod pathway, *FT*, were found to be located within flowering time QTLs of *B. napus* (Udall et al., 2006; Long et al.,

2007). Association mapping or genome-wide association studies (GWAS) were also used to study polymorphism associated with different flowering phenologies (Xu et al., 2015; Shah et al., 2018; Wu et al., 2019). These identified associations included an ortholog of *CONSTANS* (*CO*), namely *Bna.CO.C09*, a central gene in the photoperiodic pathway, which was located only 13 kb away from a significant SNP (Xu et al., 2015). Orthologs of *FLC* and *FT* (*Bna.FLC.A10* and *Bna.FT.A02*), and other floral integrators were also identified in regions where SNPs were associated with control of flowering time (Shah et al., 2018; Wu et al., 2019; Lu et al., 2019). However, despite the evidence of flowering-time-genes being conserved in *B. napus*, the presence of multiple paralogs complicated the efforts to translate the gene regulatory network from *A. thaliana*. Therefore, it is not known whether all paralogs respond to flowering inductive conditions, and how they contribute to the regulation of the floral transition in rapeseed.

In this study, we provide morphological and molecular evidences on the timing of floral transition and the formation of floral buds of winter rapeseed plants. We show that the photoperiod plays a minor role in determining flowering time in winter rapeseed. Microscopic studies and transcriptome profiling of shoot apical meristems clearly indicated that the floral transition occurred during vernalization and was marked by the activation of specific homeologs of flowering time regulator genes. Finally, we studied the effect of plant age on the vernalization response. The results showed a correlation between the upregulation of flowering time regulators and the flowering response of plants with different pre-vernalization periods, reflecting an age-dependent vernalization response in winter rapeseed.

2 Materials and Methods

2.1 Plant material and growth conditions

Plants used in this study were from the *B. napus* winter inbred line Express617, derived from the cultivar Express from NPZ Lembke KG, and Pioneer Hi-Bred International, Inc. experimental winter lines P1-P9. For the field experiment, plants from Express617 and nine Pioneer lines were sown on soil in Wulfshagen (52°N, 13°E), Germany, in August of the growing season 2016/2017. Apical buds were excised under a binocular microscope. Flowering time was measured in days from sowing to the date when 50% of the plants of one genotype had open flowers.

To study the effect of photoperiod on flowering, plants from Express617 and pioneer lines were pre-grown for three weeks in growth chambers at 20°C under long day conditions (LD 16h light/8h dark, 240 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Afterwards, plants were split into two groups that were vernalized for eight weeks under two different photoperiodic conditions 4°C/LD or 4°C/SD (8h light/16h dark). From both vernalization conditions, the plants were further split and grown under 20°C/SD or 20°C/LD. Flowering time of ten plants of one genotype from each condition was recorded when the first flower opened.

For transcriptomic and microscopic studies, plants were grown in climate chambers at 20°C under LD conditions, for three weeks. Afterwards, the plants were transferred to 4°C in LD at the same light condition for vernalization. A different set of Express617 plants was grown under the same conditions for RT-PCR analyses.

To study the effect of plant's age on the vernalization response, plants were grown for different durations at 20°C in LD conditions, then vernalized for eight weeks at 4°C in LDs. Apices were collected from plants before and during 2, 4, 6, and 8 weeks of vernalization as well as two weeks after vernalization.

2.2 Microscopic analyses of meristems

Dissected apices from Express617 plants pre-grown for three weeks at 20°C in LDs and vernalized for 3, 4, 5, 6, 7 and 8 weeks at 4°C in LDs, were fixed in 4% FAA (4% formaldehyde, 50% ethanol and 5% acetic acid) overnight. The samples were dehydrated by an ethanol series and embedded in Technovit 7100 according to the manufacturer's manual (Kulzer, Germany). For the age-dependent vernalization experiment, apices were embedded in paraffin by using a standard protocol. The plastic-embedded apices were sectioned at 3 µm and Paraplast embedded-apices were sectioned at 8 µm, using a Leica rotational microtome and stained with 0.05% toluidine blue.

2.3 Sample preparation and nucleic acid extraction

For RNA-Seq apical buds were dissected under a binocular microscope using scalpel and forceps and immediately frozen in liquid nitrogen. Shoot apices were sampled at 1, 2, 4, 5, 6, 7, 8 and 9 weeks after the start of vernalization. For the RT-qPCR, leaves and apices were sampled before vernalization and 2, 4, 5, 7, 8, and 9 weeks after the start of vernalization. Ten to twelve apical buds were pooled for one biological replicate. RNA isolation and DNase treatment were carried out according to the instruction manual provided with the peqGold Total RNA Kit (PeqLab, Germany). For Express617 genomic DNA sequencing, leaf samples were collected and frozen in liquid nitrogen. DNA was isolated according to the user manual supplied with NucleoSpin® Plant II from Macherey and Nagel (Düren, Germany).

2.4 Generation of a reference-based Express617 genome sequence

Library preparation and DNA sequencing was carried out at the IKMB (Institute for Clinical Molecular Biology, Kiel, Germany). Hundred base pair paired end reads were generated using Illumina HiSeq-2500. Paired end reads were aligned to the Darmor-bzh reference genome (Chalhoub et al., 2014) using the Burrows-Wheeler Aligner mem (BWA-MEM v.0.7.17) software

149 package (Li & Durbin, 2009) using default parameters. Single-nucleotide polymorphisms (SNPs)
150 and small insertion/deletions (indels) were called using Haplotype Caller (GATK v4.0.8.1)
151 (McKenna et al., 2010) and filtered using the recommended parameters. The VCF variants were
152 applied to create a consensus sequence of the Express617 genome using bcftools (1.9-51-g20a170e)
153 (Li, 2011). BRAKER1 (Hoff et al., 2016) was used for gene structure annotation using RNA-Seq
154 data reads as transcript evidence. Genes were functionally annotated by comparing their protein
155 sequences against UniProt reference proteomes database (Pundir et al., 2017) and Pfam domain
156 search database (Finn et al., 2008).

157 **2.5 Transcriptome analysis**

158 Library preparation and RNA sequencing was carried out at the Center of Biotechnology, CeBiTec,
159 Bielefeld, Germany. RNA samples for each time point were sequenced with three repetitions using
160 Illumina HiSeq-1500. A modified version of the Tuxedo protocol (Trapnell et al., 2012) was used to
161 analyze RNA-Seq data and to determine differential gene expression. Single end RNA-Seq reads
162 were mapped to the Express617 genome using STAR (v2.6.0a.) (Dobin et al., 2013). For
163 transcriptome assembly, the RNA-Seq read alignments were used as an input file to run Cufflinks
164 (v2.2.1) (Trapnell et al., 2012). The 24 different Cufflinks assemblies from eight different time
165 points were merged using the Cuffmerge module. Statistical analysis and determination of
166 differentially expressed genes between the different data points was performed using Cuffdiff
167 modules, which considered replicated data and sequencing biases. *P-values* were adjusted for
168 multiple testing using the Benjamini-Hochberg false discovery rate method (Trapnell et al., 2010).
169 Data visualization and pairwise comparison of the number of differentially expressed genes
170 between different data points was carried out using CummeRbund (v2.24.0) (Goff et al., 2012).

2.6 Expression Analysis by RT-qPCR

RT-qPCR was performed for three biological replicates each with three technical repeats for each time point. RT-qPCR was carried out using Platinum™ SYBR™ Green qPCR SuperMix (ThermoFischer Scientific). Expression levels were calculated with the comparative ΔΔCt method (Livak & Schmittgen, 2001). Primers used for gene expression analysis are listed in Table S1. *Bna.ACTIN2* and *Bna.GAPDH* were used as endogenous controls to normalize gene expression levels.

3 Results

3.1 Flowering-related phenology of winter rapeseed under growth chamber and field conditions

We analyzed flowering time of ten different winter rapeseed genotypes, from which nine were winter rapeseed experimental lines from Pioneer Hi-Bred and the 10th line was the inbred accession Express617. Seeds were sown in late-August 2016 on an experimental field of Pioneer Hi-Bred in Wulfshagen, Germany. We observed in mid-November that plants already developed floral buds at apical meristems (Fig. S1a). However, flowering was only observed in a timespan from end of April to the first week of May in the following year, implying that after floral initiation, the plants went through a dormant stage, in which the floral buds did not further develop. The accessions flowered between 241 to 251 days after sowing (Fig. S1b), indicating that the floral transition at apical meristems and the appearance of open flowers were separated by about 160 days.

To analyze whether shortening of photoperiods in winter contributed to the formation of this dormant stage after floral transition, we grew all accessions under controlled environmental conditions. Rapeseed plants were grown under LD conditions for three weeks, and then they were exposed to different photoperiods during and after vernalization. Since eight weeks of 2° to 12°C

were shown to be optimal conditions for vernalization of European winter rapeseed cultivars (Filek et al., 2007), we vernalized the rapeseed plants for eight weeks at 4°C and recorded the opening of the first flower as a measure for flowering time. Plants that were exposed always to LD conditions showed the highest synchronization and flowered 20 to 25 days after the end of vernalization with P8 as the earliest accession (Fig. 1a). Plants exposed to SD after LD vernalization (LD-SD), started to flower after 35 days in the earliest accessions in a less synchronized way. Especially Express617, P1, P5, P6 and P9 showed a broad range in flowering time with more than 10 days between the earliest and latest flowering individuals (Fig. 1b). Vernalization under SD conditions, with subsequent growth under LD (SD-LD), delayed flowering in some accessions and enhanced it in others, with an average of 35 days to flowering (Fig. 1c). Plants vernalized in SD and further grown at 20°C in SD (SD-SD) showed the latest flowering, with an average of 65 days, and were least synchronized with the exception of P8 that flowered in a very narrow range (Fig. 1d). This indicated that SD conditions during and after vernalization delayed flowering. The longer the plants were exposed to 8-hour photoperiods, the later they flowered. However, the delay in flowering that was observed under controlled environmental conditions was not as extreme as in the field, indicating that SD photoperiods are not mainly contributing to the large time gap between the floral transition at SAMs and the opening of the first flowers under field conditions.

3.2 Histologic and transcriptomic changes mirroring the transition to flowering during vernalization

The formation of floral buds in autumn and the early flowering response after vernalization in LD suggested that the floral transition occurred soon after a short duration of cold. Therefore, to analyze the timing of the floral transition of Express617 in more detail, we used longitudinal sections of SAMs for microscopic studies and apical buds for an RNA-Seq study during the course of vernalization.

To study morphological changes at the SAM, apices were dissected 3, 4, 5, 6, 7 and 8 weeks after the start of vernalization at 4°C in LDs. After five weeks of vernalization, the SAM was still in a vegetative stage (Fig. 2c), but had significantly increased in size compared to the SAM in the third and fourth weeks of vernalization (Fig. 2a, 2b). One week later, the meristem appeared more dome shaped, resembling an inflorescence meristem (Fig. 2d), indicating that the floral transition at the SAM had started. Finally, floral buds became visible in the 7th week of vernalization and were more clearly visible in the 8th week (Fig. 2e, 2f).

Due to the highly redundant and dynamic rapeseed genome with high levels of genetic variation, represented by nucleotide polymorphisms and copy number variations between different cultivars (Schiessl et al., 2017; Wu et al., 2019), accurate transcript quantification and representation of homologous genes could be challenging. Therefore, and to avoid any mapping discrepancies, we generated an Express617 genome sequence that we used as a reference for our RNA-Seq study instead of the Darmor-*bzh* genome sequence (Chalhoub et al., 2014). The annotated Express617 genome sequence was generated using Darmor-*bzh* as a reference together with the RNA-Seq reads as transcript evidences. Thereby we found 4,012,249 short sequence variants between the two genomes, including 355,966 in coding regions (Fig. S2). The final annotation of Express617 contained 110,912 genes, with assigned functions based on homology to entries in the UniProt and Pfam databases.

For the RNA-Seq study, plants were grown for three weeks at 20°C/LDs followed by vernalization at 4°C/LDs. Apices were dissected under a binocular microscope at 1, 2, 4, 5, 6, 7, 8 and 9 weeks after start of vernalization, with three biological replicates of pools of 12 apices each. In total, we detected expression (RPKM≥1) of about 45,000 genes in the annotated Express617 genome sequence at least one of the nine time points (Fig. S3a). *B. napus* orthologs of *A. thaliana* SAM

188 marker genes such as *SHOOT MERISTEMLESS* (*STM*), *CLAVATA1* (*CLV1*) and *CLAVATA2*
189 (*CLV2*) (Table. S2) were detected in all samples (Fig. S4a-c), indicating that the detection of
190 expression of meristem-specific genes was possible. Interestingly, pairwise comparison of
191 expression levels at the eight time points showed that the highest number of differentially expressed
192 genes (log2 fold change ≥ 1) was between the fifth and sixth week of vernalization, indicating that a
193 profound change had taken place between these two time points, which was in accordance with our
194 morphological analyses (Fig. S3b).

To analyze the floral transition molecularly, we aimed to identify biologically relevant gene expression profiles that could be correlated to the morphological changes at the meristem. Using three different assumptions for expression profiles, we identified genes that were preferentially or specifically expressed at a certain stage during vernalization. The first profile comprised genes with RPKM >2 in the first week of vernalization, which decreased significantly in the next four weeks and remained unexpressed (RPKM < 1) for the rest of the vernalization period (Fig. S5a). Next, we looked at genes whose expression levels increased significantly in the first four weeks of vernalization and continued to increase their expression levels afterwards (Fig. S5b). Finally, the third profile comprised genes whose expression started after the formation of the inflorescence meristem and continued to increase until the end of the vernalization period (Fig. S5c).

The first profile comprised 103 genes, including orthologs of the floral repressor and vernalization responsive gene *FLC*. Only three out of eight *Bna.FLC* homeologs were represented in this profile, namely *Bna.FLC.A10*, *Bna.FLC.A02*, and *Bna.FLC.A03a*. The transcript levels of these *Bna.FLC* paralogs decreased significantly in the first four weeks of vernalization and remained below detection levels until the end of the vernalization period (Fig. 3a). Interestingly, *Bna.FLC.A10* and *Bna.FLC.A02* were proposed to be the main genes that contribute to the vernalization requirement in winter rapeseed (Long et al., 2007; Raman et al., 2016; Wu et al., 2019; Schiessl et al., 2019; Yin et al. 2020). By looking at all *Bna.FLC* expression profiles we found that *Bna.FLC.C02*,

Bna.FLC.C03 and Bna.FLC.C09a were not expressed in any of the stages of vernalization we analyzed. *Bna.FLC.A03b* was also significantly downregulated in the first four weeks of vernalization; however, it remained stably expressed in the following weeks (Fig. 3a). On the contrary, the *Bna.FLC.C09b* paralog showed an opposite pattern, where its expression was upregulated in the first four weeks of vernalization and continued to increase until the end of the vernalization period (Fig. 3a).

The second profile comprised 34 genes, including homeologs of *SOC1*, a key floral integrator and downstream target of *FLC* in *A. thaliana*. Out of six *Bna.SOC1* homeologs, *Bna.SOC1.A05* and *Bna.SOC1.C04-random* exhibited a significant upregulation in the first four weeks of vernalization, and continued to increase expression across the vernalization period (Fig.3b). Four other *Bna.SOC1* genes showed only a very subtle increase in expression during cold treatment. The second profile also included homeologs of the SPL transcription factor *SPL5*. Two out of five *Bna.SPL5* homeologs, *Bna.SPL5.A05* and *Bna.SPL5.C05,* increased their expression significantly after two weeks of vernalization and continued to increase similarly to *Bna.SOC1.A05* and *Bna.SOC1.C04-random*, whereas three additional *Bna.SPL5* homeologs showed no expression (Fig. 3c). Among the genes that exhibited an expression pattern similar but a bit delayed relative to *Bna.SOC1* and *Bna.SPL5* were orthologs of the *FUL* gene (Fig. 3d), which regulates the transition to flowering redundantly with *SOC1* in *A. thaliana* (Melzer et al., 2008; Torti et al., 2012). An ortholog of the transcription factor gene *FANTASTIC FOUR 2 (FAF2), Bna.FAF2.C05*, which showed an increased expression during floral transition in *A. thaliana* (Torti *et al.* 2012), was also strongly upregulated in the meristems of Express617 during vernalization (Fig. 3e). The downregulation of *Bna.FLC* genes in the first four weeks of vernalization, accompanied by an early and significant upregulation of certain *Bna.SOC1* and *Bna.SPL5* genes, followed with the change in meristem size and structure, indicated that the floral transition was initiated within four weeks after the shift to 4°C, and culminated by the formation of an inflorescence meristem after additional two weeks. In *A.*

thaliana, the expression of the floral meristem identity gene *APETALA 1(AP1)* defines the initiation of flower development (Hempel et al., 1997; Lohmann & Weigel, 2002). None of the orthologs of *AP1* were represented in this profile, nor were they detected in the first six weeks of vernalization, suggesting that floral determination and the formation of floral meristems had yet to occur. The sequence information of the studied genes and their identity with their corresponding Arabidopsis orthologs are shown in Table. S3.

Our Express617 genome sequence annotation covers 58 *Bna.SPL* genes that encode the SBP-box (Pfam: PF03110), which is consistent with a report from Cheng et al. (2016) that describes 58 SBP-box genes in the *B. napus* genome. We analyzed the expression patterns of orthologs of five *A. thaliana* clades of *SPL* genes *SPL3/SPL4/SPL5, SPL9/SPL15, SPL2/SPL10/SPL11, SPL6* and *SPL13A*. Interestingly, two homeologs of *Bna.SPL15* (*Bna.SPLA07* and *Bna.SPL.C06*) were significantly upregulated in the first four weeks of vernalization, but remained relatively stable afterwards (Fig. 3f). Most of the identified *Bna.SPL* genes were stably expressed in the meristems of Express617 plants (Fig. S6). Only *Bna.SPL3.C03*, *Bna.SPL8.C05* and *Bna.SPL8.A10* were upregulated during vernalization, although at relatively low levels (Fig. S6).

The *B. napus* orthologs of *AP1* were detected in the third profile, marking the floral determination at the meristems of Express617 plants after eight weeks of vernalization. *Bna.AP1* homeologs were only detectable after eight weeks of vernalization and continued to increase their expression levels in the following week (Fig. 3g, Table. S4). The upregulation of *B. napus* orthologs of floral organ identity genes, such as *APETALA 3 (Bna.AP3)* and *PISTILLATA (Bna.PI),* after nine weeks of vernalization (Fig. 3h, i, Table. S4) confirmed the onset of flower development at this stage.

Since our transcriptome analysis was focused on the vernalization response at the apical meristem, we used quantitative RT-PCR to compare the cold response in apical meristems and leaves of

Express617 plants. We studied the overall expression profiles of *Bna.FLC*, *Bna.SOC1*, *Bna.FUL*, *Bna.SPL5* and *Bna.SPL15* in different biological samples, before and during vernalization. Interestingly, no significant difference was observed in the expression pattern or the expression levels of the studied genes between apices and leaves (Fig. 4a-e), except for *Bna.SPL5* and *Bna.SPL15*. Transcripts of *Bna.SPL5* genes were not detectable in leaves at any of the time points studied, indicating that their activity is restricted to the meristem (Fig. 4d) and *Bna.SPL15* transcripts, in leaves, were detected only after nine weeks of vernalization (Fig. 4e).

3.3 Pre-vernalization growth period impacts flowering time in winter rapeseed

It has been shown for other Brassicaceae with an obligatory vernalization requirement, that a juvenile phase is needed to be overcome before plants become responsive to vernalization. Therefore, we analyzed the flowering response for Express617 plants of different developmental age in response to cold. The plants were pre-grown for different durations under LDs at 20°C, then exposed to eight weeks vernalization at 4°C and returned to 20°C.

Although flowering was induced in all plants, an age effect was clearly visible in their flowering response. Plants with three weeks of pre-vernalization growth flowered in a synchronized manner, 21 days after vernalization. With two weeks of pre-vernalization growth, flowering remained synchronized but was one week delayed. Only nine days of pre-vernalization growth desynchronized and further delayed flowering to 36 days after vernalization. Finally, seven days of pre-vernalization growth caused a drastic delay in flowering, where plants flowered very asynchronously, about 72 days after vernalization (Fig. 5a).

We then tested whether flowering time after vernalization was correlated with expression profiles of *Bna.FLC*, *Bna.SOC1* and *Bna.SPL5* genes, in plants of different pre-vernalization growth durations, before, during and after vernalization. The levels of *Bna.FLC* dropped during vernalization at a similar rate (Fig. 5b), regardless of plants' age at vernalization. Therefore, late flowering in younger

vernalized plants seemed to be determined downstream of *Bna.FLC.* Interestingly, *Bna.FLC* was upregulated again after vernalization, and this upregulation was not restricted to younger plants, but was also visible in all other samples. On the contrary, the expression of *Bna.SOC1* and *Bna.SPL5* showed a high correlation with the age at which the plants encountered vernalization. In three-weeks old plants at vernalization, *Bna.SOC1* was induced within two weeks of vernalization and further increased significantly reaching a peak after six weeks of vernalization. In plants that were one week younger at vernalization, *Bna.SOC1* expression levels increased at a slower pace, and reached a peak after eight weeks of vernalization. In plants that were nine- and seven-days old at vernalization, the upregulation of *Bna.SOC1* was further slowed down, suggesting that *Bna.SOC1* homeologs might play a role in the age dependent vernalization response. The *Bna.SPL5* homeologs showed a more pronounced delay compared to *Bna.SOC1*, and in nine and seven-days old plants at vernalization, the upregulation took place after vernalization (Fig. 5d). Therefore, both gene families showed an age specific response, in which the *Bna.SPL5* genes seem to act after the *Bna.SOC1* genes.

Histological analysis of the SAMs, two weeks after vernalization, showed that plants with two weeks preculture had already visible floral buds developed (Fig. S7a), whereas plants with nine-days preculture had a less advanced inflorescence with visible floral meristems on the flanks of the inflorescence meristem (Fig. S7b), while at plants with one-week preculture the SAM was dome shaped resembling an early stage of inflorescence meristem development (Fig. S7c). Moreover, expression analysis showed that *Bna.AP1* was already induced in the SAMs of plants with three, two weeks and nine days of preculture, two weeks after vernalization, while the expression remained very low in plants with one-week of preculture (Fig. S8), indicating that shorter growth periods before vernalization delay the floral transition.

4 Discussion

Besides barley and wheat, rapeseed is one of the most important winter crops in the northern hemisphere. The onset of flowering in these winter crops strictly depends on a prolonged exposure of cold during winter, which renders plants competent to flower by creating a mitotically permanent epigenetic memory that involves the stable downregulation of a repressor (Bouché et al., 2017). This mandatory requirement ensures that plants flower in spring and not under unfavorable winter conditions. The role of vernalization for flowering has been studied in several members of the Brassicaceae family, including the alpine perennial *A. alpina* and winter annual accessions of *A. thaliana*. Despite being a close relative to *A. thaliana*, little is known about the timing of floral transition and the genes that regulate this process in apical meristems of *B. napus*. Here we show that the production of floral buds in autumn is due to the relatively rapid response of winter rapeseed plants to cold treatment, where the initiation and completion of floral induction occurs within eight weeks of vernalization. SD photoperiods during vernalization did not have a major impact on flowering time and SD after vernalization could not maintain the dormant state of the floral buds. We also show that winter rapeseed plants have an age dependent response to vernalization that is regulated downstream of *Bna.FLC*.

SD photoperiods are not the main determinant of winter dormancy

During their life cycle, winter rapeseed plants are exposed to a range of continuously changing environmental conditions, represented mainly by changes in temperatures and photoperiod throughout the seasons of the year. The development of floral buds in mid-November and opening of the flowers in late April of the following year implied an active apical growth period in autumn that was followed by a cessation of growth in winter, leading to an extreme delay in floral buds' emergence and further flower development. Floral transition in autumn and overwintering of floral buds was also reported for other winter rapeseed cultivars grown in the UK (O'Neill et al., 2019). Since rapeseed floral buds are not encased by layers of bud scales like dormant tree buds, autumn

driven vernalization might jeopardize the whole reproductive cycle of rapeseed plants, as the floral structures have also to acquire winter hardiness to survive cold winters. In woody species, the seasonal arrest of bud development is primarily based on day length (Olsen et al., 1997; Rohde & Bhalerao, 2007), where SD photoperiods after floral induction, in buds, induce seasonal dormancy through modulating gibberellic acid metabolism (Barros et al., 2012). SD photoperiods, during and/ or after vernalization, did contribute to a delay in flowering in winter rapeseed, however, this delay was not as pronounced as under natural conditions, indicating that low temperatures are essential for longer periods of growth cessation after floral bud formation. The two successive stages "autumn-driven floral induction" and "winter-driven growth cessation" are suggestive for an intricate transcriptional and metabolic activity in meristems and their surrounding tissues to prevent damage of reproductive structures by freezing temperatures.

Molecular changes correlated with the floral transition during vernalization

To study how molecular changes at the SAM are correlated with the floral transition during cold, we performed an RNA-Seq analyses during vernalization. The early response to cold in Express617 apices was reflected by the downregulation of certain *Bna.FLC* genes, accompanied by the gradual upregulation of specific *Bna.SOC1, Bna.SPL5* and *Bna.FUL* genes, and the increase in meristem size within five weeks of vernalization. In *A. thaliana*, *SOC1* is the earliest gene to be detected in the meristem upon floral induction (Samach et al., 2000; Borner et al., 2000), and both, *SOC1* and *FUL* are needed to maintain flowering at apical meristems (Melzer et al., 2008). The upregulation of *Bna.SOC1* and *Bna.SPL5* homeologs, specifically *Bna.SOC1.A05, Bna.SOC1.C04-random, Bna.SPL5.A05 and Bna.SPL5.C05,* had started already after two weeks of vernalization (Fig. 3b, c), before *Bna.FLC* genes were completely downregulated, and the meristems exhibited floral commitment, which is reached after six weeks of vernalization in Express617 (unpublished results). This suggests that the expression of the *Bna.SOC1* and *Bna.SPL5* genes either has to reach a certain threshold or that the molecular changes induced by these genes are slowed down in the cold to

285 establish floral commitment only after several weeks. In *A. alpina* accessions with an obligate
286 vernalization requirement, adult plants undergo floral transition after five weeks of vernalization
287 under controlled environmental conditions, and additional seven weeks are required for the
288 formation of floral buds (Wang et al., 2009b). In Express617 plants, the upregulation of orthologs
289 of floral organs identity genes, such as *AP3* and *PI,* which mark the initiation of floral organ
290 development, was observed only one week after the expression of *Bna.AP1*. The relatively short
291 duration between the upregulation of *Bna.AP1* and the orthologs of floral organ identity genes,
292 indicates that, unlike in *A. alpina*, flower primordia of winter rapeseed plants acquire a floral fate
293 very rapidly without the need of continuous exposure to cold.

294 In addition to *Bna.SOC1* and *Bna.SPL5* we saw in other gene families that some genes were
295 preferentially expressed. For instance, for *Bna.FLC*, *Bna.FAF2*, and *Bna.SPL* gene families only
296 some members showed a differential expression, whereas the others showed no differences or were
297 not expressed (Fig. 3 and S6). Previous studies have shown that polyploidy creates an expression
298 bias, where certain homeologs from one ancestor are preferentially and highly expressed relative to
299 other homeologs (Grover et al., 2012; Combes et al., 2013). However, we saw no preferential
300 expression of genes coming either from the *B. rapa* or the *B. oleracea* ancestor. In fact, we saw that
301 for *Bna.SOC1* and *BnaSPL5* the two predominantly expressed genes were from both the A and C
302 genomes (Fig. 3b, 3c).

In *A. thaliana*, other *SPL* genes such as *SPL3*, *SPL4* and *SPL9* are also induced in the shoot apex during reproductive development (Wang et al., 2009a) and have been shown to be involved in the regulation of the floral transition (Xu et al., 2016). The orthologues of these genes were indeed expressed in the apex of Express617 plants (Fig. S7), but were, except for *SPL5* and *SPL3*, not significantly induced during floral transition. *SPL* genes, with miR156 binding sites, are known to be regulated post-transcriptionally. Therefore, even if there was no significant change in transcript

levels of *Bna.SPL15* or other *Bna.SPL* genes during floral transition, the protein could be accumulating as the levels of miR156 decrease Hyun *et al.* (2016).

Age-dependent response to vernalization in winter rapeseed

The ancestors of *B. napus*, *B. rapa* and *B. oleracea*, are differently responsive to vernalization. *B. rapa* is already responsive at seed germination, whereas *B. oleracea* needs several weeks of growth prior to vernalization to become responsive (Friend, 1985). *A. thaliana* is also responsive to vernalization already in the seedling stage, whereas in the perennial relative, *A. alpina*, floral commitment in response to cold needs a pre-vernalization growth phase. While younger *A. alpina* plants did not respond to cold and did not flower, five weeks old plants responded to vernalization treatments and flowered (Wang et al., 2011).

A very late sowing of winter rapeseed in the field might lead to non-flowering or a low flowering response in the following year. In this study, we found that Express617 plants with short pre-vernalization times, of 7 or 9 days, flowered much later than 3 weeks old plants. A shorter pre-vernalization period, did not only delay flowering, but also desynchronized it compared to three weeks pre-vernalization growth period (Fig. 5a). RT-qPCR analyses showed that *Bna.FLC* genes were significantly downregulated upon vernalization regardless of plant's age (Fig. 5b), indicating that these genes might not be responsible for the late flowering of plants with a short pre-vernalization time. The upregulation of both *Bna.SOC1* and, to a greater extent, *Bna.SPL5* genes, was delayed in plants with shorter pre-vernalization times (Fig. 5c), suggesting that *Bna.SOC1* and *Bna.SPL5* integrate age-related signals downstream or in parallel to *Bna.FLC* genes. Interestingly, the block of flowering in *A. alpina* plants with short pre-vernalization period occurred downstream of *AaSOC1*, as the mRNA levels of *AaSOC1* increased during vernalization with both 2- and 8-weeks of pre-treatment (Wang et al., 2011). In addition, *Bna.SOC1* expression increased despite the increase of *Bna.FLC* expression after cold, indicating that once winter rapeseed plants encounter

326 enough cold exposure, they flower independent of *Bna.FLC.* A stable silencing of *FLC* in *A.*
327 *thaliana* and of *PEP1* in *A. alpina* depended on the length of the vernalization treatment (Shindo et
328 al., 2006; Lazaro et al., 2018). Insufficient vernalization lead to unstable silencing of *FLC* and
329 *PEP1*, therefore delayed flowering (Shindo et al., 2006; Duncan et al., 2015). However, the
330 reactivation of *Bna.FLC* in apical buds in Express617 plants, after cold, was not limited to plants
331 with short pre-vernalization times, where floral commitment was not yet established, indicating that
332 the *Bna.FLC* genes might have additional functions at later stages of development.
333
334 Flowering time is a yield-associated trait (Shi et al., 2009). Studies aimed at mapping genomic
335 regions associated with flowering time, enabling breeders to develop new germplasms with allelic
336 combinations that exhibit optimal flowering time to maximize yield in a certain geographical
337 region. Until recently, QTL mapping and GWAS were the main approaches followed to determine
338 the genetic loci associated with flowering time in rapeseed. In these studies, flowering time was
339 measured as the interval between the date of sowing and the date when the first flowers opened on
340 50 % of the plants (Long et al., 2007; Raman et al., 2013; Wang et al., 2016; Shah et al., 2018). Our
341 study demonstrated that the process of floral induction at the meristem of winter rapeseed, which is
342 established by low temperatures, early during its growing season, and the process of flower
343 emergence later in spring, are two different processes separated by a long winter. Therefore,
344 phenotyping flowering time by the first opened flower might not necessarily lead to the
345 identification of flowering time regulators. Instead, genes that control the long dormant-like stage,
346 or growth and stem elongation and senescence might be identified. Interestingly, genes involved in
347 ethylene biosynthesis and signaling, were found to be in genomic regions with a strong selective-
348 sweep between winter and spring types (Wu et al., 2019). In consistence with the later flowering
349 being associated with higher yield, recently, it has been shown that warmer temperatures in
350 October, which lead to later floral induction in winter rapeseed, were associated with higher yields

(Brown et al., 2019; O'Neill et al., 2019). Therefore, more emphasis on the cold duration required for floral transition in different cultivars might identify genetic loci associated with longer vegetative growth and finally higher yields. Although SD conditions did not contribute to a major delay in flowering of the winter rapeseed lines studied, however, SDs have been shown to inhibit further inflorescence development in certain Northern European populations of the perennial *Arabidopsis lyrata* (Kemi et al.,2019). This might also be the case in winter rapeseed plants grown under SDs. Therefore, further studies concerning the effect of photoperiod on rapeseed inflorescence development, which is of great importance for rapeseed breeding and improvement, should be carried out. Furthermore, the expression patterns of the *Bna.SOC1.A05/C04-random* and *Bna.SPL5.A05/C05* homeologs are strongly associated with floral transition at the meristem and can be used as potential transcriptional markers to score early or late floral transition in different winter rapeseed genotypes. Finally, mutant analyses and functional studies will help to elucidate the role of these genes for the initiation of flowering.

5 Data availability

The RNA-Seq data and the Express617 genome sequence and annotation have been deposited in NCBI's SRA under the project number PRJNA647273 (https://dataview.ncbi.nlm.nih.gov/object/PRJNA647273?reviewer=78pleb6f8k4tgcisav246to3eh) and PRJNA668034 (https://submit.ncbi.nlm.nih.gov/subs/bioproject/). The expression data can be provided upon request.

6 Author Contribution

SM designed and performed experiments, conducted the data analysis and wrote the manuscript. AK designed the bioinformatics pipeline and proofread the manuscript. DH performed the RNA-

Seq library preparation and sequencing. BW designed RNA sequencing experiments and edited the manuscript. SM designed and performed experiments and wrote the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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553

8 Figures

Fig. 1 Effect of photoperiod on flowering time of winter rapeseed. Box-plots showing variation for days to flowering in ten different rapeseed genotypes grown under four different photoperiodic conditions. Flowering time was recorded as opening of the first flower. Ten plants per accessions were analyzed. LD-LD, 4°C /16h light followed by 20 °C/16h light, LD-SD, 4°C /16h light followed by 20 °C/8h light, SD-LD, 4°C /8h light followed by 20 °C/16h light, SD-SD, 4°C /8h light followed by 20 °C/8h light.

Fig. 2 Floral transition at the shoot apical meristem of Express617 plants during vernalization. Toluidine blue−stained longitudinal sections through apices of Express617 plants collected weekly during vernalization at 4°C under LD conditions. (a) to (c) Meristem of Express617 plants after 3, 4 and five weeks of vernalization. (d) Dome shaped meristem resembling an inflorescence meristem after six weeks of vernalization. (e) to (f) an inflorescence meristem with floral meristem after seven and eight weeks of vernalization. Bar = $100\mu m$, black arrows shows floral meristem.

Fig. 3 Transcriptomic changes mirroring the transition to flowering in shoot apical meristems. (a) Expression profiles of *FLC* orthologs that belong to profile I and their paralogs. (b) to (e) Expression profiles of genes, that belong to profile II. The genes included orthologs of *SOC1, SPL5 FUL and FAF2.* (f) Expression profiles of orthologs of *SPL15*. (g) to (i) Expression profiles of genes, *Bna.AP1*, *Bna.AP3* and *Bna.PI.* Error bars represent the lower and upper bounds of the 95% confidence interval of the abundance of the corresponding gene isoform.

Fig. 4 Comparing the expression of flowering time genes between leaves and meristems. Apices and leaves from winter rapeseed Express617 were harvested before (BV) and 2, 4, 5, 7, 8-

and 9-weeks during vernalization. Gene expression was quantified relative to *Bna.Actin*. Error bars were defined by the SEM of three biological samples.

Fig. 5 Age-dependent delay of flowering. (a) Flowering time of Express617 plants, with different days of pre-culture, with 20 to 24 plants per group. 21 days pre-culture, 14 days pre-culture, 9 days pre-culture and 7 days pre-culture. (b) to (d) Relative expression analysis of *Bna.FLC, Bna.SOC1* and *Bna.SPL5* in apices of Express617 plants with four different pre-culture durations. Expression was normalized to *Bna.GAPDH.* Error bars were defined by the SEM of three biological samples. Light blue block corresponds to the vernalization period.

9 Supporting Information

Fig. S1 Flowering of winter rapeseed under field condition.

Fig. S2 Variation between Express617 and Darmor-*bzh*

Fig. S3 Total number of expressed and differentially expressed genes

Fig. S4 Expression profiles of shoot apical meristem marker genes.

Fig. S5 Studied gene expression profiles in RNA-seq.

Fig. S6 Expression profiles of orthologs of the five clades of *SPL* genes.

Fig. S7 Shoot apical meristem of Express617 plants two weeks after vernalization.

Fig. S8 Relative expression of *Bna.AP1* in SAM of plants with different preculture periods

Table S1 Primers used in this study.

Table S2 Sequence data of *Brassica napus* meristem marker genes compared to their Arabidopsis orthologs.

Table S3 Genes that were analyzed in this study and their sequence identity with Arabidopsis.

Table S4 Sequence data of *Brassica napus* floral meristem and floral organ identity genes compared to their Arabidopsis orthologs.

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