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Copy-specific expression pattern of major flowering time genes in oilseed rape (*Brassica napus*)

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LIST OF ABBREVIATIONS

AGL24= agamous-like 24
AP1 = apetala 1
AV = after vernalization
BV = before vernalization
CAL= cauliflower
CO= constans
COLDAIR = cold assisted intronic noncoding RNA
C_t = crossing threshold
E= efficiency
FD= flowering locus D
FKF1= flavinin-binding kelch repeat F-Box protein 1
FLC= Flowering locus C
FLK = flowering locus K
FRI= frigida
FT = flowering locus T
FUL = fruitfull
FUL = short vegetative phase
GA = gibberellic acid
GI= gigantean 1 or 2
LD = luminidependens
LF= less fragmented
LFY = leafy
MF= more fragmented 1 or 2
NE = not expressed
PEBP: phosphatidyl ethanolamine-binding protein
qRT-PCR= quantitative real-time PCR
RQ = relative quantity
SD = standard deviation
SOC1= suppressor of overexpression of constans 1
TFL1= terminal flower 1
T_m = melting temperature
UTR = untranslated region
UV = ultraviolet light
WAS=weeks after sowing
WGT = whole genome triplication

1 INTRODUCTION

1.1 *Brassicas*

The genus *Brassica* belongs to the tribe *Brassiceae* that is part of the *Brassicaceae* family [1]. This family is one of the most diverse with 338 genera and 3,709 species [2]. Most of the research in this family has been focused on *Arabidopsis thaliana* [3] that has been selected due to some useful characteristics, among them a small genome size, fast life cycle and many seeds per generation. This genome was the first flowering plant to have its genome sequenced (The Arabidopsis Genome Initiative, 2000). Other members of this family that have called the attention due to its economic influence are *Brassica, Raphanus,* and *Sinapis* [4].

The *Brassica* species can be categorized into vegetables, oilseeds, condiments, and fodder. In 1935, U established the first relationship between the most important *Brassica* species. This relationship is known as the "Triangle of U" [5]. Here, it was proposed that the species with the higher chromosome numbers *B. napus* (AACC, n=19), *B. carinata* (BBCC, n=17) and *B. juncea* (AABB, n=18) originated from an interspecific hybridization of the diploid genomes of *B. nigra* (BB, n=8), *B. oleracea* (CC, n=9) and *B. rapa* (AA, n=10) (Figure 1).

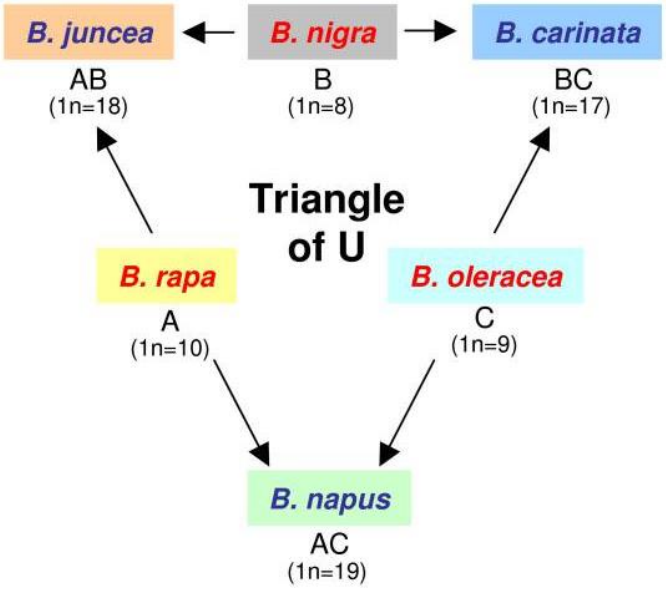

Figure 1. The Triangle of "U". The amphidiploid genomes are in the vertices of the triangle and the diploid genomes in the edges. The relationships are indicated by arrows [6].

1.2 Evolution of the *Brassicaceae*

Among the *Brassicaceae,* approximately 50 % of the species are considered to be polyploid [7]. Based on studies performed in *A. thaliana,* the *Brassicaceae* ancestor experienced three ancient whole-genome duplication events [7]. The oldest event thought to have occurred during Late Jurassic or Early Cretaceous period and seems to have effect in the division between monocot-eudicot [8]. The second and third occurred approximately 70 million years ago (Mya) and 40 Mya, respectively [9]. These different events of polyploidization may have helped the performance and adaptability to extreme environment governing ~65 Mya [9]. These three WGD events have been shown to be shared with *Brassica* [10]. Later on, an additional extensive whole-genome triplication (WGT), that happened approximately 13-17 million years ago, occurred in *Brassica* [11–13]. After WGT, the triplicated fragment suffered fractionation and reshufflings within the genome [1]. Three subgenomes have been described as an effect of WGT in both *B. rapa* and *B. oleracea*. Each subgenome has been named according to the gene density from high to low as: Less fragmented (LF), More Fragmented 1 (MF1) and More Fragmented 2 (MF2) [14]. Characteristics of the LF subgenome are a stronger gene expression and less non-synonymous mutations compared to MF1 and MF2 [14]. This WGT event had a big influence on speciation and expansion, affecting diversity within *Brassica* [1]. Plants from the genus *Brassica* have a broad spectrum of morphotypes. For example, the species *B. oleracea* comprises different varieties for human consumption such as var. *acephala* has large leaves with normal inflorescence appearance (e.g. kale); var. *gemmifera* has buds like small balls (e.g. Brussels sprouts) and var. *botrytis* has long leaves with inflorescences (e.g. cauliflower) [15]. This complexity has allowed the study of several phenomena such as vegetative development [16] and flowering time [17].

The divergence between *B. rapa* and *B. oleracea* has been estimated to be 3.75 Mya [18]. Later on, approximately 7500 years ago, by hybridization followed by allopolyploidization, these diploids gave origin to *B. napus* L. [19]. The exact origin and place of occurrence is still nowadays on debate.

As consequence of these different duplications events the *Brassica* genome shows the presence of multiple gene copies. A big part of the retained genes after duplication events tend to be: ribosomal proteins, protein kinases and transcription factors [20]. The final fate of the gene can be for example the gain of a new function

by mutation (neofunctionalization) [21]. Another reason for the retention can be specialization or subfunctionalization, where a specific copy can be required at a specific time or organ [21].

1.3 *Brassica napus* as oilseed rape/ canola

In *Brassica,* mainly 4 species have been widely used as oilseed crop: *B. juncea, B. carinata, B. rapa,* and *B. napus* [22]*.* Among these, *B napus* stands out due to its high yield level and relatively high oil production [23]. *B. napus,* also called rapeseed, is the third producer in the oil vegetable market, only after soybean and palm oil. The European Union, China, and Canada are leading as main producers of oilseed rape (USDA, September 2017). Most of the production of oils go to usage in salads or cooking, margarine and shortenings [24] as well as into biodiesel production.

Rapeseed, as well as others members of the *Cruciferae*, has the natural characteristic of containing 20-40% of erucic acid [25] and high glucosinolates in the meal. These compounds are characterized as undesired or antinutritional for human and animal consumption. Because of this and the necessity to develop a domestic supply of oil, Canadian scientist started working on rapeseed a well-agronomic ally adapted plant [23]. Initially, Canadians grew rapeseed for use like lubricants of naval craft engines during the war [26]. But, at the same time, they started to develop the possibility to use products of rapeseed for human or animal consumption.

The first breakthrough came in 1959 when a low erucic acid rapeseed line was identified [23]. Then, the effort shifted to transfer this characteristic into their well-adapted superior cultivar [23]. Nine years later, the first low-erucic acid rapeseed was released, a cultivar called “Oro” [23]. The efforts to improve the meal quality of rapeseed obtained after oil extraction continued. The main concern was the high amount of glucosinolates. These compounds, beyond giving a bitter taste to the meal, also affect the health of animals. During the late sixties, a low-glucocinolate trait was identified by Keith Downey [23]. As before, this characteristic was introduced into the low-erucic acid lines. By doing this, the first low-erucic acid (<2 %) low-glucosinolates (<26.5 µmol/g) content rapeseed came into market [25]. Later on, the name “canola” (Canadian oil low acid) was adopted (1978) [23]. Three years later, 87 % of the rapeseed produced in Canada was canola type [25]. In Canada,

the rapeseed produced is spring type, meaning: planting in the spring with harvest completed in the same year (annual) [26]. This is accompanied by a shorter development period, to escape the harsh weather conditions, that as consequence also makes the breeding program short. So, all progress in oil quality done by Canadian scientist was carried out in spring type's cultivars. When these new desirable characteristics of low-erucic and low glucosinolate contents were wanted to introgress in cultivars of Europe, the scenario became a bit more difficult. In Europe, because the weather conditions allow it, most of the rapeseed cultivars are winter types. Unlike spring types, they are biannual, with a longer developmental period due to vernalization. This, as consequence, also extended the production period of each generation. It took longer, but after 15 years of breeding gave origin to the first low-erucic-acid European rapeseed [23]. Later on, in 1986, the double zero quality cultivar became available in Europe.

Even though a big effort has been made to improve the oil quality to make it suitable for human consumption, this is not the only market where oilseed rape can take part of. For example, in industry, a high content of erucic acid in oil can be a desired characteristic [24]. The property of long polymethylene chains in erucic acid makes it a very good lubricant in, for example, in steel casting processes [24]. Because of this, also some high erucic acid rapeseed cultivars have their niche in the industry. Nowadays, the breeding programs focus mainly to improve oil content, fatty acid composition, meal quality and yield [27].

1.4 Its flowering time

Throughout evolution, angiosperms (flowering plants) have evolved specialized organs to facilitate reproduction. The transition from the vegetative stage into the reproductive one contains the essential step of flower and reproductive organ formation (figure 2). This step allows the plant to proceed with sexual reproduction for either outcrossing or self-pollinated plants. Due to this fact, synchronization of flowering time is crucial, especially for outcrossing species, in order to allow the maximum amount of successful pollination events and therefore increasing seed production. All of the different stimuli can be external as well as internal signals.

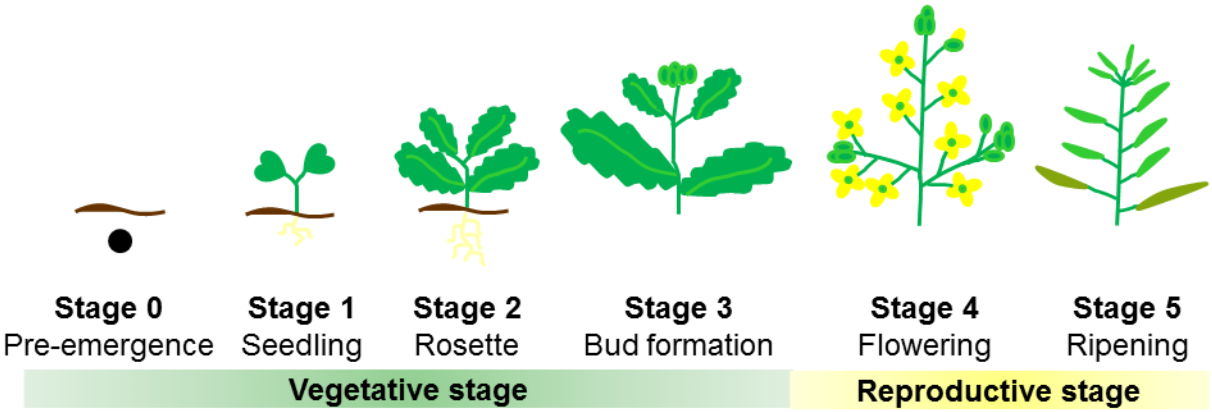

Figure 2. Growth stages of *B. napus.*

Based on the vernalization requirements to flower, in rapeseed is possible to find three different growing groups: winter, semi-winter and spring types (table 1). Another important factor affecting flowering in *B. napus* is photoperiod. Rapeseed is able to flower under long day conditions.

Table 1. Different types of *B. napus.*

	Spring	**Semi winter**	**Winter**
Growing areas	Canada, Europe, Australia	China, Japan	Europe
Sown in	Spring	Later summer – early autumn	
Vernalization requirements	None	2-8 weeks (intermediate)	8-14 weeks (strong or full)
Winter hardy	No	No	Yes

Most of the research done to reveal the processes involved in flowering have been carried out in *A. thaliana,* which is a facultative long day plant with most of the ecotypes growing as winter annuals [28]. Based on these studies, the flowering process can be divided into two groups of genes: (i) the genes involved in changing vegetative meristem into floral meristem and (ii) the ones related to the flower formation [28]. Even though the flower formation occurs at the shoot apical meristems of the plant, the environmental conditions are sensed by the leaves [29].

Five pathways have been identified in *Arabidopsis* related to environmental factors related to flowering (figure 3, [30]). Nevertheless, the ultimate end of all these pathways is to connect with the genes that will give origin to flowers [31] procuring the right conditions. Two of the most representative genes related to flower formation are transcription factors APETALA1 (AP1) and LEAFY (LFY) [31,32]. In the case of AP1, acts locally determining the formation of petals and sepals [32].

A convergence point among the different pathways is the so-called flower integrator genes. These genes are FLC, SOC1, and FT as the major key regulator [33].

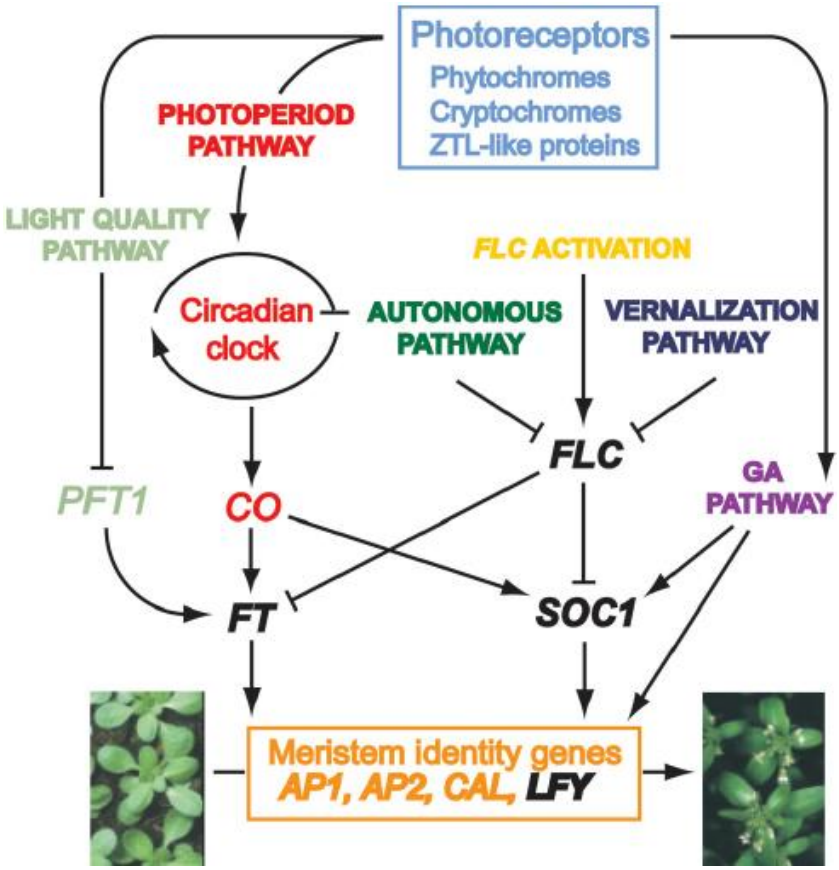

Figure 3. Genetic control of flowering time in Arabidopsis. The scheme shows the known pathways involved in flowering regulation and the interactions between them and the floral integrator genes. Photoreceptors act at least at three different levels: entraining the circadian clock, repressing PFT1 expression and probably modulating GA biosynthesis. FLC integrates signals from several pathways and repress SOC1 and FT. CO, a circadian clock regulated gene, activates both FT and SOC1. SOC1 also integrates signals from FLC, CO and the GA pathway. Both FT and SOC1 activate meristem identity genes causing the transition from vegetative to reproductive development at the shoot apical meristem. Floral integrator genes are represented in black bold fonts. Arrows indicate activation and bars depict inhibition [30].

1.4.1 Photoperiod pathway

One of the first factors that were suggested and tested to be important for flowering time was the day length. The relative length of the day is an important factor for growth and development of plants, especially in sexual reproduction where some plants could not produce flowers if the day length was not within certain limits [34]. According to this, the flower initiation in response to day length has been used to classify plants into three categories: (i) short-day plants (SDP) flower with less than a certain number of hours of light in a day; (ii) long-day plants (LDP) with more than a certain number of hours of light in a day; and (iii) day-neutral plants (DNP) which flower regardless the photoperiod [35].

Later on, it was studied how plants can sense the type of light. By studying bean's seedlings, it was observed the movement rhythms in leaves following the light and that plants kept the movements even after being put to darkness and conclude that the "biological clock" of plants is somewhat independent of the day cycle (light or dark) [36]. Nowadays there is a more elaborated theory regarding the coordination between the internal clock and environmental conditions controlling the circadian rhythm and a biological activity [37].

Most of the advances of revealing the components of the photoperiod pathway have been done in the model facultative LDP *Arabidopsis thaliana*. By studying X-ray mutants of *A. thaliana* the first insights into the genes related to photoperiod flowering were found. Initially, four mutant lines that were named as *co* (*constans*), *gi1* (*gigantean 1*), *gi2* (*gigantean 2*) and *ld* (*luminidependens*) showed to be flower later than the wild-type strain [38]. Also, these late-flowering mutants have more biomass and produced more seeds compared to the wild type, so the delay in flowering was not caused by a mutation in genes related to the plant growth. In 1991, though the analysis several mutant lines of *A. thaliana* under short and long-day conditions and postulated that *CO, GI* and *FT* genes are major controllers of the pathway in response to long-days photoperiod [39].

The expression levels of *CO* are regulated by the circadian clock, having the peak of expression during long days at the end of the day and during short days after nightfall [40]. The accumulation of *CO* transcripts at the end of long days requires the FLAVININ-BINDING, KELCH REPEAT, F-BOX PROTEIN 1 (FKF1) protein. This protein encodes an F-box protein that has been related to the degradation of proteins through ubiquitination [41]. FKF1 interacts with GI and its stability depends on light

[42,41]. Hence, this complex might be targeting the degradation of a negative regulator of *CO* mRNA and by this increasing the levels at the end of the day [41,1].

Two of the most important genes within the flowering pathways are *FT* and *TERMINAL FLOWER 1* (*TFL1*) belonging to the phosphatidylethanolamine-binding protein (PEBP) gene family [43,44]. These two genes have contrasting functions where *FT* acts as a promoter of flowering [45] and *TFL1* represses it [46]. *CO* directly targets and activates *FT* and the transcription factor *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) [47].

The development of flowers starts with FT protein production in the leaves. The protein transported throught the phloem to the shoot apical meristem. In here FT protein interacts with Bzip protein FLOWERING LOCUS D (FD) forming a heterodimer [48,47]. This interaction induces the expression of flower-specific targets such as MADS-domain protein *APETALA1* (*AP1)* and *FRUITFULL* (*FUL*) together with *LEAFY* (*LFY*) at the shoot apex [41] that will initiate the floral meristem formation. Other genes acting as transcription factors also actively promote the induction of floral meristems such as *CAULIFLOWER (CAL), FRUITFULL (FUL), SHORT VEGETATIVE PHASE (SVP)*, *AGAMOUS-LIKE24 (AGL24),* and *SOC1* [49].

1.4.2 Vernalization pathway

Vernalization can be defined as “the acquisition or acceleration of the ability to flower by a chilling treatment” [50], although not all plants require this step to flower. Annual (or spring type) plants have a facultative response to vernalization, whereas biannual (winter type) plants require vernalization to trigger flower formation. Once the plant has achieved the vernalized state, the changes are maintained stable but not passed to the next generation [51]. Because of this, the vernalization model involves epigenetic changes that are reset before passing the genetic information to the next generation [52,51].

When the different ecotypes of flowering time in *A. thaliana* with different vernalization requirement were studied, two genes were identified: *FLOWERING LOCUS C* (*FLC*) and *FRIGIDA* (*FRI*) [53–55]. These two genes act synergistically to delay flowering [51]. *FLC* encodes a MADS domain protein and its expression is localized to the shoot and root tips [51,51,54]. The expressed protein avoids

flowering by repressing *FT, SOC1* and *FD* [56]. In *Arabidopsis,* the major determinant of the flowering time variation was explained by an allelic variation of *FRI* [57] and *FLC* levels [58]. In *B. napus,* four homologues of *FRI* were identified by PCR-cloning from a BAC library [59] and sequence capture [60]. All four homologues were found to be expressed. *Bna.FRI.a* was shown to be a major determinant of flowering time by linkage analysis and association mapping [59]. Initially, at least five *FLC* were identified in *B. napus* winter cultivar Colombus [61]. When these *Bn.FLCs* were transformed into *Arabidopsis* plants, flowering time was significantly delayed from 20 days up to 7 months, and some plants never flowered [61]. Alternatively, these differences could also be explained by similarities with *At.FLC,* allowing a better recognition and interaction within floral induction of *Arabidopsis* [61]. Later on, by sequence capture, 10 copies of *FLC* were identified in *B. napus,* but not all of them are thought to be functional [60].

As discussed above, the level of *FLC* decreases by vernalization treatment. The epigenetic vernalized state is due to histones modifications of *FLC* [62–64]. VERNALIZATION *INSENSITIVE3* (*VIN3*) is the first player in decreasing, during vernalization, the expression levels of *FLC* [64]. After returned to normal temperature conditions, two genes have been identified responsible for the epigenetic maintenance, *VERNALIZATION1* (*VER1*) and *VERNALIZATION2* (*VRN2*) [65,66]. This epigenetic modification is maintained through mitotic division but resets during embryogenesis [67]. By doing this, the FLC levels are restored in the next generation so they will have to undergo vernalization to flower.

The downregulation of FLC is not only governed by epigenetic modifications, but also by the intervention of long non-coding RNAs. This group of antisense long non-coding transcripts are named *COOLAIR* [68] and are part of the autonomous pathway.

1.4.3 Autonomous pathway

The autonomous pathway has the role of promoting flowering independent of the day-length [69]. The genes that participate in this pathway have been elucidated by analyzing different mutants that flower late under all photoperiodic conditions but responded to vernalization [70,71]. The components of this pathways are

LUMINIDEPENDENS (LD), FLOWERING LOCUS D (FLD), FVE, FY, FLOWERING LOCUS K (FLK), FPA, and *FCA* [69] and *REF6* [72]. These genes have a common target that showed to be *FLC* [73] and hence, promoting flowering indirectly. Because of this, vernalization pathway and autonomous pathway are thought to work in parallel to affect mRNA levels of *FLC* [72]. In this case, *FLC* gets epigenetically regulated by *FLD* and *FVE* through histone deacetylation [69].

1.4.4 Gibberellic acid pathway

Gibberellic acid (GA) is a phytohormone that regulates, among other processes, seed germination, leaf expansion, stem elongation, pollen maturation, and induction to flower [74,74]. There are over hundred different GA described, but only a few have some biological activity. The remaining are non-bioactives working as precursors [75]. Even though GA mediates flowering under certain circumstances, this role is very dependent on the species. In flowering mediated by GA is regulated by DELLA proteins [76]. When the levels of bioactive GA are low, this comes accompanied by an accumulation of DELLA proteins that act delaying flowering independent of photoperiod [76].

1.4.5 Aging

Carbohydrates function as a long-distance signaling in floral induction [28]. An important factor that plays in favor to form flower is aging. The underlying mechanism controlling vegetative phase change and the competence to flower are miRNA156 and SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE (SPL) [77]. Another miRNA that has called the attention is miRNA172 [77]. In this case, the abundance of both miRNAs depends on the shoot age and at the same time are able to influence flowering [77]. The quantity of miRNA156 is higher in younger than older plants and vice-versa for miRNA172 [77]. The miRNA172 was predicted to target APETALA2 (AP2) acting as an activator of flowering and floral development [77,78]. Another factor that affects the flowering based on aging is the transcription factor TEMPRANILLO (TEM) [77]. In *Arabidopsis,* TEM1 binds directly to the FT promoter repressing transcription, delaying flowering [77].

1.5 Aims of the study

Flowering is a very important part of plants life. Recognizing the right conditions to flower is crucial to ensure a good set of flowers and eventually seed formation. Understanding the mechanism underlying flowering time processes can help to breed for better environmentally adapted plants. This could also help to reduce the breeding time by identifying the gene/s involved in vernalization requirements.

The aim of the study is to investigate the gene expression of two major flowering time genes: *FLC* and *FT*. In the case of *FLC*, it has been shown that in *A. thaliana* plays a role in inhibiting flowering before vernalization. On the other hand, *FT* promotes flowering. Both genes are related to differences observed in flowering time in different species. These genes, unlike in *Arabidopsis,* are largely duplicated in *B. napus*. This gene redundancy can affect, for example, functionality, and tissue-specific or time-dependent gene expression. Based on the *B. napus* reference genome there are 9 *FLC* and 6 *FT* annotated copies. The main activities carried out in the present project are: (i) to design specific primer pairs for each one of the *FLC* and *FT* copies, suitable for qRT-PCR, (ii) to analyze the gene expression of *FLC* and *FT* genes in *B. napus* genotypes under two different conditions: vernalization and aging.

2 MATERIALS AND METHODS

2.1 Plant material and treatment

The oilseed rape (*Brassica napus)* genotypes used in the experiments are part of the diversity set ERANET-ASSYST. The selected genotypes are the winter types Manitoba (ASSYST023) and Lisabeth (ASSYST033); unusual winter type Mansholt (ASSYST112), and spring types Girosa (ASSYST330) and Korall (ASSYST339). Hereafter the genotypes will be referred to as winter 1, winter 2, unusual winter, spring 1, and spring 2. The unusual winter genotype is winter-hardy with the unusual characteristic that without vernalization ~ 90 % of the plants are able to flower. When this genotype is vernalized 100 % of the plants are able to flower.

Six pots per genotype with 3-4 seeds were sown. The pots were left to germinate at the greenhouse under a photoperiod of 16/8 h day/night with a temperature of 20 °C during the day and 17 °C at night. The seeds germinated one week after sowing. At this time, the seedlings were singularized. Three weeks after this, the plants were transferred to bigger pots to allow a better development.

Seven weeks after sowing (WAS) the first sample was collected (before vernalization sample). One mature leaf per plant was cut and immediately frozen into liquid nitrogen. The samples were then stored at -80 °C until processing.

Immediately after the first sampling, three plants per genotype were transferred to the cool room for vernalization treatment. The others three plants were kept in the greenhouse as a not vernalized treatment. The vernalization regime was eight weeks at 5 °C with a photoperiod of 8/16 h day/night.

Once the vernalization treatment was completed, the plants were removed from the cool room and the second leaf sample was collected (after vernalization). At the same time, a leaf sample was taken from the not vernalized plants kept at the greenhouse (sample 15 WAS). As before, one leave was collected from each plant and immediately frozen in liquid nitrogen and stored at -80 °C until processing. After the last sampling, the plants were discarded.

2.2 RNA extraction and quantification

Total RNA was extracted by using the NucleoSpin miRNA kit (Macherey-Nagel, Düren, Germany). Initially, the samples were crushed using a mortar and pestle under liquid nitrogen. Approximately 50 mg of sample was placed in a 2 mL nuclease-free centrifuge tube and 800 µL of Trizol (Ambion by Life Technologies, Carlsbad, CA, USA) was added and homogenized with 5 min incubation at room temperature. After this, 160 µL of chloroform was added and homogenized with 3 min incubation at room temperature. The samples were centrifuged at 4 °C for 15 min at 12,000 g. After centrifugation, 400 µL of supernatant was transferred to a new tube and 1mL of Buffer MX was added. Both solutions were mixed and transferred onto the NucleoSpin RNA column placed in a 2 mL collection tube. The column was then centrifuged 30 sec at 12,000 rpm. After this, the membrane containing the bound RNA was desalted using 350 µL of Buffer MDB. The column was centrifuged 1 min at 12,000 rpm. After this, the flow-through was discarded and 100 µL of RNase-free rDNase were directly added to the membrane. The tubes were kept open and the DNase treatment was incubated for 30 min at RT. After DNase treatment, the membrane was first washed with 600 µL of Buffer MW1 and the tubes were centrifuged 30 sec at 12,000 rpm. A second wash was done using 700 µL of MW2 and the tubes were centrifuged 30 sec at 12,000 rpm. A third wash was done using 250 µL of MW2 and a centrifugation of 2 min at 12,000 rpm. After this was, the total RNA bound to the membrane was eluted in 50 µL of nuclease-free H_2O.

The total RNA was quantified using Qubit 2.0 Fluorometer software v3.10 (Invitrogen by Life Technologies, Carlsbad, CA, USA) with the Qubit RNA Broad Range Assay kit (Thermo Fisher Scientific, Cleveland, OH, USA). The quality of the RNA was measured using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). The RNA samples were stored at -80 °C until further usage.

2.3 DNase treatment

All steps were performed in nuclease-free centrifuge tubes. Before cDNA synthesis, the samples were treated again with DNase to ensure a DNA free sample. Initially, 4 µg of total RNA was DNase treated using DNase I RNase-free kit (Thermo Fisher

Scientific, Cleveland, OH, USA). For each of the RNA samples, a dilution of 400 ng/µL in 15 µL was prepared with RNase-free H_2O. After this, the following reagents were added: 10 µL of RNA sample (400 ng/µL), 2 µL of 10x Reaction Buffer, 4 µL of DNase I RNase-free, and 4 µL of RNase-free H_2O. These reagents were gently mixed with the micropipette and placed in the thermomixer comfort (Eppendorf, Hamburg, Germany) at 37 °C for 30 min. The reaction was stopped by adding 50 mM ethylenediaminetetraacetic acid to a final concentration of 5 mM. The reaction was also heat inactivated at 65 °C for 10 min.

2.4 cDNA synthesis

The cDNA for each sample was synthesized by using 1 µg of total RNA, DNase treated, and the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Cleveland, OH, USA). In a 96 well Non-Skirted PCR plate (4titude, Berlin, Germany), 1 µg of total RNA was mixed with 1 µL of Oligo dT primers and nuclease free H_2O to a final volume of 12 µL. The samples were incubated at 65 °C for 5 min in the GenAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). Afterwards, the samples were cooled down on ice and briefly centrifuged. Later on, the following reagents were added in the order respectively: 4 µL of 5X Reaction Buffer, 1 µL of RiboLock RNase inhibitor, 2 µL of 10 mM dNTP mix, 1 µL Revert Aid M-MulV RT enzyme (20 µL final volume). The samples were gently mixed and briefly centrifuged. Three controls were also included in each of the cDNA batches prepared. The controls were prepared as the samples but adding different templates. The positive control was prepared using 100 ng of Control GAPDH RNA provided in the kit. In the case of the non-template control, the volume of the template was replaced with nuclease-free H_2O. The last control, Reverse transcriptase, was prepared with a random sample as the template without RevertAid M-MulV RT enzyme.

The reverse transcriptase reaction was carried out in the GenAmp PCR system 9700 thermal cycler for 2 hours at 42 °C. A final step of enzyme inactivation at 70 °C for 5 min was also included. Once the reactions were finished, each cDNA sample was quantified by using Qubit 2.0 Fluorometer (Invitrogen by Life Technologies, Carlsbad,

CA, USA) with the Qubit DNA High Sensitivity Assay kit (Thermo Fisher Scientific, Cleveland, OH, USA). The cDNA samples were then storage at -80 °C until usage.

2.5 Primer design

The sequences used in this project were obtained from the *B. napus* Darmor-Bzh reference genome v.4.1 [19]. The specific primers for Bna.*FLC* or Bna.*FT* genes were designed by using Primer-BLAST [79] with the following modifications from the default settings: PCR product size: 70-200 bp; # of primers to return: 30; Database: custom. The new database "custom" included the different gene copies for either Bna.*FLC* or Bna.*FT* without the "query" sequence. The specificity of the primers obtained was checked in an alignment done with CLUSTAL multiple sequence alignment by MUSCLE (ver. 3.8, http://www.ebi.ac.uk/Tools/msa/muscle/). The melting temperature T_m of the specific primers was then checked with Primer Express Software v.3.0.1 (Applied Biosystems by Life Technologies, Foster City, CA, USA). The optimal T_m value was set up to be 60 °C based on the real-time machine manufacturer's recommendation. If required, the 5' end tail of the primer was modified by adding or removing nucleotides. The primers were then ordered to Invitrogen or Mycroshynth AG to be synthesized.

For the endogenous control, the initial primers were kindly provided by Dr. Christian Obermeier (Plant Breeding Institute, Justus-Liebig University, Gießen). These primers were designed by using the *Brassica rapa* ubiquitin/ribosomal protein mRNA complete CDS sequence (GenBank: L21898.1). The 5' tail from both primers was modified by adding 1 nucleotide to obtain a T_m value closer to 60 °C.

Upon arrival, the primers were dissolved in Milli Q H_2O to a final concentration of 100 µM and stored at -20 °C until use.

2.6 Efficiency of the primers

The template sample used for the determination of the primer efficiency was a pool of all the cDNA samples in the study. To create this pool, 2 µL of each cDNA sample

was placed into a common tube. This pooled sample was then diluted with Milli Q H_2O in a 1:10 ratio, aliquoted into 67 µL and used to prepare further dilutions. The first standard curve was determined with the following dilutions: 1:10; 1:100; 1:1,000; 1:10,000; 1:100,000; and 1:1,000,000. The final curve use for efficiency calculation was determined using 1:10, 1:50, 1:100, 1:200, 1:500 and 1:1,000.

2.7 Real-time PCR

Initially, each cDNA sample was diluted into 1:30 using Milli Q H_2O in a final volume of 300 µL. This dilution was then aliquoted in 67 µL and stored at -80 °C until usage. Before every qRT-PCR plate, new dilutions for the standard curve were made from the 1:10 cDNA pool. This curve was determined using the following points: 1:10, 1:50, 1:100, 1:200, 1:500, and 1:1,000. The primers were also prepared in advance to a stock concentration of 3 µM.

The qRT-PCR was carried out in a 384-well plate format (4titude, Berlin, Germany). Each 384-well plate contained 30 samples with 1 or 2 specific target primer pairs and the endogenous control ubiquitin primers. Together with this, one standard curve per primer pair was included. Each point was run in 3 technical replicates. For each primer, at least 3 non-template controls (H_2O as the template) were included. Each real-time PCR reaction well contained: 5 µL of 2X FastStart Universal SYBR Green Master (Rox) (Roche Diagnostics GmbH, Mannheim, Germany), 1 µL of each primer to a final concentration of 0.3 µM, and 3 µL of cDNA template. The total reactions per primer pair were prepared as a master mix. Then, the master mix and the diluted cDNA samples were distributed into a 96-well plate. This 96-well plate and the 384-well plate were placed into the Biomek 4000 Automated Laboratory Workstation (Beckman Coulter, Indianapolis, IN, USA) were the final reaction was plated. Lastly, the plate was briefly centrifuged and sealed with Clear Weld Seal Mark II (4titude, Berlin, Germany).

The plates were run in ViiA 7 Real-time PCR System (Applied Biosystems, Foster City, CA, USA, software v.1.2.2). The run method was as follows: initial denaturation (94 °C for 2 min), amplification and quantification program with 40 cycles (95 °C for 20 sec, 60 °C for 30 sec, 72 °C for 30sec), and a final extension (72 °C for 5 min). At the end, the melting curve program was carried out with a continuous fluorescence

measurement between 55 and 95 °C. The program used to obtain the efficiencies and C_t values was QuantStudio™ Real-Time PCR Software v1.1 (Applied Biosystems, Foster City, CA, USA).

2.8 Data analysis

2.8.1 Crossing threshold (C_t) point calculation

To be able to analyze all the samples together, the first step of the analysis was to merge the two plates containing the 60 samples. This was done by averaging the automated threshold values obtained by QuantStudio™ Real-Time PCR software. This average value was then manually changed in the QuantStudio Software and all the C_t values were recalculated. This processed was done individually for each of the targets, including the endogenous control ubiquitin. The new C_t values were then exported to an Excel data sheet. In there, the C_t values for the three technical replicates were evaluated based on the standard deviation. In the recommended criteria the standard deviation should be ≤ 0.5 C_t. In the case that the standard deviation was > 0.5 C_t one of the technical replicates was eliminated. When two out of the three technical replicated had no Ct value (undetermined) the sample was categorized as not expressed (NE). At the end of this evaluation, a minimum of two (ideal three) technical replicates per sample point were used in the analysis.

2.8.2 Analysis

The calculation was done genotype-wise using the 3 biological replicates. In some of the cases, if 2 out of the 3 biological replicates had no detectable gene expression, the final value reported is “not expressed” (NE). For any calculation, the C_t value of the target gene corresponds to *FT* or *FLC* gene copy expression. The endogenous control C_t value corresponds to ubiquitin.

The normalized gene expression was calculated using the ΔCt method (equation 1.1). The values obtained were then transformed into fold changes (equation 1.2).

$$\Delta Ct = Ct\ target\ gene - Ct\ endogenous\ control \qquad \text{eq. 1.1}$$

$$Fold\ change = 2^{-\Delta Ct} \qquad \text{eq. 1.2}$$

To calculate the ratio or relative expression quantity the Pfaffl method was used [80]. Unlike ΔCt equation, this method includes the efficiency value for calculation. The efficiencies (*E*) for each primer pair were calculated as in equation 2.1. The slope value was obtained from the QuantStudio software calculations based on the amplification of the standard curve (described in 2.6). Then, an average of primer efficiency was calculated from 3-4 independent efficiencies values. This average value was used into the Pfaffl method (equation 2.2) and for any other calculation process that included efficiency value into the formula.

$$E = 10^{\left(-\frac{1}{slope}\right)} \qquad \text{eq. 2.1}$$

$$ratio = \frac{\left(E_{target}\right)^{\Delta Ct_{target(Control-Sample)}}}{\left(E_{reference}\right)^{\Delta Ct_{reference(Control-Sample)}}} \qquad \text{eq. 2.2}$$

The Pfaffl formula uses samples defined as “control” and “sample” for calculation. This method was only used on *FLC* genes, where the control was arbitrary selected as “before vernalization” (or 7 WAS), and the sample as “after vernalization” (15 WAS). In this calculation, the “control” acquires an arbitrary value of 1 and the “sample” value is calculated depending on this. The standard deviation (SD) for Pfaffl method was calculated as shown in equation 3.1. Here, R stands for the Ratio value obtained in the Pfaffl formula. The relative quantity (RQ) was calculated as in equation 3.2.The SD RQ for target or endogenous was calculated like in equation 3.3. The data was then graphed using RStudio software (v.1.0.153).

$$SD = \sqrt{R\left(\frac{SD\ RQ_{endogenous}}{RQ_{endogenous}}\right)^2 + \left(\frac{SD\ RQ_{target}}{RQ_{target}}\right)^2} \qquad \text{eq.3.1}$$

$$RQ = E^{(Ct\ control - Ct\ sample)} \qquad \text{eq. 3.2}$$

$$SD\ RQ = SD_{Ct} \cdot RQ \cdot log_e(E) \qquad \text{eq. 3.3}$$

2.8.3 Statistical analysis

To be able to determine if the differences observed in the data were statistically significant a *t*-test was carried out. The compare data was the result of the fold-change of the delta-Ct method. The comparisons were made between 7 vs 15 WAS or before and after vernalization. The *t*-test was set up to be paired and one tail with a significance $p \geq 0.05$. The one tail test was selected based on the information in *Arabidopsis* FLC genes will respond to vernalization by down relating gene expression. A similar effect was expected in *B. napus.*

2.9 Fragment analyzer

The specificity and fragment sizes for each qRT-PCR primer pair product was analyzed in the Fragment Analyzer *INFINITY* Automated CE System (Advanced Analytical, Heidelberg, Germany). From each one of the primers, one qRT-PCR sample per genotype was selected based on the amount of expression. The samples for all *FLC* genes and ubiquitin were taken from “before vernalization” stage. In the case of *Bna.FT.A02,* the samples were taken from “after vernalization”. From each sample, two technical replicated from qRT-PCR was run into the fragment analyzer. Due to space issues, for ubiquitin, only 1 technical replicate per genotype was run.

The samples and gel were prepared using DNF-910 dsDNA Reagent Kit following the manufacturer’s instruction. Briefly, the gel was prepared by mixing the intercalating dye plus dsDNA 810 separation gel. In a 96 well plate, the markers for 35 bp and 1500 bp were prepared. The samples were prepared in another plate by adding 2 µL of qRT-PCR product mixed with dilution buffer 1X TE up to 200 µL. Later, the 100 bp ladder was loaded into one of the wells of the sample plate. All the plates were loaded into the fragment analyzer and run using the software Fragment Analyzer v. 1.0.0.5 (Advanced Analytical, Heidelberg, Germany). The fragment sizes were then calculated with ± 5 % accuracy using the software PROSize 2.0 v. 1.2.1.1 (Advanced Analytical, Heidelberg, Germany).

2.10 Primer test

Initially, the primers were tested in a standard PCR. For this, the PCR reaction was prepared as follows: 1.2 µL of magnesium chloride (25mM), 2 µL 10x Key buffer (VWR, Belgium), 1 µL of each primer (10 µM), 1 µL of dNTPs (10mM), 0.5 µL Taq DNA Polymerase (VWR, Belgium), and 1 µL of template. The volume was completed up to 20 µL with Milli Q water. The cDNA tested was diluted with Milli Q H_2O to a ratio 1:10. Genomic DNA (gDNA) from a double haploid line derived from a cross between Express617 and V8 was used as positive control. The gDNA was diluted to 10 ng/µL and used as the template for the PCR.

The reactions were prepared as a master mix and distributed in a 96-well plate. The wells were then closed with cap strips and briefly centrifuged. The plate was then placed into the GeneAmp PCR System 9700 (Applied Biosystems by Life Technologies, Foster City, CA, USA). The thermal program used was: initial denaturation (94 °C for 3 min), 30 cycles of amplification (94 °C for 3 sec, 60 °C for 30 sec, 72 °C for 15 s), and a final extension (72 °C for 5 min) with an ending cooling step at 4 °C. Once the PCR was finished, the samples were run on a 2 % agarose gel with 0.5 % TBE and Midori Green. The samples were run together with a 100 bp DNA ladder (Invitrogen by Life Technologies, Carlsbad, CA, USA). After running for 90 min at ~100V, the gel was visualized under UV light and a photo was taken.

3 RESULTS

3.1 Literature search for specific primers

FLC and *FT* are important genes for flowering time regulation and therefore were already subject of different gene expression analysis in *Brassica*. Because of this, the first step in this project was to look for primers already available in scientific publications. Unfortunately, in most of the cases analyzed, the primers were not able to differentiate between paralogs. As an example, the primers used in [81] were designed based on only 4 copies of *B. napus FLC* genes. From the list of primers in this publication, only *Bna.FLC.A10* was specific for the sequences of Darmor-Bzh. In figure 5 there is one example of the primer pair specifically designed to amplify *Bna.FLC.A02*. In the sequence alignment of *Bna.FLCs* sequences of Darmor-Bzh reference genome is possible to observe that the forward primer is common at the 3'end for most of the *FLC* sequences. In the case of the reverse, is more likely to amplify other copies such as *Bna.FLC.A03b* or *Bna.FLC.C02.* The analysis for the remaining primers is in table 2.

When reviewing the literature for *Bna.FT* genes [82], a similar situation was observed (Table 3).

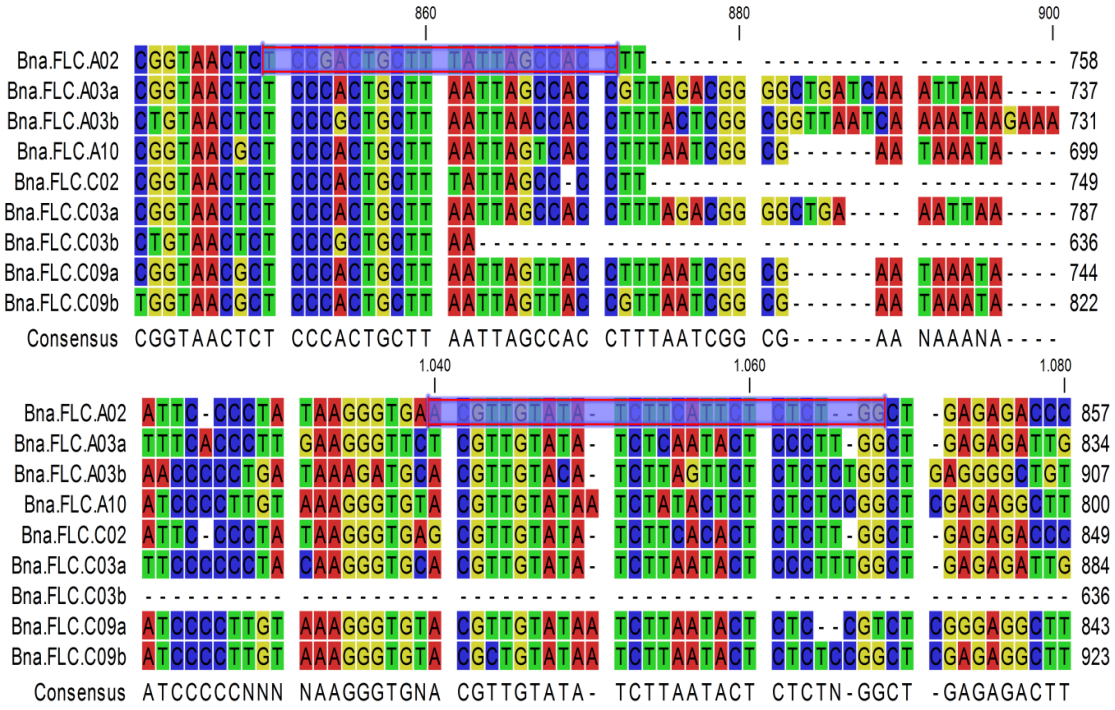

Figure 4. Primer specifically designed for *Bna.FLC.A02* copy [81]. The primers are marked with a blue box. The alignment was done using CLC sequence Viewer v. 7.8.1 (QIAGEN Aarhus A/S).

Table 2. Primer analysis from [81]**.**

Target		Primer sequence (5'-3')	Size (bp)	Comments
FLC.A10	Forward	GCTCCCACTGCTTAATTAGTC	136	Specific for Darmor-Bzh
	Reverse	CGAGCCGGAGAGAGAGTATAG		
FLC.A2	Forward	TCCGACTGCTTTATTAGCCACC	112	Possibility to amplifly *FLC.A03a* and *FLC.A10*
	Reverse	CCAGAGAGAATGAAGATATACAACGT		
FLC.A3a	Forward	AAATGTCGGTGGTGTAAGCGTG	199	Possibility to amplify *FLC.*A10
	Reverse	TTCGGCTCCCGCAAGATTC		
FLC.A3b	Forward	TGCCCTGGATCTTCAGTCAAAAT	112	Could amplify FLC genes C03 and A02
	Reverse	GAGGGAATCCACGCTTACATCAT		

Table 3. Primer analysis from [82]**.**

Paralog		Primer (5'-3')	Size (bp)	Comments
FT .A2	Forward	GTTGTAGGAGACGTTCTTGAATGT	179	Possibility to amplify C06 and A07
	Reverse	TCTGGATCCACCATAACCAAAGTA		
FT.C2	Forward	GTTGTAGGAGACGTTCTTGAATGT	179	Only one base different 3'end C06(reverse)
	Reverse	TCTGGATCCACCATAACCAAAGTG		
FT.A7.a	Forward	CCCACCTCGGGAATTCATCGTC	146	Only one base different 3'end "C06b"(reverse)
	Reverse	TAAACCGCAGCCACGGGAAGGCC		
FT.A7.b	Forward	AATGAGATTGTGTCTTACGAGAA	159	Only one base different 3'end "C06b"(reverse)
	Reverse	AAGGCCGAGATTGTATAGCGCG		
FT.C6.a	Forward	CCCACCTCGGGAATTCATCGTC	146	Specific
	Reverse	TAAACCGCAGCCACGGGAAGGCT		
FT.C6.b	Forward	AATGAGATTGTGTCTTACGAGAA	159	Specific
	Reverse	AAGGCCGAGATTGTATAGCGCA		

After reviewing this and other scientific publications it became clear that the best option was to design new primers.

3.2 Specific primer design for FLC and FT genes in *B. napus*

In the case of *FLC* gene, there are 9 annotated sequences in the Darmor-Bzh reference genome. Five of this copies are part of the C subgenome and the remaining four copies correspond to the A subgenome. In the case of *FT* gene, two belong to the A subgenome and four are part of the C subgenome.

At the beginning of the analysis, only the coding sequences were used to design the primers. Unfortunately, these sequences were too identical (table 4) and for some of the copies it was not possible to obtain specific primers. This is why, if available, the 5' and 3' untranslated region (UTR) region was also included (table 5). The primers were designed by using the online software Primer-BLAST [79]. This tool had some determined options that were modified. From this software, a list of primers was obtained that included information such as the position of the primers, %CG content, and product size, among others.

One of the parameters modified was the number of primer pairs expected to obtain. In an ideal case, the software will give a maximum of 30 pairs. This was not the case for all the copies analyzed. In the case of *Bna.FLC.A02,* there were 22 primer pairs obtained and mainly localized towards the 5' end of the sequence. For *Bna.FLC.A03a, Bna.FLC.A03b, Bna.FLC.A10, Bna.FLC.C09a,* and *Bna.FLC.C09b* there were 30 primers combinations obtained but all of them localized at the 3'end of the sequence. For *Bna.FLC.C02* and *Bna.FLC.C03b* there were also 30 pairs but most of them localized at the 5' end of the sequence. The copy *Bna.FLC.C03a* showed to be more similar to the other paralogs. In this case, only 4 possible primer combinations were obtained. All of them localized at the very end of the 3'end sequence.

Table 4. Percent Identity Matrix - created by Clustal2.1 for FLC copies.

Bna.FLC.	***A03b***	***C03b***	***A10***	***C09b***	***C09b***	***A03a***	***C03a***	***A02***	***C02***
A03b	100	96.5	85.62	85.45	85.96	88.49	88.66	88.32	86.63
C03b	96.5	100	84.62	79.18	84.97	87.59	87.76	86.71	84.97
A10	85.62	84.62	100	97.98	98.65	87.71	87.71	88.49	87.31
C09b	85.45	79.18	97.98	100	98.65	87.54	87.54	88.32	86.97
C09a	85.96	84.97	98.65	98.65	100	88.05	88.05	88.83	87.48
A03a	88.49	87.59	87.71	87.54	88.05	100	98.48	91.2	89.17
C03a	88.66	87.76	87.71	87.54	88.05	98.48	100	91.2	89.17
A02	88.32	86.71	88.49	88.32	88.83	91.2	91.2	100	97.46
C02	86.63	84.97	87.31	86.97	87.48	89.17	89.17	97.46	100

In the case of *FT* genes, from *Bna.FT.A02, Bna.FT.C02,* and *Bna.FT.C04* there were 30 primer pair combinations obtained. The localization was towards the middle of the sequence. For *Bna.FT.A07* and *Bna.FT.C02* also 30 primers combinations were obtained but localized mainly at the 3' end. In the case of *Bna.FTC06,* there were also 30 primer pairs but localized at the 5' end of the sequence.

The specificity of the primers obtained was later double check manually in nucleotide alignment done with MUSCLE. Each possible primer was located in the alignment and the specificity was checked at the 3'end. If this part of the sequence was at least 1 nucleotide unique among the other paralogs, this primer was considered specific. This was done for each one of the copies of *Bna.FLC* and *Bna.FT* genes. Based on this, the qRT-PCR primer pairs were chosen. In most of the cases, only one of the primers was specific. The other had the characteristic of being common for the paralogs. Once the primers arrived, they were initially tested in a conventional PCR.

Table 5. Sequence used for specific primers design for *FLC* and *FT* genes in *B. napus.*

Gene	5'UTR (bp)	CDS (bp)	3'UTR (bp)	Total (bp)
Bna.FLC.A02	160	591	181	932
Bna.FLC.A03a	113	594	354	1061
Bna.FLC.A03b	106	591	258	955
Bna.FLC.A10	81	594	184	859
Bna.FLC.C02	152	591	156	899
Bna.FLC.C03a	167	594	306	1067
Bna.FLC.C03b	-	636	-	636
Bna.FLC.C09a	126	594	608	1328
Bna.FLC.C09b	108	690	272	1070
Bna.FT.A02	58	528	284	870
Bna.FT.A07	57	528	353	938
Bna.FT.C02a	-	609	-	609
Bna.FT.C02b	57	528	280	865
Bna.FT.C04	-	528	-	528
Bna.FT.C06	-	351	-	351

3.3 Initial primer test

The functionality and specificity of the qRT-PCR primers were tested initially in a conventional PCR. In here, two different templates were used: (1) cDNA and (2) genomic DNA (gDNA). In the case of *Bna.FLC* genes in the A subgenome, all the primers amplified a fragment from gDNA close to the excepted size (figure 3). At the same time, the copies *Bna.FLC.A03b* and *Bna.FLC.A10* amplified additional bigger fragments. Together with this, the primers for *Bna.FLC.A03b* copy seemed to have formed dimers in the reaction. This is visible as a "cloud" under the specific band (figure 6, blue arrow). All the copies were also amplified from cDNA. This initial result gave an indication of the levels of transcripts present in the sample. For example, *Bna.FLC.A10* had a higher band intensity compared to the other copies which hint a higher gene expression.

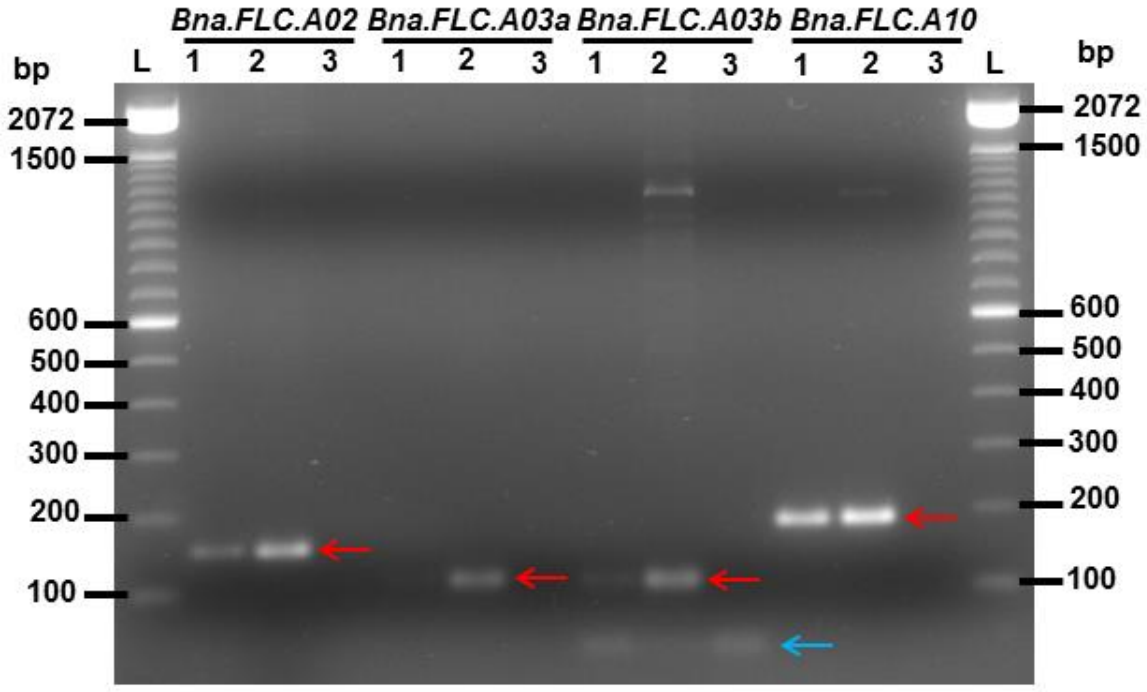

Figure 5. Agarose gel with PCR product from specific primers designed for *Bna.FLC* A subgenome. The wells correspond to products from (1) cDNA, (2) gDNA, or (3) non-template control. The L stands for 100 bp DNA marker, with sizes in base pair (bp). The fragments amplified matching the expected sizes are pointed with a red arrow. The suspected primer-dimer is pointed with a blue arrow.

In the case of the *Bna.FLC* C subgenome (figure 7), the primer amplification from gDNA produced more unspecific bands than in A subgenome (figure 6). Especially the primers specifically designed to amplify *Bna.FLC.C02* gene produced many

different size bands. This showed that, at least when gDNA was used as the template, this primer pair was not specific enough. But, at the same time, the expected fragment size was also visible. From the specific primers *Bna.FLC.C03b* the amplified band in gDNA had a bigger size (>2,072 bp) that did not match the expected size (figure 4, green arrow). This larger fragment is explained by the fact that the primers were located flanking intron 1. This intron is the largest of the *FLC* genes, and in this copy has an extension of 2,229 bp. In the case of *Bna.FLC.C09b* there was also an unspecific big band at ~550 bp (figure 4, yellow arrow). In this case, the only plausible explanation was that the primers were not specific enough.

When the primers for the *Bna.FLC* C subgenome were tested from cDNA, they only amplified a faint band in the copies *Bna.FLC.C02* and *Bna.FLC.C09b.* The remaining copies did not show any fragment at all.

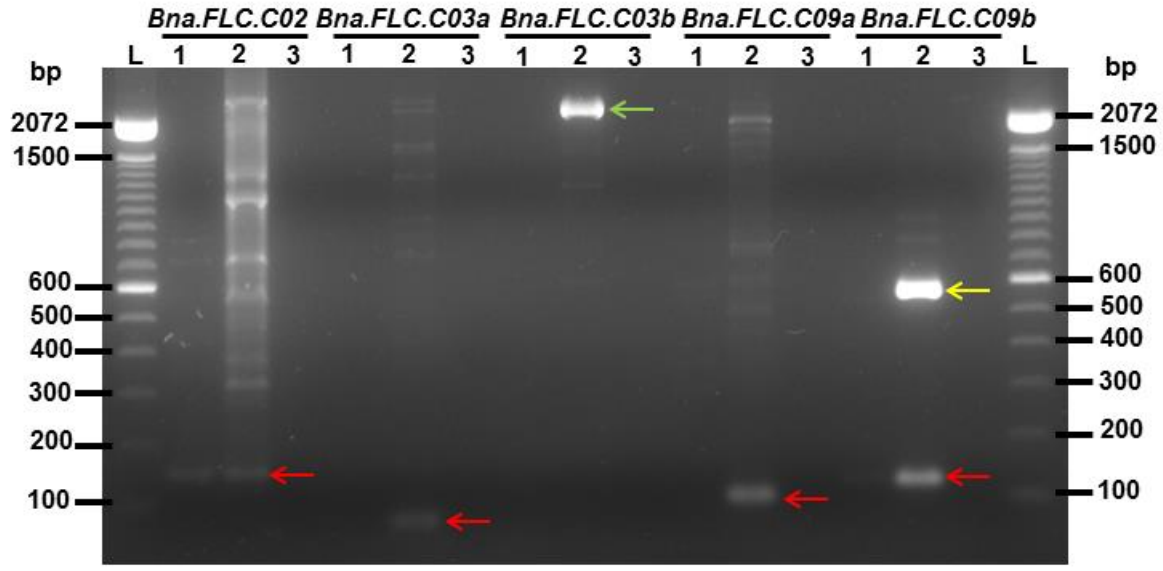

Figure 6. Agarose gel with PCR product from specific primers designed for *Bna.FLC* C subgenome. The wells correspond to products from (1) cDNA, (2) gDNA, or (3) non-template control. The L stands for 100 bp DNA marker, with sizes in base pair (bp). The fragments amplified matching the expected sizes are pointed with a red arrow. The bigger fragment size amplified due to the location of the primers is pointed in with a green arrow. The very intense unspecific band from *Bna.FLC.C09b* is pointed with a yellow arrow.

For *FT* genes, the specific primers designed were also tested in PCR using gDNA and cDNA as template (figure 8). In this case, from the 6 annotated *Bna.FT* copies

only 5 amplified from gDNA. The copy *Bna.FT.C06* did not produce any intense band but rather many faint, more likely unspecific bands. On the other hand, from the other 5 primer pairs, a single clear band was produced that matched the expected size. The only difference was that due to the position of the primers flanking two introns, the primer pair *Bna.FT.C04* amplified a bigger fragment. When cDNA template was used, only the copy *Bna.FT.A02* amplified a faint fragment visible only under direct UV light exposure.

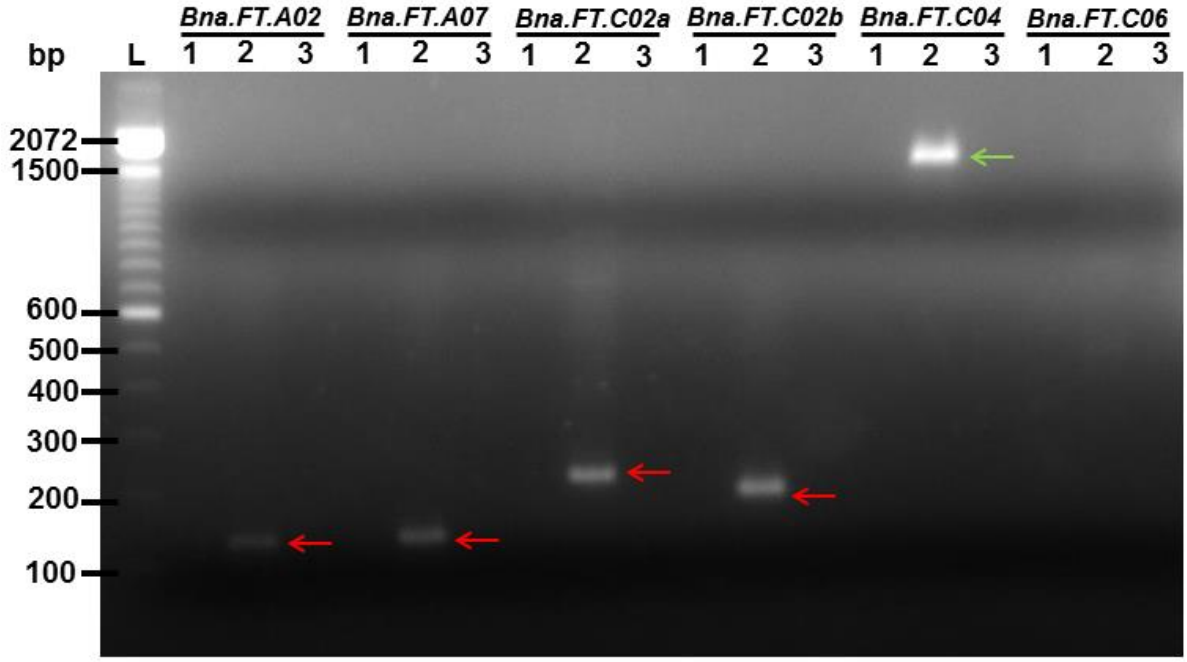

Figure 7. Agarose gel for FT primers. Gel with PCR product from specific primers designed for *Bna.FT*. The wells correspond to products from (1) cDNA, (2) gDNA, or (3) non-template control. The L stands for 100 bp DNA marker, with sizes in base pairs (bp). The fragments amplified matching the expected sizes are pointed with a red arrow. The bigger fragment size amplified due to the location of the primers is pointed in with a green arrow.

From the initial PCR test was clear that most of the primers worked because at least amplified some band from genomic. But, at the same time, the specificity of the primers was not sufficient enough due to many unintended bands from gDNA. Because of this reason, it was decided to double check one of the most important parameters in a PCR, the melting temperature (T_m).

The T_m was checked with Primer Express software. This software has been specifically developed to work with ViiA 7 and QuantStudio real-time PCR systems.

After checking the T_m of the primers with Primer Express it was clear that there were some differences in the T_m of Primer-BLAST versus Primer Express. In some of the cases, the difference was up to 4 °C. In most of the cases, the primers that showed to have many unspecific bands also had a T_m <60 °C in Primer Express.

Because of this reason, it was decided to adjust the primers and increase the T_m if necessary. To do this, the 5'-end of the primer was modified by adding or removing nucleotides to achieve a T_m as close to 60 °C as possible. At the same time, some of the primers were completely redesigned because they did not amplify any product. The final set of primers used in qRT-PCR is listed in table 6.

Table 6. List of specific primers designed for qRT-PCR.

Target	#	Sequence 5'-3'	Fragment size (bp)
Bna.FLC.A02	F3	CGGCGAGAGTTGAAACCGAAT	74
	R17	CTTCCCATGGCTTCTGTCTCC	
Bna.FLC.A03a	F4	TCATGGCGAAGAAGCCTACC	105
	R4	ACAACCTCTATGCGTTGTGGA	
Bna.FLC.A03b	F3	CCTGTAACTCTCCCGCTGCTT	124
	R2	AGTAAAGGTGGTTAATTAAGCTGCGA	
Bna.FLC.A10	F23	GCCGAAGCTGATAATATGGATGTC	70
	R23	GTGGGAGCGTTACCGGAAGA	
Bna.FLC.C02	F3	GAGAGCTGAACCGAACCGAA	143
	R3	TTGCGTCGTTTGGAGAAGGT	
Bna.FLC.C03a	F2	TGAGTTTGCTTGTGTGTTCTTCC	70
	R2	GTTGCAAATTAGACGGCTGGC	
Bna.FLC.C03b	F1	ATCTGTCGGGCTTCTCGTTG	96
	R1	ACCATGTCGGGTGTTCCATC	
Bna.FLC.C09a	F3	GATTTATGCAGCGTGGGACAGT	98
	R3	GAGGCTCGCTCAGGTTTGGTA	
Bna.FLC.C09b	F2	TCGAGAGGCTTCGGGTGTAA	123
	R2	GATACACAAGCAAGCTCGAAGTGA	
Bna.FT.A02	F	CTTACGAGAGTCCAAGGCCC	127
	R18	ACGGGAAGGCCGAGATTGTA	
Bna.FT.A07	F3	TGGTGCGTGATGCAATGTTTT	132
	R3	GCCACTGGTTGTTTTTCTAGTCC	
Bna.FT.C02a	F6	AAACCAACGGTCGAGATCGG	105
	R6	CACCAAAACCCCAAAGCAGT	
Bna.FT.C02b	F6	CGATACGAGCAATGAACGGTG	195
	R6	TTCCGAATGTTAAGATACTGTTTGC	
Bna.FT.C04	F6	CAAGTCCAAGCAACCCTCAC	169
	R6	TGTCTTCCGAGTTGCCAGAAT	
Bna.FT.C06	F2	CCCGACGAATTTCTCCCTCG	125
	R2	GAGGTGGGCCTTGGATTCTC	
Ubiquitin	99 F1	CTTCTTCGGCCTCAACTGGTT	101
	99 F2	GAAGATGATCTGCCGCAAGTGT	

The primers for the endogenous control ubiquitin were kindly provided by Dr. Christian Obermeier. This pair had been tested before and showed to be stable in other experiments. Because of this reason, it was also used in this experiment. By reviewing the T_m of this primer pair, the 5'-end was also modified by adding 1 nucleotide to each end. The final primer pair used in this study is also listed in table 6.

As mentioned before, the primers position differed depending on the copy. In the case of *Bna.FLC* genes, most of the variability was found at the 3 and 5'-UTR region. For *Bna.FT* the variability was found to be more within the coding region. The relative position of the primers is represented in figure 9 and 10.

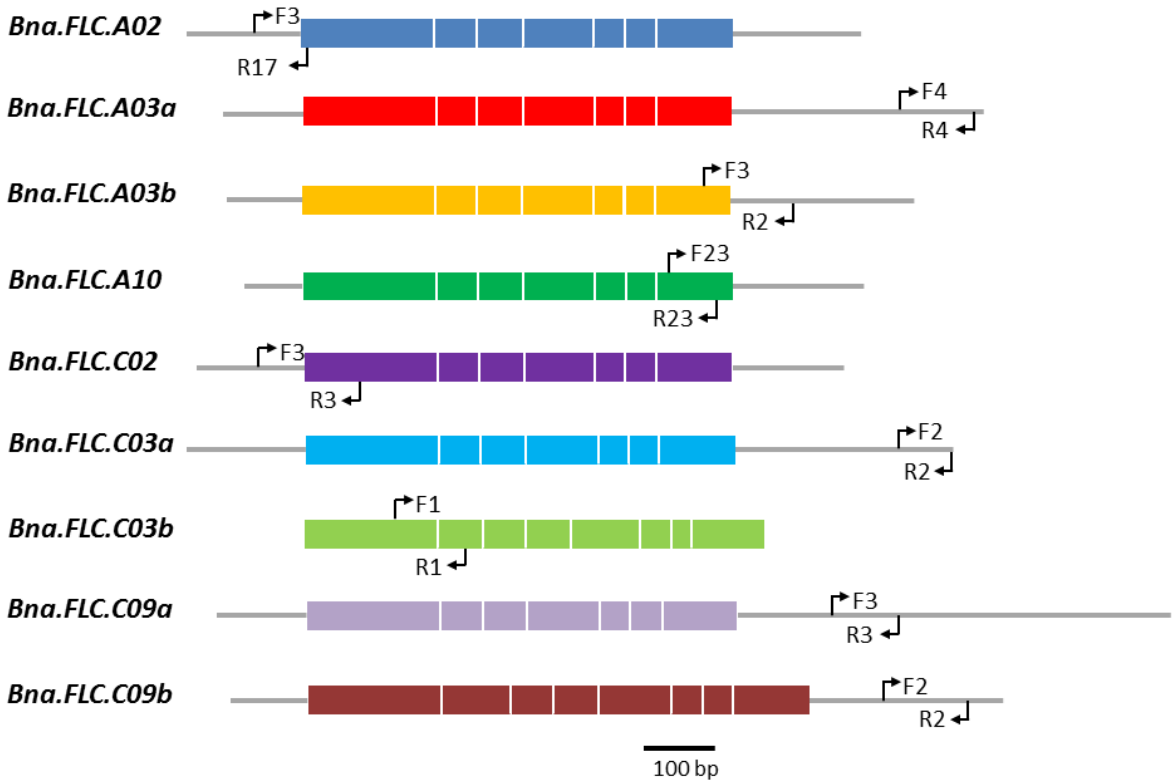

Figure 8. Relative primer position for *Bna.FLC* paralogs. Exons are shown as rectangles, the 5' and 3' UTR are represented as lines.

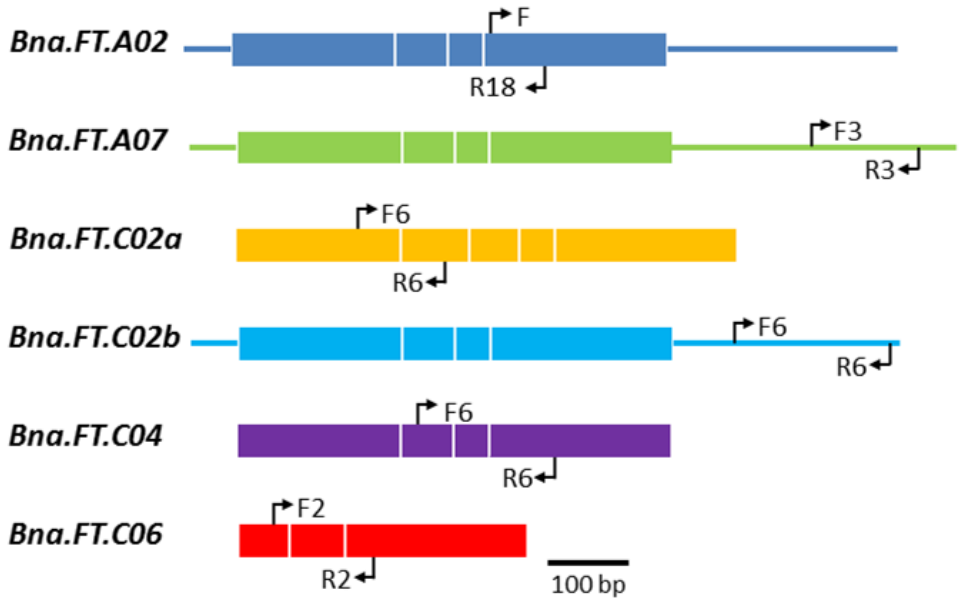

Figure 9. Relative primer position for *Bna.FT* paralogs. Exons are shown as rectangles, the 5' and 3' UTR are represented as lines.

3.4 Initial test for qRT-PCR

Initially, in order to find the best dilution for sample measurement, a wide standard curve was used. This curve consisted of cDNA pool dilutions between 1:10 and 1:1,000,000 and was tested in real-time PCR. In this case, most of the *FLC* copies just amplified in the 1:10 dilution and just a few at 1:100 and 1:1,000. Also, the copy *Bna.FLC.C03b* did not amplify above the threshold. In the case of *FT* genes, only the primers specific for *Bna.FT.A02* amplified a product at 1:10. The remaining 5 primers pairs did not amplify any product at all in any concentration. Because of this reason, it was decided to just continue with the qRT-PCR analysis only using *Bna.FT.A02.*

In the case of ubiquitin, it was possible to visualize amplification until dilution 1:10,000. Due to these results, the dilution for the cDNA samples was set to 1:30. Also, because the specific targets did not amplified beyond 1:1,000 the standard curve for efficiency calculation got narrowed. The new standard curve consisted of: 1:10, 1:50, 1:100, 1:200, 1:500, and 1:1,000 dilutions of pooled cDNA. The final efficiency obtained for each primer pair is listed in table 7.

Table 7. Percentage of efficiency for each primer pair used in qRT-PCR.

Target	Primer #	% of efficiency	Standard deviation
Bna.FLC.A02	F3/R17	92	± 2.8
Bna.FLC.A03a	F4/R4	90	± 8.9
Bna.FLC.A03b	F3/R2	104	± 11.8
Bna.FLC.A10	F23/R23	95	± 2.5
Bna.FLC.C02	F3/R3	93	± 3.8
Bna.FLC.C03a	F2/R2	94	± 11.8
Bna.FLC.C09a	F3/R3	90	± 9.2
Bna.FLC.C09b	F2/R2	100	± 15.8
Bna.FT.A02	F/R18	93	± 15.6
Ubiquitin	99 F1/R1	91	± 1.1

3.5 Gene expression analysis: before vernalization treatment

3.5.1 *Bna.FLC* gene expression before vernalization: differential expression between winter and spring types.

As mentioned before, from the 9 annotated *FLC* copies in *B. napus* only 8 were expressed in the samples used here. In this part, *FLC* gene expression levels were measured in leaves of before vernalization stage plants. Remarkably, not all the *Bna.FLC* copies were expressed at the same level. Most of the FLC copies showed a low level of expression at this point (figure 10A). Exceptions to this were the copies *Bna.FLC.A10* and *Bna.FLC.C02.* Interestingly, the copy *Bna.FLC.A10* was differentially expressed between winter and spring types. Here, winter types had a higher gene expression than spring types, with a 19-fold difference. The other highly expressed copy, *Bna.FLC.C02*, also showed a difference of gene expression between winter and spring types (figure 10B). In this case, the difference was lower, but still significant, with a 2.8-fold change. Another copy that showed differential expression between winter and spring genotypes was *Bna.FLC.A03b.* This gene was not expressed in spring types and showed a low gene expression in winter and usual winter genotypes (figure 10A).

For winter types, when comparing *Bna.FLC.A10* versus the other copies in the A subgenome there was a big difference. Compared to *Bna.FLC.A02, Bna.FLC.A03a* and *Bna.FLC.A03b* there were 86, 29 and 33-fold differences, respectively. These

differences in expression were smaller in spring types. In the case of *Bna.FLC.A02* there is a 6.3-fold difference and in *Bna.FLC.A03a,* no difference was observed.

In the case of *Bna.FLC.C02,* there was also a notorious difference of expression between paralogs in winter types. When this copy was compared with *Bna.FLC.C03a, Bna.FLC.C09a* and *Bn.FLC.C09b* the difference of expression was 28, 41 and 38-fold change. When comparing the same copies in spring type the differences were 7.3, 6.4 and 8.8-fold change. So, the tendency of this copy of being more expressed in winter types was also maintained in spring types.

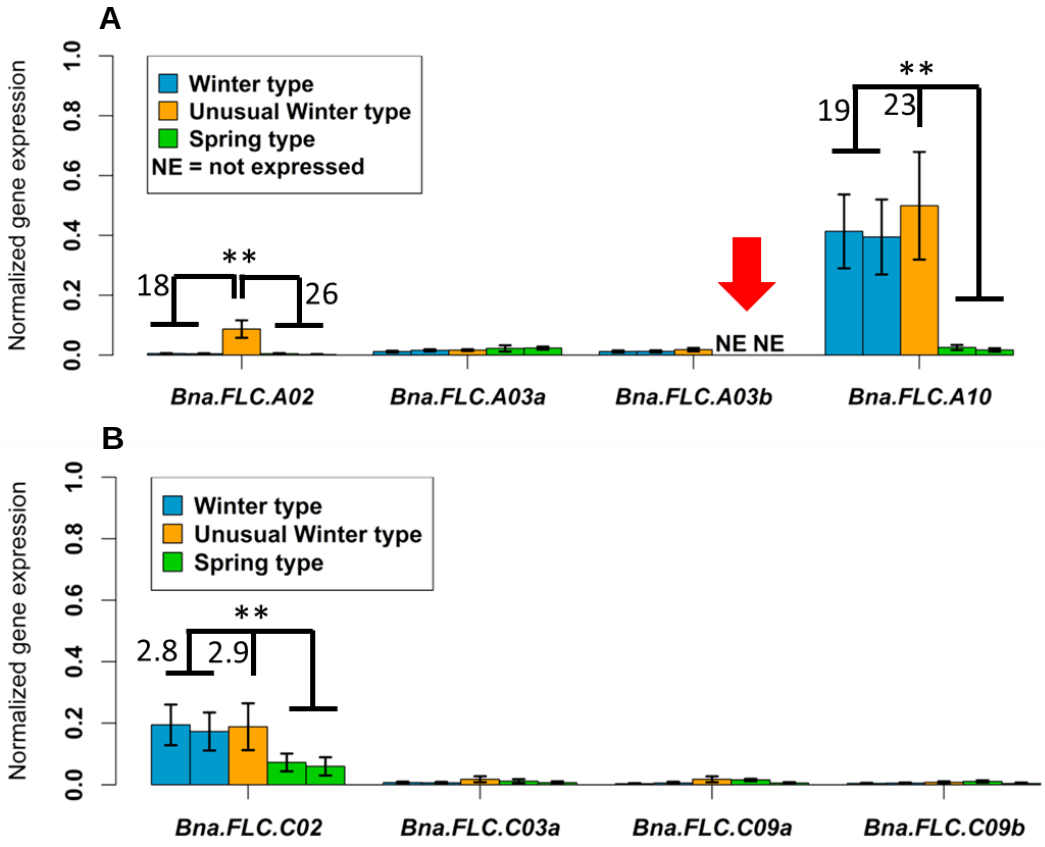

Figure 10. Normalized gene expression of *Bna.FLC* genes in before vernalization stage. (A) A subgenome, and (B) C subgenome. The experimented were performed in triplicate. The data are presented as mean ± SD. * p ≤ 0.05, ** p ≤ 0.01. Numbers next to the lines represent fold-changes

When comparing the others 6 copies in winter versus spring types, the differences were not so strong. In the case of *Bna.FLC.A02, Bna.FLC.A03a, Bna.FLC.C03a,* and *Bna.FLC.C09b* the differences are lower than 2-fold change. Interestingly, in the case of *Bna.FLC.C09a* the gene expression was 2.3-fold higher in spring than winter types.

When analyzing the performance of the unusual winter type versus winter and spring, it overall behaved as winter type. The exception to this was the gene expression of *Bna.FLC.A02.* In this gene, the amount of mRNA in unusual winter type was 18 and 26-fold higher than in winter and spring types, respectively. A minor difference was observed in the copies *Bna.FLC.C03a* and *Bn.FLC.C09a.* In this case, the unusual winter type had a higher gene expression with 2.9 and a 3.9-fold difference with winter types.

3.5.2 *Bna.FT.A02* gene expression before vernalization

In the initial PCR and qRT-PCR tests, it was only possible to detect the gene expression of *Bna.FT.A02.* The remaining copies only amplified in PCR using gDNA and were not included in the gene expression analysis.

The gene expression of *Bna.FT.A02* was analyzed in before vernalization genotypes (figure 11). In spring types, *Bna.FT.A02* gene expression was generally very low. Despite this, in genotype spring 1 the gene expression was higher than in spring 2 with a 5.8-fold difference. In winter and unusual winter samples there was no detectable gene expression. This is in agreement with spring genotypes getting ready to flower, unlike winter types.

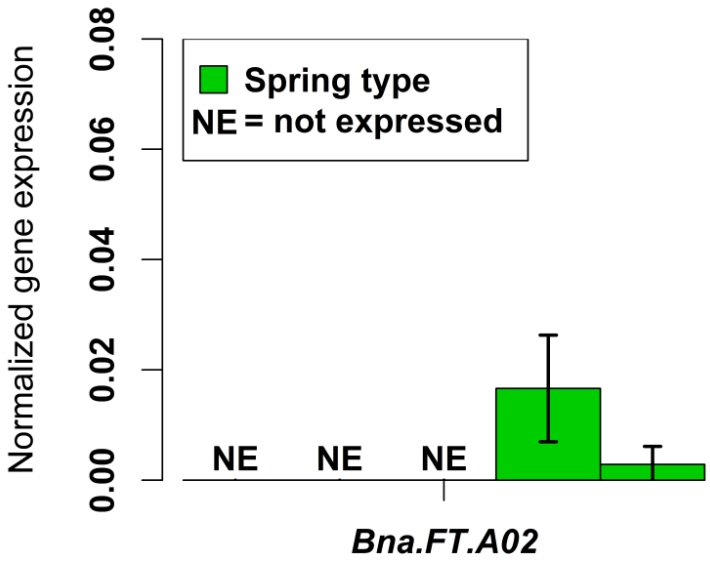

Figure 11. Gene expression of *Bna.FT.A02* in before vernalization stage. The experimented were performed in triplicate. The data are presented as mean ± SD.

3.6 Gene expression analysis: vernalization treatment

3.6.1 Response to vernalization of FLC genes

Seven weeks after sowing, the plants were transferred to the cold chamber and kept them in vernalization for eight weeks. An after vernalization leaf sample was collected immediately after vernalization and the gene expression was measured. In the case of *A. thaliana, FLC* gets downregulated in response to vernalization. Overall, most of the FLC copies of *B. napus* responded to vernalization and the gene expression observed was very low (figure 12). *Bna.FLC* from the A subgenome had a lower gene expression than C subgenome, the opposite scenario than before vernalization (figure 12A and 12B). Surprisingly, the differential gene expression between winter and spring types observed before vernalization in *Bna.FLC.A10* was no longer present (figure 12A). Interestingly, *FLC* in spring types also got downregulated in response to vernalization.

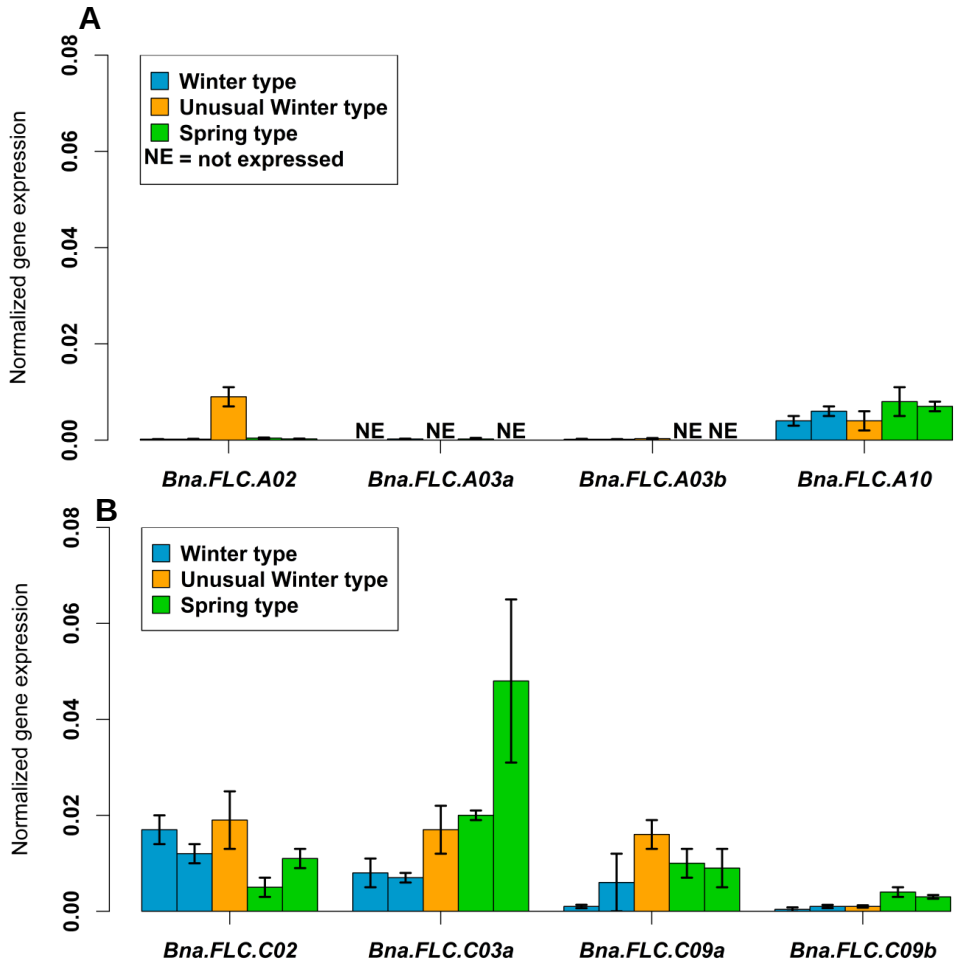

Figure 12. Normalized gene expression of *Bna.FLC* genes in after vernalization stage. (A) A subgenome, and (B) C subgenome. The experimented were performed in triplicate. The data are presented as mean ± SD.

When the comparison was made between before and after vernalization gene expression the degree of response depended on the gene. Arbitrary, the before vernalization was set as the reference and therefore had the value 1. Values that are <1 indicate a lower gene expression and vice versa.

The copy *Bna.FLC.A02* responded to vernalization by downregulating its gene expression in all genotypes (figure 13A). In winter types, it got downregulated an average of 18-fold. In the unusual winter type, it responded with a lower extent with 8.4-fold downregulation. In spring types, it responded as well but at different levels with downregulation of 12 and 5.4-fold compared to before vernalization. The copy *Bna.FLC.A03a* responded strongly to vernalization (figure 13B). In the genotype winter 1, unusual winter and spring 2 was not possible to detect gene expression within range of detection (NE). The remaining winter 2 and spring type 1 had a very low gene expression with a downregulation of 57 and 112-fold, respectively.

As before, the copy *Bna.FLC.A03b* showed not expression in spring types (figure 13C). In winter and unusual winter types, it showed a strong downregulation in response to cold treatment. For winter 1, winter 2 and unusual winter type the downregulation was 63, 85 and 72-fold, respectively.

The copy *Bna.FLC.A10* that showed to be differentially expressed before vernalization also showed differences in response to vernalization (figure 13D). For winter types, it got a downregulated 79 and 57-fold. In the case of unusual winter type, the downregulation was stronger (118-fold). Spring types responded mildly with 3.6 and 2.2-fold downregulation.

The copy *Bna.FLC.C02* also responded in all the genotypes analyzed (figure 14A). For winter types, it got downregulated by 9.1 and 13-fold. Unusual winter types reacted very similar to the observed in winter types, with a downregulation of 9.1-fold. For spring types, spring 1 responded more strongly with a downregulation of 16-fold compared to the 4.2-fold downregulation of spring 2.

Interestingly, the copy *Bna.FLC.C03a* did not respond to the cold treatment (figure 14B). The levels of gene expression in winter and usual types were very similar to the observed in before vernalization stage. For the spring types, spring 1 showed to have almost the same level of gene expression. On the other hand, spring 2 showed a much higher transcripts level with a 9.7-fold change.

The copy *Bna.FLC.C09a* showed a mild downregulation in almost all the genotypes (figure 14C). For winter and unusual winter types, the downregulation was close to 2-fold. For spring types, spring 1 showed a mild downregulation as well. In spring type 2, this copy was mildly upregulated by 1.7-fold.

Finally, the copy *Bna.FLC.C09b* also responded to cold (figure 14D). For winter and unusual winter types the changes were very similar with a downregulation between 6.3 and 15-fold. For spring types, it also responded but to a smaller extent with 3.1 and 1.1-fold decrease. To test if the difference observed was significant, a t-test was carried out. The results are summarized in table 10 and 11.

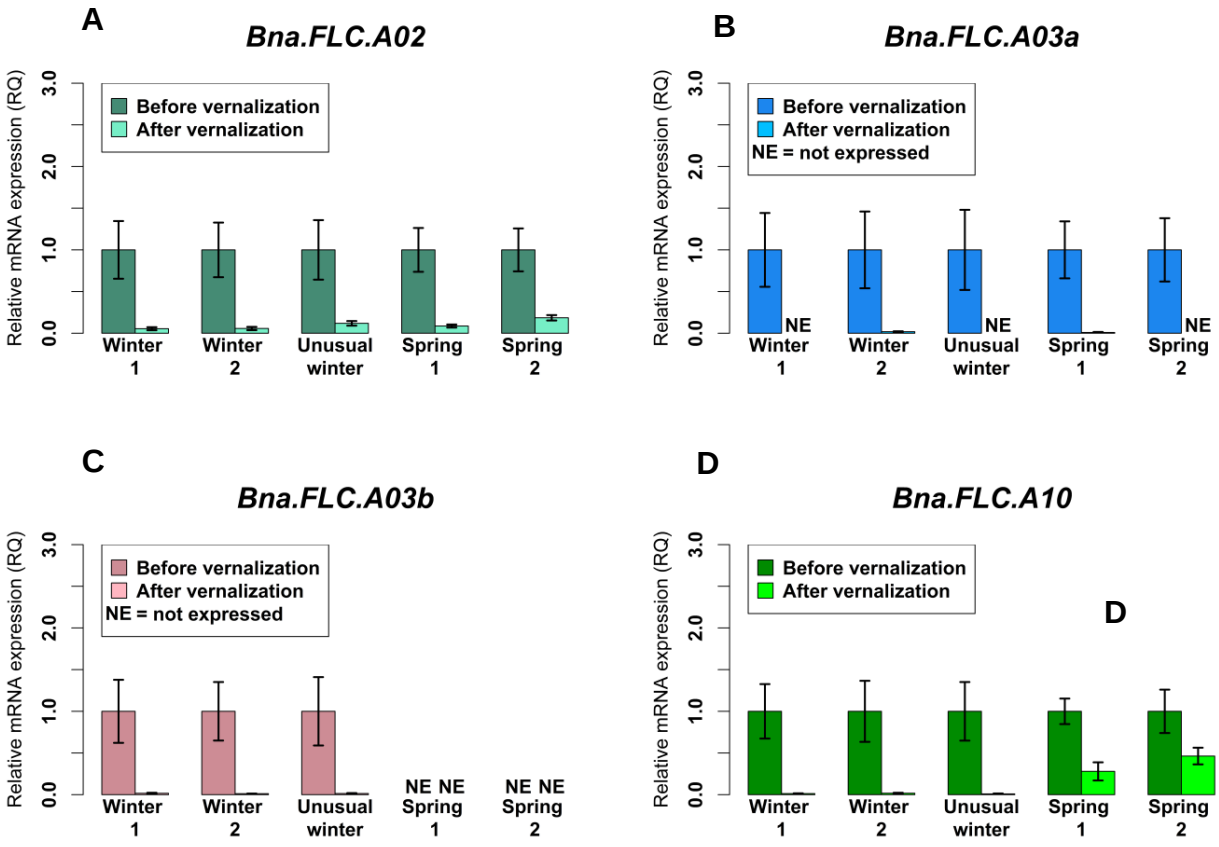

Figure 13. Gene expression before and after vernalization in *B. napus* A subgenome. The experimented were performed in triplicate. The data are presented as mean ± SD.

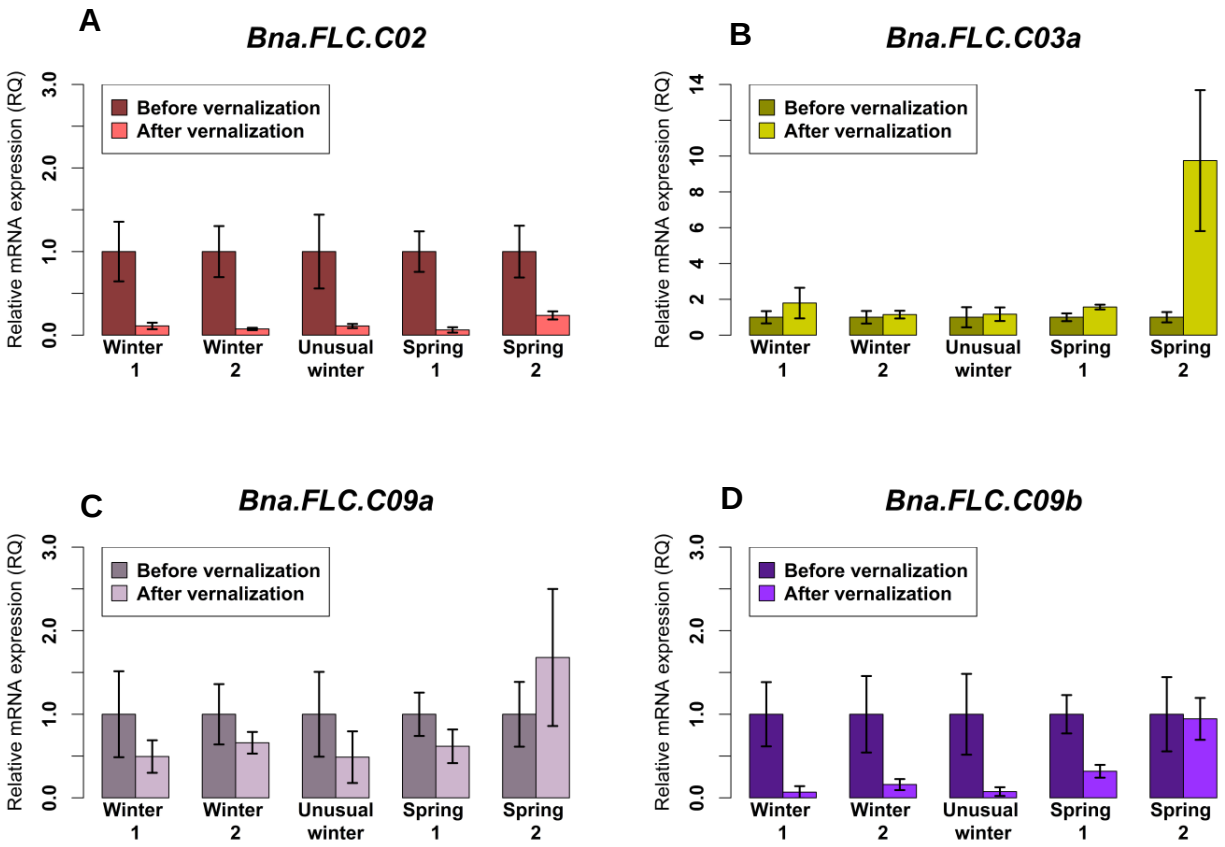

Figure 14. Gene expression before and after vernalization in *B. napus* C subgenome. The experimented were performed in triplicate. The data are presented as mean ± SD.

Table 8. *t*-test results comparing before (BV) and after vernalization (AV) gene expression of *Bna.FLC* subgenome A. The media (M) and standard deviation (SD) values are shown. The significant p-values (p) are marked with an asterisk (p≤0.05).

Genotype		*Bna.FLC.A02*		*Bna.FLC.A03a*		*Bna.FLC.A03b*		*Bna.FLC.A10*	
		M	**SD**	**M**	**SD**	**M**	**SD**	**M**	**SD**
Winter 1	BV	0.004	0.001	0.011	0.003	0.010	0.002	0.371	0.128
	AV	1.93E-04	2.1E-05	-	-	1.8E-04	7.6E-05	0.004	0.001
	p	**0.008***		**-**		**0.008***		**0.019***	
Winter 2	BV	0.004	0.001	0.016	0.004	0.014	0.003	0.415	0.068
	AV	1.94E-04	7.0E-05	2.2E-04	8.5E-05	1.7E-04	3.7E-05	0.006	0.001
	p	**0.011***		**0.057**		**0.005***		**6.31E-05***	
Unusual winter	BV	0.095	0.044	0.018	0.003	0.019	0.008	0.535	0.242
	AV	0.009	0.002	-	-	2.9E-04	1.3E-04	0.004	0.002
	p	**0.041***		**-**		**0.028***		**1.44E-04***	
Spring 1	BV	0.006	0.002	0.029	0.010	-	-	0.028	0.003
	AV	4.04E-04	1.2E-04	2.3E-04	2.3E-04	-	-	0.008	0.003
	p	**0.017***		**0.022***		**-**		**0.018***	
Spring 2	BV	0.002	0.001	0.021	0.001	-	-	0.014	0.003
	AV	2.52E-04	5.6E-05	-	-	-	-	0.007	0.001
	p	**0.031***		**-**		**-**		**0.030***	

Table 9. *t*-test results comparing before (BV) and after vernalization (AV) gene expression of *Bna.FLC* subgenome C. The media (M) and standard deviation (SD) values are shown. The significant p-values (p) are marked with an asterisk (p≤0.05).

Genotype		*Bna.FLC.C02*		*Bna.FLC.C03a*		*Bna.FLC.C09a*		*Bna.FLC.C09b*	
		M	**SD**	**M**	**SD**	**M**	**SD**	**M**	**SD**
Winter 1	BV	0.176	0.045	0.004	0.001	0.003	0.001	0.004	0.001
	AV	0.017	0.003	0.008	0.003	0.001	3.6E-04	3.8E-04	4.4E-04
	p	**0.014***		**0.133**		**0.005***		**0.021***	
Winter 2	BV	0.195	0.061	0.006	0.002	0.005	3.5E-04	0.005	0.001
	AV	0.012	0.002	0.007	0.001	0.006	0.006	0.001	3.5E-04
	p	**0.018***		**0.081**		**0.377**		**0.001***	
Unusual winter	BV	0.210	0.109	0.018	0.014	0.019	0.015	0.009	0.005
	AV	0.019	0.006	0.017	0.005	0.016	0.003	0.001	2.6E-04
	p	**0.051**		**0.475**		**0.393**		**0.002***	
Spring 1	BV	0.084	0.027	0.013	0.002	0.017	0.001	0.013	0.004
	AV	0.005	0.002	0.020	0.001	0.010	0.003	0.004	0.001
	p	**0.017***		**0.025***		**0.059**		**0.001***	
Spring 2	BV	0.050	0.018	0.004	0.002	0.005	0.000	0.004	0.002
	AV	0.011	0.002	0.048	0.017	0.009	0.004	0.003	3.9E-04
	p	**0.038***		**0.020***		**0.096**		**0.018***	

3.6.2 Response of FT to vernalization

In the samples before and after vernalization, the overall gene expression of *Bna.FT.A02* was very low (figure 15). In winter types, gene expression was not observed before vernalization and very low after vernalization. As seen before in figure 11, the unusual winter type did not express *Bna.FT.A02* in any of the samples analyzed. In the case of spring types, both showed a very low gene expression in before vernalization samples and no expression after vernalization.

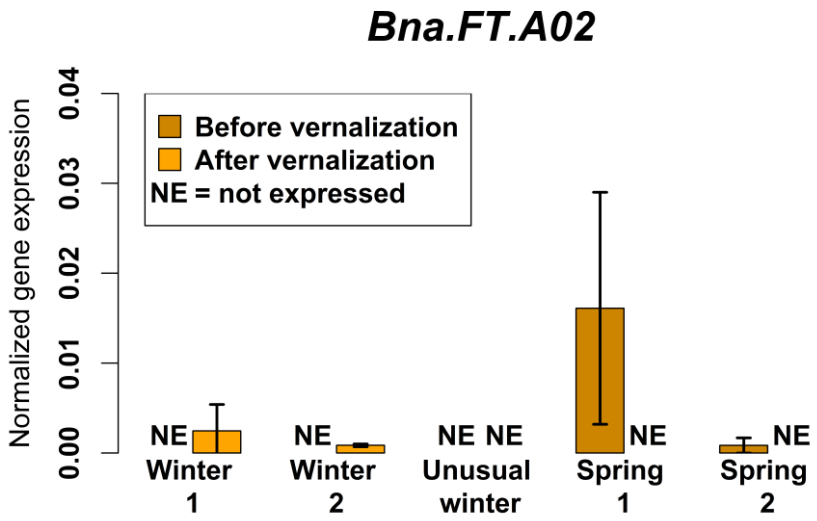

Figure 15. Normalized gene expression for *Bna.FT.A02* in before and after vernalization. The experimented were performed in triplicate. The data are presented as mean ± SD.

3.7 Gene expression analysis in response to aging

3.7.1 Response to aging of *Bna.FLC*

To test how aging affected the gene expression of *Bna.FLC* gene copies, samples were collected at two different developmental points: 7 and 15 weeks after sowing (WAS). During this period the plants were kept at the greenhouse with long day regime and temperature of 20 and 17 °C during day and night. At the time of the second sample collection some genotypes were starting to flower. In the case of spring 1 genotype, 2 out of the three plants were flowered at different levels. In spring 2 genotype, the plants had only buds developed. The winter and unusual winter genotypes at this stage showed no signs of flowering.

In the case of *Bna.FLC.A02* (figure 16A) there was a slight increment in the gene expression for winter and unusual winter types. They behave very similar with values ≤2-fold change. For spring types the gene expression was different according to the genotype. In the case of spring 1 the gene expression was very different between biological replicates. This is reflected in the high standard deviation in the graph. In the case of spring 2 the gene expression showed to be much higher than at 7 WAS. Here the difference was 3.9-fold change.

For *Bna.FLC.A03a* (figure16B) there was a differential decrease in gene expression for most of the genotypes. For winter 1, the decrease of gene expression was 3.4-fold. For winter 2 and unusual winter there was no major difference with a <2-fold change. Interestingly, this copy showed a major decrease in spring 1 with a 6.1-fold difference. In spring 2 there was no difference in gene expression.

The copy *Bna.FLC.A03b*, as mentioned before, was not expressed in spring samples. In winter types the gene expression had a mild increment on time although the values were <2-fold different. For the unusual winter type the gene expression did not show a major difference (figure 16C). For the most expressed *FLC* copy *Bna.FLC.A10* overall did not change throughout time. The amount of transcript was maintained for all the genotypes analyzed (Figure 16D).

The copy *Bna.FLC.C02,* the second most expressed copy, showed a small increment in gene expression in winter and unusual winter genotypes (<2-fold, figure 17A). For spring 1, this copy showed to have a big difference in gene expression between biological replicates. In spring 2, the copy suffered a moderate increment in gene expression throughout time with a 2.3-fold difference.

For *Bna.FLC.C03a,* the copy had an increment in gene expression in winter types with 1.9 and 3.2-fold difference. In unusual winter type, there was no difference observed. The increment in gene expression was more pronounced in spring types. Here, spring 1 and spring 2 had a 4.4 and 2.7-fold difference (figure 17B).

The copy *Bna.FLC.C09a* had no difference in gene expression for winter types. Interestingly, the unusual winter type had a big decrease in gene expression with 8.3-fold difference. Spring 1, as before, had very different levels of expression between biological replicates. In spring 2 it showed an increment in gene expression with 2.7-fold difference (figure 17C).

Finally, *Bna.FLC.C09b* showed a minor decrease in gene expression for winter types with a ≤2-fold difference (figure 17D). This copy in unusual winter type behaved similarly to *Bna.FLC.C09a* with a decrease in gene expression of 3.7-fold. In spring types, the amount of transcript got increased in spring 2 by 2-8-fold. Also here the genotype spring 1 had a different behavior among biological replicates. To analyze if the difference observed is statistically significant a *t*-test was carried out. The values used for the comparison were the fold changes of delta-C_t method. A summary of these results is in table 8 and 9.

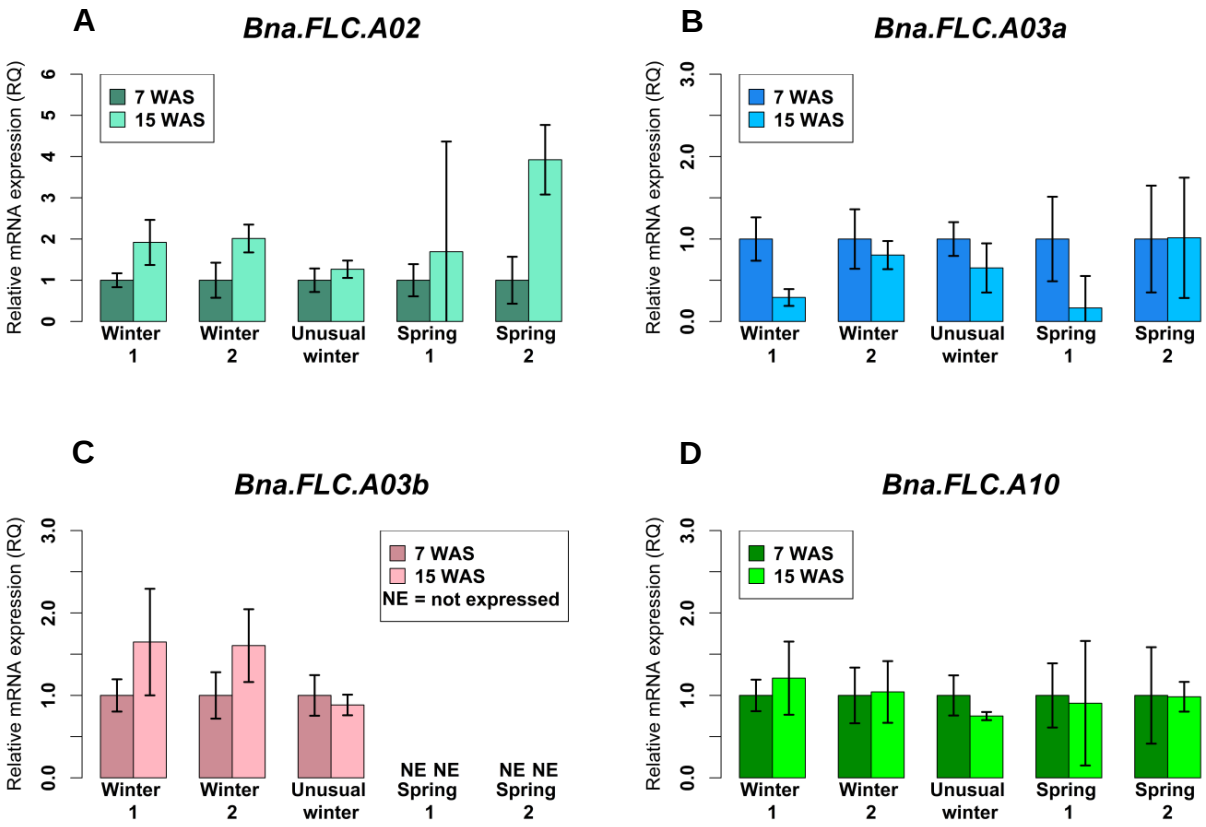

Figure 16. Gene expression comparison between 7 and 15 weeks after sowing (WAS) for *Bna.FLC* C subgenome. The experimented were performed in triplicate. The data are presented as mean ± SD.

Table 10. *t*-test results comparing 7 and 15 weeks after sowing (WAS) gene expression of *Bna.FLC* subgenome A. The mean (M) and standard deviation (SD) values are shown. The significant p-values (p) are marked with an asterisk (p≤0.05).

Genotype		*Bna.FLC.A02*		*Bna.FLC.A03a*		*Bna.FLC.A03b*		*Bna.FLC.A10*	
		M	**SD**	**M**	**SD**	**M**	**SD**	**M**	**SD**
Winter 1	7 WAS	0.006	0.001	0.012	0.004	0.014	0.004	0.456	0.128
	15 WAS	0.012	0.002	0.003	0.001	0.022	0.002	0.549	0.089
	p	**0.012***		**0.042***		**0.058**		**0.240**	
Winter 2	7 WAS	0.005	0.002	0.015	0.003	0.011	0.004	0.374	0.183
	15 WAS	0.009	0.001	0.012	0.003	0.017	0.005	0.394	0.173
	p	**0.050***		**0.244**		**0.086**		**0.408**	
Unusual winter	7 WAS	0.079	0.006	0.015	0.002	0.017	0.003	0.463	0.136
	15 WAS	0.102	0.019	0.010	0.004	0.015	0.003	0.331	0.003
	p	**0.125**		**0.091**		**0.271**		**0.388**	
Spring 1	7 WAS	0.004	0.002	0.016	0.005	-	-	0.023	0.012
	15 WAS	0.014	0.012	0.009	0.007	-	-	0.027	0.019
	p	**0.164**		**0.069**		**-**		**0.439**	
Spring 2	7 WAS	0.003	0.001	0.026	0.007	-	-	0.020	0.007
	15 WAS	0.010	0.003	0.029	0.020	-	-	0.019	0.002
	p	**0.039***		**0.367**		**-**		**0.301**	

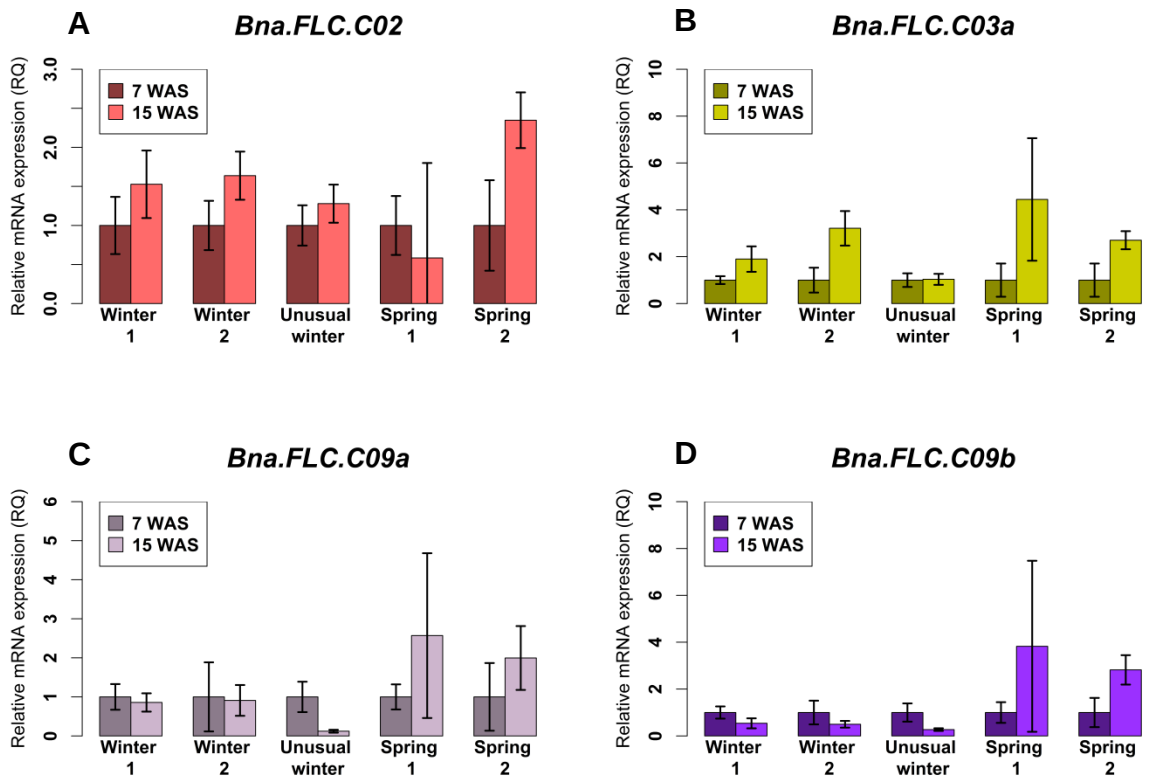

Figure 17. Gene expression comparison between 7 and 15 weeks after sowing (WAS) for *Bna.FLC* C subgenome. The experimented were performed in triplicate. The data are presented as mean ± SD.

Table 11. *t*-test results comparing 7 and 15 weeks after sowing (WAS) gene expression of *Bna.FLC* subgenome C. The media (M) and standard deviation (SD) values are shown. The significant p-values (p) are marked with an asterisk (p≤0.05).

Genotype		*Bna.FLC.C02*		*Bna.FLC.C03a*		*Bna.FLC.C09a*		*Bna.FLC.C09b*	
		M	**SD**	**M**	**SD**	**M**	**SD**	**M**	**SD**
Winter 1	7 WAS	0.213	0.089	0.009	0.002	0.004	0.002	0.005	0.001
	15 WAS	0.319	0.083	0.019	0.005	0.003	0.001	0.003	4.9E-04
	P	**0.102**		**0.017***		**0.176**		**0.067**	
Winter 2	7 WAS	0.151	0.066	0.006	0.005	0.003	0.001	0.005	0.002
	15 WAS	0.243	0.055	0.018	0.003	0.004	0.002	0.002	0.001
	P	**0.077**		**0.009***		**0.295**		**0.018***	
Unusual winter	7 WAS	0.167	0.033	0.018	0.007	0.008	0.003	0.007	0.002
	15 WAS	0.217	0.055	0.018	0.005	0.002	4.3E-04	0.002	3.2E-04
	P	**0.199**		**0.492**		**0.036***		**0.003***	
Spring 1	7 WAS	0.061	0.032	0.010	0.010	0.014	0.005	0.009	0.003
	15 WAS	0.101	0.092	0.038	0.012	0.052	0.037	0.053	0.041
	P	**0.306**		**0.083**		**0.128**		**0.126**	
Spring 2	7 WAS	0.070	0.040	0.009	0.006	0.006	0.005	0.005	0.003
	15 WAS	0.154	0.028	0.019	0.003	0.010	0.005	0.013	0.004
	P	**0.061**		**0.062**		**0.237**		**0.093**	

3.7.2 Response to aging of *Bna.FT.A02*

In the case of *Bna.FT.A02,* the gene expression was different depending on the genotype but overall at 7 WAS showed a low or not gene expression. For instance, in winter genotypes this gene was not expressed at 7 WAS. After 8 weeks, the levels of transcript increase to a detectable level but still remained very low. When comparing the gene expression average of winter genotypes vs spring 1 or spring 2 there were 472 and 917-fold difference, respectively. In the case of unusual winter type, the *Bna.FT.A02* gene was not expressed in any of the stages analyzed. On the other hand, spring types showed a different behavior. In the 7 WAS sample, the spring types showed a higher amount of transcripts compared to winter and unusual winter types but still low. When comparing the gene expression at 7 WAS between spring types there was a small difference. Here, spring type 1 had a higher level of expression with a 3.6-fold difference with spring type 2. In 15 WAS sample, the genotype spring 1 showed an increment in gene expression in an average of 28.5-fold change but also showed a high difference between biological replicates. Spring 2 genotype showed also an increment of gene expression thought-out development. When comparing gene expression in this genotype 7 WAS vs 15 WAS there is 198-fold difference, with the later having the highest levels of transcripts (figure 18).

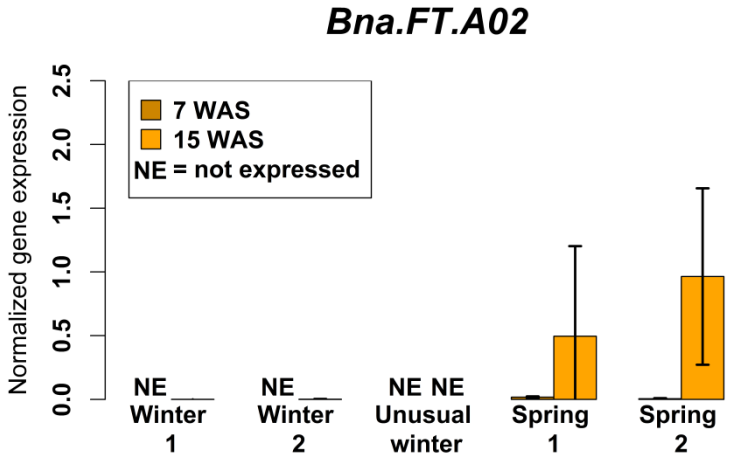

Figure 18. Gene expression levels of *Bna.FT.A02.* The experimented were performed in triplicate. The data are presented as mean ± SD.

3.8 Confirmation of primer specificity

The products from the qRT-PCR were run into the Fragment Analyzer high-resolution gel. In this case, the size of the product was calculated. This technique has an error ±5 % which was taken into account.

This resulted in the size as expected for almost all the primers analyzed (figure 19, table 12). The only primer pair that showed to have a different size than the expected was *Bna.FLC.C09a.* This copy had a ~ 8 % smaller size than the expected. Because of this reason is likely that the difference in size could be due to: (i) the primers are amplifying another gene, (ii) the annotated CDS sequence in the reference genome is not the correct, (iii) or this copy has an alternative splicing not describe in the reference genome. If these primers are going to be used in future studies the best option would be to test them with another technique to ensure specificity, using for example sequencing.

Table 12. Fragment sizes expected (E) and obtained for specific primers design to amplify *FLC*, *FT* and *ubiquitin* genes. Genotypes: winter 1 (W1), winter 2 (W2), unusual winter (UW), spring 1 (S1), spring 2 (S2).

Target gene	E	Fragment size per genotype sample (bp)						
		W1	W2	UW	S1	S2	Average	±5 %
Bna.FLC.A02	**74**	71	72	72	72	72	72	
Bna.FLC.A03a	**105**	99	100	99	98	99	99	√
Bna.FLC.A03b	**124**	120	119	119	-	-	119	√
Bna.FLC.A10	**70**	68	68	68	68	68	68	√
Bna.FLC.C02	**143**	135	135	135	135	135	135	√
Bna.FLC.C03a	**70**	74	74	73	74	74	74	√
Bna.FLC.C09a	**98**	90	90	90	90	89	90	±8%
Bna.FLC.C09b	**123**	119	121	119	121	120	120	√
Bna.FT.A02	**127**	126	127	127	126	127	127	√
Ubiquitin	**101**	100	100	99	99	100	100	√

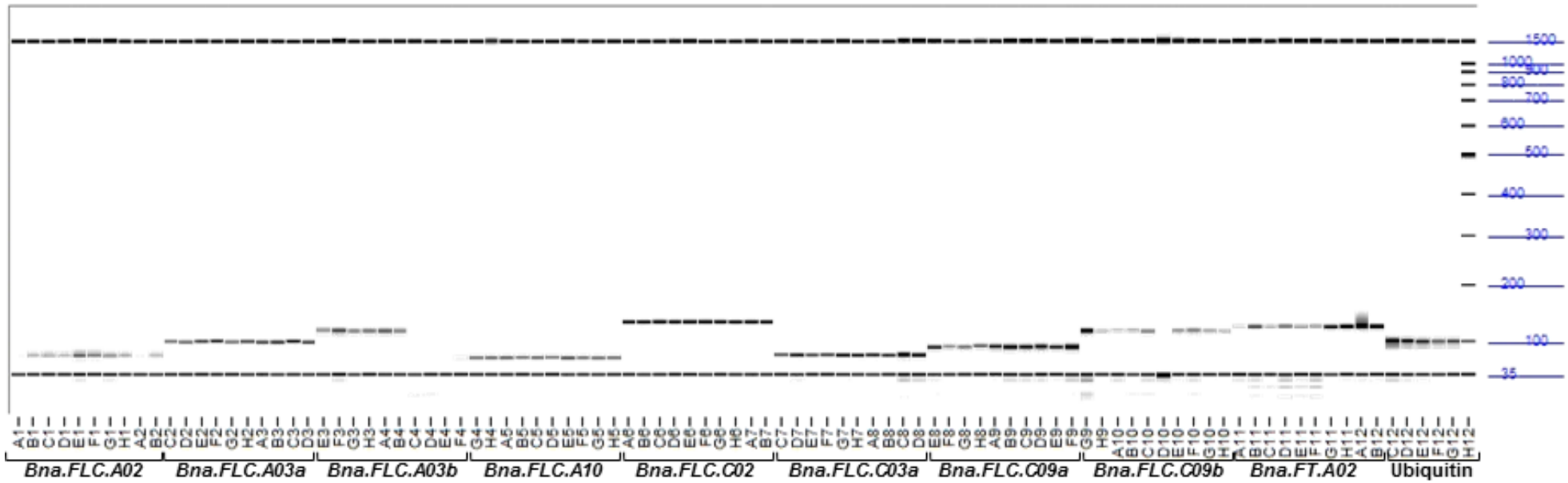

Figure 19. Fragment analyzer gel. Per primer pair one sample per genotype was run with two technical replicates. For ubiquitin there is only one technical replicate was used. The order is: winter 1, winter 2, unusual winter, spring 1, and spring 2.

4 DISCUSSION

4.1 Not all the *Bna.FLC* genes are expressed

In *B. napus* there are 9 annotated copies for *FLC* genes. In this study, only 8 out of the 9 copies were expressed. The copy that showed no expression, *Bna.FLC.C03b,* in a previous study [60] showed a lack of promoter region. Also in [81], this gene was not expressed either in winter, semi-winter or spring genotypes and it's more likely to be a pseudogene.

4.2 *Bna.FLC* differentially expressed between winter and spring genotype

The gene expression of *Bna.FLC* homologues were measure in before vernalization stage in leaves of 7 weeks after sowing plants. Overall, the gene expression of *Bna.FLC* homologues were low. The exceptions to this were the copies *Bna.FLC.A10* and *Bna.FLC.C03.* At the same time, the copies *Bna.FLC.A10, Bna.FLC.A03b* and *Bna.FLC.C02* showed to be differentially expressed between winter and spring types. This is in correlation with what was observed in [61]. In here, the overall *Bna.FLC* gene expression was higher in winter compared to spring genotypes in before vernalization stage. In the present study, the copies *Bna.FLC.A10* and *Bna.FLC.C02* also had higher gene expression in winter than in spring genotypes. This is not the first time that the copy *Bna.FLC.A10* has been shown to have importance in flowering. Zou et al., analyze a natural rapeseed population of winter, semi-winter and spring genotypes. Here, they identified a 621-bp insertion in the upstream region of *Bna.FLC.A10* in most of the winter types. This insertion corresponds to a *Tourist*-like MITE that strongly associated with vernalization requirements [83]. This new insertion could be associated with the increment in gene expression especially in this copy that presented the highest gene expression of all the *Bna.FLC* homologues. This high level of expression and the differential gene expression between winter and spring genotypes of *Bna.FLC.A10* suggest an important role in differentiating between annual and biannual plants.

The copy *Bna.FLC.A03b* was only expressed in winter and unusual winter types and completely absent in spring genotypes. In previous studies, four quantitative trait loci (QTL) related to flowering time have been identified in double haploid (DH) population mapping [81]. These QTLs were located close to *Bna.FLC* homologues.

From Tapidor/Ningyou7 DH population, the QTLs were located close to *Bna.FLC.A03b, Bna.FLC.A10.* In the other mapping population Skipton/Ag-Spectrum DH population, the QTLs are close to *Bna.FLC.A03a* and *Bna.FLC.C02.* Furthermore, a 25-fold higher expression in winter types compared to a semi-winter type was also determined [81]. Even though, in the present study this copy was not so highly expressed, the differentially expressed characteristic between winter and spring types might have some influence in vernalization requirement. Nevertheless, the direct relation between *Bna.FLC* homologues and vernalization requirements still need to be asses.

It is important to remember that *FLC* genes have been shown to act in a dosage-dependent manner. The difference between Zou and the present study regarding the levels of expression could be explained by the age of the plants. Zou analyzed the gene expression after 4 weeks. In the present study, the analysis was carried out after 7 weeks. Nonetheless, this gene might have an important role at the beginning of the plant development. Later on, it gets downregulating by aging, letting the plant flower. This could be in concordance with what was observed in one of the winter types analyzed. In winter 1, without vernalization treatment, after 8 weeks the levels of *Bna.FLCA03b* got downregulated up to 3.4-fold. This was the only copy that shows some impact throughout aging in the gene expression levels.

4.3 *Bna.FLC.C03a* vernalization insensitive

In the present study, the gene expression analysis showed that seven *Bna.FLC* homologues are expressed in winter, unusual winter and spring genotypes before vernalization. One, *Bna.FLC.A03b,* was only present in winter and unusual winter. Also, when the gene expression was compared with after vernalization, most of the genes responded at different degrees by downregulating the gene expression of *FLC.* This suggests a similar role in vernalization like *FLC* from *Arabidopsis.* The exception to this was the copy *Bna.FLC.C03a.* This copy showed a different patter of expression after vernalization. Unlike its homologues, this copy significantly increments the gene expression when exposing to vernalization. This might suggest a different role than inhibiting flowering. Together with this, when the gene expression was measured 7 vs 15 WAS, the gene expression also showed a significant increment. In *Arabidopsis,* FLC has shown to have other regulatory roles

different than inhibiting flowering. For example, in *Arabidopsis* it was found that *FLC* has potentially more than 500 binding sites, especially located in the promoter region of genes [84]. Most of the targets are related to developmental pathways [84]. It was also shown here that *FLC* can inhibit as well as promote gene expression depending on the target [84].

4.2 Expression of FT genes

There are 6 annotated copies of *FT* gene in the *B. napus* reference genome. Initially, when the primers were tested in a normal PCR, most of the FT specific primers amplified a product from gDNA template. The only copy that did not amplify any visible product was *Bna.FT.C06.* When the primers were tested in cDNA, only the copy *Bna.FT.A02* amplified a visible product. Because of this initial result, this was the only copy analyzed by qRT-PCR. It can be concluded that only *Bna.FT.A02* has been expressed at this early stage. The *FT* copies have been analyzed before at the promoter level [85]. Here, they identified the presence of a transposable element in the copy *FT-C02* (*Bna.FT.C02b)* possibly responsible for the silencing of this gene. It is possible that the absence of gene expression in some samples of the present study could be explained in a similar way.

When this copy was analyzed before vernalization, it was only detected in spring genotypes. This is consistent with the fact that spring types are able to flower without vernalization and *FT* levels are not repressed by *FLC*. At the same time, this copy had different levels of transcripts between spring 1 and spring 2 (figure 11). This level of transcript was consistent with the fact that spring 1 genotype flowered earlier than spring 2 after 15 weeks.

When the levels of *Bna.FT.A02* were measured at 15 WAS there was a big difference of expression between biological replicates, especially in spring 1 genotype, denoted by the large SD. This is consistent with the observation that the 3 biological replicates did not flower at the same time. The spring 2 genotype showed more uniformity in the gene expression and flower pattern. This genotype was not flowering at the time of the sample collection but the plants had formed buds ready to flower. Interestingly, the winter types even though they were not vernalized, they showed a minimum of gene expression. In this case, for example, the levels of *FLC* gene expression could have been affected by the autonomous pathways. By

decreasing the *FLC* levels though vernalization, the *FT* gene expression levels would have increased once return to long day conditions.

After vernalization, the *FT* levels were different in winter than in spring. While the copy was not expressed before vernalization in winter types, but slightly afterward, it was the other way round for spring types. While the pattern in spring types is most likely a direct result of the photoperiodic conditions, the expression pattern in winter types is more likely to be controlled via the vernalization (no expression before vernalization) and the aging pathway (after vernalization inhibition has been released). The possible reason of this is that *FT* genes are expressed in response to photoperiod and the remaining copies might have an increment in gene expression once the plants comeback to long day regime.

5 SUMMARY

Copy-specific expression pattern of major flowering time genes in oilseed rape (*Brassica napus*)

Plants possess many pathways to sense the right time to flower to ensure reproduction. The main pathway related to the seasonal stage is the vernalization pathway. One of the main players in this pathway is *FLOWERING LOCUS C* (*FLC*). This gene encodes a transcription factor that acts as a repressor of the main floral activator *FLOWERING LOCUS T (FT)*. When exposed to cold, *FLC* gets downregulated due to epigenetic modifications. Here, it was analyzed the gene expression of *Brassica napus FLCs* and *FT* homologues. The two different condition tested were vernalization and aging. The gene expression was measure through qRT-PCR in five different genotypes: 2 winter, 1 unusual winter, and 2 spring types. In *B. napus* reference genome there are 9 annotated *FLC* copies and 6 *FT*. In the case of *FT* genes, only the copy *Bna.FTA02* was expressed in the samples. The other copies were not expressed. For *FLC*, *Bna.FLC.C03b* was not expressed in any of the samples. Moreover, the gene expression level was different among the remaining genes. Most of the *Bna.FLC* genes were weakly expressed before vernalization. Only *Bna.A10* and *Bna.C02* had considerably higher levels of transcripts. In addition, not all the copies responded equally to cold. In almost all the copies there was a decrease in the transcript levels when compared with before vernalization. The response intensity was also different among the copies. While *Bna.FLC.A03a* responded most strongly, the copy *Bna.FLC.C03a* appears to be not cold responsive. One of the major findings was the differential expression of the copy *Bna.FLC.A10*. In before vernalization stage, it had high levels of expression in winter types but low expression in spring types. Because of this, the copy suggests an important role in the differentiation between annual and biannual plants.

In the present study, only five genotypes were tested. In future studies, the test of gene expression in more genotypes is a major interest. Also, to investigate if there is some nucleotide variation between *FLC* sequences from winter and spring types that might explain the differences in gene expression. Another interesting point would be to test the gene expression in more tissues. Under this scenario, maybe some of the *FLC* low expressed copies have a different patter of expression.

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7 DECLARATION

Thesis: "Copy-specific gene expression pattern of major flowering time genes in oilseed rape (*Brassica napus*)"

I declare that this thesis has been written by me and that it has not been submitted, in whole or in part, in any previous application for any other degree or professional qualification. The work presented is entirely my own except where stated otherwise by reference or acknowledgment using standard referring practices.

Daniela R. Quezada Martínez

Gießen, November 28, 2017.