Study of the C genome QTL affecting flowering time in spring oilseed *Brassica napus* under a short-day condition

by

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Abstract

Brassica napus, mostly known as canola, is an important oilseed crop in Canada. Earliness of flowering and maturity are one of the important traits for growing spring canola in North America. These traits can be improved through identification of the genes and alleles affecting flowering time and understanding their effect on other traits. In this study, near-isogenic lines (NILs) carrying flowering time QTL alleles of *Brassica oleracea* or *B. napus* which located on C1 or C9 chromosome were evaluated under 10 hr photoperiod condition for morphological and root and shoot biomass traits to understand the effect of these QTL alleles on the other traits. The NILs carrying C1 or C9 allele of *B. oleracea* flowered earlier, as expected, and no visible difference was observed between the NILs and the recurrent parent for leaf characteristics. However, the NILs showed significant difference for plant height, shoot and root biomass indicating that the flowering time QTL alleles may exert pleiotropic effect on these traits or QTL affecting these traits are located in the same genomic region. This thesis research also included expression analysis of putative flowering time genes from C5 and C9 flowering time QTL. For this, a set of early- and late-flowering *B. napus* lines developed from a *B. napus* × *B. oleracea* interspecific cross were used, and expression analysis of 30 flowering genes from C5 (15) and C9 (15) QTL was carried out on leaf and shoot apex tissue collected from the plants grown under 10 hr photoperiod condition. A majority of the genes showed a greater expression at night than in morning, and significant differential expression was observed for *CO*, *PHYA*, *FT*, *AP2*, *SOC1* and *TEM1*; this suggests that these genes might be involved in flowering time variation between the early and late flowering lines. The leaf materials of the above-mentioned early- and late-flowering plants were also used in a proteomic study to identify the proteins involved in flowering time variation. This study identified the proteins involved in photosynthesis, pigmentation, phytohormone signaling, stress response, ROS, ion binding, mRNA binding, protein binding and many others. Thus, this

thesis research showed that the flowering time alleles can exert effect on other morphological and physiological traits, and the research also identified the genes and proteins potentially involved in the regulation of flowering time in canola under short-day photoperiod conditions.

Preface

This dissertation is submitted by Juthy Abedin Nupur for the degree of Master of Science in Plant Science. Dr. Habibur Rahman contributed to the research design, interpretation of the findings, and useful comments and recommendations for writing and finalizing all thesis chapters.

For chapter II, Juthy Abedin Nupur conducted all experiments in growth chamber for evaluating near isogenic lines (NILs) for days to flowering, leaf characteristics, plant height, root and shoot biomass. Juthy Abedin Nupur collected data, analyzed, and wrote the chapter. For the development of NILs used in this chapter, the F1, BC1 and BC2 generation populations were produced by the Canola Program of the University of Alberta and BC3, BC4, BC4F2 and BC4F3 generations NILs population were developed by Karanjot Gill under the supervision of Dr. Habibur Rahman.

For chapter III, Juthy Abedin Nupur carried out the whole experiment, such as growing the lines in growth chamber under 10 hr photoperiod condition, RNA extraction, designing qPCR primers, performing qPCR and gene expression analysis. Dr. Aleya Ferdausi helped in designing the experiment for this chapter. The lines used in this experiment were developed by Azam Nikzad in the Canola program of University of Alberta under the supervision of Dr. Habibur Rahman.

For chapter IV, Dr. Glen Uhrig conducted the LC-MS/MS analysis and prepared the materials and methods. Juthy Abedin Nupur conducted the experiment, collected, and prepared samples, sorted data, performed other analysis such as GO, KEGG pathway and association network. Dr. Dinesh Adhikary and Dr. Kawalpreet Kaur helped in data analysis.

Dedicated

To

My beloved husband Md. Abir Ul Islam

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List of Symbols and Abbreviations

=	Equal
%	Percent
/	Per
<	Less than
>	Greater than
±	Plus/minus
$\mu molg^{-1}$	Micromoles per gram
2*n*	Diploid number of chromosomes
ANOVA	Analysis of variance
BC1	First backcross generation
BC1F1	First filial generation after first backcross
$BC1F_x$	x^{th} generation of BC1-derived population
bp	Base pairs
℃	Degree Celsius
Canola	Canadian oil low acid
cm	Centimeter
cM	Centimorgan
cv.	cultivar
df	Degrees of freedom
DH	Double haploid
DNA	Deoxyribonucleic acid
DTF	Days to flowering
e.g.	For example

F1	First filial generation
g	Gram
g^{-1}	Per gram
GC	Guanine cytosine
L.	Linnaeus
LG	Linkage group
LSmeans	Least-squares means
Mb	Million base pairs
ml	Milliliter
NIL	Near isogenic line
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
PH	Plant height
P-value	Probability value
qRT-PCR	Real-Time Quantitative Reverse Transcription PCR
QTL	Quantitative trait loci
r	Pearson's correlation coefficient
RB	Root biomass
RNA	ribonucleic acid
S.E.	Standard Error
SB	Shoot biomass
USDA	United States Department of Agriculture
Δ	Delta

Chapter 1. Literature Review

1.1 Introduction of Canola (Rapeseed)

Brassica oilseed crop is the second largest oilseed crop in the world following soybean (USDA, 2022). According to the analysis carried out by LMC International, the contribution of canola to the Canadian economy has increased by $7.2 billion, or 35%, in the last 10 years, and currently, it contributes about $30 billion to the economy of this country (www.canolacouncil.org). In 2021, total canola production in Canada was 13.8 million metric tonnes where Saskatchewan, Alberta and Manitoba contributed 49%, 32% and 18% of the total production, respectively (Canola Council of Canada, 2023).

The word "rape" in "rapeseed" comes from the Latin word rapum, which means "turnip" (Shahidi, 1990). The traditional rapeseed cultivars contained a significant amount of erucic acid (>40% of the total fatty acids) in its oil and a high content of glucosinolates (>80 µmol/g) in its seed meal; both of these seed constituents are undesirable. The presence of erucic acid in edible oil causes fatty deposits in heart and skeletal muscles as well as impaired growth, while feeding animals this seed meal causes liver disease and reduce growth and weight gain (for review, see Przybylski, 2011). These two undesired seed constituents were reduced genetically through traditional plant breeding in Canada, and the word "canola" was used for the Brassica oilseed crops (*Brassica napus, Brassica rapa* and *Brassica juncea)* which seed oil is almost free from erucic fatty acid (<2%) and seed meal contain <30 µmol glucosinolates per g seed meal (www.canolacouncil.org). The canola quality Brassica oilseed crops first became available in the 1970's in Canada. Today, it's oil is primarily used as an edible oil; however, biodiesel is also produced from this oil (Qian et al., 2013) and its seed meal serves as a good source of protein in animal feed (Ivanova et al., 2016).

1.2 Origin and evolution history of *Brassica* species

Brassicaceae family, which also known as Cruciferae, includes 338 genera and 3709 species. This family includes economically important diverse plant species which are sources of vegetables, edible and industrial oils, forage, and condiments (for review, see Al-Shehbaz et al., 2006). Moreover, this family also includes the well-known model flowering plant *Arabidopsis thaliana* (2*n* = 10) (for review, see Schmidt et al., 2001). The genus *Brassica* includes the vegetable crops such as cabbage, cauliflower, brussels sprouts, kale, broccoli, and kohlrabi which belongs to *B. oleracea* L. (CC, 2*n* = 18), turnip and Chinese cabbage belonging to *B. rapa* L. (AA, 2*n* = 20), oilseed crops rapeseed or canola belonging to *B. napus* L. (AACC, 2*n* = 38), Indian mustard belonging to *B. juncea* L. (AABB, 2*n* = 36), Ethiopian mustard belonging to *B. carinata* (BBCC, 2*n* = 34), and black mustard belonging to *B. nigra* L. (BB, 2*n* = 16) (Figure 1-1) (for review, see Gupta and Pratap, 2007).

The Brassicaceae family underwent rapid diversification following its split from the ancestral tribe Aethionemeae (Courvreur et al., 2010), and the species diversity has been greatly influenced by polyploidization and interspecific hybridization events (Lihová et al., 2006). The split between *Brassica* and *Arabidopsis* occurred about 43.2 million years ago (MYA). The first diploid *Brassica* species to emerge was *B. nigra* which occurred about eight MYA, followed by the separation of *B. rapa* and *B. oleracea* from their common ancestor about 3.75 MYA. These three diploid *Brassicas* naturally hybridised among themselves and the three allopolyploids *B. napus, B. juncea*, and *B. carinata* evolved about 0.75 MYA (for review, see Laha et al., 2020). Thus, two types of polyploidization exists in *Brassica* species; the ancient polyploidization which exists in the genome of the three diploid species *B. rapa, B. oleracea* and *B. nigra* and occurred during their evolution, and the other is allopolyploidization in *B. napus*, *B. juncea* and *B. carinata* which

resulted from mating between the diploid species, (for review, see Osborn, 2004). The allotetraploid species *Brassica napus* evolved through interspecific hybridization between *Brassica rapa* and *Brassica oleracea* (Figure 1-1) (U, 1935). The genus *Brassica* share evolutionary relationship with *Arabidopsis* (Yang et al., 1999), and a high degree of genome co-linearity exists between *Brassica* and *Arabidopsis;* therefore, the knowledge of the *Arabidopsis* genome can be used to understand the *B*. *napus* genome (Schmidt et al., 2001).

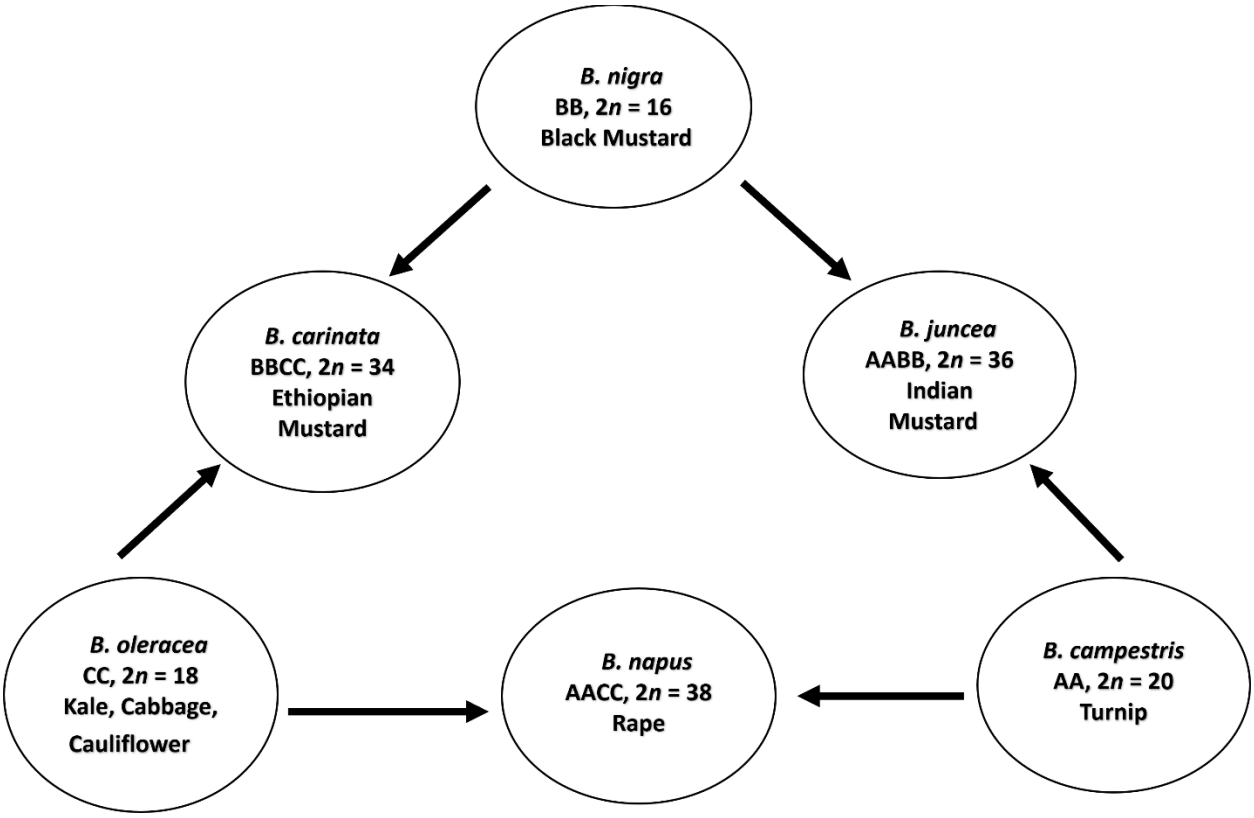

Figure 1-1. Important *Brassica* crops and their genome relationship (adapted from Gupta and Pratap, 2007)

1.3 Flowering in *Brassica napus*

Flowering is an important event of crop reproduction. Different environmental cues such as light, water, temperature, and plant hormone exert influence on this event (Lee et al., 2018). This

is an important adaptive feature of seed crops, and the timing of flowering affects crop yield (Nelson et al., 2014). For reproduction to be successful, flowering needs to occur under suitable environmental conditions for the development of seeds (for review, see Andrés and Coupland, 2012).

QTL (Quantitative trait loci) mapping of flowering time in *Brassica* species has been carried out by several researchers to understand the genetic basis of this trait. Flowering time QTLs have been reported from almost all chromosomes of this species. A list of flowering QTLs mapped in *Brassica* summarized in Table 1-1.

Table 1-1. List of QTL affecting flowering time in *Brassica*

Cross	Linkage group	No. QTL affecting flowering time	Position (cM)	Position (Mb)	Population/remarks	Reference
B. rapa						
Late flowering x early flowering	R2, R3	Two	43, 18	-	F_2	Axelsson et al., 2001
Early flowering x late flowering	A07	One	-	15.48-16.54	F_2	Qu et al., 2022
Chinese cabbage x Chinese cabbage	A02, A08	Six	31.2-33.7, 61.6 - 66.7	-	Doubled haploid (DH)	Liu et al., 2016
Rapid cycling x vegetable type Chinese cabbage	R01, R02, R03, R06, R07, R08, R10	Eight	-	-	$F_{2/3}$, DH, BC1	Lou et al., 2007
Late bolting DH line x Chinese DH line	LG1, LG2, LG3, LG4, LG5, LG9, LG10	Ten	-	-	DH	Nishioka et al., 2005
Biennial x Annual	LG2, LG8	Two	-	-	F_2	Teutonico and Osborn, 1995
B. oleracea						
Cabbage x broccoli	LG2, LG6, LG8	Three	-	-	F_2	Camrago and Osborn, 1996
var. *alboglabra* x var. *italica*	O2, O3, O5 (2), O9 (2)	Six	81, 12, 31, 42, 88 (Average position from two trial)	-	DH	Bohuon et al., 1998
Annual x Biennial	O2 (2), O3, O6, O8,	Six	49.9-67.1,0-20.3, 8.2-27.3, 3.217.6, 28.6- 37.1, 48.1- 57.7	-	DH	Okazaki et al., 2007
var. *italica* x var. *alboglabra*	O1, O2, O3, O5, O9	Eleven	0-30.3, 30.3-38.1, 78 ± 9, 0-30.3, 30.3 – 75, 0-34.2, 0-20, 20- 39, 42-58, 70-102, 20-46	-	Recombinant backcross substitution lines	Rae et al., 1999
Late flowering × early flowering	O3, O9	Two	13, 18	-	F_2	Axelsson et al., 2001
B. napus						
Spring x Spring	N2, N5, N6, N11, N18	Three main effect and	-	-	DH	Cai et al., 2008

Cross	Linkage group	No. QTL affecting flowering time	Position (cM)	Position (Mb)	Population/remarks	Reference
		four pairs of epistatic QTLs				
Winter x Spring	C04, C08	Two	-	14.56, 9.43	F_2	ArifUzZaman et al., 2016
Early flowering x late flowering spring	C08	One	-	0.9	BC_3F_2, BC_4F_2	Tang et al., 2023
Spring *B. napus* x *B. oleracea*	C02, C05	Two	-	5.1-10.7, 26.1-31.8	Inbred	Nikzad et al., 2023
Early flowering x late flowering	A02, C02	Two	-	0.13, 0.21	NILs	Chen et al., 2018
Intertribial hybrid x Spring	A02, A07	Four	5.31, 13.41-14.41, 114.71, 118.01	0.11-1.5, 1.5-4.3, 15.95-16.68, 16.17-18.91	DH	Shen et al., 2018
Semi-winter x Spring	A03, A07, C04, C06, C07, C09	Twelve	0.01, 69.21, 73.81,74.71,81.91,82.71,44.61 ,69.01,112.71,117.81, 101.21, 106.21	5.7-8.1,14-15.1,15.23-17.27,17.27-18.81,19.93-21.60, 21.6-21.91, 5.1-5.9, 33.67-34.61,40.02-40.51,40, 45.82-47,47	RILs	Yu et al., 2019
High stalk late flowering x dwarf early flowering mutant	LG2, LG3, LG6, LG13, LG17	Six	-	-	$F_{2:3}$	Mei et al., 2009
Spring x Winter	A2, C6	Six major QTLs (Total 55 QTls)	60.74, 69.01, 54.84, 60.27, 64.11, 66	-	DH	Li et al., 2018
Spring x Spring	C1, C2, C3, C6	Five	89, 96, 10.1, 38.5, 3.6, 147, 157.2	21.17-41.13 (C1), 44.46 (C2)	DH	Rahman et al., 2017
Spring x Spring	C1, C2, C8, C9	Six	101.4, 102.6, 104.2, 106.8, 6.8, 38.5, 5.6, 32.5, 27.6	21-41 (C1), 0.65 (C9)	DH	Rahman et al., 2018
Annual x Biennial	LG9, LG12, LG16	Three	-	-	DH, F_1, BC1	Ferreira et al., 1995
Summer x Summer	A02, A07, C031	Three	13.9, 82, 12.1	-	DH	Nelson et al., 2014
Winter x Semi-winter	N1, N2, N3, N9, N10, N12, N16, N18, N19	Forty-two	-	-	DH, F_2	Long et al., 2007

Cross	Linkage group	No. QTL affecting flowering time	Position (cM)	Position (Mb)	Population/remarks	Reference
Semi-spring x Semi-spring	A2, A3, A4, A6, A7, C2, C3, C5, C6, C8	Twenty	-	-	DH	Raman et al., 2013

Rahman et al. (2011) reported that *B. oleracea* flowers very late as compared to *B. napus*, however, this species carries alleles that can improve the earliness of flowering in *B. napus* under a short-day condition. By using a spring *B. napus* mapping population carrying C genome contents of *B. oleracea*, Rahman et al. (2017, 2018) mapped QTL affecting flowering time on C1 and C9 chromosome. The C1 QTL allele of *B. oleracea* improve the earliness in *B. napus* without being affected by photoperiod, while the C9 QTL improve earliness only under a 10-hour photoperiod condition. Near-isogenic lines (NILs) for these two loci have been developed by this research group for further investigation (Gill, 2021). Nikzad et al. (2023) also reported flowering QTL from C2 and C5 chromosome *B. napus* where the early flowering allele of C5 introgressed from *B. oleracea*.

Genetic and molecular analyses of flowering time in plants have identified four major pathways involved in the control of this trait, and this includes the photoperiodic, autonomous, vernalization, and hormonal pathways. Interaction of genes from these pathways can play a role for expression of some of the genes involved in floral initiation and various signal transduction which concerted effect ultimately regulate the flowering time (Mockler et al., 2004). A brief discussion of these pathways is given below along with a diagram showing different flowering genes from these pathways (Figure 1-2).

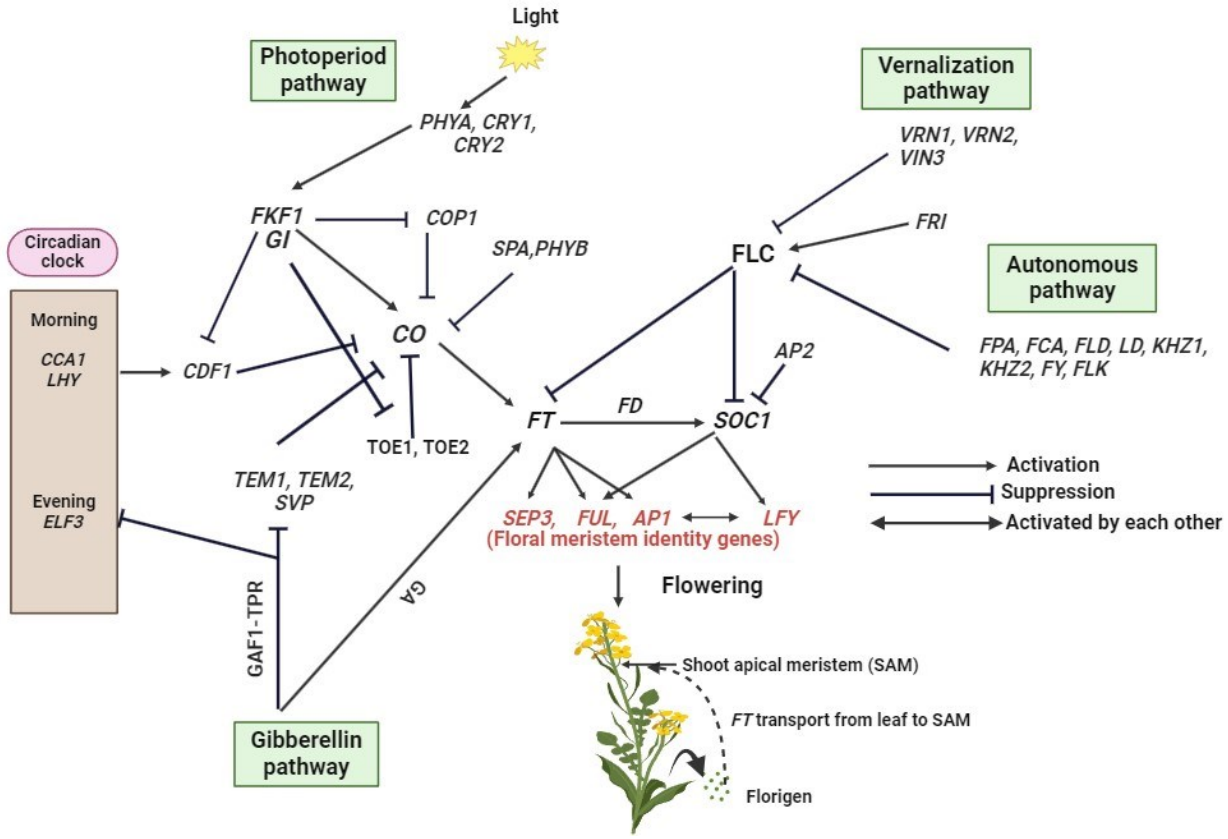

Figure 1-2. A simple schematic diagram of the flowering pathways in *Arabidopsis thaliana* (modified from Kim et al., 2020). *CCA1 = CIRCADIAN CLOCK ASSOCIATED 1, LHY = LATE ELONGATED HYPOCOTYL, ELF3 = EARLY FLOWERING3, PHYA = PHYTOCHROME A, CRY1 = CRYPTOCHROME 1, CRY2 = CRYPTOCHROME 2, FKF1 = FLAVIN-BINDING KELCH REPEAT F-BOX PROTEIN 1, GI = GIGANTIA, COP1 = CONSTITUTIVE PHOTOMORPHOGENIC 1, CO = CONSTANS, SPA = SUPPRESSOR OF PHYA-105, PHYB = PHYTOCHROME B, CDF1 = CYCLING DOF FACTOR1, TEM1 = TEMPRANILLO1, TEM2 = TEMPRANILLO2, SVP = SHORT VEGETATIVE PHASE, TOE1 = TARGET OF EAT1, TOE2 = TARGET OF EAT2, FT = FLOWERING LOCUS T, FD =* bZIP transcription factor*, SOC1 = SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1, AP2 = APETALA2, SEP3 = SEPALLATA3, FUL = FRUITFULL, AP1 = APETALA1, LFY = LEAFY,* GA = Gibberellic Acid*,* GAF1-TPR = GAI ASSOCIATED FACTOR 1-TOPLESS RELATED*, VRN1 = VERNALIZATION1, VRN2 = VERNALIZATION2, VIN3 = VERNALIZATION INSENSITIVE3, FRI = FRIGIDA, FLC = FLOWERING LOCUS C , FPA = FLOWERING LOCUS PA, FCA = FLOWERING LOCUS CA, FLD = FLOWERING LOCUS D, LD = LUMINIDEPENDENS ,* KHZ1 = zinc-finger and K homology domain protein 1 , KHZ2 = zinc-finger and K homology domain protein 2*, FY = FLOWERING LOCUS Y , FLK = FLOWERING LOCUS KH DOMAIN*.

1.3.1 Autonomous pathway

In autonomous pathway, plants can flower after a certain phase of vegetative development and without responding to any external cues. In this case, the activity of the central flowering repressor gene *FLC* is inhibited and thus speeds up flowering regardless of the length of the day (for review, see Cheng et al., 2017). This pathway includes the genetic components which controls RNA processing and epigenetic mechanisms that results in downregulation of floral repressor *FLOWERING LOCUS C (FLC)* (for review, see Simpson, 2004). This pathway has been linked to several other genes such as, *FLOWERING LOCUS CA (FCA), FLOWERING LOCUS D (FLD), FLOWERING LOCUS KH DOMAIN (FLK), FLOWERING LOCUS PA (FPA), FLOWERING LOCUS VE (FVE), FLOWERING LOCUS Y (FY),* and *LUMINIDEPENDENS (LD)* (Lim et al., 2004; Mockler et al., 2004; Simpson, 2004; Marquardt et al., 2006). According to Yan et al. (2020), the RNA-binding proteins, KHZ1 (zinc-finger and K homology domain protein 1) and KHZ2 (zinc-finger and K homology domain protein 2), are involved in the regulation of this pathway through repressing the splicing efficiency of the *FLC* pre-mRNA.

1.3.2 Vernalization pathway

Vernalization is the process through which plants get the ability to flower after being exposed to cold temperatures for a period (for review, see: Amasino, 2005; Kim et al., 2009; Sung et al., 2006). Basically, it enhances flowering in winter growth habit plants after prolonged winter or cold period. The requirements of the low temperature as well as the length of the cold period for vernalization varies from species to species. The genes involved in vernalization pathway are activated only when the plants are exposed to a low temperature, such as about 4 ºC (for review, see Kim et al., 2009). Vernalization induces a stable change in plants which stay till next spring to initiate flowering (for review, see: Sung et al., 2006; Amasino, 2005). The gene *FRIGIDA (FRI)*

is the activator of the floral repressor FLC (for review, see Schmitz and Amasino, 2007). The major genes involved in the vernalization pathway are *VERNALIZATION INSENSITIVE3 (VIN3)*, which is responsible for initiating changes in chromatin structure and express only after prolong exposure to cold, DNA-binding protein *VERNALIZATION1 (VRN1)* and the polycomb-group protein *VERNALIZATION2* (*VRN2)*; all of these are needed for a stabile silencing of the *FLC* (for review, see Sung and Amasino, 2004).

Vernalization creates epigenetic modification of histone proteins of *FLC* chromatins, such as demethylation of H3 at Lys9 (H3K9) and Lys27 (H3K27), which ultimately silence the expression of *FLC* and keep this repressed until spring. Moreover, it has been reported that the LIKE HETEROCHROMATIN PROTEIN 1 (LHP1) is required to maintain the epigenetically suppressed state of the *FLC* in *A. thaliana*. After prolonged exposure to cold, the LHP1 become abundant at the *FLC* chromatin. For this, activity of the LHP1 is needed to maintain an elevated levels of H3K9 di-methylation at the *FLC* chromatin for vernalization (Sung et al., 2006).

1.3.3 Gibberellin pathway

Hormones are a class of signaling molecules which control the growth and development of plants and regulate the behavior of the plants in response to the environment. The hormone response pathway in plants is regulated at various levels such as at biosynthesis, metabolism, perception and signaling, and the plants also carry a special capacity to spatially regulate the dispersion of the hormones (for review, see Anfang and Shani, 2021). Among the different plant growth hormones, gibberellins influence the growth and differentiation of the organs. All vascular plants as well as some bacterial and fungal species produce gibberellins (for review, see Hedden, 2020). Gibberellin improves flowering by triggering the floral integrator genes *FLOWERING LOCUS T (FT), SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1)*, and *LEAFY*

(LFY). A group of genes encoding DELLA proteins functions as negative regulators in the gibberellic acid (GA) pathway and co-activates the transcription factor *GAI ASSOCIATED FACTOR 1 (GAF1)*. GA changes this *GAF1* complex from transcriptional activator to a suppressor which supress the floral repressor genes *EARLY FLOWERING3 (ELF3), SHORT VEGETATIVE PHASE (SVP), TEMPRANILLO1 (TEM1),* and *TEMPRANILLO2* (*TEM2*) and thus promotes flowering (Fukazawa et al., 2021).

1.3.4 Photoperiod pathway

Photoperiodism is the ability of the plants to identify the day length (photoperiod) and regulate the physiology of the plant in response to day length (for review, see Kobayashi and Weigel, 2007). This determines if a plant will flower and produce seeds or not (for review, see Cai et al., 2008). Depending on the photoperiod, plants are classified into three groups: long-day plant that flowers when day-length passes the critical photoperiod, short-day plants which starts flowering if day-length is less than critical photoperiod, and day-neutral plants which flower irrespective of day length (for review, see Andrés and Coupland, 2012). According to Major (1980), the long-day plants flower faster when the photoperiod increases while flowering in a short-day plant fasten as the photoperiod decreases. Upon receiving adequate amount of photoperiod, initiation of flowering occurs due to the production of floral stimulus florigen. Leaf phloem companion cells generates this floral stimulus and transport through phloem tissue to the shoot apical meristem where floral development occurs (Abe et al., 2015).

The gene *CONSTANS (CO)*, a crucial floral regulator, activates the expression of the *FT* for the induction of flowering and, thus, play the central role in the photoperiod pathway. This gene controls flowering time by integrating the circadian clock and light signal (Cheng and Wang, 2005). Circadian clock system receives various external light information, which enables the

plants to assess the day length changes (for review, see Shim et al., 2017). In morning, the *CYCLING DOF FACTOR1 (CDF1)* protein supress the expression of *CO* and delay flowering (Imaizumi et al., 2005), and the expression of *CDF1* is induced by *CIRCADIAN CLOCK ASSOCIATED (CCA1)* and *LATE ELONGATED HYPOCOTYL (LHY*) clock genes (Niwa et al., 2007). On the other hand, degradation of *CO* starts at night due to dimerization of *CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1)* in late afternoon. However, the activation of *FLAVIN-BINDING KELCH REPEAT F-BOX PROTEIN 1 (FKF1)* in the late afternoon suppresses the *COP1* as well as the effectiveness of the *CDF1* which causes earliness in flowering under a long day condition (Lee et al., 2018; Imaizumi et al., 2005).

1.4 Photoperiod sensitivity and early flowering in *Brassica napus*

The onset of flowering in many flowering plants is frequently influenced by seasonal variations including day length (photoperiod) (Abe et al., 2015). The photoperiod, or the length of daily light phase, varies throughout the year because of earth's rotation and axial tilt. Plants depend on this regular fluctuation to maximize their survival via seasonal evolution (for review, see Gendron and Staiger, 2023). Most of the spring *B. napus* cultivars grown in Canada and Europe are photoperiod sensitive; they flower early under a long-day condition but flowers late under a short-day condition. However, genetic variation for photoperiod sensitivity can be found in spring canola cultivar (for review, see Cai et al., 2008). Gomez and Miralles, (2011) demonstrated the prospect of manipulation of photoperiod sensitivity for the adjustment of vegetative and reproductive phase in oilseed *B. napus*, and thus, increasing seed yield by increasing the length of reproductive phase at the cost of vegetative phase.

The earliness of flowering and maturity are one of the most important traits for growing spring canola in North America (Rahman et al., 2018). King and Kondra (1986) reported that the

minimum optimal photoperiod for Canadian spring *B. napus* is about 18 hours while day length in most of the canola growing regions in Canada is about 15 to 17 hours. This indicates that the minimum optimum photoperiod cannot be met for the canola cultivars grown in Canada to promote its flowering. Therefore, identification of flowering QTL alleles which improves the earliness of flowering under a short-day condition is needed.

1.5 Some important flowering genes

Flowers are produced due to the expression of the regulatory genes called floral meristem-identity genes; these genes dictate the cells in the shoot apical meristems to induce floral initiation (for review, see Coen and Meyerowitz, 1991; Pidkowich et al., 1999). A total of five floral meristem identity gene have been discovered and this includes *LEAFY (LFY), APETALA1 (AP1), CAULIFLOWER (CAL), APETALA2 (AP2)* and *UNUSUAL FLORAL ORGANS (UFO)*; among these *LFY* and *AP1* play the key roles as flowering initiator. The lack of expression of any one of these will result in the plant to continue with vegetative growth (for review, see Pidkowich et al., 1999).

A group of genes that govern the floral transition and serve as the meeting point of many flowering pathways are called floral integrator genes (for review, see Andrés and Coupland, 2012). *FLOWERING LOCUS T (FT)* is a major floral integrator gene; however, some other genes such as *SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1)* and *LEAFY (LFY)* also acts as floral integrator (Abe et al., 2005). Floral integrator can respond to various signals (e.g., gibberellin) in plants through changing their expression and thus promote or delay flowering (Andrés et al., 2014). Sometimes, a flowing time gene can serve a dual function - act as a floral meristem identity gene as well as flowering time gene, such as the *LEAFY* gene. This gene is known as floral meristem identity gene; however, its expression can be activated by the plant hormone gibberellin and thus

can enhance the flowering; in that sense it also plays a role of flowering time gene (Blázquez et al., 1997). A change in expression of the flowering integrator *FT* can alter the entire flowering process (for review, see Blümel et al., 2015), and a difference in the promoter region of this gene can produce changes in flowering time in *B. rapa* (Wei et al., 2022).

Jaeger et al. (2013) reported that the primordia on the sides of the shoot apical meristem are programmed to create flowers during blooming rather than producing leaves, and this transition is regulated by *FLOWERING LOCUS T (FT)* and *TERMINAL FLOWER1* (*TFL1*) through *FD* and *FD PARALOG*. According to Abe et al. (2005), *FD* is a bZIP (basic leucine zipper) containing transcription factor which is produced in shoot apex and is essential for functionality of the *FT* to initiate flowering through activating the floral identity gene *AP1*. *FT* and *FD* proteins coexist through protein interaction and is involved in transcriptional activation of the floral meristem identity gene *AP1*. From these two examples, it is evident that *FD* transcription factors helps *FT* to promote flowering by connecting with floral meristem identity genes. To date, several genes have been discovered from different flowering pathways which directly or indirectly improve or delay flowering; some of the flowering genes are summarized in Table 1-1.

Table 1-2. List of flowering genes and their involvement in flowering pathways

Gene	Function	Pathway	References
FLOWERING LOCUS T (FT)	Floral integrator	-	Kobayashi et al., 1999
AGAMOUS Like24 (AGL24)	Floral integrator	-	Adal et al., 2021; Liu et al., 2008
SEPALLATA3 (SEP3)	Floral development	-	Adal et al., 2021
SUPPRESSOR OF OVEREXPRESSION OF CO1 (SOC1)	Floral integrator	-	Hong et al., 2013; Liu et al., 2008
APETALA1 (AP1)	Floral meristem identification	-	Mandel and Yanofsky, 1995; Yant et al., 2010
APETALA2 (AP2)	Negatively regulates flowering	-	Yant et al., 2010

Gene	Function	Pathway	References
LEAFY (LFY)	Floral meristem identification	-	Mandel and Yanofsky 1995; Blázquez et al., 1997
SHORT VEGETATIVE PHASE (SVP)	Repression of flowering transition	-	Hartmann et al., 2000
CONSTANS (CO)	Promotes flowering	Photoperiod	Cheng and Wang, 2005; Kobayashi et al., 1999; Robert et al., 1998
TEMPRANILLO (TEM)	Directly repress *FT*	Photoperiod, Gibberellin	Osnato et al., 2012; Castillejo and Pelaz, 2008
PHYTOCHROME A (PHYA)	Promotes early flowering	Photoperiod	Bagnall et al., 1995; Reed et al., 1994
PHYTOCHROME B (PHYB)	Delays flowering	Photoperiod	Bagnall et al., 1995; Reed et al., 1994
CRYPTOCHROME 1 (CRY1), CRYTOCHROME 2 (CRY2)	Promotes *FT* transcription and flowering	Photoperiod	Du et al., 2020a
TARGET OF EAT1 (TOE1), TARGET OF EAT2 (TOE2)	Negatively controls *FT* transcription	Photoperiod	Du et al., 2020a
GIGANTEA (GI)	Helps in *CO* transcription	Photoperiod	Fornara et al., 2009
CYCLING DOF FACTOR (CDF)	Delays flowering through inhibiting *CO* transcription	Photoperiod	Fornara et al., 2009
FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1)	Regulates daily *CO* expression by repressing *CDF*	Photoperiod	Imaizumi et al., 2005; Song et al., 2014
CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1)	Assist in early flowering	Photoperiod	Jang et al., 2008
SUPPRESSOR OF PHYA-105 (SPA)	Stabilizes *CO* expression for floral induction	Photoperiod	Laubinger et al., 2006
TREHALOSE-6-PHOSPHATE SYNTHASE (TPS)	Regulates flowering	Photoperiod	Zhang et al., 2022
VERNALIZATION INSENSITIVE 3 (VIN3)	Floral induction in cold	Vernalization	Kyung et al., 2022
FLOWERING LOCUS C (FLC)	Repress flowering	Vernalization	Kim et al., 2022
VERNALIZATION 1(VRN1), VERNALIZATION 2 (VRN2)	*VRN1* enhances reproductive development at shoot apex and *VRN2* delays flowering till vernalization is complete	Vernalization	for review, see Trevaskis et al., 2007

1.6 Investigation of flowering QTL using near isogenic lines (NILs)

Different mapping population have been used to identify QTL affecting a trait, and this includes recombinant inbred lines (Yu et al., 2019), doubled haploid (DH) lines (Li et al., 2018; Rahman et al., 2018; Raman et al. 2013), and near isogenic lines (NILs) (Cai et al., 2022). Among these, NILs carry a common genetic background and differ for only a small genomic region or for the alleles of the gene of interest. This makes NILs an excellent material for investigating the effect of the gene on the trait as well as to understand the genetic and molecular mechanism of the trait (Castro et al., 2010; Mia et al., 2019; Wang et al., 2019). NILs have been used in various studies, such as to fine map a minor flowering time QTL in *B. napus* (Cai et al., 2022), Fusarium crown rot resistance in barley (Gao et al., 2019), major-effect QTL for fruit shape in cucumber (Pan et al., 2022), tiller inhibition gene in wheat (Wang et al., 2022), grain length gene in rice (Chen et al., 2023), and grey leaf spot resistance in maize (Benson et al., 2015). Several researchers also used NILs for transcriptome analysis to identify the putative candidate genes for different traits, such as pre-harvest sprouting resistance in wheat (Wang et al., 2021), flowering time in rice (Hori et al., 2021), photoperiod sensitivity under long day condition in maize (Song et al., 2019), floral transition in maize (Du et al., 2020b), panicle development in rice (Liang et al., 2022), and seed oil content in *B. napus* (Wang et al., 2016).

1.7 Gene expression study for quantification of mRNA (messenger RNA)

In the post-genome era, reverse transcription quantitative PCR (RT-qPCR) has become an important tool for conducting quantitative analysis of RNA and gene expression. In this, RNA is transcribed in reverse into DNA. This combined with real time PCR or quantitative PCR (qPCR) (i.e., qRT-PCR) is used for quantification of RNA or DNA, and this is extensively used in gene expression studies. This method has almost replaced the gene expression analysis using Northern

blotting and RNase protection assays (for review, see VanGuilder et al., 2008). The accuracy of the quantitative real-time PCR (qRT-PCR) results also depends on the reference gene used for normalization (Xu et al., 2014).

Brassica genomes evolved through polyploidization of an ancestral genome; hence, multiple copies of a gene can be found in *Brassica* species (Axelsson et al., 2001). Therefore, there is plethora of chances to occur variation in gene expression resulting in wide phenotypic variation for traits including variation for flowering time (for review, see Osborn, 2004). To date, several gene expression studies have been conducted to unveil the changes in gene regulation at different phases of plant growth and development. For instance, Schiessl et al. (2019) studied nine copy of *B. napus* flowering gene *FLC* and found differential expression for only three genes (*Bna.FLC.A03b, Bna.FLC.A10 and Bna.FLC.C02*) between winter and spring growth habit types. Jones et al. (2018) reported that about 67% (931/1,380) of the flowering time genes expressed in *B. napus*, where 23% of the expressed genes showed shoot apex tissue-specific expression while only 7% of the genes showed leaf-specific expression. While working with duplicated homologues of the shoot meristem identity gene *TERMINAL FLOWER1* (*TFL1)*, this research group showed that diverged expression patterns of this gene is due to differences in *cis*-regulatory elements. Huang et al. (2019) reported that a higher expression of *BcMAF2* (*MADS Affecting Flowering*) can delay flowering in *B. rapa* ssp. *chinensis* by activating the *BcTEM1* (*Tempranillo 1*). According to Kim et al. (2013), mutation in *HISTONE DEACETYLASE 9 (HDA9)* increases the expression of *AGL19 (AGAMOUS-like 19)* without affecting the *CO, SOC1* or *FLC* genes, and this results in early flowering in *Arabidopsis* under a short-day condition. Mutation in *HISTONE ACETYLTRANSFERASE 1 (HAC1)* gene epigenetically increases the expression of the flowering repressor gene *FLC* and delays flowering in *Arabidopsis* (Deng et al., 2007). Gene expression

analyses have also been carried out to identify the putative candidate genes for other traits, such as clubroot resistance in *B. napus* (Summanwar et al., 2021), fusarium crown rot severity in wheat (Liu et al., 2010), flowering time in soybean (Thakare et al., 2010), and seed oil content in *B. napus* (Zhu et al., 2012). Thus, gene expression analysis discloses the abundance of the transcripts, i.e., the mRNA, of a gene at a particular stage of plant growth and development, and this knowledge can be used to identify the candidate genes controlling the trait of interest.

1.8 Proteomics analysis

Proteomics includes the study of the structure, function, composition, and interaction of the proteins, and their activities in the cells of an organism (for review, see Alterovitz et al., 2006). While genomics provides an understanding of the genes, their location and expression pattern, proteomics provided understanding of the products of the genes produced by the cell; thus, the biological engines that sustain life are the proteins (Humphery-Smith et al., 1997). Therefore, an understanding of the protein expression or abundance, composition, interactions, and changes are important to extend our knowledge of the molecular mechanisms underlying the plant phenotypes. The proteins are incredibly dynamic. Their abundance in a cell depends on environment and the stage of plant development, where a variety of protein modifications and network configurations occurs (Mergner and Kuster, 2022). These proteins keep a cellular homeostasis within a certain environment by regulating the physiological and metabolic pathways (for review, see Eldakak et al., 2013). Proteomics can serve as the key link between transcriptomics and metabolomics (for review, see Salekdeh and Komatsu, 2007). Therefore, an understanding of the proteins that play a role in growth and development will help to improve our crop plants using molecular and biotechnological approaches.

To date, several investigations have been carried out on photoperiod responsive flowering time in different crops. For instance, Li et al. (2017) performed comparative proteome analysis between two photoperiod sensitive maize accessions M9 and SM9 (M9 was sensitive at vegetative stage and SM9 was sensitive at tasseling stage) by growing under short- (10 hr light), long- (16 hr light) and neutral (12 hr light/12 hr dark) conditions. They detected 4,395 proteins of which 401 and 425 were differentially abundant in the leaves of M9 and SM9. They also demonstrated that a crosstalk between photoperiod pathway, circadian clock rhythm and high light density signals takes place, and the light-responsive or the dark-responsive or both types of proteins are involved in photoperiod response. Song et al. (2019) reported 5259 proteins from a comparative study between photoperiod insensitive and photoperiod sensitive near isogenic maize lines where only six proteins were found to be involved in photoperiodic flowering. Seaton et al. (2018) investigated photoperiodic responses in *A. thaliana* at the protein level and found a coordinated change of abundance in proteins of photosynthesis and primary and secondary metabolism, and this was consistent with increased metabolic activity in long photoperiods. In the case of *B. napus*, very limited information can be found at proteome level to understand the molecular basis of flowering. Mao et al. (2021) provides some insights into this through proteome and transcriptome analysis. They found differential abundance of flowering, hormone as well as stress related proteins between the early flowering mutant and wild type lines and provided evidence that differential expression of the flowering-, hormone- and stress-related genes to be the main reason for early flowering in the mutant line. From this proteome analysis, they also identified fructose-bisphosphate aldolase 2, glycine-rich RNA-binding protein 7, heat shock protein, and germin 3 which regulates the physiological activities and might contribute to early flowering.

Several proteomic studies have been carried out to understand the molecular basis of other traits in *B. napus*. For example, Adhikary et al. (2022) detected 784 differentially abundant proteins (DAPs) in roots of clubroot resistant and susceptible plants at 7-, 14- and 21-days post inoculation with *Plasmodiophora brassicae* to identify the proteins to be involved in clubroot resistance in canola. They reported several proteins involved in calcium dependent signaling pathways or related to reactive oxygen species (ROS), dehydrins, lignin, thaumatin, and phytohormones, and this included 73 DAPs which are found to be orthologous to clubroot resistance proteins from eight clubroot resistance loci including the A3 and A8 chromosome loci where the major clubroot resistance genes are located. Wang et al. (2022) reported 760 DAPs related to anther development, oxidative phosphorylation, and programmed cell death in a cytoplasmic male sterile *B. napus* line; among these seven could also be detected using transcriptome analysis and might play a role in male sterility. Other proteome studies in *B. napus* includes understanding the genetic control of seed germination (Gu et al., 2016), arsenic toxicity effects (Farooq et al., 2021), drought stress response (Koh et al., 2015), salt stress response (Shu et al., 2022), and pollen germination (Sheoran et al., 2009).

1.9 Research objectives

The University of Alberta Canola Program has developed *B. napus* NILs for flowering time QTL located on C1 and C9 (Gill, 2021). In addition to this, this program has also developed an advanced generation inbred line population of *B. napus* canola from different *B. napus* × *B. oleracea* interspecific crosses. In these populations, the early flowering allele has been introgressed from *B. oleracea* (Rahman et al. 2011, 2017, 2018; Nikzad et al. 2019, 2023). These NILs and the advanced generation inbred line population were used in this MSc thesis research to understand the effect of the flowering time QTL alleles of these population under a short-day condition, as well as to

understand the molecular basis of flowering time variation. Furthermore, *B. napus* lines collected from various sources were evaluated for days to flowering under a short-day condition to identify the early flowering lines for use in breeding. For this, the following studies were undertaken:

Study I: Investigate the effect of the C1 and C9 flowering time QTL alleles on days to flowering and other morphological traits under a short-day condition.

Study II: Investigate the expression of the genes from C5 and C9 flowering QTL region to identify the putative flowering time genes.

Study III: Decipher the molecular basis of flowering time through proteome analysis of early and late flowering recombinant inbred lines derived from *B. napus* × *B. oleracea* interspecific cross.

Chapter 2. Evaluation of the *Brassica napus* near isogenic lines (NILs) for days to flowering and different morphological traits

2.1 Introduction

Brassica oilseed crop is second biggest oilseed crop in the world (USDA, 2022). In 2022, total canola (*B. napus*) seed production in Canada was 18.17 million metric tonnes (Canola Council of Canada, 2023). It is primarily used for edible oil; however, biodiesel is also produced from this oil (Qian et al., 2013) and its seed meal (a by-product after oil extraction) serves as a source of protein for use in animal feed (Ivanova et al., 2016). The oilseed *B. napus* has been adapted for growing in a wide geographical region through manipulation of its flowering time and growth habit. Three ecotypes of this species, viz. winter, semi-winter, and spring types can be found (Wu et al., 2019). The spring type is grown in Canada under long-day condition. Flowering in this crop is delayed under a short-day condition (Cai et al., 2008).

Flowering time is an important agronomic trait that influences seed production. A vigorous plant may result from a delayed floral induction. In this case, the plant can be harvested in a short crop growing season like in Canada. On the other hand, plants that flower early often lack sufficient energy for growth and development to produce high yield (for review, see Matías-Hernández et al., 2014). According to Habekotté (1997), a crop with the greatest potential for high seed yield could be one that mature late but flower early, i.e. receive a longer grain filling period. Different morphological traits such as days to first flowering, plant height, number and length of primary and secondary branches, number of secondary branches, total number of siliques per plant, number of siliques in main branch, silique length and seed weight play an important role in the formation of seed yield in *B. napus* (Islam et al., 2020). Climate and adaptation to day-length also exerts a considerable effect on flowering time, plant height, and seed yield (Schiessl et al., 2015). Plant

height in canola is an important morphological characteristic. Reduced plant height is an important trait in canola breeding, especially for the development of hybrid cultivars, which frequently are taller (Cuthbert et al., 2009; Rahman et al., 2017). Also, reduced height and robust stalk also improve lodging resistance and harvest index, and allow more effective use of fertilizer, pesticide, and water (for review, see Sun et al., 2016). Furthermore, root and aboveground biomass also play an important role in plant's performance (Kebede and Rahman, 2019). Root is an important feature of the plant which plays a vital role in the uptake of nutrients and water and anchors the plant for mechanical support (Smith and De Smet, 2012).

Among the different morphological traits, earliness of flowering and maturity are one of the most important traits for growing spring canola in North America (Rahman et al., 2018). Days to flowering is controlled by multiple gene loci (Raman et al., 2013; Nikzad et al., 2023; Rahman et al., 2017; Rahman et al., 2018) and the trait often correlates well with days to maturity (Nikzad et al., 2019). Different mapping population have been used to identify QTL affecting a trait, and this includes recombinant inbred lines (Yu et al., 2019), doubled haploid (DH) lines (Li et al., 2018; Rahman et al., 2018; Raman et al. 2013), and near isogenic lines (NILs) (Cai et al., 2022). Among these, NILs carry a common genetic background but differ for only a small genomic region or for the alleles of the gene of interest. This makes NILs an excellent material for investigating the effect of a gene on the trait as well as to understand the genetic and molecular basis of the trait (Castro et al., 2010; Mia et al., 2019; Wang et al., 2019). NILs have been used in various studies, such as to fine map flowering time QTL in *B. napus* (Cai et al., 2022), *Fusarium* crown rot resistance in barley (Gao et al., 2019), major-effect QTL for fruit shape in cucumber (Pan et al., 2022), tiller inhibition gene in wheat (Wang et al., 2022), grain length gene in rice (Chen et al., 2023), and grey leaf spot resistance in maize (Benson et al., 2015).

The objective of this study was to evaluate two sets of *B. napus* NILs differing for days to flowering QTL alleles of C1 and C9 chromosome to understand the effect of these alleles on morphological traits under a short-day (10 hr photoperiod) condition.

2.2 Materials and method

2.2.1 Plant material

Two sets of NILs were used in this study, and they were developed for the flowering time QTL located on C1 and C9 chromosomes where the early-flowering QTL alleles have been introgressed into a *B. napus* from *B. oleracea*. The C1 and C9 NILs were developed through recurrent backcrossing using the spring canola cv. Hi-Q as recurrent parent and the early flowering canola line RIL-144, carrying the *B. oleracea* alleles, as the donor parent. Thus, the NILs were developed in the genetic background of Hi-Q, and they carried either Hi-Q allele or *B. oleracea* allele. To develop the NILs, marker assisted selection for QTL marker allele was carried out in each generation, and the NILs were developed after four backcrossing followed by two times of self-pollination (BC_4F_3) (Gill, 2021). The following NIL families (self-pollinated single plants) were used in this thesis research: 10 NIL families carrying the C1 QTL allele of Hi-Q which are designated as 'HiQ-HiQ:C1', seven NIL families carrying the C1 QTL allele of RIL-144 which are designated as 'HiQ-Ole:C1', eight NIL families carrying the C9 QTL allele of Hi-Q which are designated as 'HiQ-HiQ:C9', and one NIL family carrying the C9 QTL allele of RIL-144 which is designated as 'HiQ-Ole:C9'.

2.2.2 Phenotypic evaluation of the NILs

Three to five plants of each NIL families were grown in a growth chamber together with the parents Hi-Q and RIL-144 as checks, and average value of the plants was used for statistical analysis. The experiment was repeated four times which constituted four replications. The

following environmental conditions were maintained in the growth chamber: constant temperature of 20 ℃ throughout the day and night, and 10 hr photoperiod with light intensity of 450 µE (mV) m^{-2} s^{-1}. The NILs were grown in 18-cells trays (7 cm × 7 cm × 7 cm, L × W × D) filled with Sunshine Professional Growing Mix (Sun Gro Horticulture Canada Ltd, Seba Beach, Canada). The following phenotypic data were collected: Leaf color, leaf shape, leaf margin indentation, presence or absence of leaf lobes, number of lobes, plant height at first flowering, days to first flowering, and aboveground and root biomass. All these traits were recorded when the plants were at first open flower stage. Leaf characteristics were recorded visually following Canola Test Guideline (Version 2009/05, https://www.upov.int/fr/publications/tg-rom/tg036/tg_36_6_corr.pdf) using the following scale: Leaf margin, 1 to 3 scale where 1 = undulating, 2 = round and 3 = sharp; Leaf color, 1 to 4 scale where 1 = light green, 2 = medium green, 3 = dark green, 4 = blue green; and Leaf lobe, 1 to 2 scale where 1 = absent and 2 = present. Count for the number of leaf lobes was based on projection of the leaf blade with gaps between them. Plant height was measured in cm from the ground to the tip of the plant. For biomass, the plants were cut at the cotyledonary leaf point visible at the base of the stem and was used for aboveground biomass, and the plant parts below the cotyledonary leaf point was used for root biomass. Running tap water was used to wash roots until soil and other debris were removed from all primary, secondary, and tertiary roots. The aboveground plant parts and the washed root parts were placed in paper bags and dried in an oven for eight weeks at 50 ℃ temperature for dry weight (g).

2.3 Statistical Analysis

Analysis of variance (ANOVA) was performed with software program ‘R’ (R studio team R, 2021) where lmer function of ‘lme4’ package was used to fit a fixed effect model for each trait. Least square mean (LSmean) values were calculated by using ‘lsmeans’ package of R and for

comparing the mean values for significant difference Tukey's test was used. Pearson's correlation coefficients and principal component analysis were calculated using R.

2.4 Results

2.4.1 Correlation between the four replications for different morphological traits

Coefficient of correlation (r) between the four replications for days to flowering, plant height, root biomass, and shoot biomass data varied from 0.86 to 0.92, 0.74 to 0.90, 0.79 to 0.87, and 0.52 to 0.86, respectively (Figure 2-1). These values were positive and highly significant ($p < 0.001$) suggesting that the NILs behaved consistently for these traits across the four replications.

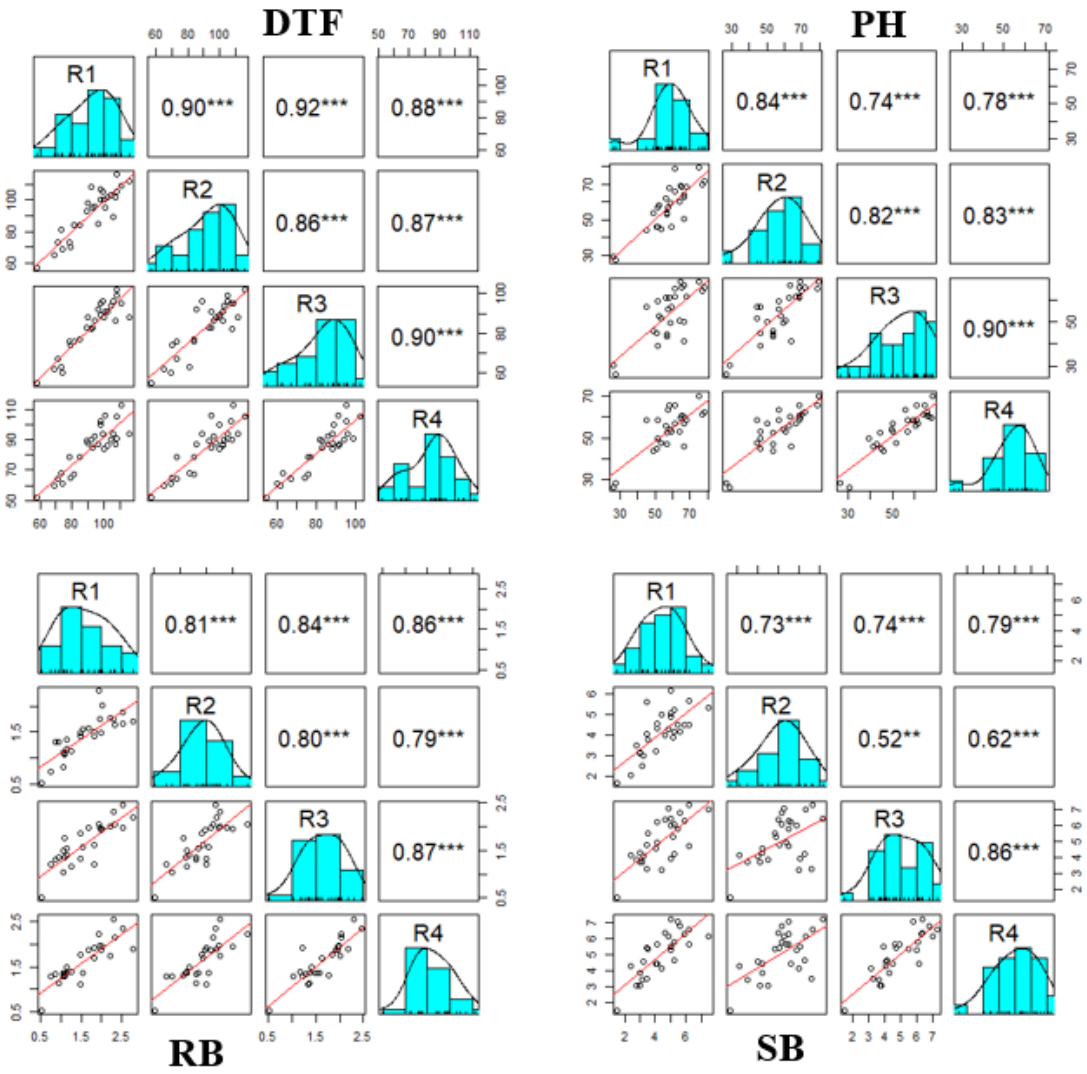

Figure 2-1. Correlation between the four replications of the *Brassica napus* near isogenic lines (NILs) for days to flowering (DTF), plant height (PH), shoot biomass (SB) and root biomass (RB). The experiments were conducted in a growth chamber at 10 hr photoperiod and 20 °C constant temperature. R1, R2, R3 and R4 indicates the four replications

2.4.2 Analysis of variance and comparison among NILs

The leaves of the recurrent parent Hi-Q were lobeless, while the donor parent RIL-144 had about seven lobes. The leaf characteristics of all NILs was almost similar to the recurrent parent Hi-Q, as could be expected. No visible difference between the two parents was found for leaf color and leaf margin indentation, and this also reflected in the NIL families (Figure 2-2).

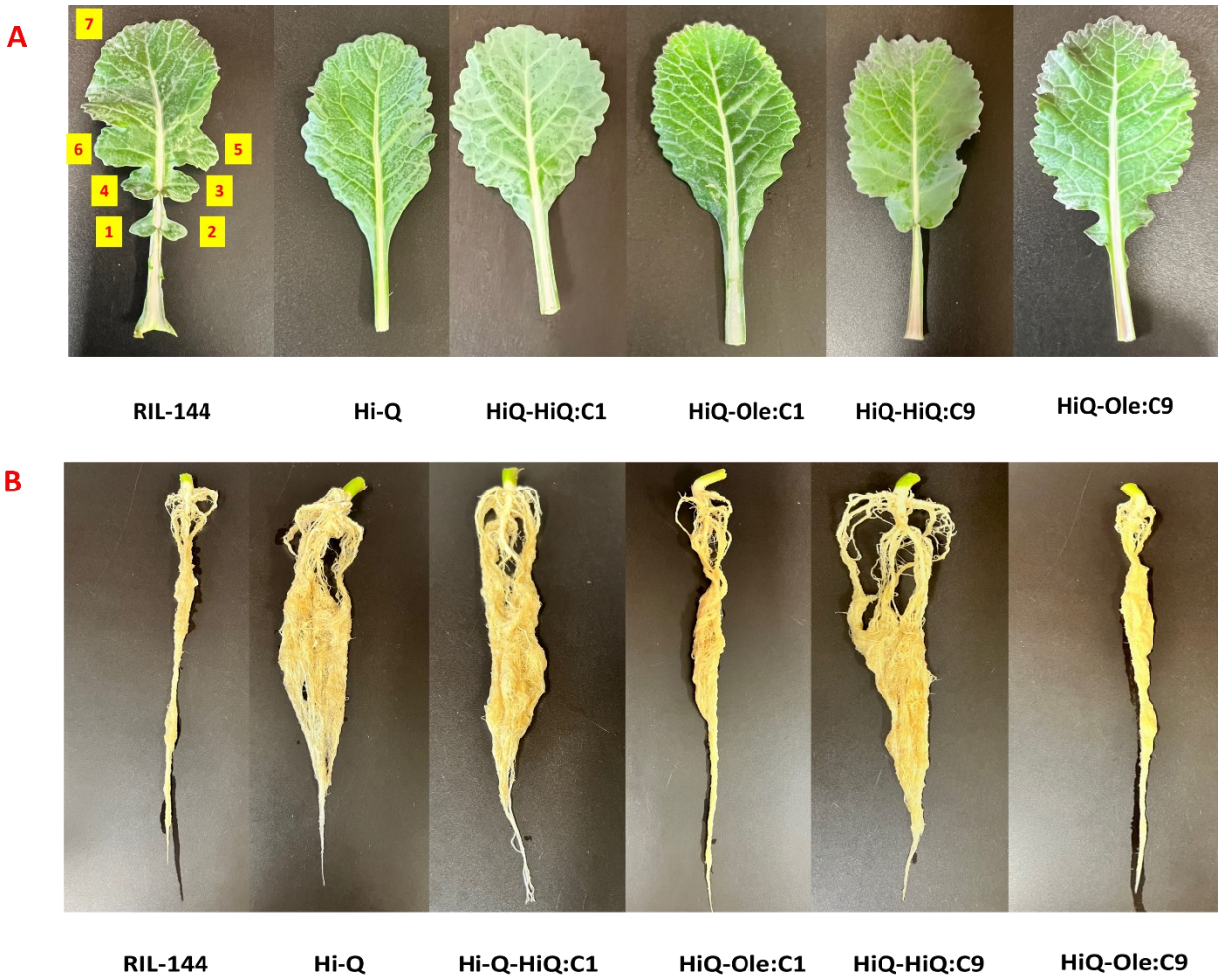

Figure 2-2. Representative (A) leaf and (B) root samples of the *Brassica napus* recurrent parent Hi-Q and the donor parent RIL-144, and the near-isogenic lines (NILs) carrying C1 allele of Hi-Q (HiQ-HiQ:C1) or RIL144 (HiQ-Ole:C1), and the NILs carrying C9 allele of Hi-Q (HiQ-HiQ:C9) or RIL144 (HiQ-Ole:C9) in genetic background of Hi-Q

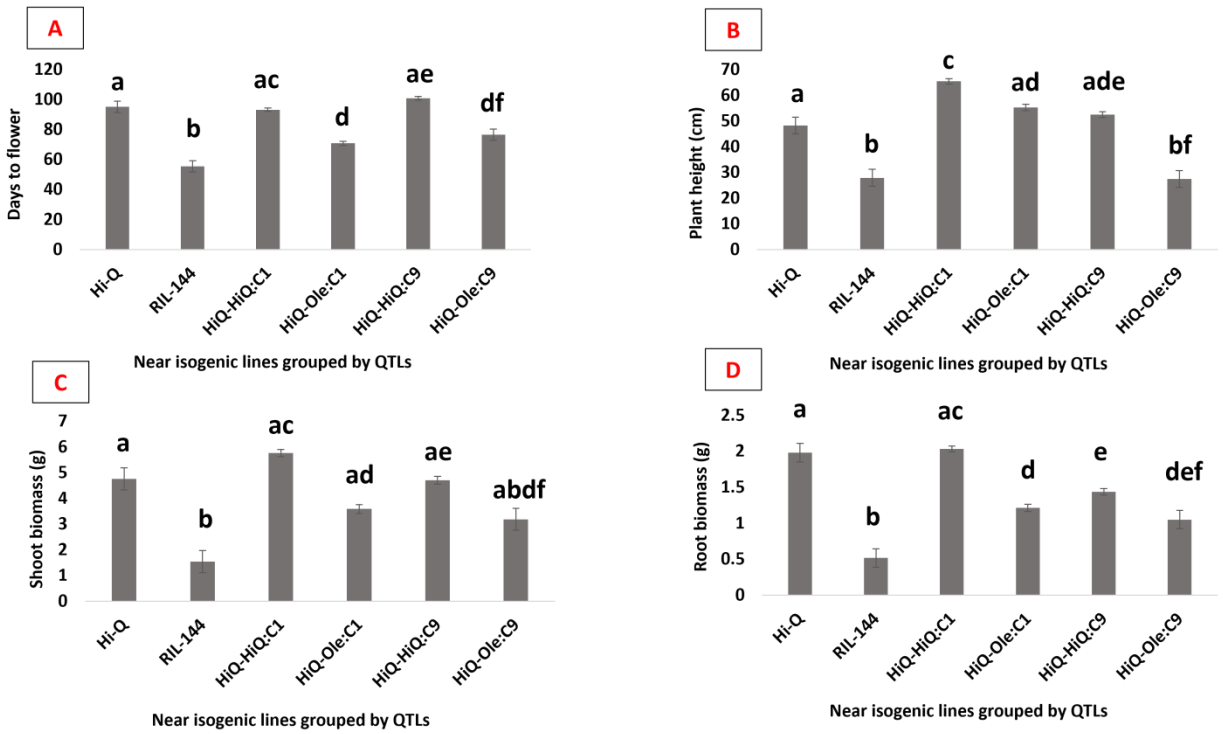

Figure 2-3. Least square mean and S.E. (±) of the four groups of *Brassica napus* near-isogenic lines (NILs) carrying C1 allele of Hi-Q (HiQ-HiQ:C1) or RIL-144 (HiQ-Ole:C1), and the NILs carrying C9 allele of Hi-Q (HiQ-HiQ:C9) or RIL-144 (HiQ-Ole:C9) in the genetic background of Hi-Q, and the recurrent Hi-Q and the donor parent RIL-144 for days to flowering (A), plant height (B), shoot biomass (C) and root biomass (D). The populations were grown at 10 photoperiod and 20 °C constant temperature conditions

Analysis of variance showed the presence of significant variation between the NILs carrying Hi-Q or RIL-144 alleles of C1 or C9 for all morphological traits, viz. days to flowering, plant height, shoot biomass, and root biomass) (Appendix 2-1). Among the two parents, the recurrent parent Hi-Q flowered significantly later ($t = 7.58$, $p < 0.0001$) and was taller ($t = 4.39$, $p < 0.0004$), and also produced significantly greater amount of shoot ($t = 5.30$, $p < 0.0001$) and root ($t = 8.03$, $p < 0.0001$) biomass than the donor parent RIL-144.

In case of the C1 NILs, significant difference was found between the NILs carrying the Hi-Q (HiQ-HiQ:C1) or RIL-144 allele (HiQ-Ole:C1) for days to flowering ($t = 11.95$, $p < 0.0001$), plant height ($t = 6.13$, $p < 0.0001$), shoot biomass ($t = 10.03$, $p < 0.0001$) and root biomass ($t =$

12.54, p <0.0001). NILs carrying the RIL144 allele (HiQ-Ole:C1) flowered significantly earlier than the NILs carrying Hi-Q allele (HiQ-HiQ:C1) (70.8 ± 1.45 vs. 93.2 ± 1.19 days); however, they flowered later than the early flowering parent RIL-144 (55.5 ± 3.7 days) (Figure 2-3A & Appendix 2-2). Plant height of both groups of C1 NILs was close to the recurrent parent Hi-Q, as expected for the NILs developed for flowering time gene (Figure 2-3B and Appendix 2-2). The HiQ-Ole:C1 NILs produced lower amounts of shoot and root biomass than the recurrent parent Hi-Q; however, the difference was statistically significant (t = 5.53, p <0.0001) only for root biomass (1.214 ± 0.0505 g vs. 1.98±0.1287 g) (Figure 2-3C and Figure 2-3D; Appendix 2-2).

In case of the C9 QTL, the NILs carrying RIL-144 allele (HiQ-Ole:C9) flowered significantly (t = 6.21, p <0.0001) earlier (76.5 ± 3.7 days) than the NILs carrying the Hi-Q allele (HiQ-HiQ:C9) (100.9 ± 1.31 days) as well as the recurrent parent Hi-Q (t = 3.57, p < 0.006) (95.2 ± 3.7 days); however, flowering of the HiQ-Ole:C9 NILs was significantly later than the early flowering donor parent RIL-144 (55.5 ± 3.7 days) (Figure 2-3A and Appendix 2-1). Plant height of the HiQ-HiQ:C9 NILs was not significantly different (t = -1.2, p <0.804) from the recurrent parent Hi-Q; however, height of the HiQ-Ole:C9 NILs was significantly shorter than Hi-Q (t = 4.5, p < 0.0003) and was similar to the height of the donor parent RIL-144 (Figure 2-3B). The two types of NILs (HiQ-HiQ:C9 and HiQ-Ole:C9) were not significantly different from each other for both shoot and root biomass (Figure 2-3C and Figure 2-3D; Appendix 2-2). When compared with the recurrent parent, they were not significantly different from Hi-Q for shoot biomass; but were different for root biomass (t = 3.98, p < 0.0017 for Hi-Q vs. HiQ-HiQ:C9 and t = 5.10, p <0.0001 for Hi-Q vs. HiQ-Ole:C9).

2.4.3 Principal component analysis

PCA explained a total of 82.36 % of the total variation, where the PC1 explained 65.63 % and PC2 explained 16.73 % of the variation. The first PC separated the NILs carrying the flowering allele of Hi-Q or RIL-144 as well as the two parents into separate groups (Figure 2-4 & Appendix 2-3). On the other hand, PC2 separated only the HiQ-Ole:C1 and HiQ-Ole:C9 NILs (Figure 2-4 and Appendix 2-3).

The length of the vector for days to flower was relatively longer as compared to the other traits, indicating that this trait was the major factor differentiating the NILs. The vectors for shoot and root biomass had an angle close to zero ($r = 0.78$, $p < 0.001$) suggesting that these two traits exhibited similar variation. The trait pairs, days to flower and shoot biomass ($r = 0.46$, $p < 0.001$), days to flower and root biomass ($r = 0.46$, $p < 0.001$) and days to flower and plant height ($r = 0.36$, $p < 0.001$) had an acute angle (<90◦) suggesting that their variation was positively correlated (Figure 2-4 and Figure 2-5).

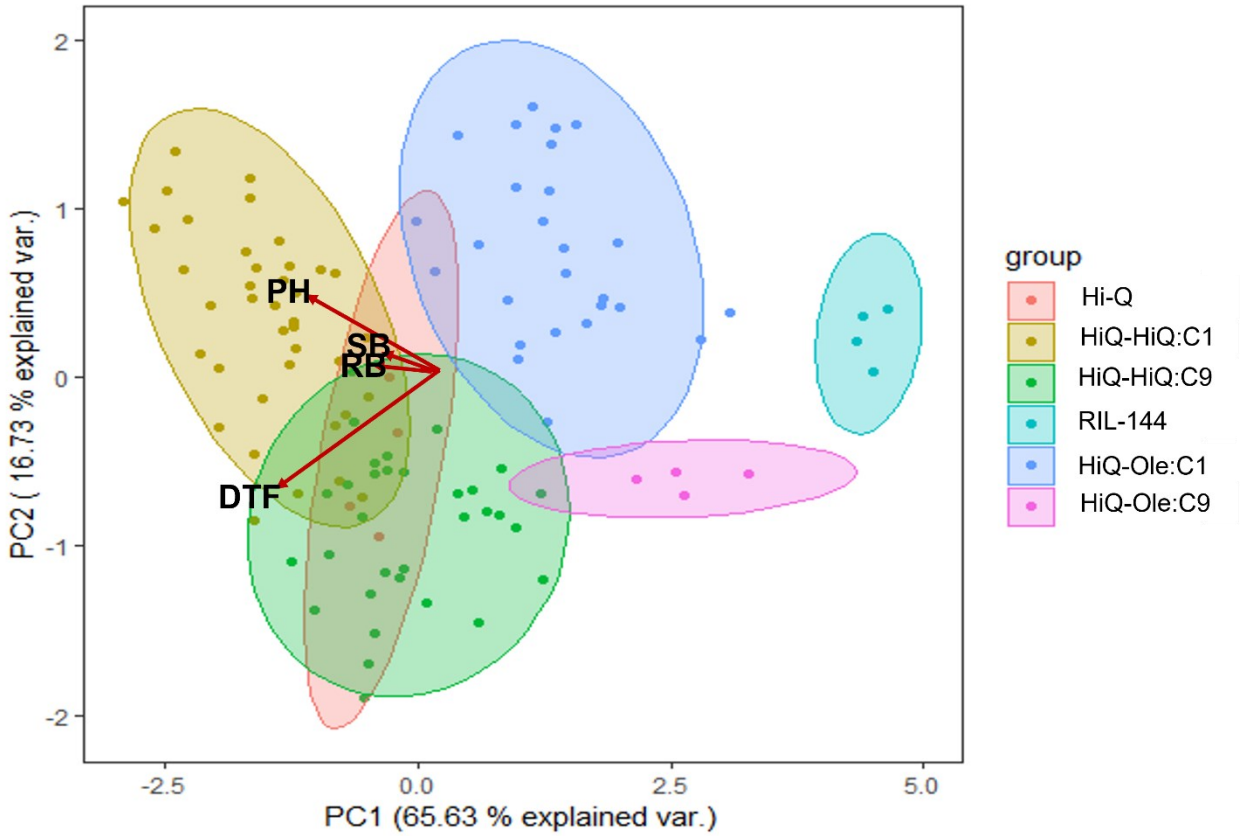

Figure 2-4. Principal component analysis biplot of the *Brassica napus* near isogenic lines (NILs), developed through crossing and backcrossing of the spring canola cv. Hi-Q as the recurrent parent and the early flowering line RIL-144 as the donor parent, illustrating the distribution of the NILs

characterized by different traits in the space of the two principal components (PCs). DTF = days to flower PH = plant height; SB = shoot biomass; RB = root biomass

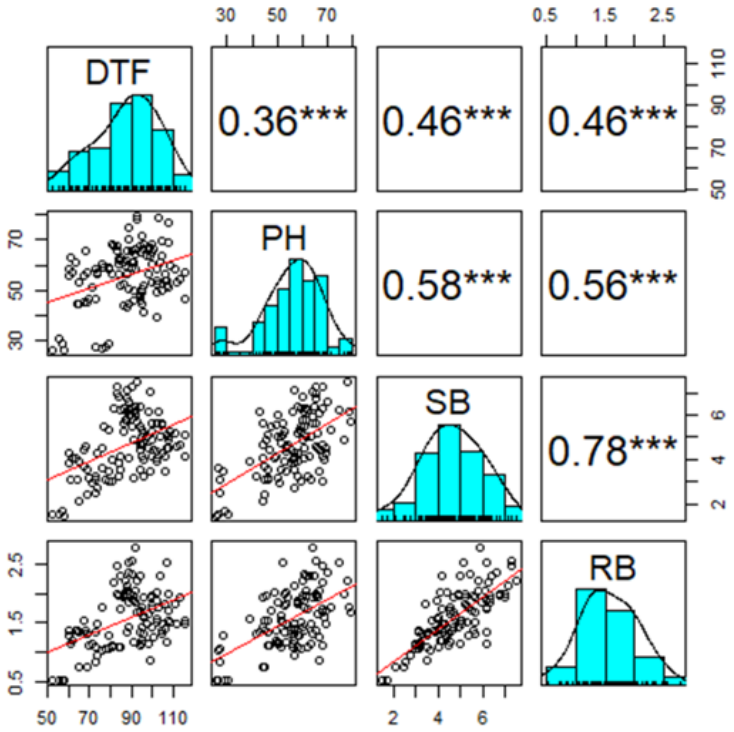

Figure 2-5. Correlation between days to flowering (DTF), plant height (PH), shoot biomass (SB), and root biomass (RB) in the *Brassica napus* near isogenic lines (NILs) carrying flowering time QTL alleles of C1 or C9 chromosome. The experiments were conducted in a growth chamber at 10 photoperiod and 20 °C constant temperature throughout.

2.5 Discussion

This research is focused on understanding the effect of flowering time on other morphological traits that contribute to crop productivity. The effect of a gene or QTL can be better understood by using NILs for the gene or QTL. In this study, two NIL populations differing for QTL alleles of C1 and C9 chromosome were used to evaluate for days to flowering and other morphological traits such as leaf characteristics, plant height, and root and shoot biomass. The NILs were developed through recurrent backcrossing the F_1's to Hi-Q four times followed by self-pollination for two generations. At this stage, it was expected that NILs would carry 96.87 % of

the Hi-Q genome. Therefore, they were expected to differ mainly for days to flowering. Indeed, the NILs differed for days to flowering under short day conditions, as expected. Also, no visible difference between the NILs could be found for leaf color, margin and lobes when compared with Hi-Q. However, these NILs showed significant difference for plant height, and root and shoot biomass under short day photoperiod; for example, the NILs carrying early flowering allele of RIL-144 showed reduced plant height as compared to NILs carrying the late flowering allele of Hi-Q. It was reported by Schiessl et al. (2015) that some of the genomic regions affecting flowering time can also affect plant height, and this agrees with the results from this study.

By using a doubled haploid mapping population derived from a cross between RIL-144 and Hi-Q and growing under different photoperiod (10, 14, 16 and 18 hour) conditions, Kebede and Rahman (2019) found a significant positive correlation between root biomass and days to flowering which indicates that the flowering time QTL allele also influences the root biomass. In this study, the NILs carrying the Hi-Q allele, viz. HiQ-Ole:C1 and HiQ-Ole:C9, also showed an increased root biomass as compared to the NILs carrying the RIL-144 allele. Hammond et al. (2009) identified one QTL on C1 affecting shoot dry matter in *B. oleracea*; however, physical position of this QTL cannot be found in literature to compare with the position of the C1 flowering time allele.

In conclusion, it is apparent that flowering time QTL influence some other traits like plant height and plant biomass, which could be due to co-localization of the QTL affecting these traits or pleotropic effect of the flowering time QTL on the other traits. Fine mapping of these QTL regions needs to be done to get a better understanding of the flowering time as well as the above-mentioned traits.

Chapter 3. Gene expression analysis of the putative flowering genes from the C5 and C9 QTL region of spring *Brassica napus*

3.1 Introduction

Brassica napus L., generally known as canola, rapeseed, or oilseed rape, second most important source of vegetable oil in the world after soybean (USDA, 2022). It provides not only oil for human food but also protein-rich meal for use in animal feed, as well as renewable material for biodiesel production and industrial use (for review, see Raboanatahiry et al., 2021). *Brassica napus* is an allotetraploid species that evolved about 7500 years ago from hybridization between *B. rapa* and *B. oleracea* (Chalhoub et al., 2014).

Flowering time is one of the most important traits that play a role in crop evolution, domestication, and local adaptation, and the trait in canola is controlled by a variety of genes and influenced by environment (Raman et al., 2013). Based on the need of vernalization (exposure to less than 4 ºC) for flowering, *B. napus* canola can be grouped into winter, spring, and semi winter types (Shah et al., 2018). The spring type does not require vernalization and takes about 4-5 months from seeding to harvest in North America and Europe, but up to 6-7 months in subtropical regions like Australia (reviewed in, Rahman et al., 2018). The winter types are mostly cultivated in Central and Northern Europe and this type require about eight weeks of vernalization for flowering (Shah et al., 2018) whereas the semi-winter types grown in China need exposure to low temperature for a shorter period (about four weeks) (reviewed in, Sun et al., 2017, Shah et al., 2018). According to Habekotté (1997), early flowering, longer grain-filling period, and high leaf area index make a significant contribution to high seed yield in oilseed *B. napus*.

The most effective method of illuminating the genetic underpinnings of a quantitative trait is quantitative trait loci (QTL) mapping (Wang et al., 2016). Several researchers have developed genetic linkage maps to identify the key QTL and locate the genes involved in the control of a trait in oilseed *B. napus*. To date, several flowering time QTL have been identified in *B. napus* by different researchers (e.g. Mei et al., 2009; Tang et al., 2023; Nikzad et al., 2023; Raman et al., 2013; Rahman et al., 2018; Liu et al., 2016; Chen et al., 2010; Long et al., 2007). However, the regulatory mechanisms (pathways) that affect flowering time have been extensively investigated in the model plant *A. thaliana* (Zhang et al., 2022; Kyung et al., 2022, Kim et al., 2022; Du et al., 2020; Del Olmo et al., 2019), and relatively fewer studies carried out in *B. napus* (Matar et al., 2021; Li et al., 2018; Nelson et al., 2014). *Brassica* and *A. thaliana* are both cruciferous plants and closely related; therefore, the knowledge of a gene gained from *Arabidopsis* can be used to understand the gene in *Brassica* (Schiessl et al., 2017). Theoretically, for each of the *Arabidopsis* gene, three copies can be found in the *Brassica* genome (Lagercrantz, 1998). The presence of multiple copies of a gene in a plant can result in functional differences among the homologous copies, and this can increase the complexity of the regulatory networks of the trait, such as flowering time (Li et al., 2018). Years of research have uncovered several fascinating pathways that regulate the timing of floral induction in plants. One such mechanism is the perception of day length in leaves, which results in the production of a mobile signal called florigen that encourages the induction of flowering at the shoot apical meristem (for review, see Amasino et al., 2010). In this, the flowering time gene *CONSTANS* (*CO*) play a central role where it upregulates the expression of *FT (FLOWERING LOCUS T)* and *SOC1* (*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*) genes; activation of these two genes occur in the leaf, and FT protein transports to shoot apical meristem where it interacts with FD (bZIP transcription factor) and form FT-FD

dimer which activates *SOC1* and several other floral meristem identity genes, such as *LFY (LEAFY), AP1 (APETALA1)*, *FUL* (*FRUITFULL*) and *SEP3* (*SEPALLATA3*), in shoot apical meristem and promote flowering (Collani et al., 2019; Kim, 2020; Kinoshita and Richte, 2020). Genes like *CO (CONSTANS), GI (GIGANTIA), FKF1 (FLAVIN-BINDING, KELCH REPEAT, F-BOX), FT (FLOWERING LOCUS T), CDF1 (CYCLING DOF FACTOR 1), COP1 (CONSTITUTIVE PHOTOMORPHOGENIC 1), SPA (SUPRESSOR OF PHYA 105), CRY1 (CRYPTOCHROME 1), CRY2 (CRYPTOCHOME 2), PHYA (PHYTOCHROME A), PHYB (PHYTOCHROME B), TOE1 (TARGET OF EAT1), TOE2 (TARGET OF EAT2), TEM1 (TEMPRANILLO 1)* and *AP2 (APETALA 2)* are mostly involved in photoperiod pathway. *GI* and *FKF1* interact with each other to prevent the degradation of *CO* by *CDF1* and make it more stable. *CRY1*, *CRY2* and *PHYA* also stabilize the *CO* by inhibiting the physical interaction of *COP1*/*SPA* with *CO*. On the other hand, *PHYB* destabilizes the *CO* causing late flowering through ubiquitination. *TOE1*, *TOE2*, *TEM1* and *TEM2* act as a repressor of *FT* expression (For review see, Kim, 2020). *AP2,* another floral repressor gene, delays flowering by repressing the expression of *FT* and *SOC1* (Yant et al., 2010).

In a research project carried out in the Canola Breeding-research Program of the University of Alberta, an advanced generation inbred *B. napus* population was developed from six different *B. napus* × *B. oleracea* interspecific crosses; many of these *B. napus* lines flowered earlier than the *B. napus* parent (Nikzad et al., 2019). Based on flowering data from 10 field trials, Nikzad et al. (2023) identified a QTL on C5 affecting flowering time where the allele introgressed from *B. oleracea* contributed to earliness. By using the same population, Gill (2021) identified two QTL, one on C5 and the other on C9, affecting flowering time under a short-day condition, where the *B. oleracea* alleles improved the earliness; in this regard, these two QTL are unique in *B. napus*.

Therefore, investigation of these two QTL at a molecular level will not only extend our knowledge on this trait but will also identify the candidate genes involved in flowering, and this knowledge can be used to develop gene-based molecular markers for use in breeding. Thus, the objective of this study was to investigate the expression of the putative flowering time genes from these two QTL regions for their involvement in flowering under a short-day condition.

3.2 Materials and method

3.2.1 Plant material and growing conditions

As mentioned above, Nikzad et al. (2019, 2023) developed an advanced generation inbred *B. napus* population from six different *B. napus* × *B. oleracea* interspecific crosses, where a some of the lines of *B. napus* × *B. oleracea* var. *capitata cv*. Bindsachsener cross flowered earlier than the *B. napus* parent under a 10 hr photoperiod condition (Gill 2021). Seven early-flowering and seven late flowering lines of this interspecific cross were used in the present study. Trays containing 32- cell with pot size of 7 cm × 7 cm × 9 cm (L × W × D) filled with Sunshine Professional Growing Mix (Sunshine Horticulture, 15831 N.E. Bellevue, USA) were used for growing plants and were placed in a growth chamber set at 10 hr photoperiod, 20 ℃ constant temperature, and light intensity of 450 μE (mV) m^{-2} s^{-1}. Nine plants of each line were grown; the plants were monitored and watered daily, and fertilization was done once a week.

3.2.2 Sample collection for quantitative real-time PCR (qRT-PCR)

Two types of samples, leaf at rosette stage and shoot apex at bolting stage, were collected from the early and late flowering lines in bulks in the morning (fours hours after turning on the lights) and at night (four hours after turning off the lights), where each bulk included three plants of each line, i.e. 7 × 3 = 21 plants. The experiment included three biological replicates with two

technical replicates for leaves and three biological replicates without any technical replication for shoot apex. Thus, the total number of samples (bulks) for qRT-PCR analysis was 2 types of lines (early and late) × 2 times points (morning and night) × 3 biological replicates × 2 technical replicates = 24 for leaf, and 2 types of lines × 2 times points × 3 biological replicates = 12 for shoot apex.

3.2.3 Identification of putative flowering genes from the QTL regions and qRT-PCR

Arabidopsis flowering gene orthologues in *Brassica* were identified using the BRAD database (http://www.brassicadb.cn/#/FlowerGene/) (Chen et al., 2022). Sequences of these genes were retrieved (13 February 2023) and blast searched in EnsemblPlants (https://plants.ensembl.org/Multi/Tools/Blast) to identify the flowering genes in the *B. napus* genome. From this list, genes from C5 and C9 chromosome were retained, and their locations and sequences were obtained from GENOSCOPE (https://www.genoscope.cns.fr/brassicanapus/). Based on this, 15 putative flowering genes from each of the C5 and C9 QTL regions to be involved in photoperiod and autonomous pathway were selected for qRT-PCR analysis.

Gene-specific primer pairs for the 15 genes from about 22-39 Mb region of C5 and 15 genes from about 24-40 Mb region of C9 were designed using Primer3Plus v3.3 (Untergasser et al., 2007); the list of the primers presented in Appendix 3-1. For endogenous control, housekeeping gene Ubiquitin-Conjugating Enzyme 10 (*UBC10*) from *B. napus* was used. The $\mathbf{2}^{-\Delta\Delta Ct}$ method was used for calculating relative expression level of each gene (Livak and Schmittgen, 2001).

The above-mentioned 24 bulks of leaf and 12 bulks of shoot apex samples were used for qRT-PCR analysis. For this, samples were stored at -80 °C for RNA extraction, and total RNA of the samples was extracted using QIAGEN RNeasy® plant mini kit (QIAGEN, Inc., Valencia, CA)

and DNase treatment was performed out using DNase I, RNase-free kit in accordance with manufacturer's instructions (ThermoFisher Scientific, USA). Concentration of the RNAs was measured using a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific, USA) and cDNA synthesis was carried out using RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, USA) following manufacturer's guidelines. The qRT-PCR was performed on a StepOne Plus real-time PCR system (Life Technologies, Burlington, Canada) with the use of FASTSYBR Green mix from Applied Biosciences (ThermoFisher Scientific, USA).

3.3 Statistical analysis

Two-way ANOVA followed by Fishers's LSD multiple comparisons test was carried out using GraphPad Prism version 9.5.1 for Windows, GraphPad Software, Boston, Massachusetts USA (https://www.graphpad.com/).

3.4 Results

A total of 151 genes to be involved in flowering were identified from the C5 and C9 chromosomes. This included 7 floral meristem identity genes, 12 floral integrator genes, 69 genes to be involved in photoperiod pathway, 8 genes to be involved in autonomous pathway, 24 genes to be involved gibberellin pathway, 18 genes to be involved in vernalization pathway, three temperature-responsive genes, one aging–related gene, and five genes to be involved in gibberellin, autonomous and photoperiod pathway, one gene to be involved in photoperiod and vernalization pathway and three genes to be involved in photoperiod and gibberellin pathway (Table 3-1). The details of these genes presented in Appendix 3-2.

Table 3-1. List of flowering genes and their copy number found in *Brassica napus* chromosome C5 and C9 from BLAST search

Pathway/types of gene	Gene	Chromosome (copy number of gene)	Total No. copies
Floral meristem identity	*AP1*	C5 (2), C9 (2)	4
Floral meristem identity	*LFY*	C9	3
Floral integrator	*FT*	C5	1
Floral integrator	*SOC1*	C5 (3), C9 (4)	7
Floral integrator	*AGL24*	C5 (3), C9 (1)	4
Photoperiod	*AP2*	C5 (2), C9 (4)	6
Photoperiod	*CO*	C5 (1), C9 (4)	5
Photoperiod	*CDF1*	C5	4
Photoperiod	*COP1*	C9	1
Photoperiod	*CRY1*	C9	1
Photoperiod	*CRY2*	C9	1
Photoperiod	*DNF*	C5	2
Photoperiod	*FKF1*	C9	1
Photoperiod	*GI*	C5 (2), C9 (1)	3
Photoperiod	*PHYA*	C5 (3), C9 (1)	4
Photoperiod	*PHYB*	C5 (4), C9 (2)	6
Photoperiod	*SPA*	C5 (7), C9 (3)	10
Photoperiod	*SPY*	C5 (1), C9 (6)	7
Photoperiod	*TEM1*	C5 (2), C9 (2)	4
Photoperiod	*TEM2*	C5 (2), C9 (2)	4
Photoperiod	*TOE1*	C9	4
Photoperiod	*TOE2*	C5 (1), C9 (2)	3
Photoperiod	*TPS1*	C5 (1), C9 (2)	3
Autonomous	*CSTF64*	C9	3
Autonomous	*CSTF77*	C5 (1), C9 (1)	2
Autonomous	*FVE*	C5	1
Autonomous	*FY*	C5	2
Gibberellin	*GAI*	C5 (4), C9 (3)	7
Gibberellin	*GID1A*	C5 (1), C9 (1)	2
Gibberellin	*GID1B*	C5 (1), C9 (1)	2
Gibberellin	*GID1C*	C5 (1), C9 (1)	2
Gibberellin	*GNC*	C5	3
Gibberellin	*GNL*	C9	2
Gibberellin	*RGA*	C5 (4), C9 (2)	6
Vernalization	*FLC*	C5 (2), C9 (2)	4
Vernalization	*FRI*	C9	1
Vernalization	*VRN1*	C5	5
Vernalization	*VRN2*	C5 (1), C9 (2)	3

Pathway/types of gene	Gene	Chromosome (copy number of gene)	Total No. copies
Vernalization	*VIN3*	C5	2
Vernalization	*LHP1*	C5 (1), C9 (2)	3
Age	*PRC2 (CLF)*	C9	1
Temperature	*SVP*	C5	3
Gibberellin, autonomous, photoperiod	*GA1*	C5 (1), C9 (4)	5
Photoperiod, vernalization	*SMZ*	C5	1
Photoperiod, Gibberellin	*SPL*	C5 (2), C9 (1)	3
Total			**151**

3.4.1 Expression of the genes from C5 QTL

Of the 15 flowering genes from the C5 QTL region, seven showed differential expression in leaf and shoot apex of the early and late flowering bulks at night, and this included *CO (BnaC05g22130D), FT (BnaC05g25360D), PHYA (BnaC05g45140D), AP2 (BnaC05g23110D), SPA (BnaC05g30140D), SPA (BnaC05g38270D)* and *TOE2 (BnaC05g32750D)* (Figure 3-1A). Differential expression of *CO* and *PHYA* could not be detected in morning; however, expression of the remaining five genes could be detected at this time point. At night, the *CO* showed a significantly greater expression in both leaf and shoot apex of the early flowering bulk (1.51-fold in leaf; 1.42-fold in shoot apex) as compared to the late flowering bulk (0.65-fold in leaf; 0.77-fold in shoot apex), whereas the *PHYA* showed a greater expression only in leaf of the early bulk (8.95-fold) as compared to the late bulk (1.70-fold) (Figure 3-1A). The remaining five genes (*FT*, *AP2*, two *SPA* and *TOE2*) showed an opposite pattern of expression at night as compared to their expression in morning (Figure 3-1A and 3-1B). Among these, *FT* showed a greater expression in shoot of the early flowering bulk as compared to the late flowering bulk at night; however, its expression in morning was significantly greater in both leaf and shoot of the late flowering bulk (leaf: 29.6-fold vs. 4.15-fold; shoot: 4.6- vs. 1.4-fold). *AP2*, on average, also showed a greater

expression in morning as compared to night. In the morning, this gene showed a significantly greater expression in both leaf and shoot of the late flowering bulk than the early flowering bulk (Figure 3-1A and 3-1B). On the other hand, this gene showed a greater expression in both tissues of the early flowering bulk at night. Expression of the two *SPAs* and *TOE2* was quite similar in both early and late flowering bulks (Figure 3-1A and 3-1B). These three genes showed a relatively greater expression in leaf as compared to shoot at night (Figure 3-1A), while a greater expression in shoot as compared to leaf in morning (Figure 3-1B); however, no significant difference between the early and late flowering bulks could be found for these genes.

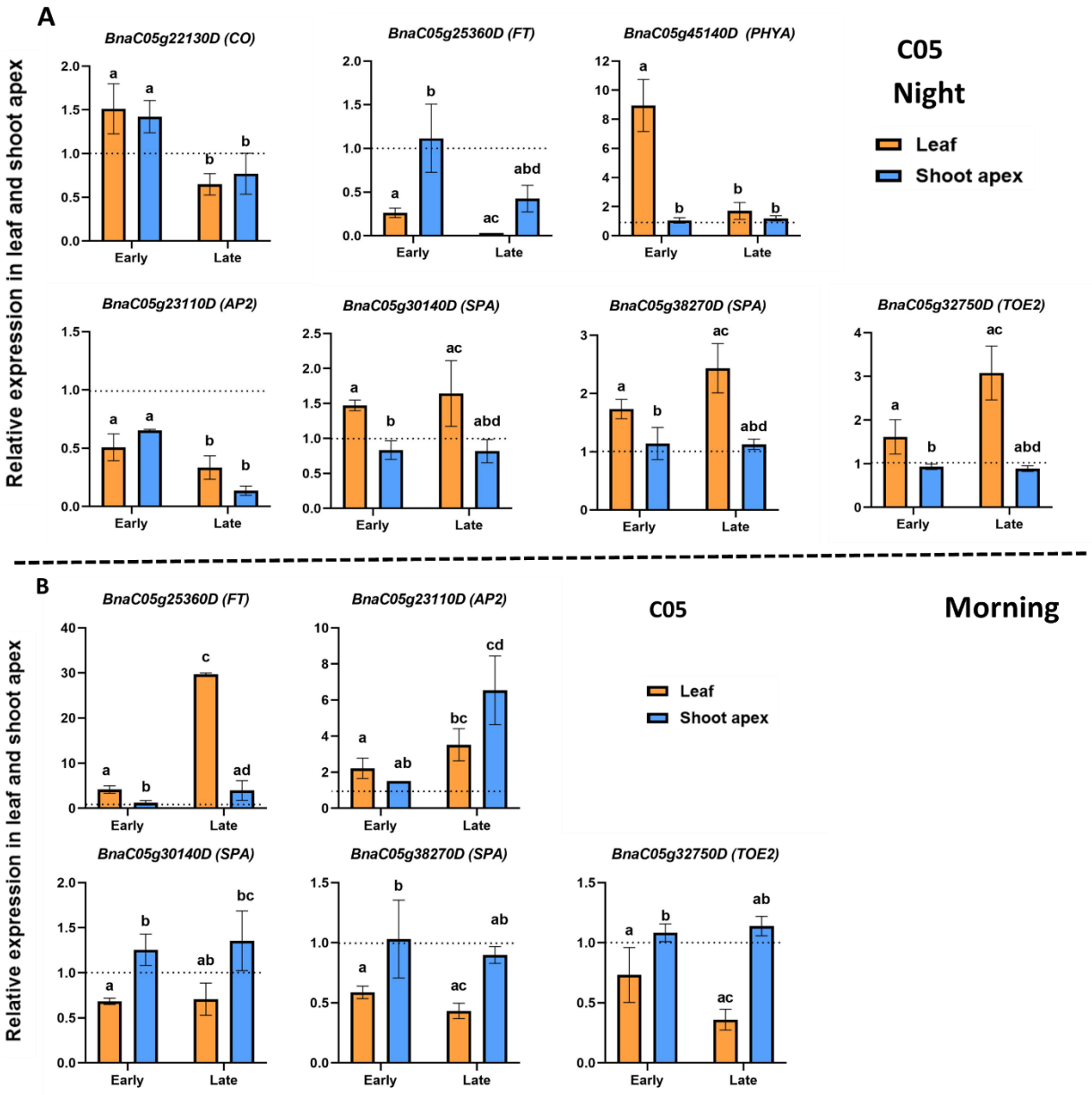

Figure 3-1. Relative expression of flowering genes from the C5 QTL in early-and late-flowering lines (bulks) of *Brassica napus* canola (A) at night (4 hours after the light turned off), and (B) in the morning (4 hours after the light turned on) under a 10-hour photoperiod condition; mean of three biological replicates ± standard error (SE) presented. Different letters indicate significant difference between the mean values ($p < 0.05$). Expression level was normalized to the control for each time point and indicated by dotted line at expression level = 1.0

3.4.2 Expression of the genes from C9 QTL

In case of the genes from the C9 QTL region, five of the 15 genes showed differential expression in leaf and shoot apex of the early and late flowering bulks at night, and this included *SOC1 (BnaC09g29450D), SOC1 (BnaC09g42060D), GI (BnaC09g38380D), TOE2 (BnaC09g35430D)* and *TEM1 (BnaC09g49240D*) (Figure 3-2A). In morning, four of these genes, except *TOE2*, showed significant differential expression. An additional gene, *BnaC09g41980D (CO)*, also showed differential expression in morning; its expression in both leaf and shoot apex was significantly greater in the early flowering bulk as compared to the late flowering bulk (4.94-fold in leaf; 3.7-fold in shoot apex of the early lines) (Figure 3-2B). Among the other genes, differential expression between the early- and late-bulk was found for *SOC1 (BnaC09g29450D)*, *TOE2* and *TEM1*. *SOC1 (BnaC09g29450D)*, at night, showed a significantly greater expression in leaf and shoot of the early bulk as compared to the late bulk; however, expression of this gene was significantly greater in shoot of the late bulk as compared to the early bulk in morning (Figure 3-2A and Figure 3-2B). *TEM1* also showed contrasting expression at night and morning. Its expression at night was higher in both leaf and shoot of the early flowering bulk, but in morning, a higher expression of this gene was found in the late flowering bulk; the overall expression of this gene was greater in the morning as compared to night (Figure 3-2A and 3-2B). *TOE2* showed a greater expression in both leaf and shoot apex of the late flowering bulks at night; however, overall expression of this gene was low (Figure 3-2A).

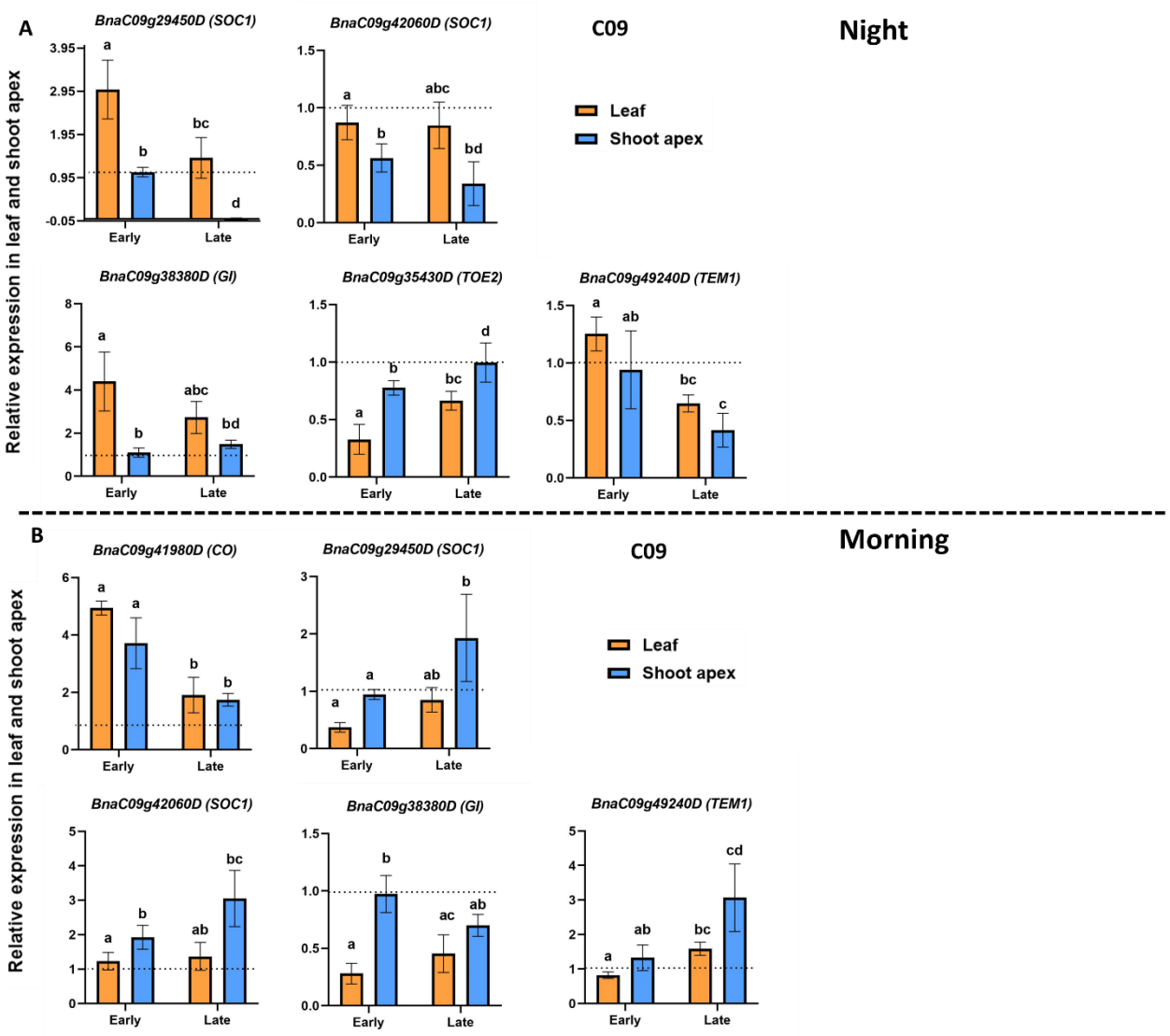

Figure 3-2. Relative expression of flowering genes from the C9 QTL in early-and late-flowering lines (bulks) of *Brassica napus* canola (A) at night (4 hours after the light turned off), and (B) in the morning (4 hours after the light turned on) under a 10-hour photoperiod condition; mean of three biological replicates ± standard error (SE) presented. Different letters indicate significant difference between the mean values ($p < 0.05$). Expression level was normalized to the control for each time point and indicated by dotted line at expression level = 1.0

3.5 Discussion

This study was carried out under a short-day condition to identify the genes from the C5, and C9 QTL regions involved in flowering time variation. Among the genes showed differential expression, relative expression of *PHYA* from C5 was very high at night while the expression of

FT and *AP2* from this QTL was very high in morning. Among the genes from the C9 QTL, high expression was found for *GI* at night, but for *CO*, *SOC1* and *TEM1* in the morning. Several other genes from these two QTL regions also showed differential expressions in leaf and shoot apex of the early- and late-flowering plants.

Among the different flowering genes from the photoperiod pathway, *CONSTANS* (*CO*) is the key floral regulator; it integrates the circadian clock and photoperiod signal and play a critical role in flowering timing variation. Suárez-López et al. (2001) found significant abundance of *CO* mRNA at night under a short-day condition, while high abundance of this mRNA at the end and beginning of the photoperiod under a long-day condition. Phytochrome A (*PHYA*) gene is one of the most important photosensor in *Arabidopsis*; this gene is involved in photoperiod pathway and improves flowering in response to Far Red light. According to Mockler et al. (2003), the abundance of *PHYA* fluctuates in *Arabidopsis* during the day under a short-day condition where an increased expression occurs in dark than in daytime; however, very little change in expression of this gene could be seen under a long-day condition. Usually, *PHYB* starts the degradation of *CO* in early morning while *PHYA* protein stabilizes *CO* during evening which directly activates the expression of *FT* under a long-day condition (Valverde et al., 2004). Among the 15 genes from the C5 QTL that we investigated in this study, a high expression of *PHYA* as well as a moderate expression of *CO* was found at night in leaf of the early flowering bulk. This demonstrates that a greater expression of these photoperiod pathway genes from the C5 QTL played an important role on flowering in the early-flowering lines.

The *AP2* is known to express in shoot apical meristem (Würschum et al., 2006) where it represses the day-length dependent expression of the floral integrator gene *FT* as well as *SOC1* (Yant et al., 2010) and, thus, delay flowering in *Arabidopsis*. In this study, while working with the

Brassica C genome we also found a greater expression of *AP2* but a lower expression of *FT* (*BnaC05g25360D*) in shoot apex of the late flowering bulk in morning, and this validates that the knowledge of this gene from *Arabidopsis* holds true also in *Brassica*.

In case of the genes from the C9 QTL, expression of *CO* (*BnaC0941980D*) in morning was significantly higher in the early flowering bulk as compared to the late flowering bulk; however, no differential expression of this gene could be detected between these two bulks at night. The higher expression of *CO* in the morning is difficult to explain based on our current knowledge that the greatest expression of this gene occurs at night under a short-day condition, while at the end and the beginning of the photoperiod under a long-day condition (Suárez-López et al., 2001). It is possible that an allelic form of this gene evolved in the Brassica C genome (*B. oleracea*) during evolution of this species from *Arabidopsis*; however, experimental confirmation of this will be needed.

Two additional genes from the C9 QTL, viz. *SOC1* (*BnaC09g29450D*) and *TEM1*, showed significant differential expression between the early- and late-flowering bulks. Expression of *SOC1* is known to be strongly dependent on the autonomous pathway and improve flowering in *Arabidopsis* (Moon et al., 2005). In this study, we also found a greater expression of *SOC1* (*BnaC09g29450D*) at night in both leaf and shoot apex of the early flowering bulk as compared to the late bulk. In case of *TEM1*, it regulates flowering time in *Arabidopsis* by repressing the expression of *FT* (Castillejo and Pelaz, 2008). In this study, we also found significantly lower expression of *TEM1* in both leaf and shoot of the early flowering bulk as compared to the late flowering bulk in the morning. A decreased expression of *TEM1* in the early flowering plants indicate that a mutant allele of this gene might be present in *B. oleracea* which has been introgressed into the materials used in this study.

Other genes *SPA* (*BnaC05g30140D*)*, SPA (BnaC05g38270D), TOE2 (BnaC05g32750D)*, *SOC1 (BnaC09g42060D), GI (BnaC09g38380D),* and *TOE2 (BnaC09g35430D)* also showed differential expression across the two time points and between the samples and bulks. This implies that these genes might have also contributed to the flowering in this population; however, understanding of the detailed mechanism of their involvement was beyond the scope of the present study.

In conclusion, it is apparent that *CO* (*BnaC05g22130D, BnaC0941980D), PHYA (BnaC05g45140D), FT (BnaC05g25360D), AP2 (BnaC05g23110D), SOC1* (*BnaC09g29450D*) and *TEM1* (*BnaC09g49240D*) contributed to the flowering time variation between the early- and late-flowering lines under 10 hr photoperiod condition. Among these, the *CO*, which is known to be the main gene in the photoperiod pathway, apparently played a crucial role in early flowering in this population. The *PHYA* from the C5 QTL and *TEM1* from the C9 QTL might have also played an important role in flowering time variation between the early- and late-flowering lines.

Chapter 4. Proteome study of the spring *Brassica napus* lines carrying genome contents of *B. oleracea*

4.1 Introduction

The study of proteins, translated from genes, is one of the advanced methods for understanding the global gene expression and their functional processes which is known as scientific proteomics (for review, see Eldakak et al., 2013). The proteome is the whole collection of proteins that are expressed by a genome in a cell type or a type of tissue at a specific time. Each organism carries a distinct genome; however, the proteome may vary under various conditions and can differ in different tissues of the same organism. This is one of the contrasting differences between the concepts of the proteome and the genome. Surprisingly, the number of proteins can outnumber the number of genes in an organism (for review, see Wilkins et al., 1995). Several proteomic studies have been carried out on different plant parts or tissues for various purposes, such as for tracking developmental changes (e.g. Fan et al., 2023; Zhang et al., 2012) or to understand the impact of environmental factors on protein patterns (e.g. Liu et al., 2023; Feng et al., 2023) or to investigate the molecular mechanism of disease resistance (e.g. Adhikary et al., 2024; Sharma et al., 2007).

Flowering is one of the critical stages of crop yield development, and the timing of flowering is crucial in attaining optimal yield. A large number of genes and their interactions control this trait in oilseed *B. napus* (for review, see Schiessl, 2020). To date, most of the in-depth studies on flowering time was carried out on the model plants *A. thaliana*, which is the progenitor of *Brassica*, and mostly using transcriptomics approaches (e.g. Jian et al., 2019; Shah et al., 2018); only a few studies has been carried out using other 'omics' approaches, such as epigenomics (e.g. Xue et al., 2023; Poza-Viejo et al., 2022) and proteomics (e.g. Mao et al. 2021; Krahmer et al. 2018; Seaton

et al. 2018). One of the unique benefits of the proteomics study is its ability to consider post-translational modifications and demonstrating how the proteins affect crop yield; however, this is not possible using transcriptomics or epigenomics. Krahmer et al. (2018) carried out proteome analysis to understand the post translational control of flowering gene *GI (GIGANTIA)* in *Arabidopsis*. Seaton et al. (2018) investigated the photoperiod response in *A. thaliana* at proteome level and found a coordinated changes in proteins governing photosynthesis, primary and secondary metabolism, as well as pigment biosynthesis. They also found an increased metabolic activity in the plants under long photoperiod conditions. Mao et al. (2021) carried out a proteomic study on flowering time and showed that expression of several flowering time and hormone related proteins might result in early flowering phenotype in *B. napus*.

It is well established today that the *Brassica* genomes evolved from *Arabidopsis* through extensive genome duplications, rearrangements, and changes in the genomes (for review, see Lagercrantz, 1998). In case of flowering time, most of the genes found in *Arabidopsis* remained conserved in *B. napus*; however, some change including the loss of original function had occurred during evolution of this species (for review, see Schiessl, 2020). Therefore, an understanding of the molecular basis of flowering time variation in *B. napus* at transcriptome and proteome level will allow to improve this trait through molecular plant breeding.

In a research project, the Canola Program of the University of Alberta developed an advanced generation *B. napus* inbred population from different *B. napus* (AACC) × *B. oleracea* (CC) interspecific crosses; many of these lines flowered earlier than the *B. napus* parent (Nikzad et al., 2019). Based on this pedigree, it is expected that the early flowering *B. napus* lines carry early flowering alleles which have been introgressed from the late-flowering species *B. oleracea* and the alleles are located in their C genome. Indeed, this has been confirmed through QTL

mapping of this trait by growing the population under field conditions as well as under a short-day condition in a growth chamber (Gill, 2021; Nikzad et al., 2023). In this regard, these early-flowering *B. napus* lines are novel canola genetic resources. The purpose of this study was to examine the changes in protein expression in the above-mentioned early and late flowering *B. napus* lines to identify the proteins playing a role in flowering time variation in this population.

4.2 Materials and method

4.2.1 Plant material and growing condition

Seven most early and most late flowering *B. napus* advanced generation inbred lines, which were used in Chapter 3, were used for proteome analysis. For this, nine plants of each line were grown in a growth chamber set at 10 hr photoperiod with light intensity of 450 µE (mV) m^{-2} s^{-1} and 20 ℃ constant temperature throughout the light and dark hours.

4.2.2 Sample collection for proteomics

Leaf samples of the early and late flowering lines were collected at rosette stage and at two time points; (i) morning, i.e. 4 hours after light turned on, and (ii) night, i.e. 4 hours after light turned off. Bulk sample of the early and late flowering lines were prepared by including three plants of each of the lines. Thus, each of the early and late bulk included 21 plants (7 lines × 3 plants); the number of replications of the experiment was three. Thus, the total number of samples (bulks) for proteomics study was 2 types of lines (early and late) × 2 times points × 3 biological replicates = 24. The samples were frozen at -80 ^{0}C until use.

4.2.3 Protein extraction for proteomic analysis

Analysis of the samples for protein profile was carried out in the laboratory of Dr. Glen Uhrig, Department of Biological Science, University of Alberta. For this, frozen leaf tissue samples were crushed in liquid nitrogen with a mortar and pestle. 100 mg of ground tissue was dissolved in a solution containing 50 mM HEPES-KOH pH 8.0, 50 mM NaCl, and 4% (w/v) SDS at a 1:2 (w/v) ratio to extract the protein. The protein extract was alkylated with 30 mM iodoacetamide (IA) and reduced with 10 mM dithiothreitol (DTT). Using a KingFisher APEX (ThermoScientific) automated sample preparation system, peptide pools were created following Leutert et al. (2019). Samples were digested with sequencing grade trypsin (V5113; Promega) and acidified to a final concentration of 5% (v/v) with formic acid. After being digested, samples were finalized with formic acid (A117, Fisher) at a concentration of 0.5% (v/v) in the acidification process. The desalination of peptides was carried out as described by Uhrig et al. (2019) utilizing an OT-2 liquid handling robot (Opentrons Labworks Inc.) fitted with Omix C18 pipette tips (A5700310K; Agilent). Prior to re-suspension in 3.0% (v/v) ACN / 0.1% (v/v) FA and before MS injection, desalted peptides were dried and kept at -80 °C.

4.2.4 Nanoflow LC-MS/MS analysis

Thermo Scientific's Fusion Lumos Tribrid Orbitrap mass spectrometer was used to analyze the peptides using the BoxCarDIA technique in data independent acquisition (DIA) mode (Mehta et al., 2022). A 25 cm Easy-Spray PepMap C18 Column (ES902; Thermo-Scientific) was used to separate one microgram of re-suspended peptide that had been injected using an Easy-nLC 1200 system (LC140; ThermoScientific). The peptide was eluted with a solvent B gradient (0.1% (v/v) FA in 80% (v/v) ACN), consisting of 4-41% B, using a 40-minute nonlinear gradient as described by Mehta et al. (2022). Two multiplexed targeted SIM scans of 10 BoxCar windows each were

used to accomplish the BoxCarDIA MS1 analysis. At 100% normalized AGC targets per BoxCar isolation window, detection was carried out at 120,000 resolutions. Twenty-eight 38.5 m/z windows with a 1 m/z overlap and an AGC target value of 2000% were used for the MS2 analysis, which was carried out at a resolution of 30,000.

4.2.5 Functional annotation and enrichment analysis of differentially abundant proteins

The N-acetyl variable was not modified while processing any of the BoxCarDIA files with Spectronaut v17 (Biognosys AG) using default parameters. Using the publicly available *B. napus* Westar proteome, spectra were searched (Song et al., 2020). Significantly altering differentially abundant proteins were identified and adjusted for multiple comparisons (Bonferroni- corrected *p*-value < 0.05; *q*-value). The predicted proteins were annotated with gene descriptions from the Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.genome.jp/kegg/), Pfam (Mistry et al., 2020) and SwissProt (https://www.expasy.org/resources/uniprotkb-swiss-prot) databases to better understand their biological functions. The PANTHER Overrepresentation Test in the Gene Ontology database (http://geneontology.org/docs/go-enrichment-analysis/) was used for gene ontology analysis utilizing the TAIR (https://www.arabidopsis.org) hit IDs corresponding to the important *B. napus* proteins (*q*-value 0.05). The parameters employed in this investigation were statistical test type FISHER, correction technique FDR (False Discovery Rate), and significance level of 0.05. In the end, the Database for Annotation, Visualization, and Integrated Discovery (DAVID) was utilized to analyze the KEGG pathways using the TAIR IDs (Jr et al., 2003). After multiple testing was performed with Benjamini-Hochberg correction (Benjamini and Hochberg, 1995), the KEGG enriched categories with the FDR-corrected *p*-value 0.05 were taken into consideration for further research. All selected potential flowering time DAPs

gene symbol were searched in STRING database (https://string-db.org/) in *Arabidopsis thaliana* to see the association network among them.

4.3 Results

4.3.1 Leaf proteome profile difference due to genotype and time point

The leaf proteomes of the early and late flowering lines (as bulk) at two different time points were analyzed. A total of 472 proteins were differentially accumulated in the early flowering samples when compared with the late flowering samples (early vs. late) of which 275 and 197 DAPs were observed at morning and night, respectively (Table 4-1). Among these, 33 DAPs were detected at both time points (Supplementary Table S4.1a and Figure 4-1A) where 15 proteins showed an increased abundance, and 14 proteins showed a decreased abundance (Supplementary Table S4.1a). Remaining four DAPs were found to be contrasting in their abundance at these two time points (Supplementary Table S4.1a). Among the unique DAPs (DAPs which were only found at morning early vs. morning late comparison and night early vs. night late comparison), 77 DAPs showed an increased abundance, and 165 DAPs showed a decrease abundance at morning, while 95 DAPs showed an increased abundance, and 69 DAPs showed a decreased abundance at night between early vs. late comparison (Supplementary Table S4.1b). When comparison was made for the early- and late-flowering genotype separately at two different time points (morning early vs. night early, and morning late vs. night late), a total 280 proteins were differentially accumulated of which 170 and 110 DAPs were observed in the early- and late-flowering bulk, respectively. Among these, only seven DAPs were detected in both bulks (Supplementary Table S4.1c and Figure 4-2A). Frequency distribution of the proteins, either increasing or decreasing in abundance, from all the above-mentioned comparisons is represented in figure 4-1B and figure 4-2B.

Table 4-1. Total count of proteins in leaves from different comparisons of the early and late flowering lines of *Brassica napus*

Comparison	Total no. of leading proteins
Morning early vs. Morning late	275
Night early vs. Night late	197
Morning early vs. Night early	170
Morning late vs. Night late	110
Total	752

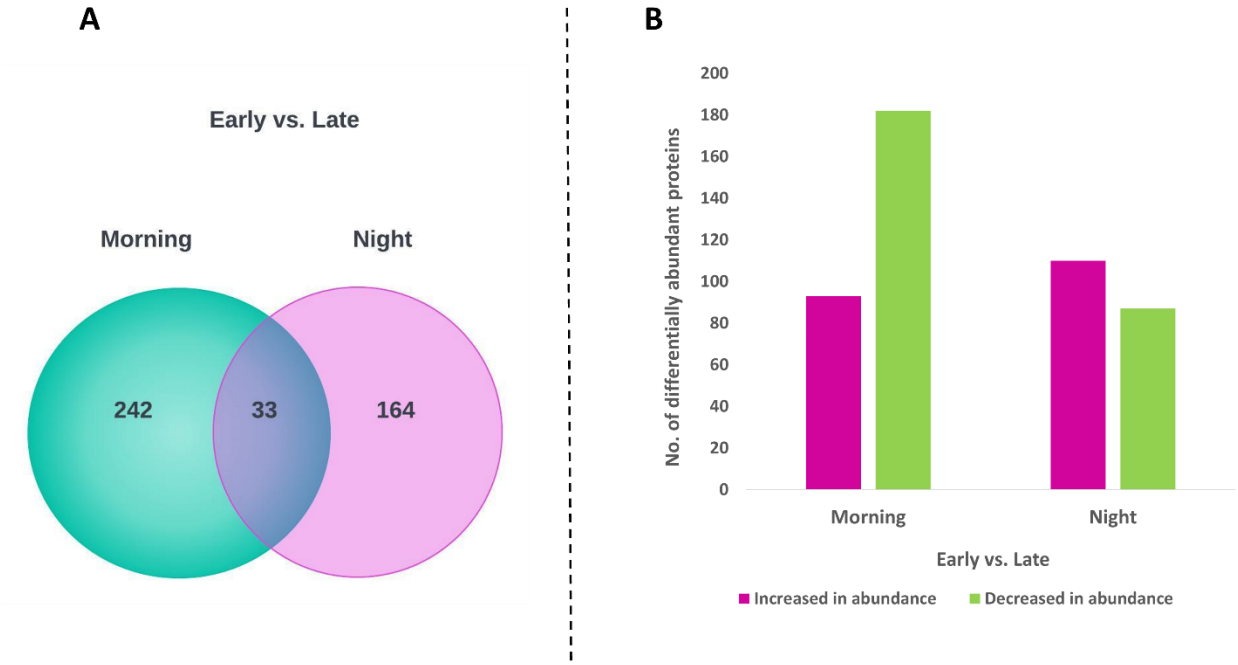

Figure 4-1. Differentially accumulated proteins (DAPs) at morning and night in early vs. late comparison in leaves of the early and late flowering lines (bulk) of *Brassica napus* under 10-hour photoperiod. ME vs. ML (ME=Morning early, ML= Morning late) and NE vs. NL (NE=Night early, NL=Night Late)

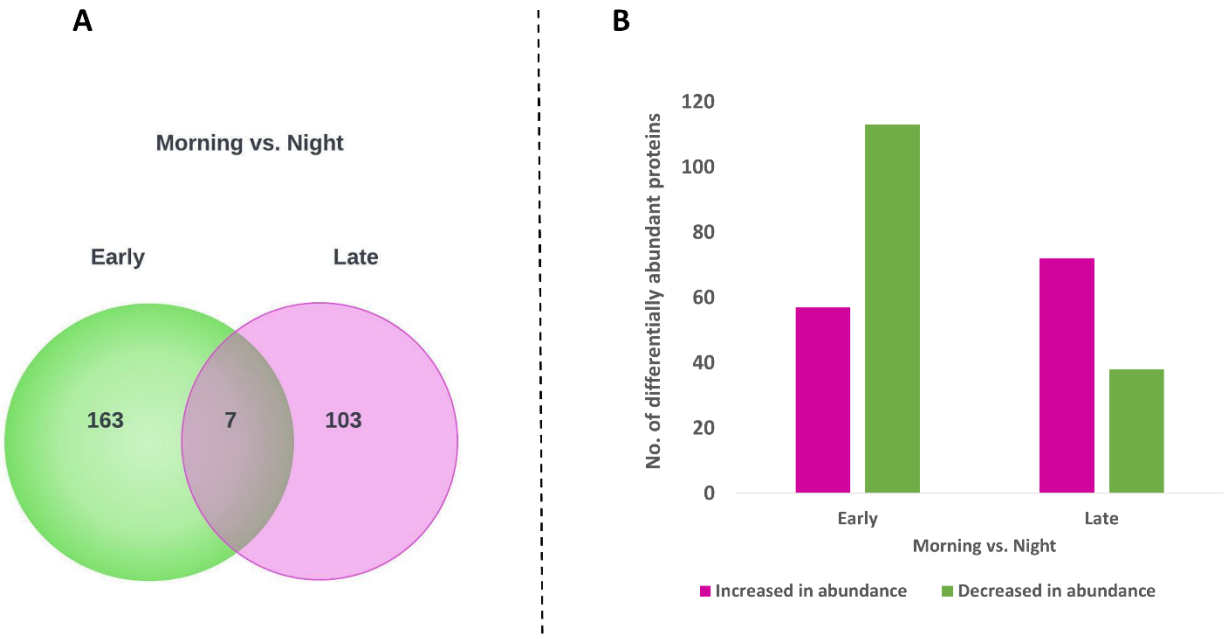

Figure 4-2. Differentially accumulated proteins (DAPs) in leaves of the early and late lines (bulk) of *Brassica napus* under 10-hour photoperiod across the two time points (morning vs. night) (ME vs. NE) and (ML vs. NL). ME=Morning early, NE=Night early; ML=Morning late. NL=Night late)

4.3.2 Temporal changes in leaf proteome of the early and late flowering lines (bulk) in response to light at two different time points

At morning, 57 and 74 DAPs showed increased abundance in the early and late flowering bulks, respectively, while 113 and 38 DAPs showed increased abundance in the early and late flowering bulks at night (Table 4-2). Among these, two DAPs showed an increased abundance, and three DAPs showed a decreased abundance in both early and late flowering lines; this type of proteins will be referred to as "shared proteins' (Supplementary Table S4.1c). Proteins that were showing a differential accumulation pattern in the early and late flowering lines were also identified, i.e., either decreased in the early flowering bulk but increased in the late flowering bulks, and vice-versa. This type of proteins hereafter will be considered as "contrasting proteins".

Only two contrasting proteins were identified between the early and late flowering lines (Supplementary Table S4.1c).

Among the contrasting proteins between the early vs. late comparison, 60S ribosomal protein L27-3-like protein *AT4G15000.1* (BnaA03T0382000WE) showed increased abundance at morning and decreased abundance at night whereas ubiquitin fold modifier 1-specific protease *AT3G48380.1* (BnaA06T0163300WE), stress-induced-phosphoprotein 1 *AT1G04190.1* (BnaA08T0295500WE) and ATP sulfurylase 1 *AT3G22890.1* (BnaC05T0334300WE) proteins showed decreased abundance at morning and increased abundance at night. Among the shared proteins of this comparison, glycine cleavage system H protein 3 *AT1G32470.1* (BnaC05T0270600WE), trypsin and protease inhibitor *AT1G72290.1* (BnaC06T0406500WE, BnaC06T0406700WE) were observed in decreased abundance at both time points while desiccation protectant protein Lea14 *AT2G44060.2* (BnaC04T0583200WE), tetraketide alpha-pyrone reductase 1 *AT1G09500.1* (BnaC08T0469900WE), rhodanese-like domain-containing protein 4 *AT4G01050.1* (BnaC09T0005800WE), reticulata-related 4 *AT5G12470.1* (BnaC09T0504400WE) observed in an increased abundance at both time points.

Table 4-2. The number of differentially accumulated proteins (DAPs) that were significantly changing in abundance (q value < 0.05) in leaves of the early and late lines (bulks) of *Brassica napus* at morning and night

Change in abundance	Total number of DAPs	Lines (bulk)	Time
Increased	57	Early	Morning
Increased	113	Early	Night
Increased	74	Late	Morning
Increased	38	Late	Night

4.3.3 GO enrichment for differentially accumulated proteins (DAPs)

To identify the putative proteins associated with flowering time variation, GO enrichment analysis was performed of the DAPs from early vs. late flowering lines at morning and night. In case of early vs. late comparison at morning, 204 GO terms were significantly enriched (FDR < 0.05); among these 97 terms belonged to Biological Process (BP) category, 45 to Molecular Function (MF) category and 62 to Cellular Components (CC) category. Analysis of the DAPs from night identified 125 significantly enriched (FDR < 0.05) GO terms; among these 48 terms belonged to Biological Process (BP) category, 11 to Molecular Function (MF) category, and 66 to Cellular Components (CC) category from the early vs. late comparison. All GO terms identified from this comparison at two time points separated based on common (found in both timepoint for early vs. late comparison) and unique (found at one specific timepoint for early vs. late comparison) GO terms, which are presented in supplementary Tables S4.2a and S4.2b. The top significant common GO terms belonging to BP category included photosynthesis (GO:0015979), response to temperature stimulus (GO:0009266), response to abiotic stimulus (GO:0009628), response to light stimulus (GO:0009416), response to stress (GO: 0006950), cellular process (GO: 0009987), organonitrogen compound biosynthetic process (GO:1901566) while MF category included oxidoreductase activity (GO:0016491), mRNA binding (GO:0003729), catalytic activity (GO:0003824) and many others (Figure 4-3 and Supplementary Table S4.2a). The unique GO term identified from early vs. late comparison at morning belonging to BP category included primary metabolic process (GO:0044238), photosynthesis, dark reaction (GO:0019685), regulation of RNA biosynthetic process (GO:2001141), detoxification of nitrogen compound (GO:0051410), photosynthetic electron transport chain (GO:0009767), chlorophyll metabolic process (GO:0015994), pigment biosynthetic process (GO:0046148), pigment metabolic process

(GO:0042440), carbon fixation (GO:0015977), carbon utilization (GO:0015976) and MF category included antioxidant activity (GO:0016209), copper ion binding (GO:0005507), sequence-specific DNA binding (GO:0043565), sugar-phosphatase activity (GO:0050308), salicylic acid binding (GO:1901149), peroxidase activity (GO:0004601), cation binding (GO:0043169), anion binding (GO:0043168) and many others (Figure 4-4A and Supplementary Table S4.2b). At night unique GO terms belonging to BP category included response to reactive oxygen species (GO:0000302), response to oxidative stress (GO:0006979), photosynthesis, light harvesting in photosystem I (GO:0009768), photosynthesis - light reaction (GO:0019684), cellular oxidant detoxification (GO:0098869) and unique GO terms belonging to MF category included structural constituent of ribosome (GO:0003735), L-ascorbate peroxidase activity (GO:0016688), poly(U) RNA binding (GO:0008266), glutathione transferase activity (GO:0004364) and others (Figure 4-4B and Supplementary Table S4.2b).

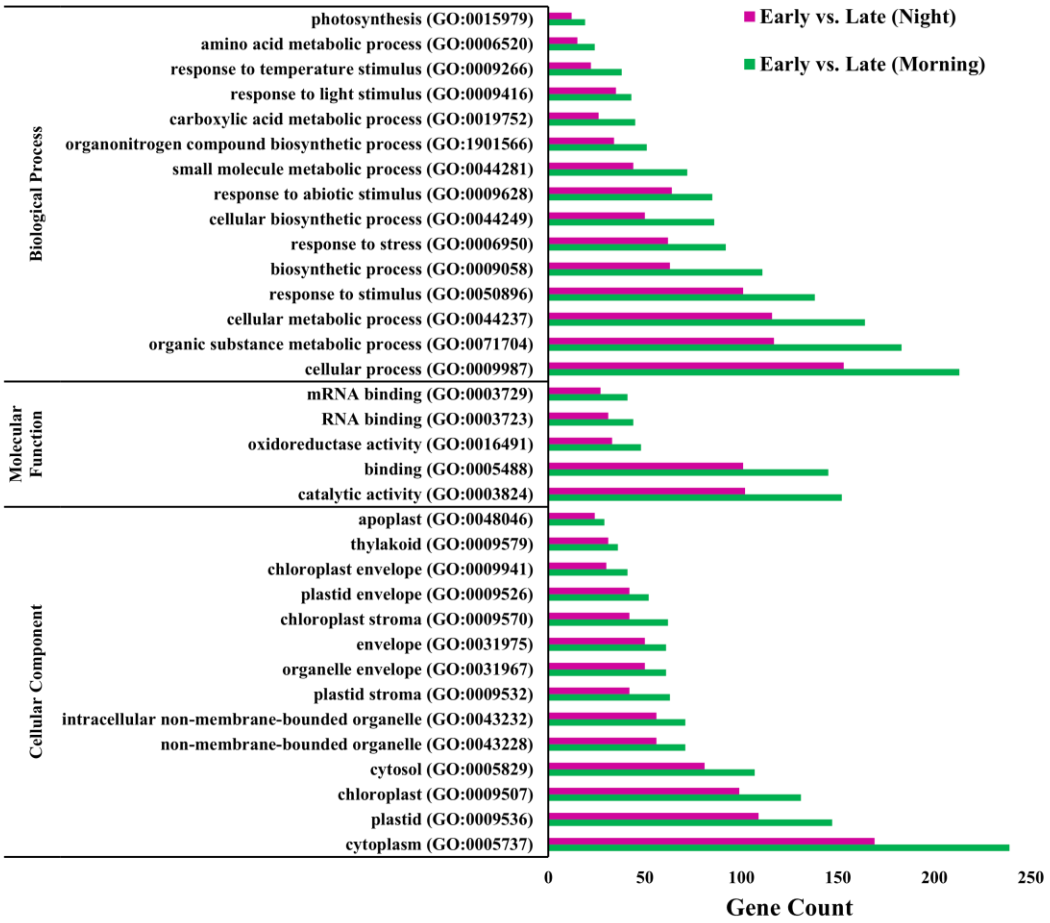

Figure 4-3. The topmost significantly (FDR<0.05) enriched and common (GO terms found in both time point for early vs. late comparison) gene ontology (GO) terms of each of the biological process (BP), molecular function (MF) and cellular components (CC) category for the differentially accumulated proteins (DAPs) identified from the comparisons of early vs. late flowering lines at morning (ME vs. ML) and early vs. late flowering lines at night (NE vs. NL) in leaves of *Brassica napus* under 10-hour photoperiod

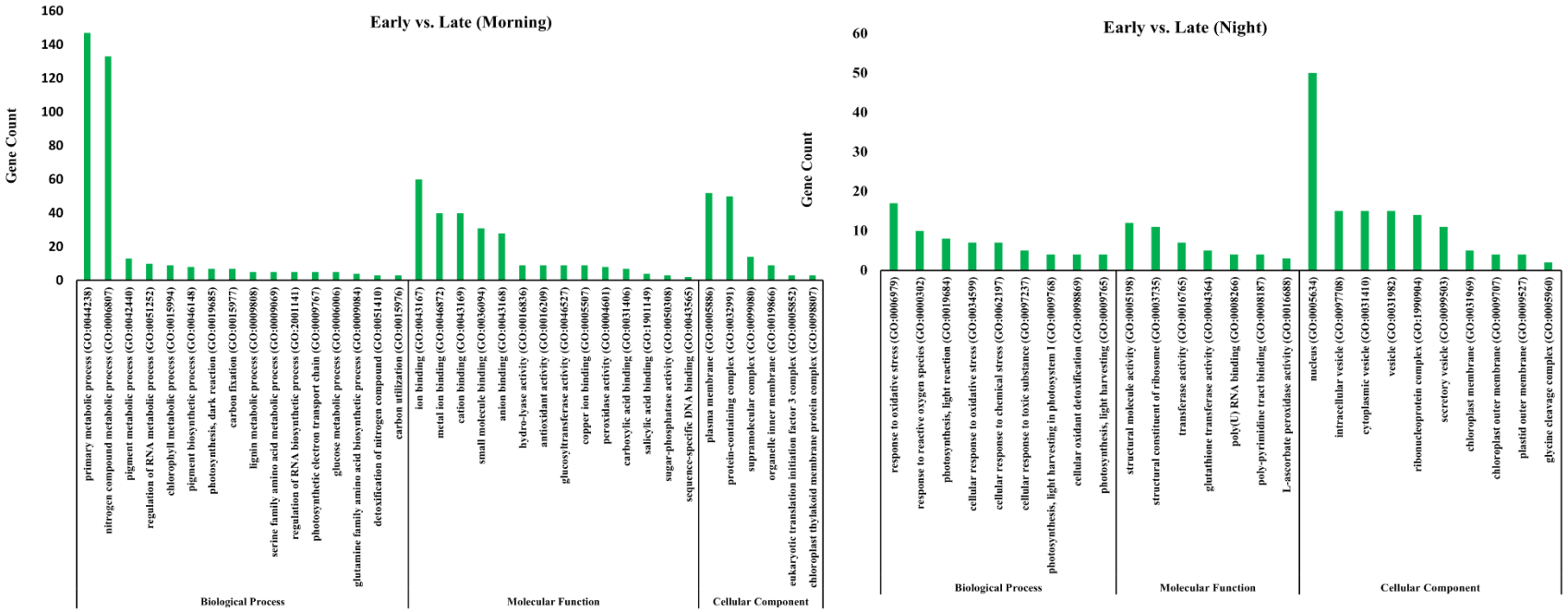

Figure 4-4. The topmost significantly (FDR<0.05) enriched and unique gene ontology (GO) terms of each of the biological process (BP), molecular function (MF) and cellular components (CC) category for the differentially accumulated proteins (DAPs) identified from the comparisons of early vs. late flowering lines at morning (A) and early vs. late flowering lines at night (B) in leaves of *Brassica napus* under 10-hour photoperiod

4.3.4 KEGG pathway enrichment of differentially accumulated proteins (DAPs)

The KEGG pathway enrichment analysis of data from the early vs. late comparison at morning showed that the significantly enriched (FDR < 0.05) 179 DAPs belonged to four pathways which were biosynthesis of secondary metabolites (60 DAPs), metabolic pathways (89 DAPs), carbon metabolism (21 DAPs) and carbon fixation in photosynthetic organisms (9 DAPs). Among these only the carbon fixation in photosynthetic organisms' pathway could not be detected at night. From early vs. late comparison at night, a total of 175 significantly enriched (FDR < 0.05) DAPs were identified belonging to 11 pathways from which glyoxylate and dicarboxylate metabolism (10 DAPs), ascorbate and aldarate metabolism (7 DAPs), glycine serine threonine metabolism (7 DAPs), pyruvate metabolism (8 DAPs), glutathione metabolism (8 DAPs), tryptophan metabolism (6 DAPs), nitrogen metabolism (5 DAPs) and photosynthesis-antenna proteins (4 DAPs) (Figure 4-5 and Supplementary Table S4.3).

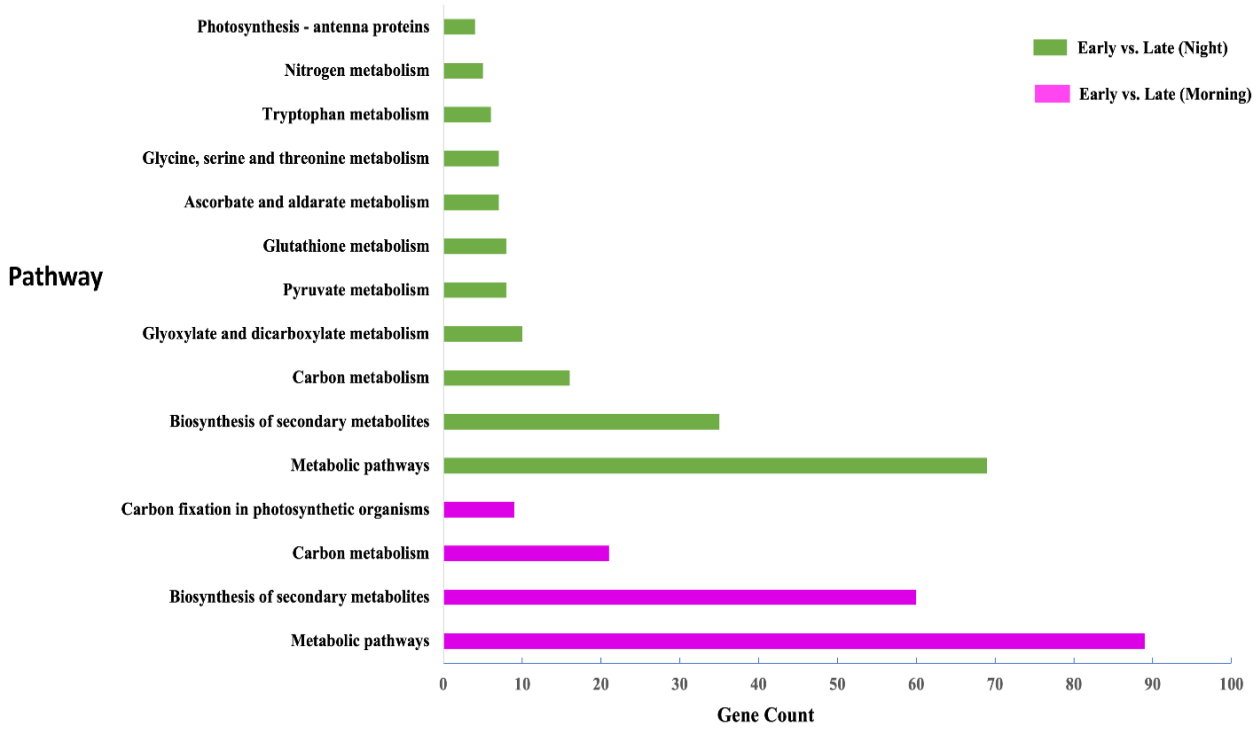

Figure 4-5. The unique significantly (FDR < 0.05) enriched KEGG pathways of the differentially accumulated proteins (DAPs) in leaves of early and late lines bulk of *Brassica napus* under 10-hour photoperiod at morning and night. The y-axis indicates the enriched KEGG pathways, and the x-axis indicates the gene count

4.3.5 Identification of putative differentially accumulated proteins (DAPs) to be involved in flowering time

For this, relative abundance of a DAP at a timepoint was compared to the abundance of the DAP at the other timepoint as control, such as a DAP showing increased abundance at morning compared to night and vice versa. Based on this, the following DAPs were identified.

Flowering time

A total of 12 DAPs were identified that could be associated with flowering time variation. Among these, six DAPs were uniquely found in the early flowering lines whereas six DAPs were uniquely found in the late flowering lines (Supplementary Table S4.4a and S4.4b). In case of the

early flowering lines, SNW/SKI-interacting protein (SKIP, BnaA07T0363300WE), zinc finger CCCH domain-containing protein 33 (ZFN1, BnaA01T0321600WE), 14-3-3-like protein GF14 nu (GRF7, BnaA05T0440000WE) and one uncharacterized mitochondrial import inner membrane translocase subunit TIM22 (BnaA06T0154500WE) carrying SAM (sterile alpha motif) showed an increased abundance at night than in morning, whereas protein plastid transcriptionally active 12 (HEMERA/PTAC12, BnaA05T0099100WE) and 14-3-3-like protein GF14 omega (GRF2, BnaA07T0117400WE) showed an increased abundance at morning compared to night (Supplementary Table S4.4a). From late flowering lines, protein transport protein SEC31 homolog B (SEC31B, BnaC08T0369400WE), eukaryotic peptide chain release factor subunit 1 (ERF1, BnaA08T0254500WE), ATP synthase protein I (ATPI, BnaA04T0190000WE) DAPs showed increased abundance at night than in morning, while ankyrin repeat domain-containing protein 2 (AKR2, BnaC01T0012600WE), receptor-like protein kinase FERONIA (FER, BnaA01T0125700WE), and cullin-1 (CUL1, BnaA03T0280800WE) DAPs showed increased abundance at morning compared to night (Supplementary Table S4.4b). To find out if there is any association of the early and late flowering proteins with previously known flowering genes, all above mentioned protein names from two groups were searched separately on STRING database in *A. thaliana* where an association of the early flowering proteins were found mostly with the genes which are responsible for promoting flowering whereas the late flowering proteins showed an association with the genes that negatively regulative flowering (Figure 4-6). This association network indicated that these proteins have interactions among themselves to a wide extent than expected for a random set of proteins of the same size and degree distribution.

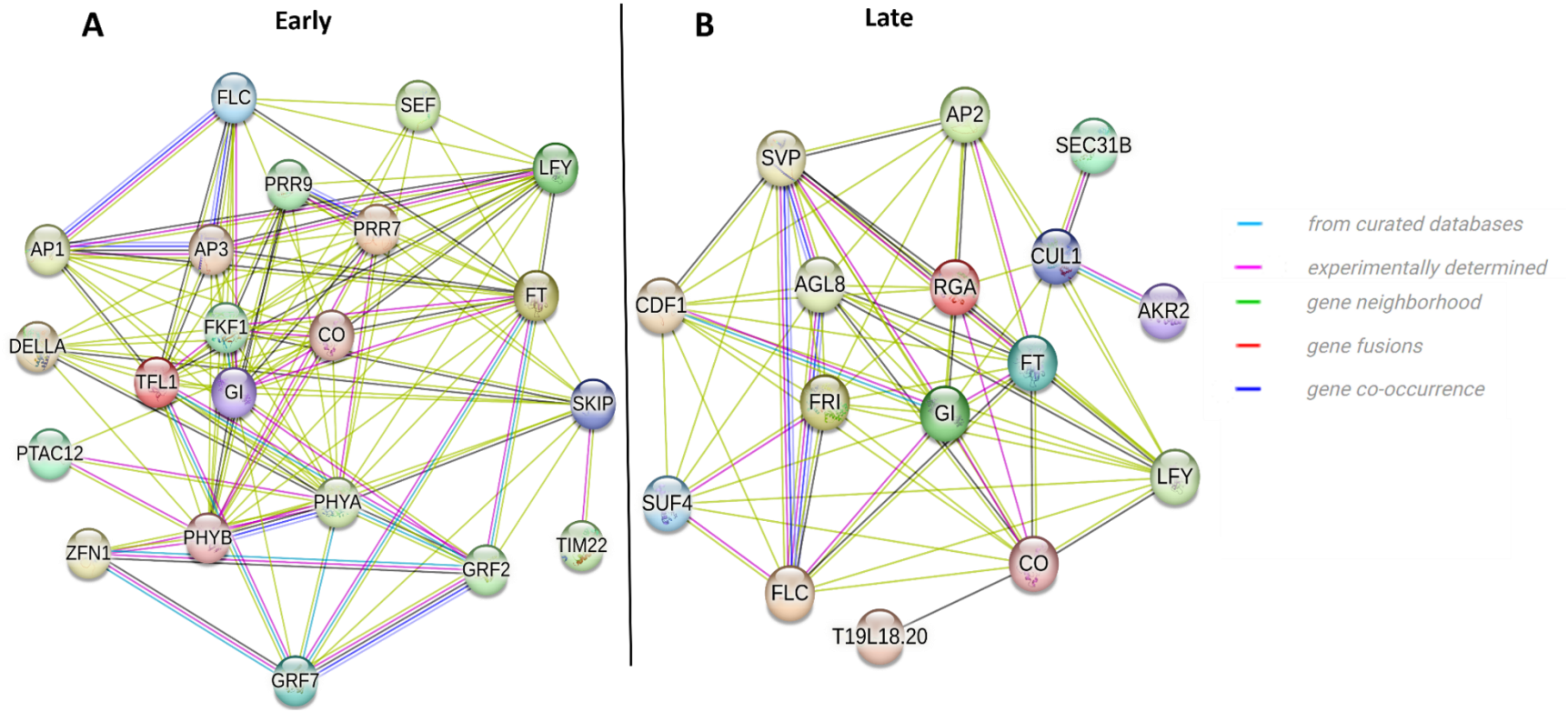

Figure 4-6. Association network (A) between the potential flowering proteins identified in early flowering lines with previously reported genes that promote flowering, and (B) between the potential flowering proteins identified in late flowering lines with previously reported genes that delay flowering from STRING database in *Arabidopsis thaliana*. Minimum confidence score considered was at least 0.152

Photosynthesis

We found eight and three (total 11) DAPs to be involved in photosynthesis in early and late flowering lines, respectively. In the early flowering lines, three DAPs showed an increased abundance at night while five DAPs showed an increased abundance in morning. On the other hand, in case of the late flowering lines, two DAPs showed an increased abundance at night while only one DAP showed an increased abundance in morning. Two DAPs namely BnaC06T0226800WE (from the early lines) and BnaA07T0183300WE (from the late lines) belongs to chlorophyll a-b binding protein 6 (LHCA1) type of photosynthetic proteins. In both lines, these two proteins showed an increased abundance at night than in morning; however, its abundance was much higher in the early flowering lines than the late flowering lines (LHCA1: 3.00 AVG Log2 Ratio in early bulk; 0.20 AVG Log2 Ratio in late bulk) (Supplementary Table S4.4a and Supplementary Table S4.4b). Among other DAPs in the late flowering lines, only one DAP, viz. protein proton gradient regulation 5 (PGR5, BnaA03T0398900WE), showed an increased abundance (1.23 AVG Log2 Ratio) involved in photosynthesis (Supplementary Table S4.4b).

Photorespiration

Three DAPs were found to be associated with photorespiration in the early flowering lines where two, viz. glycine dehydrogenase (decarboxylating) 1 (BnaC01T0034100WE) and zinc-binding dehydrogenase (BnaA06T0475300WE), showed an increased abundance at night while one DAP, viz. malate dehydrogenase (BnaA02T0112500WE), showed an increased abundance in morning. However, AVG Log2 Ratio of all three DAPs were below 1.0 (Supplementary Table S4.4a).

Stress Related

We identified 21 DAPs related to stress in the early flowering lines and 27 DAPs in the late flowering lines. In the early flowering lines, 15 DAPs showed an increased abundance at night while six DAPs showed an increased in morning. The abundance of three DAPs (AVG Log2 Ratio above 2.00), viz. cytochrome c-2 (BnaA03T0264400WE), uric acid degradation bifunctional protein TTL (BnaA02T0113100WE), senescence-associated protein (BnaA06T0347000WE), greatly increased at night, while only one DAP CS domain containing uncharacterized protein (BnaA10T0016100WE) showed an increased abundance (AVG Log2 Ratio close to or above 2.00) in morning in the early flowering lines (Supplementary Table S4.4a). In case of the late flowering lines, nine DAPs showed an increased abundance at night while of the remaining 18 DAPs showed an increased abundance in morning. Among these, eukaryotic translation initiation factor 5 (BnaA09T0348100WE) showed an increased abundance with 8.7 AVG Log2 Ratio at night followed by cyanate hydratase (BnaA07T0073200WE) and small heat shock protein Hsp20 (BnaC05T0045900WE) with AVG Log2 Ratio of about 2.00. In morning, aldose 1-epimerase (BnaA06T0168200WE), stress-induced-phosphoprotein 1 (BnaA08T0295500WE) and inositol-3-phosphate synthase (BnaC08T0394100WE) showed an increased abundance with AVG Log2 Ratio of more than 2.00 (Supplementary Table S4.4b); the Heat shock protein 90 (BnaA03T0131100WE) also showed an increased abundance, however, the level was very low (Supplementary Table S4.4b).

Reactive oxygen species

We identified eight DAPs related to reactive oxygen species in the early flowering lines and seven DAPs in the late flowering lines. In the early flowering lines, two DAPs viz. peroxidase 42 (BnaA01T0039700WE) and isocitrate dehydrogenase (BnaA03T0078000WE) showed the greatest level of abundance at night (Supplementary Table S4.4a). In case of the late flowering lines, thioredoxin X (BnaA06T0030100WE) showed an increased abundance at night while protein DJ-1 homolog C (Bnascaffold286T0039800WE) and peroxisomal membrane protein (BnaA09T0225500WE) showed an increased abundance in morning (Supplementary Table S4.4b).

Phytohormone

Compared to the late flowering lines (only two DAPs), a greater number of proteins related to phytohormone were found in the early flowering lines (12 DAPs). Of the 12 DAPs detected in the early flowering lines, 10 DAPs showed an increased abundance at night; among these cysteine proteinase 15A-like (BnaA01T0108800WE), methylmalonate-semialdehyde dehydrogenase (BnaC09T0113500WE), glutathione S-transferase (BnaA02T0240600WE), isochorismate synthase 2 (BnaA08T0232100WE), inositol hexakisphosphate and diphosphoinositol-pentakisphosphate kinase 1 (BnaA01T0331100WE), and probable leucine-rich repeat receptor-like serine/threonine-protein kinase (BnaA05T0355000WE) were the most abundant ones (Supplementary Table S4.4a). On the other hand, of the two DAPs detected in late flowering lines glutathione gamma-glutamyl cysteinyl transferase (BnaA06T0465900WE) showed an increased abundance in morning while patellin-1 (BnaA02T0196100WE) showed an increased abundance at night (Supplementary Table S4.4b).

Pigmentation Related

We identified three DAPs in the early flowering lines involved in anthocyanin biosynthesis and this included 26S proteasome non-ATPase regulatory subunit 8 homolog A (BnaA09T0130200WE), chalcone synthase (BnaC02T0452400WE), and chalcone synthase 1 (BnaC02T0452500WE). All these DAPs showed a greater abundance at night than in morning.

Ion binding

We found seven DAPs involved in copper ion binding in the early flowering lines, while four DAPs found to be involved in cobalt ion binding in the late flowering lines. In early flowering lines, methylmalonate-semialdehyde dehydrogenase (BnaC09T0113500WE), cytochrome c-2 (BnaA03T0264400WE) and plastocyanin major isoform (BnaC08T0214300WE) were the most important DAPs that increased in abundance at night, while NifU-like protein 5 (BnaA06T0023200WE) showed an increased abundance in morning (Supplementary Table S4.4a). On the contrary, the cobalt ion binding proteins 2-oxoglutarate dehydrogenase (BnaA09T0188600WE) and NADH dehydrogenase [ubiquinone] iron-sulfur protein 3 (BnaA04T0078500WE) showed an increased abundance at night and in morning respectively, in the late flowering lines (Supplementary Table S4.4b).

mRNA binding

We identified a total of 20 mRNA binding proteins of which eight in the early flowering lines and 12 in the late flowering lines. Of the eight DAPs found in the early flowering lines, four were most abundant where chlorophyll a-b binding protein 6 (BnaC06T0226800WE) and 40S ribosomal protein S3a-2 (BnaA08T0116600WE) increased in abundance at night while 60S ribosomal protein L19-1 (BnaA09T0016100WE) and 60S ribosomal protein L27-3

(BnaA03T0382000WE) increased abundance in morning. On the other hand, of the 12 DAPs found in the late flowering lines, six were most important of which eukaryotic peptide chain release factor subunit 1-2 (BnaA08T0254500WE), polyadenylate-binding protein 2 (BnaA03T0405500WE), guanine nucleotide-binding protein subunit beta-like protein (BnaC08T0419300WE) and 30S ribosomal protein S1 homolog A (BnaC07T0094700WE) showed an increased abundance at night while 60S ribosomal protein L2 (BnaC01T0248000WE) and Translation machinery associated TMA7 (BnaA06T0100000WE) showed an increased abundance in morning.

Protein binding

Thirteen DAPs were identified to be playing a role in protein binding. Seven of them were from the early flowering lines; however, four of these were of our interest. Among them, NPL4-like protein 1 (BnaC04T0251700WE) and histone H2B (BnaC09T0418200WE) showed an increased abundance at night while zinc-finger homeodomain protein 1 (BnaA02T0404700WE) and sorting nexin 2B (BnaA10T0254300WE) showed an increased abundance in morning (Supplementary Table S4.4a). In case of the late flowering lines, cyanate hydratase (BnaA07T0073200WE) showed an increased abundance at night while mitochondrial import inner membrane translocase subunit TIM22-2 (BnaC01T0153200WE) showed an increased abundance in morning (Supplementary Table S4.4b).

Others

In addition to the above mentioned DAPs, additional unique DAPs were also identified in the early flowering lines, such as the DAPS involved in cell wall modification: actin-related protein 7-like (BnaA07T0206100WE); lipid metabolism: geranylgeranyl diphosphate reductase (BnaA08T0139800WE) and glycerol-3-phosphate dehydrogenase (BnaA05T0390300WE);

microtubule association: protein MOR1 (BnaA03T0184100WE); programmed cell death: metacaspase-1 (BnaA09T0642600WE); and mRNA splicing: probable splicing factor 3A subunit 1 (BnaA06T0094600WE). All these DAPs showed a high abundance at night than in morning indicating that they might also contribute to the earliness of flowering (Supplementary Table S4.4a).

4.4 Discussion

Proteomics analysis serves the information on proteins which are translated from the genes. This is one of the best methods for comprehending the expression of genes globally and their functional mechanisms. Moreover, post-translational modifications (PTMs) are explained by studying proteins, which also yields information crucial to comprehending the biological function of an organism (reviewed in Eldakak et al., 2013). Several proteomic studies have been conducted on flowering time in various crops (Li et al., 2017; Krahmer et al., 2018; Seaton et al., 2018) but very few studies have been carried out on *B. napus* to understand the molecular mechanism of flowering time at protein level (Mao et al., 2021).

In this study, we used bulks of seven early and seven late flowering lines from an advanced generation population of *B. napus* × *B. oleracea* interspecific cross (Nikzad et al. 2019, 2023) to understand the molecular basis of flowering time variation resulting from photosensitivity. To the best of our knowledge, this is the first study on protein expression between early and late flowering spring *B. napus* lines grown under a short-day photoperiod condition. We found that the average number of DEPs increased by 1.4 times in morning than at night, and the analyses showed that the expression of certain proteins related to flowering, stress, reactive oxygen species, hormone, photosynthesis, pigment, photorespiration, ion binding, mRNA binding and protein binding significantly varied between the early and late flowering lines suggesting that they could be the

primary cause of flowering time variation. Additionally, we identified the proteins that are involved in mRNA splicing, microtubule association, cell wall modification, programmed cell death and lipid metabolism resulting in the early flowering phenotype. It was also observed that most of the DAPs showed an increased abundance at night as compared to their abundance in morning in the early flowering lines. Mao et al. (2021) also reported that stress and phytohormone related proteins can contribute to early flowering in *B. napus*, and Seaton et al. (2018) reported that the proteins related to photosynthesis contribute to photoperiod-responsive flowering in *Arabidopsis*. While working with maize, Li et al. (2017) demonstrated that dark or light responsive proteins to be involved in photoperiod response. Thus, some of the results from this study agree with the results reported by different researchers.

Flowering related

A few putative proteins were identified such as SNW/SKI-interacting protein (SKIP, BnaA07T0363300WE), zinc finger CCCH domain-containing protein 33 (ZFN1, BnaA01T0321600WE), 14-3-3-like protein GF14 omega (GRF2, BnaA07T0117400WE), protein plastid transcriptionally active 12 (HEMERA, BnaA05T0099100WE), and uncharacterized protein containing SAM domain (Sterile alpha motif, BnaA06T0154500WE) in early flowering lines which might contribute to the earliness of flowering, and proteins like protein transport protein SEC31 homolog B (SEC31B, BnaC08T0369400WE), eukaryotic peptide chain release factor subunit 1 (ERF1, BnaA08T0254500WE), ankyrin repeat domain-containing protein 2 (AKR2, BnaC01T0012600WE), receptor-like protein kinase FERONIA (FER, BnaA01T0125700WE) and cullin-1 (CUL1, BnaA03T0280800WE) in late flowering lines might contribute to the lateness of flowering.

Ski-interacting protein (SKIP) is a splicing factor involved in post-transcriptional regulation of circadian clock genes such as *PSEUDORESPONSE REGULATOR7* (*PRR7*) and *PSEUDORESPONSE REGULATOR9* (*PRR9*) in *A. thaliana* (Wang et al., 2012). It is also required for the splicing of the *serrated leaves and early flowering* (*SEF*) pre-messenger RNA (*mRNA*) and suppresses flowering by activating the *FLOWERING LOCUS C* (*FLC*). However, it has also been observed that alternative splicing of *SEF* can be regulated by SKIP and mutation in SKIP can confer early flowering phenotype under both long- and short-day photoperiod conditions (Cui et al., 2017). In this study, we found an increased abundance of the SKIP protein at night as compared to morning in the early flowering lines (Supplementary Table S4.4a); this indicates that alternative splicing might have contributed to the earliness of flowering in this population. Another protein ZFN1 or ZF1, which is a zinc finger CCCH domain-containing protein, reported to be promoting or delaying flowering in *A. thaliana* (Liu et al., 2022; Chao et al., 2014). Xu et al. (2015) reported that GRF proteins (growth regulatory factors) promote flowering in maize by repressing DELLA protein regulated through GA pathway under both under long and short-day conditions. HEMERA is known as light dependent *PHYA* gene signal transduction component, which is required for proteolysis of the photoperiod flowering gene *PHYA* (Chen et al., 2010). In this study, an increased abundance of SKIP, ZFN1, HEMERA, GRF7 was found at night than in morning where GRF2 showed an opposite pattern in the early flowering lines, i.e. increased in abundance at morning compared to night (Supplementary Table S4.4a) indicating their possible role in early flowering. An uncharacterized protein that contains SAM domain, which is also known as sterile alpha motif, showed the same pattern in their abundance as the above-mentioned proteins (Supplementary Table S4.4a). Proteins containing the SAM domain can result in oligomerization of the transcription factors (TFs) that are essentially needed for function of the master floral regulatory

gene *LEAFY (LFY)*. In this case, oligomerization of the TFs increases the sharpness of expression of the *LEAFY* gene which empowers the expression of the target genes of *LEAFY* with high affinity (*AP1*, *APETALA1*) as well as the genes having low affinity such as *AP3* (*APETALA3*), *AG* (*AGAMOUS*) and *TFL1* (*TERMINAL FLOWER1*). This demonstrated the possible role of SAM in floral development in *B. napus* (Sayou et al., 2016).

On the contrary, SEC31B protein interacts with *SUPPRESOR OF FRIGIDA 4 (SUF4)* and activates the expression of *FLC* and thus repress flowering in *A. thaliana* (Yang et al., 2023); overexpression of ERF1 protein reported to delay flowering in *A. thaliana* (Zhou et al., 2010); FER positively regulates flowering by repressing the *FLC* but mutation in this gene can also delay flowering (Wang et al., 2020a); AKR2 also positively regulate flowering by regulating the *FT* but upregulation of negative regulator genes such as *RGA*, *FRI* or *FLC* can lead to delay flowering in *Arabidopsis* (Tang et al., 2022); CUL1 can enhance *FLC* expression resulting in delayed flowering (Wang et al., 2020b). In this study, we found an increased abundance of the SEC31B and ERF1 proteins at night while an increased in abundance FER, CUL1 and AKR2 proteins in morning in the late flowering lines (Supplementary Table S4.4b); this suggests that these proteins might play a role in delayed flowering.

Photosynthesis

Plant photosystem I-light-harvesting I super complex (PSI-LHCI) outer antenna consist of four pigmentation proteins where LHCA1 (chlorophyll a-b binding protein 6) is one of them. This light harvesting complex or antenna complex transfer light energy to middle point of PSI after capturing light (Novoderezhkin and Croce, 2022). In this study, we also found photosynthesis proteins which play a role in electron transport, pigmentation, photosystem I. We identified two chlorophyll a-b binding protein 6 (LHCA1) type proteins viz. BnaC06T0226800WE (early

flowering lines) and BnaA07T0183300WE (late flowering lines) involved in photosynthesis. Both proteins showed an increased abundance at night than in morning where BnaC06T0226800WE from the early flowering lines showed a higher AVG Log2 Ratio (3.00) at night compared to the protein (BnaA07T0183300WE) detected in the late flowering lines (0.20) at night. This indicated that these proteins might have contributed to the flowering time variation in this population. Another protein, protein proton gradient regulation 5 (PGR5) was only found in the late flowering lines with an increased abundance at night than in morning (Supplementary Table S4.4b). PGR5 is a thylakoid protein and an essential component of the photosynthesis cyclic electron transport chain of photosystem I. It helps in the formation of proper proton motif force in thylakoid membrane and provides photoprotection to plants from overreduction of acceptor side of PSI (Kappel et al., 2023). The abundance of this protein might have also contributed to late flowering phenotype in the late flowering lines.

Stress Signaling

According to Lamberto et al. (2010), TTL is involved in the synthesis of S-allantoin, which assists in the mobilization of nitrogen from the source to the sink and increases stress tolerance in plants (Kaur et al., 2021). According to Wang et al. (2009), the *FTA* gene downregulates the expression of *TTL* and improves drought tolerance. Cytochrome c proteins increases mitochondrial membrane potential, ATP content and activates target of rapamycin (TOR) pathway, which regulates the cellular processes like mRNA translation, cell division, chloroplast function, anabolic metabolism, hormone responses, and autophagy, necessary for regulating growth and metabolism in energy deficient condition (Canal et al., 2024). Eukaryotic translation initiation factor (elF) proteins were reported to give resistance to plant virus (Ferguson et al., 2021*).* Protein like cyanate hydratase, also referred to as cyanase (CYN) found in this study, reported to protect *Arabidopsis*

from potassium cyanate stress (KCNO) (Qian et al., 2011). According to Wang et al. (2024) and Isaioglou et al. (2024), heat shock proteins HSP20 and HSP90 have been shown to increase *Arabidopsis's* ability to withstand heat stress. Moreover, studies conducted in *Arabidopsis* by Isaioglou et al. (2024) revealed that HSP90 is involved in the regulation of epigenetic flowering. Huang et al. (2011) observed that stress-induced phosphoprotein was produced under stressful conditions, while Sharma et al. (2020) noted that the protein myo-inositol-1-phosphate synthase (MIPS) is recognized for its ability to withstand both biotic and abiotic stresses.

In this study, we identified the following stress related proteins viz. uric acid degradation bifunctional protein (TTL, BnaA02T0113100WE), protein farnesyltransferase type-1 subunit alpha-like (FTA, BnaA01T0114700WE) and cytochrome C-2 (CYTC-2, BnaA03T0264400WE) in the early flowering lines and these proteins showed an increased abundance at night than in morning. In case of the late flowering lines, stress related proteins eukaryotic translation initiation factor 5 (elF5, BnaA09T0348100WE), cyanate hydratase (CYN, BnaA07T0073200WE) and small heat shock protein (HSP20, BnaC05T0045900WE) showed an increased abundance at night compared to morning. However, proteins such as heat shock protein 90 (HSP90, BnaA03T0131100WE), stress-induced-phosphoprotein 1 (BnaA08T0295500WE) and myo-inositol-1-phosphate synthase (MIPS, BnaC08T0394100WE) showed an opposite pattern of abundance in late flowering lines, i.e. increased in abundance at morning than night Based on this, it is likely that these stress related proteins also contributed to flowering time variation between the early and late flowering lines.

Reactive oxygen species

To mitigate the oxidative stress and ROS homeostasis, plants have evolved a variety of enzymatic and non-enzymatic scavenging mechanisms (reviewed in Abuelsoud et al., 2020).

Antioxidants such as superoxide dismutase (SOD), glutathione-S-transferase (GST), peroxidase (PX), catalase (CAT), and other ROS-scavenging antioxidants can all help to balance the buildup of ROS in plants (for review, see Saed-Moucheshi et al., 2014). In this study, we found an increased abundance of peroxidase 42 (BnaA01T0039700WE) and glutathione S-transferase (BnaA02T0240600WE) proteins in the early flowering lines at night than in morning. On the other hand, we found an increased in abundance of thioredoxin X (BnaA06T0030100WE) and catalase-2 (BnaA08T0114000WE) proteins in the late flowering lines at night than in morning; among these, the level of abundance of catalase-2 was quite low. Other proteins such as copper chaperone for superoxide dismutase (BnaC08T0174400WE), peroxisomal membrane protein PMP22 (BnaA09T0225500WE) and protein DJ-1 homolog C-like (Bnascaffold286T0039800WE) showed an increased abundance in the late flowering lines at in morning than at night. Several researchers also reported that thioredoxin (Lu and Holmgren, 2014), peroxisomes (Pan et al., 2020) and protein DJ-1 homolog C (Lin et al., 2011) provide protection against oxidative stress. Thus, the ROS related proteins that we identified in this study might have also played role in the flowering time variation.

Phytohormone

Cagnola et al. (2018) reported that jasmonic acid dependent systemic resistance as plant defense to pathogen increased by reducing the *COP1* (*CONSTITUTIVE PHOTOMORPHOGENIC 1*) nuclear activity which negatively regulates flowering under a long day photoperiod condition than under short day photoperiod in *A. thaliana*. Wang et al. (2017) reported that jasmonic acid can delay in flowering in *A. thaliana*. However, it has also been reported that a lower concentration of jasmonic acid (≈47.5 nmol l−1) increases flowering in the photo-periodically neutral plant *Spirodela polyrrhiza* under both long- and short-day conditions (reviewed in Krajnčič et al., 2006).

In this study, we identified three proteins involved in jasmonic acid and ethylene dependent resistance, viz. inositol hexakisphosphate and diphosphoinositol-pentakisphosphate kinase (BnaA01T0331100WE), probable leucine-rich repeat receptor-like serine/threonine-protein kinase (BnaA05T0355000WE) and endochitinase CH25-like (BnaC03T0326700WE) only in the early flowering lines BnaA01T0331100WE and BnaA05T0355000WE showed an increased abundance at night than in morning. Gibberellic acid is another phytohormone, which has been reported essential for inducing flowering under short day photoperiod in *A. thaliana* (Wilson et al., 1992). In this study, we found an increased abundance (7-AVG Fold Ratio) of the protein methylmalonate-semialdehyde dehydrogenases, which is regulated by Gibberellic acid signal pathway (Tanaka et al., 2005), increased at night only in the early flowering lines (Supplementary Table S4.4a).

In case of salicylic acid (SA), we identified isochorismate synthase 2 (BnaA08T0232100WE) in the early flowering lines and patellin-1 (BnaA02T0196100WE) in the late flowering lines where both showed an increased abundance at night than in morning. SA mostly originates from isochorismate in *Arabidopsis thaliana*, which is generated from chorismate by isochorismate synthase1 (ICS1) and isochorismate synthase2 (ICS2) (Garcion et al., 2008). Patellin-1 (BnaA02T0196100WE) also reported as a salicylic acid binding protein (Manohar et al., 2015). Salicylic acid can promote flowering under both stressed and non stressed conditions and an absence of salicylic acid can result in late flowering in *A. thaliana* (Martinez et al., 2004). Here, low abundance of patellin-1 at night in late flowering lines and high abundance of isochorismate synthase 2 at night in early flowering lines might have played role in flowering time variation in the *B. napus* lines.

Pigment Related

Anthocyanins serve as sunscreens, antioxidants, metal chelators, and play a critical role in delaying leaf senescence. Plant hormones like abscisic acid, jasmonic acid, ethylene, and gibberellin can regulate anthocyanin synthesis, either positively or negatively (for review, see Li and Ahammed et al., 2023). In addition to that, the redox condition of the plastoquinone (PQ) pool within the PET (photosynthetic electron transport) chain can also influence anthocyanin biosynthesis after integrating information captured from light (for review, see Das et al., 2011). In this study, we identified three anthocyanin biosynthesis related proteins only in the early flowering lines and this included 26S proteasome non-ATPase regulatory subunit 8 homolog A (BnaA09T0130200WE), chalcone synthase (BnaC02T0452400WE) and chalcone synthase 1 (BnaC02T0452500WE); all these proteins showed an increased abundance at night compared to in morning (Supplementary Table S4.4a). It was reported by Lepistö et al. (2009) that anthocyanin accumulation can decrease under both short- and long-day photoperiod. However, in this study, we found an increased abundance of anthocyanin related proteins at night in the early flowering lines; this suggests that these proteins might have played a role in flowering time variation.

Ion binding

In this study, we found two different types of ion binding proteins in the early and late flowering lines. In the case of the early flowering lines, we identified a few copper ion binding proteins whereas in the late flowering lines we identified a few cobalt ion binding proteins.

Copper (Cu), a vital cofactor for metalloproteins involved in photosynthetic electron transport and oxidative stress response, is highly demanded by plant chloroplasts. It is a component of plastocyanin (Pc), a significant Cu-protein that helps with electron transport during

the first steps of photosynthetic processes. Furthermore, Cu is a component of the stroma-based Cu/Zn-superoxide dismutase (Cu/Zn-SOD), which guards against reactive oxygen species (ROS) produced during photosynthesis. While too much Cu can be harmful and hinder photosynthesis, too little Cu causes chlorosis and lowers photosynthetic activity in developing leaves (for review, see Yruela, 2013). In *A. thaliana*, *SQUAMOSA PROMOTER BINDING-LIKE7* (*SPL7*) facilitates the activation of miR398 under Cu deficit. Degradation of the mRNA encoding copper/zinc superoxide dismutase is aided by miR398. Other microRNAs that assist in controlling the amount of copper in plants, such as miR397, miR408, and miR857, are also activated by *SPL7* (Yamasaki et al., 2009). *SPL7* is a flowering time gene which is involved in aging pathway, and it was reported to promote flowering through directly upregulating the *SEPALLATA3* (*SEP3*) (Gou et al., 2019). Furthermore, it was also reported that copper deficiency can cause delay in flowering which is associated with reduced expression of *FLOWERING LOCUS T* (*FT*) (Rahmati and Vatamaniuk, 2020). In this study, we found the proteins involved in copper ion binding viz. lysyl-tRNA synthetase (BnaA06T0023200WE), methylmalonate-semialdehyde dehydrogenase (BnaC09T0113500WE), plastocyanin-like (BnaC08T0214300WE) and cytochrome C-2 (BnaA03T0264400WE) in the early flowering lines where only lysyl-tRNA synthetase (BnaA06T0023200WE) showed an increased abundance in morning compared to night; abundance of the other three proteins increased at night than morning. All these copper binding proteins might have played a role in early flowering.

In this study, we found a cobalt ion binding proteins viz. 2-oxoglutarate dehydrogenase (BnaA09T0188600WE) in the late flowering lines and this protein showed an increased abundance at night than in morning. This protein has been reported to delay flowering upon increased

expression while its reduced activity shows early flowering phenotype in tomato (Araújo et al., 2012). Thus, it is probable that BnaA09T0188600WE played a role in the lateness of flowering.

In conclusion, to our knowledge, this is the first proteome-level study using spring *B. napus* lines carrying genome contents of *B. oleracea* to identify proteins involved in flowering time variation. Our findings showed that the variation in flowering time can be caused by a variety of factors including photosynthesis, ROS, stress-related proteins, phytohormones, pigmentation proteins, ion binding proteins, and possibly crosstalk between them. This study also provided evidence for the involvement of proteins associated to protein binding, glucosinolate biosynthesis, lipid metabolism, cell wall modification, programmed cell death, and mRNA binding and splicing in flowering time. Thus, the findings of this study provide a deeper understanding of the putative proteins involved in flowering time variation and set the base for functional validation of the genes and proteins involved in flowering time; the knowledge can eventually be used in molecular breeding to manipulate flowering time in the breeding of *B. napus* canola cultivars.

Chapter 5. General Discussion and Conclusion

5.1 General Discussion

Brassica oilseed is the world's second largest oilseed crop, following soybean (USDA, 2022). Canada is one of the leading producers of canola, where Saskatchewan accounting for most of the production recently, followed by Alberta and Manitoba, with respective contributions of 49%, 32%, and 18% of the total production (Canola Council of Canada, 2023). Oil of this crop is mainly used for edible purposes; however, biodiesel also be produced from this oil (Qian et al., 2013). In addition to this, it is also a good source of protein for animal feed (Ivanova et al., 2016).

Flowering time is one of the most important traits that play a role in crop evolution, domestication, and local adaptation, and the trait in canola is controlled by a variety of genes and influenced by environment (Raman et al., 2013). The times of flowering and maturity are one of the most important traits for growing spring canola in North America (Rahman et al., 2018).Quantitative trait loci (QTL) mapping has been carried out by several researchers to understand the genetic control of flowering time in *B. napus* (Qu et al., 2022; Nikzad et al., 2023; Rahman et al., 2017; Rahman et al., 2018). Among different mapping population, NILs are considered as a good material for investigating the effect of a gene or QTL on the trait as well as to understand the genetic and molecular basis of the trait, because NILs share a common genetic background but differ for only a small genomic region or for the alleles of the gene of interest (Castro et al., 2010; Mia et al., 2019; Wang et al., 2019). As a result, NILs may be employed for accurate mapping of a QTL, identification of candidate genes for days to flowering, and for the development of genetic markers for use in breeding. This thesis research was focused on evaluating two *B. napus* NIL populations to investigate the effect flowering time QTL on other morphological traits under a short-day photoperiod (10 hour) condition. These two sets of NILs were developed

for flowering time QTL located on C1 and C9 chromosome through recurrent backcrossing using cv. Hi-Q as the recurrent parent and the early flowering canola line RIL-144 as the donor parent where the early-flowering alleles have been introgressed into *B. napus* from *B. oleracea* (Gill, 2021). It was expected that these NILs only vary for days to flowering; however, I found that the NILs also varied for other morphological traits such as plant height, shoot and root biomass. However, there was no difference in terms of leaf characteristics among the NILs as well as with the recurrent parent Hi-Q. This difference might be due to pleiotropic effect of the flowering time QTL on other traits or co-localization of multiple QTL in the same genomic region.

Brassica genomes evolved from an ancestral genome through polyploidization; hence, multiple copies of a gene can be found in *Brassica* species (Axelson et al., 2001). Therefore, there is plethora of chances to occur variation in gene expression resulting in wide phenotypic variation for traits including variation for flowering time (for review, see Osborn, 2004). To date, several gene expression studies have been conducted to reveal the molecular basis of flowering time variation (Jones et al., 2018; Huang et al., 2019; Kim et al., 2013). For example, Schiessl et al. (2019) studied nine copy of the *B. napus* flowering gene *FLC* and found differential expression between winter and spring growth habit types only for three copies (*Bna.FLC.A03b, Bna.FLC.A10 and Bna.FLC.C02*). My thesis research also included expression analysis of putative flowering time genes from C5 and C9 QTL associated with flowering using advanced generation inbreed *B. napus* population developed by Nikzad et al. (2019, 2023) where some of the lines of *B. napus* × *B. oleracea* var. capitata cv. Bindsachsener cross flowered earlier than the *B. napus* parent under a 10-hr photoperiod condition (Gill 2021). Seven early-flowering and seven late flowering lines of this interspecific cross were used as bulk namely early flowering bulk and late flowering bulk. I studied expression of 30 putative flowering genes from C5 (15) and C9 (15) QTL in leaf and shoot

apex tissue in the early and late flowering lines and found a greater expression for a greater number of genes at night than in morning. This included the *CO*, *PHYA*, *FT*, *AP2*, *SOC1* and *TEM1* which might be responsible for flowering time variation between early and late flowering lines, and these genes were also reported to be controlling flowering time (Suárez-López et al., 2001; Valverde et al., 2004; Würschum et al., 2006; Moon et al., 2005; Castillejo and Pelaz, 2008). Furthermore, this also confirms that C5 and C9 QTL regions are involved in the control of flowering time.

Proteome analysis has become a valuable technique for understanding plant gene function (for review, see Canovas et al., 2004). Till now, several proteomic studies have been conducted on flowering time in various crops (Li et al., 2017; Song et al., 2019; Seaton et al., 2018) but very few studies have been carried out in *B. napus* (Mao et al., 2021). While genomics provides an understanding of the genes, their location and expression pattern, proteomics provide an understanding of the products of the genes produced by the cell; thus, the biological engines that sustain life are the proteins (Humphery-Smith et al., 1997). Therefore, an understanding of the protein expression or abundance, composition, interactions, and changes at different time points are important to extend our knowledge of the molecular mechanisms underlying the plant phenotypes. For a comparative proteomic study to understand the molecular mechanism behind the flowering time variation at proteome level, I used the leaf tissue of from early and late flowering lines used in the gene expression analysis chapter. From this thesis research, I found information on putative proteins involved in flowering, photosynthesis, pigmentation, phytohormone signaling, stress response, ROS, ion binding, mRNA binding, protein binding and many other proteins related to flowering time variation that could be responsible for early and late flowering in spring *B. napus.* Certain results of this study align with those of previous studies: stress and phytohormone-related proteins may facilitate early flowering in *Brassica napus* (Mao

et al., 2021); photosynthesis-related proteins may contribute to photoperiod-responsive flowering in *Arabidopsis* (Seaton et al., 2018); and photoperiod response in maize (Li et al., 2017) involves light- or dark-responsive proteins.

5.2 Conclusion

In conclusion, this thesis research resulted in the following outcomes: -

- ✓ The genomic regions of C1 and C9 chromosome affecting flowering time also affects other traits such as plant height and plant biomass, which might be due to pleiotropic effect or co-localization of multiple QTL in the same genomic region.
- ✓ Identified flowering time genes from C5 and C9 chromosomes through expression analysis of the genes under a short-day condition; this also confirms that the C5 and C9 QTL affects flowering time in spring *B. napus* under short-day condition.
- ✓ Identified several proteins that might affect flowering time variation in *B. napus*.

5.3 Future Research

- ✓ The NILs carrying flowering time QTL located on C1 and C9 chromosomes can be used for fine mapping of this trait.
- ✓ The flowering time genes identified from the C5 and C9 chromosomes can be used in overexpression or knock out studies such as functional characterization through transformation of *A. thaliana* to confirm the exact role of these genes.
- ✓ Functional validation of the putative proteins identified from this study need to be carried out to elucidate their involvement in flowering time variation in *B. napus.*

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Appendices

Appendix 2-1. Analysis of variance for different morphological traits

Days to flower					
Source of variation	Df	Sum of square	Mean Square	F value	Pr (>F)
NILs	5	19071	3814	69.48	<2e-16***
Residuals	103	5654	55		
Plant height					
NILs	5	10537	2107.4	49.56	<2e-16***
Residuals	103	4380	42.5		
Shoot biomass					
NILs	5	124.9	24.98	34.11	<2e-16***
Residuals	103	75.44	0.732		
Root biomass					
NILs	5	18.374	3.675	55.45	<2e-16***
Residuals	103	6.826	0.066		

Appendix 2-2. Least square mean and S.E. (±) of the four groups of *Brassica napus* near-isogenic lines (NILs) HiQ-HiQ:C1, HiQ-Ole:C1, HiQ-HiQ:C9 and HiQ-Ole:C9 and their recurrent parent (RP) Hi-Q and donor parent (DP) RIL144 for days to flowering, plant height, shoot biomass, and root biomass. The populations were grown at 10 photoperiod and 20 °C constant temperature conditions. HiQ-Hi-Q:C1 = NILs carrying Hi-Q-allele of C1 QTL; HiQ-Ole:C1 = NILs carrying the C1 QTL allele of RIL-144; HiQ-HiQ:C9 = NILs carrying the C9 QTL allele of Hi-Q; HiQ-Ole:C9 = NILs carrying the C9 QTL allele of RIL-144.

NILs group	**No. of NIL families**	**Days to flower**		**Plant height (cm)**		**Shoot biomass (g)**		**Root biomass (g)**	
		Range	**Mean± SE**	**Range**	**Mean± SE**	**Range**	**Mean± SE**	**Range**	**Mean± SE**
Hi-Q	1	87.9-102.6	95.2 ± 3.7	41.7-54.6	48.2± 3.26	3.909-5.61	4.76± 0.428	1.725-2.235	1.98±0.1287
RIL-144	1	48.2-62.8	55.5± 3.7	21.5-34.4	27.9± 3.26	0.696-2.39	1.54±0.428	0.262-0.773	0.517±0.1287
HiQ-HiQ:C1	10	90.9-95.6	93.2± 1.19	63.4-67.5	65.4± 1.04	5.494-6.04	5.77±0.137	1.95-2.114	2.032±0.0412
HiQ-Ole:C1	7	67.9-73.7	70.8± 1.45	52.8-57.8	55.3± 1.28	3.259-3.92	3.59±0.168	1.114-1.314	1.214±0.0505
HiQ-HiQ:C9	8	98.3-103.5	100.9± 1.31	50.3-54.8	52.5± 1.15	4.41-5.01	4.71±0.151	1.346-1.527	1.436±0.0455
HiQ-Ole:C9	1	69.2-83.8	76.5± 3.7	20.9-33.9	27.4± 3.26	2.341-4.04	3.19±0.428	0.795-1.305	1.05±0.1287

Appendix 2-3. Coefficients values for different traits after principal component analysis (PCA) for the four components

Variables	PC1	PC2	PC3	PC4
Days to flower	-0.42	-0.89	-0.18	0.01
Plant height	-0.48	0.39	-0.79	0.04
Shoot biomass	-0.55	0.17	0.38	-0.72
Root biomass	-0.54	0.17	0.45	0.69

Appendix 3-1. List of 30 flowering gene specific qRT-PCR primer

Chromosome	Gene symbol	Gene ID	Description / other designations	Position (bp)	Sequence (Forward)	GC %	Tm (°C)	Sequence (Reverse)	Tm (°C)	Product Size (bp)
C05	*SOC1*	*BnaC05g21200D*	*SUPPRESSOR OF OVEREXPRESSION OF CO1*	14940965 to 14943819	AATTCATGCTCGACCAGCTC	50	60.4	TTGAGTTGGAGTGGCATCTG	59.8	115
C05	*CO*	*BnaC05g22130D*	*CONSTANS*	15999676 to 16000398	CGTTGGATCACCATCATCAG	50	59.9	TGTCTCCATGCTCTTGCATC	60	85
C05	*FT*	*BnaC05g25360D*	*FLOWERING LOCUS T*	20195173 to 20195580	TCCTTGTAGCCCTGCTTTTG	50	60.4	AAGCCCAACCAACCTTGTAG	59.1	146
C05	*GI*	*BnaC05g26340D*	*GIGANTIA*	22348141 to 22350766	AGTCGGTTGAAGGACAATGC	50	60.1	TGATTCGAGCCAACTGTGTC	59.8	86
C05	*AP2*	*BnaC05g23110D*	*APETALA 2*	17200884 to 17201714	AGCTTGCCACACAAGAAACC	50	60.3	CCATCCATTTCGAGCTAACC	59.5	128
C05	*PHYB*	*BnaC05g36390D*	*PHYTOCHROME B*	35604584 to 35609052	TTTCTTGCAGATCCCGAGTC	50	60.3	CTCTTTCGCCTTCGTGAAAC	60	88
C05	*PHYA*	*BnaC05g36120D*	*PHTOCHROME A*	35297575 to 35299544	TTTGCGCAGTCTGTATGCTC	50	60.2	CCTCCATCACCATTTTCACC	60.2	108
C05	*CDF1*	*BnaC05g24380D*	*CYCLING DOF FACTOR*	18760033 to 18761546	ATTGGATAGCTGGTGGAACG	50	60	AAGATCAGCGGTTCTTGCAG	60.5	144

Chromosome	Gene symbol	Gene ID	Description / other designations	Position (bp)	Sequence (Forward)	GC %	Tm (°C)	Sequence (Reverse)	Tm (°C)	Product Size (bp)
C05	*SPA*	*BnaC05g38270D*	*SUPPRESSOR OF PHYA-105*	37058879 to 37062179	CTTTCCTTGAGGGTTTGTGC	50	59.7	GCGAAAAACTCTCCATCTCG	60	136
C05	*SPA*	*BnaC05g30140D*	*SUPPRESSOR OF PHYA-105*	29180958 to 29182927	TGAAGTGTCGTCGTTTGAGG	50	59.9	AAGCTGAGCGGAAGATTGAC	59.6	124
C05	*SPL*	*BnaC05g35380D*	*SQUAMOSA PROMOTER BINDING PROTEIN LIKE*	34659757 to 34659968	TTTTGCCTCTCCCCTTTCTC	50	60.4	GGTGAATTTCTGGGTGGAAG	59.4	71
C05	*SVP*	*BnaC05g33070D*	*SHORT VEGETATIVE PHASE*	32541446 to 32542768	TAGTGAAGCCCGTCGAAATG	50	61.2	TCTTCCCACCAATTCTCAGG	60	147
C05	*TEM2*	*BnaC05g41640D*	*TEMPRANILLO 2*	39284968 to 39287221	ATGAGTCGGGAAAGTCTTGG	50	59.1	TGAGGTGCTTGTCTTTGACG	60	105
C05	*TOE2*	*BnaC05g32750D*	*TARGET OF EAT2*	32237515 to 32244460	TGCCAGAAGGTATGGTTTCC	50	59.9	AACTGATGGAGGATCGCAAC	60.1	84
C05	*PHYA*	*BnaC05g45140D*	*PHYTOCHROME A*	41108924 to 41110567	CGCGGAATGTAGGATTGAAG	50	60.6	CAGCACCAAAACCAACACTG	60.2	128
C09	*SOC1*	*BnaC09g42060D*	*SUPPRESSOR OF OVEREXPRESSION OF CO1*	43784336 to 43787341	TGGAACGGTACCAGAAATGC	50	60.9	ATCCTCCCCAAGAAGATTCC	59.3	149
C09	*SOC1*	*BnaC09g22090D*	*SUPPRESSOR OF OVEREXPRESSION OF CO1*	19226813 to 19228894	GCAATGTAGCATGGCAGATG	50	60.3	TCTGGGATGTTGTTGCAGAG	59.8	75
C09	*TOE2*	*BnaC09g35430D*	*TARGET OF EAT2*	38788784 to 38792270	TGGAAGGAACTCCAAGATGG	50	60	GGTGAAAAGAACGCAGAAGG	59.9	126
C09	*CO*	*BnaC09g41980D*	*CONSTANS*	43739067 to 43740068	AGATGGGGTTGTTCCACTTC	50	58.8	AATTGGGGAAGCTTCTGAGG	60.6	109
C09	*GI*	*BnaC09g38380D*	*GIGANTIA*	41273280 to 41275833	GAGCACGGCCTGAAAATTAG	50	59.8	ACCAGCAAGTCCTCCAAATG	60.1	84
C09	*LFY*	*BnaC09g33870D*	*LEAFY*	37098616 to 37099569	CCAAAACAGGGGTTGATGAC	50	60.2	CAACGAACTCGATGCTGAAG	59.6	116
C09	*LFY*	*BnaC09g21610D*	*LEAFY*	18807332 to 18809352	ACGGTCAGATCCAAGATTCG	50	60.1	TCGGTTTTGATCCAGGAGAC	60	89
C09	*FKF1*	*BnaC09g43510D*	*FLAVIN-BINDING, KELCH REPEAT, F-BOX 1*	44783028 to 44785881	CCTGTTTCCACCATTCCAAC	50	60.2	TGGAACGTTTGGCTAGAACC	60.1	70
C09	*SOC1*	*BnaC09g29450D*	*SUPPRESSOR OF OVEREXPRESSION OF CO1*	32089088 to 32091133	CAGCAAAGAATGCTGGTGTC	50	59.4	TGGCTCCTTTCTCAACAACC	60.2	129
C09	*PHYB*	*BnaC09g37430D*	*PHYTOCHROME B*	40709757 to 40719200	TTACCAGCGTTGAAGGAACC	50	60.1	CTATCCTGAATGCCGAAAGG	59.7	76
C09	*TEM1*	*BnaC09g49240D*	*TEMPRANILLO 1*	47732744 to 47734847	AACAGCACGCGGAGAAATAC	50	60.3	AGCACTTGCCTAACTCGTCTTC	60.1	108
C09	*COP1*	*BnaC09g27790D*	*CONSTITUTIVE PHOTOMORPHOGENIC 1*	29654647 to 29662362	TTACACCGGAAACGTTAGGC	50	60	TTCATCTCACGGTGCTCTTG	60	126
C09	*CRY1*	*BnaC09g25510D*	*CHRYPTOCHROME 1*	25053375 to 25056584	TAGGCCGTCCTTTCTCAATG	50	60.2	TATCCGCAACACACTTGGAC	59.6	132

Chromosome	Gene symbol	Gene ID	Description / other designations	Position (bp)	Sequence (Forward)	GC %	Tm (℃)	Sequence (Reverse)	Tm (℃)	Product Size (bp)
C09	*CRY2*	*BnaC09g23430D*	*CRYPTOCHROME 2*	20936480 to 20940278	TCGTTATCGTTGAGGGCTTC	50	60.2	AACACGGCTCTGTTTCCATC	60.1	70
C09	*SPA*	*BnaC09g26140D*	*SUPPRESSOR OF PHYA-105*	25996302 to 26001334	ACTATCCAGCCGTGGAAATG	50	60	AAATGGCCTGTCCTGTTGTC	60	138

Appendix 3-2. List of the flowering genes found in *Brassica napus* chromosomes C5 and C9 through BLAST search

Gene symbol	Annotation	Pathway/types of gene	Reference	Arabidopsis orthologue	Chromosome	Gene (*B.napus*)	Position (bp)
LHP1	*LIKE HETEROCHROMATIN PROTEIN 1*	Vernalization	Mylne et al., 2006	*AT5G17690*	C05	*BnaC05g16580D*	10373397 to 10374161
SPL	*SQUAMOSA PROMOTER BINDING PROTEIN-LIKE*	Photoperiod, Gibberellin	Jung et al., 2012	*AT2G47070*	C05	*BnaC05g17330D*	11038842 to 11041873
SOC1	*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*	Floral integrator	Moon et al., 2005	*AT2G45660*	C05	*BnaC05g17630D*	11372772 to 11373492
GI	*GIGANTEA*	Photoperiod	Fornara et al., 2009	*AT1G22770*	C05	*BnaC05g18020D*	11778931 to 11784461
VRN2	*VERNALIZATION 2*	Vernalization	Trevaskis et al., 2007	*AT4G16845*	C05	*BnaC05g18110D*	11831647 to 11837178
PHYB	*PHYTOCHROME B*	Photoperiod	Bagnall et al., 1995	*AT2G18790*	C05	*BnaC05g19520D*	13006374 to 13006805
SPA	*SUPPRESSOR OF PHYA-105*	Photoperiod	Laubinger et al., 2006	*AT1G53090*	C05	*BnaC05g20060D*	13557370 to 13562918
SPA	*SUPPRESSOR OF PHYA-105*	Photoperiod	Laubinger et al., 2006	*AT2G46340*	C05	*BnaC05g20180D*	13619977 to 13621514
TEM1	*TEMPRANILLO 1*	Photoperiod	Osnato et al., 2012	*AT1G25560*	C05	*BnaC05g20560D*	13975457 to 13976740
TEM2	*TEMPRANILLO 2*	Photoperiod	Osnato et al., 2012	*AT1G68840*	C05	*BnaC05g20560D*	13975457 to 13976740
ap2	*APETALA 2*	Photoperiod	Yant et al., 2010	*AT4G36920*	C05	*BnaC05g20830D*	14379243 to 14382230
AGL24	*AGAMOUS LIKE 24*	Floral integrator	Adal et al., 2021	*AT4G24540*	C05	*BnaC05g21200D*	14940965 to 14943819
ap1	*APETALA 1*	Floral meristem identity	Mandel and Yanofsky, 1995	*AT4G24540*	C05	*BnaC05g21200D*	14940965 to 14943819
SOC1	*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*	Floral integrator	Moon et al., 2005	*AT2G45660*	C05	*BnaC05g21200D*	14940965 to 14943819
SVP	*SHORT VGETATIVE PHASE*	Temperature	Lee et al., 2007	*AT2G22540*	C05	*BnaC05g21200D*	14940965 to 14943819
CDF1	*CYCLING DOF FACTOR1*	Photoperiod	Fornara et al., 2009	*AT5G62430*	C05	*BnaC05g22130D*	15999676 to 16000398
CO	*CONSTANS*	Photoperiod	Kobayashi et al., 1999	*AT5G15840*	C05	*BnaC05g22130D*	15999676 to 16000398
ap2	*APETALA 2*	Photoperiod	Yant et al., 2010	*AT4G36920*	C05	*BnaC05g23110D*	17200884 to 17201714

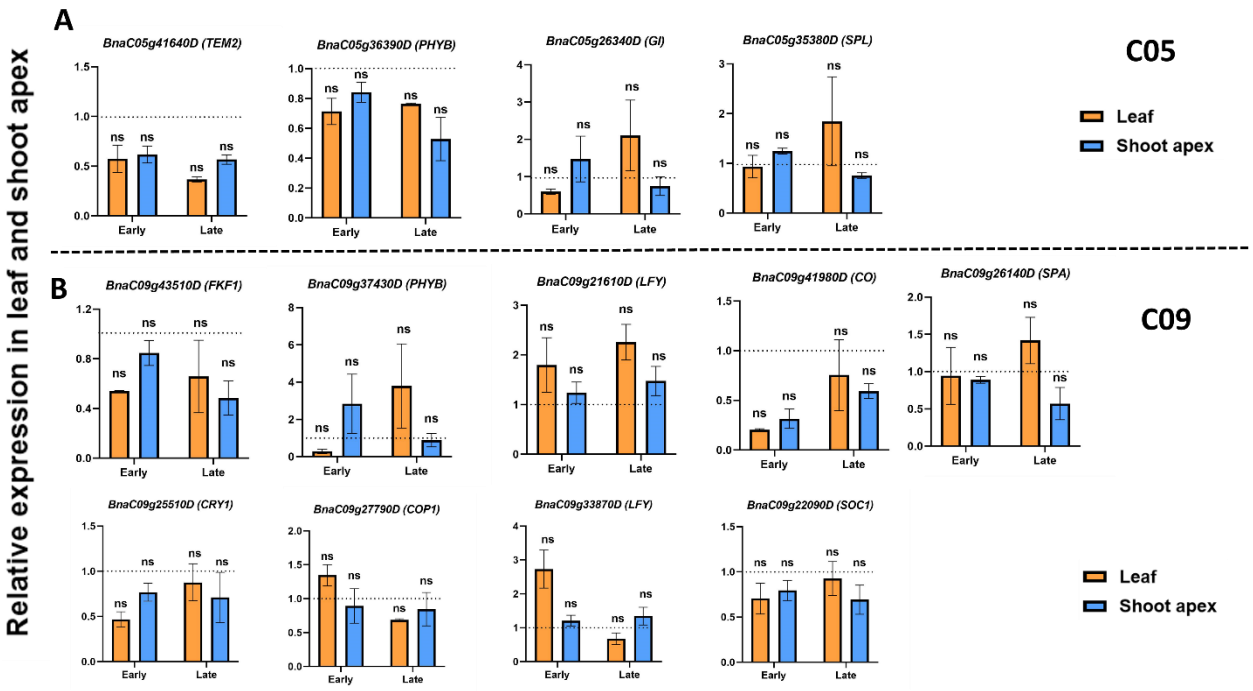
Gene symbol	Annotation	Pathway/types of gene	Reference	Arabidopsis orthologue	Chromosome	Gene (*B.napus*)	Position (bp)
TPS1	*TREHALOSE 6 PHOSPHATE SYNTHASE 1*	Photoperiod	Zhang et al., 2022	*AT1G78580*	C05	*BnaC05g24260D*	18576054 to 18579003
CDF1	*CYCLING DOF FACTOR1*	Photoperiod	Fornara et al., 2009	*AT5G62430*	C05	*BnaC05g24380D*	18760033 to 18761546
FT	*FLOWERING LOCUS T*	Floral integrator	Kobayashi et al., 1999	*AT1G65480*	C05	*BnaC05g25360D*	20195173 to 20195580
GI	*GIGANTEA*	Photoperiod	Fornara et al., 2009	*AT1G22770*	C05	*BnaC05g26340D*	22348141 to 22350766
RGA	*REPRESSOR OF GA1-3*	Gibberellin	Dill and Sun, 2001	*AT2G01570*	C05	*BnaC05g27070D*	23700003 to 23707751
GAI	*GIBBERELLIC ACID INSENSITIVE*	Gibberellin	Park et al., 2013	*AT1G14920*	C05	*BnaC05g27070D*	23700003 to 23707751
VRN1	*VERNALIZATION 1*	Vernalization	Trevaskis et al., 2007	*AT3G18990*	C05	*BnaC05g29990D*	28995901 to 28996885
SPA	*SUPPRESSOR OF PHYA-105*	Photoperiod	Laubinger et al., 2006	*AT4G11110*	C05	*BnaC05g30140D*	29180958 to 29182927
FY	-	Autonomous	Simpson et al., 2003	*AT5G13480*	C05	*BnaC05g30160D*	29190080 to 29190810
TOE2	*TARGET OF EAT 2*	Photoperiod	Du et al., 2020	*AT5G60120*	C05	*BnaC05g32750D*	32237515 to 32244460
FVE	-	Autonomous	Ausín et al., 2004	*AT2G19520*	C05	*BnaC05g32830D*	32328305 to 32333049
SVP	*SHORT VGETATIVE PHASE*	Temperature	Lee et al., 2007	*AT2G22540*	C05	*BnaC05g33070D*	32541446 to 32542768
VRN1	*VERNALIZATION 1*	Vernalization	Trevaskis et al., 2007	*AT3G18990*	C05	*BnaC05g34020D*	33561782 to 33564054
VRN1	*VERNALIZATION 1*	Vernalization	Trevaskis et al., 2007	*AT3G18990*	C05	*BnaC05g34030D*	33564807 to 33566251
VRN1	*VERNALIZATION 1*	Vernalization	Trevaskis et al., 2007	*AT3G18990*	C05	*BnaC05g34060D*	33574236 to 33576921
VIN3	*VERNALIZATION INSENSITIVE 3*	Vernalization	Kyung et al., 2022	*AT5G57380*	C05	*BnaC05g34130D*	33614589 to 33618342
SPL	*SQUAMOSA PROMOTER BINDING PROTEIN-LIKE*	Photoperiod, Gibberellin	Jung et al., 2012	*AT2G47070*	C05	*BnaC05g35380D*	34659757 to 34659968
PHYB	*PHYTOCHROME B*	Photoperiod	Bagnall et al., 1995	*AT2G18790*	C05	*BnaC05g36030D*	35223202 to 35225149
PHYA	*PHYTOCHROME A*	Photoperiod	Bagnall et al., 1995	*AT1G09570*	C05	*BnaC05g36120D*	35297575 to 35299544
VIN3	*VERNALIZATION INSENSITIVE 3*	Vernalization	Kyung et al., 2022	*AT5G57380*	C05	*BnaC05g36130D*	35300425 to 35301968
PHYA	*PHYTOCHROME A*	Photoperiod	Bagnall et al., 1995	*AT1G09570*	C05	*BnaC05g36390D*	35604584 to 35609052
PHYB	*PHYTOCHROME B*	Photoperiod	Bagnall et al., 1995	*AT2G18790*	C05	*BnaC05g36390D*	35604584 to 35609052
SPA	*SUPPRESSOR OF PHYA-105*	Photoperiod	Laubinger et al., 2006	*AT2G46340*	C09	*BnaC05g37670D*	36601942 to 36603206
CSTF64	*CLEAVAGE STIMULATING FACTOR 64*	Autonomous	Liu et al., 2010	*AT1G71800*	C09	*BnaC05g38030D*	36760699 to 36762976
SPA	*SUPPRESSOR OF PHYA-105*	Photoperiod	Laubinger et al., 2006	*AT3G15354*	C05	*BnaC05g38270D*	37058879 to 37062179
DHF	*DAY NEUTRAL FLOWERING*	Photoperiod	Liu et al.,, 2010	*AT3G19140*	C05	*BnaC05g39320D*	37867378 to 37867917
TEM1	*TEMPRANILLO 1*	Photoperiod	Osnato et al., 2012	*AT1G25560*	C05	*BnaC05g41640D*	39284968 to 39287221
TEM2	*TEMPRANILLO 2*	Photoperiod	Osnato et al., 2012	*AT1G68840*	C05	*BnaC05g41640D*	39284968 to 39287221
SPY	*SPINDLY*	Photoperiod	Tseng et al., 2004	*AT3G11540*	C05	*BnaC05g41670D*	39298853 to 39304416

Gene symbol	Annotation	Pathway/types of gene	Reference	Arabidopsis orthologue	Chromosome	Gene (*B.napus*)	Position (bp)
SPA	*SUPPRESSOR OF PHYA-105*	Photoperiod	Laubinger et al., 2006	*AT3G15354*	C05	*BnaC05g44360D*	40745132 to 40746882
PHYB	*PHYTOCHROME B*	Photoperiod	Bagnall et al., 1995	*AT2G18790*	C05	*BnaC05g44830D*	40959659 to 40962677
PHYA	*PHYTOCHROME A*	Photoperiod	Bagnall et al., 1995	*AT1G09570*	C05	*BnaC05g45140D*	41108924 to 41110567
DHF	*DAY NEUTRAL FLOWERING*	Photoperiod	Liu et al.,, 2010	*AT3G19140*	C05	*BnaC05g45200D*	41138340 to 41142595
VRN1	*VERNALIZATION 1*	Vernalization	Trevaskis et al., 2007	*AT3G18990*	C05	*BnaC05g45830D*	41597253 to 41600516
GAI	*GIBBERELLIC ACID INSENSITIVE*	Gibberellin	Park et al., 2013	*AT1G14920*	C05	*BnaC05g46410D*	41905712 to 41910737
RGA	*REPRESSOR OF GA1-3*	Gibberellin	Dill and Sun, 2001	*AT2G01570*	C05	*BnaC05g46410D*	41905712 to 41910737
GID1A	*GA INSENSITIVE DWARF 1A*	Gibberellin	Willige et al., 2007; Ariizumi et al., 2008	*AT3G05120*	C05	*BnaC05g46680D*	42053535 to 42055305
GID1B	*GA INSENSITIVE DWARF 1B*	Gibberellin	Willige et al., 2007; Ariizumi et al., 2008	*AT3G63010*	C05	*BnaC05g46680D*	42053535 to 42055305
GID1C	*GA INSENSITIVE DWARF 1C*	Gibberellin	Willige et al., 2007; Ariizumi et al., 2008	*AT5G27320*	C05	*BnaC05g46680D*	42053535 to 42055305
SMZ	*SCHLAFMUTZE*	Photoperiod, Vernalization	Dill and Sun, 2001	*AT3G54990*	C05	*BnaC05g46840D*	42109699 to 42115891
SPA	*SUPPRESSOR OF PHYA-105*	Photoperiod	Laubinger et al., 2006	*AT4G11110*	C05	*BnaC05g46940D*	42196240 to 42198116
FLC	*FLOWERING LOCUS C*	Vernalization	Kim et al., 2022	*AT5G10140*	C05	*BnaC05g47210D*	42317940 to 42326606
CSTF77	*CLEAVAGE STIMULATING FACTOR 77*	Autonomous	Liu et al.,, 2010	*AT1G17760*	C05	*BnaC05g47750D*	42606155 to 42607641
RGA	*REPRESSOR OF GA1-3*	Gibberellin	Dill and Sun, 2001	*AT2G01570*	C05	*BnaC05g47760D*	42615531 to 42616893
GAI	*GIBBERELLIC ACID INSENSITIVE*	Gibberellin	Park et al., 2013	*AT1G14920*	C05	*BnaC05g47760D*	42615531 to 42616893
GAI	*GIBBERELLIC ACID INSENSITIVE*	Gibberellin	Park et al., 2013	*AT1G14920*	C05	*BnaC05g47770D*	42616977 to 42617162
RGA	*REPRESSOR OF GA1-3*	Gibberellin	Dill and Sun, 2001	*AT2G01570*	C05	*BnaC05g47770D*	42616977 to 42617162
GA1	*GA REQUIRING 1*	Gibberellin, autonomous, photoperiod	Revees and Coupland, 2001	*AT4G02780*	C05	*BnaC05g47900D*	42655110 to 42658073
AGL24	*AGAMOUS LIKE 24*	Floral integrator	Adal et al., 2021	*AT4G24540*	C05	*BnaC05g48320D*	42836637 to 42840706
AP1	*APETALA 1*	Floral meristem identity	Mandel and Yanofsky, 1995	*AT4G24540*	C05	*BnaC05g48320D*	42836637 to 42840706
FLC	*FLOWERING LOCUS C*	Vernalization	Kim et al., 2022	*AT5G10140*	C05	*BnaC05g48320D*	42836637 to 42840706
SOC1	*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*	Floral integrator	Moon et al., 2005	*AT2G45660*	C05	*BnaC05g48320D*	42836637 to 42840706
SVP	*SHORT VGETATIVE PHASE*	Temperature	Lee et al., 2007	*AT2G22540*	C05	*BnaC05g48320D*	42836637 to 42840706
FY	-	Autonomous	Simpson et al., 2003	*AT5G13480*	C05	*BnaC05g48330D*	42840775 to 42843694
CDF1	*CYCLING DOF FACTOR1*	Photoperiod	Fornara et al., 2009	*AT5G62430*	C05	*BnaC05g50970D*	1850228 to 1858642
CDF1	*CYCLING DOF FACTOR1*	Photoperiod	Fornara et al., 2009	*AT5G62430*	C05	*BnaC05g50980D*	1865679 to 1866893

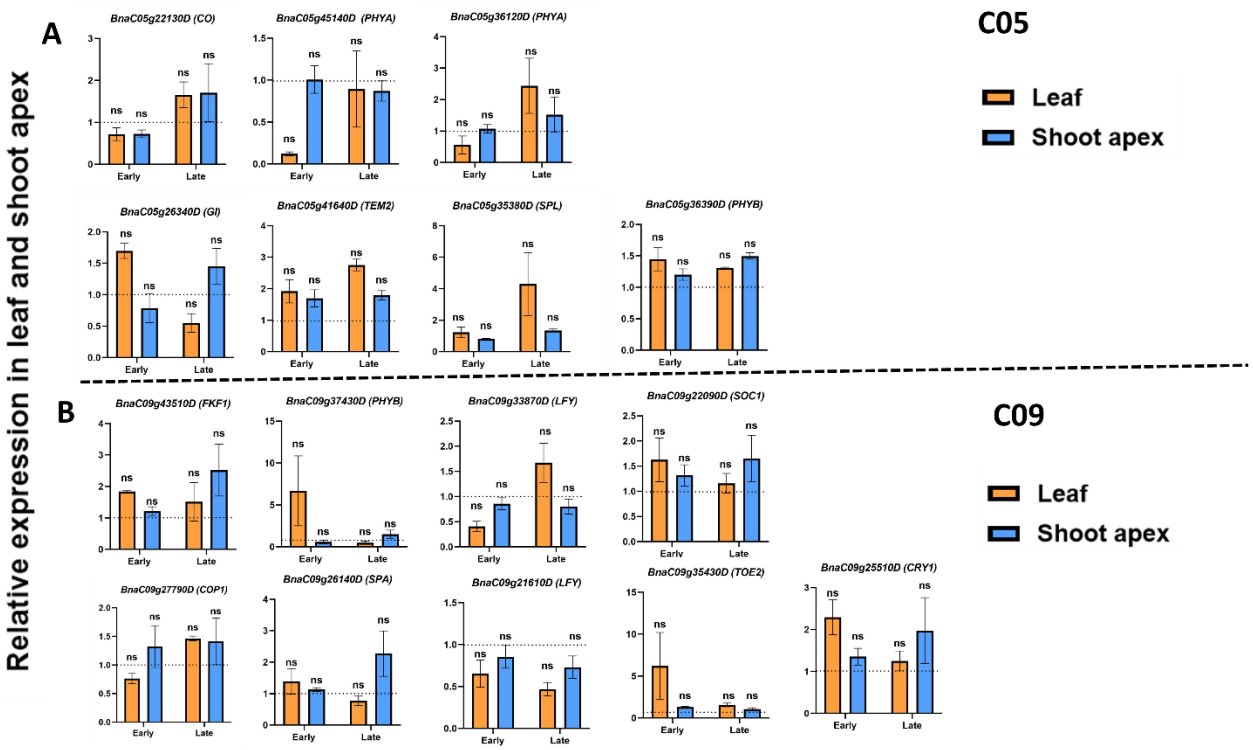
Gene symbol	Annotation	Pathway/types of gene	Reference	Arabidopsis orthologue	Chromosome	Gene (*B.napus*)	Position (bp)
AP2	*APETALA 2*	Photoperiod	Yant et al., 2010	*AT4G36920*	C09	*BnaC09g13430D*	10071530 to 10072870
TOE1	*TARGET OF EAT 1*	Photoperiod	Du et al., 2020	*AT2G28550*	C09	*BnaC09g13430D*	10071530 to 10072870
TOE2	*TARGET OF EAT 2*	Photoperiod	Du et al., 2020	*AT5G60120*	C09	*BnaC09g13430D*	10071530 to 10072870
AGL24	*AGAMOUS LIKE 24*	Floral integrator	Adal et al., 2021	*AT4G24540*	C05	*BnaC09g14450D*	11078581 to 11084314
PHYA	*PHYTOCHROME A*	Photoperiod	Bagnall et al., 1995	*AT1G09570*	C09	*BnaC09g15170D*	11743929 to 11744832
LHP1	*LIKE HETEROCHROMATIN PROTEIN 1*	Vernalization	Mylne et al., 2006	*AT5G17690*	C09	*BnaC09g17770D*	14464001 to 14467207
GA1	*GA REQUIRING 1*	Gibberellin, autonomous, photoperiod	Revees and Coupland,2001	*AT4G02780*	C09	*BnaC09g18210D*	14886879 to 14890928
AP2	*APETALA 2*	Photoperiod	Yant et al., 2010	*AT4G36920*	C09	*BnaC09g18780D*	15673822 to 15675786
GA1	*GA REQUIRING 1*	Gibberellin, autonomous, photoperiod	Revees and Coupland,2001	*AT4G02780*	C09	*BnaC09g19200D*	16049632 to 16057942
ap2	*APETALA 2*	Photoperiod	Yant et al., 2010	*AT4G36920*	C09	*BnaC09g20100D*	17191504 to 17192394
GA1	*GA REQUIRING 1*	Gibberellin, autonomous, photoperiod	Revees and Coupland,2001	*AT4G02780*	C09	*BnaC09g20240D*	17310298 to 17311047
GNC	*GATA,NITRATE-INDUCIBLE,CARBON-METABOLISM*	Gibberellin	Richter et al., 2010	*AT5G56860*	C09	*BnaC09g20930D*	18155376 to 18157028
GNL	*GNC-LIKE*	Gibberellin	Richter et al., 2010	*AT4G26150*	C09	*BnaC09g20970D*	18219346 to 18223023
SPY	*SPINDLY*	Photoperiod	Tseng et al., 2004	*AT3G11540*	C09	*BnaC09g21570D*	18777031 to 18786165
LFY	*LEAFY*	Floral meristem identity	Mandel and Yanofsky, 1995	*AT5G61850*	C09	*BnaC09g21610D*	18807332 to 18809352
TPS1	*TREHALOSE 6 PHOSPHATE SYNTHASE 1*	Photoperiod	Zhang et al., 2022	*AT1G78580*	C09	*BnaC09g21620D*	18827053 to 18828967
AP1	*APETALA 1*	Floral meristem identity	Mandel and Yanofsky, 1995	*AT4G24540*	C09	*BnaC09g22090D*	19226813 to 19228894
SOC1	*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*	Floral integrator	Moon et al., 2005	*AT2G45660*	C09	*BnaC09g22090D*	19226813 to 19228894
SPA	*SUPPRESSOR OF PHYA-105*	Photoperiod	Laubinger et al., 2006	*AT2G46340*	C09	*BnaC09g22310D*	19604849 to 19608835
TOE1	*TARGET OF EAT 1*	Photoperiod	Du et al., 2020	*AT2G28550*	C09	*BnaC09g22500D*	19787003 to 19793258
CRY2	*CRYPTOCHROME2*	Photoperiod	Du et al., 2020	*AT4G08920*	C09	*BnaC09g23430D*	20936480 to 20940278
VRN2	*VERNALIZATION 2*	Vernalization	Trevaskis et al., 2007	*AT4G16845*	C09	*BnaC09g23660D*	21177620 to 21179445
SOC1	*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*	Floral integrator	Moon et al., 2005	*AT2G45660*	C09	*BnaC09g23670D*	21207656 to 21211437
PHYB	*PHYTOCHROME B*	Photoperiod	Bagnall et al., 1995	*AT2G18790*	C09	*BnaC09g24780D*	23018030 to 23022170
CRY1	*CRYPTOCHROME1*	Photoperiod	Du et al., 2020	*AT1G04400*	C09	*BnaC09g25510D*	25053375 to 25056584
SPA	*SUPPRESSOR OF PHYA-105*	Photoperiod	Laubinger et al., 2006	*AT2G46340*	C09	*BnaC09g26140D*	25996302 to 26001334

Gene symbol	Annotation	Pathway/types of gene	Reference	Arabidopsis orthologue	Chromosome	Gene (*B.napus*)	Position (bp)
FRI	*FRIGIDA*	Vernalization	Chen et al., 2022	*AT4G00650*	C09	*BnaC09g27290D*	29041826 to 29043953
PRC2(CLF)	*Polycomb repressine complex 2 (Curly Leaf)*	Age	Dotto et al., 2018	*AT5G51230*	C09	*BnaC09g27450D*	29249093 to 29254077
VRN2	*VERNALIZATION 2*	Vernalization	Trevaskis et al., 2007	*AT4G16845*	C09	*BnaC09g27450D*	29249093 to 29254077
COP1	*CONSTITUTIVELY PHOTOMORPHOGENIC 1*	Photoperiod	Jang et al., 2008	*AT2G32950*	C09	*BnaC09g27790D*	29654647 to 29662362
GA1	*GA REQUIRING 1*	Gibberellin, autonomous, photoperiod	Revees and Coupland,2001	*AT4G02780*	C09	*BnaC09g28950D*	31554389 to 31559135
SOC1	*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*	Floral integrator	Moon et al., 2005	*AT2G45660*	C09	*BnaC09g29450D*	32089088 to 32091133
TPS1	*TREHALOSE 6 PHOSPHATE SYNTHASE 1*	Photoperiod	Zhang et al., 2022	*AT1G78580*	C09	*BnaC09g31560D*	34482619 to 34484576
CO	*CONSTANS*	Photoperiod	Kobayashi et al., 1999	*AT5G15840*	C09	*BnaC09g32640D*	35796727 to 35798212
GNC	*GATA,NITRATE-INDUCIBLE,CARBON-METABOLISM*	Gibberellin	Richter et al., 2010	*AT5G56860*	C09	*BnaC09g33380D*	36593529 to 36595688
GNL	*GNC-LIKE*	Gibberellin	Richter et al., 2010	*AT4G26150*	C09	*BnaC09g33380D*	36593529 to 36595688
LFY	*LEAFY*	Floral meristem identity	Mandel and Yanofsky, 1995	*AT5G61850*	C09	*BnaC09g33870D*	37098616 to 37099569
AP2	*APETALA 2*	Photoperiod	Yant et al., 2010	*AT4G36920*	C09	*BnaC09g35430D*	38788784 to 38792270
TOE1	*TARGET OF EAT 1*	Photoperiod	Du et al., 2020	*AT2G28550*	C09	*BnaC09g35430D*	38788784 to 38792270
TOE2	*TARGET OF EAT 2*	Photoperiod	Du et al., 2020	*AT5G60120*	C09	*BnaC09g35430D*	38788784 to 38792270
SPA	*SUPPRESSOR OF PHYA-105*	Photoperiod	Laubinger et al., 2006	*AT3G15354*	C09	*BnaC09g36940D*	40302733 to 40306030
GNC	*GATA,NITRATE-INDUCIBLE,CARBON-METABOLISM*	Gibberellin	Richter et al., 2010	*AT5G56860*	C09	*BnaC09g37230D*	40586458 to 40589243
PHYB	*PHYTOCHROME B*	Photoperiod	Bagnall et al., 1995	*AT2G18790*	C09	*BnaC09g37430D*	40709757 to 40719200
GAI	*GIBBERELLIC ACID INSENSITIVE*	Gibberellin	Park et al., 2013	*AT1G14920*	C09	*BnaC09g37510D*	40758607 to 40761865
RGA	*REPRESSOR OF GA1-3*	Gibberellin	Dill and Sun, 2001	*AT2G01570*	C09	*BnaC09g37510D*	40758607 to 40761865
GI	*GIGANTEA*	Photoperiod	Fornara et al., 2009	*AT1G22770*	C09	*BnaC09g38380D*	41273280 to 41275833
CSTF64	*CLEAVAGE STIMULATING FACTOR 64*	Autonomous	Liu et al., 2010	*AT1G71800*	C09	*BnaC09g39490D*	42069094 to 42074207
CSTF64	*CLEAVAGE STIMULATING FACTOR 64*	Autonomous	Liu et al., 2010	*AT1G71800*	C09	*BnaC09g39590D*	42186499 to 42188878
SPY	*SPINDLY*	Photoperiod	Tseng et al., 2004	*AT3G11540*	C09	*BnaC09g39760D*	42343068 to 42343564
SPY	*SPINDLY*	Photoperiod	Tseng et al., 2004	*AT3G11540*	C09	*BnaC09g39770D*	42344589 to 42345080
SPY	*SPINDLY*	Photoperiod	Tseng et al., 2004	*AT3G11540*	C09	*BnaC09g39800D*	42350862 to 42351411

Gene symbol	Annotation	Pathway/types of gene	Reference	Arabidopsis orthologue	Chromosome	Gene (*B.napus*)	Position (bp)
SPY	*SPINDLY*	Photoperiod	Tseng et al., 2004	*AT3G11540*	C09	*BnaC09g39950D*	42409562 to 42409855
LHP1	*LIKE HETEROCHROMATIN PROTEIN 1*	Vernalization	Mylne et al., 2006	*AT5G17690*	C09	*BnaC09g40240D*	42751940 to 42753879
GAI	*GIBBERELLIC ACID INSENSITIVE*	Gibberellin	Park et al., 2013	*AT1G14920*	C09	*BnaC09g40240D*	42751940 to 42753879
GAI	*GIBBERELLIC ACID INSENSITIVE*	Gibberellin	Park et al., 2013	*AT1G14920*	C09	*BnaC09g40420D*	42871776 to 42873786
RGA	*REPRESSOR OF GA1-3*	Gibberellin	Dill and Sun, 2001	*AT2G01570*	C09	*BnaC09g40420D*	42871776 to 42873786
SPY	*SPINDLY*	Photoperiod	Tseng et al., 2004	*AT3G11540*	C09	*BnaC09g41740D*	43591313 to 43593570
CO	*CONSTANS*	Photoperiod	Kobayashi et al., 1999	*AT5G15840*	C09	*BnaC09g41980D*	43739067 to 43740068
CO	*CONSTANS*	Photoperiod	Kobayashi et al., 1999	*AT5G15840*	C09	*BnaC09g41990D*	43745679 to 43747139
AGL24	*AGAMOUS LIKE 24*	Floral integrator	Adal et al., 2021	*AT4G24540*	C09	*BnaC09g42060D*	43784336 to 43787341
ap1	*APETALA 1*	Floral meristem identity	Mandel and Yanofsky, 1995	*AT4G24540*	C09	*BnaC09g42060D*	43784336 to 43787341
SOC1	*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*	Floral integrator	Moon et al., 2005	*AT2G45660*	C09	*BnaC09g42060D*	43784336 to 43787341
FKF1	*FLAVIN-BINDING,KELCH REPEAT,F-BOX 1*	Photoperiod	Song et al., 2014	*AT1G68050*	C09	*BnaC09g43510D*	44783028 to 44785881
SPL	*SQUAMOSA PROMOTER BINDING PROTEIN-LIKE*	Photoperiod, Gibberellin	Jung et al., 2012	*AT2G47070*	C09	*BnaC09g43740D*	44888933 to 44892530
LFY	*LEAFY*	Floral meristem identity	Mandel and Yanofsky, 1995	*AT5G61850*	C09	*BnaC09g46090D*	46112547 to 46118713
FLC	*FLOWERING LOCUS C*	Vernalization	Kim et al., 2022	*AT5G10140*	C09	*BnaC09g46500D*	46345350 to 46350092
FLC	*FLOWERING LOCUS C*	Vernalization	Kim et al., 2022	*AT5G10140*	C09	*BnaC09g46540D*	46366645 to 46371180
CO	*CONSTANS*	Photoperiod	Kobayashi et al., 1999	*AT5G15840*	C09	*BnaC09g46950D*	46557269 to 46559280
CSTF77	*CLEAVAGE STIMULATING FACTOR 77*	Autonomous	Liu et al.,, 2010	*AT1G17760*	C09	*BnaC09g48030D*	47046174 to 47050264
GID1A	*GA INSENSITIVE DWARF 1A*	Gibberellin	Willige et al., 2007; Ariizumi et al., 2008	*AT3G05120*	C09	*BnaC09g48990D*	47580634 to 47584764
GID1B	*GA INSENSITIVE DWARF 1B*	Gibberellin	Willige et al., 2007; Ariizumi et al., 2008	*AT3G63010*	C09	*BnaC09g48990D*	47580634 to 47584764
GID1C	*GA INSENSITIVE DWARF 1C*	Gibberellin	Willige et al., 2007; Ariizumi et al., 2008	*AT5G27320*	C09	*BnaC09g48990D*	47580634 to 47584764
TOE1	*TARGET OF EAT 1*	Photoperiod	Du et al., 2020	*AT2G28550*	C09	*BnaC09g49160D*	47671458 to 47673844
TEM1	*TEMPRANILLO 1*	Photoperiod	Osnato et al., 2012	*AT1G25560*	C09	*BnaC09g49240D*	47732744 to 47734847
TEM2	*TEMPRANILLO 2*	Photoperiod	Osnato et al., 2012	*AT1G68840*	C09	*BnaC09g49240D*	47732744 to 47734847
TEM1	*TEMPRANILLO 1*	Photoperiod	Osnato et al., 2012	*AT1G25560*	C09	*BnaC09g50700D*	48449412 to 48450620
TEM2	*TEMPRANILLO 2*	Photoperiod	Osnato et al., 2012	*AT1G68840*	C09	*BnaC09g50700D*	48449412 to 48450620



Appendix 3-3. Relative expression of flowering genes from (A) C5 QTL and (B) C9 QTL in early-and late-flowering lines (bulks) of *Brassica napus* canola at night (4 hours after the light turned off), mean of three biological replicates ± standard error (SE) presented. Different letters indicate significant difference between the mean values ($p < 0.05$). Expression level was normalized to the control for each time point and indicated by dotted line at expression level = 1.0



Appendix 3-4. Relative expression of flowering genes from (A) C5 QTL and (B) C9 QTL in early- and late-flowering lines (bulks) of *Brassica napus* canola at morning (4 hours after the light turned on), mean of three biological replicates ± standard error (SE) presented. Different letters indicate significant difference between the mean values ($p < 0.05$). Expression level was normalized to the control for each time point and indicated by dotted line at expression level = 1.0

Please refer to the attached files for supplementary tables

Supplementary Table S4.1a. List of differentially accumulated proteins (DAPs) in leaves of early and late flowering Brassica napus lines (early vs. late) at morning and night under 10-hour photoperiod. The same proteins detected at both time points are listed here.

Supplementary Table S4.1b. List of differentially accumulated proteins (DAPs) in leaves of early and late flowering Brassica napus lines (early vs. late) at morning and night under 10-hour photoperiod. The unique proteins detected at two different time points are listed here**.**

Supplementary Table S4.1c. List of differentially accumulated proteins (DAPs) in leaves of early and late flowering Brassica napus lines at morning compared to night (morning vs. night) under 10-hour photoperiod. The same and unique proteins detected in both samples are listed here.

Supplementary Table S4.2a. The significantly enriched and common gene ontology (GO) terms of the differentially accumulated proteins (DAPs) in leaves of early and late flowering lines (early vs. late) at morning and night.

Supplementary Table S4.2b. The significantly enriched and unique gene ontology (GO) terms of the differentially accumulated proteins (DAPs) in leaves of early and late flowering lines (early vs. late) at morning and night.

Supplementary Table S4.3. The significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of the differentially accumulated proteins (DAPs) in leaves of early and late flowering lines (early vs. late) at morning and night.

Supplementary Table S4.4a. The important and significantly ($q<0.05$) differentially accumulated proteins (DAPs) in leaves to be involved in flowering time at morning and night when compared in early flowering *Brassica napus* lines. The DAPs were identified in the early flowering lines while comparing with their respective controls (ME vs. NE and NE vs. ME, ME= Morning early and NE =Night early). Red color indicates these proteins were mainly prioritized in results section and most important among these proteins were discussed in this chapter.

Supplementary Table S4.4b. The important and significantly ($q<0.05$) differentially accumulated proteins (DAPs) in leaves to be involved in flowering time at morning and night in late flowering *Brassica napus* lines. The DAPs were identified in the late flowering lines while comparing with their respective controls (ML vs. NL; NL vs. ML; ML=Morning late and NL=Night Late). Red color indicates these proteins were mainly prioritized in results section and most important among these proteins were discussed in this chapter.