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Mutations in *FT-*, *TFL1-*, and *FRI* paralogs of rapeseed (*Brassica napus* L.) and their effect on flowering time and heterosis

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Abbreviations

℃	Degree Celsius
µg	Microgram
µl	Microliter
A	Adenine
A. thaliana	*Arabidopsis thaliana*
A1	*APETALA1*
B. carinata	*Brassica carinata*
B. juncea	*Brassica juncea*
B. napus	*Brassica napus*
B. oleracea	*Brassica oleracea*
B. rapa	*Brassica rapa*
BAC	Bacterial artificial chromosome
BLAST	Basic local alignment search tool
bp	Base pairs
C	Cytosine
CAMK	Ca^{2+}/calmodulin-dependent protein kinases
CBF1	*CREPEAT/DRE BINDING FACTOR1*
CDF1	*CYCLINGDOFFACTOR1*
cdkc;2	Cyclin-dependent kinase C
cDNA	Complementary DNA
CEL1	CELERY ENDONUKLEASE 1
CMS	Cytoplasmic male sterility
CO	*CONSTANS*
Col	Columbia
CTAB	Cetrylmethylammoniumbromid
DH	Double haploid
DNA	Deoxyribonucleic acid
dNTP	Di nucleotide tetra phosphate
EDTA	Ethylenediaminetetraacetic acid
ELF3	*EARLY FLOWERING 3*
EMS	Ethyl methanesulfonate
F_1	First generation
F_2	Second generation
FAE1	*FATTY ACID ELONGASE 1*
FCA	*FLOWERING LOCUS CA*
FLC	*FLOWERING LOCUS C*
FLD	*FLOWERING LOCUS D*

FLK	*FLOWERING LOCUS K HOMOLOGY DOMAIN*
FPA	*FLOWERING LOCUS PA*
FRI	*FRIGIDA*
FT	*FLOWERING LOCUS T*
FVE	*FLOWERING LOCUS VE*
FY	*FLOWERING LOCUS Y*
g	Gram
G	Guanine
GA	*Gibberellins*
GI	*GIGANTEA*
L.	Linn é
LD	*LUMINIDEPENDES*
LDs	Long days
L*er*	Landsberg *erecta*
LFY	*LEAFY*
L. japonicus	*Lotus japonicus*
LSD1	Lys-Specific Demethylase 1
M_1	First generation after mutagenesis treatment
M_2	Second generation after mutagenesis treatment
M_3	Third generation after mutagenesis treatment
min	Minute
ml	Milliliter
mM	Millimolar
MSL-GMS	Male Sterility Lembke-Genic Male System
Mya	Million years ago
NCBI	National Center for Biotechnology Information
nM	Nanomolar
NGS	Next-generation sequencing
PCR	Polymerase chain reaction
PEBP	Phosphatidylethanolamine-binding domain
pH	Potential hydrogenii
Phot	Phototropins
PIF	*PHYTOCHROME INTERACTING FACTOR*
postV	After vernalization
PRC2	*Polycomb Repressive Complex2*
P-TEFb	Positive transcription elongation factor b
preV	Before vernalization
Resyn	Resynthesized rapeseed
RFLP	Restriction fragment length polymorphism

RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
RT-qPCR	Reverse transcription quantitative real time PCR
SAM	Shoot apical meristem
SDs	Short days
sec	Second
SFT	*SINGLE FLOWER TRUSS*
SNP_S	Single nucleotide polymorphisms
SOC1	*SUPPRESSOR OF CONSTANS1*
SP	*SELF PRUNING*
T	Thymine
T-DNA	Transfer DNA
TE	Tris EDTA buffer
TFL1	*TERMINAL FLOWER 1*
TILLING	Targeting-induced local lesions in genomes
TNDH	Tapidor-Ningyou DH
Tris	Tris (hydroxymethyl) amino methane
VIN3	*VERNALIZATION INSENSITIVE3*
VRN1	*VERNALIZATION1*

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1 General introduction

1.1 The history of rapeseed cultivation

Rapeseed (*Brassica napus* L.) is a major oilseed crop grown worldwide for the production of human-consumption vegetable oil, animal feed, biodiesel, and as a source of protein meal (Röbbelen et al., 1989; USDA, 2011). The world production of rapeseed is growing rapidly. According to the Food and Agriculture Organization of the United Nations (FAO), more than 60 million tons were produced in the year 2013, which is twice the amount ten years ago and within the last decade this amount was almost doubled (http://www.worldoil.com/).

Due to the diversity of geographic environments where this crop is cultivated, rapeseed cultivars can be classified as: i) Spring types grown in geographical regions such as Canada, Northern Europe and Australia, ii) Winter types grown in Western Europe, and iii) Semi-winter types grown in central China. Regarding climatic requirements and growth cycle, spring types, which are sown after winter, reach flowering rapidly within the same season. Winter types, which are sown in autumn, can overwinter in vegetative growth and reach their reproductive phase only after a prolonged cold period (vernalization). On the other hand, semi-winter types survive mild winters and can reach the reproductive phase without vernalization (Becker et al., 1995; Diers and Osborn, 1994).

Brassica napus (AACC, n = 19) is an allopolyploid species which originated from natural hybridization between the diploid species *B. rapa* (genome AA, n = 10) and *B. oleracea* (CC, n = 9). Other species from the genus Brassica can hybridize forming other allopolyploid species such as *B. juncea* (AABB, n = 18) and *B. carinata* (BBCC, n = 17) as reported by U (1935). Rapeseed is predominantly a self-pollinating plant, however outcrossing rates range between 10 and 50%. Most probably, *B. napus* arose 7500-12500 years ago along the coastline of the Mediterranean Sea in southern Europe where native *B. rapa* and *B. oleracea* habitats overlap (Chalhoub et al., 2014).

For cultivation, rapeseed was first domesticated as an oil crop back in the 13th century in Europe. For a long period, rapeseed was grown mainly for producing lamp oil and later on for industrial lubrication (Kimber and McGregor, 1995). For human consumption, rapeseed was not found to be appropriate because of its high glucosinolate and erucic acid content. However, along the course of the past three decades rapeseed became also a major crop for humans. This rapid advance resulted from a very fast breeding success. The first major breakthrough in rapeseed breeding came with the initial 0-quality cultivars with erucic acid levels of less than 1% (Stefansson and Hougen, 1964). Subsequently, the first erucic acid-free variety, a spontaneous mutant of the German cultivar “Liho”, was released in Canada in the early 1970s. Thereafter, in 1974, the variety ‘Tower’ was released as the first 00-quantity spring rapeseed (zero erucic acid and low glucosinolate content). This event marked the beginning of oilseed rape (canola) to become the most important oil crop in temperate regions.

As further important achievements, high oleic-, low linolenic-acid types (HOLLi) were developed (Hamann et al., 2007).

Despite the later advances, still there are barriers to overcome to optimize the breeding possibilities for rapeseed. One of the most difficult obstacles to overcome is linked to the different vernalization requirements of elite materials developed to adapt to specific environments. Due to this obstacle, gene introgression among cultivars with different climatic requirements for flowering is a challenging task (Rahman et al., 2011). Therefore, advances on understanding the flowering time control in rapeseed are imperative.

1.2 Morphology and breeding of rapeseed

The herbaceous plant species *Brassica napus* L. belongs to the Brassicaceae family, a monophyletic group of about 340 genera and 3350 species distributed worldwide (Al-Shehbaz, 1984). *B. napus* flowers have four distinct petals, six stamens (tetradynamous), and one pistil that join at a specialized region of the stem called the receptacle (Polowick and Sawhney, 1986). Rapeseed leaves possess a short stem in the juvenile stage, and when maturing an erect branching stem of up to 2m high is formed. The Brassica inflorescence is racemose, unbranched and indeterminate showing lateral flowers with visible pedicels forming in sequential axil that are separated by visibly identifiable internodes (Elzebroek and Wind, 2008).

As a facultative outcrossing (10-50%) but predominantly self-pollinating species, rapeseed is traditionally bred by classical line-breeding methods. Thus, the majority of rapeseed cultivars are pure lines derived from breeding schemes designed for self-fertilizing crops (Becker et al., 1992). As alternative method, homozygous, doubled haploid (DH) lines are highly valuable for the use in plant breeding programs saving time traditionally invested in back-crossings. Thanks to the establishment of microspore culture techniques, the use of *B. napus* DH lines has become common practice in commercial breeding programs and has already resulted in numerous cultivars (Thomas et al., 2003). DH lines are today widely used for production of homozygous parental lines for breeding of hybrids and development of mapping populations in rapeseed.

Together with the development of molecular marker techniques, few studies on QTL mapping have been carried out in *B. napus* DH lines. For instance, the Tapidor/Ningyou7 DH population (TN-DH), which derived from a cross between a European winter cultivar and a Chinese semi-winter cultivar, has become a valuable resource (Qiu et al., 2006). The high stringency genetic linkage map of TN-DH consists of 277 loci, and is widely used in quantitative genetic analysis. Qiu et al. (2006) identified reproducible QTL for seed oil content and erucic acid content with the TN-DH population, Long et al. (2007) detected 36 statistically significant level (SL-QTL) and six micro-real QTL (MR-QTL) associated with

flowering time in the TN-DH and its derived reconstructed F_2 population were evaluated in 11 environments. Also within the same population (TN-DH), Shi et al. (2009) identified 85 QTL for seed yield along with 785 QTL for eight yield-associated traits from 10 natural environments.

Due to the short history of rapeseed cultivation (since 13th century) (Gómez-Campo, 1999), the germplasm pool of rapeseed used for breeding has a narrow genetic diversity compared to the parental species *B. rapa* and *B. oleracea*. This narrow genetic basis limits considerably the rapeseed cultivars improvement through conventional methods. However, introgressions from the two original direct ancestors or other relative species have been used to broaden the genetic variation of oilseed rape for many years (Becker et al., 1995; Qian et al., 2006). Becker et al. (1995) compared the genetic variation within resynthesized (Resyn) rapeseed with a world-wide collection of oilseed rape cultivars and proposed Resyn lines as a valuable source for broadening the genetic variation in present *B. napus*. Additionally, it has been proven that the introgression of Asian *B. rapa* genes can widen the genetic diversity of rapeseed (Qian et al., 2006). More recent studies have found that the introgression of winter rapeseed cultivars can widen the genetic diversity of the Canadian spring rapeseed (Kebede et al., 2010).

The current goals of rapeseed breeding include winter hardiness, plant height, lodging resistance, resistance to blackleg disease, verticillium wilt and sclerotinia, very low contents of erucic acid and glucosinolates, high oil content and marketable seed yield. After the widespread adoption of 00-quality as the accepted standard for the use of the crop in human and animal nutrition, the major focus in breeding efforts retuned to improving the seed and oil yield along with yield stability (Friedt and Snowdon, 2009).

Intercrossing different plant varieties frequently leads to hybrid offspring with yield performance higher than the mid parent value, a phenomenon known as heterosis (Schnable and Springer, 2013). Also, heterosis effects may be reflected in cell size, plant height, leaves size, and root development, among other traits. Generally, three classical models have been posited to explain heterosis (Birchler et al., 2010). The most widely accepted explanation is based on the assumption that hybrid vigor results from bringing together an assortment of favorable dominant genes (dominance model) (Lippman and Zamir, 2007; Schnable and Springer, 2013). According to this theory, alleles that contribute to vigor and growth are dominant, whereas the recessive alleles may be neutral, harmful or deleterious to the individual. However, a serious of findings suggests that dominance mechanisms may not explain all heterosis. Additionally, the overdominance theory states that the heterozygous genotypes outperform the homozygous ones and that the loss of heterozygosity in inbred progeny results in inbreeding depression (Hochholdinger and Hoecker, 2007; Springer and Stupar, 2007). Although many overdominance QTL have been identified through genetic mapping experiments, further studies have revealed several examples supporting a third

model, called the pseudo-overdominance model. It is actually a simple case of dominance complementation, in which two recessive mutations are linked *in trans*, or in repulsion. This type of complementation in the hybrid resembles overdominance, because of the tight linkage of the loci (Birchler et al., 2010; Lippman and Zamir, 2007). Both of the dominance and overdominance hypotheses are based only on single-locus theory. In addition, epistasis also plays a major role as the genetic basis of heterosis (Luo et al., 2001; Yu et al., 1997). In rapeseed, heterosis has been widely exploited with the aid of cytoplasmic-(CMS) and genetic male sterility systems (GMS) (Frauen et al., 2003; Ogura, 1968). As a proof of success, hybrid breeding in rapeseed reached in 2010 more than 950.000 ha in Germany which equals 65% of the total acreage area (Brauer and Angenendt, 2012). However, despite these significant advances, our knowledge about the genetic control of heterosis in the redundant rapeseed genome is still at its infancy.

1.3 The importance of flowering time in Brassica domestication and breeding

In flowering plants, the transition from the vegetative to the reproductive phase is a key developmental step. Controlling the timing of this transition is especially important in crop plants with high agricultural productivity. The timing of flowering and a plant's requirement for and responsiveness to vernalization are major factors in regional climatic adaptation of elite germplasm. Furthermore, it was demonstrated that flowering time genes affect hybrid vigor and thus are likely to impact on yield (see section 1.7). For example, it was found that growth vigor in allotetraploid hybrids of *Arabidopsis* was caused by repression of the circadian clock genes *CCA1* and *LHY* (Jung and Müller, 2009). Repression or mutation of *CCA1* was also shown to result in increased production of chlorophyll and starch (Ni et al., 2009).

The genus Brassica covers a wide array of important agricultural and horticultural plants. It varies greatly in flowering time and includes biennials (cabbage) and annuals (cauliflower), as well as species that can be grown both as spring and as winter crops (rapeseed). This characteristic has led to many attempts to control flowering times. For annual plants, early flowering can cause severe losses in yield and reduction of quality because generative organs are produced in expense of vegetative tissues. For example, in Chinese cabbage (*B. rapa* ssp. *pekinensis*) the vegetative stage was extended and flowering was substantially delayed by overexpression of *FLC* or its homologs (Kim et al., 2007), which was proposed as a general means for increasing biomass yield (Salehi et al., 2005). Rapeseed has been adapted to grow in a wide range of climates. This natural flowering time variation has been thought to evolve as a mechanism that allowed crucifers to withstand cold winters and high summer temperatures (Shindo et al., 2005a). However, as mentioned before, this wide variety in flowering time also impairs breeding with non-adapted elite materials. For instance, late flowering in rapeseed can be a problem in the northern hemisphere where cold temperatures

from late fall to early spring limit the spring cultivation period. Novel flowering time characteristics can be generated through targeted genetic modification by transformation, or after mutagenesis. By using a transgenic approach, Chandler et al. (2005) showed that a single MADS box gene accelerated flowering and seed ripening in spring rape plants. Moreover, many of the floral regulatory genes have been identified, their sequences can be used by breeders as functional markers for selecting favorable genotypes (Andersen and Lubberstedt, 2003). As an example, introduction of genes into European rapeseed from different geographical origins is hampered by non-adapted flowering time traits and requires extensive backcrossing (Qian et al., 2007). Allelic variants at the *BnFLC10* locus account for most of the flowering time variation between spring- and winter-type rapeseed and can be used by breeders as functional markers for selecting favorable genotypes (Hou et al., 2012). Therefore, the modulation of flowering time is considered to be a practical means to improve the agronomic value of Brassica crops.

1.4 Genetic analysis of rapeseed

The model plant *Arabidopsis thaliana* is belonging to the Brassicaceae family, a fact that has favored the transfer of knowledge to species like *B. napus* and its ancestors. This model plant possesses small genome (157 Mbp, n = 5). Efficient transformation systems exist and a diverse range of genetic and genomic resources is available together with a complete genome sequence (Johnston et al., 2005). *B. napus* and its two progenitors, *B. rapa* and *B. oleracea* are believed to share a common ancestor with *A. thaliana* (Wang et al., 2011b). During the evolution of *B. rapa* and *B. oleracea* the genome triploidization occured (Lysak et al., 2005). As a related species, *B. napus* has a 1.2 Gbp genome predicted to encode about 100,000 genes (Bancroft et al., 2011; Chalhoub et al., 2014). Comparative studies between Arabidopsis and Brassica species revealed extensive duplication within Brassica genomes and segmental relationships were identified indicative of a mixture of single, duplicated, and triplicated genome segments relative to Arabidopsis (Sharma et al., 2014). Parkin et al. (2005) mapped over 1000 RFLP (restriction fragment length polymorphism) loci on the 19 linkage groups of *B. napus* and compared their positions with the Arabidopsis genome. They identified 21 syntenic blocks within the Arabidopsis genome, which can be duplicated and rearranged in the present-day *B. napus* genome. Most of these conserved segments were found in six copies, which strongly confirm *Brassica* diploid species evolved from a hexaploid (Parkin et al., 2005). As an extension of the above mentioned study, Schranz et al. (2006) proposed 24 crucifer genomic blocks (A-X) derived from the comparative mapping studies between *Brassica* and *A. thaliana*, which are now widely accepted. These conserved blocks are linked to a proposed ancestral genome (2n = 2x = 16) which represents a useful framework for comparative genomics across the Brassicaceae. Cai et al. (2012) constructed a linkage map of *B. napus* using SSR markers with the aid of *B. rapa* and *B. oleracea* genome sequences, and then attempted to identify homologous loci in Arabidopsis. 385 SSR loci deduced from *B. napus* exhibited synteny to *A. thaliana* genes. Moreover, based on a high density genetic map

with SNP and SSR markers, a comparative analysis of *B. napus* genomes and its progenitor species and the Arabidopsis genome was performed. Based on genetic distances, approximately 70.1% (1,736.9 cM) of the genetic components in the newly formed genome of *B. napus* was derived from the corresponding chromosomes of *B. rapa* and *B. oleracea.* Another 17.7% (438.3 cM) stem from homoeologous chromosome reciprocal translocations between the A and C genomes, and only 3.6% (90.4 cM) from nonhomologous chromosomes by intra- and inter-genomic translocations (Cai et al., 2014).

With the advancement of next-generation sequencing (NGS) technology, it has become possible to economically re-sequence whole genomes or generate large amount of transcriptome data in a short time. The Solexa sequencing system was used to generate transcriptome sequences in *B. napus* cultivars Tapidor and Ningyou7 and to discover single nucleotide polymorphism (SNPs) between the cultivars (Trick et al., 2009). Furthermore, the *B. napus* genome was dissected by leaf transcriptome sequences of the parental and the mapping population and an SNP linkage map comprising 23,037 markers was constructed (Bancroft et al., 2011). Also a SNP Infinium array was used to construct a high-density integrated genetic map consisting of 5,764 SNP and 1603 PCR markers to investigate the polymorphism and linkage disequilibrium among different *B. napus* collection (Delourme et al., 2013). Recently, the homozygous *B. napus* genome of European the winter cultivar 'Darmor-bzh' was assembled with long-read (>700 bp) 454GS-FLX + Titanium and Sanger sequences (Chalhoub et al., 2014). They generated 68,405,795 reads, which equalled to a 21.2× fold coverage of the estimated 1,130 Mb genome of *B. napus*. Finally, they assigned most of the 20,702 *B. napus* scaffolds to either the A_n (314.2 Mb) or the C_n (525.8 Mb) subgenomes via unique mapping of ~5× non-assembled 454 sequences from *B. rapa* ("Chiifu") or *B. oleracea* ("TO1000"). The *B. napus* A_n and C_n subgenomes are largely colinear to the corresponding diploid A_r and C_o genomes, with asymmetric gene distribution (42,320 and 48,847, respectively) and 93% of the diploid gene space in orthologous blocks. They identified 34,255 and 38,661 orthologous gene pairs, respectively, between the A_n and C_n subgenomes and their respective progenitor genomes. Comparison of A_n-A_r and C_n-C_o orthologous gene pairs suggested a divergence 7500 to 12,500 years ago, indicating formation of *B. napus* after this date. Synteny with Arabidopsis confirmed the triplicated mesoploid structure of the A_n and C_n subgenomes. Together with the recent polyploidisation that confers to an aggregated 72× genome multiplication since the origin of angiosperms. The subgenomes A_n and C_n would lead to the functional, and epigenetic cross-talk due to homeologous exchanges.

Studies about the possible sub-functionalization of gene paralogs in rapeseed are still imperative and new approaches must be applied. Reverse genetics has proved to uncover the function of candidate genes in plants by analyzing developmental effects resulting from sequence variation in a gene of interest via sequencing, or from perturbing the function of target genes (Alonso and Ecker, 2006; Gilchrist and Haughn, 2010). As a very well

established reverse genetic strategy, TILLING (Targeting-induced local lesions in genomes) allows the rapid and inexpensive detection of point mutations. With TILLING, a library of DNA samples from thousands of individuals can be screened in a high-throughput manner (Colbert et al., 2001; McCallum et al., 2000b). DNA is pooled from multiple mutagenized individuals to increase throughput and reduce costs. Thereafter PCR and a subsequent mismatch-specific endonuclease restriction are used to amplify and identify mutations within a targeted region of the gene of interest (Oleykowski et al., 1998). TILLING was initially developed as a functional genomics tool in model plants, such as *A. thaliana* (McCallum et al., 2000a, b; Till et al., 2006a) and *L. japonicus* (Perry et al., 2003). However, since the original report, the TILLING process has been adapted to many other crops such as rice (Till et al., 2007), maize (Till et al., 2004), sorghum (Blomstedt et al., 2012), wheat (Slade et al., 2005), oilseed rape (Wang et al., 2008), soybean (Cooper et al., 2008) and potato (Elias et al., 2009). In rapeseed, Harloff et al. (2012) successfully identified mutations in genes involved in the sinapine biosynthesis mutants demonstrating the power of this technique to asses complex genomes.

A standard systematic gene nomenclature system for the *Brassica* genus has been adopted universally (Østergaard and King, 2008). This work is valuable for genomics initiatives such as TILLING populations, genome-wide expression studies and integration of linkage maps of Brassica. Categories are listed in descending order of significance from left to right: genus - species - genome - gene name - locus. The syntax proposed is of the form: <GENUS> [<species>]<GENOME>|<X>.<NAME>.<locus>. Here, < > surrounds categories, [] indicates an optional item and | denotes "or". For example, an expected orthologue of the Arabidopsis *FRIGIDA* (*FRI*) gene isolated from the A genome of *B. napus* would be designated as *BnaA.FRI.a*.

1.5 The regulation of flowering time in *Arabidopsis thaliana*

The transition from the vegetative to the reproductive stage of a plant is a complex biological process controlled by a large group of flowering time genes that respond to environmental and endogenous stimuli (Jack, 2004). In the model plant *A. thaliana* four regulatory pathways comprising more than100 flowering genes have been extensively studied: i) photoperiodic-, ii) vernalization-, iii) autonomous-, and iv) gibberellin- pathway (Amasino, 2010; Fornara et al., 2010; Romera-Branchat et al., 2014). The photoperiodic or long-day pathway promotes flowering by activating genes that encode proteins involved in light perception (Hayama and Coupland, 2003; Reeves and Coupland, 2000). The most important regulators of this pathway are *CO* (*CONSTANS*) and *FT* (*FLOWERING LOCUS T*). CO is a zinc-finger protein that can only accumulate and stability during inductive LDs (long days) (Putterill et al., 1995). *CO* is expressed in the vasculature of leaves and activates the expression of *FT* under long-day (LD) conditions but not under short days (SD) conditions (Suarez-Lopez et al., 2001; Turck et al., 2008a; Valverde et al., 2004). In turn, the FT protein acts as a transmissible signal for

flowering. This protein (florigen) is produced in leaves and then transported to developing meristems. Under long day conditions, the CO protein accumulates in the leaves and induces expression of *FT* in the phloem companion cells. The FT protein is transported within the sieve tubes to the shoot apex, where it forms a heterodimer with the FD protein. The FD/FT complex activates expression of *SOC1* (*SUPPRESSOR OF CONSTANS1*) and *AP1* (*APETALA1*), which leads to floral initiation whereas the TFL1 protein similarly binds to FD to repress downstream genes such as *AP1* and *LFY* (*LEAFY*) in the central zone of the meristem (Hanano and Goto, 2011). Both *TFL1* and *FT* are phosphatidylethanolamine binding protein (PEBP) family members that are similar to mammalian PEBPs (Ahn et al., 2006). Although the *TFL1* gene sequence shares highly similar (71%) to *FT*, *TFL1* acts antagonistically by delaying flowering (Hanzawa et al., 2005). In Arabidopsis, *TFL1* is responsible for maintaining the inflorescence in an indeterminate state, with loss of *TFL1* function resulting in the production of terminal flowers (Bradley et al., 1997).

In addition to day length, vernalization is another cue that strongly affects seasonal flowering patterns. Vernalization pathway is defined as a process regulated by exposure to prolonged cold periods. In winter-annual Arabidopsis accessions, vernalization-responsive flowering is triggered by the interaction of two genes, *FRI* (*FRIGIDA*) and *FLC* (*FLOWERING LOCUS C*) (Choi et al., 2011; Csorba et al., 2014; Song et al., 2012). *FRI* encodes a protein with two coiled-coil motifs and inhibits floral transition in *Arabidopsis* (Johanson et al., 2000). *FRI* promotes histone H3 lysine-4 trimethylation (H3K4me3) at the *FLC* locus to upregulate its expression (Jiang et al., 2009). Recent work has shown that FRI acts as a scaffolding protein interacting with FRL1 (FRIGIDA LIKE1), FES1 (FRIGIDA ESSENTIAL1), SUF4 (SUPPRESSOR OF FRIGIDA4), and FLX (FLC EXPRESSOR) to form a transcription activator complex (FRI-C) that includes both general transcription and chromatin-modifying factors. Each component of FRI-C has a specialized function. SUF4 binds to a cis-element of the *FLC* promoter, FLX and FES1 have transcriptional activation potential, and FRL1 and FES1 stabilize the complex (Choi et al., 2011). Notably, Ding et al. (2013) found that FLX4 physically interacts with FRI and FLX through distinct domains and that FLX and FLX4 show a synergistic enhancement of transcriptional activation. Interestingly, most proteins of the FRI-C complex act to promote *FLC* expression, even in the absence of *FRI*. Thus, components of FRI-C play a role in the regulation of *FLC* expression in both winter-annual and rapid-cycling strains of Arabidopsis (Ding et al., 2013). *FLC* encodes a MADS-box transcription factor that functions to repress flowering by directly blocking the transcription of *FT*, *SOC1*, and *FD* (Searle et al., 2006). The process of vernalization involves three stages: i) before exposure to cold, *FLC* expression level are low, ii) cold-induced *FLC* silencing, and iii) epigenetic maintenance of the *FLC* silenced state when plants return to warm temperatures (Song et al., 2013). Upon exposure to a prolonged cold period *FLC* interacts negatively with *VERNALIZATION INSENSITIVE3* (*VIN3*), a gene encoding a plant-specific component of the Polycomb Repressive Complex 2 (PRC2) which generates trimethylation of histone 3 lysine 27 (H3K27me3) and is essential for stable epigenetic silencing of *FLC* (Angel et al., 2011; De

Lucia et al., 2008). A recent study demonstrates that long intronic noncoding RNA (long ncRNAs) is also required for the vernalization mediated epigenetic repression of *FLC*. Heo and Sung (2011) showed a sense strand of *FLC* first intron named COLDAIR physically associates with the histone methyltransferase subunit of PRC2 and targets PRC2 to *FLC*. By contrast, a group of long antisense RNAs is expressed from a promoter at the 3' end of *FLC* called COOLAIR, which exists in several forms due to the use of different polyadenylation sites (Marquardt et al., 2014; Swiezewski et al., 2009). It has been demonstrated that the reduction of *COOLAIR* transcription by *cdkc;2* (cyclin-dependent kinase C) disrupts a *COOLAIR*-mediated repression mechanism that increases *FLC* expression. This disruption then feeds back to indirectly increase *COOLAIR* expression. This tight interconnection between sense and antisense transcription, together with differential promoter sensitivity to P-TEFb (positive transcription elongation factor b), is central to quantitative regulation of this important floral repressor gene (Wang et al., 2014).

The autonomous pathway stimulates flowering independent of environmental cues and includes genes such as *FCA* (*FLOWERING LOCUS CA*), *FPA* (*FLOWERING LOCUS PA*), *FY* (*FLOWERING LOCUS Y*), *FLK* (*FLOWERING LOCUS K*), *FLD* (*FLOWERING LOCUS D*), *FVE* (*FLOWERING LOCUS VE*), *LD* (*LUMINIDEPENDES*), and *REF6* (*RELATIVE OF EARLY FLOWERING 6*) (Kim and Sung, 2014; Simpson, 2004). Mutants are late flowering because they are unable to down regulate *FLC* expression (Baurle et al., 2007). Largely, the autonomous pathway comprises a combination of components associated with RNA binding/processing or chromatin modification (Rataj and Simpson, 2014). Four genes *FCA, FPA, FY,* and *FLK* mediate RNA regulatory processes. FCA and FPA act as RNA-binding proteins that control flowering and RNA silencing, FPA functions redundantly with FCA to control the expression of alternatively polyadenylated antisense RNAs of *FLC* (Bäurle and Dean, 2008; Hornyik et al., 2010). *FY*, the Arabidopsis homologue of the essential yeast RNA 3' processing factor Pfs2p, has previously been shown to interact directly with *FCA* leading to down-regulation of *FLC* (Manzano et al., 2009). Another study found that *fy* can partially suppress *FLC* expression in an *fca* null background and promote proximal polyadenylation site selection usage in the absence of *FCA* (Feng et al., 2011). *FLK* encodes a putative RNA binding protein with K homology motifs (Lim et al., 2004). In contrast, other autonomous pathway genes *FLD, FVE, REF6* and *LD* are involved in the regulation of the chromatin modification level (He, 2012). *FLD* encodes a plant ortholog of the human LSD1 (Lys-Specific Demethylase 1) protein that is involved in H3K4 demethylation by interacting with HDA6 (HISTONE DEACETYLASE6) (Jiang et al., 2007; Yu et al., 2011). FVE is an Arabidopsis homologue of the retinoblastoma-associated protein that has been shown to be involved in the deacetylation of the FLC chromatin (Ausin et al., 2004). Further studies indicated that FVE can bind to the *FLC* chromatin as well as the *COR15A* (*COLD REGULATED 15A*) chromatin as a large multiprotein complex of approximately 1.0 MDa regulating both flowering time and cold response (Jeon and Kim, 2011). REF6 encodes a jumonji domain-containing protein belonging to a certain class of histone demethylases (Lu et

al., 2011). *LD* encodes a homeodomain protein that regulates *FLC* expression by H3K4 demethylation and H3 deacetylation (Domagalska et al., 2007). It was also proposed that LD binds to SUF4 to suppress its activity in the absence of FRI, thereby preventing SUF4 from acting on *FLC* (Kim et al., 2006).

The last pathway involves GA (gibberellic acid) biosynthesis. Application of exogenous GA to plants results in a dramatic promotion of flowering in short days where the photoperiodic pathway is inactive (Hytonen et al., 2009; Moon et al., 2003; Wilson et al., 1992). Under SDs, GAs activates transcription of *SOC1* and *LFY* (*LEAFY*) at the shoot meristem. GAs influences gene expression by initiating the degradation of DELLA proteins (Willige et al., 2007). This removal of DELLA proteins releases transcription factors that are otherwise prevented from binding DNA by DELLAs, including PIF4 (PHYTOCHROME INTERACTING FACTOR 4) and PIF5 (De Lucia et al., 2008; Feng et al., 2008).

1.6 Regulation of flowering time in *B. napus*

Generally, a single Arabidopsis gene is represented by 3 to 6 paralogs in the allopolyploid *B. napus* genome (Schranz et al., 2006). Research on the molecular identification of flowering time genes in *B. napus* has led to the identification of several genes. Wang et al. (2011a) identified four *FRI* homologues in *B. napus* by BAC libraries screening and PCR-based cloning: *BnaA.FRI.a*, *BnaX.FRI.b*, *BnaX.FRI.c* and *BnaX.FRI.d*. Among them, *BnaA.FRI.a* was mapped to a region on chromosome A03 which co-localizes with a major flowering time-QTL (flowering time quantitative trait locus) in multiple environments in a widely used DH population (Tapidor x Ningyou7, TNDH). Further on, association analysis of *BnaA.FRI.a* revealed that six SNPs, including at least one at a putative functional site, and one haplotype block correlated with flowering time differences between ecotypes from different locations in a world-wide collection of 248 accessions. These finding suggested that *BnaA.FRI.a* is a major determinant of flowering time and also contributes to the differentiation between growth types in oilseed rape. After the publication of the full genome sequence, four *FRI* orthologes have been confirmed, *BnaA.FRI.a, BnaX.FRI.b, BnaX.FRI.c* and *BnaX.FRI.d* located at chromosomes A03, A10, C09, and C03, respectively. In *B. oleracea,* two *FRI* orthologues (*BoFRIa* and *BoFRIb*) have been found. Polymorphic regions on exon I of *BoFRIa* were identified adjacent to the conserved block of 37 amino acids defining the FRI proteins.

To investigate the role of *FLC* in the *B. napus*, five *FLC*-related sequences were isolated from the winter *B. napus* cultivar Colombus (*BnFLC1-5*) (Tadege et al., 2001). All of the five *BnFLC* constructs delayed flowering after transformation varying from 3 weeks to more than 7 months relative to untransformed *Ler*. This variation in the degree of late-flowering suggests that some of the genes could be more important than the others in *B. napus*. The different *BnFLC* genes show differential expression in leaves, stems and shoot tips. This work

gave first evidence that *BnFLC* genes account for the major vernalization responsive flowering time differences in the different cultivars of *B. napus* in a manner analogous to that of *FLC* in Arabidopsis ecotypes. Further on, Zou et al. (2012) identified nine *FLC* homologues (*BnFLC*) in *B. napus*. The *BnFLC* homologues were mapped to six chromosomes. All of the *BnFLC* homologues were located in the collinear region of *FLC* in the Arabidopsis genome except *BnFLC.A3b* and *BnFLC.C3b*, which were mapped to non-collinear regions of chromosomes A3 and C3, respectively. Four of the homologues were associated significantly with QTL for flowering time in two mapping populations. The *BnFLC* homologues showed distinct expression patterns in vegetative and reproductive organs, and at different developmental stages. *BnFLC.A3b* was differentially expressed between the winter-type and semi-winter type cultivars. Nine *FLC* orthologues have been confirmed after full genome sequencing of rapeseed. In addition, Hou et al. (2012) cloned the gene *BnFLC.A10* from a flowering time QTL. They identified 12 polymorphic sites between *BnFLC.A10* parental alleles of the TN-DH population in the upstream region and in intron 1. Among them, one of the polymorphic sites upstream of *BnFLC.A10* is strongly associated with the vernalization requirement of rapeseed. This polymorphic site is derived from a (MITE) insertion/deletion in the upstream region of *BnFLC.A10*. The MITE sequence was not present in the *BnFLC.A10* gene in spring-type rapeseed. Thereby, it was suggested this allelic diversity caused by (MITE) insertion/deletion upstream of *BnFLC.A10* is one of the major causes of differentiation between winter and spring genotypes in rapeseed.

Additionally, six *FT* gene orthologs where identified in *B. napus,* each one homologous to a common ancestral block (E) of the Arabidopsis chromosome (Wang et al., 2009). Four of the six regions were present within inverted duplicated regions of chromosomes A7 and C6 in rapeseed. The coding sequences of *BnFT* paralogues showed 92-99% identities to each other and 85-87% identity with that of Arabidopsis. However, two of the paralogues on chromosomes A2 and C2, *BnA2FT* and *BnC2FT*, were found to lack the distinctive CArG box that is located within intron I that has been shown in Arabidopsis to be the binding site for the FLC protein. Three *BnFT* paralogues (*BnA2FT*, *BnC6FTa* and *BnC6FTb*) were associated with two major QTL clusters for flowering time. One of the QTLs encompassing two *BnFT* paralogues (*BnC6FTa* and *BnC6FTb*) on chromosome C6 was resolved further using near isogenic lines, showing different alleles of both genes to promote flowering. Complementarily, association analysis of the three *BnFT* paralogues across 55 cultivars of *B. napus* showed that the alleles detected in the original parents of the TNDH population were ubiquitous amongst spring and winter type cultivars of rapeseed (Wang et al., 2009). Moreover, Wang et al. (2012a) reported the comparison of the *FT* promoter sequences in *B. napus*. Three conserved blocks A, B and C, which were found to be essential for *FT* activation by *CO* in Arabidopsis (Adrian et al., 2010; Liu et al., 2014), were identified within the *FT* upstream region. Notably, insertion of a DNA transposable element (TE) and a retro-element in *FT* upstream blocks A and B contributed to significant structural divergence between the A and C genome orthologues. *BnA2FT* was found to be transcribed in all leaf samples from different

developmental stages and different photoperiod treatments, whereas *BnC2FT* was not transcribed. Silencing of *BnC2FT* appeared to result from TE insertion and consequent high levels of cytosine methylation in TE sequences within upstream block A.

So far, investigation on *TFL1* orthologs in Brassica is still at its infancy. At least four copies of *TFL1*-like (*TERMINAL FLOWER-1*) genes which show close sequence homology to FT were found in the *B. napus* genome (Mimida et al., 1999). However, only three copies (*BnTFL1-1*, *BnTFL1-2* and *BnTFL1-3*) were available for sequence information (Mimida et al., 1999). Insertions of a stretch of sequences in these regions distinguished *BnTFL1-2* from the other two clones, indicating that *BnTFL1-1* and *BnTFL1-3* may have been duplicated from a single ancestral gene. The unique sequence found in *BnTFL1 -2* was conserved in the *TFL1*-like genes from *B. rapa* and the sequences of *BnTFL1-1* and *BnTFL1-3* were very similar to those of the genes from *B. oleracea*, indicating that *BnTFL1-2* may have originated from the AA genome and the other two genes from the CC genome. The later information was complemented that a total of five *BnTFL1* hits have been found after full genome sequencing of rapeseed.

Gene duplication events during plant speciation are a remarkable finding from comparative genomics analyses. Paralogs may retain functions of the ancestral genes and thus act redundantly and/or additively due to the increased protein dosage gene copies may have several evolutionary fates including sub- or neo-functionalization (Conant and Wolfe, 2008). Furthermore, due to random gene loss during the advent of the allopolyploid genome, segmental duplications, and additional genomic rearrangements, several pseudo genes evolved (Jiang et al., 2011; Parkin et al., 2010; Parkin et al., 2005; Parkin et al., 2003; Town et al., 2006; Udall and Wendel, 2006). However, to my knowledge there is no report about the characterization of *B. napus* flowering time paralogs. The interaction of flowering time genes is quite clear in *Arabidopsis*, however, there is a big challenge that several gene copies are found in *B. napus* (**Figure 1**). In this thesis, I will try to answer the question, how the different paralogs of two important flowering time genes interact with each other. Furthermore, I wanted to know whether all paralogs are transcriptionally active in winter type *B. napus*. The function of three flowering time genes (*BnFT*/*BnTFL1* and *BnFRI*) was analyzed by studying a panel of EMS (ethyl methanesulfonate) mutants.

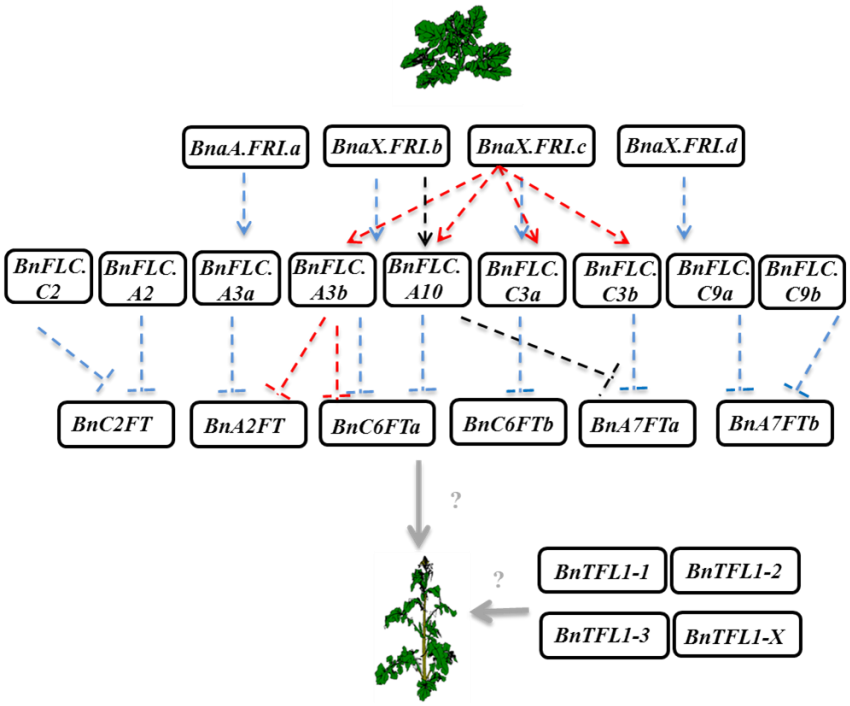

Figure 1: A putative interplay of Arabidopsis homologs of the flowering time genes *FRI* (4 paralogs), *FLC* (9 paralogs), *FT* (6 paralogs) and *TFL1* (4 paralogs) in winter type *B. napus*. Exogenous signals (cold and light) are indicated by symbols. Arrows and lines with bars, respectively indicate positive and negative regulatory actions. Three hypothetical interaction models are shown in dashed lines. Black dashed lines mean only one paralog was active, blue means all paralogs were equally active, and red means one special paralog is dominant over the others.

1.7 Pleiotropic effects of *FLOWERING LOCUS T* in plant development

Apart from effects in flowering control, flowering time genes have been shown to be associated to other pleiotropic effects in plants. As an example, besides flowering, *FT* acts as a cell autonomously-regulated timekeeper for proper opening and closure of stomatal guard cells in Arabidopsis (Kinoshita et al., 2011). Transgenic plants overexpressing *FT* in guard cells showed open stomata, whereas a loss-of-function *FT* allele, *ft-1*, exhibited closed stomata and failed to activate the H^+-ATPase in response to blue light. These results strongly suggest a new cell-autonomous role for *FT* and demonstrated that the flowering time genes *ELF3* (*EARLY FLOWERING 3*) and *FT* are involved in the regulation of H^+- ATPase by blue light in guard cells (Kinoshita et al., 2011).

Phylogenetic studies have revealed that many copies of *FT* occurred in different species due to duplications in several plant species (Pin and Nilsson, 2012a). In sunflower (*Helianthus annuus*), four *FT* paralogs (*HaFT1*, *HaFT2*, *HaFT3*, and *HaFT4*) displayed different expression patterns and carry mutations that have led to the loss and gain of functions

(Blackman et al., 2010). All four paralogs have highly similar sequences and exon-intron structure, conserved FT amino acid at two residues that functionally distinguish *FT* from *TFL1* (Ahn et al., 2006; Hanzawa et al., 2005). It was found that *HaFT2* and *HaFT4* are transcribed in leaves but *HaFT1* is expressed in the SAM (shoot apical meristem). *HaFT3* expression was not detected by sequencing reverse transcriptase (RT)-PCR products from any tissue and mutations in its coding region likely to disrupt function provide strong evidence consistent with non-functionalization or pseudo-functionalization of *HaFT3* (Blackman et al., 2010). In apple (*Malus* x *domestica*), two *FT* paralogs (*MdFT1* and *MdFT2*), appeared to promote flowering, but both genes exhibit different expression patterns along different floral transition stages, and plant tissues, suggesting sub-functionalization between them (Kotoda et al., 2010a). In maize (*Zea mays*), 15 *FT*-like genes (*ZCN*) were found in its genome. One particular *ZCN* gene, *ZCN8* was demonstrated to have a florigenic function as a maize *FT* homolog. It was shown that transgenic plants with reduced *ZCN8* expression produced much longer and wider leaves, increased stem diameter, and more tassel branches compared with their non-transgenic siblings (Lazakis et al., 2011). In sugar beet (*Beta vulgaris*), two *FT* paralogs (*BvFT1* and *BvFT2*) antagonistically regulating flowering time and growth habit have been found (Pin et al., 2010). BvFT1 shares 82% protein identity with its paralog BvFT2, from them, *BvFT2* is functionally conserved with *FT* and is essential for flowering, by contrast, *BvFT1* represses flowering and its down-regulation is crucial for the vernalization response (Pin et al., 2010). In potato (*Solanum tuberosum*), two *FT* paralogs (*StSP3D* and *StSP6A*) were proven in controlling potato floral and tuberization transitions. RNA interference (RNAi) lines transformed with *StSP3D* constructs showed a late flowering phenotype, while *StSP6A RNAi* lines flowered normally, but showed defective tuberization (Navarro et al., 2011). In tomato (*Solanum lycopersicon*), the gene *SFT* (*SINGLE FLOWER TRUSS*, *FT* ortholog) promotes a general growth arrest in addition to flowering. Constitutive expression of the *35S:SFT* gene conferred a reduction in leaf complexity, shorter internodes, thinner stems, and arrested apices together with early flowering in tomato transgenic plants (Lifschitz et al., 2006), furthermore, heterozygosity for loss-of-function alleles *sft/+* contributes up to 60% yield increase due to suppression of growth termination imposed by the *SP* (*SELF PRUNING*) gene which was proven in seven different field-based experiments (Krieger et al., 2010). In summary, it was demonstrated that *FT*-like gene have been identified in a wide range of developmental processes such as stomatal control, fruit set, vegetative growth, tuberization and yield beyond flowering time (Blackman et al., 2010; Kotoda et al., 2010a; Lazakis et al., 2011; Lifschitz et al., 2006; Navarro et al., 2011; Pin et al., 2010; Pin and Nilsson, 2012a).

1.8 Objectives and scientific hypotheses

Rapeseed is an allotetraploid species with a redundant genome compared to the model species *Arabidopsis thaliana*. In a previous study, a TILLING population (EMS-mutagenesis) for the *B. napus* winter-type accession Express 617 was established (Harloff et al., 2012). In the

present study, three major *B. napus* flowering genes (*BnFT*, *BnTFL1* and *BnFRI*) were dissected through the characterization of EMS mutants via TILLING (reverse genetics). Phenotypic differences associated to mutations on different paralogs were identified.

The present study was carried out to prove the following hypotheses: i) Point mutations in selected gene can have phenotypic effects on flowering time, yield components and even impact the expression of other downstream genes despite the redundancy of the rapeseed genome; ii) mutations in two paralogs of the major flowering time regulator *FLOWERING LOCUS-T* (*BnFT*) have different functions; iii) heterozygous mutations in *FT*/*TFL1* paralogs may impact heterosis in rapeseed.

The objectives of this investigation were: i) to screen for EMS-induced genetic variations within the *BnFT*, *BnTFL1* and *BnFRI* genes via TILLING in *B. napus*; ii) to measure the effects of point mutations in target genes on flowering time and yield-associated traits; iii) to measure the expression of other flowering genes in selected mutants; iiii) and to determine the effects of heterosis in F_1 hybrids derived from crosses between EMS mutants and their non-mutated parents.

2 Mutations in single *FT*- and *TFL1*-paralogs of rapeseed (*Brassica napus* L.) and their impact on flowering time and yield components

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2.1 Abstract

Rapeseed (*Brassica napus* L.) is grown in different geographical regions of the world. It is adapted to different environments by modification of flowering time and requirement for cold. A broad variation exists from very early-flowering spring-type to late-flowering winter cultivars which only flower after exposure to an extended cold period. *B. napus* is an allopolyploid species which resulted from the hybridization between *B. rapa* and *B. oleracea*. In *Arabidopsis thaliana*, the PEBP-domain genes *FLOWERING LOCUS-T* (*FT*) and *TERMINAL FLOWER-1* (*TFL1*) are important integrators of different flowering pathways. Six *FT* and four *TFL1* paralogs have been identified in *B. napus*. However, their role in flowering time control is unknown. We identified EMS mutants of the *B. napus* winter-type inbreed line Express 617. In total, 103 mutant alleles have been determined for *BnC6FTb*, *BnC6FTa*, and *BnTFL1-2* paralogs. We chose three non-sense and fifteen missense mutant lines (M_3) which were grown in the greenhouse. Although only two out of six *FT* paralogs were mutated, six out of eight *BnC6FTb* mutant lines flowered later as the control, whereas all five *BnC6FTa* mutant lines started flowering as the non-mutated parent. Mutations within the *BnTFL1-2* paralog had no large effects on flowering time but on yield components. F_1 hybrids between *BnTFL1-2* mutants and non-mutated parents had increased seed number per pod and total seeds per plant suggesting that heterozygous mutations in a *TFL1* paralog may impact heterosis in rapeseed. We demonstrate that single point-mutations *in BnFT* and *BnTFL1* paralogs have effects on flowering time despite the redundancy of the rapeseed genome. Moreover, our results suggest pleiotropic effects of *BnTFL1* paralogs beyond the regulation of flowering time.

2.2 Introduction

Rapeseed (*Brassica napus* L.) is a major oil crop accounting for more than 60 million tons of seed and more than 20 million tons of extracted oil worldwide (http://www.worldoil.com/). This crop is widely cultivated in several temperate regions of the world such as northern Europe, Canada, China and Australia. Adapting flowering time to regional environmental conditions has been a major target of rapeseed breeding. A broad variation exists from very early-flowering spring-type to late-flowering winter cultivars that only flower after exposure to an extended cold period, a process known as vernalization (Iniguez-Luy and Federico, 2011). In rapeseed, flowering time and yield are closely linked to each other. Several genomic regions have been reported to contain major QTL for both traits. In a doubled haploid (DH) population derived from a cross between the Chinese semi-winter line Ningyou7 and the European winter-type Tapidor, at least four flowering time QTL were suggested as indicator

QTL for yield (Long et al., 2007; Shi et al., 2009). Genetic variation within the different rapeseed types is relatively small, implying a need for wide crosses between non-adapted ecotypes to introduce traits of interest into elite materials (Girke et al., 2012). However, the introgression of genes from non-adapted ecotypes into elite cultivars is difficult due non-adapted flowering time. Therefore, it is of great interest to measure the effects of different paralogs on flowering and other characters.

Brassica napus and its close relatives *B. oleracea* and *B. rapa* belong to the family Brassicaceae which also includes the model plant *A. thaliana*. Rapeseed is an allotetraploid species originating from the spontaneous hybridization between *B. rapa* (AA) and *B. oleracea* (CC) less than 5,000 years ago (Wang et al., 2011c; Ziolkowski et al., 2006). These two progenitor species are ancient polyploids that underwent genome triplication between the *Brassica-Arabidopsis* split (~13 MYA) and their actual divergence event (~two MYA). Comparative mapping between the *Arabidopsis* and *Brassica* genomes revealed numerous homologous regions arranged in highly syntenic chromosome blocks. Many Arabidopsis genes are represented in the *B. napus* genome by at least three paralogs (Schranz et al., 2006). Due to their close phylogenetic relationship and the high economic importance of rapeseed, knowledge transfer from the model species Arabidopsis to the complex Brassica genomes constitutes a worthwhile challenge for genomics research.

In Arabidopsis, four pathways controlling flowering time have been extensively studied (Amasino, 2010). All these pathways converge at the *CO/FT* regulon (Andres and Coupland, 2012). Under long day (LD) conditions, the CONSTANS (CO) protein accumulates in leaves and induces expression of the floral integrator gene *FLOWERING LOCUS T* (*FT*) in the phloem companion cells (Moon et al., 2003; Turck et al., 2008b). FT is the long-sought "florigen" and it is reported to be a strong mobile signal triggering activation of floral identity genes in the Arabidopsis shoot apical meristem (Andres and Coupland, 2012). The FT protein is transported via the sieve tubes to the shoot apex, where it forms a heterodimer with the FD (FLOWERING LOCUS D) protein (Abe et al., 2005; Wigge et al., 2005). Interestingly, a very closely related gene, *TERMINAL FLOWER 1* (*TFL1*) plays an *FT*-antagonistic role by competing for FD, leading to a repression of floral transition (Andres and Coupland, 2012; Valverde, 2011). In Arabidopsis, *FT*-overexpressing plants and *TFL1* nonsense mutants show the same early-flowering phenotype and produce terminal flowers in the shoot apex. *TFL1* represses transcription of genes which are activated by *FT* (Hanano and Goto, 2011). In Arabidopsis, these two highly similar polypeptides belong to a family of six members characterized by the phosphatidylethanolamine-binding domain (PEBP)(Kardailsky et al., 1999). Substitutions of crucial amino acids from the *FT* and *TFL1* exon II, as well as the exchange of the exon IV led to contrasting protein functions for both polypeptides (Ahn et al., 2006; Hanzawa et al., 2005).

Apart from their major role to control flowering time, *FT* and *TFL1* orthologs have been shown to alter a variety of phenotypic characters. In tomato, the *SINGLE FLOWER TRUSS* (*FT* ortholog) and *SELF PRUNING* (*TFL1* ortholog) genes impact fruit yield heterosis. F_1 hybrids generated by crosses between loss of function *SFT* mutants and tomato wild type (WT) plants of the non-mutagenized line M82 have shown strong increment in fruit production (Krieger et al., 2010; Molinero-Rosales et al., 2004). *FT/TFL1* gene orthologs have been characterized in diploid crops such as rice (Kojima et al., 2002), pea (Hecht et al., 2011), barley (Faure et al., 2007), poplar (Bohlenius et al., 2006), and sugar beet (Pin et al., 2010) and in two polyploids, wheat (Yan et al., 2006) and potato (Navarro et al., 2011). The characterization of the *FT/TFL1* gene orthologs in polyploid plants is a special challenge because duplicated genes can build new regulation networks leading to sub- or neo-functionalization (Pin and Nilsson, 2012b).

Rapeseed has six *BnFT* paralogs (*BnA2FT, BnC2FT, BnC6FTa, BnC6FTb, BnA7FTa*, and *BnC7FTb*) sharing high sequence similarity (92-99%) in their four exons (Wang et al., 2009). It has been shown that the *BnC2FT* copy is silenced in *B. napus* and *B. oleracea* due to the insertion of a miniature inverted-repeat transposable element (MITE) in its promoter region, whereas the remaining five copies are detectable in *B. napus*, *B. rapa* and *B. oleracea* (Wang et al., 2012a). There are at least four *TFL1* paralogs in the *B. napus* genome (Mimida et al., 1999). Among them, the *BnTFL1-2* paralog shares high homology with the *B. rapa* ortholog on chromosome A10, whereas *BnTFL1-1*, *BnTFL1-3* are highly similar to their *B. oleracea* counterparts. The *BnC6FTa* and *BnC6FTb* paralogs were co-located to a major flowering time QTL detected in nine winter-cropped environments which could support their function as flowering time regulators in *B. napus* (Qiu et al., 2006; Shi et al., 2009). Until now, *B. napus FT/TFL1* homologs have not been functionally characterized.

Loss of function mutants are of particular interest since they provide valuable evidence for the role of specific genes in regulatory, developmental, biochemical and metabolic networks. Several reverse genetic approaches were implemented for functional characterization of genes in plant genomic research and is increasingly informing crop improvement. Insertional mutagenesis (T-DNA or transposon), RNA interference (RNAi) and Virus-Induced Gene Silencing (VIGS), have been used to obtain reduction-of-function or knockout mutations, and used successfully in *A. thaliana* (Long and Coupland, 1998) and rice (An et al., 2005; Waterhouse et al., 1998). However, there are particular challenges in applying these approaches to rapeseed research and crop improvement, primarily because of either reliance on Agrobacterium T-DNA vectors for transmission or lack of availability of endogenous tagging systems. TILLING is based on screening populations of pure seed line that have been treated with EMS at an appropriate concentration that cause point mutations, primarily introduces G/C to A/T transitions, followed by discovery in genes of interest using a very sensitive detection method. The main advantage is the ability to accumulate an allelic series of mutants with a range of modified functions, from wild-type to almost loss of function

(Slade et al., 2005). The M_1 plants are self-fertilized, and harvested as individuals for M_2 seed. Then leaves of M_2 individuals are used for DNA extraction. Individual DNAs are pooled in microtitre plates and pools used for mutational screening, whilst an inventory of their seeds is established for future and downstream research (Henikoff et al., 2004). The most efficient and cost-effective method has been described by Till et al. (2003). They used the enzyme, CEL1, a nuclease extracted from celery, to cleave at the site of induced mutations in heteroduplexed PCR fragments amplified with fluorescently labelled primers, and the LI-COR DNA analyzer denaturing polyacrylamide gel electrophoresis system to separate the resulting fragments. Since TILLING relies on use of chemical mutagens that induce genome lesions randomly, it is relatively easy to construct a sufficiently large mutant population that has a high probability of containing mutations in all gene loci. As a non-transgenic method for obtaining mutations within known genes, TILLING has been spread rapidly to many agricultural crops and displayed importance role into reverse genetics in crops species. TILLING has been applied in model diploid crop species like rice (Till et al., 2007), Sorghum (Blomstedt et al., 2012), and was also successfully applied rapidly into many other economically important agricultural crops with polyploid genomes, like wheat (Slade et al., 2005) and oilseed rape (Wang et al., 2008). For polyploid species *B. napus*, transgenesis is inefficient and endogenous tagging is not accepted by regulatory bodies and consumers especially in Europe. In addition, multiple copies of each gene make rapeseed very well suited for TILLING as they can tolerate very high mutation densities. Together, TILLING is better suited to rapeseed genomic research and trait improvement than other methods reported to date.

This study had two major aims. First, we aimed to uncover the role in flowering time control of different *FT* and *TFL1* paralogs in *B. napus* by analyzing EMS-treated offspring with missense and splice-site mutations within selected paralogs. We demonstrate that single mutations can change the onset of flowering in *B. napus* despite the redundancy of its allopolyploid genome. Moreover, we postulated that *BnTFL1* mutations also affect seed yield components in rapeseed. We found increased seed yield in F_1 plants carrying a mutated *BnTFL1* allele on the Express 617 background. Our data suggest that EMS-generated alleles may constitute a new resource to broaden the genetic basis of rapeseed breeding.

2.3 Materials and Methods

2.3.1 Mutation screening

A total of 3488 M_2 plants of the Express 617 EMS-population were screened by TILLING as described by Harloff and coauthors (2012). Gene specific primers were designed for *BnC6FTa* (FJ848915.1), *BnC6FTb* (FJ848917.1), and *BnTFL1-2* (AB017526.1) (**Supplementary table S1**). For primer design and comparative analysis, *B. rapa* and *B. oleracea* genome sequences were downloaded from (http://brassicadb.org/brad/downloadOverview.php) and (http://ocri-genomics.org/bolbase/),

respectively. For the *BnC6FTb* fragment, specific primers were designed which include exon 1, and exon 3/4 in a separate fragment. For *BnC6FTa*, specific primers were designed flanking exon1. For the *BnTFL1-2*, specific primers flanking the whole gene were designed.

Plant genomic DNA arrayed in two dimensional 8-fold pools was amplified by direct or nested PCR. Forward and reverse primers were 5'-end labeled with 700 nm (DY-681) or 800 nm (DY-781) IRD fluorescence dyes, respectively (Biomers, Ulm, Germany, www.biomers.net). The primer mixture contained the forward primer labeled with DY-681 (biomers, www. biomers.net), the unlabeled forward primer, the reverse primer labeled with DY-781 (biomers) and the unlabeled reverse primer in a ratio of 3:2:4:1 and were added in a final concentration of 168 nM. PCR reactions were carried out in a total volume of 20 µl containing 0.5 units *Taq* polymerase (Invitrogen), 1 × PCR buffer, 1.5 mM $MgCl_2$ and 0.2 mM of each dNTP. To test whether the target region was amplified successfully in each well of the PCR plate, 3 µl of each reaction were separated by gel electrophoresis in a total volume of 10 µl containing 1x loading dye (20 mM Tris pH 8.0, 0.5 mM EDTA, 10 mM acetic acid, 5% glycerol, 0.01% (w/v) bromphenol blue) in 1% (w/v) agarose. In each row, 3 µl FastRuler™ Middle Range DNA Ladder (Fermentas) was used as a size standard. Gel electrophoresis was carried out at 120V for 7 minutes. PCR amplifications with labelled oligos were done using the following profile: 95 ℃ 5 min; 35 cycles of 95 ℃ 30 sec, 60 ℃ 45 sec, 72 ℃ 90 sec; 72 ℃ 10 min.

After gene-specific amplification, the remaining 17 µl of each PCR reaction were subjected to the following program in a thermal cycler: 95 ℃, 10 min, 95 ℃ to 85 ℃ with an increment of -2 ℃/cycle, 2 sec for each temperature, 85 ℃ to 25 ℃ with an increment of -0.5 ℃/cycle, 2 s for each temperature. The ramp rate was reduced to 1 ℃/sec. Subsequently, the sample plate was placed on ice and 6 µl of endonuclease *CEL1* was extracted from celery and stored at -80 ℃ as reported (Till et al., 2006b) and 5.4 µl digestion buffer (10 mM $MgSO_4$, 10 mM Hepes, pH 7.5, 10 mM KCl, 200 ng/ml BSA, 0.2% (v/v) Triton X-100) were added to each well. Heteroduplex digestion was carried out at 45 ℃ for 15 min and stopped by adding 5 µl of 0.25 M EDTA, pH 8.0 to each well of the assay plate on ice.

Sample purification was carried out as described in the published TILLING protocol (Till et al., 2006b) using Sephadex G50-fine (GE Healthcare, www.gehealthcare.com). Polyacrylamide gels were prepared with KB ^{Plus} 6.5% Gel Matrix (LI-COR Biosciences) according to manufacturer's instructions. Prior to loading to the gel, 28 µl of the purified digestion products were mixed with 4 µl formamide loading dye (96% (v/v) formamide, 38.5 µM EDTA, 0.01% (w/v) bromphenol blue) and the sample volumes were reduced to approximately 5 µl by incubating the plate for 20 to 25 min at 95 ℃. Sizing standards 50-700 bp IRDye 700 and 50-700 bp IRDye 800 (LI-COR Biosciences, www.licor.com) were denatured for 3 min at 95 ℃. After a pre-run of 20 min, 0.5 µl aliquots of samples and sizing standards were loaded using 100-tooth membrane combs and separated in a LI-COR 4300

DNA analyzer (LI-COR Biosciences) for 3:15 to 4:15 hours at 1,500 V, 40 mA and 40 W. Gel images were analyzed using the software GelBuddy (http://www.proweb.org/gelbuddy/).

After positive pools had been identified, single plant PCR was carried out in a total volume of 30 µl with 0.5 units of *Taq* polymerase (Invitrogen) respectively, each containing 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of dNTPs, 150 nM of unlabeled primers of target genes, 5 ul of each PCR product were checked on 1% (w/v) agarose gels, and 5 µl GeneRuler™ 1 kb DNA Ladder (Fermentas) was used as a size standard. Gel electrophoresis was carried out at 120 V for 20 minutes. After band size confirmation, the remaining 25 µl of PCR product were used to sequence for SNPs confirmation. Sequences were analyzed with the CLC-bio Main Workbench sequence alignment tool (CLC bio, Aarhus, Denmark). Mutation frequencies *F* [1/kb] were calculated using amplicon sizes corrected by 100 bp for LI-COR gel border effects according to the formula:

$$F[1/\mathrm{kb}] = 1 \Big/ \left(\frac{(\mathrm{ampliconsize\,[bp]} - 100) \times (\mathrm{number\,of\,M_1\,plants})}{(\mathrm{number\,of\,mutations}) \times 1{,}000} \right)$$

2.3.2 Plant materials and greenhouse experiments

Seedlings of M_3 lines and Express 617 (controls) were grown in the greenhouse at constant temperature (22 ℃) under long days (LD, 16h light/8h dark) for four weeks. Express 617 is an inbreed line (F_{11}) originated from the rapeseed winter-type cultivar Express (Harloff et al., 2012). Subsequently, plants were vernalized for 8 weeks at 4 ℃ under LD conditions in a cold chamber. Of each M_3 line, thirty plants were grown. After vernalization, plants were returned to the initial greenhouse conditions and transplanted to 11 × 11cm pots. M_3 plants and Express 617 controls were arranged in randomized blocks. Plant positions on the greenhouse were indexed and linked to randomly generated numbers using the Microsoft Excel software. Selected M_3 lines were crossed with the male sterile (MS) line MSL007 (NPZ, Hohenlieth, Germany) using homozygous M_3 plants as a pollinators. F_1 plants and Express 617 controls were grown in the greenhouse under the conditions mentioned above. F_2 populations were produced by crossing M_3 homozygous plants from a selected *BnC6FTb* mutation ($BnC6FTb_{G2154A}$) and non-mutagenized Express 617 plants. In each greenhouse experiment, the following phenotypic characters were measured according to the BBCH scale (http://www.jki.bund.de/en/startseite/veroeffentlichungen/bbch-codes.html): first non-cotyledonal leaves (NCL, BBCH10), rosette plant (BBCH30), visible floral buds (BBCH50), first open flower (BBCH60), and end of flowering (BBCH69). Plants that did not grow beyond NCL (BBCH10) were excluded from the experiment. Plant height, number of branches, initial flowers, filled pods, seed number and seed weight were recorded for each plant separately.

2.3.3 DNA isolation and genotyping

Total DNA was extracted from young leaves using a CTAB protocol (Morjane et al., 1994). 50 mg freeze-dried leaves material was ground into a fine powder in liquid nitrogen, dispersed in 1250 µl of pre-warmed extraction solution (75ml 2 × CTAB mit 50 ml H_2O and mit 300 ul ß-mercaptoethanol for 100 samples), and incubated at 65 ℃ for 30 minutes with occasional mixing by gentle swirling. After cooling the suspension on ice for 5 min, 500 µl of chloroform/isoamylalcohol (24:1, v/v) was added; the solution was then mixed by inversion and centrifuged at 14000 rpm for 15 min at room temperature. The supernatant was transferred to a new tube. The DNA was precipitated with 700 µl of cold isopropanol and then was kept at -20 ℃ overnight. DNA was centrifuged at 13000 rpm for 10 min at 4 ℃. The supernatant was discarded, and the DNA was transferred into a new micro centrifuge tube containing 500 µl washing solution I. After 5-10 min incubation at room temperature, the supernatant was again discarded before addition of 500 µl washing solution II. After 5 min incubation at room temperature the supernatant was discarded. After air-drying the DNA-pellet for about 30 to 40 min, DNA was eluted into 100 µl TE buffer. In order to remove RNA, 1 ul RNase (10 µl, 10 µg/ml, Fermentas, www.fermentas.de) treatment was applied for 60 min at 37 ℃. DNA concentration was determined by spectrometry (NanoDrop, www.nanodrop.com). DNA quality was checked by 1% agarose gel electrophoresis. For genotyping mutant lines, genomic DNA from single plants was amplified by PCR using unlabeled primers. PCR was done essentially as described in the previous paragraph. Five µl of each PCR product were loaded on 1% (w/v) agarose gels. Upon band size confirmation, the remaining 25 µl of PCR product were sequenced via Sanger capillary sequencing. The sequences were analyzed with the CLC-Bio software (CLC bio, Aarhus, Denmark) using the sequence assembly viewer tool.

Extraction solution	
Tris-buffer, pH 7.5	200 mM
NaCl	1.4 M
EDTA pH 8.0	20 mM
CTAB	2% (w/v)
ß-mercaptoethanol*	0.2% (v/v)
Washing solution I	
Natrium acetate	200 mM
Ethanol	76% (v/v)
Washing solution II	
Ammonium acetate	10 mM
Ethanol	76% (v/v)

* added immediately before use

2.3.4 Tissues sampling and RT-qPCR

Young leaves of M_3 plants and Express 617 controls were sampled at four developmental stages, as described above. Genomic DNA sequences from the different flowering time genes analyzed were retrieved from the non-redundant NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/). Individual sequences were loaded to the CLC-bio main workbench version 6.0 (http://www.clcbio.com), and groups of paralogs were aligned with the help of the internal alignment routine. Two main strategies were applied for expression analysis: i) primers were designed in conserved regions within groups of paralogs for detecting joint gene expression, and ii) copy-specific primers were designed for the members of selected paralog genes (**Supplementary table S1**). Total RNA was extracted using the RNeasy kit (QIAGEN, www.qiagen.com) according to the manufacturer's protocol. The RNA concentration was determined by spectrometry (Nano Drop; Thermo Scientific, Wilmington, USA) and quality was checked by agarose gel electrophoresis. Total RNA was treated with DNAse I (Fermentas Inc., Maryland, USA). First-strand cDNA was synthesized using Oligo$(dT)_{18}$ primers and the M-MuLV Reverse Transcriptase (Fermentas).

Quantitative real-time RT-PCR (RT-qPCR) was performed with SYBR qPCR Super mix w/ROX (Invitrogen Corporation, Carlsbad, USA) using a CFX96 Real-Time System (Bio-Rad Laboratories GmbH, München, Germany). Reactions were performed in a total volume of 15 µl containing 100 nM of each primer and 2 µl of diluted cDNA templates, and amplified using the following cycling conditions: 95 ℃ for 3 min, 40 cycles of 95 ℃ for 10 s, 60 ℃ for 30 s, and 72 ℃ for 30 s, followed by 95 ℃ for 10 min. A melting curve was generated using a temperature range from 65 ℃ to 95 ℃ with increments of 0.5 ℃ every 5 s. For each sample at least three technical replications were performed. For data analysis, the mean C_t value of the target gene was normalized against the average C_t value of two housekeeping genes (*BnGADPH-3* and *BnB-Tub*). Calculation of relative expression values was carried out following Pfaffl (2001) after extracting main C_t values via CFX manager software (Bio-Rad Laboratories GmbH, München, Germany). Melting curves showed a single peak for each target with PCR products. 'No template controls' (NTCs), included in triplicates for each target in each run, were either not amplified (C_t values below baseline threshold) or had very high (>36-39) Ct values, with melting curves peaking at clearly distinguishable temperatures (76.0-78.0 ℃ and 75.5-77.0 ℃) from the respective PCRs with templates. In each analysis, the relative expression value for the reference sample has been set to 1. Normalized expression was averaged over two biological replicates and three technical repetitions in each case. Standard curves for the target and housekeeping genes are based on dilution series of purified cloned fragments for each gene.

2.3.5 Sequence diversity analysis

For analyzing sequence diversity within the *BnC6FTb* and *BnTFL1-2* genes, genomic DNA from one-hundred accessions of the *B. napus* ASSYST panel was amplified with paralog-specific primers and sequenced via Sanger method. We selected 117 lines from the *B. napus* ASSYST diversity set (Bus et al., 2011) including winter, semi-winter and spring types which had been phenotyped in several environments worldwide (**Supplementary table S2)**. Lyophilized leaf samples harvested from young plants were used for DNA isolation with the NucleoSpin Plant II DNA isolation kit (Macherei & Nagel, Germany), following the manufacturer's instructions. PCR amplifications were carried out with paralog-specific primers as follows: 95 ℃ 5 min; 35 cycles of 95 ℃ 30 sec, 60 ℃ 45 sec, 72 ℃ 90 sec; 72 ℃ 10 min. Sequences resulting from single band amplicons were assembled and aligned using the CLC-bio main workbench software (CLC bio, Aarhus, Denmark) and the resulting FASTA alignment was loaded into the software TASSEL (http://www.maizegenetics.net) for identification of polymorphic SNPs.

2.3.6 Mutant materials generation

Hand crosses were performed to developing materials during experiments. i): *BnC6FTb*, *BnC6FTa*, and *BnTFL1-2* mutants were crossed with Express 617. ii): Back-cross of $BnC6FTb_{G2154A}$ mutant. iii): cross between *BnC6FTb*, *BnC6FTa*, and *BnTFL1-2* mutants to generate double mutants. iv): *BnC6FTb*, *BnC6FTa*, and *BnTFL1-2* mutants were crossed with male-sterile (MSL007) winter-type oilseed rape plants. Details of the numbers of plants crossed, as well as seeds obtained for the F_1 and F_2 stages are deposited in **Supplementary file 3**.

i) Cross with donor line of selected families

Verified homozygous mutants (M_3) were crossed with the donor *B. napus* accession Express 617. After crossing and selfing of F_1 plants (confirmed heterozygous plants), progenies segregating for the mutated alleles can be obtained (F_2). By sowing, phenotyping, and genotyping two or more F_2 families (harboring the same EMS-generated allele) derived from independent crossings, a phenotype-genotype correlation can be tested via nested-ANOVA statistical tests in the given mutant family.

ii) Back-cross of selected families

Backcrossing is a classical programme to remove the undesirable background mutation load is a prolonged procedure, however, it is expensive in both time and resources. This is especially true where a genus such as Brassica produces large plants with a relatively long generation time. Each backcross generation reduces the mutation load by 50%.

iii) Generation of double mutants

With the aim to analyze the possible additive effects in mutations, crosses producing double mutants have been carried out on selected lines.

iv) MS crossing
Crossings between verified homozygous mutants and male-sterile (MSL007) winter-type oilseed rape plants were carried out aimed to test heterosis effects.

2.4 Results

2.4.1 Paralog-specific expression of five *BnFT* genes

We carried out a RT-qPCR experiment to measure the paralog specific expression of six *BnFT* paralogs in leaves of the winter-type inbred line Express 617 during the transition to reproductive stages. Samples were taken from greenhouse-grown plants at three different stages of development (BBCH30 before and after vernalization and BBCH50). Relative expression values for each paralog were calculated after Ct normalization using *BnGAPDH* as a reference gene. Leaf samples at BBCH30 before vernalization (preV) were used as reference samples for relative expression calculations. At BBCH30 before vernalization (BBCH30-preV), four *BnFT* paralogs (*BnC6FTa/b* and *BnA7FTa/b*) were weakly expressed (**Figure 2**), whereas two transcripts were not expressed (*BnC2FT* and *BnA2FT)*. Moreover, *BnA2FT* was only highly expressed at BBCH60 after floral transition (data not shown), whereas *BnC2FT* showed no expression at all. After vernalization (BBCH30-postV), *BnC6FTa/b* and *BnA7FTa/b* expression was higher in rosette plants, but differences between paralogs were obvious. *BnC6FTb* showed the largest relative expression level (~ 9-fold). At BBCH50 (visible floral buds), the *BnC6FTb* and *BnA7FTb* paralogs showed the largest fold-induction levels (~ 13-fold) (**Figure 2**). In leaves at BBCH 60 (first flower open), all paralogs with exception of *BnC2FT* showed very high relative expression levels (>2,000-fold), where *BnC6FTa* showed the highest relative expression (data not shown).

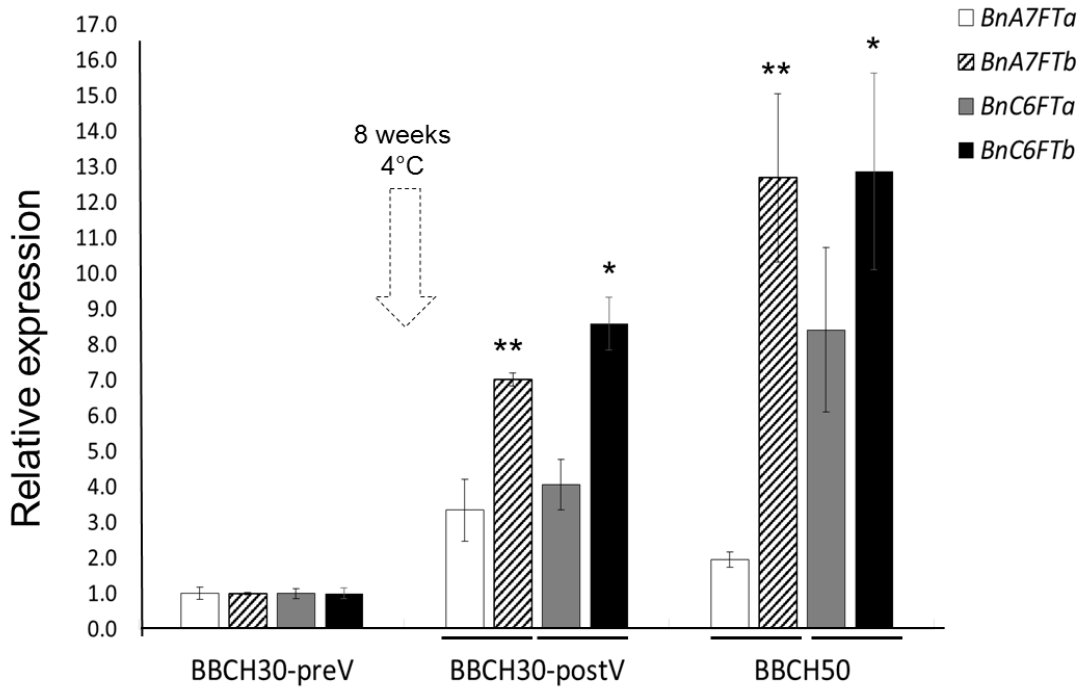

Figure 2: Relative expression of five *BnFT* paralogs in Express 617 plants at three developmental stages before and after vernalization (dotted arrow). Plants at BBCH30 were analyzed before vernalization (preV) and after vernalization (postV). The time point BBCH30-preV was taken as reference for calculation of relative expression in all target genes. Two biological replicates and three technical repetitions were analyzed for each time point. Error bars indicate the standard error of the mean of the relative expression values. Expression levels of target *BnFT* genes were normalized against *BnGAPDH* total expression. C_t values of the paralogs *BnA2FT* and *BnC2FT* were below the detection threshold at BBCH30 and BBCH50. Significant differences ($P<0.05$) are depicted by asterisks. Differences in relative expression between the *BnC6FTa*/*BnC6FTb* (*) and *BnA7FTa*/*BnA7FTb* paralogs (**) at each time point were tested via *t*-test. Lines at the base of the bars indicate the comparison pairs. All samples were taken between zeitgeber 11h and 12h in each developmental stage. Express 617 plants reached BBCH30-preV ~30 days after sowing. BBCH30-postV was registered ~90 days after sowing. BBCH50 was registered ~107 days after sowing.

2.4.2 EMS mutations in *BnFT* and *BnTFL1* paralogs

We screened our EMS population to measure the flowering time effect of mutations within the *BnFT* paralogs *BnC6FTa* (FJ848915.1) and *BnC6FTb* (FJ848917.1). In Arabidopsis, apart from FT, other PEBP proteins such as TFL1 regulate flowering by competing with FT for its binding targets (Mimida et al., 2001). Therefore, we developed primers for the *BnTFL1-2* (ABO17526) gene assigned to *B. rapa* chromosome A10. In total, 3,488 M_2 plants were screened by TILLING for EMS-induced mutations in *BnC6FTa/b* and *BnTFL1-2*.

Table 1: EMS mutations in three flowering time genes detected by TILLING of the winter-type inbred line Express 617.

	BnC6FTa	***BnC6FTb***	***BnTFL1-2***
Number of paralogs in rapeseed	6	6	4
Total number of M_2 plants screened	3488	3488	2092
Sequence screened by TILLING (bp)	1023	1767	1292
Nonsense mutations	0	1	2
Missense mutations	6	15	10
Splice site mutations	0	1	1
Total number of mutations	14	55	34
Mutations/kb	72	30	24
M_3 families selected for phenotyping	5	9	5
Total number of mutations		103	

The BnC6FTb paralog was screened by two different fragments, 1021 bp and 746 bp in size.

We generated paralog-specific PCR amplicons covering between 50% (*BnC6FTa*) and 100% (*BnC6FTb* and *BnTFL1-2*) of the open reading frames. The *BnC6FTa* fragment covered exon I and intron I. The two *BnC6FTb* fragments covered exon I/intron I and exons III/IV (**Figure 3**). The *BnTFL1-2* fragment covered all four exons. We identified 55, 14, and 34 single nucleotide mutations in the *BnC6FTb*, *BnC6FTa*, and *BnTFL1-2* genes, respectively. Forty-three mutations are located in introns, nineteen are silent mutations, and three are located within the UTRs (**Table 1**). Mutation rates ranged between 1/72 kb and 1/24 kb per 1,000 plants. The names of the mutant alleles contain the nucleotide substitution and nucleotide position (**Table 2**).

Table 2: Nucleotide position and amino acid changes due to EMS mutations in 18 missense/nonsense mutations in three *B. napus* flowering time regulators.

Gene	Mutation	Exon	Amino acid substitution	Mutant code
BnC6FTa	G37A	Exon I	Gly13Arg	*C6FTa*$_{G37A}$
	G52A	Exon I	Val17Lle	*C6FTa*$_{G52A}$
	C74T	Exon I	Ser25Leu	*C6FTa*$_{C74T}$
	G104A	Exon I	Arg35lys	*C6FTa*$_{G104A}$
	G163A	Exon I	Glu55Lys	*C6FTa*$_{G163A}$
BnC6FTb	G17A	Exon I	Arg6Lys	*C6FTb*$_{G17A}$
	G124A	Exon I	Asp42Asn	*C6FTb*$_{G124A}$
	C666T	CArG Box	CArG Box	*C6FTb*$_{C666T}$
	G1968A	Exon III	Trp98Stop	*C6FTb*$_{G1968A}$
	G2009A	Intron III	Splice site	*C6FTb*$_{G2009A}$
	G2122A	Exon IV	Arg112Lys	*C6FTb*$_{G2122A}$
	G2133A	Exon IV	Gly116Arg	*C6FTb*$_{C2133T}$
	G2154A	Exon IV	Val123Met	*C6FTb*$_{G2154A}$
BnTFL1-2	G52A	Exon I	Val18Lle	*TFL1-2*$_{G52A}$
	C518T	Exon II	Pro83Ser	*TFL1-2*$_{C518T}$
	G750A	Exon III	Gly105Arg	*TFL1-2*$_{G750A}$
	G851A	Exon IV	Val108Met	*TFL1-2*$_{G851A}$
	C965T	Exon IV	Gln146Stop	*TFL1-2*$_{C965T}$

We identified one nonsense mutation in exon III of the *BnC6FTb* gene (*BnC6FTb*$_{G1968A}$) leading to a truncated protein by substitution of a tryptophan by a stop codon (position 88). Another mutation (*BnC6FTb*$_{G2009A}$) resulted in a splice-site deletion leading to a truncated protein by interrupting the junction between exons III and IV. The *BnTFL1-2*$_{C965T}$ mutation in Exon III results in the substitution of a glutamine by a stop codon (position 146). Moreover, we detected numerous missense mutations in *BnC6FTa* (6), *BnC6FTb* (15), and *BnTFL1-2* (10).

We decided to focus on splice site- and missense-mutations which are most likely to affect the protein function. All observed missense mutations were compared to the SIFT database (http://sift.jcvi.org) in order to evaluate the impact of the amino acid substitutions on the protein function (data not shown). According to this analysis, we selected 18 M_3 lines for

growth experiments in the greenhouse (5 *BnC6FTa*, 8 *BnC6FTb*, and 5 *BnTFL1-2* mutations) (**Figure 4**).

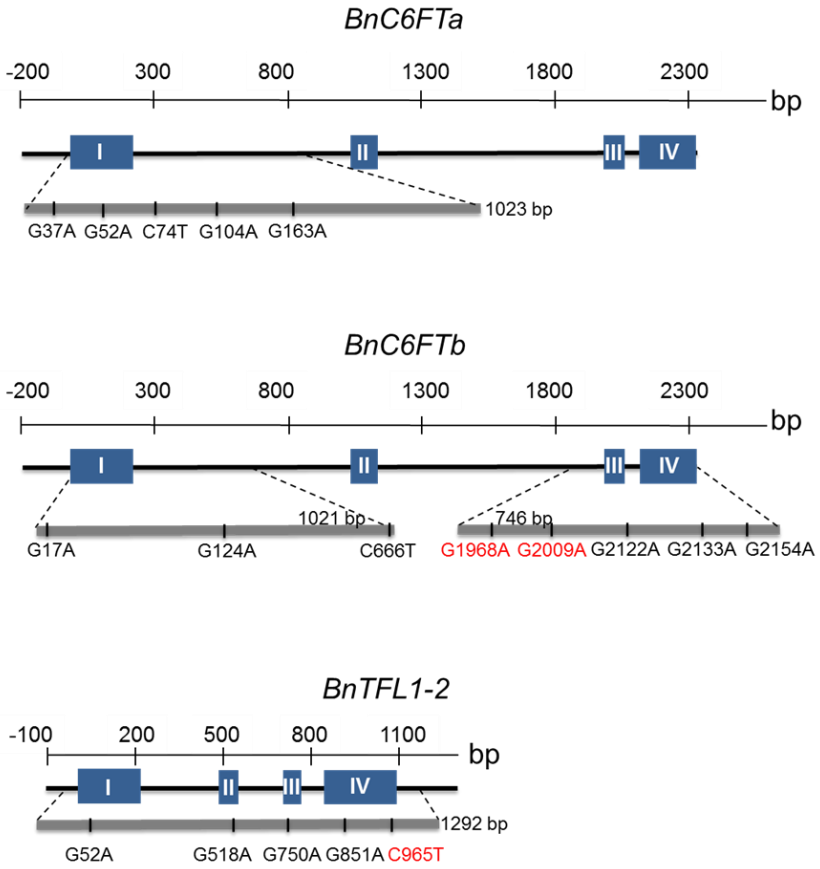

Figure 3: Graphical presentation of 18 point mutations in two *FT* (*BnC6FTa*, *BnC6FTb*) and one *TFL1* (*BnTFL1-2*) paralogs of the *B. napus* winter-type cultivar Express 617. The exon (blue boxes) - intron (black lines) structure of each gene is shown. Locations of SNPs are depicted by the grey bar below. Numbers refer to the position of the mutations from the START codon. Only STOP/splice-mutations (red) and selected missense mutations are shown.

2.4.3 Phenotypic characterization of *BnFT* and *BnTFL1-2* mutants

First, we confirmed the genotype of each selected M_2 plant by Sanger sequencing. Then, M_3 lines were grown in the greenhouse together with non-mutagenized Express 617 plants as a control. The genotype of each M_3 plant was also confirmed by Sanger sequencing (**Table 3**). According to the mother plants state in M_2 generation (homozygous or heterozygous mutant), there are two distribution patterns for the EMS-generated alleles can be expected: i) 1:2:1 segregation, or ii) fully mutant homozygous (fixed families). In *BnC6FTb*, three families showed non-segregating mutant alleles distribution, whereas five families segregated for the mutated allele (**Table 3**). In *BnC6FTa*, four families did not segregate for the EMS-generated

SNPs, whereas two families segregated for the mutated *BnC6FTa* alleles. From *BnTFL1-2,* three out of five families segregated for the mutated *BnTFL1-2* alleles. The deviation from the expected genotypic proportions (1:2:1 segregation ratio) in segregated families was determined via chi^2 test. All segregated families of three target genes displayed the expected 1:2:1 ratio except the family *BnC6FTb*$_{G17A}$ (P-Value < 0.05) (**Table 3**).

Table 3: M_3 families originating mutations in three flowering time genes and their segregation into three genotypic classes (*FT FT*: wild-type; *FT ft*: heterozygous; *ft ft*: homozygous mutants). M_3 seeds had been produced after selfing M_2 plants of the Express 617 EMS population.

Gene	Mutation	*FT FT*	*FT ft*	*ft ft*	χ2 test for H_0 = 1:2:1 (*FT FT* : *FT ft* : *ft ft*)	Seed code
BnC6FTb	*BnC6FTb*$_{G2154A}$	0	0	16		110104
BnC6FTb	*BnC6FTb*$_{G2122A}$	0	0	29		114619
BnC6FTb	*BnC6FTb*$_{G2009A}$	0	0	28		114620
BnC6FTb	*BnC6FTb*$_{C2133T}$	4	8	8	2.400	110103
BnC6FTb	*BnC6FTb*$_{C666T}$	5	8	5	0.222	110106
BnC6FTb	*BnC6FTb*$_{G17A}$	0	5	6	6.636	110109
BnC6FTb	*BnC6FTb*$_{G124A}$	6	8	3	1.118	110110
BnC6FTb	*BnC6FTb*$_{G1968A}$	2	15	5	0.155	114623
BnC6FTa	*BnC6FTa*$_{G163A}$	0	0	25		114614
BnC6FTa	*BnC6FTa*$_{G104A}$	0	0	7		114615
BnC6FTa	*BnC6FTa*$_{G37A}$	0	0	18		114617
BnC6FTa	*BnC6FTa*$_{G52A}$	5	10	3	0.667	114612
BnC6FTa	*BnC6FTa*$_{C74T}$	2	13	5	2.700	114613
BnTFL1-2	*BnTFL1*$_{G750A}$	0	0	19		114640
BnTFL1-2	*BnTFL1*$_{G52A}$	0	0	24		114641
BnTFL1-2	*BnTFL1*$_{G851A}$	5	13	3	1.571	114629
BnTFL1-2	*BnTFL1*$_{C518T}$	5	12	8	0.760	114633
BnTFL1-2	*BnTFL1*$_{C965T}$	8	10	6	1.000	114637

The phenological development of *BnC6FTa* and *BnC6FTb* lines was clearly different. All five *BnC6FTa* mutants flowered as the control, whereas six out of eight *BnC6FTb* mutants flowered later (**Figure 4**). The *BnC6FTb*$_{G1968A}$ mutants (stop mutation) showed a flowering delay of ca. 18 days, while *BnC6FTb*$_{G2009A}$ splice-site mutants flowered 29 days later as the control. Interestingly, 40% and 54% of the *BnC6FTb*$_{G1968A}$ and *BnC6FTb*$_{G2009A}$ M_3 mutants, respectively, did not bolt at all (**Figure 5**). *BnC6FTb* missense mutants started flowering 7 days (*BnC6FTb*$_{C2122A}$) up to 26 days (*BnC6FTb*$_{G17A}$) later as the control.

To evaluate the effect of background mutations on flowering time, we produced an F_2 population by crossing *BnC6FTb*$_{G2154A}$ M_3 plants with non-mutagenized Express 617.

BnC6FTb$_{G2154A}$ M_3 missense mutants gave higher hybrid seed yield as the stop mutants and they flowered 15 days later as the control. *BnC6FTb*$_{G2154A}$ M_3 plants showed a reduced number of initial flowers in comparison to other M_3 mutants, however most flowers were fertile. A total of 26 F_2 plants encompassing all three genotypic classes were grown in the greenhouse together with Express 617. In agreement with M_3 observations, homozygous F_2 mutants (*ft ft*) flowered 13 days later than F_2 siblings homozygous for the wild-type allele (*FT FT*) which did not show any significant differences in flowering time as compared to non-mutagenized Express 617 (**Figure 6**).

Apart from flowering time, reduced fertility was also apparent, mostly in *BnC6FTb* M_3 plants (**Figure 7**). For *BnC6FTb* mutants, the number of filled pods decreased in six out of eight *BnC6FTb* mutants ranging from 64.49% (*BnC6FTb*$_{G2009A}$) up to 89.87% (*BnC6FTb*$_{G124A}$). Two mutants *BnC6FTb*$_{G124A}$ and *BnC6FTb*$_{G1968A}$ showed 63.68%, 87.24% more initial flowers compared to Express 617, respectively (**Figure 7**). Plant height in *BnC6FTb* mutants remained similar to Express 617, however, seeds number decreased in most (7/8) *BnC6FTb* mutants ranging from 85.16% (*BnC6FTb*$_{G2009A}$) up to 100% (*BnC6FTb*$_{G124A}$). The similar tendency was conserved in seeds weight, ranging from 89.99% (*BnC6FTb*$_{G2009A}$) up to100% (*BnC6FTb*$_{G124A}$) compared to Express 617(**Figure 7**). For *BnC6FTa* mutants, *BnC6FTa* $_{G163A}$ M_3 plants were shorter (31.39%) than Express 617, and four out of five mutants showed decrease in plant dry weight between 25.02% (*BnC6FTa*$_{G37A}$) and 55% (*BnC6FTa*$_{G163A}$). Seeds number decreased ranging from 42.07% (*BnC6FTa*$_{G37A}$) to 78.22% (*BnC6FTa*$_{G163A}$). Seeds weight decrease ranged from 36.06% (*BnC6FTa*$_{G37A}$) to 73.47% (*BnC6FTa*$_{G163A}$) (**Figure 7**).

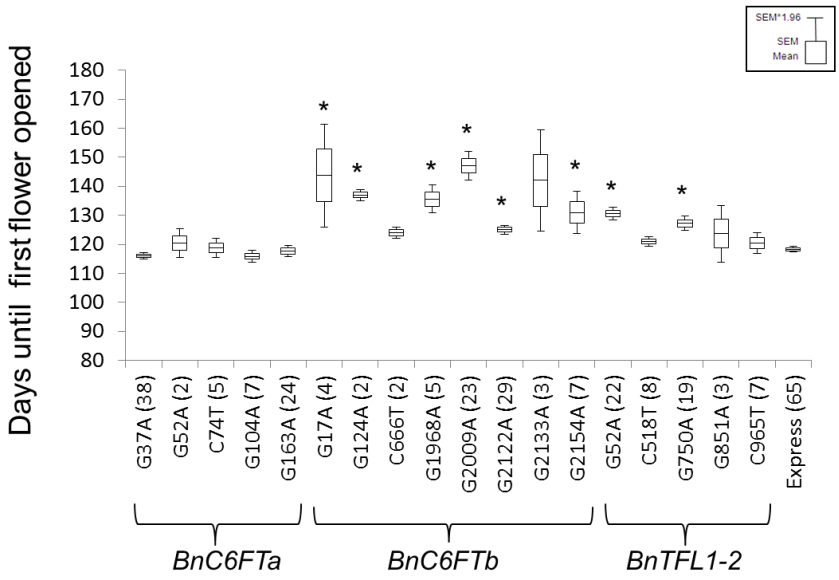

Figure 4: Flowering time point of 18 *BnC6FT a/b* and *BnTFL1-2* mutants grown in the greenhouse at constant temperature (22 ℃), and LD (16h light) after vernalization (4 ℃, 16h light, 8 weeks). Days to flowering

(BBCH60) was measured in M_3 plants homozygous for the EMS allele. The non-mutagenized donor line Express 617 was used as a control. The number of plants analyzed is written in brackets. Differences in flowering time between homozygous mutants and control plants were tested via *t*-test. Significant differences ($P<0.05$) are depicted by asterisks.

In *BnTFL1* mutants, the STOP mutation *BnTFL1-2*$_{C965T}$ did not lead to a major delay in flowering time. In contrast, the missense mutants *BnTFL1-2*$_{G52A}$ and *BnTFL1-2*$_{G750A}$ flowered ~10 days later than the control (**Figure 4**). Since, the stop mutation is close to the end of the *BnTFL1-2* gene, a functional protein may still arise after translation. Furthermore, *BnTFL1*$_{G750A}$ mutants exhibited modifications in plant architecture which gave us a reason to select them for crossing experiments. *BnTFL1*$_{G750A}$ mutants developed normally during the early growth phase until reaching BBCH50 (visible floral buds). The internode elongation phase was much longer as compared to Express 617, as a consequence, mutant plants were not able to stand by themselves after BBCH50. In this M_3 line, the flower development limited the continuous growth of the floral meristem, whereas side branches continued flowering. Four out of five *BnTFL1-2* mutants increased branches number ranged from 43.50% (*BnTFL1-2*$_{G750A}$) to 70.41% (*BnTFL1-2*$_{C965T}$) (**Figure 7**). All *BnTFL1-2* mutants showed lower seeds number (63.88%-95.65%) and seeds weight (58.91%-96.89%) compared to Express 617 (**Figure 7**).

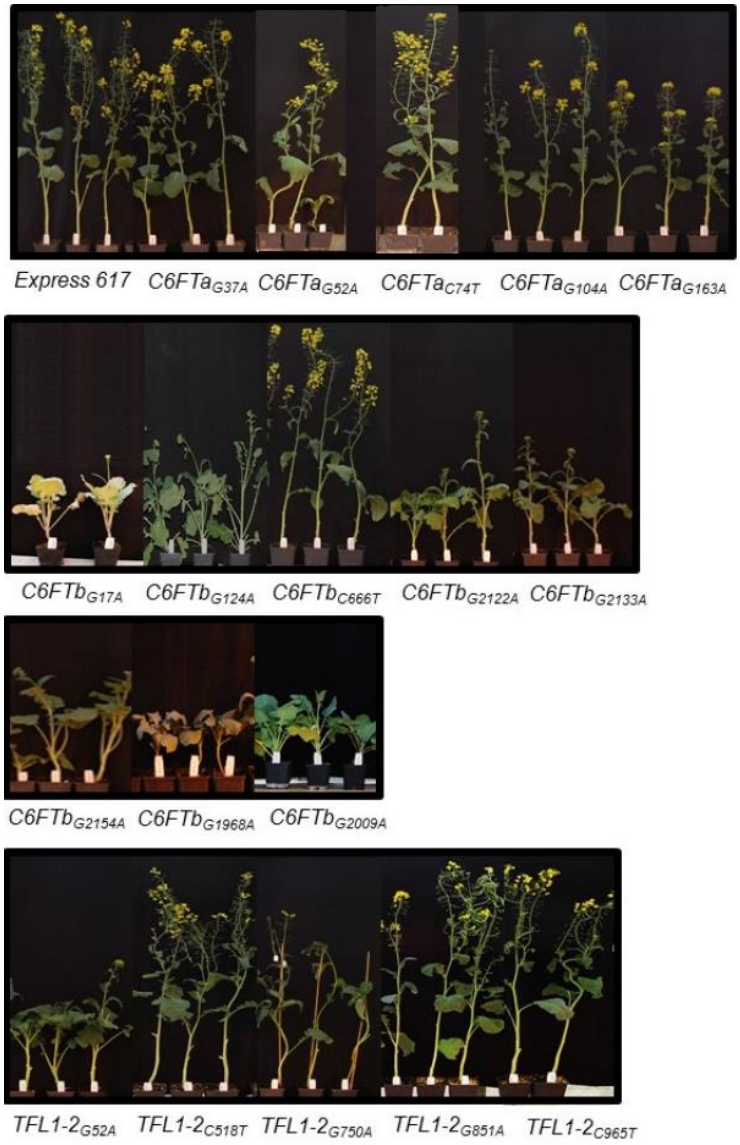

Figure 5: Phenotypes of 18 *B. napus BnC6FTa/b* and *BnTFL1-2* EMS M_3 lines. Photos were taken as the non-mutagenized Express 617 plants started flowering (top left). Plants were grown in the greenhouse at constant temperature (22 ℃), and LD (16h light) after vernalization (4 ℃, 16h light, 8 weeks).

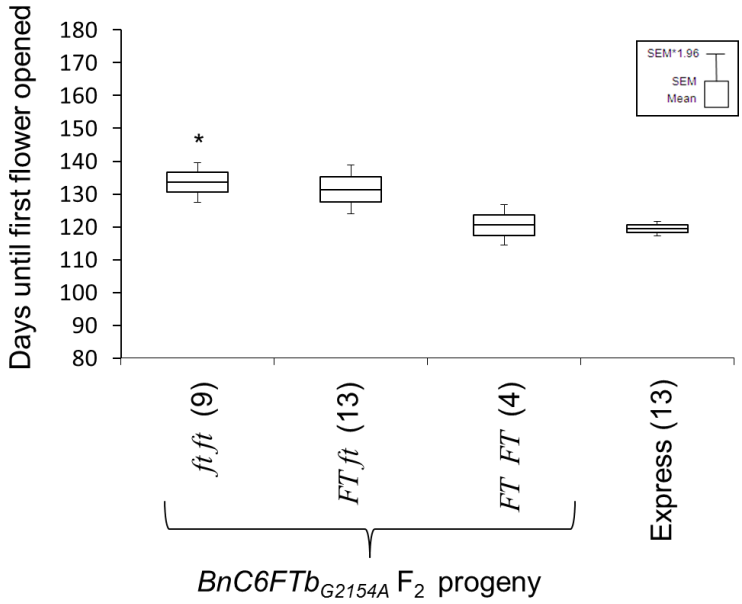

Figure 6: Flowering time point of three genotypes (homozygous mutants *ft ft*, heterozygous *FT ft*, wild-type *FT FT*) from the *BnC6FTb*$_{G2154A}$ F_2 population compared to non-mutagenized Express 617 plants. All plants were grown in the greenhouse at constant temperature (22 ℃) and LD (16h light) after vernalization (4 ℃, 16h light, 8 weeks). Days to flowering (BBCH60) was measured for each individual plant. The number of plants analyzed is written in brackets. Differences in flowering time between homozygous mutants and control plants were tested via *t*-test. Significant differences ($P<0.05$) are depicted by asterisks.

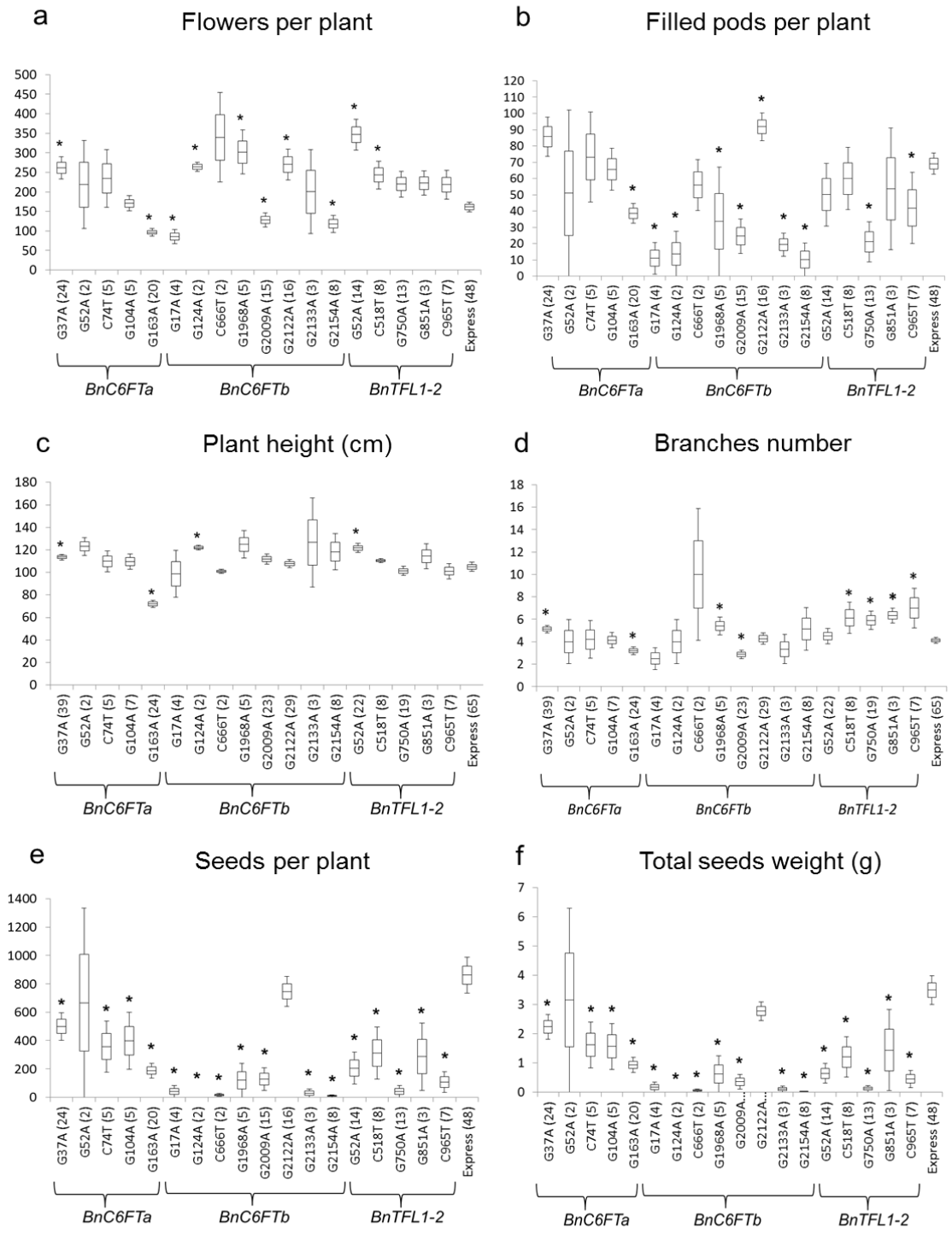

Figure 7: Traits performance of 18 *BnC6FT a/b* and *BnTFL1-2* mutants grown in the greenhouse at constant temperature (22 ℃), and LD (16h light) after vernalization (4 ℃, 16h light, 8 weeks). Total flowers per plant, total filled pods per plant, plant height, branches number, total seeds per plant and seed weight per plant were measured in M_3 plants homozygous for the EMS allele. The non-mutagenized donor line Express 617 was used as a control. The number of plants analyzed is written in brackets. Differences between homozygous mutants and control plants were tested via *t*-test. Significant differences ($P<0.05$) are depicted by asterisks.

2.4.4 The EMS mutations in *BnC6FTb* and *BnTFL1-2* paralogs are located in highly monomorphic regions of exon III and exon IV

To investigate the genetic structure of those *BnFT* and *BnTFL1* paralogs with paramount impact on flowering time, we analyzed the sequence diversity of *BnC6FTb* and *BnTFL1-2* in *B. napus* by sequencing their complete exons III and IV in 117 *B. napus* inbreed lines from different geographic origins and growth types. Sequences selected for analysis in each gene after quality trimming are deposited in **Supplementary data**. While *BnC6FTb* exon III turned out to be highly conserved, exon IV exhibited larger sequence diversity. Within 41 bp exon III of the *BnC6FTb* gene, only a single polymorphism was found at position 2004 which corresponds to an allele frequency of 1%. The EMS mutation G1968A resides within a sequence domain which is monomorphic among all accessions investigated. For *BnC6FTb* (exon IV), six polymorphic regions were found with minor allele frequencies of <5.0%. The EMS-generated alleles (positions G2122, G2133, and G2154) are residing in monomorphic sequences (**Figure 8**).

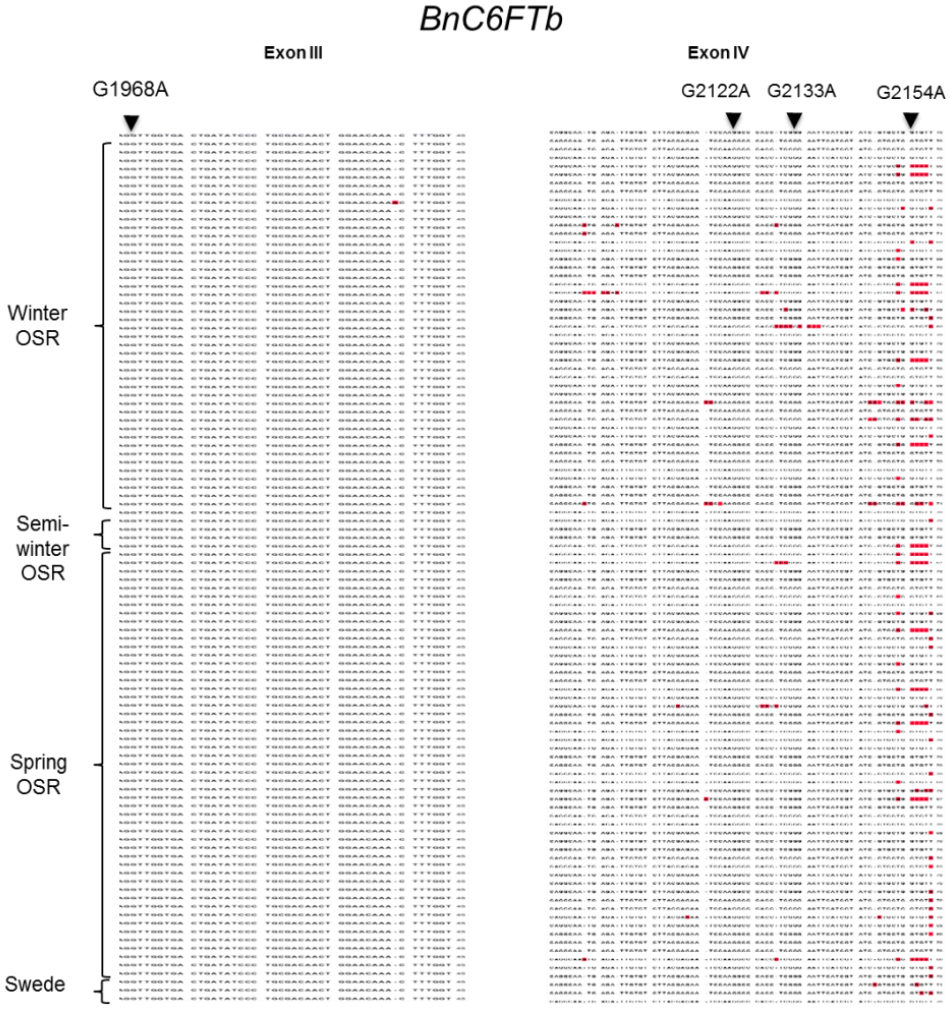

Figure 8: Sequence analysis of two exons of the *BnC6FTb* paralog from *B. napus*. One-hundred and seventeen oilseed rape cultivars from a collection of 500 accessions of different origins and growth types were sequenced. For *BnC6FTb*, 103 sequences with optimal length were further selected for further analysis Nucleotide polymorphisms are marked in red. Positions of EMS-mutations in Express 617 mutants (sequences not shown) are depicted by arrows.

In contrast to *BnFT* genes, a higher variability in *BnTFL1-2* exon III than in exon IV was found. In exon III the SNP showing the largest variation was a C insertion at position 731. The EMS mutation G750A which is also located within this exon, was located in a fully monomorphic domain. For *BnTFL1-2* exon IV, only a T/C polymorphism was found at position 1030. In conclusion, there is a high degree of sequence conservation within the analyzed sequences. Our EMS treatment created novel sequence variations within these highly conserved regions (**Figure 9**). FASTA-formatted sequences for each gene are deposited in **Supplementary data**. All sequences have been submitted to NCBI (www.ncbi.nlm.nih.gov) (accession numbers KJ533546 - KJ533625 and KJ533626 - KJ533728).

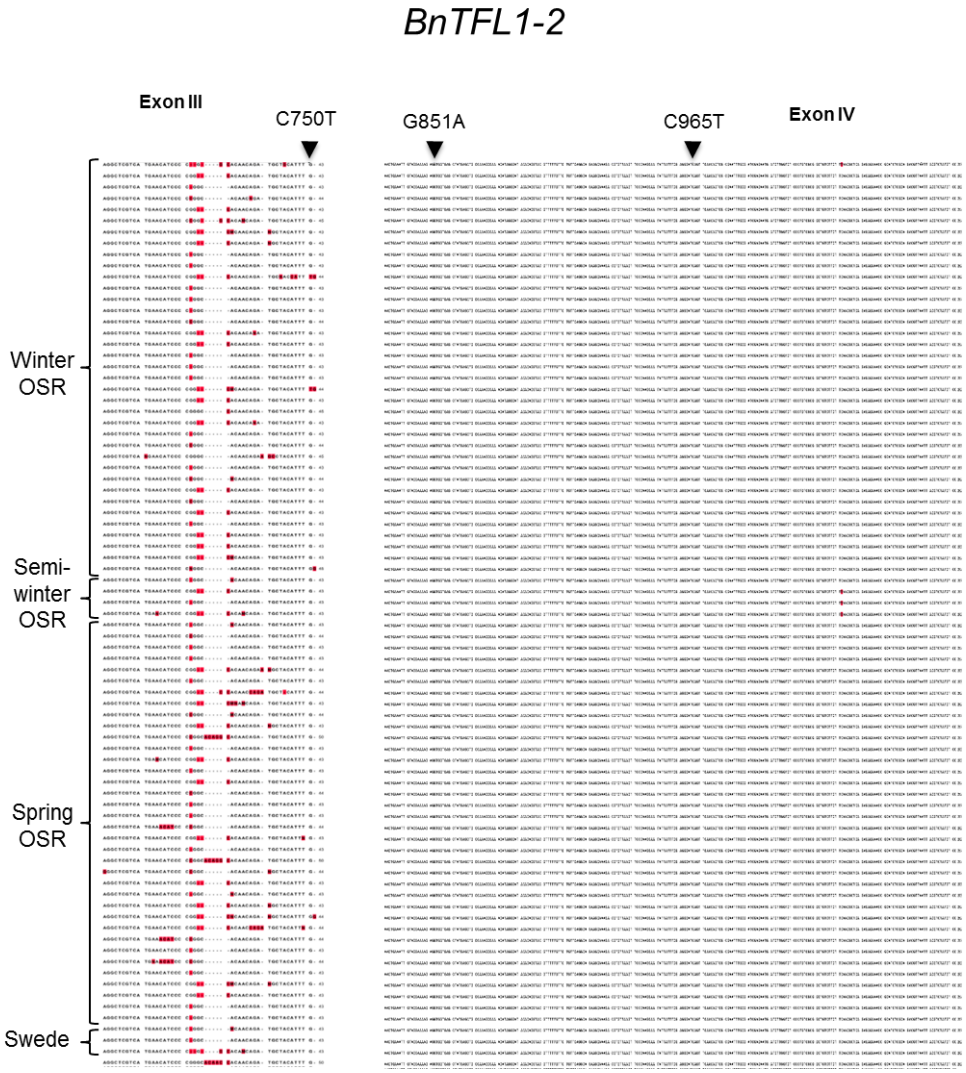

Figure 9: Sequence analysis of two exons of the *BnTFL1-2* paralog in *B. napus*. One-hundred and seventeen oilseed rape cultivars from a collection of 500 accessions of different origins and growth types were sequenced. For the *BnTFL1-2,* 80 sequences with optimal length were further selected for further analysis. Nucleotide polymorphisms are marked in red. Positions of EMS-mutations in the Express 617 mutants (sequences not shown) are depicted by arrows.

2.4.5 A *BnC6FTb*-splice-site mutation impacts the expression of other flowering time genes in leaves

We reasoned that a loss of function of the *BnC6FTb* paralog directly impacts the transcriptional activity of other major flowering time regulators downstream of *BnFT*. To test this hypothesis, we selected the $BnC6FTb_{G2009A}$ mutant because, first the G2009A SNP causes a splice-site mutation that leads to a truncated protein, and second, $BnC6FTb_{G2009A}$ M_3 plants are characterized by a marked flowering delay of about 29 days compared to the Express 617 control (**Figure 4**).

We chose *BnAP1* and *BnSOC1* as putative downstream targets of *BnFT* genes based on our knowledge from Arabidopsis (Kaufmann et al., 2010; Yoo et al., 2005). We measured their expression in leaves. *BnC6FTb*$_{G2009A}$ M_3 plants were grown in the greenhouse under constant temperature and LD conditions. For expression analysis, young leaves of three different plants were taken at stages BBCH30 (pre and post-vernalization), BBCH50, and BBCH60. Arabidopsis *AP1* and *SOC1* sequences were BLASTed against *B. oleracea* and *B. rapa*. High homology hits were aligned and primers were designed from highly conserved regions. Subsequently, joint expression of all paralogs was measured by RT-qPCR. Gene expression levels of *BnAP1* and *BnSOC1* were normalized using *BnGAPDH* and *BnB-Tub* genes.

We detected altered transcriptional activities of *BnAP1* in *BnC6FTb*$_{G2009A}$ mutants when compared to Express 617 control plants. Control Express 617 plants at BBCH30 (preV) were used as reference sample for relative expression calculations. At rosette stages (BBCH30), *BnAP1* expression in the M_3 mutant was higher than in control plants, while at BBCH50 and BBCH60 relative expression levels were 10% to 40% lower (**Figure 10**). Before vernalization (BBCH30-preV), *BnSOC1* expression in mutants was 2.5-fold higher than in Express 617. After vernalization, we detected a reduction of *BnSOC1* in *BnC6FTb*$_{G2009A}$ mutants compared to the control. When the first flower opened, the difference in expression between *BnC6FTb*$_{G2009A}$ mutants and Express 617 was at its maximum. The altered expression in leaves indicates that a single *BnC6FTb* mutation may affect other major flowering time regulators. We expect that both genes are expressed in the shoot apical meristem as well.

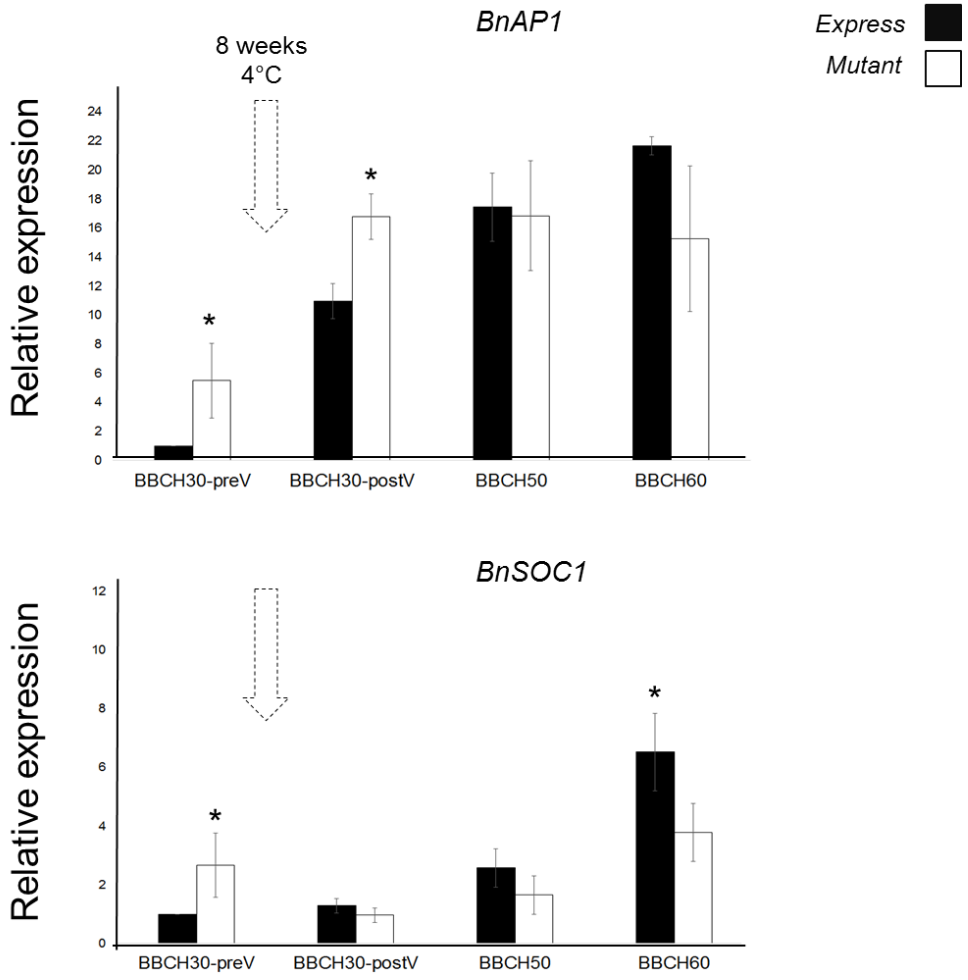

Figure 10: Joined expression analysis of two *BnFT* downstream targets in the *BnC6FTb*$_{G2009A}$ mutant (open boxes) and Express 617 (filled boxes). Four developmental stages were analyzed before and after vernalization (dotted arrows). Plants at BBCH30 were analyzed before vernalization (preV) and after vernalization (postV). Two biological replicates (M_3 plants) and three technical repetitions were analyzed for each time point. Error bars: standard error of the mean for biological replicates. C_t of target genes was normalized against the *BnGAPDH* and *BnB-Tub* total expression. The time point BBCH30-preV in Express 617 control plants was taken as reference sample for calculation of relative expression. Differences in relative expression were pairwise tested (control Vs M_3 line) via *t*-test. Significant differences ($P<0.05$) are depicted by asterisks. All samples were taken between zeitgeber 11h and 12h.

2.4.6 Performance of F_1 hybrids using the *BnTFL1-2* mutants as parents

In tomato, mutations in *FT* and *TFL1* orthologs accounted for fruit yield heterosis in F_1 hybrids (Krieger et al., 2010). We made an initial experiment to address the question whether *B. napus* orthologs might have a similar function. For producing F_1 hybrids, we selected *BnC6FTb*$_{G2009A}$ and *BnTFL1-2*$_{G750A}$ homozygous M_3 mutants as pollinators due to their late

flowering phenotype in conjunction with an altered inflorescence (lower number of fertile flowers, **figure 7**).We crossed homozygous M_3 plants with the male-sterile (MS) line MSL007. The MSL007 line (MSL-Express) is an isogenic line of Express that carries the male sterility Lembke (MSL) genic male sterility system (Basunanda et al., 2010). Thus, no F_1 heterosis was expected, except effects due to EMS mutations.

F_1 hybrids were vernalized and grown in the greenhouse with the parental lines and Express 617. Both F_1 hybrids showed differential effects. *BnC6FTb*$_{G2009A}$ hybrids showed no significant differences in seed number per plant and total seed weight in comparison to Express 617 as the best parent. In contrast, *BnTFL1-2*$_{G750A}$ F_1 hybrids had significantly higher number of seeds/plant (20%) and total seed weight (40%) as compared to the best parent (**Figure 11, 12**). Although the effects of background mutations cannot be ruled out these results could indicate that *BnTFL1* mutations impact heterosis in *B. napus*.

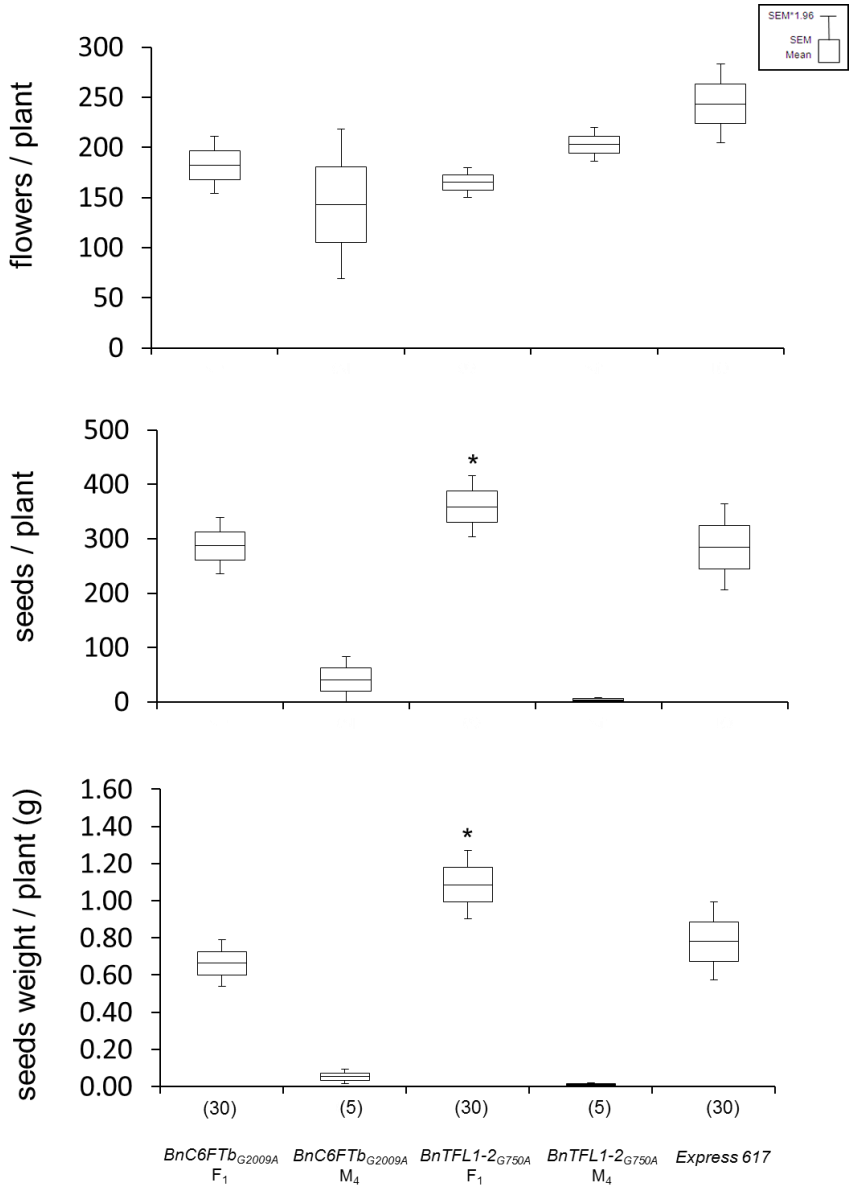

Figure 11: Growth experiments with vernalized F_1 hybrids after crossing two mutants with a male sterile isogenic line (MSL007). Each F_1 was obtained from two different crossing experiments. Mutant parents (M_4 lines), and Express 617 were used as controls. Yield components were determined on single plants grown in the greenhouse. The number of plants analyzed is given in brackets. Differences in flowers, seed number and seed weight per plant against Express 617 were tested via *t*-test. Significant differences ($P<0.05$) are depicted by asterisks. Growth conditions: 22 ℃, 16h light, greenhouse. Vernalization: 4 ℃, 16h light, 8 weeks.

Figure 12: Phenotypes of *B. napus BnC6FTb* and *BnTFL1-2* F_1 hybrids. Photos were taken as the non-mutagenized Express 617 plants matured (top right). Plants were grown in the greenhouse at constant temperature (22 ℃), and LD (16h light) after vernalization (4 ℃, 16h light, 8 weeks).

2.5 Discussion

In the present study, more than 100 EMS-alleles have been found for three flowering time genes of *B. napus.* Based on previous reports, the average EMS-mutation frequencies are expected to be lower in diploid species (~1/380 kb) than in polyploids (~1/50 kb) (Till et al., 2007; Wang et al., 2012b). In this study, mutation frequencies ranged between 1/24 kb and 1/72 kb. Using the same EMS-population, mutation frequencies ranged between 1/12 to 1/22 kb for sinapine biosynthesis genes (Harloff et al., 2012). Although the observed *BnFT* /*BnTFL1* mutation frequencies are slightly lower, our results are in the range expected for polyploid species. The present mutants will be a valuable resource to study flowering regulatory networks in polyploids and they can be introduced into breeding programs.

Our aim was to provide data about the function of *FT* and *TFL1* paralogs in rapeseed. We found that, despite the redundancy of mutations in a single gene, either nonsense or missense mutations in the *BnC6FTb* gene resulted in a marked flowering delay.This supports our hypothesis that *BnFT* paralogs contribute differently to flowering time regulation. A large plethora of Arabidopsis reports on *FT* loss-of-function mutants have established a robust correlation between *FT* mutations and flowering time delay in Arabidopsis (Andres and Coupland, 2012). Contrasting with expectations based on Arabidopsis *TFL1*-phenotypes, *BnTFL1-2* mutants showed a slight delay in flowering time. In Arabidopsis, a single amino acid change in *TFL1* (*tfl1-1*$_{Gly105Asp}$) led to early flowering and limited the development of indeterminate inflorescence by promoting the formation of a terminal floral meristem (Bradley et al., 1997). On the other hand, F_1 hybrids derived from crosses between *BnTFL1-2* M_3- and rapeseed MS lines showed increased seed yield compared to *BnC6FTb* F_1 hybrids

and Express 617 controls. Thus, although the role of *BnTFL1-2* involving flowering time regulation is not likely to be conserved compared to its Arabidopsis ortholog, *TFL1-2* appears to be involved in yield-related traits as reported for its tomato ortholog *SP* (Jiang et al., 2013b). As we mention in the following sections, confirming this hypothesis is a must for new research approaches.

During evolution, duplicated genes may undergo dosage adjustments (Conant and Wolfe, 2008; Papp et al., 2003), non-functionalization, or sub-/neo-functionalization (Force et al., 1999). We wanted to know whether different *BnFT* and *BnTFL1* paralogs gained different function by studying their phenotypes and their transcriptional activities. Through digital gene expression analyses, differential expression within early generations of re-synthesized- (F_1-F_4) and natural *B. napus* accessions has been reported (Birchler and Veitia, 2010). Three highly similar genes encoding endoplasmic reticulum-bound sn-glycerol-3-phosphate acyltransferase-4 (*BnGPAT4-C1*, *BnGPAT4-C2*, and *BnGPAT4-A1*) showed different expression patterns and altered epigenetic features (Chen et al., 2011) which is in accordance with the assumption that in polyploids orthologous genes are frequently expressed in a non-additive manner (Jiang et al., 2013a). We have also observed marked differences in the expression of six *BnFT* paralogs in support of the non/sub-functionalisation hypothesis. In regard to their position within a major flowering time QTL (Wang et al., 2009), *BnC6FTb* paralogs seem to play the most important role as flowering time regulators in winter type *B. napus*. More evidence has been given by the expression analysis of putative *FT* downstream targets *BnAP1* and *BnSOC1* in late-flowering *BnC6FTb*$_{G2009A}$ mutants. *SOC1* encodes a MADS-box transcription factor, acting as a floral integrator (Lee and Lee, 2010). The gene *SOC1* gene is expressed in the shoot apical meristem, and *SOC1* mutations lead to late flowering phenotype (Borner et al., 2000). However, *SOC1* is also expressed in vegetative organs (leaves) (Hepworth et al., 2002). A reduction in *SOC1* mRNA was detected in the meristem of late-flowering *ft-7* (Trp138Stop) *Arabidopsis* Ler background (Searle et al., 2006). In the future, the activities of these genes shall also be studied in the shoot apical meristem.

The phenotypic studies presented here gave further support to our assumption that *BnFT* paralogs do not contribute equally to flowering time regulation. In sugar beet (*Beta vulgaris*), two *FT* paralogs (*BvFT1* and *BvFT2*) were reported to antagonistically regulate flowering time (Pin et al., 2010). Knockdown of the *FT* potato paralog *StSP3D* resulted in a late flowering phenotype, while knockdown of the second paralog *StSP6A* had no effect on flowering time but on tuberization (Navarro et al., 2011). In line with these findings, we observed differential effects of *BnC6FTa* and *BnC6FTb* mutations. The strong effect of *BnC6FTb* as a flowering time regulator in rapeseed has been confirmed. To which extend *BnC6FT*a and *BnFT* paralogs are involved in flowering time control needs to be investigated in the future (e.g. by using other EMS mutants). As determined by our expression analyses, the strongest case of non-functionalization is shown by the lack of expression of the *BnC2FT*

copy. This result is in full congruence with Wang and coauthors' report (2012a) where this gene copy was neither expressed in *B. napus* nor in *B. oleracea*. A series of recent studies has demonstrated that beyond flowering time control, FT-like proteins act as mobile or cell-autonomous proteins that mediate other developmental processes, such as growth, plant architecture, and tuber formation (Carmona et al., 2007; Kinoshita et al., 2011; Navarro et al., 2011). In contrast to our *BnFT* mutant results, a previous analysis of sinapine biosynthesis mutants from the same EMS population as in our study, phenotypic or physiological effects had been observed only in double mutants (Harloff et al., 2012).

Although yield heterosis is regarded as a quantitative trait, single genes can contribute to heterotic effects through overdominance, such as the Arabidopsis *Erecta* locus (Moore and Lukens, 2011). As a first example of single gene overdominance, the yield of *sft-4537*/+ heterozygous tomato plants was increased by up to 60% in comparison to their parents after crossing high yielding M82 inbred plants with low-yielding homozygous loss of function mutants (*sft-4537*) (Krieger et al., 2010). In our work, F_1 hybrids carrying a *BnTFL1-2* mutant allele had a higher seed yield as the Express 617 parent. Our study delivers the first insights about potential *TFL1*-related heterosis in *B. napus*. In the future, experimental data are needed to verify this hypothesis. We tested our initial hypothesis by crossing mutants with the non-mutated donor line. Although, the data point at *BnTFL1* as a major gene for heterosis in rapeseed the possible impact of background mutations must be analysed by additional hybrid combinations. Moreover, we will sequence the *BnTFL1-2* loci from rapeseed lines with high and low combining ability (Qian et al., 2007). If our prelimary greenhouse data will be confirmed by field experiments this study will offer new perspectives for a hybrid breeding strategy which make use of *BnTFL1-2* sequence variations.

2.6 Acknowledgements

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3 Point mutations in a *FRIGIDA* homologue show pleiotropic effects in rapeseed (*Brassica napus* L.)

3.1 Abstract

The *FRIGIDA* (*FRI*) gene acts as a key regulator in *Arabidopsis* by inhibiting floral transition through activation of the *FLOWERING LOCUS C* (*FLC*). In rapeseed, four *FRI* orthologs have been identified (*BnFRI*), among them, the *BnaA.FRI.a* paralog had been co-localized within a major flowering time QTL. To further characterize its function, we identified 14 mutant alleles of the *BnaA.FRI.a* gene via TILING. Several missense mutations revealed differential effects regarding flowering time as well as yield components, such as plant height, flower number and seed weight. One M_3 line, $BnFRI_{G1278A}$ flowered four days earlier, showed increase in plant height, and a decrease of filled pods (49.06%) compared to the non-mutagenized donor line Express 617. Additionally, joint expression of *BnFRI* was reduced in buds and flowers of M_3 plants when they started flowering. Furthermore, the expression of the downstream targets *BnFLC*, *BnSOC1*, and *BnAP1* was altered in $BnFRI_{G1278A}$ M_3 plants. Copy-specific expression of *BnFLC* paralogs showed that a single *BnFRI* point mutation is affecting mainly three *BnFLC* paralogs, shedding new light on the regulatory relationships between *FRI* and *FLC* in a polyploid species. We demonstrate that point mutations in a single gene copy have pleiotropic effects beyond the regulation of flowering time in a complex allopolyploid genome.

3.2 Introduction

Oilseed rape (*Brassica napus* L.) is a major oilseed crop worldwide (http://www.worldoil.com/). It is grown in temperate regions of the world such as northern Europe, Canada, China, and Australia. Adaptation to different environments and regional climatic conditions in oilseed rape cultivars involves variation in the regulation of flowering time (Becker et al., 1995). Therefore, identification of the genetic factors that promote or inhibit flowering is important for oilseed rape breeding.

Phylogenetically, *B. napus* (AACC, n=19) is a young allopolyploid species that contains the genomes of two closely related species: *B. rapa* (AA, n=10) and *B. oleracea* (CC, n=9). These two species are themselves paleopolyploids that branched out from a common ancestor with *A. thaliana* about 13-20 Mya. As a consequence of the repeated polyploidization events, single Arabidopsis orthologs are represented by three to six *B. napus* paralogs (Schranz et al., 2006).

In Arabidopsis, vernalization-, photoperiod-, autonomous-, and gibberellin-dependent flowering time pathways have been characterized (Andres and Coupland, 2012). Natural Arabidopsis accessions have evolved two main life cycle strategies. Winter-annuals germinate in autumn, survive winter as a rosette and flower in spring. In contrast, summer-annuals (rapid-cycling) flower rapidly in the absence of vernalization, germinate in spring and

complete their reproduction cycle in the same growing season. Allelic variation at the gene *FRI* has been proposed as the main candidate gene to explain variation in flowering time among natural accessions of *A. thaliana*. Due to the diverse climates and environments where *Brassica* species are grown, the vernalization pathway is expected to play a central role on the oilseed rape flowering time control. In Arabidopsis winter ecotypes, the vernalization pathway has two major upstream regulators, *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*) (Andres and Coupland, 2012). *FRI* alleles with large effects on flowering time have been described in a number of independent mapping populations and near isogenic lines. Dominant alleles of *FRI* confer late flowering, which is reversed to earliness by vernalization (Johanson et al., 2000; Sanda et al., 1997). *FRI* interacts epistatically with *FLC* to determine the time to flowering (Jiang et al., 2009). It encodes a novel protein including 609 amino acids with two coiled-coil domains. Molecular analysis showed that *FLC* encodes a transcription factor, which when upregulated by *FRI*, acts as a flowering inhibitor but can be offset by cold treatment during the rosette stadium (Michaels and Amasino, 2001; Sheldon et al., 2000). For activation, FRI recruits at least four *FLC*-specific regulators to form the FLC-activator complex (Choi et al., 2011). Once transcriptionally active, *FLC* inhibits flowering by keeping the floral integrator *FT* in a repressed state. During vernalization, *FLC* expression levels are reduced allowing *FT* to be transcribed. As a consequence, flowering in Arabidopsis ecotypes carrying *FRI* and *FLC* functional alleles only occurs after a prolonged cold exposure (vernalization). Naturally occurring *FRI* null alleles have evolved independently multiple times (Le Corre et al., 2002). In fact, allelic variation in the *FRI* and *FLC* genes explains the largest flowering time differences among *Arabidopsis* ecotypes (Shindo et al., 2005b). As an example, *FRI* loss of function alleles are reported to determine the early flowering time phenotype on the Columbia (Col) and Landsberg *erecta* (L*er*) ecotypes (Johanson et al., 2000). Thus, *FRI* sequence variation may be key factor for flowering time regulation in other *Brassica* species.

In the ‘omics’ era, whole genome sequence comparisons between *Arabidopsis* and *Brassica* species have unraveled substantial sequence conservation (Schranz et al., 2006). Applying this approach, two *FRI* orthologs have been identified in the *B. oleracea* genome, *BolC.FRI.a and BolC.FRI.b* (Irwin et al., 2012). From these two paralogs, *BoFRIa* contains two coiled-coil domains whereas *BoFRIb* is predicted to contain only one coiled-coil domain. *BoFRIb* is highly conserved among different genotypes of *B. oleracea*, but a polymorphic region in exon 1 that included two deletions of seven and three amino acids was identified in *BoFRIa*. Only *BoFRIa* was functionally verified by complementation analyses in *Arabidopsis* mutants, providing evidences for sub-functionalization. Four *FRI* homologs have been identified in *B. napus* by BAC library screening and PCR-based cloning (*BnaA.FRI.a, BnaX.FRI.b, BnaX.FRI.c* and *BnaX.FRI.d*) (Wang et al., 2011a). All copies were confirmed after publication of the *B. napus* genome reference sequence (Chalhoub et al., 2014). Complementing sequence-based cloning, Wang et al. (2011a) integrated selected *BnFRI* paralogs into a genetic map containing flowering time QTL, and carried out association

studies on natural collections. As outcome result, the *BnaA.FRI.a* copy co-localized with a major flowering time QTL on chromosome A3. Further on, *BnaA.FRI.a* natural variation at amino acid positions 198, 254, 324, 348, and 417 correlated with flowering time differences between ecotypes from different locations world-wide. With the support of these observations, the *BnaA.FRI.a* paralog was postulated as a candidate gene for further analyses.

In the present work, two major hypothesis concerning the importance of *BnaA.FRI.a* in the rapeseed flowering time regulation were tested: i) sequence variations in the *BnaA.FRI.a* paralog correlate with flowering time in *B. napus*, and ii) missense mutations in a single *BnFRI* paralog can lead to phenotypic effects beyond flowering. Consequently, we determined the effects of EMS-induced *BnaA.FRI.a* point mutations in the oilseed rape winter inbred line Express 617. Complementing the findings of Wang et al. (2011a), a conserved region spanning from exon I to exon III was selected and screened via TILLING (Till et al., 2006b). We identified 14 *BnaA.FRI.a* EMS-generated alleles. From them, six M_3 lines were further characterized in the greenhouse. The *BnaA.FRI.a* missense mutations correlated with differences in flowering time and yield components. Additionally, differential gene expression was observed for five out of nine *BnFLC* paralogs. Point mutations in a single *FRI* paralog also show effects on expresson levels of at least three *BnFLC* paralogs. Understanding *BnFRI* opens new horizons for rapeseed breeding, like $BnFRI_{G1278A}$ could accelerate flowering by four days. The mutant alleles have already been introduced into the elite cultivar Express 617, which represents a new source of genetic variation for rapeseed breeding.

3.3 Materials and Methods

3.3.1 Mutation screening

In total, 2640 M_2 plants representing 695 M_1 plants of the Express 617 population were screened for *BnaA.FRI.a* point mutations. The screening procedure followed the methodology reported by Guo et al. (2014). For *BnaA.FRI.a* amplification, Gene Bank sequence JN936850 was used. Initially, a 1788 bp fragment was amplified with the primer pair BnFRI-A261 and BnFRI-N020, subsequently this amplicon was used for a second nested PCR obtaining a 1070 bp fragment with primer combination BnFRI3-seq and BnFRI-RT-rev (**Supplementary table S1**).

3.3.2 Plant materials and greenhouse experiments

From fourteen initial TILLING families, six *BnaA.FRI.a* M_3 lines (**Table 2**) were chosen for phenotyping in greenhouse trials. From each line, 30 plants were grown in 35-well plates (3 x 3 cm). Plants of the donor line Express 617 were used as non-mutagenized control. Seedlings were first grown for four weeks under long day conditions (16h day/8h dark). Subsequently, plants were vernalized for eight weeks in a climate chamber (5 ℃, 16h day/8h dark). After

vernalization, plants were transferred to 9 × 9 cm pots and grown in the greenhouse under LD conditions. All plants were randomized to reduce position effects. The phenotypic characters were measured at BBCH stages BBCH10, BBCH30, BBCH50, BBCH60 and BBCH69. Plant height, branches number, total pods per plant, filled pods per plant, seed weight, seed number, and plant dry weight were recorded at BBCH79 (all pods have reached their maximum size). Plants that did not grow beyond BBCH10 were excluded from the experiments. M_3 line *BnFRI*$_{G1278A}$ was crossed with Express 617 and an F_2 generation was produced after selfing the F_1. Plants of the F_2 population segregating for the *BnFRI*$_{G1278A}$ allele were grown in the greenhouse under long day conditions (16h day/8h dark) as well as non-mutagenized Express 617.

3.3.3 DNA isolation and genotyping

For genotyping, total DNA was extracted from young leaves using a CTAB protocol. The detailed procedure followed the methodology reported by Guo et al. (2014). Genomic DNA was amplified by PCR using unlabeled primers BnFRI3-seq and BnFRI-RT-rev. PCR product were checked by agarose gel electrophoresis and Sanger sequencing as described under chapter 2.3.3.

3.3.4 Expression analysis by RT-qPCR

M_3 plants and Express 617 controls were grown in the greenhouse under long day conditions as mentioned above. Young leaves of M_3 *BnFRI*$_{G1278A}$ plants and Express controls were sampled at four developmental stages (see chapter 3.3.2). In addition, buds and flowers were sampled at BBCH60. Total RNA was extracted with the RNeasy kit (QIAGEN, www.qiagen.com) according to the manufacturer's protocol. Primer sequences were obtained from DNA sequences from the non-redundant NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/) which was downloaded to the CLC-bio main workbench version 6.0 (http://www.clcbio.com). Both CLC-bio main workbench and Oligonucleotide Properties Calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html) were used for designing primers. Groups of paralogs were aligned with the help of the internal alignment routine. Two main strategies were applied for expression analysis. Non-specific primers were designed from conserved regions within groups of paralogs for detection of joint gene expression (*BnFRI, BnFLC, BnSOC1* and *BnAP1*) (**Supplementary table S1**). Copy specific primers were designed for five out of nine *BnFLC* paralogs (*BnFLC.A10*, *BnFLC.A3a*, *BnFLC.A3b*, *BnFLC.A2*, and *BnFLC.C2*) which had been described by Tedege (2001) and Zou et al. (2012). The RT-qPCR was performed as described by Guo et al. (2014).

3.4 Results

3.4.1 EMS mutations in the *BnaA.FRI.a* paralog

We expected that point mutants in the *BnaA.FRI.a* gene have a major impact on flowering time because of its co-localization with a major flowering time QTL on chromosome A3 (Wang et al., 2011a). Therefore, we developed two specific primers which amplified a fragment 1070 bp in size which represents 49% of the translated region of the *BnaA.FRI.a* gene (exon I/II/III) (**Figure 13**). We identified 14 *BnaA.FRI.a* mutations including one nonsense, one splice site and five missense mutations (**Table 4**). The mutation rate is 1/48 kb per 1,000 plants. A nonsense mutation in exon I (*BnFRI* $_{C841T}$) leads to a truncated protein by substitution of a tryptophan by a stop codon (position 281) whereas the *BnFRI* $_{G908A}$ mutation results in a splice-site deletion leading to an altered protein by interrupting the junction between exons I and II (**Table 5**). For phenotypic analysis, we selected one nonsense- and five missense- mutations for the greenhouse experiment (**Figure 13**).

Table 4: EMS mutations in the flowering time gene *BnaA.FRI.a* detected by TILLING of the winter-type inbred line Express 617.

	BnaA.FRI.a
Number of paralogs in rapeseed	4
Total number of M_2 plants screened	2640
Sequence screened by TILLING (bp)	1070
Number of nonsense mutations	1
Number of missense mutations	5
Number of splice site mutations	1
Total number of mutations	14
Mutations/kb	48
M_3 families selected for phenotyping	6

Table 5: Nucleotide position and amino acid changes due to EMS mutations in seven missense/nonsense mutations in the *B. napus* flowering time regulator *BnaA.FRI.a*. The mutation code contains the position and the nucleotides that had been changed (e.g. C841T means C mutates to T at position 841, and numbers are set relative to the start of the coding sequence).

Mutation	Exon	Amino acid substitution	Mutant code
C841T	Exon I	Gln281Stop	*BnFRI* $_{C841T}$
G908A	Intron I	Splice site	*BnFRI* $_{G908A}$
G1278A	Exon II	Glu327Lys	*BnFRI* $_{G1278A}$
G1285A	Exon II	Arg329Lys	*BnFRI* $_{G1285A}$
G1601A	Exon III	Asp404Asn	*BnFRI* $_{G1601A}$
G1712A	Exon III	Glu441Lys	*BnFRI* $_{G1712A}$
G1731A	Exon III	Arg447Lys	*BnFRI* $_{G1731A}$

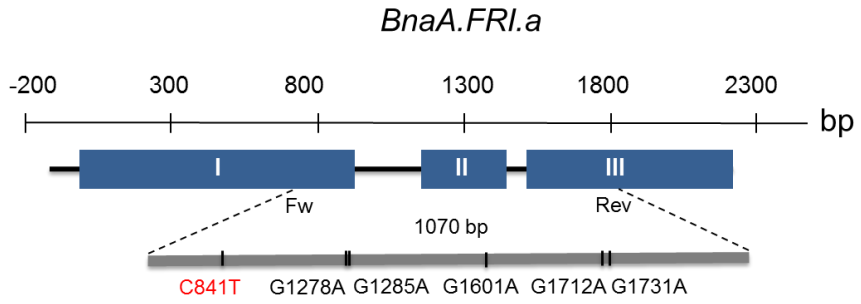

Figure 13: Graphical presentation of six point mutations in the *BnaA.FRI.a* paralog of the *B. napus* winter-type cultivar Express 617. The exon (blue boxes) - intron (black lines) structure of each gene is shown. Locations of SNPs are depicted by the grey bar below. Numbers refer to the position of the mutations from the START codon. Only STOP-mutations (red) and selected missense mutations are shown. The grey bar represents the PCR fragment used for TILLING.

3.4.2 Genotypic characterization of *BnaA.FRI.a* mutants

The M_3 populations were expected to segregate into three genotypic classes *FRI FRI*, *FRI fri* and *fri fri* as 1:2:1 ratio if its M_2 mother plant was heterozygous mutant where *FRI* stands for the *BnaA.FRI.a* gene. M_3 families were fixed for the mutant allele if their M_2 mother plants had been homozygous for the mutation. We isolated DNA from M_3 plants of each population. The DNA was amplified with the primer combination BnFRI3-seq and BnFRI-RT-rev (**Supplementary table S1**) and the amplicons were subsequently sequenced (**Table 6**). As a result, three M_3 families segregated for the EMS-alleles whereas the remaining three M_3 families did not segregate (**Table 6)**. Segregation in *BnFRI*$_{C841T}$ family deviated significantly

from the expected 1:2:1 ratio. Interestingly, the family *BnFRI*$_{C841T}$ segregating for the STOP mutation did not have any homozygous mutant (**Table 6**).

Table 6: Segregation for *BnaA.FRI.a* EMS mutations in M_3 families (*FRI FRI*: wild-type; *FRI fri*: heterozygous; *fri fri*: homozygous mutants). M_3 seeds had been produced after selfing M_2 plants of the Express 617 EMS population. Express 617 was used as a control.

Mutation	Plants sown	Gener-ation	Germinat-ion rates	*FRI FRI*	*FRI fri*	*fri fri*	χ2 test for H_0 = 1:2:1 (*FRI FRI*:*FRI fri*:*fri fri*)	Seed code
BnFRI$_{G1278A}$	30	M_3	40%	0	0	12		110097
BnFRI$_{G1285A}$	30	M_3	73.3%	0	0	22		110098
BnFRI$_{G1712A}$	30	M_3	36.7%	0	0	11		110100
BnFRI$_{G1601A}$	30	M_3	90%	9	10	8	1.889	110099
BnFRI$_{G908A}$	30	M_3	63.3%	4	8	7	1.421	110096
BnFRI$_{C841T}$	15	M_2	66.7%	8	2	0	17.2*	110094
Express 617	30	-	50%	15	0	0		

* α=0.05

3.4.3 Phenotypic characterization of *BnaA.FRI.a* mutants

Selected *BnaA.FRI.a* M_3 lines were grown in the greenhouse under long day conditions along with non-mutagenized Express 617 plants. The *BnFRI*$_{G1278A}$ M_3 plants flowered four days earlier than Express 617. In contrast, *BnFRI*$_{G1601A}$ and *BnFRI*$_{G1712A}$ flowered 19 and 6 days later than the control, respectively. Interestingly, *BnFRI*$_{G1285A}$ M_3 flowered at the same time as the Express 617 control. This mutation is next to the position of another mutation (*BnFRI*$_{G1278A}$) which flowered four days earlier than Express 617 (**Figure 14**). The mutants also differed by plant height and seed set. The *BnFRI*$_{G1278A}$ and *BnFRI*$_{G1285A}$ M_3 plants were 20 ($\pm$ 4.07) and 22 ($\pm$ 2.03) cm longer than the controls respectively (**Figure 14**) and the number of flowers/plant (BBCH 69) as compared to Express 617 was 72.37 and 55.54% higher in *BnFRI*$_{G1285A}$ and *BnFRI*$_{G1712A}$ M_3 mutants, respectively (**Figure 14**). However, the proportion of filled siliques ('pods') was between 24.4% (*BnFRI*$_{G1285A}$) and 88.55% (*BnFRIG1601A*) lower in all mutants (data not shown).

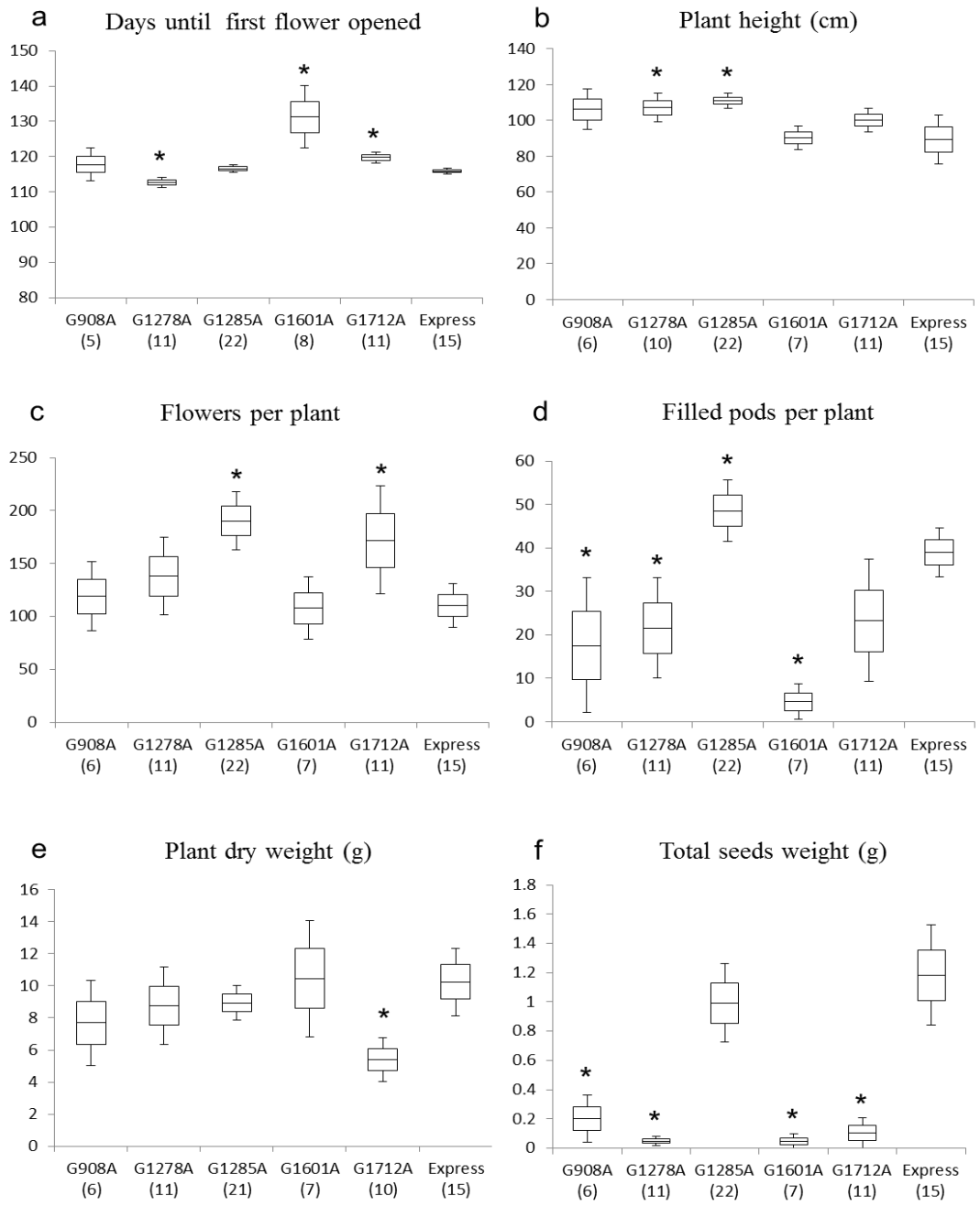

Figure 14: Box plot diagrams showing differences between *BnFRI* mutants and Express 617. The onset of flowering and yield parameters have been analysed. Five *BnFRI* M_3 mutants homozygous for the EMS allele were grown in the greenhouse at constant temperature (22 ℃), and LD (16h light) after vernalization (4 ℃, 16h light, 8 weeks). The non-mutagenized donor line Express 617 was used as a control. The number of plants analyzed is written in brackets. Differences between homozygous mutants and control plants were tested via *t*-test. Significant differences ($P<0.05$) are depicted by asterisks.

Figure 15: Phenotypes of five *B. napus BnFRI* M_3 lines. Photos were taken at BBCH79 when nearly all pods have reached their maximum size in the non-mutagenized Express 617 plants. Plants were grown in the greenhouse at constant temperature (22 ℃) under LD conditions (16h light) after vernalization (4 ℃, 16h light, 8 weeks).

3.4.4 A *BnaA.FRI.a* point mutation impacts the expression of downstream targets

We suspected that *BnaA.FRI.a* alleles with altered function may impact the transcriptional activity of other flowering time genes. To test this hypothesis, we selected the early-flowering (four days) *BnFRI*$_{G1278A}$ M_3 mutant (**Figure 14**) for expression analyses. We measured the expression of *BnFRI* and its downstream targets *BnFLC*, *BnAP1* and *BnSOC1* in *BnFRI*$_{G1278A}$ plants. For expression analysis, young leaves of at least two different plants were taken at stages BBCH30 (pre- and post-vernalization), BBCH50, and BBCH60. Buds and flowers were also sampled at BBCH60. RT-qPCR primers were designed from highly conserved regions among paralogs of the candidate genes (joint expression). Expression levels were normalized against the *BnGAPDH* and *BnB-Tub* housekeeping genes.

The joint expression of *BnFRI* was decreased by 50.98% in mutant floral bud and 48.44% in mutant flower compared to Express 617, respectively. Also a 39.97% reduction in *BnFLC* expression was observed in *BnFRI*$_{G1278A}$ mutant plants (leaves, BBCH30) compared to Express 617 before vernalization. As expected, *BnFLC* expression was drastically reduced by 84.0% in Express 617 leaves and reduced by 80.4% in *BnFRI*$_{G1278A}$ M_3 leaves (BBCH30) after vernalization. After onset of the first flower (BBCH60), *BnFLC* expression increased by 473.5% (Express 617) and 615.7% (*BnFRI*$_{G1278A}$ M_3) compared to BBCH50 (**Figure 16**). At this stage, *BnFLC* expression was increased by 62.0% in *BnFRI*$_{G1278A}$ M_3 leaves compared to Express 617(**Figure 16**).

The *BnSOC1* gene expression was increased by 61.1% in $BnFRI_{G1278A}$ mutants compared to Express 617 at BBCH60 (**Figure 16**). Interestingly, the expression of *BnAP1* was increased by 702.7% in mutant leaves before vernalization compared to Express 617 (**Figure 16**). These results show that a single point mutation in one out of four *BnFRI* paralogs alters flowering time by transcriptional activation or silencing of downstream genes.

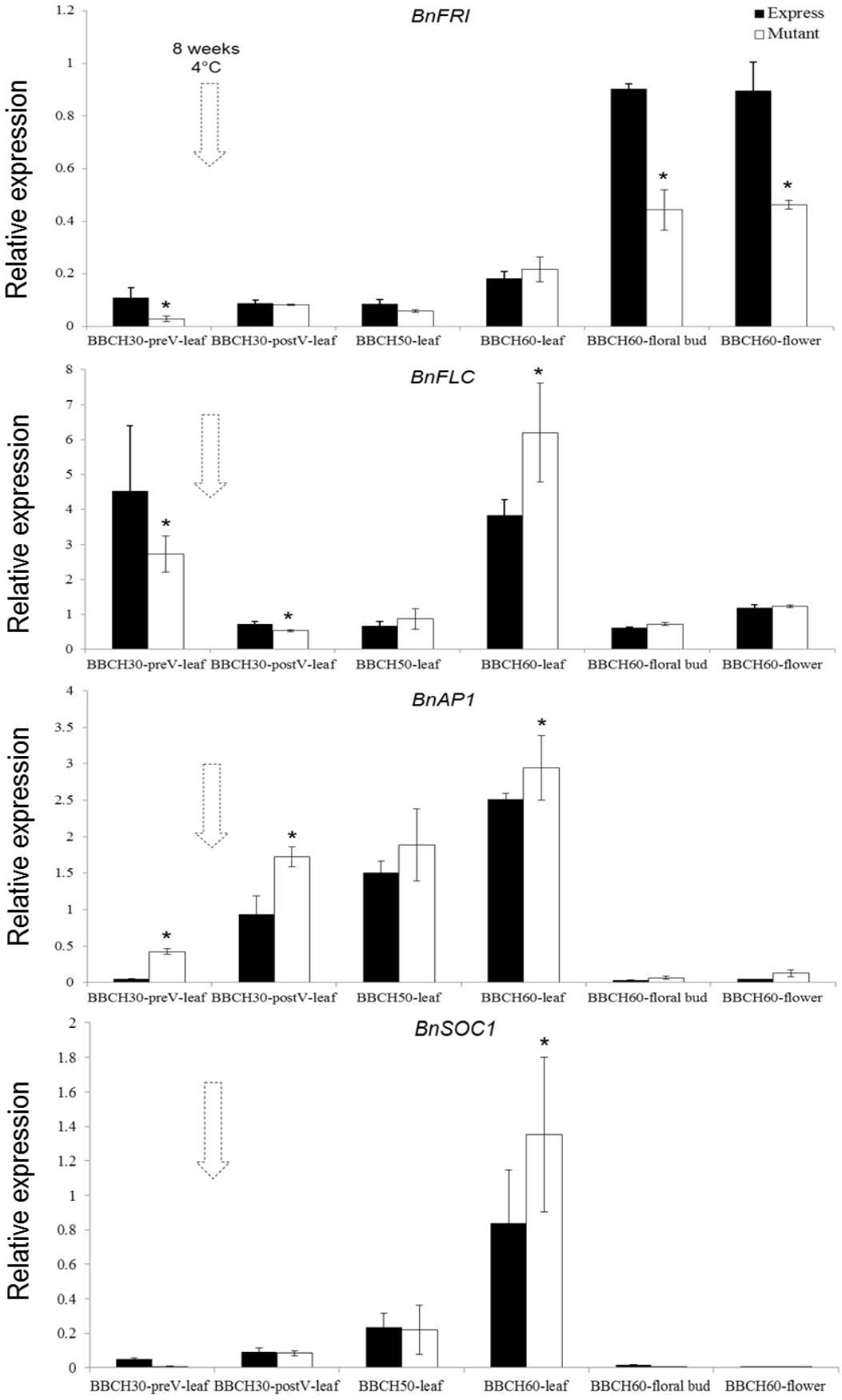
BnFRI
Express
Mutant
8 weeks
4°C
Relative expression
BnFLC
BnAP1
BnSOC1
BBCH30-preV-leaf
BBCH30-postV-leaf
BBCH50-leaf
BBCH60-leaf
BBCH60-floral bud
BBCH60-flower

Figure 16: Joint expression analysis of *BnFRI, BnFLC, BnAP1,* and *BnSOC1* in $BnFRI_{G1278A}$ M_3 mutants (open boxes) and Express 617 (filled boxes). Plants were grown in the greenhouse under LD conditions. Four developmental stages and three tissues were analyzed before and after vernalization (dotted arrows). Plant leaves at BBCH30 were analyzed before vernalization (preV) and after vernalization (postV). At least two biological replicates (M_3 plants) and three technical replicates were analyzed for each time point. Error bars: standard error of the mean for at least two biological replicates. The C_t of target genes was normalized against the *BnGAPDH* and *BnB-Tub* total expression. The time point BBCH30-preV in Express 617 control plants was taken as a reference sample for calculating the relative expression. Differences in relative expression were pairwise tested (control Vs M_3 line) via *t*-test. Significant differences ($P<0.05$) are depicted by asterisks. All samples were taken between zeitgeber 11h and 12h in each developmental stage.

3.4.5 Paralog-specific expression analyses of *BnFLC*

We had observed differential expression of *BnFLC* in the $BnFRI_{G1278A}$ mutant. The question was whether the five different *BnFLC* paralogs react in the same way or whether the $BnFRI_{G1278A}$ mutation had a paralog specific effect on *BnFLC* expression. Therefore, we analyzed the expression of *BnFLC* paralogs individually in Express 617 and $BnFRI_{G1278A}$ M_3 plants. We developed *BnFLC* paralog-specific RT-qPCR primers based on five out of nine *B. napus FLC* paralogs. We selected those paralogs because they were differentially expressed in spring and winter cultivars (Tadege et al., 2001; Zou et al., 2012). Moreover, paralog-specific analysis of the other paralogs is extremely difficult due to their high sequence similarity. As a result, three paralogs analysed showed different expression rates in Express 617 (**Figure 17**) while *BnFLC.A10* and *BnFLC.A3b* were not expressed at all. At BBCH30 (before vernalization), *BnFLC.A3a* and *BnFLC.A2* were highly expressed whereas *BnFLC.C2* showed a much lower relative expression. After vernalization (BBCH30-postV), *BnFLC.A3a*, *BnFLC.A2*, and *BnFLC.C2* reduced by 89.0%, 98.0% and 85.0%, respectively (**Figure 17**). This expression dynamics is congruent with the expectations of an active *FLC* paralog responding to cold treatment in a winter type *B. napus*. However, a drastic increase in expression was observed for *BnFLC.A3a* at the latest sampling stage. At BBCH60, *BnFLC.A3a* expression increased by 308.3% in leaves compared to BBCH50 (**Figure 17**).

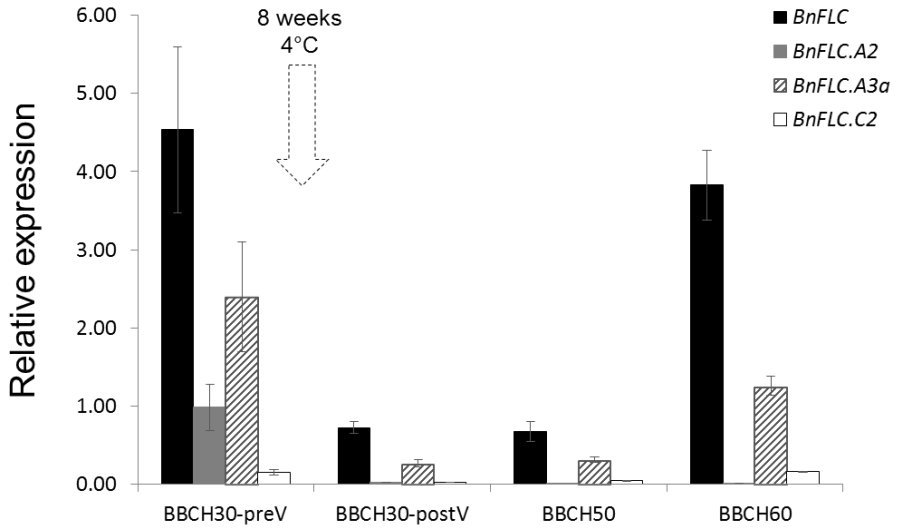

Figure 17: Relative expression of three *BnFLC* paralogs in Express 617 plants at four developmental stages before and after vernalization (dotted arrow). Plants at BBCH30 were analyzed before vernalization (preV) and after vernalization (postV). The time point BBCH30-preV was taken as reference for calculation of relative expression in all target genes. A minimum of two biological replicates and three technical repetitions were analyzed for each time point. Error bars indicate the standard error of the mean of the fold-induction values. Expression levels of target *BnFLC* genes were normalized against *BnGAPDH* and *BnB-Tub* total expression. C_t values of the paralogs *BnFLC.A10* and *BnFLC.A3b* were below the detection threshold. Samples were taken between zeitgeber 11h and 12h at each developmental stage.

In a next experiment, we compared the expression of the two most expressed *BnFLC* copies (*BnFLC.A2* and *BnFLC.A3a*) between the $BnFRI_{G1278A}$ M_3 line and Express 617. In summary, remarkable differences between the M_3 line and Express 617 were found. Before vernalization, both paralogs were less expressed in mutant plants (*BnFLC.A2*, -0.5 fold; *BnFLC.A3a*, -1.37 fold). After vernalization (BBCH30), the transcript levels of *BnFLC.A2* and *BnFLC.A3a* decreased by 89.0% - 98.9% ($BnFRI_{G1278A}$ M_3 and Express 617) until BBCH50. Interestingly, *BnFLC.A3a* showed an equal increase of expression at BBCH60 in the mutant and in Express 617 while *BnFLC.A2* expression remained very low (**Figure 18**).

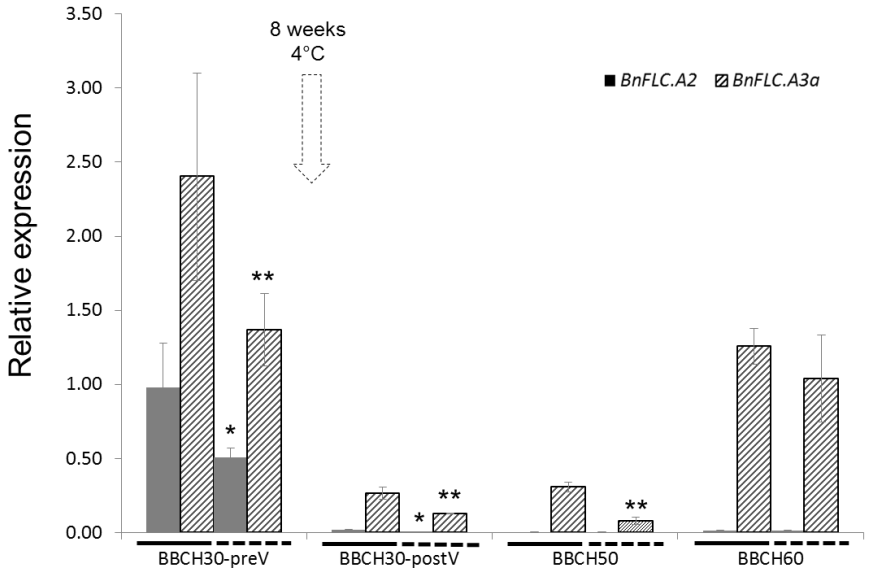

Figure 18: Relative expression of two *BnFLC* paralogs in the $BnFRI_{G1278A}$ mutant and Express 617 plants at four developmental stages before and after vernalization (dotted arrow). Samples underlined with a solid or dashed line were collected from Express 617 controls and mutants, respectively. Plants at BBCH30 were analyzed before vernalization (preV) and after vernalization (postV). The time point BBCH30-preV was taken as reference for calculation of relative expression in all target genes. At least two biological replicates and three technical repetitions were analyzed for each time point. Error bars indicate the standard error of the mean of the fold-induction values. Expression levels of target *BnFLC* genes were normalized against *BnGAPDH* and *BnB-Tub* total expression. Significant differences ($P<0.05$) are depicted by asterisks. Differences in relative expression between *BnFLC.A2*/Mutant-*BnFLC.A2* (*) and *BnFLC.A3a*/Mutant-*BnFLC.A3a* (**) at each time point were tested via *t*-test. Lines at the base of the bars indicate the comparison pairs. All samples were taken between zeitgeber 11h and 12h in each developmental stage.

3.5 Discussion

We studied the effects of single *BnFRI* mutations on the phenological development of rapeseed and on the transcriptional activity of downstream targets. *FRI* encodes a protein with two coiled-coil motifs between amino acids 55-100 and 405-450 and is part of the vernalization pathway that promotes flowering after a long period of cold (Johanson et al., 2000). In Arabidopsis, winter-annuals (late-flowering) contain functional *FRI* alleles (*FRI-wt*), whereas summer-annuals (early flowering) generally carry nonfunctional alleles. Two distinct deletions in *FRI* are believed to confer nonfunctional alleles. The Columbia allele (*FRI-Col*) contains a 16 bp-deletion (exon 1) leading to a premature stop codon and, thus, a truncated protein missing a part of the C-terminal. But the most frequent *FRI* mutation in nature is a 376 bp-deletion combined with a 31 bp-insertion in the promoter as observed in Landsberg *erecta* (*FRI-Ler*) (Johanson et al., 2000). Notably, a recent study provides the first robust evidence that the *FRI-Ler* allele induces *FLC* expression, delays flowering time and confers sensitivity to vernalization in contrast to the true null *FRI-Col* allele. Nevertheless, *FRI-Ler* has a weaker

effect than the fully functional *FRI-wt* allele (Schmalenbach et al., 2014). Many early flowering accessions carry loss-of-function *fri* alleles containing one of two deletions of *FRI-Col* or *FRI-Ler*. However, some accessions (Shakhdara, Kondara, and Kz-9) categorized as early flowering types do not carry these deletion (Gazzani et al., 2003; Le Corre et al., 2002). In this case, the flowering time is suggested to depend on the epistatic interaction between *FRI* and *FLC* (Jiang et al., 2009). *FLC* encodes a MADS-box transcriptional activator that binds to the promoter of *SOC1* and to the first intron of *FT* to block flowering by repressing their transcription (Gu et al., 2013; Helliwell et al., 2006). In addition, it has been reported FLC binds to more than 500 target sites in the *Arabidopsis* genome, indicating that it has roles in many developmental pathways (Deng et al., 2011). Choi et al. (2011) elucidated the *FRI*-mediated *FLC* regulation mechanism in Arabidopsis. They found that FRI protein functions as a scaffold polypeptide recruiting chromatin-modifying factors via the FRI-C activator complex. In a previous report, *BnaA.FRI.a* had been co-localized with a major flowering time QTL on chromosome A3. Specific primer combinations were designed for this paralog and finally we found five missense mutations of *BnaA.FRI.a* in Express 617 population via TILLING. We expected missense mutation could result in early flowering in *B. napus*. Interestingly, we observed one M_3 $BnFRI_{G1278A}$ with a Glu to Lys substitution which resulted in four days earlier flowering. We analysed the effect of this single mutation by the expression analysis of *BnFRI* and its downstream targets *BnFLC, BnAP1* and *BnSOC1* in this early flowering $BnFRI_{G1278A}$ mutant. We found that the $BnFRI_{G1278A}$ mutant flowered earlier by differentially regulating downstream targets. Even before vernalization, a 40.0% reduction in *BnFLC* expression was observed in mutant leaves, and both *BnAP1* and *BnSOC1* expression was higher in mutant leaves at BBCH60 which can well explain early flowering of M_3 $BnFRI_{G1278A}$. Moreover, in agreement with M_3 observations, homozygous F_2 mutants (*fri fri*) flowered two days earlier than F_2 siblings homozygous for the wild-type allele (*FRI FRI*) which did not show any significant differences in flowering time as compared to non-mutagenized Express 617 (Rizvi *et al*, data not shown).

Our results highlight the importance of single amino acid exchanges due to EMS mutagenesis. We compared the *FRI* homologs from (amino acid sequences) between *Arabidopsis*, *B. napus* and *B. rapa* (**Figure 19**). We found that the EDRF motif (positions 327 to 330 amino acid) to be highly conserved among *B. napus* and other Brassica species. As a protein involved in a complex conformation, phosphorylation of certain amino acids may lead to changes in the protein function. To test whether that could be the case for the Glu327Lys substitution, we screened a database for computational prediction of phosphorylation sites using our mutated and wild type *BnaA.FRI.a* sequences (http://gps.biocuckoo.org). We found a single different serine/threonine kinases phosphorylation sites between Glu327Lys and the non-mutated protein, which can impair or activate a signaling cascades. Here, the EDRF/KDRF motif mutation created an extra phosphorylation target site for Ca^{2+}/calmodulin-dependent protein kinases (CAMK) at the serine residue at position 331 (**Supplementary table S3**). In tobacco plants over-expression of a Ca^{2+}/calmodulin-dependent protein kinase (*NtCBK1)* lead to

flowering time alterations (Hua et al., 2004). Thus, we propose EDRF as a candidate motif to be further studied to understand the *BnaA.FRI.a* protein function.

Beyond flowering time, the mutants also differed by plant height and seed set comparing to Express 617. It should not be surprising, because a number of genetic mutations that regulate flowering time also have shown pleiotropic effects on leaf shape, trichome density, and inflorescence architecture (Suh et al., 2003). Pleiotropic effects of *FRI* on drought resistance have been reported (McKay et al., 2003). In addition, Lovell et al. (2013) presented evidence that *FRI* pleiotropically affects growth rate, water use efficiency as well as flowering time in *A. thaliana*. In that study, *FRI* exhibited 'adaptive' pleiotropy. 'Null' (non functional) alleles resulted in a drought escape phenotype owing to low water use efficiency, fast growth rate and early flowering relative to the ancestral adaptive strategy. Moreover, Scarcelli et al. (2007) investigated the adaptive value of *FRI* in an outbred population of *A. thaliana* produced by intermating 19 accessions. They showed that null alleles have negative pleiotropic effects on fitness by reducing the number of nodes and branches on the inflorescence. Although nonfunctional *FRI* alleles increased seed production by reducing flowering time, it also decreases seed production by reducing the number of branches. These antagonistic effects cancel each other out, resulting in no overall effect of *FRI* genotype on fitness.

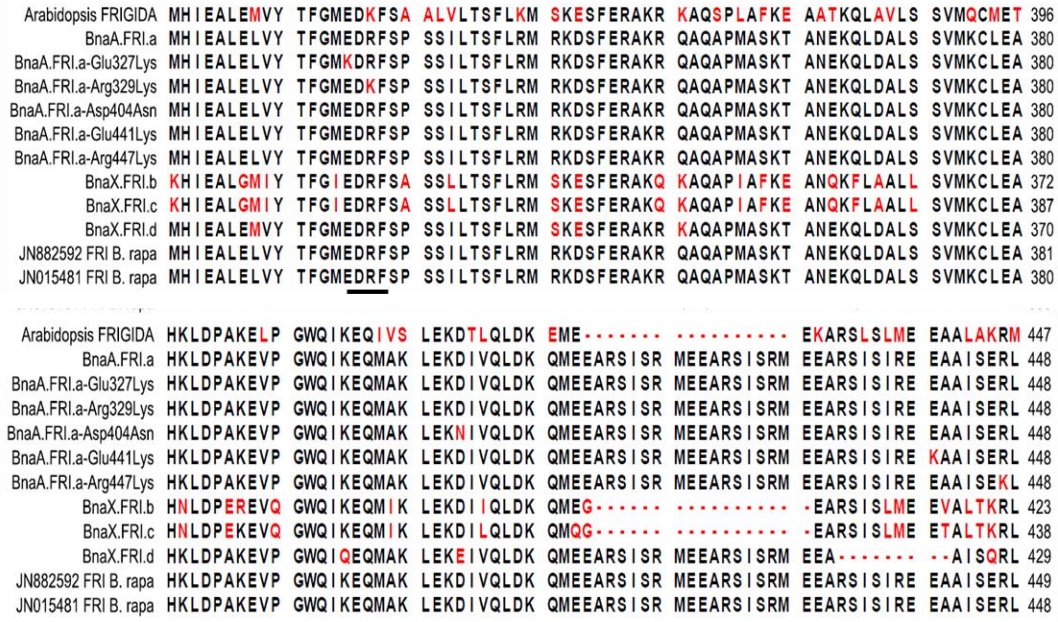

Figure 19: Amino acid seuences of the *Brassica BnaA.FRI.a* gene family. The EMS-generated alleles (red) of *BnaA.FRI.a* in the present study are located within regions with different degree of conservation. Amino acid substitutions Glu327Lys and Arg329Lys are located in a highly conserved EDRF motif (aa 327-330) in the central FRI domain (exon II).

In the following, we will discuss the question, how *BnFRI* mutations could alter the

expression of *BnFLC*. In Arabidopsis, *FLC* is constitutively expressed prior to vernalization by activation of *FRI*. To initiate the transition to flowering, *FLC* is subsequently silenced in response to prolonged cold, making *FT* accessible for activation by the photoperiod pathway. In recent years, we gained a deeper understanding about the interaction between *FRI* and *FLC*. FRI interacts with at least four more proteins to activate the *FLC* locus, such as FRL1, FES1, SUF4, and FLX. Each component of FRI-C has a specialized function. SUF4 binds to a *cis*-element of the *FLC* promoter, FLX and FES1 have transcriptional activation potential, and FRL1 and FES1 stabilize the complex (Choi et al., 2011). Although present in only a single copy in Arabidopsis, *FLC* has four copies each in *B. rapa* and *B. oleracea* and even nine copies in *B. napus* (Okazaki et al., 2007; Zhao et al., 2010; Zou et al., 2012). In *B. napus*, cDNA sequences of *FLC* homologues (*BnFLC1-5*) have been isolated and their ectopic expression delays flowering in *A. thaliana* (Tadege et al., 2001; Zou et al., 2012). Zou et al. (2012) found *BnFLC* homologues showed distinct expression patterns in vegetative and reproductive organs at different developmental stages. Interestingly, they also found that the *BnFLC.A3b* gene is tightly linked to a cold- and dehydration-responsive gene *BnCBF.A3* (*CREPEAT/DRE BINDING FACTOR*), and it is loosely linked to *BnFRI.A3*. A tendency for co-expression of *BnFRI.A3* and *BnFLC.A3b* at different developmental stages in non-vernalized Ningyou7 plants further suggested that the adjacent *BnFRI.A3* and *BnFLC.A3b* genes probably interact. They suggest the clustering of *FLC-FRI-CBF1* in Brassica might be subjected to selection to adjust the flowering time in biennial plants. Searching the reference genome sequence, Chalhoub et al. (2014) found homeologous exchanges (HEs) to be associated with loss or retention of different *FLC* paralogs in the divergent *B. napus* morphotypes. Different *FLC* homologs lie within HEs, from C_n2 to A_n2 in the Asian semiwinter oilseed forms Yudal and Aburamasari and C_n9 to A_n10 in late-flowering Swedes. These loci correspond to important QTLs for vernalization requirement and flowering time (Zou et al., 2012). In accordance with previous results, the expression magnitudes were very different between paralogs in Express 617 and $BnFRI_{G1278A}$ M_3 plants. Before vernalization, *BnFLC.A3a* and *BnFLC.A2* transcriptional activities were 57.0% and 51.7% in $BnFRI_{G1278A}$ M_3 plants as compared to the Express control whereas *BnFLC.C2* was 55.4%. With regard to *BnFLC* expression, previous studies did not give consistent results. *BnFLC.A3b* was highly expressed in the vernalized winter-type Tapidor leaves (Zou et al., 2012). But it was also 25-fold higher expressed in non-vernalized winter-type rapeseed (Tapidor) compared with a semi-winter type cultivar (Ningyou7). *BnFLC.A10*, showed a relative low expression in vernalized Tapidor leaves (Zou et al., 2012). In contrast, *BnFLC.A10* and *BnFLCA3b* were not detectable in our experiment. Quite interestingly, our observations on $BnFRI_{G1278A}$ M_3 plants do not support the one to one interaction model. We observed that at least three *BnFLC* paralogs (*BnFLC.A3a*, *BnFLC.A2* and *BnFLC.C2*) show expression alterations related to a missense mutation in a single *BnFRI* paralog. Thus, *BnFRI-BnFLC* interactions are indeed more complex in this allopolyploid species.

Finally, we will discuss the mutation frequencies from this study in comparison with earlier reports. In general, mutation frequencies in polyploids (~1/50 kb) are expected to be 10 times higher than in diploid species (~1/380 kb) (Wang et al., 2012b). In *B. napus*, mutation frequencies in the *fatty acid elongase1* gene (*FAE1*) ranged between 1/130 kb (0.3% EMS) and 1/42 kb (0.6% EMS) (Wang et al., 2008). Harloff et al. (2012) reported that mutation frequencies ranged from 1/12 kb to 1/60 kb in two rapeseed populations. Recently, Gilchrist et al. (2013) have used the TILLING technique to identify 432 unique mutations in 26 different genes in *B. napus* cv. DH12075 population. Mutation density in their TILLING population ranged from 1/56 kb to 1/308 kb depending on the locus investigated. In the present study, we obtained an average mutation frequency of 1/48 kb (1% EMS) population which is in the range expected for rapeseed. Of these, thirteen (93%) were G/C to A/T transitions and one was A/T to G/C transitions (**Table 5**) which is consistent with the mode of action of EMS (Burns et al., 1986) and similar to that reported in *Arabidopsis* (Greene et al., 2003) and *B. napus*.

In conclusion, we have demonstrated that *BnaA.FRI.a* has effects on flowering time and yield components within the redundant background of an allopolyploid genome. Moreover, a $BnFRI_{G1278A}$ mutation was found to affect at least three *BnFLC* paralogs, shedding new light into the regulatory relationships between *FRI* and *FLC* in *B. napus*. Moreover, we provide new non-transgenic *B. napus* alleles with altered flowering time for rapeseed breeding.

3.6 Acknowledgements

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4 Closing discussion

This study deals with members of three gene families controlling flowering time in *B. napus*. Paralogs were identified and phenotypic and expression studies were carried out on different EMS mutants. The aim of this study was to provide data about the function of *BnFT*, *BnTFL1* and *BnFRI* in *B. napus*. The main findings of the present work can be summarized as follows:

- *BnFT* paralogs contribute differently to flowering time regulation. Either nonsense or missense mutations in the *BnC6FTb* gene resulted in a marked flowering delay, whereas no flowering time reduction in *BnC6FTa* mutant lines.

- *BnTFL1-2* paralogs had no large effects on flowering time but on yield components. F_1 hybrids between *BnTFL1-2* mutants and non-mutated parents had increased seed numbers per pod and total seeds per plant suggesting that heterozygous mutations in a *TFL1* paralog may impact heterosis in rapeseed.

- *BnaA.FRI.a* missense mutations revealed differential effects regarding flowering time as well as yield-associated traits.

- The splice site mutation $BnC6FTb_{G2009A}$, or even a missense mutation $BnFRI_{G1278A}$ could affect the expression level of downstream flowering time regulators *BnSOC1* and *BnAP1*.

TILLING was successfully applied for flowering time genes in winter type *B. napus*. Based on previous reports, average EMS-mutation frequencies are expected to be low in diploid species (approximately 1/380 kb), whereas mutation frequencies in polyploids are expected to be higher (Wang et al., 2012b). For example, with one mutation per 24 kb reported in hexaploid wheat, one per 15 kb in allopolyploid rapeseed (Harloff et al., 2012), 40 kb in tetraploid wheat (Slade et al., 2005), 300 kb in rice (Till et al., 2007), 100 kb in barley (*Hordeum vulgare* L.) (Caldwell et al., 2004), and 170 kb in Arabidopsis (Greene et al., 2003). However, genome redundancy can have an effect on the EMS mutation density. In *B. rapa* mutation frequencies were relatively high (1/60 kb) for diploid species (Stephenson et al., 2010). Although considered diploid, the *B. rapa* genome can be regarded as ancient triploid, harboring in average three copies per *Arabidopsis* gene. In the present study, a total of 117 mutations for the *BnC6FTb*/a, *BnTFL1-2* and *BnaA.FRI.a* genes were detected via TILLING in a European winter rapeseed Express 617. Mutation frequencies ranged from 1/24 kb (*BnC6FTa*) to 1/72 kb (*BnTFL1-2*) for target genes, which is in the expected range for polyploid species. Greene et al. (2003) found that 99% of mutations from alkylation of guanine induced by EMS are G/C-A/T transitions, of the 117 mutations we obtained from screening Express 617 TILLING population, 5.98% lead to a nonsense or splice site transcript and 30.78% resulted in amino acid changes. Among them, 116 represented G/C to A/T

transitions and one represented an A to C transversion (*BnFRI* $_{A1510G}$), which is a close fit to previous reports that EMS mutagenesis primarily induces G/C to A/T transitions.

Since the past two decades, the *FT* and *TFL1* genes have been extensively studied in *Arabidopsis.* Along several approaches based on knockout mutations, several missense mutants have been also characterized. The missense *FT* mutation (*ft3*$_{Arg119His}$) was conferring a late-flowering phenotype under long days (LD, 16 h light) in the L*er* background (Kobayashi et al., 1999). In a subsequent study, a single amino acid exchange in a *TFL1* mutant (*tfl1-1*$_{Gly105Asp}$) leaded to early flowering and limited the development of the normally indeterminate inflorescence by promoting the formation of a terminal floral meristem (Bradley et al., 1997). Contrasting with expectations based on Arabidopsis *TFL1*-phenotypes, *BnTFL1-2* mutants showed a slight delay in flowering time. However, mutations of *BnTFL1-2* also showed changes in inflorescence meristem growth types as *tfl1-1* in Arabidopsis. On the other hand, we observed that F_1 hybrids derived from crosses between *BnTFL1-2* M_3- and an MS line showed increased seed yield, while *BnC6FTb*$_{G2009A}$ F_1 hybrids displayed no effects. Thus, although the role of *BnTFL1-2* involving flowering time regulation is not likely to be conserved compared to its Arabidopsis ortholog, *TFL1-2* appears to be involved in yield-related traits as reported for its tomato ortholog *SP* (Jiang et al., 2013b). For *BnaA.FRI.a* mutants, the M_3 line *BnFRI*$_{G1278A}$ flowered earlier (four days) than the control plants, and the expression of *BnFRI*, as well as its downstream targets *BnFLC, BnSOC1* and *BnAP1* were altered in leaves even before vernalization.

The generation of several paralogous copies resulted from gene duplication events. Paralogs may retain functions of the ancestral genes, and thus act redundantly and/or additively due to the increased protein dosage, but they may also develop non-, sub- or neo-functionalisation (Force et al., 1999). Non-functionality occurs when a given mutation aborts either the expression or protein function of a gene. By contrast, sub- and neo-functionalization are adaptive processes that result in paralogous copies with either conserved functions, but different temporal or special expression patterns, or different functions (Pin and Nilsson, 2012b). I wanted to know whether different *BnFT* paralogs gained different function by studying their phenotypes and their transcriptional activities. Based on our hypothesis, we checked the expression of putative *BnFT* downstream target genes *BnSOC1* and *BnAP1* in the late-flowering *BnC6FTb*$_{G2009A}$ mutants. Interestingly, we detected a reduced expression of *BnSOC1* in *BnC6FTb*$_{G2009A}$ mutants compared to the control after vernalization, and its maximum at BBCH60. *BnC6FTb*$_{G2009A}$ mutants showed a decrease on expression of *BnSOC1* in young leaves at BBCH 60 compared to Express 617. Another *FT* putative downstream gene, *AP1* is the major floral meristem identity gene that is uniformly expressed in young flower primordia, and later becomes localized to sepals and petals (Mandel et al., 1992). We found that *BnAP1* expression was higher in the *BnC6FTb*$_{G2009A}$ mutants at rosette stages, while its expression level was lower (around 40%) in leaves when first flower appears. In summary, *BnC6FTb*$_{G2009A}$ M_3 confers strong flowering time phenotypic effects by altering the

regulation of at least two downstream targets in *B. napus.*

Our results gave further support to the assumption that *BnFT* paralogs do not contribute equally to flowering time regulation. In sunflower (*Helianthus annuus*), four *FT* paralogs (*HaFT1-4*) displayed different expression patterns (Blackman et al., 2010). It was found that *HaFT1* affects developmental timing through interference with the function of paralog *HaFT4*. *HaFT3* was proposed as a non-functional paralog because lack of expression after mutation had no effect. In apple, two *FT* paralogs (*MdFT1* and *MdFT2*), appeared to promote flowering, however, both genes showed distinct expression patterns along different floral transition stages and plant tissues, suggesting a sub-functionalization patterns between them (Kotoda et al., 2010b). For neo-functionalization, supporting results have been published in sugar beet and potato. Two *FT* paralogs (*BvFT1* and *BvFT2*) antagonistically regulating flowering time have been found in sugar beet (Pin et al., 2010). From them, *BvFT2* is functionally conserved with *FT* and is essential for flowering. On the other hand, the second *FT* paralog (*BvFT1*) represses flowering and its down-regulation is crucial for the vernalization response (Pin et al., 2010). Two *FT* paralogs of potato provide another striking example of neo-functionalization (*StSP3D* and *StSP6A*). *RNAi* lines transformed with *StSP3D* constructs showed a late flowering phenotype, while *StSP6A RNAi* lines flowered normally, but showed defective tuberization (Navarro et al., 2011). As determined by our expression analyses, all six *BnFT* paralogs were differently expressed. Furthermore, lack of expression of the *BnC2FT* copy suggests strong evidence of non-functionalization (**Figure 20**). This finding is consistent with previous report by Wang et al. (2012a). In their report, *BnC2FT* was not transcribed at all either under vernalizing or non-vernalizing conditions in *B. napus* nor in *B. oleracea*.

Our data also shed new light on the regulatory relationships between *FRI* and *FLC* in *B. napus*. Tadege and co-authors (2001) initially isolated five *BnFLC* paralogs and found differences in expression among them in oilseed rape leaves. All five *BnFLC* genes delayed flowering ranging from three weeks to more than seven months once transferred into the Arabidopsis *Ler* background. Furthermore, Zou et al. (2012) found nine *BnFLC* paralogs in the winter-type cultivar Tapidor. The *BnFLC* homologues showed distinct expression patterns in vegetative and reproductive organs, and at different developmental stages (Zou et al., 2012). In the same work it was demonstrated that one out of nine *BnFLC* paralogs (*BnFLC.A10*) co-localized with a flowering time QTL in the TNDH population. Allelic diversity of *BnFLC.A10* caused by a MITE insertion was associated with vernalization requirement in rapeseed (Hou et al., 2012). In good agreement with those reports, multiple *FLC* paralogs displayed different expression patterns in this study. Choi et al. (2011) found that FRI acts as a scaffold protein interacting with FRL1, FES1, SUF4, and FLX to form a transcription activator complex (FRI-C), and proposed a model for FRI-C-mediated transcriptional activation of *FLC*. They showed that the *fri* mutation causes early flowering with reduced *FLC* expression similar to *frl1*, *fes1*, *suf4*, and *flx*, which are mutants of FLC-specific regulators. In rapeseed, it was proposed that *BnFRI.A3* and *BnFLC.A3b* probably interact to

adjust flowering time (Zou et al., 2012). Our observations on *BnFRI*$_{G1278A}$ M_3 plants do not support this one to one interaction model (**Figure 20**). It was found that at least three *BnFLC* paralogs show expression alterations related to a missense mutation in a single *BnFRI* paralog. Thus, interactions between *BnFRI* and *BnFLC* are indeed more complex in this allopolyploid species.

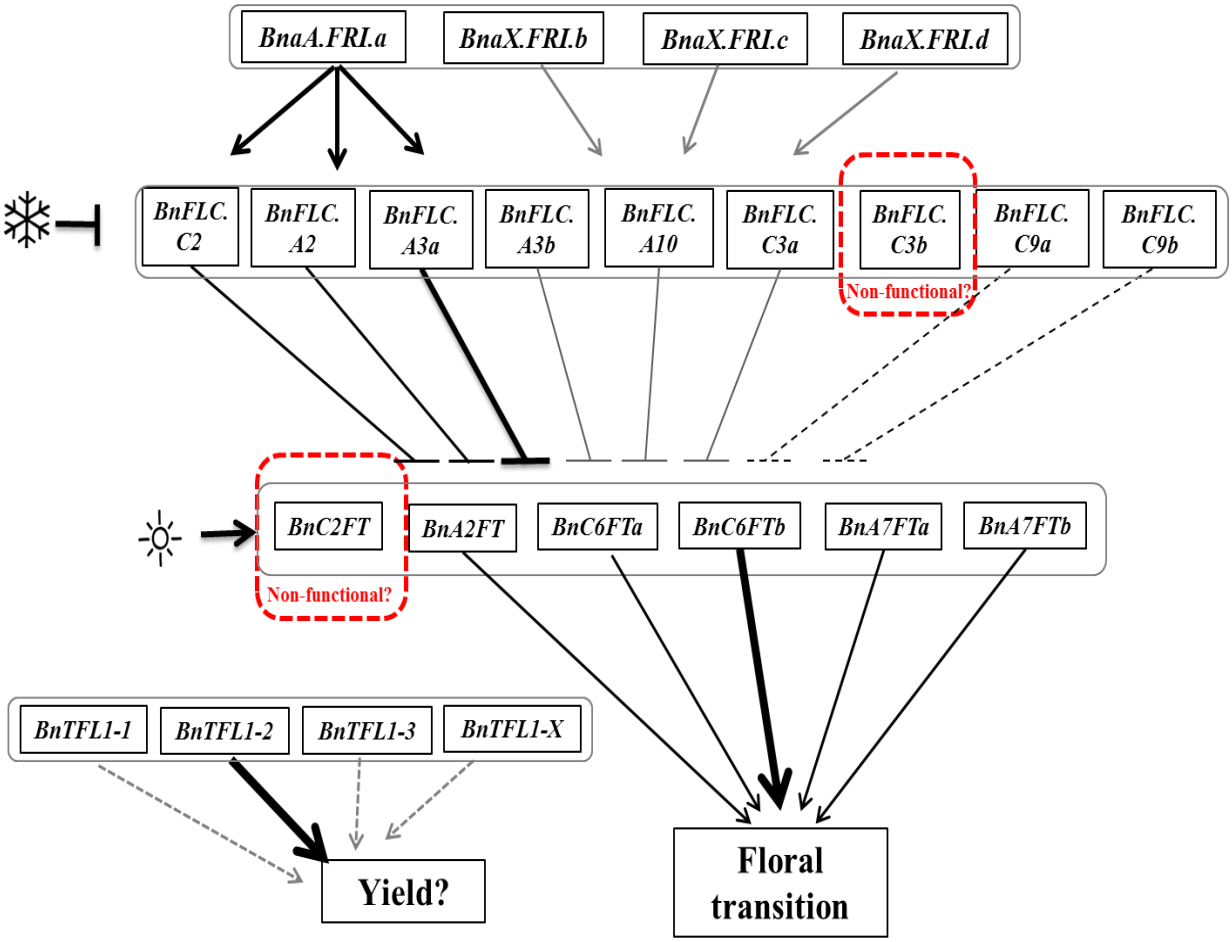

Figure 20: Suggested model of regulation of flowering time gene paralogs in winter type *B. napus* according to my experimental data (black solid line) and according to recent reports (grey solid line). Grey dashed line means no data available. Exogenous signals (cold and light) are indicated by symbols. Positive regulation is depicted with arrows. Negative regulation is depicted with truncated lines.

Together with former studies (Hou et al., 2012; Tadege et al., 2001; Wang et al., 2012a; Wang et al., 2011a; Zou et al., 2012) of paralog expression of flowering time regulators in *B. napus*, I am proposing a model of regulation among *BnFRI*, *BnFLC*, *BnFT* and *BnTFL1* paralogs in winter type *B. napus* (**Figure 20**). Our data suggest that the influence of each paralog in flowering time is not equal. It was postulated that *BnaA.FRI.a* (bigger thicker arrow) is a major *BnFLC* activator (Wang et al., 2011a). At least three *BnFLC* paralogs (*BnFLC.A3a*, *BnFLC.A2* and *BnFLC.C2*) show altered expression related to this paralog. *BnFLC.A3b* was

differentially expressed between the winter-type and semi-winter type, and *BnFLC.C3b* might be a pseudogene because it contains stop codons created by inserted nucleotides in exon 2 and exon 7 (Zou et al., 2012). Hou et al. (2012) found *BnFLC.A10* is the gene underlying *qFT10-4*, the QTL for phenotypic diversity of flowering time in the TN-DH population, they further found that winter rapeseeds had a 621-bp insertion in the upstream region of *BnFLC.A10*, whereas the 621-bp fragment was absent in the spring types. *BnC6FTb* showed a strong effect as a flowering time regulator in rapeseed but *BnC6FTa* did not in this study. Lack of expression of *BnC2FT* suggests this paralog was not functional in winter rapeseed. Quite interesting, we found that *BnTFL1-2* paralog had no large effect on flowering time but on yield components.

The superiority of hybrids (called heterosis) has benefited agriculture and fascinated geneticists for over 100 years (Crow, 1948; Duvick, 2001). Several hypotheses to explain heterosis were presented soon after plant breeders propelled efforts to dissect its genetic and molecular bases (Birchler et al., 2003; Hochholdinger and Hoecker, 2007; Lippman and Zamir, 2007; Schnable and Springer, 2013; Springer and Stupar, 2007). Generally, three classical models have been proposed to explain heterosis: i) dominance, ii) overdominance and pseudo-overdominance model, and iii) epistasis (Birchler et al., 2010; Charlesworth and Willis, 2009; Jones, 1917; Luo et al., 2001; Paschold et al., 2012; Yu et al., 1997). Since, the hybrid vigor phenomenon was revealed by Darwin (1876) more than a century ago, the typical thinking about heterosis (quantitative trait) is that it involves many genes, and this is certainly true in most cases. However, several studies of single-gene overdominance have been documented in Arabidopsis (Moore and Lukens, 2011) and crops such as maize (Hollick and Chandler, 1998) and tomato (Krieger et al., 2010). In Arabidopsis, the *erecta* locus may account for a substantial amount of heterosis in some crosses between pairs of *Arabidopsis* ecotypes (Moore and Lukens, 2011). In maize, the *pl* (*purple plant*) locus, which contributes to anthocyanin content, encoding a transcriptional regulator of anthocyanin pigment synthesis, heterozygote displays a phenotype that is greater anther pigmentation than either homozygote (Hollick and Chandler, 1998). In tomato, heterozygosity at *SFT* (*SINGLE FLOWER TRUSS*, an ortholog of *A. thaliana FT*) contributes to 60% yield increase due to suppression of growth termination imposed by the *SP* (*SELF PRUNING*) gene (Krieger et al., 2010). In addition, the tomato *TFL1* homolog (*SP*), has been reported to control the switch between determinate and indeterminate growth, changing the whole plant architecture and having impact in yield (Carmel-Goren et al., 2003; Thouet et al., 2008). These findings suggest that single heterozygous mutations may improve productivity in other agricultural organisms.

Hybrid breeding relies on crossing parents from two differing heterotic groups. However, adapted oilseed rape breeding material has a very narrow genetic basis (Girke et al., 2012). The results from my study suggest a potential *TFL1*-related heterosis in *B. napus*, however, more experiments are needed for verifying this hypothesis. First, prelimary greenhouse data will have to be confirmed by field experiments. Second, additional hybrid combinations

should be analyzed to dissect the possible impact of background mutations in the *BnTFL1* mutant. In addition, I propose to sequence the *BnTFL1-2* loci from rapeseed lines with high and low combining ability (Qian et al., 2007). A strategy to harness *TFL1*-related heterosis could be applied in oilseed rape breeding. Moreover, the mutants will be a valuable resource to study flowering regulatory networks in polyploids and they can be a new resource to broaden the genetic basis of rapeseed breeding.

5 Summary

The transition from vegetative to reproductive development is a key developmental step in the life cycle of a flowering plant. Floral transition is a complex biological process, which is controlled by multiple flowering genes that respond to environmental cues and endogenous signals. *Arabidopsis thaliana* is an excellent model system for the Brassicaceae plant family. This study deals with three major flowering time regulators from oilseed rape (*Brassica napus*), an allopolyploid species which originated from natural hybridization between *B. rapa* and *B. oleracea*.

The aim of this study was to identify and characterize *B. napus* orthologs of the *A. thaliana* flowering time genes *FT*, *TFL1* and *FRI*. First, I searched the NCBI and Brassica databases to identify paralogs of *FT*, *TFL1* and *FRI* in rapeseed. Then, I designed paralog specific primers to search for mutations in three genes (*BnC6FTa/b*, *BnTFL1-2*, *BnaA.FRI.a*) using a TILLING mutant population of the European winter type oilseed rape Express 617. Altogether, 117 mutants were identified. Four non-sense and twenty missense mutant lines (M_3) were further characterized. Homozygous M_3 lines were produced by selfing M_2 plants. They were grown in the greenhouse and their flowering time as well as yield components were analysed.

BnFT paralogs contributed differently to flowering time regulation. Despite the redundancy of mutations in a single gene, either nonsense or missense mutations in the *BnC6FTb* gene resulted in a marked flowering delay, whereas all five *BnC6FTa* mutant lines started flowering as the non-mutated parent. Mutations within the *BnTFL1-2* paralog had no large effects on flowering time but on yield components. F_1 hybrids between *BnTFL1-2* mutants and non-mutated parents had increased seed numbers per pod and total seeds per plant suggesting that heterozygous mutations in a *TFL1* paralog may impact heterosis in rapeseed. Mutations within the *BnaA.FRI.a* paralog showed pleiotropic effects beyond the regulation of flowering time.

Moreover, the expression of the mutant alleles and their downstream genes were analysed. To my surprise, a splice site mutation of *BnC6FTb*, and even a missense mutation of *BnaA.FRI.a* had an effect on the transcriptional activities of two downstream flowering time regulators, *BnSOC1* and *BnAP1* despite the presence of multiple paralogs. Moreover, the expression of three *BnFLC* paralogs was altered due to a missense mutation in a single *BnFRI* paralog. This demonstrates multiple interactions between *BnFRI and BnFLC* paralogs in an allopolyploid species.

This study unraveled the function of different *FT*, *TFL1* and *FRI* paralogs in *B. napus*. It provides plant material with new mutant alleles that may constitute a valuable resource to

broaden the genetic basis of rapeseed breeding. Furthermore, it indicates a possible role of *BnTFL1* mutations in heterosis.

6 Zusammenfassung

Der Übergang von der vegetativen zur reproduktiven Entwicklung ist ein wichtiger Entwicklungsschritt im Lebenszyklus einer Blütenpflanze. Die Blühinduktion ist ein komplexer biologischer Vorgang, der durch mehrere Blühgene gesteuert wird, die auf Umweltreize und endogene Signale reagieren. *Arabidopsis thaliana* ist ein ausgezeichnetes Modellsystem für die Pflanzenfamilie der Brassicaceae. Diese Studie befasst sich mit drei wesentlichen Blühregulatoren aus Raps (*Brassica napus*), einer allopolyploiden Art, die durch natürliche Hybridisierung aus *B. rapa* und *B. oleracea* entstanden ist.

Das Ziel dieser Studie war die Identifizierung und Charakterisierung von *B. napus* Orthologen der *A. thaliana* Blühzeittgene *FT*, *TFL1* und *FRI*. Zuerst durchsuchte ich die NCBI und Brassica Datenbanken, um Paraloge von *FT*, *TFL1* und *FRI* in Raps zu identifizieren. Dann entwarf ich paralog-spezifische Primer für die Suche nach Mutationen in drei Genen (*BnC6FTa/b*, *BnTFL1-2*, *BnaA.FRI.a*) unter Verwendung einer TILLING Mutantenpopulation aus der europäischen Winterrapssorte Express 617. Insgesamt wurden 117 Mutanten identifiziert. Vier *non-sense* und zwanzig *missense* Mutantenlinien (M_3) wurden näher charakterisiert. Homozygote M_3 Linien wurden durch Selbstung von M_2 Pflanzen erzeugt. Sie wurden im Gewächshaus angezogen und sowohl der Blühzeitpunkt als auch Ertragskomponenten analysiert.

BnFT-Paraloge trugen unterschiedlich zur Regulation des Blühzeitpunkts bei. Trotz der Redundanz von Mutationen in einem einzigen Gen führten sowohl *non-sense* als auch *missense* Mutationen im Gen *BnC6FTb* zu einer deutlichen Verzögerung der Blüte, während alle fünf *BnC6FTa* Mutantenlinien zur gleichen Zeit wie die nicht mutierten Eltern blühten. Mutationen innerhalb des *BnTFL1-2*-Paralogs hatten keine großen Auswirkungen auf den Blühzeitpunkt, jedoch auf die Ertragskomponenten. F_1-Hybriden zwischen *BnTFL1-2* Mutanten und nicht mutierten Eltern wiesen eine erhöhte Samenzahl pro Schote und pro Pflanze auf, was darauf hindeutet, dass heterozygote Mutationen in einem *TFL1*-Paralog eine Auswirkung auf die Heterosis in Raps haben könnten. Mutationen innerhalb des *BnaA.FRI.a*-Paralogs zeigten zusätzlich zur Regulation des Blühzeitpunkts pleiotrope Effekte.

Darüber hinaus wurde die Genexpression der mutierten Allele und ihrer nachgeschalteten Gene analysiert. Zu meiner Überraschung hatten eine *splice site* Mutation von *BnC6FTb* und sogar eine *missense* Mutation von *BnaA.FRI.a* einen Einfluß auf die Transkriptionsaktivitäten der zwei nachgeschalteten Blühregulatoren *BnSOC1* und *BnAP1* trotz des Vorhandenseins mehrerer Paraloge. Des weiteren wurde die Expression von drei *BnFLC* Paralogen durch eine *missense* Mutation in einem einzigen *BnFRI* Paralog verändert. Dies zeigt, dass vielfältige Wechselwirkungen zwischen *BnFRI* und *BnFLC* Paralogen in einer allopolyploiden Art bestehen.

Diese Studie entschlüsselte die Funktion verschiedener *FT*-, *TFL1*- und *FRI*-Paraloge in *B. napus*. Sie lieferte Pflanzenmaterial mit neuen mutierten Allelen, das eine wertvolle Ressource darstellen kann, um die genetische Basis der Rapszüchtung zu erweitern. Darüber hinaus verweist sie auf eine mögliche Rolle von *BnTFL1* Mutationen bei der Heterosis.

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8 Supplementary data

The supplementary material is available on a disk attached to this thesis. It contains the following files:

Supplementary table S1: PCR primers used in this study for screening EMS-mutations and for expression analysis by RT-qPCR

Supplementary table S2: *B. napus* accessions selected for *BnC6FTb* and *BnTFL1-1* sequencing

Supplementary table S3: Predicted kinase target sites in $BnFRI_{G1278A}$ and WT protein sequences

Supplementary file 1: A complete list of all primer sequences

Supplementary file 2: TILLING raw data

Supplementary file 3: A complete list of seed materials and accession numbers used in this study

Supplementary file 4: Greenhouse experiments raw data

Sequencing raw data

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10 Curriculum Vitae

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LIST OF PUBLICATIONS:

Guo, Y., Harloff, H. J., Jung, C., and Molina, C. Mutations in single *FT*- and *TFL1*-paralogs of rapeseed (*Brassica napus* L.) and their impact on flowering time and yield components. ***Frontiers in Plant Science 5, 282 (2014).***

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