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Molecular genetics and physiological analyses of the transition to flowering in winter rapeseed

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List of Abbreviations

%	Percent
°C	Degrees Celsius
µm	Micro-meter
<i>A. alpina</i> , <i>Aa</i>	<i>Arabis alpina</i>
<i>AG</i>	<i>AGAMOUS</i>
<i>AGL6</i>	<i>AGAMOUS-like 6</i>
<i>AGL19</i>	<i>AGAMOUS-like 19</i>
<i>AGL24</i>	<i>AGAMOUS-like 24</i>
<i>API</i>	<i>APETALA 1</i>
<i>AP3</i>	<i>APETALA 3</i>
<i>A. thaliana</i> , <i>At</i>	<i>Arabidopsis thaliana</i>
<i>AS1</i>	<i>ARABIDOPSIS PHANTASTICA-LIKE 1</i>
<i>Am</i>	<i>Antirrhinum majus</i>
<i>AV</i>	After vernalization
<i>Bam</i>	Binary alignment map
<i>BLAST</i>	Basic Local Alignment Search Tool
<i>bp</i>	Base pair
<i>B. cretica</i>	<i>Brassica cretica</i>
<i>B. incana</i>	<i>Brassica incana</i>
<i>B. napus</i> , <i>Bna</i>	<i>Brassica napus</i>
<i>B. nigra</i>	<i>Brassica nigra</i>
<i>B.oleracea</i>	<i>Brassica oleracea</i>
<i>B. rapa</i>	<i>Brassica rapa</i>
<i>BWA</i>	Burrow Wheelers Aligner
<i>BV</i>	Before vernalization
<i>Cas</i>	CRISPR-associated
<i>CHIP</i>	Chromatin immunoprecipitation
<i>CLV1</i>	<i>CLAVATA 1</i>
<i>CL2</i>	<i>CLAVATA 2</i>
<i>CO</i>	<i>CONSTANS</i>
<i>CRISPR</i>	Clustered Regularly Interspaced Short Palindromic Repeats
<i>DN</i>	Day neutral
<i>DH</i>	Doubled haploid
<i>DNA</i>	Deoxyribonucleic acid
<i>FAA</i>	Formaldehyde-acetic acid alcohol
<i>FAF2</i>	<i>FANTASTIC FOUR 2</i>
<i>FD</i>	<i>FLOWERING LOCUS D</i>
<i>FRI</i>	<i>FRIGIDA</i>
<i>FLC</i>	<i>FLOWERING LOCUS C</i>
<i>FLM</i>	<i>FLOWERING LOCUS M</i>
<i>FT</i>	<i>FLOWERING LOCUS T</i>

<i>FUL</i>	<i>FRUITFULL</i>
FM	Floral meristem
FTi	Flowering time
<i>GA2</i>	<i>GIBBERELLIN 2-OXIDASE 2</i>
<i>GA2x1</i>	<i>GIBBERELLIN 2-OXIDASE 1</i>
<i>GAPDH</i>	<i>GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE</i>
GWAS	Genome wide association study
h	Hour
IGV	Integrative Genome Viewer
IM	Inflorescence meristem
InDel	Insertion/Deletion
Kb	Kilo bases
LD	Long day
<i>LFY</i>	<i>LEAFY</i>
miR156	MicroRNA-156
MADS	MCM1 AGAMOUS DEFICIENS SRF
NCBI	National Center for Biotechnology Information
MITE	Miniature Inverted-repeats Transposable Element
<i>NF-YC4</i>	<i>NUCLEAR FACTOR Y, SUBUNIT C04</i>
PAV	Presence/absence variation
PCR	Polymerase chain reaction
PcG	Polycomb group
<i>PI</i>	<i>PISTILLATA</i>
<i>PRR9</i>	<i>PSEUDO-RESPONSE REGULATOR 9</i>
<i>PEPI</i>	<i>PERPETUAL FLOWERING 1</i>
<i>PhyB</i>	<i>PHYTOCHROME B</i>
PHD	Plant Homeodomain
QTL	Quantitative trait locus
RFLP	Restriction Fragment Length Polymorphism
RNA-Seq	Ribonucleic acid sequencing
RPKM	Read per kilobase per million
RT-qPCR	Realtime quantitative polymerase chain reaction
Sam	Sequence alignment map
SAM	Shoot apical meristem
SD	Short days
SEM	Standard error of mean
<i>SEP3</i>	<i>SEPALLATA 3</i>
SNP	Single nucleotide polymorphism
<i>SOC1</i>	<i>SUPRESSOR OF OVEREXPRESSION OF CONSTANS 1</i>
<i>SPL</i>	<i>SQUAMOSA PROMOTER BINDING PROTEIN-LIKE</i>
SRA	Sequence Read Archive
<i>STM</i>	<i>SHOOT MERISTEMLESS</i>
STAR	Spliced Transcripts Alignment to a Reference
SV	Structural variation

<i>SVP</i>	<i>SHORT VEGETATIVE PHASE</i>
TILLING	Targeting Induced Local Lesions IN Genomes
<i>TFL1</i>	<i>TERMINAL FLOWER 1</i>
<i>TFL2</i>	<i>TERMINAL FLOWER 2</i>
TNDH	Tapidor and Ningyou7 doubled haploid
VCF	Variant Call Format
<i>VIN3</i>	<i>VERNALIZATION INSENSITIVE 3</i>
<i>VRN1</i>	<i>VERNALIZATION 1</i>
<i>VRN2</i>	<i>VERNALIZATION 2</i>

1 General introduction

1.1 Floral transition at the shoot apical meristem

While plants and animals are remarkably similar in their basic metabolism and cellular structure, they bear almost no resemblance when it comes to development. A fundamental difference between plants and animals is that the main body features and organ systems in higher animals are laid out during embryo development, while those of higher plants are established later in their lifecycle. A mature plant embryo is simple, consisting mainly of two organ systems, an axis, with a root meristem and a shoot meristem, and cotyledons. However, although simple, the germinating mature embryo, termed as seedling, is armed with a multitude of sensors and mechanisms that allow it to respond to different environmental and endogenous signals. In seedlings, the tip of the axis accommodates the shoot apical meristem (SAM). Cells differentiating from the SAM are the ultimate source of all postembryonic aerial structures of a plant. A remarkable feature of the SAM lies not only in its capability to maintain itself through continuous division and differentiation of cells, but also through its ability to undergo several distinct transitions in identity during the lifecycle of a plant (Irish & Nelson, 1991; Pidkowich et al., 1999; Steeves & Sussex, 1989). A major transition in identity occurs when the SAM switches from a vegetative meristem into a reproductive meristem, in a process known as floral transition. The timing of this major switch is not arbitrary, as it takes place under precise environmental and developmental control, and determines the reproductive success of the plant. In flowering plants such as *Arabidopsis thaliana*, floral transition transforms the vegetative meristem into an inflorescence meristem (IM), marking the beginning of the reproductive development and the termination of any further vegetative growth. The IM produces floral meristems, which in turn give rise to flowers.

In a process as complicated as floral transition, timing is everything. Correct flowering time ensures that plants flower in the most favorable environmental conditions for outcrossing and optimal seed setting. Early flowering, that is a short vegetative period, leads to lower yield potentials; however, delayed flowering might lead to premature termination of seed development by hot summers or onset of the winter season (Worland et al., 1998). In addition, early flowering before winter can be detrimental for yield as it enhances the vulnerability of a plant to frost damage. Therefore, understanding the factors that cause flowering time variation is essential to predicting yield variation in crops.

1.2 *Brassica napus*, genetic architecture, evolution and breeding

As a recent allotetraploid, *Brassica napus* (rapeseed, canola, rutabaga, swede, AACC, $2n = 38$) was formed by a spontaneous hybridization of two diploid species, *Brassica rapa* (AA, $2n = 20$) and *Brassica oleracea* (CC, $2n = 18$), around 7500 years ago (Friedt et al., 2018). No true wild *B. napus* forms are known (Friedt et al., 2018). The two progenitors are ancient diploids that evolved into morphologically diverse subspecies with distinct genome structures, therefore, the precise identities of the two ancestors of *B. napus* remain unknown. Recent comparative genomic studies, in addition to previous archeological and linguistic studies, suggested that the A subgenome originated from the ancestor of European turnip while the C subgenome originated from a common ancestor of several subspecies of *B. oleracea* (Lu et al., 2019). The first assembled *B. napus* genome, of the winter rapeseed cultivar ‘Darmor-bzh’, comprised 101,040 gene models,

with most orthologous genes from *B. rapa* and *B. oleracea* maintained as homologous pairs in the sequenced genome. At the chromosome level, homologous exchange, especially at syntenic regions, is prevalent between the two subgenomes (Chalhoub et al., 2014), leading to genome restructuring and creating a basis for natural variation in *B. napus*.

The allotetraploid genome of *B. napus* is a driving force for its broad adaptation to natural environments, climatic zones and latitudes (Chalhoub et al., 2014). As a crop, *B. napus* appeared in Europe as winter rape around the year 1600 (Campo & Prakash, 1999), and was mainly cultivated for its oil-rich seeds (Campo & Prakash, 1999). It was then developed and spread to England in the late 18th century, and was introduced to China in the 1930s (Qian et al., 2006). As a result, three different growth types of rapeseed are present nowadays. Winter types, grown mainly in Northern Europe, are biennials that have an obligate vernalization requirement to flower. Spring types, however, are annuals that flower without vernalization, and are grown mainly in Canada and Australia. Semi winter types are grown in China and are adapted to moderate winter temperatures and have a mild vernalization requirement (Leijten et al., 2018).

During the short history of *B. napus* cultivation, the antinutritional compounds in the seeds, mainly erucic acid and aliphatic glucosinolates, have been reduced dramatically, whereas seed yield and seed oil content were remarkably improved (Snowdon et al., 2007). After the introduction of the so-called 00-rapeseed cultivars (minimal erucic acid and low glucosinolates) the production of rapeseed skyrocketed, and rapeseed oil became the third most consumed vegetable oil after palm and soybean oil. Currently, in addition to being a source of vegetable oil, it is also used for biodiesel production, and the residual meals after oil extraction are used as a protein rich animal feed.

Genetic variation between and within species is indispensable for breeding and improvement of crops. However, intensive breeding and selection, and the introduction of 00-rapeseed cultivars, in addition to the limited germplasm represented by very few known landraces, caused a substantial reduction in the genetic diversity of rapeseed (Friedt et al., 2018). Therefore, several efforts have been made to broaden the genetic fundament in rapeseed, exploiting both interspecific and intraspecific variations. For instance, novel resistance alleles have been introduced to rapeseed by crossing elite cultivars with related species from the Brassicaceae family, such as *B. rapa* and *B. nigra*, followed by successive backcrossing (Friedt et al., 2018). Re-synthesis of rapeseed using *B. rapa* and *B. oleracea* has also been employed to introduce new phenotypic and allelic variation into the rapeseed germplasm (Friedt et al., 2018). In addition to *B. rapa* and *B. oleracea*, wild *Brassica* species such as *B. incana* and *B. cretica* have also been used to generate resynthesized rapeseed lines (Jesske et al., 2013). Molecular marker analysis showed that the resynthesized *B. napus* lines are genetically divergent from *B. napus* cultivars, and are therefore a valuable source for novel genomic variation (Becker et al., 1995; Jesske et al., 2013). Existing intraspecific variation has also been employed to produce genetically diverse populations that broadened the genetic diversity in rapeseed breeding and research. These populations were derived from different parents depending on the varying trait. For instance, the Tapidor/Ningyou7 and Lynx-037DH/Monty028DH doubled haploid (DH) populations were used to develop high density consensus maps to identify genomic regions associated with flowering time, in addition to oil content and quality (Friedt et al., 2018). Beyond natural variation, mutagenesis, whether random mutagenesis, for example by TILLING, or targeted mutagenesis, for instance by

CRISPR-Cas, have also been proven to be effective strategies to increase genetic variation of certain agronomic traits in rapeseed (Braatz et al., 2017; Karunarathna et al., 2020; Sashidhar et al., 2020).

Like many members of the Brassicaceae family, rapeseed is a facultative outcrossing species (Becker et al., 1992), where open pollinated or hybrids can be developed. Nevertheless, as hybrids out-perform open pollinating varieties in terms of yield, most of the new varieties registered today are hybrids (Friedt et al., 2018). Moreover, hybrids display higher and more stable oil content. They also tend to have a faster and more vigorous development in fall, thus a better winter survival (Friedt et al., 2018). Major goals of rapeseed breeding are winter hardiness, disease resistance (mainly against blackleg disease, *Verticillium* wilt and sclerotinia) low erucic acid and glucosinolate content, and most importantly, increasing oil content and seed yield (Friedt & Snowdon, 2010).

1.3 Arabidopsis as a model plant for studying the genetic basis of flowering time

In the last four decades, *Arabidopsis* has become a central genetic reference for understanding the molecular mechanism of flowering time in plants in general, and in Brassicaceae in particular. *Arabidopsis* is a facultative long day plant that flowers earlier under long day conditions, but it also responds to several environmental and endogenous signals that influence flowering in other species, making it an excellent model to decipher the complex network of signaling pathways that control flowering time. One of the most important environmental factors that regulate flowering time in temperate regions is temperature. Several plant species, including winter annuals of *Arabidopsis*, require a long period of low temperature in order to undergo floral transition, a process known as vernalization (Bouché et al., 2017). Plants that require vernalization to flower, encode a repressor that impairs floral induction by directly repressing flowering promoting genes (Boss et al., 2004).

Under long-day conditions, summer annuals of *Arabidopsis* flower rapidly and complete their lifecycle within few weeks, whereas, winter annuals remain vegetative for months. However, after 6 -12 weeks at 4°C, winter annuals flower rapidly when returned to long-days 20°C (Andrés & Coupland, 2012). Genetic studies in *Arabidopsis* demonstrated that winter annuals encode functional alleles of two loci, *FLOWERING LOCUS C (FLC)* and *FRIGIDA (FRI)*. The *FRI* locus was first described by Napp-Zinn (1955) who examined the progeny of a cross between a late flowering winter accession and an early flowering summer accession. Later, *FRI* was cloned by map-based cloning and was shown to encode a coiled-coil plant-specific protein (Johanson et al., 2000). *FRI* interacts with several transcription factors to form a transcription activator complex, *FRI-complex (FRI-C)*, that activates the transcription of *FLC* (Choi et al., 2011).

FLC has been shown to be the master regulator of the vernalization-dependent flowering. *FLC* is a MADS-box transcription factor that acts as a transcriptional repressor (Michaels & Amasino, 1999; Sheldon et al., 1999). *FLC* is expressed in most parts of the plant (Sheldon et al., 1999) and the protein has over 500 binding sites, mostly located in the promoter regions of genes (Deng et al., 2011). The function of *FLC* has been shown to be mutually dependent on another MADS-box transcription factor, *SHORT VEGETATIVE PHASE (SVP)* (Li et al., 2008). *FLC* and *SVP* proteins form a heterodimer that directly represses main floral integrators in leaves and meristems (Li et al., 2008). Vernalization

leads to an epigenetic silencing of *FLC* by histone methylation (Bastow et al., 2004). Vernalization-driven methylation, at the *FLC* locus, is mediated by the activity of chromatin-modifying complexes. One of the main DNA-binding proteins, that is expressed only during vernalization, is *VERNALIZATION INSENSITIVE 3 (VIN3)*. *VIN3* is a plant homeodomain protein, involved in the deacetylation of *FLC* leading to histone methylation and the formation of heterochromatin at the *FLC* locus (Sung & Amasino, 2004). Other genes that are required for *FLC* methylation are *VERNALIZATION 1 (VRN1)* and *VERNALIZATION 2 (VRN2)*. *VRN1* is a DNA binding protein with a B3-domain, while *VRN2* is a member of Polycomb group (PcG) proteins (Gendall et al., 2001). Unlike *VIN3*, *VRN1* and the *VRN2* are constitutively expressed, and are involved in maintaining the repressed state of *FLC* after vernalization (Sung & Amasino, 2004). This cold-mediated mitotically-stable epigenetic silencing of *FLC*, releases the repression from its downstream targets, including several floral integrators, rendering the plant more competent to flower.

The vernalization requirement overrides daylength-mediated floral induction because *FLC* represses *FLOWERING LOCUS T (FT)*. *FT* is a core systemic signal that promotes flowering in response to long photoperiods (Searle et al., 2006). *FT* was isolated by activation-tagging, where T-DNA mutants flowered earlier and independently of photoperiod (Kardailsky et al., 1999; Kobayashi et al., 1999). Under long-day (LD) conditions, the transcriptional regulator, *CONSTANS (CO)*, induces expression of *FT* in the vascular tissues of leaves (An et al., 2004). The FT protein that has been identified as the long sought florigen, is transported through the phloem from the leaves to the SAM, where it interacts with a basic leucine-zipper transcription factor, *FLOWERING LOCUS D (FD)* (Corbesier et al., 2007). CHIP-sequencing (Chromatin immunoprecipitation-sequencing), in addition to earlier genetic studies, demonstrated that the FT-FD complex can directly bind and activate the floral meristem identity genes *FRUITFULL (FUL)* and *APETELA 1 (API)* to induce floral transition in Arabidopsis (Abe et al., 2005; Wigge et al., 2005).

While *FLC* is a master regulator of vernalization-dependent flowering, another MADS-box gene, termed *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1)*, is considered the ultimate switch at which several flowering signals converge. *SOC1* was isolated by a mutagenesis screen where *soc1* mutants suppressed the early flowering phenotype caused by overexpression of *CO* (Onouchi et al., 2000). *SOC1* was also isolated by activation-tagging where its overexpression partially rescued the late flowering phenotype of an Arabidopsis line with a functional *FRI* allele (Lee et al., 2000). *SOC1* is a target of *FLC*, therefore, its expression is strongly regulated by vernalization (Michaels et al., 2003; Moon et al., 2003). Upon downregulation of *FLC*, *SOC1* is upregulated in the SAM, and this meristematic expression is effective in promoting floral transition (Lee & Lee, 2010). *SOC1* is also a downstream target of *FT* and *CO* (Michaels et al., 2005; Searle et al., 2006; Yoo et al., 2005), hence, it acts as a cross-link between the vernalization and photoperiod pathways.

As a major floral integrator, *SOC1* binds to a plethora of genes including several floral meristem identity genes and floral homeotic genes (Immink et al., 2012), thereby, regulating flowering time and floral patterning. *SOC1* is expressed in the inflorescence meristem and in leaf primordia, however, it is excluded from floral primordia but reappears in later stages of flower development (Borner et al., 2000; Samach et al., 2000). *SOC1* interacts with *FUL*, to regulate determinacy of all meristems, and together, *SOC1*

and *FUL*, are involved in the prevention of secondary growth and longevity in annual plants (Melzer et al., 2008). It has also been shown that *SOC1* interacts with another MADS-box transcription factor, *AGAMOUS-LIKE 24* (*AGL24*), and that the heterodimerization of *SOC1* and *AGL24* is a key mechanism to activate the expression of the floral meristem identity gene *LEAFY* (*LFY*) (Lee et al., 2008).

In addition to temperature and photoperiod, *SOC1* integrates signals from an endogenous flowering pathway that is primarily defined by the developmental age of the plant (Lee & Lee, 2010). This age-dependent endogenous pathway involves a set of transcriptional regulators that are mostly microRNA-targeted transcription factors, known as *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) factors (Wang et al., 2009). In *Arabidopsis*, there are 16 *SPL* genes, of which, 11 are negatively regulated by microRNA156 (*miR156*) through transcript cleavage (Cardon et al., 1999; Guo et al., 2008). *miR156* is implicated in the juvenile-to-adult vegetative phase transition; it is highly expressed early in development and decreases as plants age (Wu & Poethig, 2006). However, *miR156* is not the sole regulator of *SPL* genes. For instance, *SPL3*, *SPL4* and *SPL5* are induced under long-day conditions, and are downregulated in *co* and *ft* mutants, suggesting that they act downstream of *FT* and *CO* at the shoot apex (Schmid et al., 2003). *SPL3/4/5* have also been shown to be downstream targets of *SOC1* (Jung et al., 2012). In addition, *SPL3* and *SPL15* have been shown to be downstream targets of *FLC* (Deng et al., 2011).

SPL15 is a key floral integrator that, similarly to *SOC1*, integrates age, temperature, photoperiod and phytohormone gibberellin signals to coordinate floral initiation (Bergonzi et al., 2013; Hyun et al., 2016). *SPL15* has been functionally characterized in *Arabidopsis*, as well as in its perennial relative, *Arabis alpina*. In *Arabidopsis*, *SPL15* promotes flowering under non-inductive short-day (SD) conditions, in a gibberellin-dependent manner, and together with *SOC1*, a complex is formed to activate downstream targets and finally to promote floral transition (Hyun et al., 2016). In *A. alpina*, *SPL15* is repressed by the *FLC* ortholog, *PERPETUAL FLOWERING 1* (*PEP1*), and its mRNA is targeted by *miR156*, hence, the activity of *AaSPL15* determines the age at which *A. alpina* is responsive to vernalization (Hyun et al., 2019).

Ultimately, after receiving inductive signals, the floral transition will commence and the vegetative meristem will convert into an inflorescence meristem. The inflorescence meristem will, in turn, give rise to floral meristems. This second phase of floral transition is governed by a set of downstream targets of *SOC1* and other meristem identity genes. Two main genes act synergistically to promote floral identity of the developing inflorescence meristem, *API* and *LFY* (Mandel & Yanofsky, 1995; Weigel et al., 1992). Double mutants of these genes develop shoots instead of flowers at the inflorescence (Huala & Sussex, 1992; Weigel et al., 1992). *LFY* encodes a plant-specific transcription factor that is expressed in very early floral primordia, promoting floral identity and determinacy (Weigel et al., 1992). In addition to being a direct downstream target of *SOC1*, *LFY* is also activated by the phytohormone GA (Weigel et al., 2000), and was proposed to be a downstream target of *SPL3* (Yamaguchi et al., 2009). *API* is a MADS-box transcription factor, which is expressed only in floral meristems and directs floral organ development (Gustafson-Brown et al., 1994). Once the floral transition is established, *API* represses the expression of *SOC1* in floral primordia, creating a sort of feedback loop in choice of floral versus inflorescence identity (Liu et al., 2007). At later stages of floral development, *API* activates the floral homeotic genes, *APETALA 3* (*AP3*),

PISTILLATA (PI) and *AGAMOUS (AG)*, thus regulating floral organ development (Kaufmann et al., 2010).

Flowers, the final product of the floral transition, are organized in concentric whorls of sepals, petals, stamens and carpels. The process by which distinct organ identities are specified by unique combinatorial spatio-temporal expression of the homeotic floral organ identity genes constitute the “ABC” model of flower organ development (Coen & Meyerowitz, 1991). In early stages of flower development, the ABC model genes are expressed throughout the developing floral buds. However, in later stages they become constrained to specific whorls (Irish, 2017). *API* together with *APETALA 2 (AP2)*, an EREBP (Ethylene-Responsive Element Binding Protein) family transcription factor, become restricted to the first and the second whorls of the flower, providing the A function. *AP3* and *PI* are restricted to the third whorl, providing the B function, and *AG* is only expressed in the fourth whorl providing the C function. Later, it has been shown that the activity of the B and C functions require another set of three closely related MADS-box genes termed *SEPALLATA1/2/3 (SEP1/2/3)* (Pelaz et al., 2000). Figure 1 summarizes the gene regulatory network that controls the process of floral transition at the shoot apical meristem of Arabidopsis.

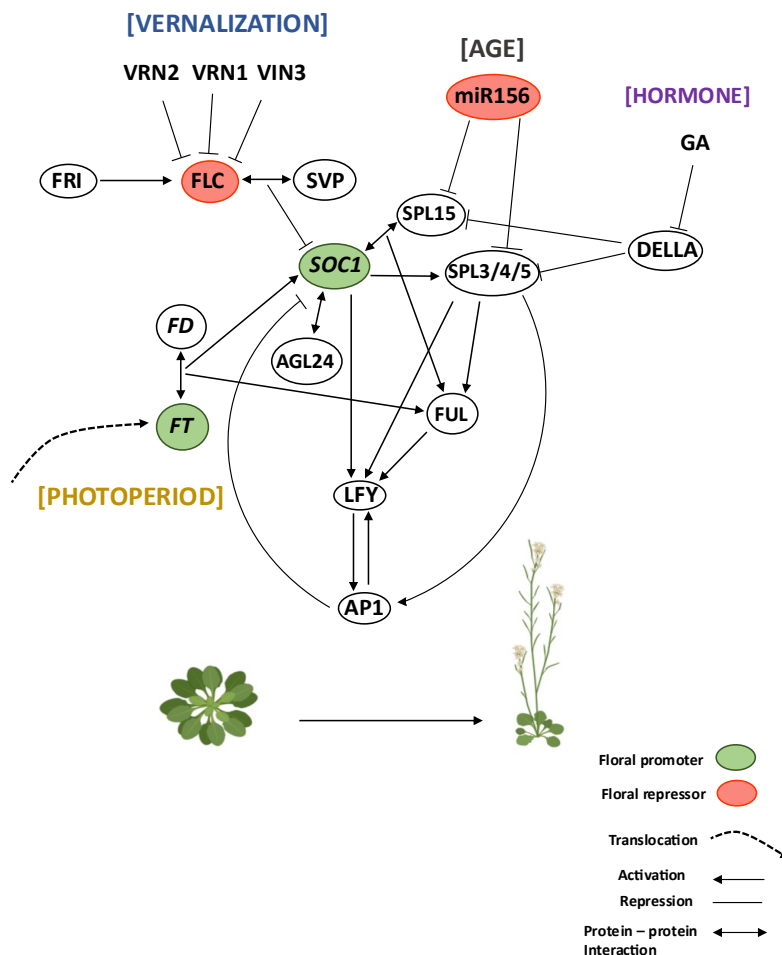


Figure 1: An overview of the flowering-time gene regulatory network at the SAM of Arabidopsis. The figure demonstrates molecular interactions between genes in the SAM involving different flowering time pathways (in square brackets) at the SAM of Arabidopsis. The main floral repressors are marked in red, and main floral integrators are marked in green. The network includes the floral integrator FT which is translocated from the leaves to the SAM.

1.4 Photoperiod, vernalization and juvenility in the genus *Brassica*

The plant family Brassicaceae, is proposed to have arisen within the past 100 million years with more than 3600 species distributed across the globe (Nikolov et al., 2019). Despite its species richness and diversity, members of this family are recognizable with their conserved cross-like floral structure, which inspired their botanical name Cruciferae (Nikolov et al., 2019). As members of the Brassicaceae family, *Brassica* species bear tetramerous flowers with four sepals, four alternating petals and six stamens. The inflorescence is a raceme, and the fruit is a silique with two valves each exposing a row of black or brown seeds arranged on each side of the replum (Friend, 1985).

Although highly conserved at the level of flower morphology, centuries of adaptation, selection and hybridization, lead to a great variation in the vegetative morphologies in *Brassica* species, in addition to considerable diversity in their environmental and developmental requirements for flowering (Friend, 1985). *Brassica* species are either long-day (LD) or day-neutral (DN) plants (Friend, 1985). The LD response can be obligate such as in Ethiopian mustard (*B. carinata*), or facultative as in Chinese cabbage and turnip (*B. rapa*) (Friend, 1985). The DN response is observed, mostly, in rosette plants that require vernalization, such as cabbage and kohlrabi (*B. oleracea*) (Friend, 1985).

The vernalization requirement in *Brassica* can be obligate, where plants will stay vegetative for years if not exposed to low temperatures, or facultative (preferential), where plants will eventually flower without low-temperature treatment, although low-temperature can accelerate flowering (Friend, 1985). The optimal duration of exposure to low temperature varies greatly between different *Brassica* species, and between varieties of the same species. For instance, the optimal duration for vernalization in European winter rapeseed is between 8-10 weeks at 2- 12°C (Filek et al., 2007). *Brassica* species differ in the way they respond to the vernalization treatment. In certain species, such as Brussels sprouts (*B. oleracea* var. *gemmifera*), there is a direct response to low temperatures and flower initiation starts during the cold. In the case of Brussel sprouts, however, if plants were transferred to higher temperatures before the formation of an inflorescence meristem, they will remain vegetative (Friend 1985). Such response to vernalization is termed as “direct-effect” vernalization. The optimal temperature for “direct effect” vernalization ranges from 10 to 15°C (Friend, 1985). In other *Brassica* species, such as Chinese cabbage (*B. rapa* var. *pekinensis*), where the germinating seedlings are responsive to vernalization, cold treatment is effective even if simultaneous flower initiation was restricted. In these species, flower initiation will be manifested after the cold treatment, thus, it is termed as “after-effect” vernalization.

In addition to photoperiod and vernalization, juvenility has been also shown to influence flowering. A juvenile phase is defined as the interval between germination of a seed and the ability of seedlings to respond to inductive environmental signals (Friend, 1985). The length of the juvenile phase varies between cultivars of the same plant. The end of the juvenile phase can be scored morphologically, by examining leaf numbers, stem diameter or corm diameter as in kohlrabi. Variation in the length of the juvenile phase is of economic importance. A long juvenile phase helps in preventing premature inflorescence initiation, which might occur in response to low-temperatures encountered soon after sowing.

1.5 Studies on flowering-time regulation in *B. napus*

Early genetic studies on *Brassica* flowering time, were carried out by analyzing segregating populations derived from crosses between annual and biennial cultivars, using RFLP (Restriction Fragment Length Polymorphism) mapping (Camargo & Osborn, 1996; Ferreira et al., 1995; Teutonico & Osborn, 1995). The studies identified two major flowering time QTLs in *B. rapa* and *B. oleracea*, and one major and a minor flowering time QTL in *B. napus*. Later, the map positions of the identified QTLs were compared between the *Brassica* species. The chromosomal segments were found to be conserved between *B. napus* and *B. rapa*, but no evidence of conservation was found in *B. oleracea* (Camargo & Osborn, 1996). The mapped QTL positions were also compared to syntenic regions in *Arabidopsis* (Osborn et al., 1997), where they showed strong homology to the tops of chromosome 5 and chromosome 4, where *FLC* and *FRI*, respectively, are located.

Tadegé et al. (2001) cloned five *Bna.FLC* homologs and showed that they delayed flowering in *Arabidopsis*, therefore, demonstrating that *Bna.FLC* genes are orthologs of the *Arabidopsis FLC* gene. Further studies identified nine *FLC* homologs in rapeseed, out of which, four were associated significantly with flowering-time QTLs and one homolog, *Bna.FLC.A03*, was differentially expressed between winter and semi-winter types (Zou et al., 2012). Moreover, the insertion of a tourist-like miniature inverted-repeat transposable elements (MITE), upstream of *Bna.FLC.A10* gene was found to be associated with vernalization requirement (Hou et al., 2012), and another 810-bp MITE insertion in exon 7 of *Bna.FLC.A02* was found to be associated with a spring habit of rapeseed plants (Yin et al., 2020). Finally, Schiessl et al. (2019) compared the expression patterns of all nine *FLC* orthologs between winter and spring rapeseed cultivars, and showed that only three copies, *Bna.FLC.A03b*, *Bna.FLC.A10* and *Bna.FLC.C02*, were differentially expressed between the two growth-types.

In addition to *FLC*, four *FRI* homologs were identified in *B. napus* by BAC library-screening and PCR-based cloning (Wang et al., 2011). One of the homologs, *Bna.FRI.A03*, co-localized with a major flowering-time QTL, in a doubled haploid population derived from a cross between a winter rapeseed cultivar, Tapidor, and a semi-winter cultivar, Ningyou7 (TNDH). Wang et al. (2011) identified polymorphisms in a functional domain of *Bna.FRI.A03*, that were associated with flowering time variation in 248 rapeseed accessions. *Bna.FRI.A03* was also functionally characterized in the Columbia (Col-0) accession of *Arabidopsis* carrying a functional *FLC* gene and a non-functional *FRI* (*FLC/fri*) gene. The study showed that both, number of days to flowering and *FLC* expression levels, were significantly higher in transgenic *Arabidopsis* carrying *Bna.FRI.A03* from Tapidor compared to transgenic plants carrying *Bna.FRI.A03* from Ningyou7 (Yi et al., 2018).

Other functional studies, further confirmed the conserved role of *Arabidopsis* flowering-time orthologs in *B. napus*. For instance, a splice-site mutation in one of the *Bna.FT* paralogs (*Bna.FT.C06b*) delayed flowering in winter rapeseed (Guo et al., 2014), and a stop-codon mutation in one of the *API* orthologs (*Bna.API.A02*), lead to the development of secondary floral buds instead of sepals, confirming its role as a floral meristem identity gene in rapeseed (Shah et al., 2018a). A list of *B. napus* flowering time genes that have been functionally characterized is shown in Table 1. Several other genes that are involved in floral transition in *Arabidopsis*, such as *CO*, *VIN3*, *VRN2*, in addition to other paralogs of *FT*, have also been shown to be aligned within *B. napus* flowering-time QTLs or on interacting loci, implying a role in flowering-time regulation in rapeseed. A list of major

flowering-time QTL studies, the DH populations used, and flowering-time genes aligned within or associated with these QTLs are shown in Table 2.

Table 1: List of flowering time genes that have been functionally characterized in *B. napus*.

Arabidopsis name	<i>B. napus</i> orthologue	Method	Phenotype	Reference
<i>FLC</i>	<i>Bna.FLC</i>	Over-expression of five <i>Bna.FLC</i> in Arabidopsis	Late flowering	(Tadege <i>et al.</i> , 2001)
<i>FRI</i>	<i>Bna.FRI.A03</i>	Overexpression in Arabidopsis (<i>FLC/fri</i>)	Late flowering	(Yi <i>et al.</i> , 2018)
<i>FT</i>	<i>Bna.FT.C06b</i>	EMS induced splice-site mutation in Express617	Late flowering	(Guo <i>et al.</i> , 2014)
<i>TFL1</i>	<i>Bna.TFL1.A10</i>	EMS induced nonsense mutation in Express617	Increased total number of seeds per plant	(Guo <i>et al.</i> , 2014)
	<i>Bna.TFL1.C03</i>	CRISPR-Cas9 induced mutations	Early flowering	(Sriboon <i>et al.</i> , 2020)
<i>API</i>	<i>Bna.API.A02</i>	EMS induced nonsense mutation in Express617	Development of secondary floral buds	(Shah <i>et al.</i> , 2018a)

Table 2: Flowering time QTL studies in rapeseed.

FTi orthologs within QTL	DH population	QTL	Reference
<i>Bna.AGL19.A01a</i> <i>Bna.VR2.A01</i> <i>Bna.PHY2.A01</i>	Tapidor/Ningyou7	qFT1-1	(Long et al., 2007)
<i>Bna.FD.A01</i> <i>Bna.AP2.A01</i>		qFT1-2	
<i>Bna.AP1.A02</i> <i>Bna.CAL.A02</i>		qFT2-4	
		qFT3-2*	
<i>Bna.CO.A10</i>		qFT10-3	
<i>Bna.FT.C06a</i> <i>Bna.FT.C06b</i> <i>Bna.AP1.C06a</i>		qFT16-3	
<i>Bna.AGL18.C08</i>		qFT18-2	
<i>Bna.FLC.C09a</i> <i>Bna.FLC.C09b</i>		qFT19-4	
<i>Bna.TFL2.C09</i>		qFT19-3	
<i>Bna.FRI.A03</i>		Tapidor/Ningyou7	
<i>Bna.CAL.A02</i> <i>Bna.AP1.A02</i> <i>Bna.GA2x1.A02</i>	Skipton/Ag-Spectrum	Qdtf(f).wwai-A2	(Raman et al., 2013)
<i>Bna.FRI.A03</i> <i>Bna.FLC.A3b</i> <i>Bna.TFL1.A03</i> <i>Bna.VIN3.A03</i>		Qdtf(f).wwai-A3	
<i>Bna.SVP.A04</i>		Qdtf(f).wwai-A4	
		Qdtf(f).wwai-A9*	
<i>Bna.FLC.C02</i>		Qdtf(f).wwai-C2b	
<i>Bna.FLC.C03b</i>		Qdtf(f).wwai-C3a	
		FQ1*	(Luo et al., 2014)
		FQ2*	
<i>Bna.FT.A07b</i>		FQ3	
		FQ4*	
<i>Bna.FT.A07</i>	Lynx-037DH/ Monty-028DH	brPb-660356	(Nelson et al., 2014)
<i>Bna.FLC.C03b</i>		brPb-809530	

<i>Bna.TFL2.A02</i> <i>Bna.CO.A02</i>		brPb-809917	
	Polo/Topas	qFLR-A1a*	(Javed et al., 2016)
<i>Bna.FY.A02</i> <i>Bna.AGL6.A02</i> <i>Bna.PRR.A02</i>		qFLR-A2b	
		qFLR-A2c*	
		qFLR-A10b*	
		qFLR-C3c*	
	Nos. 5246 /4512	cqDTFA2a*	(Liu et al., 2016)
<i>Bna.AP1.A07</i>		cqDTFA7a	
		cqDTFC2a*	
		cqDTFC8*	
<i>Bna.PRR9.A02</i>	Y689/WESTAR	cqFT.A02-1	(Shen et al., 2018)
<i>Bna.FT.A02</i>		cqFT.A02-2	
<i>Bna.FLM.A03</i>	APL01/Holly	qFT.A3-1	(Yu et al., 2019)
<i>Bna.GAD1.A07</i>		qFT.A7-2	
<i>Bna.AS1.C06</i>		qFT.C4-2	
<i>Bna.GA5.C07</i>		qFT.C7-1	
	GH06/P174	qFTA02*	(Jian et al., 2019)
<i>Bna.CIB1.A06</i> <i>Bna.NF-YC4.A06</i>		qFTA06	
		qFTA08*	
<i>Bna.FY.C02</i> <i>Bna.PRR7.C02</i> <i>Bna.UBP13.C02</i>		qFTC02	
<i>Bna.FLC.A02</i> <i>Bna.FT.A02</i>	Cabriolet/Darmor	ChrA02-QTL	(Tudor et al., 2020)

*QTL where known flowering time genes were not found.

Genome-wide association studies (GWAS), using a high-density marker set, such as the *Brassica* 60 k-Illumina Infinium SNP array, were also conducted to further investigate the genetic architecture of flowering-time in rapeseed (Raman et al., 2016; Schiessl et al., 2015; Xu et al., 2015). More recently, whole genome sequencing of worldwide collections of rapeseed germplasm was also used to investigate the genetic variation underlying the evolution and flowering-time divergence among the three rapeseed ecotypes (Lu et al., 2019; Wu et al., 2019). Wu et al. (2019) reported global genomic variation by re-sequencing 991 rapeseed accessions, and studied genomic selective signals that were produced by natural or artificial selection of rapeseed. Several selective sweeps were found between spring and winter types, semi-winter and winter types, and spring and semi winter types. The strongest selective-sweep was found between spring and winter types, on a region on chromosome A10, where 56 annotated genes, including *Bna.FLC.A10*, are located. The second strong selective-sweep was found on a region on chromosome A02. The region included *Bna.FT.A02* and other genes involved in flowering-time control and ethylene biosynthesis and signaling. The GWAS study also showed that SNPs in the same regions of chromosomes A02 and A10, were significantly-associated with flowering time. Another recent study, employed the genome sequences of 588 *B. napus* accessions, and reported strong selective signals in regions harboring orthologs of genes involved in maintenance of floral organ identity and regulation of floral meristem growth (Lu et al., 2019). A list with major flowering-time GWAS, the technology used and the flowering-time genes in association are shown in **Table 3**.

Table 3: Flowering time GWAS in rapeseed

FTi orthologs in association	Method	Reference
<i>Bna.CCA1</i> <i>Bna.FT</i> <i>Bna.FUL</i>	158 winter rapeseed cultivars genotyped with 60K-Illumina Infinium SNP array	(Schiessl et al., 2015)
<i>Bna.CO.C09</i> <i>Bna.FLC.A10</i> <i>Bna.VIN3.A02</i> <i>Bna.SVP.A09</i>	523 rapeseed genotypes genotyped with 60 k-Illumina Infinium SNP array	(Xu et al., 2015)
<i>Bna.FT</i> <i>Bna.FLC.A02</i> <i>Bna.FRI</i> <i>Bna.FUL</i> <i>Bna.CO</i> <i>Bna.PHYB</i>	Genotyping by sequencing DArT (Diversity Array Technology)	(Raman et al., 2016)
<i>Bna.AGL24.A04</i> <i>Bna.AGL18.A04</i> <i>Bna.PIE.A04</i> <i>Bna.FLC.A03</i> <i>Bna.FRI.A03</i>	448 inbred rapeseed lines genotyped with 60 K-Illumina Infinium SNP array	(Wang et al., 2016)
<i>Bna.FUL.A09</i> <i>Bna.FUL.C07</i> <i>Bna.SOCI.A03</i> <i>Bna.SPL15.A04</i> <i>Bna.FLC.A03a</i> <i>Bna.VIN3.A02</i>	158 winter-type accessions using 21,623 unique SNP markers from the <i>Brassica</i> Infinium 60K SNP Chip	(Shah et al., 2018b)

<i>Bna.FLC.A02</i> <i>Bna.FLC.C02</i>	Whole genome sequencing of 588 rapeseed accessions	(Lu et al., 2019)
<i>Bna.FLC.A10</i> <i>Bna.FT.A02</i>	Whole genome sequencing of 991 rapeseed accessions	(Wu et al., 2019)
<i>Bna.FLC.A10</i> <i>Bna.FLC.C02</i> <i>Bna.FLC.A02</i>	PAV-GWAS using Illumina and PacBio sequencing for 16 rapeseed accessions	(Song et al., 2020)

1.6 Transcriptome studies on flowering-time regulation in rapeseed

In addition to genomic studies, studying the transcriptional activity of genes, in different tissues, under different conditions or during a developmental time series, is a robust as well as indispensable approach to infer the function and biological activity of a gene. Moreover, whole transcriptome analysis is ideal to study the transcriptional activity of a set of genes during a certain developmental process, and is especially valuable in polyploids as it can distinguish between very similar paralogs of a gene.

Whole transcriptome sequencing has been used to study the control of flowering time, and to investigate the spatio-temporal expression dynamics of gene paralogs in *B. napus* (Jones et al., 2018; O'Neill et al., 2019; Shah et al., 2018b). The first study on paralog-specific expression dynamics of flowering-time regulating genes was conducted using a leaf transcriptome of the semi-winter rapeseed line, Ningyou7, at different developmental stages (Shah et al., 2018b). The study combined transcriptome data with association mapping, and reported 36 genes that were differentially expressed before and after vernalization and were in association with flowering time, seed yield or both. The reported genes included orthologs of *SOC1* (*Bna.SOC1.A05* and *Bna.SOC1.A03*), *SPL15* (*Bna.SPL15.A04*) and *FUL* (*Bna.FUL.A09* and *Bna.FUL.C07*). A second study used a transcriptome time series from apices and leaves across the development of a spring rapeseed cultivar, Westar (Jones et al., 2018). The study showed that flowering-time genes are preferentially retained relative to other genes in the *B. napus* genome, and that more than 60% of these genes exhibited a divergent expression pattern relative to their homologs in the *B. napus* genome. A case study for the rapeseed ortholog of the meristem identity gene *TFL1*, showed that differences in the promoter region could explain the expression divergence.

1.7 Scientific hypotheses and objectives

In the field, winter rapeseed plants proceed through floral transition during autumn and over-winter with floral buds in a manner similar to perennials. Therefore, my aim was a comprehensive understanding of the cold-driven floral transition in winter rapeseed, through analyzing the timing of floral transition at morphological and molecular levels, and to determine the gene cascade that responds early to the cold signal at the SAM.

The study was carried out based on the hypothesis that winter rapeseed plants, at a certain developmental age, respond to vernalization very early and undergo floral transition during cold, and that the temporal expression of orthologs of floral integrators marks the different stages of the meristem development.

I wanted to answer the following questions:

- When do winter rapeseed plants initiate floral transition?
- Which genes mark the initiation of floral transition and the following developmental stages of the SAM?
- How does plant's age affect the transcriptional activity of orthologs of floral integrators and the timing of floral transition?
- Do sequences of flowering time genes differ between spring and winter types?
- If there were any structural variation in flowering time genes, from which *B. napus* progenitor did this variation originate? and what is its functional impact?

2 Transition to flowering in winter rapeseed during vernalization

Plant, Cell and Environment 2020

2.1 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.

2.2 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 (Amasino, 2004; Chouard, 1960). 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 *SOCI* 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 *Arabidopsis 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., 2017).

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* (Long et al., 2007; Udall et al., 2006). Association mapping or Genome-wide association studies (GWAS) were also used to study polymorphism associated with different flowering phenologies (Shah et al., 2018; Wu et al., 2019; Xu et al., 2015). 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 (Lu et al., 2019; Shah et al., 2018; Wu 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.

2.3 Materials and Methods

2.3.1 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 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 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). 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.

2.3.2 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.

2.3.3 Sample preparation and nucleic acid extraction

For RNA-Seq and RT-qPCR 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).

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

2.3.5 Transcriptome analysis

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

2.3.6 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 $\Delta\Delta C_t$ method (Livak & Schmittgen, 2001). Primers used for gene expression analysis are listed in Supplementary Table 1. *Bna.ACTIN2* and *Bna.GAPDH* were used as endogenous controls to normalize gene expression levels.

2.4 Results

2.4.1 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 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 (Supplementary Figure 1a). 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 (Supplementary Figure 1b), 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 (Figure 2a). 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 (Figure 2b). 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 (Figure 2c). 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 (Figure 2d). 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.

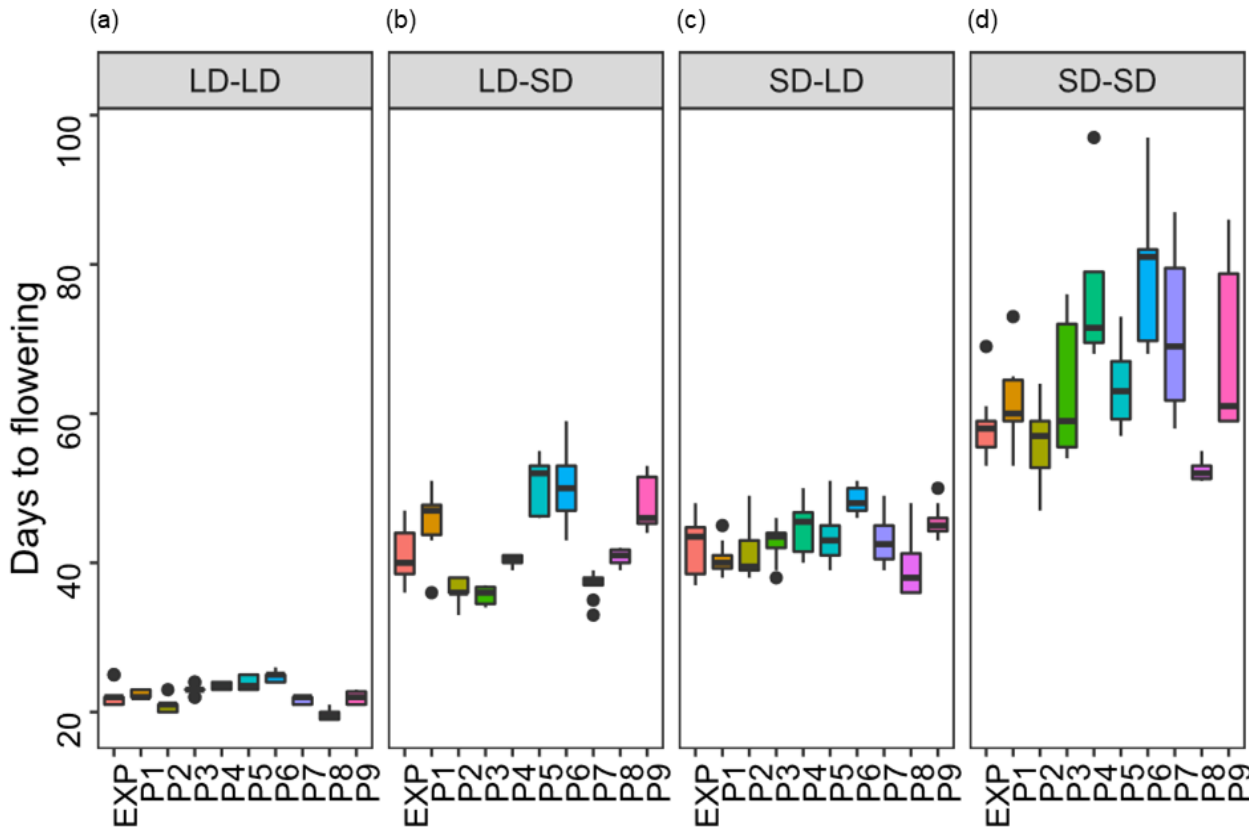


Figure 2: Effect of photoperiod on flowering time of winter rapeseed. Box-plots showing variation for days to flowering in ten different rapeseed genotypes grown under four different photoperiodic conditions. Flowering time was recorded as opening of the first flower. Ten plants per accessions were analyzed. 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.

2.4.2 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. After five weeks of vernalization, the SAM was still in a vegetative stage (Figure 3c), but had significantly increased in size compared to the SAM in the third and fourth weeks of vernalization (Figure 3b, c). One week later, the meristem appeared more dome shaped, resembling an inflorescence meristem (Figure 3d), 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 (Figure 3e, f).

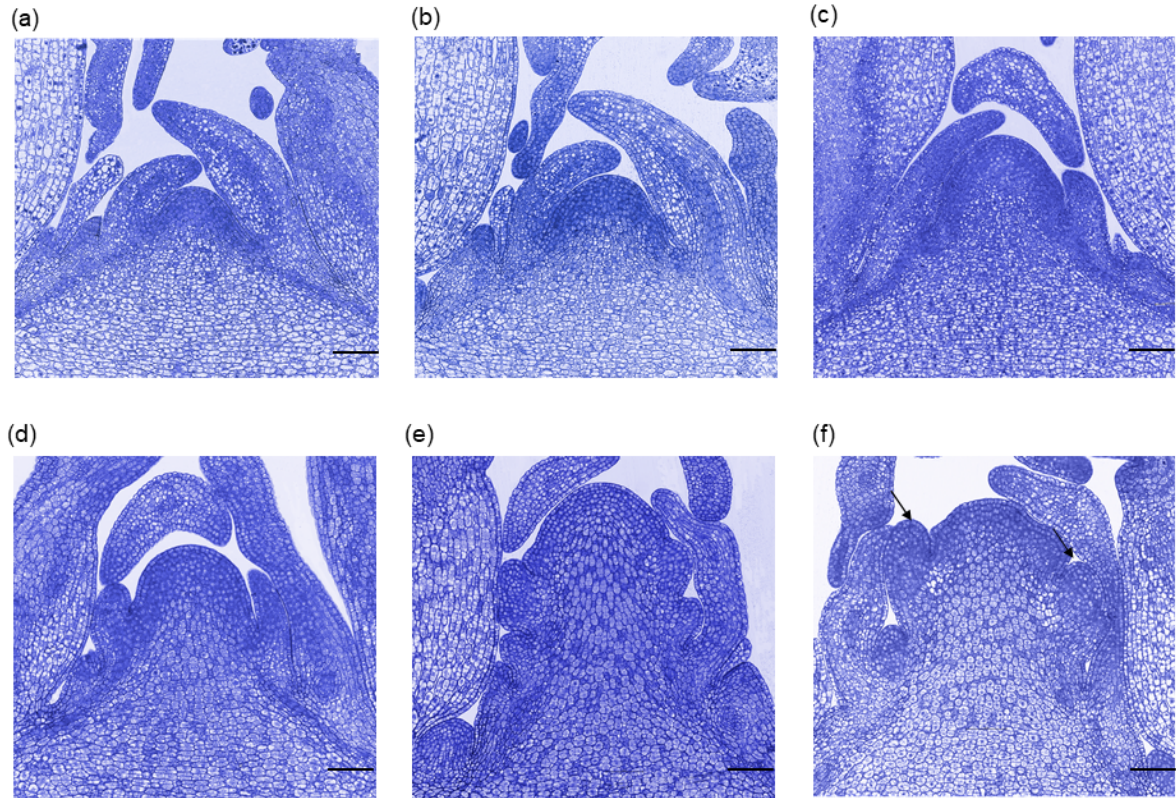


Figure 3: Floral transition at the shoot apical meristem of Express617 plants during vernalization.

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 μ m, black arrows shows floral meristem.

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 (Supplementary Figure 2). 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 ($RPKM \geq 1$) of about 45,000 genes in the annotated Express617 genome sequence at least one of the nine time points (Supplementary Figure 3a). *B. napus* orthologs of *A. thaliana* SAM marker genes such as *SHOOT MERISTEMLESS (STM)*, *CLAVATA1 (CLV1)* and *CLAVATA2 (CLV2)*

(Supplementary Table 2) were detected in all samples (Supplementary Figure 4), indicating that the detection of expression of meristem-specific genes was possible. Interestingly, pairwise comparison of expression levels at the eight time points showed that the highest number of differentially expressed genes (\log_2 fold change ≥ 1) was between the fifth and sixth week of vernalization, indicating that a profound change had taken place between these two time points, which was in accordance with our morphological analyses (Supplementary Figure 3b).

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 (Supplementary Figure 5). 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 (Supplementary Figure 5b). 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 (Supplementary Figure 5c).

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 (Figure 4a). 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; Schiessl et al., 2019; Wu 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 (Figure 4a). 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 (Figure 4a).

The second profile comprised 34 genes, including homeologs of key floral integrators in *A. thaliana* and downstream targets of *FLC*, *SOC1*. 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 (Figure 4b). 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 (Figure 4c). 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 (Figure 4d), 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 (Figure 4e). 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 (API)* defines the initiation of flower development (Hempel et al., 1997; Lohmann & Weigel, 2002). None of the orthologs of *API* 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 Supplementary Table 3.

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) 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 (Figure 4f). Most of the identified *Bna.SPL* genes were stably expressed in the meristems of Express617 plants (Supplementary Figure 6). Only *Bna.SPL3.C03*, *Bna.SPL8.C05* and *Bna.SPL8.A10* were upregulated during vernalization, although at relatively low levels (Supplementary Figure 6).

The *B. napus* orthologs of *API* were detected in the third profile, marking the floral determination at the meristems of Express617 plants after eight weeks of vernalization. *Bna.API* homeologs were only detectable after eight weeks of vernalization and continued to increase their expression levels in the following week (Figure 4g, Supplementary Table 4). The upregulation of *B. napus* orthologs of floral organ identity genes, such as *APETALA 3* and *PISTILLATA*, after nine weeks of vernalization (Figure 4h, i, Supplementary Table 4) confirmed the onset of flower development at this stage.

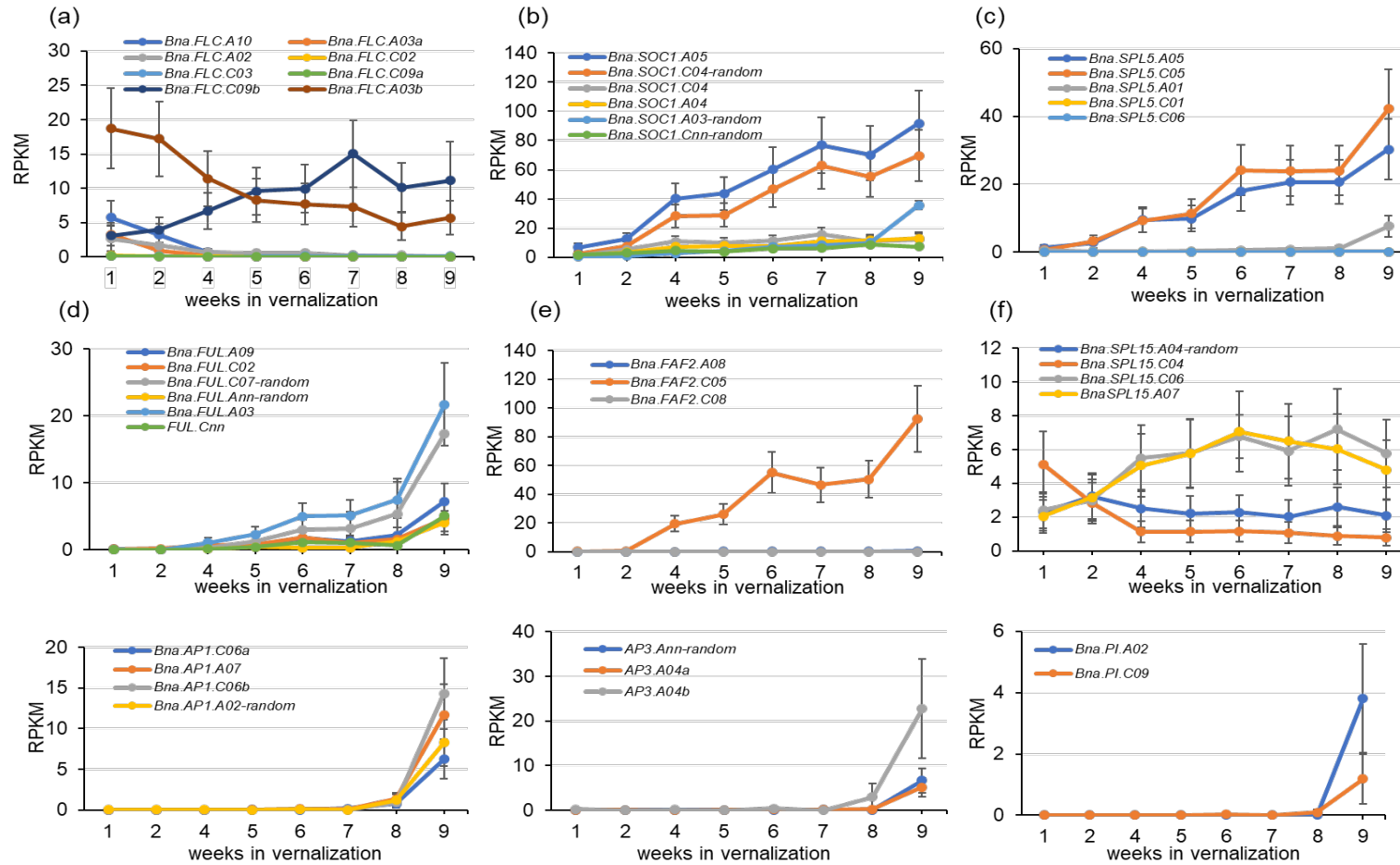


Figure 4: Transcriptomic changes mirroring the transition to flowering in shoot apical meristems. (a) Expression profiles of *FLC* orthologs that belong to profile I and their paralogs. (b) to (e) Expression profiles of genes, that belong to profile II. The genes included orthologs of *SOC1*, *SPL5* *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.

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 (Figure 5a-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 (Figure 5d) and *Bna.SPL15* transcripts, in leaves, were detected only after nine weeks of vernalization (Figure 5e).

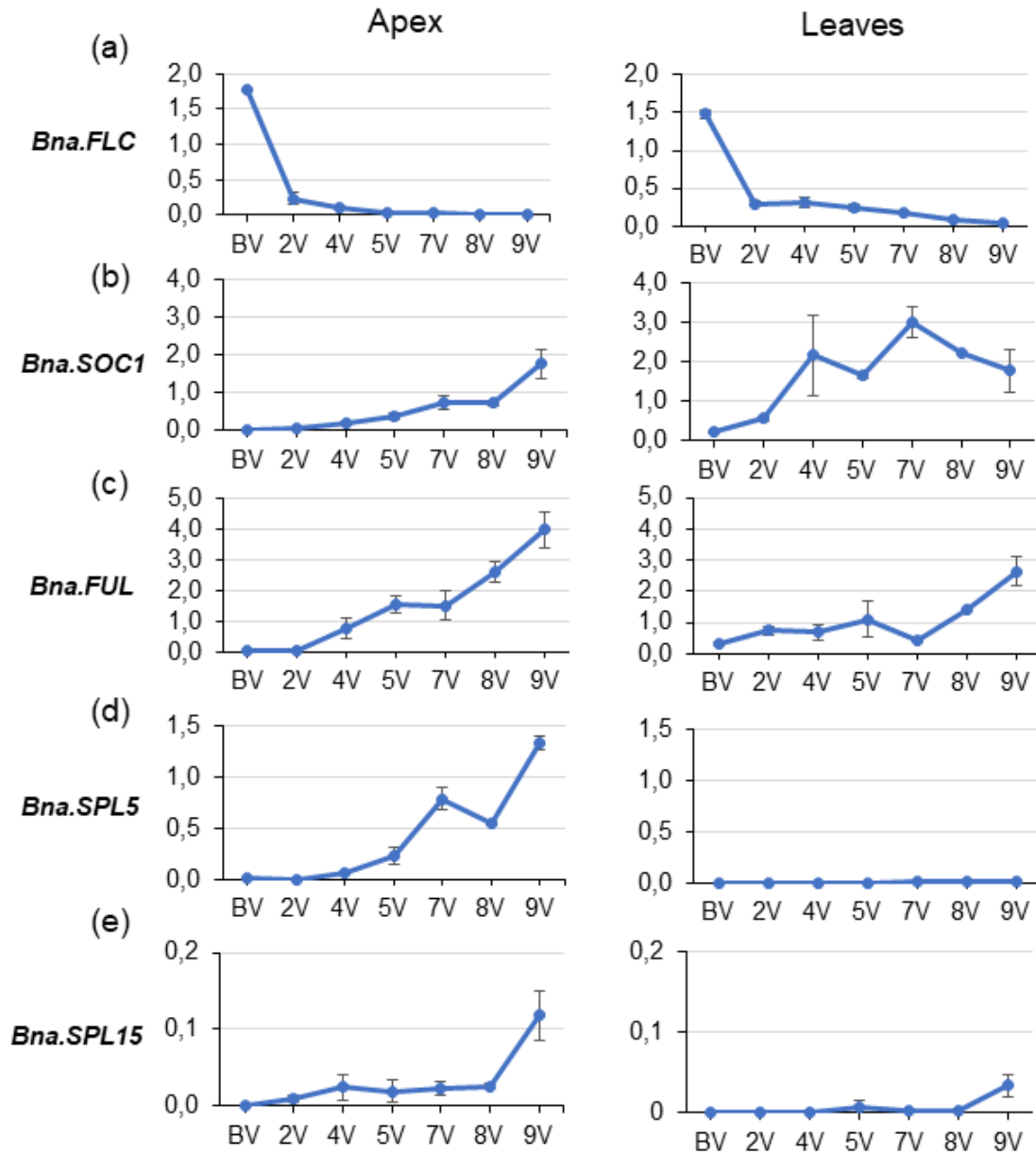


Figure 5: Comparing the expression of flowering time genes between leaves and meristems. Apices and leaves from winter rapeseed Express617 were harvested before (BV) and 2, 4, 5, 7, 8- and 9-weeks during vernalization. Gene expression was quantified relative to *Bna.Actin*. Error bars were defined by the SEM of three biological samples.

2.4.3 Pre-vernialization 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-vernialization growth flowered in a synchronized manner, 21 days after vernalization. With two weeks of pre-vernialization growth, flowering remained synchronized but was one week delayed. Only nine days of pre-vernialization growth desynchronized and further delayed flowering to 36 days after vernalization. Finally, seven days of pre-vernialization growth caused a drastic delay in flowering, where plants flowered very asynchronously, about 72 days after vernalization (Figure 6a).

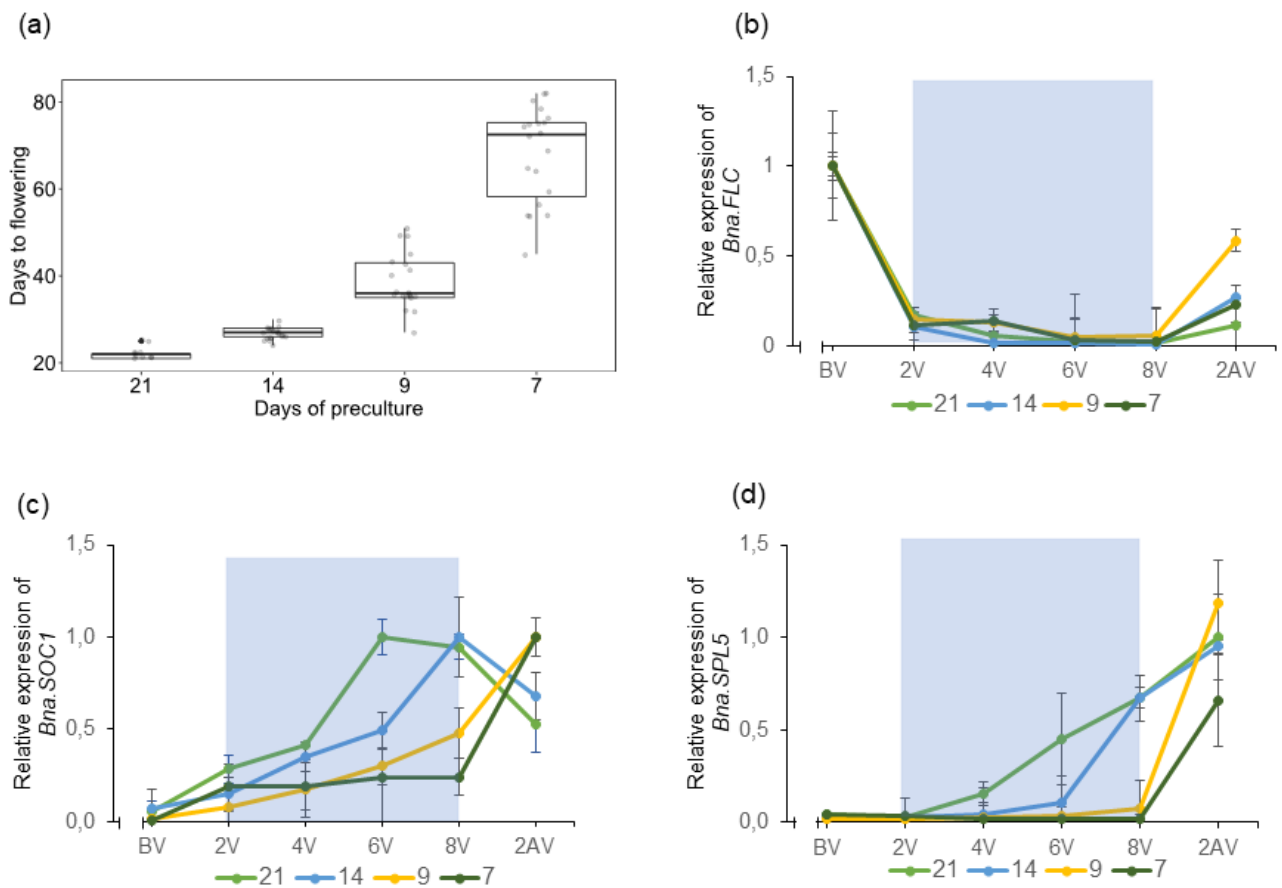


Figure 6: Age-dependent delay of flowering. (a) Flowering time of Express617 plants, with different days of pre-culture, with 20 to 24 plants per group. 21 days pre-culture, 14 days pre-culture, 9 days pre-culture and 7 days pre-culture. (b) to (d) Relative expression analysis of *Bna.FLC*, *Bna.SOC1* 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.

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 (Figure 6b), 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 (Figure 6d). 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 SAM, two weeks after vernalization, showed that plants with two weeks preculture had already inflorescence meristem with emerging floral buds (Supplementary Figure 7a), plants with nine-days preculture had a less advanced inflorescence meristem with visible floral meristems on its flanks (Supplementary Figure 7b), while plants with one-week preculture the SAM was dome shaped resembling an early stage of inflorescence meristem development (Supplementary Figure 7c). Moreover, expression analysis at SAM showed that *Bna.API* was already induced in the plants with three, two weeks and nine days of preculture, two weeks after vernalization, while it remained very low in plants with one-week of preculture (Supplementary Figure 8). These results further confirm that shorter growth periods before vernalization delay floral transition.

2.5 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*.

2.5.1 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.

2.5.2 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 (Borner et al., 2000; Samach 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 (Figure 4b, 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 establish floral commitment only after several weeks. In *A. alpina* accessions with an obligate vernalization requirement, adult plants undergo floral transition after five weeks of vernalization under controlled environmental conditions, and additional seven weeks are

required for the formation of floral buds (Wang et al., 2009b). In Express617 plants, the upregulation of orthologs of floral organ identity genes, such as *AP3* and *PI*, which mark the initiation of floral organ development, was observed only one week after the expression of *Bna.API*. The relatively short duration between the upregulation of *Bna.API* and the orthologs of floral organ identity genes, indicates that, unlike in *A. alpina*, flower primordia of winter rapeseed plants acquire a floral fate very rapidly without the need of continuous exposure to cold.

In addition to *Bna.SOC1* and *Bna.SPL5* we saw in other gene families that some genes were preferentially expressed. For instance, for *Bna.FLC*, *Bna.FAF2*, and *Bna.SPL* gene families only some members showed a differential expression, whereas the others showed no differences or were not expressed (Figure 4, Supplementary Figure 6). Previous studies have shown that polyploidy creates an expression bias, where certain homeologs from one ancestor are preferentially and highly expressed relative to other homeologs (Combes et al., 2013; Grover et al., 2012). However, we saw no preferential expression of genes coming either from the *B. rapa* or the *B. oleracea* ancestor. In fact, we saw that for *Bna.SOC1* and *Bna.SPL5* the two predominantly expressed genes were from both the A and C genomes (Figure 4b, c).

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 (Supplementary Figure 6), 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).

2.5.3 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 (Figure 6a). RT-qPCR analyses showed that *Bna.FLC* genes were significantly downregulated upon vernalization regardless of plant's age (Figure 6b), 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 (Figure 6c), 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 enough cold exposure, they flower independent of *Bna.FLC*. A stable silencing of *FLC* in *A. thaliana* and of *PEP1* in *A. alpina* depended on the length of the vernalization treatment (Lazaro et al., 2018; Shindo et al., 2006). Insufficient vernalization lead to unstable silencing of *FLC* and *PEP1*, therefore delayed flowering (Duncan et al., 2015; Shindo et al., 2006). However, the reactivation of *Bna.FLC* in apical buds in Express617 plants, after cold, was not limited to plants with short pre-vernalization times, where floral commitment was not yet established, indicating that the *Bna.FLC* genes might have additional functions at later stages of development.

Flowering time is a yield-associated trait (Shi et al., 2009). Studies aimed at mapping genomic regions associated with flowering time, enabling breeders to develop new germplasms with allelic combinations that exhibit optimal flowering time to maximize yield in a certain geographical region. Until recently, QTL mapping and GWAS were the main approaches followed to determine the genetic loci associated with flowering time in rapeseed. In these studies, flowering time was measured as the interval between the date of sowing and the date when the first flowers opened on 50 % of the plants (Long et al., 2007; Raman et al., 2013; Shah et al., 2018; Wang et al., 2016). Our study demonstrated that the process of floral induction at the meristem of winter rapeseed, which is established by low temperatures, early during its growing season, and the process of flower emergence later in spring, are two different processes separated by a long winter. Therefore, phenotyping flowering time by the first opened flower might not necessarily lead to the identification of flowering time regulators. Instead, genes that control the long dormant-like stage, or growth and stem elongation and senescence might be identified. Interestingly, genes involved in ethylene biosynthesis and signaling, were found to be in genomic regions with a strong selective-sweep between winter and spring types (Wu et al., 2019). In consistence with the later flowering being associated with higher yield, recently, it has been shown that warmer temperatures in October, which lead to later floral induction in winter rapeseed, were associated with higher yields (Brown *et al.*, 2019; O'Neill *et al.*, 2019). 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. 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. 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.

2.6 Data availability

The DNA- and RNA-Seq data, and the Express617 genome sequence and annotation have been deposited in NCBI's SRA under the project number PRJNA647273 and PRJNA668034, respectively. The expression data can be provided upon request.

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2.8 Supporting Information

Supplementary Figure 1: Flowering of winter rapeseed under field condition.

Supplementary Figure 2: Variation between Express617 and *Darmor-bzh*

Supplementary Figure 3: Total number of expressed and differentially expressed genes

Supplementary Figure 4: Expression profiles of shoot apical meristem marker genes.

Supplementary Figure 5: Studied gene expression profiles in RNA-seq.

Supplementary Figure 6: Expression profiles of orthologs of the five clades of *SPL* genes.

Supplementary Figure 7: Shoot apical meristem of Express617 plants two weeks after vernalization.

Supplementary Figure 8: Relative expression of *Bna.API* in SAM of plants with different preculture periods

Supplementary Table 1: Primers used in this study.

Supplementary Table 2: Sequence data of *Brassica napus* meristem marker genes compared to their Arabidopsis orthologs.

Supplementary Table 3: Genes that were analyzed in this study and their sequence identity with Arabidopsis.

Supplementary Table 4: Sequence data of *Brassica napus* floral meristem and floral organ identity genes compared to their Arabidopsis orthologs.

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Chapter 2

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3 Natural variation in the promoter region of *Bna.SOC1.A05* is associated with winter habit in rapeseed

3.1 Abstract

During the course of rapeseed domestication and breeding, genetic diversity of this species allowed to adapt it to different eco-geographical regions and to shape its useful traits. Structural variations, including presence/absence variations, are thought to play a major role in the genetic diversity and phenotypic plasticity of rapeseed. In this study, we detected a 598-bp presence/absence variation within the promoter region of an orthologue of a major flowering time gene and a downstream target of *FLC*, *SOC1*. The 589-bp sequence originated from *B. rapa*, likely inserted upstream of *Bna.SOC1.A05* after the hybridization of the two rapeseed ancestors and was selected for winter rapeseed adaptation to temperate regions. The process resulted in a strong reduction in nucleotide diversity in the genomic region harboring *Bna.SOC1.A05* and upstream sequences. Cis-regulatory element analysis showed that the insertion contains an ACGTG box, which has been shown to be the strongest binding motif for the HY5 transcription factor in *Arabidopsis*. In addition, expression analysis showed that expression levels of *Bna.SOC1.A05* are lower in accessions carrying the insertion compared to the ones that have no insertion.

3.2 Introduction

Rapeseed (*Brassica napus*, $A_nA_nC_nC_n$) is an allotetraploid crop formed by spontaneous interspecific hybridization of two diploid species; *Brassica rapa* (A_nA_n) and *Brassica oleracea* (C_nC_n) around 7500 years ago (Chalhoub et al., 2014). Rapeseed was evolved rapidly to one of the most important oil crops grown worldwide. Its dynamic allopolyploid genome plays a prime role in its diversification and adaptation to different natural environments. According to vernalization requirement, *B. napus* can be divided into three different growth types; winter, semi-winter and spring rapeseed (Zou et al., 2019). Winter types are grown mainly in Europe; they have an obligate vernalization requirement, and are suggested to be the original type of rapeseed (Lu et al., 2019). Spring types are grown in Australia and North America and flower without any vernalization requirement. Semi-winter types were formed gradually after the introduction of winter types into China in the twentieth century, where they were adapted to flower after a short period of vernalization (Qian et al., 2006). The divergence in growth types of rapeseed accessions has been associated mainly to allelic variation, represented by short (SNPs or InDels) or large-scale structural variations (SVs), within or upstream of flowering time genes especially in vernalization responsive genes and downstream targets such as *Bna.FLC* (*FLOWERING LOCUS C*), *Bna.FRI* (*FRIGIDA*) and *Bna.FT* (*FLOWERING LOCUS T*) (Wang et al., 2011; Wu et al., 2019).

Whole genome sequencing of *B. napus* revealed a highly complex and redundant genome, with high frequency of homologous exchanges between the two subgenomes (Chalhoub et al., 2014). Homologous exchanges can be translocations, duplications and deletions,

which led to extensive presence/absence variations (PAVs) and segmental deletions, and represent an additional source of genetic diversity (Hurgobin et al., 2018; Schiessl et al., 2017). Several studies have revealed the association of PAVs with important agronomic traits such as disease resistance and oil content in rapeseed (Gabur et al., 2018; Liu et al., 2012; Qian et al., 2016; Stein et al., 2017). For instance, deletion of the Arabidopsis orthologous *NON-YELLOWING 1 (NYE1)* gene (*Bna.NYE.A01*) was associated with increased chlorophyll content, which simultaneously affected oil-content and adaptation to cooler environments (Qian et al., 2016). Transposable elements are an important driving force for genomic diversity. It can affect gene functions through insertions into coding regions, or influence gene expression through insertion into regulatory elements or by affecting local chromatin structure (Anderson et al., 2019; Chuong et al., 2017; Lisch, 2013). For instance, in rapeseed, the insertion of a tourist-like miniature inverted-repeat transposable elements (MITE), upstream of the *Bna.FLC.A10* gene was found to be associated with vernalization requirement (Hou et al., 2012). Moreover, a 810-bp MITE insertion in exon 7 of *Bna.FLC.A02* silenced the gene and was found to be associated with a spring habit of rapeseed plants lacking the MITE insertion in *Bna.FLC.A10* (Yin et al., 2020). Recently, a study revealed that up to 10% of genes in the rapeseed genome are affected by SVs, for which half of these variations are between 100 to 1000 bp long (Chawla et al., 2020). The study also reported a SV upstream of *Bna.FT.A02* that was associated with the eco-geographical distribution of different rapeseed cultivars. Therefore, studying SVs in flowering time genes might be a useful approach to further understand the history of evolution and adaptation of rapeseed and it might also facilitate the development of molecular markers to differentiate between different growth types.

Assuming that genomic variation in other flowering time genes might have played a role in the evolution and divergence of rapeseed, we analyzed SVs within and upstream of orthologs of key downstream targets of *Bna.FLC* genes. Therefore, we used the comprehensive resource of genomic sequences of a worldwide collection of 991 rapeseed germplasms (Wu et al., 2019). We uncovered a previously unidentified sequence insertion upstream of *Bna.SOCI.A05*, which is associated with vernalization requirement. We also confirmed this variation in the ASSYST *B. napus* diversity set (Bus et al., 2011). Further analysis suggested that the sequence was inserted in the promoter region of *Bna.SOCI.A05* after the merge of the two ancestral genomes. Evidence of a selective sweep was reflected by the dramatic reduction of nucleotide diversity in the region harboring this insertion compared to its surrounding region. The strong co-segregation of this SV with vernalization behavior indicated an additionally and potentially crucial role of this genomic re-arrangement for the eco-geographical adaptation of rapeseed.

3.3 Materials and Methods

3.3.1 Plant materials and growth conditions

For expression analysis, the two spring rapeseed accessions that carry the insertion, Bingo and Adamo, and the winter inbred line Express617, from the ERANET-ASSYST panel (Bus et al., 2011), in addition to the spring accession Haydn that lacks the insertion, were used. Seeds were sown on soil in 9 cm pots and grown under greenhouse conditions in 16h light/8h dark cycles at 20°C. Flowering time was measured in days from sowing to opening of the first flower.

3.3.2 RNA extraction and expression analysis

For RT-qPCR, leaves and shoot apices were sampled at 2, 4 and 6 weeks after sowing. Apical buds were dissected under a binocular microscope using scalpel and forceps and immediately frozen in liquid nitrogen. Eight to ten 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).

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 $\Delta\Delta C_t$ method (Livak & Schmittgen, 2001). Primers used for gene expression analysis are listed in Supplementary Table 1. *Bna.ACTIN2* was used to normalize gene expression levels.

3.3.3 Transcriptome analysis

For RNA-Seq analyses, the raw RNA-Seq reads of 72 rapeseed accessions including 31 winter, and 41 spring types from an associative transcriptomics study (Harper et al., 2012; Havlickova et al., 2018) were downloaded from SRA NCBI (<https://www.ncbi.nlm.nih.gov>) using parallel-fastq-dump. The reads were mapped to the Express617 genome (Matar et al., 2020) using STAR (Dobin et al., 2013) (v.2.5.4). For transcriptome assembly, the RNA-Seq read alignments were used as an input file to run Cufflinks (v2.2.1) (Trapnell et al., 2012). The Cufflinks module, Cuffnorm, was used to generate normalized transcript expression levels (normalized FPKM) from all rapeseed accessions studied. Expression levels of six *Bna.SOC1* genes were plotted in RStudio (RStudio Team, 2020), using the data visualization package ggplot2 (Wickham, 2011).

3.3.4 Sequence analysis and detection of presence/absence variation

Whole genome sequencing data of 80 rapeseed accessions were downloaded from the SRA NCBI using parallel-fastq-dump. Reads of each accession were mapped to the Darmor-*bzh*-based genome assembly of winter rapeseed Express617 using Burrows-Wheeler Aligner mem (BWA-MEM v.0.7.17) software package (Li & Durbin, 2009). Visual detection of read alignments were performed using the Integrative genome viewer (IGV). Per base depth of coverage was calculated for each accession at the genomic region of six *Bna.SOC1* orthologues in the Express617 genome, including 3000 bp upstream, using Samtools (Li et al., 2009). PCR using genomic DNA from the ASSYST *B. napus* diversity set of different spring and winter rapeseed accessions (Bus et al., 2011), followed by Sanger sequencing was used to further confirm the presence/absence variation. Sequence alignment was visualized on the multiple sequence alignment visualization and editing program Jalview (Waterhouse et al., 2009).

NSITE-PL (Solovyev et al., 2010) was used to analyze the putative promoter region and the composition of functional motifs upstream of *Bna.SOC1.A05* other *Bna.SOC1* orthologues and *At.SOC1*.

3.3.5 Phylogenetic and nucleotide diversity analysis

For evolutionary analyses, SNPs within 10.5-kb genomic fragments including the insertion and 5000-bp flanking sequence, were extracted from whole genome SNP files of

1007 accessions. A phylogenetic tree was constructed using Tassel software package (v5.2.59), and visualized using iTOL. To study whether the upstream region of *Bna.SOC1.A05* was under selection, nucleotide diversity (π) was calculated for chromosome A05 using VCFtools (v0.1.13) in a 1000 bp sliding window with 100-bp steps in 80 rapeseed accessions (Wu et al., 2019).

3.3.6 Screening of homologous sequences in *B. napus* and *B. rapa* genomes

To identify homologous sequences, the insertion sequence was queried against the Express617 and *B. rapa* (Chiifu-401-42) reference genomes using BLAST+ package from NCBI. Circos software package (Krzywinski et al., 2009) was used to visualize the position of the insertion sequence in *B. napus* and *B. rapa* genomes. RepeatMasker Web Server (Smit et al., 2013-2015) and MITE-Tracker (Crescente et al., 2018) were used to search for putative repetitive and transposable elements. Multiple sequence alignments and phylogenetic analyses of the homologous sequences from *B. napus* and *B. rapa* were performed using the multiple sequence alignment program Clustal Omega (McWilliam et al., 2013).

3.4 Results

3.4.1 A 598-bp insertion upstream of *Bna.SOC1.A05* is associated with winter habit

The Express617 genome assembly contained six orthologues of the floral integrator *SOC1* (

Supplementary Table 5). As recently shown, the *Bna.SOC1.A05* and *Bna.SOC1.C04-random* genes were highly induced in shoot apical meristems during the floral transition under vernalization conditions (Chapter 2). To study SVs between different rapeseed cultivars, within or upstream of *Bna.SOC1* genes, we used the whole genome sequencing reads of 40 winter and 40 spring accessions, with an average sequence depth ranging from 5 to 23 (Supplementary Table 6). The reads were aligned to our Darmor-bzh reference-based genome assembly of the winter rapeseed cultivar Express617 (Chapter 2), using Bwa-mem (v.0.7.17). We first carried out a visual detection of read alignments using IGV with a mapping quality threshold of 5. We observed that certain accessions had no reads that mapped upstream to a distinct region of *Bna.SOC1.A05*. A closer examination showed that more than 87% of the spring accessions had no reads mapping to this region, while in 85% of the winter accessions reads mapped to it (Supplementary Figure 9A, B). Therefore, we concluded that this SV upstream of *Bna.SOC1.A05* segregates with the growth types of *B. napus* accessions, and that it might play a role in the vernalization requirement.

To further ascertain the boundaries of this SV we used Samtools to calculate the depth of coverage in the genomic regions including the gene sequence and 3000-bp upstream of it. Average sequence coverage was higher in winter types compared to spring types because certain spring types had low sequencing depth (Figure 7B, Supplementary Table 6). A closer examination showed that 1.8-kb upstream of *Bna.SOC1.A05* (Figure 7A) the sequencing coverage in spring accessions gradually dropped to zero, while in winter accessions it remained at the same level (Figure 7B, C).

Low sequencing depth is not always reliable to detect SVs, therefore, we further validated the PAV upstream of *Bna.SOC1.A05* via PCR with flanking primers and using genomic DNA from spring and winter accessions of the ASSYST-PANEL (Bus et al., 2011). The variation could be clearly distinguished by PCR, and showed that an insertion is present in 84% of the winter types but absent in 90% of the spring types (Supplementary Table 7). Out of the 60 tested accessions from the ASSYST panel, 22 accessions were common with accessions from the whole genome sequencing data. The PCR results of the 22 rapeseed accessions were in accordance with the whole genome sequencing data.

Based on SNP data of a 10.5-kb region flanking the insertion, we performed a phylogenetic analysis and found that the 80 rapeseed accessions were clustered into two groups that corresponded to winter and spring ecotypes, which suggested that the genomic variation in the region containing the insertion and *Bna.SOC1.A05* contributed to growth-type diversification (Supplementary Figure 11).

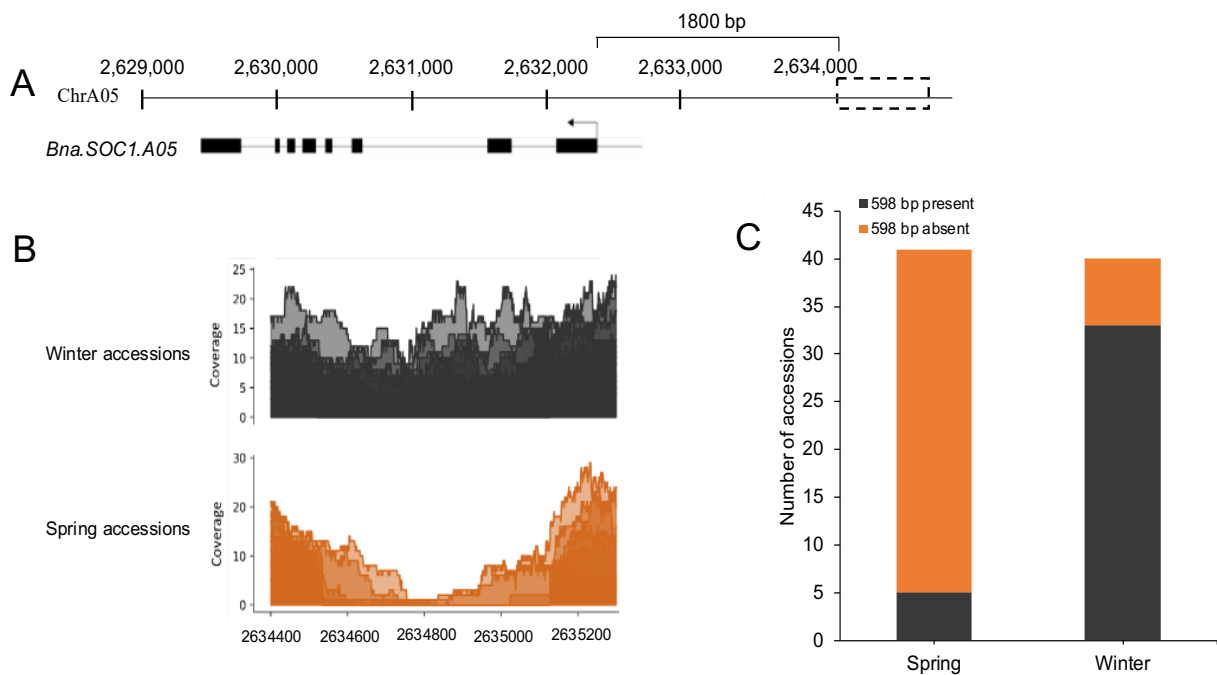


Figure 7: Presence/absence variation upstream of *Bna.SOC1.A05* is associated with growth type. (a) Genomic region harboring *Bna.SOC1.A05* and the presence/absence variation in Express617 genome (dotted box). (b) Per base depth of coverage in the region 2,634,400 to 2,635,200, showing drop in coverage in the spring accessions. (c) Stacked plot showing the presence of the insertion in 85% of the winter accession and deletion in 87% of the spring accessions studied.

3.4.2 The 598-bp insertion upstream of *Bna.SOC1.A05* originated from *B. rapa*

The PCR products from different rapeseed genotypes were sequenced using Sanger sequencing and aligned. The insertion upstream of *Bna.SOC1.A05* and the region flanking the insertion site showed a high sequence identity in all accessions (98-100%)

(Supplementary Figure 10), indicating that this intergenic region is conserved among the different rapeseed genotypes. The insertion sequence had no sequence similarity to repetitive and transposable elements on the RepeatMasker web server. In addition, the MITE annotating software, MITE Tracker (Crescente et al., 2018) did not annotate the insertion sequence as a putative MITE. Further analysis sequence analysis showed that the first 151 nucleotides are only present in these sequences, however, the following 313 nucleotides, showed a high sequence identity (e-value < 7e-29) with the gene *SIMILAR TO RCD ONE 2 (SRO2)* from Arabidopsis. SRO2 protein have a poly (ADP-ribose) polymerase (PARP) domain, which is signature domain known to modify a variety of proteins by attaching the ADP-ribose-moiety from NAD⁺ to the target molecule (Kraus & Lis, 2003). Analysis of cis-regulatory elements in the inserted fragment uncovered an ACGTG box, which has been shown to be the strongest element for binding the ELONGATED HYPOCOTYL 5 (HY5) transcription factor in Arabidopsis (Burko et al., 2020) (Figure 8A). It is worth mentioning that four functional homologs of the *SRO2* gene are present with two copies in each subgenome of rapeseed.

To trace the origin of the 598-bp DNA fragment, we performed a local blast search and identified three homologous sequences of the insertion sequence (e-value = 0.0) that were present only in the A subgenome (Figure 8b, Supplementary Table 8), indicating that the sequence might have originated from *B. rapa*. A local blast search of the insertion sequence identified four homologous sequences (e-value < 9.41e-75) in the *B. rapa* genome (Chiifu-401-42) (Supplementary Table 9). However, none of the homologous sequences that were identified in *B. rapa*, were located on chromosome A05 (Figure 8b), and the upstream region of *Br.SOC1.A05* was almost 100% identical to the upstream sequence of *Bna.SOC1.A05* in the spring rapeseed accessions. We also analyzed sequences of other *B. rapa* accessions, including winter turnip that is suggested to be the ancestor of *B. napus*, but no insertion was found on chromosome A05, indicating that the sequence was inserted upstream of *Bna.SOC1.A05* after the hybridization of the two ancestral genomes. Chromosome A06 in *B. rapa* carries two copies of the insertion, while the other copies are present on chromosomes A10 and A09 (Figure 8B).

To estimate the evolutionary relationship between the identified homologous sequences from *B. napus* and *B. rapa* genomes, we conducted a phylogenetic analysis using multiple sequence alignment of the eight identified sequences. Phylogenetic analysis of the homologous sequences resulted in four distinct pairs (Figure 8c), where the sequence on chromosome A06 in *B. rapa* (Br.ChrA06a) and its surrounding sequences showed 100% identity to that on Bna.ChrA06, indicating that the sequence on Bna.ChrA06 is the same as that on Br.ChrA06. The other sequence on *B. rapa* A06 (Br.ChrA06b) showed a high sequence identity to the insertion sequence on Bna.ChrA09-random (Figure 8c). Insertions on chromosomes A04 and A05 in *B. napus* formed a separate clade, and showed less sequence identity to sequences on Br.ChrA10 and Br.ChrA09 compared to that on Br.ChrA06 (Figure 8c, Supplementary Table 9). The phylogenetic analysis suggested that the sequences on Bna.ChrA04 and Bna.ChrA05 are more likely to be recent copies of the insertion sequence on Br.ChrA06, rather than a reshuffling of the sequences on Br.ChrA10 and Br.ChrA09.

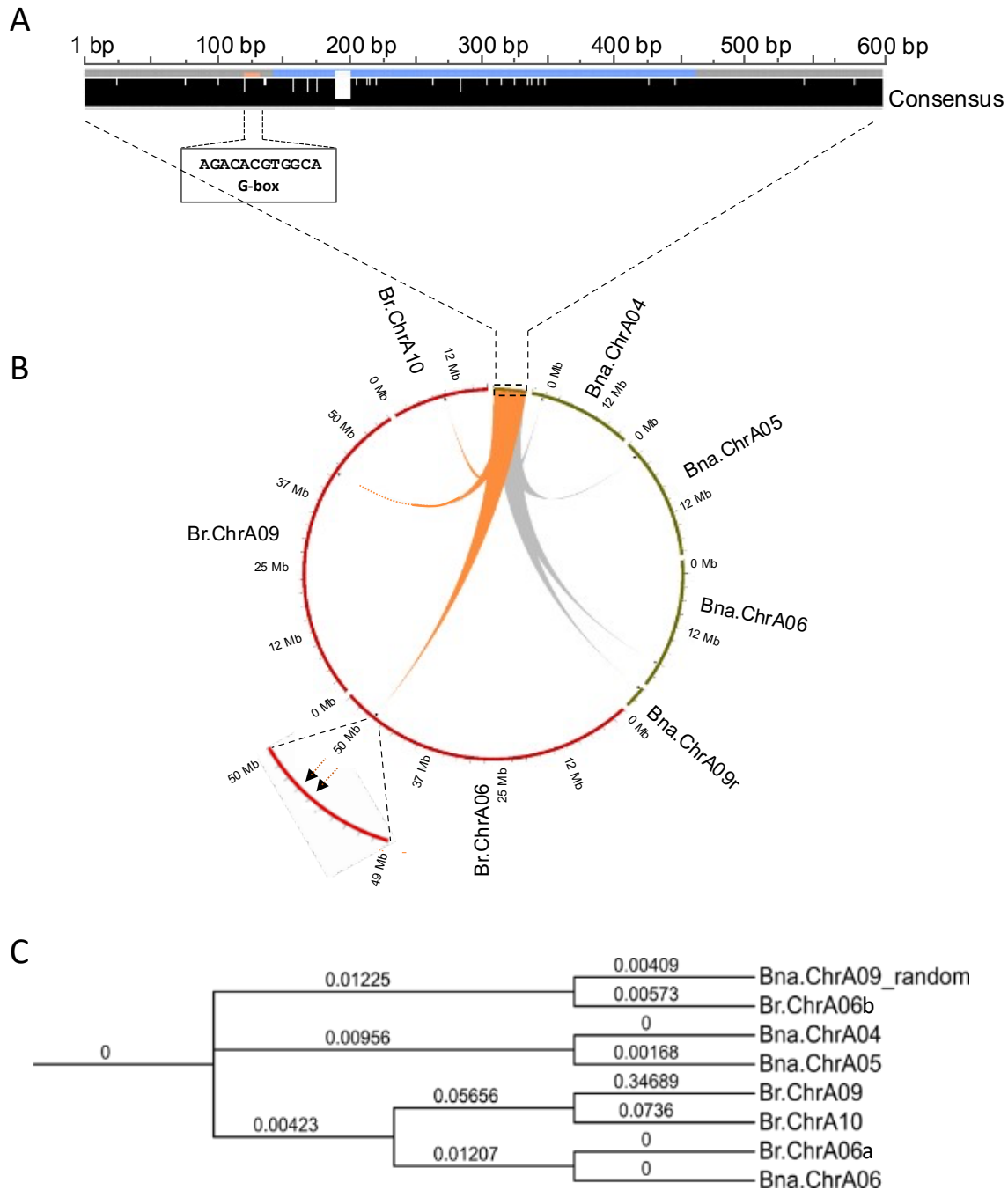


Figure 8: Sequence analysis and genomic positions of the insertion-homologous sequences in *B. napus* and *B. rapa*. (A) Multiple sequence alignment of the four homologous sequences from *B. napus*, showing consensus (black bar) with a high modal residue per column, and the position of the G-box HY5 binding motif. Blue colour in the middle of the insertion sequence corresponds to the region matching to *SRO2*. (B) Circos plot showing genomic positions of the the insertion-homologous sequences on chromosomes of *B. rapa* (red) and *B. napus* (green). (C) Phylogenetic tree showing the evolutionary relationship between the eight identified sequences in the genomes of *Bna. napus* and *B. rapa*.

3.4.3 Reduced nucleotide diversity in the genomic region surrounding *Bna.SOC1.A05*

To determine whether the genomic region harboring the insertion was potentially affected by selection, we used whole genome SNP information, of the 80 rapeseed accessions studied, to analyze nucleotide diversity in the region around *Bna.SOC1.A05* (*BnaA05g05010D*), compared to the nucleotide diversity of 40-kb surrounding that region (Figure 9A). The rapeseed accessions exhibited dramatically reduced nucleotide diversity in a genomic region of 15 kb (Figure 9B, C). In addition to *Bna.SOC1.A05*, a homolog of the MADS-box gene *AGAMOUS-LIKE 6* (*AGL6*) (*BnaA05g05000D*) is also present in this region (Figure 9a). The low level of diversity in the genomic region suggests that the region has evolved under functional constraints.

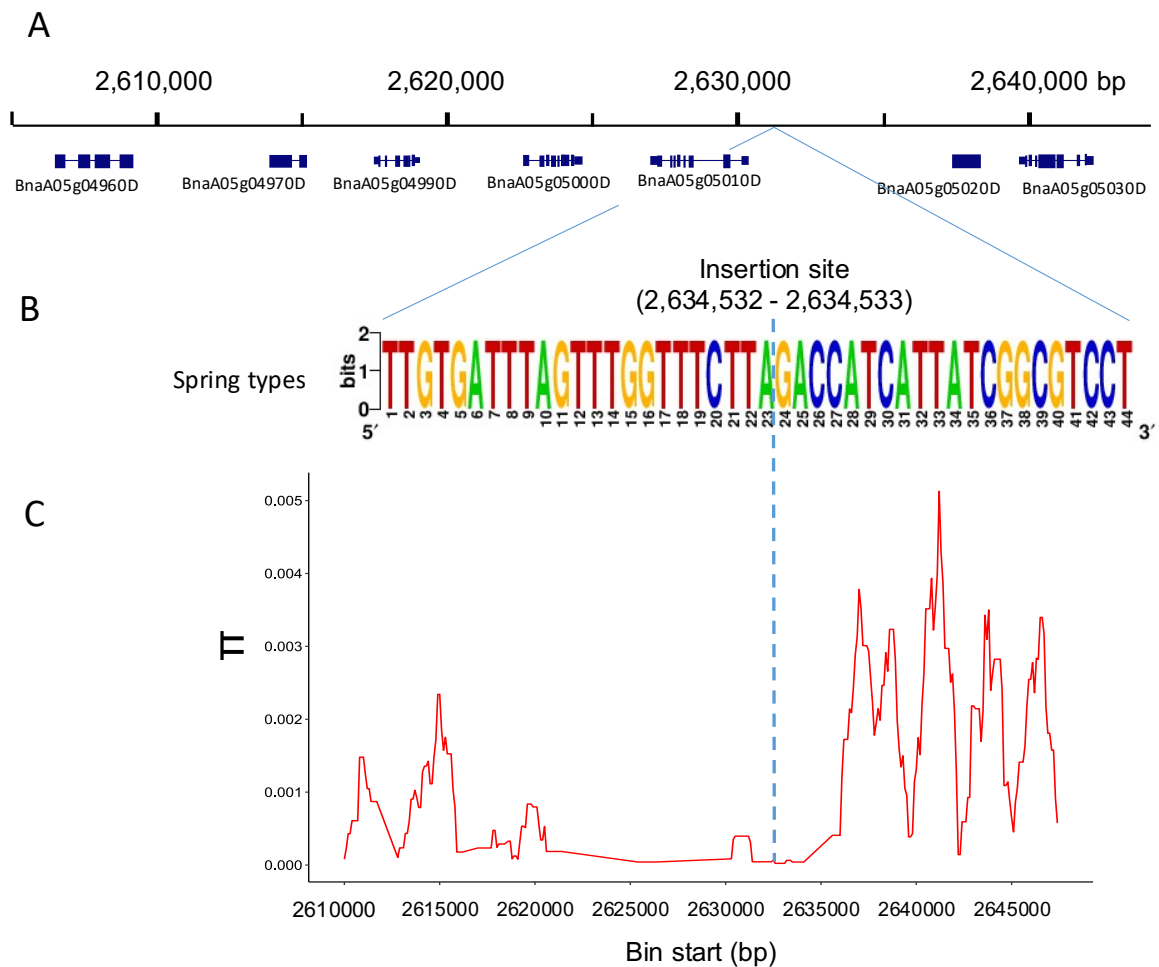


Figure 9: Nucleotide diversity across the the insertion site on chromosome 5. (A) 0.4-Mb genomic region surrounding the insertion sequence, and the genes annotated in this region according to Darmor-*bzh* reference genome. **(B)** The consensus sequence covering the breakpoint of the presence/absence variation in 18 rapeseed accessions. The breakpoint of the PAV is located between 2,634,532 and 2,634,533 in Darmor-*bzh* reference genome. **(C)** Nucleotide diversity (π) calculated based on SNP data of the 80 studied rapeseed accessions showing a reduced nucleotide diversity in the region 2,620,000 to 2,635,000 surrounding the PAV.

3.4.4 *Bna.SOC1.A05* is highly expressed in spring types lacking the insertion

To get an idea whether the PAV is associated with transcriptional activity of the *Bna.SOC1.A05* gene, we compared the expression of three spring accessions and one winter rapeseed accession. Two spring accessions that carried the insertion upstream of *Bna.SOC1.A05* were from the ERRANET-ASSYST panel and the third spring accession was Haydn that has no insertion. The winter accession was Express617 that carries the insertion. We analyzed expression levels in apices of two, four and eight weeks old plants and found that the expression of *Bna.SOC1.A05* was consistently lower in Bingo and Adamo compared to Haydn (Figure 10A). In fact the expression levels of *Bna.SOC1.A05* was more comparable to that of the winter accessions Express617 (Figure 10A). In addition, Haydn flowered around 55 days after sowing, while Adamo and Bingo flowered 5 and 10 days later (Figure 10B). However, the expression of *Bna.SOC1.A05* did not

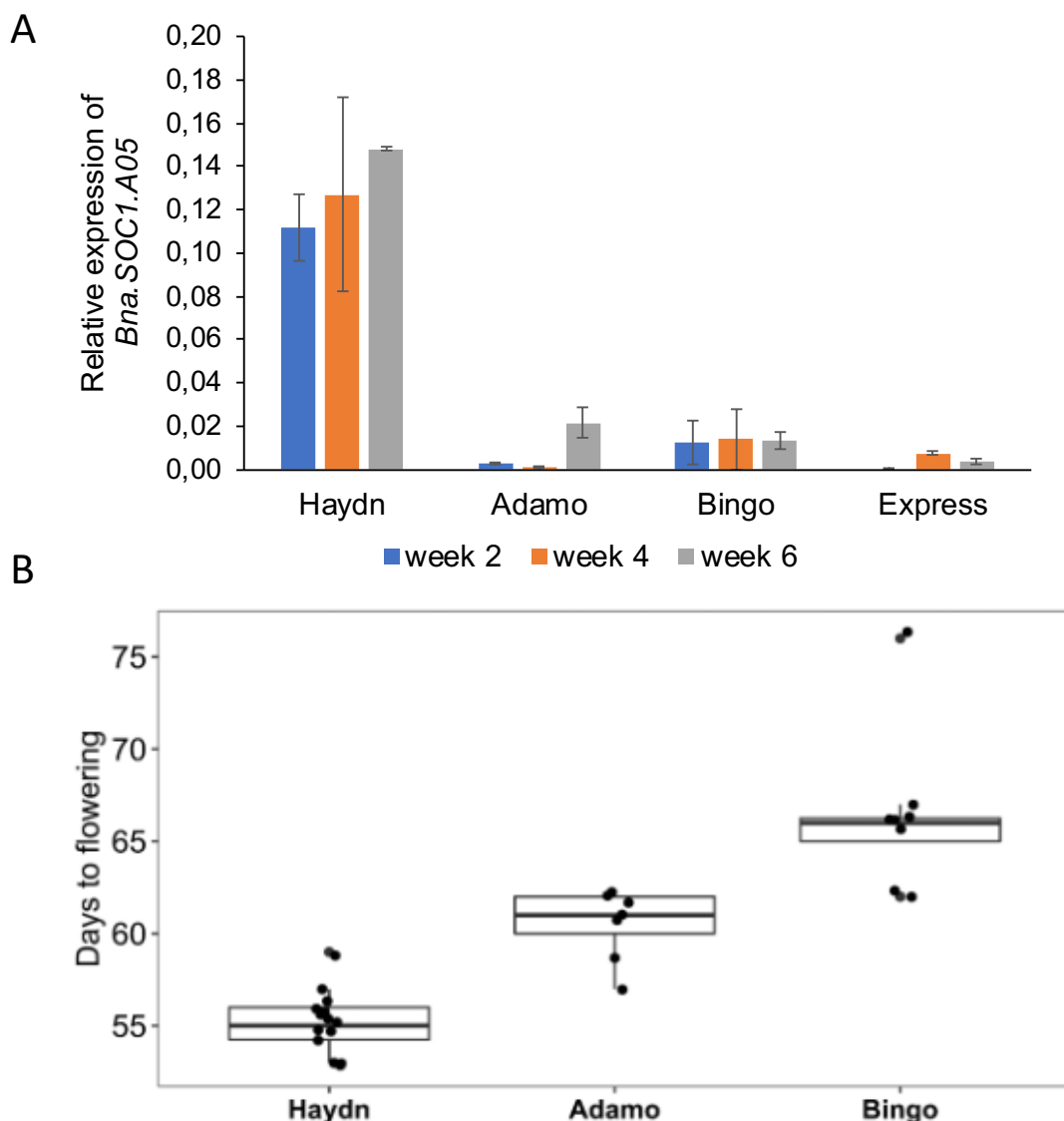


Figure 10: Expression and flowering time variation in different rapeseed accessions. (A) Expression levels of *Bna.SOC1.A05* in the apices four different rapeseed accessions, 2, 4, and six weeks after sowing. Expression was normalized to *Bna.Actin2*. Error bars represent SEM of three biological replicates. (B) Days to flowering determined by scoring the opening of the first flower in three different spring accessions.

explain the difference in flowering time between Bingo and Adamo, as Adamo was earlier flowering, but exhibited lower expression levels of *Bna.SOC1.A05*.

To further study the expression level of *Bna.SOC1.A05* in comparison to other *Bna.SOC1* genes, we analyzed the leaf transcriptome of 72 rapeseed accessions, including 31 winter and 41 spring accessions. The whole transcriptome reads (Havlickova et al., 2018) were downloaded from SRA NCBI and mapped to the Express617 genome assembly. The 60 ASSYST panel accessions that were genotyped for the PAV were also included in RNA-seq analysis.

The expression levels of all *Bna.SOC1* genes in winter accessions were significantly lower than the expression of *Bna.SOC1* in spring accessions, except for *Bna.SOC1.C04* (Figure 11). This is expected considering that flowering in winter types is vernalization dependent, and that winter types are expected to have higher levels of *Bna.FLC* (Schiessl et al., 2019). In spring types, *Bna.SOC1.A03-random* and *Bna.SOC1.A04* showed the highest expression levels compared to other *Bna.SOC1* genes (Figure 11). Interestingly, in winter types, *Bna.SOC1.A05* was the only gene that was not detected before vernalization (Figure 11). In a transcriptome analysis of the winter accession during the course of vernalization (Chapter 2), *Bna.SOC1.A05* was the first gene to be induced during floral transition. This tight vernalization-controlled expression of *Bna.SOC1* further reinforces its pivotal role in vernalization-driven floral transition in winter rapeseed (Chapter 2).

Further analysis of *Bna.SOC1* expression showed that the expressions levels *Bna.A03-random* and *Bna.SOC1.A04* were higher in Adamo compared to Bingo (Figure 11), which might explain the earlier flowering of Adamo.

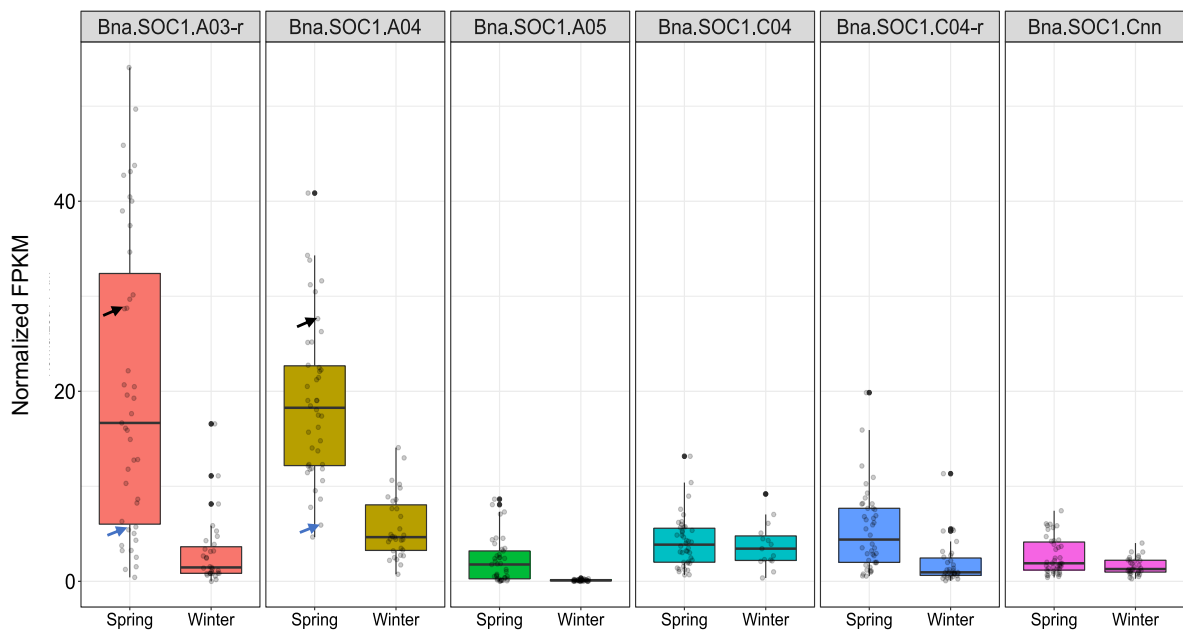


Figure 11: Expression levels of *Bna.SOC1* genes in spring and winter rapeseed accessions. Boxplot of normalized FPKM values of the six *Bna.SOC1* paralogs in leaf transcriptome of 31 winter and 41 spring rapeseed accessions. Black arrow corresponds to the normalized FPKM values of Adamo, while blue arrows correspond to the normalized FPKM values of Bingo.

3.5 Discussion

In addition to SNPs and InDels, SVs, represented mainly by PAVs are prevalent in the *B. napus* genome, and are believed to be a driving force for its diversification and adaptation to different environments. Several studies have investigated the genetic variation of flowering-time divergence among the three rapeseed ecotypes. Most of the studies focused on the orthologs of *FLC*, as major genes contributing to the vernalization requirement, thus to the divergence between spring and winter types. PAVs upstream of certain *Bna.FLC* genes have been shown to be associated with flowering-time divergence in rapeseed (Hou et al., 2012; Yin et al., 2020), (Lu et al., 2019; Wu et al., 2019).

Since downstream targets of *Bna.FLC* genes, might have also played a role in the evolution and diversification of flowering-time in rapeseed, we analyzed SVs within and upstream of orthologs of key downstream targets of *Bna.FLC*. Utilizing the comprehensive resource of genomic sequences of a worldwide collection of 991 rapeseed accessions, we looked for SVs within or upstream of orthologs of *Bna.SOC1* genes. Detection of SVs requires a high sequencing depth, therefore, we started to analyze only accessions that had a sequencing depth of 10X or more. However, out of 991 accessions 188 were spring types, from which only very few had a sequencing depths of more than 10X (Wu et al., 2019). Therefore, our set of spring types included accessions with sequencing depth of 5X and 6X (Supplementary Table 6).

SOC1 is a main floral integrator and a downstream target of *FLC* in Arabidopsis (Lee & Lee, 2010; Searle et al., 2006). In a previous study, we showed that two *Bna.SOC1* paralogs, *Bna.SOC1.A05* and *Bna.SOC1.C04-random*, are significantly induced during vernalization (Chapter 2), suggesting a significant role of these two genes in floral transition. Analysis of the upstream regions of the *Bna.SOC1* genes, uncovered a 598-bp PAV upstream of *Bna.SOC1.A05*. The PAV is located 1.8-kb upstream of the transcription start site, suggesting that it is part of the promoter region of *Bna.SOC1.A05*. We used genomic DNA of accessions from the ERANET-ASSYST panel to confirm the PAV by PCR. More than 90% of the tested spring accessions lacked the insertion, while around 84% of the winter accessions had the insertion (Supplementary Table 7), indicating that this PAV with its significant association to the two growth types might play a role for vernalization driven flowering in winter rapeseed plants.

Although it is in an intergenic region, sequencing of the insertion from different rapeseed accessions showed that the sequence is highly conserved, which suggested that the insertion is of functional importance and might contain certain motifs that are relevant to the activity of the downstream gene. Motif analysis revealed a CACGTG box, which is known to be a binding site for the transcription factors HY5 (Burko et al., 2020). In Arabidopsis, *SOC1* mediates the crosstalk between cold response and flowering through regulating cold-responsive (COR) genes (Seo et al., 2009). *SOC1* is known to be a downstream target of HY5, but through the binding motif CACGTA (Lee et al., 2007), which was also present in the promoter region of *Bna.SOC.A05*. Using SNP data from Wu et al. (2019) we observed that the sequence conservation is not only in the inserted sequence but extended to the surrounding genomic region. This extremely low nucleotide diversity was probably caused by a selective sweep including the presence of the insertion in winter types while it was absent in spring types.

PAVs in the promoter regions can affect the expression level or pattern of the corresponding downstream gene. Changes in expression might be due to new cis-

regulatory elements that are introduced to the region, or due to the elimination of certain motifs that were present at the breakpoint of the insertion. Changes in expression could also be a result of DNA methylation levels in the inserted sequence. In our analysis, the expression level of *Bna.SOC1.A05* in the two accessions that contained the insertion was significantly lower than in the spring accessions that lacked the insertion. Since the expression levels of *Bna.SOC1.A05* did not correlate with flowering time, we analyzed the expression levels of other *Bna.SOC1* paralogs using leaf transcriptome data from Havlickova et al. (2018). Interestingly, *Bna.SOC1.A05* was the only gene that was not expressed in two weeks old winter types plants, further indicating that it might be involved in the vernalization driven flowering response in winter rapeseed. In spring types, *Bna.SOC1.A03* showed the highest expression levels, with a higher level in Adamo compared to Bingo, which is in line with the observed flowering time in these two varieties. However, these transcriptome data were from two weeks old plants and might not reflect the activity of the genes directly before and during the transition to flowering.

Further analyses revealed that the inserted sequence has three homologous sequences in the *B. napus* A genome, which were also found in *B. rapa*, indicating that the sequences originated from this ancestor. Surprisingly, analyses of homologous sequences in *B. rapa* showed that the sequences are not located on the same chromosomes as in *B. napus*, except for one sequence present on chromosome A06, indicating that these sequences might be mobile by a hitherto unknown mechanism. As the upstream region of *Br.SOC1.A05* was similar to that in spring types, it is evident that this 598-bp sequence was introduced to the upstream region of *Bna.SOC1.A05* after the hybridization event. The known PAVs upstream of certain *Bna.FLC* genes are MITE-like transposable elements, that were activated after the generation of *B. napus* and got introduced to the upstream regions of *Bna.FLC* also after hybridization (Hou et al., 2012). However, as our analyses showed that the 598-bp DNA sequence upstream of *Bna.SOC1.A05* is not a transposable element, we were further intrigued to determine how the new insertion appeared on different chromosomes in the *B. napus* genome. Phylogenetic analyses suggested that the sequences in *B. napus* are more likely to be recent copies of the insertion sequences on chromosome A06 in *B. rapa*, as they showed much higher sequence identity to the insertion on Br.ChrA06 compared to the ones on Br.ChrA09 and Br.ChrA10.

The strong association of this PAV with growth type, its high sequence conservation, in addition to association with expression indicate that this PAV is of functional relevance. Why this insertion is selected for in winter types, and whether it affects the cross-talk between cold response and flowering time, through *Bna.SOC1.A05*, in winter rapeseed has yet to be investigated.

3.6 Supplementary materials

Supplementary Figure 9. Screenshot from Integrative Genomics Viewer (IGV) showing read alignment results in a 7-kb region (track 1, top) within and upstream of *Bna.SOC1.A05* (track 17, bottom). A: Sequence coverages of 15 spring accessions mapped to Express617. B: Sequence coverages of 15 winter accessions mapped to Express617.

Supplementary Figure 10: Figure 9: Multiple sequence alignment of the insertion in 18 winter accessions from ERANET-ASSYST panel showing a high sequence conservation between the accessions.

Supplementary Figure 11. Phylogenetic Neighbor-joining tree. The tree was constructed based on SNP data in 10.5-kb genomic region surrounding the insertions, using Tassel and plotted using iTOL. Green color represents winter accessions and orange color represents spring accessions. Red branch represents accessions without the insertion while blue branch represents accessions with insertion.

Supplementary Table 5: Chromosome position of six homologs of *SOC1* in Express617 genome.

Supplementary Table 6: List of winter and spring accessions from the whole genome genome sequenced 991 that were analyzed for the presence absence variation upstream of *Bna.SOC1*

Supplementary Table 7: List of accessions from ERANET ASSYST *B. napus* diversity set tested by PCR for the presence or absence of the insertion.

Supplementary Table 8: Results of local blast of the insertion sequence upstream of *Bna.SOC1.A05* against Express617 genome.

Supplementary Table 9: Results of local blast of the insertion sequence upstream of *Bna.SOC1.A05* against *B. rapa* Z1 genome.

3.7 References

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4 Closing Discussion

Understanding the stages of reproductive development and the genetic factors that control this process is essential to optimize yield and produce varieties that are better adapted to the changing climate. A global gene expression profiling of the SAM, accompanied with detailed histological analyses of the stages of meristematic development of winter rapeseed Express617 during floral transition was performed in this study. The aims were to determine the timing of floral transition and to identify gene paralogs that mark each stage of meristem development. Although some previous studies on rapeseed flowering-time included transcriptome analysis (Jones et al., 2018; O'Neill et al., 2019; Shah et al., 2018), such high resolution of physiological and molecular analysis of the floral transition during the course of vernalization was still lacking.

The results of this study provide comprehensive insights of cold-driven floral transition in winter rapeseed, and open new avenues for further flowering time and yield related studies. In this work, I showed through histological analysis, that winter rapeseed plants, at a certain developmental age, initiate floral transition in the first five weeks of vernalization. Through transcriptome analysis, I identified the genes that are associated with different stages of the flowering response, and showed that specific paralogs of *Bna.SOC1* (*Bna.SOC1.A05* and *Bna.SOC1.C04-random*) and *Bna.SPL5* (*Bna.SPL5.C05* and *Bna.SPL5.A05*) are induced during the early events of the floral transition. Moreover, I investigated the age-dependent responsiveness to vernalization and showed that pre-vernalization growth periods affect the timing of the floral transition in winter rapeseed, and that *Bna.SOC1* and *Bna.SPL5* genes respond to age-related signals downstream of *Bna.FLC*. My study on genomic variation in flowering time genes, between spring and winter types, uncovered a novel presence/absence variation upstream of *Bna.SOC1.A05* that is associated with growth type in rapeseed.

4.1 Autumn driven vernalization

I started this work based on an observation that winter rapeseed plants developed floral buds by mid-November in the fields in Schleswig Holstein. This observation led to the assumption that winter rapeseed plants respond to vernalization very early and undergo floral transition during cold. To confirm this hypothesis, I carried out a weekly histological analysis of the SAM during the course of vernalization. The assay revealed that developmental changes at the SAM, represented mainly by changes in meristem size and structure, took place in the first five to six weeks of vernalization. Within eight weeks, the meristem resembled an inflorescence meristem bearing floral meristems on its flanks.

While it is thought that vernalization requirement is an adaptive trait that prevents flowering before winter (Bouché et al., 2017), winter rapeseed plants with an obligate vernalization requirement rather vernalize before winter. Recently, a field study on flowering-time in winter rapeseed reported also that winter rapeseed plants undergo floral transition in autumn and over-winter with floral buds similar to perennials (O'Neill et al., 2019). Therefore, autumn-driven vernalization seems to be an adaptive life history strategy in winter types of *B. napus*. Vernalization before winter has also been observed in northern Sweden for winter annual *Arabidopsis* accessions, which complete their vernalization before snowfall (Duncan et al., 2015).

Although overwintering with floral buds is similar to life history of perennial plants, the winter dormancy observed in biennial rapeseed plants is fundamentally different from that of perennials. Perennials detect the reduction of photoperiods in autumn, which induces a cessation of growth prior to winter (Rohde & Bhalerao, 2007). The most recognizable characteristic of short-day induced growth cessation is the production of buds that encase the apical meristem and leaf primordia (Karlberg et al., 2011). This type of dormancy is known as endodormancy, and it does not require cold to be induced, but it can rather occur under growth conducive conditions (Preston & Sandve, 2013). Another distinguishing feature of endodormancy is that growth will not be reactivated, unless dormancy-breaking signals, such as long winter cold, are perceived by the plant (Maurya & Bhalerao, 2017). In biennial rapeseed, short photoperiods before or after formation of floral buds do not induce any sort of dormancy (Figure 2, Chapter 2). Moreover, a long winter cold is not required for flowering in spring. Winter projects a period of ecodormancy, which is a type of dormancy provoked by unfavorable environmental conditions (Lang, 1987). This ecodormancy can be broken by subjecting biennial rapeseed plants to warm temperatures as shown in Chapter 2 under controlled conditions.

The observation of the development of floral buds in mid-November was based on one-year field experiment. Knowing that temperatures in autumn can vary between the years, and flowering time is a quantitative trait which is highly dependent on the environmental conditions, I suggest to conduct additional field studies with different winter accessions varying in flowering time.

4.2 Whole transcriptome analysis: From a vegetative meristem to an inflorescence meristem and then a flower

To further study how developmental changes at the SAM are reflected at the molecular level, I generated an RNA-Seq data series of the SAM during the floral transition and performed an RNA-Seq data analysis with the following assumptions; 1) cold-driven repression of orthologs of floral repressors takes place early during vernalization, 2) floral activators are induced in the first five weeks of vernalization correlating with the onset of floral transition 3) Floral meristem identity genes are induced after the formation of an inflorescence meristem. Based on these assumptions I filtered out the expression data with three expression profiles that correlated with the morphological changes at the meristem. The first expression pattern corresponded to an early response to the cold signal through the downregulation of floral repressors at the SAM of Express617 plants. The profile coincided with expression patterns of three orthologs of the main floral repressor *FLC*, which was the first indication that winter rapeseed plants are rendered competent to flower. However, the downregulation of floral repressors is not sufficient for floral transition to take place, but it increases the competency of plants to respond to flowering promoting signals which leads to the induction of floral activators. On a quest for finding the early floral activators at the SAM, another expression profile was filtered for genes that were significantly upregulated during the first weeks of vernalization. Orthologs of key regulators of the floral transition, such as the MADS-box genes *SOC1* and *FUL* were found with this pattern. The early induction of orthologs of floral meristem identity genes following the repression of *Bna.FLC*, might be the key molecular event leading to the initiation of floral transition at the SAM of Express617 plants. The orthologs of *SPL5*, *Bna.SPL5.A05* and *Bna.SPL5.C05* were also induced in parallel to *Bna.SOC1*, suggesting their role in early events of floral transition in winter rapeseed. Interestingly, only two *Bna.SOC1* genes, namely *Bna.SOC1.A05* and *Bna.SOC1.C04*-

Closing discussion

random where highly induced in the meristem. Although *Bna.SOC1* and *Bna.SPL5* genes were significantly upregulated during the first four weeks of vernalization, initiation of floral transition, represented by increase in meristem size, took place a week later. The time gap might suggest that a threshold of expression level of floral activators has to be reached to initiate the developmental changes that were observed in the SAM.

In Arabidopsis, a SOC1-SPL module, where SOC1 promotes the expression of certain SPL genes including SPL5 by binding to their promoter region, promotes flowering under inductive signals (Jung et al., 2012). The SOC1-SPL module also mediates flowering under non-inductive photoperiodic conditions through gibberellins (Jung et al., 2012). The fact that both genes are induced during floral transition in winter rapeseed, and knowing that winter rapeseed plants also flower under SD-conditions (Figure 2, Chapter 2), suggests the conservation of this SOC1-SPL module in rapeseed. Further functional analysis that includes production of *Bna.SOC1* mutants followed by expression analysis of *Bna.SPL5* is a crucial step in confirming the SOC1-SPL module in rapeseed. Other studies such as yeast-one-hybrid experiments are essential to confirm the interaction of certain *Bna.SOC1* proteins to the promoter regions of *Bna.SPL5.A05* and *Bna.SPL5.C05*. As only two *Bna.SPL5* genes were induced, cloning the promoters of all *Bna.SPL5* genes for a protein-DNA interaction assay will confirm whether this specific induction is due to selective binding of *Bna.SOC1* encoded proteins to the promoter regions of *Bna.SPL5.C05* and *Bna.SPL5.A05* only. Such follow-up experiments are crucial for further understanding how the regulatory mechanism leading to floral transition evolved in the allopolyploid *B. napus*. They might clarify whether a observed phenotype in this polyploid species might be a mere additive effect of all gene homologs present in the genome, or due to certain gene homologs that respond to environmental and endogenous signals and shape the phenotype of the polyploid species. Moreover, my expression analysis experiment in leaves of Express617 plants showed that despite the parallel upregulation of *Bna.SOC1* in leaves, *Bna.SPL5* genes were not expressed outside of the meristem, indicating that a possible SOC1-SPL module in *B. napus* is restricted to meristems and that other factors might be needed for the meristem-specific induction of *Bna.SPL5*.

The third expression pattern corresponded to genes whose expression was restricted to the time-point after the formation of the inflorescence meristem. The question was, when did Express617 plants attain floral determination? And when was flower development initiated? Certain genes known as floral meristem identity genes, such as *API*, which is expressed in the floral meristems, are known to define the end of the process of floral induction and the initiation of flower development in Arabidopsis (Hempel et al., 1997; Torti et al., 2012). The orthologs of these genes in rapeseed, *Bna.API*, were not detected at the SAM in the first seven weeks of vernalization. This indicated that the process of floral induction had yet to be completed. Four *API* homologs were induced after eight weeks of vernalization and were followed by the induction of orthologs of floral organs identity genes indicating that at eight weeks of vernalization, winter rapeseed plants attain floral determination and a week after, they initiate flower development.

Based on the analysis of SAM whole transcriptome data, during the course of vernalization, I could draw a clear molecular time-line that defines each stage of meristem development, and answer the questions concerning the timing of floral transition and the genes that mark the initiation and maintenance of this process (Figure 12). In brief, the time-line can be summarized as follows; in the first four weeks of vernalization winter

rapeseed plants acquire flowering competence by repression of *Bna.FLC* genes. After six weeks of vernalization, which are sufficient for Express617 plants to flower (Shah, 2018), the inflorescence meristem was formed and plants attained floral commitment, meaning that they will flower independently of the environmental cues. Two weeks after the formation of the inflorescence meristem, floral meristems were formed, which was supported by the expression of *Bna.AP1* genes. One-week later orthologs of floral organ identity genes were induced indicating the initiation flower development. Such detailed analyses of the timing of floral transition and floral determination in winter rapeseed, at a molecular and histological level, have not been reported so far. In this study, I could also show that not all gene homologs respond to flowering inductive conditions. Functional analyses of these genes, in addition to determination of their spatial expression at the SAM using *in-situ* hybridization, will further broaden our knowledge about the architecture of the gene regulatory network controlling flowering time in winter rapeseed.

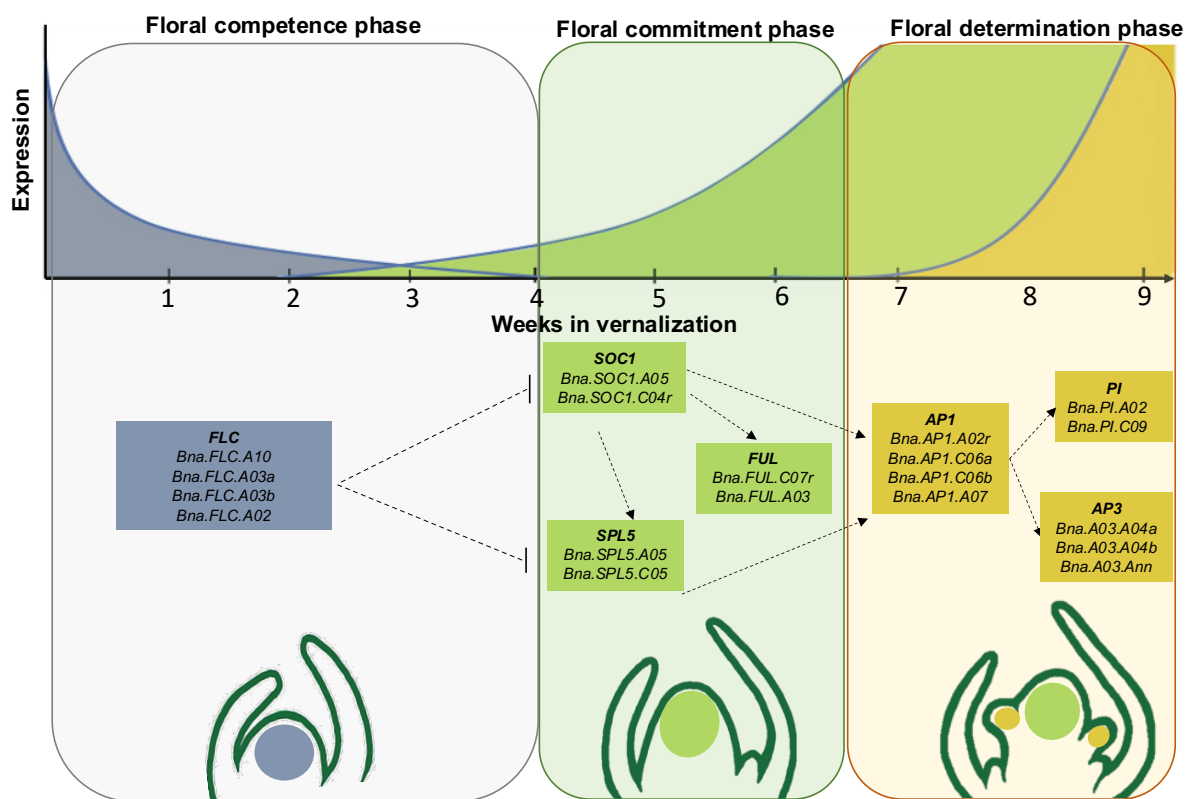


Figure 12: Stages of the process of floral transition and proposed regulatory mechanism at the SAM of winter rapeseed plants during vernalization. The floral competence phase represents the first four weeks of vernalization as the orthologs of the floral repressor *FLC* are downregulated. At the end of this phase, rapeseed plants acquire competence to flower. The floral commitment phase starts as the orthologs of the floral activators are upregulated in SAM. At the end of this phase, the SAM meristem is an inflorescence meristem and plants become committed to flowering irrespective of the inductive signal. The floral determination phase starts as the orthologs of the floral meristem identity gene *AP1* are upregulated, indicating the formation of floral meristems. At the end of this phase the upregulation of orthologs of floral organs identity genes mark the initiation of flower development. Colored boxes contain the Arabidopsis genes and the *B. napus* orthologs that were identified in the transcriptome study. Dotted arrows correspond to hypothetical transcriptional activation by certain genes. Colored areas in the schematic meristem figures correspond to the genes that are expressed in the meristem in a certain phase and their proposed spatial expression.

4.3 Age-dependent vernalization response

In the first research chapter, I was also aiming to study the plant age on the responsiveness to vernalization. This experiment was encouraged by previous studies on juvenility and age-dependent vernalization response in *Arabidopsis* and its perennial relative *A. alpina* (Bergonzi et al., 2013; Hyun et al., 2016). Interestingly, the aspects of juvenility and plant age have also been reported for certain forms of the ancestral species of *B. napus*, *B. rapa* and *B. oleracea* (Friend, 1985). To study the effect of plant age on vernalization-driven floral induction Express617 plants of different ages were exposed to vernalization at the same time (7 days, 9 days, 14 days and 21 days). I analyzed gene expression at SAM before, during and after vernalization, performed a histological study of the meristem after vernalization and recorded flowering time. As *Bna.SOC1* and *Bna.SPL5* were the main genes to be highly induced during floral transition, I studied their expression using RT-qPCR. The results revealed that floral transition and, consequently, flowering was delayed in younger plants. At the molecular level, the induction of *Bna.SOC1* and *Bna.SPL5* correlated with the delay of flowering time, even though the *Bna.FLC* genes were downregulated in the first weeks of vernalization. These results are a clear indication that the downregulation of *Bna.FLC* is not sufficient for the induction of flowering time genes like *Bna.SOC1* and *Bna.SPL5*, and that other endogenous signals must also be integrated. Moreover, these results suggested that *Bna.SOC1* and *Bna.SPL5* can be used as transcriptional markers to infer the timing of the floral transition. Histological analysis further supported my finding, that floral transition is delayed in younger plants. The experiment also shed light on the type of vernalization effect observed in *B. napus*. In plants that were one-week old at the time of vernalization, *Bna.SOC1* and *Bna.SPL5* were induced after vernalization, and the SAM was still in a very early stage of floral transition, but the plants did flower, indicating that the low-temperature treatment is effective even though concurrent floral transition was restricted. This is a typical “after-effect” vernalization, where flowering will still occur even if flower initiation did not take place during vernalization. It is worth mentioning that, biennial winter turnip, which is suggested to be the origin of the A genome in *B. napus* (Lu et al., 2019), also has an “after-effect” type of vernalization (Friend, 1985).

4.4 A novel presence/absence variation is associated with growth types in rapeseed

The second chapter of my research project was based on a hypothesis that genomic variation at downstream targets of *Bna.FLC* might have played a role in the diversification and adaptation of different rapeseed ecotypes. Therefore, I took advantage of the comprehensive whole genome sequencing of 991 rapeseed accessions (Wu et al., 2019) to study genomic variation within or upstream of putative downstream targets of *Bna.FLC*. A reliable SV analysis requires a relatively high sequencing depth. Therefore, I started the analyses with accessions with a sequencing depth of 10X or more. However, from the 991 accessions, very few spring accessions had a sequencing depth of more than 10X, therefore I had to use the available sequencing data irrespective of a low sequencing depth, and I subsequently validated the PAV by PCR.

In *Arabidopsis*, FLC has over 500 binding sites in the promoter region of genes (Deng et al., 2011). Knowing that *Bna.SOC1* genes are putative targets of *Bna.FLC*, and that their meristematic activity is strongly correlated with timing of floral transition (Chapter 2), *Bna.SOC1* genes were the first ones to be analyzed. By looking at the read alignment from different accessions, a PAV was uncovered, which turned out to be associated with

the spring and winter growth-types. The analyses showed that the PAV corresponds to a 598-bp DNA sequence that is located 1.8-kb upstream of the transcription start of *Bna.SOC1.A05*. The sequence is present in 85% of the winter types while absent in 90% of the spring types. To further validate the PAV, I used genomic DNA of rapeseed accessions from ERANET-ASSYST panel for a PCR and subsequent sequencing analysis and found that the sequence was highly conserved in different rapeseed accessions. Local blast search uncovered three homologous sequences that are present only in the A-subgenome, indicating that the sequences originated from *B. rapa*. A closer examination at the upstream region of *Br.SOC1.A05* showed that the region is identical to the upstream region of *Bna.SOC1.A05* in spring accessions, suggesting that the 598-bp sequence was inserted upstream of *Bna.SOC1.A05* after the hybridization event. SNP data analysis showed that the genomic region containing *Bna.SOC1.A05* and its upstream region exhibit a very low nucleotide diversity compared to its surrounding regions, which further suggested that this genomic region evolved under functional constraints. I then analyzed whether the PAV had any impact on *Bna.SOC1.A05* expression. Expression levels of *Bna.SOC1.A05* were higher at different time points in the spring accession Haydn that lacks the insertion, compared to two spring accessions Bingo and Adamo with the insertion. The strong association with growth type, the high sequence conservation, in addition to association with expression, indicated that this PAV might be of functional relevance. However, its evolution and role in flowering time divergence needs to be further elucidated. Therefore, I suggest promoter-reporter gene fusion studies to investigate whether the two *Bna.SOC1.A05* promoters, with and without the insertion, affect the expression of *Bna.SOC1.A05*. To investigate the protein-DNA interaction at the identified binding-motif in the insertion, CHIP-qPCR can be advantageous to elucidate the transcription factors that might bind to this putative regulatory region.

4.5 Flowering time: A new perspective of a yield associated trait

The final question would be, how can the results of this research project be implemented to improve rapeseed cultivation and yield performance? In the pursuit of improving yield, researchers and breeders have used the “onset of flowering”, an easily scorable trait, as a major determinant for yield. Although the timing of emergence and opening of the flower do affect the performance and yield of a plant, the key developmental switch that determines the production of dry matter is actually the timing of floral transition (Jung & Müller, 2009). In my research project, and in accordance with a recent report about flowering time of winter rapeseed (O'Neill et al., 2019), I showed that winter rapeseed plants respond to cold within few weeks, hence, in northern Germany and other regions of the northern hemisphere, plants sown in late August undergo floral transition and produce floral buds in autumn. Therefore, between September and mid-November, a great proportion of yield of winter rapeseed plants is being decided. This gap between flower initiation and its emergence and opening, caused mainly by winter cold, suggests that these two processes are controlled by different environmental cues, and might be under different selection pressures. In other words, selecting for early or late flowering varieties in spring, might not necessarily lead to the selection of varieties that undergo early or late floral transition in autumn. The idea that the two traits, timing of floral transition and timing of flower opening, might not be determined by the same genetic factors, opens new avenues for winter rapeseed yield improvement and broadens the pool of selection. The correlation between warmer Septembers and higher yields of winter rapeseed (Brown et al., 2019), further reinforces on the idea that later floral transition might lead to higher yields. Considering that the dormancy observed in winter rapeseed plants is not an

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endodormancy, a slight rise in temperatures in October and November after floral transition had taken place, might lead to the emergence of flower buds. Flower buds are tender and prone to damage from cold and frost, therefore, their emergence could be detrimental to rapeseed yields.

Although variation in the timing of floral transition sounds a very difficult trait to score for, at least compared to flower opening, considering the advancement in phenotyping techniques in the recent years, scoring for such an important trait should not be out of question. Moreover, my study on the expression of *Bna.SOC1.A05*, which showed that it is the only *SOC1* ortholog that is not expressed before vernalization in winter rapeseed (Chapter 3) and that its induction clearly correlates with the initiation of floral transition during cold (Chapter 2), in addition to a previous study which showed the association of *Bna.SOC1.A05* with yield (Shah et al., 2018), highlight the potential of this gene to be used as transcriptional marker to score for the initiation of floral induction. If scoring for the timing of floral transition will be implemented to improve winter rapeseed performance in the future, certain questions concerning the effect of onset of winter on the initiation of floral transition, the consequences of extremely delayed floral transition on the onset and synchronization of flowering time in spring, in addition to the effect of climate change, which might lead to warmer Septembers, on the timing of floral transition, and thereby yield, should be answered.

5 Summary

Rapeseed (*Brassica napus*) is one of the most important oil crops grown worldwide. Flowering time optimization is one of the main goals in rapeseed breeding, therefore, knowledge about the gene regulatory network that control the timing of floral transition will have a substantial impact on rapeseed breeding strategies. In this study, comprehensive physiological and molecular analyses of the cold-driven floral transition in winter rapeseed were carried out.

In the first experiment, I carried out weekly histological analyses of the shoot apical meristems of winter rapeseed plants during vernalization. The analyses revealed that meristem size and structure changed after five to six weeks of vernalization. Within eight weeks, the meristem resembled an inflorescence meristem bearing floral meristems on its flanks. Therefore, this study provided a physiological evidence that winter rapeseed plants respond to cold incredibly early and undergo floral transition during vernalization.

To study the floral transition at the molecular level, I generated an RNA-seq data series of the SAM during vernalization and performed expression analyses. The RNA-seq reads were mapped to a Darmar-bzh-based Express617 genome assembly. I analyzed three expression profiles to identify genes associated with different stages of the flowering response at the SAM. Thereby, I could identify genes that responded fast to the cold treatment. With the histological and transcriptome analyses I could draw a molecular time-line defining different stages of meristem development. Finally, two paralogs each of *Bna.SOC1* and *Bna.SPL5* were identified as genes that might regulate the initiation and maintenance of the floral transition.

Additionally, I studied the effect of plant age on vernalization-dependent floral induction. I analyzed gene expression at SAMs before, during and after vernalization, performed a histological study of the meristem after vernalization, and recorded flowering time. Expression and histological analyses showed that floral transition was delayed when plants were vernalized 7 days after sowing. Expression analysis showed that this delay was independent of the *Bna.FLC* genes. The expression levels of *Bna.SOC1* and *Bna.SPL5* paralogs again correlated with timing of the floral transition, indicating that these genes are targets of the age dependent cold-induced floral transition.

In the second research chapter I analyzed genomic sequences of putative downstream targets of *Bna.FLC* using whole genome sequencing reads from 80 rapeseed accessions and genomic DNA of the rapeseed ASSYST panel. Thereby, I detected a 598-bp presence/absence variation within the promoter region of *Bna.SOC1.A05*. Subsequent analyses showed that the presence/absence variation is associated with the winter and spring growth types of rapeseed. Furthermore, I showed that the 589-bp is located 1.8-kb upstream of *Bna.SOC1.A05* and originated from *B. rapa*. However, the insertion upstream of *Bna.SOC1.A05* likely occurred after the hybridization of the two rapeseed ancestors. The analysis of SNP data from 80 rapeseed accessions showed that the genomic region containing *Bna.SOC1.A05* and its upstream region exhibit a very low nucleotide diversity compared to their surrounding regions, indicating that this region was under selection pressure. Cis-regulatory element analysis showed that the insertion contained a binding motif for the HY5 transcription factor. Finally, expression analysis showed that mRNA levels of *Bna.SOC1.A05* were lower in accessions carrying the insertion compared to the

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ones that had no insertion, indicating that *Bna.SOC1A05* might play an additional role in vernalization requirement of winter rapeseed plants.

6 Zusammenfassung

Der Raps (*Brassica napus*) ist eine der wichtigsten Öl-Kulturpflanzen weltweit und eines der Hauptziele der Rapszüchtung ist die Optimierung des Blühzeitpunktes. Daher hat ein grundlegendes Verständnis der Regulationsmechanismen, die den Zeitpunkt der Blütenbildung steuern, eine erhebliche Bedeutung für Rapszüchtungsstrategien. Dafür wurden in dieser Studie umfassende physiologische und molekulare Analysen der vernalisationsabhängigen Blühinduktion beim Winterraps Express617 durchgeführt.

Im ersten Teil der Arbeit führte ich wöchentliche histologische Analysen der apikalen Sprossmeristeme von Winterrapspflanzen während der Vernalisation durch. Die Analysen ergaben, dass sich die Größe der apikalen Meristeme nach fünf bis sechs Wochen Vernalisation änderte. Innerhalb von acht Wochen hatte sich das apikale Meristem zu einem Infloreszenz Meristem umgewandelt, das an seinen Flanken Blühmeristeme abgliederte. Daher lieferte diese Studie einen physiologischen Beweis dafür, dass Rapspflanzen im Winter sehr früh auf Kälte reagieren und während der Vernalisation zur Blütenbildung induziert werden.

Um die Blühinduktion auf molekularer Ebene zu untersuchen, habe ich während der Vernalisation eine RNA-Seq basierte Transkriptom Analyse der apikalen Sprossmeristeme durchgeführt. Die RNA-Seq-Reads wurden dazu auf eine Darmor-bzh-basierte Express617 Genomassemblierung gemappt. Es wurde drei Expressionsprofile analysiert, um Gene zu identifizieren, die mit verschiedenen Stadien der Blühinduktion am apikalen Sprossmeristem assoziiert sind. Dadurch konnte ich Gene identifizieren, die schnell auf die Vernalisation reagierten. Mit den histologischen Untersuchungen und den Transkriptom Analysen konnte ich Expression von bestimmten Genen mit verschiedenen Stadien der Meristem Entwicklung korrelieren. Schließlich wurden je zwei Paraloge Gene von *Bna.SOC1* und *Bna.SPL5* identifiziert, die bei der Induktion und der Aufrechterhaltung der Blütenbildung eine wesentliche Rolle spielen könnten.

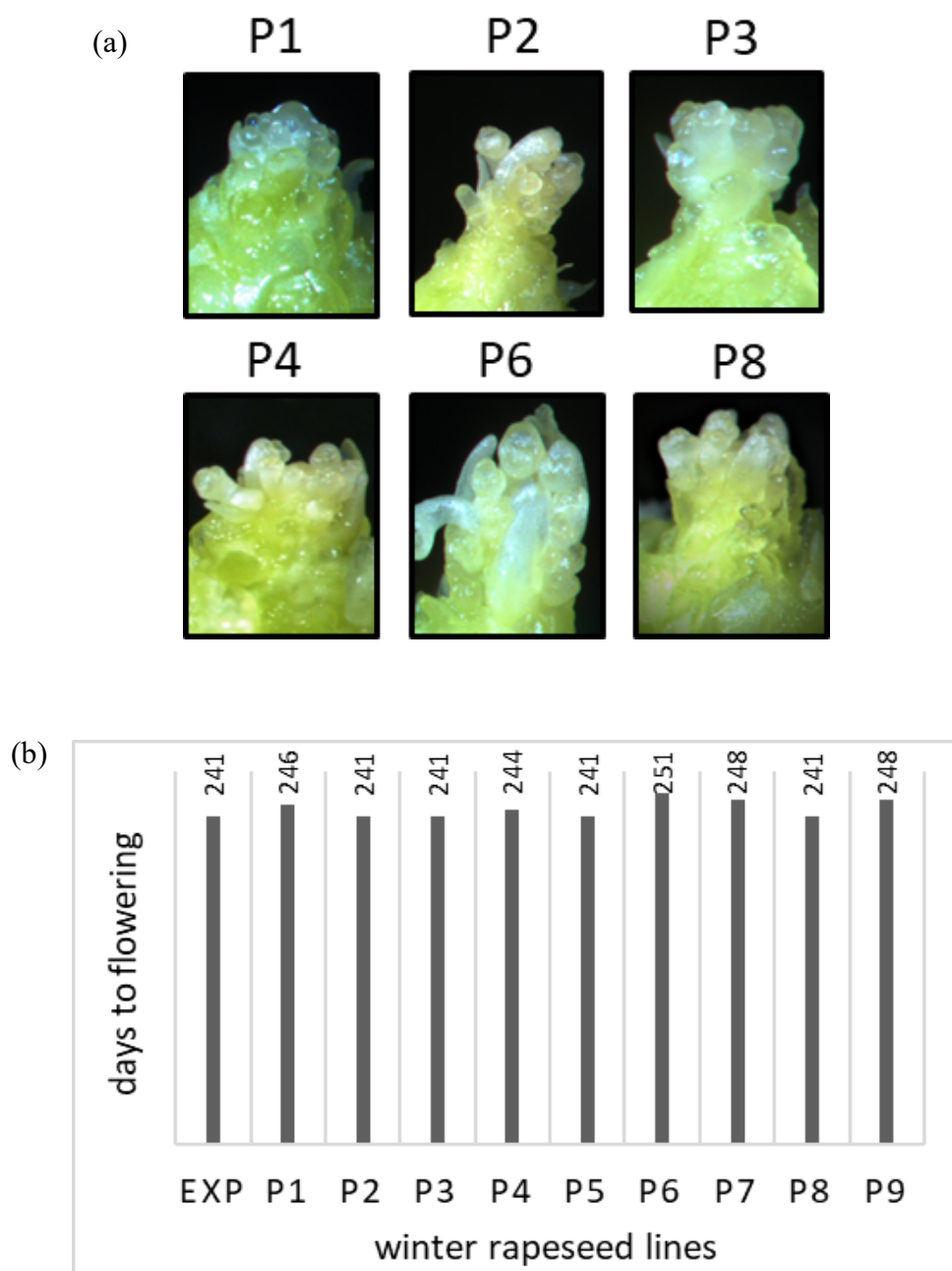
Zusätzlich untersuchte ich den Einfluss des Pflanzenalters auf die vernalisationsabhängige Blüteninduktion. Dazu analysierte ich die Genexpression in apikalen Sprossmeristemen vor, während und nach der Vernalisation und führte nach der Vernalisation eine histologische Untersuchung des Meristems durch. Die Expressions- und histologischen Untersuchungen zeigten, dass der Blütenübergang verzögert war, wenn die Pflanzen bereits 7 Tage nach der Aussaat vernalisiert wurden. Dabei war die Blühverzögerung unabhängig von den *Bna.FLC*-Genen, korrelierte aber wieder mit den Expressionsdaten von *Bna.SOC1* und *Bna.SPL5* paralogen Genen. Dies deutet darauf hin, dass diese Gene die altersabhängige kälteinduzierte Blühinduktion regulieren.

Im zweiten Teil der Arbeit analysierte ich genomische Sequenzen von durch *Bna.FLC* regulierter Gene unter Verwendung von Genomsequenzierungen von 80 Rapsvarietäten und PCR Analysen von genomischer DNA des Raps-ASSYST-Panels. Dabei fand ich eine Insertion von 598 Basenpaaren innerhalb der Promotorregion von *Bna.SOC1.A05*. Nachfolgende Analysen zeigten, dass die Insertion mit den Winterrapsvarietäten assoziiert war. Darüber hinaus habe ich gezeigt, dass Insertion 1,8 kb oberhalb vom Transkriptionsstart von *Bna.SOC1.A05* liegt und sehr wahrscheinlich von *Brassica rapa* stammt. Die Insertion oberhalb von *Bna.SOC1.A05* erfolgte jedoch wahrscheinlich erst nach der Hybridisierung der beiden Rapsvorfahren. Die Analyse von SNP-Daten von 80

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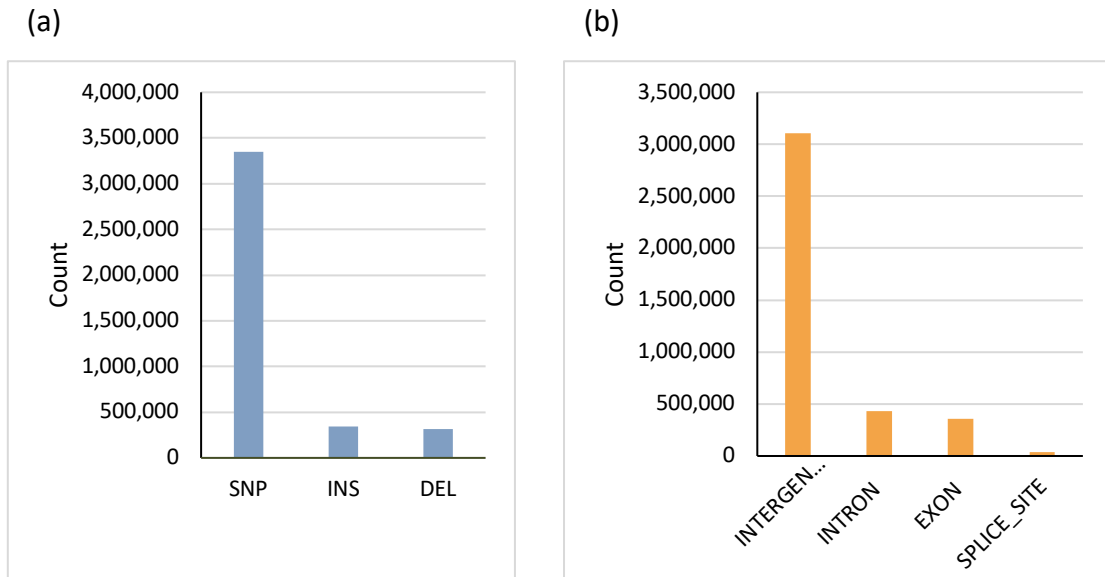
Raps-Akzessionen zeigte, dass eine 40 kb Genomregion um *Bna.SOC1.A05* im Vergleich zu einer größeren Region eine sehr geringe Nukleotid-Diversität aufweisen. Das könnte ein Hinweis darauf sein, dass diese Region unter Selektionsdruck stand. Die Analyse der Cis-regulatorischen Elemente zeigte, dass die Insertion ein Bindungsmotiv für den HY5-Transkriptionsfaktor enthält. Schließlich zeigte die Expressionsanalyse, dass die mRNA-Level von *Bna.SOC1.A05* bei Akzessionen mit der Insertion niedriger waren als bei solchen, die keine Insertion hatten. Das könnte darauf hinweisen, dass *Bna.SOC1.A05* eine wichtige Rolle bei der vernalisationsabhängigen Blütenbildung von Winterrapspflanzen spielen könnte.

7 Appendix

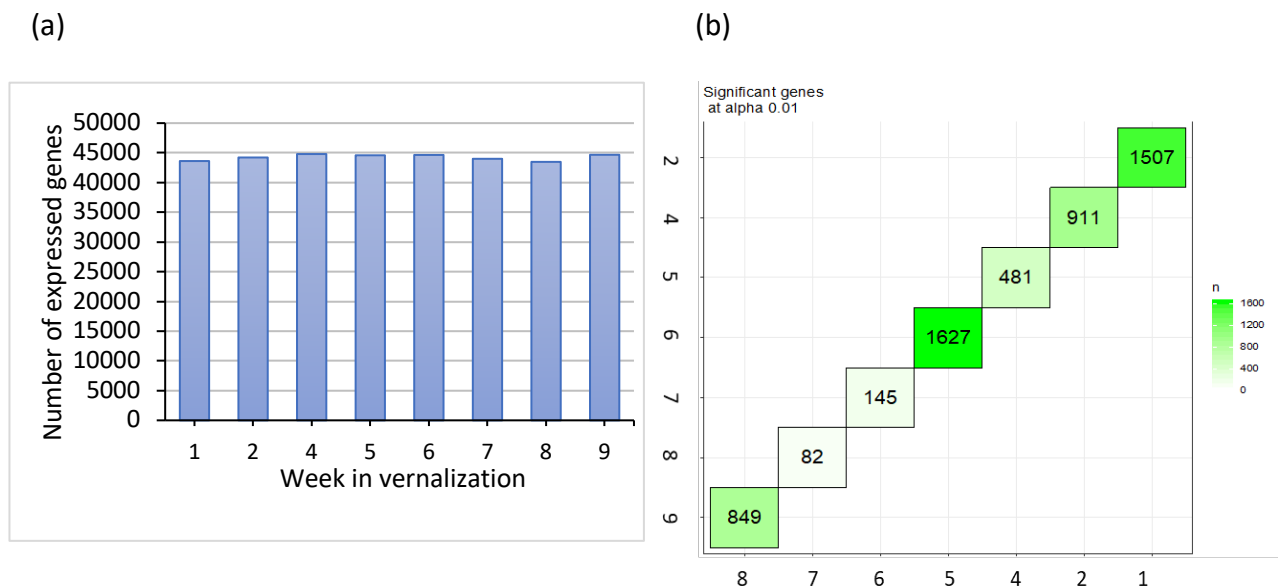


Supplementary Figure 1: Flowering of winter rapeseed under environmental condition. (a): Dissected apices of genotypes P1, P2, P3, P6, and P8 in mid-November 2017, observed using a binocular microscope. The images show the presence of floral primordia. (b) Days to flowering of ten different winter rapeseed genotypes sown in the field in August 2017. Flowering was recorded when 50% of the plants had an open flower on the main inflorescence.

Appendix

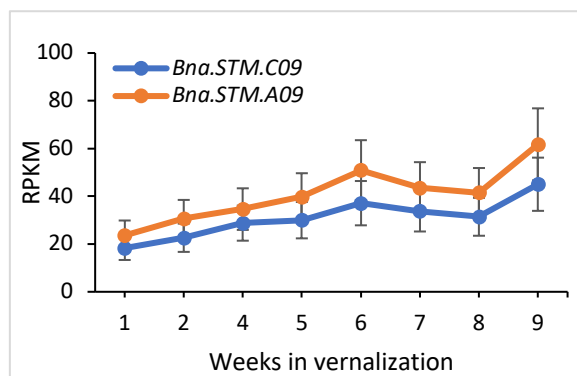


Supplementary Figure 2: Variation between Express617 and Darmor-bzh. (a) Number of variants between Express617 and Darmor-*bzh*. (b) Distribution of nucleotide variation in different genomic regions.

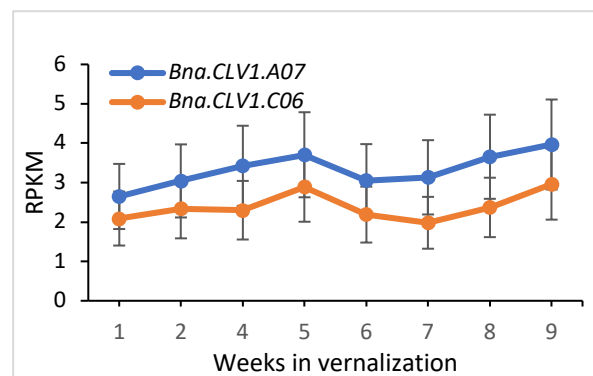


Supplementary Figure 3: Total number of expressed and differentially expressed genes. (a) Number of expressed genes ($RPKM \geq 1$) at each time point. (b) Pairwise comparison of the number of differentially expressed (\log_2 fold change ≥ 1) genes between two time point. RPKM = Reads Per Kilobase of transcript, per Million mapped reads.

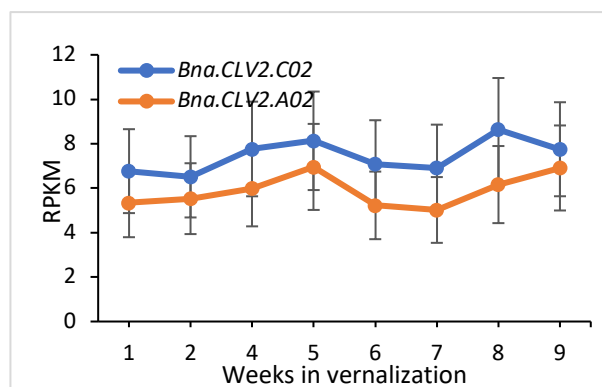
(a)



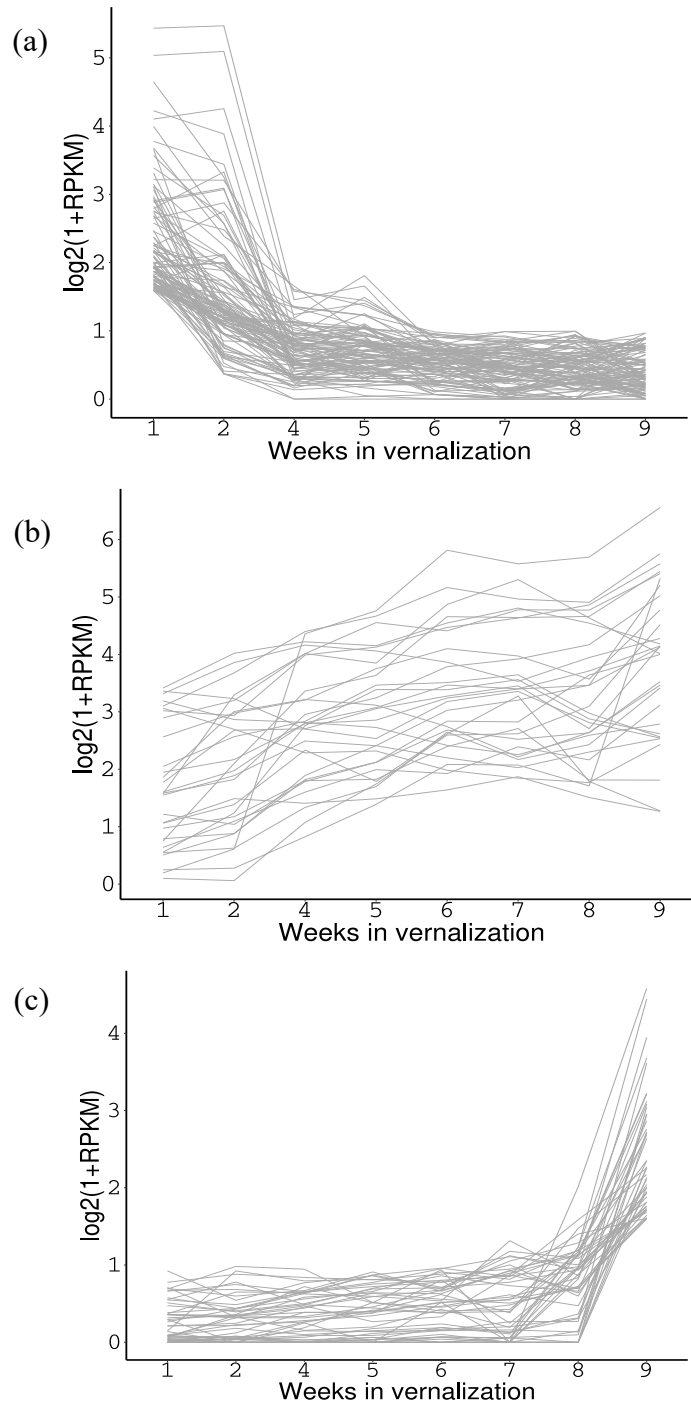
(b)



(c)



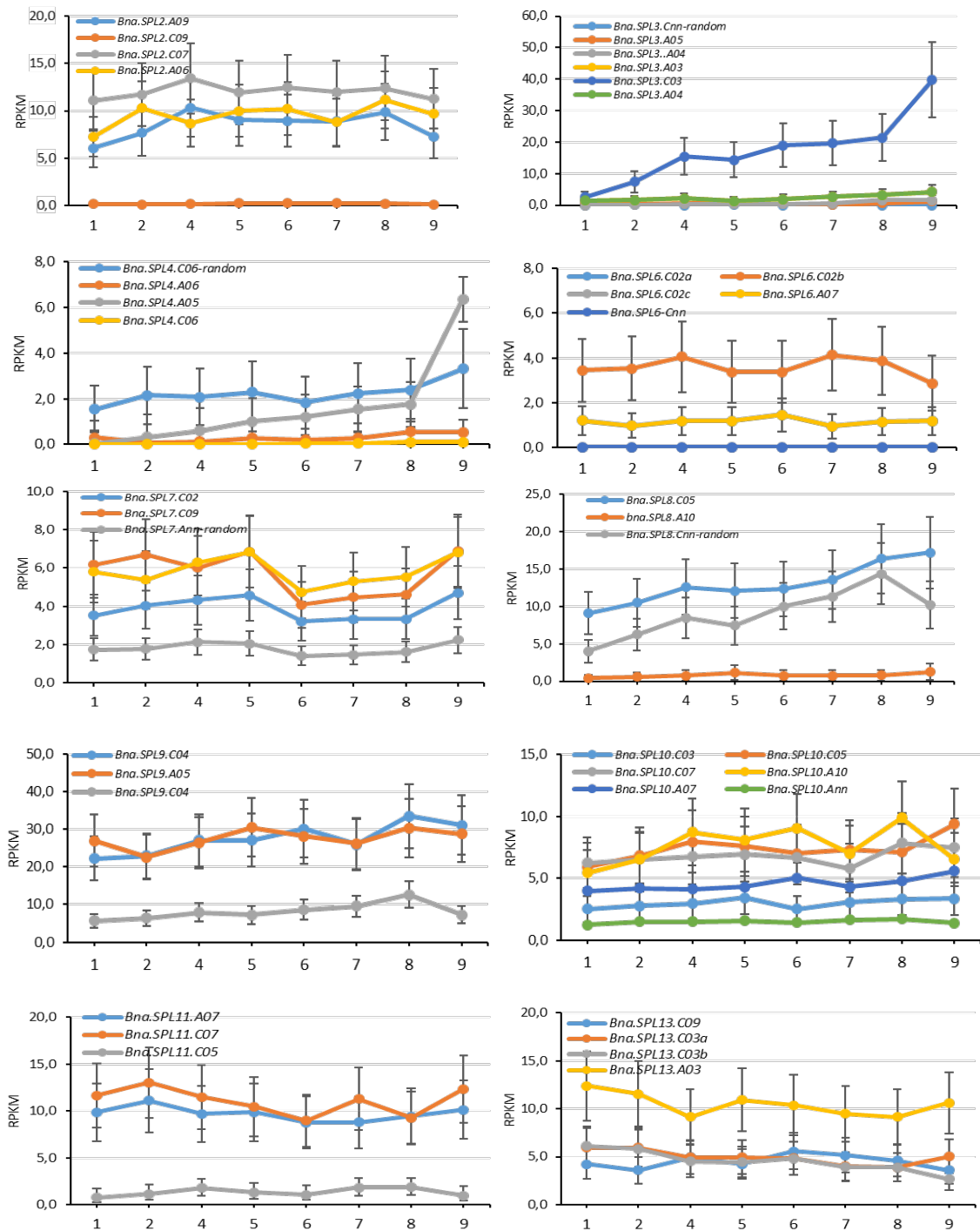
Supplementary Figure 4: Expression profiles of shoot apical meristem marker genes. (a) to (c): Expression profiles of orthologs of *SHOOT MERISTEMLESS (STM)*, *CLAVATA 2 (CLV2)* and *CLAVATA 1 (CLV1)* present in Express617 genome. Error bars represent the lower and upper bounds of the 95% confidence interval of the abundance of the corresponding gene isoform.



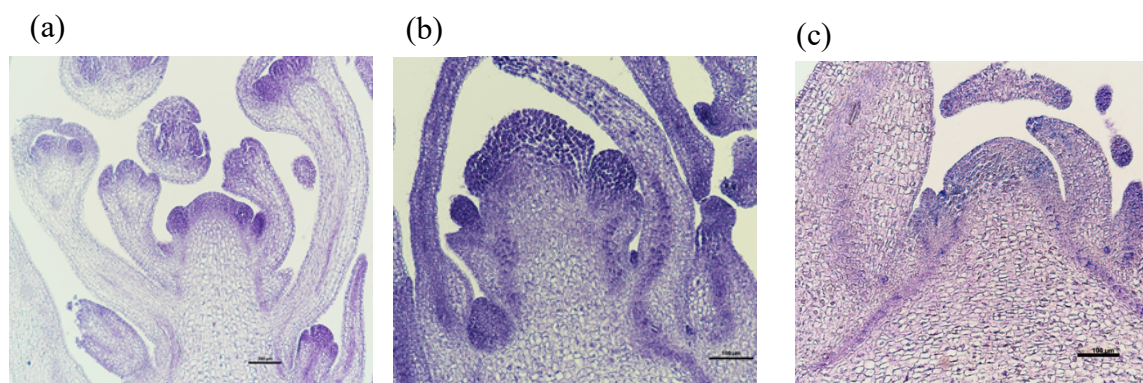
Supplementary Figure 5: Studied gene expression profiles in RNA-Seq. (a) Expression profiles of genes with $RPKM \geq 2$ at the first week of vernalization, but reduced significantly after four weeks until they were not expressed ($RPKM < 1$), and continued to be not expressed across the entire vernalization period. (b) Expression profiles of genes that were significantly upregulated in the first four weeks of vernalization, and continued to increase without any significant drop. (c) Expression profile of genes that were not expressed before seven weeks of vernalization but were expressed ($RPKM \geq 1$) after seven or eight weeks and continued to increase in the following weeks.

Expression level is indicated on the y-axis as $\log_2(1+RPKM)$. Time points are represented on the x-axis: 1-9 weeks in vernalization.

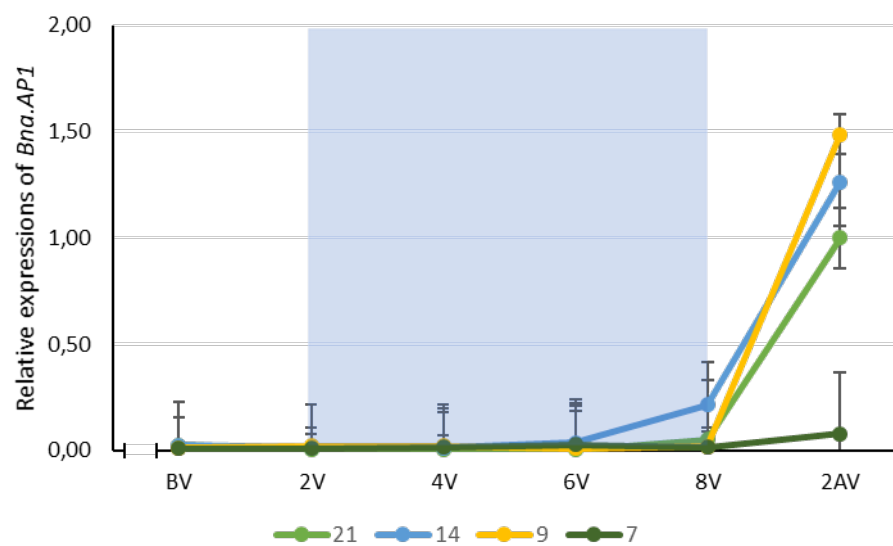
Appendix



Supplementary Figure 6: Expression profiles of SPL genes. (a) to (c): Expression profiles of orthologs of five SPL gene clades present in Express617 genome. Error bars represent the lower and upper bounds of the 95% confidence interval of the abundance of the corresponding gene isoform.



Supplementary Figure 7: Toluidine blue stained longitudinal sections of apices of Express617 plants collected two weeks after vernalization (4°C/LD). (a) SAM of a plant with two weeks pre-growth before vernalization. (b) SAM of a plant with nine days pre-growth. (c) SAM of a plant with one-week pre-growth. Bar = 100 µm.



Supplementary Figure 8: Relative expression of *Bna.AP1* in SAM of plants with 21-days, 14-days, 9-days and 7-days pre-vernalization growth period. Expression was normalized to *Bna.GAPDH*. Error bars were defined by the SEM of three biological samples. BV= Before vernalization, V= Weeks in vernalization, AV= After vernalization. Color code represent the different pre-growth periods.

Supplementary Table 1: List of primers used for Expression analysis using RT-qPCR

Primer name	Primer sequence 5'-3'	Gene family
SM12_fwd	CTCTGATCATCTTCTCTCCTAAGG	<i>Bna.SOC1</i>
SM12_rev	GATCCTATGCCTTCTCCCAAGAG	
SM14_fwd	GAGCATTAGGTCAAGAAAGAACC	<i>Bna.FUL</i>
SM14_rev	GGAGGTTAAGCAGTATTGGGG	
SM67_fwd	GAGAACAAAAGTAGCCGACAAG	<i>Bna.FLC</i>
SM67_rev	CAGGTCATCGCCGCGGAGAAG	
SM68_fwd	ATGGAGGGTCAGAGATCACAGCG	<i>Bna.SPL5 (C05 and A05)</i>
SM68_rev	ATCGACCAGGCACAGCCGCAAG	
SM102_fwd	ACTAGTCGTAAGTTGATGACAGC	<i>Bna.SPL15 (A07 and C06)</i>
SM102_rev	CTGGCAAAACCTTTGGTGAAGA	
SR160_fwd	GGACCAGCAAACCATGGCCACACTG	<i>Bna.AP2.A02</i>
SR159_rev	GCAGCCAAGGTTACAGTTGTAAACGGG	
GC1	CCGCTTCCTTCAACATCATT	<i>Bna.GAPDH</i>
GC2	GTCGCAGCTTTCTCGAGTCT	
Act_1	TCTGGTGATGGTGTGTCTCA	<i>Bna.Actin</i>
Act_2	GGTGAACATGTACCCTCTCTCG	
SM100_fwd	CTATAGTTTTGTTTTGAAAGGGAAAC	<i>Bna.SOC.A05</i>
SM100_rev	AGAAACTTCAGCATCACAAAGA	

Supplementary Table 2: *B. napus* meristem marker genes compared to their Arabidopsis orthologs.

<i>Arabidopsis name</i>	Express617 gene id	<i>B. napus</i> paralog	Genomic sequence length (bp)	Coding sequence identity to At (%)	Amino acid sequence identity to At (%)
<i>STM</i>	g43269	<i>Bna.STM.A09</i>	2880	89.06	83.68
	g147116	<i>Bna.STM.C09</i>	2924	88.63	83.42
<i>CLV1</i>	g36899	<i>Bna.CLV1.A07</i>	3042	82.12	87.93
	g122490	<i>Bna.CLV1.C06</i>	3036	82.29	87.93
<i>CLV2</i>	g6417	<i>Bna.CLV2.A02</i>	2213	84.95	87.08
	g165675	<i>Bna.CLV2.C02</i>	2162	84.53	87.68

Supplementary Table 3: Studied *B. napus* genes compared to their *A. thaliana* orthologs.

<i>Arabidopsis gene</i>	Express617 Gene id	<i>B. napus</i> paralogs	Genomic sequence length (bp)	Coding sequence At gene (%)	Amino acid sequence identity with At gene (%)
<i>FLC</i>	g52652	<i>Bna.FLC.A10</i>	4314	87.77	85.86
	g63440	<i>Bna.FLC.C02</i>	3266	86.26	82.74
	g10869	<i>Bna.FLC.A03a</i>	2941	87.60	83.33
	g5020	<i>Bna.FLC.A02</i>	3518	87.94	85.28
	g156712	<i>Bna.FLC.C09b</i>	4333	88.31	84.38
	g12074	<i>Bna.FLC.A03b</i>	4886	79.29	76.77
	g156716	<i>Bna.FLC.C09b</i>	4449	87.74	85.03
	g75658	<i>Bna.FLC.C03</i>	3146	87.44	82.83
<i>SOC1</i>	g22155	<i>Bna.SOC1.A05</i>	3438	92.27	95.33
	g168970	<i>Bna.SOC1.C04-random</i>	3685	92.71	95.33

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	g158435	<i>Bna.SOC1.A03-random</i>	3609	91.77	94.39
	g199819	<i>Bna.SOC1.Cnn-random</i>	2427	92.56	92.06
	g102010	<i>Bna.SOC1.C04</i>	2417	92.56	92.06
	g112238	<i>Bna.SPL5.C05</i>	612	88.64	77.35
	g25419	<i>Bna.SPL5.A05</i>	615	88.82	76.24
<i>SPL5</i>	g62610	<i>Bna.SPL5.C01</i>	623	88.80	80.71
	g4322	<i>Bna.SPL5.A01</i>	626	88.43	80.00
	g117024	<i>Bna.SPL5.C06</i>	624	77.31	77,31
	g42295	<i>Bna.FUL.A09</i>	3197	93.14	91.60
	g74831	<i>Bna.FUL.C02</i>	4621	93.30	93.31
	g172616	<i>Bna.FUL.C07-random</i>	3501	93.02	92.47
<i>FUL</i>	g177301	<i>Bna.FUL.Ann</i>	3359	94.48	94.14
	g15158	<i>Bna.FUL.A03</i>	3131	94.34	92.08
	g204708	<i>Bna.FUL.Cnn</i>	2165	92.54	92.18
	g118701	<i>Bna.SPL15.C06</i>	1261	74.06	70.15
	g35197	<i>Bna.SPL15.A07</i>	1249	73.59	69.35
<i>SPL15</i>	g96875	<i>Bna.SPL15.C04</i>	1287	74.37	63.93
	g159926	<i>Bna.SPL15.A04-random</i>	1238	83.80	58.46
	g134327	<i>Bna.FAF2.C08</i>	653	83.70	66.67
<i>FAF2</i>	g41472	<i>Bna.FAF2.A08</i>	650	82.86	74.77
	g102383	<i>Bna.FAF2.C05</i>	653	83.80	72.82

Supplementary Table 4: *B. napus* floral meristem and floral organ identity genes compared to their Arabidopsis.

<i>Arabidopsis name</i>	Express617 gene id	<i>B. napus</i> paralog	Genomic sequence length (bp)	Coding sequence identity At (%)	Amino acid identity with At (%)
<i>API</i>	g120662	<i>Bna.API.C06a</i>	3658	93.13	94.14
	g121485	<i>Bna.API.C06b</i>	3701	93.13	94.53
	g36010	<i>Bna.API.A07</i>	3760	93.00	93.42
	g157955	<i>Bna.API.A02-random</i>	3578	92.87	94.14
<i>PI</i>	g51865	<i>Bna.PI.A10</i>	2069	95.70	92.31
	g155309	<i>Bna.PI.C09</i>	2103	90.59	91.35
<i>SEP2</i>	g4846	<i>Bna.SEP2.A01</i>	1857	90.91	92.86
	g26444	<i>Bna.SEP2.A05</i>	2426	90.65	91.57
	g113599	<i>Bna.SEP2.C05</i>	2062	91.24	92.80
<i>AP3</i>	g186420	<i>Bna.AP3.Ann</i>	2112	91.94	93.51
	g18028	<i>Bna.AP3.A04</i>	1870	90.13	91.81
	g46995	<i>Bna.AP3.A09</i>	1915	89.84	90.95

Supplementary Table 5: Chromosome position of six homologs of *SOCI* in Express617.

<i>BnaSOCI</i>	Chromosome	Start	End
	A05	2629203	2632641
	A04	18741547	18743985
	C04	48087210	48089627
	C04-random	867265	870950
	A03-random	902396	906005
	Cnn-random	35200534	35202961

Supplementary Table 6: List of winter and spring accessions from the whole genome sequenced 991 accessions that were analyzed for the presence/absence variation upstream of *Bna.SOCI*.

Accession No.	Name	Origin	Growth type	Sequenced depth	SRR_id	insertion
26	SI 524	Czechoslovakia	winter	10	SRR7601059	yes
147	Darmor	France	winter	10	SRR7601146	Yes
148	Bienvenue	France	winter	10	SRR7601145	No
152	Titus	France	winter	9	SRR7601152	Yes
301	Planet	Germany	winter	12	SRR7601120	Yes
387	Wrg 15	Germany	winter	9	SRR7600976	Yes
397	Hermes	Germany	winter	9	SRR7601107	Yes
487	Gesunder	Germany	winter	10	SRR7601073	Yes
565	Bnw 1.82/86	GDR	winter	9	SRR700963	No
572	SR 413	GDR	winter	12	SRR7600968	No
590	Bnw 1.63/83	GDR	winter	9	SRR7600866	Yes
593	Bnw 9	GDR	winter	10	SRR7600896	Yes
631	SR 417	GDR	winter	9	SRR7631348	Yes
632	Bnw 18	GDR	winter	9	SRR7631349	Yes
636	Ir 1	Hungary	winter	13	SRR7631344	Yes
648	Sinapri	Italy	winter	9	SRR7631222	Yes
665	Michinoku	Japan	winter	10	SRR7631424	yes
694	Mansholt	Netherlands	winter	10	SRR7630985	Yes
697	Barcoli	Netherlands	winter	9	SRR7630988	Yes
705	Marana	Netherlands	winter	9	SRR7630980	Yes
711	Bladkool	Netherlands	winter	10	SRR7631375	No
716	Stego	Netherlands	winter	9	SRR7631367	No
724	Giant Rape	New Zealand	winter	13	SRR7631188	Yes
725	Rangi	New Zealand	winter	9	SRR7631099	Yes

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735	Niemerczanski 4292	Poland	winter	9	SRR7631331	Yes
736	Pn 3/89	Poland	winter	9	SRR7631330	Yes
738	Rod S-5125/79	Poland	winter	10	SRR7631434	Yes
770	K 626/72	Poland	winter	9	SRR7631236	Yes
773	K 2040	Poland	winter	12	SRR7631241	No
781	Pur 1	Poland	winter	11	SRR7631389	Yes
787	Boh 585, Boh 5	Poland	winter	9	SRR7631014	Yes
795	Mah 587	Poland	winter	9	SRR7631006	Yes
819	CR 851	Russian Federation	winter	11	SRR7631313	Yes
937	Sv 66/1109	Sweden	winter	15	SRR7631089	yes
942	Sv 7473	Sweden	winter	10	SRR7631204	Yes
339	Santana	Germany	winter	9	SRR7600829	Yes
703	Blako	Netherlands	winter	9	SRR7630986	Yes
235	Liquanta	Germany	winter	15	SRR7600789	Yes
408	Bk 28/78	Germany	winter	9	SRR7600731	Yes
255	Liraglu	Germany	winter	16	SRR7600652	Yes
27	Slapska, Slapy	Czechoslovakia	winter	5	SRR7601056	No
651	CR 2262	Italy	spring	9	SRR7631051	Yes
336	Pura	Germany	spring	9	SRR7601151	yes
37	Ceska Krajowa	Czechoslovakia	spring	6	SRR7600915	No
63	Galant	USSR	spring	5	SRR7600993	No
70	Kruglik	USSR	spring	6	SRR7600994	No
80	Wesway	Australia	spring	6	SRR7600831	No
88	Tribute	Canada	spring	5	SRR7600924	No
90	Oro	Canada	spring	5	SRR7600926	No
100	Altex	Canada	spring	6	SRR7600757	No
103	Target	Canada	spring	5	SRR7600762	No
108	Westar	Canada	spring	5	SRR7600759	No

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113	Andor	Canada	spring	8	SRR7600775	No
122	Mozart	Denmark	spring	7	SRR7600782	No
132	Bingo	Denmark	spring	5	SRR7600947	Yes
146	Brio	France	spring	9	SRR7601147	Yes
175	Orpal, Crop	France	spring	15	SRR7600954	No
189	Erglu	Germany	spring	5	SRR7601085	No
190	Adamo	Germany	spring	6	SRR7601084	Yes
250	Korinth	Germany	spring	6	SRR7600785	Yes
303	Aurora	Germany	spring	5	SRR7601118	No
335	Mali	Germany	spring	9	SRR7600827	No
336	Pura	Germany	spring	9	SRR7601151	No
348	Futura	Germany	spring	6	SRR7600930	No
377	Loras	Germany	spring	6	SRR7600970	No
430	Lisandra	Germany	spring	5	SRR7600906	No
447	Liraspa	Germany	spring	7	SRR7601040	No
460	Lirawell	Germany	spring	5	SRR7600679	No
496	Liho	Germany	spring	5	SRR7600699	No
529	Gisora	Germany	spring	6	SRR7601035	No
707	Hankkija's Lauri	Netherlands	spring	6	SRR7631371	No
764	Zachodni	Poland	spring	12	SRR7631070	No
895	Korall	Sweden	spring	6	SRR7631299	No
903	Niklas	Sweden	spring	6	SRR7631214	No
913	Gulliver	Sweden	spring	8	SRR7631036	Yes
927	Omega	Sweden	spring	13	SRR7631442	No
926	Olga	Sweden	spring	6	SRR7631441	Yes
962	Swabi-B11	Pakistan	spring	12	SRR7631407	No
965	Islamsbad	Pakistan	spring	9	SRR7631406	No
981	Chamkani	Pakistan	spring	9	SRR7631132	No
1093	CR 2264	China	spring	23	SRR7631402	No

Supplementary Table 7: List of accessions from ERANET ASSYST *B. napus* diversity set tested by PCR for the presence or absence of the insertion.

Accession	Assyst number	Growth type	Insertion
WESTAR DH	BnASSYST-240	Spring	No
NIKLAS	BnASSYST-253	Spring	No
TARGET	BnASSYST-254	Spring	No
ERGLU	BnASSYST-263	Spring	No
Liho	BnASSYST-271	Spring	No
Marnoo	BnASSYST-278	Spring	No
Olga	BnASSYST-280	Spring	No
Tribute	BnASSYST-284	Spring	No
Wesway	BnASSYST-285	Spring	No
Oro	BnASSYST-287	Spring	No
Mozart	BnASSYST-302	Spring	No
Adamo	BnASSYST-312	Spring	Yes
Altex	BnASSYST-313	Spring	No
Andor	BnASSYST-314	Spring	No
Bingo	BnASSYST-317	Spring	Yes
Erake	BnASSYST-325	Spring	No
Futura	BnASSYST-327	Spring	No
Galant	BnASSYST-328	Spring	No
Giant Xr707	BnASSYST-329	Spring	No
Gisora	BnASSYST-330	Spring	No
Granit	BnASSYST-333	Spring	No
Gulliver	BnASSYST-334	Spring	Yes
Hankkija's Lauri	BnASSYST-335	Spring	No

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Korall	BnASSYST-339	Spring	No
Korinth	BnASSYST-340	Spring	No
Kosa	BnASSYST-341	Spring	No
Kruglik	BnASSYST-342	Spring	No
Lirafox	BnASSYST-344	Spring	No
Lirasol	BnASSYST-345	Spring	No
Liraspa	BnASSYST-346	Spring	No
RAFAL DH1	BnASSYST-098	Winter	Yes
Lirabon	BnASSYST-119	Winter	No
Leopard	BnASSYST-128	Winter	Yes
Matador	BnASSYST-160	Winter	Yes
Panter	BnASSYST-166	Winter	Yes
CANARD	BnASSYST-185	Winter	No
Silona	BnASSYST-201	Winter	No
SLAPSKA, SLAPY	BnASSYST-212	Winter	No
Dwarf Essex	BnASSYST-193	Winter	Yes
Express617	BnASSYST-078	Winter	Yes
Nunsdale	BnASSYST-197	Winter	Yes
Matador	BnASSYST-160	Winter	Yes
Savannah	BnASSYST-021	Winter	Yes
Kromerska	BnASSYST-150	Winter	Yes
Rodeo	BnASSYST-014	Winter	No
Rapid	BnASSYST-015	Winter	Yes
Ladoga	BnASSYST-024	Winter	Yes
Capitol	BnASSYST-028	Winter	Yes
Tenor	BnASSYST-054	Winter	Yes

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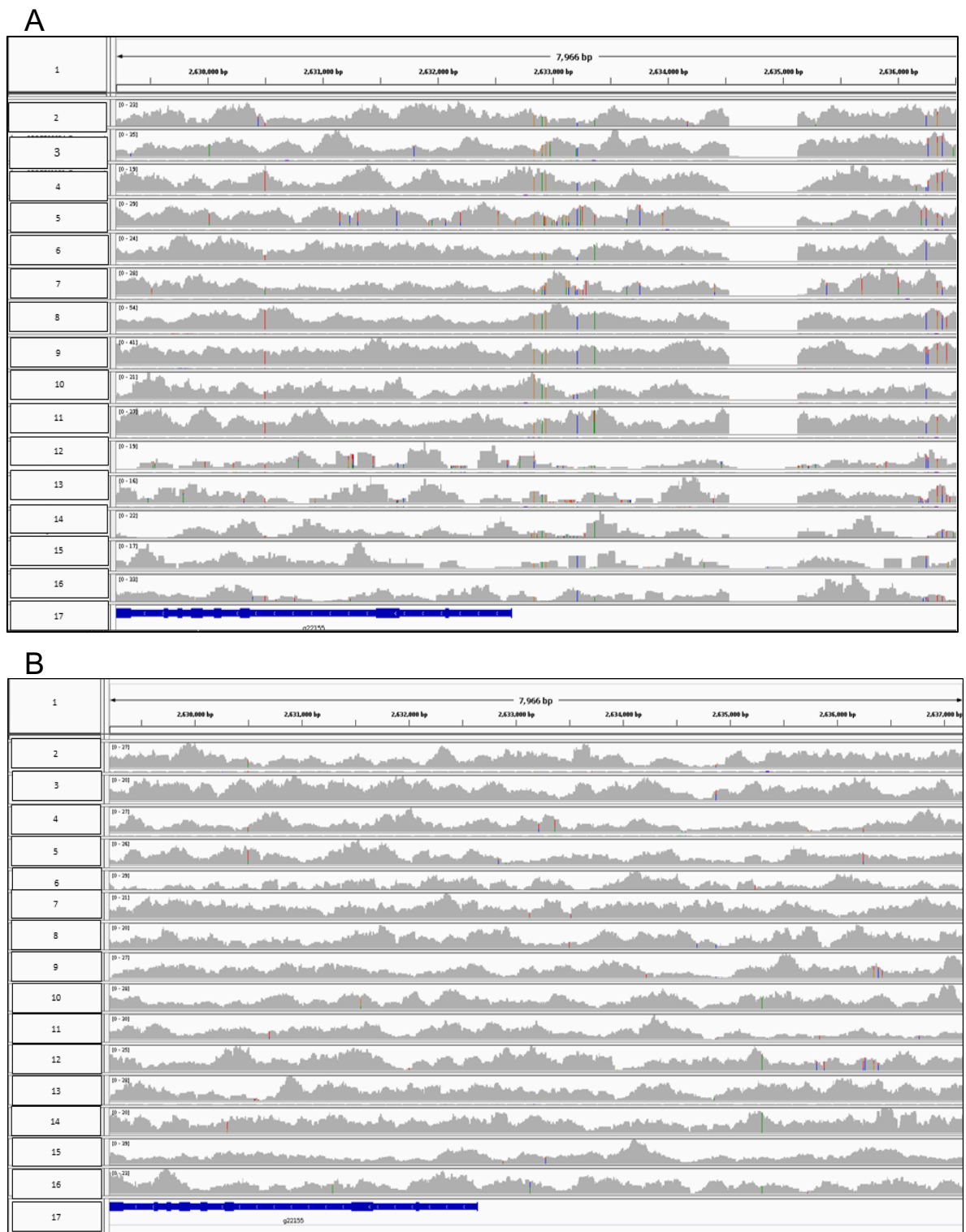
Cooper	BnASSYST-026	Winter	Yes
Samourai	BnASSYST-113	Winter	Yes
Lipid	BnASSYST-034	Winter	Yes
Smart	BnASSYST-046	Winter	Yes
SWGospel	BnASSYST-052	Winter	Yes
Tenor	BnASSYST-054	Winter	Yes
SW Sinatra	BnASSYST-059	Winter	Yes
Nugget	BnASSYST-067	Winter	Yes
Maplus	BnASSYST-117	Winter	Yes
AMBER X COMMANCHE DH LINE	BnASSYST-089	Winter	Yes
GROENE GRONINGER SNIJMOES	BnASSYST-218	Winter	Yes

Supplementary Table 8: Results of local blast of the insertion sequence upstream of *Bna.SOC1.A05* against Express617 genome.

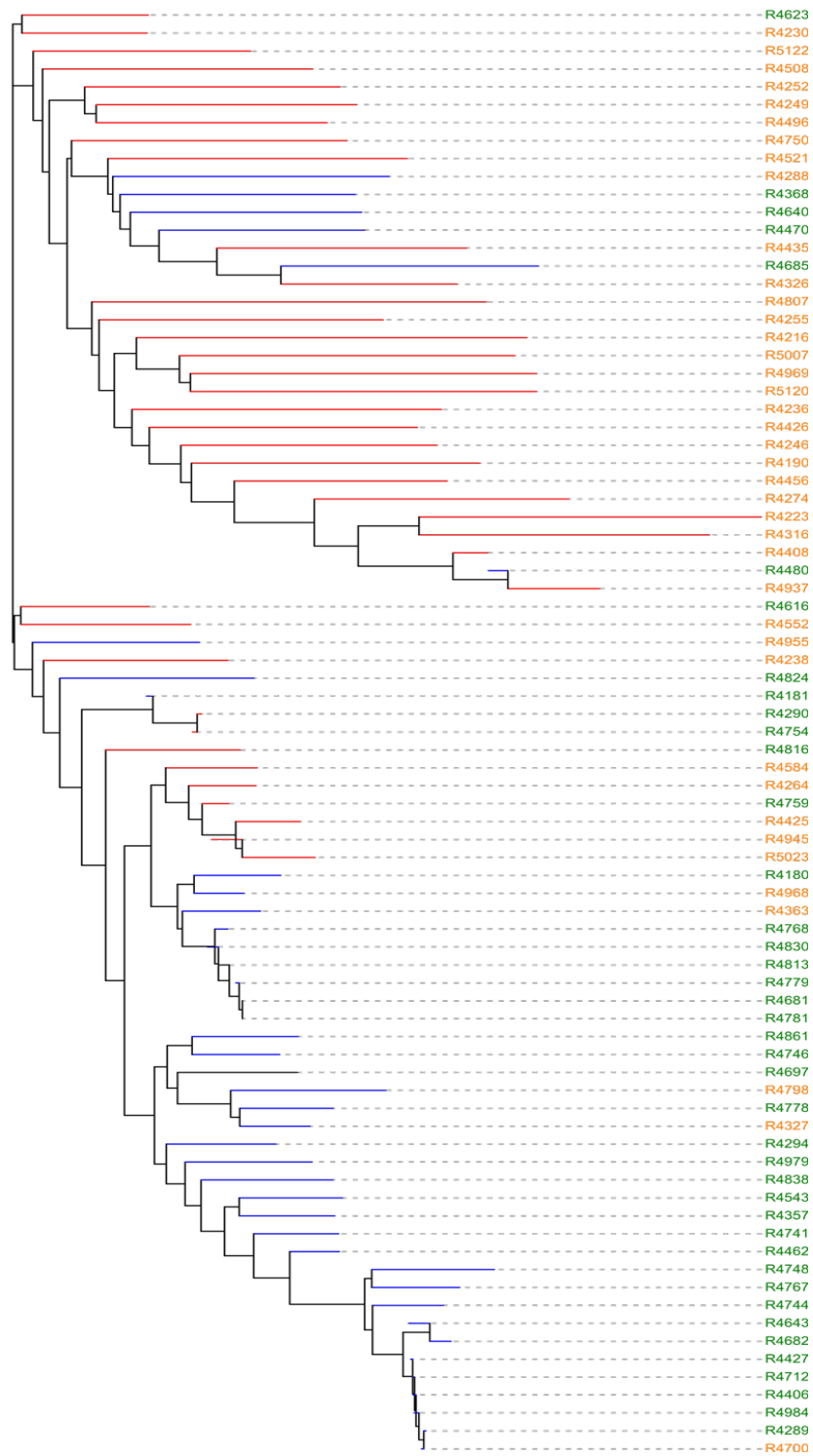
Chromosome	Start	End	Identity	E-value
A05	2634532	2635132	100	0.0
A04	2135909	2136509	99.834	0.0
A06	20055057	20054459	97.496	0.0
A09-random	305568	306178	95.581	0.0

Supplementary Table 9: Results of local blast of the insertion sequence upstream of *Bna.SOC1.A05* against *B. rapa* Z1 genome

Chromosome	Start	End	Identity	E-value
A06	49417216	49416618	97.496	0.0
A06	49315549	49316161	95.269	0.0
A10	9637785	9638281	93.964	0.0
A09	43559843	43559539	83.654	9.41e-75



Supplementary Figure 9: Screenshot from Integrative Genomics Viewer (IGV) showing read alignment results in a 7-kb region (track 1, top) within and upstream of *Bna.SOC1.A05* (track 17, bottom). A: Sequence coverages of 15 spring accessions mapped to Express617. B: Sequence coverages of 15 winter accessions mapped to Express617.



Supplementary Figure 11: Phylogenetic Neighbor-joining tree. The tree was constructed based on SNP data in 10.5-kb genomic region surrounding the insertions, using Tassel and plotted using iTOL. Green color represents winter accessions and orange color represents spring accessions. Red branch represents accessions without the insertion while blue branch represents accessions with insertion.

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9 Supplementary data on CD/DVD

The following supplemental data are available on a DVD and can be distributed upon request

File name	Content	Format
Genes in manuscript	Coding sequences of all studied flowering time genes	.clc
Express617 genome	Whole genome sequence	.fasta
	annotation file	.gff3
	protein sequences	.fasta
	CDs sequences	.fasta
Insertion sequence homologs	Homologous sequences of the insertion in <i>B. rapa</i> and <i>B. napus</i>	.clc
Meristem images	Histological analysis	.nd2 and .tiff
RNA-Seq	Expression analysis of flowering time genes	.txt
Realtime expression	Ct values for Realtime-qPCR data in chapters 2 and 3	.xlsx
ASSYST panel sequencing data	Sanger sequencing of the PAV	.clc

10 Curriculum Vitae and Publications

10.1 Curriculum vitae

Personal Data

Name: Sarah Matar

Date of birth: 10th of November 1993

Place of birth: Otterndorf, Germany

Nationality: German

Education

01/2017 – 02/2021 Ph.D. student at the Plant Breeding Institute, Kiel University

Topic: Molecular genetics and physiological analyses of the transition to flowering in winter rapeseed

10/2014 - 01/2017 Master of AgriGenomics, Kiel University

Master's thesis at the Molecular Phytopathology Lab, Kiel University

Topic: Investigation of the anti-fungal activity of transgenic Arabidopsis plants over-expressing PDF2.2.

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10.1 Publications and declaration of own contribution

Sarah Matar, Avneesh Kumar, Daniela Holtgräwe, Bernd Weisshaar and Siegbert Melzer.
The transition to flowering in winter rapeseed during vernalization. August 28, 2020.
Plant, Cell and Environment. Accepted.

11 Declaration of own contribution

Chapters 2 of this thesis was prepared as a manuscript for publication. The respective declarations of co-authorship are presented on the following page. Meanwhile, chapter 3 includes the work of the doctoral candidate alone.

Declaration of co-authorship

If a dissertation is based on already published or submitted co-authored articles, a declaration from each of the authors regarding the part of the work done by the doctoral candidate must be enclosed when submitting the dissertation.

1. Doctoral candidate

Name: Sarah Matar

2. This co-author declaration applies to the following article:

The transition to flowering in winter rapeseed during vernalization

The extent of the doctoral candidate's contribution to the article is assessed on the following scale:

- A. Has contributed to the work (0-33%)
- B. Has made a substantial contribution (34-66%)
- C. Did the majority of the work independently (67-100%)

3. Declaration on the individual phases of the scientific work (A,B,C)	Extent
Concept: Formulation of the basic scientific problem based on theoretical questions which require clarification, including a summary of the general questions which, it is assumed, will be answerable via analyses or concrete experiments/investigations	B
Planning: Planning of experiments/analyses and formulation of investigative methodology, including choice of method and independent methodological development, in such a way that the scientific questions asked can be expected to be answered	C
Execution: Involvement in the analysis or the concrete experiments/investigation	C
Manuscript preparation: Presentation, interpretation and discussion of the results obtained in article form	C

4. Signature of all co-authors

Date	Name	Signature
10.11.20	Avneesh Kumar	
27.10.20	Daniela Holtgräwe	
28.10.20	Bernd Weisshaar	
10.11.20	Siegbert Melzer	

5. Signature of doctoral candidate

Date	Name	Signature
10.11.20	Sarah Matar	

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